ISSN 0026-265X

Volume 25, Number 2, June 1980

Microchemical Journal

devoted to the application of microtechniques in all branches of science

Editor: Al Steyermark

Published under the auspices of the American Microchemical Society by

ACADEMIC PRESS
New York and London

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Volume 25, Number 2, June 1980

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Published quarterly by Academic Press, Inc.,
111 Fifth Avenue, New York, New York 10003.
1980: Volume 25. Price: $73.00 U.S.A.;
$86.00 outside U.S.A. All prices include
postage and handling.
(Information about reduced price for personal subscriptions placed
by members is available from the American Microchemical Society.)
All correspondence and subscription orders should be sent to the office of the
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Microchemical Journal, Volume 25, Number 2, June 1980

Briefs

Potentiometric Determination of Boron in Graphite Microspheres for Nuclear Engineering. PIER LUIGI BULDINI, *C.N.R.—Lamel Laboratory, Via de' Castagnoli 1, 40126 Bologna, Italy,* AND DONATELLA FERRI, *Chemical Institute G. Ciamician, University of Bologna, 40126 Bologna, Italy.*

The acidimetric determination of boron via the boric acid–mannitol complex is applied to the analysis of boron-loaded graphite microspheres used in nuclear engineering.

Microchem. J. **25**, 143–146 (1980).

Sensitive Spectrophotometric Determination of Molybdenum(VI) with Pyrogallol Red and Cetyltrimethylammonium Ions. CZESŁAW WYGANOWSKI, *Institute of General Chemistry, Technical University, 90-924 Łódź, Poland.*

Microgram amounts of molybdenum are determined by the proposed method. The composition of the complex is determined.

Microchem. J. **25**, 147–152 (1980).

The Determination of Lecithin and Total Choline-Containing Phospholipids in Amniotic Fluid Employing Enzymes as Reagents. J. D. ARTISS*,† T. F. DRAISEY,*,† R. J. THIBERT,*,†,‡ B. ZAK,*,‡ AND K. E. TAYLOR, **Department of Chemistry, University of Windsor, Windsor, Ontario N9B 3P4, and †Department of Pathology, Salvation Army Grace Hospital and Windsor Western Hospital Centre, Windsor, Ontario, Canada; and ‡Department of Pathology, Wayne State University, School of Medicine, Detroit, Michigan 48201.*

Two assays which employ enzymes as reagents have been described. Both are quick, precise, involve no extractions, and are amenable to automation. Interferences have been studied and appear to be negligible.

Microchem. J. **25**, 153–168 (1980).

Optimum Acidity and Masking of Redox Reactions. B. W. BUDESINSKY, *Phelps Dodge Corporation, Morenci, Arizona 85540.*

Theoretical relationships for the estimation of optimum acidity and masking of redox reactions are developed on the basis of the coefficients of side reactions. The concentrations of reacting components are calculated from optimum conditions and the values of standard redox potentials.

Microchem. J. **25**, 169–175 (1980).

Surface Analysis of Thick Gold Films by X-Ray Fluorescence Using the Base Metal as an Internal Reference. RONALD LEE FOSTER AND PETER F. LOTT, *Department of Chemistry, University of Missouri–Kansas City, Kansas City, Missouri 64110.*

A procedure is described for the determination of the amount of gold clad on a brass base. The method does not require dissolution of the sample and utilizes the presence of copper in the base metal as an internal standard.

Microchem. J. **25**, 176–178 (1980).

Isolation of Starch Granules and Starch-Granule-Bound Glucan Synthetase from Cotton Leaves. CHONG W. CHANG, *Agriculture Research, Science and Education Administration, USDA, Western Cotton Research Laboratory, Phoenix, Arizona 85040.*

A simple sucrose density technique is described for starch-granule isolation from leaves. Starch-granule-bound glucan synthetase (EC 2.4.1.21) was isolated from phenolic-rich plant tissue.

Microchem. J. **25**, 179–185 (1980).

Comparison of High-Performance Liquid Chromatography Electrochemical Detectors for the Determination of Aporphines, Catecholamines, and Melatonin. D. W. HUMPHREY, M. E. GOLDMAN, R. E. WILCOX, C. K. ERICKSON, AND R. V. SMITH, *Drug Dynamics Institute and Division of Pharmacology, College of Pharmacy, University of Texas at Austin, Austin, Texas 78712.*

Modifications of the Bioanalytical Systems flow cell, formerly described, gave increased sensitivity toward certain catecholic compounds, decreased baseline noise, and allowed for serial coupling of detectors.

Microchem. J. **25**, 186–195 (1980).

Determination of Alkoxyl Groups on Submilligram Samples. MICHAEL G. KOVALYCSIK AND AL STEYERMARK, *Department of Chemistry, Newark College of Arts and Sciences, Rutgers—The State University, Newark, New Jersey 07102.*

A study was made of the applicability of the official method using standardized apparatus for the determination of alkoxyl groups on sample sizes in the submilligram range. Acceptable results were obtained on 0.1-mg samples.

Microchem. J. **25**, 196–199 (1980).

Microtitration of Various Anions with Quaternary Ammonium Halides Using Solid-State Electrodes. WALTER SELIG, *Lawrence Livermore Laboratory, University of California, Livermore, California 94550.*

Many solid-state electrodes were found to respond as endpoint detectors in the potentiometric titration of large inorganic and organic anions with quaternary ammonium halides.

Microchem. J. **25**, 200–208 (1980).

Resorcinol as a Reagent for Zinc. R. H. DINIUS AND J. M. BAKER, *Department of Chemistry, Auburn University, Auburn, Alabama 36830.*

The use of the zinc ion-catalyzed autoxidation or hydrogen peroxide oxidation of resorcinol to determine zinc concentration has been examined.

Microchem. J. **25**, 209–218 (1980).

Microgram Determination of Vanadium from Natural Resources. Y. K. AGRAWAL AND K. P. S. RAJ, *Pharmacy Department, Faculty of Technology and Engineering, M. S. University of Baroda, Kalabhavan, Baroda 390 001, India,* AND M. S. SANT, *Pathology Department, Medical College, Jabalpur (M.P.), India.*

Vanadium has been extracted and determined from a number of natural resources by liquid–liquid extraction technique using *N*-*m*-tolyl-*o*-methoxybenzohydroxamic acid.

Microchem. J. **25**, 219–222 (1980).

Analytical Reactions of Substituted Cyanoferrates. 3. Spectrophotometric Determination of Azide Ion in an Aqueous Medium. M. C. MEHRA AND R. GARVIE, *Chemistry Department, Université de Moncton, Moncton, New Brunswick E1A 3E9, Canada.*

The pentacyanoamminoferrate(II) ion and N_3^- in acidic condition produce a colored species absorbing at 555 nm that is useful in the spectrophotometric determination of the latter in an aqueous medium. A number of common anions including Cl^-, SO_4^{2-}, and NO_3^- do not interfere.

Microchem. J. **25**, 223–227 (1980).

Analytical Reactions of Substituted Cyanoferrates. IV. Spectrophotometric Determination of Beryllium(II), Gallium(III), and Indium(III) in Solution. M. C. MEHRA AND G. VERRET, *Chemistry Department, Universite de Moncton, Moncton, New Brunswick E1A 3E9,* Canada.

The ions accelerate formation of colored pentacyanoazidoferrate(III) species in alkaline media. The reaction is nonselective, but some cations and anions can be tolerated at comparable concentrations.

Microchem. J. **25**, 228–231 (1980).

An Improved Selective Fluoride Electrode. H. M. STAHR, P. F. ROSS,* AND W. HYDE, *Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Iowa State University, Ames, Iowa 50011, and *USDA, APHIS, NADC, Ames, Iowa 50011.*

The major cause of failure for an Orion Research 9409A fluoride-selective electrode observed in routine use in the authors' laboratories is loss of contact with the LaF_3 crystal. Opening the electrode and replacing the electrolyte restores electrodes that suffer from this effect.

Microchem. J. **25**, 232–234 (1980).

Spectrophotometric Determination of Copper as Tetraphenylarsonium or Tetraphenylphosphonium Thiocyanate–Cuprate. B. THAMHINA, *Laboratory of Analytical Chemistry, Faculty of Science and Mathematics, University of Zagreb, Strossmayerov trg 14, 41000 Zagreb, Yugoslavia.*

The spectrophotometric determination of copper(II) with thiocyanate by extraction of the tetraphenylarsonium and tetraphenylphosphonium ion-association complexes is described.

Microchem. J. **25**, 235–239 (1980).

Spectrophotometric Determination of Chloride, Bromide, and Iodide with an Improved Mercury–Iron–Thiocyanate Method. WOLFGANG J. KIRSTEN AND INGER LINDHOLM-FRANZÉN, *Department of Chemistry, Swedish University of Agricultural Sciences, S-750 07 Uppsala, Sweden.*

Stable reagents for the determination have been developed.

Microchem. J. **25**, 240–245 (1980).

Investigation of *m*-Nitroaniline Formation during the Zimmermann Reaction. C. S. Ho and K. G. Blass, *Department of Chemistry, University of Regina, Regina, Saskatchewan, S4S 0A2 Canada.*

The Zimmermann reaction for the determination of 17-ketosteroids was tested under both room-temperature and steam-distillation reaction conditions. *m*-Nitroaniline was isolated from the residue of the steam distillation, but was not found under the room-temperature conditions.

Microchem. J. **25**, 246–253 (1980).

Interference of Potassium on Barium Measurements in the Inductively Coupled Plasma. S. Nikdel and J. D. Winefordner, *Department of Chemistry, University of Florida, Gainesville, Florida 32611*

The study was made to determine the influence of an ionizer (potassium) upon the Ba-atom and Ba-ion emission signals produced in an inductively coupled plasma as a function of height above the load coil and as a function of inductively coupled plasma input power.

Microchem. J. **25**, 254–256 (1980).

MICROCHEMICAL JOURNAL 25, 143–146 (1980)

Potentiometric Determination of Boron in Graphite Microspheres for Nuclear Engineering

PIER LUIGI BULDINI AND DONATELLA FERRI*

C.N.R.—Lamel Laboratory, Via de' Castagnoli 1, 40126 Bologna, and
**Chemical Institute G. Ciamician, University of Bologna, 40126 Bologna, Italy*

Received July 17, 1979

INTRODUCTION

Because of the great importance attached to boron doping of graphite for nuclear engineering, much interest has been shown in reliable methods of analysis. Various techniques have been devised to evaluate the boron content in graphite; usually spectrography (*4–6*), neutron activation analysis (*1, 3*), and spectrophotometry (*2, 9, 10*) are used. The well-known acidimetric determination of boron via the boric acid–mannitol complex was also applied to similar materials (*8*) and this method seemed to be the most suitable and simple for boron determination in graphite microspheres.

The fabrication procedure for the graphite microspheres, developed by Le Carbone Lorraine (92 Gennevilliers, France), was an application of the so-called "powders method": the graphite kernels were grown from an intimate mixture of graphite powder (about 2 μm in diameter), appropriate amounts of B_4C grains (about 10 μm in diameter), and an organic binder inside a rotating drum. The initial density of the kernels (around 1.0 g/cm^3) was increased, in order to obtain the required physical properties, by successive stages of high-pressure impregnation with organic liquid followed by cockerization. The microsphere coating was deposited by acetylene pyrolysis, thereby obtaining a pyrocarbon coating thickness between 20 and 40 μm with a density between 1.7 and 1.8 g/cm^3. The graphite microspheres had an overall diameter of between 920 and 1180 μm. They were coated with a pyrolytic carbon layer to prevent the chance for inclusion of humidity, as well as to avoid powderization during handling.

MATERIALS AND METHOD

Reagent-grade chemicals were used throughout. Sodium hydroxide, 0.1 and carbonate free, was prepared according to Strouts *et al.* (*7*).

Grind (Spex Model 8000 Mixer/Mill) the sample in a 60-ml agate container for 30 min so that all of the material is able to pass through a 150-mesh sieve. Fuse 1 g of anhydrous sodium carbonate into a zirconium

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crucible and after cooling add 1 g of sodium peroxide, over which a portion (10–250 mg, containing at least 0.5 mg of boron) of the ground sample is placed. Then add 1 g of sodium peroxide and 1 g of anhydrous sodium carbonate, cover the crucible, and fuse the mixture by warming at 600°C until the peroxide reaction subsides and then to 800°C in order to complete the sodium carbonate reaction also.

Care must be taken that there is no mechanical loss of sample due to excessive gassing. When the fusion is complete, remove from the heat and rotate the crucible, allowing the melt to solidify on the sides.

After cooling, place the zirconium crucible and its cover in a 400-ml beaker and leach them with warm double-distilled water. After complete dissolution of the melt, remove the zirconium crucible and its cover and neutralize the solution to methyl red with 16–18 ml of 1:1 hydrochloric acid. Cover the beaker and heat the solution gently for about 15 min to completely remove carbon dioxide. Keep the solution acid by adding 1:1 hydrochloric acid if necessary.

Cool the solution and adjust its pH to 3–3.5; then titrate potentiometrically with 0.1 *M* carbonate-free sodium hydroxide until a pH value of about 8 in order to detect the first endpoint (about pH 5–5.5). Add 5.0 g of mannitol and titrate to pH 9–9.5 with 0.1 *M* carbonate-free sodium hydroxide.

The stoichiometric quantity of titrant consumed by the boric acid–mannitol complex may be evaluated by determining the difference between the two inflection points (see *t* in Fig. 1).

The contribution due to mannitol acidity is determined by titrating a

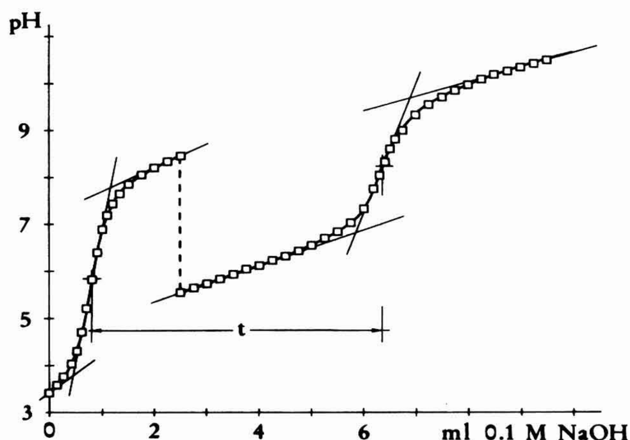

FIG. 1. Potentiometric titration of the boric acid–mannitol complex. *t* indicates the quantity of titrant consumed by the complex. To obtain the true value, it is necessary to subtract the mannitol acidity contribution.

mannitol blank and subtracting it from the obtained value. Each milliliter of 0.1 *M* carbonate-free sodium hydroxide corresponds to 1.08 mg of boron in the sample.

RESULTS AND DISCUSSION

Table 1 shows the results obtained for some samples. To compare these results, the boron content specified by Le Carbone Lorraine and that obtained at the European Joint Nuclear Research Centre of Ispra (Italy) are also reported.

Various types of alkaline fluxes were tried (Na_2CO_3, $Na_2CO_3 + NaNO_3$, $NaOH + NaNO_3$, $NaOH + Na_2O_2$, $Na_2CO_3 + Na_2O_2 + NaOH$, and so on) but the best results were obtained by dissolving the sample with the oxidizing alkaline flux suggested. Na_2CO_3 permits attainment of satisfactory results if, before the fusion, the sample is sintered in a platinum crucible with sodium carbonate at 600°C for several hours to destroy the graphite matrix, but this procedure is more time consuming without obtaining advantages. Other types of crucibles may also be used with the suggested flux. When using platinum crucibles it is convenient, to minimize the flux attack, to line them with a layer of fused anhydrous sodium carbonate before adding sodium peroxide with the graphite sample; moreover, avoid that the subsequent fusion is unduly prolonged. When using nickel crucibles, the only difference in the procedure is that after complete dissolution of the melt, it is necessary to filter off any nickel oxide present and wash the residue to avoid losses.

Accurate grinding of the sample is essential in order to obtain well-reproducible results. This fact may be explained by the inhomogeneity of the sample which contains both the boron carbide and the pyrolytic carbon external shield. On the other hand grinding is necessary because of the great inertness of the microsphere to each flux, so results may be scattered due to an incomplete fusion.

According to Tereshko (*8*), EDTA has been proven to be a masking agent for metallic impurities, but no advantage was found due to both the

TABLE 1
DETERMINATION OF BORON IN GRAPHITE MICROSPHERES

	Boron content (%)					
Sample	Le Carbone Lorraine	J.N.R.C., Ispra	Found	N^a	s	S_r (%)
78 BS 11	4.50	4.43 ± 0.05	4.52	10	±0.02	0.45
S 7	12.65	11.05 ± 0.05	10.95	10	±0.05	0.45
16/2	12.65	—	10.80	6	±0.05	0.46
16/2/140	12.00	11.05 ± 0.04	10.96	5	±0.03	0.27

[a] Number of determinations.

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low level of metallic impurities present in nuclear-grade graphite and the less definite detection of the inflection points. Nuclear activation analysis of the graphite which was used in manufacturing samples evidenced less than 3000 ppm (w) of impurities, which included about 100 ppm (w) of hydrogen, 1000 ppm (w) of oxygen, and 20 ppm (w) of boron.

SUMMARY

The well-known acidimetric determination of boron via the boric acid–mannitol complex is applied to the analysis of boron-loaded graphite microspheres used in nuclear engineering. After the sample is accurately ground, it is fused with an oxidizing alkaline flux. The melt is dissolved with warm water, the solution is acidified to completely remove carbon dioxide, and the boric acid is titrated potentiometrically with carbonate-free sodium hydroxide after its complexing with mannitol.

The determination of at least 0.2% boron in graphite is carried out with an average error of less than 1%.

ACKNOWLEDGMENTS

The authors wish to thank Dr. P. Azzoni (Comitato Nazionale per l'Energia Nucleare, Bologna) and Dr. G. Serrini (EURATOM, Ispra) for their helpful discussions and suggestions.

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MICROCHEMICAL JOURNAL 25, 147–152 (1980)

Sensitive Spectrophotometric Determination of Molybdenum(VI) with Pyrogallol Red and Cetyltrimethylammonium Ions

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Received August 8, 1979

INTRODUCTION

Pyrogallol Red has been used for the determination of a number of metal ions such as aluminium (*11*), silver (*2*), indium (*5*), and gallium (*10*). The sensitivity of the methods can be increased by using triple systems, e.g., quaternary long-chain amines showing surface activity.

Cetyltrimethylammonium bromide, cetyldimethylbenzylammonium chloride, and dimethyldioctadecylammonium was applied to the determination of vanadium (*9*), tin (*12*), and molybdenum (*8*) with Pyrogallol Red. This paper describes a sensitive method for the determination of molybdenum, based on the formation of a colored complex with Pyrogallol Red in the presence of cetyltrimethylammonium ions.

EXPERIMENTAL

Reagents and Apparatus

Molybdenum(VI) stock solution: 1 mg Mo/ml. Dissolve 2.5220 g of sodium molybdate in water 1 ml of concentrated hydrochloric acid, and dilute with water to 1 liter.

Molybdenum(VI) dilute standard solution: 10 μg Mo/ml. Dilute 1 ml of molybdenum(VI) stock solution to 100 ml.

Pyrogallol Red, sodium salt (PR): 4.8×10^{-4} *M*. Dissolve 50 mg of the reagent in 125 ml of ethanol and dilute with water to 250 ml.

Cetyltrimethylammonium bromide (CTA): 4×10^{-3} *M*. Dissolve 0.3750 g of the reagent in water and dilute with water to 250 ml.

Buffer solution (pH 3.6). Dissolve 82.0 g of sodium acetate, 3.7 g of ethylenediaminetetraacetic acid disodium salt (EDTA), and 87.7 g sodium chloride in water, adjust the pH to about 3.6 with dilute sulfuric acid by using a pH meter, and dilute this solution to 1 liter with water. This buffer is 1 *M* in acetate, 0.01 *M* in EDTA, and 1.5 *M* in sodium chloride.

Spectrophotometer Varian Techtron Model 635, 10-mm cells. N-512 pH meter (Polish).

Procedure

Dilute a sample of test solution, containing 5 to 20 μg of molybdenum,

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to about 20 ml in a beaker. Adjust the pH to 3.6 with dilute hydrochloric acid, add 20 mg ascorbic acid and 5 ml of buffer solution. Transfer this solution to a 50-ml standard flask. Allow the solution to stand for 5 min, then add 1 ml of Pyrogallol Red solution and 5 ml of CTA, fill with water to the mark and mix. After 10 min, measure the absorbance of the solution at 600 nm against a blank as a reference.

RESULTS AND DISCUSSION

Conditions of Molybdenum Determination

Molybdenum ions react with Pyrogallol Red in the acid range and forms a complex with a Mo:PR molar ratio of 1:2 (*8*). In the presence of cetyltrimethylammonium ions a bathochromic shift of the absorption maximum from 540 to 600 nm takes place. The absorption spectra of the Mo–PR–CTA complex (λ_{max} = 600 nm), the Mo–PR complex (λ_{max} = 540 nm), and the PR (λ_{max} = 480 nm) are shown in Fig. 1.

The effect of pH on the color development of the molybdenum complex was examined by measuring the absorbance of a colored solution at 600 nm. The results are shown in Fig. 2, in which it is shown that maximum intensity can be obtained in the pH range 3.5 to 4.4.

The dependence of the absorbance of the complex on the Pyrogallol Red concentration was examined at a constant concentration of CTA equal to 4×10^{-4} *M*. The concentration of the reagent (PR) varied in the range 4.8×10^{-6} to 3×10^{-5} *M*. The maximum absorbance was observed when the molar excess of Pyrogallol Red over molybdenum was somewhat higher than 2.

The effect of changes in the concentration of CTA on the absorbance of the molybdenum complex was determined by measuring the absorbance.

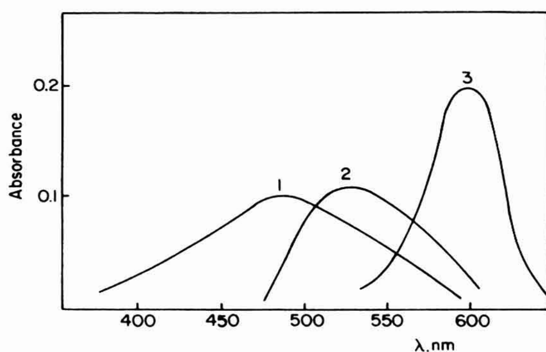

FIG. 1. Absorption spectra (pH 3.6) of: (1) Pyrogallol Red (PR—C = 9.6×10^{-6} *M* measured against water; (2) Mo–PR complex—C_{Mo} = 2×10^{-5} *M*, C_{PR} = 4×10^{-5} *M* measured against a blank; (3) Mo–PR–CTA complex—C_{Mo} = 2×10^{-6} *M*, C_{PR} = 1.4×10^{-5} *M*, C_{CTA} = 4×10^{-4} *M* measured against a blank.

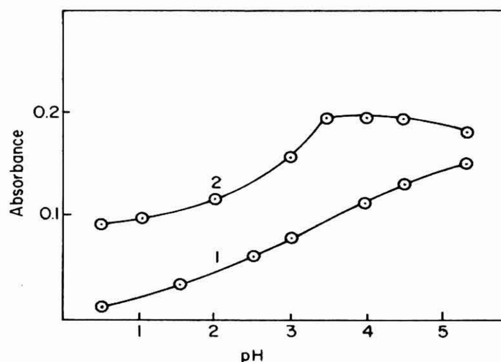

FIG. 2. Effect of pH at 600 nm (C_{Mo} = 0.2 μg/ml, C_{PR} = 9.6 × 10^{-6} *M*, C_{CTA} 4 × 10^{-4} *M*) of: (1) PR + CTA measured against water; (2) Mo–PR–CTA measured against blank.

The molar ratio of molybdenum to PR was constant and equal to 1:2.5. The maximum absorbance can be obtained over the range 2–4 × 10^{-4} *M* CTA.

Calibration curve, Sensitivity, and Precision

Calibration curves for the molybdenum determination were prepared by the procedure. Beer's law was obeyed over the range 0.1–0.4 μg/ml of molybdenum. The molar absorptivity was 9.00 × 10^4 at 600 nm.

The present method is very sensitive. Its sensitivity is much greater than that of the methods using Pyrogallol Red with dimethyldioctodecylammonium (molar absorpitivity = 4.20 × 10^4) (*8*), bromoPyrogallol Red (*4*), Catechol Violet with CTA (*1*), Gallein with CTA (*6*), and 2,2′-dihydroxybenzophenone thiosemicarbazone (*7*).

The precision of the method was evaluated for a concentration of 10 μg of molybdenum: the standard deviation is 0.011 and the relative error, ±2.5%.

Effect of Foreign Ions

The influence of foreign ions on the determination of molybdenum was ascertained by the standard procedure. The most commonly encountered ions were added individually to a solution containing 10 μg of molybdenum. The following metal ions did not interfere up to a 1000-fold weight excess: bismuth, iron aluminium, arsenic(III), cobalt, zinc, magnesium, cadium, manganese, nickel, lead, barium, thorium, beryllium, thallium; 500-fold: chromium, rhenium; 300-fold: tellurium; 100-fold: copper, zirconium, palladium, platinum, selenium(IV); 50-fold: uranium(VI), tin(IV); 10-fold: mercury, silver, titanium, vanadium, antimony(III). Chloride, fluoride, bromide, nitrate, and sulfate do not interfere, while tungsten, tartrate, iodide, citrate, and oxalate do interfere.

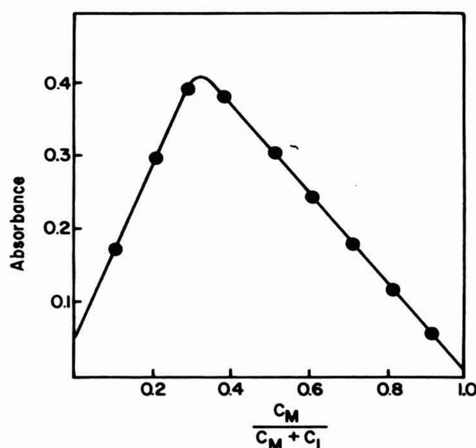

FIG. 3. Composition of the Mo–PR complex by Job's method (pH 3.6, λ = 600 nm). $C_{CTA} = 4 \times 10^{-4}$ *M*, $C_{Mo} + C_{PR} = 3 \times 10^{-5}$ *M*.

Before determination of the molybdenum, separation of molybdenum in the presence of tungsten and vanadium was carried out. Separation of molybdenum by extraction with benzoin α-oxime can be used (*3*).

Separation of Molybdenum

To 5 ml of 5% sulfuric acid, containing 5–20 μg of molybdenum, add 2 ml of KH_2PO_4 solution (1 ml = 1 mg KH_2PO_4) and 30 mg of ferrous

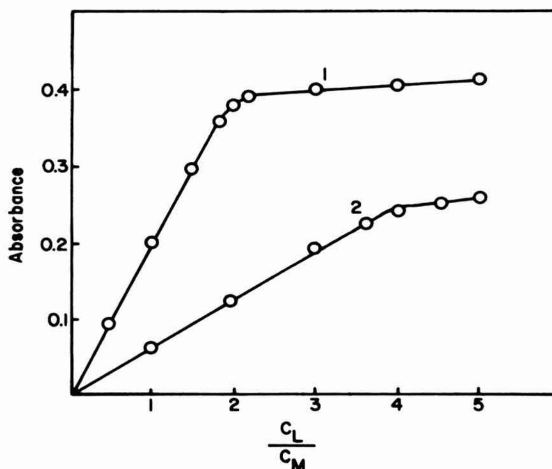

FIG. 4. Determination of the molar ratio of PR to molybdenum (1) and of CTA to molybdenum (2) at pH 3.6, λ = 600 nm by the spectrophotometric titration method. (1) $C_{Mo} = 0.4$ μg/ml, $C_{CTA} = 4 \times 10^{-4}$ *M*, $C_{PR} = 4.8 \times 10^{-4}$ *M*. (2) $C_{Mo} = 0.4$ μg/ml, $C_{PR} = 1.4 \times 10^{-5}$ *M*, $C_{CTA} = 4 \times 10^{-5}$ *M*.

ammonium sulfate hexahydrate. After 5 min, add 5 ml of 0.2% benzoin α-oxime in chloroform and shake the mixture for 2 min. Wash the chloroform phase with 1.5 *M* hydrochloric acid. Transfer the extract into a beaker, destroy the sample by heating with 0.5 ml of concentrated sulfuric acid and nitric acid. Dilute with 5 ml of water. The determination of molybdenum should be carried out according to the recommended procedure.

Complex Formation

Job's method of continuous variations and the spectrophotometric titration (*13*) have been used to establish the molar ratio of the molybdenum complex in the presence of CTA. The results are shown in Figs. 3 and 4. The 1:2 metal-ligand molar ratio, under the experimental conditions given, was ascertained by both methods, whereas the molar ratio of molybdenum to CTA in the ternary complex was determined by spectrophotometric titration (Fig. 4). The composition of the complex in the presence of CTA may therefore be formulated as Mo:PR:CTA = 1:2:4.

SUMMARY

Pyrogallol red in the presence of cetyltrimethylammonium bromide is proposed for the spectrophotometric determination of microgram amounts of molybdenum. The sensitivity of the color reaction between molybdenum and Pyrogallol Red has been greatly increased by the sensitizing action of cetyltrimethylammonium bromide ($\epsilon_{600\ nm}$ = 90,000). Beer's law is obeyed over the range 0.1−0.4 μg/ml of molybdenum. The composition of the complex may therefore be formulated as Mo:PR:CTA = 1:2:4.

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MICROCHEMICAL JOURNAL 25, 153–168 (1980)

The Determination of Lecithin and Total Choline-Containing Phospholipids in Amniotic Fluid Employing Enzymes as Reagents

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Received August 30, 1979

INTRODUCTION

Perhaps the most common contemporary approach to the diagnosis of respiratory distress syndrome (RDS) is the thin-layer chromatographic separation of phospholipids extracted from amniotic fluid followed by the determination of the lecithin-sphingomyelin ratio. The concentrations of the latter have often been determined by calculating the areas of the separated phospholipids after visualization by charring, molybdate reaction, or dye coloring. Alternatively, some have measured the volumes of the spots by colorimetry at appropriate wavelengths after the visualizing step, using densitometers. There are reported difficulties with the latter instruments which include the use of opaque matrices; the variability in reactivity of different phospholipids and the variation in spot sizes pose difficult problems for a densitometer. In an effort to develop a simpler, somewhat less empirical way in which to determine the concentration of the phospholipids, it was decided that it might be possible to use two phospholipases to generate choline from lecithin or choline from lecithin, lysolecithin, and sphingomyelin, three of the major pulmonary surfactants (7). By such means these compounds could be easily assayed with sequential reactions catalyzed by choline oxidase to generate hydrogen peroxide and horseradish peroxidase to catalyze an oxidative coupling of 4-aminoantipyrene and sodium 2-hydroxy-3,5-dichlorobenzenesulfonate (DCBS).[1] Consequently, two assays have been developed, one which measures lecithin and a second which measures total choline-containing phospholipids (TCCP).

[1] Nonstandard abbreviations used: TCCP, total choline-containing phospholipids; PL-D, phospholipase D; PL-D(c), phospholipase D (cabbage); PL-D(m), phospholipase D (microbial); COD, choline oxidase; POD, peroxidase; 4-AAP, 4-aminoantipyrene; DCBS, sodium 2-hydroxy-3,5-dichlorbenzenesulfonate; DMG, β,β-dimethylglutarate.

0026-265X/80/020153-16$01.00/0
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MATERIALS AND METHODS

Samples

Amniotic fluid samples were obtained by transabdominal amniocentesis and, if necessary, were stored at 4°C for no more than 8 hr before the lecithin and/or TCCP concentrations were determined. Unless otherwise stated, the samples were vortexed to obtain a homogeneous suspension prior to removal of aliquots for the assays.

Reagents

L-α-Dipalmitoyl phosphatidylcholine (synthetic) was purchased from Calbiochem, LaJolla, California 92037.

Phospholipase D (cabbage), [phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4] and sphingomyelin (bovine brain) were purchased from Sigma Chemical Company, St. Louis, Missouri 63178.

Choline oxidase (from *Arthrobacter globiformis*), [no EC number assigned] and phospholipase D (from *Streptomyces chromofuscus*), [phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4] were purchased from Toyo Jozo Co., Ltd., Matsuda-Yaesu-Dori Bldg, 1-10-7 Hacchubori, Chuo-Ku, Tokyo, Japan.

Peroxidase (from horseradish), [hydrogen-peroxide oxidoreductase, EC 1.11.1.7] was purchased from bmc Diagnostics/Biochemicals Ltd., 1475 Begin, St. Laurent, Quebec H4R 1V8, Canada.

The enzyme units quoted in the text are used as defined by the various suppliers.

Apparatus

Spectrophotometers, Model 35 and Acta MVI, Beckman Instruments, Inc., Analytical Instruments, 901 Oxford St., Toronto, Ontario M8Z 5T2, Canada.

Methods

Working acetate buffer. Prepare the solution to contain 2 g/liter Triton X-100, 8 mmol/liter 4-aminoantipyrene (4-AAP), 2 mmol/liter sodium dodecyl sulfate, and 91.7 mmol/liter calcium chloride dihydrate in 100 mmol/liter, pH 5.5 acetate buffer. This solution is stable for at least 2 months when stored in a brown bottle at 4°C.

Phospholipase D (cabbage) solution. Prepare this solution to contain 1.1×10^4 U/liter phospholipase D (PL-D(c)) in β,β-dimethylglutarate buffer (DMG) 10 mmol/liter, pH 7.0, containing 1 g/liter bovine serum albumin. This reagent is stable for 2–5 days when stored in a brown bottle at 4°C and at least 8 hr at room temperature.

Stock Tris–HCl buffer for the lecithin assay. Prepare this solution to contain 2 g/liter Triton X-100 and 18 mmol/liter DCBS in 400 mmol/liter,

pH 8.0 Tris–HCl buffer. This reagent is stable for at least 2 months when stored in a brown bottle at 4°C.

Choline oxidase reagent for the lecithin assay. Prepare this solution to contain 2.8×10^3 U/liter choline oxidase (COD) and 6×10^3 U/liter peroxidase (POD) in stock Tris–HCl buffer. This reagent is stable for at least 21 days when stored in a brown bottle at 4°C.

Stock Tris–HCl buffer for the TCCP assay. Prepare this solution to contain 2 g/liter Triton X-100, 2.9 mmol/liter 4-AAP, 1 g/liter bovine serum albumin, 1×10^3 U/liter COD, and 1×10^4 U/liter POD in 50 mmol/liter, pH 7.8 Tris–HCl buffer. Use this solution for reagent and sample blanks.

Working Tris–HCl buffer for the TCCP assay. Prepare this solution to contain 8×10^2 U/liter microbial phospholipase D (PL-D(m)) in stock Tris–HCl buffer. This reagent is stable for at least 5 days when stored at 4°C in a brown bottle. Over a 7-day period at 4°C the rate of color production may decrease by about 25%.

DCBS reagent. Prepare this solution to contain 154 mmol/liter DCBS in 50 mmol/liter, pH 7.8 Tris–HCl buffer containing 2 g/liter Triton X-100.

Stock Triton X-100 solution. Prepare this solution to contain 5 g/liter Triton X-100 in distilled deionized water.

Lecithin standard. Prepare this solution to contain 200 μmol/liter L-α-lecithin in stock Triton X-100 solution. Twelve to eighteen hours of constant stirring may be necessary to effect complete solution of this material.

Sphingomyelin standard. Prepare this solution to contain 100 mg/liter sphingomyelin in stock Triton X-100 solution.

Sodium 2-hydroxy-3,5-dichlorobenzenesulfonate was prepared by a modification of a previous synthesis (*2*). Five grams of 2,4-dichlorophenol are melted in a 25-ml round-bottom flask equipped with a reflux condenser and thermometer. Four milliliters of 30% fuming sulfuric acid are added slowly so that the reaction mixture temperature does not exceed 150°C. Upon addition of the acid, the mixture is allowed to cool to 100°C where it is maintained for 36 hr. The mixture is then added with mixing to 25 g of NaCl and allowed to cool to room temperature. Upon filtering under reduced pressure the crude product is mixed with 200 ml of water, adjusted to about pH 10 with NaOH(s), and brought to a boil. Undissolved material is removed by filtering hot through Hyflo Super Cel under reduced pressure. The volume of the filtrate is reduced by boiling until crystallization begins; the solution is then allowed to cool to 4°C, filtered, and recrystallized once from water. The yield, based on 2,4-dichlorophenol, is 83%. Nuclear magnetic resonance and microanalysis data was previously reported (*2*).

Procedure

The assay protocol for the lecithin assay is as follows: mix 300 μl of working acetate buffer with 100 μl of sample or standard. The hydrolysis reaction is initiated with the addition of 100 μl of PL-D(c) solution. The hydrolysis reaction mixture is allowed to sit at room temperature for 15 min at which time 500 μl of COD reagent is added and mixed. This mixture is incubated for 12–15 min at 37°C. The absorbance is measured in 1-cm cuvettes at 510 nm.

Blanking is performed as follows: the instrument is blanked with a reagent blank containing all of the reagents except that DMG buffer is substituted for the PL-D(c) solution. A PL-D blank is prepared by substituting stock Triton X-100 solution as a sample. A sample blank is prepared in a manner similar to that of the reagent blank (DMG buffer without PL-D(c)). The absorbance of the PL-D blank is subtracted from all tests and standards while the absorbance of the sample blanks is subtracted from the readings of their respective samples.

The assay protocol for the TCCP assay is as follows: 1 ml of working Tris–HCl buffer is added to and mixed with 100 μl of DCBS reagent.[2] The reaction sequence is initiated upon addition of 100 μl of sample or standard. This mixture is incubated for 12–15 min at 37°C. The absorbance is measured in 1-cm cuvettes at 510 nm.

Blanking is performed as follows: the instrument is blanked with a reagent blank which utilizes the stock Tris–HCl buffer in place of the working Tris–HCl buffer and stock Triton X-100 solution is used in place of the sample. A PL-D(m) blank is composed of the assay reagents except that stock Triton X-100 solution is employed as a sample. Sample blanks are prepared by substituting the stock Tris–HCl reagent for the working Tris–HCl reagent. The absorbance of the PL-D blank is subtracted from all tests and standards while the absorbance of the sample blanks are subtracted from their respective samples.

RESULTS AND DISCUSSION

The lecithin assay which is described here has been optimized previously (*2*). However, in order to extend the working range of the assay the sample size was decreased and the volume of two of the buffers was increased, while the relative proportions of the reagent constituents were maintained. A typical standard curve for this modified assay has, by linear regression analysis of 10 standards in duplicate ranging from 20 to 200

[2] It is not necessary to separate the DCBS from either the stock or working Tris–HCl buffers; however, the shelf life of these reagents is extended considerably by doing so. Alternatively, aliquots of the stock and working Tris–HCl buffer may be premixed with the DCBS reagent prior to performing the assay and thus obviating one pipetting for each assay.

TABLE 1
RATE OF LECITHIN[a] HYDROLYSIS BY PL-D(m)[b]

U/assay PL-D(m)	Δ Absorbance (per min)	Time required for full color development (min)
2	1.02	2
1	0.75	3
0.8	0.44	7
0.7	0.36	8
0.6	0.18	13
0.5	0.07	15

[a] A 200 μmol/liter lecithin standard was used as sample.
[b] In the presence of 4 U/assay COD and 8 U/assay POD.

μmol/l lecithin, the following characteristics: $m = 0.004$, $b = -0.013$, and $r = 0.9997$.

By doubling the volume of the DMG buffer the PL-D(c) preparation is totally soluble, whereas a slight turbidity was reported earlier (*2*). The partially insoluble material previously noted readily dissolved in this increased buffer volume.

Because the PL-D(m) and COD are the most costly enzymes employed in the assays, it is economically advantageous to use as little of these enzymes as possible while maintaining a reasonable incubation period. Under the described conditions for the TCCP assay, it was found that 0.8 U per assay of PL-D(m) was sufficient for complete hydrolysis of a 200 μ*M* lecithin standard within 8 min (Table 1). However, variations in the quality of different shipments of the enzyme have been encountered and consequently this is used only as a guide. The rate of color development is routinely checked with each new shipment, which obviates the necessity of actually determining the specific activity of the enzyme for each new batch.

Because the PL-D(m) is the first enzyme in the TCCP assay sequence, the phospholipid hydrolysis has been made rate limiting. One unit per assay COD was found to be sufficient to oxidize the generated choline at a rate which is slightly faster than it is produced (Table 2). A large excess of the enzyme is not used as it is relatively expensive and has not proven to be necessary. Horseradish peroxidase, however, is comparatively inexpensive and readily available with a relatively high specific activity; consequently it is used in at least a 10-fold excess (10 U per assay).

Calcium ion has been reported (*8, 11*) to be an activator of PL-D(m) activity. Figure 1 demonstrates the effects of calcium on the rate of color production in the TCCP assay. There is no apparent increase in the rate of

TABLE 2
RATE OF OXIDATION OF GENERATED CHOLINE[a] BY COD[b]

U/assay COD	Δ Absorbance (per min)	Time required for full color development (min)
1.5	0.44	7
1.3	0.44	7
1.0	0.42	7
0.8	0.33	9
0.7	0.30	11

[a] The choline was generated from a 200 μmol/liter lecithin standard by 0.8 U/assay PL-D(m).
[b] In the presence of 8 U/assay POD.

color production (lecithin hydrolysis) with the inclusion of calcium in the assay mixture. In fact, there would appear to be a slight inhibitory effect. For this reason calcium is not included in the reaction mixture. This apparent discrepancy may be due to the use of organic solvents by the previous workers (*8, 11*) to emulsify the phospholipids.

The effects of incubation temperature on the rate (Fig. 2A) and degree (Fig. 2B) of color development is presented for both the TCCP and the color development step of the lecithin procedures. It would appear from Fig. 2A that the rate of color development for the lecithin procedure increases with temperature over the range studied. While the maximal rate for the TCCP procedure occurs at 37°C, from this one could infer that the higher temperatures exert an inhibitory effect on the PL-D(m). Figure 2B demonstrates the effects of temperature on the maximal absorbance at 510 nm. It is apparent that the extent of color production is near constant with temperature for the lecithin assay. The TCCP assay, however, seems to have its greatest color development at 30°C and falls off sharply after 37°C. On the basis of rate and convenience 37°C was chosen as the incubation temperature for color development in both assays.

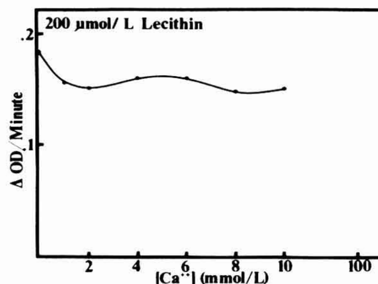

FIG. 1. The effects of calcium ion, in mmol/liter of reaction mixture, on the TCCP assay.

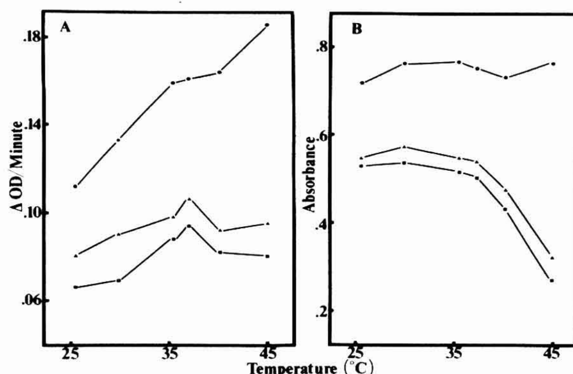

FIG. 2. The effects of incubation temperature on the rate (A) and maximal color development (B) for 0.1 g/liter lecithin with the lecithin assay (●), 0.1 g/liter lecithin (▲), and 0.1 g/liter sphingomyelin (■) with the TCCP assay.

Typical standard curves for lecithin and sphingomyelin with the TCCP assay by linear regression of seven standards ranging from 0 to 100 mg/liter show an $m = 0.0049$, $b = -0.0083$, and $r = 0.9996$ for lecithin and an $m = 0.0048$, $b = -0.008$, and $r = 0.9996$ for sphingomyelin. Since it has not been possible to find a source of pure sphingomyelin to use as a standard, the assay is standardized with lecithin and if it is required the average molecular weight for the sphingomyelin may be calculated. From the above data, the molecular weight of this particular source of sphingomyelin was determined to be 752.4.

The TCCP procedure has been developed for equilibrium analysis. However, as with the lecithin assay (*2*), it was found that kinetic determinations are possible. In order to make measurements more convenient, however, it is necessary to decrease the quantities of the enzymes used by about twofold.

Table 3 demonstrates the within-run reproducibility of the TCCP procedure with a 100 mg/liter sphingomyelin standard. Statistical analysis on this data shows the coefficient of variation to be a respectable 0.58%. As

TABLE 3
WITHIN-RUN REPRODUCIBILITY OF THE TCCP ASSAY[a]

Absorbance at 510 nm of a 100 mg/liter sphingomyelin standard			
0.420	0.420	0.422	0.421
0.419	0.421	0.420	0.428
0.416	0.422	0.421	0.423
0.422	0.420	0.425	0.421
0.420	0.420	0.419	0.423

[a] $\bar{x} = 0.421$; SD $= 2.46 \times 10^{-3}$; CV $= 0.58\%$.

with the lecithin assay (*2*), one would expect the reproducibility of an amniotic fluid sample to be worse than the pure standard. However, it is felt that this inevitable problem arises because of a lack of homogeneity in the sample and not with the assay itself.

As reported previously (*2*), PL-D(c) does not utilize sphingomyelin as a substrate under the described reaction conditions. Further studies have shown that L-α-lysophosphatidyl choline (palmitoyl lysolecithin) is not hydrolyzed under the described assay conditions by this PL-D. The microbial PL-D, however, shows a much broader specificity, and hydrolyzes lecithin, sphingomyelin, and lysolecithin with comparable rates. Neither of these phospholipases will utilize L-α-glycerophosphoryl choline as a substrate.

In Fig. 3 the linearity of the two assays is demonstrated. Serial dilutions of a high sample (measured lecithin concentration = 181.9 μmol/liter, TCCP concentration = 213.6 μmol/liter) with a low sample (lecithin concentration = 24.2 μmol/liter, TCCP concentration = 29.8 μmol/liter) were made and carried through both the lecithin and TCCP procedures. Serial dilutions of the high sample were also made with the stock Triton X-100 solution. The best fit lines were calculated by linear regression analysis; the coefficients of correlation for these four lines ranged from 0.9991 to 0.9994. The low sample was also mixed with equal volumes of a series of lecithin standards (Fig. 4). The resulting coefficient of correlation was 0.9997 for the lecithin assay and 0.9990 for the TCCP assay. Considering both of these figures, it is apparent that both assays are linear and that the aqueous standards prepared in the stock Triton X-100 solution react similarly to the amniotic fluid samples.

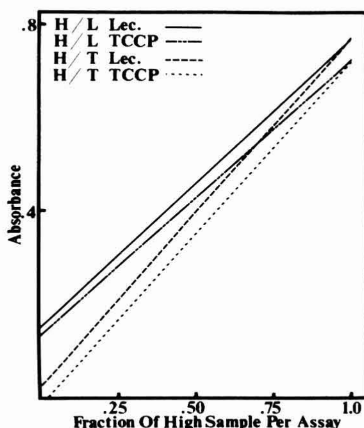

FIG. 3. The effects of serial dilution of a high (H) amniotic fluid with a low (L) fluid and the same high fluid with stock Triton X-100 solution. Lec. corresponds to the lecithin assay.

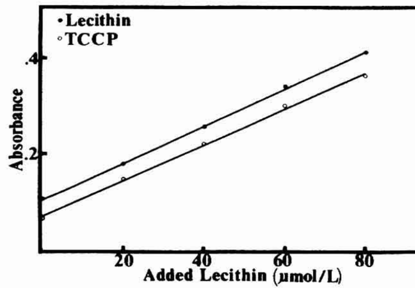

FIG. 4. The effects of the addition of a series of lecithin standards to a low amniotic fluid sample, for both the lecithin and TCCP assays.

Hemoglobin and bilirubin have been reported to be interferents in oxidase–peroxidase coupled reactions. Both of these materials have been examined extensively, particularly by Perlstein *et al.* (*12, 13*), in other coupled assays. For this reason it seemed unnecessary to carry out an extensive investigation of these interferences. However, as a matter of completeness a brief study was conducted.

Figure 5 presents a series of spectra of an amniotic fluid sample, carried through the lecithin assay, with and without added hemoglobin. The final sample concentration of hemoglobin was about 1 g/liter. This level can be considered to correspond to a severely contaminated sample. It is obvious that this amount of hemoglobin has added considerably to both the blank and test spectra, as compared to the unhemolyzed spectra, when scanned against water. However, when scanned against their respective blanks there is negligible, if any, difference in the resulting spectra at 510 nm,

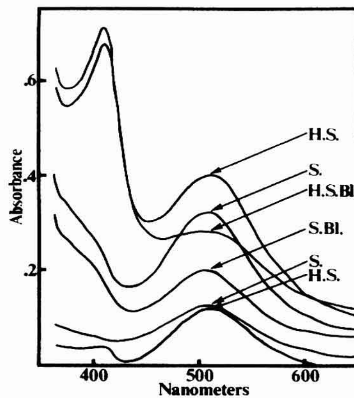

FIG. 5. The spectra of an amniotic fluid sample contaminated with hemoglobin (H.S.), the same sample without hemoglobin (S.) and their respective blanks (H.S.Bl. and S.Bl.). The upper four spectra were generated by scanning against water while the lower two are the difference spectra of the samples scanned against the corresponding blank.

was was predicted by Perlstein and co-workers (*12, 13*). It is also important to note that the manner in which the hemoglobin was obtained separated it only from cellular debris and no attempt was made to remove the plasma. Of particular interest is that the amount of plasma phospholipid which would be present as contamination in this severely hemolyzed sample is low enough not to interfere with the assay. Badham and Worth (*3*) have reported, however, that contamination by blood alters inorganic phosphate as well as *L/S* ratio determinations in amniotic fluid.

In considering the effects of hemolysis on the assay it was felt that the effects of blood cell membranes as a possible source of substrate for either of the phospholipases should also be studied. Figures 6A, B, and C correspond to the spectra of the TCCP and lecithin assays of the same sample which was used to generate Fig. 5. However, in this instance the contaminating species were the cell membranes which correspond to that amount of blood necessary to obtain 1 g/liter hemoglobin. There is an apparent increase in absorbance throughout the spectra of both the contaminated sample and sample blank, presumably due to turbidity caused by the suspended cellular debris. However, when the sample and contaminated sample were scanned against their respective blanks the generated spectra are similar for both assays. Under the conditions of these

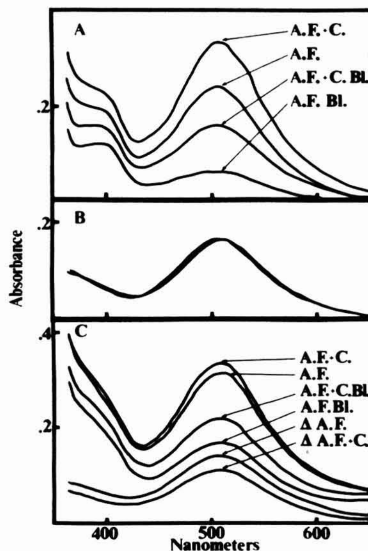

FIG. 6. The spectra of an amniotic fluid contaminated with blood cell debris (A.F.+C.), the same sample uncontaminated (A.F.), and their respective blanks (A.F.+C.Bl. and A.F.Bl.) as scanned against water for the TCCP (A) and lecithin (C) assays. The difference spectra for the TCCP assay are shown in B and for the lecithin assay are labelled ΔA.F. and ΔA.F.+C. for the uncontaminated and contaminated samples.

assays it is apparent that neither of the phospholipases employed can utilize membrane-bound phospholipids as substrate.

The manner in which bilirubin interferes in oxidase–peroxidase coupled reactions is somewhat complex. Apart from contributing its characteristic spectrum to the final color, it also behaves as a hydrogen donor for the peroxidase coupling reaction, thus reducing the expected concentration of the intended chromogen (*12, 13*), a loss which appears to be compensated for by residual bilirubin and perhaps the products of the bilirubin reaction. Fortunately, bilirubin concentrations in normal amniotic fluid are relatively low, 0.1–0.3 mg/liter (0.17–0.51 μmol/liter) (*6*). Anaokar *et al.* (*1*) reported a 45.09% increase in an apparent lecithin concentration of 51 mg/liter (69.5 μmol/liter) with their enzymic assay, at a bilirubin level of 4.0 mg/liter (6.8 μmol/liter). Figure 7 is indicative of the effects of bilirubin with the described lecithin procedure. If the absorbance of the 80 μmol/liter lecithin standard is compared to that of the same standard which has been contaminated with bilirubin, to a sample concentration which corresponds to 100 mg/liter (170 μmol/liter), an extremely high level which is not likely to be encountered, there is an apparent increase of about 19% in the absorbance at 510 nm. However, when the pure and contaminated standards are scanned against their respective blanks there is an apparent decrease in absorbance of about 9%

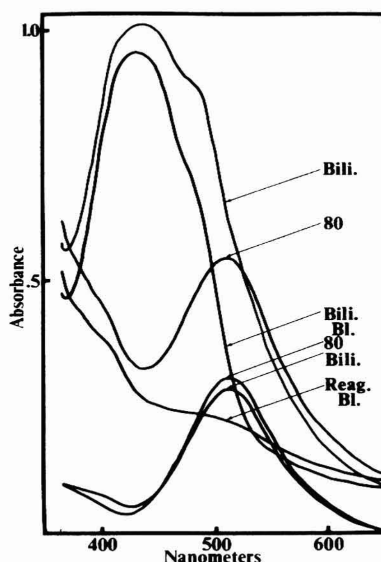

FIG. 7. The spectra of an 80 μmol/liter lecithin standard (80) and the same standard contaminated with bilirubin to a sample concentration of 100 mg/liter (Bili.), and their respective blanks (Reag. Bl. and Bili. Bl.). The lower two curves labeled 80 and Bili are the difference spectra, whereas the other curves were generated by scanning against water.

due to this very high bilirubin concentration. This apparent decrease corresponds to about 6–7 μmol/liter lecithin, which is not likely to have any clinical significance. The discrepancies between our results and those of Anaokar *et al.* (*1*) may be due to differences in the wavelength of measurement, their necessity to extract the aqueous phase, differences in sample to reagent ratios, and their lack of a sample blank.

Currently, a clinical investigation as to the applicability of these assays in the prediction of RDS in the neonate is being conducted. At present the data is limited; however, some of it is presented in order to indicate the concentration ranges of lecithin and TCCP which have been encountered.

Figure 8 is a plot of lecithin and TCCP concentrations versus *L/S* ratios as determined by the commercially available Gelman ITLC SG system. With respect to the lecithin data, there appears to be a high and low group of points separated by about 10 μmol/liter between 35 and 45 μmol/liter. The data available pertaining to TCCP concentrations is quite limited; however, it is also presented in Fig. 8. As with the lecithin concentrations, it is expected that a gap in the data in the region of 40–70 μmol/liter will develop and that upon the accumulation of more data a cutoff value, which will distinguish the mature from the immature fetal lung, may be assigned. There are two lines on this figure which connect replicate lecithin analysis, performed one week apart, on two different patients. A third line connects the TCCP values which correspond to the lower set of lecithin values. There is a considerable increase in the upper pair of points over the one-week period. Upon delivery there was no indication of RDS in this neonate. However, in the case of the lower two sets of data,

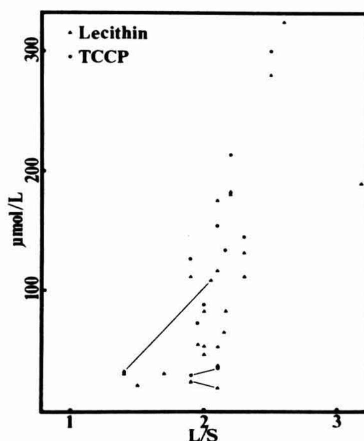

FIG. 8. A plot of lecithin (▲) and TCCP (●) concentrations versus the *L/S* ratio of amniotic fluid samples. (Two samples (△) were severely hemolyzed thus making the *L/S* ratios questionable.)

delivery was induced on the basis of the *L/S* ratio of 2.05. This neonate did develop a mild RDS, as might be predicted by the lecithin and TCCP results.

Based on 10 samples there appears to be a direct correlation between the lecithin and TCCP concentrations. Regression analysis yields a coefficient of correlation of 0.9847 with a slope of 1.022 and an intercept of 24.7 μmol/liter on the TCCP axis.

It is anticipated that it will eventually be possible, as more samples are assayed, to determine a cutoff value which will be indicative of RDS. Lecithin and total choline-containing phospholipid concentrations determined by other procedures (*1, 4, 5, 7, 9, 10, 14, 15*) appear to fall into the same ranges as ours. Discrepancies which arise are probably due to differences in procedures and especially sample treatment. In particular are the effects of centrifugation. The data presented above pertain to samples which were not centrifuged, but rather thoroughly mixed to obtain, as nearly as possible, a homogeneous suspension.

The current literature (*3, 5, 10, 14, 15*) pertaining to the effects of centrifugation on the lecithin and sphingomyelin concentrations in amniotic fluid is somewhat ambiguous. Railsback and Haller (*14*) report that, although the absolute concentrations of lecithin and sphingomyelin are decreased by centrifugation, the *L/S* ratio remains unaltered. Conversely, two groups (*5, 10*) have reported that the apparent lecithin concentration is decreased more by centrifugation than is the sphingomyelin. Lindback and Frantz (*10*) and Thom *et al.* (*15*) report that the phospholipid values obtained from uncentrifuged samples are clinically more significant. The latter two groups, as well as Badham and Worth (*3*), report that centrifugation of the sample is not necessary. The experience of this laboratory has been that even brief centrifugation at low *g* forces decreases the apparent concentration, not only of lecithin, but also of the total choline-containing phospholipids. Perhaps of greater significance is the finding that samples centrifuged under identical conditions do not show identical decreases in either the lecithin or TCCP concentrations (Table 4). Initial studies have shown that by resuspending the pellet in the stock Triton X-100 solution it is possible to recover some of the lost phospholipids. The investigation into the effects of centrifugation on the analysis of choline-containing phospholipids in amniotic fluid is not yet complete; however, in considering the apparent decreases in concentration as well as the lack of interference by materials such as hemoglobin and blood cells, pretreatment of the sample by centrifugation appears to be of questionable merit.

For the most part phospholipid concentrations in amniotic fluid have, in the past, been expressed in terms of weight per unit volume. This leads to results which are difficult to interpret because of different molecular

TABLE 4

DECREASES IN LECITHIN AND TCCP CONCENTRATIONS UPON CENTRIFUGATION

Patient	Lecithin uncentrifuged (μmol/liter)	Lecithin centrifuged[a] (μmol/liter)	Centrifuged / uncentrifuged (%)	TCCP uncentrifuged (μmol/liter)	TCCP centrifuged[a] (μmol/liter)	Centrifuged / uncentrifuged (%)
1	279.7	182.5	65.2	300.0	240.6	80.2
2	181.9	118.6	65.2	213.6	140.6	65.8
3	24.2	22.2	91.7	29.8	19.8	66.4
4	82.6	61.6	74.6			
5	188.5	151.5	80.4			
6	110.8	99.0	89.4	144.6	125.2	86.6
7	124.2	95.4	76.8			
8	191.9	145.5	75.8	200.2	162.5	81.2

[a] Centrifuged at 100*g* for 10 min at 4°C.

weights of the various phospholipids as well as different molecular weights of individual classes due to different acyl chain substitutions. However, in all of the materials which are determined by these assays the choline content is constant, and therefore makes standardization on a μmol/liter basis more quantitative.

A cold acetone precipitation step has not been incorporated into either of these assays for basically the same reasons expressed by Anaokar *et al.* (*1*). Following the procedures as described here one of the assays may be performed easily within 45 min or both within 60 min. It is felt that the inclusion of an acetone precipitation step would probably prolong the time required to complete the assays to at least 12 hr and possibly introduce serious error.

Although the basic enzyme sequence for the lecithin assay is the same as that of Anaokar *et al.* (*1*), the proposed assay has several advantages. The assay requires approximately 1/25th as much PL-D(c) per assay, probably due to different buffer systems, the order of addition of the reagents, and the use of SDS, to obtain complete hydrolysis in one-half the incubation time at a lower temperature. The necessity for plastic tubes has been obviated by the inclusion of BSA in the PL-D solution. The substitution of DCBS for phenol has increased the sensitivity so that not only is the sample volume employed quite small, but also there is no need to extract the chromogen. Of considerable importance is the fact that hemoglobin, cellular debris, and bilirubin pose no apparent threat for interference.

Thus far there is no apparent clinical difference between the lecithin and TCCP assays. Consequently the only advantages which the TCCP assay has over the lecithin determination is that it is somewhat simpler and faster to perform.

SUMMARY

Two assays which employ enzymes as reagents have been described for the determination of lecithin and TCCP in amniotic fluid. Both of these assays are relatively quick, simple, inexpensive, precise, involve no extractions, and are amenable to automation.

Hemoglobin, blood cell membranes, and bilirubin, common interferences in *L/S* ratio determinations, have been studied. Their effects on these two assays appear to be negligible.

It can be inferred from preliminary studies that the described assays correlate well with each other, and with the clinical status of the fetus. The values obtained for lecithin and TCCP cmncentrations are in the same range as previous reports employing different procedures.

ACKNOWLEDGMENTS

Supported in part by a research grant to R.J.T. from the National Sciences and Engineering Research Council of Canada. We gratefully acknowledge the technical assistance of Mr. Edwin Olivero for the performance of the TLC *L/S* ratios.

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MICROCHEMICAL JOURNAL 25, 169–175 (1980)

Optimum Acidity and Masking of Redox Reactions

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Received October 1, 1979

INTRODUCTION

In comparison with precipitation, complexation, and solvent extraction, the redox reactions are far more complicated from thermodynamic and kinetic point of views. This is well illustrated by the example of the classical reaction between permanganate and oxalic acid which is still uncertain in its intermediate steps (*6*). Therefore we will limit our attention just to the final equilibria in aqueous solution.

The effect of the redox potential value on the quantitative course of reactions is well known and has already been discussed (*1*). Also the optimum acidity conditions of redox titrations have been briefly discussed (*3*). In this paper we will extend the discussion to include even the excess of oxidant or reductant and the optimum of masking.

THEORETICAL

Optimum acidity. If we have the redox reaction

$$aA + bB + hH = eE + fF + gH_2O, \tag{1}$$

where H is always hydrogen ion and A an oxidant and B a reductant, then the equilibrium constant β of that reaction is given by the well-known equation

$$\log \beta = nF(\epsilon_A^0 - \epsilon_B^0)/(2.303RT), \tag{2}$$

where n is the number of electrons transferred in reaction (1), F is the Faraday constant (96,487 C), ϵ_A^0, $\epsilon_B{}^0$ are the standard potentials of the half-reactions involving A and B, R is the gas constant (8.3143 J°K^{-1} mol^{-1}), and T is the absolute temperature (in °K).

Expressing the same constant in concentrations we get

$$\begin{aligned}\beta &= [E]^e[F]^f[H_2O]^g[A]^{-a}[B]^{-b}[H]^{-h} \\ &= (f/e)^f c_E{}^{e+f}(c_{AO} - c_E a/e)^{-a}(c_{BO} - c_E b/e)^{-b}[H_2O]^g\ \phi_{AB},\end{aligned} \tag{3}$$

where the side-reaction function

$$\phi_{AB} = [H]^h \alpha_{A(Y)}^{-a}\alpha_{B(Y)}^{-b}\alpha_{E(Y)}^{e}\alpha_{F(Y)}^{f}. \tag{4}$$

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Y is the ligand of a complexing medium (from solvent, buffer, or masking reagent), c_Y, c_E, c_F ($c_E/e = c_F/f$) are the total concentrations of the corresponding species, and c_{AO} and c_{BO} are the total concentrations *before* the start of the redox reaction. Furthermore we have

$$\alpha_{X(Y)} = \sum_0^V \sum_0^W \beta_{vw}[H]^v(c_Y/\alpha_{Y(H)})^w, \tag{5}$$

where X = A, B, E, F, $c_Y >> c_{AO}, c_{BO}$, and

$$\alpha_{Y(H)} = \sum_0^U \beta_u[H]^u. \tag{6}$$

The optimum acidity of reaction (1) is given by the condition

$$\phi_{AB} = \text{maximum}. \tag{7}$$

It can be calculated by iteration introducing the sequence of values $pH_s = pH_o + s\Delta pH$ (pH_o and ΔpH are the optional starting value and the value of the gradient, respectively) into Eqs. (4)–(6). The first value for which $\phi_{AB(s+1)} < \phi_{AB(s)}$ indicates the optimum acidity pH_s.

Assuming c_{AO} and c_Y as optional and

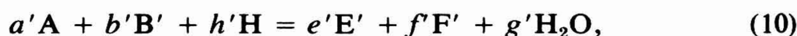
$$0.999c_{AO}/a = c_E/e, \tag{8}$$

we can calculate the value c_{BO} for the acidity optimum from Eq. (3):

$$c_{BO} = \left\{10^{3a}c_{AO}^{e+f-a}(e/a)^e(f/a)^f[H_2O]^g/(\beta\ \phi_{AB})\right\}^{1/b} + c_{AO}b/a. \tag{9}$$

If we need to calculate the starting concentration of the oxidant, we simply reverse the meaning of A and B and reverse the signs of ϵ_A^0, ϵ_B^0 in (2). .

Optimum masking. If we have besides reaction (1) the side reaction



$$a'A + b'B' + h'H = e'E' + f'F' + g'H_2O, \tag{10}$$

we have as in (2) for the equilibrium constant

$$\log \beta' = n'F(\epsilon_A^0 - \epsilon_{B'}^0)/(2.303RT), \tag{11}$$

where n' is the number of electrons transferred in (10) and $\epsilon_{B'}^0$ is the standard potential of the corresponding half-reaction involving B$'$. The meaning of the other symbols is the same as in (2). Employing the concentrations to express β' we have

$$\beta' = [E']^{e'}\ [F']^{f'}\ [H_2O]^{g'}\ [A]^{-a'}[B']^{-b'}\ [H]^{-h'}$$
$$= (f'/e')^{f'}\ c_{E'}^{e'+f'}\ (c_{AO} - c_{E'}a'/e')^{-a'}\ (c_{B'O} - c_{E'}\ b'/e')^{-b'}\ [H_2O]^{g'}\phi_{AB} \tag{12}$$

so that using the condition of quantitative masking

$$c_E/e = 1000c_{E'}/e', \tag{13}$$

we get by combination of (3) and (12) and elimination of [A] [see the similar equation in (*4*)]

$$c_{B'O} = \{(\beta^{a'}/\beta'^{a})\,(a/e)^{a'e}\,(a/f)^{a'f}\,(e'/a)^{ae'}\,(f'/a)^{af'}\,10^{-3a(e'+f')}$$

$$c_{AO}^{a(e'+f')-a'(e+f)}\,(c_{BO} - c_{AO}b/a)^{a'b}\,[H_2O]^{ag'-a'g}\,\psi_{BB'}\}^{1/(ab')} \tag{14}$$

where the masking function

$$\psi_{BB'} = \alpha_{B'(Y)}^{ab'}\,\alpha_{E(Y)}^{a'e}\,\alpha_{F(Y)}^{a'f}\,\alpha_{B(Y)}^{-a'b}\,\alpha_{E'(Y)}^{-ae'}\,\alpha_{F'(Y)}^{-af'}\,[H]^{a'h-ah'}$$

$$= \phi_{AB}^{a'}/\phi_{AB'}^{a}. \tag{15}$$

The functions $\alpha_{B'(Y)}$, $\alpha_{E'(Y)}$, $\alpha_{F'(Y)}$ have the same meaning as described in (*5*).

The optimum masking is given by the condition

$$\psi_{BB'} = \text{maximum}. \tag{16}$$

That value is calculated by a similar iteration procedure as described for the value of ϕ_{AB}. Again the values of c_{AO} and c_Y are optional; $\psi_{BB'}$ is calculated first together with the optimum pH from (15) and then the value of ϕ_{AB} from (4) and c_{BO} from (9). Finally $c_{B'O}$ (which represents the maximum concentration that can still be masked) is calculated from (14).

To calculate the starting concentration of an oxidant to be masked we need just to reverse the meaning of A and B′ and to reverse the signs of ϵ_A^0 and $\epsilon_{B'}^0$ in (11).

RESULTS AND DISCUSSION

Typical dependence of log ϕ_{AB} and log $\psi_{BB'}$ on pH is presented in Fig. 1. The maximum can also occur as the negative value of log ϕ_{AB} or log $\psi_{BB'}$ (the masking of iodide reaction of Cu^{2+} or Fe^{3+} with EDTA is an illustrative example; see Table 2).

Calculated conditions of optimum acidity of some reactions are given in Table 1. Conditions of optimum masking are shown in Table 2. In both cases a reasonable agreement with the experiment can be seen.

As Eqs. (4) and (15) indicate, the optimum acidity and masking do not depend on the values of standard redox potentials and the concentration c_{AO} (that means the substance to be determined). This behavior is similar to precipitation, complexation, and solvent extraction (*3, 4*).

In some cases, if the maximum of the curves log $y = f(\text{pH})$ ($y = \phi_{AB'}$, $\psi_{BB'}$) is too flat, it is useful to know the threshold of the maximum. That can be estimated on the basis of the relationship

$$\log y_{s+1} \leqslant \log y_s + 0.01. \tag{17}$$

TABLE 1
OPTIMUM ACIDITY OF SOME REDOX REACTIONS[a]

Reaction	c_Y	c_A	c_B	pH_{opt}	
				Calculated	Found
$UO_2^{2+} + 2Fe^{2+} + 4H^+ = U^{4+} + 2Fe^{3+} + 2H_2O$	0.1[b]	0.010	0.020	8.5	8.5–9.4 (*9*)
$2Fe^{3+} + Sn^{2+} = 2Fe^{2+} + Sn^{4+}$	1–5[c]	0.100	0.050	1–5[d]	2–6[d] (*14*)
$2MoO_4^{2-} + Sn^{2+} + 4H^+ = 2MoO^{3+} + Sn^{4+} + 2H_2O$	2–6[c]	0.100	0.050	2–6[d]	3–6[d] (*5*)
$VO^{2+} + Fe^{2+} + 2H^+ = V^{3+} + Fe^{3+} + H_2O$	0.1[b]	0.010	0.010	7.8	8.0–9.4 (*9*)
$MoO_4^{2-} + Fe^{2+} + 2H^+ = MoO_3^- + Fe^{3+} + H_4O$	11.6[e]	0.010	0.010	4.8–11.6[f]	5–12[f] (*12*)
$UO_2^{2+} + 2Fe^{2+} + 4H^+ = U^{4+} + 2Fe^{3+} + 2H_2O$	11.6[e]	0.010	0.020	6.8–11.6[f]	6–12[f] (*11*)
$Fe^{3+} + Co^{2+} = Fe^{2+} + Co^{3+}$	0.01[g]	0.001	0.001	3.5	3–5 (*15*)
$2Fe^{3+} + Hg_2^{2+} = 2Fe^{2+} + 2Hg^{2+}$	0.5[h]	0.010	0.005	0.3	0.1–0.5 (*2*)

[a] For the values of stability constants see Ref. (*13*); for standard redox potentials see Ref. (*8*).
[b] Tiron.
[c] Chloride.
[d] Molarity of hydrochloric acid.
[e] Phosphate.
[f] Molarity of phosphoric acid.
[g] *o*-Phenanthroline.
[h] Thiocyanate.

TABLE 2
OPTIMUM MASKING OF SOME REDOX REACTIONS[a]

Reactions	c_Y	c_{AO}	c_{BO}	$c_{B'O}$	pH_{opt} Calculated	pH_{opt} Found
$4I^- + 2Cu^{2+} = I_2 + 2CuI$[d]	0.1[b]	1.0	0.11	0.017	4.0	3.5–4.0 (*7*)
$2I^- + 2Fe^{3+} = I_2 + 2Fe^{2+}$	0.3[b]	1.0	0.10	0.010	4.0	
	0.5[b]	1.0	0.10	0.003	4.0	
$10I^- + 2MnO_4^- + 16H^+ = 5I_2 + 2Mn^{2+} + 8H_2O$	0.1[c]	0.01	0.0020	0.095	2.5	2.5–3.0 (*10*)
$4I^- + 2Cu^{2+} = I_2 + 2CuI$						
$10I^- + 2MnO_4^- + 16H^+ = 5I_2 + 2Mn^{2+} + 8H_2O$	0.01[c]	0.01	0.0020	0.097	2.3	2.5–3.0 (*10*)
$2I^- + 2Fe^{3+} = I_2 + 2Fe^{2+}$						

[a] See footnote *a* of Table 1.

[b] Fluoride.

[c] EDTA.

[d] It was interchanged $c_{AO} \longleftrightarrow c_{BO}$, $a \longleftrightarrow b$ and $\log \beta = n(\epsilon^0_{Cu} - \epsilon^0_I)F/(2.303RT)$ in Eq. (9).

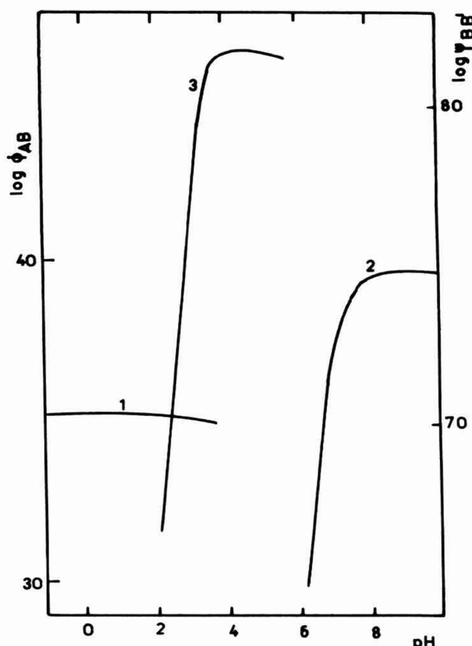


FIG. 1. The dependence $\log y = f(\mathrm{pH})$ ($y = \phi_{AB}, \psi_{BB'}$) of some reactions: $2Fe^{3+} + Hg_2^{2+} = 2Fe^{2+} + 2Hg^{2+}$ in potassium thiocyanate (*1*); $UO_2^{2+} + 2Fe^{2+} + 4H^+ = U^{4+} + 2Fe^{3+} + 2H_2O$ in tiron (*2*); $4I^- + 2Cu^{2+} = I_2 + 2CuI$, $2I^- + 2Fe^{3+} = I_2 + 2Fe^{2+}$ in potassium fluoride (*3*). For the other data see Tables 1 and 2.

The developed theory represents a useful tool in the estimation of optimum acidity and masking of redox reactions. The remaining concentration data can be calculated from those conditions and the values of standard redox potentials.

Finally, we should only repeat [see (*4*)] that the possible disagreement of the theory with the experiment does not lie in an inadequate theory of computation but in an inadequate knowledge of the equilibria involved and their constants. That refers particularly to the values of standard redox potentials in specific conditions of reaction.

SUMMARY

Theoretical relationships for the estimation of optimum acidity and masking of redox reactions are developed on the basis of the coefficients of side reactions. The concentrations of reacting components are calculated from optimum conditions and the values of standard redox potentials. A good agreement of the theory and experiment is shown on several illustrative examples.

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MICROCHEMICAL JOURNAL 25, 176–178 (1980)

Surface Analysis of Thick Gold Films by X-Ray Fluorescence Using the Base Metal as an Internal Reference

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Received October 20, 1979

INTRODUCTION

The common methods for the determination of gold have been reviewed by Beamish and Van Loon (*1*) and these methods such as the spectrophotometric, atomic absorption, volumetric, and gravimetric procedures require a dissolution of the sample as well as possibly a separation step. The atomic absorption determination of gold is subject to chemical interferences when gold is present in low concentration in solutions having a high total ion concentration such as 10,000 ppm (*2*). Such interference was observed also by us and four other laboratories in an attempt to determine the amount of gold in gold-"filled" manufactured objects. Franken analyzed a prepared thin film Ni–Au two-phase system formed by evaporation of the metals on a quartz substrate and found the fluorescence technique to be more reliable than the diffractometric method (*3*).

Techniques using X-ray fluorescence analysis have not been reported for the analysis of gold films on a metal base without dissolution of the sample. The X-ray fluorescence procedure to be described for the analysis of thick gold films was found to be free of interference and does not require any chemical treatment of the sample once a standard sample is available as a reference in setting instrument conditions. The samples were ball-point pen caps and bodies; "gold filled" refers to the manufacturing process whereby a thin gold overlay is rolled or clad onto one side only of the base metal.

MATERIALS AND METHODS

Standard gold solution, 0.94 mg Au/ml, prepared by dissolving 18.8 mg of $HAuCl_4 \cdot 3H_2O$ in water and diluting to 10 ml.

Philips XRD 3000 X-ray spectrograph with Cr source, LiF analyzing crystal, and Philips sample cups.

PROCEDURE

The gold-filled object—the pen casing—was prepared by slitting one side and opening the metal cylinder into a sheet. For the purpose of

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determining the amount of gold on the sheet and to have a reference standard, a sample weighing 1.5499 g was placed in a 250-ml beaker, covered with concd. HCl, and heated. Concentrated HNO_3 was added dropwise until dissolution was completed. The solution was evaporated to near dryness and then transferred by washing with distilled water into a 50-ml volumetric flask and diluted to volume with water. Then 5-ml aliquots of this solution were pipetted into a series of four plastic X-ray sample cups; 0, 0.50, 1.00, and 1.50 ml of the standard gold solution was added respectively to the cups and sufficient water to bring each sample up to a total volume of 6.5 ml. The sample cups were covered with "Mylar" film and placed with the film side facing the X-ray tube. The gold $L\beta_1$ (31.19° 2θ) and Cu$K\alpha$ (44.96° 2θ) peaks were scanned.

A linear regression calculation of the gold/copper ratios versus the amount of added gold showed a coefficient of variance of 0.994 and extrapolation showed 39.3 mg of Au to be present for a sample content of 2.54% Au.

This analyzed sheet was now employed as a reference standard for the determination of gold in other equivalent samples. The sheets were cut to fit into the rectangular opening of the standard Philips sample mount. The gold/copper ratios of the samples were measured and compared to the 2.54% gold standard sheet for which the instrument conditions were set to produce a 90% of full-scale deflection.

DISCUSSION

The cladding process produces a gold film (approximately 5 mg/cm^2) on one side of the metal. When the sample is placed in the spectrograph with the gold surface facing the X-ray tube, the pattern for both Au and Cu is observed.

However, when the sample is placed with the brass surface facing the tube, only the brass pattern is observed and, hence, the sample appears "infinitely thick" for copper. Consequently in the normal scan where the gold side faces the tube, the copper content of the backing metal becomes in effect an internal standard due to the homogenizing effect of the penetration depth of the excitation source beam. That the copper can be validly used as an internal standard is demonstrated by the lack of the Au$L\beta_1$ peak when the sample is reversed in orientation in the holder and scanned through the angles for the peak without a response. This indicates the sample is infinitely thick for copper and that the copper content of the backing will limit the observed Cu$K\alpha$ intensity. From this, identically prepared and scanned samples should demonstrate Au/Cu peak ratios variable only with the amount of deposited gold. A control check on the backing can be conducted by reversing the specimen and scanning the alloy metal peaks to insure copper content uniformity against the stan-

dard. In practice, a backing alloy should not show significant variation. If significant variation is suspected, then a representative range of specimens should be analyzed by dissolving the samples and adding known amounts of gold as was done initially in determining the amount of gold present on the gold-filled sheet. The solid samples are also scanned. Then a calibration curve can be generated by plotting the Au/Cu ratio for these materials against the amount of gold.

SUMMARY

A procedure has been developed for the determination of the amount of gold clad on a brass base. The method does not require dissolution of the sample and utilizes the presence of copper in the base metal as an internal standard.

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MICROCHEMICAL JOURNAL 25, 179–185 (1980)

Isolation of Starch Granules and Starch-Granule-Bound Glucan Synthetase from Cotton Leaves[1]

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Received October 22, 1979

INTRODUCTION

Two forms of glucan synthetase (ADPglucose: 1,4-α-D-glucan 4-α-D-glucosyltransferase, EC 2.4.1.21), soluble (*1, 7–10, 15, 16*) and starch-granule bound (*2–4, 12*), have been reported. Isolation and characterization of the soluble enzyme are well documented. However, only limited information is available for the preparation of starch-granule-bound glucan synthetase (*3, 12*). The basis of the reported procedures (*3, 12*) is homogenization of leaves or seeds in cold water followed by sedimentation of starch granules by repeated low-speed differential centrifugation at about 350*g*. The technique was tested with cotton leaves and found to be unsuitable because this preparation was heavily contaminated with broken chloroplasts, as judged by microscopic observation.

Determination of glucan synthetase activity was necessary in order to study the pathway of starch synthesis in cotton. A simple sucrose density technique allowed the successful isolation of purer enzyme-bound starch granules. With this preparation a high specific activity (more than 100-fold greater than the previously reported procedure) could be measured from a minute amount of starch-granule-bound protein (about 20 μg) without time-consuming purification. Some properties of this enzyme preparation were determined in order to verify the feasibility of the technique with cotton leaves, which are rich in enzyme-interfering substances (*11*).

MATERIALS AND METHODS

Plant material. Glandless cotton plants (*Gossypium hirsutum L.*, cv. Coker 100) were grown as previously described (*5*).

Reagents (all reagents were prepared with water unless otherwise indicated):

Borate (boric acid–sodium borate) buffer, 0.2 *M*, pH 7.6, containing 0.005 *M* glutathione (reduced)

Glycine–NaOH buffer, 0.25 *M*, pH 8.5

[1] Mention of a specific trade name is made for identification only and does not imply endorsement by the United States Department of Agriculture.

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Sucrose, 65% (w/w) in 0.2 *M* borate buffer, pH 7.6
EDTA, 0.02 *M*
Glutathione, 0.25 *M* (reduced)
KCl, 0.5 *M*
Adenosine diphosphate glucose, 0.025 *M*
Uridine diphosphate glucose, 0.025 *M*
Dinitrophenylhydrazine, 0.1% (w/v)
NaOH, 10 *N*
Ethanol, 95% (w/v)
Amylopectin, 2.5% (w/v)
Pyruvate kinase, 15 IU.

Procedure for isolation of starch granules and enzyme purification. All procedures were carried out near 4°C. Seven grams of fresh cotton leaves was homogenized in 50 ml of 0.2 *M* borate buffer, pH 7.6, containing 0.005 *M* glutathione. The homogenization was conducted twice in an Omni-Mixer. A short pause (30 sec) was made after the first 30 sec. The material was squeezed through two layers of Miracloth and the filtrate was centrifuged at 11,500*g* for 20 min in a SorVall SS-34 rotor. The surface of the residue was carefully triturated with a glass rod and the loosened broken chloroplasts were repeatedly rinsed with homogenizing medium. The pellet then was resuspended in 15 ml of homogenizing medium and centrifuged at 20*g* for 2 min in a horizontal head (clinical centrifuge, GT-2). The residue was discarded, and the supernatant was centrifuged again at 20*g*. Five-milliliter portions of the final supernatant were layered onto 15 ml of 65% sucrose in each of three centrifuge tubes (capacity of 34 ml) and then an additional 10 ml of homogenizing medium was added to each tube. The tubes were spun at 4,000 rpm for 5 min and then at 10,000 rpm for an additional 10 min in a Beckman SW 25.1 rotor (*14*). Three distinctively separated layers were observed: a top slightly green large volume, a thin dark-green middle band, and the cushion of 65% sucrose, in which visibly white starch granules were suspended. The starch suspension was drained directly into 30 ml of homogenizing medium in a graduate (100 ml) after puncturing the bottom of the centrifuge tube. The top layer (about 35 ml) of the uniform starch granule suspension was removed with a pipette. The remaining bottom-most layer (about 5 ml) was discarded. The diluted suspension was well mixed and centrifuged at 27,000*g* for 10 min. The sediment was washed once with homogenizing medium. The starch granules were suspended in acetone at −15°C and centrifuged at 4°C (*3*). This was repeated twice, after which the material was dried in a vacuum.

Assays. Protein associated with starch granules and soluble enzyme protein were determined by the procedure of Lowry *et al.* (*13*). Glucan synthetase was assayed according to Tanaka and Akazawa (*16*) with minor modifications. Standard reaction mixture contained (in μmoles):

glycine–NaOH buffer, pH 8.5, 25; EDTA, 0.2; glutathione, 2.5; amylopectin, 0.25 mg; KCl, 5; ADPglucose, 0.25; and enzyme suspension or solution, 0.1 ml, in a total of 0.25 ml. After incubation at 30°C for 10 min the reaction was terminated by immersing the mixture in a boiling water bath for 1 min. The amount of ADP or UDP liberated was determined by the pyruvate kinase method (*12*). Assay mixtures containing starch granules were frequently stirred with glass rods for determinations of protein (*13*) and enzyme activity (*15*).

Starch determination. Acetone-washed starch granules were dissolved in 7.2 *M* perchloric acid. This solution was then adjusted to 4.8 *M* by the addition of water. The polymer content was determined with anthrone as previously described (*6*).

RESULTS AND DISCUSSION

The time course of enzymic activity demonstrated that the release of ADP proceeded at a constant rate during the incubation period of 10 min (Fig. 1). Also the activity of various starch-granule-bound enzyme dilutions was found to be linear up to about 120 μmole ADP released (Fig. 2). Therefore the data permitted the expression of enzyme activity as moles ADP released per unit enzyme protein in 10 minutes.

Total and specific activities of starch-granule-bound glucan synthetase did not change significantly in spite of successive washing with homogenizing buffer (Table 1). The data showed that the enzyme was firmly bound to starch granules. The comparatively high purity of the enzyme is shown in Table 2 (Method A). About 95% of the total crude sample preparation was composed of starch and the enzyme specific activity was about 100 times greater in Method A as compared with Method

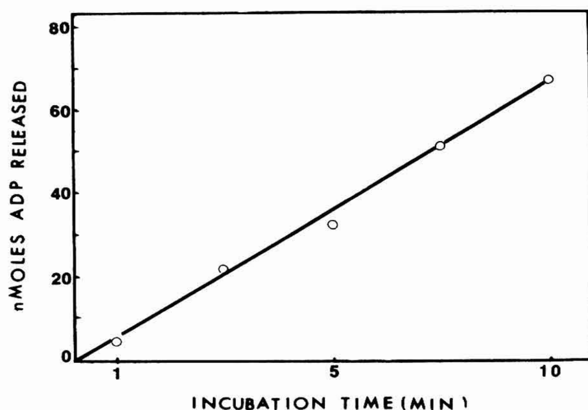

FIG. 1. Time-course activity of starch-granule-bound glucan synthetase. Activity was determined with 0.1 ml of starch granule suspension containing 31 μg protein as described in the text.

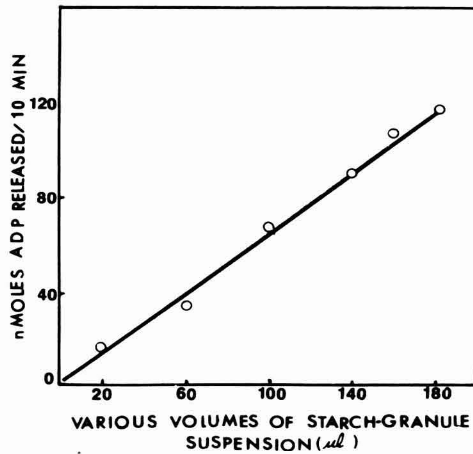

FIG. 2. Effects of enzyme concentration on the activity of starch-granule-bound glucan synthetase. Activity was determined as described in the text, except that the assay mixture contained various amounts of enzyme preparation. One-tenth milliliter of suspension contained 35 μg protein.

B. This difference was attributed to the fact that the starch granule preparation by Method B contained less starch (about 30% of the total dry weight) and more protein (about 34%) than by Method A. The major impurity was probably contributed by broken chloroplasts, since a large green-brown deposit surrounded by a ring of white starch was visible after

TABLE 1
THE BINDING CAPACITY OF GLUCAN SYNTHETASE TO STARCH GRANULES[a]

Wash No.	Activity of glucan synthetase	
	Total[b]	Specific[c]
Before wash	828	2.37
1st	807	2.41
2nd	807	2.34
3rd	809	2.40
4th	805	2.44
5th	806	2.46
After the final wash	804	2.44

[a] One milliliter of starch granule suspension containing 350 μg protein was prepared. This suspension was consecutively washed with 0.5 ml of homogenizing buffer by centrifugation. The activity released into the wash buffer was determined by measuring 0.1 ml sample according to the standard procedure. With this value the residual enzyme activity bound to starch granules was calculated.

[b] nmoles ADP released/10 min.

[c] μmoles ADP released/10 min/mg protein.

TABLE 2
PROPERTIES OF STARCH-GRANULE-BOUND GLUCAN SYNTHETASE PREPARED BY THE PRESENT TECHNIQUE (METHOD A) AND THE PREVIOUSLY REPORTED PROCEDURE (9) (METHOD B)

	Method A	Method B
Crude starch granules		
Dry weight (mg)	25	25
Starch content (mg) by Anthrone method (*16*)	23.8	7.9
Protein content (mg) by Lowry *et al.* (*13*)	0.24	8.4
Total enzyme activity in total starch[a]	539	179[b]
Enzyme activity/mg protein[a]	2246	21.3

[a] Enzyme activity was expressed as nmoles ADP released per 10 minutes.
[b] Starch granules prepared by Method B (before acetone treatment) were further purified according to Method A. Then activities with total starch granules were determined.

the final centrifugation. The acetone treatment of such a preparation would remove only phytol moieties from chlorophyll but leave other impurities such as membranes, lamellae, and associated proteins behind.

In order to demonstrate that this enzyme was not the soluble glucan synthetase bound to starch granules, pH optima of the two forms of the enzyme were investigated. The pH optimum of starch-granule-bound glu-

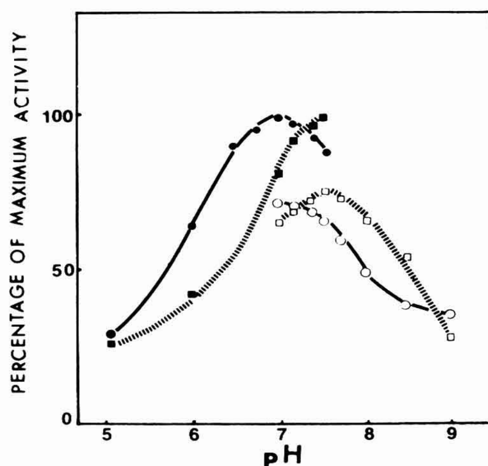

FIG. 3. Activity of starch-granule-bound (■IIIIIII■) and soluble (●——●) glucan synthetase influenced by different pHs of citrate–phosphate buffer. Activity of starch-granule-bound (□IIIIIII□) and soluble (○——○) glucan synthetase affected by different pHs of tris (maleate)–NaOH buffer. The concentration of each buffer was 0.08 *M*. Various pHs were normalized for activity determinations by the addition of 0.5 *M* glycine–NaOH buffer, pH 8.5, which was optimum for ADP determination by pyruvate kinase. All other conditions were similar to the standard assay procedure. Soluble glucan synthetase was isolated by the procedure of Hawker *et al.* (9).

can synthetase was about 7.5, whereas that of soluble glucan synthetase was about 7.0 (Fig. 3). This difference was not great, but was consistent in repeated experiments. These pH optima were determined by assaying the activities in two different buffers. Each buffer influenced enzyme activity differently, but a single separate pH optimum was obtained for each form of the enzyme.

Additional evidence for separate forms of the enzyme was obtained, when the enzymes were assayed with two different enzyme substrates. The specific activity of starch-granule-bound glucan synthetase assayed with UDPG was about 2.5-fold greater than that with ADPG (top, Fig. 4). In contrast, the specific activity of soluble glucan synthetase assayed with UDPG was more than 7.5-fold greater than that with ADPG (bottom, Fig. 4). Both forms of the enzyme, however, showed higher activities with UDPG than with ADPG. The data were in contrast with those from soybean leaves, which showed a greater activity with ADPG than with UDPG (*3*).

The data shown in Fig. 3 and 4 demonstrated that cotton leaves contained two forms of glucan synthetase. Therefore the present procedure

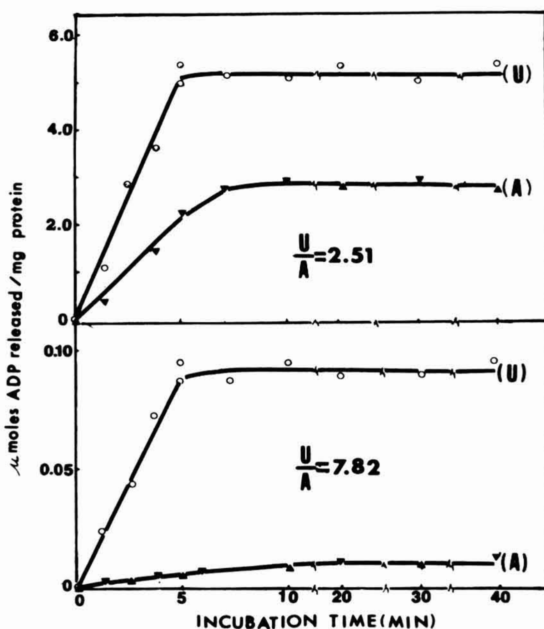

FIG. 4. Time-course activities of starch-granule-bound (top) and soluble (bottom) glucan synthetases assayed with UDPG (U) or ADPG (A). The activity was determined with 0.1 ml of starch granule suspension (34 μg protein) or soluble enzyme (922 μg protein) as described in the text. The activity determined with UDPG to that with ADPG (U/A) at 5-min incubation time was calculated from each enzyme assay.

isolated starch-granule-bound glucan synthetase, which was free from soluble glucan synthetase.

SUMMARY

A simple sucrose density technique has been developed for starch granule isolation from leaves. With this procedure starch-granule-bound glucan synthetase (EC 2.4.1.21) was successfully isolated from phenolic-rich plant tissue. This enzyme is firmly bound to starch granules and free from soluble glucan synthetase. The enzyme specific activity is more than 100-fold higher than that prepared by the previously reported procedure.

ACKNOWLEDGMENTS

The author thanks Dr. G. Guinn for his critical review for the manuscript and Mr. S. Bortman for his technical assistance.

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MICROCHEMICAL JOURNAL 25, 186–195 (1980)

Comparison of High-Performance Liquid Chromatography Electrochemical Detectors for the Determination of Aporphines, Catecholamines, and Melatonin

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Received October 27, 1979

INTRODUCTION

High-performance liquid chromatography with electrochemical detection is becoming a widely used procedure for the quantitative determination of readily oxidizable compounds in a variety of matrices (*1, 3, 4*). The utility of this method was first investigated in our laboratories during the development of sensitive and selective analytical methods for apomorphine (*6*) and melatonin (*2*) in biological specimens. In our earliest work with a commercially available electrochemical detector, we encountered difficulty with the formation of bubbles in the flow cell and connecting tubes, especially when organic modifiers where used to improve elution characteristics. Furthermore, the design of the only commercially available detector (Bioanalytical Systems, Inc.) at the time (*4*) would not allow serial coupling of detectors for selective detection of chromatographically unresolved peaks. Our designs incorporate the reference electrode into the flow pattern of the detector cell. In this report, details are provided on the development of the modified cells. Additionally, comparisons of the performance characteristics of the new detectors with two commercially available cells are reported along with an evaluation of two currently commercially available electronic control units.

MATERIALS AND METHODS

Chemicals. Apomorphine and the other aporphine compounds, apocodeine, isoapocodeine, and *N-n*-propylnorapomorphine, were obtained as described earlier (*5*). The catecholamines, dopamine, epinephrine, and norepinephrine, and melatonin, were obtained from Sigma Chemical Company (St. Louis, Mo). Buffer components were of analytical reagent grade and the water used was deionized and double distilled in glass. The organic modifiers used were of distilled-in-glass grade (Mal-

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linckrodt Chemicals). All mobile phases were filtered through glass fiber pads (Whatman, GF/F) prior to use.

Equipment. For the aporphine work, a Tracor 995 pump and a Waters μ-Bondapak phenyl column (30 cm × 4 mm i.d.) were used. Samples were introduced through a Rheodyne 7120 loop injector. A pulse damper was installed between the pump and injector to remove excessive baseline variations. Aporphines were eluted using methanol–acetonitrile–KH_2PO_4 (5:15:80) as mobile phase. Electrode potentials were maintained at 0.9 V (Ag/AgCl reference), except where noted, on a glassy carbon working electrode. Periodically, electrode potentials were checked with a calibrated Simpson 461 Multimeter.

For the catecholamine and melatonin studies, a Milton Roy pump and glass column (50 cm × 2.1 mm i.d.) packed with Vydac SCX (Separations Group, Hesperia, Calif.) cation exchange resin were used. Samples were introduced through a Rheodyne 7010 injection port. A pulse damper was installed between pump and injector. The mobile phase contained 5.30 g sodium acetate, 5.75 g citric acid monohydrate, 2.40 g sodium hydroxide, and 1.05 ml glacial acetic acid per liter (pH = 5.3). The carbon paste working electrode (wax binder) potential was maintained at 0.65 or 0.90 V depending on the compound being measured.

Detector designs. Two commercially available electrochemical detector units, Bioanalytical Systems, Inc. (BAS) LC-2A (*4*) and Brinkmann Instruments E611, were tested. The Brinkmann system was operated in the amperometric mode.

The modified flow cells are shown in Fig. 1. The reference and auxiliary electrodes were force-fitted after careful drilling of Teflon blocks. In some cases, Teflon tape was used to insure a tight seal at the electrode junctions. The overall dimensions of the modified cells allowed facile interchanging with Bioanalytical Systems cell bases (containing working electrodes).

Comparisons of modifications with Bioanalytical Systems cell. A standard plot of amount of compound versus peak response of the various compounds was run with the modified cells. The LC-2A controller was shut off and the flow cell was rapidly taken apart and reestablished in the configuration of the Bioanalytical Systems cell. The controller was then turned on and allowed to reestablish a stable baseline before a second standard plot was generated with the same standard solutions. The changeover time was usually less than 3 min. The only components changed were the upper cell block with reference electrode. The working electrode was not cleaned or polished during the changeover and the amplification was kept constant. Each data point was run in triplicate.

Comparison of commercial detectors. Comparisons were made by using each flow cell sequentially with both controller units. The working

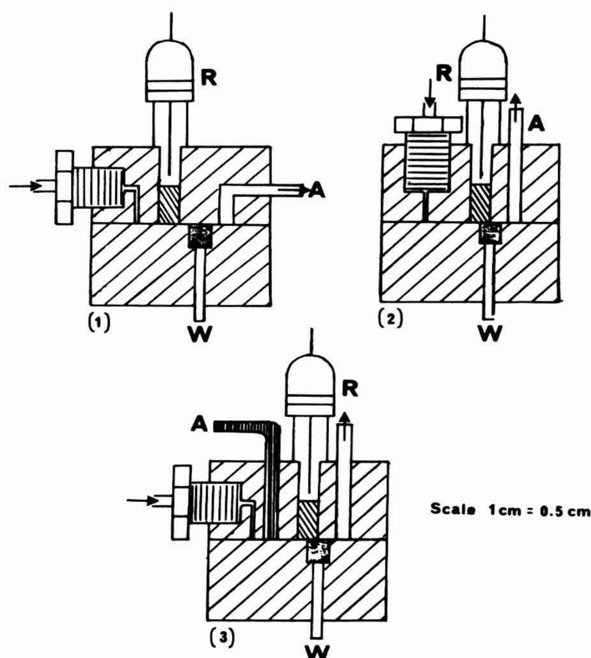


FIG. 1. Flow cell modifications Nos. 1, 2, and 3. A, auxiliary electrode and outlet (unless indicated); R, reference electrode; W, working electrode.

TABLE 1

COMPARISON OF BAS AND BRINKMANN DETECTORS[a]

Flow cell	Compound	Response[b]	
		BAS controller	Brinkman controller
BAS	Apomorphine	26.0 ± 1.0	27.0 ± 1.0
	Dopamine	81.0 ± 1.7[d]	79.7 ± 1.5[d]
	Epinephrine	89.7 ± 0.6[c,d]	83.3 ± 3.1[d]
	Melatonin	172.3 ± 3.1[d]	176.8 ± 6.6[d]
	Norepinephrine	141.7 ± 0.6[d]	136.7 ± 4.2[d]
Brinkmann	Apomorphine	37.0 ± 0.6	33.0 ± 1.0
	Dopamine	15.0 ± 1.7[c]	6.3 ± 0.6
	Epinephrine	7.3 ± 1.5[c]	3.3 ± 0.6
	Melatonin	177.3 ± 0.6[a]*	136.0 ± 5.0[a]*
	Norepinephrine	12.7 ± 1.5[c]	3.7 ± 0.6

[a] Detectors at same working potentials (apomorphine 0.9 V, catecholamines 0.65 V, melatonin 0.9 or 1.2 V (*)) and amplification on both detectors 20 nA/V at all times.

[b] Peak heights in mm ± SD for equal picomolar amounts injected (range = 0.2 to 0.5 pmole).

[c] Significantly greater (one-tail t test, p = 0.05) response between controller units.

[d] Significantly greater response between cells with the same controller (one-tail t test, p = 0.05).

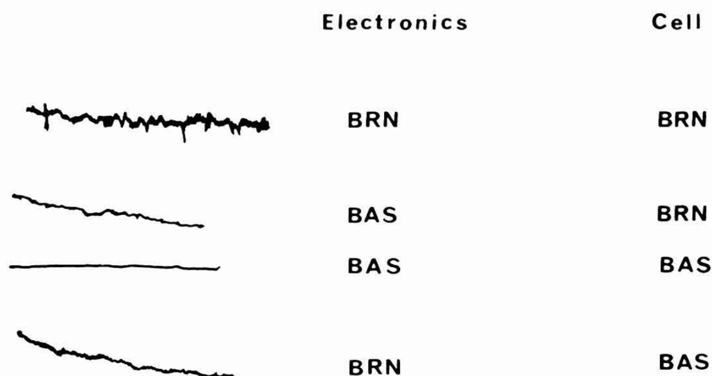


FIG. 2. Comparison of baselines obtained with Brinkmann (BRN) and Bioanalytical Systems (BAS) detectors and controllers. Column, μ-Bondapak phenyl; mobile phase, methanol–acetonitrile–0.05 M KH_2PO_4 (5:15:80); flow, 1.5 ml/min; attenuation, 10 nA/V; potential, 0.90 V.

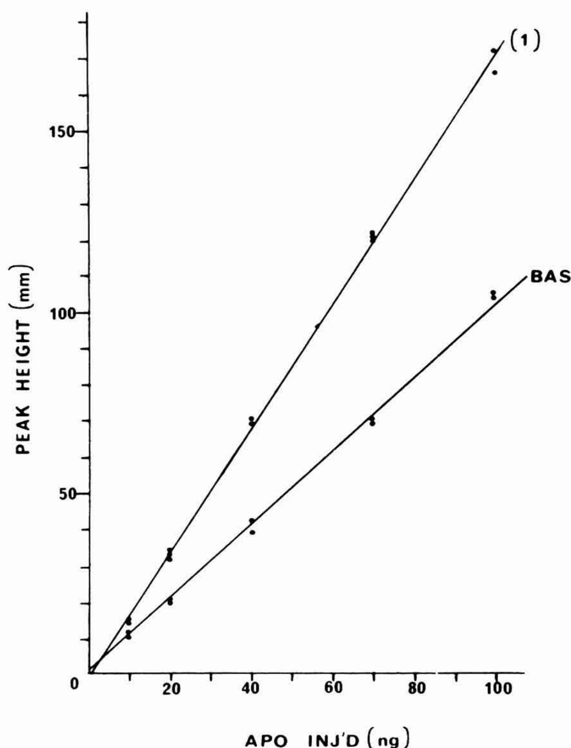


FIG. 3. Comparison of flow cell modification No. 1 and Bioanalytical Systems cell (BAS) using apomorphine (APO) as test compound. Column, μ-Bondapak phenyl; mobile phase; methanol–acetonitrile–0.05 M KH_2PO_4 (5:15:80); flow, 1.5 ml/min; attenuation, 10 nA/V; potential, 0.90 V.

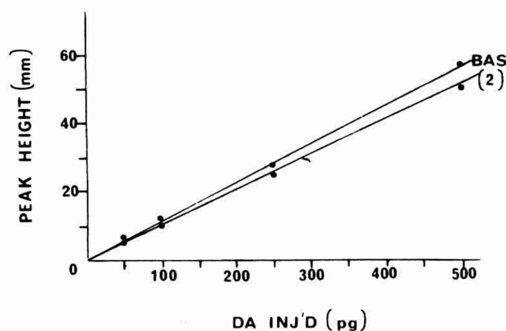


FIG. 4. Comparison of flow cell modification No. 2 and Bioanalytical Systems cell (BAS) using dopamine (DA) as test compound. Column, Vydac SCX; mobile phase, acetate–citrate buffer (pH 5.3); flow, 0.67 ml/min; attenuation, 1 nA/V; potential, 0.65 V.

electrode potential and amplification of signal were maintained as constants between the controller units. All measurements were repeated at least in triplicate.

The analysis of the data generated in this study was performed with an analysis of variance (ANOVA) program within the SPSS statistical package.

RESULTS AND DISCUSSION

Comparison of commercial detectors. Both flow cell and electronic components were significantly different ($p = 0.01$) when tested by two-

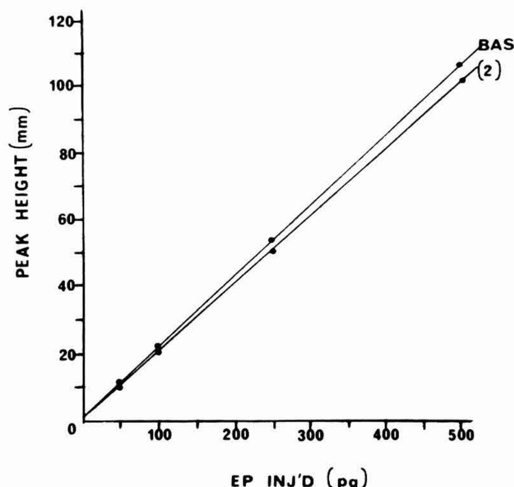


FIG. 5. Comparison of flow cell modification No. 2 and Bioanalytical Systems flow cell (BAS) using epinephrine (EP) as test compound. Column, Vydac SCX; mobile phase, acetate–citrate buffer (pH 5.3); flow rate, 0.67 ml/min; attenuation, 1 nA/V; potential, 0.65 V.

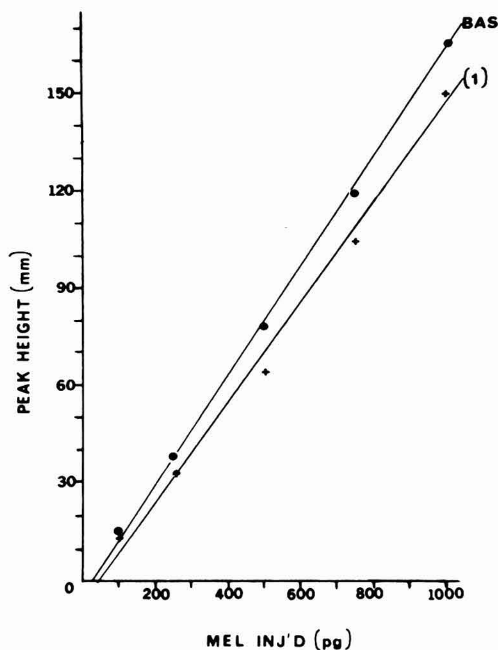


FIG. 6. Comparison of flow cell modification No. 1 and Bioanalytical Systems flow cell (BAS) using melatonin (MEL) as test compound. Column, Vydac SCX; mobile phase, acetate–citrate buffer (pH 5.3); flow 0.67 ml/min; attenuation, 1 nA/V; potential, 0.90 V.

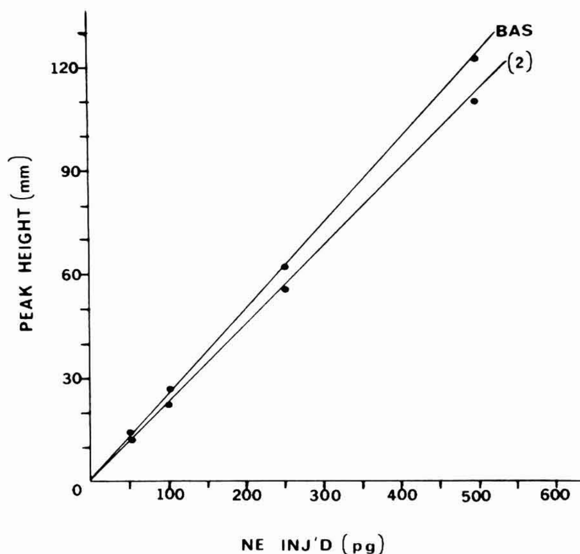


FIG. 7. Comparison of flow cell modification No. 2 and Bioanalytical Systems flow cell (BAS) using norepinephrine (NE) as test compound. Column, Vydac SCX; mobile phase, acetate–citrate buffer (pH 5.3); flow rate, 0.67 ml/min; attenuation, 1 nA/V; potential, 0.65 V.

way ANOVA. As demonstrated in Table 1, the Bioanalytical Systems electronic unit shows a significantly (one-tail t test, $p = 0.05$) better response for epinephrine with both flow cells and for dopamine, melatonin, and norepinephrine than when the Brinkmann flow cell is used. For all comparisons, both detectors were set at an amplification of 20 nA/V. For measurement of relevant physiological levels of these compounds a sensitivity of 2–20 times greater (depending upon the compound being analyzed) is typically needed. The Brinkmann system is unusable at these settings because of erratic baselines.

The comparison of the different cells within electronic components is also interesting (Table 1). All comparisons using a totally aqueous mobile phase show the Bioanalytical Systems flow cell to be significantly better ($p = 0.05$). The Brinkmann flow cell also seemed to be noisier with either

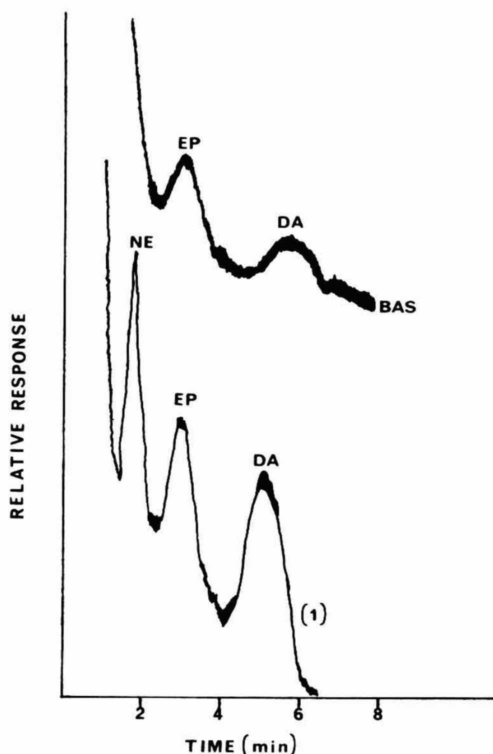


FIG. 8. Responses with Bioanalytical Systems (BAS) and modification No. 1 flow cells to a catecholamine mixture consisting of norepinephrine (NE), epinephrine (EP), and dopamine (DA). Top: Bioanalytical Systems cell, attenuation 1 nA/V; potential, 0.65 V. Bottom: flow cell modification No. 1, attenuation 0.2 nA/V. The NE peak is lost in the solvent front in the Bioanalytical Systems cell chromatogram. Column, Vydac SCX; mobile phase, acetate–citrate buffer (pH 5.3); flow rate, 1 ml/min; amount of each catecholamine injected, 50 pg.

electronic unit as shown in Fig. 2. Similar observations were made when new or freshly repolished glassy carbon working electrodes were used in the Brinkmann flow cell so that the differences in Brinkmann working electrodes are not likely to be significant in this study.

Comparisons of modified flow cells with the Bioanalytical Systems flow cell. Representative plots of response versus amount of compound injected are shown in Figs. 3–7. None of the modified cells showed consistently better response than the Bioanalytical Systems flow cell at the settings used. The modified cells (Fig. 1) did, however, show less baseline noise which allows for greater attenuation and increased sensitivity with equivalent baseline noise (see Fig. 8). The new cells can also be coupled for serial measurements (Fig. 9) without serious loss in resolution. Similar coupling is impossible with the Bioanalytical System cell because of band spreading in the reference component.

The aporphines showed markedly lower responses than the other compounds tested. This is attributable to the extent of organic modifiers (particularly acetonitrile) necessary for the development and separation of these compounds (see Fig. 9). In contrast, a totally aqueous media which aids in the development of ion currents, as used for the separation of the

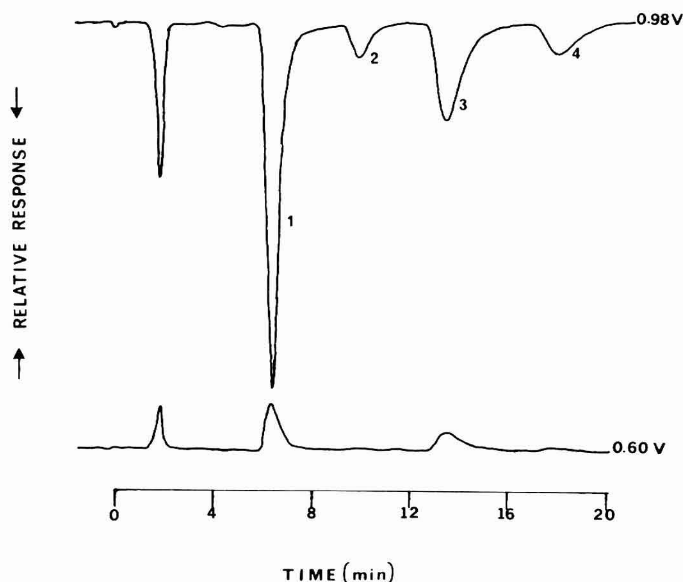


FIG. 9. Responses of two flow cells (modification No. 2) connected in series toward a mixture of aporphines: 1, apomorphine; 2, isoapocodeine; 3, *N*-*n*-propylnorapomorphine; 4, apocodeine. Top: second flow cell in series; electrode potential, 0.98 V. Bottom: first flow cell in series, electrode potential, 0.60 V. Both cells held at same attenuation (20 nA/V) with two Bioanalytical Systems LC-2A electronics units. Column, μ-Bondapak phenyl; mobile phase, methanol–acetonitrile–0.05 *M* KH_2PO_4 (5:15:80); flow rate, 1.5 ml/min.

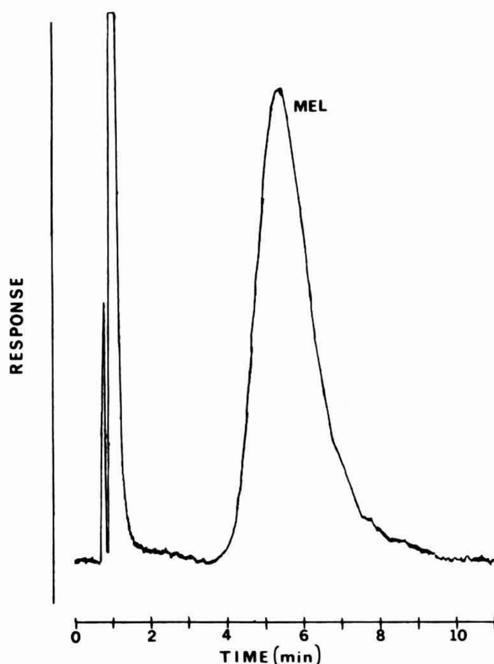
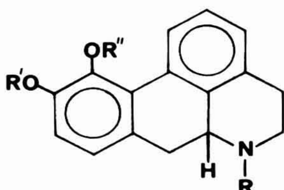
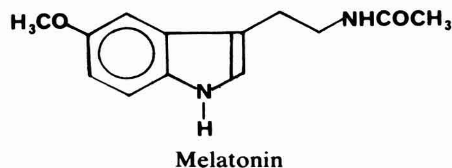


FIG. 10. Development of 2.0 ng of melatonin (MEL) using totally aqueous mobile phase. Column, Vydac SCX; mobile phase, acetate–citrate buffer (pH 5.3); flow rate, 0.67 ml/min; detector, Bioanalytical Systems flow cell and LC-2A controller unit; attenuation, 1 nA/V; potential, 0.90 V.

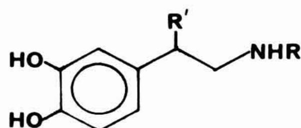
catecholamines (Fig. 8) and melatonin (Fig. 10), provides for inherently improved sensitivities.





Melatonin

Apomorphine: R = CH_3; R′ = R″ = H
Apocodeine: R = R′ = CH_3; R″ = H
Isoapocodeine: R = R″ = CH_3; R′ = H
N-*n*-Propylnorapomorphine; R = $CH_2CH_2CH_3$; R′ = R″ = H



Dopamine: R = R′ = H
Epinephrine: R = CH_3; R′ = OH
Norepinephrine: R = H; R′ = OH

SUMMARY

A Bioanalytical Systems detector showed a greater sensitivity for low-level determinations of apomorphine, norepinephrine, epinephrine, dopamine, and melatonin compared to a Brinkmann Instruments system. Modifications of the Bioanalytical Systems flow cell, which have been developed in our laboratories, increase the sensitivity toward certain catecholic compounds, generally decrease baseline noise, and allow for serial coupling of detectors.[2]

ACKNOWLEDGMENTS

This research was supported by Grant NS-12259 from the National Institute of Neurological and Communicative Disorders and Stroke. Fellowship support for D.W.H. from Hoffmann-La Roche, Inc. is gratefully acknowledged. The authors are also grateful to the Sterling Winthrop Research Institute for a generous sample of *N-n*-propylnorapomorphine. Furthermore, we are indebted to Brinkmann Instruments for providing the equipment used in the detector and system controller unit comparisons.

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[2] While this work was in progress, Bioanalytical Systems, Inc. began manufacture of a modified electrochemical cell which is similar to the ones described in Fig. 1 of this paper.

MICROCHEMICAL JOURNAL 25, 196–199 (1980)

Determination of Alkoxyl Groups on Submilligram Samples[1]

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Received November 29, 1979

INTRODUCTION

The American Chemical Society's Committee on Microchemical Apparatus (*6, 7*) recommended specifications for an apparatus for the determination of alkoxyl groups. The apparatus was later adopted by the American Society for Testing and Materials (*6, 8*) as a standard. A method using this apparatus was submitted for collaborative study by the Association of Official Analytical Chemists[3] (*5, 6*) and the results were such that the method was adopted as the official method of that Society. The recommended sample size for this determination is approximately 3 mg (*6*).

Belcher and co-workers (*1*) developed a method for the determination of alkoxyl groups on sample sizes in the order of 50 μg. The apparatus employed was a scaled-down version of a conventional piece. The results obtained from the analysis of 10 organic compounds gave accuracy generally within ±0.3% of theory.

Bylund *et al.* (*2*) reported successful determinations of only methoxyl groups on sample sizes down to 10 μg. However, no data were given to show the accuracy and precision of their method.

The above methods have drawbacks in that they require delicate and specialized pieces of apparatus. The modified Clark apparatus is commercially available[4] and less fragile. It was therefore decided to test the official method using the modified Clark apparatus for applicability to the analysis of samples in the submilligram-size range.

MATERIALS AND METHODS

A standard modified Clark apparatus (*5–8*) was used in this study. The official method was used with the exception that for all samples in the 0.1-mg range, tin sample carriers were employed (*3, 4, 6*). This was neces-

[1] Part of this paper was presented by A.S. at the Eastern Analytical Symposium, New York City, October 31, 1979. It is also part of the thesis of M.G.K. presented in partial fulfillment for the Degree of Master of Science in Rutgers University, June 1979.

[2] To whom all inquiries should be addressed.

[3] Originally called Association of Official Agricultural Chemists.

[4] Arthur H. Thomas Company, Philadelphia, Pa.

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TABLE 1

RESULTS OBTAINED USING STANDARD APPARATUS (5–8) AND ADOPTED METHOD (5–7)

Compound	Theoretical % alkoxyl	n^a	Sample-size range (mg)	$\bar{x}$ (%)[a]	$\bar{x}$ – theory (%)	SD[a]
Vanillin	20.40 methoxyl	4	1.901–2.022	20.39	−0.01	0.13
Vanillin	20.40 methoxyl	4	0.990–1.145	20.43	+0.03	0.21
Vanillin	20.40 methoxyl	4	0.335–0.990	20.36	−0.04	0.21
Ethyl *p*-aminobenzoate	27.28 ethoxyl	4	1.978–2.104	27.46	+0.18	0.07
Ethyl *p*-aminobenzoate	27.28 ethoxyl	4	0.995–1.103	27.55	+0.27	0.26
Ethyl *p*-aminobenzoate	27.28 ethoxyl	4	0.450–0.543	27.47	+0.19	0.24

[a] n = Number of determinations; $\bar{x}$ = mean value; SD = standard deviation.

TABLE 2

RESULTS OBTAINED USING STANDARD APPARATUS (5–8) AND ADOPTED METHOD (5–7) WITH TIN SAMPLE CARRIERS (3–6)[a]

Compound	Theoretical % alkoxyl	n[a]	Sample-size range (mg)	$\bar{x}$ (%)[a]	$\bar{x}_i$ – theory (%)	SD[a]
Vanillin	20.40 methoxyl	4	0.0802–0.1116	20.48	+0.08	0.11
Ethyl *p*-aminobenzoate	27.28 ethoxyl	4	0.0888–0.1242	27.36	+0.08	0.16
p-Ethoxyacetanilide	25.15 ethoxyl	4	0.0940–0.1175	25.25	+0.10	0.23
3-*O*-Methylglucose	15.98 methoxyl	4	0.1012–0.1198	16.12	+0.14	0.27
Methyl *p*-aminobenzoate	20.51 methoxyl	4	0.0885–0.1060	20.42	–0.09	0.27
Anisic acid	20.40 methoxyl	4	0.0948–0.1068	20.31	–0.09	0.21

[a] n = Number of determinations; $\bar{x}$ = mean value; SD = standard deviation.

sary because a Cahn electrobalance was used instead of a microchemical balance for greater accuracy in this sample range. In the absence of the customary platinum boat, a small piece of platinum wire was added to the hydroiodic acid mixture to aid in preventing bumping (*6*).

RESULTS AND DISCUSSION

Tables 1 and 2 show the compounds analyzed; the calculated percentage alkoxyl; n, the number of determinations; the sample size range in milligram; $\bar{x}$, the mean value for each group; $\bar{x}$ − theory, the deviation of the mean from the theoretical value; and SD, the standard deviation of each group. A standard deviation of 0.3 and a deviation from theory of ±0.3% are considered to be acceptable (*5*, *6*).

Table 1 shows the results obtained with the official procedure using the modified Clark apparatus on samples of vanillin and ethyl *p*-aminobenzoate, respectively. It includes the sample sizes grouped into ranges of 2, 1, and 0.5 mg. Acceptable results were obtained for both compounds in each of the sample-size ranges.

Table 2 shows the results obtained on various compounds using the same procedure, with 0.1-mg samples being weighed in tin sample carriers. The compounds analyzed were vanillin, ethyl *p*-aminobenzoate, 3-*O*-methylglucose, *p*-ethoxyacetanilide, methyl *p*-aminobenzoate, and anisic acid. In each case, acceptable results were obtained.

Determinations on samples in the 0.05-mg range were attempted using the above procedure, but the results were unacceptable in both accuracy and precision.

SUMMARY

A study was made of the applicability of the official method using the modified Clark apparatus for the determination of alkoxyl groups on sample sizes in the submilligram range. The official method was found to give acceptable results on 0.1-mg samples, using tin boats as sample carriers.

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MICROCHEMICAL JOURNAL 25, 200–208 (1980)

Microtitration of Various Anions with Quaternary Ammonium Halides Using Solid-State Electrodes[1,2]

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Received December 4, 1979

INTRODUCTION

In a recent series of papers (*6–10*) we have described the use of quaternary ammonium halides in the potentiometric precipitation titration of many large inorganic and organic anions. EMFs were monitored with commercially available liquid-membrane indicator electrodes and a double-junction reference electrode. A limitation of the methods was their strict applicability in aqueous media only. This restriction is imposed by the nature of the ion exchangers of the ion-selective electrodes (ISEs) which are soluble in organic solvents.

We have since discovered that several solid-state ISEs also respond to many of the inorganic and organic anions previously investigated. This obviates the limitation of operating in a strictly aqueous medium. In this paper we report the feasibility of titrating a large variety of such anions with quaternary ammonium halides using commercially available solid-state ISEs such as the iodide, cyanide, and thiocyanate electrodes as endpoint sensors. The reverse titration of quaternary ammonium halides with anions such as dodecylsulfate is also feasible.

EXPERIMENTAL

The titrant was approximately 0.01 *N* hexadecylpyridinium chloride (CPC, Aldrich Chemical Co.), prepared by dissolving the required amount in hot water and diluting to volume with cold distilled water. Other titrants

[1] Work performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore Laboratory under Contract W-7405-ENG-48.

[2] This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Department of Energy, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights. Reference to a company or a product name does not imply approval or recommendation of the product by the University of California or the U.S. Department of Energy to the exclusion of others that may be suitable.

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that can be used are hexadecyltrimethylammonium bromide (CETAB, Aldrich), hexadecyltrimethylammonium chloride (CETAC, Eastman), and diisobutylphenoxyethyl(dimethyl)benzylammonium chloride (Hyamine 1622, Rohm and Haas). The titrants were standardized vs sodium dodecylsulfate (SDS, Bio-Rad Laboratories, electrophoresis purity reagent). All other chemicals were of the highest purity available from various commercial sources.

The titration system was controlled by a Tektronix 4051 graphics system, as previously described (*4*). EMFs were monitored by a double-junction reference electrode and the following indicator electrodes made by Orion Research Inc.: 94-35 bromide electrode, 94-48 cadmium electrode, 94-17 chloride electrode, 94-29 cupric electrode, 94-06 cyanide electrode, 94-53 iodide electrode, 94-82 lead electrode, 94-16 silver/sulfide electrode, and 94-58 thiocyanate electrode. A Beckman 39271 platinum thimble electrode and a 6-mm-diameter carbon rod were also used as indicator electrodes.

Stirring was provided by a magnetic stirrer. The stirring motor was separated from the titration vessel by a water cooling plate and an aluminum plate connected to a ground. Titrations were performed at ambient temperature (23 ± 1°C). Samples containing approximately 0.025 mmol were pipetted into a 50-ml beaker containing a Teflon-coated stirring bar. They were diluted to 25 ml with distilled water prior to titration. All titrations were carried out at 0.5 ml/min. Titration endpoints were calculated by the second derivative method previously described (*4*), or as described below.

RESULTS AND DISCUSSION

In previous reports (*6*–*9*) we have studied the use of commercially available liquid-membrane electrodes in the potentiometric titration of many large inorganic and organic anions. Examples of some inorganic anions are perchlorate, fluoroborate, hexafluorophosphate, hexachlorostannate, and hexachloroplatinate; examples of organic anions are nitroform, cyanotriphenylborate, tetraphenylborate, picrate, and dodecylsulfate. A particularly useful application, in our opinion, is the use of these electrodes in the potentiometric titration of detergents and soaps which heretofore were determined only with specially prepared electrodes (*10*).

An obvious limitation on the use of liquid-membrane electrodes is their applicability in aqueous media only. Many organic anions are not sufficiently soluble in aqueous solution and thus cannot be determined by the methods previously described.

We have discovered that for many of the compounds previously determined using liquid-membrane electrodes practically any commercially

available indicator electrode can serve as endpoint sensor. This is in agreement with Vytras (*12*) who found that the valinomycin potassium electrode has the function of a universal electrode sensitive to monovalent positively charged ions.

For the starting point in our investigation we have used the microtitration of sodium cyanotriphenylborate vs CPC because we found previously (*9*) that this compound, when titrated vs CETAB, yielded endpoint breaks greater than 300 mV. Although sodium tetraphenylborate yields even larger endpoint breaks, it is less soluble in water and on standing the solutions become turbid. Titration curves for cyanotriphenylborate vs CPC using various electrodes are shown in Figs. 1a and 1b. In most cases the titration curves had similar shapes to those obtained using two platinum electrodes polarized by a constant current in the nonaqueous titration of weak organic acids (*5, 11*). They have the shape of an inverted V, exhibiting a more or less sharp peak after a steep rise in potential, and then decline in EMF. Only the carbon electrode did not exhibit such a curve. It therefore is possible to calculate endpoints according to two methods:

(1) the usual calculation of the maximum change in EMF vs titrant increment for which we use the second derivative method according to Savitsky and Golay (*1*),

(2) the point of maximum EMF according to Shain and Svoboda (*11*). Both methods are programmed into the computer and thus can easily be used for each determination. It is, of course, mandatory to standardize the titrant according to the method to be used in the final determination.

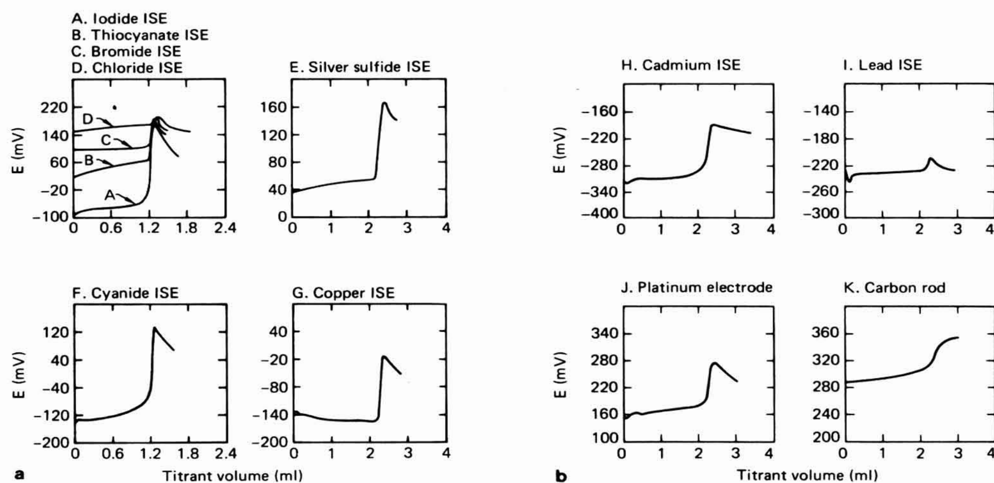

FIG. 1. Titration curves for cyanotriphenylborate vs CPC using various solid-state indicator electrodes.

We have investigated various methods of conditioning the indicating electrode. At first we assumed that immersion of the electrode for some time in the solution to be analyzed was required. This, however, was not necessary. Best results were obtained by slightly abrading the electrode surface prior to each run with 3/0 emery paper (Buehler Ltd., Evanston, Ill.). This was particularly required for the electrodes not yielding very large breaks.

Statistics for the standardization of CPC vs cyanotriphenylborate using the iodide, cyanide, thiocyanate, bromide, and chloride electrodes are presented in Table 1. Both methods for endpoint calculation are shown. When using the method of the maximum EMF the normalities are slightly lower because the titrant volumes are slightly larger. Endpoint breaks were largest using the iodide and cyanide electrodes. However, the values for the magnitude of the endpoint breaks are approximate only: With solid-state electrodes they vary considerably more than with the previously used liquid-membrane electrodes. Nevertheless, the precision (as indicated by the standard deviations) is quite satisfactory. The magnitude of the endpoint breaks varies directly with the size of the anions, I >Br> Cl. With the other electrodes insufficient replicates were run to calculate standard deviations; the magnitude of the endpoint breaks, however, is shown in Table 2. It seems that any material which can conduct an electric current can serve as an endpoint sensor in the titration of cyanotriphenylborate vs CPC.

The reverse titration, of quaternary ammonium halides vs SDS, is also feasible. Statistics for the standardization of approximately 0.01 *N* CPC vs SDS using the iodide ISE are shown in Table 3. We have also assayed another lot of CPC by titration with SDS and compared the use of the fluoroborate and iodide electrodes in Table 4. The average results differed by 0.23% relative. CETAC also was determined by titration with SDS using the iodide and silver/sulfide electrodes.

TABLE 1

STATISTICS FOR THE STANDARDIZATION OF ~0.02 *N* CPS VS SODIUM CYANOTRIPHENYLBORATE USING VARIOUS SOLID-STATE ISEs

	Second derivative			Maximum EMF		
Electrode	Normality, mean	Standard deviation	No. of replicates	Normality, mean	Standard deviation	Endpoint break[a] (mV)
Iodide	0.02045	0.00006	4	0.01948	0.00009	230
Cyanide	0.02048	0.00022	4	0.01966	0.00023	250
Thiocyanate	0.01996	0.00005	4	0.01857	0.00004	150
Bromide	0.02014	0.00004	4	0.01927	0.00012	90
Chloride	0.01985	0.00014	4	0.01903	0.00011	25

[a] Endpoint breaks are approximate only; see text.

TABLE 2

ENDPOINT BREAKS FOR OTHER SOLID-STATE ISEs IN TITRATION OF SODIUM CYANOTRIPHENYLBORATE VS 0.02 *N* CPC

Electrode	Endpoint break[a] (mV)
Cupric	140
Cadmium	120
Silver sulfide	110
Platinum	100
Carbon	45
Lead(II)	20

[a] Endpoint breaks are approximate only; see text.

TABLE 3

STATISTICS FOR THE STANDARDIZATION OF ~0.01 *N* CPC VS SDS USING THE IODIDE ISE

Endpoint calculation mode	Normality, average	Standard deviation	No. of replicates
Second derivative	0.01016	0.00004	5
Maximum EMF	0.00968	0.00005	5

The various inorganic and organic anions previously reported titratable vs CETAB and CPC using liquid-membrane electrodes (*7, 9, 10*) were then subjected to several qualitative runs on the microscale to assess the applicability of the new method. The results are summarize in Table 5. Note that the compounds not titratable using the iodide ISE were previously found titratable using the fluoroborate electrode. All compounds were titrated at the optimum pH as previously determined. In Table 6 we present a qualitative classification of the sharpness and magnitude of endpoint breaks obtained using the iodide ISE. Representative titration curves are shown in Fig. 2 for anions yielding sharp breaks, in Fig. 3 for those yielding medium breaks, and in Fig. 4 for those yielding small breaks. It is noteworthy that while most of the organic compounds previously found titratable using the fluoroborate ISE could also be determined using the iodide ISE, only about one-half of the inorganic anions could be

TABLE 4

STATISTICS FOR THE ASSAY OF CPC BY TITRATION WITH SDS: COMPARISON OF FLUOROBORATE AND IODIDE ELECTRODES

Electrode	Assay (%) deviation	Standard deviation	No. of replicates
Fluoroborate	97.58	0.18	4
Iodide	97.34	0.07	3

TABLE 5
SUMMARY OF COMPOUNDS INVESTIGATED IN TITRATION WITH CPC USING THE IODIDE ELECTRODE

	Titratable compounds	Compounds not titratable[a]
Inorganic compounds	Permanganate	Iodide
	Perrhenate	Periodate
	Persulfate	Perchlorate
	Hexafluorophosphate	Fluoroborate
	Hexafluoroarsenate	Hexafluoroantimonate
	Tetrachloroplatinite	Tetrachloroaurate
	Hexachloroplatinate	Tetrachlorothallate
	Tetrachlorostannite	Tetrachloropalladite
	Hexachlorostannate	Tetrachloromercurate
	Bismuth tetrachloride	Hexachloroiridate
	Ferricyanide(III)	Ferrocyanide(II)
	Hexachloroosmiate	Dichromate
Organic compounds	Nitroform	2,4-Dinitrobenzenesulfonate decylsulfate
	Tetraphenylborate	Octylsulfate
	Cyanotriphenylborate	2-Naphthalenesulfonate[b]
	Picrate	Chromotropic acid (4,5-dihydroxynaphthalene-2,7-disulfonate)[b]
	Picryl sulfonate	Arsenazo I [*o*-(dihydroxy-3,6-disulfonaphthalene-2,7-bisazo)bisbenzene-arsonic acid][b]
	2,4,5-Trichlorobenzene-sulfonate	
	Bromphenol blue	
	Bromcresol purple	
	Laurate	
	Myristate	
	Oleate	
	Stearate	
	Dodecylsulfate	
	Tetradecylsulfate	
	Dodecylbenzenesulfonate	
	Dioctylsulfosuccinate	

[a] Compounds not titratable were titratable using the fluoroborate ISE.
[b] Not previously reported, but titratable using the fluoroborate ISE.

titrated using the latter electrode. We have at present no explanation for this.

Sodium stearate was previously found insufficiently water soluble (*10*) and was titrated while the solution was hot. We have now dissolved this compound in 10 ml of ethyl ether plus 5 ml of methanol and diluted the solution to 100 ml with water. The partially nonaqueous solution was found titratable vs CPC using the iodide ISE. Duplicated determinations yielded recoveries of 99.90 and 99.70%. The titration of SDS vs CPC using the iodide ISE was found feasible in a medium containing 20% (v/v) of ethanol although smaller breaks were obtained than in a totally aqueous

TABLE 6

QUALITATIVE CLASSIFICATION OF POTENTIOMETRIC BREAKS OBTAINED WITH IODIDE ELECTRODE

Large, sharp breaks (>100 mV)	Small, sharp breaks (<100 mV)	Medium breaks (50–100 mV)	Small breaks (<50 mV)
Tetraphenylborate	2,4,5-Trichlorobenzenesulfonate	Hexafluoroarsenate	Permanganate
Cyanotriphenylborate	Bromphenol blue	Picrate	Perrhenate
Bromcresol purple	Dioctylsulfosuccinate	Dodecylbenzenesulfonate	Persulfate
	Dodecylsulfate	Myristate	Hexachloroosmiate
	Stearate		Tetrachloroplatinite
	Tetradecanoate		Hexachloroplatinate
	Hexafluorophosphate		Tetrachlorostannite
			Hexachlorostannate
			Ferricyanide(III)
			Bismuth Tetrachloride
			Nitroform
			Dodecanoate
			Laurate

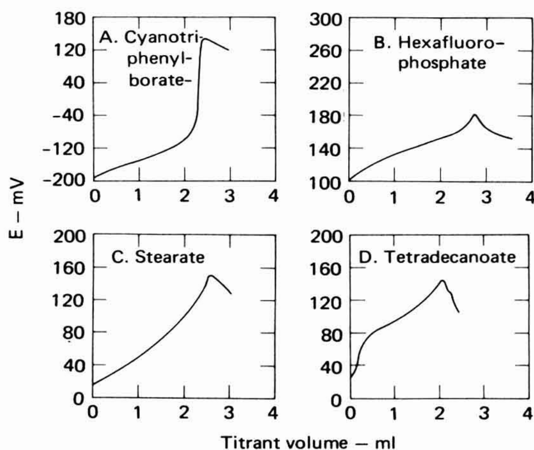

FIG. 2. Titration curves for some anions yielding sharp breaks with the iodide ISE.

medium. Therefore the solid-state ISEs can be used as endpoint detectors in partially nonaqueous media. The solubility of the precipitates must, however, be investigated in each case. Many of the quaternary ammonium salts are more soluble in organic solvents than in water (thus tetraphenylborates are soluble in acetone).

SUMMARY

Many solid-state electrodes were found to respond as endpoint detectors in the potentiometric titration of large inorganic and organic anions with quaternary ammonium halides. The best response was obtained with the iodide and cyanide electrodes although practically any electrode can function as endpoint sensor. The titrants were hexadecylpyridinium chloride and hexadecyltrimethylammonium chloride; hexadecyltrimethylammonium bromide and Hyamine 1622 may also be used. Some inorganic anions thus titratable are

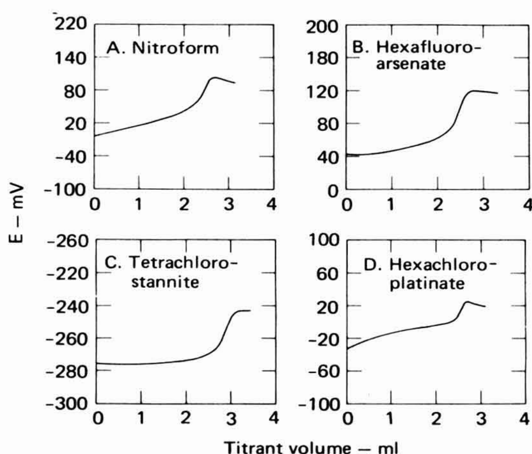

FIG. 3. Titration curves for some anions yielding medium breaks with the iodide ISE.

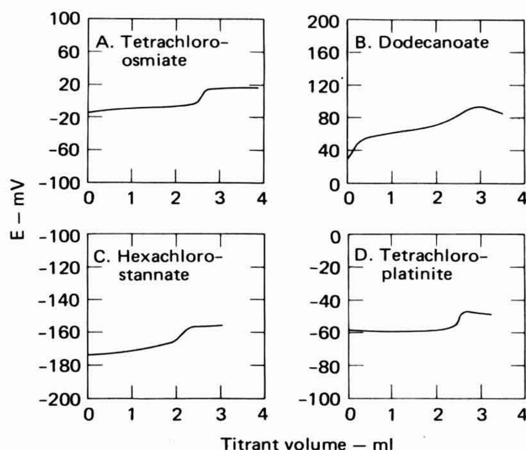

FIG. 4. Titration curves for some anions yielding small breaks with the iodide ISE.

perrhenate, persulfate, ferricyanide, hexafluorophosphate, and hexachloroplatinate. Examples of organic anions titratable are nitroform, tetraphenylborate, cyanotriphenylborate, picrate, long-chain sulfates and sulfonates, and some soaps. The reverse titration of quaternary ammonium halides vs dodecylsulfate is also feasible. Some titrations are feasible in a partially nonaqueous medium.

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MICROCHEMICAL JOURNAL 25, 209–218 (1980)

Resorcinol as a Reagent for Zinc

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Received December 15, 1979

INTRODUCTION

Pollard and co-workers (*10*) pointed out several years ago that development of chromatography for use in the quantitative analysis of inorganic substances has lead to a change in the criteria which govern the selection of reagents used for the spectrophotometric determination of cations (*4, 5*).

Prior to the development of good separation techniques for essentially all the cations specificity of the color-forming reagent for the particular cation of interest was of major importance, but now that the cation of interest can be separated from possible interfering cations with relative ease the significance of specificity of reagent has decreased. The criteria on which reagents are selected for the determination of individual cations after chromatographic separations are sensitivity and convenience.

Many reagents have been briefly investigated and tentative analytical procedures proposed but not utilized due to lack of specificity; the catalytic action of zinc ion on the darkening of resorcinol (1,3-dihydroxybenzene) in the presence of air is such a procedure. It was first proposed as an analytical reagent for the colorimetric determination of zinc by Campoly Cerdun and Puente (*2*). Later Lambert proposed resorcinol as a colorimetric reagent for the determination of copper (*6*).

In this report we wish to clarify the nature of the reactions involved and the condition of reagent concentration, pH, and reaction time that allow the greatest reproducibility and sensitivity for the determination of zinc. A modification which makes this readily available economical reagent somewhat more useful is offered.

NATURE OF THE REACTION

The oxidation of resorcinol has been studied for a long time in connection with the formation of dyes (*1*). The structure of these products has only recently been elucidated (*7, 8*). Since resorcinol cannot give rise to *o*- or *p*-quinoial products on removal of two electrons its behavior in the first oxidation step is that of a monophenol with an oxidation potential lowered by the presence of the second hydroxyl group. The oxidation of resorcinol in alkaline solution produces a mixture of polymers (I)

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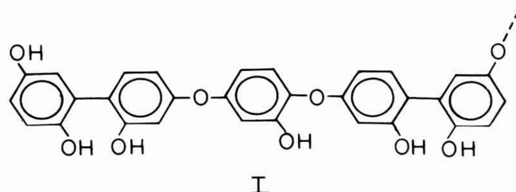
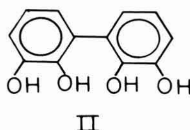
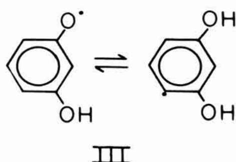


containing only a small percentage of dimer, 2,4,2′,4′-tetrahydroxybiphenyl (II)

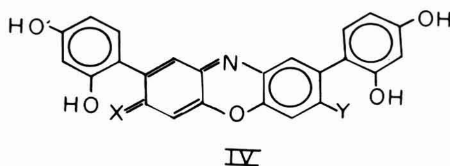


The formation of the dimer has been considered as evidence that the biresorcinate radical (III)



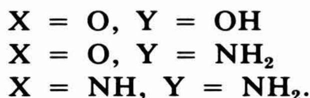
is involved in the reaction.

Autoxidation of resorcinol in the presence of ammonia produces various dyes. These consist of mixtures of 7-hydroxy- and 7-aminophenoxazones and 7-aminophenoxazines (IV)

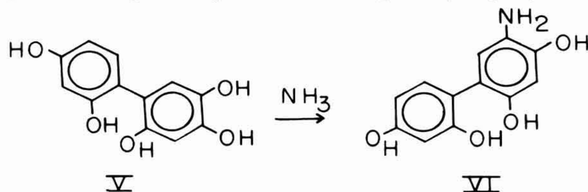


where

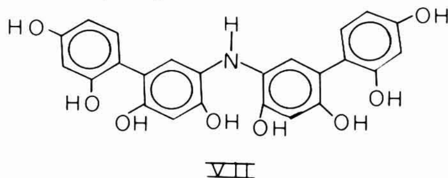
$$X = O,\ Y = OH$$
$$X = O,\ Y = NH_2$$
$$X = NH,\ Y = NH_2.$$



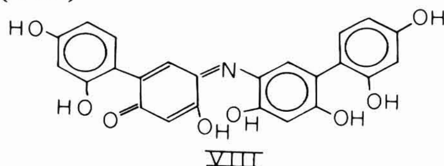
Ammonia participates in the autoxidation of resorcinol by replacing a hydroxyl group of the 2,4,5,2′,4′-pentahydroxybiphenyl (V) intermediate yielding 2,4,2′,4′-tetrahydroxy-5′-aminobiphenyl (VI)



After the diphenylamine (VII) has been oxidized



to the indophenol (VIII)



ring closure to the phenoxazones and phenoxazines (IV) can take place.

The substitution of hydroxyl groups by amino groups and condensations are all apparently free-radical-initiated processes. Hydrogen peroxide or oxygen each may serve to initiate the same series of reactions.

The kinetics of the oxidation of resorcinol in pH 5 solutions by hydrogen peroxide catalyzed by copper ions has been investigated and a Michaelis–Menton type mechanism proposed (e.g., a ternary complex of substrate oxidant and catalyst) (*3*). However, the extrapolation of the results of these studies to oxidations taking place at higher pH values may be questioned.

Colorimetric procedures based on the autoxidation of resorcinol have been described for zinc, the reaction taking place in an ammoniacal system, and for copper, the reaction in a phosphate system (*2, 6*). Sensitivities are reportedly in the tenths of a part per million range; however, neither spectra for the system nor optimum pH for the reaction medium have been reported. There is also disagreement between the two procedures described as to the buffer salts best suited for the determination.

In this work the pH at which the oxidation progressed most rapidly was determined. A comparison of carbonate, phosphate, ammonia, and borate buffer systems at the optimum pH, in terms of rapidity of the reaction, was made. The use of oxygen (air) versus hydrogen peroxide was assessed and a comparison of this metal-ion-catalyzed reaction for the determination of zinc and copper was made.

EXPERIMENTAL

Materials

Reagent-grade chemicals, J. T. Baker Company, Baker analyzed reagents, without further purification were used throughout this work. Water was passed through cation and anion exchange resin columns fol-

lowed by distillation from a potassium permanganate still. Stock solutions of all reagents were prepared by direct weighing. The concentrations desired for the various experiments were obtained by standard dilution techniques.

Stock resorcinol solution was 0.200 *M* and had a shelf life of approximately 2 weeks. Stock buffer solutions were as follows:

(a) ammonia/ammonium chloride, pH 10, 2.0 *M* in ammonia and ammonium;
(b) dibasic phosphate/tribasic phosphate, pH 10, 0.40 *M* in total phosphate;
(c) bicarbonate/carbonate, pH 10, 0.40 *M* in total carbonate;
(d) borax system adjusted to pH 10 with 6.0 *M* NaOH, 0.025 *M* in tetraborate.

Stock solutions of zinc chloride and copper(II) chloride were both 0.20 *M* in the respective salts. Hydrogen peroxide was 3% reagent grade approximately 0.88 *M*.

Spectra

Two spectrophotometers were used: (1) A Beckman DU equipped with a Gilford Instruments Company photometer and (2) a Cary 17 recording spectrophotometer.

Procedures

The objective of these experiments was to determine the combination of reagent concentrations, pH, and reaction time that gave the greatest reproducibility and sensitivity for the determination of zinc ion concentration. The concentration of zinc was determined by correlation with the absorbance of the products resulting from the zinc-catalyzed autoxidation of resorcinol. All experiments and measurements were carried out at ambient room-temperature conditions. Experiments were accomplished by making appropriate dilutions of the stock solutions described above.

The products of the autoxidation of resorcinol have absorption spectra that are pH dependent, pK = 5−7.5 (*9*). They may be measured under acidic or basic conditions. The oxidation products and residual resorcinol are stable under acidic conditions and absorption spectra may be measured without concern for further changes. If the spectra are measured under basic conditions care must be exercised that significant changes do not occur in the spectra. This can be accomplished by removing oxygen from solution with ascorbic acid, flushing the solution with nitrogen gas, or by working rapidly. In basic solutions there is a well-defined absorption maximum occurring at 610 nm that depends upon elapsed time of reaction and presence of zinc ion, at constant resorcinol and buffer concentrations. This absorption maximum is shifted to 480 nm in an acidic solution (below pH 4).

RESULTS AND DISCUSSION

Determination of Optimum pH

The pH dependence of the zinc-ion-catalyzed autoxidation of resorcinol was determined in solutions of constant ammonia and ammonium ion concentrations (e.g., $[NH_3] + [NH_4^+] = 0.020\ M$). The pH of these solutions was adjusted to the desired value ranging from 6 to 12 by adding 6 *M* NaOH or 6 *M* HCl as was needed. Zinc ion was constant at 0.004 *M*. The absorbances were measured in acidified solutions at 480 nm. The results of these measurements are plotted in Fig. 1. The reaction was allowed to proceed for 1 hr before recording the spectra.

In Fig. 1 the maximum occurs at pH 10; this corresponds to the pH for maximum rate of reaction because all solutions reacted for the same length of time and absorbance is taken as proportional to reaction product concentration. Resorcinol has equilibrium dissociation constants corresponding to $pK_{a_1} = 9$ and $pK_{a_2} = 11$. As a consequence of these values for the equilibrium constants the principal molecular species in pH 10 resorcinol solution is the biresorcinate ion ($C_6H_5O_2^-$). We presume that this is the species which undergoes the zinc-catalyzed oxidation reaction.

Effect of Diverse Buffer Salts

A comparison of the rate of the zinc-catalyzed resorcinol oxidation reaction in carbonate, borate, phosphate, and ammonia buffer systems at pH 10 was made. The comparison was in terms of the absorbance of the solution for similar reaction times. The reaction took place in all four buffers, but the rate was in increasing order carbonate $<$ borate $<$ phosphate $\sim$ ammonia. The reaction products in the ammonia system are different as indicated earlier and the extinction coefficients of the products are apparently greater. Therefore we continued to use an ammonia buffer system for the metal-ion-catalyzed reaction.

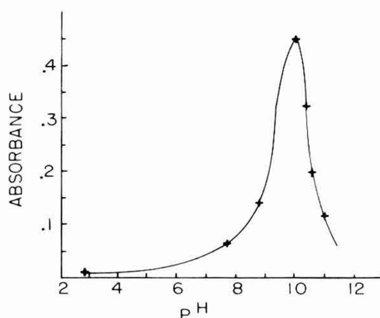


FIG. 1. Effect of pH on the autoxidation of resorcinol, catalyzed by zinc ion. Absorbance measured in pH 2 solutions at 480 nm.

Background Effect

The occurrence of the noncatalytic autoxidation of resorcinol leads to the same final products as the catalytic reaction. As a result of the noncatalytic reaction the overall rate (V) and consequently the overall absorbance will be the sum of the rates or abşorbances of two reactions, catalytic (V_c) and ordinary (V_0). Or the rate of the catalyzed reaction will be the difference between the total overall reaction rate and the uncatalyzed reaction, $V_c = V - V_0$, or in terms of the corresponding absorbances of the products formed, $A_c = A - A_0$. As long as $V - V_0$ is large the error will be relatively small. As V_c ($V - V_0$) decreases the variation of V and V_0, as measured in terms of absorbance, will become significant and if $\alpha = \Delta V/V$, where ΔV is the variance of the measured variable (i.e., velocity of reaction), then V_c must exceed $\alpha V + \alpha V_0$ (if V and V_0 are not too different, then $V_c \geqq 2\alpha V$) for a significant measurement. If we assume measured absorbances (A) are proportional to velocities ($A \approx V$), then $\alpha = \Delta A/A$. It is generally accepted that the most precise absorbance measurements are in the range of 0.15 to 1.0 for an indeterminant error of 0.005, typical of most spectrophotometers (*11*). An ideal measurement based on the foregoing would have an absorbance blank, the uncatalyzed reaction, of 0.15 and for the catalyzed reaction an absorbance of 1.0. We were unable to satisfy these limits using oxygen as an oxidant without an excessive reaction time.

Table 1 shows absorbance values for three different experimental conditions leading to different dissolved oxygen concentrations and illustrating how absorbance varied.

Column 2 of Table 1, labeled "Quiescent closed," tabulates the absorbance of solutions prepared by making dilutions of stock solutions into 50-ml volumetric flasks to give the concentrations indicated at the bottom of the table. The flasks were immediately closed with ground-glass stoppers and shaken thoroughly for 1 min. After the initial shaking the flasks were not disturbed till the absorbance was measured 1 hr later. Entry of more oxygen (air) into the flask was restricted by the tight-fitting groundglass stopper.

Column 3 of Table 1 shows the absorbances of solutions prepared in the same manner as those used for column 2. These solutions differed in that after preparation and shaking the solutions were transferred to 50-ml Erlenmeyer flasks. The solution was protected from the entry of dust but oxygen entry was not inhibited; oxygen could diffuse into the surface of the solutions. After the transfer to the Erlenmeyer flasks the solutions were not disturbed till the absorbances were measured.

Solutions for the absorbances tabulated in column 4 were prepared the same as those for column 2 measurements, but in addition small Teflon-covered magnetic stirring bars were inserted and the solutions stirred

TABLE 1
EFFECT OF DEPLETED OXYGEN CONCENTRATION[a]

Zinc concentration (*M*)	Absorbance		
	Quiescent, closed	Quiescent, open	Open and stirred
0.0	0.105	0.105	0.206
4×10^{-4}	0.120	0.210	0.306
4×10^{-3}	0.160	0.310	0.460

[a] Solution composition: 0.08 *M* resorcinol, 0.2 *M* $NH_3 + NH_4^+$, pH 10 buffer, 60-min reaction time, 1-cm pathlength.

continuously to ensure complete solution equilibrium with oxygen from the air during the entire reaction time.

Comparison of the absorbance values tabulated in columns 2, 3, and 4 indicates a depletion of oxygen in the solution as the reaction proceeds. But when the oxygen concentration is held constant by maintaining air saturation (column 4) the rate of the catalyzed to uncatalyzed rate is not great enough to satisfy the criteria established above, 1.0/0.15. In Fig. 2 is a Beer's law plot of a zinc ion concentration series obtained under quiescent closed conditions.

Hydrogen Peroxide Oxidant

Seeking to obtain absorption values closer to the optimum identified above the substitution of hydrogen peroxide for oxygen was attempted. To use hydrogen peroxide we need to determine (a) if zinc is a catalyst for the oxidation of resorcinol and (b) the optimum hydrogen peroxide concentration. In Fig. 3 absorbance values are plotted of pH 10 buffered, 0.08 *M* resorcinol solution, zinc ion concentration fixed at 0.004 *M* with hydrogen peroxide variable. Figure 3 shows that the upper limit of zinc ion

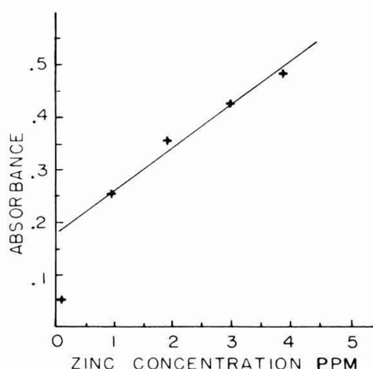


FIG. 2. Effect of zinc ion on the autoxidation of resorcinol.

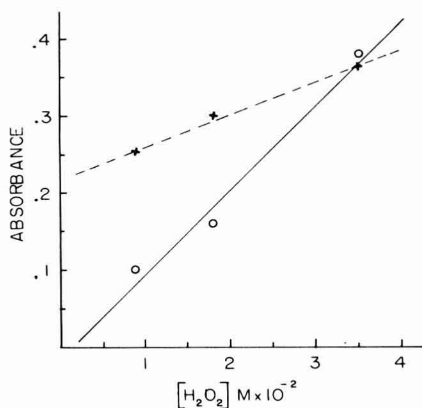


FIG. 3. Effect of hydrogen peroxide concentration on rate of oxidation of resorcinol. (– – –) Zinc ion concentration 4.0 × 10^{-3} F; (——) no zinc ion present.

concentration to be measured determines the maximum hydrogen peroxide concentration used. If the uncatalyzed rate, which depends on hydrogen peroxide concentration, is excessive it will "swamp" the catalyzed rate; consequently there is a maximum hydrogen peroxide concentration. The relative reaction rates, catalyzed vs uncatalyzed, should approach 1.0/0.15 (~6.5). From this figure it is inferred that a hydrogen peroxide concentration in the range of 5 × 10^{-3} to 2 × 10^{-2} *M* would approach a satisfactory ratio for the catalyzed to uncatalyzed reaction rates.

In Fig. 4 the absorbances of a series of solutions of variable zinc ion concentration are shown. In these solutions resorcinol was 0.08 *M*, hydrogen peroxide was 5 × 10^{-3} *M*, and the ammonia/ammonium buffer was 0.2 *M* at pH 10. The reaction times for the series were approximately 1 hr. It is necessary to maintain at least a 20-fold molar excess of hydrogen

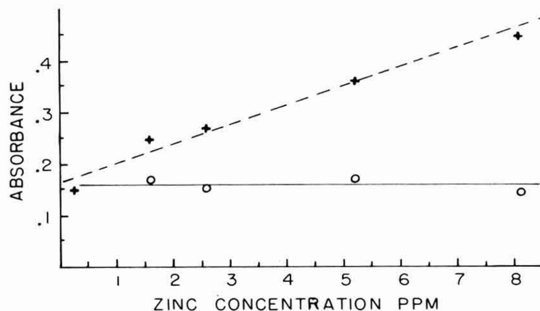


FIG. 4. Effect of zinc ion concentrations on oxidation of resorcinol by hydrogen peroxide. (– – –) Variable zinc ion; (——) no zinc ion present.

peroxide to zinc ion to ensure a linear relation between the absorbance of the solution and zinc concentration. The linear relationship between absorbance and zinc ion concentration, Beer's law type behavior, exists over only a limited zinc ion concentration range for the resorcinol hydrogen peroxide/pH system described here; Fig. 4 encompasses that range, approximately 0 to 8 ppm zinc. The average deviation of four series of measurements similar to those of Fig. 4 yields a value of 10.6%. Using Fig. 4 as a basis for analysis the least-squares slope and intercept are respectively 0.0371 ppm^{-1} and 0.160 absorbance. If an overall variance of 20% is accepted as a reasonable value for the system as described, the minimum to maximum range of zinc concentration that can be reliably measured is 0.86 ~ 9 ppm.

The catalytic properties of zinc and copper(II) ions in the system described above were compared by substituting copper for zinc. Copper is approximately 100-fold more effective as a catalyst for the reaction; consequently the procedure described above is approximately 100 times more sensitive with respect to copper.

As was indicated earlier many of the transition metals will catalyze the oxidation of resorcinol by hydrogen peroxide; if present they would interfere. The resorcinol oxidation reaction is useful because clean separations of metal ions may be made by means of ion-exchange chromatography (*4*, *5*).

SUMMARY

The use of the zinc-ion-catalyzed autoxidation or hydrogen peroxide oxidation of resorcinol (1,3-dihydroxybenzene) to determine zinc concentration has been examined. The nature of the reaction products involved, the conditions of reagent concentration, pH, and reaction time have been specified. The procedure will yield results with a relative average deviation of 10% over the concentration range of 0.9 ppm to approximately 8 ppm. It is an inexpensive and convenient reagent and procedure.

ACKNOWLEDGMENT

This research was supported in part by the Office of Water Research and Technology of the U.S. Department of the Interior through the Water Resources Research Institute of Auburn University (Project A-036-ALA).

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MICROCHEMICAL JOURNAL 25, 219–222 (1980)

Microgram Determination of Vanadium from Natural Resources

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Received October 19, 1977

INTRODUCTION

Vanadium is widely distributed in nature and is expected to have a vital role in plant and animal metabolism. In the present work a method for the determination of the vanadium content in a number of naturally occurring animal and plant materials by liquid–liquid extraction technique using *N-m*-tolyl-*o*-methoxybenzohydroxamic acid (*N-m*-T-*o*-MBHA) (as the complexing agent for vanadium) is described. The details of the rapid extraction and spectrophotometric determination of vanadium in microgram quantities have been described elsewhere (*1*).

Vanadium is extracted from chloroform solution of *N-m*-T-*o*-MBHA from 6 to 8 *M* HCl. The violet extract has a maximum absorbance at 550 nm with a molar absorptivity 6.5×10^3. The extracted complex obeys Beer's law over the range of 0.05 to 15 μg of vanadium.

EXPERIMENTAL

The reagent *N-m*-T-*o*-MBHA was synthesized as described earlier (*2*). It was recrystallized before use and its purity was tested by elemental analysis, TLC, IR, and UV spectra. A 0.1 *M* reagent solution in ethyl alcohol-free chloroform was used for extraction of vanadium.

A standard vanadium (V) solution was prepared by dissolving 0.6260 g of ammonium meta vanadate (Analar) in a liter of double-distilled water and its vanadium content was determined volumetrically.

Absorbance at 550 nm was measured with a double beam Carry-14 spectrophotometer using 10-mm cells.

Preparation of samples. Depending upon the concentration of the vanadium in samples, 20 to 50 g of material was digested with an excess of

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TABLE 1
CALIBRATION DATA IN THE DETERMINATION OF VANADIUM[a]

Vanadium concentration (μg/ml)	Absorbance	Standard deviation
0.05	0.006	±0.002
0.10	0.012	±0.003
0.20	0.026	±0.001
0.50	0.0631	±0.001
2.00	0.255	±0.002
4.00	0.510	±0.001
5.00	0.638	±0.001
10.00	1.275	±0.002
15.00	1.915	±0.002

[a] Maximum absorbance 550 nm.

perchloric and nitric acid. The hot solution was filtered and then centrifuged off any silicious residue. The filtrate was evaporated and diluted to 100 ml with 12 *M* HCl.

Calibration curve. An aliquot of standard vanadium (V) solution was placed in a 100-ml separatory funnel. Adjustment of the aqueous phase to

TABLE 2
VANADIUM IN NATURAL RESOURCES

Series number	Sample	Vanadium found (μg/ml)	Number of determinations	Standard deviation
1.	Potato sample 1	5.72	10	±0.05
2.	Potato sample 2	6.82	8	±0.09
3.	Onion sample 1	7.45	10	±0.05
4.	Onion sample 2	6.83	10	±0.02
5.	Sea water	5.72	10	±0.01
6.	Lake water	1.53	8	±0.02
7.	Carrots	3.55	8	±0.02
8.	Spinach	6.35	8	±0.02
9.	Cabbage	5.75	10	±0.02
10.	Nonripe mango skin	1.54	9	±0.03
11.	Ripe mango skin	3.55	10	±0.03
12.	Tobacco	2.05	9	±0.01
13.	Corn leaves	1.05	9	±0.01
14.	Tomato	1.55	8	±0.04
15.	Rat liver	0.05	12	±0.01
16.	Rat kidney	0.01	12	±0.01
17.	Rat bone	0.01	12	±0.01
18.	Rice	1.00	10	±0.02
19.	Peas (leaves)	0.75	12	±0.01

25 ml of 6 to 8 *M* hydrochloric acid with concentrated hydrochloric acid and water was made. Then 5 ml of 0.1 *M* chloroform solution of reagent (*N*-*m*-T-*o*-MBHA) was added and the funnel was shaken for 5 min. Aqueous phase was then removed and the organic phase was transferred to an Erlenmeyer flask containing anhydrous sodium sulfate. The flask was swirled to remove the droplets of water. Then the organic layer was diluted to 25 ml and absorbance of clear organic phase was recorded at 550 nm against a reagent blank as reference. Finally a graph was plotted between absorbance and concentration of vanadium (0.5 to 15 μg). Typical data for a calibration curve is recorded in Table 1.

Extraction of vanadium from natural samples. Into a 100-ml separatory funnel 10 ml of sample solution, 10 ml of blank (standard vanadium), and 10 ml of 12 *M* HCl were taken. Then 0.1 *M* potassium permanganate solution was added drop wise until the pink color persists for 5 min and then 10 ml of 0.1 *M* oxalate and citrate solution was added. The 10 ml of 0.1 *M* reagent solution (in chloroform) was added and contents were shaken for 5 min. The phases were allowed to settle and the violet organic layer was separated and transferred into a 25-ml volumetric flask after drying over anhydrous sodium sulfate. Extraction was repeated twice to ensure the complete recovery of vanadium. Finally the extracts were diluted to 25 ml with chloroform and the absorbance was measured at 550 nm. The concentration was calculated from calibration curve and the difference between sample and blank gave the vanadium content in samples.

RESULTS AND DISCUSSION

The data on the calibration curve for vanadium are given in Table 1. The interference of various foreign ions in the determination of vanadium was studied. It was observed that the following ions (mg/50 ml) did not interfere with the determination of vanadium: Ag^{+} (100 mg), Be^{2+} (100 mg), Mg^{2+} (100 mg), Ca^{2+} (100 mg), Sr^{2+} (100 mg), Ba^{2+} (100 mg), Cu^{2+} (80

TABLE 3
ANALYTICAL DATA FOR VANADIUM DETERMINATION

Vanadium concentration taken (μg/ml)	Vanadium found (μg/ml)		
	Present method	Polarographic method	Ref. (*3*)
0.05	0.05 ± 0.01	0.05 ± 0.01	—[a]
0.20	0.21 ± 0.01	0.20 ± 0.01	0.20 ± 0.02
0.50	0.50 ± 0.01	0.50 ± 0.01	0.50 ± 0.01
5.00	5.02 ± 0.02	5.01 ± 0.01	5.02 ± 0.02
10.00	9.99 ± 0.01	10.01 ± 0.01	10.03 ± 0.02

[a] Cannot be determined.

mg), Cd^{2+} (100 mg), Zn^{2+} (80 mg), Hg^{2+} (100 mg), Pb^{2+} (100 mg), Mn^{2+} (80 mg), Co^{2+} (80 mg), Ni^{2+} (80 mg), UO_2^{2+} (75 mg), Ga^{3+} (100 mg), La^{3+} (100 mg), Fe^{3+} (80 mg), As^{3+} (100 mg), Cr^{3+} (70 mg), Sb^{3+} (80 mg). In $^{3+}$ (80 mg), Th^{4+} (80 mg); 80 to 100 mg of chloride, fluoride, acetate, phosphate, oxalate and citrate. The interferences of Ti^{4+} (70 mg), Zr^{4+} (70 mg), Mo^{6+} (80 mg), and W^{6+} (70 mg) was eliminated by adding oxalate and citrate prior to the extraction of vanadium.

The present method is very rapid, selective, and sensitive. Generally the plants contain the higher amount of molybdenum and titanium (*3, 4*) which intereferes with the determination of vanadium. However, the interference of Mo and Ti was eliminated by adding oxalate and citrate ions. The data on vanadium from natural resources are given in Table 2. The vanadium content found in rat kidney and rat bone is almost background (0.01 μg/ml: Table 2).

A comparison of the present method with other methods for the determination of microgram levels of vanadium is shown in Table 3.

SUMMARY

Vanadium has been extracted and determined from a number of natural resources by liquid–liquid extraction technique using *N*-*m*-tolyl-*o*-methoxybenzohydroxamic acid.

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MICROCHEMICAL JOURNAL 25, 223–227 (1980)

Analytical Reactions of Substituted Cyanoferrates

3. Spectrophotometric Determination of Azide Ion in an Aqueous Medium

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Received June 18, 1979

INTRODUCTION

The colorless pseudohalide ion N_3^- has been found to undergo complexation reactions with several cations. Aqueous reactions of azide ion have been explored and physicochemical data established using electrometric and photometric procedures (*2, 3, 6, 10–12*). However, there are very few analytical procedures reported for its analysis. The reaction of N_3^- ion with Fe^{3+} has been investigated by several authors and perhaps is the best-known direct-colored reaction for this ion (*1, 9*). Neves *et al.* (*7*) have proposed a $Cu^{2+}-N_3^-$ reaction that absorbs in uv, though it is reported to be less sensitive than the $Fe^{3+}-N_3^-$ reaction. Pepkowitz (*8*) has determined N_3^- by measuring the N_2 gas evolved when it is treated in acidic medium with a reducing agent.

The azide ion produces a violet-colored species with pentacyanoamminoferrate(II) in faintly acidic conditions. This reaction has been employed by Jaselskis (*5*) in analytical determination of pentacyanoamminoferrate(II) species. We have further extended the usefulness of this simple reaction and employed it in microanalysis of N_3^- in an aqueous medium. The use of pentacyanoamminoferrate ion has been previously made in our laboratory in the analysis of cations (*4*).

MATERIALS AND METHODS

Reagents

(1) Sodium azide 0.001 *M* was prepared by dissolving 0.0325 g of AR-quality salt in 500 ml of deionized distilled water.

(2) Sodium pentacyanoamminoferrate(II) (PCAF) 0.005 *M* was prepared by dissolving 0.680 g of salt from Fisher Scientific Company in 500 ml of deionized distilled water.

(3) Ammonium acetate/acetic acid buffer was prepared by dissolving 62.5 g of CH_3COONH_4 in 70 ml 5 *M* CH_3COOH and diluting to 250 ml.

(4) The solutions of other ions at 0.01 *M* were prepared similarly by dissolving their sodium salts in deionized distilled water.

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The spectrophotometric data were recorded on Spectronic-70 Bausch and Lomb spectrophotometric using 1-cm matched rectangular quartz cells. The data were obtained in transmission using a digital attachment and were converted to absorbance by using the standard conversion table.

Procedure

Place 10 ml of PCAF reagent in a 50-ml standard flask and add 1 ml of buffer and dilute to approximately 30 ml. Add a desired aliquot of azide solution to yield concentration in the final volume ranging from 1×10^{-5} M to 1.5×10^{-4} M. Complete the volume with deionized distilled water and set aside for 30 min. Prepare a blank in a similar manner without azide ion in solution. Measure the absorbance of the violet-blue complex against blank at 555 nm and prepare a calibration curve using different azide concentrations. Determine the effects of experimental parameters such as acidity, period of contact, and foreign ions in a similar manner at a fixed azide concentration.

RESULTS AND DISCUSSION

The reaction between azide ion and PCAF produces a violet-blue species, which is pH dependent. However, the color development reaches its maximum intensity within 30 min after mixing of reagents as seen in experimental data in Table 1. The pH dependency on the other hand is evident in Fig. 2, wherein pH range 4.5–6 seems most appropriate. The color reaction in alkaline medium ($pH > 9$) is likewise inhibited considerably as in the acidic medium. Hence a buffered medium at pH 5 controlled with ammonium acetate/acetic acid was found appropriate and employed throughout.

The analytical sensitivity of the reaction extends down to 10^{-5} M range or 0.42 ppm azide concentration, which compares favorably with ferric azide reaction. The analytical precision is excellent since no more than 2% coefficient variation was noticed at the concentrations analyzed. The experimental data of this reaction are shown in Fig. 1 and Table 2. The presence of some foreign ions is also tolerable. The quantitative runs with 2.5 ppm N_3^- and 25 ppm of Cl^-, NO_3^-, SO^{2-}_4, PO^{3-}_4, OCN^-, ClO_4^-, ASO^{3-}_4, SO^{2-}_3, $Fe(CN)^{3-}_6$, and $Fe(CN)^{4-}_6$ show no adverse effects on azide absorbance data. The ions Br^-, I^-, SCN^-, $S_2O^{2-}_3$, and CrO^{2-}_4, on the other hand,

TABLE 1

VARIATION OF ABSORBANCE OF PCAF–N_3^- WITH TIME[a]

Time (min)	5	10	15	20	25	30	60	90	120	240
Absorbance	0.096	0.129	0.141	0.147	0.148	0.149	0.150	0.150	0.150	0.149

[a] 10^{-3} M PCAF, 10^{-4} M N_3^-, pH 5.04, λ 555 nm.

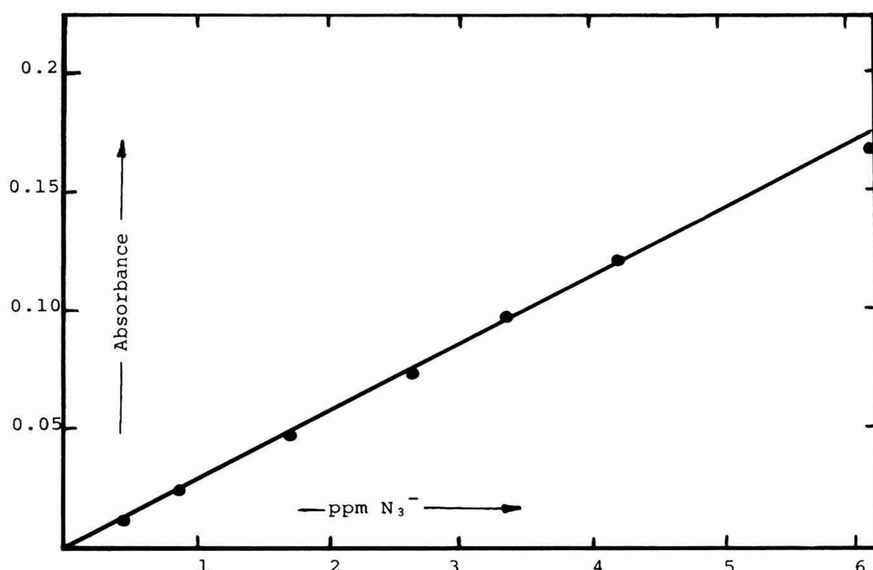
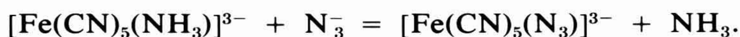


FIG. 1. Calibration curve for N_3^- determination.

have positive effects on N_3^- absorbance with maximum effects noticed with $S_2O^{2-}_3$ and SCN^- ions. The reaction is practically inhibited in the presence of CN^- ions. The ions NO_2^-, N_2H_4, and NH_2OH at the same concentration reduce the absorbance to nearly half and are thus also interferences.

The reaction between PCAF and N_3^- probably stems from displacement of NH_3 by N_3^- in the complex molecule:



$$[Fe(CN)_5(NH_3)]^{3-} + N_3^- = [Fe(CN)_5(N_3)]^{3-} + NH_3.$$

The colored pentacyanoazidoferrate(III) species absorbs at 555 nm. Such a mechanism along with oxidation of Fe^{2+} to Fe^{3+} in the coordination

TABLE 2
CALIBRATION CURVE FOR N_3^-[a]

Concn N_3^- (ppm)	Net absorbance	No. of determinations
1. 0.42	0.012	4
2. 0.84	0.024	4
3. 1.68	0.049	5
4. 2.52	0.073	4
5. 3.36	0.097	5
6. 4.20	0.121	6
7. 6.30	0.167	5

[a] 10^{-3} *M* PCAF, pH 5.03, 30-min contact, 50-ml volume.

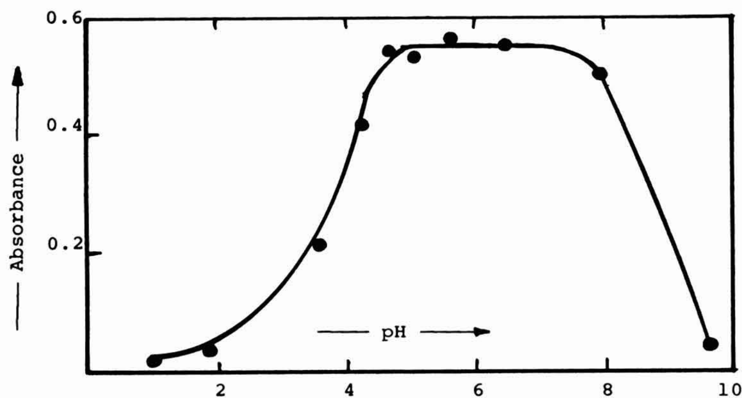


FIG. 2. pH effect on absorption of PCAF$-N_3^-$ reaction.

sphere has been suggested by Jaselskis (*5*). The standard continuous variation and mole ratio techniques applied to this reaction show a break in the curve at a 2:1 proportion between PCAF and N_3^- ion, though not very distinct. Perhaps N_3^- in this reaction acts as a bridging ligand between two PCAF molecules, although an NH_3 molecule is necessarily displaced from the coordination sphere of the complex cyanoferrate to produce colored species. The PCAF–azide complex has a molar absorption 1.2×10^3 liter mol^- cm^{-1}, which is inferior to ferric–azide complex reported to be 2.9×10^3 (*3*). Nonetheles rapidity and noninterference from common anions such as Cl^-, NO_3^-, and SO^{2-}_4 render this procedure still useful in microanalysis of azide in an aqueous medium.

SUMMARY

The pentacyanoamminoferrate(II) ion and N_3^- in acidic condition produce a colored species absorbing at 555 nm that is useful in the spectrophotometric determination of the latter in ån aqueous medium. The results show a linear response for N_3^- in 0.42 to 4.2 ppm range under controlled experimental conditions. Many common anions including Cl^-, SO^{2-}_4, and NO_3^- do not interfere, and color development attains maximum intensity in 30 min.

ACKNOWLEDGMENTS

The authors express their sincere thanks to the Conseil de Recherches, Université de Moncton and the National Sciences and Engineering Research Council of Canada for their continued financial support and encouragement.

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MICROCHEMICAL JOURNAL 25, 228–231 (1980)

Analytical Reactions of Substituted Cyanoferrates

4. Spectrophotometric Determination of Beryllium(II), Gallium(III), and Indium(III) in Solution

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Received July 14, 1979

INTRODUCTION

In a previous study it was shown that azide (N_3^-) ion reacts with pentacyanoamminoferrate(II) in solution to produce a colored species absorbing at 555 nm. However this reaction is inhibited under alkaline conditions (*1*). Further studies have shown that some nontransition metals react with pentacyanoamminoferrate(II) and azide ions in alkaline condition to regenerate the pentacyanoazidoferrate(III) complex. Based on this observation a spectrophotometric procedure for Be^{2+}, Ga^{3+}, and In^{3+} ions is proposed. The reaction is not selective, yet it is sensitive for these ions in the parts-per-million range.

MATERIALS AND METHODS

Reagents

1. Sodium pentacyanoamminoferrate(II), PCAF, 0.05 *M*, was prepared by dissolving 1.355 g of the salt in 100 ml deionized double-distilled water. This was prepared fresh each day.
2. Sodium azide, 0.1 *M*, was prepared by weighing 6.51 g of the compound in 1 liter of deionized double-distilled water.
3. Sodium chloride, 0.1 *M*, was similarly prepared by dissolving 5.850 g in 1 liter of water.
4. Buffer solution was prepared by dissolving 47.67 g borax with 4 g NaOH in 500 ml of water.
5. The solutions of cations in each case at 100 ppm were prepared by dilution from the 1000-ppm Fisher certified stock solutions for atomic absorption work.
6. Absorbance data were recorded on a Spectronic-70, Bausch & Lomb spectrophotometer, using 1-cm matched cells. The electronic attachment indicated data in percentage transmission, which were converted to absorbance using the standard conversion table.

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Procedure

Pour 1 ml PCAF solution into a 50-ml standard flask and add 2 ml of buffer and 2 ml of NaCl solution and dilute to 35 ml with deionized distilled water. Add 5 ml of the azide solution and complete the volume. This serves as a blank in all investigations. Repeat the same procedure for a cation study by adding 0.1 to 10 ml of the 100-ppm metal solution before completing the volume. Set aside the blank and metal-treated solutions for 1 hr. Measure the absorbance of the metal-treated solution against a blank at 555 nm and trace a calibration curve for quantitative work. Determine the effects of other experimental parameters in a similar manner at a fixed cation concentration.

RESULTS AND DISCUSSION

The cations Ga^{3+} and In^{3+} show a linear response in the ranges 0.4–3 and 0.4–4.0 ppm, respectively, whereas for Be^{2+} the analytical data to 10 ppm fall on a curve (Fig. 1). Detection of these ions even at 0.1 ppm is possible but quantification is not feasible since below a 0.4-ppm concentration the analytical curves hold more or less nonlinear responses.

These data were obtained at about pH 9.5 after an equilibration period

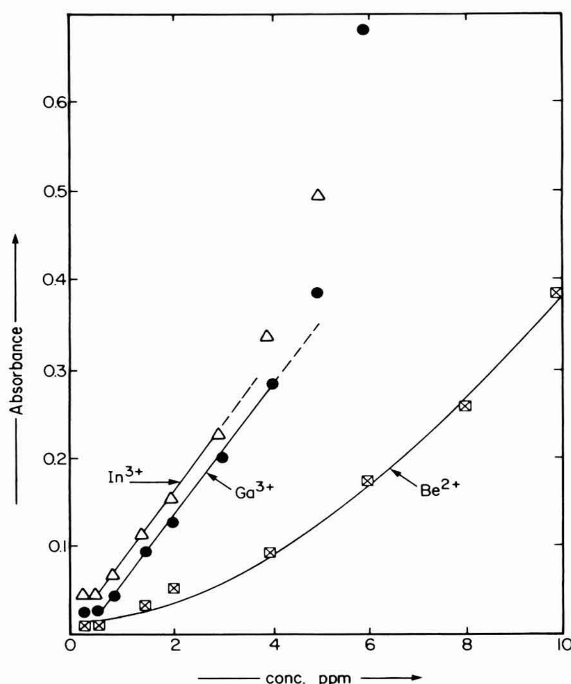

FIG. 1. Analytical response curves for Ga^{3+}, In^{3+}, and Be^{2+} ions in solution.

of 1 hr. The preliminary experiments had shown that color development occurs in the pH range 8–11, but the absorbance of the blank increases somewhat at about pH 8 and decreases slightly around pH 11. Hence an optimal pH 9.5 was chosen for convenience. Likewise, an equilibration period extending from 0.5 to 3 hr had no ill effects on the absorbance of the system and therefore a 1-hr equilibration was fixed to facilitate laboratory manipulations.

On examining the analytical data in Table 1, it is evident that the reaction sensitivity follows the order $In^{3+} > Ga^{3+} > Be^{2+}$. In any event the net absorbance in each case is sufficiently distinct from that of the blank to be useful in microanalysis of these cations.

Use of the PCAF–azide displacement reaction for Ga^{3+}, In^{3+}, and Be^{2+} analysis has never been envisaged and this study thus enhances the use of substituted cyanoferrates as analytical reagents and adds yet another analytical procedure for these cations.

The reaction, however, has limited use since some other cations also accelerate pentacyanoazidoferrate(III) formation. The effects of diverse ions were examined using a 5-ppm concentration in each case, following the general experimental procedure described. The absorbance in each case was compared against Be^{2+} absorbance (least sensitive of the three) at the same concentration. The results show that not more than 5% variation is caused by the cations Cs^{+}, Mg^{2+}, Ca^{2+}, Cd^{2+}, Ge^{4+}, Tl^{+}, Pb^{2}, Au^{3+}, V^{5+}, Cr^{3+}, and Fe^{3+}, while positive contributions over 10% occur with Al^{3+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+} ions. The cations Ag^{+} and Hg^{2+}

TABLE 1
ABSORBANCE DATA FOR Ga^{3+}, In^{3+}, AND Be^{2+} ANALYSIS AT pH 9.5 AND 555 nm

Metal concn (ppm)	Ga^{3+}		In^{3+}		Be^{2+}	
	No. det.[a]	Net abs.[b]	No. det.	Net abs.	No. det.	Net abs.
0.2	8	0.030	6	0.043	8	0.014
0.4	8	0.028	6	0.044	8	0.015
0.8	8	0.046	6	0.067	—	—
1.4	8	0.093	8	0.112	8	0.037
2.0	4	0.127	6	0.151	8	0.053
3.0	4	0.200	8	0.227	—	—
4.0	8	0.282	8	0.335	8	0.091
5.0	4	0.383	6	0.490	—	—
6.0	4	0.680	—	—	8	0.172
8.0	—	—	—	—	8	0.255
10.0	—	—	—	—	8	0.380

[a] Number of determinations.
[b] Net absorbance.

offer a negative interference. The anions Cl^-, Br^-, NO_3^-, OCN^-, SCN^-, $S_2O_3^{2-}$, SO_4^{2-}, and PO_4^{3-}, even at 100 ppm, have no serious effects on the system, but a general increase of the order of 5% occurs in the blank absorbance. In order to circumvent this inconvenience all determinations were made in Cl^- medium (~150 ppm). This treatment maintained a constant ionic force in the medium and in general improved the precision within individual observations. The anions CN^- and EDTA hold a negative effect on the system and as such are interferences.

The results thus show that Be^{2+} in the parts-per-million range can be analyzed using the PCAF$-N_3^-$ reaction in the presence of alkali and alkaline earth metals at a comparable concentration. The cations Ga^{3+} and In^{3+} likewise can be determined in the presence of their immediate neighbors and some other heavy elements. It remains, however, that a preseparation is required for any of these ions if analysis in the presence of a large number of diverse elements is envisaged.

SUMMARY

A new spectrophotometric method for microanalysis of Be^{2+}, Ga^{3+}, and In^{3+} in solution has been developed. These ions accelerate formation of colored pentacyanoazidoferrate(III) species (absorbing at 555 nm) under alkaline conditions. Ga^{3+} and In^{3+} have been quantified in the ranges 0.4–4.0 and 0.4–3.0 ppm, respectively, while for Be^{2+} the range extends to 10 ppm. The reaction is nonselective, but some cations and anions can be tolerated at comparable concentrations.

ACKNOWLEDGMENTS

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MICROCHEMICAL JOURNAL 25, 232–234 (1980)

An Improved Selective Fluoride Electrode

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Received November 5, 1979

INTRODUCTION

The fluoride-selective electrode composed of a lanthanum fluoride crystal and a counter connection has been devised by Frant and Ross and marketed by Orion Research Inc. Studies of lanthanum fluoride by Sher *et al.* (*2*) and Vesely (*6*) and studies of fluoride electrodes by Stahr and Clardy (*3*) have shown conduction through the crystal is truly ionic and surprisingly rapid. The mechanism has been further clarified and some early proposals verified by Van den Winkel *et al.* (*5*).

The response of the electrode "decays" with use. That is, it becomes more sluggish in its response until it is impractical to use. The time period for the response to change can be as little as 1 to 6 months after purchase. The response time becomes as much as 10 times longer than normal. Normal response times are characterized by Mertens *et al.* (*1*). Response *t* 90% as defined by this article becomes 8–10 min instead of 1–2 min for 0.1–1 ppm fluoride level. If one must buy a new electrode every month, it can be an extremely expensive analysis.

EXPERIMENTAL

Apparatus. Orion fluoride electrode Model 94-09, a Corning Research pH meter and/or an Orion Model 701 pH meter.

Reagents. Calcium chloride, hydrochloric acid, and silver nitrate—Fisher reagent grade.

Electrolyte solution. (*4*): Double-distilled water was saturated with calcium chloride. It was then made 1 *M* in hydrochloric acid and 1 or 2 drops of a 1 *M* $AgNO_3$ solution was added.

Procedure. A fluoride electrode that had become unusable due to its long time constant was cut open at the uppermost connection. The electrode is shown in Fig. 1. The lower-level internal connection (the one that touches the lanthanum fluoride crystal) was saturated with electrolyte solution. About 1 ml of electrolyte was added to the tube which forms the outer electrode sheath. The electrode was reassembled and taped together.

Electrode response curves are shown in Fig. 2. The uppermost curve is a new electrode as received. The other curves are for restored electrodes.

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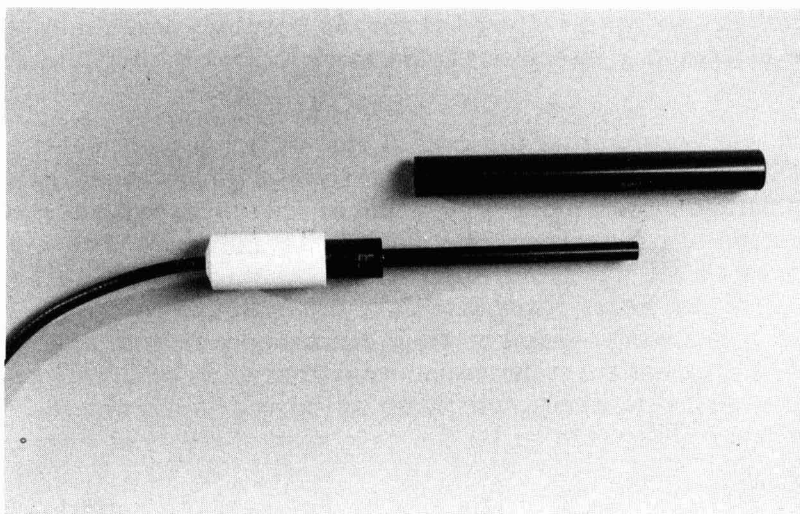

FIG. 1. Sectioned fluoride electrode.

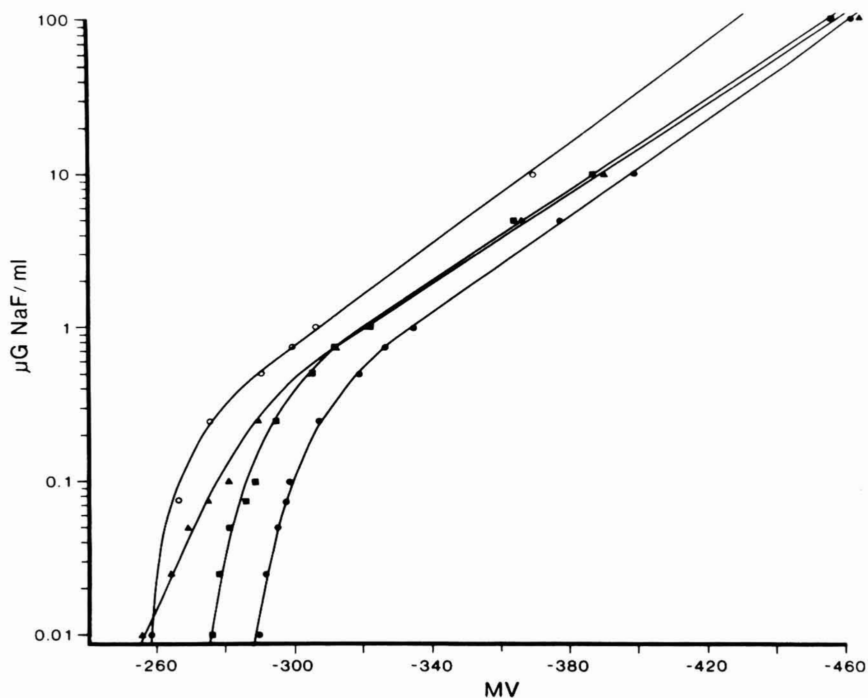

FIG. 2. Response curves of restored fluoride electrodes.

The response time for a restored electrode becomes essentially the same as a new electrode, *t* 90% is 1–2 min as described by Martens *et al.*(*1*).

DISCUSSION

After it was learned that the crystal was truly ionic permeable to fluoride ion (*2, 3, 6*), the lengthening of response time was attributed to a series-resistance (connection-resistance) increase, which makes the concentration-sensitive response difficult to measure. The equivalent components of the system are described by Van den Winkel (*1*). The separation of the series resistance as a separate entity may be done as described in this work. Whether the precise mechanism of failure is electrolyte displacement from the connective fiber wick or drying out of the electrolyte, the new electrolyte addition solves the problem.

This was demonstrated to be the case with a practical restoration of four electrodes. The restored electrodes worked as well as a new electrode. The EMF for the electrode may be shifted slightly after recharging. This may be explained as due to the charging effect of the electrode itself (*1, 3, 5, 6*). Since we first discovered this procedure, we have restored three different electrodes with success.

CONCLUSION

Fluoride electrodes may be restored and used indefinitely by a simple electrolyte regeneration technique.

SUMMARY

The major cause of failure for an Orion Research 9409A fluoride-selective electrode observed in routine use in our laboratory is loss of contact with the LaF_3 crystal. Opening the electrode and replacing the electrolyte restores electrodes that suffer from this effect.

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MICROCHEMICAL JOURNAL 25, 235–239 (1980)

Spectrophotometric Determination of Copper as Tetraphenylarsonium or Tetraphenylphosphonium Thiocyanate–Cuprate

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INTRODUCTION

The extraction of an ion pair formed between a metal thiocyanate and tetraphenylarsonium or tetraphenylphosphonium has been used successfully for the determination of cobalt (*1*, *8*), palladium (*7*), tungsten (*2*, *3*), niobium, (*4*, *5*, *9*), molybdenum (*10*), and titanium (*11*). These methods indicate that the conditions for the extraction of an arsonium or phosphonium ion pair are usually less critical than those for the free complex, and that the arsonium or phosphonium ion pair complex in inert solvents is usually more stable than the free complex.

In this paper a selective and reproducible extraction–spectrophotometric method for the determination of copper is described. It is based on the extraction of copper as an ion-associated complex, formed between the copper(II) thiocyanate and tetraphenylarsonium or tetraphenylphosphonium ion, which is determined spectrophotometrically in chloroform.

Ellis and Gibson (*6*) described a method for the determination of copper based on the extraction of copper as a complex formed between the copper(II) thiocyanate and triphenylmethylarsonium ion which is determined spectrophotometrically in *o*-dichlorobenzene.

The method described here has some advantages in comparison with the previous one (*6*): it is more sensitive, and the intensity of the "color" independs on the concentration of chloride present and on the sequence in which the reagents are added.

MATERIALS AND METHODS

Reagents

Standard copper solution (0.01 *M*) was prepared by dissolving copper sulphate in distilled water. The solution was standardized gravimetrically with cuprone (*12*). Solutions of lower concentrations were obtained by dilution. Tetraphenylarsonium and tetraphenylphosphonium chloride

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(analytical grade, Fluka) were dissolved in reagent-grade chloroform. All chemicals used were of analytical purity.

Apparatus

A Perkin–Elmer Coleman 124 spectrophotometer with 1-cm cells was used.

Procedure

A solution containing 30–500 μg of copper(II), 0.6 ml of 3 M H_2SO_4, 1 ml of 25% (w/v) KSCN, and water up to 10 ml was placed in an Erlenmeyer flask. After the addition of 10 ml of 8×10^{-3} M tetraphenylarsonium or tetraphenylphosphonium chloride in chloroform the solution was shaken for 15 min with a mechanical shaker. After the phases were separated, the absorbance of the organic phase was measured at 465 nm against a reagent blank.

RESULTS AND DISCUSSION

Optimal Conditions for Extraction

The copper(II) complex formed with thiocyanate in sulfuric acid solution can be extracted with tetraphenylarsonium (TPA) or tetraphenylphosphonium (TPP) chloride in chloroform as a reddish complex that has a maximum absorbance at 465 nm. TPA or TPP dissolved in water can be added in aqueous phase and then extraction is carried out with chloroform. However, all experiments showed that copper(II) thiocyanate is better extracted with TPA or TPP dissolved in chloroform. The effects of the concentration of sulfuric acid, potassium thiocyanate, and TPA or TPP chloride were studied and the results obtained are shown in Fig. 1.

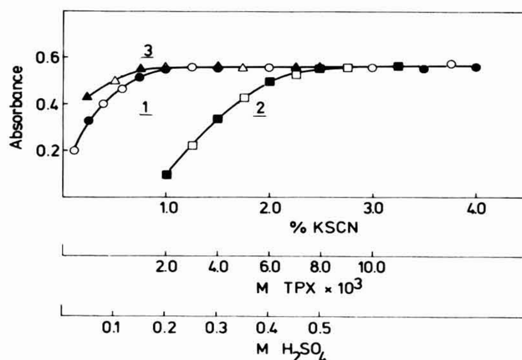

FIG. 1. Dependence of Cu–SCN–TPX complex formation on concentration of (1) KSCN, (2) TPX, and (3) H_2SO_4. Copper conc. 2×10^{-4} M. X = As (empty symbols) and X = P (full symbols).

Optimal concentrations are 0.15–0.50 M H_2SO_4, 1–4% KSCN, and molar ratio of TPA(TPP):Cu must be at least 40. The extracted complex in chloroform obeys Beer's law in the range of 3–50 ppm of copper(II) with an accuracy of ±2%, except at the extreme lower end of the range, where the percentage variation is somewhat higher. Absorbance of the organic phase is stable for at least 3 hr and independs on the sequence in which the reagents are added. The molar absorptivity of the colored extract at 465 nm is 2.8×10^3 liters mol^{-1} cm^{-1}. The photometric sensitivity, as defined by Sandell, is 0.022 μg of Cu cm^{-2}.

Influence of Foreign Ions

Chloride, sulfate, and bromide in 5000-fold amounts do not interfere with copper. Nitrate, phosphate, cyanide, fluoride, acetate, and tartrate are tolerated in a 1000-fold excess, perchlorate in a 100-fold excess, and oxalate in a 10-fold excess. Ammonium, potassium, sodium, magnesium, calcium, strontium, barium, chromium(III), nickel, and manganese(II) do not interfere in a 1000-fold excess. In the procedure described above, up to a 100-fold excess of tungsten(VI) and gallium(III), a 10-fold excess of uranium(VI) and thorium(IV), a 5-fold excess of cadmium, zirconium(IV), and niobium(V), and a 2-fold excess of cobalt, mercury, and zinc have no influence. Large amounts of gallium, thorium, cadmium, zirconium, niobium, cobalt, zinc, and mercury decrease absorbance because they consume TPA or TPP for their own extraction and decrease the concentration of TPA or TPP and the extraction of copper. To prevent this, a higher concentration of TPA or TPP must be used. Molybdenum(VI), iron(III), and palladium(II) interfere and must be removed before copper determination.

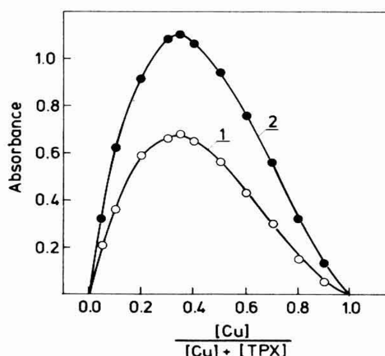

FIG. 2. Determination of complex composition by Job's method. 0.18 M H_2SO_4, 2.5% KSCN. (1) [Cu] + [$(C_6H_5)_4As$] = 4×10^{-3} M and (2) [Cu] + [$(C_6H_5)_4P$] = 5×10^{-3} M.

Composition of the Extracted Complexes

The ratio of copper to TPA or TPP was determined by Job's method of continuous variation. The results obtained show that the molar ratio of copper to onium ion is 1:2 (Fig. 2). This indicates that the anionic copper–thiocyanate complex, bearing two negative charges, must be present in aqueous solutions. It was not possible to determine the ratio of copper to thiocyanate, but the above results show that most probably the copper(II)–thiocyanate anion is $[Cu(NCS)_4]^{2-}$. When onium salts are added, complexes of formula $[(C_6H_5)_4X]_2[Cu(NCS)_4]$ (X = As, P) are formed and transferred into the organic phase.

SUMMARY

The spectrophotometric determination of copper(II) with thiocyanate by extraction of the tetraphenylarsonium and tetraphenylphosphonium ion-association complexes is described. The extracted complexes in chloroform have a maximum absorbance at 465 nm, obey Beer's law in the range of 3–50 ppm of copper, and are stable for at least 3 hr. The molar absorptivity of the method is 2.8×10^3 liters mol^{-1} cm^{-1}. The compositions of the extracted complexes were studied in solution and they are $[(C_6H_5)_4X]_2[Cu(NCS)_4]$ (X = As, P).

ACKNOWLEDGMENT

The author is grateful to Miss Z. Dupin for technical assistance.

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MICROCHEMICAL JOURNAL 25, 240–245 (1980)

Spectrophotometric Determination of Chloride, Bromide, and Iodide with an Improved Mercury–Iron–Thiocyanate Method

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Received December 29, 1979

INTRODUCTION

For the determination of low concentrations of halides a fast and reliable spectrophotometric measuring method was needed. The mercury–iron–thiocyanate method (*2, 3, 6*) appeared suitable. Experiences with different reagents led to the method described below, which uses two stable reagents. Conditions for the determination of chloride, bromide, and iodide were investigated.

METHOD

Reagents

Mercury thiocyanate solution. Dissolve 0.20 g of mercury(II) thiocyanate, $Hg(SCN)_2$, in 150 ml of ethylene glycol monomethyl ether or monoethyl ether and dilute to 500 ml with glacial acetic acid.

Iron solution. Dilute 77 ml of 70% perchloric acid to 150 ml with water. Dissolve 6.0 g of ammonium iron(III) sulfate, $FeNH_4(SO_4)_2 \cdot 12\ H_2O$, in this solution and dilute to 500 ml with glacial acetic acid.

All reagents and solvents were used Merck, Darmstadt pro analysi except for the monoethyl ether, which was for synthesis.

Procedure

To 5.00 (1.00) ml of the halide solution in water add 10.0 (2.00) ml of mercury thiocyanate solution, mix, and add 10.0 (2.00) ml of iron solution. Mix and measure after 10 min in a 10 (50) mm cuvette at 460 nm.

The free figures above give the reagent volumes and cuvette length for halide corresponding to 0–600 μg of chloride, those in parentheses for halide corresponding to 0–25 μg.

RESULTS

Spectrophotometric calibration curves obtained with standard solutions of chloride, bromide, and iodide at different temperatures are shown in Fig. 1, which also shows a calibration curve for chloride obtained with

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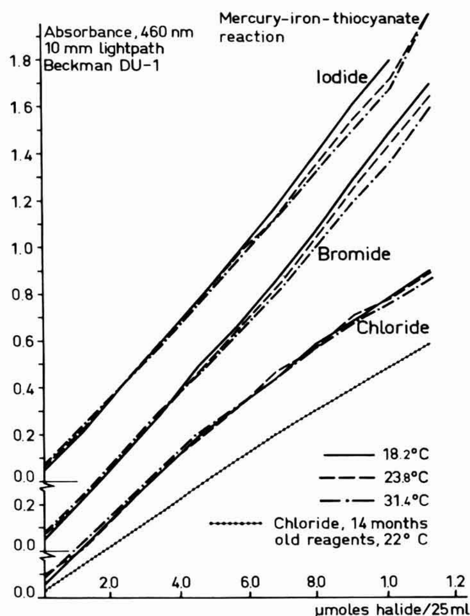

FIG. 1. Influence of temperature. Reagents and halide solutions were kept in a water bath at the reported temperatures. They were mixed according to the described procedure and held further 9 min in the water bath. The solution was then quickly poured into the cuvette and measured.

14-month-old reagents that had been kept in a cupboard at room temperature. Calibration curves obtained with different water contents of the measuring solutions are shown in Fig. 2. The curves are not far from being parallel, which means that systematical volumetric errors have little influence upon the analytical results.

Figure 3 shows the stability of the color with time.

The molar absorptivity of the measuring solutions containing up to 170 μmole/liter of halide is for chloride 3.4×10^3, for bromide 3.9×10^3, and for iodide 4.0×10^3. Above this concentration there is a slight decrease of the values for chloride and a slight increase of those for bromide and iodide.

In order to test the applicability of the method a series of halogen determinations in organic compounds was carried out using the earlier described modification of the Schöniger flask combustion method (*4*). The results are reported in Table 1. We tried to simplify the combustion method further by weighing the samples into aluminum capsules. This was not entirely satisfactory. We have, however, no possibility to continue the experimental work in the reasonably near future.

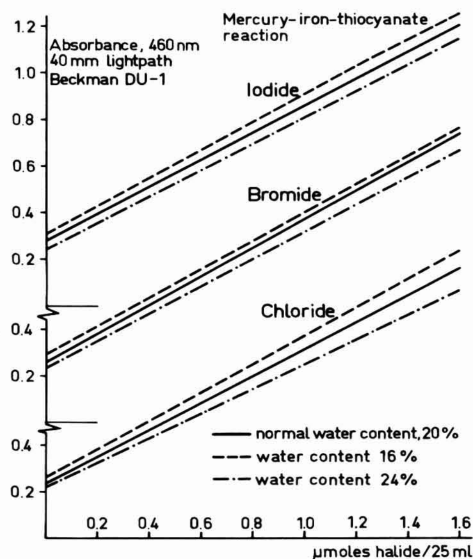

FIG. 2. Influence of water content. The experiments were carried out as described under Procedure, except that different volumes of halide solutions, containing the same total amount of halide, were used, and the total volume was finally made up to 25 ml with glacial acetic acid.

DISCUSSION

We started our experiments with described methods that used nitric acid to create the necessary acidity for the reaction. The mercury(II) thiocyanate was dissolved in different organic solvents, and in some cases the reaction itself was carried out in an organic solvent solution. Con-

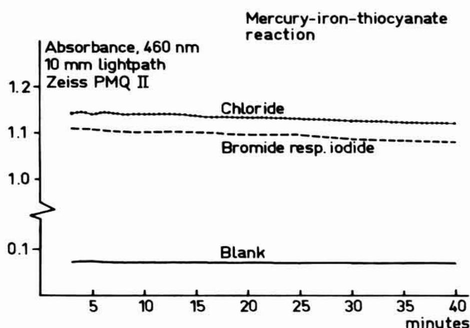

FIG. 3. Stability of color with time. Temperature 22°C; chloride solution, 9.02 µmole chloride/25 ml; bromide and iodide solutions, 6.76 µmole halide/25 ml; 10 min after the mixing was considered to be the most suitable time for the measurement.

TABLE 1
ANALYSES OF ORGANIC COMPOUNDS[a]

Substance	Sample wt (μg)	Results: Found (%)	Calculated (%)	Deviation (%)
1-Chloro-2,4-dinitrobenzene	855.1	17.3	17.5	−0.2
	898.5	17.7	17.5	+0.2
	962.6	17.5	17.5	0.0
	1153.0	17.5	17.5	0.0
	1389.1	18.3	17.5	+0.8*
	1367.2	17.2	17.5	−0.3
Hexachlorobenzene	259.1	71.4	74.7	−3.3*
	326.6	75.0	74.7	+0.3
	234.8	74.5	74.7	−0.2
4-Chlorobenzoic acid	873.5	22.1	22.7	−0.6
	1150.7	22.8	22.7	+0.1
	1149.6	22.9	22.7	+0.2
	705.7	20.3	22.7	−2.4*
7-Iodo-8-oxyquinoline-5-sulfonic acid	1488.9	36.5	36.2	+0.3
	1574.4	36.4	36.2	+0.2
	1520.7	35.3	36.2	−0.9
4-Iodoaniline	1185.1	57.6	57.9	−0.3
p-Bromoacetanilide	1152.0	35.2	37.3	−2.1*
	1431.2	37.4	37.3	+0.1
	1242.3	37.6	37.3	+0.3
	1115.1	37.8	37.3	+0.5
	1022.2	38.2	37.3	−0.9
	1024.9	37.3	37.3	0.0
	1572.6	37.2	37.3	−0.1
3-Bromo-2-thiophenic acid	1296.3	38.9	38.6	+0.3
	921.2	39.4	38.6	+0.8
	914.8	39.1	38.6	+0.5
Hexabromoethane	539.8	94.2	95.2	−1.0
	418.8	95.1	95.2	−0.1
	666.6	95.8	95.2	+0.6

[a] The samples were weighed out in aluminum capsules, Reinhardt, CH 4125 Riehen, 136 Rainallee, Switzerland, No. 396, wrapped into paper, and burned. Chloride was absorbed in 5 ml of water, bromide and iodide in 0.5% hydrazine hydrate solution in water. In analyses marked * capsules fell down, which caused incomplete combustion.

tradictory results were frequently obtained with different batches of the same reagent. Not only the results were different, but the color was stable in some cases, in others it faded or it increased. Similar experiences were reported also by Elsheimer and Kochen (*1*). Results of experiments indicated that the nitric acid reacted with small impurities in the organic

solvents in different ways, and in some cases probably even with the solvents themselves. It appeared, therefore, desirable to use an acid with a lower reactivity at room temperature and to use solvents that are resistant to oxidation and that are easy to obtain in a very pure state. Kulhánek and Fišer (*5*) use perchloric acid, which is very stable at room temperature, and most modern methods for the microdetermination of chloride use acetic acid as solvent. Very pure acetic acid is easily available, and it is highly resistant to oxidation.

It was more difficult to find a suitable solvent for the mercury thiocyanate, but analytical-grade ethylene glycol monomethyl ether was finally found to give stable solutions. Ethylene glycol monoethyl ether dissolved the mercury thiocyanate as well, and a specimen, Merck, for synthesis, was also found satisfactory. The reported work was carried out with this specimen. Small amounts of precipitate were obtained when solutions of mercury thiocyanate in the organic solvent were allowed to stand for a week. The reagent with the acetic acid, however, is stable for several months at room temperature.

The iron solution contains a high concentration of perchloric acid together with a high concentration of acetic acid. Spoons and spatulas with drops of the solution were held into the flame of a Bunsen burner. The solution evaporated quietly in the flame without puffing. There seems to be no risk for explosions even under severe conditions.

SUMMARY

Stable reagents for the spectrophotometric mercury–iron–thiocyanate method for the determination of halide have been developed. The molar absorptivity for concentrations up to 170 μmole/liter of halide is for chloride 3.4×10^3, for bromide 3.9×10^3, and for iodide 4.0×10^3. Above this concentration there is a slight decrease of the absorptivity for chloride and a slight increase for bromide and iodide.

ACKNOWLEDGMENTS

Skilled technical assistance from Cecilia Cornejo and Ann-Britt Nyman with a part of the work is gratefully acknowledged.

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MICROCHEMICAL JOURNAL 25, 246–253 (1980)

Investigation of *m*-Nitroaniline Formation during the Zimmermann Reaction

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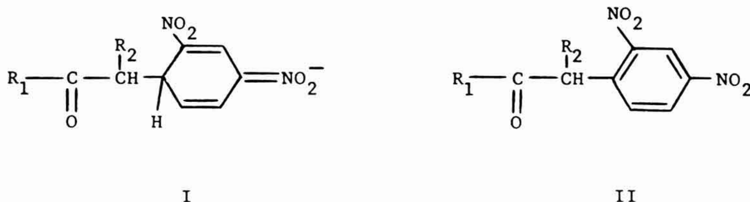
Received January 4, 1980

INTRODUCTION

In 1904, Reissert isolated and identified *m*-nitroaniline as one of the products formed during the steam distillation of acetone and *m*-dinitrobenzene under alkaline conditions (*10*). This phenomenon was confirmed in 1962 by King and Newall (*6*). In 1965, Nambara and Katō (*7*) extended these findings by identifying *m*-nitroaniline as a major product of the reaction between dehydroisoandrosterone and *m*-dinitrobenzene under alkaline conditions. The formation of *m*-nitroaniline had been recognized as evidence of an oxidation step which may account for the conversion of structure **I** to structure **II** (*4, 6*). This was also in agreement with other research groups that had concluded that excess *m*-dinitrobenzene acted as an oxidizing agent (*5, 7, 9*).



In contrast to the above conclusions, recent investigation of the Zimmermann reaction in this laboratory clearly shows that *m*-nitroaniline is not formed under room-temperature reaction conditions, as commonly employed in clinical laboratories for the determination of 17-ketosteroids (*3*). A detailed investigation is presented herein.

MATERIALS AND METHODS

Δ^5-Androsten-3β-ol-17-one (dehydroisoandrosterone, dehydroepiandrosterone) was purchased from Sigma Chemical Company, St.

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Louis, Missouri. Certified reagent grades of acetone, benzene, chloroform, ether, *m*-dinitrobenzene (DNB), and potassium hydroxide were obtained from Fisher Scientific, Fair Lawn, New Jersey. Practical grade of *m*-nitroaniline (NA), from Eastman Kodak Company, Rochester, New York, was twice recrystallized prior to use. Purity was established by TLC. Absolute methanol (ACS grade) was from the J. T. Baker Chemical Company, Phillipsbury, New Jersey. Infrared spectroscopy grade potassium bromide was purchased from Matheson, Coleman & Bell, Norwood, Ohio.

Thin-layer chromatography (TLC) was performed on Quanta/Gram precoated TLC plates (Q5F, 20 × 20-cm size) from Quantum Industries, Fairfield, New Jersey. Developed TLC plates were examined under shortwave uv light (253.7 nm) with a Universal UV Unit from the Gelman Instrument Company, Ann Arbor, Michigan. Steam distillation experiments were performed in a Quickfit distillation apparatus obtained from Canadian Laboratories Supplies, Winnipeg, Manitoba, Canada. Ether extracts were evaporated with a Büchi Model R rotary vacuum evaporator from Brinkmann Instruments, Rexdale, Ontario, Canada. Infrared spectra were recorded with a Perkin–Elmer Model 268 infrared grating spectrometer from Perkin Elmer Canada, Ltd., Montreal, Quebec, Canada. Photography was by the Audio-Visual Service, University of Regina, Regina, Saskatchewan, Canada.

A stock solution of alkaline DNB was prepared by dissolving 2.0 g of DNB and 1.0 g of potassium hydroxide in a 50-ml volumetric flask which was filled to volume with absolute methanol (*6*). Ten milliliters of this solution was pipetted into each of three Erlenmeyer flasks. To the first flask, 1.0 ml of acetone was added giving an acetone/DNB ratio of approximately 5:1. To the second and third flasks, 0.2 ml and 0.04 ml of acetone were added, giving approximate acetone/DNB ratios of 1:1 and 0.2:1, respectively. The solutions were allowed to react for 60 min at room temperature in the dark. Thereafter, 20 ml of distilled water was added to each flask, followed by extraction with two 10-ml volumes of ether. The extracts of each flask were immediately evaporated to dryness under vacuum at 30°C with a Büchi rotary evaporator. Five milliliters of ether was added to each flask to reconstitute the dry residues, producing concentrated ether extracts for the subsequent TLC analyses. Concentrated ether extracts of each of the following reagent blank solutions were similarly prepared: 10.0 ml of alkaline DNB; 1.0 ml acetone and 0.4 g DNB in 10.0 ml of absolute methanol; and 2 mg NA and 0.2 g potassium hydroxide in 10.0 ml of absolute methanol. Twenty microliters of each of the concentrated ether extracts was spotted on a Quanta/Gram precoated TLC plate, 3.0 cm from the left edge and at intervals of 2.5 cm. Chromatographic separation was performed in a CHROMAGRAM Developing Apparatus

from Fisher Scientific, Winnipeg, Mannitoba, Canada. The solvent system consisted of benzene and ether (50:50, v/v) (*8*). One hundred milliliters of freshly prepared solvent was transferred to the chromatography trough. The chromatography chamber was assembled and the solvent system was allowed to migrate for 60 min. The TLC plate was air-dried and examined under shortwave uv light. The plate was photographed and R_f values were calculated.

A second series of acetone and DNB reaction mixtures was similarly prepared as described above. The reaction mixtures were incubated at room temperature for 60 min. Thereafter, 20 ml of distilled water was added to each flask and steam distillation was performed. Each of the following reagent blank solutions was similarly treated and subjected to steam distillation: 10.0 ml alkaline DNB; and 1.0 ml acetone with 0.4 g DNB dissolved in 10.0 ml absolute methanol. Preparation of concentrated ether extracts, TLC separation, photography, and establishment of R_f values were performed as previously described above.

The Zimmermann reaction for the determination of 17-ketosteroids was evaluated at room temperature and at elevated temperature, similar to the acetone test procedure described above. Reaction mixtures containing dehydroepiandrosterone/DNB ratios of 5:1, 1:1, and 0.2:1 were prepared by dissolving 3.432, 0.686, and 0.137 g of dehydroepiandrosterone in each of three 10-ml volumes of stock alkaline DNB solution, respectively. The ether extraction for the room-temperature study required a total of 50 ml of ether in order to achieve two separate phases. Each of the following blank solutions was similarly tested: 10.0 ml of alkaline DNB stock solution; 0.686 g of dehydroepiandrosterone and 0.2 g of potassium hydroxide dissolved in 10.0 ml of absolute methanol; and 3.432 g of dehydroepiandrosterone and 0.4 g of DNB dissolved in 10.0 ml of absolute methanol. Preparation of concentrated ether extracts, TLC separation, photography, and establishment of R_f values were performed as previously described above.

An alkaline solution was prepared to contain acetone and DNB in approximately 0.2:1 molar ratio as previously described. The solution was allowed to react for 24 hr at room temperature in the dark. Preparation of the ether extract and subsequent chromatographic separation were performed as described above. An alkaline solution containing dehydroepiandrosterone and DNB in a 0.2:1 molar ratio was similarly prepared and tested as described above.

NA standard recovery studies were performed. An alkaline solution was prepared to contain acetone and DNB in a 5:1 ratio as previously described. The solution was allowed to react for 60 min at room temperature in the dark. Two milligrams of NA was added and allowed to dissolve. A concentrated ether extract was prepared and TLC separation

was performed as previously described. The above procedure was similarly performed for each of the following alkaline solutions: 1:1 molar ratio of acetone and DNB; and 5:1 and 1:1 molar ratios of dehydroepiandrosterone and DNB.

Preparative TLC was performed to isolate the yellow product which had the same R_f value and color as the NA standard. After chromatographic separation, the yellow band was scraped off the chromatogram. Five milliliters of chloroform was employed to elute the yellow product from the silica gel. The chloroform extract was concentrated to approximately 1 ml under a stream of nitrogen at room temperature. A potassium bromide disk of 1.5-mm diameter was prepared to contain 10 μl of the concentrated chloroform extract. The chloroform was allowed to evaporate and the disk was formed under vacuum pressure. The infrared spectrum of the disk was recorded on a Perkin–Elmer Model 268 infrared grating spectrometer. The spectrum was compared to spectra obtained from a similarly prepared NA standard potassium bromide disk.

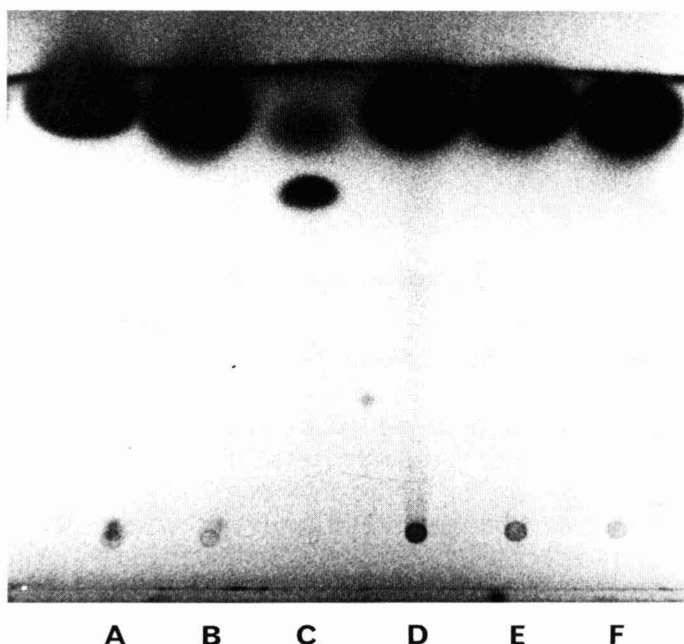

FIG. 1. Thin-layer chromatographic separation of concentrated ether extracts of each of the following room-temperature reacted solutions: (A) DNB + KOH; (B) acetone + DNB (5:1 molar ratio)*; (C) NA + KOH; (D) acetone + DNB (5:1 molar ratio)* + KOH; (E) acetone + DNB (1:1 molar ratio)* + KOH; (F) acetone + DNB (0.2:1 molar ratio)* + KOH. *Approximate molar ratios are presented. Refer to text for complete details.

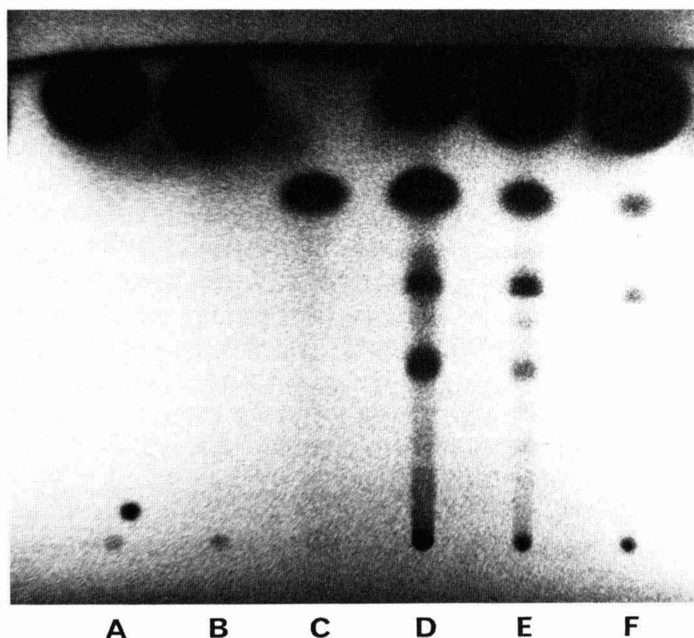

FIG. 2. Thin-layer chromatographic separation of concentrated ether extracts from steam distillation residues of reacted solutions containing: (A) DNB + KOH; (B) acetone + DNB (5:1 molar ratio)*; (C) NA + KOH**; (D) acetone + DNB (5:1 molar ratio)* + KOH; (E) acetone + DNB (1:1 molar ratio)* + KOH; (F) acetone + DNB (0.2:1 molar ratio)* + KOH. *Approximate molar ratios are presented. **The above test solution was not subjected to steam distillation. Refer to text for complete details.

RESULTS AND DISCUSSION

The present research investigation attempted to reproduce the King and Newall procedure (*6*) for the isolation of NA from the steam distillate of the reaction between acetone and DNB in alkaline medium. But the ether extraction was not successful, because the addition of ether formed only one phase with the steam distillate. However, NA was isolated from the steam distillation residue by ether extraction and subsequent TLC separation. Under the experimental conditions of this laboratory, the average R_f values ($n \geqslant 18$) for DNB, NA, and dehydroepiandrosterone were 0.90, 0.71, and 0.61, respectively. The NA was observed to migrate between the DNB and other unidentified reaction products below R_f 0.52 (see Figs. 1–3). The large separation distances and the yellow color of the NA spots simplified the identification process. Furthermore, the detection limit for NA, when observed under shortwave uv light (253.7 nm), was established to be below 1 μg.

The thin-layer chromatogram depicted in Fig. 2 shows that NA was

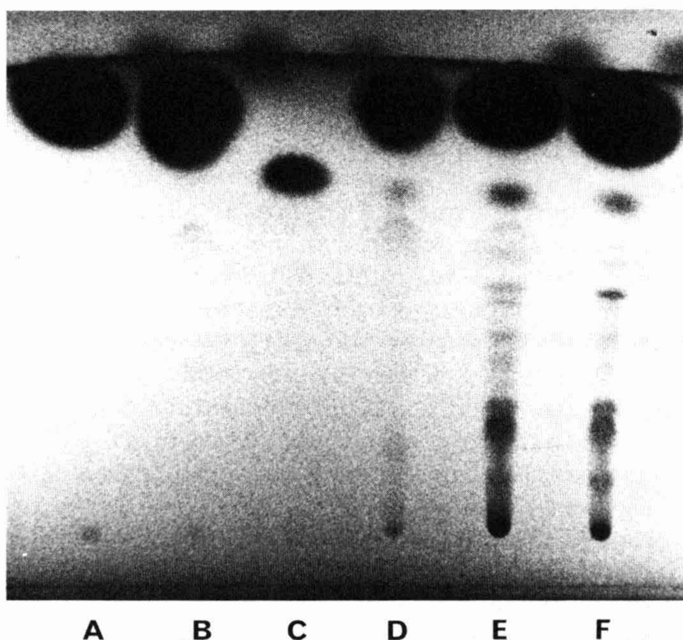

FIG. 3. Thin-layer chromatographic separation of concentrated ether extracts from steam distillation residues of reacted solutions containing: (A) DNB + KOH; (B) dehydroepiandrosterone + DNB (5:1 molar ratio); (C) NA + KOH**; (D) dehydroepiandrosterone + DNB (5:1 molar ratio) + KOH; (E) dehydroepiandrosterone + DNB (1:1 molar ratio) + KOH; (F) dehydroepiandrosterone + DNB (0.2:1 molar ratio) + KOH. **The above test solution was not subjected to steam distillation. Refer to text for complete details.

present for acetone/DNB molar reaction ratios of 5:1, 1:1, and 0.2:1, while NA was not isolated from similarly tested steam-distilled reagent blanks. Preliminary identification of NA was by comparison of R_f values and yellow spot color. The analysis of infrared spectra of known and of TLC-isolated test material conclusively confirmed that NA was present in the steam-distilled residues.

In contrast, the results depicted in Fig. 1 clearly indicate that NA was not formed during the room-temperature reaction between DNB and acetone under alkaline conditions. The investigation was performed in triplicate for each of the acetone/DNB molar ratios tested (5:1, 1:1, and 0.2:1).

The TLC results of the steam-distilled residues of the dehydroepiandrosterone reaction with DNB in alkaline medium are depicted in Fig. 3. NA was isolated from the 5:1, 1:1, and 0.2:1 dehydroepiandrosterone/DNB reaction mixtures. Similarly, NA was also present for each of the three acetone/DNB ratios tested. However, the NA spot size for the acetone/DNB-reacted solutions was observed to increase with increasing

acetone concentration, while the NA spot size was observed to decrease for the 5:1 dehydroepiandrosterone/DNB reaction (see Fig. 3). These test results were confirmed by duplicate and subsequent triplicate analyses. The observed decrease is presently unexplained.

With the present extraction procedure and under the established TLC detection limit, NA was not isolated for the room-temperature reaction between dehydroepiandrosterone and DNB in alkaline medium. The TLC results were similar to those depicted in Fig. 1; however, dehydroepiandrosterone was observed to migrate at R_f 0.61. Furthermore, in another investigation the room-temperature reaction time was extended to 24 hr. NA was not isolated for either the acetone nor the dehydroepiandrosterone reactions with DNB in alkaline media. To evaluate the recovery process, NA was added to room-temperature reacted solutions at similar concentration to that previously isolated by other investigators (*6*). The NA was easily isolated from the dry residue by ether extraction and TLC.

The formation of NA has been described as evidence of an oxidation step required for the conversion of structure I to structure II (*4, 6*). However, the conversion of DNB to NA was not observed to occur to any appreciable extent, under the reaction conditions employed, during a previous polarographic investigation (*2*). Furthermore, the present TLC studies show that elevated temperatures are required for the formation of NA. A similar phenomenon has been reported for the Jaffé reaction, creatinine reacting with alkaline picrate. At elevated temperatures, picramic acid has been proposed (*1*), while under room-temperature conditions, such does not occur (*11*).

The present investigation provides further experimental evidence to elucidate the mechanism of the Zimmermann reaction, as performed in clinical chemistry laboratories for the determination of 17-ketosteroids. The results presented herein clearly show that NA was not present in the room-temperature test material. Thus contrary to the conclusions of previous investigators (*4, 6*), NA cannot be considered as evidence of the conversion of structure I to II. If structure II is formed under room-temperature reaction conditions, an alternative mechanism must be sought to account for such.

SUMMARY

The Zimmermann reaction for the determination of 17-ketosteroids was tested under both room-temperature and steam-distillation reaction conditions. *meta*-Nitroaniline was isolated from the residue of the steam distillation by ether extraction and thin-layer chromatography. Conclusive identification was by infrared spectroscopy. In contrast, *m*-nitroaniline was not formed under room-temperature reaction conditions, even when allowed to react for 24 hr. Similar results were also obtained for the reaction between acetone and *m*-dinitrobenzene under alkaline conditions. In conclusion, the results indicate

that *m*-nitroaniline formation cannot account for the conversion of structure I to structure II under room-temperature reaction conditions as investigated herein.

ACKNOWLEDGMENTS

The authors thank the Natural Sciences and Engineering Research Council of Canada for financial support of this work. Special thanks are extended to Dr. H. E. Robertson, Director of Laboratories, and Dr. A. E. Emery, Chief of Chemistry, Provincial Laboratories Saskatchewan Health, Regina, for providing instrumentation and technical guidance during the infrared spectroscopy studies.

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MICROCHEMICAL JOURNAL 25, 254–256 (1980)

Interference of Potassium on Barium Measurements in the Inductively Coupled Plasma[1]

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Numerous reports indicate the lack of chemical interferences (condensed-phase interferences, such as those related to the lack of complete vaporization, ionization interferences, compound formation interferences, etc.) in the inductively coupled plasma (ICP) when used as an emission source (*4–7, 9*). However, there have been several reports (*2, 3, 8*) which indicate subtle chemical-type interferences, particularly in low-power ICPs, such as those operated at powers below 1 kW but also in the ICP system operated under normal analytical conditions. Barnes and Nikdel (*1*) via theoretical calculations have shown that there should be incomplete vaporization (only ~70% complete) of a spherical Al_2O_3 particle (10^{-3} mm diameter) at a height of 60 mm above the coil of a 1.5-kW ICP when operated with a carrier gas flow rate of 1.5 liter/min and over when assuming a plasma temperature of 8000–10,000°K. The latter temperature is several thousand degrees too high based upon more recent experimental temperature measurements (*4–7, 9*).

Our interest in chemical interferences in the ICP was enhanced because of the observations of light scatter from water droplets at rather low heights in the ICP (*10*). Therefore, the present study was performed to determine the influence of an ionizer (potassium) upon the Ba-atom and Ba-ion emission signals produced in an ICP as a function of height (z) above the lead coil and as a function of ICP input power. A spatial area resolution of 0.03 mm^2 was achieved in the present study. All instrumentation and experimental conditions have been described previously (*11*). Analytical-grade reagents were used to prepare the stock solutions of barium and potassium and diluted to volume with doubly deionized water.

The results of these studies are shown in Table 1 where the ratios of Ba(I) with K to Ba(I) without K, Ba(II) with K to Ba(II) without K, Ba(II) without K to Ba(I) without K, and Ba(II) with K to Ba(I) with K are given

[1] Research supported by AF-AFOSR-F44620-76-C-0005.

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0026-265X/80/020254-03$01.00/0

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TABLE 1

VARIATION OF BARIUM-ATOM EMISSION (λ = 553.5 nm) AND BARIUM-ION EMISSION (λ = 455.4 nm) WITH VARIATION OF ICP INPUT POWER, OBSERVATION HEIGHT, AND PRESENCE OF POTASSIUM[a]

Observation height (mm)	Input power (kW)	$\frac{B}{A}$[b,c]	$\frac{D}{C}$[b,c]	$\frac{C}{A}$[b,d]	$\frac{D}{B}$[b,d]
12	0.7	4	4	3	3
15	0.7	6	3	5	2
18	0.7	7	2	8	2
21	0.7	6	2	7	2
24	0.7	5	1	7	1
27	0.7	6	1	9	2
30	0.7	5	0.6	9	1
12	1.1	4	3	4	4
15	1.1	5	4	10	7
18	1.1	8	5	50	28
21	1.1	8	3	25	10
24	1.1	6	2	42	14
27	1.1	5	2	74	23
30	1.1	4	1	100	29
12	1.5	3	3	11	10
15	1.5	3	4	102	124
18	1.5	1	5	170	180
21	1.5	4	5	320	390
24	1.5	3	3	1100	930
27	1.5	3	2	1200	700
30	1.5	2	2	440	310

[a] Ba(II) or Ba(I) signals measured for 100 μg/ml Ba with no K or with 1000 μg/ml K added.

[b] A = Ba(I) emission with no K; B = Ba(I) emission with K added; C = Ba(II) emission with no K; D = Ba(II) emission with K added.

[c] Values given have standard deviations of $\leq$ 0.2.

[d] Values given have percentage relative standard deviation of $\leq$ 10%.

for several observation heights (z = 12–30 mm) and for three plasma input powers (0.7, 1.1, and 1.5 kW). By inspection of the results in Table 1, it is clear that: (i) Ba(II) emission exceeds Ba(I) emission at all heights; (ii) Ba(II) and Ba(I) emission signals are greater in the presence of K; (iii) Ba(I) emission with or without K decreases by a factor of ~2 to 10× as the height increases from 12 to 30 mm; (iv) Ba(II) emission without K generally increases by a factor of ~2 to 6× as the height increases from 12 to 30 mm; (v) Ba(II) emission with K decreases by a factor of ~5× at 0.7 kW and increases by a factor of ~4× or less at 1.1 and 1.5 kW as the height increases from 12 to 30 mm.

Clearly, the effects noted are smaller than in combustion flames (*3, 4*) but, nevertheless, interference effects exist. These effects are most likely a complex interaction of the ICP temperature variation, condensed-phase

vaporization interference, and an ionization interference similar to the problems noted by Kornblum and DeGalan (*8*) in a prior study with a low-power plasma. No attempt was made to determine an exact model to explain these effects. However, it is quite clear that at the normal height of ≈24 mm, the emission signals of Ba(II) or Ba(I) with or without K are not extremely sensitive to variation in heights. However, the Ba(II) and Ba(I) emission signals are quite sensitive to variation in plasma input power and to the presence of K. Therefore, even though the ICP is much less susceptible to ionization and condensed-phase interferences than flames, it is clear that one should at least determine the susceptibility of a given analyte emission to variation in the concentration of species comprising the sample matrix. Finally, matrix matching of samples and standards in most real analytical problems is probably still necessary even when using the ICP as an emission source.

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MICROCHEMICAL JOURNAL 25, 257–263 (1980)

BOOK REVIEWS

Chromatography of Synthetic and Biological Polymers, Vol. 1. Column Packings, GPC, GF and Gradient Elution. Edited by ROGER EPTON. Halsted Press, New York, 1978. ix + 368 pp., $42.50.

This is Volume 1 of a two part series and is based upon a Chemical Society Symposium held at the University of Birmingham, U.K. July 7–9, 1976. The papers are divided into three sections: General Developments, Preparative and Industrial Scale Chromatography, and Specialized Applications, Theory, and Techniques.

The eleven chapters in part 1 comprise over half of this volume and cover polysaccharide supports, inorganic packings, three types of gel packings as well as polymer structures, mechanisms of fractionation and thin-layer gel chromatography, and service problems in GPC. Each of these chapters is extensively illustrated with graphs, tables, molecular structures and diagrams. Ample up-to-date (1976) references are included at the end of each. This assemblage of international authors has followed no set pattern, but each chapter achieves the goal of presenting the important recent developments in the science of macromolecular fractionation along with a substantial background.

The three chapters in part 2 deal with preparative gel permeation chromatography, continuous chromatography, and column fractionation by gradient elution. Each is amply specific with working details, diagrams, schematic, charts, and references.

Part 3 consists of fourteen short papers on specific topics, theory, and techniques.

The fact that workers in this field are divided into physical scientists who are mostly concerned with the synthesis and mechanics of polymers on the one hand and life scientists who are mostly concerned with the applications of these polymers on the other hand is clearly noted in this volume.

This is a rather expensive but well put together book. It should provide service as a reference book for some time.

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Separation and Purification, 3rd Edition. Edited by E. S. PERRY AND A. WEISSBERGER. John Wiley and Sons, New York, 1978. xi + 438 pp., $30.00.

Purity is a somewhat elusive concept. What may be a high state of purity for some applications may be grossly impure for others. The development of ever more sensitive methods of analysis repeatedly has shown that samples once thought pure actually contained many contaminating elements. The purpose of this book is to review and discuss the concept of purity and methods for separation and purification of chemical compounds. This book is volume XII of the series entitled Techniques of Chemistry edited by A. Weissberger.

The book includes seven chapters, a name index, and a subject index. The first chapter, prepared by M. Zief and A. Barnard, Jr., discusses the concept and reality of purity. In chapter 2, L. Snyder reviews criteria for selecting the solvent to be used in separation procedures. E. G. Scheibel presents the theory and practical application of the liquid–liquid extraction technique of separation. In chapter four, P. Brown and A. Krstulovic thoroughly review all aspects of the use of ion-exchange chromatography as a separation procedure.

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The widely used procedure known as affinity chromatography or ligand-specific chromatography is discussed by S. May in chapter five. The use of centrifugal force as a separation technique is reviewed by C. Ambler and F. Keith in chapter six. The last chapter, by C. Oulman and E. Baumann, examines in detail the use of filtration as a separation procedure.

The editors have done an excellent job of selecting topics and authors and assembling this excellent text. The contents have been revised and updated for this 3rd edition and four of the chapters are completely new. This book should prove to be a valuable source of information for individuals involved in chemical analysis.

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Nuclear Microanalysis. By VLADO VALKOVIĆ. Garland, New York, 1977. xii + 415 pp., $27.00.

This book describes some fundamental laws of radioactivity. This is followed in a logical order by chapters on nuclear reactions, charged-particle activation analysis, and neutron activation analysis. Finally, the author treats the subject of charged-particle induced X-ray emission spectroscopy. Each chapter is rich in references and, therefore, is excellent from a reference standpoint. Those working or intending to work in this field will find the book valuable.

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Environmental Analysis. Edited by G. W. EWING. Academic Press, New York, 1977. xiii + 344 pp., $18.50.

Identification of environmental pollutants and quantification of the levels present in the environment have been and continue to be challenging tasks for the analytical chemist. At the Third Annual Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies, a large number of papers were presented dealing with the problems of environmental analysis. For this volume, the editor has selected papers from those presented at this meeting covering a variety of aspects of environmental analysis.

The book includes 23 papers and a brief subject index. These papers can be roughly grouped into those dealing with air pollutants, those dealing with water pollutants, and those dealing with other topics. The group of papers on air pollutants includes six papers on topics such as the application of infrared spectrometry to the analysis of air pollutants, the measurement of airborne lead, the use of laser Raman spectroscopy for environmental analysis, the use of anodic stripping voltammetry for analysis, and the analytical use of the tunable semiconductor laser. The second group includes papers on the mass contribution of the atmosphere to bodies of water; methods for measuring adenosine triphosphate, phenolic compounds, organics, arsenic, selenium, ammonia; methods for the analysis of beach sand components and contaminants; and the use of remotely sensed and locally acquired optical data for the determination of water quality. The last group of papers includes articles on the application of methods such as flame resonance spectrometry, chromatography using a nondispersive atomic fluoresence detector, inductively coupled argon plasma optical emission spectroscopy, poly(dithiocarbamate) chelating ion-exchange resin chromatography,

and gas chromatography–mass spectometry, plus the use of green tea leaves and pepperbush leaves as environmental pollution standards and an evaluation of a personal monitoring device for vinyl chloride exposure.

The editor has selected papers which should prove of interest to many of the individuals responsible for the analysis of environmental samples. This volume should certainly be examined for possible inclusion in professional libraries.

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Scanning Electron Microscopy of Human Reproduction. Edited by E. S. E. HAFEZ. Ann Arbor Science Publishers, Ann Arbor, Michigan, 1978. *xii* + 233 pp., $34.00.

"Scanning Electron Microscopy [SEM] of Human Reproduction" is the fourth in the series entitled "Perspectives in Human Reproduction." The volume contains papers presented at the Workshop on SEM of Human Reproduction in conjunction with the World Congress of Fertility and Sterility in Miami, Florida, April 1977. It consists of 15 chapters subdivided into 4 sections: Methodology, Andrology, Gynecology, and Products of Conception. A total of 94 well-reproduced, high-quality electron micrographs and some 284 updated references are collected and cited in this volume.

The first two chapters by E. S. E. Hafez and P. S. Sherman are devoted entirely to the discussion of the methodology and instrumentation of SEM. Several methods such as pinning, fixation, dehydration, drying, mounting, metal coating, and viewing with the SEM are concisely described. In addition, some useful clinical applications of SEM in the areas of physiology and pathology of reproduction; hematological status of the newborn; diagnostic implication of infertility, carcinoma, fetal prematurity, and certain birth defects; etc., are particularly mentioned.

Chapters 3 through 6 consider the applications of SEM to the study of andrological problems. In Chapter 3, C. J. Connell discusses the process of spermatogenesis in relation to the structure and function of the Sertoli cells and the seminiferous tubules. The morphology of spermatozoa and its functional significance are demonstrated briefly by Hafez in Chapter 4. Chapter 5 by P. F. Tauber, D. Propping, and L. J. D. Zaneveld illustrates the phenomenon of semen coagulation and liquefaction, the physiological importance and morphology of the human and animal coagula. The following chapter by E. Spring-Mills and A. L. Jones then provides an electron micrographic review of the general structure and function of the prostate gland.

Section III deals with the problems of gynecology. There are six chapters in this section. The fundamental structure of the ovary, particularly the granulosa cells, is first presented in Chapter 7 by S. Makabe and Hafez. Then, M. Oshima, H. Okamura, and Hafez follow with an extensive description of the SEM microscopical structure of the human oviduct–uterus and endometrium, and their physiological mechanisms and functional significance. Fifteen excellently reproduced electron micrographs are provided for clear illustration. In Chapter 9, the SEM characteristics of the uterotubal junction are reviewed by H. E. Fadel. Investigations of proliferative phase, secretory phase, and postmenopausal period are reported. The finding is that the morphological appearance of the surface epithelium of the uterotubal junction is strongly indicative of an active physiological role in the reproductive processes.

The cervix, which is a site for one of the most common forms of cancer in women, has been extensively studied by SEM. The surface ultrastructure of cervical epithelium by SEM including original columnar, original squamous, and metaplastic squamous is revealed by J. M. Allen and J. A. Jordan in Chapter 10. In Chapter 11, the rheological characteristics and

ultrastructure of cervical mucus by SEM are discussed by Zaneveld, Tauber, Propping, and Hafez. It has been discovered that cervical mucus has a three-dimensional micellar ultrastructure which is consistent with the nuclear magnetic resonance properties of this material. In addition, the chemical, biophysical, and physiological properties of cervical mucus are quite variable during different phases of the ovarian cycle. Chapter 12 by S. M. Noonan and L. Weiss is concerned with the morphological investigation of normal amniotic fluid cells by light microscopy, transmission electron microscopy, and SEM. It is anticipated that the knowledge obtained will also provide a basis for future examination of abnormal amniotic fluid cells.

Finally, the last three chapters show the SEM of the products of conception. The SEM of inactive or resting adult mammary gland tissue (e.g., stroma and parenchyma) which was obtained from women undergoing reduction mammoplasty is demonstrated by Spring-Mills and J. J. Elias. A comparison study of the SEM of a 41-day-old embryo with a 61-day-old fetus ectoderm was conducted by J. H. L. Watson and M. A. Kamash and is illustrated in Chapter 14. The volume concludes with a discussion of the SEM of umbilical cord and neonatal blood by M. I. Barnhart and J. M. Lusher. Differences between erythrocytes (RBC) from neonatal and adult blood, RBC disorders (hemolytic disease and hemolytic anemia), and causes of RBC disorders are critically reviewed.

In summary, this volume is an excellent pictorially oriented information source of SEM in human reproduction. It should constitute an invaluable guide for the pathologist, obstetrician, neonatologist, pediatrician, biological research worker, and SEM scientist.

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Chromatography of Synthetic and Biological Polymers, Vol. 2: **Hydrophobic, Ion-Exchange and Affinity Methods**. Edited by ROGER EPTON. Halstead Press, New York, 1978. *x* + 353 pp., $47.50.

This is the second of a two-volume set based on an international symposium of the Macromolecular Group of the British Chemical Society held at the University of Birmingham in the U.K. on July 7–9, 1976. It is devoted exclusively to the chromatographic fractionation of biopolymers. The book is divided into three sections as indicated in the title.

The first five chapters deal with Hydrophobic Chromatography, the second five with Ion-Exchange Chromatography, and the remaining nineteen chapters, about sixty-five per cent of the total, with Affinity Chromatography.

Each of the chapters is adequately illustrated with figures and charts, has an ample bibliography, and provides historical and/or theoretical background for the subject being discussed. While this is not a laboratory manual enough details of the procedures and equipment used are provided by the authors to enable one to make immediate applications. An interesting aspect is that a substantial number of industrial-scale examples are given in considerable detail.

Since many of the authors are pioneers in their areas their comments are worth noting.

This set should be welcomed in any laboratory planning on entering into biopolymer fractionation. The wealth of experimental details, equipment specifications, and critiques of extant methods should make these volumes welcome in established laboratories as well.

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Archaeological Chemistry II. Edited by G. F. CARTER. American Chemical Society, Washington, D.C., 1978. xi + 389 pp., $46.00.

The purposes of archaeological chemistry are not only to deduce history from the analysis and investigation of artifacts but also to include authenticity studies, identification of sources, deduction of production techniques, and dating. In order to fulfill these purposes, several important problems are to be addressed by archaeological chemists, archaeologists, and others interested in the field. Primarily these include recommended procedure for data reporting, methods of specimen handling and sampling, identification of sources of standards and synthesis of new standards, computerized data storage and retrieval, and round-robin test programs to ensure interlaboratory agreement in the analysis of archaeological objects. This volume of "Archaeological Chemistry II," Advances in Chemistry Series 171, is the fruitful results of a group of archaeological chemists organized to work toward this direction. It compiles some 20 papers presented at a Symposium which was sponsored by the Division of the History of Chemistry at the 174th Meeting of the American Chemical Society, Chicago, Illinois, August 31–September 1, 1977. The volume consists of five introductory papers on perspectives and techniques, six papers on organic materials, four papers on ceramics, and five papers on metals.

In the opening chapter of "Chemistry and Archaeology: A Creative Bond," S. V. Meschel clearly states that archaeological chemistry is an interdisciplinary endeavor–a mediator among scientific fields and between the exact sciences and the humanities. Archaeological chemists, therefore, must be flexible, versatile, and well versed in several areas of chemistry. The second paper by N. S. Baer then discusses the chemical aspects of the conservation of archaeological materials. Of special concern are typical conservation treatments for textiles, waterlogged wood, bone and ivory, cuneiform tablets, and cast and wrought marine iron.

"Radiocarbon dating," utilizing the method of carbon-14 for age determination in archaeology is comprehensively reviewed by R. E. Taylor in Chapter 3. In Chapter 4, A. M. Friedman and J. Lerner report, in detail, techniques for analysis and methods of sample preparation for using spark source mass spectrometry (SSMS) to study archaeological samples. Results of comparative studies of neutron activation and SSMS on identical samples: "the ores of origin of two series of early Peruvian artifacts" are also provided and critically illustrated. The following chapter by P. Meyers is concerned with the applications of X-ray radiography in the study of archaeological objects including the famous Bronze Horse, Sasanian silver, and a Chinese bronze vessel.

Trace element analysis using neutron activation to characterize archaeological bone is described by G. Wessen *et al.* in Chapter 6. Calcium, barium, and strontium concentrations are determined simultaneously in a short irradiation. It was interpreted as indicating that strontium and barium appear to be reliable indicators of bone origin. Chapter 7 by A. A. Hassan *et al.* considers the amino acid analysis in radiocarbon dating of bone collagen. Methods of nitrogen/carbon ratios combined with quantitative amino acid analyses in determining impurity level are presented. In Chapter 8, amino acid racemization dating, a promising new technique for dating materials of biological origin (bone and shell) which are about 1000 to several hundred thousand years old, is discussed by P. M. Masters and J. L. Bada.

In Chapter 9, chemical investigations on ancient near eastern archaeological ivory artifacts for fluorine and nitrogen composition are reported by N. S. Baer *et al.* Chapter 10 by R. F. Marschner and H. T. Wright presents the standard analytical techniques, liquid chromatographic analysis, and X-ray diffraction for study of asphalts in 70 samples from 14 Middle Eastern archaeological sites. In Chapter 11, the use of solution spectrophotometry in the visible and ultraviolet range to determine and identify the nature of dyes in archaeological and ethnographic textiles is illustrated by M. Saltzman.

Chapter 12 by J. B. Lambert and C. D. McLaughlin reviews the analysis of nine XVIIIth Dynasty Egyptian glass fragments by atomic absorption and X-ray photoelectron spectroscopy. It was confirmed that the Egyptian glass fragments have the soda lime silica matrix with high MgO and K_2O. The prime colorants are Fe, Cu, Pb, Mn, and Co, all in less than 2% (total). The following chapter by J. S. Olin deals with the applications of neutron activation analysis and X-ray diffraction analysis for elemental compositions to distinguish two distinctive groups of pottery among the majolica sherds excavated from Spanish sites in the New World. Chapter 14 by R. O. Allen and S. E. Pennell also report the use of neutron activation analysis to measure 20 trace elements in over 700 samples of soapstone from artifacts and geological outcrops. The methodology of atomic absorption spectroscopy of archaeological ceramic materials is described in Chapter 15 by V. Gritton. The detailed procedures include sample preparation, sample size, sample decomposition, standards, instrumentation, and practical applications.

A fractionation experiment to test the possible change of lead isotope ratios in the ancient manufacture of pigments was conducted by I. L. Barnes *et al.* and reported in Chapter 16. Chapter 17 also reviews the analyses of lead isotope ratios among 19 Nigerian copper alloy objects. A correlation between isotopic ratios of objects and their possible metal sources is made by theses investigators, C. L. Goucher *et al.* Chapter 18, contributed by W. T. Chase and T. O. Ziebold, is a very interesting and informative one. They illustrate the ancient Chinese Bronze compositions by utilizing a three-component plot, namely, ternary representations of a copper–tin–lead system with the aid of computer techniques. The ternary representations give new insights into the evolution of bronze alloys in China, show clearly the great control over alloy composition exerted by ancient Chinese Foundrymen. This is a long paper consisting of 18 figures (phase diagrams), 3 big data tables, and 5 computer profiles (in Appendix). Chapter 19 by S. I. Goad and J. Noakes presents the preliminary analysis of native copper ores and artifacts from the eastern U.S. by using spectrographic and activation analysis. Fifteen trace elements were identified by the optical emission spectroscopic technique and another set of fifteen trace elements were determined by neutron activation analysis.

The last paper by G. F. Carter summarizes the X-ray fluorescence analyses of the compositions of 245 Roman copper coins minted from about ca. 9 to 4 BC. The objectives of this work are multiple, that is, to determine whether (1) chemical compositions may be correlated closely with the date of manufacture; (2) different issues of coins within a given year have the same range of compositions, i.e., whether the issues were struck concurrently, separately, or only partly concurrently; and coins having either obverse or reverse die links have the same composition within experimental error. The trace elements reported include Fe, Ni, Cu, Ag, Sn, Sb, and Pb. It is one of the many excellent papers presented in this volume which enable the book to be considered an invaluable reference book in the field of archaeology.

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Lange's Handbook of Chemistry. 12th Edition. Edited by JOHN A. DEAN. McGraw–Hill Book Co., New York, 1979. xv + 1470 pp., $28.50.

This 12th edition of Lange's well-known Handbook is the second under the editorship of Dr. Dean, and is considerably improved. Structure-correlation tables for proton magnetic resonance and infrared spectroscopy, ionization potentials of molecular and radical species, potentials of reference electrodes for water-organic solvent mixtures, and a wavenumber/

wavelength conversion table have been added for the first time; coverage is expanded in 14 areas, and updating of the remaining information has added to the usefulness of this laboratory stand-by.

These handbooks get thicker and heavier year by year—a sign of progress, no doubt—but Dr. Dean has helped enormously by condensing some of the more familiar data and by restructuring the mathmatical section by omitting results now obtained almost without effort by the ubiquitous electronic hand calculator.

The new material and revised tables make this edition more than just a replacement for the old worn-out handbook. It is well worth the investment.

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MICROCHEMICAL JOURNAL 25, 264 (1980)

Erratum

Volume **24**, No. 3 (1979), in the article, "The American Microchemical Society: An Informal History," by David B. Sabine and Herbert K. Alber, pp. 265–274: Page 273, Table 2, for 1953–4, the name should read "J. S. Wiberley."

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MICROCHEMICAL JOURNAL 25, 265 (1980)

Announcements

Northeastern Association of Forensic Scientists

The Sixth Annual Meeting of the Northeastern Association of Forensic Scientists will take place in the Northern New Jersey area, October 24 and 25, 1980.

Information regarding this meeting or the Association may be obtained by writing to Mark B. Lewis, c/o Scientific Laboratory, State Campus, Building 22, Albany, New York 12226.

Thin-Layer Chromatography Symposium

A 3-day Thin-Layer Chromatography Symposium will be held on December 2, 3, 4, 1980 at the Holiday Inn, City Line Avenue (U.S. 1 and I-76), Philadelphia, Pennsylvania. The Symposium is entitled "Clinical and Environmental Applications of Thin-Layer Chromatography." There will be an instrument exhibit in conjunction with the symposium.

Speakers already committed represent laboratories doing clinical toxicological, regulatory applications and research from international locations. Submit papers before July 1, 1980, to Dr. Joseph C. Touchstone, 574 Dulles Building, 3400 Spruce Street, Philadelphia, Pennsylvania, or Dr. H. M. Stahr, Chemist, Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa 50011; Dr. David C. Fenimore, Texas Research Institute of Mental Sciences, Houston, Texas; Ms. Vicki Cooper, Sales Manager, Whatman, Inc., Bridgewell, New Jersey; Dr. Haalem Issacs, Cancer Research Institute, Frederick, Maryland 21701.

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