ISSN 0026-265X

Volume 23, Number 2, June 1978

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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Microchemical Journal

Volume 23, Number 2, June 1978

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Microchemical Journal
Volume 23, Number 2, June 1978

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Published quarterly by Academic Press, Inc.,
111 Fifth Avenue, New York, New York 10003.
1978: Volume 23: $60.00 U.S.A.; $68.00 outside U.S.A. All prices include postage.
(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 23, Number 2, June 1978

Briefs

Simultaneous Determination of Microgram Amounts of Chlorine or Bromine or Iodine and Sulfur in Organic Compounds. A. MAZZEO-FARINA AND P. MAZZEO, *Laboratori di Chimica del Farmaco dell'Istituto Superiore di Sanità, Istituto di Chimica Farmaceutica e Tossicologica della Università, Roma, Italy.*

Slight modifications of the oxygen flask methods for the halogens and sulfur are used to determine these simultaneously on samples of approximately 1 mg or less.

Microchem. J. **23**,137–141(1978).

Simultaneous Spectrophotometric Determination of Zinc and Copper with Biacetyl Bis of (4-Phenyl-3-Thiosemicarbazone). Application to the Determination of Zinc in Waste Water from a Sulfuric Acid Plant (Pyrites Process). A. G. ASUERO AND J. M. CANO, *Department of Analytical Chemistry, Faculty of Sciences and Pharmacy, The University, Seville-4, Spain.*

The properties of the zinc complex are described. The optimal conditions for a selective and sensitive spectrophotometric determination of zinc are discussed. Two photometric methods for the determination of traces of copper(II) and zinc(II) ions in a mixture have been developed.

Microchem. J. **23**, 142-150(1978).

Extraction and Spectrophotometric Determination of Platinum(IV) Using Tetramethylthiuram Disulfide. R. M. UTTARWAR AND A. P. JOSHI, *Chemistry Department, Nagpur University, Nagpur-440010, India.*

Platinum is extracted as a pale yellow complex by toluene. There is little interference from most anions and cations and the method can be satisfactorily applied for the determination of platinum in the presence of four- to eightfold excesses of ruthenium and osmium, respectively.

Microchem. J. **23**,151-159(1978).

A Rapid Microdetermination of Chlorine Dioxide in the Presence of Active Chlorine Compounds. GARRY L. WHEELER, PETER F. LOTT, AND FRANCIS W. YAU, *Department of Chemistry, University of Missouri–Kansas City, Kansas City, Missouri, 64110; and Olin Water Services, Kansas City, Kansas 66115.*

A titrimetric and spectrophotometric procedure has been developed for the determination of ClO_2 in water samples. The procedure is rapid, accurate, and free of normal interferences present in water. It is based upon the reaction of ClO_2 with substituted halophenal indicators.

Microchem. J. **23**, 160–164 (1978).

Organic Particulate Analysis of Isocyanate Compounds. D. COLIN PHILLIPS, J. D. B. SMITH, J. F. MEIER, AND T. D. KACZMAREK, *Westinghouse Research & Development Center, Pittsburgh, Pennsylvania 15235.*

The thermal decomposition of a series of isocyanate compounds has been investigated. The temperatures at which particulate or aerosol matter is emitted from heated organic substances have been measured. No obvious correlation exists between the values obtained and the melting or decomposition points of the compounds.

Microchem. J. **23**, 165–177 (1978).

Evaluation of the Chloramine-T Membrane Electrode Response in Acidic Solutions. The Determination of the pK_a of *N*-Chloro-*p*-toluenesulfonamide (Chloramine-T Acid). M. A. KOUPPARIS AND T. P. HADJIIOANNOU, *Laboratory of Analytical Chemistry, University of Athens, Athens, Greece.*

Measurements made with a combination of glass and chloramine-T ion-selective membrane electrodes in acidified chloramine-T solutions varying in ionic strength have been used to determine the dissociation constant of *N*-chloro-*p*-toluenesulfonamide (chloramine-T acid).

Microchem. J. **23**, 178–184 (1978).

Gas Chromatographic Determination of Two Isosorbide Mononitrates in Plasma. ROBERT V. SMITH AND JOSIP BESIC, *Drug Dynamics Institute, College of Pharmacy, University of Texas at Austin, Austin, Texas 78712.*

A GC/EC method is described for the determination of 2-isosorbide mononitrate and 5-isosorbide mononitrate in blood plasma. The procedure is based on extraction of ammonium sulfate-treated plasma with ether followed by a cleanup via *n*-heptane/methanol partitioning. After conversion to t-butyldimethylsilyl ethers the compounds are chromatographed on a 3% OV-17 column.

Microchem. J. **23**, 185–196 (1978).

Automatic Microanalyzers. VI. Use of a Vertical Process System. D. FRAISSE AND R. SEMET. *Service Central de Microanalyse, Centre National de la Recherche Scientifique, 2 rue Henry Dunant, 94320 Thiais, France.*

In order to automate the techniques in microanalysis, a new series of analyzers utilizing vertical processing has been developed. Three automatic microanalyzers, i.e., an oxygen, a carbon–hydrogen, and a nitrogen analyzer, are described. For each of them, the same type of vertical reactor is used and therefore, this design represents an important standardization of this kind of analyzers.

Microchem. J. **23**, 197–219 (1978).

Applications Involving Oxidation with $KBrO_3$. I. Rapid Potentiometric Method for Manganese Alone or in Steel and Some Ores. H. KHALIFA AND I. A. ISMAIL, *Faculty of Science, Zagazig University, Zagazig, Egypt, A.R.E.*

The method is based on the oxidation of the divalent cation with a known excess of potassium bromate to the tetravalent state. The unreacted oxidant as well as Mn(IV) are then reduced with sulfurous to bromide and Mn(II). The resulting bromide is titrated with silver sulfate using silver metal as the indicator electrode.

Microchem. J. **23**, 220–225 (1978).

Postulated Flaw in Densitometry. B. ZAK,[1] E. EPSTEIN,[2] AND R. WATKINS,[1] *Departments of Clinical Pathology at: [1] Wayne State University School of Medicine and Detroit General Hospital, Detroit, Michigan 48201, and [2] William Beaumont Hospital, Royal Oak, Michigan 48072.*

An effort was made to explain the reported large discrepancies between comparative spectrophotometric measurements for two separated hemoglobins.

Microchem. J. **23**, 226–236 (1977).

Spectrophotometric Evaluation of Interferences in Three Iron Reactions for the Determination of Serum Total Cholesterol. J. D. ARTISS,[1] R. J. THIBERT,[1,2] AND B. ZAK[1,2], *[1] Department of Chemistry, University of Windsor, Windsor, Ontario, Canada N9B 3P4, and [2] Departments of Pathology, Wayne State University School of Medicine and Detroit General Hospital, Detroit, Michigan 48201.*

It has been shown that all three reactions are affected by various interfering substances, such as 2-thiouracil, nitrate, azide, bromide, diethylstilbesterol, and steroids. Spectral differences between the reactions are probably due to solvent and anion effects.

Microchem. J. **23**, 237–258 (1978).

MICROCHEMICAL JOURNAL 23, 137–141 (1978)

Simultaneous Determination of Microgram Amounts of Chlorine or Bromine or Iodine and Sulfur in Organic Compounds

A. MAZZEO-FARINA AND P. MAZZEO

Laboratori di Chimica del Farmaco dell' Istituto Superiore di Sanità, Istituto di Chimica Farmaceutica e Tossicologica della Università, Roma, Italy

Received October 20, 1977

INTRODUCTION

The simultaneous microdetermination of halogens and sulfur in organic compounds has for many years been an interesting research field (*1, 3, 5–10, 16–20, 22, 23*).

Most of the methods described (*3, 6, 7, 10, 16, 17, 20, 22, 23*) utilize the combustion of the sample in a Schöniger flask (*21*), others (*1, 5, 8, 9, 19*) in a combustion tube. Sulfur is generally determined as sulfate (*1, 3, 5–7, 9, 10, 16, 17, 19, 20, 23*) by titration with barium ions; the halogens as halides (*1, 3, 5–7, 9, 10, 16, 18–20, 22, 23*) with various procedures.

We now report a general method for the rapid and simultaneous determination of microgram amounts of chlorine or bromine or iodine and sulfur in organic compounds.

The procedure is based on the combustion of the sample in a Schöniger flask, using KOH and hydrazine hydrate, which as an absorption solution is better thermodynamically than alkaline hydrogen peroxide for the reduction of iodine (*11*). The excess of hydrazine is eliminated with hydrogen peroxide after the combustion. In this way the complete reduction of halogens to halides and the oxidation of sulfur to sulfate are obtained.

The halide ions are titrated with mercuric perchlorate and diphenylcarbazone (*11–13*); the sulfate ions by the Budesinsky method (*4*), appropriately modified (*15*), with barium perchlorate and dimethylsulfonazo(III) as indicator.

MATERIALS AND METHODS

Reagents and Equipment

A. Thomas filter paper for flask combustion
0.1 *N* solution of potassium hydroxide
10% aqueous solution of hydrazine hydrate
30% hydrogen peroxide
Isopropanol

0026-265X/78/0232–0137$01.00/0
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0.05% solution of bromophenol bue in ethyl alcohol
0.5 *N* HNO_3
1% solution of *s*-diphenylcarbazone in ethyl alcohol
0.005 *N* titrated solution of mercuric perchlorate
0.1 *M* solution of potassium chloride
0.1% aqueous solution of dimethylsulfonazo(III)
0.005 *M* titrated solution of barium perchlorate
200-ml Schöniger combustion flask
2-ml Metrohm automatic microburet (tip should be fine enough so that 1 drop is approximately 0.005 ml)

Procedure

Sample containing approximately 0.005 meq of chlorine or bromine or iodine and 0.005 meq of sulfur is accurately weighed on a Mettler UM7 ultramicrobalance and then enclosed in a paper container for flask combustion of dimensions 2×2 cm. Combustion is performed in a 200-ml Schöniger flask, employing 1 ml of 0.1 *N* KOH, 2 ml of H_2O, and 2 drops of 10% hydrazine hydrate as absorption solution. After combustion is complete, the flask is shaken for 10 min and is then left to stand for 20 min. The stopper, platinum holder, and walls of the flask are washed with 5 ml of H_2O, 4 drops of 30% hydrogen peroxide are added, the solution is boiled until the volume is ca. 3 ml. After cooling, 15 ml of isopropanol and 3 drops of bromophenol blue solution are added; the solution is taken to the end point with 0.5 *N* HNO_3 and 1 drop is added in excess. After the addition of 4 drops of diphenylcarbazone solution, the halide is titrated with 0.005 *N* mercuric perchlorate to orchid-pink. The solution is decolorized with 0.1 ml of 0.1 *M* KCl, 1 drop of dimethylsulfonazo(III) solution is added, and the sulfate is titrated with 0.005 *M* barium perchlorate to persistent blue-green.

Vigorous and continuous magnetic stirring is necessary for these titrations.

It is essential to determine blank by repeating the determination of the halide and sulfur in the absence of sample and to subtract these amounts from the volumes used for the sample.

RESULTS AND DISCUSSION

The method allows rapid and simultaneous determination of microgram amounts of chlorine or bromine or iodine and sulfur in organic compounds.

The results obtained using the above procedure are in good agreement with calculated values, as shown in Table 1.

SUMMARY

A general method for the simultaneous and rapid determination of microgram amounts of chlorine or bromine or iodine and sulfur in organic compounds is described.

TABLE I
HALOGEN AND SULFUR ANALYTICAL RESULTS

Compound	Sample weight (mg)	Halogen (%) Calcd	Found	Δ	Sulfur (%) Calcd	Found	Δ
S-Benzylisothiouronium chloride, $C_8H_{11}ClN_2S$	1.0507	17.49	17.47	−0.02	15.82	15.84	+0.02
	0.8588		17.53	+0.04		15.91	+0.09
	1.0029		17.58	+0.09		15.73	−0.09
	0.9627		17.32	−0.17		15.77	−0.05
Cysteine hydrochloride monohydrate, $C_3H_{10}ClNO_3S$	0.5559	20.19	20.06	−0.13	18.26	18.27	+0.01
	0.9128		20.05	−0.14		18.05	−0.21
	1.0500		20.31	+0.12		18.37	+0.11
	0.9005		20.19	0.00		18.09	−0.17
Vitamin B_1 hydrochloride, $C_{12}H_{18}Cl_2N_4OS$	0.9415	21.02	21.00	−0.02	9.51	9.47	−0.04
	1.2935		21.06	+0.04		9.43	−0.08
	0.8993		21.15	+0.13		9.52	+0.01
	1.4415		21.07	+0.05		9.69	+0.18
6,7-Dichloro-3-methyl-2H-1,2,4-benzothiadiazine 1,1-dioxide (*2*), $C_8H_6Cl_2N_2O_2S$	0.6180	26.75	26.90	+0.15	12.09	12.16	+0.07
	0.9695		26.51	−0.24		11.93	−0.16
	0.6336		26.98	+0.23		12.32	+0.23
	1.0725		26.74	−0.01		12.12	+0.03
6,7-Dichloro-3,4-dihydro-3-methyl-2H-1,2,4-benzothiadiazine 1,1-dioxide (*14*), $C_8H_8Cl_2N_2O_2S$	0.5900	26.54	26.54	0.00	12.00	12.00	0.00
	0.6624		26.26	−0.28		12.00	0.00
	0.7184		26.57	+0.03		12.07	+0.07
	0.6000		26.46	−0.08		12.04	+0.04
Cloxacillin sodium salt monohydrate, $C_{19}H_{19}ClN_3NaO_6S$	1.7415	7.45	7.43	−0.02	6.74	6.72	−0.02
	1.7675		7.61	+0.16		6.71	−0.03
	1.7133		7.55	+0.10		6.92	+0.18
	1.9150		7.60	+0.15		6.57	−0.17
4-Bromobenzoic acid, $C_7H_5BrO_2$ + Sulfonal, $C_7H_{16}O_4S_2$	1.2250+0.9525	39.75	39.89	+0.14	28.09	28.05	−0.04
	1.1970+1.0095		39.51	−0.24		27.87	−0.22
	0.9897+0.8385		39.82	+0.07		28.10	+0.01
	0.9280+0.9540		39.92	+0.17		28.37	+0.28
5′,5″-Dibromo-*o*-cresolsulfonphthalein, $C_{21}H_{16}Br_2O_5S$	1.6930	29.58	29.56	−0.02	5.94	5.82	−0.12
	1.8275		29.76	+0.18		5.86	−0.08
	1.6210		29.42	−0.16		5.82	−0.12
	1.7630		29.73	+0.15		5.99	+0.05
2-Iodobenzoic acid, $C_7H_5IO_2$ + Sulfonal, $C_7H_{16}O_4S_2$	1.2095+1.0715	51.17	50.71	−0.46	28.09	27.97	−0.12
	1.1343+0.9845		51.31	+0.14		27.96	−0.13
	1.2690+0.8170		50.80	−0.37		28.38	+0.29
	1.1518+0.6358		51.08	−0.09		28.23	+0.14
8-Hydroxy-7-iodo-5-quinolinesulfonic acid, $C_9H_6INO_4S$	1.7975	36.14	36.20	+0.06	9.13	9.35	+0.22
	1.4550		35.75	−0.39		8.99	−0.14
	1.7573		36.32	+0.18		8.94	−0.19
	1.8940		36.34	+0.20		9.05	−0.08
2,5-Diiodothiophene, $C_4H_2I_2S$	1.1633	75.55	75.85	+0.30	9.54	9.70	+0.16
	1.2480		75.72	+0.17		9.61	+0.07
	1.3335		75.10	−0.45		9.54	0.00
	1.1580		75.12	−0.43		9.74	+0.20
S-Butyrylthiocholine iodide, $C_9H_{20}INOS$	1.5250	40.00	40.22	+0.22	10.11	10.12	+0.01
	1.6015		39.86	−0.14		10.08	−0.03
	1.4745		39.90	−0.10		9.99	−0.12
	1.5510		40.35	+0.35		10.31	+0.20

The procedure is based on the combustion of the sample, containing approximately 0.005 meq of Cl or Br or I and 0.005 meq of S, by the Schöniger flask technique, using potassium hydroxide and hydrazine hydrate as absorption solution. The excess of hydrazine is eliminated with hydrogen peroxide after combustion.

This method allows complete reduction of halogens to halides and oxidation of sulfur to sulfate.

The halide ions are titrated with mercuric perchlorate and diphenylcarbazone as indicator; the sulfate ions with barium perchlorate and dimethylsulfonazo(III) as indicator.

The results obtained are in good agreement with calculated values.

ACKNOWLEDGMENT

The authors are grateful to Mr. R. Piergallini for technical assistance.

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Simultaneous Spectrophotometric Determination of Zinc and Copper with Biacetyl Bis of (4-Phenyl-3-Thiosemicarbazone)

Application to the Determination of Zinc in Waste Water from a Sulfuric Acid Plant (Pyrites Process)

A. G. ASUERO AND J. M. CANO

Department of Analytical Chemistry, Faculty of Sciences and Pharmacy, The University, Seville-4, Spain

Received October 18, 1977

INTRODUCTION

Several vicinal α-dithiosemicarbazones have been used as analytical reagents in spectrophotometry. Most transition metals form some kind of stable colored complex with these compounds. Usually they act as chelating ligands by bonding through the sulfur and hydrazinic nitrogen atoms, because the formation of five chelate rings will increase the stability of the complex.

The bis of (thiosemicarbazones) mainly studied have been derived from glyoxal (*4*), biacetyl (*8*), dipyridylglyoxal (*2*), 1,2-cyclohexanedione (*9*), and *o*-phtalaldehide (*10*). Many investigations have been carried out in the study of coordination properties of dithiosemicarbazones (*5*). The analytical aspects of the thiosemicarbazones and semicarbazones also have been recently reviewed (*6*).

In previous studies it has been shown that the sensitivity of a thiosemicarbazone toward metal ions is enhanced by phenyl substitution at the end of the thiosemicarbazide molecule, batochromic shifts thus being produced in the maximum wavelengths of absorption of complexes formed. Compounds with the bis(phenylthiosemicarbazones) grouping, nevertheless, have received very little attention, probably because they suffer from one disadvantage: their sparse solubilities in water. Ballchsmiter (*3*) had suggested the use of these reagents for extractive spectrophotometric procedure in metal trace analysis.

The great ability which the atoms of sulfur have for coordinating metal cations imposes a more serious inconvenience to the use of these reagents, since it makes the establishment of selective methods of analysis difficult. In spite of this, sulfur-containing compounds have proved a fruitful source of analytical reagents (*12*).

Few colorimetric reagents are available for the determination of zinc.

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However, the present paper, which forms part of an investigation into the use of diphenylthiosemicarbazones as analytical reagents, deals with the study of optimal conditions for a selective and sensitive spectrophotometric method for the determination of zinc with the use of biacetyl bis(4-phenyl-3-thiosemicarbazone) (BBPT). The method has been applied to the determination of trace amounts of zinc in an industrial effluent, in which the copper content had been previously determined with the BBPT reagent (*1*).

MATERIALS AND METHODS

Salts and solvents of analytical reagent grade purity or better were used throughout. Distilled water was employed in the preparation of the stock and the standard solutions.

Biacetyl bis(4-phenyl-3-thiosemicarbazone) reagent solution at 0.033% (w/v) in dimethylformamide. The reagent is synthesized from biacetyl and phenylthiosemicarbazide (*1*).

Standardized solutions of copper and zinc.

Acetic acid–sodium acetate buffer solution pH 4.5. Dissolve 160 g of sodium acetate and 75 ml of glacial acetic acid in water and dilute to 1 liter.

All absorbance measurements were made in 1.0-cm quartz cells with a Unicam SP800 recording spectrophotometer and a Coleman 55 (digital) spectrophotometer.

The pH measurements were made with a Philips, PW 9408 pH-meter with glass-calomel electrodes.

Procedures

Determination of zinc. To a zinc solution (6–50 μg of Zn) in a 25-ml volumetric flask, add 15 ml of 0.033% (w/v) solution in dimethylformamide, 2 ml of pH 4.5 acetate buffer, and dilute to the mark with distilled water. Measure the absorbance at 440 nm against a reagent blank.

Determination of zinc and copper in mixtures—method A. To the solution of zinc and copper (6–75 μg of each) add 2 ml of acetate buffer pH 4.5, 15 ml of 0.033% (w/v) BBPT solution in dimethylformamide and dilute to the mark with distilled water. Measure the absorbances at 440 nm against a reagent blank, and at 480 or 530 nm against distilled water, and calculate the zinc and copper concentrations by solving two simultaneous equations.

Determination of zinc and copper in mixtures—method B. With a sample, the sum of zinc plus copper is determined at pH 6.7 as indicated above; with other analogous samples copper is determined alone if the selective determination of copper described in a previous paper (*1*) is used: place the sample in a 25-ml volumetric flask, add 1 ml of EDTA 0.1 *M*, dilute hydrochloric acid and 15 ml of 0.033% BBPT reagent, and dilute

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the mixture to the mark with distilled water (pH final about 2.5). The absorbance is measured at 440 or 480 nm against a reagent blank prepared simultaneously with the sample. The amount of zinc is obtained by the difference in concentration.

Determination of zinc in the waste industrial water. Follow the two techniques previously explained. Adjust the pH of the waste water filtered with a mate crucible (0.5–2.0 ml) at about 4–4.5 pH by adding sodium hydroxide. Add 2.0 ml of a solution containing sodium fluoride (0.4 g/liter) and sodium tartrate (25 g/liter). The sum of zinc plus copper is thus determined. The copper alone is determined at pH 2.5, as already indicated.

Notwithstanding, it is also possible to determine directly the zinc by using a masking procedure as indicated: to the neutralized waste water add potassium cyanure (3 mg), sodium tartrate (50 mg), and sodium fluoride (0.8 mg), before adding the buffer solution and the BBPT reagent. The absorbance from zinc in this case may be measured promptly after color development.

RESULTS AND DISCUSSION

Formation of the Zinc Complex

BBPT forms a yellow complex with zinc which has an absorption maximum at 440 nm and a wide absorption band at 400–500 nm (Fig. 1).

The absorption spectra for different zinc–reagent molar ratios shows that only one complex is formed. The complex can be extracted into chloroform, benzene or tributylphosphate, but photometric data showed that the extraction technique is not useful.

The effect of pH on the color development was studied by preparing series of solutions with 2.2 ppm of zinc varying from pH 1 to 11. The ionic strength was kept constant (0.1) by means of potassium chloride. Absorbance reached a maximum and remained constant between pH 5.5–9.9 (Fig. 2), no color appeared below pH 4.0. For the analytical procedure an acetate buffer was selected because the volume of dimethylformamide added in the preparation of the solutions increased the pH over about two units. Throughout the text pH is used to denote pH-meter reading, not the concentration of hydrogen ions in solution. The presence of acetate in solution had no effect on color formation or extraction.

The order of addition of the reagent was not found to be important. The sequence of metal ion, reagent, and buffer was adhered to during the preparation of all measured solutions.

The color was fully developed at once by adding the diphenylthiosemicarbazone reagent, and it remained stable for at least 24 hr at room temperature and in direct contact with air.

Job's curves were plotted for pH 6.7 (Fig. 3) and 10. In both cases the

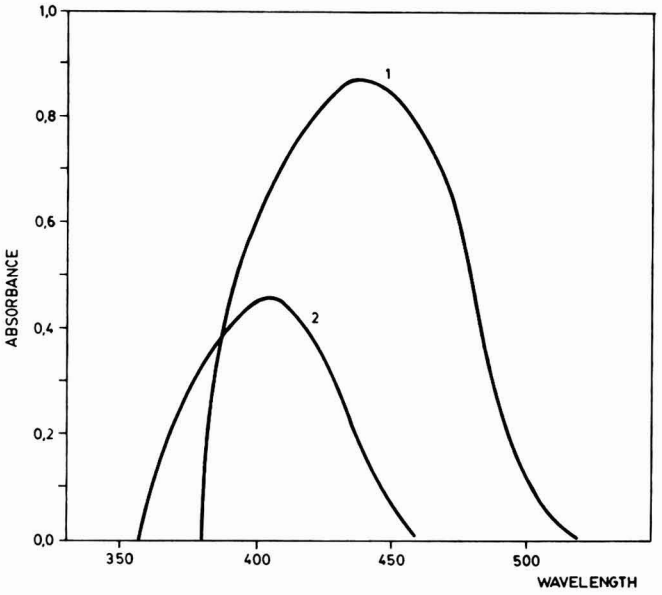

FIG. 1. Absorption spectra of the complexes of zinc with biacetyl bis(4-phenyl-3-thiosemicarbazone) at pH 6.7 (1), and biacetyl bis(4-thiosemicarbazone) at pH 10 (2); C_{Zn} = 2.5 ppm.

zinc–ligand ratios found were 1:1. The mole ratio method also was applied again confirming the reaction stoichiometry 1:1.

Spectrophotometric Determination of Zinc with BBPT

The calibration graph was prepared by using standard solutions containing from 0.15 to 2.5 ml of a 25 ppm solution of Zn(II), treated in the same way as the recommended procedure. It proved to be linear over the range investigated 0.15–2.5 ppm of Zn (absorbance about 0.05–0.83). The molar absorptivity for the complex calculated statistically from Beer's law was 21,570 $\times$ 10^3 1 mol^{-1} cm^{-1}. The Sandell sensitivity (*11*) is 0.003 μg cm^{-2}. Therefore, the method is much more sensitive for zinc than many existing methods. The method has also been advantageously compared with the other method previously reported from this laboratory with the use of a thiosemicarbazone reagent (Table 1). The optimum concentration range, evaluated by Ringbom's method was 0.6–2 ppm of zinc. The relative error (P = 0.05) of the method was $\pm$0.30%. It was calculated by measuring the absorbance of eleven samples (1.2 ppm of Zn), prepared in the same way, but with different solutions. All absorbances were within the range 0.407–0.414 (average 0.410; standard deviation 0.5 $\times$ 10^{-3}).

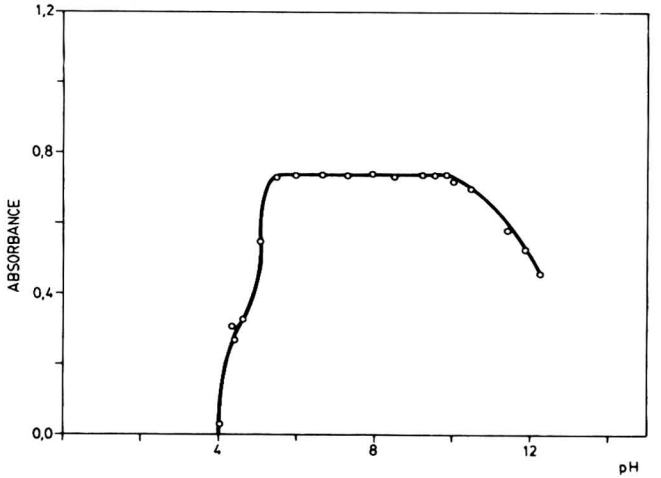

FIG. 2. Effect of pH on the formation of the zinc complex; 2.2 ppm of zinc, 440 nm, $C_{BBPT} = 5{,}4 \times 10^{-4}$ *M*.

Effect of Foreign Ions

For the determination of 1.2 ppm of zinc by this method, the foreign ions can be tolerated at the levels given in Table 2. An attempt was made to eliminate or minimize the interferences by the use of masking agents. The percentage of dimethylformamide containing the samples prevents the decomposition of metal–thiosulfate complexes. Thus, the interference of Ag(I), Pb(II), Hg(II), Au(III), Bi(III), and Cd(II) can be eliminated or

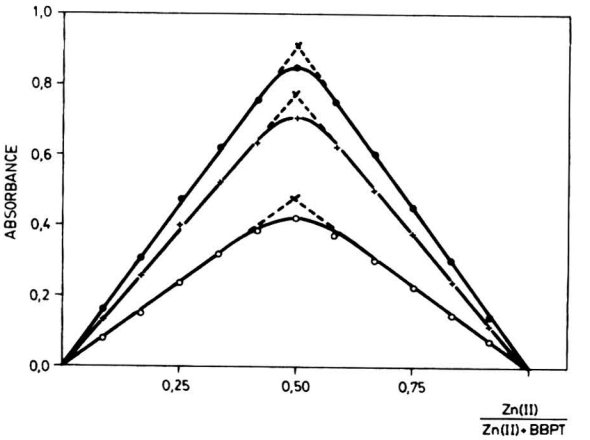

FIG. 3. Composition of Zn–BBPT complex by the continuous variation method, pH 6.7: ● 440 nm, × 460 nm, and ○ 480 nm.

TABLE 1
COMPARISON WITH EXISTING METHODS

Compound	Optimal pH	λ_{max} (nm)	ϵ^a ($\times 10^{-3}$)	Metal/ reagent	Ref.
1.2-cyclohexanedione dithiosemicarbazone	4.5–5.2	415	7.3	1:1	(9)
Phtalimide dithiosemicarbazone	8–10	450	12	2:3	(7)
Biacetyl diphenylthiosemicarbazone	5.5–9.9	440	21.5	1:1	—

[a] 1 mol^{-1} cm^{-1}.

minimized. Cyanure effectively masked the interference of 12 ppm of Ni(II) or Co(II), but only 2.5 ppm of Cu(II), if the absorbance from Zn(II) is measured promptly after color development. The results of test tolerance with masking agents are given in Table 3.

Simultaneous Determination of Zinc and Copper

The absorption spectra of the three acetate-buffered solutions prepared as described under procedures, with 2 ppm of copper, 2 ppm of zinc, and 2 ppm of copper plus 2 ppm of zinc are shown in Fig. 4. The absorption spectrum of the mixed complexes is the sum of the two separated spectra.

There is a difference of 40 and 90 nm in the wavelengths of absorption maximum of the zinc and copper complexes. In view of this the following

TABLE 2
EFFECTS OF FOREIGN IONS ON THE DETERMINATION OF 30 μg OF ZINC

Tolerance[a] (ppm)	Ion added[b]
50	Be(II), Mn(II), As(III), Al(III)
100	Tl(I), W(VI), Mo(VI), Se(IV), Th(IV), Ce(IV), UO_2(II), La(III), alkaline, and alkaline earths
120	F^-, $C_2O_4^{2-}$
150	CN^-
>1000	citrate, $B_4O_7^{2-}$, SCN^-, I^-, Br^-, PO_4^{3-}, tartrate, $S_2O_3^{2-}$

[a] The limiting value of the concentration of foreign ions was taken as that which caused an error of not more than 2.5% in the absorbance.

[b] Cations were added in the form of chlorides, nitrates, or sulfates to a maximum of 100 ppm; anions were added in the form of sodium or potassium salts.

TABLE 3
ELIMINATION OF INTERFERENCES

Foreign ion	Tolerance (original with masking)		Masking agent
Fe(III)	<0.1	20	F^-, $C_2O_4^{2-}$ (120 ppm)
Co(II), Ni(II)	<0.1	12	CN^- (150 ppm)
Cu(II)	<0.1	2.5	CN^- (150 ppm)
Al(III), As(III), Sb(III),		>120	tartrate (2500 ppm)
Mn(II)		>120	tartrate (2500 ppm)
Sn(II)		20	tartrate (2500 ppm)
Ag(I), Au(III), Hg(II),			$S_2O_3^{2-}$ (1200 ppm)
Pb(II)		>120	$S_2O_3^{2-}$ (1200 ppm)
Bi(III)	<0.2	5	$S_2O_3^{2-}$ (1200 ppm)
Cd(II)	<0.1	0.4	$S_2O_3^{2-}$ (1200 ppm)

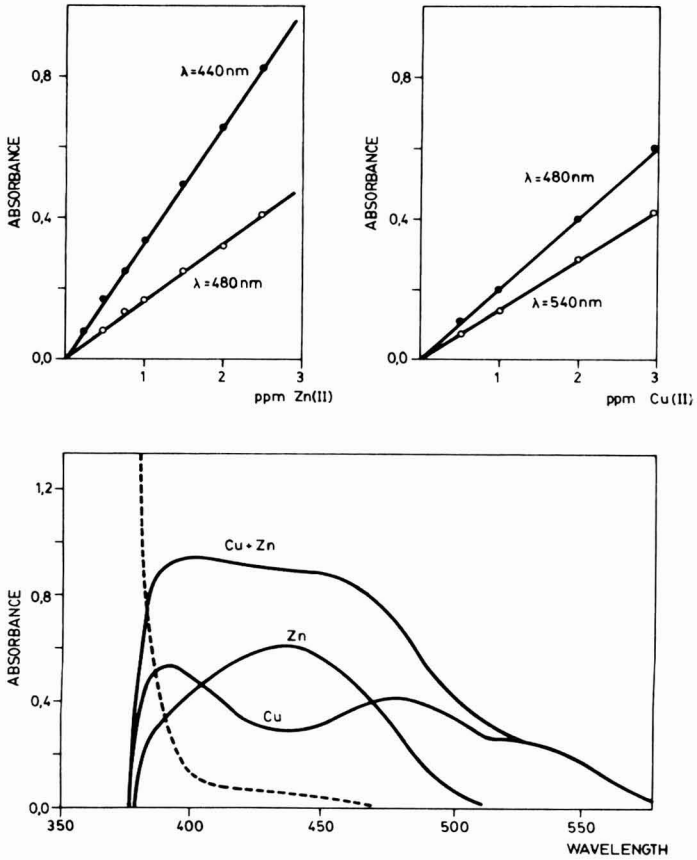

FIG. 4. Simultaneous spectrophotometric determination of traces of zinc and copper with BBPT. C_M^{2+} = 2 ppm.

equations for simultaneous determination of the two components were employed:

$$A_{440} = 9{,}02 \times 10^3\, C_{Cu} + 2{,}15 \times 10^4\, C_{Zn}, \quad (1)$$

$$A_{480} = 1{,}27 \times 10^4\, C_{Cu} + 1{,}07 \times 10^4\, C_{Zn}, \quad (2)$$

$$A_{530} = 8{,}26 \times 10^3\, C_{Cu}, \quad (3)$$

where C_{Cu} and C_{Zn} are the unknown concentrations. To evaluate the molar absorptivities of the complexes at the selected wavelengths, calibration graphs were plotted as shown in Fig. 4. When the concentration of zinc which is going to be calculated is greater than 2 ppm, Eqs. (2) and (3) are used. If the concentration of copper is less than 0.5 ppm, then (1) and (2) are used.

The results obtained for the determination of zinc and copper in synthetic mixtures of the two components were satisfactory. It is possible to determine 0.15 ppm of Zn, in the presence of 3.0 ppm of copper.

Application

The method has been applied satisfactorily to an industrial effluent from a sulfuric acid plant (pyrites process). The waste water solution was filtered through an asbestos mat contained in a Gooch crucible. The average composition of the waste water analyzed (seven samples); pH = 1.4 ± 0.1, was (ppm): chloride (300), zinc (27.8), iron (53.1), chromium (<0.05), copper (15.6), manganese (0.5), lead (<0.05), arsenic (11.0), and calcium carbonate (534).

Since it is a water rich in metallic traces, it is necessary that the proposed method be free of interferences. The content in zinc of the effluents taken in seven different days are shown in Table 4, and it is compared

TABLE 4
DETERMINATION OF THE ZINC IN WASTE WATER[a]

Zinc content (ppm)	Method A	Method B	CN^- as masking
14.0	14.2	14.3	13.7
28.2	28.9	28.5	28.0
27.0	26.4	27.2	26.7
18.2	18.2	18.7	17.9
25.6	25.9	25.6	25.9
32.8	32.4	33.0	32.9
13.8	13.5	13.6	14.0

[a] Results are the means of three determinations.

with the use of BBPT in accord with the procedures indicated in the experimental part of the study.

SUMMARY

The properties of zinc complex of biacetyl bis-(4-phenyl-3-thiosemicarbazone) (BBPT) are described. The optimal conditions for a selective and sensitive spectrophotometric determination of zinc are discussed. Two photometric methods for the determination of traces of copper(II) and zinc(II) ions in mixtures has also been developed and the methods have been applied to the determination of zinc in an industrial effluent.

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Extraction and Spectrophotometric Determination of Platinum(IV) Using Tetramethylthiuram Disulfide

R. M. UTTARWAR AND A. P. JOSHI

Chemistry Department, Nagpur University, Nagpur-440010, India

Received January 19, 1977

INTRODUCTION

Tetramethylthiuram disulfide, also known as bis(dimethylthiocarbamoyl) disulfide, has been used for the extraction and photometric studies of cobalt *(5)*, mercury *(10)*, tellurium *(17)*, and silver *(9)*. The use of this reagent for the extraction and photometric determination of copper *(7)*, palladium *(18)*, and osmium *(19)* has been reported from our laboratory.

Various reagents for the colorimetric determination of platinum have been known *(2–4, 6, 11)*. Some of the important and recently introduced reagents are acenapthenequinone monoxime *(15)* and thiobenzhydrazide *(8)*.

The present paper describes the extraction and photometric studies of platinum(IV) as a tin chloride complex of tetramethylthiuram disulfide. The reagent proves to be quite selective and platinum can be determined in the presence of a large number of cations and other platinum metals.

EXPERIMENTAL DETAILS

Standard platinum solution was prepared by dissolving chloroplatinic acid in distilled water containing sufficient quantity of hydrochloric acid to maintain acidity to 1 *M*. The solution was standardized by the usual method *(16)*. More dilute solutions of required strength were prepared from the stock solution.

Tetramethylthiuram disulfide (TMTD) solution in toluene (2.56×10^{-4} *M*) was used. Toluene, reagent grade, was distilled. Stannous chloride solution, 10% in 3 *M* HCl, was used. Absorbance measurements were made with a Beckman DU spectrophotometer using 10-mm quartz cells.

General Procedure

To a suitable aliquot of platinum solution containing 19–72 μg of platinum, 1 ml of 10% $SnCl_2$ solution was added and the volume was made up to 25 ml with 3 *M* HCl. The solution was then shaken in a separatory funnel with 10 ml of TMTD in toluene for 30 sec. The organic layer was separated and water droplets were removed with anhydrous sodium sul-

0026-265X/78/0232–0151$01.00/0
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fate. The absorbance of the pale yellow complex solution was then measured at 348 nm against reagent blank prepared under identical conditions with all the components except platinum. The amount of platinum present was then computed from the calibration curve.

RESULTS

Absorption Spectra

The absorption spectra of the Pt:TMTD complex (Pt = 50 μg, conc ratio Pt:TMTD = 1:10) against reagent blank (A) and of reagent blank against toluene (B) are shown in Fig. 1. The extent of the reaction of platinum alone with TMTD is much less but becomes rapid and quantitative in the presence of a large excess of stannous chloride. Neither stannous chloride, under the conditions reacts with TMTD, nor the Pt:$SnCl_2$ complex gets extracted in toluene. The platinum–TMTD complex shows λ_{max} at 348 nm. The absorbance of reagent blank against toluene shows a continuous decrease and is insignificant at 348 nm. The molar absorptivity of the system comes to be 1.89×10^4 when for 50 μg of platinum the absorbance is 0.485 at 348 nm with 10-mm path length.

Stability

The stability was tested by measuring color intensity at different time

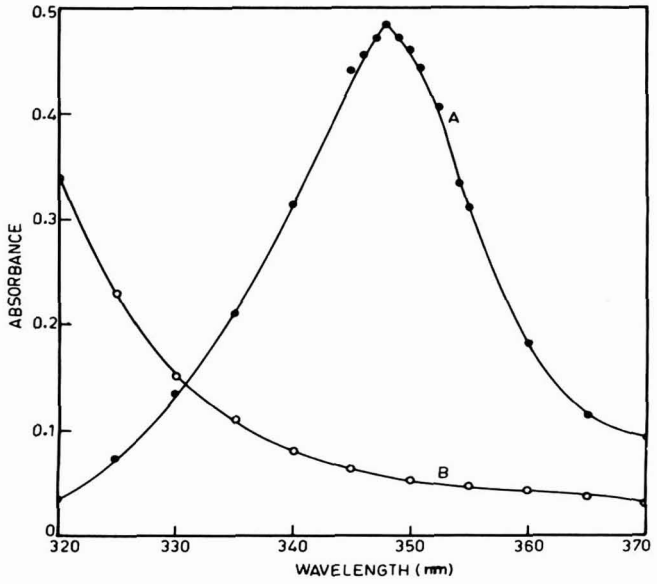

FIG. 1. Absorption spectra of Pt(IV)–TMTD complex against reagent blank. Pt(IV) = 50 μg, TMTD in toluene = 2.56×10^{-4} *M*. (A) Pt:TMTD against reagent blank. (B) Reagent blank against solvent.

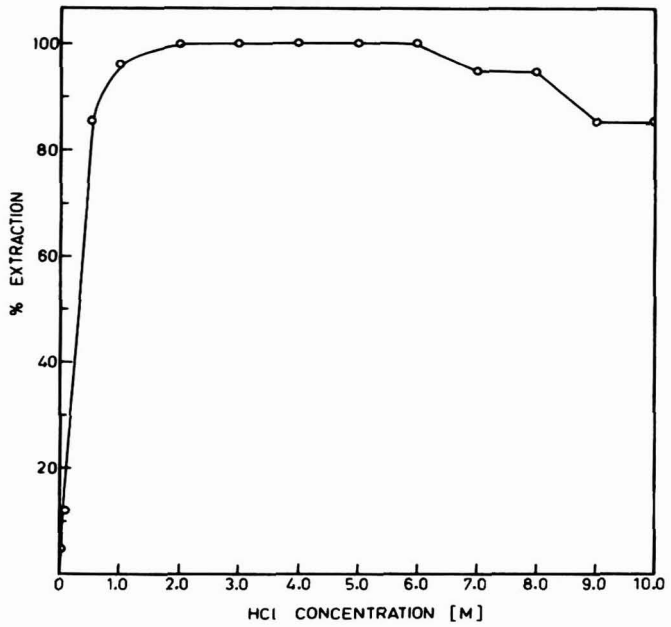

FIG. 2. Effect of acidity on the extraction of platinum. Pt(IV) = 50 μg, λ = 348 nm.

intervals. The absorbance was found to remain constant for 3 hr. The color formation is instantaneous. One shaking with 10 ml of TMTD solution in toluene for 30 sec was found to be sufficient for quantitative extraction. Changing the order of addition of stannous chloride and hydrochloric acid does not affect the complexation reaction.

Calibration Curve

By taking different concentrations of metal ions, the extraction procedure was followed and the absorbance of the organic phase was measured at 348 nm. Beer's law was obeyed in the concentration range of 0.2 to 9.0 μg of platinum/ml (Fig. 3). The optimum range for the determination of platinum as given by Ringbom plot (Fig. 4) was found to be 1.90 to 7.24 μg/ml of platinum.

Extraction as a Function of Acidity

The extraction was carried out in the acid range of 0.05 to 10 *M* of HCl. It was observed that extraction is quantitative in the range of 2–6 *M* (Table 1). Below 2 *M* and above 6 *M*, extraction decreases. At low acidities (< 0.05 *M*), extraction is negligible. Figure 2 shows the effect of acidity on the percentage of extraction.

Effect of Varying Volume and Concentration of the Reagent

The extraction of platinum was carried out by varying the volume and

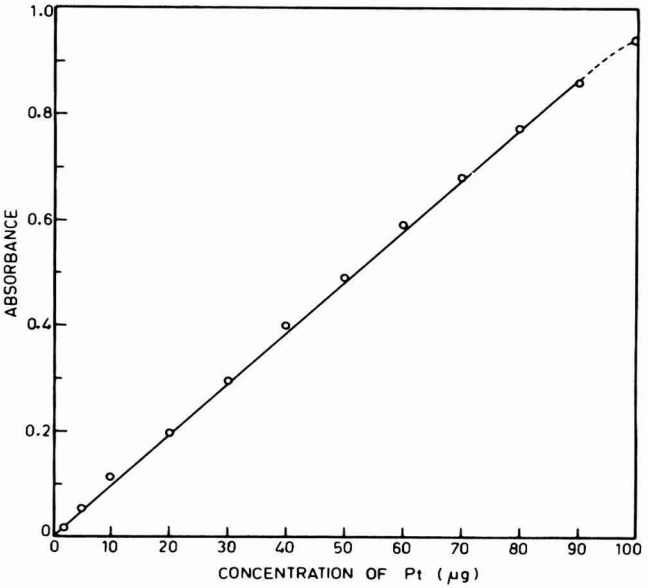

FIG. 3. Beer's law: [HCl] = 3 M, TMTD in toluene = 2.56×10^{-4} M, λ = 348 nm.

concentration of the reagent. The results obtained are given in Table 2. In the present studies, 10 ml of 2.56×10^{-4} M TMTD in toluene was used for extraction.

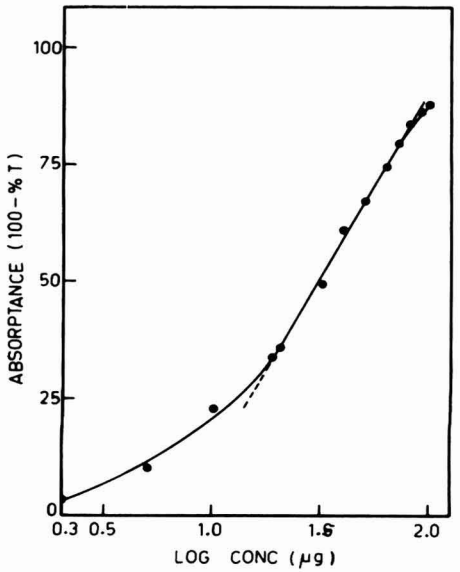

FIG. 4. Ringbom plot: [HCl] = 3 M, λ = 348 nm.

TABLE 1
EFFECT OF ACIDITY ON PERCENTAGE EXTRACTION[a]

Strength of HCl [*M*]	*E* (%)	Distribution Ratio (*D*)
0.05	5	0.13
0.1	12	0.34
0.5	85	14.16
1.0	96	60.00
2.0–6.0	100	∞
7.0	95.5	53.05
8.0	95.5	53.05
9.0	85	14.16
10.0	85	14.16

[a] Platinum (IV) = 50μg; λ = 348 nm.

Effect of Solvents

Different solvents, such as toluene, benzene, chloroform, and carbon tetrachloride, were tried for extraction. Extraction was quantitative with benzene and toluene, while with chloroform and carbon tetrachloride it was 96 and 64.5%, respectively.

Effect of Other Acids

The extent of extraction in sulfuric, perchloric, and hydrobromic acids was 68, 64, and 88%, respectively. Hydrochloric acid was most suitable because extraction was quantitative.

TABLE 2
EFFECT OF VARYING VOLUME AND CONCENTRATION OF THE REAGENT[a]

Reagent conc ($\times 10^{-4}$ *M*)	Volume of reagent (ml)	Absorbance
0.512	10	0.465
1.024	10	0.480
1.536	10	0.485
2.048	10	0.485
2.560	10	0.485
3.072	10	0.485
3.594	10	0.485
4.106	10	0.485
5.120	10	0.485
2.560	1	0.220
2.560	2	0.390
2.560	3	0.455
2.560	4	0.460
2.560	5	0.465
2.560	6	0.475
2.560	7–10	0.485

[a] Pt(IV) = 50μg; λ = 348 nm; [HCl] = 3 *M*. 1 ml of 10% $SnCl_2$ added.

Composition of the Extracted Species

The ratio of platinum to TMTD in the extracted species was determined by the method of continuous variations modified for the two phase system by Irving and Pierce *(12)*. The curves obtained indicated the formation of a 1:1, Pt:TMTD complex. This was further confirmed by the mole ratio method *(20)* modified for extraction.

Effect of Diverse Ions

The interference of several anions and cations on the extraction behavior of platinum was studied. The tolerance limit was calculated as the amount needed to cause minimum error in the recovery of platinum. It was observed that most of the anions and cations were tolerated in larger ratios while thiosulfate and sulfide, and copper, thallium, and mercury showed serious interferences (Table 3). Platinum, however, can be satisfactorily extracted in the presence of other platinum metals. Ruthenium and osmium are tolerated to four- and eightfold excess, respectively, rhodium to twofold excess, while iridium and palladium are tolerated up to a 1:1 ratio (Table 4).

From 10 different observations, the absorbance comes out to be 0.485 $\pm$ 0.005. The relative mean deviation is 0.61%. The sensitivity (Sandell) is found to be 0.010 $\mu g/cm^2$ for $\log I_o/I_t = 0.001$.

DISCUSSION

In the presence work, extraction and photometric determination of platinum(IV) with TMTD in toluene and in the presence of a large excess of $SnCl_2$ has been described. Stannous chloride is known to react with platinum, and an extensive study of the Pt(IV)–Sn(II) system has been reported by Meyer and Ayres *(13)*. The yellow color absorbs strongly at about 403 nm and has a transmission maximum at 355 nm. The color is extractable in organic solvents and exhibits the same spectral characteristics. However, it is much less stable in organic media and can be stabilized by the addition of resorcinol.

In the studies, the λ_{max} was found to be at 348 nm and the system is also sufficiently stable. This shows that the species formed is not the one reported above and it can be, therefore, concluded that TMTD is involved in complex formation.

Ayres and Meyer *(1,14)* have also indicated that in the presence of a large excess of stannous chloride (Pt:Sn more than 1:5) platinum exists in the form of a tetrapositive ion $[PtSn_4Cl_4]^{4+}$ in which platinum has zero oxidation state. It is thought that this species is involved in complex formation with TMTD in the present work.

Many reagents are known for the colorimetric determination of platinum *(11)* but a few are suited for the purpose. The *p*-nitrosodimethyl anniline method is very sensitive but less selective. It is more sensitive to

TABLE 3
TOLERANCE LIMIT OF DIVERSE IONS[a]

Foreign ions	Tolerance limit ($g \times 10^3$)	Foreign ions	Tolerance limit ($g \times 10^3$)
$EDTA^{4-}$	8	Na^+	2
$Acetate^-$	5	Mg^{2+}	2
$Malonate^{2-}$	5	Al^{3+}	3
$Succinate^{2-}$	4	K^+	5
$Citrate^{3-}$	0.5	Ca^{2+}	1.5
$Tartarate^{3-}$	5	VO^{2+}	3
$Oxalate^{2-}$	2	Mn^{2+}	2
SO_4^{2-}	5	Fe^{3+}	1
$S_2O_3^{2-}$	None	Co^{2+}	2
SO_3^{2-}	2	Ni^{2+}	2.5
CO_3^{2-}	6	Cu^{2+}	None
SCN^-	3	Zn^{2+}	2
S^{2-}	None	Ga^{3+}	1.5
Br^-	3	Sr^{2+}	3
I^-	3.5	Y^{3+}	3.5
NO_3^-	5	Zr^{4+}	3
NO_2^-	3.5	Ag^+	3
PO_4^{3-}	5	Cd^{2+}	3
$Cr_2O_7^{2-}$	9	In^{3+}	1
$Mo_7O_{24}^{6-}$	10	Ba^{2+}	4
WO_4^{2-}	5	La^{3+}	4.5
		Hg^{2+}	None
		Tl^{3+}	None
		Pb^{2+}	1
		Bi^{3+}	6
		UO_2^{2+}	7
		Ce^{4+}	4
		Th^{4+}	7.5
		Pd^{2+}	0.05
		Rh^{3+}	0.1
		Ir^{3+}	0.05
		Os^{8+}	0.4
		Ru^{3+}	0.2

[a] Platinum = 50 μg; λ = 348 nm; [HCl] = 3 *M*. 10 ml of 2.56×10^{-4} *M* TMTD in toluene.

the time of heating, change of pH, and requires more reagent. With 3,4-diaminobenzoic acid, molar absorptivity is high but the reaction takes place at 90°C and Pd, Ru, and Os interfere. The present method is less sensitive than those based on *o*-phenylenediamine and *N,N'*-bis(3-dimethylaminopropyl) dithiooxamide, but is more selective.

Recommended Procedure

Introduce an aliquot of sample solution containing 1.9 to 7.24 μg of

TABLE 4
DETERMINATION OF PLATINUM IN THE PRESENCE OF OTHER PLATINUM METALS

Pt taken (μg)	Metal ions (μg)					Pt found (μg)
	Ru	Rh	Pd	Os	Ir	
50	200	100	50	400	50	49
50				400	50	50
50	200	100	50			49

platinum into a 25-ml volumetric flask. It should not contain more than the permitted interfering ions. Add 1 ml of 10% $SnCl_2$ solution and make up to the mark with 2–6 *M* HCl. Equilibrate the solution with 10 ml of TMTD in toluene (2.56×10^{-4} *M*) for 30 sec in a separatory funnel. Separate the organic phase, dry with anhydrous sodium sulfate, and measure the absorbance at 348 nm against reagent blank. Determine the platinum content in a sample solution from the calibration curve.

SUMMARY

The extraction and photometric determination of platinum(IV) with tetramethylthiuram disulphide has been studied. Addition of an excess of stannous chloride and maintaining acidity to 2–6 *M* with HCl is necessary for quantitative extraction. With 10 ml of TMTD in toluene and equilibration for 30 sec, platinum is extracted as a pale yellow complex in the organic phase. It has λ_{max} at 348 nm and obeys Beer's law in the concentration range of 0.2 to 9.0 μg of Pt per ml. The complex is stable for 3 hr. The ratio of Pt:TMTD in the extracted species is found to be 1:1. Most of the anions and cations are tolerated in large ratio. The method can be satisfactorily applied for the determination of platinum in the presence of four- and eightfold excesses of ruthenium and osmium, respectively.

ACKNOWLEDGMENTS

Thanks are due to Professor R. H. Sahasrabudhey for facilities and to U.G.C. for providing a fellowship to one of the authors (R.M.U.).

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MICROCHEMICAL JOURNAL 23, 160–164 (1978)

A Rapid Microdetermination of Chlorine Dioxide in the Presence of Active Chlorine Compounds

GARRY L. WHEELER AND PETER F. LOTT*

Department of Chemistry, University of Missouri–Kansas City, Kansas City, Missouri 64110

AND

FRANCIS W. YAU

Olin Water Services, Kansas City, Kansas 66115

Received June 13, 1977

INTRODUCTION

Chlorine dioxide (ClO_2) is becoming increasingly popular as a water disinfectant (*5*) and selective oxidizing agent for undesirable industrial compounds (*6*). White (*7*) reviewed proposed volumetric, amperometric, and colorimetric methods for the determination of ClO_2 and found them lacking speed, sensitivity, or specificity. The oxidation of substituted phenols by ClO_2 is well documented (*1*). Consequently, an investigation was undertaken to develop an analytical reagent for ClO_2 that is unreactive to other active chlorine compounds. It was observed that the pH indicators chlorophenol red, bromothymol blue, bromophenol blue, bromocresol green, and bromocresol purple change color in the presence of free ClO_2, are not readily susceptible to reactions with other active chlorine compounds, and thus should serve as reagents for the determination of ClO_2. These five indicators were found to be suitable titrants or spectrophotometric reagents for a rapid and direct determination of 0.05 to 2.5 mg/liter of ClO_2 in the presence of other active chlorine compounds as well as commonly occurring oxidizing agents. In basic solution, 1 mol of titrant reacts with 2 mol of ClO_2 to form a colorless product. In a visual titration, the appearance of the titrant's characteristic color indicates that all of the ClO_2 has been reduced and the equivalence point has been passed. For ease in ascertaining the end point, chlorophenol red (CPR) appears to be the titrant of choice.

In the spectrophotometric procedure, the decrease in the absorbance of the reagent (575 nm for CPR) produced by ClO_2 follows Beer's law. This enables a direct spectrophotometric determination of ClO_2 to be performed in the presence of other active chlorine compounds in samples having no background interference.

* Author to whom correspondence should be addressed.

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MATERIALS AND METHODS

A 0.01 *N* standard potassium dichromate solution was prepared by dissolving 0.4903 g of $K_2Cr_2O_7$ in 600 ml of deionized water and diluting to 1 liter.

Buffer solution at pH 7.0 was prepared by dissolving 1.76 g of KH_2PO_4 and 3.64 g of $Na_2HPO_4 \cdot 2H_2O$ in 60 ml of deionized water and diluting to 100 ml.

A 0.1 *M* sodium thiosulfate solution was prepared by dissolving 0.25 g of $Na_2S_2O_3 \cdot 5H_2O$ in 80 ml of deionized water and diluting to 100 ml.

Stock ClO_2 solution of approximately 600 mg/liter concentration was prepared using a gas generating and absorbing system (*4*) and standardized by measuring the absorbance at 360 nm of a diluted aliquot (*1*).

Standard ClO_2 solutions were prepared immediately before use by diluting the required volume of stock ClO_2 solution to the desired strength with chlorine dioxide-demand-free water. Working standard ClO_2 solutions were analyzed using a colorimetric diethyl-*p*-phenylenediamine (DPD) method (*2*) and amperometric titration method (*4*).

Reagents

Approximately 3.33×10^{-4} *M* CPR titrant was prepared by dissolving 0.1436 g of chlorophenol red (Eastman Kodak) in 100 ml of 0.01 *N* NaOH and diluting to 1 liter with deionized water. The solution was allowed to stand overnight, then filtered through a 0.45-μm Millipore filter before use as a titrant. The CPR titrant was standardized by titrating, at 100–105°C, a mixture consisting of a 10.0-ml aliquot of the standard 0.01 *N* potassium dichromate solution, 15 ml of deionized water, and 25 ml of concentrated sulfuric acid either to a visual pink end point or to a potentiometric end point. For a 3.33×10^{-4} *M* CPR solution, 1 ml of CPR

TABLE 1
COMPARISON OF DIFFERENT METHODS FOR THE DETERMINATION OF CHLORINE DIOXIDE

ClO_2 taken (mg/liter)	ClO_2 found		
	DPD (mg/liter)	Amperometric (mg/liter)	CPR (titrimetric) (mg/liter)
0.20	—[a]	0.21	0.22
0.30	0.30	0.32	0.30
0.40	0.40	0.44	0.41
0.50	0.61	0.65	0.45
1.00	1.05	1.22	1.05
1.25	1.27	1.38	1.10
1.50	1.65	1.78	1.55
2.00	2.07	2.51	1.95

[a] 95% transmittance.

TABLE 2
DETERMINATION OF CHLORINE DIOXIDE IN PRESENCE OF OXIDIZING AGENTS AND OTHER CHLORINE-CONTAINING COMPOUNDS

ClO_2 taken (mg/liter)	ClO_2 found		
	Interferences	DPD (mg/liter)	CPR (titrimetric) (mg/liter)
0.50	None	0.49	0.52
0.50	—[a]	—[b]	0.53
0.70	None	0.66	0.69
0.70	—[a]	—[b]	0.68
0.85	None	0.81	0.85
0.85	—[a]	—[b]	0.85
1.25	None	1.31	1.25
1.25	—[a]	—[b]	1.25

[a] Na_2CrO_4: 5 mg/liter as CrO_4^{2-}; $Fe(NO_3)_3$: 5 mg/liter as Fe^{3+}; $NaClO_2$: 4.65 mg/liter as ClO_2^-; HOCl: 4.9 mg/liter as OCl^-; $KMnO_4$: 1 mg/liter as MnO_4^-; $KClO_3$: 5 mg/liter as ClO_3^-.
[b] Color of sample and other oxidizers resulted in extreme high readings by the DPD colorimetric method.

equals 2.5 μg of ClO_2, the standardization consumes from 6.6 to 6.8 ml of standard 0.01 *N* potassium dichromate solution. A 10-ml microburette was employed for all titrations.

Titrimetric Procedure

Transfer exactly 50.0 ml of a sample containing from 0 to 0.5 mg of ClO_2 into each of two titration vessels suitable for detection of the appropriate color change at the end point. To one aliquot add 1 ml of pH 7 buffer and mix, add 1 ml of 0.1 *M* $Na_2S_2O_3$ and mix, then add titrant until a distinct color change occurs; this is the blank value. Add 1 ml of pH 7 buffer to the second aliquot, mix, and titrate the sample to the distinct color change obtained in the blank titration.

TABLE 3
CPR SPECTROPHOTOMETRIC DETERMINATION OF CHLORINE DIOXIDE IN PRESENCE OF OTHER CHLORINE CONTAINING COMPOUNDS[a]

ClO_2 taken (mg/liter)	Absorbance (575 nm)	ClO_2 found (mg/liter)
0.00	0.70	0.00
0.40	0.59	0.40
0.60	0.51	0.61
1.20	0.40	1.18
1.60	0.31	1.54
2.40	0.13	2.28

[a] $NaClO_2$: 4.7 mg/liter as ClO_2^-; HOCl: 4.9 mg/liter as OCl^-; $KClO_3$: 5.0 mg/liter as ClO_3^-.

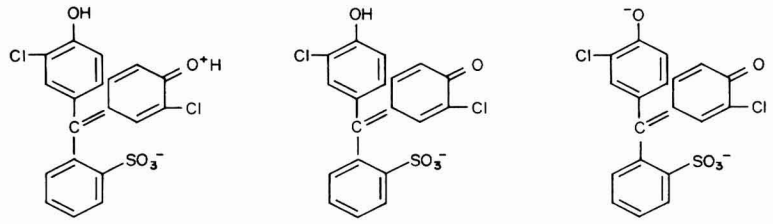
Spectrophotometric Procedure

Transfer exactly 2.0 ml of 3.33×10^{-4} *M* standard CPR solution into a 125-ml Erlenmeyer flask. Add 1.0 ml of pH 7.0 buffer and swirl to mix; a purple color will immediately develop. Add exactly 50.0 ml of sample containing 0 to 0.1 mg ClO_2 to the flask and swirl to mix. Measure the absorbance of the resulting mixture at 575 nm. A blank prepared with 2.0 ml of 3.33×10^{-4} *M* CPR, 1 ml of buffer, and 50.0 ml of deionized water results in a reading of 0.70 absorbance unit using a 1-cm cell.

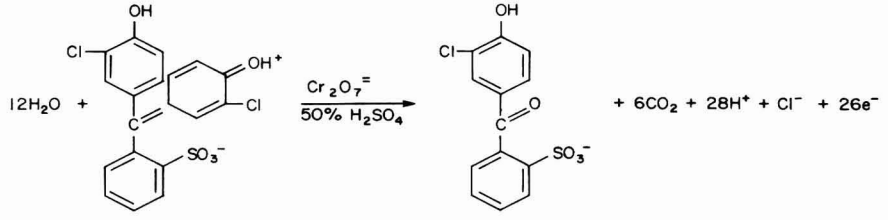
RESULTS AND DISCUSSION

Table 1 presents a comparison of the results for the determination of ClO_2 by the DPD (*2*), amperometric (*4*), and visual titrimetric methods using CPR. As can be seen the CPR method is applicable over a wider range and shows a lower deviation. Table 2 reports the results on the titrimetric determination of ClO_2 with CPR in the presence of interfering ions and Table 3 the spectrophotometric results.

By analogy to the work of Schwartzenbach (*3*), the three color forms of the indicator can be shown as:



In the presence of $K_2Cr_2O_7$ in 50% H_2SO_4 at 100–105°C, the reaction for the standardization appears to be a cleavage at the C=C bond to form the ketone, and oxidation of the ring.



This reaction for the standardization of CPR agrees well with the titer determined by the analysis of standard samples of ClO_2. It is necessary to standardize the reagent at the specified acidity and temperature to allow the reaction to proceed rapidly and to easily detect the end point. The

reaction of CPR with ClO_2 is reproducible; however, no equation is postulated for the reaction at this time; possibly an ion-pair complex is formed. In both the spectrophotometric and titrimetric determinations of ClO_2, $Na_2S_2O_3$ is added to reduce ClO_2 to give the blank value. The reaction is free from halogen interferences as indicated in Table 2. The titrimetric procedure permits the determination of ClO_2 in colored or turbid solutions where spectrophotometric interference might occur. Aqueous solutions of CPR are quite stable, showing little change in composition upon standing for 6 months. Because of its simplicity, coverage of a wide concentration range, accuracy, and ease of standardization, CPR titrimetry is possibly the procedure of choice.

It is relatively unimportant which of the halogenated phenol indicators is employed as the reagent in either the titrimetric or spectrophotometric method. Consequently, particularly in the spectrophotometric method, the reagent can be selected to minimize a background interference if the sample is colored.

SUMMARY

A titrimetric and spectrophotometric procedure has been developed for the determination of ClO_2 in water samples. The procedure is rapid, accurate, and free of normal interferences present in water. It is based upon the reaction of ClO_2 with substituted halophenol indicators.

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MICROCHEMICAL JOURNAL 23, 165–177 (1978)

Organic Particulate Analysis of Isocyanate Compounds

D. COLIN PHILLIPS, J. D. B. SMITH, J. F. MEIER, AND T. D. KACZMAREK

Westinghouse Research & Development Center, Pittsburgh, Pennsylvania 15235

Received December 3, 1977

INTRODUCTION

In previous publications in this series (*4, 5, 8*) we have described a new analytical technique, termed organic particulate analysis (OPA), which enables us to measure the temperature at which particulate (or aerosol) matter is emitted from a heated organic substance. The technique has been applied to the study of the thermal decomposition of malonic acids (9–11), amine arenesulfonates (*1*), metal acetylacetonates (*12, 13*), and diazonium compounds (*14–16*). Industrial applications have been found for the technique (*6, 10, 11, 13, 15, 16*).

In this work, we have extended our studies to include isocyanate compounds. "Splitting" or "blocked" isocyanates have been used extensively in the rubber and coatings industry (*7*). These blocked isocyanates are reaction products of isocyanates with certain "active" hydrogen compounds which exhibit limited stability at elevated temperature, but are stable at room temperature. For example, the addition products of an isocyanate with phenol would exhibit the following equilibrium behavior:

$$RNCO + C_6H_5OH \rightleftharpoons RNHCOOC_6H_5 \ .$$

Such a "blocked" isocyanate may dissociate in the temperature range 150 to 200°C to regenerate the original materials.

Several adducts of hexamethylene diisocyanate have been evaluated for "splitter" activities (*3, 19*). Such compounds have been made with ethyl malonate, acetyl acetonate, ethyl acetoacetate, hydroxylamine hydrochloride, diphenylamine, and phenol and show splitting temperatures in the range 130 to 180°C (*3, 19*).

We chose the isocyanate family for OPA studies because we expected the compounds to emit volatile particulate matter close to the "splitting" temperatures. In fact, it should be possible to "tailor-make" a blocked isocyanate to give thermoparticulation properties over a chosen temperature range (*17*).

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EXPERIMENTAL

1. Preparation of Blocked Isocyanates

The amines, ethers, alcohols, oximes, isocyanates, and diisocyanates used in the preparation of the blocked isocyanate compounds were procured from a variety of sources, such as Eastman Chemical Company, Aldrich Chemical Company, Polysciences Inc., etc. In all cases, these starting materials were used without further purification; the reactions were carried out in toluene.

In most preparations, 0.1 eq of the alcohol, oxime, amine, etc., was added to the reaction kettle fitted with a four-neck head, to which a stirrer, thermometer, condenser, and addition port were affixed. Then ~200 ml of toluene was added and 0.1 eq of isocyanate was added dropwise. In a few cases, reverse addition was used. No matter what addition was used, the second material was added dropwise and the temperature was monitored for exotherm. After addition of the second material was complete, the reaction was usually heated to 90°C and held for 1.5 to 2 hr to ensure completion of the reaction. In the case of reactions with mercaptobenzothiazole, the reaction was sluggish, so additional heating and the addition of a catalyst (stannous octoate) was used. All reactions were cooled to room temperature and petroleum ether was added to precipitate the product. The product was filtered, washed several times with petroleum ether, and dried overnight at 50°C under vacuum.

2. Preparation of Samples for Particulation

The most convenient method of sample preparation was to incorporate the isocyanate compound into an air-drying styrenated, alkydmodified epoxy varnish using the following composition: 100 parts blocked isocyanate compound, 100 parts epoxy varnish, 1.0 part cobalt naphthenate solution, and 0.25 part lead naphthenate (all parts by weight). The epoxy material, which was used in the sample preparation, was found to thermoparticulate at temperatures (*9*) well above those shown by the blocked isocyanate compounds.

The blocked isocyanate–epoxy mixtures were brushed onto thin-section aluminum strips (1 × 3 in.) and allowed to air-dry for at least 2 hr before being placed in an oven at 60°C for a 16-hr period to remove the last traces of solvent. Small portions were cut from these samples (usually measuring 1 × ¼ in. and weighing ~0.5 g) and subjected to thermoparticulation analyses.

Since the isocyanate–epoxy mixtures were air-dried at temperatures well beneath the decomposition temperatures of the various isocyanate compounds (i.e., <60°C), no interaction would occur between the unreacted epoxy resin and the organoparticulate products at the thermoparticulation temperatures.

3. Instrumentation and Organic Particulate Analysis Technique

In these studies, an ion chamber instrument (*4, 5*) was used to detect submicron particles by their influence on the output current of such an instrument. Small ions are produced by a low-level radiation source containing the particulate matter. In the absence of particulate matter, almost all the ions are collected and this results in a maximum output current of a magnitude determined by the strength of the radiation source and the ionization properties of the gas stream. When particles are present in the ionized gas stream, some ion–particle combinations take place. Because the particles are much larger than the ions, the mobility of the resultant charged particle is less, and only a few of the species are collected in the ion chamber. The result is a decrease in the output current of the ion chamber, this decrease being a function of the particle concentration and particle size. Concentrations as low as 2×10^{-10} g/liter can be detected. When temperature and ion current are monitored as described in previous publications (*4, 5, 8, 9*), this results in an organoparticulation pattern for that particular organic compound.

Two temperatures were read from the charts; the threshold temperature which corresponded to the onset of organoparticulation (as shown by an initial falloff in amplified ion current) and the temperature which signified a 50% decrease in the ion current (usually 0.8 to 0.4 mA). These values enabled an "organoparticulation temperature range" (OPTR) to be determined for each compound.

4. Mass Spectrometry

In an attempt to characterize the nature of the organoparticulates emitted from the blocked isocyanate compounds, mass-spectral studies were carried out on particulates and vapor effluents at thermoparticulation. The particulate pyrolyzate was collected on a glass fiber disk. Vapor pyrolyzate was collected on 500 mg of Porapak R (a modified divinylbenzene absorbent, obtainable from Waters Associates, Inc.) held in a thin layer between two filter disks. The overall experimental procedure, which employed a Perkin–Elmer Model 270 gas chromatograph/mass spectrometer, has been described previously (*4, 5, 9*).

RESULTS

1. Organoparticulation Data

Eighteen different blocked isocyanate compounds were evaluated in this work. The organoparticulation temperature range (OPTR) for each compound is given in Table 1. Also included in the table, for comparative purposes, are their experimental melting points or decomposition temperatures. Some of the samples (**5a**, **5b**, and **6a**) were repeats of previous

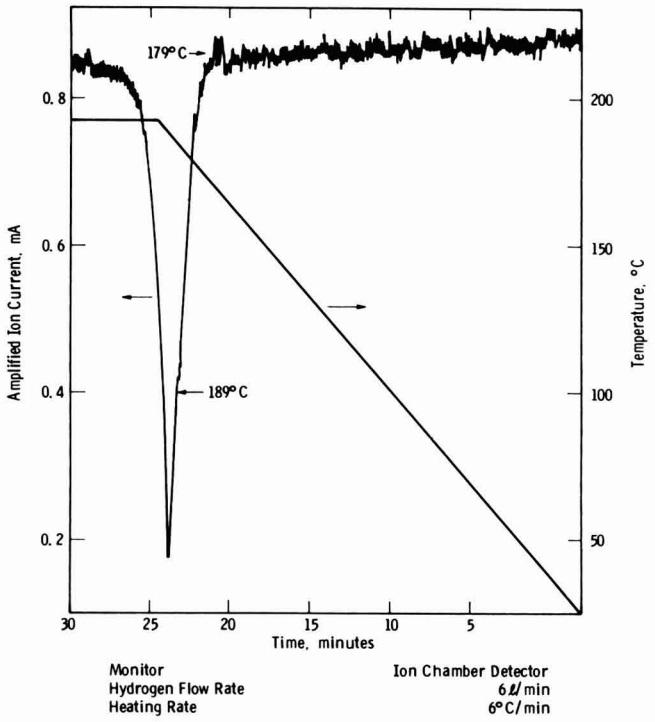


FIG. 1. Organoparticulation pattern for hexamethylene diisocyanate + phenol (sample **2**).

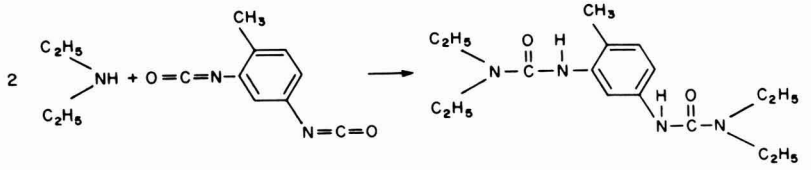
samples, where the blocked isocyanate was resynthesized each time before its OPTR was determined.

Figures 1 and 2 show typical organoparticulation responses obtained with the ion chamber detector for representative blocked isocyanate samples (**2**, hexamethylene diisocyanate plus phenol; **6a**, toluene diisocyanate plus diethylamine). From the organoparticulation patterns of these blocked isocyanates, it can be seen that very strong particulation activity occurred; in each instance, the amplified ion current fell well beneath the 50% current level on the chart.

2. Mass Spectral Results

The mass-spectral data associated with five thermoparticulated blocked isocyanate samples are shown in Fig. 3–7. Also included in these figures are standard mass spectra used as aids in the interpretation. Relevant observations on each mass-spectral chart are given below.

a. Sample **6a** *diethylamine + toluene diisocyanate*



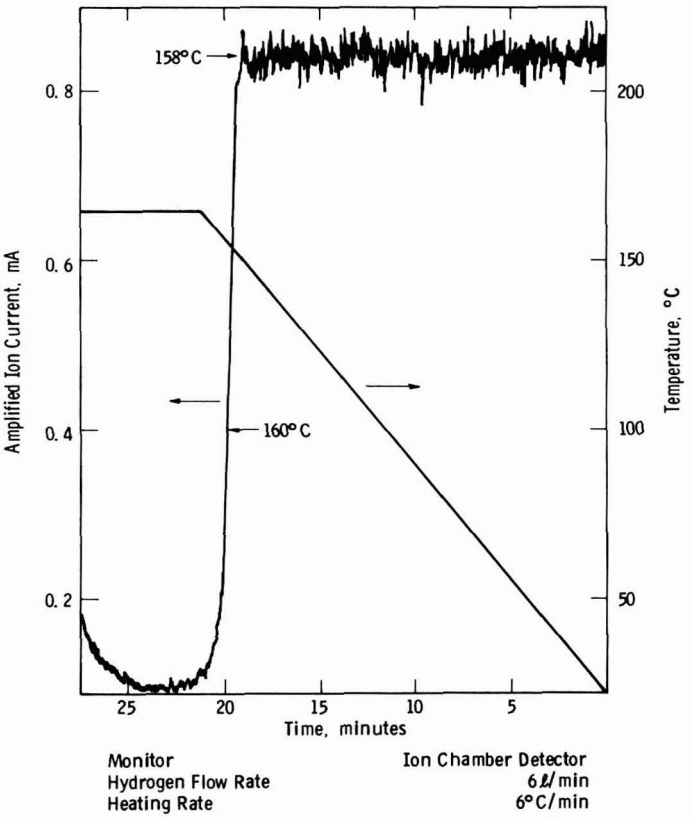
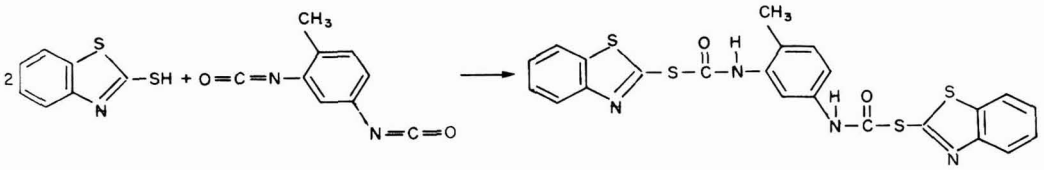


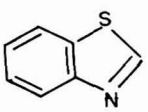
FIG. 2. Organoparticulation pattern for toluene diisocyanate + diethylamine (sample **6a**).

The mass-spectral pattern of particulate effluent from this system (Fig. 3) is that of diethylamine. The amine is not strong in the mass spectra of vapor effluent, which show primarily aromatic hydrocarbons.

b. Sample **5a**, *mercaptobenzothiazole + toluene diisocyanate*



The particulate mass-spectral signal from this system (Fig. 4) is that of benzothiazole,



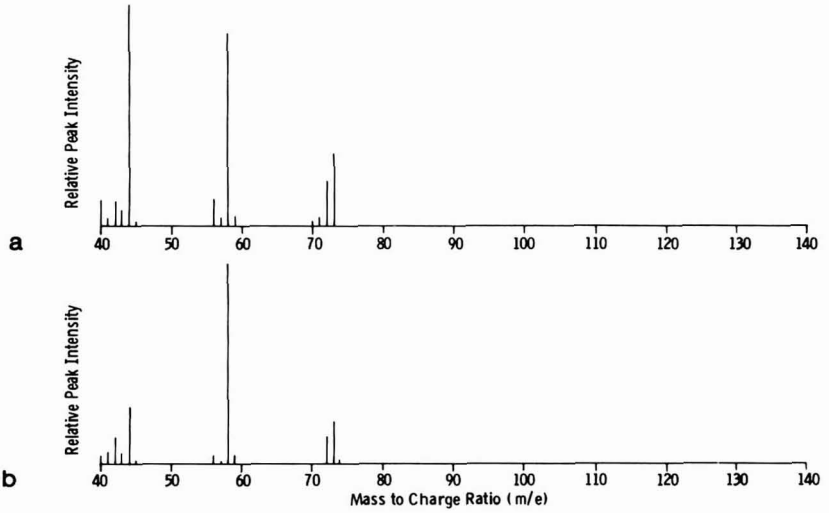
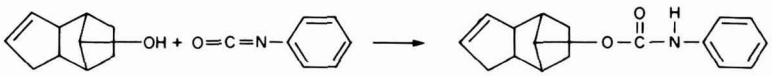


FIG. 3. (a) Mass spectra involved with particulate effluent from sample **6a** (toluene diisocyanate + diethylamine). (b) Reference spectra of diethylamine from the literature.

(i.e., of mercaptobenzothiazole minus the sulfhydryl group). The mass spectrum of mercaptobenzothiazole would have its dominant peak at a molecular weight of 167).

c. Sample **14**, *dicyclopentenyl alcohol + phenylisocyanate*



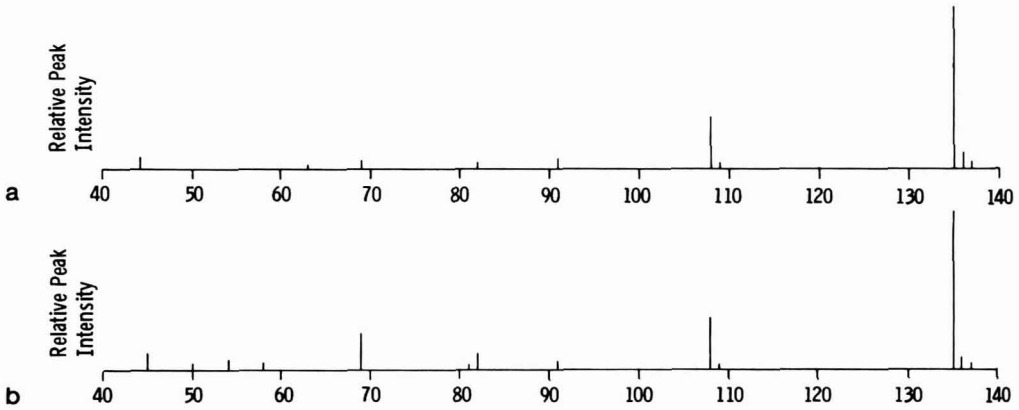


FIG. 4. (a) Mass spectra involved with particulate effluent from sample **5a** (toluene diisocyanate + mercaptobenzothiazole). (b) Reference spectra of benzothiazole from the literature.

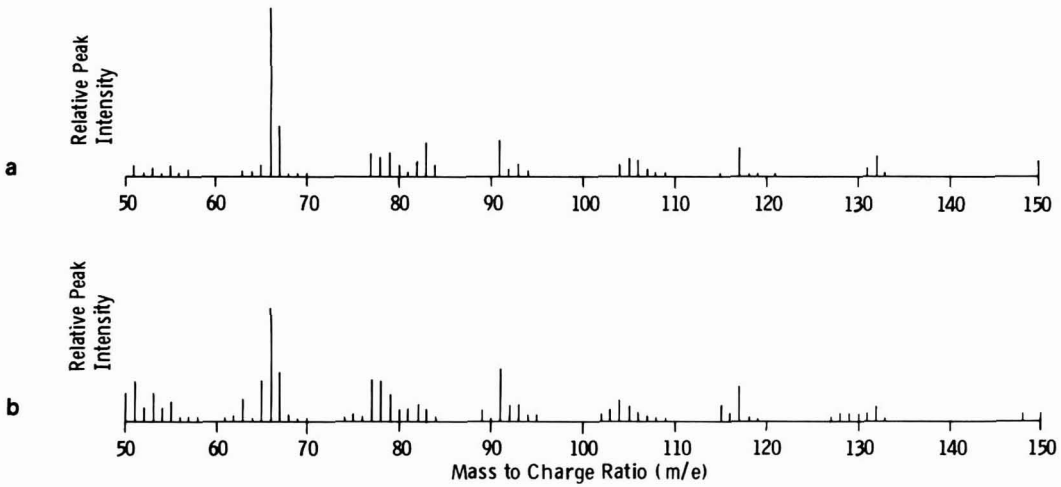
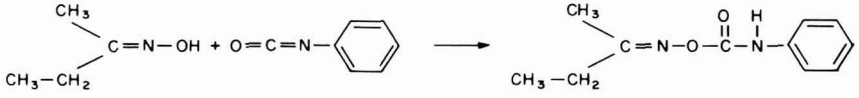


FIG. 5. (a) Mass spectra involved with particulate effluent from sample **14** (phenylisocyanate + dicyclopentenyl alcohol). (b) Laboratory sample of dicyclopentenyl alcohol.

The mass spectra of particulate effluent from this system (Fig. 5) are those of dicyclopentenyl alcohol. The dominant peak in the particulate spectra occurs at *m/e* 66 (cyclopentadiene?).

The mass spectra of vapor effluent from sample **14** are primarily due to aromatic hydrocarbon solvents and residues.

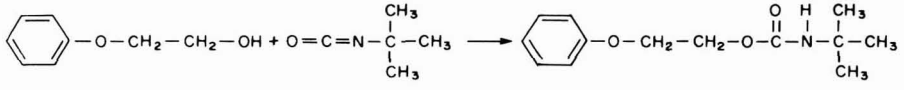
d. Sample **11**, *methylethyl ketoxime + phenylisocyanate*



The mass spectra (Fig. 6) of particulate effluent from this system show the presence of methylethyl ketoxime and aniline. There may be a small amount of phenylisocyanate indicated but the evidence at *m/e* 119 is weak.

The mass spectra of vapor effluent from this system also show the presence of methylethyl ketoxime (molecular weight = 87) and possibly aniline. Aromatic hydrocarbons (solvent?) dominate the spectra at *m/e* 91, 105, 119, and 134. Some of the signal at *m/e* 119 may be due to the phenylisocyanate molecular ion.

e. Sample **12**, *2-phenoxyethanol + t-butylisocyanate*



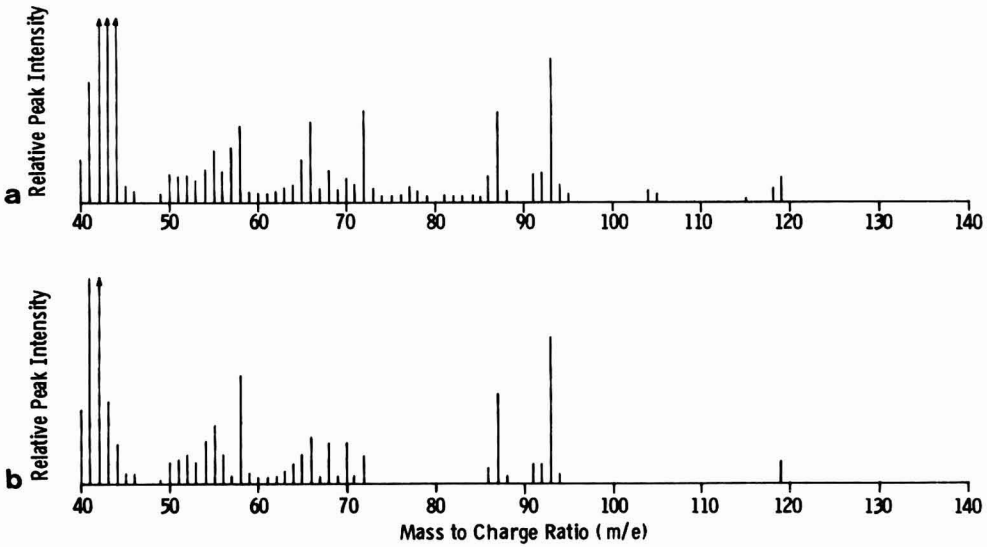


FIG. 6. (a) Mass spectra involved with particulate effluent from sample **11** (phenyl isocyanate + methylethyl ketoxime). (b) Composite reference spectra of aniline (literature), phenylisocyanate (literature), and methylethyl ketoxime (laboratory).

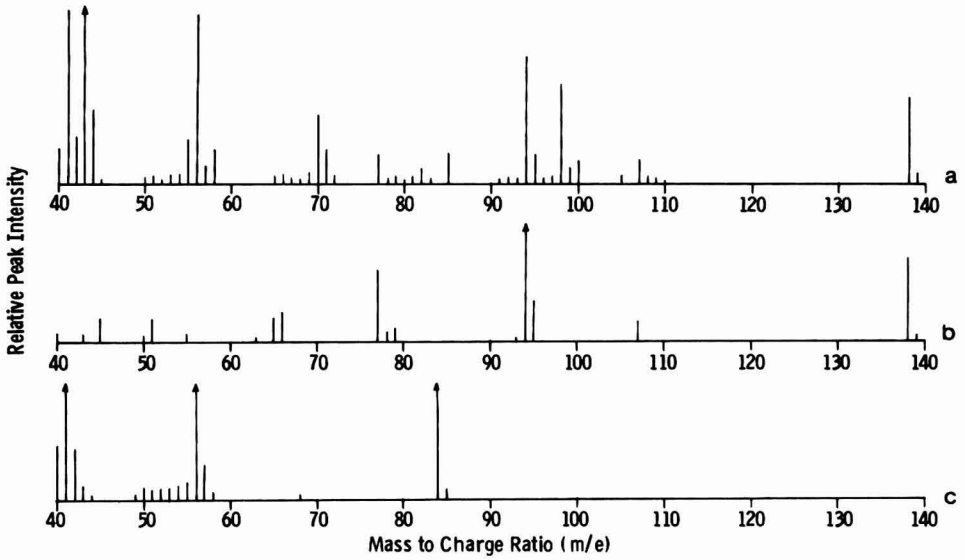


FIG. 7. (a) Mass spectra involved with particulate effluent from sample **12** (t-butylisocyanate + 2-phenoxyethanol). (b) Reference spectra of 2-phenoxyethanol from the literature. (c) Laboratory reference spectra of t-butylisocyanate.

The mass spectra of the particulate effluent of this system (Fig. 7) show the presence of 2-phenoxyethanol in the effluent. The appropriate mass peaks [at *m/e* 94 (phenol fragment) and 138 (molecular ion)] make a good distinctive fingerprint for this system.

The particulate spectra also show the presence of other species in the effluent. This evidence is primarily at *m/e* 56, 70, and 98. These masses suggest that the isocyanate (molecular weight 99) rather than the phenoxyethanol is involved. The mass spectra of Fig. 7 also show that t-butylisocyanate alone is *not* involved in this "other species."

Spectra of vapor effluent from this system are rather indistinct. There is perhaps a little phenol and a trace of 2-phenoxyethanol. Toluene appears to be the dominant feature in the spectra.

DISCUSSION

From Table 1, it can be seen that all the blocked isocyanate compounds except sample **3** (hexamethylene diisocyanate plus thiophenol) had particulation temperatures below 200°C. The lowest and highest OPTR values were shown by samples **6** (toluene diisocyanate plus diethylamine) and **15** (*p, p'*-diphenylmethane diisocyanate plus phenol); they were 154 to 159 and 190 to 194°C, respectively. A comparison of the OPTR data with the melting points (or decomposition temperatures) for these compounds in Table 1 reveals that, with the possible exceptions of samples **5**, **7**, **14**, and **15**, no obvious correlation exists among these experimentally derived quantities; this same observation has been made with other organic compounds (*1*, *4*, *9*, *12*).

Recent work in this laboratory has indicated that the particulates have to be above a certain critical size to be detected by the ion chamber method. This size was determined by passing a series of saturated fatty acids ($C_nH_{2n}O_2$, where n = 8 to 18) through the ion chamber instrument. The break in the detectability occurred between C_{10} and C_{11}. Since the molecular dimensions of the fatty acids are well established (*2*), an approximate value of 25 to 30 Å was established as the critical size of molecule which can be detected by the ion chamber instrument. This compares very favorably with a figure of 25 Å established recently for the detectability limit of a Condensation Nuclei Monitor (*18*).

Because the value of 25 to 30 Å is high compared with most simple organic molecules (where values of 6 to 12 Å are appropriate), we have previously postulated hydrogen-bonded acetic acid oligomers (*9*), acetylacetone oligomers (*12*) (through the enol form of the molecule), and tertiary amine sulfonates (*1*), to be the main components of emitted, particulate matter. A very similar situation appears to be occurring with the blocked isocyanate particulates. Most of the mass-spectral particulate analyses showed the presence of species capable of hydrogen bonding

TABLE 1

ORGANOPARTICULATION DATA AND COMPOSITION OF ISOCYANATE COMPOUNDS

Sample no.[a]	Blocked isocyanate compound used	Organoparticulation temperature range (OPTR, °C)	Blocked isocyanate decomposition or melting point (°C)[b]
1	Toluene diisocyanate + phenol	170–177	153–156
2	Hexamethylene diisocyanate + Phenol	179–189	128–131
3	Hexamethylene diisocyanate + Thiophenol	>200	132–138
4	Phenyl isocyanate + phenoxyethyl alcohol	168–174	95–97
5	Toluene diisocyanate + Mercaptobenzothiazole	161–165	153–161
5a	Toluene diisocyanate + mercaptobenzothiazole; repeat of sample 5	172–177	185–187
5b	Toluene diisocyanate + mercaptobenzothiazole; second repeat of sample 5	172–177	183–185
6	Toluene diisocyanate + diethylamine	154–159	90–95
6a	Toluene diisocyanate + diethylamine; repeat of sample 6	158–160	143–145

7	Hexamethylene diisocyanate + dimethylamine	166–171	166–170
8	Phenyl isocyanate + phenylglycidyl ether	167–171	>200
9	Phenyl isocyanate + styrene oxide	172–174	mp not determined
10	Phenyl isocyanate + butylglycidyl ether	172–176	No sharp mp
11	Phenyl isocyanate + ethylmethyl ketoxime	169–173	126–130
12	Butyl isocyanate + phenoxyethanol	157–160	75–78
13	Phenyl isocyanate + *N*-(2-hydroxyethyl)piperazine	180–185	177–181
14	Phenyl isocyanate + dicyclopentenyl alcohol	168–175	160–165
15	*p,p'*-Diphenylmethane diisocyanate + phenol	190–194	192–194
16	Butylisocyanate + 4,4'-thiodiphenol	175–178	157–162
17	Butylisocyanate + 4,4'-sulfonyldiphenol	181–183	161–166
18	Phenyl isocyanate + dimethyl glyoxime	173–176	190–195

[a] Sample prepared as 1:1 mixture in a cured epoxy resin.
[b] Obtained experimentally.

(e.g., diethylamine, methylethyl ketoxime, aniline, dicyclopentenyl alcohol, and 2-phenoxyethanol). Also, the fact that particulates of the low-boiling-point diethylamine (bp, 56°C) could be collected may reflect the tendency of this polar compound to exhibit hydrogen bonding after particulation.

This same reasoning may account for the fact that sample **3** (hexamethylene diisocyanate plus thiophenol) does not show thermoparticulation properties. In thiophenol, the very polar $-OH$ group of phenol is replaced by the far less polar $-SH$ group. If the blocked isocyanate decomposed to give thiophenol, the $-SH$ group would not hydrogen-bond nearly as much as the $-OH$ of phenol (present in samples **1** and **15**). Hence, this might explain why "phenol"-blocked isocyanates exhibit particulation properties whereas "thiophenol"-blocked compounds do not exhibit these same properties. To test this hydrogen-bonding postulate further, sample **16** was prepared; this contained butyl isocyanate plus 4,4′-thiodiphenol (two phenol groups separated by a sulfur atom). Since this compound has two polar hydroxy groups present, it should exhibit thermoparticulation properties; this was in fact found to be the case—thermoparticulation occurred at 175 to 178°C.

SUMMARY

The technique of Organic Particulate Analysis (OPA) has been employed to investigate the thermal decomposition of a series of isocyanate compounds; OPA measures the temperature at which particulate or aerosol matter is emitted from a heated organic substance. Of the eighteen isocyanate compounds investigated, seventeen showed strong organoparticulation activity below 200°C. With the possible exception of four isocyanates, no obvious correlation exists between the OPA values and the melting or decomposition point of the compound.

In an attempt to characterize the nature of the particulate matter derived from these compounds, mass-spectral data were obtained at thermoparticulation. Most of the mass-spectral particulate analyses showed the presence of very polar species (such as diethylamine) which were capable of exhibiting hydrogen-bonding properties. This hydrogen bonding gives rise to particulate matter of sufficient size to be detected by an ion chamber instrument. The ability of phenols to particulate, whereas thiophenols do not particulate, is discussed.

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Evaluation of the Chloramine-T Membrane Electrode Response in Acidic Solutions. The Determination of the pK_a of *N*-Chloro-*p*-toluenesulfonamide (Chloramine-T Acid)

M. A. KOUPPARIS AND T. P. HADJIIOANNOU[1]

Laboratory of Analytical Chemistry, University of Athens, Athens, Greece

Received January 17, 1978

INTRODUCTION

Ion-selective electrodes have been used for the potentiometric determination of equilibrium constants by ion-activity measurements (*6–8*). Recently, we reported a new chloramine-T (CAT) ion-selective membrane electrode and its applications in the potentiometric determination of various substances and in catalytic kinetic analysis (*3, 4*). The ability of the CAT electrode to monitor the activity of the chloramine-T ion, $CH_3C_6H_4SO_2NCl^-$, with a rapid response makes it a useful tool in equilibrium studies involving chloramine-T.

In this paper an evaluation of the CAT ion-selective electrode response in acidic medium and a potentiometric determination of the ionization constant of *N*-chloro-*p*-toluenesulfonamide, the chloramine-T acid, are described. The value obtained for the ionization constant K_a of this acid at 25 ± 0.2°C and at zero ionic strength is (2.5 ± 0.3) × 10^{-5}, in close agreement with reported values (*2*).

Theory of Measurements

The current view of the behavior of chloramine-T in aqueous solution is listed in the following equations (*2, 5, 1*). (The *p*-toluenesulfonyl part of the molecule $CH_3C_6H_4SO_2$ is designated by the symbol R.)

$$\underset{\text{(CAT)}}{RNClNa} \rightarrow \underset{\text{(CAT ion)}}{RNCl^-} + Na^+, \quad (1)$$

$$RNCl^- + H^+ \rightleftharpoons \underset{\text{(CAT acid)}}{RNHCl}, \quad (2)$$

$$2RNHCl \rightleftharpoons \underset{\text{(DCT)}}{RNCl_2} + \underset{\text{(TSA)}}{RNH_2}, \quad (3)$$

$$RNCl_2 + H_2O \rightleftharpoons RNHCl + HOCl, \quad (4)$$

$$RNHCl + H_2O \rightleftharpoons RNH_2 + HOCl\ . \quad (5)$$

[1] To whom correspondence should be addressed.

0026-265X/78/0232–0178$01.00/0
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The equilibrium constants are $K_a = (2.8 \pm 0.2) \times 10^{-5}$ for reaction (2), 6.1×10^{-2} for reaction (3), and 8×10^{-7} and 4.88×10^{-8} for reactions (4) and (5), respectively (*7*).

The response of the CAT electrode is strongly affected by acidity at pH values smaller than 5 (*3*) because in acidic medium the chloramine-T acid is formed which then disproportionates to give *p*-toluenesulfonamide, RNH_2, and, sparingly soluble dichloramine-T, $RNCl_2$ (reaction (3)).

If strong acid is added to a chloramine-T solution of known concentration saturated with DCT and TSA, the anion takes up hydrogen ion to form the free acid (reaction (2)), but the equilibrium in reaction (3) is not displaced. If $[RNCl^-]_o$ and $[RNCl^-]_r$ are the CAT ion concentrations before and after the addition of the acid, respectively, and [RNHCl] is the concentration of the free acid formed, the mass balance can be expressed as

$$[RNCl^-]_o = [RNCl^-]_r + [RNHCl]. \tag{6}$$

As this is a buffer system of a weak acid, we can say that

$$pH = pK_a + \log \frac{[RNCl^-]_r}{[RNHCl]}. \tag{7}$$

The CAT ion-selective electrode yields the usual Nernstian behavior, and the electrode potentials before and after the addition of the acid can be represented as

$$E_o = E' - S \log [RNCl^-]_o, \tag{8}$$

$$E_r = E' - S \log [RNCl^-]_r. \tag{9}$$

If the CAT electrode is not affected directly by the hydrogen ion, so that the electrode slope S is the same before and after acid addition, substitution of Eqs. (8) and (9) into Eq. (7) and rearrangement yield

$$\log (10^{(E_r - E_o)/S} - 1) = pK_a - pH. \tag{10}$$

From Eq. (10) it is clear that plots of the left factor of the equation vs pH should yield a linear relationship of slope -1 with intercept pK_a.

MATERIALS AND METHODS

Reagents

All solutions were prepared with bidistilled deionized water from reagent grade materials.

Chloramine-T. Chloramine trihydrate (Merck) was purified by recrystallization from distilled water (*2*) so that a purity of 99.5% was obtained.

Dichloramine-T and p-toluenesulfonamide. These reagents were prepared in mixture by acidifying 0.1 *M* CAT solution with sulfuric acid,

filtering, and washing the insoluble DCT and TSA obtained several times with distilled water.

Apparatus

The chloramine-T ion-selective electrode was constructed, used, and stored as previously reported (*3*). As reference electrode, an Orion 90-02 Ag–AgCl double-junction electrode was used with 10% KNO_3 solution in the outer chamber.

For recording the cell potential changes a Heath–Schlumberger rapid-response recording system was used. Measurements of pH were carried out with a combination glass electrode and an Orion model 801 digital/mV meter connected with an Orion model 751 digital printer. The same equipment was used for CAT electrode emf measurements.

All measurements were carried out in a 50-ml double-walled glass cell, containing the above three electrodes, at 25.0 ± 0.2°C in the absence of direct sunlight. Test solutions were magnetically stirred rapidly so that reagent mixing was obtained in a few seconds.

This system allows the potential changes and pH values to be recorded in a few seconds after acid additon.

Procedure

a. Evaluation of electrode response in acidic solutions. A 4 M sulfuric acid solution was injected with a microsyringe at intervals, in chloramine-T solutions, 1×10^{-2}, 5×10^{-3}, 2×10^{-3} and 1×10^{-3} *M*, stirred in the cell, and containing 0.10 *M* sodium sulfate for ionic strength adjustment and 0.010 *M* sodium acetate for buffer generation. Measurements of emf and pH were taken after their stabilization (about 5 min).

b. Determination of chloramine-T electrode slope at various ionic strength values. Electromotive force measurements of standard CAT solutions, 5.00×10^{-4}, 1.00×10^{-3}, 5.00×10^{-3}, and 1.00×10^{-2} *M*, saturated with DCT and TSA and containing 0.050, 0.100, 0.200, and 0.250 *M* sodium sulfate, were carried out in duplicate. All slopes were calculated by means of regression analyses on the linear part of the calibration curves.

c. Evaluation of pK_a. A small volume of 4 *M* sulfuric acid was injected rapidly with a microsyringe into a stirred 0.0100 *M* chloramine-T solution saturated with DCT and TSA and containing 0.050, 0.100, 0.200, and 0.250 *M* sodium sulfate. The pH was measured and the CAT electrode potential change was recorded for a few seconds, and the cell was quickly emptied and rinsed with water. The next acid addition was carried out after at least a 5-min stay of the CAT electrode in the new CAT solution. Ten acid additions were made for each ionic strength value so that the pH range 5.5 to 3.0 was studied. From the values of potential and pH

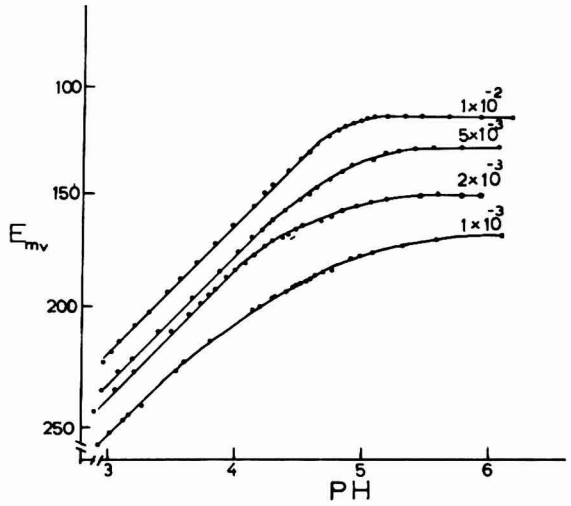

FIG. 1. Chloramine-T electrode potential at various pH levels after acid addition.

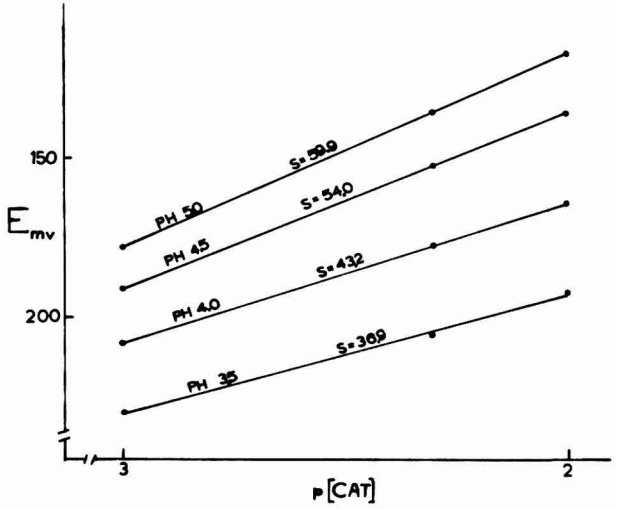

FIG. 2. Chloramine-T electrode slope at various pH levels.

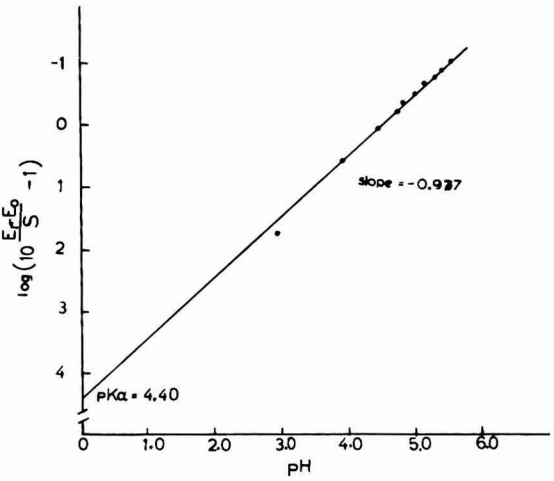

FIG. 3. Evaluation of pK_a at ionic strength 0.150 M, $T = 25 \pm 0.2$°C.

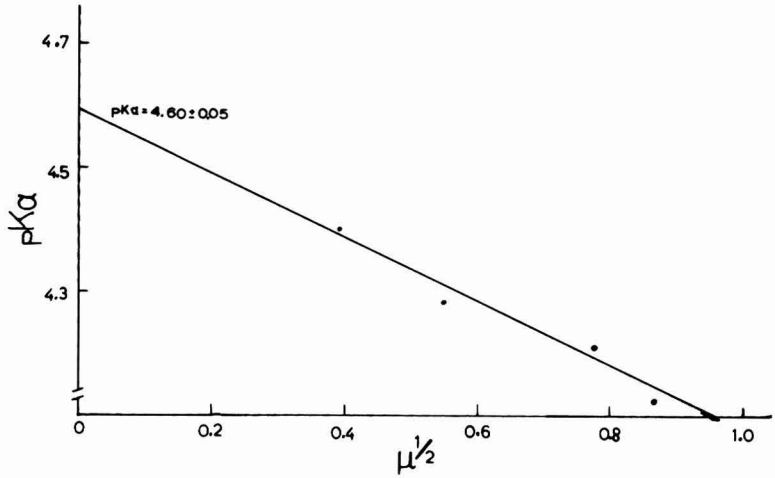

FIG. 4. Variation of equilibrium constant with ionic strength.

TABLE 1
VALUES OF pK_a AT VARIOUS IONIC STRENGTHS

Ionic strength		S^a		
μ (M)	$\mu^{1/2}$	(mV/log[CAT])	Slope[b]	Intercept[c]
0.150	0.387	59.4	−0.977	4.403
0.300	0.548	59.3	−1.000	4.282
0.600	0.775	59.2	−1.042	4.212
0.750	0.866	59.0	−1.048	4.125

[a] Experimental slope for CAT ion electrode response.
[b] Least-squares analysis, fit to linear equation.
[c] Intercept of experimental curve, least-squares analysis.

recorded, plots of log $(10^{(E_r - E_o)/S} - 1)$ vs pH are drawn for each ionic strength. The pK_a is equal to the intercept on the ordinate.

RESULTS AND DISCUSSION

The emf measurements of CAT solutions obtained at various pH levels after acid additions (procedure a) were plotted vs pH and are shown in Fig. 1. The sharp increase in potential in acidic solutions is due to a decrease of CAT ion concentration which is caused by a shift in the equilibrium between $RNCl^-$ and RNHCl (reaction (2)). Insoluble DCT and TSA were first noticed at pH 4.5. There is a drift of electrode potential below pH 5 and several minutes are necessary for stabilization. The slope of the electrode at various pH levels is shown in Fig. 2. It can be seen that the slope decreases with decreasing pH. It is evident that hydrogen ion strongly affects the CAT electrode in acidic solutions, causing drift and a different electrode response, and therefore the determination of pK_a by measuring potentials at various buffered solutions is not feasible. To overcome this difficulty the potential was measured within a few seconds after the rapid injection of sulfuric acid into the stirred CAT solution and the cell was emptied and rinsed promptly.

Every new acid addition must be carried out after a sufficient stay of the electrode in a neutral CAT solution so that any change in chloramine-T membrane can be eliminated. A typical plot of log $(10^{(E_r - E_o)/S} - 1)$ vs pH for the evaluation of pK_a is shown in Fig. 3. Values of pK_a at various ionic strengths calculated by the least-squares method are given in Table 1. These pK_a values were plotted vs the square root of ionic strength (Fig. 4), yielding a value of $pK_a = 4.60 \pm 0.05$ at zero ionic strength, corresponding to an acid ionization constant of $(2.5 \pm 0.3) \times 10^{-5}$ mole liter^{-1}. This value agrees closely with reported values (*5*). The agreement of the ionization constant with that found by other techniques indicates that the method is essentially sound. Furthermore, the use of ion-selective

electrodes in the determination of equilibrium constants is proved as a useful technique.

SUMMARY

Measurements made with a combination of glass and chloramine-T ion-selective membrane electrodes in acidified chloramine-T solutions varying in ionic strength have been used to determine the dissociation constant of *N*-chloro-*p*-toluenesulfonamide (chloramine-T acid). Experimental data agree satisfactorily with reported values.

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MICROCHEMICAL JOURNAL 23, 185–196 (1978)

Gas Chromatographic Determination of Two Isosorbide Mononitrates in Plasma

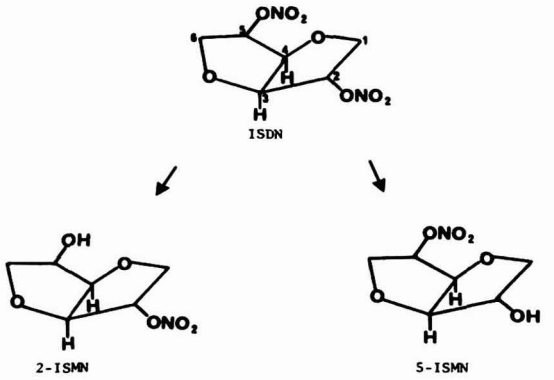
ROBERT V. SMITH[1] AND JOSIP BESIC[2]

Drug Dynamics Institute, College of Pharmacy, University of Texas at Austin, Austin Texas 78712

Received January 31, 1978

INTRODUCTION

Isosorbide dinitrate (1,4:3,6-dianhydro-D-glucitol 2,5-dinitrate; ISDN) is widely used in the treatment of angina pectoris. Like many organic nitrates, ISDN is therapeutically effective at parts-per-billion (ng/ml) blood levels in man. ISDN is rapidly metabolized in man and other animals to 2-isosorbide mononitrate (2-ISMN) and 5-isosorbide mononitrate (5-ISMN) (see Scheme 1). The combination of low therapeutic blood levels and rapid conversion to metabolites provides a significant challenge in the development of assays for ISDN, 2-ISMN, and 5-ISMN in blood plasma.



SCHEME 1. Biotransformations of ISDN in mammals.

Dietz *(5)* employed thin-layer chromatography (TLC) to detect 2-ISMN and 5-ISMN in the urine of dogs and humans following administration of ISDN. However, no rigorous attempt was made to quantitate the metabolites. Sherber *et al. (12)* employed gas chromatography (GC) with flame

[1] To whom inquiries and reprint requests should be sent.

[2] Current address: Institute for the Control of Drugs, Ulica Mose Pijade 160, 41000 Zagreb, Yugoslavia.

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ionization detection (FID) to determine ISDN in rabbit blood. However, the procedure described is of limited utility because of the modest sensitivity limits of FID (approximately 500 ng/ml) *(13)*.

Organic nitrates are very sensitively measured by electron capture (EC) detectors. When coupled with GC (GC/EC), low nanogram amounts of ISDN and its mononitrate metabolites can be selectively detected as described by Rossell and Bogaert *(9)*. A number of authors have attempted to exploit this high sensitivity (and selectivity) by developing quantitative GC/EC procedures for ISDN in blood or blood plasma. Gobbeler *(6)* described a method for ISDN in blood involving extraction with ethyl acetate; after drying over anhydrous sodium sulfate, the extract was concentrated and submitted to GC/EC using a 1% SE-30 column. Nearly quantitative recovery of low nanogram amounts of ISDN was claimed; however, no precision data were given. Additionally, obvious potential interferences are noted in the gas chromatograms of blood extracts *(6)*.

Rossell and Bogaert *(10)* measured ISDN in blood plasma using nitroglycerin as an internal standard and an extraction procedure similar to that of Gobbeler *(6)*. However, an additional cleanup step using charcoal-impregnated paper disks was employed, apparently to simplify the extracts and to remove potentially interfering substances. Nevertheless, the resulting chromatograms are very complex, indicating that significant amounts of plasma components are coextracted and subsequently chromatographed using this procedure. In our own laboratories, we have noted that analyses of complicated biological extracts by GC/EC can lead to fouling (with concomitant loss of sensitivity and spurious signal production) of EC detectors (see below).

Assinder *et al. (1, 2)* have determined ISDN in plasma by an incompletely described modification of the procedure reported by Rossell and Bogaert *(10)*. A further permutation was recently reported by Malbica *et al. (8)*, who extracted plasma with benzene and used charcoal-impregnated paper disks for cleanup. The use of benzene and the charcoal treatment probably decreases the levels of coextracted plasma constituents ultimately submitted to the GC/EC step. However, resulting chromatograms *(8)* still appear complicated and possess compounds that cochromatograph with ISDN and isoidide dinitrate, the internal standard employed. The methods noted above were either not applied to the ISMNs *(1, 2, 6, 10)* or failed *(8)* to provide satisfactory results with these metabolites.

Chin *et al. (3)* recently described a method for determining ISDN, 2-ISMN, and 5-ISMN in plasma. In their procedure, alkalinized plasma is extracted with ether which is dried (anhydrous magnesium sulfate), the dried ether extract is evaporated to dryness, and the residue is recon-

stituted in ethyl acetate prior to GC/EC using a 30% SE-30 column. The unusually highly loaded column was apparently necessary to prevent irreversible adsorption of ISDN and the mononitrates on exposed support surfaces. These authors' chromatograms appear less complex[3] than those resulting from previously noted methods. However, after repeated injections of extracts, interfering components leech through, necessitating a change in column. Furthermore, the method does not reliably eliminate an apparent plasma component of certain individuals that significantly interferes with the determination of 5-ISMN.[4]

In our hands, the Chin *et al. (3)* procedure provided good separation of ISDN, 2-ISMN, and 5-ISMN, and satisfactory calibration curves were obtained with standard reference substances. However, difficulties were experienced with detector fouling which appeared to be due in part to bleeding from the highly loaded SE-30 column. Furthermore, stated recoveries and precision *(3)* could not be duplicated.

A number of problems become apparent during the development of GC/EC procedures for ISDN and its mononitrate metabolites in plasma. The difficulties with selective recovery from biological media and irreversible adsorption of materials to GC columns have already been noted. Additionally, ISDN is quite volatile at standard temperature and pressure, creating problems of losses of compound when extracts are not evaporated extremely carefully. Like all determinations for drugs and their metabolites in biological fluids, assays for ISDN and the ISMNs should incorporate suitable internal standards for maintenance of requisite accuracy and precision. This requirement has been difficult to fulfill in the case of the ISMNs because diastereomeric mononitrates that might separate by GC are not readily available.

No suitable methodology exists for the analysis of 2-ISMN and 5-ISMN in plasma. Because these compounds do not present the problem of losses due to excess volatility, a method for their analysis was pursued initially. The procedure utilizes ether extraction, cleanup through *n*-heptane/methanol partitioning, and GC on a 3% OV-17 column as *O*-t-butyldimethylsilyl (TBDMS) derivatives. During studies of methods for the determination of 2-ISMN and 5-ISMN, techniques were uncovered that

[3] In principle, judgments of the relative complexities of extracts based on inspection of chromatograms generated by GC/EC can be tenuous because of the selectivity displayed by the EC cell. However, observations in our laboratories indicate a rank order relationship between chromatogram and extract complexities.

[4] Richard *et al.* recently noted in a letter to *Clinical Chemistry* [**22**, 2060–2061 (1976)] that a similar problem exists with a slightly modified version (essentially involving use of a 10% OV-1 column instead of a 30% SE-30 column) of the procedure described by Chin *et al. (3)*. Interferences with 5-ISMN and ISDN have also been noted by Kostenbauder *(7)* to arise in plasma that is permitted to stand several days under refrigeration.

may permit simultaneous analyses of ISDN, 2-ISMN, and 5-ISMN in plasma. These investigations are the subject of this paper.

MATERIALS AND METHODS

GC apparatus and conditions. A Hewlett–Packard model 5713A gas chromatograph equipped with a single ^{63}Ni (15 mCi) electron capture detector and a Varian Model 2100 gas chromatograph equipped with a scandium tritide (Sc ^{3}H; 100 mCi ^{3}H) detector were used as indicated. A repetition of the Chin *et al.* procedure was attempted with either coiled (Hewlett–Packard GC) or U-shaped (Varian GC) silylated (2% trimethylchlorosilane in benzene) glass columns (2-mm i.d. × 190 cm) packed with 30% SE-30 on Gas Chrom Q, 60/80 mesh (Applied Science Laboratories). These columns were conditioned at 220°C for 48 hr with a carrier gas flow of 30 ml/min prior to use. All other GC work was performed with coiled or U-shaped glass columns (2-mm i.d. × 190 cm) packed with 3% OV-17 on Chromosorb W-HP, 100/120 mesh (Analabs) which were conditioned at 275°C for 18 hr with a carrier gas flow of 50 ml/min. All columns were operated under the following conditions: injection port, 190°C (Varian GC), 160°C (Hewlett–Packard GC); oven, 160°C; detector, 250°C; carrier gas flow, (N_2) Varian GC, 60 ml/min, (Ar–methane, 95:5) Hewlett–Packard GC, 50 ml/min.

Chemicals and reagents. ISDN was obtained as a 25% mixture with lactose from the Wyeth Laboratories (Paoli, Pa.). Pure ISDN was recovered by extraction of the lactose mixture with ethyl acetate (EtOAc). After evaporation, the resulting residue was crystallized as needles from ethanol–water. The crystalline ISDN was homogeneous upon GC (see conditions above) and TLC [R_f = 0.85; silica gel GF_{254} (Analtech); CH_2Cl_2–EtOAc (3:1)] and employed as reference material. The ISMNs were obtained from the Ayerst Laboratories (Rouses Point, N.Y.) and were employed as reference standards without further purification. The 2-ISMN and 5-ISMN used were homogeneous by GC (conditions above) and TLC (2-IMSN, R_f = 0.47; 5-IMSN, R_f = 0.27; solvent system above). *N,O*-Bis(trimethylsilyl) acetamide (BSA) was obtained from Pierce Chemical Company (Rockford, Ill.). Heptafluorobutyric anhydride (HFBA) and *o*-nitrobenzyl alcohol (*o*-NBA) were used as purchased from Aldrich Chemical Company (Milwaukee, Wis.). The t-butyldimethylchlorosilane/imidazole reagent (1 *M* *t*-butyldimethylchlorosilane and 2.5 *M* imidazole in dimethylformamide) was purchased from Applied Science Laboratories (State College, Pa.). Ether, heptane, and acetonitrile were nanograde (distilled in glass) and obtained from Burdick and Jackson Laboratories (Muskegon, Mich.). EtOAc was spectroquality (Matheson, Coleman and Bell). All other solvents and reagents were of analytical reagent grade.

Assay procedure. All glassware was washed with detergent, thoroughly rinsed with water, and silanized by rinsing with 2% trimethylchlorosilane in benzene and dried at 100°C for 15 min. Standard solutions of 2-ISMN, 5-ISMN, and *o*-NBA were prepared in methanol, stored under refrigeration, and prepared fresh weekly. To four pairs of 3-ml blood plasma samples contained in 50-ml test tubes fitted with Teflon-lined plastic screw caps add duplicate 50-μl aliquots of methanol solutions containing the following: (A) 30 ng of 2-ISMN, 750 ng of 5-ISMN, 750 ng of *o*-NBA; (B) 75 ng of 2-ISMN, 1500 ng of 5-ISMN, 750 ng of *o*-NBA; (C) 150 ng of 2-ISMN, 3000 ng of 5-ISMN, 750 ng of *o*-NBA; (D) 300 ng of 2-ISMN, 4500 ng of 5-ISMN, 750 ng of *o*-NBA. The spiked plasma standards are treated with 1 ml of ether-saturated 10% ammonium sulfate solution and extracted with 15 ml of ether. After centrifugation at 2000*g* for 15 min, the ether layers are transferred to 50-ml centrifuge tubes containing 0.5 g each of anhydrous magnesium sulfate. After standing for 15 min and brief centrifugation at 2000*g*, 10-ml aliquots of the dried ether extracts are transferred to 15-ml centrifuge tubes and reduced to dryness under a stream of nitrogen. The residues are dissolved in 1-ml aliquots of *n*-heptane-saturated methanol which is partitioned twice with 1-ml portions of methanol-saturated *n*-heptane. The *n*-heptane layers are discarded and the combined methanol layers are reduced to dryness under a stream of nitrogen. The residues are treated with 50 μl of *t*-butydimethylchlorosilane/imidazole and 100 μl of acetonitrile, mixed on a Vortex mixer, and allowed to stand at room temperature for 30 min. The reaction mixtures are quenched with 250-μl aliquots of *n*-heptane-saturated methanol and the resulting polar phase is immediately partitioned with 500-μl portions of methanol-saturated *n*-heptane. After brief centrifugation at 2000*g*, 3-μl aliquots of the *n*-heptane layers are subjected to GC using a 3% OV-17 column (see above). Samples containing 2-ISMN and/or 5-ISMN are spiked with 750 ng of *o*-NBA contained in 50-μl portions of methanol and treated in a fashion identical to that for the standards.

Calculations. Peak height ratios are calculated from peak heights for the TBDMS derivatives 2-ISMN and 5-ISMN, which are separately divided by the peak height of TBDMS–*o*-NBA obtained in each chromatogram. Calibration curves are prepared by plotting peak height ratios from the standards versus the concentration of 2-ISMN and 5-ISMN in plasma, expressed in nanograms per milliliter. Values for unknown concentrations of 2-ISMN and/or 5-ISMN in sample plasma specimens are obtained by interpolation or calculated from the slope of the calibration curve.

RESULTS AND DISCUSSIONS

From a perusal of the recent literature, the GC/EC method published by Chin, *et al. (3)* appeared to be the most satisfactory for the analysis

of 2-ISMN and 5-ISMN in blood plasma. When the method was replicated in our laboratories, good development and separation of the ISMNs were observed on the recommended 30% SE-30 column. Furthermore, consistantly linear calibration curves were obtained with standard mononitrates. However, when analysis of the ISMNs in plasma was attempted, serious interferences were noted. The interfering peaks did not arise from the solvents utilized although artifacts from the plastic recepticles used to obtain standard plasma samples could not be entirely ruled out because of a lack of plasma harvested exclusively in glass.[5] Since the observed interferences were suspected to be lipid-like in nature, it was thought that an *n*-heptane/methanol partitioning step might serve as a suitable cleanup measure following initial recovery by ether extraction. Before this was pursued, however, it was deemed necessary to reinvestigate the ether extraction procedure previously reported *(3)*.

Chin *et al. (3)* note that the partition coefficients (K_D) for 2-ISMN and 5-ISMN in ether/saline are 0.50 and 0.35, respectively. We determined K_D's for 2-ISMN and 5-ISMN in ether/water and obtained comparable values of 0.44 and 0.37. However, the Chin *et al. (3)* method employs ether extraction of the ISMNs from alkalinized plasma. The K_D values determined for 2-ISMN and 5-ISMN in ether/alkali were 0.18 and 0.16, respectively. If one calculates recoveries of 2-ISMN and 5-ISMN using the K_D values and the volume ratios recommended by Chin *et al. (3)*, significantly less than quantitative recoveries are achieved in the initial ether extracted, as indicated in Table 1. Furthermore, the presence of alkali detrimentally effects the recovery of ISMN's (see Table 1).[6]

It was found that the recovery of 2- and 5-ISMN by ether extraction could be significantly improved by the use of a salting-out agent, ammoni-

TABLE 1
RECOVERIES OF 2-ISMN AND 5-ISMN FROM AQUEOUS MEDIA BY ETHER EXTRACTION

Isosorbide Mononitrate	Ether/saline		Ether/water		Ether/alkali[d]	
	K_D[a]	% Recovery[b]	K_D	% Recovery[c]	K_D	% Recovery[b]
2-ISMN	0.50	77	0.44	62	0.18	55
5-ISMN	0.35	70	0.37	58	0.16	52

[a] Reported by Chin *et al.* (3).

[b] Determined by the formula $p = K_D U/(K_D U+1)$, where K_D = partition coefficient, U = volume ratio, and p = fraction extracted. An average volume ratio of 6.75 was used.

[c] Volume ratio of 3.75 (see Materials and Methods).

[d] 1 *N* Sodium hydroxide.

[5] Rossell and Bogaert *(11)* recently identified phthalate plasticizers as potential interferences in a GC/EC analysis of ISDN in plasma.

[6] This may be due to base-catalyzed hydrolysis of the nitrate esters of 2- and 5-ISMN.

um sulfate. When employed at a concentration of 2.5%, essentially quantitative recoveries of 2-ISMN and 5-ISMN were observed when aqueous media was extracted with an approximate four-volume excess of ether.

After defining conditions for the recovery of 2-ISMN and 5-ISMN from aqueous solutions by ether extraction, an evaluation of an *n*-heptane/methanol cleanup was sought. It was found that ether extracts of plasma were simplified (as indicated from subsequent gas chromatograms) if subjected to this type of partitioning where the methanol layer was ultimately subjected to GC. Initial studies indicated that the K_D's for 2-ISMN and 5-ISMN in *n*-heptane/methanol would favor the alcohol layer. Indeed, when carefully determined, K_D^{-1}'s of 15 and 22 were observed for 2-ISMN and 5-ISMN, respectively.[7]

A shortcoming of earlier methods for the GC determination of 2-ISMN and 5-ISMN was the lack of a suitable internal standard. As indicated previously, diastereoisomeric mononitrates are not readily available for this purpose. Thus, a unique surrogate was sought. The internal standard ultimately chosen was *o*-nitrobenzyl alcohol. It was rationalized that the nitrobenzyl alcohols would possess solubility properties similar to those of the ISMNs and would, at the same time, be senstivity detected by EC. This was found to be so, as indicated below.

A second drawback associated with GC analyses of ISMNs is the irreversible adsorption of these compounds to active surfaces of GC supports. Chin *et al. (3)* attempted to overcome this difficulty by utilizing a highly loaded (30% SE-30) column. We found 30% SE-30 columns initially satisfactory. However, after a few days, significant problems were observed with detector fouling. This problem was particularly acute when the 30% SE-30 column was utilized with our Hewlett–Packard instrument. It was proposed that both the detector fouling and adsorption problems might be remedied through the use of suitable derivatives. Initially, trimethylsilyl and heptafluorobutyryl derivatives were prepared and submitted to GC. However, neither of these derivative approaches produced satisfactory GC resolution of 2-ISMN, 5-ISMN, and one of three isomeric nitrobenzyl alcohols. As an alternative, *t*-butyldimethylsilyl (TBDMS) ethers were considered. These derivatives also possess the advantage of increased hydrolytic stability *(4)* compared with their trimethylsilyl homologs. Indeed, when chromatographed on a 3% OV-17 column, excellent separation of the TBDMS derivatives of 2-ISMN, 5-ISMN, and *o*-NBA was achieved. The TBDMS derivatives were found to form rapidly at room temperature (see Fig. 1). When chromatographed after recovery from plasma using the procedure outlined in Scheme 2, no interferences from plasma constituents were observed (see Fig. 2).

[7] Since *n*-heptane is lighter than methanol, reciprocal K_D's >10 reveal marked affinities of the ISMNs for the methanol layer.

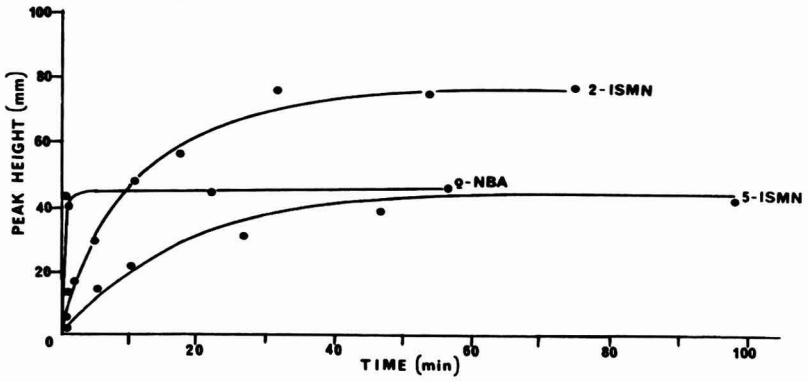
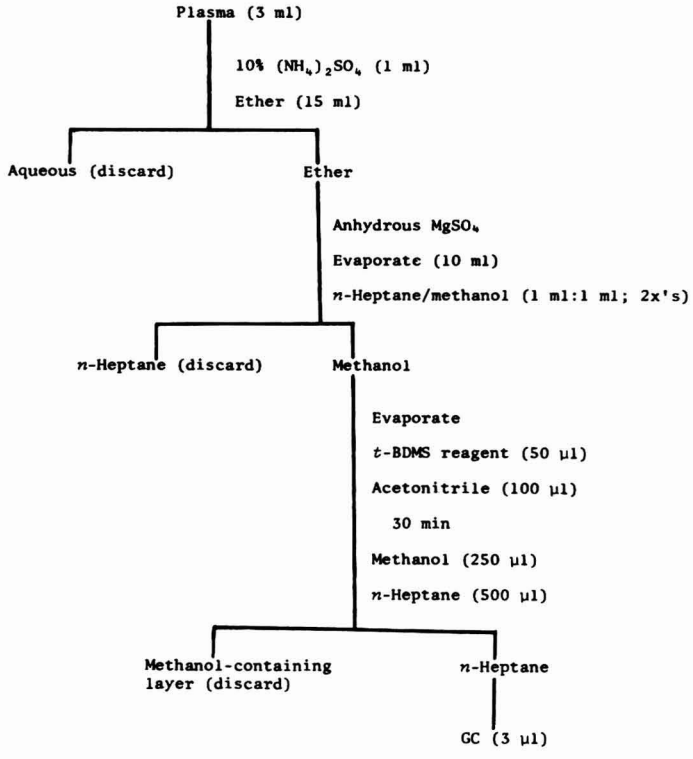


FIG. 1. Kinetics of t-butyldimethylsilylation of ISMNs and *o*-nitrobenzyl alcohol at room temperature.

The method outlined in Scheme 2 was evaluated for accuracy and precision. Standard curves prepared for 2-ISMN and 5-ISMN were consistantly linear, as indicated in Fig. 3 and 4. Absolute recoveries of the



SCHEME 2

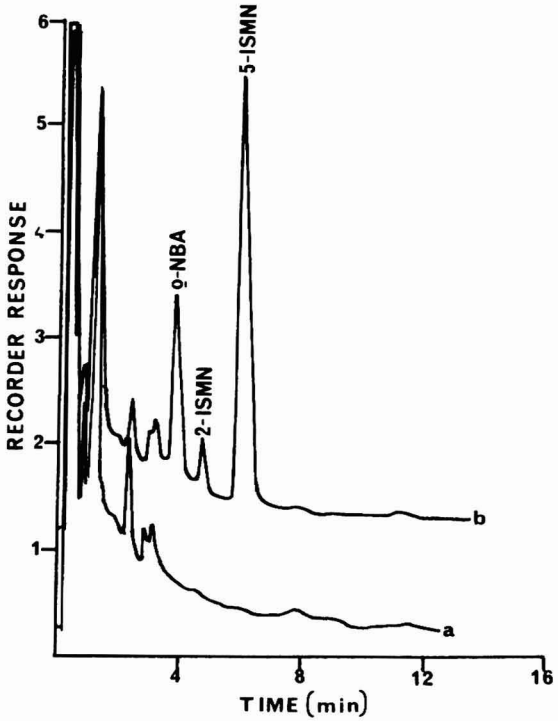


FIG. 2. Gas chromatograms (2-mm i.d. × 190 cm; 3% OV-17) derived from human plasma samples processed as outlined in Scheme 2; attenuation = 128. Legend: (a) normal plasma specimen; (b) plasma specimen containing 100 ng/ml 2-ISMN, 1500 ng/ml 5-ISMN, and 250 ng/ml *o*-NBA (internal standard).

ISMNs and *o*-NBA over the range of concentration listed in Table 2 were: 2-ISMN, 76.7% ($N = 7$); 5-ISMN, 84.8% ($N = 8$); *o*-NBA, 62.6% ($N = 8$). When evaluated for precision, the method provided excellent reproducibility, as indicated in Table 2.

The GC/EC method devised should be suitable for measuring plasma levels of 2-ISMN and 5-ISMN in man and animals and may be useful in more accurately determining the metabolic fate of these metabolites. An obvious question, however, is whether this methodology could be employed to measure mixtures of the three compounds, ISDN, 2-ISMN, and 5-ISMN.

During the development of the steps indicated in Scheme 2, it was thought that any ISDN present in the initial plasma sample would either be eliminated in the ether evaporation step[8] or would partition into the

[8] ISDN is sufficiently volatile under standard conditions to present serious loss problems during solvent evaporation steps.

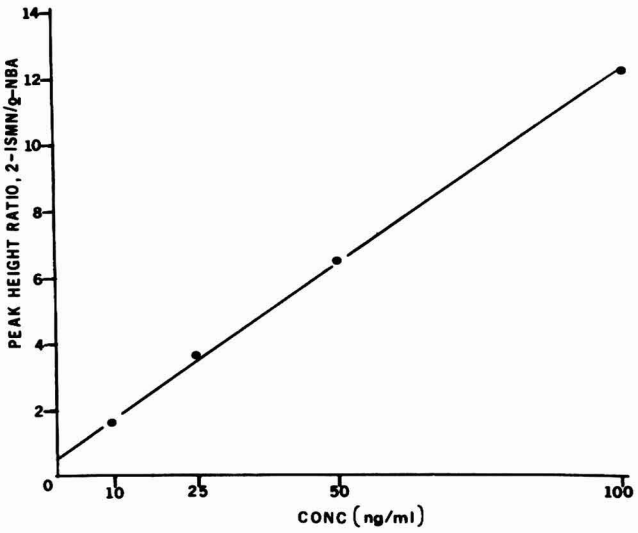


FIG. 3. Typical calibration curve for the determination of 2-ISMN in plasma. $r = 0.994$.

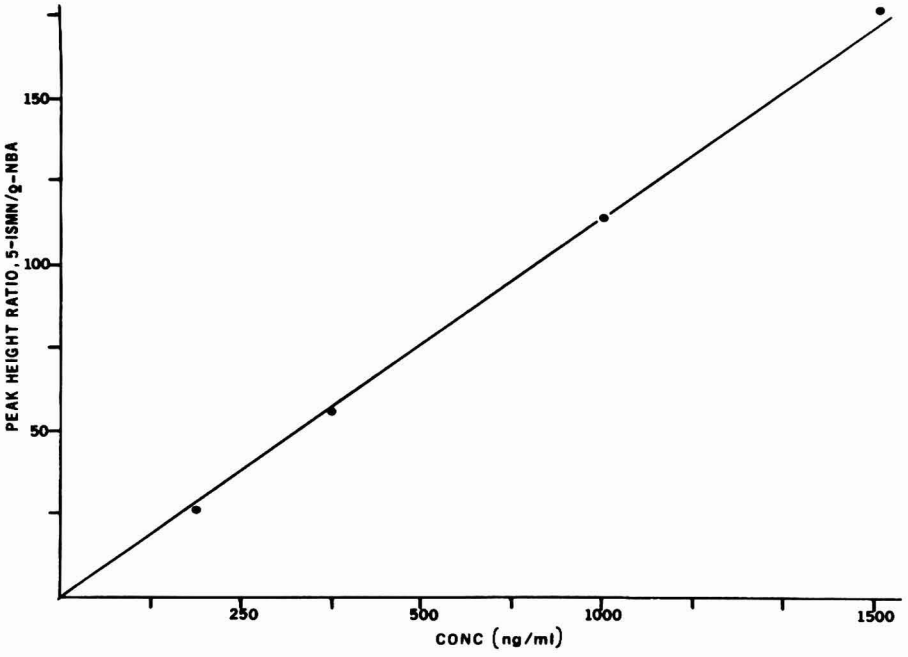


FIG. 4. Typical calibration curve for the determination of 5-ISMN in plasma. $r = 0.999$

TABLE 2
PRECISION OF GC/EC METHOD FOR ISMNs

ISMN	Plasma levels analyzed (ng/ml)	No. of determinations	Relative standard deviation (±%)[a]
2-ISMN	10–25	19	4.8
	50–100	13	6.1
5-ISMN	250–500	18	5.2
	1000–1500	14	4.6

[a] RSDs of simulated samples assayed using standard curve generated as described in Materials and Methods.

n-heptane that would subsequently be discarded. However, to our suprise, the K_D^{-1} for ISDN in *n*-heptane/methanol was determined to be >1000. Thus, it could potentially interfere in the final GC step since it cochromatographs with the TBDMS ether of 2-ISMN. Fortunately, we had previously found that it was desirable to partition the TBDMS derivatives between *n*-heptane/methanol prior to the GC step to avoid injection of excess derivation reagents (see Scheme 2). This last partitioning step completely eliminates the ISDN from the *n*-heptane layer that is ultimately submitted to GC.

The unexpected partitioning behavior of ISDN in *n*-heptane/methanol has suggested a methodology that might ultimately be developed for the analysis of ISDN in combination with the ISMNs. This lead is currently being pursued in our laboratories and will be the subject of a future report.

SUMMARY

A GC/EC method has been developed for the determination of 2-isosorbide mononitrate (2-ISMN) and 5-isosorbide mononitrate (5-ISMN) in blood plasma. The procedure is based on extraction of ammonium sulfate-treated plasma with ether followed by a cleanup via *n*-heptane/methanol partitioning. After conversion to t-butyldimethylsilyl ethers, the ISMNs are chromatographed on a 3% OV-17 column. Absolute recoveries of 76.7 and 84.8% were achieved for 2-ISMN and 5-ISMN, respectively. Using *o*-nitrobenzyl alcohol as an internal standard, relative standard deviations of 4.8 to 6.1% were observed over the concentration ranges of 10 to 100 ng/ml for 2-ISMN and 250 to 1500 ng/ml for 5-ISMN.

ACKNOWLEDGMENTS

This work was supported by a grant from the Ives Laboratories, New York, New York, and Research Training Grant M8/181/4B. 158 from the World Health Organization. The

authors are grateful to Dr. Milan Grims, Director of the Institute for the Control of Drugs, Zagreb, Yugoslavia, who helped in arranging postdoctoral training for J. Besic at the University of Texas at Austin. The authors also appreciate technical support and advice from David W. Humphrey during early stages of this work.

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MICROCHEMICAL JOURNAL 23, 197–219 (1978)

Automatic Microanalyzers

VI. Use of a Vertical Process System[1]

D. FRAISSE AND R. SEMET

Service Central de Microanalyse, Centre National de la Recherche Scientifique, 2 rue Henry Dunant, 94320 Thiais, France

Received January 28, 1978

INTRODUCTION

For the past 10 years our effort has been directed toward the development of automatic microanalyzers in our laboratory.

In order to make clear the evolution of our own techniques during this period it should be emphasized that the success of any modern quantitative analytical technique depends not only on the accuracy and precision of its results but also on its capacity to bring them forth rapidly.

Insofar as our laboratory was concerned, we had to solve three main problems:

—to give answers as quickly as possible to an increasing demand in elemental organic microanalysis;

—to develop simultaneously elemental inorganic microanalysis;

—to extend the scope of our activities to the field of the microdetermination of elements in very small quantities or, eventually, to the trace scales in organic and inorganic compounds.

The fact that the staff of our analytical service laboratory could not increase in number indefinitely meant that the only possible way to solve these problems was to improve our productivity by the introduction of automation into our analytical techniques. Our first approach was the automation of commercially available analyzers for the simultaneous microdetermination of carbon, hydrogen, and nitrogen. However, these modified apparatus were shown to be unsatisfactory, for want of universality, the more so because within the framework of our service laboratory a very large variety of compounds must be analyzed whether they contain interfering elements or not. Such limitations obliged us to keep old analyzers of the classical type still working, which brought us no solution to the above-mentioned problems. For this reason we decided to develop our own automatic analyzers.

Our initial objective was achieved by using automatic coulometric titra-

[1] This paper was presented at the International Symposium on Microchemical Techniques, Davos, May 1977.

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tion for rapid determinations of oxygen (*4*) and carbon and hydrogen (*2*). Our second step aimed at the automation of the process techniques, keeping in mind that the most difficult stage was the introduction of the sample into the reactor and the withdrawal of the sample container after use. We considered that the easiest way to carry out these operations implied the use of a vertical reactor where the sample process should take place.

As to the risk of trouble because of the presence of many used containers in the reactor, it appeared to us it could be avoided provided that no contact with the reagents took place. However, other drawbacks, such as a memory effect related to the presence of used containers, could occur, especially in oxygen determination, and prevent the application of such a principle. On the other hand the analysis of some compounds containing metals or phosphorus could lead to biased analytical results because of the adsorption of gaseous process products on the reactor walls and (or) in the residues of used containers. As a matter of fact such a nuisance appears only when the sample size is too large; it will be seen later that it is very easy to get rid of the memory effect in our analyzers.

Therefore, in the system we have developed, the weighing containers heap up in a special tube without disturbing the analyses.

This paper sums up the results of our efforts to build up our own automatic analyzers; it is divided into three parts which deal respectively with:

—the development of the so-called "automatic introduction device" (AID) which is the fundamental first step of our research work;

—the development of the so-called "automatic sample feeder" (ASF);

—the presentation of three automatic microanalyzers provided with a vertical reactor.

DESCRIPTION

Automatic Introduction Device (AID)

This system aims at making possible the introduction of many solid or liquid, stable or unstable samples of organic compounds into vertical reactors of analyzers used for determinations as different as those of CHN and O (*5*). For this purpose the system consists of two main parts: a "sample inlet device" and a "collecting tube." This device (Fig. 1) is made of Pyrex glass, its central vertical tube is provided with a lower standard ground cone joint for connection to the collecting tube, and its upper end is closed with a plastic stopper. The middle side tube provided with a screwthread joint and a plastic screwcap houses a coil spring and a movable sample holder made of Pyrex glass with an iron core sealed in one end. A Pyrex rod passing through the plastic cap holds the spring and the sample holder that enters the central vertical tube.

The device for general use (Fig. 2) is provided with lower and upper

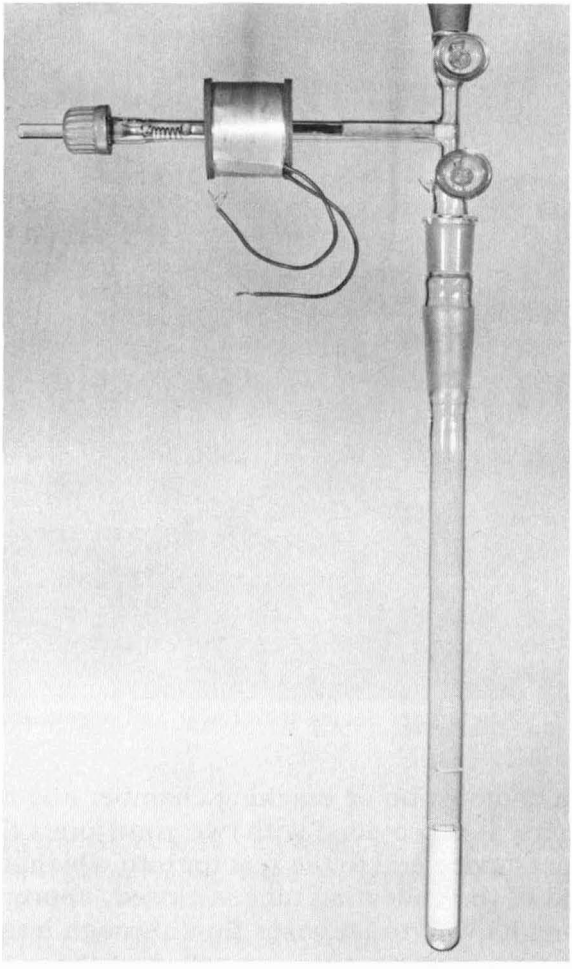

FIG. 1. Automatic introduction device (AID).

side tubes for gas flow. Another type of device is provided only with the lower side tube (Fig. 3). The choice of the appropriate type of device depends on the determination on hand. It means that the device provided with the upper and the lower side tubes is used for any determination affected by traces of air which have diffused into the AID during the sample introduction; for example, this is the case for the oxygen determination.

Collecting Tube and Reactor

The container with the sample held by the sample holder in the inlet device falls down into the collecting tube (right-hand side of Fig. 4) when the electromagnetic coil is switched on. This tube, made of transparent

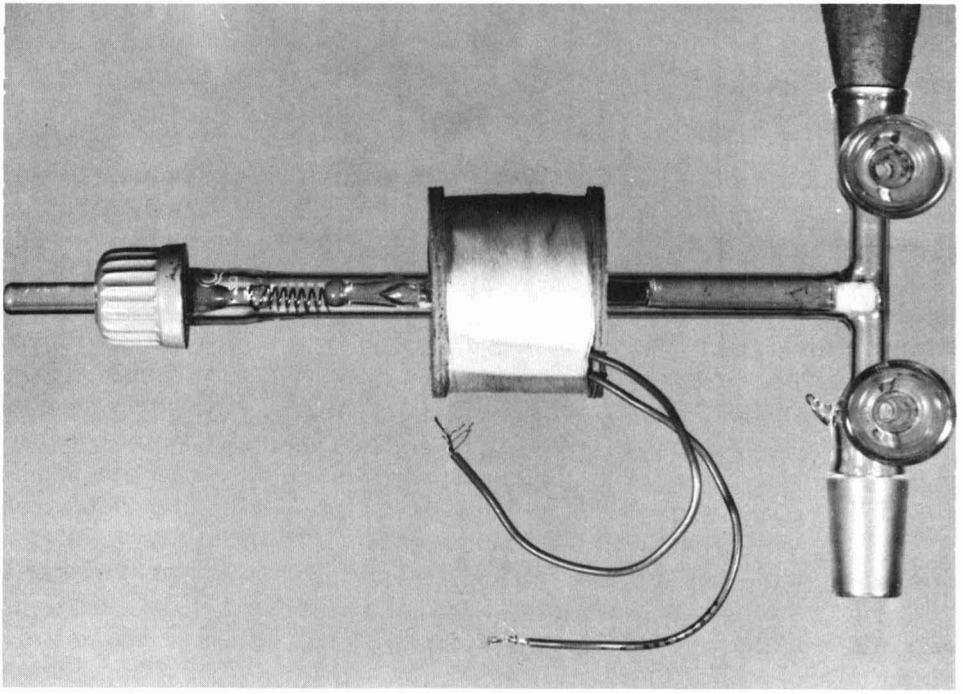

FIG. 2. Sample inlet device with lower and upper side tubes.

quartz, is both a combustion or cracking chamber and a collecting vessel for used containers. It is provided with two cone joints for its connections to the sample inlet device and to the reactor into which it is fitted. (Fig. 5). As the lower end of the collecting tube is closed, appropriate slits are cut horizontally along its wall to let gases flow through into the reactor. The assembly of the three components consisting of the sample inlet device, the collecting tube, and a reactor is shown in Fig. 6.

Sample Containers

Cylindrical microbeakers are used; they are convenient for sampling stable solid and liquid compounds. Short capillary tubes made of Pyrex glass may also be used for sampling extremely volatile liquid compounds but it is advisable to use cylindrical microbeakers even in this case because of their easier handling. These microbeakers can be made of different materials such as fused quartz, silver, or aluminum. For the sake of standardization and simplification only silver microbeakers are now used in our laboratory for sampling any solid, liquid, stable, or unstable compounds in order to carry out O, CH, or N determinations. When silver microbeakers are used a so-called collecting crucible, usually made of either tubular fused quartz or alumina, is placed inside the collecting tube

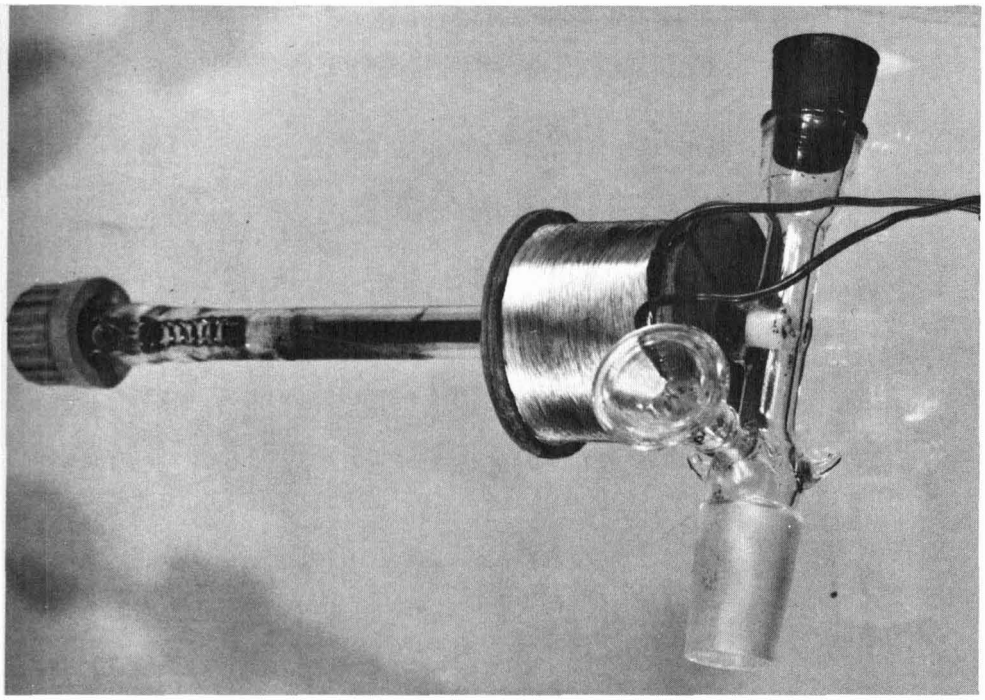

FIG. 3. Sample inlet device with a lower side tube.

on the bottom; the microbeakers can heap up in this collecting crucible, which can contain 100 or more used molten or crushed silver microbeakers.

A further advantage of this collecting crucible is that it is the only part of the AID which has to be changed for a new one when it is filled up.

Automatic Sample Feeder (ASF)

The use of the automatic introduction device, previously described, requires that the operator be present at the start of each determination in order to introduce the sample container into the device.

In order to achieve full automation of our analyzers in carrying out repetitive determinations without the intervention of an operator, we have designed and developed the automatic sample feeder (*1*), or ASF (Figs. 7 and 8). This design allows the connection to the collecting tube to be made at the location of the sample inlet device without disturbing the analyzer.

As we can see in Figs. 7 and 8, the ASF consists of a revolving magazine made of plastic material and a straight vertical tube provided with a Pyrex glass stopcock with a PTFE key which does not require lubrication; however, a Pyrex glass key should be used when the gas

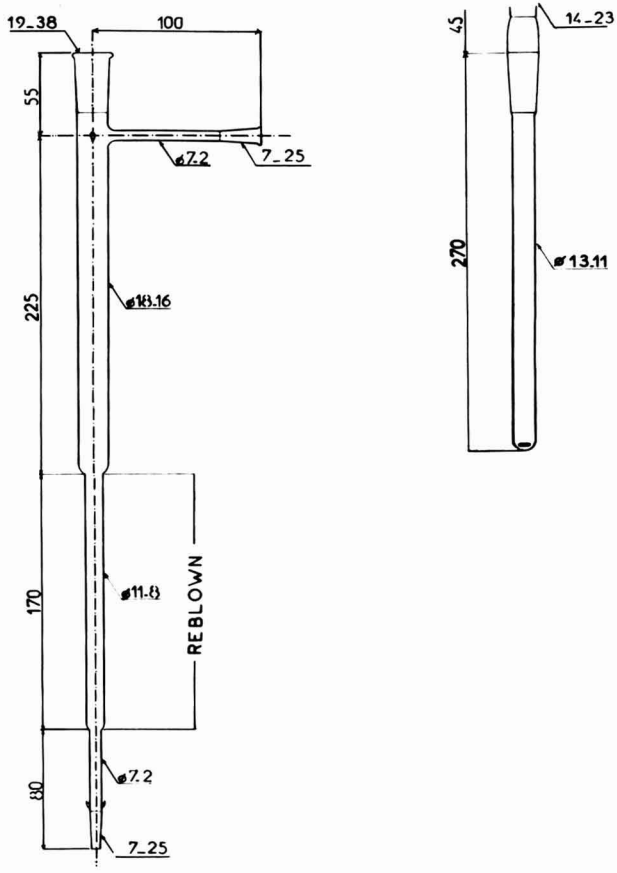

FIG. 4. Collecting tube and reactor.

pressure inside the analyzer requires more tightness; instead of bores, these keys are provided with cylindrical-bottomed cavities.

The magazine and key are simultaneously driven by the same small electric motor so that a sample container is fed stepwise into the collecting tube; first, the sample container falls off the magazine down into the key cavity and then it falls off this cavity down into the collecting tube and heaps up in the collecting crucible.

The upper limb of the stopcock vertical tube is provided with a side arm which makes it possible for any required gas to flow and purge the key cavity at will. The lower limb of this tube is also provided with a side arm which makes it possible for the used carrier gas to flow for the determination on hand.

Advantages of the AID and the ASF

The AID and the ASF show many advantages:

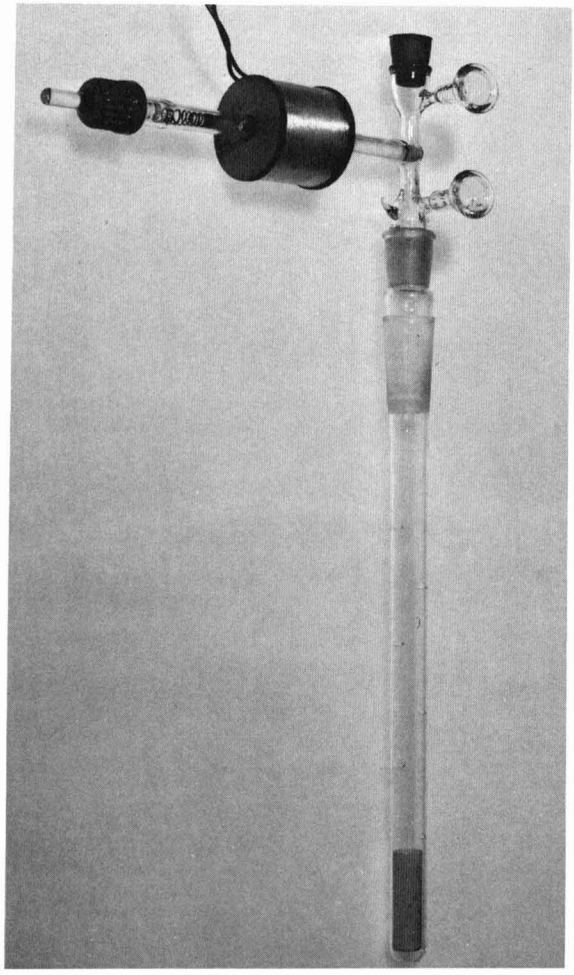

FIG. 5. Sample inlet device and collecting tube.

The first is their low cost because of their simplicity and their makeup of Pyrex glass and plastic material only. Furthermore they can be much more widely used than other systems described in the literature and they can be used with any microanalyzer with a vertical reactor, as we shall see later.

The design of our collecting tube and its collecting crucible makes it possible to change both for new ones, simultaneously or not, at any time when necessary, without effecting variations in furnace and reagent temperatures; thus, the microanalyzer is always kept ready in working condition, for any type of determination on hand. In addition, the collecting tube prevents contact between the reagents and the microbeakers, thus avoiding the nuisance of unwanted chemical reactions; moreover

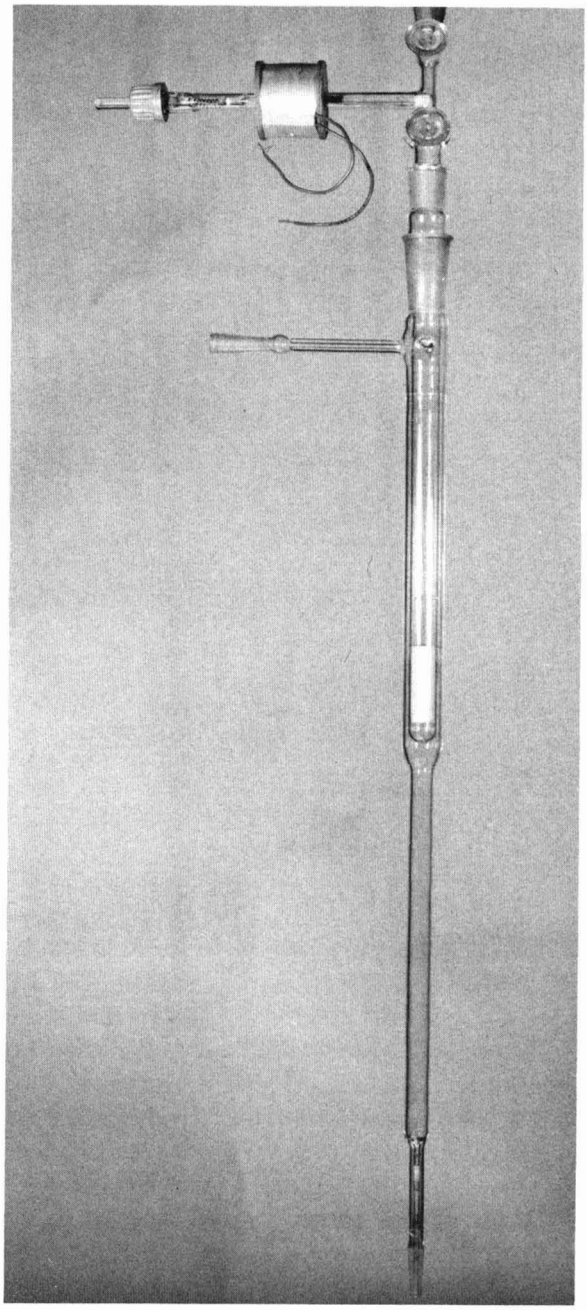

FIG. 6. Sample inlet device, collecting tube, and reactor.

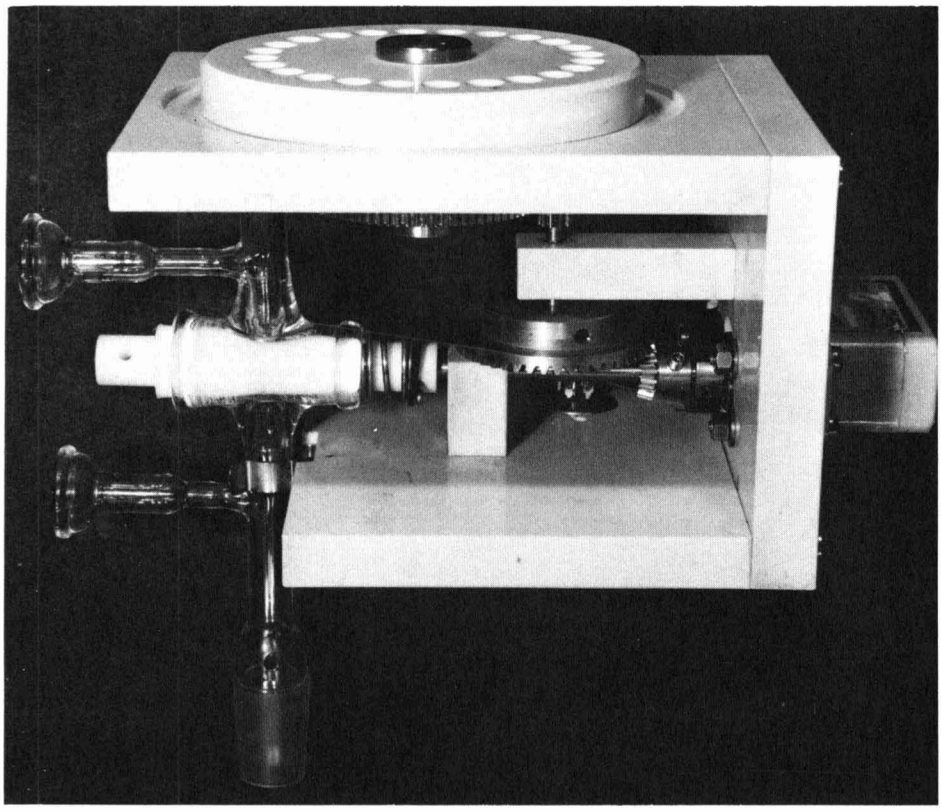

FIG. 7. Automatic sample feeder (ASF), front view.

there is no squeezing of the reagent filling in the reactor by the collecting tube.

As for the ASF a fundamental advantage is that it makes possible the introduction of a selected sample container into the AID at any time without stopping the analyzer; it allows one either to check the calibration factor of the analyzer by the analysis of a reference substance or to introduce unstable compounds immediately prior to their analysis.

APPLICATIONS AND RESULTS

Oxygen Microanalyzer

The first analyzer developed in our laboratory was the oxygen analyzer (*3*). By using the AID we have been able to determine oxygen contents in organic compounds within 8 min. Our first O-microanalyzer model, based

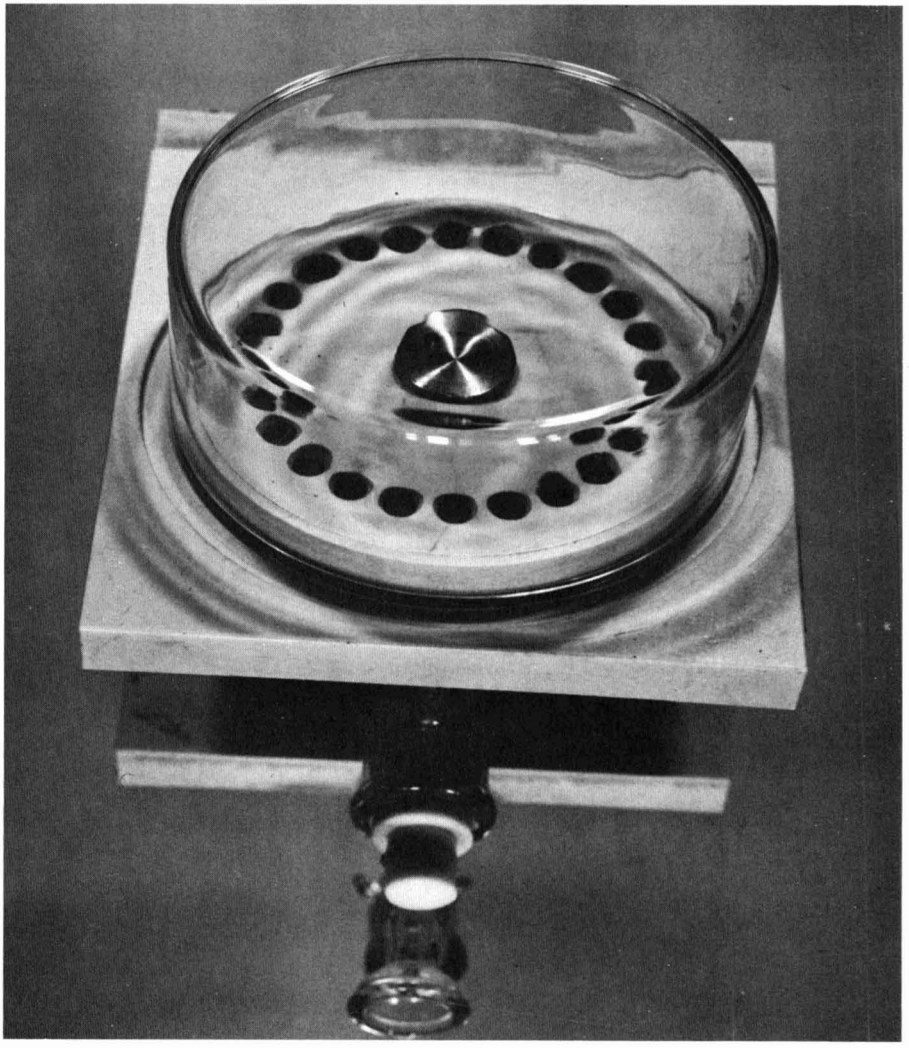

FIG. 8. Automatic sample feeder, top view.

on the Schütze–Unterzaucher principle, is shown in Fig. 9; it involves a flash pyrolysis of the sample which takes place in the collecting tube, a reduction of pyrolytic gases on carbon black at 1120°C, and then the oxidation of carbon monoxide into carbon dioxide; the latter is finally titrated by automatic protonometric coulometry. The use of this O-microanalyzer model requires the operator to introduce the sample container manually at the start of each determination.

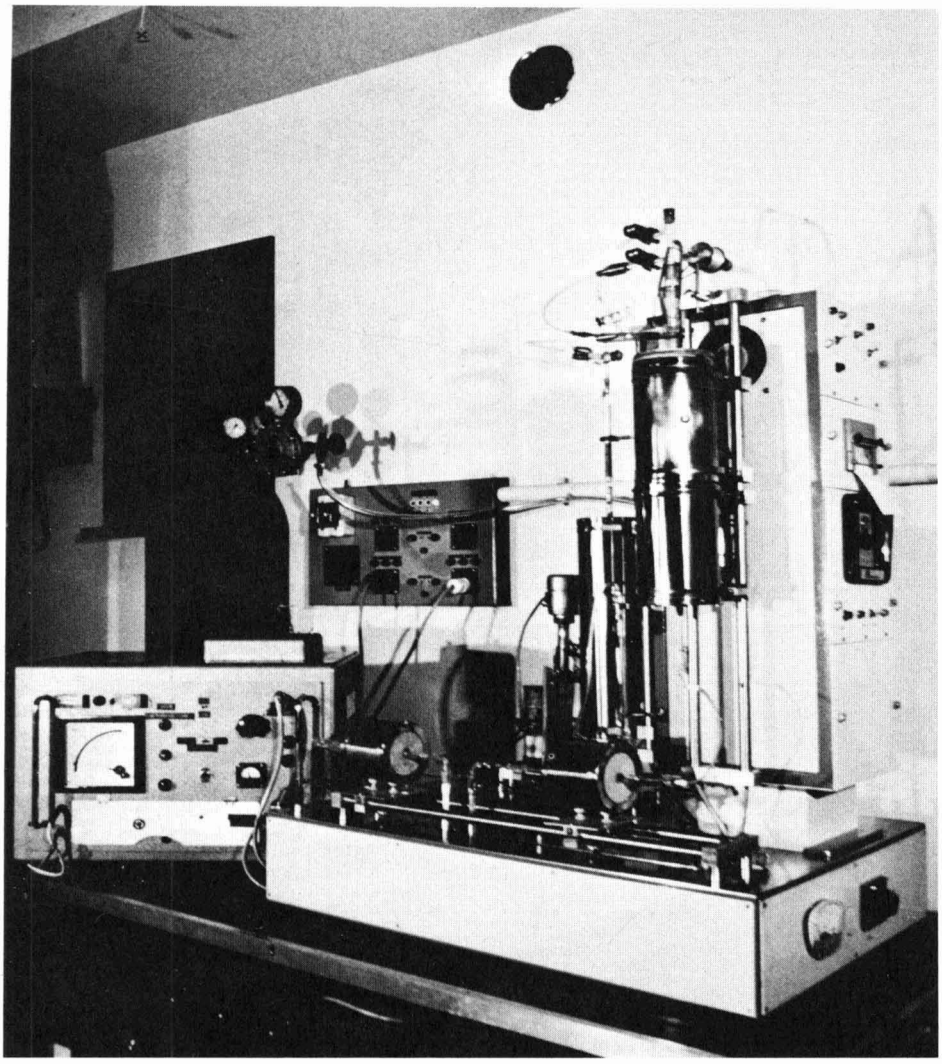

FIG. 9. O-Microanalyzer equipped with an AID.

Our second O-microanalyzer model, based on the same principle and provided with an ASF, is shown in Fig. 10. It is fully automated and makes possible the determination of oxygen in solid, liquid, stable, or unstable, organic, and some inorganic compounds within 4 to 10 min. A duration of 7 min was adopted in our Laboratory. Some results are reported in Table 1.

The results obtained for the determination of the oxygen contents in

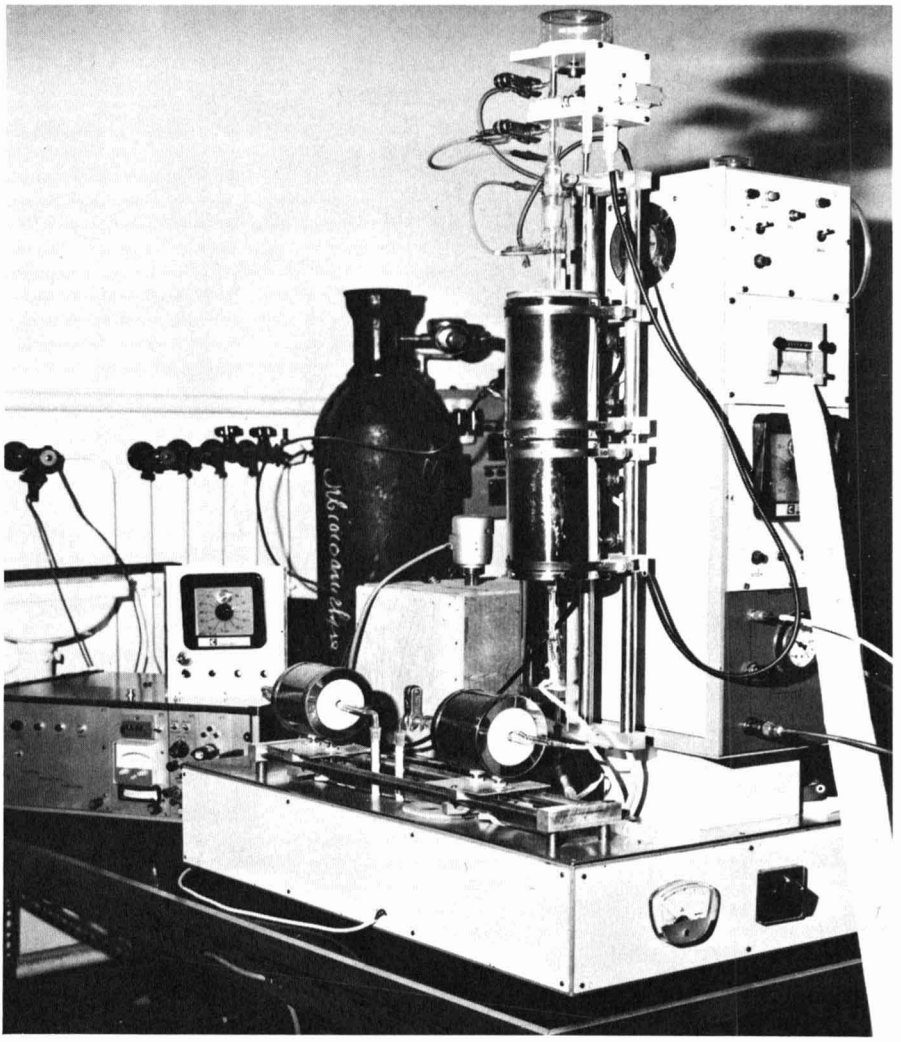

FIG. 10. O-Microanalyzer equipped with an ASF.

organic compounds containing phosphorus should be noted; a statistical study has shown that the addition to the sample of reagents such as carbon black or diphenylamine does not really improve the results; to the contrary, the values of blanks obtained by running diphenylamine alone are not quite reproducible even if the sizes of the samples are only slightly different. As a matter of fact it is only required that the temperature in the collecting tube be above 1050°C where the samples in microbeakers are

TABLE 1
OXYGEN DETERMINATION IN ORGANIC COMPOUNDS

Compound	Sample weight (mg)	% O	
		Calcd	Found
	1.459	4.14	4.13
Cholesterol	1.350		4.16
	1.827		4.14
Aminopyrine	0.819	6.92	6.92
	1.039		6.87
	0.829		6.90
Nicotinamide	3.076	13.10	13.01
	2.842		13.15
	2.471		13.13
Atropine	2.391	16.59	16.63
	2.809		16.59
	2.196		16.63
Sulfanilamide	2.129	18.58	18.64
	2.315		18.59
	1.620		18.65
Urea	1.589	26.64	26.65
	1.467		26.73
	1.590		26.63
Vanillin	1.580	31.55	31.51
	1.612		31.50
	1.400		31.49
Acetylsalicylic acid	1.636	35.52	35.55
	1.146		35.51
	1.403		35.60
Saccharose	1.312	51.42	51.62
	1.514		51.54
	1.279		51.32

pyrolyzed and that the sample weights not exceed 1.3 to 1.4 mg, whatever the oxygen and phosphorus contents in the substance analyzed may be.[2] Furthermore, the calibration coefficients should be the same for analyses of plain organic compounds and organophosphorus compounds. The results obtained for the analysis of the latter are shown in Table 1A.

[2] It should be noticed that diphenylamine is efficient only at lower temperatures, but making the temperature higher is still more efficient.

TABLE 1A
OXYGEN DETERMINATION IN ORGANIC COMPOUNDS CONTAINING PHOSPHORUS

Compound	Sample weight (mg)	%O	
		Calcd	Found
Triphenyl phosphine oxide	1.280	5.75	5.76
	1.199		5.60
	1.269		5.92
Research compound	1.216	14.36	14.09
Triphenyl phosphate	1.239	19.61	19.62
	1.206		19.58
	1.272		19.45
	1.261 + (0.6 DPA)[a]		19.50
	1.413		19.58
	1.188 + (1 DPA)[a]		19.60
Research compound	1.133	19.94	19.81
Research compound	1.076	21.14	20.98
Benzenephosphonic acid	1.301	30.36	30.16
	12.09		30.09
	1.111		30.18
Triethyl phosphate	1.114	35.14	35.16

[a] DPA: Diphenylamine.

Carbon–Hydrogen Microanalyzer

The oxygen analyzer has been working successfully in our laboratory for about 3 years and we can assert that it has contributed to an increase in our capabilities in routine analysis. This success prompted us to develop an automatic analyzer for carbon and hydrogen determination.

The determination of both elements remains a major part of our analytical work, the more so because the CNH Technicon analyzers which are in use in our laboratory are unable to analyze unstable solids or liquids, in routine analysis. Therefore, instead of a CH apparatus of the classical type, we needed a rapid automatic analyzer for CH determination in stable or unstable, liquid or solid organic compounds. In order to solve this problem we have developed the CH analyzer shown in Fig. 11 (7). A schematic view of the processing unit is shown in Fig. 12.

The technique is based on the principle of flash combustion of the sample, which takes place inside the collecting tube in a stream of pure oxygen. The collecting tube is fitted into the upper part of the vertical reactor, and the combustion gases flow through the lower part, which is filled with the appropriate reagents ensuring complete oxidation and removal of sulfur and halogen by-products.

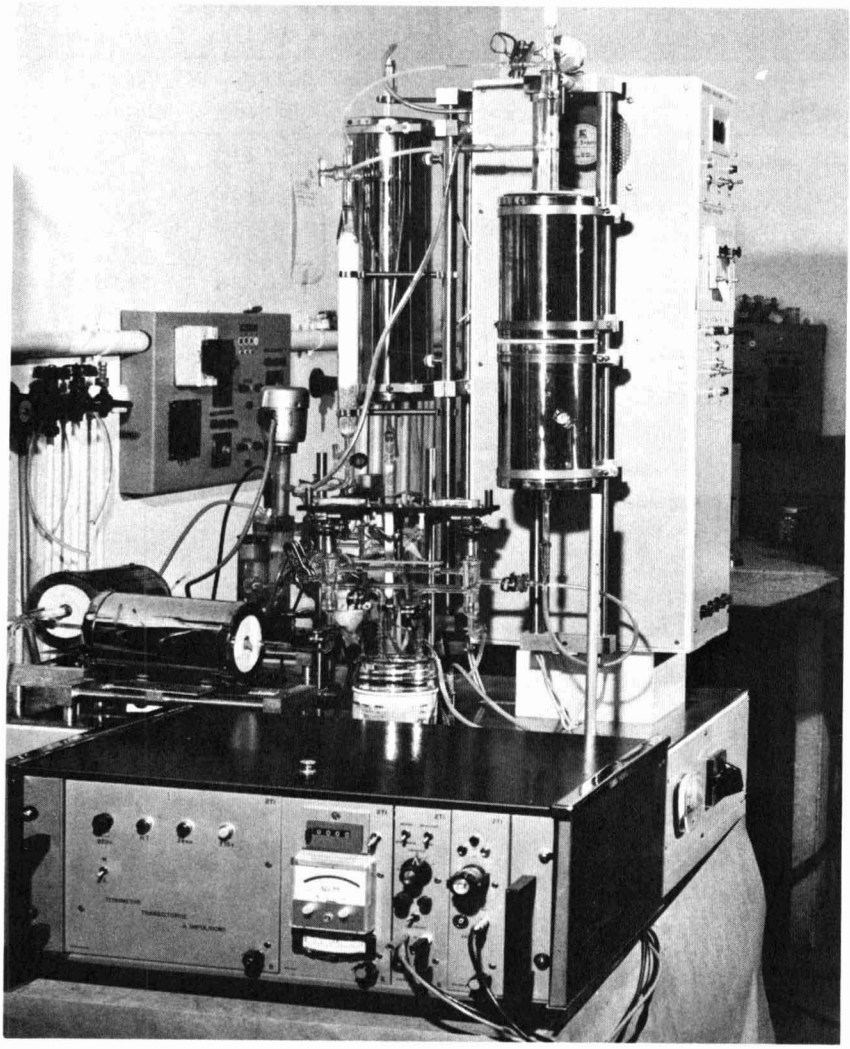

FIG. 11. CH-Microanalyzer equipped with an AID.

Water is then frozen in a cooled trap, possibly present nitrogen oxides are removed by a manganese dioxide filling in an absorption tube, and carbon dioxide is flushed by oxygen into the cathodic compartment of an absorption titration electrolytic cell.

An automatic coulometer titrates the protons generated by carbon dioxide in the cell and a digital pulse printing counter reads the equivalent quantity of electricity consumed. When this titration approaches its completion, the water frozen in the cold trap is vaporized by heating up to about 100°C and flushed by a nitrogen (instead of an oxygen) flow through

TABLE 2
CARBON AND HYDROGEN DETERMINATION IN ORGANIC COMPOUNDS

Compound	Sample weight (mg)	% C (calcd)	% H (calcd)	% C (found)	% H (found)
S-Benzylthiourea	2.042	47.41	5.47	47.43	5.48
	2.198			47.44	5.42
	1.726			47.42	5.49
Nicotinamide	1.622	59.00	4.95	58.97	4.91
	1.718			59.02	4.91
	1.191			59.02	4.93
Nitrobenzene	1.202	58.53	4.09	58.59	4.07
	1.203			58.46	4.13
	1.175			58.60	3.97
Acetophenetidin	1.383	67.01	7.31	66.96	7.22
	1.512			66.97	7.33
	1.392			67.01	7.36
Benzoic acid	1.253	68.85	4.95	68.96	4.96
	1.246			68.79	4.96
	1.322			68.88	4.97
Anthraquinone	1.217	80.76	3.87	80.79	3.96
	1.283			80.75	3.91
	1.197			80.78	3.86
Cholesterol	1.084	83.87	11.99	83.94	11.96
	0.995			83.93	11.99
	1.004			83.83	11.87
Gasoline	0.716			84.60	15.52
	0.680			84.52	15.53
	0.685			84.64	15.63

a carbon filling heated at 1120°C in order to generate carbon monoxide. The carbon monoxide is subsequently oxidized into carbon dioxide by heated copper oxide in a tube. Carbon dioxide is then flushed by the carrier gas into the cathodic compartment of an absorption–titration electrolytic cell where it is titrated as previously described.

The use of a vertical reactor, an automatic introduction device or an automatic sample feeder, and a collecting tube makes possible the full automation of the analyzer. A sequence programmer automatically switches the required functions at the correct times in the analytic cycle.

The use of the CH analyzer allows the determination of carbon and hydrogen in stable or unstable, liquid and solid, organic and inorganic compounds. This analyzer is especially useful for the analysis of volatile liquids such as petroleum products. It is also possible to determine *directly* the carbon-to-hydrogen ratio (C/H), which means the ratio of the

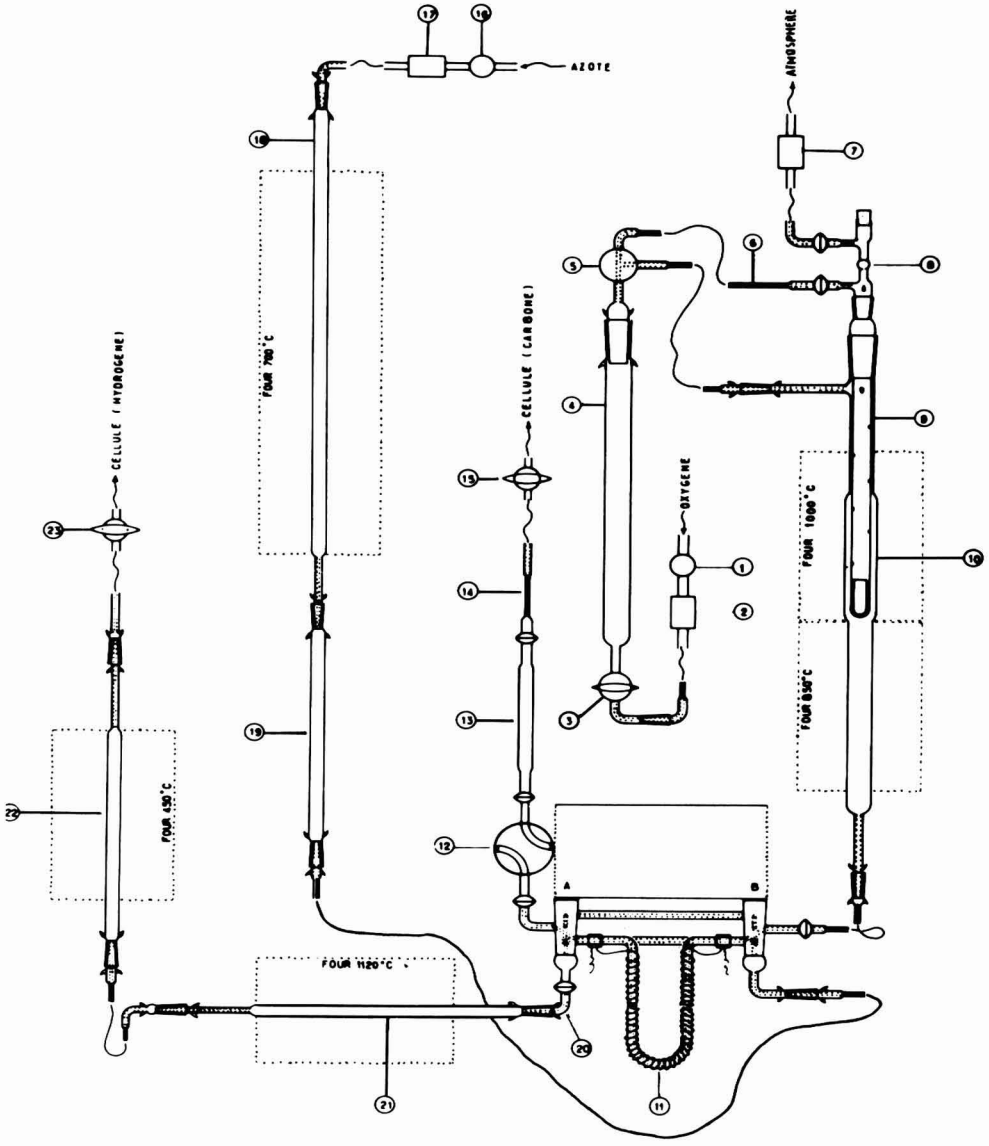

FIG. 12. CH-Microanalyzer, schematic drawing.

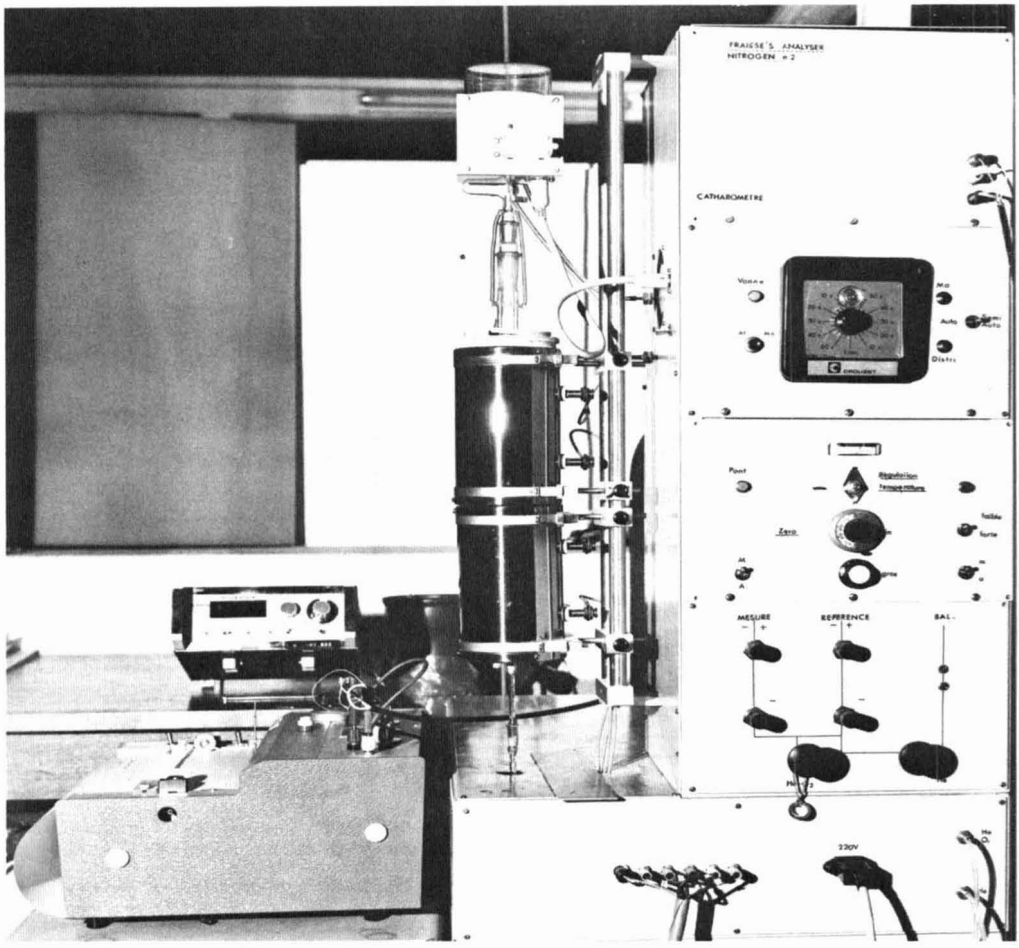

FIG. 13. N-Microanalyzer equipped with an ASF.

number of pulses for carbon to the number of pulses for hydrogen. In this C/H determination there is no need to weigh the sample for analysis. This capability of our analyzer for the analysis of petroleum products was described earlier (*8*).

The duration of one CH determination within a series is about 12 min. Some results are reported in Table 2. The precision of the results obtained within the sample weight range of 0.5 to 3 mg, depending on the carbon contents in the compounds analyzed, is shown to be equal to the precision of the classical techniques.

Nitrogen Microanalyzer

So far as nitrogen determination in organic compounds is concerned,

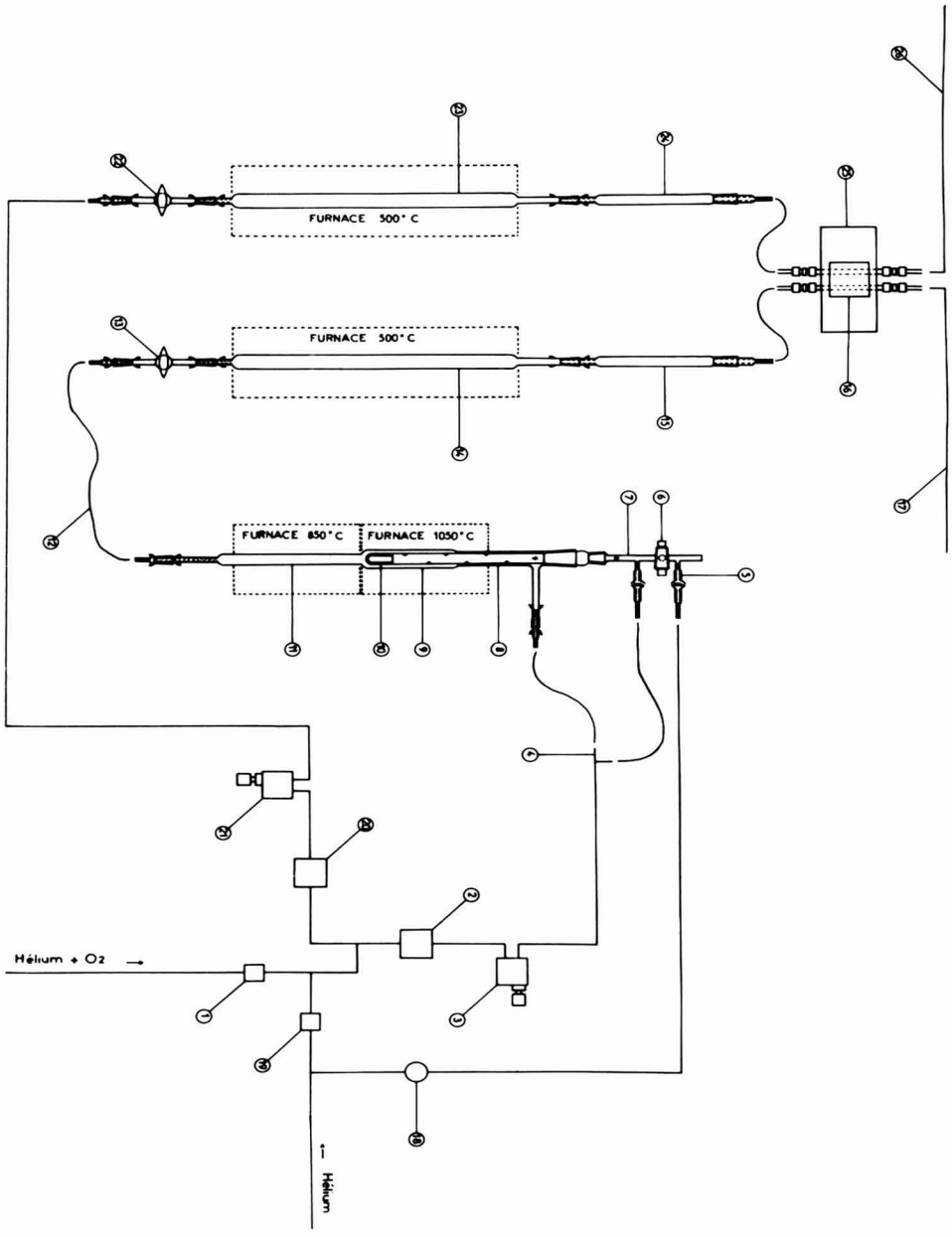

FIG. 14. N-Microanalyzer, schematic drawing.

TABLE 3
NITROGEN DETERMINATION IN ORGANIC COMPOUNDS

Compound	Sample weight (mg)	% N	
		Calcd	Found
Atropine	3.391	4.84	4.88
	2.079		4.84
	2.539		4.80
Chloracetamide	1.353	14.98	14.88
	1.330		14.85
p-Nitraniline	0.807	20.28	20.27
	1.076		20.38
Aminopyrine	0.985	18.16	18.06
	1.765		18.20
	0.905		18.20
Urea	0.788	46.65	46.75
	0.4298		46.60
	0.3161		46.63

we were faced with the same problem as that met with in the case of CH determination: Our Technicon CHN analyzers are unable to analyze unstable compounds; so instead of a classical Dumas-type apparatus, which is still used in our laboratory, we needed a rapid automatic nitrogen analyzer to solve this problem, and we have developed such an analyzer (*6*). This analyzer is shown in Fig. 13. A schematic view of the commercially available nitrogen analyzer is shown in Fig. 14. The basic principle of our technique is similar to that of the Walisch CHN analyzer (*6*).

In Fig. 13 we can see the same vertical reactor, collecting tube, and automatic sample feeder as those in the CH analyzer previously described. A flash combustion of the sample in a stream of helium with a 3% oxygen content takes place in the collecting tube and the combustion gases are flushed downward by the carrier gas through the lower part of the reactor, which is filled with appropriate reagents that ensure their complete oxidation and remove the halogen and sulfur by-products.

The gases then flow through a copper filling heated at 480°C in a tube for the purpose of reducing nitrogen oxides and removing excess oxygen. Carbon dioxide and water are then trapped out on soda asbestos and magnesium perchlorate fillings so that the only gas left in the helium stream is molecular nitrogen.

The differential thermal conductivity of the helium–nitrogen mixture is measured by comparison with pure helium using a thermal conductivity cell. The output signal from the detector is integrated and printed out automatically.

TABLE 4

CHARACTERISTICS OF THE O-, CH-, AND N-MICROANALYZERS

	O-Analyzer	CH-Analyzer	N-Analyzer
Equipment	AID or ASF	AID or ASF	AID or ASF
Reactor	Vertical reactor	Vertical reactor	Vertical reactor
Sample container	Pyrex glass capillaries *Silver* microbeakers	*Silver* or aluminium microbeakers Quartz capillaries	*Silver* microbeakers Quartz capillaries
Collecting crucible			
Material	Quartz	Quartz or alumina	Quartz
capacity	>100	>100	>100
Sample size	0.3–3 mg $m > 3$ mg; % O < 0.1	0.5–3 mg	0.5–2 mg
Range	0.1–100%	0.5–100%	0.5–100%
Precision	±0.2% O	±0.1% C ±0.1% H	±0.2% N

The use of this analyzer makes possible the determination of nitrogen in stable or unstable, liquid and solid, organic compounds. A single determination lasts about 5 to 6 min within a series. The sample size is about 0.5 to 2 mg. The technique and apparatus are suitable for the determination of nitrogen contents as low as 0.5% N.

A further study, in our laboratory, is aiming at improving this technique for the determination of traces of nitrogen without increasing the sample size. Some results of nitrogen content determination are shown in Table 3, and we can see that their precision is nearly equivalent to the result obtained with classical techniques.

Characteristics of the Oxygen, Carbon–hydrogen, and Nitrogen Microanalyzers

The main characteristics of the three microanalyzers described above are summarized in Table 4 for comparison.

CONCLUSION

In conclusion, the use of the vertical process system including a vertical reactor with a collecting tube and either an automatic introduction device or an automatic sample feeder has allowed us to automate our analyzers easily. It is now clear that the vertical system can easily replace the horizontal system, as we can accurately control the combustion or the pyrolysis of the compounds in the reactor with the collecting tube.

In addition we have shown that our vertical system is convenient with such diverse types of detectors as a coulometry cell and a thermal conductivity cell. Briefly, our vertical system is very flexible and can be applied to many elemental determinations.

SUMMARY

In order to automate the techniques in microanalysis a new series of analyzers utilizing a vertical processing has been developed. Three automatic microanalyzers, i.e., an oxygen, a carbon–hydrogen, and a nitrogen analyzer, are described. For each of them the same type of vertical reactor is used and therefore this design represents an important standardization of this kind of analyzers.

Two possibilities for sample introduction are offered. The so-called "automatic introduction device" allows the automatic introduction of the sample into the analyzer; nevertheless it requires the presence of an operator. A fully automated sample introduction is obtained by using the "automatic sample feeder." This new device can store up to 50 weighed samples and therefore the analyzer runs continuously. These analyzers can carry out an oxygen determination in 7 minutes, a carbon–hydrogen determination in 11 minutes, and a nitrogen determination in 6 minutes. The analyzers can run several hundred determinations continuously.

The results reported show a greater reproducibility than that of the results obtained using the classical analyzers, and at least equal accuracy. In addition, the new analyzers have a larger capability; e.g., the oxygen analyzer can determine the oxygen content in organic compounds containing phosphorus and the carbon–hydrogen analyzer allows the determination of the carbon to hydrogen ratio without weighing.

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MICROCHEMICAL JOURNAL 23, 220–225 (1978)

Applications Involving Oxidation with $KBrO_3$.

I. Rapid Potentiometric Method for Manganese Alone or in Steel and Some Ores

H. KHALIFA AND I. A. ISMAIL

Faculty of Science, Zagazig University, Zagazig, Egypt, A.R.E.

Received June 20, 1976

INTRODUCTION

Manganese is the most important of the ferroalloy metals. It is used where hardness and toughness are desired. Among the gravimetric methods cited in the literature is that of Shigeru (*9*), who stated that during hydrolysis of urea with urease Mn(II)-8-quinolinol complex is coprecipitated. The method is time consuming, requiring 6 to 8 hr to ensure complete precipitation. Jefferson (*5*) determined Mn volumetrically in blast furnace slags using persulfate as oxidant and arsenite as titrant. Hamya (*4*) proposed a titrimetric method for microgram amounts of Mn(II) involving amplification based on oxidizing it with an excess of iodate, masking of the unreacted oxidant with ammonium molybdate, and determining the permanganate and iodate formed iodometrically. Buzinova (*2*) titrated permanganate produced by oxidation of Mn(II) with persulphate in the presence of a mixture of cobalt and nickel sulfates amperometrically with thiosulfate at 0.6 or 0.8 V. Wechter (*10*) determined Mn potentiometrically by titrating unconsumed EDTA at pH 10 with Ca^{+2} using a tungsten–bronze indicator electrode. Anufrieva (*1*) titrated Mn(II) potentiometrically in the presence of pyrophosphate at pH 7–7.5 with permanganate using a platinum indicator and mercury iodide reference electrodes. Khalifa (*8*) determined Mn(II) in mixtures with Hg(I) and other cations by back-titration of unconsumed KI with Hg(II) using silver amalgam as the indicator electrode. The present method aims to investigate a more simple, convenient, and precise procedure for determination of manganese which is based on the oxidation of the divalent element with potassium bromate, followed by reduction of the unreacted oxidant as well as Mn(IV) with H_2SO_3. The resulting bromide is potentiometrically titrated against silver ions using silver metal as the indicator electrode. The method finds wide application in steel and some manganese and iron ores.

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EXPERIMENTAL METHODS

The water used was always deionized. The chemicals were all of the highest purity available.

Solutions. The 0.05 *M* Ag_2SO_4 was prepared by dissolving 1.348 g spectrographically pure Ag in 50 ml of 30% H_2SO_4, boiling the solution to expel SO_2. On cooling, it was made up to 250 ml with water and stored in an amber-glass bottle. It was standardized by back-titration of an excess of KI with Hg(II) using silver amalgam as the indicator electrode (*6*). Lower molarities were prepared by accurate dilution of the more concentrated solution. The 0.0484 *M* manganous sulfate was prepared by dissolving the calculated amount of $MnSO_4 \cdot H_2O$ (MW = 169.02) in water slightly acidified with 10% H_2SO_4. The solution was made up to volume with water and standardized following recommended procedures. The 0.05 *M* $KBrO_3$ (MW = 167.012) was prepared by dissolving the calculated amount in water. It was standardized potentiometrically either by (a) back-titration in a slightly acid medium of an excess of KI with Hg(II) (*7*) or (b) direct titration involving reduction with H_2SO_3 in slightly acidic medium according to: $BrO^-_3 + 3SO_2 = Br^- + 3SO_3$, boiling the solution for 5 min to expel excess of SO_2 and titrating bromide with Ag_2SO_4 in the presence of 25 ml of 5% K_2SO_4 to increase the sensitivity of the reaction and to establish equilibrium in the vicinity of the end point using silver metal as the indicator electrode.

One percent bromine was prepared by dissolving bromine in ethyl alcohol.

Apparatus. The titration assembly was as that described before (*9*) except that when using Ag^+ as the titrant the cell consisted of silver and calomel electrodes.

PROCEDURES

1. *For Mn*

Acidify Mn(II) solution (2 ml to 0.1 ml 0.0484 *M*) with 1 ml of 1% H_2SO_4, add a measured excess of $KBrO_3$ (5 to 1 ml 0.05 *M*). Boil for 15 min to produce MnO_2, maintaining the solution at constant volume with water, and to expel liberated Br_2, add a few drops of SO_2water to reduce both BrO^-_3 and Mn(IV) to Br^- and Mn (II). Boil for 10 min to expel excess SO_2, cool, add 25 ml of 5% K_2SO_4, water up to 40 ml, stir vigorously, and titrate Br^- with Ag^+. Find out $Br^- = Mn$.

2. *Analysis of Egyptian Manganese Ore (Pyrolusite)*

(a) For MnO_2. To a 20-mg sample in a 200-ml beaker add 100 ml of 5% H_2SO_4, 10 ml of 0.05 *M* KBr. Boil gently for 20 min to decompose the ore, as shown by the disappearance of the black color of Mn(IV) indicating its reduction to Mn(II), and to expel liberated Br_2, cool, add 25 ml of 5%

TABLE 1
DETERMINATION OF MANGANESE

Taken (mg)	Found (mg)	Error (%)
5.32	5.28	0.75
2.16	2.14	1.0
1.62	1.60	1.2
1.10	1.10	0.0
0.863	0.858	0.6
0.540	0.543	0.6
0.431	0.428	0.7
0.216	0.216	0.0

K_2SO_4, and titrate unreacted KBr with 0.05 *M* Ag^+. Find out KBr = Mn(IV).

(b) For total Mn. Digest 0.25 g of the ore with 100 ml of 20% (v/v) H_2SO_4 and 15 ml 30% H_2O_2. Boil gently for 30 min to decompose the ore and to destroy excess H_2O_2. Cool, filter through 540 Whatman filter paper, wash the residue thoroughly with hot water, and then fuse it with anhydrous Na_2CO_3. Dissolve the melt in the original filtrate and evaporate it to dense white fumes of SO_3. Cool, dilute with 100 ml of H_2O, filter off SiO_2 through 542 Whatman filter paper, wash thoroughly with hot water, and complete the filtrate to 250 ml.

Take a 5-ml aliquot, add 5 ml of 0.05 *M* $KBrO_3$ and proceed as follows:

(1) To obtain MnO_2, boil for 15 min to expel liberated Br_2, cool, adjust with dilute NaOH to pH 6, add several drops of SO_2 water to reduce BrO^-_3, Mn^{4+}, and Fe^{3+} to Br^-, Mn^{2+}, and Fe^{2+}, respectively. Boil for 10 min, acidify with 1 ml of 1% H_2SO_4, and continue boiling while passing a moderate stream of N_2–oxygen free gas which helps in expelling the excess of SO_2. Cool in ice water (5°C), add 25 ml of 5% K_2SO_4, stir vigorously, and back-titrate $Br^-$with 0.05 *M* $Ag^+$to obtain Br^- = total Mn.

(c) For Fe. Immediately oxidize Fe^{2+} with few drops of 1% ethanolic bromine solution. Boil for some time to expel excess Br_2, cool, and back-titrate liberated Br^- with 0.01 *M* Ag^+. Find out Br^- = Fe.

(d) For total Mn + Fe + Al. Add 5 ml of 0.05 *M* CDTA to the same aliquot and after determining Fe, leave aside for 3 min to ensure complete complexation of Al with cyclohexane diamine tetraacetic acid (CDTA) on

TABLE 2
ANALYSIS OF EGYPTIAN MANGANESE ORE

	MnO_2 (%)	MnO (%)	Fe_2O_3 (%)	Al_2O_3 (%)
(a)	61.13	3.14	1.54	1.47
(b)	61.21	3.12	1.54	1.51

cold. Buffer with 8 ml of 10% urotropine and back-titrate excess complexone with Hg(II) at pH 6.5 using silver amalgam as the indicator electrode to obtain CDTA = Fe + Al + Mn. Compute CDTA = Al by difference.

3. *Analysis of Egyptian Iron Ore Containing Manganese (A) and Low Alloy Steel (B)*

Digest 0.5 g of sample with 15 ml of conc HCl (in the case of A) or a mixture of 15 ml of conc HCl and 10 ml of 1:1 HNO_3 (in the case of B), heat gently until the reaction ceases. To the solution of (B) add a few drops of HF, boil it for 5 mins, and cool. Cautiously add to each solution 25 ml of conc H_2SO_4, heat to incipient white fumes of SO_3, cool, and dilute with 40 ml of water. Boil for 10 min with 1 ml of 30% H_2O_2 to ensure the presence of iron, manganese in the form of Fe(III) and Mn(II), respectively, and to decompose excess H_2O_2, cool, filter any insoluble residue through 542 Whatman filter paper, wash thoroughly with hot water, cool, and make the filtrates of both solutions with water up to 250 ml.

For Mn (3A) or (3B). Take 5-ml (3A) or 25-ml (3B) aliquot portions, boil each aliquot with 5 ml of 0.025 *M* $KBrO_3$, and proceed following (1) to obtain Br^- = Mn.

For Fe (3A, 3B). Continue as under (2c) to obtain Br^- = Fe. For Al (3A): Add 5 ml of 0.05 *M* CDTA to the same aliquot, after determining Fe, and proceed following (2d) to obtain CDTA = Fe + Mn + Al. Find out CDTA = Al by difference.

For Ni(3B). Take another 5-ml aliquot, add 5 ml of 0.05 *M* CDTA, buffer with urotropine, and back-titrate excess CDTA with Mg(II) at pH 6.5 to obtain CDTA = Fe + Mn + Ni. Find out CDTA = Ni.

RESULTS AND DISCUSSION

Table 1 lists the results for the determination of manganese alone, which indicate that the present method is accurate and reliable. Tables 2, 3, and 4 list the percentages of various constituents of manganese ore, iron ore, and low alloy steel analyzed by the present method (a) and by the classical one (b) *(10)*, which show good agreement with each other. The end point potential breaks are sharp and large in magnitude. Thus in titrating unreacted Br^- with Ag^+, unreacted CDTA, in the pre-

TABLE 3
ANALYSIS OF EGYPTIAN IRON ORE

	Total Fe(calculated) as Fe_2O_3)%	MnO_2 (%)	Al_2O_3 (%)
(a)	76.23	2.42	1.12
(b)	76.32	2.40	1.12

TABLE 4
ANALYSIS OF LOW ALLOY STEEL

	Mn (%)	Ni (%)
(a)	0.72	0.83
(b)	0.74	0.85

Note. (a) = By the classical methods; (b) = by the present methods.

sence and absence of AgBr with Hg^{2+}, they are on the average of 211, 94, and 112 mV/0.1 ml of titrant, respectively.

The following comments are to be taken into account:

(1) Oxidation of Mn(II) with BrO^-_3 in moderately acidic medium according to:

$$5Mn^{2+} + 2BrO^-_3 + 4H_2O \rightarrow 5MnO_2 + Br_2 + 8H^+$$

provides for a more simple, convenient, and precise method since the unreacted BrO^-_3 resists decomposition on boiling. Subsequently BrO^-_3 can be easily determined, after reduction with SO_2, by potentiometric titration of the liberated Br^- with Ag_2SO_4 using silver metal as the indicator electrode.

(2) Silver ions have no oxidizing effect on either Fe(II) or Mn(II) in the vicinity of the end point under the experimental conditions of acidity and low temperature. Oxidation with such ions occurs only at high pH values.

(3) EDTA or CDTA may exert a reducing action on Ag^+ at elevated temperature. Therefore CDTA is preferred to EDTA in complexing Al because the complexation takes place in the cold with CDTA but only with heating with EDTA.

SUMMARY

A rapid and reliable method is developed for the determination of manganese based on oxidation of the divalent cation with a known excess of $KBrO_3$ to the tetravalent state. The unreacted oxidant as well as Mn(IV) are then reduced with H_2SO_3 to Br^- and Mn(II). The resulting Br^- is titrated with Ag_2SO_4 using silver metal as the indicator electrode. K_2SO_4 is added to increase sensitivity and establish equilibrium in the vicinity of the end point. Fe(III) when present is also reduced with SO_2 to the divalent state, which can be reoxidized with Br_2. The equivalent amount of bromide is again titrated with Ag_2SO_4. The method provides for the simultaneous determination of Mn and Fe and finds application to some ores and steels.

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MICROCHEMICAL JOURNAL 23, 226–236 (1978)

Postulated Flaw in Densitometry[1]

B. ZAK,[2] E. EPSTEIN,[3] AND R. WATKINS[2]

Departments of Clinical Pathology, [2] Wayne State University School of Medicine and Detroit General Hospital, Detroit, Michigan 48201, and [3] William Beaumont Hospital, Royal Oak, Michigan 48072

Received November 16, 1977

INTRODUCTION

Recently, some discontent has been suggested with the use of scanning densitometers as the quantifying instrumentation for determining the ratio of HbA to HbA_2 (*12*). In this laboratory a similar dissatisfaction with densitometers began with the separation of serum proteins in agar gel. When using this procedure, it was noticed that the electrophoretic zone widths were not always the same size. Zone widths in this case are the dimensions of the zone perpendicular to its movement past the densitometer slit, that is, parallel to the slit. The albumin, a major concentration band, usually appeared to be the widest (*1*). This is a common phenomenon to encounter and is easily confirmed by looking at many of the reported patterns in the literature. These findings led to the development of a spectrophotometric spot scanner into which slits of variable dimensions were incorporated from modified Lowrey diaphragms (*16*) in order to ensure that pherograms might be more properly scanned by covering all zones in their stained entirety with these masking slits. It seemed that ignoring the latter phenomenon could not result in correct relative areas.

An alternative suggestion to densitometry of hemoglobins suggested by the Center for Disease Control was elution of the zones followed by colorimetic measurement of the eluates (*12*). The elution values were found to relate well to those obtained with column chromatography using DEAB-Sephadex columns, a procedure considered to be the most definitive methodology of the present (*3, 12*). However, since densitometers are obviously colorimeters, this suggestion implied that two photometers which measured the same material both by molecular absorption but in different ways, solid versus liquid sample, arrived at different results when determining what appeared to be the same

[1] The study was supported by N.I.H. Grant Number 05389-16, by a Grant-in-Aid from the Detroit General Hospital Research Corporation, Detroit, Michigan, and by a grant from the William Beaumont Hospital Research Institute Royal Oak, Michigan.

0026-265X/78/0232–0226$01.00/0
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absorbers or derivatives of those absorbers. This curious fact triggered the present investigation as a means of attempting to explain and perhaps resolve why this odd discrepancy should occur. Another important fact to consider here is that if the chromogen is a Ponceau-S derivative of hemoglobin and yields incorrect results, this might indirectly indict more complex measurements such as Ponceau-S stained serum proteins about which nothing at all was mentioned. An obvious conclusion inferred from observations such as this one is that if densitometry is bad for hemoglobin, it cannot be good for proteins or other measurements more involved than are those with just the two zones of hemoglobin to consider. Since these measurements are all requirements of a clinical chemistry laboratory, certainly an investigation of the simplest of all of them, the hemoglobins, appeared mandatory.

The area chosen for study here is one that has not been considered by those others (*12*) who claimed that densitometry is an ineffective and inaccurate way to conclude an electrophoretic procedure for HbA:HbA_2 ratios and then suggested that it be abandoned for that purpose. Therefore, the results obtained by using a slit which masks off the ends of the zones and which can in effect be smaller in that dimension than the smallest zone is the primary target of the present investigation. For if this, the simplest of electrophoretic determinations is inaccurate, the use of densitometers would probably have to be abandoned in clinical laboratories if all other common uses appear to have more complexities for measurement than do the hemoglobins. A secondary subject of the investigation was a comparison of elution to densitometry for separated duplicate patterns of hemoglobins to confirm whether elution followed by spectrophotometry results in the strikingly different values reported (*12*).

MATERIALS AND METHODS

The reagents and methods used in this study were those of the manufacturers, Gelman Instruments Co. (Ann Arbor, Mich.), for the reagents and the electrophoresis systems and the Transidyne RFT instrument (Ann Arbor, Mich.) for the densitometry. The electrophoretic hemoglobin buffer at pH 9.2 was composed of 84.9% w/w Trimethamine (Tris), 0.6% w/w EDTA, and 14.5% w/w glycine made up to a final weight of 18.3 g and then diluted to 750 ml with distilled water. This solution is stable at 2 to 8°C for 1 week. The cellulose polyacetate strips, 2.5 × 15.2 cm, were Sepraphore III of the Gelman Co. The electrophoretic conditions were 250 to 300 V for 75 min. The strips were handled wet after electrophoresis for elution where the cut out zones of the HbA_2 were eluted with 1.2 ml of water, the HbA with 10 ml, and then each read in a Gilford 300-N spectrophotometer at 415 nm. If HbS was present, it was also eluted with 10 ml of water, read at 415 nm, and used in the calculations for HbA_2.

The absorbances were totaled and the percentages of each hemoglobin were determined from these values. The densitometer scan materials were prepared by fixing and staining a second set of strips with Ponceau-S, 0.5% in 100 ml of 7.5% trichloracetic acid. These were washed with three successive rinses of 5% acetic acid for a total of 10 min, placed in a 40% V/V *N*-methyl pyrrolidone clearing solution for 3 min, and finally heated at 80 to 90°C for 20 min to dry and clear the membranes.

DISCUSSION AND RESULTS

It might be best to begin by attempting to define nomenclature for the term, width, as it is commonly expressed in densitometry. Manning described as confusing the width nomenclature for spectrophotometry (*6*) and detailed how the spectral bandwidth is used to resolve observed bandwidths. If the spectral bandwidth is small enough, the observed bandwidth is hopefully the same as the natural bandwidth of the chromogen, making the measurement of an absorber synchronous with its theoretical intrinsic sample characteristic (*6*). No one should have trouble with such precise definitions. But, the width of the rectangular masking slit of a densitometer is used to help characterize the relative "volume" of a spot (zone) actually scanned during the spectrophotometric process where absorbance represents a third dimension of volume, a third dimension in that it would be considered a measure of depth, while the area of the spot is made up of width and length. In a liquid spectrophotometric measurement, the volume is usually kept constant and the solution absorbance is the only real variable. However, in measuring the volume of a zone, in order to get the true relationship to other zones, the same relative proportion of that zone must always be determined, or in other words, the volume measured should be proportional. If it is not, then the true relationship is lost and relative values cannot relate accurately. Therefore, when the volumes related are relatively different, it becomes necessary to ensure that the entire concentration of absorber must be measured, a process which could place densitometry in harmony with total elution followed by spectrophotometry. Densitometry as it is presently performed routinely could be described as a process which considers zones to be constant in dimensions such as width but measures them often with some variance. To continue then with the width explanation, during the process of densitometry, the width of the spectral slit of the photometer is perpendicular to the masked width of the measured zone which is a confusing aspect of nomenclature, and to compound the confusion, the term used for that zone dimension is length by some (*7*) and width by others (*15*). In addition, the transduction of the measurement of the color of a zone, really a volume consideration, to the graphed areas to be integrated

mathematically may also be somewhat confusing, for here a three-dimensional measurement, involving a changing absorbance signal during the movement of absorber, is reduced to two dimensions, area, as a calibration device. Again, adding to confusing terminology: The measurement of the areas under the bell-shaped curves can be conventional using peak times the width at half height. But the width here is not derived from the width of the masking slit. If anything, it is a perpendicular dimension.

Having deliberated some about the confusion of nomenclature with respect to the term, width, there are several possibilities one might consider which could cause inaccurate relationships between 2 bands which are compared for their concentrations relative to one another. If the two zones are decidedly disproportionate in their concentrations as there could be with HbA:HbA_2, 50:1 for example, the major band could be beyond the linearity range of the measurement system. That is, the major constituent, HbA in this case, may be in excess with respect to the linear nature of calibration in either staining or measurement characteristics. If the hemoglobins are stained with a protein stain, they may not stain linearly on a weight basis, or the major protein may be understained because all of the dye may not react in linear proportion with the protein when that protein is too concentrated (*15*). It is possible and it is claimed (*11, 13*) that if one stains the hemoglobins, another protein may superimpose the HbA_2 zone and as a consequence the ratio to HbA would be altered. However, if that protein were colorless at the hemoglobin Soret band wavelength, then perhaps this latter problem could be obviated by avoiding the staining procedure to take advantage of the inherent color of hemoglobin. Still another factor to consider would be the effect of using a dense almost-opaque medium such as uncleared paper or cellulose acetate. In this case, the calibration characteristics could be nonlinear, and if this is not taken into consideration it could cause an underestimation of the major band (*10*).

In the comparative procedure to be described here, the elution technique was carried out using Soret band measurement on unstained eluates while the densitometric process was carried out on Ponceau-S stained hemoglobins with the final measurement made by scanning the cleared membrane at the Ponceau-S peak maximum. The comparison was made in this manner because that is the way elution and densitometry techniques are commonly carried out and the way the Center for Disease Control did their investigation in signaling the problem (*12*).

A comparison was therefore made between the eluted values of the constituent hemoglobins of pherograms read in a spectrophotometer and the scanned and integrated values of the hemoglobin zones determined by densitometry. Figure 1 shows the graphic correlation between these two kinds of colorimetry for normal samples. All four quadrants contain the

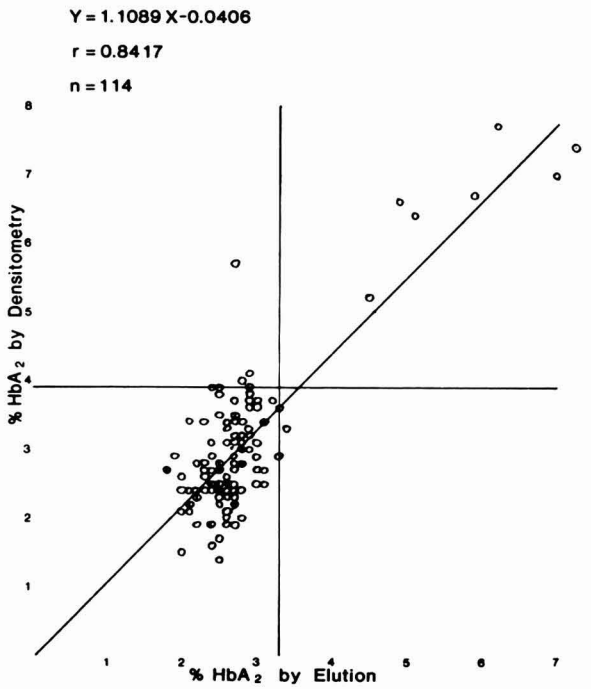

FIG. 1. Comparison of %HbA_2 in blood hemolysates separated by alkaline electrophoresis and determined by elution and subsequent spectrophotometry at 415 nm versus densitometry of Ponceau S stained zones at 520 nm.

114 samples used for this comparison but the upper right quadrant samples from thallasemia patients were eliminated from the subsequent calculation of comparison of two population of samples from normal patients. The work of Schmidt *et al.* (*12*) contained the description of wide discrepancies between what would appear to be two very similar colorimetric approaches to quantification. However, though there is some tendency to show higher HbA_2 percentages for densitometry, the finding described here for the 102 nomal duplicate samples, are not nearly so divergent as previously reported (*12*). The statistics indicate a somewhat similar dispersion of values for the measurement comparison differing in their means for normal HbA:HbA_2 ratios by 0.21% with 2.60 $\pm$ 0.28% found for elution and 2.81 $\pm$ 0.67% found for densitometry. However, the Schmidt *et al.* (*12*) values showed means of 2.56 $\pm$ 0.31% for elution of normal HbA_2 control samples and 4.65 $\pm$ 1.46% for their densitometry with a difference in means of 2.09%, while samples from patients with the β-thallassemia trait calculated out to be 4.60 $\pm$ 0.61% for elution and 7.12 $\pm$ 1.62% for densitometry, a difference of 2.52% in the means of these two populations. An interesting factor here is that the

larger number of samples of the present study showed a much smaller difference in means between the two measurement techniques compared than did either the samples or normal controls tested by the Schmidt group (*12*), and the standard deviations were also smaller.

There remains another seemingly less obvious possibility for a problem in measurement, along with those mentioned earlier which resides in the densitometer itself in terms of the ability of an instrument to include all of the areas of the stained zones when the pattern is moved across the masking slit. Since this phenomenon could also be the source of problem measurements for other densitometric tracings such as total proteins, lipoprotein–lipids, and even chromatograms, it seemed an important area to pursue. In fact, the study of this phenomenon is the primary objective of the present investigation.

Evidence exists to confirm that densitometry should be a true three dimensional measurement if relative areas for adjacent spots of electropherograms are to be quantitatively in correspondence one with the other (*8*). Most commonly, routine densitometers use rectangular masking slits whose dimension in the direction of the scan can be made quite narrow while their lane widths are also variably adjustable. The latter is sometimes smaller than the smallest band in width especially for the most common of the densitometers presently in routine use. As a consequence, the scan is made with two dimensions firmly established because the instrument scans through the zone which passed by the slit. Therefore, if these dimensions represent depth and length, the third dimension, the width, is not totally accounted for. In view of the fact that this dimension is at a maximum with the most concentrated zone usually the widest, it is fairly safe to assume that the widest band will be underestimated in proportion to its relative area with respect to the area of the smallest band. This presents a problem in terms of how one could correct for the underestimation of all bands which are larger than the smallest band.

To be able to ensure that the slit width does not encroach on the adjacent patterns, and for perhaps other reasons not clearly stated by manufacturers, it has been kept to a size which is often as small or smaller than the smallest band. In some electrophoretic separatory systems in which eight samples are simultaneously striped onto cellulose acetate, the end of the run shows that high concentration zones such as albumin are often very close to each other and are sometimes even touching. This is a dangerous analytical circumstance for a slit which might be as wide as the widest band. But when the bandwidth is narrowed to a slit measurement perhaps smaller than the smallest band, then the circumstance may also be a dangerous analytical one in view of the fact that an inanimate densitometer could not be aware of the different widths of the zones broadened a bit by diffusion as they move down their lanes. In order to

define dimensions of a zone constituent for the purpose of clarity, the continuous absorbance during densitometry is the depth, the path through the zone during densitometry is the length, and the third dimension remaining perpendicular to the path direction is the width. The use of width of a zone is derived from that dimension of the lane traversed, that is, the lane width rather than the spectral slit width.

In attempting to set up experiments in which pipetted volumes of colored materials or proteins to be stained could be studied for their comparative relative concentrations by densitometry, creep-back, and capillation errors (*2*) on paper or cellulose acetate surfaces obscured the problem. It was decided that the same kind of information could be derived more accurately from graphic representations of rectangular zones which could be drawn to exact known sizes and to which arbitrary absorbances could be assigned. In this theoretical system, all spots would be absolutely uniform for those absorbances and the boundaries defining the rectangular zones would be perfect. The effect of a fixed masking slit on those measurements would then be easily calculable. Such graphs were made using four possible alternatives for the shapes and arbitrary absorbances of the measured zones. The slit which would determine the areas of those zones to be scanned was just as arbitrarily kept to the exact dimensions of the smallest zone width. Four different graphic calculations were made as described by Fig. 2.

If one were to consider a circumstance in which several zones might be available to be scanned all of whom had identical absorbance values (the depth factor) but the masking slit which determined the areas of the zones scanned (the other two dimensions) was the size of the smallest zone or less, then all of the zones should appear to have the same total concentration of analyte. This seems clear from observing graph A in Fig. 2. Five rectangular zones are present ranging from 2 to 10 mm in width, 1 mm in length, and all identical in their absorbance characteristics. They should vary in total concentration of absorber from 2 to 10 in increments of 2 yielding a line shown as "Present" in the graph. However, inasmuch as decreasing portions of each zone would be covered by the slit, ranging from all of the zone to one-half, one-third, one-fourth, and one-fifth of the zones, it is the "Found" line which should be more representative of what the instrument would see. In the several circumstances to be described subsequently, it will be shown that a similar Present–Found situation exists in which zones are underestimated in proportion to the relationship between masking slit width and zone width. Even though the instrument would be measuring linearly, it would be reporting nonlinearly, a factor not encountered with solution measurements where volumes are fixed procedurally and absorbance is the variable directly proportional to concentration when obedience to Beer's law is obtained.

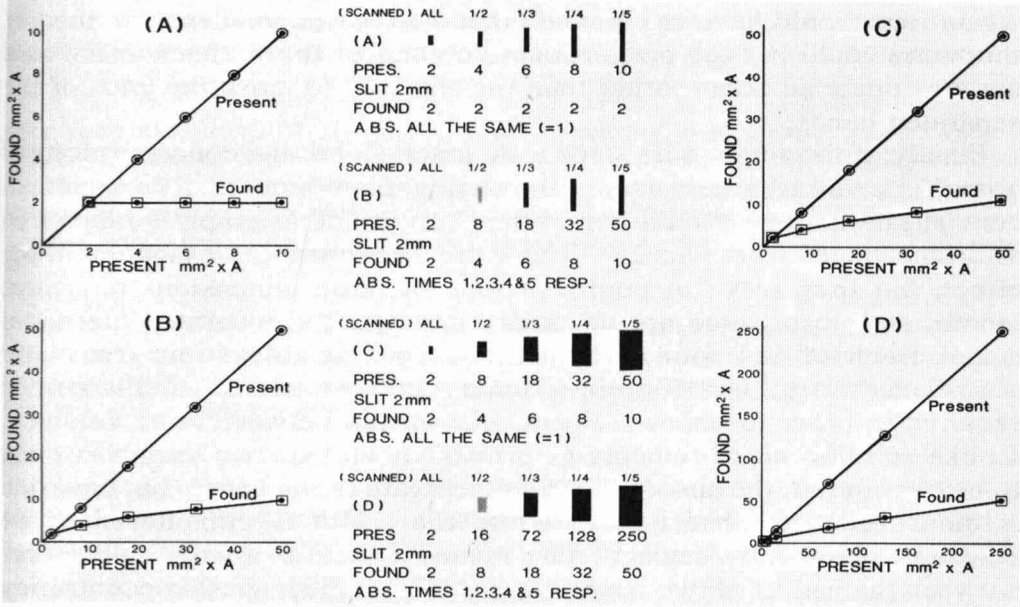

FIG. 2. Shows the graphs of theoretically established separated zones calculated for what is present and compared to what should be found if the fixed slit is small as shown. (A) Zones are variable in width, constant in length, and constant in depth (absorbance). (B) Zones are variable in width, constant in length, and variable in depth. (C) Zones are variable in width, variable in length, and constant in depth. (D) Zones are variable in width, length, and depth.

In looking at a similar circumstance in which the zone areas were the same as graph A in Fig. 2, but their absorbances varied from one time for the smallest zone to five times for the largest zone, the patterns would resemble these of the rectangular zones of graph B in Fig. 2. In this instance the arbitrary concentrations would vary from 2 to 50 and would plot as shown in the Present line. Again, a fixed slit, the size of the smallest zone width, would cause the instrument to record variable fractions of the several zones ranging from all to one-fifth resulting in the Found line shown. The underestimation of the total concentrations of each zone beyond the first one as a result of inability to cover proportional volumes of each zone which are relative for all zones must, as can be seen, result in nonlinearities unrelated to total concentrations.

If the zones were broadened in length and ranged from 1 to 5 mm, as in Fig. 2, graph C, but the absorbances were kept constant, while the widths were kept in the same proportions as in the previous two figures, the concentrations would range as in graph B across the arbitrary values of 2 to 50, and would again plot as shown for the Present line. However, the Found line should be identical to that of graph B because the same proportions of each zone would be used and calculated by the instrument.

Again, one would have to conclude that a fixed slit smaller than most of the zones could not see porportional volumes of those zones and would underestimate all zones larger than the slit used to mask the ends of the separated bands.

Finally, if the zones were sized as in graph C, but the concentrations of absorbers varied as in graph B ranging from one to five times in concentrations, one should obtain the Present line of graph D ranging in arbitrary values from 0 to 250. These are circumstances exaggerated for effect, but they serve to point out that all three dimensions of width, length, and absorbance are necessary adjuncts for accurate determinations. Each of the four circumstances of variable zone areas and absorbances result in a nonproportional representation of what would be required in order to obtain a linear relationship between zone volumes. Unlike solution measurements as previously stated, two variables exist to be considered, the absorbance and the width of the zones, but only one is measured, the absorbance, while the width is considered to be constant, perhaps by neglect. As a result, if there is a small difference between the widths of two zones measured with the highest concentration constituent diffused to a slightly wider zone, that zone will be underestimated and result in an improper ratio of the percentages of the two bands. Obviously, that could easily account for the small differences shown by our means found for the previously described hemoglobin comparison of elution-colorimetry and densitometry.

Some studies previously reported fortify the concept that if linear relative areas of separated zones are to be determined, the portions of those areas scanned must be considered (*9, 14*). In the case of electrophoresis as a process for preparing separated zones to be subsequently measured, this phenomenon appears to be overlooked or forgotten. As a consequence, quantification following separation has been attacked (*4, 12*). However, in recognizing this flaw of measurement, something can be done to avoid it (*5*). There are other problems in these kinds of measurements with opacity of medium, polychromatic light effects, and staining discrepancies between different proteins which should also be considered for their potential to create measurement difficulties and which may be responsible in part for the dissatisfaction with densitometry as a quantification factor (*4, 12*). Some efforts in those areas are under present investigation. The fact that true relative volumes of a scanned zone may not be measured should be a point of consideration for interested investigators.

SUMMARY

An effort was made to explain the reported large discrepancies between comparative spectrophotometric measurements for two separated hemoglobins. This is the simplest quantification requirement following electrophoretic separation involving either integrating

densitometry or elution followed by spectrophotometry. A graphic simulation of several exaggerated separation conditions with known areas and assigned absorbances was postulated from which the results obtained with inadequate slit geometry were calculated. The relative concentrations found were always in marked nonlinear disagreement with the linear theoretical values present. From this one could infer that true relationships between the volume relationships of separated zones are not realized if one neglects one dimension of a three dimensional measurement. Comparison between elution followed by spectrophotometry and direct spectrophotometry (densitometry) showed a smaller difference in means than did the work which stimulated this study (*12*). There is no way in which the differences in these two findings can be easily defined. However, one could infer that neglect of a zone dimension during the measurement process could be considered one source of such a measurement discrepancy. But even if nonproportional measurement of different zones to obtain relative areas of each does not describe the total problem, it should still be an important factor to keep in mind. A further study of other factors such as translucence or opacity with its Lambert effect on measurement, and the related severe turbidity factor in solution measurements is in progress.

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MICROCHEMICAL JOURNAL 23, 237–258 (1978)

Spectrophotometric Evaluation of Interferences in Three Iron Reactions for the Determination of Serum Total Cholesterol[1]

J. D. ARTISS,[2] R. J. THIBERT,[2,3] AND B. ZAK[2,3]

[2]*Department of Chemistry, University of Windsor, Windsor, Ontario, Canada N9B 3P4 and* [3]*Departments of Pathology, Wayne State University School of Medicine and Detroit General Hospital, Detroit, Michigan 48201*

Received November 25, 1977

INTRODUCTION

The development of the ferric iron reaction for the determinations of cholesterol (*28*) led to a proliferation of alterations of reagents and reaction conditions. Among these, Parekh and Jung (*17*) developed a modified iron reagent procedure for the determination of serum total cholesterol which was proposed as superior to all previously reported cholesterol methodologies including those iron procedures (*5, 7, 26*) from which it was derived. The reagent system is composed of a ferric acetate–uranyl acetate mixture in acetic acid, the protein precipitant–cholesterol extraction agent, and a ferrous sulfate glacial acetic acid mixture in concentrated sulfuric acid which when added to the previously obtained extract completes the color reagent. This would substitute for the original ferric chloride–acetic acid precipitation-extraction reagent to which sulfuric acid would be added to complete the color reagent (*5, 7, 26*). It was proposed here that the incorporation of uranyl acetate into the reagent mixture promoted complete protein precipitation while the ferrous sulfate simultaneously enhanced and stabilized color production. Although this was claimed to be a direct procedure, implying no phase separation (*25*), the ferric acetate–uranyl acetate reagent reportedly not only serves as a protein precipitant but also extracts the cholesterol from the lipoproteins, an apparent dichotomy in terminology.

Part of the rationale for the use of ferric acetate, rather than the previously reported ferric chloride (*28*), is that the presence of chloride ion imparts a yellow color to the blank due to the "partial evolution of HCl gas" (*17*), a process which supposedly caused variability in the reaction temperature and therefore in the color production of the original procedure (*26*).

[1] Supported in part by a Grant-in-Aid from the Detroit General Hospital Research Corporation and NIH Grant Number 05389-16, and National Research Council Canada.

0026-265X/78/0232–0237$01.00/0
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In a second publication, it was claimed (*9*) that the use of concentrated sulfuric acid rather than the sulfuric acid–ferrous sulfate reagent yielded falsely elevated values. In their first report (*17*) it was claimed that the ferrous sulfate had been added to enhance and stabilize color development. It is difficult to understand just how the color can be enhanced while the apparent concentration of cholesterol from serum is depressed. This intriguing phenomenon is not explained by the authors.

A comparison of reaction specificity, with respect to numerous steroids, between the proposed method (*17*) and an iron chloride reaction (*12*) was also published (*18*) in which it was concluded that the ferric acetate reagent was more specific toward cholesterol in the presence of other steroids than was a ferric chloride reaction. It was also suggested that this added specificity of the acetate versus chloride reagents could not be explained by the cholesterol reaction mechanism for iron reactions with cholesterol as proposed by the National Bureau of Standards (*4*) and in effect the validity of that mechanism was questioned.

In a more recent investigation, Jung *et al.* (*8*) suggested that the incorporation of uranyl acetate into the reagent system greatly reduces interference by noncholesterol chromogens and eliminates the interference of bilirubin. They also reported that the contaminant, bromide, increased the apparent cholesterol concentration, in their procedure by 0.43 mg/liter (1.1×10^{-2} m*M*) for each milligram of bromide per liter in the range of 200 to 2000 mg/liter (1.9–19.4 m*M*) sodium bromide.

The most recent paper (*10*) involving the ferric acetate–uranyl acetate reagent system involved a comparison of this reaction with two iron chloride procedures (*6, 26*) and the iron perchlorate reaction of Wybenga *et al.* (*24*) with respect to the interference effects of sodium azide. In this study, the ferric acetate–uranyl acetate reaction was shown to suffer minimally from the inhibiting interaction of azide when compared to other ferric iron procedures. One partial explanation for this and perhaps previous claims resides in the fact that their sample treatment and procedural dilutions resulted in a lower concentration of interference tested for the ferric acetate–uranyl acetate modification than those procedures to which it was compared.

Apart from the above-mentioned studies other reports of bromide (*2, 13, 19, 20, 23*), 2-thiouracil (*14, 19, 20, 22*), nitrate (*11, 21*), and other sterols (*15*) have been described elucidating the effects of these interferences on iron reactions for serum cholesterol determinations. Therefore, it was the purpose of this study to evaluate and compare three common iron reactions (*8, 24, 27*) with respect to the effects of these interferences and to determine if the ferric acetate–uranyl acetate procedure as proposed (*8, 9*) is indeed as unique as is claimed. In the process of comparison, the concentrations of interferences tested as well as the cholesterol con-

centrations in the final reaction mixture were kept the same for all procedures of the study. If this were not done, it would not be as easy to determine whether the reactions were discrepant with respect to the interferences or if the procedures themselves were at variance.

MATERIALS AND METHODS

Reagents

Cholesterol standard solutions (10.34 and 5.14 m*M*). Prepared from reagent grade cholesterol, recrystallized four times from absolute ethanol, and dissolved in glacial acetic acid (GAA).

Ferric perchlorate color reagent. Obtained from Dow Diagnostics, Dow Chemical, Indianapolis, Ind.

Ferric acetate–uranyl acetate color reagent. Prepared according to Jung *et al.* (*8*). One gram of ferric chloride hexahydrate was dissolved in 80 ml of water and split into two 50-ml conical centrifuge tubes with Teflon-lined screw caps. Ammonium hydroxide (3 ml) was added to each tube, mixed well, and centrifuged for 5 to 10 min at about 3000 rpm. The supernatant liquid was removed, the precipitate was washed with distilled deionized water, and centrifuged again. This procedure was repeated until a negative silver nitrate test for chloride was obtained. All of the supernatant except for about 1 ml was aspirated (this differs from the original procedure) and about 40 ml of GAA was added. Mixing was continued until all of the precipitate was dissolved and the solution was a clear dark red-brown color. This solution was transferred to a 2-liter volumetric flask, 200 mg of uranyl acetate added, and dissolved with about 1.2 liter of glacial acetic acid. This often took in excess of 6 hr. The reagent was diluted to volume with GAA and stored in a brown glass bottle.

Ferric acetate–sodium acetate color reagent. This reagent was prepared in the same manner as the ferric acetate–uranyl acetate color reagent except that the reagent was prepared to contain 0.237 m*M* sodium acetate in place of the uranyl acetate.

Sulfuric acid–ferrous sulfate reagent. In a 2-liter volumetric flask 200 mg of anhydrous ferrous sulfate was dissolved in a mixture of 200 ml of GAA and 200 ml of concentrated sulfuric acid. About 1.4 liter of concentrated sulfuric acid was added and mixed until the solution had cooled to room temperature. The reagent was diluted to volume with concentrated sulfuric acid and stored in a brown bottle.

Ferric ammonium chloride color reagent. Prepared exactly as described (*27*). Ferric ammonium chloride monohydrate (2.12 g), was dissolved in 100 ml of 80% (v/v) acetic acid. This reagent was initially turbid but cleared on standing overnight. For comparison purposes a similar reagent prepared in 1962 was tested for its reactivity and tested for its stability over this period.

Thiouracil stock solution. Prepare this solution to contain 3.1 m*M*/liter in distilled deionized water.

Sodium azide stock solution. Prepare this solution to contain 500 m*M*/liter in distilled deionized water.

Sodium nitrate stock solution. Prepare to contain 50 m*M*/liter nitrate in distilled deionized water.

Sodium bromide stock solution. Prepare this to contain 50 m*M*/liter bromide in glacial acetic acid. This solution is not stable and should be prepared immediately prior to use.

Steroid stock standard solutions. Prepare these solutions to contain 10.34 m*M*/liter Δ^5-pregnenolone, corticosterone, dihydrocholesterol, estriol, and pregnanetriol in GAA.

Diethylstilbesterol stock solution. Prepare to contain 10.34 m*M*/liter diethylstilbesterol (DES) in a 1:1 (v/v) mixture of GAA and absolute ethanol.

Alcoholic potassium hydroxide solution. Prepare by adding 6 ml of 33% (w/v) aqueous KOH to 94 ml of absolute ethanol immediately prior to use.

Petroleum ether. Reagent grade bp 69 to 90°C.

All other reagents or chemicals were of analytical grade.

Methods

Ferric acetate. Where raw serum was used or where calibration standards for these serums were run the procedure according to Jung *et al.* (*8*) was followed implicitly. Fifty microliters of serum was added and mixed well with 5.0 ml of ferric acetate–uranyl acetate color reagent in Teflon-lined screw cap culture tubes. Each sample was allowed to stand for about 5 min before centrifugation at about 3000 rpm for 5 to 10 min. Three milliters of supernatant solution was transferred to a second Teflon-lined screw cap culture tube. Sulfuric acid–ferrous sulfate reagent was added to each tube whose contents were then mixed immediately. Once the tubes cooled to room temperature, 10 to 15 min, they were either read at 560 nm, or scanned through the visible range of the spectrum against a reagent blank. All spectrophotometric measurements were performed on a Beckman ACTA MVI.

In those instances where the same sample concentration was required for all three iron color reactions, 50 μl of standard was added to 3 ml of iron reagent. Sulfuric acid–ferrous sulfate reagent (2 ml) was added and the remainder of the procedure, as above, was carried out.

Ferric perchlorate. In all instances the originally described procedure for serum (*18, 24*) was followed implicitly. Fifty microliters of serum or standard was added to 5.0 ml of the reagent solution, mixed well, and incubated for 90 sec in a heating block at 100°C. This mixture was contained in tightly closed screw-capped 1.25 × 10 cm tubes, as supplied by

Dow. After incubation the mixture was cooled in a less than 17°C water bath for 5 min. The samples were then either read at 560 nm or scanned against a reagent blank.

Ferric ammonium chloride. In all instances the originally described procedure for serum extracts (*27*) was followed. Fifty microliters of standard was added to 3 ml of GAA, mixed well, and then remixed upon the addition of 0.1 ml of ferric ammonium chloride reagent. Concentrated sulfuric acid (2 ml) was added and mixed well. This solution was contained in Teflon-lined screw cap culture tubes. Once the samples cooled to room temperature, 5 to 10 min, they were either read at 560 nm or scanned through the visible range against a reagent blank.

Pure extracts of serum were obtained by saponification and subsequent extraction with petroleum ether, as described (*1*). A 1.0-ml aliquot of the extract, representing 50 μl of serum was evaporated to dryness and the residue dissolved with either ferric acetate–uranyl acetate reagent or GAA and ferric chloride reagent.

In order to study the effect of 2-thiouracil 25 μl of the appropriate standard was added in addition to 50 μl of cholesterol standard. The various color reactions were carried out as described above.

The effects of sodium azide were studied upon addition of 25 μl of the appropriate standard solution in addition to 50 μl of cholesterol standard. The various color reactions were carried out as described above.

Nitrate was studied by adding 50 μl of the appropriate standard in addition to 50 μl of cholesterol standard. The various color reactions were carried out as described above.

Bromide enhancement was studied by the addition of 25 μl of the appropriate standard solution with and without 50 μl of cholesterol standard. The various color reactions were carried out as described above.

The necessity of having ferrous sulfate in the sulfuric acid reagent was studied by dispensing about 3.05 ml of a cholesterol color reagent–cholesterol standard mixture (1.9 ml of a 7.8 m*M* cholesterol standard to 135 ml of reagent to yield a final reaction mixture concentration equivalent to 6.56 m*M*/liter cholesterol) into 2 ml of either sulfuric acid–ferrous sulfate reagent or concentrated H_2SO_4. Following the appropriate color development period the samples were read at 560 nm against a reagent blank.

RESULTS AND DISCUSSION

The evaluation of the three iron reactions (*8, 24, 27*) was initiated by a qualitative examination of the spectra of the three reactions for cholesterol. With reference to Fig. 1, it can be readily seen that all three spectra have maxima at about 560 nm and that the general shape of the curves are

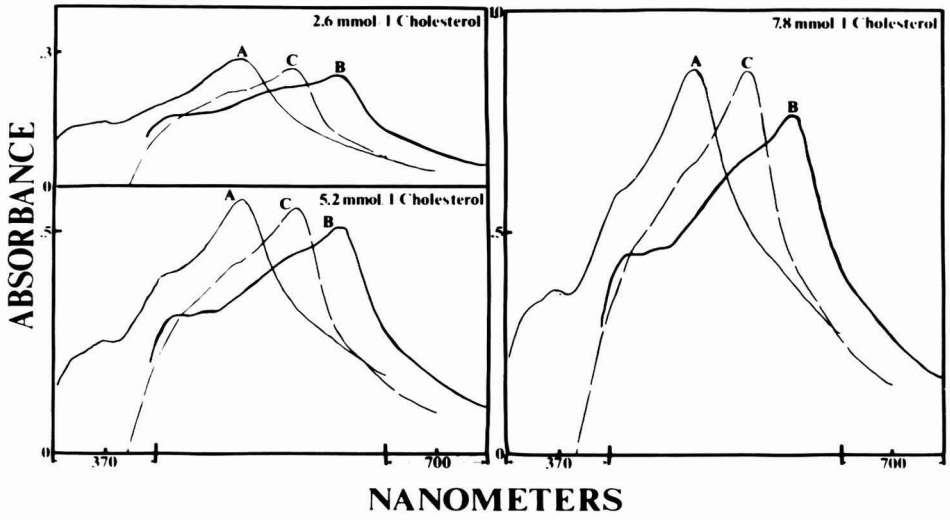

FIG. 1. Shown are the visible spectra of the ferric perchlorate (A), ferric acetate (B), and ferric chloride (C) reactions for cholesterol. Pure cholesterol standards were employed at three concentrations. Each spectrum is displaced 1 in. on the chart paper.

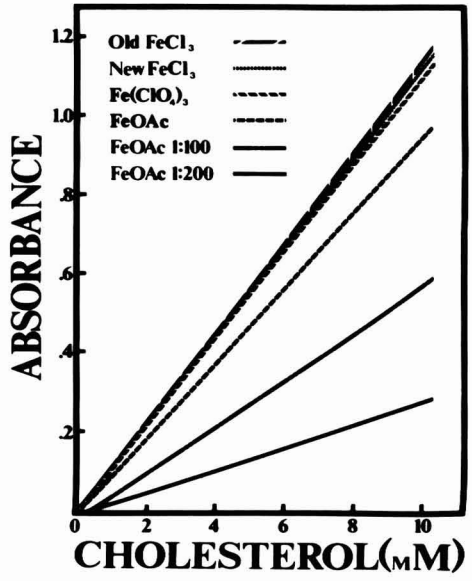

FIG. 2. Typical absorbances, as measured at 560 nm, for the iron reactions at various concentrations of cholesterol are illustrated.

TABLE 1
ABSORBANCES OBTAINED IN THE FERRIC ACETATE–URANYL ACETATE PROCEDURE WITH AND WITHOUT THE SULFURIC ACID–FERROUS SULFATE REAGENT

Absorbance	
H_2SO_4–$FeSO_4$ Reagent	Concentrated H_2SO_4
0.550	0.565
0.542	0.558
0.573	0.563
0.555	0.553
0.553	0.561
0.578	0.566
0.568	0.571
0.572	0.564
0.563	0.558
0.578	0.567
0.575	0.568
0.568	0.560
0.563	0.545
0.566	0.556
0.566	0.566
0.566	0.557
0.561	0.553
0.565	0.562
0.572	0.560
Mean 0.565	0.561

the same although the molar absorptivities are not identical for the three iron reaction modifications.

Figure 2 illustrates a series of calibration curves, the top four are representative of the three iron reactions with identical concentrations of cholesterol in the final reaction mixture. Jung *et al.* (*8*) claim that the ferric acetate–uranyl acetate reagents are stable indefinitely, a time period difficult to define, implying that this is an advantage over other iron-type reagents which are apparently less stable in their reaction characteristics. But the ferric ammonium chloride reagent used to generate the line identified as "old $FeCl_3$" was 15 yr old at the time this study was made. Fifteen years may not be "indefinitely," but one would have to admit, it certainly is a rather long time for a reagent to be stable. Repeated analyses indicate that the molar absorptivities of the ferric perchlorate and ferric chloride reactions are the same at roughtly 11,000 liter $mole^{-1}$ cm^{-1} while the molar absorptivity of ferric acetate–uranyl acetate reaction is consistently somewhat lower at about 9,500 liter $mole^{-1}$ cm^{-1}. The lower two lines in Fig. 2 are indicative of the calibration curves obtained with the

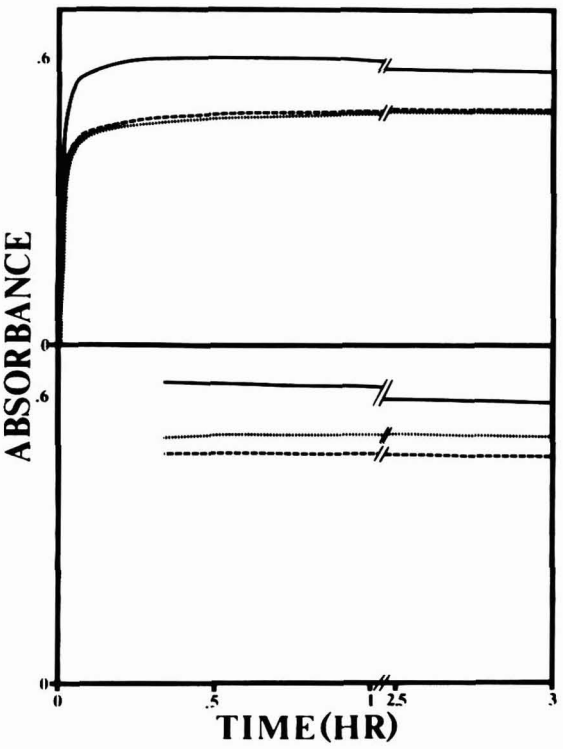

FIG. 3. The upper portion illustrates the color development, beginning at 30s. The lower portion illustrates a study initiated at 20 min following normal color development procedures.

Ferric chloride reaction ——————
Ferric acetate with $FeSO_4$ ....
Ferric acetate without $FeSO_4$ — — — — —

iron acetate–uranyl acetate reaction when the recommended serum to reagent dilutions of 1:100 (*8*) and 1:200 (*7, 13, 16, 24, 26*) are employed.

The necessity of the inclusion of ferrous sulfate in the sulfuric acid reagent was studied and the data derived is shown in Table 1 and Fig. 3. Table 1 lists the absorbances obtained when repeated analyses with an iron acetate–uranyl acetate color reagent—cholesterol standard mixture were made with either sulfuric acid–ferrous sulfate reagent or concentrated sulfuric acid. Statistical analyses of this data shows that there is no significant difference in either the means or standard deviations of the two sets of data. Therefore, the addition of ferrous sulfate apparently does not enhance the color of pure standards at 560 nm.

In Fig. 3 it can be seen that ferrous sulfate does not impart any apparent stability to the ferric acetate–uranyl acetate reaction for standards as the reactions with and without ferrous sulfate are parallel after 3 hr of color

TABLE 2
REPLICATE ANALYSES OF AN ICTERIC SERUM WITH FERRIC ACETATE REAGENTS

Determined concentration (m*M*/liter)			
Ferric acetate–Uranyl acetate	Ferric Acetate–Sodium acetate	Ferric acetate	Abell-type extract
2.95	3.15	3.44	2.76
3.02	3.39	3.36	2.82
3.05	3.39	3.39	2.84
3.00	3.36	3.33	3.82
2.97	3.23	3.33	2.84
3.00	3.00	3.31	2.79
2.92	3.44	3.33	2.69
3.00	3.72	3.28	2.82
2.97	3.36	3.36	2.79
2.97	3.33	3.33	2.71
3.00	3.36	3.31	2.82
2.79	3.07	3.31	2.74
2.61	3.23	3.36	2.71
2.97	3.46	3.26	2.89
2.95	3.28	3.23	2.71
2.97	3.36	3.23	2.74
2.87	3.36	3.20	2.79
2.92	3.26	3.31	2.71
2.89			2.61
2.89			2.79
Mean 2.92	3.31	3.31	2.76

development. This figure also illustrates that at 3 hr there is only a slight decrease in the color produced by the ferric ammonium chloride reaction.

Although Parekh and Jung (*9, 17*) claim to have developed a new direct method for the determination of serum total cholesterol it bears a great deal of resemblance to previously reported simple extraction techniques (*3, 5, 7, 24*). As with the earlier reports, Parekh and Jung (*9, 17*), Parekh and Creno (*16*), Jung *et al.* (*8*), and Parekh *et al.* (*10, 18*) employ the iron color reagent to precipitate the serum proteins and extract the cholesterol from its binding sites. It is apparent that this cannot be considered a direct procedure (*25*) nor is it therefore a new idea or concept but rather a modification of similarly reported procedures (*5, 7, 26*).

Parekh and Jung (*9, 17*) incorporated uranyl acetate into their reagent to afford complete protein precipitation and claimed that the results obtained, without the uranyl salt in the reagent, do not compare well with the Abell (*1*) extraction technique. In order to examine this claim, ferric acetate reagents were prepared incorporating (a) uranyl acetate, (b)

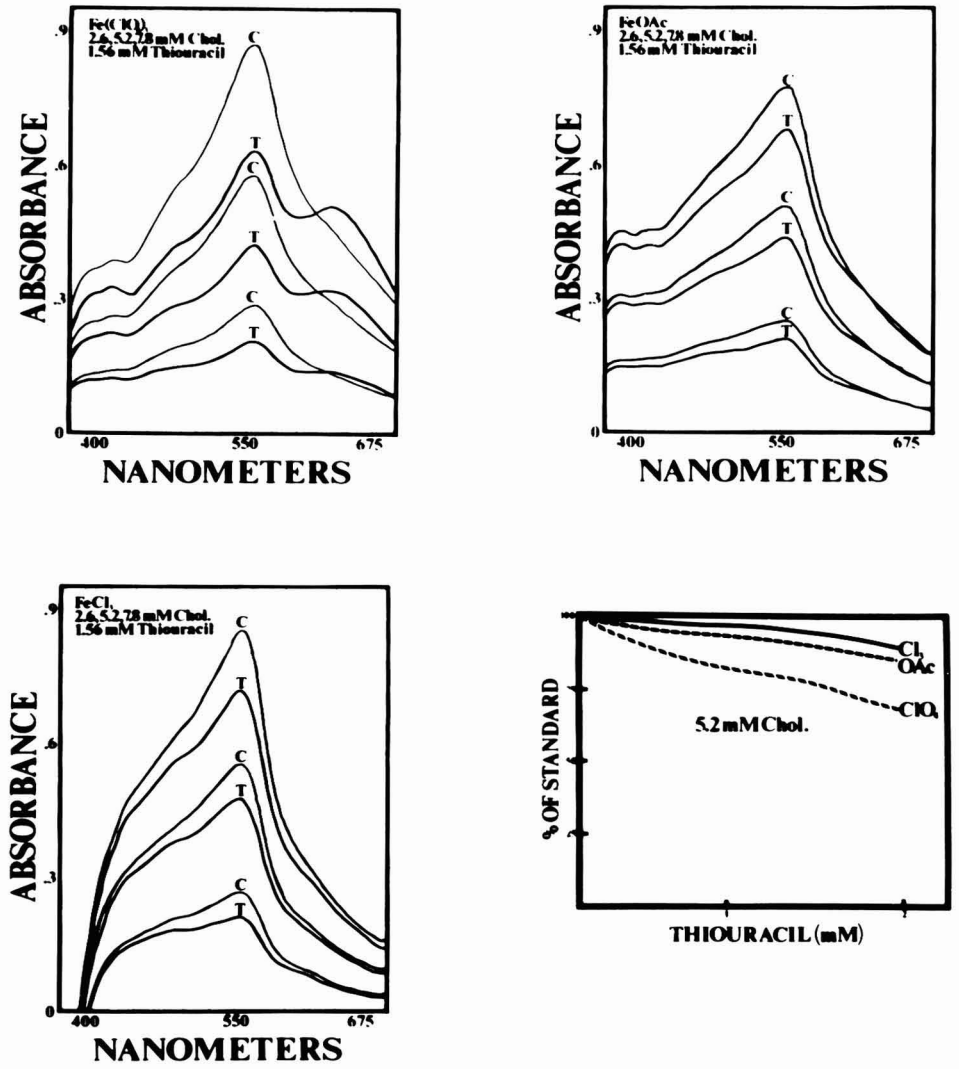

FIG. 4. The spectra generated with cholesterol standards (C) and 2-thiouracil contaminated standards (T) at three levels of cholesterol are shown for the three reactions. In the lower right the effects of 2-thiouracil with a 5.2 m*M*/liter cholesterol standard are summarized. Clockwise from lower left the ferric ammonium chloride, ferric perchlorate, and ferric acetate–uranyl acetate reactions.

sodium acetate, or (c) no metal salt other than the iron. Table 2 presents the data obtained from an icteric sera, with a bilirubin concentration of 0.44 m*M*/liter and a triglyceride of 1.7 m*M*/liter. From these data, calculations show that the standard deviations of the ferric acetate–uranyl acetate reagent, and the ferric acetate–sodium acetate reagent compare well

with the results obtained when the ferric acetate–uranyl acetate reaction was performed on the Abell-type extract. However, when no metal salt, other than the iron acetate, was employed the standard deviations did not compare with those of the petroleum ether extract. In all cases the means did not compare with the ether extract value. These data suggest that the incorporation of uranyl acetate improved the accuracy of the procedure, relative to a reagent without a second metal ion present, if one accepts the ether extraction value as the "true" value. However, the ferric acetate–uranyl acetate reagent system did not yield values exactly comparable with the Abell-type extraction procedure for this very icteric sample.

Figure 4 illustrates the spectra of the three reactions with a known interference (*14, 19, 20, 22*), 2-thiouracil. Apart from the depression of color intensity there is little spectral distortion in either the ferric acetate–uranyl acetate or ferric chloride reactions. However, a prominent shoulder appears at about 635 nm in the ferric perchlorate spectra. Although it is not evident from these spectra, it was found that 2-thiouracil does not give a blank reaction, which is in agreement with previous studies (*19, 20*). The ferric perchlorate reaction is affected the most by 2-thiouracil at its peak maximum, the ferric chloride reaction the least, and the ferric acetate–uranyl acetate reaction only slightly more than the iron chloride. The data as presented in the lower right quadrant are derived from the average of five determinations at five different thiouracil concentrations.

Kitamura and Arimatsu (*11*) reported that nitrate contamination of commercially available sulfuric acid, in Japan, led to inhibition of color formation in an iron chloride (*26*) procedure for serum cholesterol determinations. Rice (*21*) confirmed this report, but pointed out that the levels of nitrate necessary for noticeable color suppression far exceed American Chemical Society allowances. Figure 5 shows the spectral characteristics for the three iron reactions for cholesterol standards with and without contamination of the reaction with sodium nitrate. Qualitatively there are no apparent spectral changes apart from an increase in absorbance around 380 nm in the ferric acetate–uranyl acetate reaction. It is apparent from Fig. 5 that at these A.C.S. unacceptably high levels, the ferric chloride reaction is severely suppressed by contaminating nitrate while there is little or no interference in the ferric perchlorate reaction and only slight inhibition of the ferric acetate.

Although azide has lost popularity as a serum preservative because of its potential explosive properties (*6*) it has recently been reported (*10*) to cause a negative interference in the three iron reactions. For this reason a study of the effects of azide on the three reactions was carried out. As may be seen in Fig. 6 the shape of the cholesterol spectra with the azide

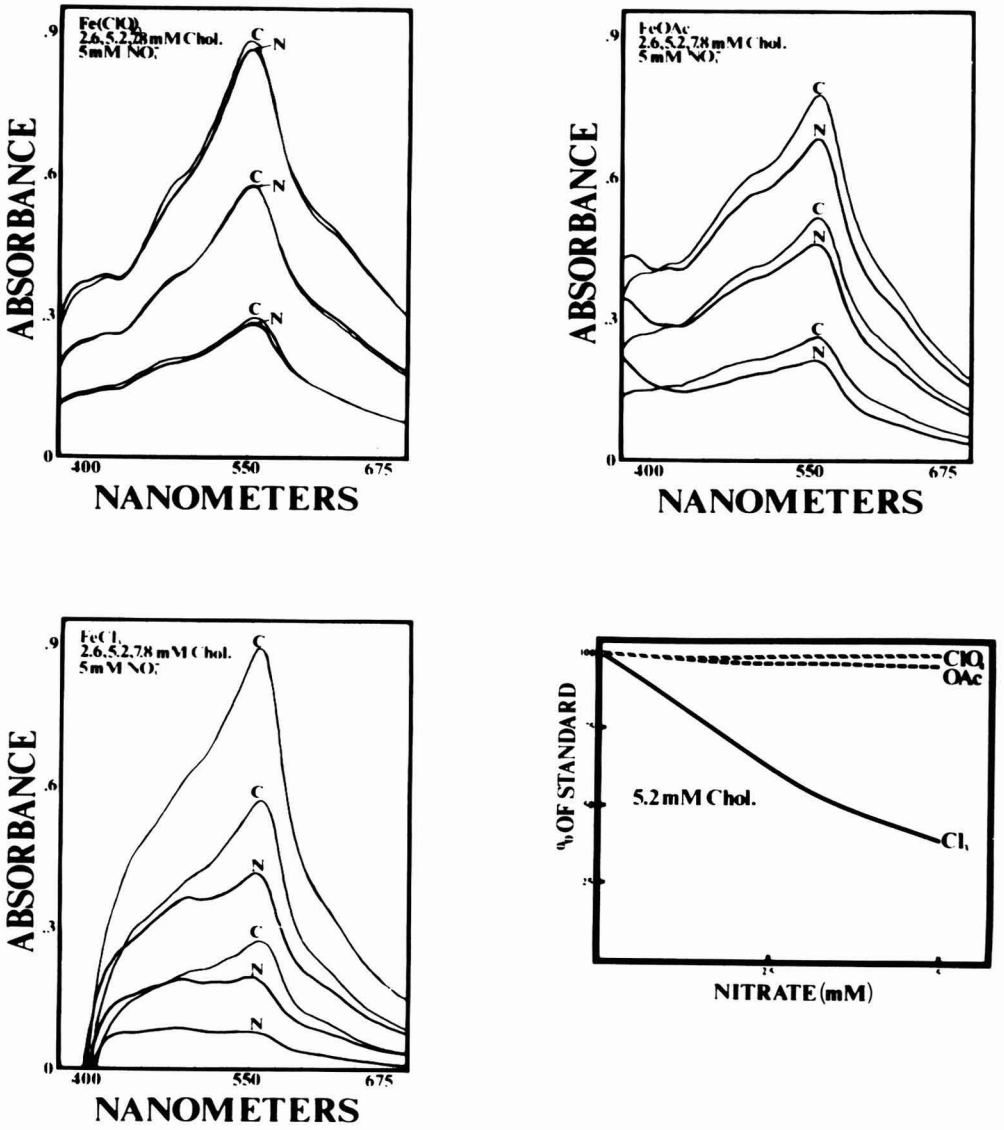

FIG. 5. The spectra generated with cholesterol standards (C) and nitrate (N), as sodium nitrate, contaminated standards are shown for the three reactions. On the lower right the effects of nitrate with a 5.2 m*M*/liter cholesterol standard are summarized. Clockwise from lower left the ferric ammonium chloride, ferric perchlorate, and ferric acetate reactions.

contamination was not altered, however, the color development was depressed considerably. The ferric perchlorate reaction suffers the most inhibition followed by the ferric chloride and the ferric acetate–uranyl acetate reactions. The effects of azide appear to plateau at about 100 m*M*

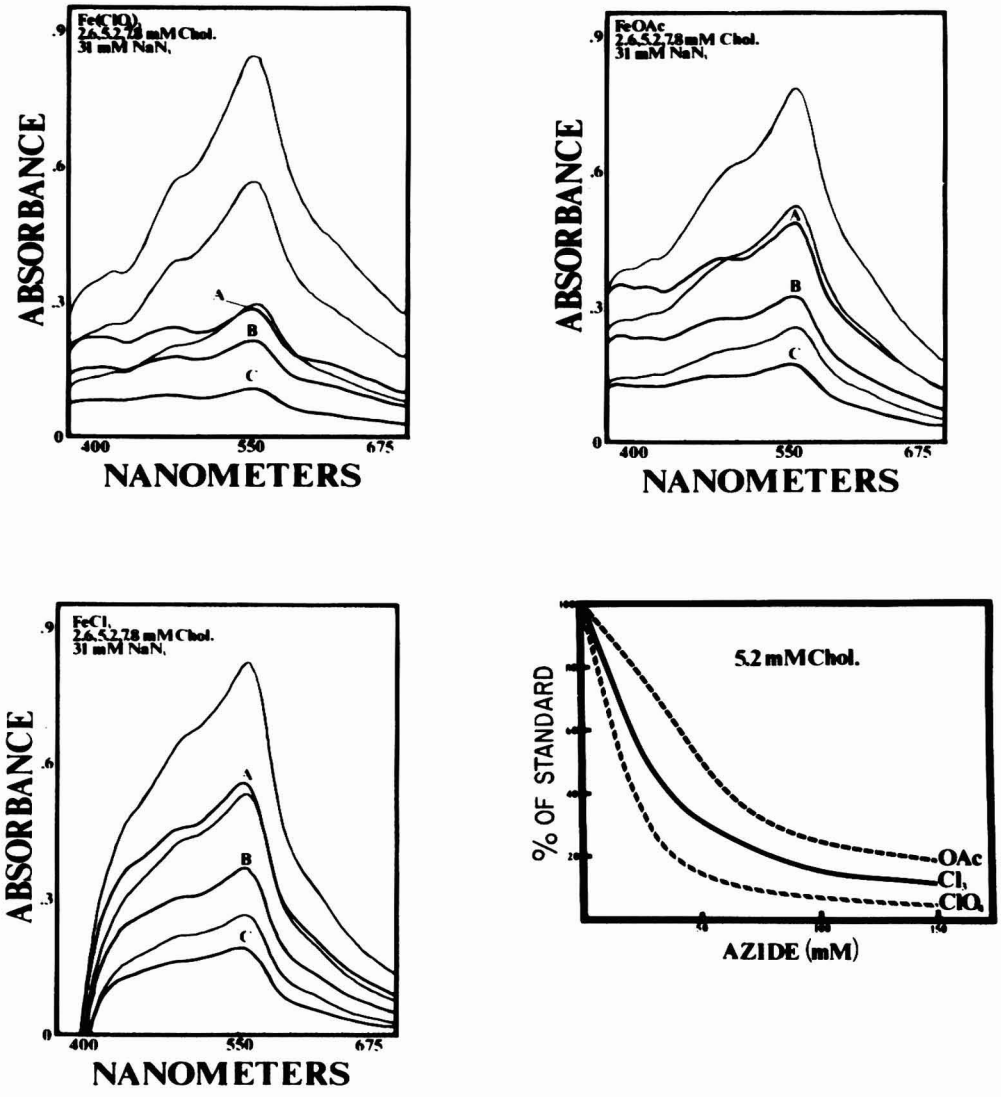

FIG. 6. The spectra generated with cholesterol standards and azide (A, B, C), as sodium azide, contaminated standards. In the lower right the effect of azide with a 5.2 m*M*/liter cholesterol standard are summarized. Clockwise from lower left the ferric ammonium chloride, ferric perchlorate, and ferric acetate reactions.

for all three reactions. It is difficult to state whether or not the data are consistent with that of Khayan-Bashi *et al.* (*10*), as the figures describing this phenomenon have abscissas which are not consistent with each other. Empirically, however, there appears to be agreement as to the order of magnitude of the interference.

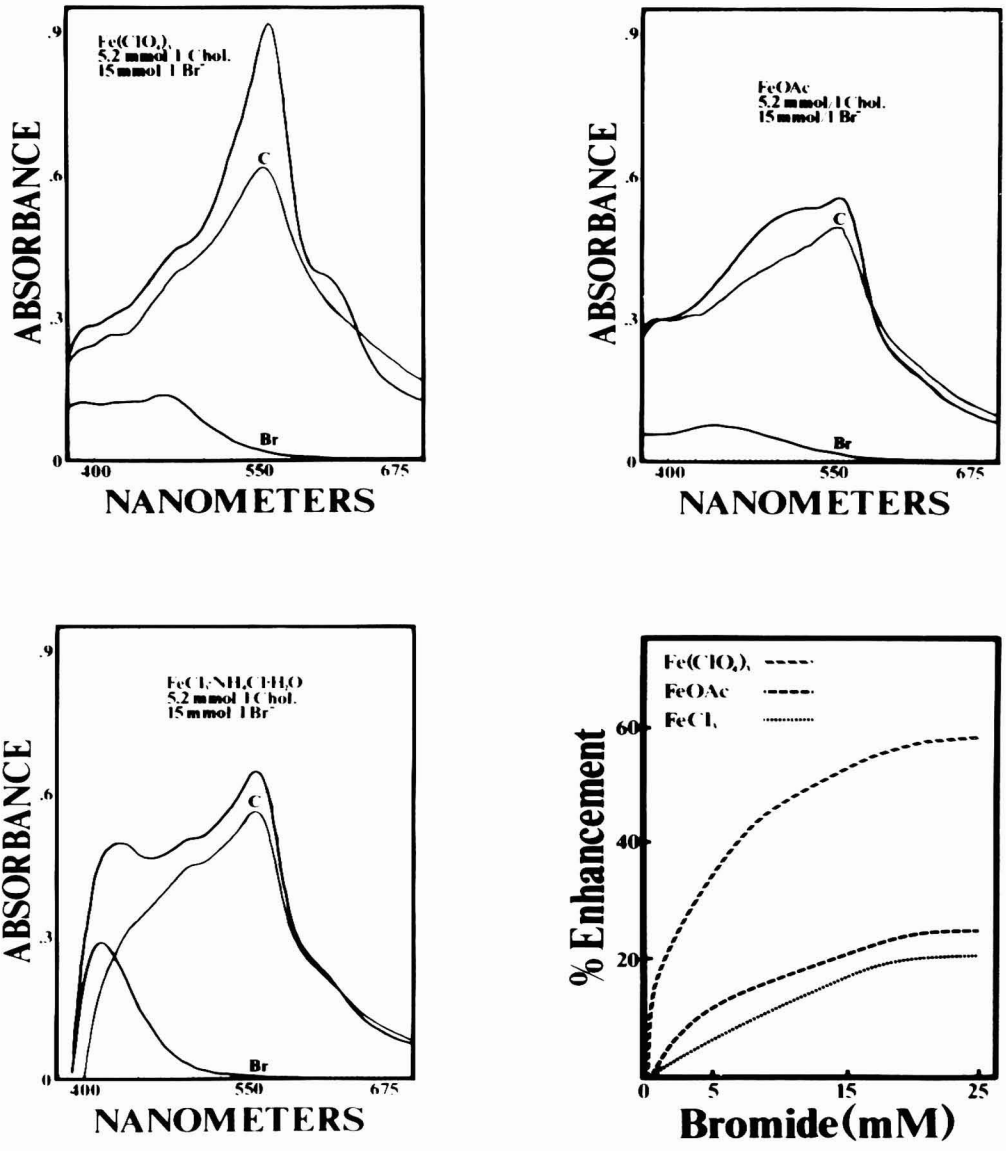

FIG. 7. Spectra of a pure cholesterol standard (C), a pure bromide (Br), as sodium bromide, and a cholesterol standard contaminated with the bromide standard are illustrated. In the lower right the effects of bromide on a 5.2 m*M*/liter cholesterol standard at 560 nm, are summarized. Clockwise from lower left the ferric ammonium chloride, ferric perchlorate,

Enhancement, due to bromide contamination, of the ferric chloride (*2, 13, 23*) and ferric perchlorate (*19, 20*) reaction has been well documented. As may be seen in Fig. 7, bromide gives a blank reaction. However, this reaction alone does not account for the enhanced spectra of a contaminated sample. All three reactions show different characteristic spectral aberrations when bromide is present. Bromide contamination is responsible for the prominent shoulders, to the 560-nm peak maxima, at 475 and 620 nm in the ferric perchlorate reaction. The ferric acetate–uranyl acetate reaction shows a hyperchromic effect for the 500-nm shoulder until at 25 m*M*/liter bromide it becomes almost as intense as the 560-nm maxima. Perhaps the most interesting change occurs with the ferric chloride reaction. There is little spectral change, apart from the obvious enhancement, except for the peak at 410 nm. As may be seen, this peak also occurs in the blank reaction and is presumably due to the formation of some sort of ferric-chloro-bromo complex. This is of interest because even at low levels of contamination this bright yellow color is readily noticeable to the unaided eye before the initiation of the color reaction. Therefore, bromide poisoned samples may be revealed when the ferric chloride procedure is utilized. In fact, of the several contaminants studied, bromide is the most likely to be encountered as a contaminating interference whereas the others are either not encountered or the levels which are encountered may be too low to affect the reaction. In the lower right quadrant of Fig. 7 the enhancement produced by bromide, on a 5.2 m*M* cholesterol standard for the three reactions is illustrated. The lines as drawn are the average results of eight determinations at six levels of bromide. It must be pointed out, however, that the reproducibility of these reactions when contaminated with bromide, is very poor. The ferric perchlorate reaction is enhanced considerably more than either of the other two reactions. It is also apparent that the phenomenon of enhancement reaches a plateau between 20 and 25 m*M*/liter bromide for all three reactions. However, owing to poor reproducibility, the inclusion of bromide for the purpose of enhancing the sensitivity of these reactions as they are described, would be unreliable.

Finally, a comparative study of steroid interferences using the ferric chloride and ferric acetate–uranyl acetate reactions was described (18). However, in this comparison, samples were diluted 1:200 with iron reagent, as before (*9, 17*), but the ferric chloride samples were left comparatively undiluted. It was decided, therefore, that a more realistic comparison would involve the same concentration of interfering steroids in the final mixtures of all three iron reactions. Two criteria were used to select the steroids to be examined: (a) those steroids which reportedly gave the greatest interference in the work of Parekh *et al.* (*18*); and (b) those steroids which were readily available in our laboratory. It should be

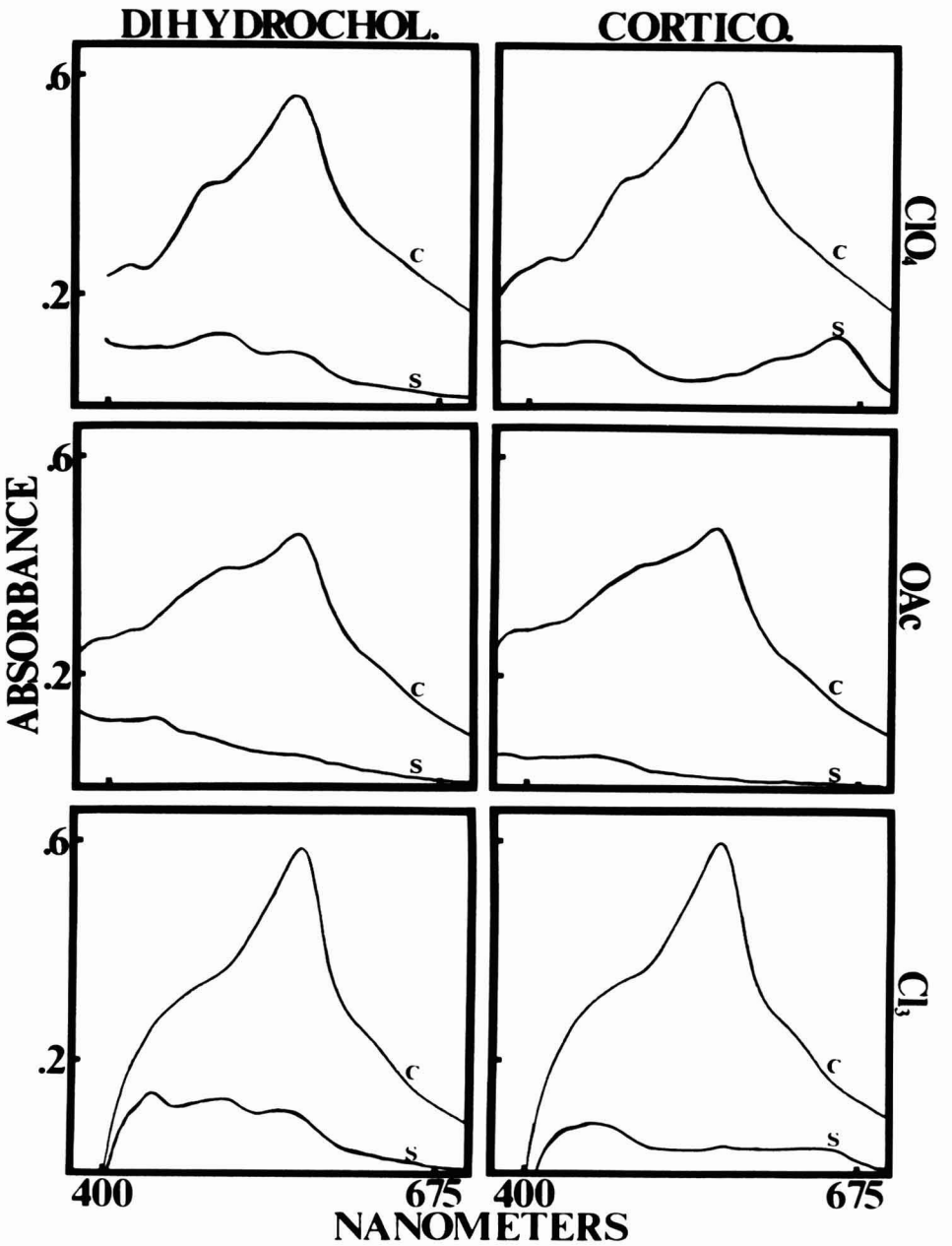

FIG. 8. Shown are the spectra of the three iron reactions with 5.2 m*M* cholesterol (C) and 10.3 m*M* dihydrocholesterol (S) on the left, and 10.3 m*M* corticosterone (S) on the right. The ferric perchlorate, ferric acetate–uranyl acetate, and ferric ammonium chloride reactions are shown, from top to bottom.

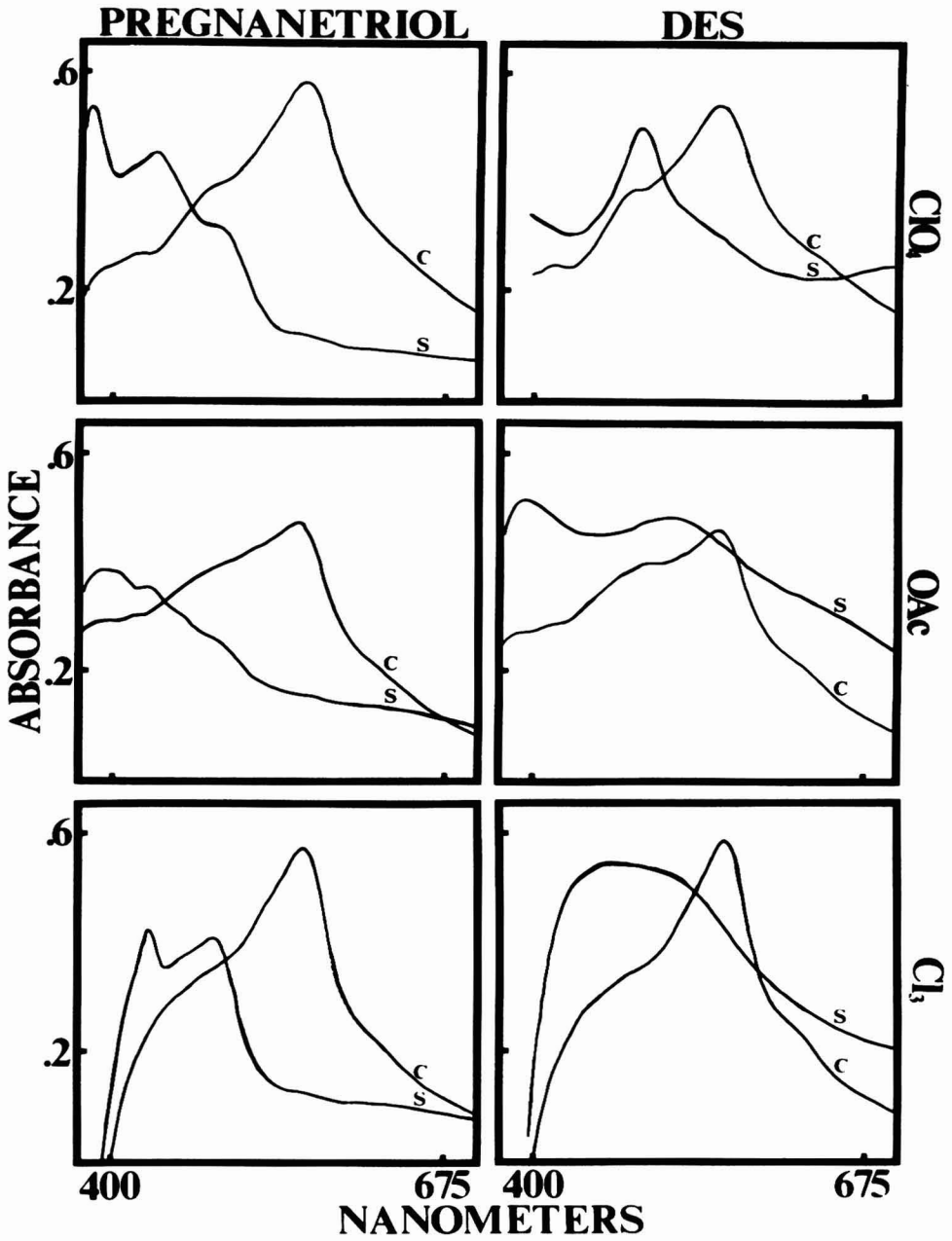

FIG. 9. Shown are the spectra of the three iron reactions with 5.2 m*M* cholesterol (C) and 10.3 m*M* pregnanetriol (S) on the left and 10.3 m*M* diethylstilbesterol (S) on the right. The ferric perchlorate, ferric acetate–uranyl acetate, and ferric ammonium chloride reactions are shown, from top to bottom.

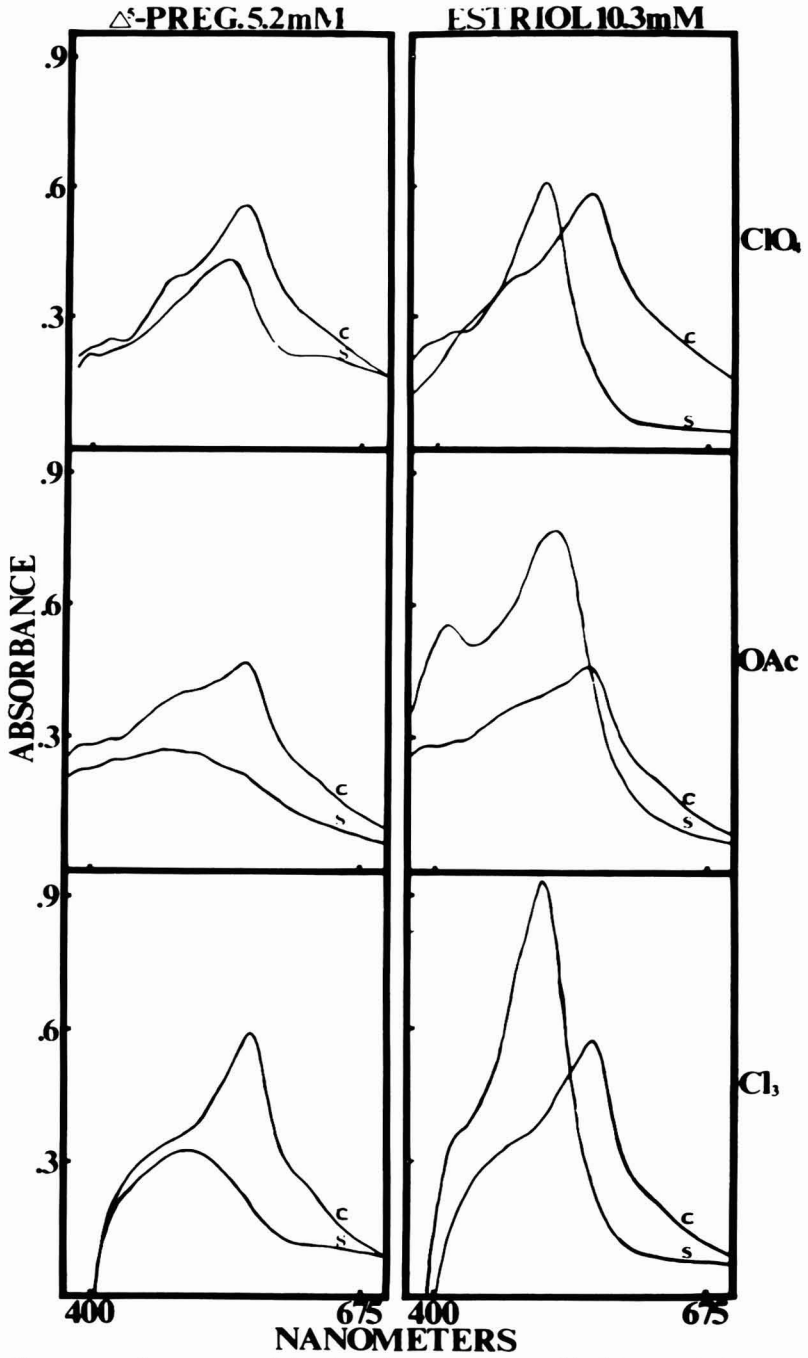

FIG. 10. Shown are the spectra of the three iron reactions with 5.2 m*M* cholesterol (C) and 5.2 m*M* Δ^5-pregnenolone (S) on the left and 10.34 m*M* estriol (S) on the right. The ferric perchlorate, ferric acetate–uranyl acetate, and ferric ammonium chloride reactions are shown, from top to bottom.

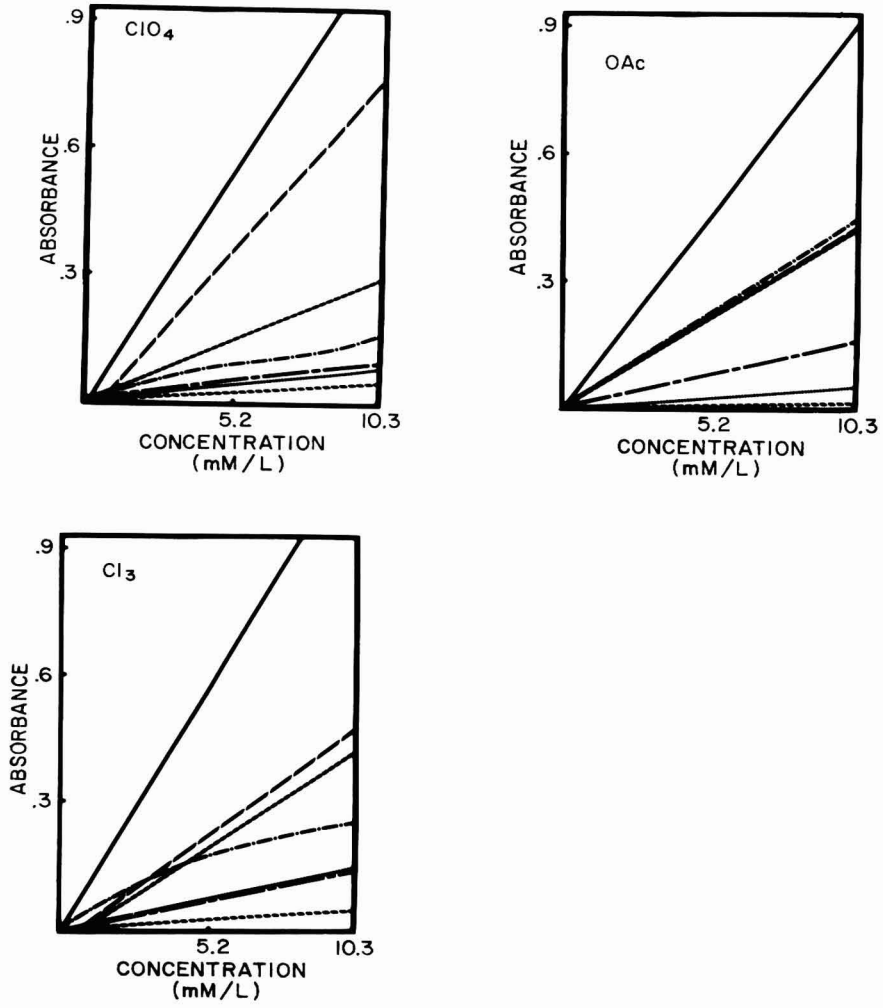

FIG. 11. Typical absorbances, as measured at 560 nm, of various steroids and diethylstilbesterol with the ferric perchlorate reaction, upper left, ferric acetate–uranyl acetate reaction, upper right, and the ferric ammonium chloride reaction, lower left.

Cholesterol
Δ^5-Pregnenolone
Diethylstilbesterol
Estriol
Pregnanetriol
Dihydrocholesterol
Corticosterone

TABLE 3
COLOR PRODUCTION BY VARIOUS COMPOUNDS WITH THE IRON REAGENTS

Compound (5.2 m*M*)	Percent of standard at 560 nm		
	Iron perchlorate	Iron acetate–Uranyl acetate	Iron chloride
Cholesterol	100	100	100
Δ^5-Pregnenolone	68	46	37
Corticosterone	3	2	4
Dihydrocholesterol	15	6	12
Estriol	13	49	29
Pregnanetriol	9	17	12
Diethylstilbesterol	26	46	32

pointed out that the concentration of the steroids used in this study are much higher than the previously described levels (*18*), but it was felt that the latter (*18*), 1.34 m*M*, were too low and their dilutions too high to have elicited any meaningful data.

Figures 8, 9, and 10 illustrate the spectra produced with five steroids and diethylstilbesterol. On making single wavelength readings (560 nm), Parekh *et al.* (*18*) concluded that the ferric acetate–uranyl acetate reaction (*17*) was more specific than the ferric chloride reaction (*25*) and that the reaction mechanism as proposed by Burke *et al.* (*4*) could not explain this increased specificity. However, a qualitative study of the spectra as presented in Fig. 8, 9, and 10 suggest that the three iron reactions produce similar, although not exactly identical, spectra for the compounds studied. It may therefore be unreasonable to dispute the proposed reaction mechanism (4) on the basis of the previous (*18*) single wavelength measurements with dilutions too great to achieve meaningful measurements.

Standard curves for these compounds, as measured at 560 nm, are presented for the three reactions in Fig. 11. Generally, the response is linear, with the exception of estriol by the ferric perchlorate and ferric chloride reactions, but not all of the curves pass through the origin. These deviations from the ideal may be explained by the location on a given spectrum where the measurement is made. Table 3 presents the data as the percent of a cholesterol standard (5.2 m*M*) on an equal molar basis. As may be seen the ferric acetate–uranyl acetate reaction is probably no more specific to cholesterol, and perhaps even a bit less specific, than either the ferric perchlorate or ferric chloride reactions for the other compounds when they are measured at 560 nm.

SUMMARY

A spectrophotometric and chemical evaluation of reported interferences for three iron reactions for the determinations of serum cholesterol has been presented. It has been shown

that all three reactions are affected by various interfering substances, such as 2-thiouracil, nitrate, azide, bromide, diethylstilbesterol, and steroids. Spectral differences between the reactions are probably due to solvent and anion effects.

The incorporation of uranyl acetate as a precipitating agent into the ferric acetate–uranyl acetate procedure did not make the results obtained comparable with the Abell-type extract of a very icteric serum. Incorporation of ferrous sulfate does not noticeably affect the intensity or stability of color development with standards.

As proposed, the ferric acetate–uranyl acetate procedure for the determination of cholesterol represents a modified iron reagent, but the reaction mechanism and the procedure described for it is neither new nor direct. The use of the ferric acetate reagent for serum cholesterol as opposed to ferric chloride, ferric ammonium chloride, or ferric perchlorate appears to have no real advantages as a color reaction. In fact the reaction is less sensitive while reagent preparation is more tedious, time consuming, and expensive than the ferric chloride procedure. Although no quantitative studies were performed, the only effects that chloride had on the reactions were in the region of 400 nm, a wavelength sufficiently far enough away from the 560-nm peak that it did not affect determinations. The concept that the ferric acetate–uranyl acetate reagent contains only acetate and sulfate anions is nullified as soon as one adds serum to the reagents.

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BOOK REVIEWS

Developments in Electron Microscopy and Analysis. Edited by J. A. VENABLES. Academic Press, New York, 1976. 537 pp., $29.00.

This book results from a conference held by the Electron Microscopy and Analysis Group of the Institute of Physics (EMAG) at the University of Bristol in England on September 8–11, 1975. There are 128 articles of varying length, each written by an expert in his particular field. It is impracticable to review each article and an overall review of the text will be presented.

The text is divided into ten sections. A detailed introduction compares, in a general way, conventional transmission electron microscopy (CTEM), the scanning electron microscope (SEM), and the scanning electron microscope used with transmission (STEM). This section is well written and is easily understood by one not versed in the field. It serves as a good introduction to the contents of the book.

The first three sections (33 articles) describe recent developments in instrumentation. A wide variety of subjects is treated, including the newer developments in the field with stress on the expansion of uses of SEM and STEM. Emphasis is placed on obtaining higher resolution by improving the "optics" of these instruments and by improved sample preparation. Routine resolution of 0.3 nm or less is obtained with the field emission scanning transmission electron microscope.

Another section applying high-resolution microscopy presents ten papers focusing on obtaining single-atom contrast images. The photographs of images obtained for MoS_2 and the individual atoms forming the thorium oxide lattice are impressive.

Other sections are devoted to analysis and data handling, including the use of the electron microscope as an electron probe in microanalysis and in obtaining diffraction patterns. Thus, all facets of the application of the electron microscope to analytical problems are explored.

A major application of the instrumentation is in the area of studying crystal structure and faults in crystal structure. This is applied to correlation of the information obtained to physical properties of the material. Special attention is paid to examination of natural minerals such as dolomite, quartz, sapphire, feldspar, pigeonite, diamond, and meteorites as typical examples. Substantial space is given to the study of metals, metal oxides, and alloys for practical reasons.

An important section deals with problems encountered with beam sensitive materials. This includes damage to various organic polymers. Of interest in this section is a paper considering catalase. It discusses problems in studying lipid protein films. This is the only article in the text which addresses itself to a biochemical problem.

This book sets out to study the newer developments in the field of the design and application of the electron microscope to imaging and analytical applications. In this it succeeds admirably. It should serve as a source book for those engaged in the use of the electron microscope in any of its applications. It belongs in the library of the University as a reference text in analytical chemistry, particularly as an instrument for submicroanalysis.

SAMUEL NATELSON, *Department of Biochemistry, Michael Reese Medical Center, 29th Street & Ellis Avenue, Chicago, Illinois 60616*

0026-265X/78/0232–0259$01.00/0
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Biochemical Analysis of Membranes. Edited by A. H. MADDY. Halsted Press, New York, 1976. ix + 513 pp., $37.50.

What this collection of reviews does, which is to discuss the techniques of membrane isolation, purification, and protein–lipid–carbohydrate analysis, it does very well. The authors are, by and large, eminent and authoritative, the level of exposition is high, and the organization of each chapter is unusually consistent for a work of this type.

The book is divided into two sections. Section 1 (Chapters 1–5) deals with the preparation and fractionation of membranes from mycoplasma, cell surfaces, mitochondria, endoplasmic reticulum, and the nuclear envelope. Section 2 (Chapters (6–12) includes discussion of electrophoretic, chromatographic, and immunologic techniques for the analysis of membrane protein, lipid, and carbohydrate. Also included in Section 2 are reviews of the solubilization of membranes (by A. H. Maddy and M. J. Dunn), of phospholipases as membrane probes (by R. F. A. Zwaal and B. Roelofsen), and of specific labels for cell surfaces (by A. L. Hubbard and Z. A. Cohn); these three chapters are the favorites of this reviewer.

If this book prompted any disappointment, it was in its essentially static viewpoint. Little recognition is given to the biochemistry of membranes as a locus of cellular metabolism. Where a membrane enzyme is mentioned it is as a marker of membrane purity. The researcher looking for detailed discussions of hormone receptors, adenylate cyclases, ATPases, or the role of phosphorylation in membrane function will not find them here. Nevertheless, as a wealth of practical information on experimental techniques of membrane analysis, this work is a valuable addition to the library of the serious membrane biochemist.

IRA WEINRYB, *USV Pharmaceutical Corporation, Tuckahoe, New York 10707*

Analysis of Essential Oils by Gas Chromatography and Mass Spectrometry. By YOSHIRO MASADA, John Wiley and Sons, New York, 1976. ix + 334 pp., $37.50.

The application of gas chromatography and mass spectrometry to the analysis of essential oils has greatly advanced our knowledge in this area. This volume presents the research efforts of the author plus those of others in this important field of study.

The first part of the book (up to page 263) is in English with Japanese summaries. Part 2 is entirely in Japanese. Part 1 consists of sixty-four brief chapters grouped under twenty headings. The book includes a series of tables as an appendix listing compounds, structure, molecular weight, boiling point, and relative retention time. In addition, the book includes an index to the essential oils discussed and to the mass spectra and gas chromatograph presented in the text. The sixty-four chapters presented in part 1 each deal with an essential oil. The plant family of the species from which the essential oil was extracted is used to group the chapters into twenty sections. Each chapter includes the extraction process used to prepare the oil, commercial uses of the oil, a physical and chemical description of the oil, a gas chromatogram and mass spectra for the oil if available, and a list of references. Although the text of part 2 is in Japanese, the figures, tables, and references are in English.

The author has performed a highly valuable service in compiling this book on essential oils. It will be an essential guide to further research by students and professionals concerned with perfumes, spices, and flavors.

DONALD F. LOGSDON, JR., *USAF Occupational and Environmental Health Laboratory, McClellan AFB, California 95652*

The Colonial Physician & Other Essays. By WHITFIELD J. BELL, JR. Science History Publications, New York, 1975. 229 pp. $6.95. $5.00. Paperback.

These 15 essays by W. H. Bell, Jr., were selected by the American Association for the History of Medicine and published as a book to commemorate their 50th anniversary. The fact that chemistry was a basic ingredient of the Colonial physician's lore makes them of interest to chemists of all disciplines. Those who have a real interest in the history of science will find this book fascinating and very informative.

DAVID B. SABINE, *185 Old Broadway, Hastings-on-Hudson, New York 10706*

Principles and Techniques of Scanning Electron Microscopy, Vol. 5, **Biological Applications.** Edited by M. A. HAYAT. Van Nostrand Reinhold, New York, 1976. xiv+ pp. $24.50.

Since its inception in 1974, this multivolume treatise, the *Principles and Techniques of Scanning Electron Microscopy (SEM),* has successfully reflected the growth of SEM in instrumentation as well as in methodology. Volume 5 of this continuous treatise thus fulfills a particularly pressing need to keep the readers abreast of the remarkable expansion of the field in recent years and the ever-growing importance of its contributions to the understanding of many problems in the biological and biomedical sciences. It should serve not only as an authoritative international source to cover important new development but also as a guide and survey to save a newcomer the tedious search for information scattered in the biological literature.

In brief, this volume consists of 10 chapters with material covering the preparation of normal and infarcted myocardial tissue, the removal of resins from specimens for SEM, sectioned specimens, immunolatex spheres as cell surface markers for SEM, SEM autoradiography, intermicroscopic (LM, SEM, TEM) correlation, correlative scanning and high-voltage TEM of single cells, backscattered electron imaging (application of atomic number contrast), scanning X-ray and proton microscopy, and SEM applications to cereal science and technology. The format and basic approach in this volume are similar to those in the previous four volumes. The methods and techniques presented have been tested for their reliability, applicability, and versatility, and are the best of those currently available. The instructions for the preparation and use of various solutions, media, stains, coats, and apparatus are straightforward, complete, and easily adoptable. In addition, some new viewpoints concerning the current problems and the potential usefulness of a new method are also critically described and suggested. Each chapter is provided with an exhaustive list of references with complete titles. Full author and subject indexes are available at the end of the volume.

In the first chapter, Ashraf and Sybers describe the optimal techniques (such as fixation, the cryofracture method for specimen preparation, drying, mounting and coating, and observation in SEM) for preparation of myocardial tissue for study with the SEM and discuss the problems in interpretation of the results obtained from normal and ischemic heart.

The next chapter by W. B. Winborn presents a special technique for the removal of polymerized epoxy resin from samples of tissue for use as a single specimen for LM, TEM, and SEM study. Use of the epoxy removal techniques has the potential for offering a new dimension in the examination of tissues, particularly in immunochemical and cytochemical aspects of research.

Methods of sectioning specimens, which can fulfill the need for precise analyses of

internal structures of tissues and cells, are illustrated in detail in Chapter 3 by M. M. Laane. Some operational and procedural precautions are also clearly demonstrated. Then Molday, in Chapter 4, discusses the synthesis of acrylic latex microspheres. methods for coupling antibodies to these spheres, and applications of these new reagents as cell surface antigenic markers for SEM examinations.

Chapter 5 by Hodges and Muir reviews and summarizes the current information on the qualitative and quantitative analyses of biological autoradiograms with SEM. It has a potential value for further study.

The instrumentation required and the general methods for the precise correlation of images from LM, SEM, STEM, and TEM are presented by Geissinger in Chapter 6. The following chapter by Barber also considers the techniques for correlating the data obtained from high-voltage microscopy of single cells with that from the SEM. The advantages and limitations of several methods of intermicroscopic correlation are critically discussed. Both chapters deserve to be thoroughly studied from the economic as well as scientific points of view.

Chapter 8 by DeNee and Abraham is concerned with the ease of direct correlation in the SEM between natural surface topography as seen in the secondary electron (SE) image and underlying histology and cytology as revealed by atomic number contrast in the backscattered electron (BSE) image. Theory, techniques, and applications are comprehensively discussed.

Scanning X-ray and proton microscopy, new techniques, seem to show promise as a useful way to view small specimens, combined with chemical element discrimination, enormous depth of field,, minimal radiation damage, good penetrating power, moderate resolution, and operating in an atmospheric environment, etc., and are concisely introduced by P. Horowitz in Chapter 9. This should be found useful for numerous problems in cell biology, metallurgy, geology, and environmental studies.

The last chapter, by Y. Pomeranz, reviews the application of SEM to the studies of the structure of cereal grains, processing of cereal grains, malting and brewing, and changes during storage of cereal grains and starches. This chapter will be of interest to plant breeders, plant pathologists, cereal chemists, and food processors.

In closing, this volume has been completed over the years through the joint effort of twelve distinguished author–scientists. The materials and information presented and cited are comprehensive, most valuable, and up-to-date. The figures and micrographs for demonstration and illustration are produced in an extremely clear and lucid form. Paper, printing, and cloth binding are of good quality. There are no typographical errors. It is highly recommended for research scientists, electron microscopic technicians, biochemists, biologists, biophysicists, and other structural specialists as an invaluable working reference book.

GEORGE W. C. HUNG, *Woodson–Tenent Laboratories, Hill Top Testing Services, American Biomedical Corporation, Memphis, Tennessee 38101*

An Introduction to Bio-Inorganic Chemistry. Edited by DAVID R. WILLIAMS. Charles C Thomas, Springfield, Illinois, 1976. x + 402 pp. $24.50.

Dr. Williams has assembled an excellent book on the subject of metal ions in biological systems. Some 23 international researchers and teachers in chemistry, biochemistry, pathology, and medicine have contributed to this volume. It is divided into three areas as follows. Section I discusses the general principles of bio-inorganic chemistry; Section II describes the experimental methods used to produce the facts that resulted in these principles; and Section III outlines the applications of these principles to medicine.

Section I begins by discussing modern concepts on the evolution of life from the elements distributed over the Earth's surface (Chapters 1 and 2). Chapters 3–6 concentrate on ion–ligand bonding with discussions of the unique matching involved in the bondings and a highlighting of the metalloenzymes and mixed ligand complexes. Chapters 7 and 8 focus on the present state of knowledge of the reactions and roles of three bio-metals—iron, calcium, and magnesium. These two chapters are of particular importance for students.

Section II describes a variety of techniques for trace element analysis, including that of ion-selective electrodes (Chapter 12), crystallography (Chapter 13), and neutron activation analysis and atomic absorption spectroscopy (Chapter 14). The section concludes with two chapters related to the investigation of biological membrane "structure" by membrane transport (Chapter 15) and bio-oscillations (Chapter 16).

Section III discusses the bio-medical applications of bio-inorganic chemistry. In Chapter 18 an in-depth review of medical, biochemical, and inorganic aspects of one metal-dependent disease (Wilson's disease) is given. Chapter 19 provides an overview of several dozen diseases associated with metal ions as well as describing the therapeutic uses of metals, ligands, and complexes. Chapter 20 describes 19 essential and nonessential *in vivo* elements that produce cytotoxic effects and gives information on the chelating ligands in current use to treat these conditions. The information given in Chapters 19 and 20 is reason enough to obtain this text.

I highly recommend this book to advanced students (graduate level) in inorganic chemistry, biochemistry and medicine, as well as researchers in these fields because it is a well-written text of definite value to anyone interested in the role of metal ions in biological systems.

CAROLE R. SAWICKI, *Environmental Research Center, Environmental Protection Agency, Research Triangle Park, North Carolina 27711*

Siebentes Colloquium über metalkundlich Analyse mit besonderer Berücksichtigung der Elektronenstrahl-Mikroanalyse. Springer-Verlag, Vienna/New York, 1975. vii + 480 pp. $74.00.

The Seventh Colloquium on Metallographic Analysis was held in Vienna on October 23–25, 1974. The publishers are to be congratulated on making the proceedings of that meeting available by October 1975. Some 23 contributions are included, all but one in German, with 273 illustrations, of which a few are in color. This meeting focused on advances in microprobe analysis; consequently, the majority of papers relate to the use of electron or X-ray beam techniques, Auger electron spectroscopy, etc.

This 1974 colloquium celebrated the 65th birthday of Walter Koch, a pioneer for over 40 years in the art and science of metallography, and the author of over 130 technical publications and 2 monographs. Professor Hans Malissa, in a three-page foreword, highlights Doctor Koch's achievements as a staff member of the Krupp Research Institute, then the Max Plank Institute for Iron Research, and recently as Director of the Chemical Laboratories of the August–Thyssen Metallurgical Works and also Honorary Professor at the University of Cologne. In a 21-page lecture, Professor Koch recalls the yesteryears of metallographic analysis and delineates the revolution of the past decade with the introduction of the microprobe, electron raster microscope, and recently, the secondary ion mass spectrometer.

Technical libraries with holdings in materials science and metallurgy should consider purchase of this paperback volume, unless they have already received it as Supplementum VI as part of a *full* subscription to *Mikrochimica Acta*.

This collection of papers should be welcomed by workers applying energy-dispersive surface techniques in diverse sectors of materials science, including ceramics, steels, alloys generally, geochemical samples, electronic devices, microstructures and inclusions, and crystallography.

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