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INTRODUCTION TO AIR CHEMISTRY

By **SAMUEL S. BUTCHER** / **ROBERT J. CHARLSON**
Bowdoin College / *University of Washington*
Brunswick, Maine / *Seattle, Washington*

Here is a book on atmospheric chemistry specifically designed for those with no experience in the field – one that will introduce the reader to *successful* approaches to this important field. It covers air pollution, chemical cycles, and the chemical and physical behavior of trace atmospheric constituents. It uses the principles of both analytical chemistry and meteorology to cope with the most crucial problems of our atmospheric environment.

The first two chapters introduce the reader to air chemistry and outline the basic chemical and meteorological principles used throughout the book. The authors then discuss general methods of obtaining and evaluating air chemical data, emphasizing some of the analytical methods now available. Next, they deal with three main classes of chemical compounds (those containing sulfur, nitrogen, and carbon) which must be considered in the study of atmospheric trace constituents – covering significant atmospheric reactions, global budgets, and selected methods of analyzing these compounds. The final chapter describes the physical characteristics of aerosols.

CONTENTS:
Introduction. Summary of Chemical Principles. Sampling and Collection. Treatment of Data. Special Methods of Analysis. The Atmospheric Chemistry of Sulfur Compounds. Nitrogen Compounds and Ozone. Carbon Compounds. Aerosols. Appendixes. Author Index–Subject Index.

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Briefs

Spectrophotometric Microdetermination of Iron(III) by *tris*-Pyrrolidone(5)-Hydroxamato(2)-Chelate. M. Pećar, N. Kujundžić, H. Meznarić, and I. Jelcic, *Department of Chemistry, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Yugoslavia.*

Conditions for the determination of iron(III) with neutral *tris*-PIH-chelate are described. Under these conditions, Beer's law is obeyed. A large number of ions, including iron(II), do not interfere.

Microchem. J. **20,** 401–408 (1975).

Indirect Spectrophotometric Determination of Perchlorate Using Nitron as Reagent. Salah Shahine and Soad Khamis, *Chemical Research Laboratory, Faculty of Engineering, Ain Shams University, Abbassia, Cairo, Egypt.*

Perchlorate is quantitatively precipitated as nitron perchlorate in the presence of excess nitron and the excess determined as nitron cobaltothiocyanate. The absorbance of the complex is measured at 625 nm.

Microchem. J. **20,** 409–414 (1975).

Photometric Microdetermination of Acetyl Groups in Organic Compounds. H. Trutnovsky Alfy B. Sakla, and S. Abou Taleb, *Medizinisch-Chemisches Institut and Pregl-Laboratorium der Universität Graz, Universitätspl. 2, A 8010 Graz, Austria.*

A method for the determination of acetyl groups is described which combines features of the known transesterification and the hydroxamate methods. All acetyl groups, attached to oxygen or nitrogen, are converted to methyl acetate, which is distilled off and determined photometrically as the iron complex of the acethydroxamic acid. A slightly modified technique permits the selective determination of acetyl groups in the presence of formyl groups.

Microchem. J. **20,** 415–420 (1975).

Determination of Organic Substances by Oxidation with Permanganate. XIX. Analysis of Mixtures of Malonic and Oxalic Acids and of Malonic and Formic Acids. A. Berka, M. Kořínková, and J. Barek, *Department of Analytical Chemistry, Charles University, Albertov 2030, 128 40 Prague 2, Czechoslovakia.*

A method for the analysis of mixtures of the acids has been developed. It is based on stepwise oxidation with alkaline permanganate.

Microchem. J. **20,** 421–427 (1975).

Spectrophotometric Study of Influences on the Direct Ferric Perchlorate Method for the Determination of Serum Cholesterol. MARIE T. PERLSTEIN,[1] R. J. THIBERT[1,2,3], AND B. ZAK[2,3], *Department of Chemistry, University of Windsor, Windsor, Ontario, Canada*[1], *Department of Pathology, Wayne State University School of Medicine, Detroit, Michigan,*[2] *and Detroit General Hospital, Detroit, Michigan*[3].

A spectrophotometric study of several influences on a direct colorimetric determination of serum cholesterol has been described. Interferences on *in vitro* and *in vivo* types were considered and it was found that certain compounds, such as, bromides, uracils, and bilirubin could exert both positive and negative interfering influences on the reaction of ferric perchlorate with cholesterol in an ethyl acetate–ethanol–sulfuric acid medium.

Microchem. J. **20,** 428–439 (1975).

Microdetermination of Fluorine in Organic Compounds by Oxygen Flask Combustion and Conductometric Titration. AKIO NARA, NOBUKO KOBAYASHI, KAZUKO HASEGAWA, AND KATSUKO HONBA, *Tokyo Research Laboratories, Kowa Company, Ltd., Noguchi-cho, Higashimurayama-city, Tokyo, Japan.* AND SHIGEO BABA, *Tokyo College of Pharmacy, Kitashinjuku, Tokyo, Japan.*

Calcium acetate was used as the titrant and the titration conditions were studied regarding the concentration of the organic solvent, pH, and effect of coexisting foreign ions in the sample solution.

Microchem. J. **20,** 440–448 (1975).

Radioactive Decontamination by Bacterial Growth. GREG FRESHWATER, DON SENS, AND ERIC JAMES, *Department of Chemistry, University of South Carolina, Columbia, South Carolina 29208.*

Decontamination of apparatus is done by the growth of *Escherichia coli* in media with limiting quantities of inorganic phosphate.

Microchem. J. **20,** 449–452 (1975).

Ultramicrodetermination of Nitrogen in Organic Compounds. IV. Thermal Behavior of a Gas in a Nitrometer due to Local Variations of Temperature. KEIKICHI MIYAHARA, *Shionogi Research Laboratory, Shionogi & Co., Ltd., Fukushima-ku, Osaka, 553 Japan.*

In order to be able to determine nitrogen more accurately, the variations of local temperature in the nitrometer were studied. Based upon the findings, a new nitrometer was designed which was equipped with a water jacket.

Microchem. J. **20,** 453–461 (1975).

The Preconcentration, Separation, and Spectrophotometric Determination of Traces of Silicate. ALI BAZZI AND D. F. BOLTZ, *Department of Chemistry, Wayne State University, Detroit, Michigan 48202.*

A method for the determination of traces of silicate based on the use of a strong base anion exchange in a preconcentration step and a batch-reactive stripping agent technique prior to spectrophotometric determination has been proposed. The reaction of molybdate and silicate in acidic solution to form 12-molybdosilicic acid permits recoveries exceeding 90% in analyzing solutions containing 0.02–0.1 μg of silicon per ml.

Microchem. J. **20,** 462–467 (1975).

The Indirect Atomic Absorption Spectrometric Determination of Several Organic Bases Using Molybdophosphoric Acid. STEPHEN J. SIMON AND D. F. BOLTZ, *Department of Chemistry, Wayne State University, Detroit, Michigan 48202.*

Results are reported of an investigation the the applicability of atomic absorption spectrometry for the determination of several organic bases after their reaction with molybdophosphoric acid. The final step in each procedure is the measurement of the molybdenum incorporated in the molybdoheteropoly anion. This molybdenum is equivalent to the amount of organic constituent associated with the complex.

Microchem. J. **20,** 468–475 (1975).

TLC of Substituted Pyridines. Hydroxy Derivatives. B. MATKOVICS, ZSUZSANNA FÁTRAY, AND L. M. SIMON, *Biochemical, Genetical Groups of "A.J." University, Szeged, Hungary.*

The primary aim of the investigation was to identify and determine the monohydroxypyridines formed in vitro chemical and enzymatic hydroxylation experiments.

Microchem. J. **20,** 476–482 (1975).

Extraction-Visible Spectrophotometric Method for Determination of Nitrate. Application to Water Analysis. M. ŠIROKI, Z. ŠTEFANAC, AND LJ. MARIĆ, *Laboratory of Analytical Chemistry, Faculty of Science, The University of Zagreb, Strossmayerov trg 14, 41000 Zagreb, Yugoslavia.*

The method is based on the extraction of nitrate with tetraphenylphosphonium chloride and the exchange of the nitrate in the colorless extract by intensely colored vanadium(V)-4-(2-pyridylazo)resorcinol complex.

Microchem. J. **20,** 483–491 (1975).

Procedure for Preparing Infrared Spectra on Gas Chromatographic Fractions. THOMAS H. PARLIMENT, *General Foods Technical Center, White Plains, New York 10625.*

A convenient technique is described for the preparation of microinfrared spectra on materials which elute from a gas chromatograph. The technique involves trapping the sample on KBr and then pressing this material in a die between two portions of pure KBr. Samples as small as 10 μg and as low boiling as 150°C produce acceptable spectra. Since no solvents are employed, solvent band compensation is unnecessary.

Microchem. J. **20,** 492–494 (1975).

An Automatic One- to Eight-Sample Applicator for Fast Qualitative and Quantitative Microelectrophoresis of Plasma Proteins, Hemoglobins, Isoenzymes, and Cross-Over Electrophoresis. B. W. GRUNBAUM, *Environmental Physiology Laboratory, University of California, Berkeley, California 94720.*

The system consists of a flexible eight-sample applicator with a well integrated complimentary 32-sample holder and cell cover. The system permits simultaneous pickup and transfer of one to eight samples and the concurrent electrophoresis of up to 24 specimens on a single cellulose acetate membrane or an agarose gel supporting medium.

Microchem. J. **20,** 495–510 (1975).

A Specific Microcrystalline Test for Indolamine Derivatives. PAUL J. CASHMAN AND JOHN I. THORNTON, *School of Criminology, University of California, Berkeley, California 94720.*

Ethylenediamine was reacted with 11 indolamines and found to give very characteristic crystalline forms. The sensitivity is of the order of 50 μg of LSD.

Microchem. J. **20,** 511–518 (1975).

The Nephelometric Determination of Selenium with 2,3-Diaminonaphthalene. ROBERT E. SMITH AND PETER F. LOTT, *Department of Chemistry, University of Missouri-Kansas City, Kansas City, Missouri 64110.*

A nephelometric determination of selenium has been developed in the concentration range from 10 to 300 ppm based on the reaction of Se(IV) with 2,3-diaminonaphthalene.

Microchem. J. **20,** 519–522 (1975).

Spectrophotometric Study of Drug Interferences in an Aqueous 17-Ketosteroid Reaction. C. S. FELDKAMP,[1] E. EPSTEIN,[2] R. J. THIBERT,[1,3] AND B. ZAK,[1] *Departments of Pathology, Wayne State University School of Medicine,*[1] *and Detroit General Hospital,*[1] *Detroit Michigan and Wm. Beaumont Hospital,*[2] *Royal Oak, Michigan, and the Department of Chemistry, University of Windsor,*[3] *Windsor, Ontario, Canada.*

A spectrophotometric study of two reported drug interferences in an aqueous Zimmermann reaction for 17-ketosteroids has been described. One drug, Tegretol, did not interfere spectrally with the 17-ketosteroid determination because its Zimmermann reaction spectrum was displaced far enough towards the ultraviolet so there was no significant superimposition of its spectrum with that of 17-ketosteroids. The other drug, Cephalothin, proved to be no problem analytically, because, even though it was Zimmermann-reactive, it was unextractable from acid hydrolyzed urines and therefore could not interfere in the color-forming step.

Microchem. J. **20,** 523–533 (1975).

Determination of Hexafluoropropan-2,2-Diol in its Binary Mixtures with Water. NOAH LOTAN AND SARAH EHRLICH-ROGOZINSKI, *Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel and* RODNEY S. ROCHE, *Biopolymer Research Group, Department of Chemistry, University of Calgary, Calgary, Alberta, Canada.*

A visual titration method for the determination of hexafluoropropan-2,2-diol (HFPD) in HFPD-water mixtures is suggested. The procedure involves the titration of the weakly acidic HFPD with sodium methoxide, using dimethylformamide or ethanol as solvent and thymol blue as indicator. The results are in good agreement with the ones obtained by potentiometric titration in water.

Microchem. J. **20,** 534–538 (1975).

Investigation of the Mechanism of Visualization of Fatty Acids with New Fuchsine in TLC by Means of IR Spectroscopy. JÓZEF ŚLIWIOK AND BOŻENA KOCJAN, *Institute of Chemistry, Silesian University, 9, Szkolna Street, 40-006 Katowice, Poland.*

With the new data presented, the possibility exists to distinguish between high and low hydrophobic fatty acids.

Microchem. J. **20,** 539–543 (1975).

Decimilligram-Scale Determination of Sulfur in Organic Compounds. A. MAZZEO-FARINA AND P. MAZZEO, *Laboratori di Chimica dell'Istituto Superiore di Sanitá-Istituto di Chimica Farmaceutica e Tossicologica della Universitá-Roma, Italy.*

The work on the determination of sulfur in organic compounds using oxygen flask combustion followed by titration of the sulfate with barium perchlorate using dimethylsulfonazo III as indicator has been extended to the decimilligram scale.

Microchem. J. **20,** 544–546 (1975).

The Application of Thin-Layer and Gas Chromatography to Identification of the Oxidation Products of *n*-Alkylaromatic Hydrocarbons. JÓZEF ŚLIWIOK AND LEONARD OGIERMAN, *Institute of Chemistry, Silesian University, 40-006 Katowice, Poland.*

On the basis of the presented chromatographic investigations, the partial identification of the oxidation products for the selected *n*-alkylaromatic hydrocarbons is given.

Microchem. J. **20,** 547–552 (1975).

MICROCHEMICAL JOURNAL **20**, 401–408 (1975)

Spectrophotometric Microdetermination of Iron(III) by *tris*-Pyrrolidone(5)-Hydroxamato(2)-Chelate

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University of Zagreb, Zagreb, Yugoslavia

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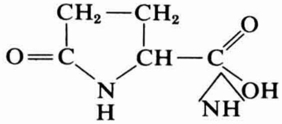
INTRODUCTION

Among numerous methods for determination (*10*) of iron(III), spectrophotometry occupies a prominent place. Simple cations often interfere with colorimetric reagents. Some reagents respond only to iron(II) or do not conform well to Beer's law. Thus it appeared worthwhile to examine hydroxamates.

H. Lossen (*11*), the discoverer of hydroxamic acids, noticed that these form colored compounds with iron(III) salts. This observation was utilized in various spectrophotometric methods for the determination of iron(III) with hydroxamic acids or with compounds easily converted into hydroxamic acids (*1–3,5–9,13*). Various aliphatic, cyclic, and even *N*-heterohexacyclic hydroxamic acids, which formed red colors with iron(III) and other cations, were examined. Thus this study was also extended to *N*-heteropentacyclic hydroxamic acids. Pyrrolidone(5)-hydroxamic(2)-acid (PIH) (*16*) was chosen, not only because of its pyrrol skeleton and the relationship with proline, but also because of the characterization of its iron(III) chelates (*14,15*) and relation to blood chemistry.



The existence of all three theoretically possible iron(III) *mono-*, *bis-*, *tris*-chelates was proved (*14*). Their stability (*15*) constants $\log K_1 = 1.49$, $\log K_I K_{II} = 1.55$ and $\log K_I K_{II} K_{III} = 0.21$, respectively, or $\log K_1 = 10.14$, $\log K_1 K_2 = 18.85$, $\log K_1 K_2 K_3 = 26.16$ which points to the great influence of pH on the formation as a consequence of the weak ionization of PIH $pK_a = 8.65$ (*14*). Therefore, the *mono*-chelate is favored at lower pH and higher iron(III) concentration, with a shifting of the absorbance maximum to longer wavelengths. At higher pH and higher PIH concentration, the *tris*-chelate is favored and the absorbance maximum is narrowed permitting a sharp differentiation of *mono*- and *tris*-chelate only. For the spectrophotometric determination of iron(III), the *tris*-chelate is obviously more suitable.

EXPERIMENTAL METHODS

Reagents

All chemicals and reagents used in this work were "pro-analysi" grade.

Pyrrolidone(5)-hydroxamic(2)-acid (*14,16*). A solution of 10 g of glutamic acid dissolved in 75 ml methanol was saturated with dry hydrochloric acid while boiling under reflux. After introduction of a further 75 ml methanol, the heating and introduction of hydrochloric acid were continued for a further 3 hr. The solution was evaporated at 30°C and 12 torr and then diluted with 30 ml methanol. The hydrochloride of the dimethylester of glutamic acid was treated with a solution of ammonia in chloroform to liberate the free ester. The ammonium chloride was filtered off and the solvent was distilled from the ester. The methanolic hydroxylamine solution was added and the combined solution stored in a freezer for 4 days. The PIH precipitate was then filtered and one more fraction was obtained by evaporation of the filtrate. The raw product was recrystallized from the smallest possible quantity of boiling water. The fine crystals of PIH were filtered and dried over P_2O_5 at 100°C/12 torr. An additional fraction was obtained from the mother liquor after addition of additional 50 ml methanol. PIH = $C_5H_8O_3N_2$; molecular weight = 144.1. It is recommended that a fresh 0.2 *M* solution be prepared daily.

Acetate buffer. 0.2 *M* solution, pH = 5.0.

Estimation Procedure

One milliliter 0.2 *M* PIH and 5 ml 0.2 *M* acetate buffer were added to 1.00 ml $(0.6\text{–}3.3) \times 10^{-3}$ *M* iron(III) solution. The volume was adjusted to 10.00 ml. Absorbances were measured at 430 nm using 1 cm cells, using an Opton PMQ II spectrophotometer (double-monochromator). The iron(III) was estimated from the absorbance (A) by means of the calibration line or using the formula: molar concentration of iron(III)/10 ml:

$$\mathrm{Fe}^{3+} = \mathrm{A} \times 3.57 \times 10^{-4} \qquad \text{(Eq. 1)}$$

or

$$\mu\mathrm{g\ iron(III)/10\ ml} = \mathrm{A} \times 199 \qquad \text{(Eq. 2)}$$

The blank was prepared by filling 1 ml 0.2 *M* PIH and 5 ml 0.2 *M* acetate buffer, pH = 5, to 10.00 ml with redistilled water.

RESULTS AND DISCUSSION

The optimum pH = 5 for this determination follows from Fig. 1, the optimum wavelength $\lambda = 430$ nm from Fig. 2, and the concentration PIH $= 2 \times 10^{-2}$ *M* for completely converting iron(III) into the *tris*-chelate from Fig. 3. The range of iron(III) concentration of $(0.6\text{–}3.3) \times 10^{-4}$ *M* is in the range of optimum accuracy for the spectrophotometric determination (A = 0.155–0.920); this is shown in Fig. 4. Since this calibration curve is linear and passes through the origin (Lambert-Beer Law), the iron(III) may be either estimated from the calibration curve, or calculated from the absorbance using Eqs. 1 and 2. The molar absorptivity of the *tris*-chelate, A = 2800, may be accurately deduced from Fig. 3 and Fig. 4 and applied to the derivation of the coeffients in Eqs. 1 and 2.

As seen from Table 1, including 51 determinations from ten series, the

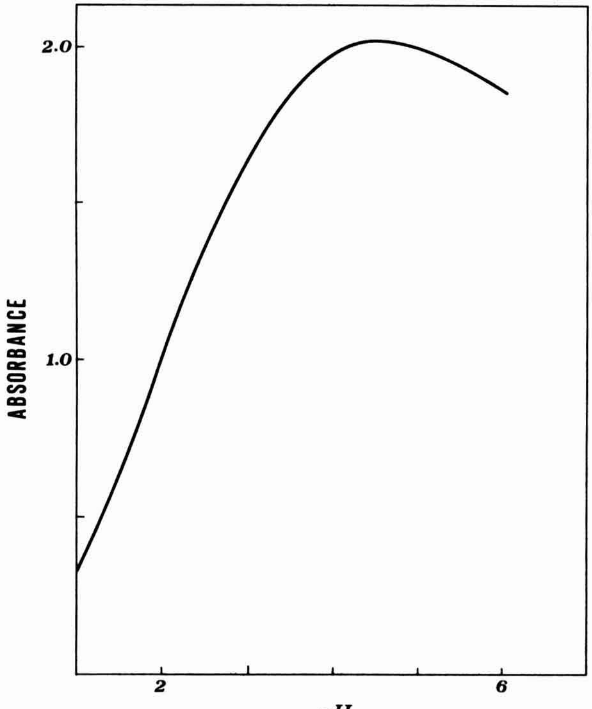


Fig. 1. pH optimal. Iron(III) concentration 1×10^{-3} *M*. Fe:PIH ≈ 1:3. $\lambda = 450$ nm. pH adjusted by HCl and NaOH.

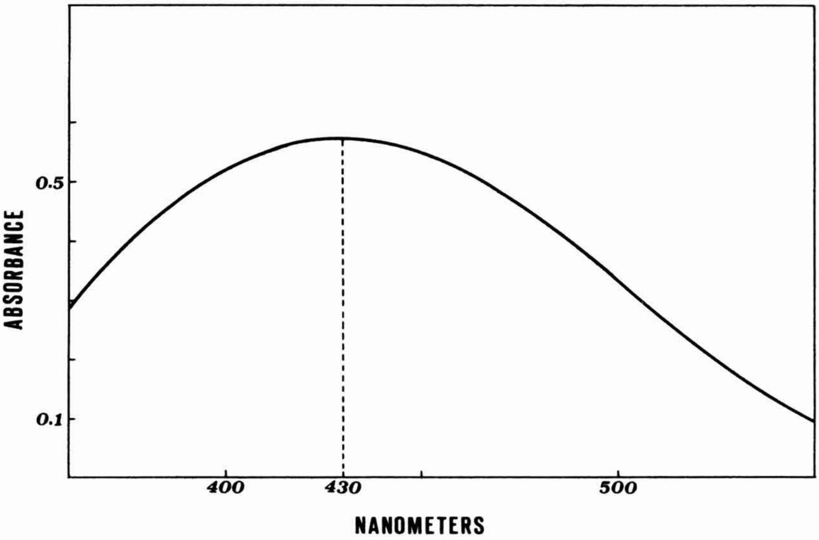


FIG. 2. Optimal wavelength. Fe(III) concentration 2×10^{-4} *M*. PIH concentration 2×10^{-2} *M*. Acetate buffer, pH = 5.

relative accuracy of the proposed procedure was found to be ±0.48% by estimation from the calibration curve. The corresponding accuracy using Eq. 1 and/or 2 was found to be ±1.02%. In three series of ten determinations, each in Table 2, the deviation from the mean was $d = \pm 0.53\%$, and the overall SD was $\sigma = 0.69\%$.

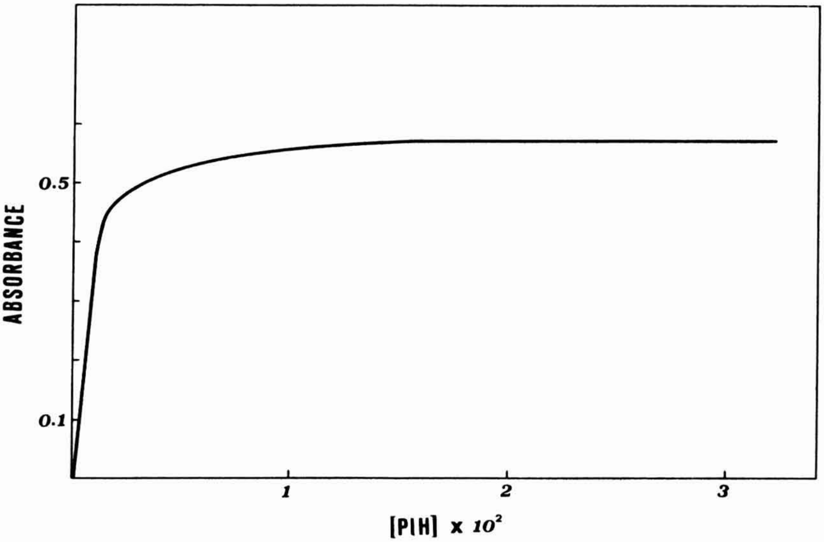


FIG. 3. Quantitative converting of iron(III) to *tris*-chelate. Fe(III) concentration 2×10^{-4} *M*. Acetate buffer, pH = 5. λ = 430 nm.

TABLE 1

ACCURACY OF PROCEDURE

Series	No. of determinations	Taken		Found average			
				By calculation from A = m		From calibration curve	
		$[Fe^{3+}] \times 10^4$	$\mu g\ Fe^{3+}$	$[Fe^{3+}] \times 10^4$	$\Delta Fe^{3+}\%$	$[Fe^{3+}] \times 10^4$	$\Delta Fe^{3+}\%$
I	3	0.50	27.9	0.49	−2.00	0.49	−2.00
II	10	1.00	55.9	1.02	+2.00	1.01	+1.00
III	3	1.25	69.8	1.26	+0.80	1.25	±0.00
IV	3	1.50	83.8	1.49	−0.67	1.49	−0.67
V	3	1.75	97.7	1.75	±0.00	1.75	±0.00
VI	10	2.00	111.7	2.01	+0.50	2.00	±0.00
VII	3	2.25	125.7	2.20	−2.22	2.20	−2.22
VIII	3	2.50	139.6	2.50	±0.00	2.50	±0.00
IX	3	2.75	153.6	2.78	+1.09	2.75	±0.00
X	10	3.00	167.6	3.02	+0.67	3.00	±0.00
Average		—		—	±1.02	—	±0.48

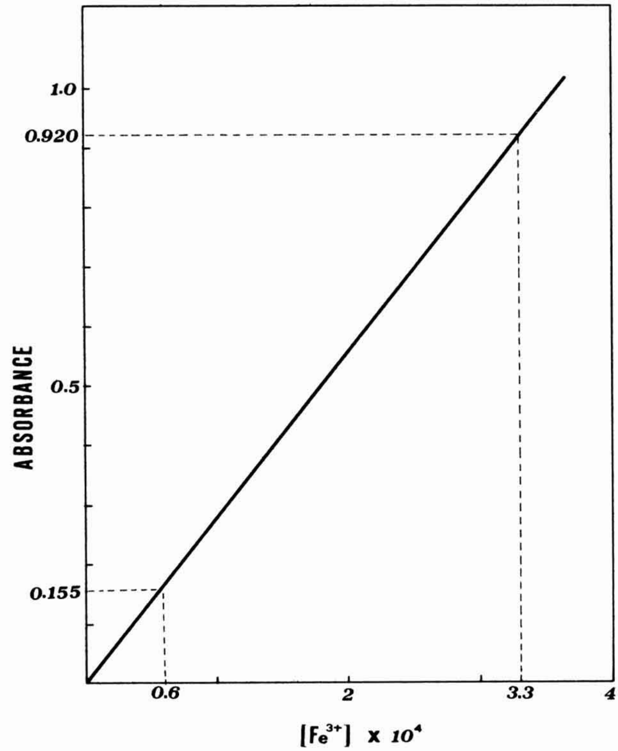


FIG. 4. Optimal range of iron(III) concentration. PIH concentration 2×10^{-2} *M*. Acetate buffer, pH = 5. λ = 430 nm.

None of the following ions at 0.1 *M* in the final observed solution interfered with the procedure for the determination of iron(III): Fe^{2+}, Al^{3+}, Cr^{3+}, Pb^{2+}, Hg^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mg^{2+}, and Ag^{+}. These species, either remain optically clear in the visible spectrum, or are compensated by the reference solution. Interferences are caused by U^{6+} larger than 1.3×10^{-4} *M*, giving an absorbance incre-

TABLE 2
PRECISION OF PROCEDURE

Series	Taken µg Fe^{3+}	Average A = m	Average Found µg Fe^{3+}	d %	σ %
II	55.9	0.285	56.7	±0.73	0.85
VI	111.7	0.564	112.2	±0.70	0.91
X	167.6	0.845	167.6	±0.17	0.23
Average	—	—	—	±0.53	

TABLE 3

SENSITIVITY OF TRIS-CHELATE REACTION FOR IRON (III)

	Visual	Spectrophotometric
Limit of identification μg Fe^{3+}	3.49	0.84
Limit of dilution = = 1 g Fe^{3+} : ml of solution	1:2.86 × 10^6	1:3.58 × 10^6
pD (*12*)	6.46	6.55

ment of 0.006; V^{5+} larger than 3.1×10^{-5} *M*, giving an absorbance increment of 0.008; Mo^{2+} larger than 1.6×10^{-5} *M*, giving an absorbance increment of Δ 0.008; while copper(II) in higher concentration causes a green PIH–chelate precipitate. Anions that normally compete for iron(III), fluoride, and tartrate in their concentrations less than 10^{-3} M do not interfere with the procedure for the determination of iron (III); while oxalate at 1.6×10^{-5} M causes an absorbance increment of −0.010, and citrate at 7.8×10^{-6} M also causes a negative interference of $\Delta A = -0.010$.

According to Table 3 the visual and spectrophotometric limits of dilution are very close. Thus the proposed procedure would lend itself well to either spectrophotometric or visual estimation of iron(III).

SUMMARY

The optimum conditions for the spectrophotometric determination of iron(III) with neutrial *tris*-PIH-chelate are: pH = 5, λ = 430 nm, PIH concentration 2×10^{-2} *M*, and iron (III) concentration $(0.6\text{–}3.3) \times 10^{-4}$ *M*. Under these conditions the suggested method conforms to Beer's law and the molar absorptivity is 2800. The relative accuracy is ±0.48% based upon evaluation from the calibration curve and ±1.02% based upon algebraic equations. The precision is ±0.53% as deviation from the mean, and 0.69% as SD.

The suggested method suffers from no interference from Fe^{2+}, Al^{3+}, Cr^{3+}, Pb^{2+}, Hg^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mg^{2+}, Ag^{+}, F^{-}, and tartrate, but interference is caused by U^{6+}, V^{5+}, Mo^{2+}, Cu^{2+}, (in larger concentrations) or by oxalate and citrate. The method may be used either as a sensitive spectrophotometric or visual method.

ACKNOWLEDGMENTS

We are grateful to the Alexander von HUMBOLDT Foundation for kindly granting us Opton spectrophotometer PMQ II. This work was taken in hand by the first mentioned author in course of his fellowship awarded by the Foundation at the Max PLANCK Institute for Medical Research Heidelberg, Supported by the Republic Foundation for scientific work of the S. R. Croatia.

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MICROCHEMICAL JOURNAL **20**, 409–414 (1975)

Indirect Spectrophotometric Determination of Perchlorate Using Nitron as Reagent[1]

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INTRODUCTION

The mono-acid base nitron, $C_{20}H_{16}N_4$, can be used for the gravimetric determination of perchlorate (*2*), the compound formed having a solubility of approximately 0.008 g per 100 ml of very dilute acid solutions at ordinary temperature. The method is applicable down to 0.08–0.1 g of perchlorate.

When the gravimetric method was applied on the microscale low results were obtained. In the present work, however, a difference spectrophotometric method is proposed. An excess of the reagent is added to the sample solution, and the excess reagent is determined in the supernatant liquid after separating off the precipitate. The determination of excess nitron is based on the formation of the blue ion-association complex nitron cobaltothiocyanate. The latter compound is extractable in a mixed solvent consisting of 3 volumes of cyclohexanone and 7 volumes of carbon tetrachloride, in the presence of excess thiocyanate. The extract has an absorption maximum at 625 nm.

EXPERIMENTAL

Apparatus and Reagents

All spectral measurements were made with a Carl Zeiss Jena "Spekol" spectrocolorimeter, using 1.00-cm glass cuvettes.

Nitron reagent. An approximately 0.025 *M* solution was prepared by dissolving 4 g of nitron (Merck reagent) in 5 ml of warm 5% acetic acid and diluting to 500 ml with distilled water. The reagent solution was filtered and kept in a brown bottle. The reagent remained unchanged for a long period.

Standard perchlorate solutions. A solution containing 5 mg of perchlorate per ml was prepared from dry AR. ammonium perchlorate.

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[1] Dedicated to Prof. R. J. Magee.

From this solution, a series of diluted standards containing 0.1–2 mg of perchlorate per milliliter was prepared.

Cobalt sulfate. An approximately 0.05 *M* solution was prepared from AR. $CoSO_4 \cdot 7H_2O$.

Potassium thiocyanate. A 4 *M* solution of the AR. salt was used.

Mixed solvent. 30% (V/V) cyclohexanone in carbon tetrachloride was prepared from the AR. solvents.

Calibration Curve

Transfer 2 ml portions of the standard perchlorate solutions containing from 0.1–2 mg of perchlorate per milliliter to microbeakers or centrifuge tubes (10-ml capacity). Add to each 1 drop of 2 *M* sulfuric acid, warm, and add 2 ml of the nitron reagent measured with a pipette. Cool, and allow to stand in ice for 1 hr. Filter through a sintered glass funnel or centrifuge off the precipitate. Withdraw 2 ml of the supernatant liquid and dilute with 30 ml of distilled water in a 100 ml separatory funnel. Add 2 drops of 1 *M* sulfuric acid, 10 ml of 0.05 *M* cobaltous sulfate and 5 ml of 4 *M* potassium thiocyanate solutions, and mix. Extract the blue complex with four 5-ml portions of 30% cyclohexanone in carbon tetrachloride. Combine the extracts and dilute to 25 ml with the solvent. Determine the absorbance at 625 nm in a 1-cm cell against the solvent.

Carry out a blank by treating 1 ml of the same nitron reagent with cobaltous sulfate and potassium thiocyanate, extracting the complex as described above and measuring the absorbance against the solvent.

Subtract the absorbance values of the perchlorate standards from that of the blank. Draw the relation between the obtained values ($A_{\text{difference}}$) and the perchlorate-ion concentration. A straight line passing by the origin will be obtained (Fig. 1).

Determination of Perchlorate

Treat 2 ml of the test solution with an equal volume of the reagent and continue as previously mentioned. Carry out the blank using the same reagent solution. Find the difference in absorbances, and read off the perchlorate-ion concentration from the calibration graph.

Elimination of Interfering Ions

Dissolve the weighed sample in a suitable solvent and treat with the proper reagent as shown below. Make up to the appropriate volume with distilled water. Use this as the test solution.

Bromide and iodide. Add chlorine water dropwise to the boiling solution until the color of bromine or iodine disappears.

Nitrite. Treat with powdered hydrazine sulfate or urea.

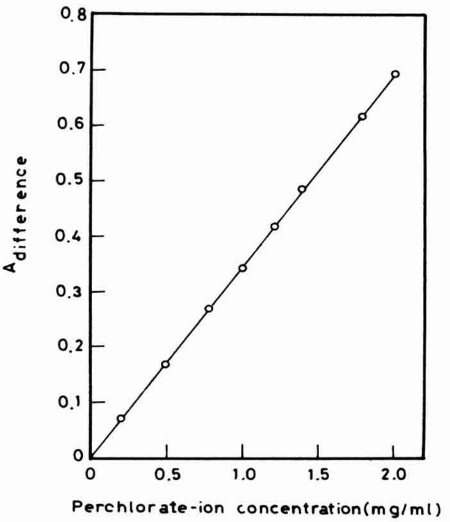

FIG. 1.

Chromate. Reduce with powdered hydrazine sulfate or with 10% sulfurous acid.

Chlorate. Reduce with 10% sulfurous acid or 10% formaldehyde.

RESULTS AND DISCUSSION

Nitron acetate forms precipitates with a considerable number of anions (*2*). Magee *et al.* (1) have confirmed that these precipitates are salts of ionic structure.

The solubility product of nitron perchlorate is 3.4×10^{-8}, which is just low enough for gravimetric work on the semimicro scale. On the micro scale, however, errors resulting from the washing of the precipitate becomes appreciable. The difference method described above allows the determination of amounts of perchlorate as small as 0.1 mg. It obviates errors which would otherwise have been associated with the washing of such a small precipitate.

Nitron acetate reacts with a cobaltous salt in the presence of thiocyanate to form a blue precipitate of nitron cobaltothiocyanate [(Nitron · $H)_2Co(CNS)_4$]. This formula was elucidated by photometric titration using standard solutions of nitron and cobalt. The blue compound dissolves readily in a mixed solvent consisting of 30% cyclohexanone in carbon tetrachloride to give a blue solution which is used for the spectrophotometric determination of nitron. Cyclohexanone alone extracts cobalt thiocyanate as well as nitron cobaltothiocyanate, while carbon tetrachloride alone extracts none of them. The mixed solvent, on the other hand, extracts only nitron cobaltothiocyanate. The extraction can

TABLE 1

1.964	1.978	1.982	1.986	1.994
1.994	1.996	1.996	2.004	2.014
Mean value				1.991 mg
Standard deviation for a single determination				±0.020 mg
Coefficient of variation				±1.00%

be done in the pH range 1–7. The optimum concentration of thiocyanate is about 0.4 *M*. At much lower concentrations nitron cobaltothiocyanate is not completely extracted, and at much higher concentrations cobalt thiocyanate is partially extracted together with the compound.

The Precision of the Method

The results for a series of ten determinations are shown in Table 1. The figures are calculated as the amount of perchlorate found for a sample containing 1.990 mg of this ion.

Analysis of Inorganic Perchlorates

As a test of the applicability of the method described, representative inorganic perchlorates were analyzed. The test solutions of these salts were previously standardized against EDTA except sodium perchlorate which was prepared from the dried AR. material. The results obtained are recorded in Table 2. These results are considered to be satisfactory, the maximum error being about 1%.

TABLE 2
DETERMINATION OF INORGANIC PERCHLORATES

Sample	Concentration (*M*)	mg of ClO_4^- Calc.[a]	mg of ClO_4^- Found	% Error
Sodium perchlorate (dried)	0.0100	0.995	0.995	0.00
	0.0050	0.497	0.500	+0.60
Magnesium perchlorate	0.0048	0.955	0.955	0.00
	0.0024	0.477	0.472	−1.05
Zinc perchlorate	0.0046	0.915	0.920	+0.46
	0.0023	0.457	0.455	−0.44
Cadmium perchlorate	0.0052	0.993	0.988	−0.51
	0.0026	0.496	0.498	+0.42

[a] Calculated on the basis of EDTA titrations of the salt solutions except for sodium perchlorate.

TABLE 3

EFFECT OF FOREIGN ANIONS (ClO_4^- TAKEN AS NH_4ClO_4 1.990 mg)

Foreign anion	Concentration (M)	Added as	Removed with	ClO_4^- found (mg)	% Error
Cl^-	0.01	NaCl	—	2.000	+0.50
	0.05		—	1.990	0.00
	0.10		—	1.985	−0.26
	0.10		—	2.00	+0.50
Br^-	0.01	KBr	—	2.388	+20.00
	0.10		Chlorine water	2.000	+0.50
	0.10		Chlorine water	1.990	0.00
I^-	0.01	KI	—	3.980	+100.00
	0.10		Chlorine water	1.995	+0.26
	0.10		Chlorine water	2.010	+1.05
NO_2^-	0.01	KNO_2	—	2.452	+23.31
	0.10		Hydrazine hydrate	1.990	0.00
	0.10		Urea	1.980	−0.52
CrO_4^{2-}	0.01	K_2CrO_4	—	2.628	+32.06
	0.10		Hydrazine sulfate	1.990	0.00
	0.10		Sulfurous acid	2.000	+0.50
ClO_3^-	0.01	$KClO_3$	—	2.584	+29.84
	0.10		Sulfurous acid	2.000	+0.50
	0.10		Formaldehyde	1.980	−0.52

Interferences

Sulfate, phosphate, fluoride, and oxalate do not form precipitates with nitron. They also have no effect on the spectrophotometric determination of the reagent. Chloride does not interfere up to a concentration of 0.1 M (Table 3). Bromide, iodide, nitrite, chromate, and chlorate interfere by forming precipitates of varying solubilities with nitron. Bromide and iodide can be eliminated with chlorine water, nitrite with hydrazine sulfate or urea, chromate with hydrazine sulfate or sulfurous acid and chlorate with sulfurous acid or formaldehyde; the procedures are outlined above. To test the effectiveness of these procedures, synthetic mixtures were analyzed before and after treatment. All treatments proved to be successful, the maximum error was about 1% (Table 3). Nitrate forms a sparingly soluble precipitate with nitron and must be absent. It may be decomposed with Devarda's alloy, but the method will become laborious.

SUMMARY

An indirect method is outlined for the spectrophotometric determination of small amounts of perchlorate. Perchlorate is quantitatively precipitated as nitron perchlorate in

the presence of excess nitron. After separating off the precipitate, the excess reagent is determined in the supernatant liquid as nitron cobaltothiocyanate. The latter compound is extracted in 30% cyclohexanone in carbon tetrachloride and the absorbance of the extract measured at 625 nm. The method is simple, reproducible, and accurate to ± 1%. The interferences of other anions were also investigated.

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MICROCHEMICAL JOURNAL **20**, 415–420 (1975)

Photometric Microdetermination of Acetyl Groups in Organic Compounds

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The photometric determination of acetyl groups by conversion to the acethydroxamic acid and formation of the colored iron complex is an interesting alternative to the conventional saponification methods (*1,3,5,7*).

The high sensitivity of the photometric finish and the short reaction times offer advantages, but, as acid chlorides and anhydrides react in the same way as do esters and, as different other compounds may also form colored iron complexes, the selectivity is not satisfactory.

To develop a generally applicable method for the microdetermination of acetyl groups the following features need to be added: 1. Increased selectivity against acid chlorides and anhydrides as well as against acyl groups other than acetyl; 2. sensitivity for acetyl groups attached to nitrogen; 3. no interference by other functional groups which might produce colored iron complexes.

Those three features can be achieved with minimum complications by adding a preliminary transesterification in a closed vessel. Heating with a great molar excess of methanol and *p*-toluenesulfonic acid converts all acyl groups to their methyl esters. This method, described by Spingler and Markert (*8*) for their gas chromatographic acetyl determination is fast and reliable.

But as the transesterification was performed in sealed glass ampoules, this method was to inconvenient to gain popularity.

By other methods of transesterification (*4,9,10*) the methyl acetate produced is highly diluted with excess methanol.

In our experiments, vessels with screw-cap closures with Teflon liners from Sovirel, France (SVL 15) proved sufficiently tight at 100°C to be used instead of sealed vessels.

A quick cryogenic distillation can separate the ester from the saponi-

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fied sample and the toluenesulfonic acid, due to the high volatility of methyl acetate; only methyl formiate and propionate are sufficiently volatile to interfere seriously. That means that also anhydrides and chlorides of other acids than acetic and propionic will not interfere as their esters do not distill over.

The reaction between methyl acetate and hydroxylamine proceeds quickly even in methanolic medium at room temperature. The advantages of anhydrous ether as a solvent as recommended by Bayer and Reuther (*1*) are more than compensated by the rather tedious technique necessary. The ether solvent is necessary to ensure uniform conversion rates of the different acyl groups they investigated. Due to the transesterification and distillation in our method, methyl acetate is the only compound to convert to hydroxamic acid. We found, that the well known (*1,5*) preparation of the hydroxylamine reagent could be improved by replacing the sodium hydroxide by lithium hydroxide. Lithium chloride is very soluble in methanol, thus no filtration is necessary.

As methyl acetate reacts very quickly with hydroxylamine, no boiling is necessary; 15 min at room temperature are sufficient (*5*). The interference from formic acid can be overcome by acidifying the reaction mixture after the 15 min and leaving it for 60 min. Formhydroxamic acid decomposes in acidic medium completely (*2,6*). The color is developed by addition of $FeCl_3$ in anhydrous methanol. Crystallized $FeCl_3$ can be used, the quantity of water introduced this way does not exceed the stability limit (maximum 2% H_2O) of the red–violet modification of the iron complex which is best suited for photometric work as found by various authors. This solution must be acid to prevent precipitation of $Fe(OH)_3$ by the excess LiOH but to high acidity reduces the color stability (*5*).

We found that the conventionally used perchloric acid can be replaced by glacial acetic acid. Thus the quantity of the acid can be sufficiently high without danger of making the solution too acidic. Also all possible problems in mixing concentrated perchloric acid and methanol are avoided. As free acetic acid does not react with hydroxylamine, acetic acid can be used for the acetyl group determination.

The absorbancy is measured at 530 nm, and the acetyl content determinated from a calibration graph, prepared by analysis of pure pentaacetylglucose.

EXPERIMENTAL

Apparatus

The apparatus for cryogenic distillation, Fig. 1, consists of a simple distilling bridge with stopcock and SVL 15 connectors. A Dewar flask with dry-ice acetone or a suitable thermoelectric cooler. A photometer set at 530 nm wavelength.

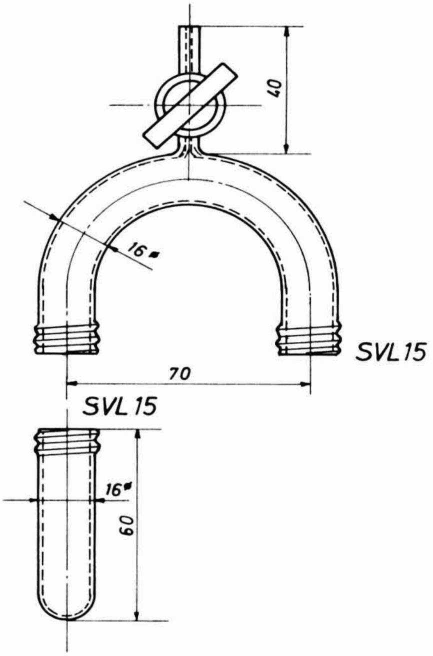

FIG. 1.

Reagents

0.3 M p-toluenesulfonic acid. Dissolve 5.7 g in dry methanol and make to 100 ml.

0.75 M hydroxylamine hydrochloride. Dissolve 5.2 g solid in dry methanol and make to 100 ml.

1.25 M lithiumhydroxide. Dissolve 3.0 g lithium hydroxide in dry methanol make to 100 ml and centrifuge. Decant the clear solution and store protected from CO_2.

0.0005 M ferric chloride. Dissolve 1.35 g of crystallized ferric chloride in dry methanol, add 100 ml glacial acetic acid, and add methanol to 1 l.

Procedure

Weigh 2–5 mg sample according to the expected acetyl content into a dry reaction vessel and add 1 ml of toluenesulfonic acid. Prepare a blank at the same time. Close the vessels and heat for 30 min to 70° for *O*-acetyl, or for 1 hr to 100°C in the case of *N*-acetyl compounds. Cool with dry ice acetone or the thermoelectric cooler to at least −25°C. Open and attach to the distilling bridge. Connect an empty reaction vessel to the other side of the distilling bridge, evacuate quickly with a water pump, and close the stopcock. Remove the sample vessel from the cold bath and heat it slightly with warm water while cooling the other

TABLE 1

	CH$_3$CO %		Number of acetyl groups	
Compound	Calc.	Found	Calc.	Found
2-Phenyl-(3′-0–acetyl-1′, 2′-didesoxy-5′,6′-0-isopropyliden-α-D-glucofurano)[2′,1′:4,5] 2-oxazolin	11,48	11,82 12,12	1,0	1,03 1,06
Methyl-2,3,4,6-tetra-0-acetyl-β-D-glucopyranosid	47,51	47,44 47,93	4,0	3,99 4,03
Benzyl-3,4,6-tri-0-acetyl-2-benzamido-2-desoxy-β-D-glucopyranosid	25,85	23,58 25,28	3,0	2,74 2,93
Benzyl-3,4-di-0-acetyl-2-benzamido-6-0-benzoyl-2-desoxy-β-D-glucopyranosid	12,29	12,67 11,92	2,0	2,06 1,94
Benzyl-3-0-acetyl-2-benzamido-4,6-0-benzyliden-2-desoxy-β-D-glucopyranosid	6,90	6,23 7,30	1,0	0,90 1,06
3-Acetoxy-1-(4-hydroxy-3,5-dimethylphenyl)-1-propanon	18,22	19,09 18,82	1,0	1,05 1,03
3-Acetoxy-1-(4-acetoxy-3,5-dimethylphenyl)-1-propanon	30,93	30,78 31,28	2,0	1,99 2,02
(2*RS*,3*RS*)-2-(4-Acetoxy-3,5-dimethylphenyl)-3,6,8-trimethylchroman	12,72	13,31 12,32	1,0	1,05 0,97
5,6-Di-0-acetyl-2-0-benzyl-3-desoxy-L-threo-hex-2-enono-1,4-lacton	25,72	25,67	2,0	2,00
Benzyl-3,4-di-0-acetyl-2-benzylcarbamoyl-2-desoxy-α-D-glucopyranosiduronsäure-methylester	16,70	16,63 16,62	2,0	1,89 1,99
α-D-Glucose pentaacetate	55,13	55,28 54,98 55,38	5,0	5,01 4,98 5,02
Octaacetylcellobiose	50,70	51,31 51,01	8,0	8,09 8,04
Hexaacetylmannitol	59,45	59,30 59,41 59,43	6,0	5,98 5,99 5,99
Phenacetine	24,02	23,86 24,15	1,0	0,99 1,01
Acetanilide	31,58	31,95 31,72	1,0	1,01 1,00
1-Acetylimidazole	39,05	38,38 38,48	1,0	0,98 0,99

(*Continued*)

TABLE 1 (*Continued*)

Compound	CH_3CO %		Number of acetyl groups	
	Calc.	Found	Calc.	Found
3β-Acetoxy-5-pregnen-16-one-20	11,99	11,99 12,51	1,0	1,00 1,04
4-Acetoxy-5-acetyl-6-methyl-2-pyridon	20,57	21,19 20,93	1,0	1,03 1,02
4-Acetoxy-3-benzyl-1-(2′-pyridyl)-1,2-dihydro-2-chinolon	11,94	11,20	1,0	0,94
4-Acetoxy-3-äthyl-1-(2′-pyridyl)-1,2-dihydro-2-chinolon	13,96	14,22	1,0	1,02
6-Acetoxy-5-benzyl-4-hydroxy-2-phenyl-2,3 dihydro-1H-benzo de isochinolin-1,3-dion	9,84	8,88	1,0	0,90

vessel. Within 10–15 min methyl acetate and methanol distill over completely. Open the stopcock, remove the receiver, and add 1 ml of hydroxylamine hydrochloride and 1 ml lithium hydroxide. Close with a screw cap and set aside for 15 min. Wash with the iron solution into a 25-ml volumetric flask and add to volume. Read the absorbancy at 530 nm, correct for the blank, and calculate the acetyl content from a calibration graph obtained by analysis of pure pentaacetylglucose. The interference of formyl groups can be avoided by a slightly modified technique. The reaction mixture is acidified with 0.5 ml of concentrated hydrochloric acid after the reaction with hydroxylamine and kept at room temperature for 1 hr. Formhydroxamic acid is destroyed completely and after addition of 5 ml of LiOH solution, the procedure is as usual. A separate calibration graph is necessary as also a partial decomposition of acethydroxamic acid takes place.

RESULTS

Some results of test compounds are given in Table 1.

ACKNOWLEDGMENT

The authors are very indebted to Prof. Dr. Th. Kappe, Inst. for Organic Chemistry, University of Graz, and to Doz. Dr. H. Griengl, Inst. for Organic Chemistry, Technical University of Graz for numerous test compounds.

SUMMARY

A method for the microdetermination of acetyl groups is described which combines features of the known transesterification and the hydroxamate methods. All acetyl groups attached to oxygen or nitrogen are converted to methyl acetate which is distilled off and determined photometrically as the iron complex of the acethydroxamic acid. A slightly modified technique permits the selective determination of acetyl groups in the presence of formyl groups.

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MICROCHEMICAL JOURNAL **20,** 421–427 (1975)

Determination of Organic Substances by Oxidation with Permanganate

XIX. Analysis of Mixtures of Malonic and Oxalic Acids and of Malonic and Formic Acids[a]

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INTRODUCTION

Different courses of the reactions of malonic, oxalic, and formic acids (*4*) with permanganate in acidic and alkaline media were employed for the analysis of mixtures of malonic and oxalic acids and malonic and formic acids.

EXPERIMENTAL

Reagents

Potassium permanganate, 0.1 *N* (equiv. = mol/3).

Potassium bichromate, 0.1 *N*.

Ferrous sulfate, 0.1 *N* in 0.5 *N* sulfuric acid. The titer was determined daily by potentiometric titration of a standard potassium bichromate solution.

Malonic acid. A 1.25×10^{-2} *M* solution was prepared by dissolving an accurately weighed amount of the pure substance in distilled water and diluting to 1 l.

Oxalic acid. 1.25×10^{-2} *M*, 7.5×10^{-3} *M* and 5.0×10^{-3} *M* solutions were prepared by dissolving accurately weighed amounts of the pure substance in distilled water and diluting to 1 l.

Formic acid. 1.2×10^{-2} *M* and 6×10^{-3} *M* solutions were prepared and their titre was determined manganometrically (*6*).

Sodium pyrophosphate, solid and a saturated solution.

Manganese(II) sulfate, 0.5 *M*.

Sodium carbonate, 2 *M*.

Sulfuric acid, 8 *N*, 4*N*.

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[a] Part XVIII: The oxidation of malonic acid. *Microchem. J.*, **20,** 353–359 (1975).

PROCEDURES AND RESULTS

Mixtures of Malonic and Oxalic Acids

Malonic and oxalic acids can be determined in the presence of each other as follows:

A mixture of the two acids is oxidized by 4-hr action of excess permanganate in a sodium carbonate medium, under the conditions described in a previous paper (*4*); malonic acid is oxidized according to the equation

$$\mathrm{HOOC{-}CH_2{-}COOH} + 2\ \mathrm{MnO_4^-} \rightarrow \mathrm{(COO)_2^{2-}} + \mathrm{CO_2} + 2\ \mathrm{MnO(OH)_2} \quad (1)$$

Oxalic acid, both the amount originally present in the mixture and that formed by the oxidation of malonic acid, remains unchanged.

The amount of malonic acid is determined by determining the manganese dioxide formed according to Eq. (1). The manganese dioxide is filtered off from the reaction mixture, converted into the pyrophosphate complex of tervalent manganese and titrated with a standard ferrous sulfate solution (*1*). The ferrous sulfate consumption found in this way (value A) must be corrected for the amount of manganese dioxide formed by the decomposition of the permanganate in sodium carbonate under the conditions for the determination of malonic acid. This amount is determined from the blank (value B) and is subtracted from A; hence $C = A - B$, where C is the amount of ferrous sulfate corresponding to the amount of manganese dioxide actually formed during the oxidation of malonic acid and, consequently, to the amount of malonic acid.

Oxalic acid present in the mixture and formed by the oxidation of malonic acid is determined in the filtrate after separation of manganese dioxide (see the above paragraph) by oxidation with manganese dioxide in an acidic medium (*3*). The manganese dioxide is prepared by the reduction of the permanganate present in the filtrate with a manganese(II) salt. The oxidation of oxalic acid with manganese dioxide in acidic media proceeds according to the equation

$$\mathrm{(COOH)_2} + \mathrm{MnO(OH)_2} + 2\ \mathrm{H^+} \rightarrow 2\ \mathrm{CO_2} + 3\ \mathrm{H_2O} + \mathrm{Mn^{2+}} \quad (2)$$

Hence the loss of active oxygen from manganese dioxide corresponds to the overall amount of oxalic acid.

The amount of active oxygen in manganese dioxide at disposal for the oxidation of oxalic acid is determined as follows. The permanganate is heated for 4 hr in an alkaline medium in the absence of an oxidizable substance. The active oxygen corresponding to the permanganate is determined in the filtrate after separation of manganese dioxide formed by the decomposition of the reagent by performing the blank determination;

the B value is also found in the same way, i.e., the permanganate is converted into the pyrophosphate complex of tervalent manganese and titrated with a ferrous sulfate standard solution (consumption D). The amount of active oxygen corresponding to the permanganate consumed for the oxidation in the alkaline medium (E) is subtracted from this value. This is calculated from the known amount of manganese dioxide formed by the reduction of the reagent by the substance to be determined, i.e., from the value already known (C). As 1 mole of manganese dioxide is formed from 1 mol of permanganate during reduction of permanganate in alkaline media and since 5 and 2 equivalents of reductant are required for the reduction of 1 mol of permanganate and 1 mol of manganese dioxide, respectively, it follows that $E = (5/2)C$. Further, $F = D - E$, where F is the amount of active oxygen corresponding to the manganese dioxide available for the oxidation of oxalic acid.

The amount of active oxygen, corresponding to unreacted manganese dioxide after the oxidation of oxalic acid is again determined by titration with a ferrous sulfate standard solution after converting the active substance into the pyrophosphate complex of tervalent manganese (consumption G). Then $H = F - G$, where H is the amount of ferrous sulfate standard solution corresponding to the amount of manganese dioxide consumed for the oxidation and thus also to the overall content of oxalic acid.

If the amount of oxalic acid formed by the oxidation of malonic acid is subtracted from the overall amount of oxalic acid, the amount of the acid originally present in the mixture is found. The amount of oxalic acid formed by the oxidation of malonic acid is calculated directly in milligrams, if the determined amount of malonic acid in milligrams is multiplied by a factor of 0.8653, that is, by the ratio of the molecular weights of oxalic and malonic acids.

It is thus evident that values A, B, D, and G are determined by titration and the rest are calculated. All of values A–H are given in terms of milliliters of a 0.1 N ferrous sulfate standard solution.

It follows from the stoichiometry of the malonic acid reaction with permanganate in sodium carbonate that 1 ml of 0.1 N ferrous sulfate corresponds to 2.6015 mg malonic acid; the amount of malonic acid in mg can thus be calculated from the relationship

$$X = 2.6015 \cdot C$$

From the stoichiometry of the reaction of oxalic acid with manganese dioxide it follows that 1 ml of 0.1 N ferrous sulfate corresponds to 4.5020 mg oxalic acid. The overall amount of oxalic acid in mg is thus given by

$$Y = 4.5020 \cdot H$$

The amount of oxalic acid originally present in the mixture, in milligrams, is given by the difference between the amount of oxalic acid found, in milligrams, and the amount of oxalic acid formed by the oxidation of malonic acid in an alkaline medium (the X value calculated above is multiplied by a factor of 0.8653). This can be expressed by the relationship

$$Z = Y - 0.8653 \cdot X$$

or

$$Z = 4.5020 \cdot H - 2.2511 \cdot C$$

The analysis of a mixture of malonic and oxalic acids and the evaluation of the results are very simple. A single standard solution, 0.1 N ferrous sulfate, is employed. Both acids are determined in a single sample solution, and the values determined from the blank measurement are also obtained from a single initial solution.

The procedure for the determination of mixtures of malonic and oxalic acids was verified as follows:

To 20 ml 2 M sodium carbonate, 10.00 ml 0.1 N potassium permanganate was added, followed by 5.00 ml 1.25×10^{-2} M malonic acid and 5.00 ml 1.25×10^{-2} M, or 7.5×10^{-3} M or 5.0×10^{-3} M oxalic acid. The mixture was stirred thoroughly and refluxed for 4 hr on a boiling water-bath. Then the flask was cooled to laboratory temperature under running water, the manganese dioxide formed was filtered off on an S 4 frit, washed thoroughly with 10 × 10 ml distilled water and dissolved on the frit in a fresh mixture of saturated sodium pyrophosphate, 4 N sulfuric acid and 0.5 M manganese(II) sulfate (6 : 1 : 2). The pyrophosphate complex of tervalent manganese formed was determined reductometrically by titration with a 0.1 N ferrous sulfate standard solution using diphenylamine indicator (*1*) (consumption A). The filtrate after separation of manganese dioxide was neutralized by 10 ml 8 N sulfuric acid, 5 ml 0.5 M manganese(II) sulfate were added and, after the formation of manganese dioxide, 8 N sulfuric acid was added to give a resultant concentration of 2 N. Oxalic acid was then oxidized with manganese dioxide for 30 min at laboratory temperature, stirring vigorously with a magnetic stirrer. After 30 min, the unreacted manganese dioxide was converted into the pyrophosphate complex of manganese(III) by adding 4.5 g of solid sodium pyrophosphate and was titrated immediately with a 0.1 N ferrous sulfate standard solution using diphenylamine indicator (consumption G). (In order to determine the time required for the oxidation of oxalic acid with manganese dioxide in an acidic medium, the dependence of the degree of oxidation on time was measured. Thirty minutes suffice for quantitative oxidation of 10.00 ml of

TABLE 1
The Analysis of Mixtures of Malonic and Oxalic Acids

Malonic acid (mg)			Oxalic acid (mg)		
Taken	Found	SD	Taken	Found	SD
6.504	6.377	0.087	5.628	5.498	0.011
6.504	6.413	0.065	3.374	3.280	0.160
6.504	6.433	0.033	2.251	2.189	0.056

1.25×10^{-2} *M* oxalic acid under the given conditions; this time must be doubled for the two lower concentrations.)

Blank determination was performed in parallel with the determination itself. After 4 hr heating, the manganese dioxide formed by the reagent decomposition was determined in the blank solution employing the above procedure (consumption *B*) and permanganate in the filtrate was reduced by a manganese(II) salt to manganese dioxide that was titrated as the pyrophosphate complex of manganese(III) with a 0.1 *N* ferrous sulfate standard solution using diphenylamine indicator (consumption *D*).

The results of the analysis of mixtures of malonic and oxalic acids are given in Table 1. The standard deviations given were calculated from the results of seven measurements.

Mixtures of Malonic and Formic Acids

The fact that malonic acid is oxidized to oxalic acid by excess permanganate in an alkaline medium (*4*) according to equation (1), while formic acid is oxidized as far as carbon dioxide and water (*2,5,7*), according to the equation

$$3\ HCOOH + 2\ MnO_4^- + 2\ H^+ \rightarrow 3\ CO_2 + 2\ MnO(OH)_2 + 2\ H_2O \quad (3)$$

was utilized for the determination. Oxalic acid, formed by the oxidation of malonic acid, can be quantitatively oxidized in an acidic medium (Eq. (2)).

The amount of manganese dioxide formed according to equations (1) and (3), proportional to the amount of the two acids to be determined, and the amount of manganese dioxide consumed in the oxidation of oxalic acid according to equation (2), which is proportional to the original amount of malonic acid alone, are evaluated.

The procedure for the analysis of mixtures of malonic and formic acids is the same as the above procedure. The blank values (*B,D*) have the same significance as above, while the other measured quantities (*A,G*) have a different significance, although the procedure for their determination is the same as in the analysis of mixtures of malonic and oxalic acids. The calculated values (C,E,F,H) are obtained from relationships formally identical with those given above. The significance of the individual quantities:

A corresponds to the amount of manganese dioxide formed both by the reduction of permanganate with formic and malonic acids in a sodium carbonate medium and by the reagent decomposition under these conditions.

B corresponds to the amount of manganese dioxide formed solely by the reagent decomposition in sodium carbonate during the blank determination.

$C = A - B$ corresponds to the amount of manganese dioxide formed solely by the reduction of permanganate by formic and malonic acids in sodium carbonate.

D corresponds to the amount of potassium permanganate present in the blank solution after 4 hr heating in sodium carbonate.

$E = (5/2)C$ corresponds to the amount of permanganate consumed in the oxidation of the two acids in an alkaline medium.

$F = D - E$ corresponds to the amount of manganese dioxide available for the oxidation of oxalic acid.

G corresponds to the amount of unreacted manganese dioxide after the oxidation of oxalic acid.

$H = F - G$ corresponds to the amount of manganese dioxide consumed in the oxidation of oxalic acid.

$2\ H$ corresponds to the amount of manganese dioxide formed by the reduction of permanganate in sodium carbonate by malonic acid alone (it follows from comparison of Eq. (1) and (2) that the amount of manganese dioxide formed by the reduction of permanganate by malonic acid in sodium carbonate is twice that consumed for the oxidation of oxalic acid).

As 1 ml 0.1 N ferrous sulfate corresponds to 5.2030 mg malonic acid or 3.45225 mg formic acid, the amount of malonic acid in mg can be calculated from the relationship

$$X = 5.2030\ H$$

and that of formic acid from

$$Y = (C - 2\ H)\cdot 3.45225$$

The procedure for the determination of mixtures of malonic and formic acids was verified analogously as for mixtures of malonic and oxalic acids, with the difference that 5.00 ml $1.25 \times 10^{-2}\ M$ malonic acid and 5.00 ml $1.2 \times 10^{-2}\ M$ or $0.6 \times 10^{-2}\ M$ formic acid were employed. The results of the analyses of mixtures of malonic and formic acids are given in Table 2.

TABLE 2
THE ANALYSIS OF MIXTURES OF MALONIC AND FORMIC ACIDS

Malonic acid (mg)			Formic acid (mg)		
Taken	Found	SD	Taken	Found	SD
6.504	6.270	0.088	2.785	2.797	0.119
6.504	6.348	0.096	1.393	1.398	0.045

DISCUSSION

This paper studies the possibility of analysis of mixtures of various organic acids on the basis of redox reactions, provided that these reactions are well-defined and lead to different reaction products with the possibility of quantitatively detecting the individual reaction stages. As can be seen from the examples given, in some cases the reactions can be favorably influenced by simply varying the acidity of the reaction medium. This is advantageous when the oxidation of some of the substances studied is stepwise, and the individual stages can be monitored quantitatively, e.g., with the oxidation of malonic acid with permanganate. It follows from the results obtained that by a suitable choice of reaction conditions even the action of oxidants as strong as permanganate can be made selective to a certain degree.

SUMMARY

A method for the analysis of mixtures of malonic and oxalic and malonic and formic acids has been developed. The method is based on stepwise oxidation with excess permanganate in alkaline [Eqs. (1) and (3)] and acid [Eq. (2)] media.

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Spectrophotometric Study of Influences on the Direct Ferric Perchlorate Method for the Determination of Serum Cholesterol

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INTRODUCTION

Manual determinations for serum cholesterol can be lengthy and difficult procedures if it is necessary to use tedious preliminaries such as saponification, extraction, and digitonide or tomatide precipitation before color development can be performed; or they may be simple procedures in which serum is added to but one reagent in a relatively uncomplicated methodology. In the routine circumstance, the lengthy procedures may prove too involved, which can lead to serious errors, while the simplest procedures may result in values which have been subjected to chemical interference by either naturally occurring serum constituents (*2,4,13*) *in vitro* additions to the sample (*4,9*) browning reactions (*8,15*) or *in vivo* additions from drugs given to the patient (*3,5*). A simple and direct modification of a method for the quantitative determination of total cholesterol in serum was described (*12*) in which the sample was mixed with a stable reagent that consisted of ferric perchlorate dissolved in a mixture of sulfuric acid and ethyl acetate. This mixture was substituted for a previously described acetic-sulfuric acid medium (*13*) in an effort to avoid a Hopkins-Cole reaction (*2*) between a glyoxal impurity in glacial acetic acid and the tryptophane present in whole serum. Should such a side-reaction occur in which a peak maximum in spectrum could be obtained for tryptophane and in which its spectrum superimposed the measurement wavelength of cholesterol, then the requirement for acetic acid purity might be too stringent to maintain and the price too expensive to bear when routine large volume determinations are needed. The procedural simplicity of the ferric perchlorate method was appealing, and, therefore, a spectrophotometric investigation of this modification of an iron reaction for cholesterol was carried out. Several compounds of both endogenous and exogenous origin known to interfere in other procedures involving iron reactions with cholesterol were studied along with the heating conditions described in the procedure. The heating step was looked at because heating the reaction mixture was apparently required to shorten the reaction time of the system, and improve its specificity. Lengthy reaction times (30 min at room temperature) gave rise to nonspecific color formation. The interferences were looked at because the test modification might respond differently than the original procedure (*14*) to the presence of those potential interferences, for such a difference in reaction had previously been shown for bromide (*2*). Clear, lipemic, jaundiced, and hemolyzed serums were subjected to direct reaction and compared to the same reaction carried out on purified residues of the samples that were obtained by saponification of the cholesterol esters followed by extraction of the resultant free cholesterol. The latter system of isolation of cholesterol in a purified state is accepted as the reference technique when used in conjunction with the Liebermann-Burchard reaction (*1*). By means of difference spectrophotometry using a double beam system, the background of irrelevant absorption of the directly reacted sample was obtained by simultaneous scanning against the reaction products of the residues which served as the blanks for the directly treated samples. All of the possible distinct interferences were therefore allowed to react in their native environment according to the described conditions of the procedure (*12*). This seemed like the simplest way to determine irrelevant absorption at all wavelengths rather than to look for all of the potential interferences individually. Only those interferences occurring at the measurement wavelength are of any analytical significance anyway.

MATERIALS AND METHODS

Reagents

Cholesterol standard solution (2000 mg/l). Dissolve 200 mg of cholesterol in 100 ml of glacial acetic acid.

Ferric perchlorate color reagent. Dissolve 520 mg of ferric perchlorate (nonyellow, containing excess $HClO_4$) in 600 ml of purified ethyl acetate contained in a 2-l Erlenmeyer flask. Cool to 4°, add 400 ml of cold concentrated sulfuric acid in small portions, mixing after each portion is added. Do not allow the temperature to exceed 45°C. This reagent so prepared is reported to be stable for at least 1 yr when stored in an amber bottle at 25°C or 2 yr when refrigerated (*12*).

Propylthiouracil and thiouracil stock solutions. Prepare 1000 mg/l aqueous solutions of each.

Sodium bromide stock solution. Prepare this solution to contain 100 mEq/1 of Br^- in distilled water.

Bilirubin stock solution. Dissolve 20 mg bilirubin in chloroform, dilute to 1 l and refrigerate until used.

Alcoholic potassium hydroxide solution. Prepare this reagent by adding 6 ml of 33% aqueous KOH to 94 ml of absolute ethanol immediately before use.

Petroleum ether. Reagent Grade B.P. 63°–75°C.

Apparatus

Heating blocks. For all studies except where otherwise specified, a Dow heating block, calibrated at 100°C, was used as the heat source (heating block B). For comparison purposes, the time and temperature studies also utilized a Technilab Co. heating block (heating block C), and a precision water bath.

Methods

The originally described procedure for serum (*12*) was followed implicitly. Fifty microliters of serum was added to 5.0 ml of the reagent solution and reacted for 90 sec in a heating block or water bath. The mixture was contained in a tightly closed screw capped tube of 1.25 × 10 cm. If Parafilm was used to cap the tubes, unusual spectra resulted. Afterwards, the mixture was cooled in a water bath at 17°C for 5 min before scanning the resultant spectra of the mixtures in a double beam recording spectrophotometer.

In order to study the effect of bilirubin, aliquots of the bilirubin stock solutions were added and evaporated to dryness. Fifty microliters of glacial acetic acid or a cholesterol standard solution were added to the residue to dissolve it, followed by the addition of 5.0 ml of the color reagent. The well-mixed solution was heated, cooled, and the spectrum scanned as described. Uracil solutions were added as 50 μl aqueous aliquots of several concentrations made up by diluting the stock standards with distilled H_2O.

Pure extracts of serum were obtained by saponification and subsequent extraction with petroleum ether, as described (*1*). A 6.0 ml portion of the extract representing 0.3 ml of serum was evaporated and the residue dissolved in glacial acetic acid (0.3 ml). Fifty microliters of the resultant solution was then subjected to the color development step.

RESULTS AND DISCUSSION

The spectrum generated by reaction of a pure standard solution with the ferric perchlorate reagent exhibits a maximum at 563 nm and a shoulder at 475 nm, which is characteristic of the described iron reaction with cholesterol (*11*). The molar absorptivity at 563 nm is of the same magnitude as that exhibited by the ferric chloride reaction, and repeated

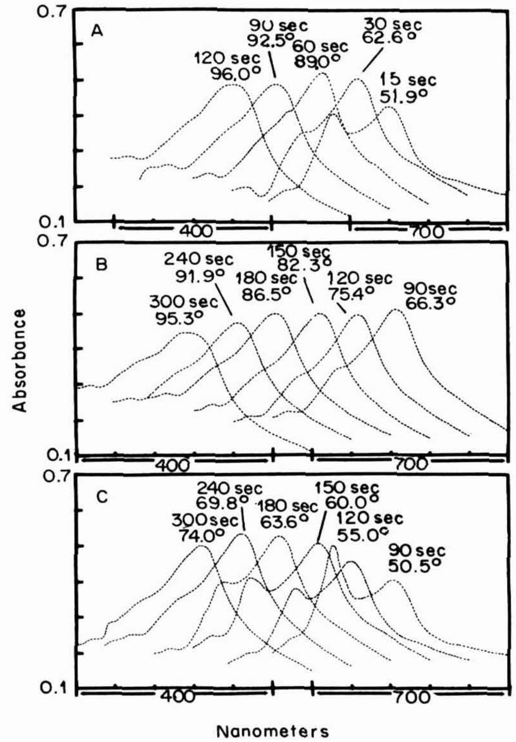

FIG. 1. The effect of time and temperature on the resultant and varied spectra of a cholesterol standard solution (2000 mg/l) are shown. In block A are the spectral results obtained when using a precision water bath as the heat adjusted to 100°C. In *B* and *C* are the varied spectra obtained on using two heating blocks adjusted to 100°C (*B* is a Dow heating block, *C* is a Technilab heating block).

analysis confirms that the reaction Beer's Law to a concentration of at least 4000 mg/l.

Wybenga (*12*) indicated that the reaction temperature was critical in the color development phase. Therefore, this phenomenon was studied by considering how one might heat a sample when on reading stated directions, it leads one to infer that several heating choices may be acceptable. For example, a heating block of one kind or another or a boiling water bath might appear to be interchangeable alternatives. When this assumption of interchangeability was examined, it was found that heating a sample under that appeared to be similar conditions of time and temperature did not produce similar spectral graphings. The varied results obtained from such apparent dissimilar sample treatments are described by Fig. 1. The effects of time and temperature on the spectrum of a 2000 mg/l standard solution are demonstrated. Each spectrum, manually displaced laterally for visual clarity, was graphed for several time intervals at which the temperature was simultaneously recorded. Figure 1A contains the results obtained when using a boiling water bath, and illustrates that a heating time of 60 sec produces the characteristic peak absorbance (*11*) at 563 nm at a final reaction temperature of 89°C. On continuing the reaction, the temperature rises to 96°C at 2 min, but the absorbance decreases below what was achieved at the more optimum time and temperature. Figures 1B and 1C show a similar spectral study on the course of the reaction when using two different heating blocks both calibrated at 100°C. In each case maximum color was developed when the final reaction temperature was greater than 65°C. However, the time required to achieve this maximum was different and longer for the block used in Fig. 1C than the one used in Fig. 1B. At the lower temperatures encountered with the heating block of Fig. 1C, a sequence of spectral structures became evident when the reaction was carried out more slowly as is shown here. At lower temperatures and shorter reaction times, a discrete peak at 475 nm was present, while at higher temperatures and longer reaction times the peak at 560 nm decreased in absorbance value. These curves were obtained by using several aliquots of the same standard, scanning each after heating for the indicated time period, and then cooling as described under procedure. The explanation of the different results obtained with the two heating blocks appears to be reasonably simple. The test tubes fit snugly into heating block B, and the spaces were deep enough so that all of the liquid reacting was surrounded, which was not the case with heating block C. In the latter case, the holes in the block were more shallow and the fit of the tube not as tight (although block C was advertised as proper for the size of tubes employed in this study). The interferences to be drawn from the data obtained is that the reaction achieves maximum color when the mixture is heated between 66°C and 89°C as previously described (*9*). However, an indiscriminate choice of heating systems can result in spectral differences under what appear to be fixed and similar reaction conditions. At higher temperatures, such as those achieved when sample heating takes place in a boiling water bath for 90 sec, the reaction is also linear to 4000 mg/l, but the absorbances decrease. The results reported here show that the reaction conditions can be varied to suit the facilities available, but apparently similar though actually dissimilar reaction conditions could make inter laboratory comparisons difficult.

Since the reaction proceeded more slowly in heating block C, it facilitated following the course of the reaction spectrophotometrically. Figure 1C illustrates the progression of this reaction which appears to be taking place in several steps. A band corresponding to one structural form appears at 475 nm and is then converted into another structure absorbing at 563 nm. At the same time, there is also some evidence of the development of spectral character at 423 nm. If one includes some of the other spectra of Figs. 1A and 1B which are different than those of Fig. 1C, such as the 15-sec spectrum of Fig. 1A, it becomes rather simple to visualize the progression of the reaction from a spectrophotometric point of view. These observations are in agreement with the spectrophotometric studies on the ferric chloride reaction as recently described (*11*) by the Bureau of Standards which, on visual comparison, results in the inference that the ferric perchlorate reaction has a similar, probably identical chemistry, and that the same molecular species are generated.

When bilirubin is dissolved into an acid oxidizing medium it is rapidly converted to biliverdin. This results in a positive absolute interference when direct serum reactions for cholesterol are used in which overlapping spectra for cholesterol and bilirubin are generated. Biliverdin exhibits a broad absorption maximum peaking at 675 nm in the described medium, but it has minimum absorption at 563 nm where cholesterol measurements by iron reactions are made.

Since there appears to be some overlap between the cholesterol and biliverdin when both are generated in the same solution by the iron perchlorate reaction, it seemed important to study the potential quantity of interference by bilirubin in this direct serum reaction. The individual and mixed results obtained with bilirubin and cholesterol in the iron reaction are shown in Fig. 2A. Curve (C + B) was generated from the reaction of a mixture containing a cholesterol concentration of 2000 mg/l and a bilirubin content of 800 mg/l. When the mixture was scanned against a cholesterol standard of 2000 mg/l as a blank, curve (C + B) − C, the biliverdin spectrum resulted. When the same mixture was reacted and scanned against a reacted bilirubin blank of 800 mg/l, curve (C + B) − B, the cholesterol spectrum was regenerated. It can be determined from these observations that the two compounds are reacting independently and that the effect of each on the other is truly additive. Varying concentrations of bilirubin were added to a constant concentration of 2000 mg/l of cholesterol. When the concentration of bilirubin reached 300 mg/l, the cholesterol concentration was only moderately elevated by a factor of 12.6%. From the results of these studies, it is obvious that the positive interference caused by high bilirubin concentrations is relatively minimal for the ferric perchlorate system for cholesterol, and since the interference is linear with concentration, it is easily correctable. In addition, in looking at the spectra obtained with cholesterol and bilirubin, it would undoubtedly be a relatively simple process to use simultaneous equations to compensate for the irrelevant absorbance caused by the formation of biliverdin in the reaction process. However, in most cases such a small correction could

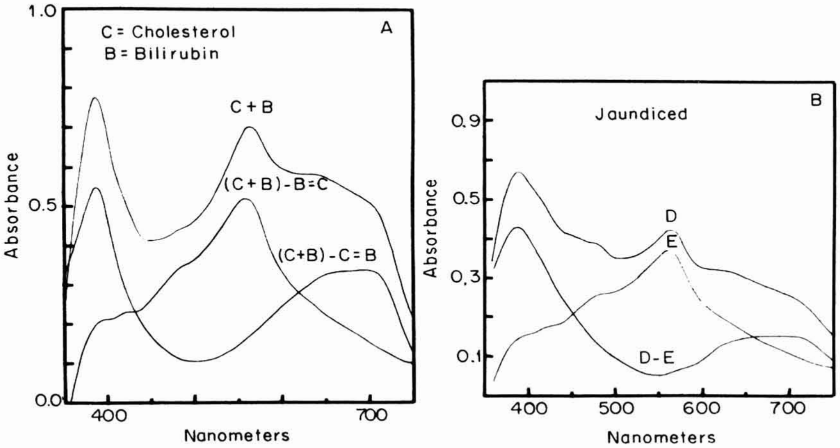

FIG. 2. In Fig. 2A, the additive effect of bilirubin on the $Fe(ClO_4)_3$ reaction with cholesterol is shown as $(C + B)$. When the mixture was scanned using reacted bilirubin as a blank, the residual spectrum for cholesterol of $(C + B) - B = C$ resulted. When the mixture was scanned using reacted cholesterol as a blank, the residual spectrum for bilirubin $(C + B) - C = B$ resulted. Figure 2B shows the direct reaction spectra obtained with a jaundiced serum, containing 358 mg/l of bilirubin (curve *D*), with an Abell extract of that serum (curve E) and a background of residual absorbance (curve *D–E*).

be ignored for all practical purposes except when bilirubin concentration was high, for example, over 10 mg per dl.

A comparison of the spectra obtained from an Abell extract and a direct reaction with severely jaundiced serum having a bilirubin value of 358 mg/l is shown in Fig. 2B. Curve D is the spectrum of the mixture containing all irrelevant absorption while curve E is the spectrum obtained with the purified residue. At this elevated value, the direct cholesterol concentration is only 13% higher than the extract, which agrees with the results obtained with the *in vitro* addition of bilirubin to a standard (Fig. 2A). Interestingly, when spectrum E is subtracted from spectrum D (Fig. 2A-B), the difference curve closely approximates a biliverdin spectrum.

Thiouracil is a compound which has been reported as an interference in iron reactions for cholesterol (*7,9*). Therefore, thiouracil and propylthiouracil, a therapeutic analogue, were both investigated for their effect in the ferric perchlorate reaction. Figure 3 shows the spectra obtained by introducing thiouracil or propylthiouracil into the reaction. Scan A for each is the spectrum of reaction for a cholesterol standard solution, while scan B is the spectrum obtained by the addition of 5.0 mg/l thiouracil or propylthiouracil, respectively, to the same standard cholesterol solution. There seems to be little effect with this concentration of contaminant, but on repeated analysis, this amount of either uracil de-

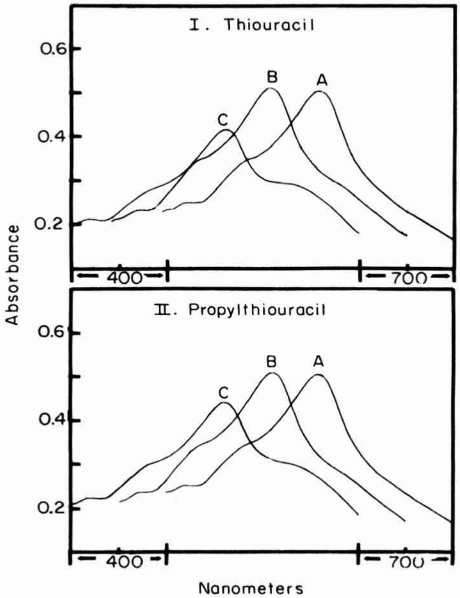

FIG. 3. Each curve *A* shows the reaction spectrum for cholesterol, while the spectra obtained with the identical cholesterol concentration contaminated with 5.0 mg/l and 50 mg/l of thiouracil and propylthiouracil are shown as curves *B* and curves *C*, respectively.

rivative had a maximum effect statistically of supressing the color development by approximately 2%. At higher concentrations such as 50 mg/l as shown in scan C, both uracils diminish the absorbance by approximately 12%, and, interestingly, a new shoulder becomes evident at 620 nm indicating that the drug somehow is involved in the reaction.

Bromide ion is also a reported interference in iron reactions for cholesterol (*5,6,10*). Previous investigators who studied this phenomenon (*6*) found that bromide interference could be eliminated by modifying the reaction medium to a mixture of ethanol, phosphoric acid, and sulfuric acid. Since the present reagent contains ethyl acetate, rather than acetic acid, the potential effect of bromide ions as an interference was of particular interest. When varying concentrations of bromide were introduced into the reaction medium as contaminants, the amount of color was continually increased to a maximum of 150% of the expected absorbance with the addition of 15 mEq/l of bromide where the hyperchromic effect appeared to plateau. The spectrum of a cholesterol standard (2000 mg/l) containing bromide ions (20 mEq/L) is shown in Fig. 4, curve B. Curve A is the spectrum of the reacted standard alone, while curve C was generated by reaction with a solution containing 20 mEq/l of bromide. Bromide has a spectrum of its own, but it does not form a color in this

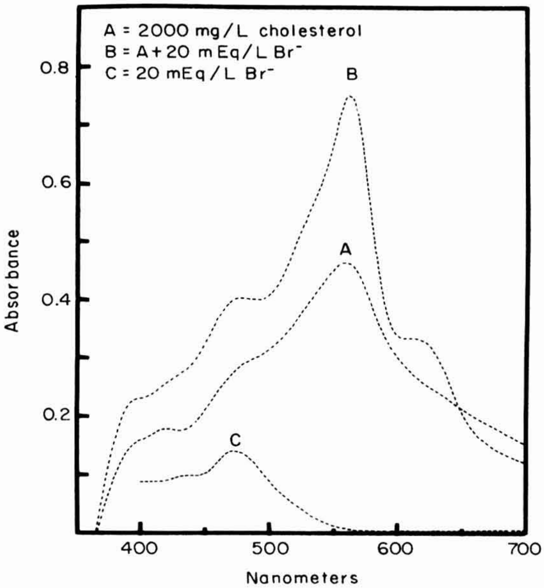

FIG. 4. The effect of bromide on the $Fe(ClO_4)_3$ reaction of cholesterol is shown here. Spectrum A is the reaction obtained with pure cholesterol, spectrum C is the reaction obtained with bromide while spectrum B shows the hyperchromic effect of the reaction of the same amount of cholesterol when it was contaminated with the bromide.

system which absorbs at 563 nm. If one adds curves A plus C, it does not result in curve B, which indicates that bromide somehow causes a hyperchromic effect on the spectrum obtained with cholesterol, so that in the presence of bromide there are some differences in the chemistry taking place. Since plateau formation can be achieved for the reaction, the addition of bromide at a plateau concentration might be of value to increase sensitivity and to eliminate the interference with the determination when bromide is ingested. It is interesting to note that, like the uracils, fine structure changes occur in the spectral region of 600–700 nm indicating that the reaction itself is affected.

Serum samples were saponified with alcoholic KOH and then extracted with petroleum ether as described in the Abell procedure (*1*). The extracts were then evaporated to dryness, and the residues were dissolved with glacial acetic acid and reacted with the iron perchlorate reagent. This afforded a way to compare the direct serum reaction with the purified extract as a measure of background absorption. However, if the reagent was added directly to the residue, low results were obtained due to incomplete solution by the iron perchlorate reagent. Therefore glacial acetic acid (50 μl per ml of petroleum ether extract evaporated) was required to solublize the residue. Better precision was also obtained if a large volume of petroleum ether extract, larger than is needed for the

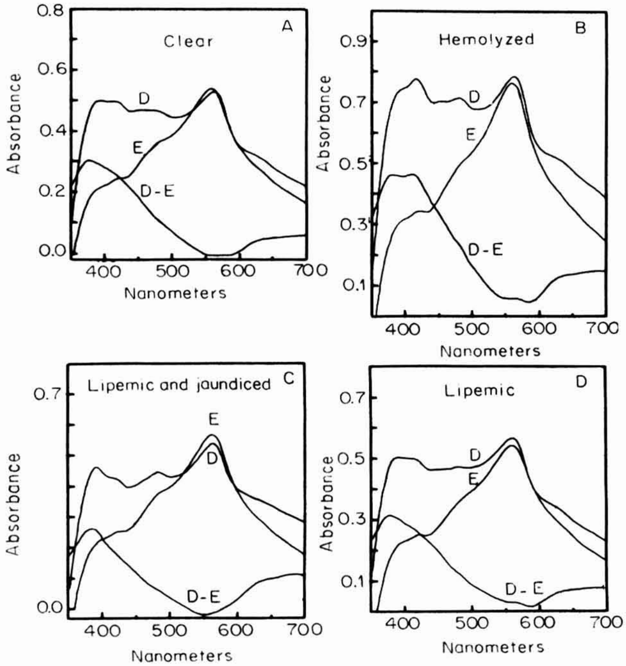

FIG. 5. Spectra designated as *D* are from direct $Fe(ClO_4)_3$ reactions in various serum types, spectra designated as *E* are from reactions on the residues of purified extracts of those serums while spectra designated as (*D–E*) are the difference curves representing the irrelevant absorptions of the individual direct reactions.

determination, was evaporated, dissolved in acetic acid, and appropriate aliquots subjected to color development as described under procedure.

Figure 5 shows the results obtained using representative samples selected from a large number of treated specimens of clear, hemolyzed, lipemic, and lipemic-jaundiced serums. In each quadrant, curve D is the spectrum of the direct reaction, curve E is that of the extract and D-E is the difference between the two and represents the irrelevant absorption due to other reactions, including perhaps some browning reactions. The peak absorbances of the clear serum superimpose and are near identical. In the same manner, Figs. 5B–D illustrate the results obtained with the other kinds of serums that often pose problems in direct determinations. Figure 5B represents a hemolyzed serum where the hemoglobin value was 1340 mg/l and shows little difference at peak maximum. Figure 5C is a serum that was both lipemic and jaundiced (120 mg/l bilirubin), but the peak obtained with the Abell extract is slightly higher than that of the direct reaction, an error that is sometimes encountered when handling (or mishandling) low boiling petroleum ethers. This example was included in order to show that this is an easily encountered phenomenon when handling volatile solvents. A severely lipemic serum is shown in Fig. 5D where a difference of 4.0% at peak maximum is evident in the direct minus extract scan.

In this investigation, the primary concern was with the chemical perturbations imposed under the varied circumstances studied here. As a result of the data generated during this experience, it can be concluded that the one-step method of determining cholesterol with the described one-piece reagent (12) is rapid, simple, and reasonably accurate.

SUMMARY

A spectrophotometric study of several influences on a direct colorimetric determination of serum cholesterol has been described. Interferences of *in vitro* and *in vivo* types were considered, and it was found that certain compounds such as bromide, uracils, and bilirubin could exert both positive and negative interfering influences on the reaction of ferric perchlorate with cholesterol in an ethyl acetate-ethanol-sulfuric acid medium. Some interferences such as bilirubin are noncompeting side reactions which are absolute in their interference capabilities, while others such as the uracils and bromide have an entirely different influence. In the latter circumstance, the fine structures of spectra are altered by a nonadditive phenomenon of the reactant and hyperchromic (bromide), and hypochromic (uracils) effects take place which appear to result in relative rather than absolute errors.

ACKNOWLEDGMENT

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MICROCHEMICAL JOURNAL 20, 440–448 (1975)

Microdetermination of Fluorine in Organic Compounds by Oxygen Flask Combustion and Conductometric Titration

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INTRODUCTION

Although many methods have been known for the determination of fluoride, direct colorimetry by the Alizarin Complexone method by Belcher and others (*2*), is commonly used because of its high sensitivity. On the other side, with the development of automatic titration apparatus, conductometric titration (*1,7,9*), (*14*) and potentiometric titration (*3,19*) have been also used increasingly. Recently potentiometric titration with a fluoride-selective electrode has been reported (*6,12*). We have been interested in these electric titration methods and have attempted the microdetermination of fluorine in organic compounds by the combination of flask combustion and conductometric titration. Titrants hitherto used in this conductometric titration involved solutions of aluminum chloride (*9*), lanthanum acetate (*9,14*), and thorium nitrate (*1,7*).

It has been recalled that the classic method for the determination of fluoride was gravimetry (*11*) involving the precipitation of calcium fluoride, which was also used inversely for the determination of calcium ion (*15*). Therefore we attempted the titration of fluoride using a solution of calcium salt as an easy-to-handle reagent. As its counter ion, acetate was selected because of its counterbalanced ionic mobility, and the titration conditions were examined in view of the concentration of organic solvent, acidity, and effect of coexisting foreign ions in the sample solution. Microdetermination of fluorine in organic compounds was then attempted by the use of this titration method in combination with the oxygen flask combustion, and a satisfactory result has been obtained.

MATERIALS AND METHODS

Apparatus

Automatic titration apparatus. A Metrohm potentiograph E-336 was equipped with the Conductoscope E-165 and Conducto-Cell TEA-608.

Cation exchange resin column. A chromatographic tube (1 × 10 cm), provided with a stopcock at the lower end, was filled with Amberlite IR-120 to a depth of 4 cm.

Combustion flask (17). A commercial egg-shaped flask of 100-ml capacity (Pyrex and quartz) with a ground glass stopper suspending a platinum mesh holder was used.

Filter paper pieces (17): Toyo Roshi No. 6, a round filter paper of 7 cm diameter was cut into 12 equal pieces.

Reagents

0.01 M *sodium fluoride solution.* 41.99 mg of reagent grade sodium fluoride was accurately weighed and dissolved in 100 ml of water.

0.005 M *calcium acetate solution* (50% (*v*/*v*) *isopropanol solution*). About 0.44 g of calcium acetate monohydrate dissolved in 250 ml of water was diluted to 500 ml with isopropanol and standardized with the sodium fluoride solution.

p-*fluorobenzoic acid and fluoroacetamide.* These were the standard samples for elemental analysis (Kishida Chemicals Ltd., Osaka, Japan).

Methylene blue and methyl red reagents. Ethanolic 1% solution.

Others. Acetone, and 0.1 *M* and 0.01 *M* ammonia water.

Procedure

A few milligrams of the sample containing more than 190 μg of fluorine was weighed on the filter paper piece and burned in the quartz combustion flask, in the conventional manner, with 5 ml of water as the absorption liquid. After combustion, the absorption liquid was transferred into a 50-ml beaker, and the flask was washed out with 11 ml of water and then with 24 ml of acetone, combining these washings with the absorption liquid. Two drops each of methyl red and methylene blue reagents were added to the beaker as indicators and the solution was adjusted to pH 6–7, coarsely with 0.1 *M* and finely with 0.01 *M* ammonia water (the solution becomes bluish violet). This solution is then set in the automatic conductometric titration apparatus and titrated with 0.005 *M* calcium acetate.

RESULTS AND DISCUSSION

Concentration of Organic Solvent in Sample Solution

A sample solution containing 3 ml of 0.01 *M* sodium fluoride and 37 ml of water was titrated with 0.005 *M* calcium acetate and a very indiscernible inflection in the titration curve was obtained. In such case, addition of an organic solvent is often tried as a means of improving the sharpness of the inflection, and we have already experienced good results with the use of sample solution and titrant made up to 40%–50%

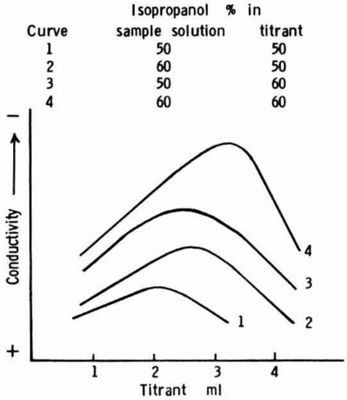

FIG. 1. Effect of concentration of isopropanol on conductometric titration of fluoride.

isopropanol solution for conductometric titration of sulfate ion (*18*). Therefore, we have repeated this procedure by the addition of isopropanol to the titrant and sample solution of 3 ml of 0.01 *M* sodium fluoride solution, and the effect of concentration of the solvent on the titration curve was examined. Isopropanol concentration of 50% in the titrant and that of 50% and 60% in the sample solution still gave dull inflections in the titration curves and the central points of inflections appeared earlier than the equivalence point of 3 ml as illustrated in Fig. 1. The shape of the inflection was somewhat improved when the isopropanol concentration of the titrant and the sample solution were raised to 60% although the inflection point did not coincide with the equivalence point. Further increase in isopropanol concentration was not tried because it would cause low electric conductivity. Experiments were therefore made with combination of solvent systems for titrant–sample solution, i.e., acetone–acetone, acetone–isopropanol, and isopropanol–acetone, and the titration were carried out with various concentrations of these systems. As a result, a favorable titration curve was obtained with 50% isopropanol in the titrant and 60% acetone in the sample solution, and the inflection coincided well with the equivalence point (Fig. 2). It was revealed that the inflection points were not very discernible at below 50% acetone concentration, and the points appeared at lower volumes of the titrant than that of the equivalence. Higher concentrations of 70% and 80% reproduced the inflection points at the equivalence point, but the sharpness was markedly reduced.

Addition of ethanol in the conductometric titration of fluoride with lanthanum acetate (*9,14*) was also reported, but the present method resulted in no advantage using this solvent.

It is generally believed that, in the titration of fluoride, slightly soluble or slightly dissociable complex will be formed by the reaction of the

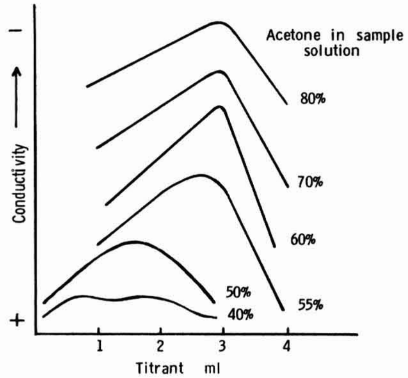

FIG. 2. Conductometric titration of fluoride with titrant containing 50% isopropanol.

fluorine ion with the multivalent cation (*9*). In the present method of titration, the reaction system of complex formation was presumed from the mode of the titration curve which was susceptible to the changes of the solvent concentration or changes of the pH of the solution, as will be described later. However, the present method was based on the reaction for gravimetry involving precipitation of calcium fluoride (*11,15*) and, in our experiment, the end-point appeared to be around the equivalence point where 1 mole of calcium ion was consumed by 2 moles of fluorine ion under optimum titration conditions. Lingane (*10*) also considered the reaction system to be based on the formation of calcium fluoride (CaF_2) for the potentiometric titration of fluoride with calcium chloride as the titrant. We have therefore considered the present method to be based on the reaction system of $Ca^{2+} + 2F^- \rightarrow CaF_2$. It seemed however, due to the relatively large solubility of calcium fluoride in water (solubility product; 1.7×10^{-10}), that the titration exhibited a somewhat complex behavior in the dilute solution provided for the present experiment. Details of these problems are not still elucidated, but future examintion should be required.

Effect of Acidity

Three milliliters of 0.01 *M* sodium fluoride solution was loaded on a cation exchange resin column, and the column was eluted several times with 2–3 ml of water (total amount of water used: 13 ml). To the effluent solution was added 24 ml of isopropanol. This sample solution was adjusted to various pHs by the addition of a small amount of ammonia water, and solutions were titrated. As shown in Fig. 3, inflection of the titration curve was very sharp at pH 7–5 and the end-point coincided with the equivalence point. At above pH 8, the inflection became dull, resulting in poorer reproducibility, and the end-point moved to the positive side. At a lower pH, inflection of the titration curve became

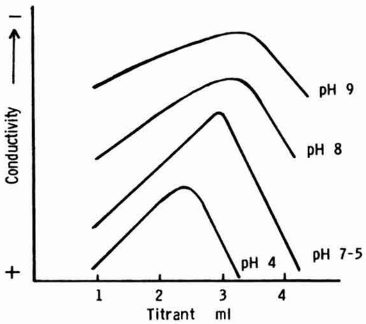

FIG. 3. Effect of pH on conductometric titration of fluoride with calcium acetate.

sharper, but the end-point moved to the negative side. The pH range of 5–7 seemed to be the optimum during the titration.

Limit of Fluoride Concentration

From the foregoing experimental results, titration was carried out with sample solutions of pH 5–7 and acetone concentration of 60%, and with titrant of 50% isopropanol solution of 0.005 *M* calcium acetate to examine the lowest limit of fluoride in the sample. The minimum quantity with a detectable end-point in phe titration curve was 8×10^{-6} *M*, but a linear relationship between amounts of fluoride and calcium acetate consumed was attained at about 1×10^{-5} *M* of fluoride (Fig. 4). It was suggested therefore that the sample taken should contain more than 190 μg of fluorine.

Effect of Coexisting Ions

In conductometric titration, presence of foreign ions not incorporated with the reaction should be rejected as possible, but diverse ions often accompany analyses of organic samples as contaminants. Consequently, examinations were made of the effect of a few ions which are generally believed to coexist. Figure 5 illustrates titration curves obtained by additions of 1×10^{-5} to 1×10^{-4} *M* of chlorine, bromine, nitrate, or acetate ions, and it was found that, irrespective of the kinds of anions present,

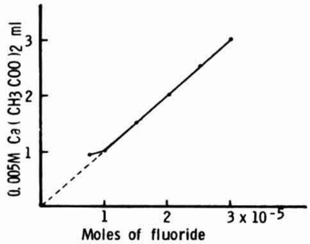

FIG. 4. Moles of fluoride in sample vs. consumption of 0.005 *M* calcium acetate titrant.

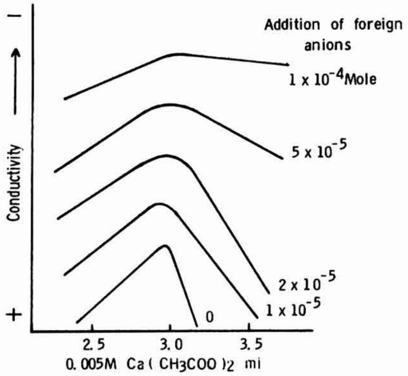

FIG. 5. Effect of foreign anions on conductometric titration fluoride.

clear end-points were obtained when the quantities of these ions added were less than 5×10^{-5} M. At 1×10^{-4} M, the end-point became considerably indiscernible. Coexistence of these contaminant ions below 5×10^{-5} M was desirable, but organic samples containing contaminants at higher levels are infrequently encountered.

Coexistence of sulfate and phosphate ions are naturally not desirable, even in minute quantities. Consequently, the present method is not applicable to samples containing these ions.

Effect of Carbon Dioxide Produced in Combustion Flask

Based on the results of the foregoing examinations, determination of fluorine in organic compounds was then attempted with the oxygen flask combustion method. In this case, production of large amounts of carbon dioxide during the combustion of filter paper produced the question of an interfering effect with conductometric titration. A test was carried out to burn the filter paper piece, with water or 0.1 M ammonia water as the absorption liquid, and then 3 ml of 0.01 M sodium fluoride was added (after cation resin treatment in the case of ammonia water). The test solution was adjusted to pH 5–7, and the solution was titrated. In either case, absolutely no effect was observed on the typical titration curve shown in Fig. 3. It was therefore thought that the effect of carbon dioxide produced by filter paper burned could be excluded from further consideration.

Flask Material and Absorption Liquid

Many discussions have been made on the flask materials for combustion of fluorine-containing compounds, and quartz flasks are generally recommended (*2,4,8,16,20*), because the use of Pyrex or other hard

TABLE 1
ANALYTICAL ERRORS OF ORGANIC FLUORINE USING DIFFERENT MATERIALS OF COMBUSTION FLASK AND ABSORPTION LIQUIDS[a]

Quartz flask		Pyrex flask	
Absorption liquid		Absorption liquid	
0.1 *M* NH_4OH Error (%)	H_2O Error (%)	0.1 *M* NH_4OH Error (%)	H_2O Error (%)
−0.14	−0.12	−1.01	−1.75
+0.03	+0.05	−1.46	−1.28
−0.11	+0.10	−0.57	−0.84
−0.18	−0.05	−1.53	−1.66
−0.08	+0.15	−0.73	−1.62

[a] Sample: *p*-fluorobenzoic acids; F% = 13.56.

glasses containing boron would produce stable fluorine–boron complexes and result in lower determination values (*16*). There are also numerous reports of the use of water as the absorption liquid (*4,8,16,20*), but Ferrari and others (*5*) used alkaline absorption liquid. Therefore, we attempted the determination of fluorine in *p*-fluorobenzoic acid using quartz and hard-glass flasks, either with water and 0.1 *M* ammonia water as the absorption liquid. In the case of ammonia water, the absorption liquid was treated with cation exchange resin before the titration. A list of the analytical errors, as shown in Table 1, indicated that either absorption liquid was evenly effective when the quartz flask was used, while the use of a Pyrex flask introduced 1–1.6% negative errors. The result clearly indicated that the quartz flask was preferable to the hard-glass one, and water could be conveniently used as the absorption liquid, in spite of our prediction that the alkaline solution might prevent the formation of boron–fluoride complex.

In the titration process, use of a polyethylene cell instead of a Pyrex glass cell has been reported (*16*), but comparative use of the two cells did not show any diffrence between each other, and good result was obtained with either cell. Even when the sample solution was allowed to stand in the glass cell for 2 hr before the titration, no loss of the fluorine ion was observed. Another test was carried out that the sample was burned in the Pyrex flask and the absorption liquid was allowed to stand in the same flask for 30, 60, and 120 min, but practically the same fluorine values were obtained with different standing times. Consequently, formation of boron fluoride is assumed to take place instantaneously during the combustion of the sample. Use of the Pyrex glass cell could be therefore allowed for the titration.

TABLE 2
ANALYTICAL RESULTS OF FLUORINE IN ORGANIC COMPOUNDS

p-fluorobenzoic acid, F% = 13.56		Fluoroacetamide, F% = 24.66	
Found		Found	
F (%)	Error (%)	F (%)	Error (%)
13.72	+0.16	24.72	+0.06
13.49	−0.07	24.45	−0.21
13.78	+0.22	24.74	+0.08
13.59	+0.03	24.54	−0.12
13.75	+0.19	24.44	−0.22
13.42	−0.14	24.71	+0.05
13.60	+0.04	24.81	+0.15
13.54	+0.02	24.55	−0.11
SD (%): 0.14		SD (%): 0.13	

Analytical Results of Organic Sample

Determination of fluorine in *p*-fluorobenzoic acid and fluoroacetamide was carried out with a quartz combustion flask. As shown in Table 2, the results obtained were satisfactory with the error within ±0.3% and SD of 0.14%. There are several examples of the addition of inorganic salts like potassium perchlorate, or carbohydrates like sucrose and paraffin wax (*13*), but, for the present samples, such addition seemed unnecessary.

SUMMARY

For the conductometric titration of fluoride with calcium acetate, solvent system in sample solution and titrant, concentration, and acidity of sample solution were examined to establish the titration conditions. Results of these examinations were transferred to the microdetermination of fluorine in organic compounds using oxygen flask combustion method.

Comparative examination between quartz and Pyrex flasks for the combustion of fluorine samples indicated that good results were obtained by the use of the former flask, whereas the use of the latter one gave negative values of 1.0–1.6% due to the formation of boron fluoride during the combustion.

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Radioactive Decontamination by Bacterial Growth

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INTRODUCTION

The removal of radioactive contamination from laboratory equipment is a time-consuming and expensive procedure requiring repeated washing with strong detergents, sulfochromic acid, or other corrosive agents (*2,4*). The use of such protocols is not applicable to the decontamination of aluminum rotor caps used in the preparative ultracentrifuge because corrosion will result.

We use large quantities of ^{32}P for *in vivo* labeling of the DNA of various bacteriophages and consequently experience heavy contamination of rotor caps during the subsequent purification of the phage. We report a facile, novel, and efficacious procedure for the decontamination of rotor caps and other apparatus by the growth of *Escherichia coli* in media with limiting quantities of inorganic phosphate.

MATERIALS AND METHODS

Aluminum ultracentrifuge caps for the 50 Ti and 50.2 Ti rotors were purchased from Beckman Instruments, Atlanta, Georgia; casamino acids were obtained from Difco; other chemicals were of reagent quality and were purchased from Fisher Scientific Co. or Sigma Chemical Co.

Bacteria and Growth Procedures

An auxotrophic strain JC411E, with the genotype *leu argG met lac aroE argR$^+$ his str$^+$*, (kindly supplied by Dr. George Jacoby) was utilized in this work. General growth of bacteria was in L medium (*5*); however, bacterial growth for decontamination was in S1 medium (Table 1) minus KH_2PO_4 with nucleic acid bases and tryptophan at the concentrations described (*3*) and with the addition of 3.3 ml/l of dephosphorylated 20% casamino acids.

Dephosphorylated casamino acid was prepared by the addition of 1.9 ml of 1.0 *M* $CaCl_2$ to 20 ml of 20% casamino acid. The precipitated calcium phosphate was removed by centrifugation at $6000 \times g$ for 10

TABLE 1
S1 MEDIUM

0.1 M Tris HCl, pH 7.4	2 m*M* $MgSO_4$
7 m*M* NaCl	0.2 m*M* $CaCl_2$
10 m*M* NH_4Cl	4 mg/l vitamin B_1
3 m*M* KH_2PO_4	20 ml/l 20% glucose

min and the resulting supernatant sterilized by filtration through an 0.44 μ Millipore filter.

Decontamination Procedure

Ultracentrifuge caps which had been used for several preparative ultracentrifugation runs with more than 10 mCi quantities of ^{32}P were washed with SDS/water until no more radioactivity could be easily removed. Radioactivity was determined by Cerenkov counting (*1*) in a Beckman LS 230 Scintillation Spectrometer.

Caps to be decontaminated were placed in a 250-ml beaker containing 100 ml of S1 medium with growth supplements (without KH_2PO_4) and the medium was innoculated with approximately 10^9 *E. coli* from an overnight culture grown in L medium. The beaker was covered with a petri dish lid and incubated at 37°C for 2 days. The caps were removed, briefly rinsed with distilled water and the remaining radioactivity ascertained by determination of Cerenkov radiation.

RESULTS AND DISCUSSION

Ultracentrifuge caps which had been used for the purification of approximately 5×10^{13} bacteriophage λ labeled with ^{32}P to a specific activity of about 2×10^5 cpm/μg DNA were used in this work. Each cap had been used for six equilibrium centrifugation runs with 1.5 density cesium chloride. These caps were washed by the normal procedure utilized by the dishwasher; they were then scrubbed vigorously with a 1% SDS solution, rinsed five times with distilled water and remaining radioactivity determined by counting Cerenkov radiation (Table 2). This

TABLE 2
RESIDUAL RADIOACTIVITY AFTER WASHING WITH 1% SDS

		^{32}P CPM after washes				Total
Cap	Initial ^{32}P CPM	1	2	3	4	^{32}P CPM removed (%)
1	3.56×10^6	2.36×10^6	2.05×10^6	1.84×10^6	1.67×10^6	53
2	3.89×10^6	2.59×10^6	2.26×10^6	1.98×10^6	1.84×10^6	53

TABLE 3
RESIDUAL RADIOACTIVITY AFTER *E. coli* GROWTH

Cap	Initial ^{32}P CPM	^{32}P CPM after *E. coli* growth: 1st Cycle	^{32}P CPM after *E. coli* growth: 2nd Cycle	Total ^{32}P CPM removed (%)
1	2.77×10^6	6.07×10^5	1.69×10^5	94
2	3.02×10^6	1.39×10^6	3.90×10^5	87

procedure was repeated three more times. The data presented in Table 2 shows that some 30% of the contamination was removed by the first vigorous wash; however, little more was removed in the subsequent washes. In addition it is clear that much contamination will be left remaining in inaccessible areas of the caps. This radioactivity will only be determined with low efficiency by the procedure used in this work. The results obtained by the use of the decontamination protocol described in this work are shown in Table 3.

Two ultracentrifuge caps with a previous history of exposure to radioactivity similar to that used in the experiment shown in Table 2 were placed in a beaker of S1 medium and bacteria added as described; after 2 days at 37°C the caps were removed and rinsed briefly with distilled water; the remaining radioactivity was determined by Cerenkov counting and correlated to the level of radioactivity originally present. The caps were then placed in 100 ml of fresh media, bacteria were once more added, and after 2 days of additional growth, residual radioactivity on the caps was determined as described. The data presented in Table 3 indicated that between 70% and 80% of the radioactivity originally present is removed by each cycle of growth of *E. coli*.

The procedure of decontamination described is relatively noncorrosive and requires little effort compared to vigorous scrubbing. It is considerably less damaging to the ultracentrifuge caps than the use of strong decontaminating solutions. It is applicable to any other laboratory apparatus exposed to radioactive contamination and, by the use of mutant strains, this procedure may be used to remove ^{3}H, ^{14}C, and ^{35}S contamination. ^{3}H leucine contamination may be removed facilely by the growth of a leucine auxotroph and ^{3}H uridine by the use of a uridine auxotroph. It is more difficult to quantitate the decontamination of ^{3}H leucine, as scintillation counting procedures have to be used to determine this isotope, and there is leaching of the radioactivity by the fluor; however, we have qualitative data which confirms the efficiency of this protocol under these conditions.

ACKNOWLEDGMENTS

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MICROCHEMICAL JOURNAL **20,** 453–461 (1975)

Ultramicrodetermination of Nitrogen in Organic Compounds

IV. Thermal Behavior of a Gas in a Nitrometer due to Local Variations of Temperature

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INTRODUCTION

Three problems must be considered when measuring the nitrogen volume in a nitrometer: the errors of volume, temperature, and pressure. The error of pressure in using the nitrometer for ultramicrodetermination was recently described by the author (*2*). This report concerns the expansion and contraction of a gas due to temperature variation.

Generally, the temperature of nitrogen in the nitrometer is measured with a thermometer hung next to the graduated tube of the nitrometer. However, the local variations of temperature, e.g., the temperature rise caused by the opening near the sample heater, must not be neglected. There are many methods of directly measuring the temperature of the alkali solution in the nitrometer as well as that of nitrogen. For example, the temperature of the alkali solution can be measured by inserting a thermometer into the upper glass bulb (*4*), that of the alkali solution near the lower side tube of the nitrometer by setting a small thermometer inside the lower parts of the wide stem of the measuring tube (*1*), and that of the alkali solution inside the leveling bulb by inserting a thermometer. However, these methods are not always regarded as the real measurement of the gas temperature in the nitrometer.

In both microanalysis and ultramicroanalysis, local temperature variation must be avoided, and especially in using a nitrometer which has a slender graduate tube, temperature measurement requires extra care. Therefore, we decided to investigate the variation of local temperature in the nitrometer by using a thermistor in order to obtain more accurate analytical results in ultramicroanalysis. Investigation indicated the necessity of using a water jacket in order to remove the effect of local temperature variation.

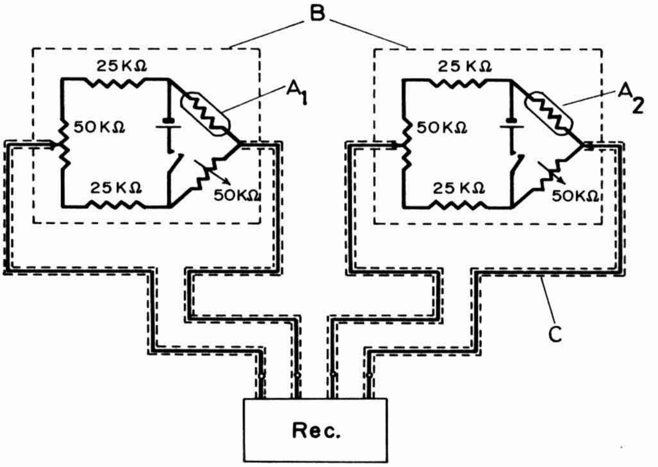

FIG. 1. Electrical circuit for detecting variations of local temperature of the nitrometer. A_1, Thermistor 1, R at 0°C: 95.73 KΩ, B: 3655°K; A_2, thermistor 2, R at 0°C: 101.5 KΩ, B: 3675°K; B, sealed box; C, sealed wire.

METHOD

Temperatures were measured simultaneously at two positions of the nitrometer as shown by the electrical circuit diagram in Fig. 1, and the results were recorded on a polyrecorder. The temperature–resistance characteristics of the two thermistors incorporated in this circuit show general exponential decreasing curve, but, especially in the range of 12–40°C, they almost form a straight line. The sensitivity of this bridge with thermistor 1 is 13.04 mV/°C and that of this bridge with thermistor 2 is 12.95 mV/°C.

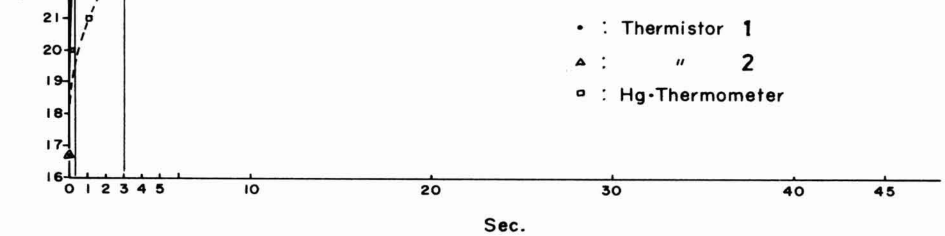

Fig. 2. Time constants (T) of thermometers. Thermistor 1 and 2: T = 0.25 sec; mecury thermometer: T = 3.0 sec.

TABLE 1
ANALYTICAL ERROR CAUSED BY TEMPERATURE ERROR

R	5	10	20	30	40	50	60
R[a]	0.017	0.034	0.068	0.102	0.136	0.170	0.240

[a] $\Delta R = R \cdot \Delta t/(273 + t)$; R: Nitrogen content (%); t: Temperature of nitrogen, 20°C; Δt: Temperature error, 1°C.

The response times of the thermistors and the mercury thermometer used in this investigation are shown in Fig. 2. The time constants, T, of both thermistors 1 and 2 are 0.25 sec, and that of the mercury thermometer is 3.0 sec.

Analytical errors cased by temperature error, 1°C, are shown in Table 1. Table 1 indicates that analytical error, ΔR, increases in proportion to nitrogen content.

In this experiment we used the nitrometer immersed in water jacket, as shown in Fig. 3, which makes it very easy to measure the temperature at each part, B, C, D, E, etc., signed in Fig. 3.

For forced heat convection, warm air, 40°C, was blown from a hair dryer at a distance of 20 cm from a ventilating hole.

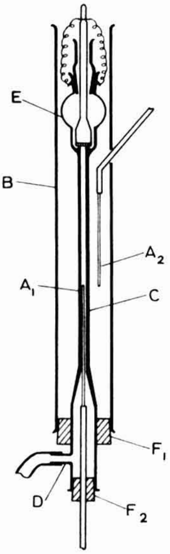

FIG. 3. Apparatus for measuring temperature inside and outside the nitrometer. A_1, Thermistor-1; A_2, thermistor-2; B, Water-jacket; C, calibrated measuring tube; D, lower side tube; E, upper glass bulb; F_1 and F_2, gum stoppers.

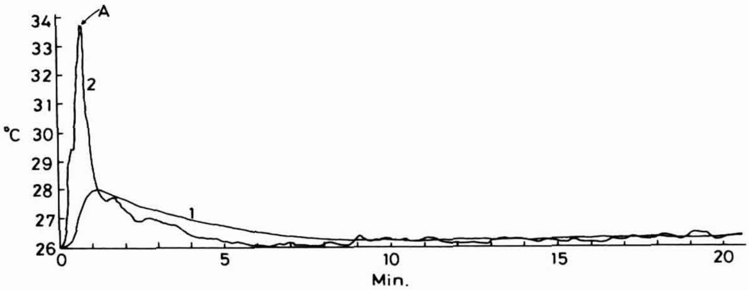

FIG. 4. Temperature changes outside and inside the graduated tube after blowing warm air. Temperature of warm air, 40°C; blowing time, 40 sec; room temperature, 26°C. 1. Inside the graduated tube without the water jacket. 2. Outside the graduated tube.

MEASUREMENT AND DISCUSSION

Seven experiments, from A to G, were conducted to evaluate the water jacket in removing local temperature variation.

Experiment A. The variation of local temperature of the nitrometer without a water jacket was measured. Thermistor 1 was put into the graduated tube and thermistor 2, which was maintained at the same level, was hung in the vicinity of the graduated tube. This is the usual way of determining nitrogen temperature. Warm air, 40°C, was blown on to these two thermistors for 40 sec at room temperature, 26°C (Fig. 4). When the warm air was stopped after 1 min, the difference of temperature between 1 and 2 was 6–7°C but 1 min later, both thermistors gave almost the same temperature, which gradually decreased to room temperature.

Experiment B. All conditions except the time of blowing were the same as in experiment A; the time of blowing was 5 min. Fig. 5 shows that both temperatures were almost the same when the warm air was stopped, and 5 min later, they became stable and coincided with each

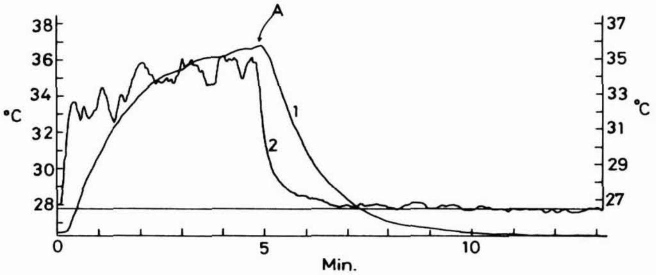

FIG. 5. Temperature changes outside and inside the graduated tube after blowing warm air. Left ordinate, temperature of thermistor 1; right ordinate, that of thermistor 2. Temperature of warm air, 40°C; blowing time, 5 min; room temperature, 26.5°C. 1 and 2 are the same as described in Fig. 4.

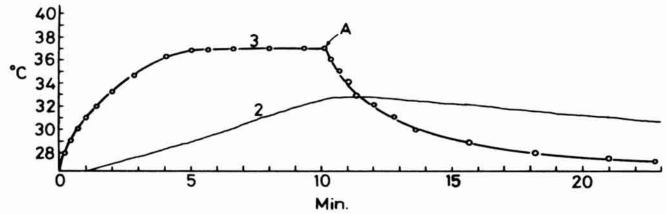

FIG. 6. Temperature changes outside the graduated tube and inside the upper glass bulb. Temperature of warm air, 40°C; blowing time, 10 min; room temperature, 26.5°C. 2. Inside the upper glass bulb. 3. Thermometer suspended along the nitrometer.

other. Experiments A and B, showed that, if local temperature variation existed at the time the nitrogen volume was read, they could become an error factor.

Experiment C. The temperature of the solution filling the nitrometer was measured. The nitrometer did not have a water jacket. Thermistor 2 was put into the upper glass bulb, the mercury thermometer was put outside the graduated tube, and warm air was blown at them for 10 min (Fig. 6). After 5 min, the mercury thermometer registered a constant temperature, while the temperature in the upper glass bulb continued to increase gradually and 10 min later reached a level 4°C lower than thermometer. Nine minutes after the warm air was stopped, the thermometer registered a that of the constant temperature, while the thermistor temperature gradually continued to decrease and was 3.5°C higher than the former.

Experiment D. All conditions except the position of thermistor 2 were the same as in experiment C. Thermistor 2 was put in the lower side tube (Fig. 7). Both curves of temperature change were similar to those of C, but the difference of temperature was larger owing to the large volume of solution in the nitrometer.

Experiment E. The nitrometer did not have a water jacket. Thermistor

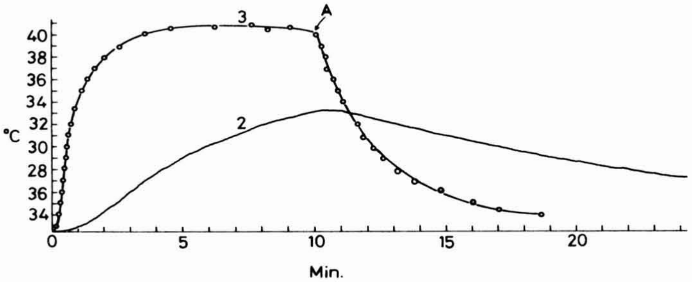

FIG. 7. Temperature changes outside the graduated tube and inside the lower side tube. Temperature of warm air, 40°C; blowing time, 10 min; room temperature, 22.5°C. 2. The lower side tube. 3. Thermometer suspended along the nitrometer.

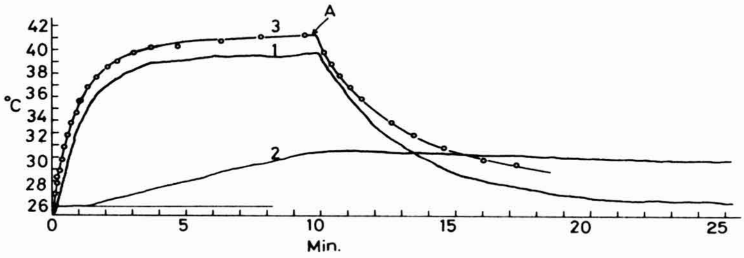

FIG. 8. Temperature changes outside and inside the graduated tube and inside the leveling bulb. Temperature of warm air, 40°C; blowing time, 10 min; room temperature, 25.7°C. 1. Inside the graduated tube without the water jacket. 2. Inside the leveling bulb. 3. Thermometer suspended along the nitrometer.

2 was put into the solution in the leveling bulb, the mercury thermometer was put outside the graduated tube, and thermistor 1 was put into the graduated tube. Temperatures at the three positions were simultaneously measured. The temperature variations in and outside the graduated tube were analogous to those of B during the blowing period, but the difference between the temperatures in the leveling bulb and outside the graduated tube was about 8.5°C when the blowing was stopped (Fig. 8). The temperature in the leveling bulb decreased slightly, about 1°C/15 min, after the warm air was stopped. This was because the volume of the solution in the leveling bulb was greater than that in the other parts.

This test indicated that if variation in local temperatures occurs, it is difficult to remove the disparity between the temperature inside of the graduated tube and the other positions.

Experiment F. The nitrometer had a water jacket (Fig. 3). Thermistor 1 was put into the graduated tube and thermistor 2 was hung at the same level outside the water jacket. Warm air was blown at these two thermistors for 11.5 min (Fig. 9). The difference between the temperatures outside the water jacket and in the graduated tube of the nitrometer with the water jacket was large (8°C). One minute after the blowing was

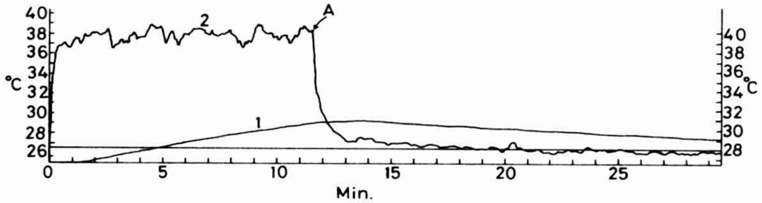

FIG. 9. Temperature changes outside the water jacket and inside the graduated tube. Right ordinate, temperature of thermistor 1; left ordinate, that of thermistor 2. Temperature of warm air, 40°C; blowing time, 11.5 min; room temperature, 26.5°C. 1. Inside the graduated tube equipped with a water jacket. 2. Outside the water jacket.

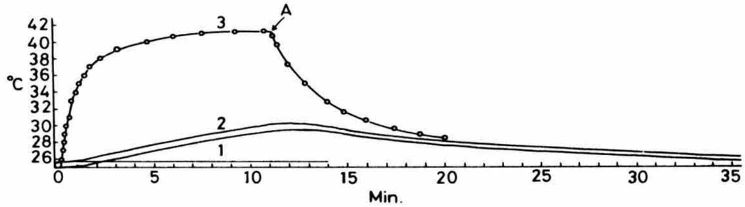

FIG. 10. Temperature change outside and inside the water jacket and inside the graduated tube. Temperature of warm air, 40°C; blowing time, 11 min; room temperature, 25.7°C. 1. Inside the graduated tube equipped with a water jacket. 2. Inside the water jacket. 3. Thermometer outside the water jacket.

stopped, the relationship was reversed, and 10 min later the difference was about 3°C.

Experiment G. The nitrometer had a water jacket. Thermistor 1 was put into the graduated tube, thermistor 2 was put into the water jacket, and the mercury bulb of the thermometer, which was maintained at the same level as the two thermistors, was put outside the water jacket. The temperature of the three positions was measured simultaneously (Fig. 10). The baselines for thermistors 1 and 2 have completely coincided. In Fig. 10, however, the baseline for thermistor 2 is shifted 0.7° above that of thermistor 1 to make clear that the time dependence of the two thermistors is the same. The warm air current caused the temperature inside the graduated tube to increase parallel with the temperature inside the water jacket; when the warm air was stopped the increases were about 4.5°C and after that they decreased gradually in parallel. Therefore no difference of temperature arose. On the other hand, the difference between the temperatures outside the water jacket and the former two was about 12°C at the time the warm air was stopped.

Therefore, we can regard the temperature inside the water jacket installed in the nitrometer as the accurate temperature of nitrogen, if the interval between reading the volume and reading the temperature is not long, even if crude variations of local temperature occur in the nitrometer.

NITROMETER

The nitrometer equipped with a water jacket for the sealed tube method is illustrated in Fig. 11. It consists essentially of three parts, the water jacket, A, the calibrated measuring tube, B, and a receiving chamber, C. The water jacket is tapered to fit the body joint at the wide stem of the bottom of the measuring tube. It is a 35-mm diameter tube of 350-mm overall length. The mercury thermometer, D, is suspended along the measuring tube on the hook at the bottom of the upper glass bulb, E. The measuring tube is a glass capillary tube of about 1.0 mm in-

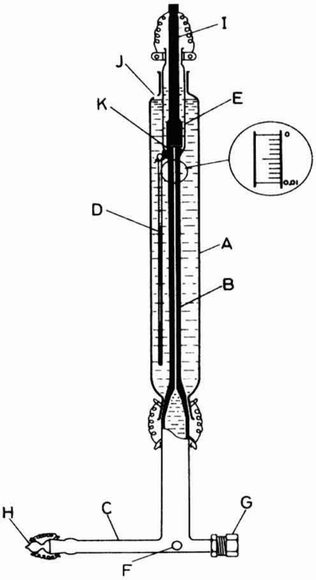

FIG. 11. Nitrometer equipped with a water jacket. A, Water jacket; B, calibrated measuring tube; C, receiving chamber; D, mercury thermometer; E, upper glass bulb; F, glass side-arm; G, metal stuffing-box type joint; H, glass stopper; I, plunger; J, hole; K, nozzle.

ternal diameter. It is about 25 cm long, with a capacity of about 0.20 ml, and is marked off in 0.001 ml graduations numbered as shown. The bottom of the capillary tube widens out into the stem which is joined near one end of the receiving chamber which has been described previously (*2*). At this same junction, a short glass side-arm, F, is joined at right angles to the other tubes. This is connected by a Tygon tube to a leveling bulb. The end of the receiving chamber nearest the junction of the tubes is blocked with a self-sealing rubber cap held on by a metal stuffing-box type joint, G, the other end has a ground glass stopper, H.

The analytical procedure has been described in previous papers (*2,3*).

SUMMARY

To more accurately measure nitrogen temperature for ultramicrodetermination of nitrogen, the variation of local temperature in the nitrometer was investigated. Two or three parts of the nitrometer were simultaneously measured. Based on our results, we designed a new improved nitrometer equipped with a water jacket.

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The Preconcentration, Separation and Spectrophotometric Determination of Traces of Silicate*

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INTRODUCTION

In the spectrophotometric determination of nonmetals by spectrophotometry, one is frequently concerned with the limited sensitivity and selectivity of the available methods. Preconcentration and separation is often a practical approach in this situation. This paper reports the results of a study on the preconcentration, separation, and spectrophotometric determination of silicate at the ppb level. A strong base anion exchange resin, in hydroxyl form, and packed in a polyethylene column; an acidic molybdate eluting reagent, and the near-infrared heteropoly blue spectrophotometric were used.

Cation exchange resins have been used to remove metal ions (*6*,*8*) prior to determination of silicate and for the preconcentration of traces of metal ions (*7*,*9*). Weakly basic anion exchange resins have been used to separate phosphate (*1*,*4*) and chromate (*8*) from silicate. Strongly basic anion exchange resins have been investigated for the isolation of silicate at the milligram level (*10*) and at the microgram level (*3*,*5*). Osmun and Wirth (*5*) reported difficulty in removing the retained silicate.

MATERIALS AND METHODS

Reagents

Standard silicate solution. Dissolve 10.127 g of sodium silicate, $Na_2SiO_3 \cdot 9H_2O$, in distilled water containing 10 ml of silica-free ammonia solution and dilute to 1000 ml. Standardize this solution gravimetrically. Use a microburet to transfer sufficient standardized silicate solution to 1000-ml volumetric flask containing 5 ml of silica-free ammonia

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solution, so that on dilution to volume the solutions contains 10.0 μg of silicon/ml. Store the stock and dilute silicate solutions in polyethylene bottles.

Molybdate solution (10% w/v). Transfer 10 g of ammonium paramolybdate, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, to a 100-ml volumetric flask, dilute to the mark and store in a polyethylene bottle.

Tartaric acid solution (10% w/v). Transfer 10 g of tartaric acid to a 100-ml volumetric flask, dilute to volume, and store in a polyethylene bottle.

Sulfuric acid solution (*4*N). Transfer 11 ml of concentrated sulfuric acid (sp. gr. = 1.83; 95% by wt.) to a 100-ml volumetric flask containing distilled water and dilute to volume. Transfer sulfuric acid solution to polyethylene bottle for storage.

1-Amino-2-naphthol-4-sulfonic acid solution. Dissolve 25 g of sodium hydrogen sulfite in 200 ml of water. Dissolve 2 g of anhydrous sodium sulfite and 400 mg of 1-amino-2-naphthol-4-sulfonic acid in 25 ml of water. Mix these two solutions and store in polyethylene bottle.

The water used throughout this study was redistilled using a glass condenser fitted internally with polyethylene tubing and stored in polyethylene containers.

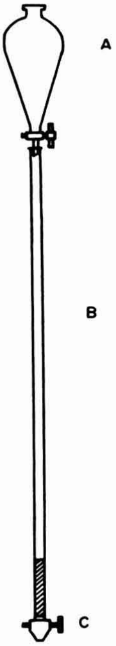

FIG. 1. Polyethylene microcolumn. A. 125-ml polypropylene funnel, B. Polyethylene tube, 40 cm in length × 6 mm (i.d.), C. Teflon stopcock.

Apparatus

The spectrophotometric measurements were made with a Cary 14 spectrophotometer. The pH measurements were made with a Corning Model 110 pH meter equipped with glass and calomel electrodes.

A microcolumn constructed of 6-mm polyethylene tubing and fitted with a Teflon stopcock was used. The construction details and dimensions of the column are shown in Fig. 1.

Procedure for Preparation of Ion Exchange Column

Transfer 1 g of a strongly basic anion exchange resin, Bio-Rad AG-2-X8 (20–50 mesh) in Cl-form, to a polyethylene beaker containing double distilled water and allow to stand for 24 hr. Insert a small wad of cotton (~10 mg) above stopcock in the polyethylene microcolumn. Transfer the resin to the column. Add 50 ml of 4% sodium hydroxide to the funnel at top of column and adjust flow rate to 3–4 ml/min. Wash the resin bed with double distilled water until the pH of effluent is less than 9.

Procedure for Preparation of Calibration Plot

Transfer 0, 1.0, 2.0, 2.5, 3.0, 4.0, 5.0, and 6.0 ml of the standard silicate solution (10.0 μg of Si per ml) to a polyethylene beaker and dilute to about 30 ml with redistilled water. Add 4 ml of the 10% molybdate reagent and adjust to pH 1.6 with 4 *N* sulfuric acid, usually 0.8–1.0 ml is sufficient. After 10 min, transfer these solutions to 50-ml volumetric flasks. Add 5 ml of the 10% tartaric acid reagent, mix, and add 0.5 ml of the 1-amino-2-naphthol-4-sulfonic acid solution. Dilute to volume with redistilled water and mix thoroughly. After 20 min, measure the absorbance at 815 nm in 1-cm absorption cells using distilled water in the reference cell. Construct a calibration plot by plotting absorbance vs. μg of silicon per ml.

Procedure for Preconcentration, Separation, and Determination of Silicate

Transfer stepwise 250 ml or 500 ml of aqueous samples containing 0.02 to 0.1 μg of silicon/ml to the microcolumn and adjust flow rate to 9–10 ml/minute. After the sample solution has passed through the column, wash the column with 10 ml of a very dilute silica-free ammonia solution. The resin bed is then pushed out of the column into a polyethylene beaker. Add 30 ml of double distilled water and 4 ml of 10% molybdate reagent. Adjust pH to 1.6 using 4 *N* sulfuric aicd. After stirring for 10 min, filter the solution and collect filtrate in 50-ml volumetric flask. Add 5 ml of the 10% tartaric acid solution and 0.5 ml of the 1-amino-2-napthol-4-sulfonic acid solution. Dilute to volume with redistilled water and mix thoroughly. After 20 min measure the absorbance at 815 nm in 1-cm cells, using a distilled water in the reference cell.

RESULTS AND DISCUSSION

Precautions. A polyethylene column must be used, inasmuch as the sodium hydroxide used to convert the resin to hydroxyl form attacks glass to give irreproducible blanks. The use of a glass wool or sintered glass in the bottom of the column has to be avoided. All solutions and the double distilled water are stored in polyethylene bottles. The silica-free ammonia is prepared by bubbling ammonia through double distilled water.

Preconcentration. The preconcentration of silicate was studied using three sample solutions corresponding to 100, 40, and 20 ppb of silicon. Using 250-ml or 500-ml samples the concentration factor was 5 or 10, as the final volume of the colored solution was 50 ml. Because this resin has a total exchange capacity of 3.5 meq/g, the column has ample capacity for much larger sample volumes if more dilute solutions are to be analyzed.

Separation. As reported previously (*5*) and as observed in this investigation, it is difficult to elute final traces of silicate from a strongly basic anion exchange resin column using sodium hydroxide. In order to avoid prolonged contact time or the use of large volumes of eluting reagent, the technique of removing the resin from the column and using an acidic molybdate eluting solution to form molybdosilicic acid to expedite

TABLE 1
RESULTS OBTAINED WITH PROPOSED METHODOLOGY

Volume of sample solution (ml)	Concentration factor	Si present, (ppb)	Si taken (μg)	Si found (μg)	Percentage recovery
250	5	100	25.0	24.8	99
250	5	100	25.0	24.5	98
250	5	100	25.0	24.8	99
250	5	100	25.0	24.5	98
250	5	100	25.0	24.0	96
250	5	40	10.0	9.5	95
250	5	40	10.0	9.5	95
250	5	40	10.0	9.2	92
250	5	40	10.0	9.3	93
250	5	40	10.0	9.4	94
500	10	20	10.0	9.2	92
500	10	20	10.0	9.1	91
500	10	20	10.0	9.1	91
500	10	20	10.0	8.8	88
500	10	20	10.0	8.8	88

recovery was utilized. Thus, the reactive stripping agent technique is superior to the displacement technique in preconcentration of trace constituents.

Spectrophotometry. The classical heteropoly blue method was employed in the determinative step (*2*). The molybdosilicic acid formed in removal of the silicate from the anion exchange resin is reduced to give a heteropoly blue complex whose absorbance is measured at 815 nm. Although the molar absorptivity for this method is 1.72×10^4 liter $\text{mole}^{-1}\ \text{cm}^{-1}$, further sensitivity could be achieved by using 5-cm absorption cells.

Recovery of traces of silicate. The batch-reactive stripping agent technique gave satisfactory recoveries at the ppb level of silicate in the sample solutions. These data are summarized in Table 1. Slightly lower results were obtained at the 20 ppb and 40 ppb levels. In general, the suggested method has a relative standard deviation of about 3% and an average recovery of 90% or above when preconcentrating, separating, and determining silicate in the parts per billion range.

SUMMARY

A method for the determination of traces of silicate based on the use of a strong base anion exchange in a preconcentration step and a batch-reactive stripping agent technique prior to spectrophotometric determination has been proposed. The reaction of molybdate and silicate in acidic solution to form 12-molybdosilicic acid permits recoveries exceeding 90% in analyzing solutions containing 0.02 to 0.1 μg of silicon/ml.

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The Indirect Atomic Absorption Spectrometric Determination of Several Organic Bases Using Molybdophosphoric Acid

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INTRODUCTION

Heteropoly acids have been used extensively as precipitants for organic bases. Jean (*4*) indicated some of the organic compounds which can be determined by reaction with heteropoly acids. In general, alkaloids and other nitrogen heterocyclic compounds can be determined. These methods utilizing heteropoly acids usually involve gravimetry or colorimetry. Each method has its limitations.

This paper reports the results of an investigation on the applicability of atomic absorption spectrometry to the determination of several organic bases after their reaction with molybdophosphoric acid. The final step in each procedure is the measurement of the molybdenum incorporated in the molybdoheteropoly anion. This molybdenum is equivalent to the amount of organic constituent associated with the molybdoheteropoly complex. Two high molecular weight alkaloids, strychnine and brucine, react with molybdophosphoric acid to give precipitates. The excess molybdophosphoric acid is masked with citric acid allowing extraction of the remaining organo-molybdophosphate complex. Following a wash step to remove any excess reagent, the molybdate equivalent to the organic base is stripped by a basic buffer and measured by atomic absorption spectrometry. Attempts to selectively extract the organo-molybdophosphate complex in the presence of excess molybdophosphoric acid were unsuccessful and gave highly irreproducible results. A lower molecular weight organic base, quinoline, also reacts with molybdophosphoric acid to give a precipitate. However, this precipitate is unstable in the presence of complexing agents such as citric acid. In this case, the analytical approach is to centrifuge the precipitate from solution, remove the excess molybdate by washing, dissolve the precipitate in a basic buffer, and determine the molybdenum equivalent to the quinoline by AAS. The two techniques investigated should be applicable to a large variety of similar organic bases.

MATERIALS AND METHODS

Reagents

Standard phosphate solution (100 μg P/ml). Dissolve 2.200 g of potassium dihydrogen phosphate, KH_2PO_4, in distilled water and dilute to 1l. Dilute a 50.00-ml aliquot of this solution to 250 ml with distilled water.

Sodium molybdate solution (6% w/v). Dissolve 15.0 g of reagent grade sodium molybdate dihydrate, $Na_2MoO_4 \cdot 2H_2O$, in distilled water and dilute to volume in a 250-ml volumetric flask.

Sulfuric acid (7.2 N). Add slowly 100 ml of concentrated sulfuric acid to approximately 350 ml of distilled water; cool, stir, and dilute to 500 ml.

Sulfuric acid (2.4 N). Add slowly 16.7 ml of concentrated sulfuric acid to 100 ml of distilled water; cool, stir and dilute to 250 ml.

Sodium citrate solution (10% w/v). Dissolve 10.0 g of reagent grade sodium citrate, $Na_3C_6O_7H_6$, in distilled water and diluted to volume in a 100-ml volumetric flask.

Buffer solution. Dissolve 53.5 g of reagent grade ammonium chloride in 500 ml of distilled water. Add 70 ml of ammonium hydroxide and dilute to 1 l with distilled water. The final pH is about 9.3.

Standard strychnine sulfate solution (500 μg strychnine/ml). Dissolve 641.5 mg of strychnine sulfate (U.S.P.) in distilled water and dilute to volume in a 1 l volumetric flask. Dilute as necessary for standard working solutions. Store in a dark, cool place.

Standard brucine solution (200 μg brucine/ml). Dissolve 200.0 mg of brucine (Matheson, Coleman and Bell) in 2–3 ml of absolute ethanol and dilute to volume with distilled water in a 1-l volumetric flask. Dilute as necessary for standard working solutions. Store in a dark, cool place.

Standard quinoline solution. Weigh exactly 0.5 ml of reagent grade quinoline (Fischer Scientific), dissolve in approximately 2.5 ml of absolute ethanol, transfer to a 1-l volumetric flask, and dilute to volume with distilled water. Calculate the concentration of this solution and dilute to as necessary for standard working solutions. Store in a dark, cool place.

Solvent. Methyl isobutyl ketone. All aqueous solutions were stored in polyethylene bottles.

Apparatus

The atomic absorption measurements were made with a Beckman Model 1301 Atomic Absorption Accessory, a Beckman DB-G grading spectrophotometer equipped with a Beckman potentiometric recorder and a Beckman laminar flow burner. The molybdenum hollow cathode lamp was neon filled and supplied by Beckman.

General Procedure for Determination of Brucine or Strychnine

Transfer to a 60-ml separatory funnel 4 ml of the 6% sodium molybdate solution, 1 ml of the standard phosphate solution, and 1.0 ml of the 7.2 *N* sulfuric acid. Mix and wait 5 min for complete formation of molybdophosphoric acid. Add the sample containing up to 1.12 mg of brucine, or 1.00 mg of strychnine, and dilute to approximately 20 ml. Swirl to mix and wait 10 min for complete formation of the alkaloid–molybdophosphate complex. Add 4.0 ml of the 10% sodium citrate solution, swirl to mix, and wait 3 to 5 min. Extract the alkaloid–molybdophosphate complex with 10 ml of methyl isobutyl ketone. Shake for 1 min, allow the phases to equilibrate, and remove and discard the lower aqueous phase. Wash the organic extract containing the alkaloid and the molybdophosphoric acid (presumably in a soluble form since no evidence of a precipitate is visible) three times with 10-ml portions of 1.0 *N* sulfuric acid. For each wash, shake the separatory funnel with the dilute acid wash for 1 min, allow the phases to equilibrate, then remove, and discard the lower aqueous phase. After the final wash, rinse the tip of the separatory funnel with distilled water to remove any traces of excess molybdate. Add 10 ml of the ammoniacial buffer solution, shake for 1 min and let stand for approximately 1 hr or until the basic buffer completely decomposes the molybdophosphoric acid. Decomposition is evident when the organic phase loses the characteristic yellow color of molybdophosphoric acid. Shake the separatory funnel again for 1 min, allow the phases to equilibrate, and drain the aqueous phase into a 25-ml volumetric flask. Add another 5-ml portion of the buffer solution and shake again. Drain this aqueous phase into the same volumetric flask and dilute to the mark with distilled water. Use the atomic absorption spectrometer to determine the molybdenum concentration of the aqueous buffer solutions. Adjust the current to 15 mA for the hollow cathode lamp. Use a slit width of 0.15 mm and adjust the monochromator wavelength to obtain maximum transmittance at 313 nm. Use a reducing air–acetylene flame with a support gas pressure of 21 psi and an acetylene pressure of 7 psi; adjust the fuel flow rate while aspirating 20 ppm of molybdenum solution until maximum absorbance is obtained. Also, adjust the burner position for maximum absorbance reading while aspirating the 20 ppm of molybdenum solution. Aspirate the known and unknown solutions and record the per cent transmittance. Aspirate water between all samples to clean the premix chamber and check the base line. Prepare calibration graph(s) and determine the strychnine and/or brucine content of the unknown solutions.

Alternate procedure. After the three washes of the MIBK extract containing the equivalent molybdophosphoric acid, it is possible to directly aspirate the MIBK extract into the AAS burner to determine the molybdenum equivalent to the strychnine or brucine. This will result in an enhancement in the sensitivity of the alkaloid determination and avoid the necessity of the approximate 1 hr wait for the decomposition and stripping of the molybdophosphoric acid. However, the major disadvantage of this alternate procedure is the necessity of preparing a new set of standards each day rather than reusing the buffer extracts.

General Procedure for Determination of Quinoline

Add to a 10-ml centrifuge tube, 2 ml of the 6% sodium molybdate reagent, 1 ml of the standard phosphate solution, and 1.0 ml of 2.4 *N* sulfuric acid. Mix and wait 5 min for complete formation of the molybdophosphoric acid. Add 2 ml of sample solution containing up to 400 μg of quinoline. Mix and place the centrifuge tube in boiling water for about 5 min. Centrifuge for a sufficient time (5–10 min) to ensure that a tightly compacted precipitate is collected at the base of each tube. Carefully decant the clear solution. Wash the precipitate in each tube once with 5 ml of 1.0 *N* sulfuric acid. Centrifuge again and carefully decant the wash solution.

Dissolve the precipitate in 10 ml of the basic buffer solution and transfer to a 25-ml volumetric flask. Rinse the centrifuge tube with another 5 ml portion of the buffer solution and transfer to the same 25-ml volumetric flask. Dilute the flask to the mark with distilled water.

Adjust the atomic absorption spectrometer as previously described in the preceding general procedure. Aspirate known and unknown solutions and prepare a calibration graph in order to determine the amount of quinoline in the unknown samples.

RESULTS AND DISCUSSION

Determination of Brucine or Strychnine

Strychnine concentration. Figure 1 shows a typical calibration graph obtained for the indirect atomic absorption spectrometric determination of strychnine. The graph shows slight curvature for the 0 to 40 ppm of strychnine concentration range (on the basis of a final volume of 25 ml). The sensitivity for 1% absorption is 0.65 ppm of strychnine.

Brucine concentration. The corresponding graph for brucine showed a slight curvature at higher concentrations and is applicable to the determination of 0–45 ppm of brucine (on the basis of a final volume of 25 ml). The sensitivity for 1% absorption is 0.75 ppm of brucine.

Effect of acidity, order of reagent addition, and time on the formation of the alkaloid–molybdophosphate complex. In a series of solutions varying from 0.18 to 1.4 *N* sulfuric acid, it was found that a molybdate solution prestripped of phosphate gave no turbidity with either strych-

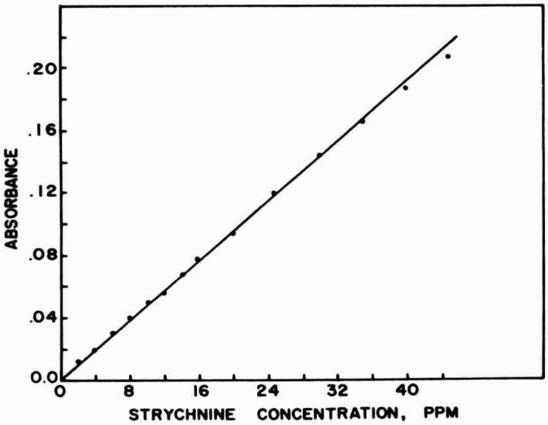

FIG. 1. Typical calibration graph for the indirect AAS determination of strychnine.

nine or brucine, while molybdophosphoric acid forms a precipitate with either alkaloid over this entire range of acidities. A final acidity of 0.36 *N* sulfuric acid was used in this procedure to allow complete formation of the alkaloid–molybdophosphate complex within a period of 10 min.

The order of reagent addition which was found to enable rapid complex formation is as described in the general procedure. In this method, the strychnine or brucine sample is added 5 min after the addition of the molybdate, phosphate, and acid solutions. Although the rate of formation of molybdophosphoric acid is quite rapid under the conditions used, the 5-min waiting period ensures that molybdophosphoric acid is formed before the sample is added. Under these conditions, the alkaloid–molybdophosphate complex formation is complete in the initial aqueous solution after 10 min, so that the excess molybdophosphoric acid may be destroyed with citrate at any time after this. The extraction should then be performed several minutes after addition of the citrate solution.

Effect of citrate on molybdophosphoric acid and the alkaloid–molybdophosphate complex. It is necessary to mask the excess molybdophosphoric acid after the formation of the strychnine or brucine–molybdophosphate complex. Several publications have shown that complexing agents such as citric acid (1–3), tartaric (2), and oxalic acid (1,6) destroy molybdophosphoric acid by complexing the excess molybdate necessary for molybdoheteropoly acid formation. In this work it was found that 4 ml of a 10% citrate solution quickly masks the excess molybdophosphoric acid but affects the strychnine or brucine–molybdophosphate complex at a much slower rate. The effect of citrate on the strychnine and brucine–molybodophosphate complexes was tested by extracting the remaining complex at various times after citrate addition and continuing with the general procedure. Table 1 shows the results of this study.

TABLE 1
EFFECT OF ELAPSED TIME BETWEEN CITRATE ADDITION AND THE INDIRECT AAS DETERMINATION OF STRYCHNINE AND BRUCINE

Time (min)	Absorbance due to 20.0 ppm of strychnine	Absorbance due to 16.0 ppm of brucine
2	0.094	0.050
5	0.093	0.050
10	0.094	0.052
20	0.092	0.048
30	0.086	0.043

As Table 1 indicates, no real effect is evident on the complex until approximately 20 min after the addition of the citrate. In this procedure, extraction is recommended in about 5 min after the addition of the sodium citrate solution. Once extracted the equivalent molybdophosphoric acid was found to be stable in MIBK for at least 24 hr.

Extraction, wash, and retrograde extraction. The strychnine or brucine–molybdophosphate complex is completely extracted with one 10-ml portion of MIBK. It is believed that the complex is soluble in this solvent since no evidence of any turbidity was evident. Only the characteristic yellow color of molybdophosphoric acid was present. Spectrophotometric scans of the ultraviolet region failed to show traces of the original alkaloid in either phase or the acid washes, since the characteristic wavelength of maximum absorbance of the alkaloids is obliterated by the molybdate and citrate absorptivities. Thus, the ultraviolet absorption spectra are of no analytical value in this method.

The excess molybdate was found to be completely removed with three 10-ml washes with 1.0 *N* sulfuric acid. The MIBK extract containing the equivalent molybdophosphoric acid is than decomposed when contacted with a basic buffer. However, the rate of decomposition is slow (approximately 1 hr). The washed MIBK extracts can be aspirated directly into the AAS burner. This will result in enhanced sensitivity due to a more efficient nebulization of MIBK over water and a higher concentration of the molybdophosphoric acid equivalent to the original alkaloid.

Stiochiometry. Assuming that 3 moles of the protonated strychnine or brucine react with 1 mole of molybdophosphoric acid to give $(C_{21}H_{22}N_2O_2H)_3$-$PMo_{12}O_{40}$ for strychnine molybdophosphate and $(C_{23}H_{26}N_2O_4H)_3PMo_{12}O_{40}$ for brucine molybdophosphate, the stoichiometric amount (weight basis) of each alkaloid to equivalent molybdenum is as follows: strychnine: Mo = 1 : 1.12, brucine: Mo = 1 : 0.97. Table 2 shows data obtained by analyzing solutions containing known amounts of each alkaloid, and a comparison of calculated and experimentally de-

TABLE 2

COMPARISON OF CALCULATED MOLYBDENUM CONTENT TO FOUND MOLYBDENUM CONTENT FOR VARIOUS CONCENTRATIONS OF STRYCHNINE AND BRUCINE

Strychnine	Brucine added (ppm)	Molybdenum calculated (ppm)	Molybdenum found (ppm)
2.00		2.2	2.2
	4.00	3.9	4.0
6.00		6.7	6.6
	12.00	11.6	11.0
8.00		8.9	8.7
	16.00	15.5	15.0
10.00		11.2	11.0
	32.00	31.0	30.0
14.00		15.7	15.7

termined amounts of molybdenum. The slightly lower values for the experimentally determined molybdenum content is most likely due to a slight decomposition of the alkaloid–molybdophosphate complex by citric acid. However, these data indicate that the proposed 3:1 stoichiometry holds for each complex.

Precision. An estimate of the precision of the method was obtained by analyzing six samples of 20.00 ppm of strychnine and six samples of 16.00 ppm of brucine according to the recommended general procedure. For the determination of strychnine the mean absorbance value was 0.0902 at 313 nm. The SD was 0.0022 absorbance unit, or a relative SD of 2.5%. For the determination of brucine, the mean absorbance value was 0.0440 at 313 nm with a SD of 0.0013 absorbance unit, or a relative SD of 3.3%.

Determination of Quinoline

Quinoline concentration. The calibration graph for quinoline is linear from 2 to 16 ppm of quinoline (on the basis of a final volume of 25 ml). Attempts to eliminate the curvature of the calibration graph at the lower concentrations of quinoline (<2 ppm of quinoline) were unsuccessful. This curvature is probably due to a slight solubility loss.

Effect of acidity, order of reagent addition, and time on the formation of quinoline–molybdophosphate. The formation of the quinoline–molybdophosphate complex was found to take place at acidities ranging from 0.18 to 1.44 *N* sulfuric acid. An acidity of 0.40 *N* sulfuric acid used in this procedure allows a rapid formation of the quinoline molybdophosphate complex. Variations in acidity are permissible, provided the acidity is within the specified range.

The order of reagent addition must ensure that molybdophosphoric acid is formed before the quinoline sample is added. Although the rate of formation of molybdophosphoric acid is quite rapid under the conditions used, the solution was allowed to stand for 5 min before adding the sample. The order of reagent addition used was molybdate reagent, phosphate solution, acid, and sample. Maximum formation of the quinoline–molybdophosphate complex occurs in about 5 min while digesting the precipitate. A longer digestion period resulted in no significant increase in absorption.

Washing and dissolution of quinoline–molybdophosphate. The quinoline molybdophosphate precipitate is washed once with a 5-ml portion of 1.0 *N* sulfuric acid to remove any traces of the molybdate reagent. One wash was found sufficient to give a negligible blank and retain the precipitate. A larger number of washes led to a steadily decreasing absorbance due to a slight dissolution of the precipitate.

The quinoline molybdophosphate precipitate is unstable in basic solution. Thus, 10 ml of the basic buffer solution was found to be sufficient to completely dissolve the precipitate. A second 5-ml portion of base serves to rinse the centrifuge tube.

Stoichiometry. Quinoline molybdophosphate has been shown by Perrin (*5*) to be a 3 : 1 complex. On this basis, an amplification factor of 1 : 2.97 for quinoline to molybdenum is possible, indicating good sensitivity by atomic absorption spectrometry.

Precision. An estimate of the precision of this method was obtained by analyzing six solutions each containing 9.00 ppm of quinoline. A mean absorbance value of 0.0818 was obtained at 313 nm. The standard deviation was 0.0013 absorbance unit, or a relative standard deviation of 1.6%.

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TLC of Substituted Pyridines.
XII. Hydroxy Derivatives.

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In our *in vitro* chemical and enzymatic hydroxylation experiments with pyridine (1,2), it proved necessary to develop rapid and well-reproducible methods of separation and identification in order to study the transformation products formed from the pyridine.

In this case too the best method was found to be TLC.†

To date not many literature data are known as regards the TLC separation of substituted pyridines (3).

In the present paper we report on the separation and quantitative determination of substituted pyridines.

MATERIALS AND METHODS

The substituted pyridines used were in part commercial products of Merck (Darmstadt, FRG) and of Aldrich-Europe (Brussels, Belgium), and in parts the gifts of EGA-Chemie KG (D-7924 Steinheim (Abuch Kreis-Heidelheim).

The solvents were the purest preparations of Reanal (Budapest, Hungary), and were further purified by distillation.

In the TLC, Kieselegel G nach Stahl (Merck) adsorbent was used in a layer thickness of 0.25 mm. The layer was prepared with a Desaga layering apparatus. The plates were pretreated as described previously (4). In accordance with requirements, 5 × 20, 10 × 20 or 20 × 20 cm plates were employed. Ascending TLC was performed in all cases. The average running distance of the solvent front was 17 cm.

The materials were generally transferred to the start point in the form of a solution in ether.

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† Abbreviations used: TLC = thin-layer chromatography; 2-OH-pyridine = 2-hydroxypyridine, etc.; 2-NH_2-pyridine = 2-aminopyridine, etc.; picolinic acid = pyridine-2-carboxylic acid; nicotinic acid = pyridine-3-carboxylic acid; isonicotinic acid = pyridine-4-carboxylic acid.

After drying, development and detection were carried out with the Van Urk reagent, 0.05% alcoholic fluorescein solution (3), iodine vapor and by UV lamp (Camag).

With the Van Urk reagent the hydroxypyridines give various shades of pink or yellow, e.g., 3-OH-pyridine gives a lemon-yellow color.

With 0.05% alcoholic fluorescein, on the other hand, greens and browns predominate, e.g., 3-OH-pyridine gives a dark-brown color.

The solvents used in the separations were the following:

I. acetone
II. n-butanol—acetic acid—water (4:1:1)
III. ethyl acetate
IV. benzene—methanol (8:2)
V. ethyl acetate—methanol—acetic acid (75:20:5)
VI. n-butanol—acetic acid—water (12:3:5)
VII. chloroform—ethanol (9:1)
VIII. ethyl acetate—methanol—formic acid (80:10:10)

Systems I–VI were used most frequently.

Since the above separations primarily served for the identification and quantitative determination of the *in vitro* chemically and enzymatically hydroxylated products, a method was elaborated for quantitative recovery from the layer plate, and a calibration curve too was prepared. In these cases the spots outlined under the UV lamp were scraped off and extracted with 5 ml ethanol each. These extracts were used for back-measurements. The calibration curves were prepared via extraction by a similar method. Thus, the adsorption losses could be taken into consideration.

At the characteristic absorption maxima the calibration curves exhibited linearity for pyridine in the range 40–160 μg/ml, for pyridine-HCl in the range 60–230 μg/ml, and for 2-, 3- and 4-OH-pyridines in the range 50–190 μg/ml.

The UV spectra of pyridine and all the substituted pyridines were recorded at a concentration of 10^{-3} M in ethanolic solution at 190–330 nm, using a Unicam SP 1800 UV spectrophotometer.

RESULTS AND DISCUSSION

The absorption spectra of the various compounds in ethanol are depicted as follows: pyridine and monohydroxypyridines (Fig. 1); pyridine and monoaminopyridines (Fig. 2); pyridine and monocarboxypyridines (Fig. 3); and pyridine, 2,3-di-OH-pyridine and 3-OH-pyridine-N-oxide (Fig. 4).

The R_f values of pyridine and the monohydroxypyridines are listed in Table I.

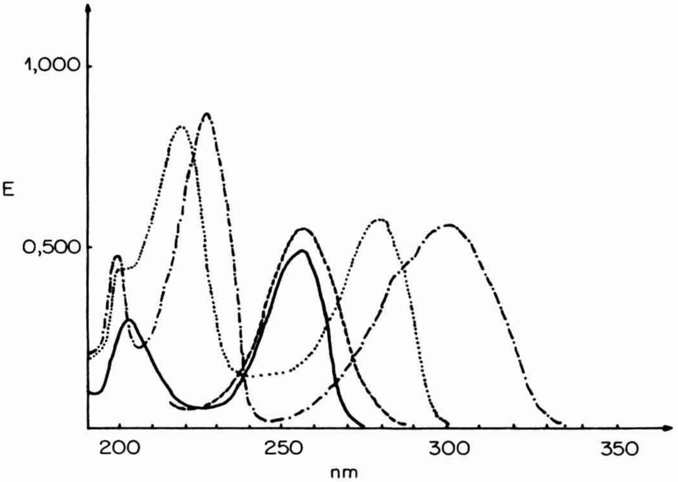

FIG. 1. UV absorption spectra of pyridine (—), 2-OH-pyridine (–·–·–), 3-OH-pyridine (···) and 4-OH-pyridine (---) at a concentration of 10^{-3} M in ethanol.

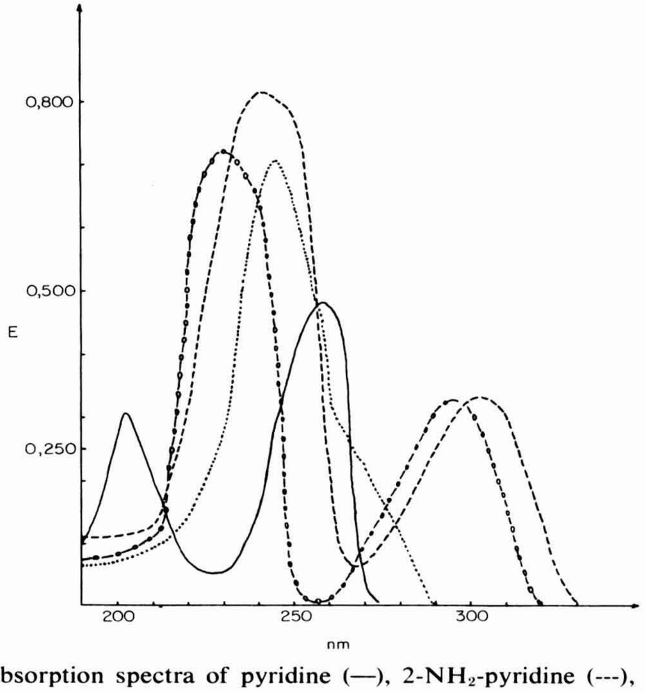

FIG. 2. UV absorption spectra of pyridine (—), 2-NH$_2$-pyridine (---), 3-NH$_2$-pyridine (-·-·-·) and 4-NH$_2$-pyridine (···) at a concentration of 10^{-3} M in ethanol.

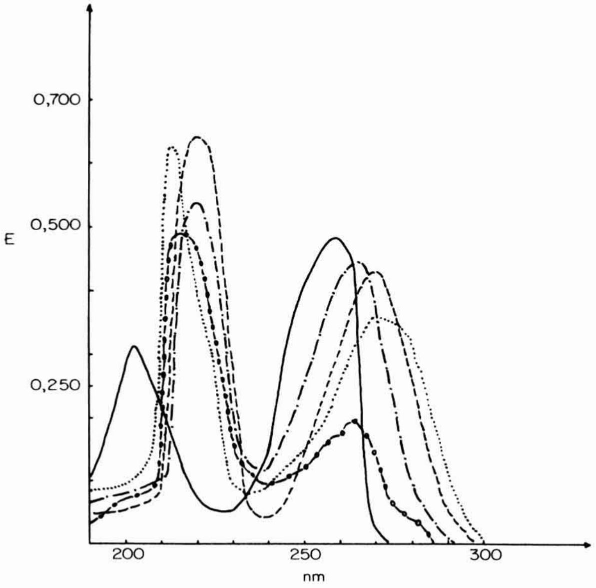

FIG. 3. UV absorption spectra of pyridine (—), 2-pyridinecarboxylic acid (–·–·–), 3-pyridinecarboxylic acid (–·–·–), 4-pyridinecarboxylic acid (···) and nicotinic acid N-oxide (---) at concentrations of 10^{-3}M in ethanol.

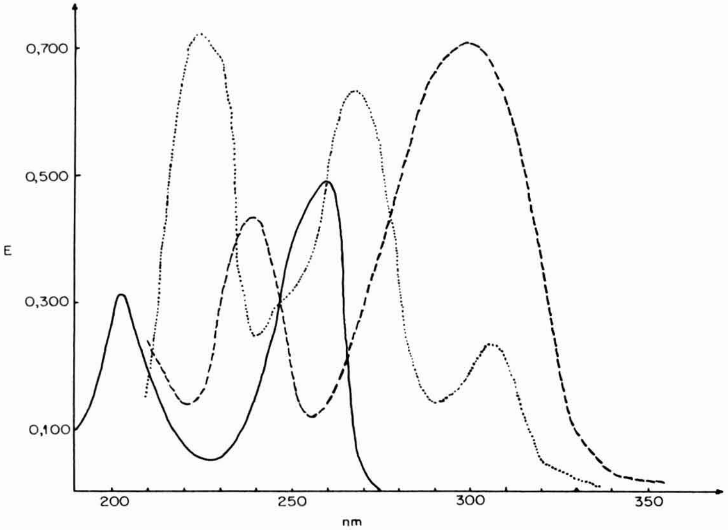

FIG. 4. UV absorption spectra of pyridine (—), 2,3-di-OH-pyridine (---) and 3-OH-pyridine-N-oxide (···) at a concentration of 10^{-3}M in ethanol.

TABLE 1

R_f VALUES OF MONOHYDROXYPYRIDINES IN SYSTEMS I–VI

	R_f values					
Substances	I	II	III	IV	V	VI
pyridine	0.80	0.56	0.50	0.73	0.78	0.73
2-OH-pyridine	0.53	0.62	0.12	0.41	0.59	0.80
3-OH-pyridine	0.71	0.47	0.45	0.43	0.66	0.88
4-OH-pyridine	0.07	0.39	0.04	0.17	0.19	0.37

Table II contains the R_f values of the monohydroxypyridines and some other monosubstituted pyridines in the running mixtures I and II.

Figures 5 and 6 show the spots on the chromatograms for the above two systems.

The R_f values of the monohydroxypyridines, 2,3-di-OH-pyridine and 3-OH-pyridine-N-oxide are given in Table III.

The isolation of the monohydroxypyridines and their recovery from the layer are pH-sensitive processes. Recovery is most favoured at pH 8.0.

Of the above-listed systems, I and II are the most suitable for the separation of the substituted pyridines.

SUMMARY

Investigations were made to determine the TLC properties of monosubstituted pyridines. The primary aim was to identify and quantitatively

TABLE 2

R_f VALUES OF MONOHYDROXYPYRIDINES AND OTHER MONOSUBSTITUTED PYRIDINES (MONOAMINES AND MONOCARBOXYLIC ACIDS) IN SYSTEMS I AND II

	R_f values	
Substances	I	II
nicotinic acid	0.92	0.69
isonicotinic acid	0.00	0.62
nicotinic acid N-oxid	0.00	0.33
picolinic acid	0.28	0.40
2-NH_2-pyridine	0.75	0.49
3-NH_2-pyridine	0.60	0.39
4-NH_2-pyridine	0.17	0.42
pyridine-HCl	0.05	0.24
2-OH-pyridine	0.53	0.62
3-OH-pyridine	0.71	0.47

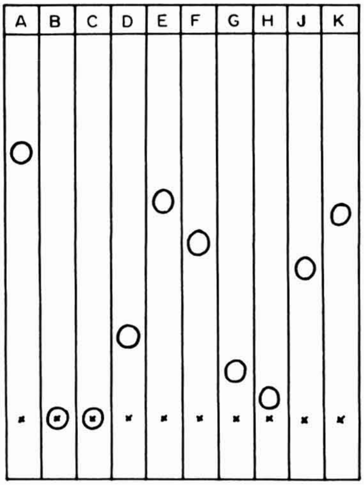

FIG. 5. TLC of nicotinic acid (A), isonicotinic acid (B), nicotinic acid N-oxide (C), picolinic acid (D), 2-NH_2-pyridine (E), 3-NH_2-pyridine (F), 4-NH_2-pyridine (G), pyridine-HCl (H), 2-OH-pyridine (J), 3-OH-pyridine (K) in solvent I.

determine the monohydroxypyridines formed during *in vitro* chemical and enzymatic hydroxylation experiments.

TLC studies were carried out not only on the monosubstituted pyridines, but also on 2,3-di-OH-pyridine and 3-OH-pyridine-N-oxide.

The UV absorption spectra of pyridine and its substituted derivatives were recorded at a concentration of 10^{-3} M in ethanol, and were compared.

The best TLC detection of these derivatives was elaborated too.

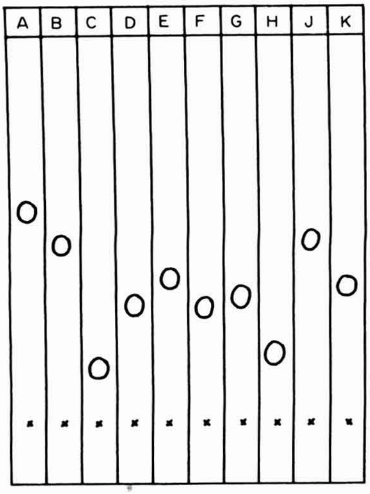

FIG. 6. TLC of the compounds in Fig. 5 in system II.

TABLE 3

R_f Values of Monohydroxypyridines, 2,3-di-OH-Pyridine and 3-OH-Pyridine-N-Oxide in Systems I–VIII

	R_f values							
	I	II	III	IV	V	VI	VII	VIII
Substances	in different solvents							
pyridine	0.80	0.56	0.50	.073	0.78	0.73	0.58	0.31
2-OH-pyridine	0.53	0.62	0.12	0.41	0.59	0.80	0.75	0.61
3-OH-pyridine	0.71	0.47	0.45	0.43	0.66	0.88	0.57	0.32
2,3-di-OH-pyridine	0.09	0.54	0.17	0.11	0.59	0.71	0.04	0.70
3-OH-pyridine-N-oxide	0.03	0.58	0.01	0.27	0.48	0.58	0.11	0.58

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MICROCHEMICAL JOURNAL 20, 483–491 (1975)

Extraction-Visible Spectrophotometric Method for Determination of Nitrate

Application to Water Analysis

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INTRODUCTION

The determination of nitrate is difficult in the presence of interfering ions. Numerous methods, based mainly on the nitration and oxidation of organic compounds, or on reduction of nitrate to nitrite or ammonia were described. Of particular importance is nitrate determination in drinking water. As, in excessive amounts it causes the infant methemoglobinemia, limitation of the nitrate concentration in drinking water is imposed. Several methods for nitrate control in drinking water have been recommended (*2,5,7,8*) but none of them is generally preferable.

In this paper an extraction visible-spectrophotometric method is described, based on the extraction of nitrate with tetraphenylphosphonium chloride (*1,3*) followed by the exchange of nitrate in the extract with intensely colored vanadium(V)-4-(2-pyridylazo)resorcinol (PAR) complex and spectrophotometric measurement of the latter. The coloration of the extract is very intensive and changes rapidly with the concentration enabling even visual colorimetric estimation of nitrate.

MATERIALS AND METHODS

Reagents

All the chemicals and solvents were of analytical purity. Chloroform with 0.5% ethanol was used without further purification and drying.

0.1 *M* sodium nitrate (100 ml) was prepared by dissolving 0.8499 g $NaNO_3$ in water. Solutions of lower concentrations were obtained by dilution.

$2 \cdot 10^{-2}$ *M* aqueous solution of tetraphenylphosphonium chloride (100 ml) was prepared by dissolving 0.7497 g $(C_6H_5)_4PCl$ in water.

$1.5 \cdot 10^{-2}$ *M* silver sulfate (100 ml) was obtained by dissolving 0.4677 g Ag_2SO_4 in water (kept in darkness).

Buffered solution of vanadium(V)-4-(2-pyridylazo)resorcinol complex $5 \cdot 10^{-4}$ *M* was prepared by mixing 100 ml $1 \cdot 10^{-3}$ *M* ammonium vanadate, 50 ml $2 \cdot 10^{-3}$ *M* PAR solution and 50 ml 1 *M* acetate buffer pH 6.

$1 \cdot 10^{-2}$ *M* ammonium vanadate (1000 ml) was obtained by dissolving 0.1170 g NH_4VO_3 in water.

$2 \cdot 10^{-3}$ *M* PAR solution (100 ml) was prepared by dissolving 0.0510 g monosodium salt of 4-(2-pyridylazo)resorcinol in water.

Buffer solution (pH 6) was prepared by addition of 1 *M* acetic acid to 1 *M* sodium acetate until a solution with pH 6 was obtained.

Apparatus

Spectra were recorded on a Perkin-Elmer Coleman 124 spectrophotometer. Absorbance measurements were taken on a Beckman DU-2 spectrophotometer, but a filter photometer might be used as well.

Procedure

The water sample (1 ml) was transfered into a separatory funnel, and 1 ml $2 \cdot 10^{-2}$ *M* tetraphenylphosphonium chloride and 3 ml $1.5 \cdot 10^{-2}$ *M* Ag_2SO_4 solution was added. Extraction was performed twice with 5 ml of chloroform by shaking for about 1 min. The layers were separated and the organic phases collected in another separatory funnel. 5 ml $5 \cdot 10^{-4}$ *M* vanadium–PAR complex buffered solution was added and shaken again for 1 min. The colored chloroform phase was filled up to 10 ml in a volumetric flask. The absorbance was measured at 560 nm in a 1-cm cell, against the blank prepared analogously with 1 ml of distilled water.

The described procedure is shown to be suitable for water samples containing not more than 30 mg/l of nitrate. Otherwise the sample has to be diluted under that level.

RESULTS AND DISCUSSION

The extraction of anions with tetraphenylarsonium and tetraphenylphosphonium cation was systematically investigated by Bock and co-workers (*1*). It was found then, that with these extractants, nitrate could be extracted into chloroform. Based on this extraction ability Burns, Fogg, and Willcox (*3*) have worked out a method for the determination of nitrate. The authors have not realized the initial idea to exchange the nitrate in the colorless tetraphenylphosphonium extract by an intensely colored anion and to measure the absorbance of the latter. Instead, they utilized the UV absorption maximum of tetraphenylphosphonium cation at 269 nm.

On the basis of our experience with the extraction behavior of intensely colored metal–PAR complexes, we proposed the suitability of vanadium–PAR complex for the exchange of nitrate in the colorless tetraphenylphosphonium extract. This proposal was confirmed, and the exchange was found to be fast and quantitative, leaving an intensive red-violet coloration of the extract. The color intensity is very stable and proportional to nitrate initially present in aqueous solution.

The method involves two stages:

1. extraction of nitrate with Ph_4PCl into chloroform according to equation:

$$[(C_6H_5)_4P]^+_{aq} + NO_3{}^-_{aq} \rightleftharpoons \underset{\text{colorless}}{[(C_6H_5)_4P]NO_{3\,org}}$$

2. the exchange of the nitrate in the colorless chloroform extract by vanadium–PAR complex (*6*):

$$[(C_6H_5)_4P]NO_{3\,org} + [VO_2R]^-_{aq} \rightleftharpoons \underset{\text{red-violet}}{[(C_6H_5)_4P]\ [VO_2R]_{org}} + NO^-_{3\,aq}$$

where R refers to divalent ion of 4-(2-pyridylazo)resorcinol, abbreviated as H_2R.

For the first stage of extraction, the principle as given by Burns and co-workers (*3*) was followed, but the working conditions were changed. The volumes were transfered from macro to semimicro scale, the concentration of reagents mainly increased, and silver ion was added as sulfate resulting with smaller blanks than acetate. Moreover, the volume ratio of the phases was changed, bearing in mind that the distribution ratio is not very high (*1,4*).

Spectra

The visible spectra of the colored $[Ph_4P][V–PAR]$ extract are shown in Fig. 1 along with the UV-spectra of the colorless $[Ph_4P]NO_3$ extract. Absorption maximum in the first spectra occurs at 558 nm with molar absorptivity $3.6 \cdot 10^4$. The same absorbance value was obtained in the wavelength range 555–562 nm. Molar absorptivity of the $[Ph_4P]NO_3$ extract at 269 nm is about one order of magnitude lower than that of the colored extract at 560 nm. Moreover, since the absorption maximum at 269 is very narrow it must be located very precisely in order to obtain reliable absorbance values. Thus, small shifts effected by solvents and impurities can cause significant spectrophotometric errors when nitrate is determined by the UV-spectra of $[Ph_4P]NO_3$ extract.

Effect of Reagent Concentration

The effect of concentration of tetraphenylphosphonium chloride and vanadium–PAR complex on the absorbance of colored extract was stud-

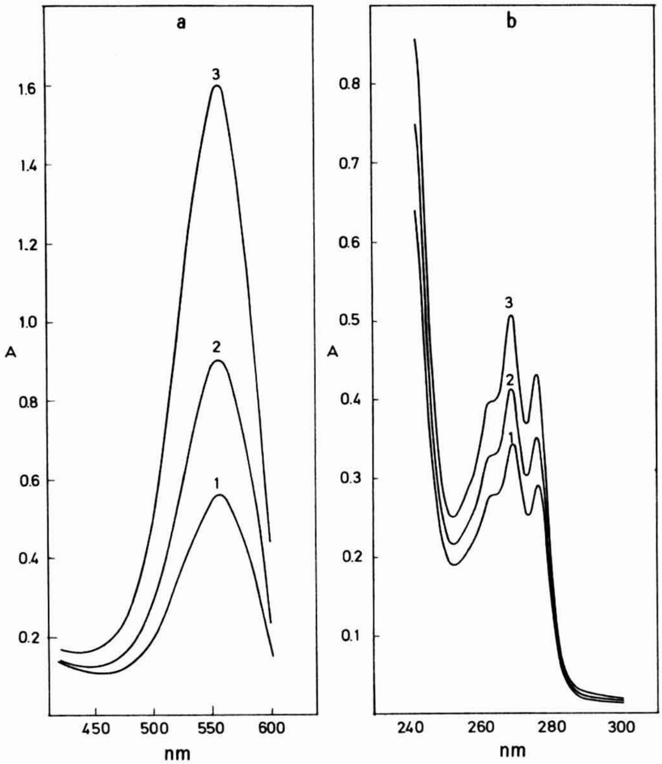

FIG. 1. The VIS spectra of [Ph_4P][V–PAR] extract (a) and the UV spectra of [Ph_4P]NO_3 extract (b) at three different concentrations.

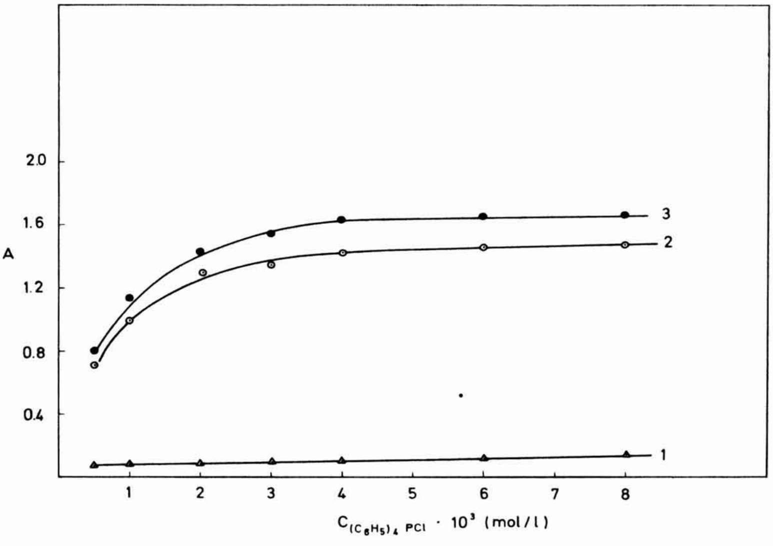

FIG. 2. The effect of $(C_6H_5)_4PCl$ concentration in the aqueous phase. 1. blank, 2. $NaCO_3$ solution, 3. drinking water sample.

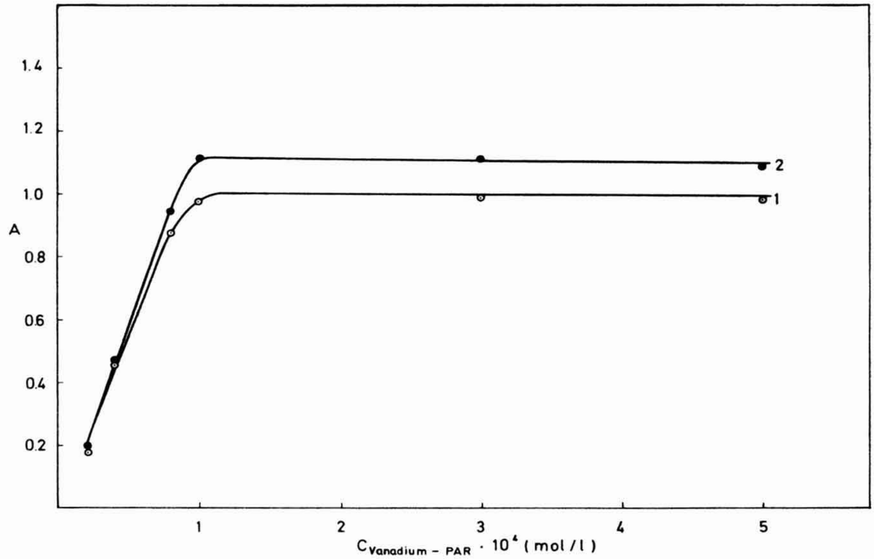

FIG. 3. The effect of vanadium–PAR concentration 1. $NaCO_3$ solution, 2. drinking water sample.

ied. As shown in Fig. 2, maximal and nearly constant extraction was obtained at relatively high concentration of tetraphenylphosphonium chloride. The quantitative exchange of nitrate with the vanadium–PAR complex in the second extraction stage was obtained at the fivefold molar excess of the latter. Above this value the absorbance of the extract was independent of vanadium–PAR complex concentration in the aqueous phase (Fig. 3).

To eliminate the interferences of chloride from tetraphenylphosphonium chloride, as well as chloride and some other interfering ions present in water samples, silver ion was added for precipitation and thus prevented their extraction. By addition of silver ion as sulfate, instead of as acetate as in the original UV-method, the blank absorbance of the colored extract was reduced to about one-third. This is in agreement with the distribution ratio values for tetraphenylphosphonium ion obtained under identical conditions. An excess of silver ion is necessary to minimize the extraction of interfering ions. For chloride, with highest solubility product, a constant small excess is required as shown in Fig. 4. The absorbance of the extract for blank and nitrate solution remained independent of silver ion concentration above Ag^+/Cl^- molar ratio 1.2.

Effect of pH

Extraction of nitrate and most inorganic anions as tetraphenylphosphonium salts is practically independent from the pH (*1,3*).

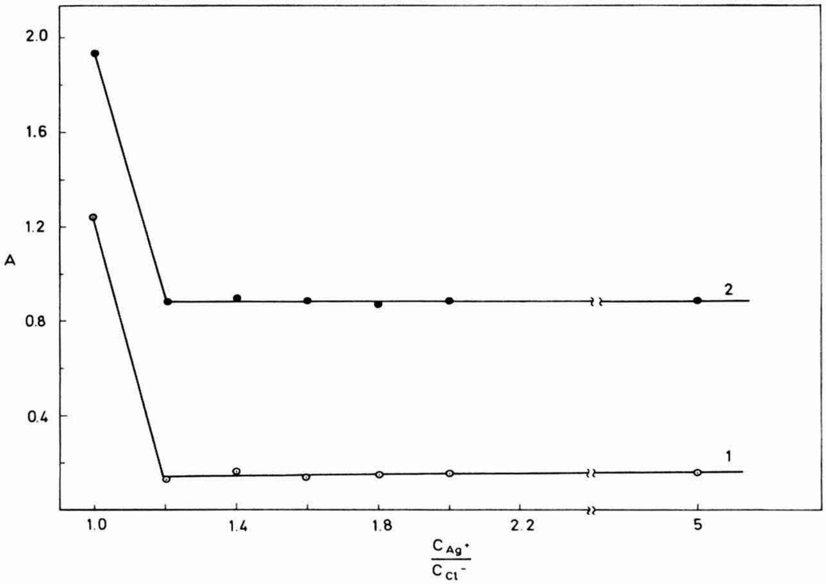

FIG. 4. The effect of silver ion concentration. 1. blank, 2. $NaNO_3$ solution.

Thus pH of solution in the first extraction stage of the method described, is not critical. However, in the second extraction stage, the pH of aqueous phase must be about 6 because of pH-dependent equilibria in V-PAR complex formation and extraction. The maintenance of optimal pH was achieved with acetate buffer, whose anions are present in solution at nitrate exchange stage do not change the absorbance of the extract.

Other Extraction Variables

By shaking the systems for 1–20 min in the first and second extraction stages, respectively, it was shown that equilibria were achieved after a 1-min shaking period. The extraction of nitrate with tetraphenylphosphonium chloride is far from being complete in single extraction, even with a large excess of extractant. It also significantly depended on the volume ratio of solvents. It was found that nearly complete extraction of nitrate was achieved by double extraction with equal volumes of chloroform and at phases volume ratio 1 : 1.

Interferences

The spectral interferences are less pronounced in VIS method described here than in the original UV-method, but the interferences of foreign ions are the same, because there is no essential difference in the first extraction stage. Cations and most of anions present in drinking water are not extractable and do not interfere (*3*). Although chloride,

TABLE 1
THE EFFECT OF INTERFERING IONS

Interfering ion (mg/l)	NO_3^- added (mg/l)	NO_3^- found (mg/1)	
Cl^-			
0	12.40	12.42	+0.02
106		12.58	+0.18
462		12.14	−0.24
570		13.60	+1.20
Br^-			
0	12.40	12.30	−0.10
240		12.14	−0.24
1040		12.08	−0.32
1280		12.59	+0.19
I^-			
0	12.40	12.51	+0.11
381		12.08	−0.32
1650		12.08	−0.32
2030		12.80	+0.40
NO_2^-			
0	12.40	12.48	+0.08
0.46		12.56	+0.16
0.92		12.58	+0.18
1.15		13.40	+1.40
2.30		14.53	+2.13
4.60		15.05	+2.65

bromide, and iodide are extractable they can be removed by addition of silver ion. Large amount of these anions can be tolerated as evident from Table 1. Nitrite is extracted with Ph_4PCl, but to a much smaller extent than nitrate. Moreover it is present in water at relatively small concentration and does not interfere seriously up to nitrite/nitrate ratio 1/10.

Beer's Law, Sensitivity, Reproducibility, and Accuracy

The calibration graph shown in Fig. 5 proved to be linear all over the range suitable for spectrophotometric measurements, corresponding to 1–30 mg NO_3/l of water sample. The effective molar absorptivity of the colored extract at 560 nm is $3.6 \cdot 10^4$ 1 $mol^{-1}cm^{-1}$.

The reproducibility of the results expressed as relative SD is about ±2% in the range between 5 and 30 mg NO_3/l and about ±7% in the lower nitrate concentration range.

The accuracy of the method was tested with series of prepared samples as well as with sample of drinking water. The composition of prepared samples and results obtained are given in Table 2. The sample of drinking water with optimum nitrate concentration considering brucin,

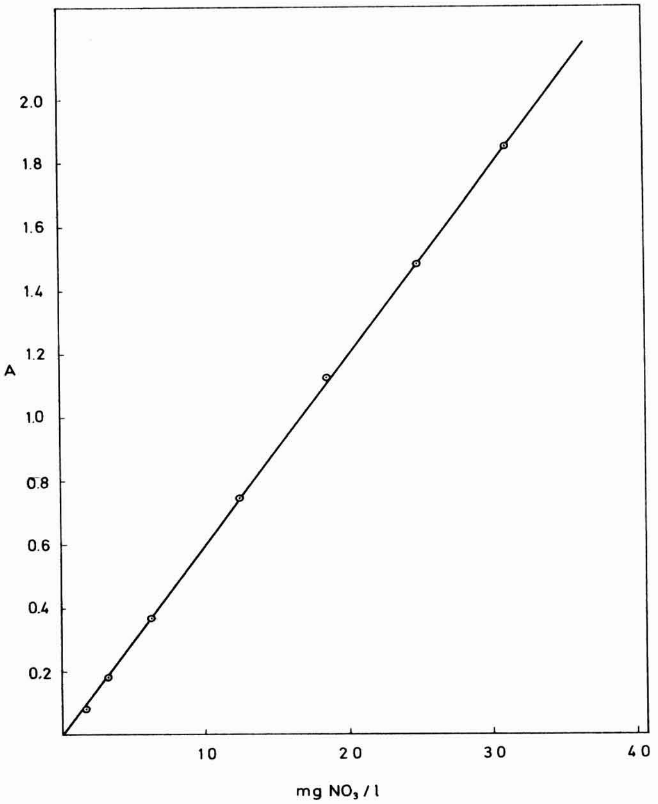

FIG. 5. The calibration graph.

TABLE 2
ANALYSES OF KNOWN SOLUTIONS[a]

NaNO$_3$ added to:	Concentration (mg/l) NO$_3^-$ 12.4		NO$_3^-$ found (mg/l)
NaHCO$_3$	HCO$_3^-$	100	12.3
		200	12.4
NaHPO$_4$	HPO$_4^-$	10	13.0
		100	11.8
(NH$_4$)$_2$SO$_4$	NH$_4^+$	1	12.9
		100	12.2
Synthetic water 1	Cl$^-$	320	12.8
	Total hardness	83	
	Carbonate hardness	20	
Synthetic water 2	Cl$^-$	130	
	Total hardness	100	12.5
	Carbonate hardness	14	
Synthetic water 3	Cl$^-$	260	
	Total hardness	80	12.0
	Carbonate hardness	175	

[a] Synthetic water samples contain Ca^{2+}, Mg^{2+}, Na$^+$, Cl$^-$, SO$_4^{2-}$, HCO$_3^-$, and CO$_3^{2-}$. Total and carbonate hardness given in mg CaO/1, Total hardness determined by EDTA-titration.

$Ph_4P\text{-}NO_3$, UV, and our method was chosen and analyzed. With five not simultaneous determinations, the following results were obtained: 19.8 ± 0.3, 19.2 ± 0.5, and 19.6 ± 0.3 mgNO_3/l with brucine, UV, and the method described here, respectively. It is evident that the accuracy and reproducibility of all three methods do not differ greatly in the optimum concentration range. However, the UV method is far less accurate and less reproducible at lower and the brucine method at higher concentration.

SUMMARY

An extraction-visible spectrophotometric method for determination of nitrate is described. The method is based on the extraction of nitrate with tetraphenylphosphonium chloride and the exchange of the nitrate in the colorless $Ph_4P\text{-}NO_3$ extract by intensely colored vanadium(V)-4-(2-pyridylazo)resorcinol complex. The color intensity of this extract is very stable and reproducible. Absorption maximum appears at 560 nm with molar absorptivity $3.6 \cdot 10^4$ l mol^{-1}cm^{-1}. The application of the method to water analysis was investigated and the procedure for determination of nitrate in drinking water is developed.

ACKNOWLEDGMENT

The authors are grateful to Mrs. M. Kaleb for carrying out numerous analyses.

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MICROCHEMICAL JOURNAL **20**, 492–494 (1975)

Procedure for Preparing Infrared Spectra on Gas Chromatographic Fractions

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There is a need for a technique which produces infrared spectra from small quantities of volatile materials which elute from a gas chromatograph. When individual components are present in quantities greater than 50 μg, the sample can be condensed and run neat as a capillary film between salt plates. When only smaller quantities are available, the most convenient procedure is to dissolve the condensed sample in a solvent such as chloroform or carbon tetrachloride and place the solution in an ultramicrocavity cell. Solvent band compensation becomes very difficult under these conditions.

To achieve maximum infrared sensitivity, it is necessary to confine the sample to a small cross-sectional area. Sparagana and Mason (*2*) concluded that KBr techniques are the most satisfactory for preparing spectra of solid material when only microquantities are available. Curry *et al.* (*1*) described a syringe technique which produced acceptable micro KBr pellets from 0.5 μg of certain barbiturates. These authors indicated, however, that incorporation of components with high vapor pressure resulted in a very substantial sample loss and concluded that their technique has greatest utility for high boiling materials.

The technique described below produces acceptable spectra from samples as low boiling as 150°C and has the additional advantages of a low sample size requirement and absence of spurious solvent bands. In this procedure the sample is actually trapped on KBr and this matrix is then sandwiched between two windows of KBr which reduces losses by vaporization.

EXPERIMENTAL

Materials and Apparatus

A Wilks Model 34 Micro KBr Press Kit was used to prepare the micro samples utilizing the 2-mm die set (Wilks Scientific Corp., South Norwalk, Conn.). Spectra were obtained on a Perkin-Elmer Model 521 Spectrophotometer utilizing a 4× refracting beam condenser. KBr powder was obtained from Harshaw Chemical Company, Solon, Ohio.

Micro KBr Technique

Powdered KBr is lightly packed into a 5-cm length of melting point capillary (about 1 mm inside diameter) to give a plug 2–3 mm long located near one end. When the component of interest elutes from the gas chromatograph, the capillary is inserted into the exit port so that the KBr section is external to the chromatograph. A tight seal between the gas chromatograph and capillary is necessary due to the back pressure created. After the peak elutes, the tube is removed, and the KBr is extruded through the end of the capillary which was adjacent to the gas chromatograph. The KBr is deposited in a well which is formed in the 2-mm die as described below. (A paper clip makes a convenient extruding device.) The die is lightly pressed and then a small portion of fresh KBr is added, and the pellet pressed with normal force. In this fashion the sample is sandwiched between pure KBr and is not expressed out of the pellet during pressing. The pellet will be somewhat cloudy if the collected sample is a liquid at room temperature, but it will pass sufficient infrared energy to produce a usable spectrum. The die is placed in a beam condenser and the spectra prepared according to the manufacturer's instructions for microsamples.

The well in the 2-mm die is formed by pressing a small amount of pure KBr in the die. A minimum amount of KBr should be used, just enough to produce a solid, very thin clear window.

RESULTS AND DISCUSSION

Using the technique described, it has been possible to obtain usable infrared spectra on 10 μg or less of a sample. Figure 1 gives a typical spectrum obtained from 10 μg of methyl hexanoate (bp 150°C). Spectral features expected, such as a strong carbonyl band (1740 cm^{-1}) methyl and methylene groups (2960, 2940 cm^{-1}) and a C–O stretch (1170 cm^{-1}), are all readily apparent. When materials with a boiling point less than 200°C are being trapped, the collection efficiency can be markedly

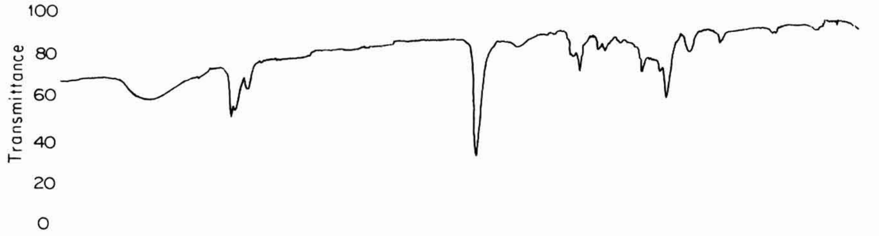

FIG. 1. Infrared spectrum of 10 μg methyl hexanoate.

increased by holding a chip of dry ice against the KBr section of the glass capillary.

Moisture must be scrupulously avoided when handling the KBr or a broad band will be found at 3450 cm^{-1}. For the same reason, the KBr used should be as dry as possible. A control pellet, made by the procedure herein described, will normally exhibit a –OH band of about 10%–25% T and can be used for a background correction. Since the pellets are normally not transparent, there is some loss of energy at higher frequencies, however, this loss should not exceed 25% T.

It should be stressed that when the KBr is extruded from the capillary, it must be through the end of the tube which was previously adjacent to the exit of the gas chromatograph. In this fashion, any component which condensed before reaching the KBr will be recovered and transferred to the die.

SUMMARY

A convenient technique is described for the preparation of microinfrared spectra on materials which elute from a gas chromatograph. The technique involves trapping the sample on KBr and then pressing this material in a die between two portions of pure KBr. Samples as small as 10 μg grams and as low boiling as 150°C produce acceptable spectra. Since no solvents are employed, solvent band compensation is unnecessary.

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MICROCHEMICAL JOURNAL 20, 495–510 (1975)

An Automatic One- to Eight-Sample Applicator for Fast Qualitative and Quantitative Microelectrophoresis of Plasma Proteins, Hemoglobins, Isoenzymes, and Cross-Over Electrophoresis

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INTRODUCTION

In 1963 Grunbaum *et al.* (*6*) described in this journal a new design of a compact apparatus for electrophoresis, utilizing cellulose acetate as the supporting medium. This apparatus, now manufactured and distributed by Beckman Instruments, Inc., of Fullerton, California, is widely used in both clinical and research laboratories. One important feature of the apparatus is a controlled tensioning device that holds the cellulose acetate membrane taut and parallel to the base of the unit without any support in the center of the membrane. This arrangement is important in that it prevents pooling of liquid and retains a uniform moisture content in the membrane. A second feature is an indexing device operating semiautomatically which provides for placing a given sample on any one of 24 fixed positions on the membrane.

Since eight samples are electrophoresed simultaneously, this is equivalent to placing a row of samples in any one of three different positions relative to the cathode and anode. A single-sample applicator used with the above system permits an even, reproducible, quantitative application of a 0.25 μl sample.

It is time consuming to apply one specimen at a time. Also, the samples applied first are subject to local diffusion and mobility (due to convection currents) while the subsequent samples are being applied. This becomes serious when high resolution is required in comparative electrophoresis of unknown specimens.

Several types of multiple-sample applicators have been proposed recently by manufacturers of electrophoresis equipment. None of these have an integrated electrophoretic assembly which takes into account the indispensable features in the design of the equipment published in 1963 (*6*).

This paper describes the uses of a newly designed eight-sample applicator with a complimentary 32-sample holder and an indexed cell cover. This paper describes the usefulness of the eight-sample applicator in clinical, forensic, genetics, and biology laboratories, and microchemistry, in general.

Equipment

The electrophoretic cell and bridge assembly, which holds the cellulose acetate membrane taut at controlled tension, is unchanged at present. The design which modifies the earlier equipment features an eight-sample applicator, a new cell cover, and a new sample holder. All of these parts, when assembled, are well integrated.

The eight-sample applicator. The eight-sample applicator, shown in Fig. 1, will pick up eight different specimens from corresponding wells

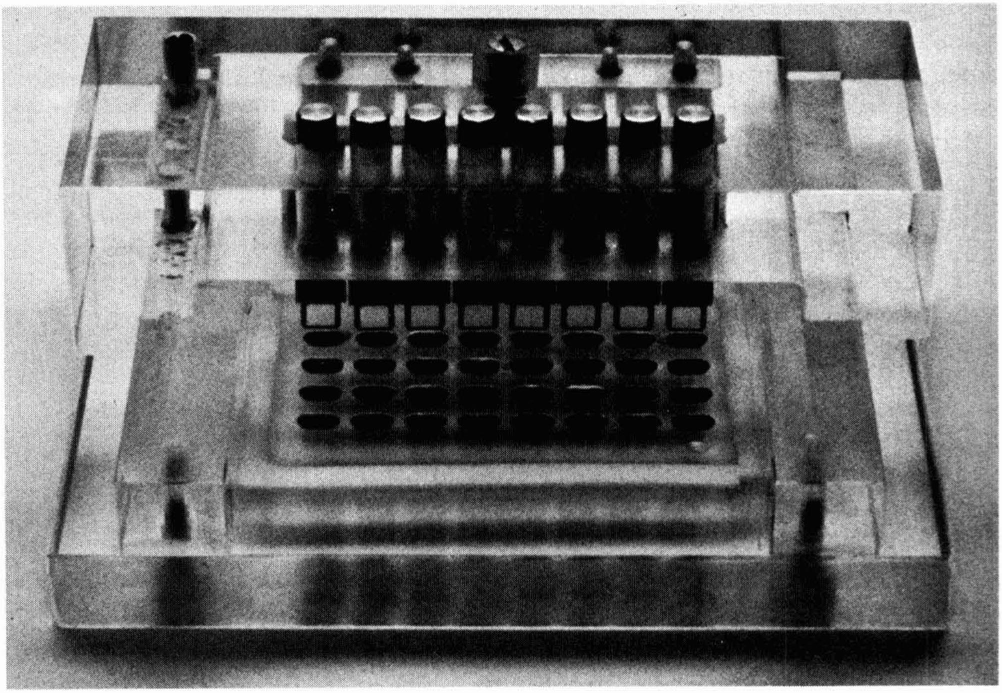

FIG. 1. The eight-sample applicator rests on top of the sample holder in row D. The applicator tips are in their normal retracted position. When the central button is slowly depressed, all the individual tips touch the surface of the fluid in the corresponding wells simultaneously and fill by capillary action. Each one of the eight applicator tips can also be actuated separately by depressing the shaft in which the tip is inserted. In front and to the right of row A, a dimple indicates the position of sample No. 1. Around the periphery of the sample wells is a disposable filter paper gasket saturated with saline.

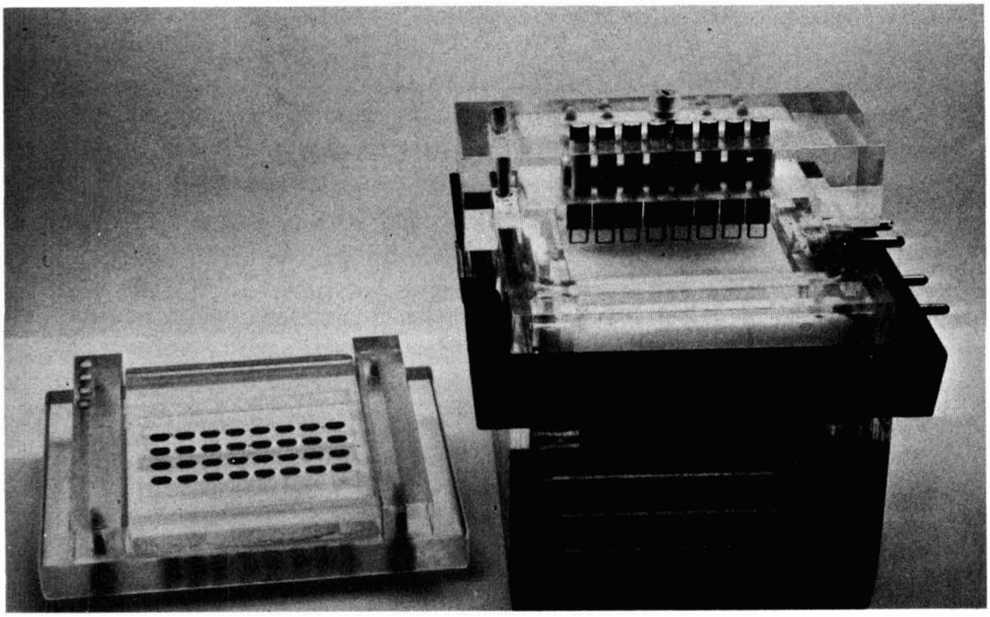

FIG. 2. To the right the eight-sample applicator, loaded with samples, rests in one of the 10 predetermined positions along the special cell cover. By fully depressing the central button, the eight applicator tips fall freely onto the supporting medium (cellulose acetate membranes or agarose gel). The samples are transferred by capillarity. The sample holder is pictured to the left. It is covered by a heavy plate resting on a saturated filter paper gasket to prevent evaporation from the specimens in the wells. Four index holes on the left shoulder of the sample holder determine the row of samples that will be transferred.

containing hemolysate, serum, plasma, whole blood, bloodstain extract, or any other tissue extract, as if they were one single sample. When the central button is depressed, the applicator tips touch the surface of the fluid and fill by capillary action. Normally the tips hold between 0.2 and 0.25 μl, depending on the viscosity of the liquid sample. The applicator is then placed on the specially modified cell cover, shown in Fig. 2, so that it fits a predetermined slot or position relative to the electrodes. All eight specimens are then transferred in one motion. When the central button is fully depressed, each one of the applicator tips falls freely and independently onto the cellulose acetate membrane. This feature is of utmost importance for the best and most complete sample transfer. If the eight-sample applicator were rigid, some of the samples might "hang up" because of the occasional imperfections in the tautness of a membrane over its entire width. Many times it is necessary to apply increasing amounts of a given specimen onto the same spot on the supporting medium, such as in a case of very low solute concentration; then each of the eight-sample applicators can be actuated separately. Each of the applicator tips can be readily replaced with tips of larger volume capacities, or tips can be inserted at alternate positions to allow longer sample application zones.

The same multiple applicator may be used with agarose gel or starch gel as the supporting medium. No prior slot formation is required, as is usually the case with various gels, because the applicators cut through the gel and transfer all eight specimens in a single operation.

The cell cover. Fractionation of proteins or some other charged molecules on certain media, especially on cellulose acetate, is a function of the voltage, electroendosmosis, and a number of other controllable variables. Thus, the resultant vector of an electrophoretic separation is the sum of the electrophoretic vector and electroendosmotic vector. Therefore, the placement of the sample on the supporting medium relative to the cathode and anode will affect and determine the degree of separation and resolution of the individual components. Flexibility was built into the cell cover (Fig. 2) to permit placing samples in any of ten fixed positions spaced at equal intervals between the cathode and anode. Since the total distance of an electrophoretogram in many instances occupies only a small portion of the supporting medium, it is possible to electrophorese not just eight samples, but 16 or 24 at a time by selecting the appropriate slots along the cell cover. Such utilization of space in an electrophoretogram is not only extremely economical and fast, it is also more accurate and reproducible in comparative electrophoresis. For example, in phenotyping the genetic variants of enzyme systems (*2*) it is imperative that identification of an unknown genotype be compared with known standards under absolutely identical conditions. This requires the simultaneous application of all samples and concurrent electrophoresis of whatever variables are involved at the time.

The sample holder. There is a need in the modern clinical laboratory to determine the presence and quantity of specific blood proteins which may reflect pathologic conditions if they vary significantly from a homeostatic base line. In addition to the major protein fractions, such as albumin, alpha, beta, and gamma globulins, it is now customary to analyze for such specific proteins as the isoenzymes of lactic acid dehydrogenase, alkaline phosphatase, lipoproteins, creatine phosphokinase, and perhaps others. All of these proteins can be determined by simple electrophoresis. Normally such determinations are done in succession and, depending on the method used, may take from several hours to several days, and require comparatively large volumes of blood. The blood or serum must be kept alternately at room temperature and under refrigeration. Some of the parameters of interest may undergo some degree of deterioration, the extent of which is unpredictable. To avoid this uncertainty and be able to produce a true physiologic profile in the shortest possible time with the minimum amount of blood sample, a procedure is being developed in this laboratory to analyze all samples concurrently for as many parameters as possible.

The sample holder shown in Figs. 1 and 2 serves an important function in this respect. The holder presently contains four rows of eight wells each. Each well is labeled by both letter and number, such as A-1 or D-8. The well is oval in shape and rounded on the bottom; it holds up to 25 μl of the sample. Disposable capillaries are used to charge the wells with fresh samples. When the desired number of samples are placed into the wells (up to 32), electrophoresis can proceed within less than a minute. Prior to filling the wells, electrophoretic cells are set up containing the proper buffers needed for the determination of the desired constituents. Each cell is properly labeled. At this moment the eight-sample applicator picks up eight individual specimens and transfers them simultaneously to the supporting medium and at a predetermined position (from 1 to 10), closer to either the negative or positive electrodes, as described above.

Since the electrophoretic methodologies employed use a very small portion of the sample in the well, the remaining sample should be protected against dust or concentration by evaporation. This is done by placing a disposable filter paper gasket saturated with 0.9% saline around the perimeter of all the wells and covering the gasket with a heavy plate. The air space above the sample wells is thus very small and saturated with moisture. The sample holder with the remaining specimens can then be placed in the refrigerator for temporary storage in case it should become necessary to repeat the analysis of some specimens. Even if the determinations must be done in succession because of lack of adequate equipment to perform all analyses concurrently, the specimens will remain well preserved up to 24 hr.

Forensic Science Applications

The sample holder is useful in the criminalistics or forensic science laboratory. In civil or criminal cases involving bloodstains, it is of primary importance to establish the species origin of the blood. If the blood is not human, knowledge of the contributing species may still help in the investigation. When a bloodstain is submitted for analysis, it must first be extracted because the chemically reacting constituents are present only in the soluble portion. Bloodstain scrapings or particles are placed directly into individual wells in the sample holder and covered with a solvent such as water, saline, or some buffer. Depending on the age and dryness of a stain, the penetration of solvent may require anywhere from several minutes to perhaps 12–16 hr. Occasionally it may be necessary to suck the fluid in the well into a capillary and expel it several times. This facilitates the extraction. Eventually, the undissolved portion of the stain settles to the bottom of the well in the sample holder. The supernatant liquid can now be subjected to two analytical procedures, namely, cross-over electrophoresis and phenotyping of polymorphic enzymes.

1. Cross-over electrophoresis. Cross-over electrophoresis will determine the species origin. The principle of this procedure depends on an immunochemical reaction between an antigen, the unknown stain, and known species specific antisera. This is conveniently and rapidly performed on cellulose acetate using the eight-sample applicator in conjunction with the sample holder. In cross-over electrophoresis, antigens and antibodies are placed in a supporting medium in close proximity and are forced to move towards each other, thus forming a complex molecule which is eventually seen as a precipitin. An antibody having little negative electric charge in a basic buffer migrates towards the cathode (the negative electrode) due to electroendosmosis. Antigens having greater electrophoretic mobility migrate towards the anode in spite of electroendosmosis. Conventionally, cross-over electrophoresis is carried out in agar gel or agarose gel. Round wells are formed and filled on the anodic side with antiserum and with antigen on the cathodic side. The filling of individual wells can be a tedious and lengthy process. The slightest damage of the gel by nicking will certainly produce artifacts. With the equipment described here, up to 24 unknown specimens can be tested against an antiserum in a very uniform, sensitive, and rapid fashion. No prior slot formation is required. Samples are placed simultaneously on the cellulose acetate membrane, and, in the next slot on the anodic side, a parallel row of antiserum is applied. This arrangement utilizes the maximum immunochemical reactivity of the available molecules and improves the sensitivity over that in gels. However, while gels, being transparent, produce visible precipitin bands within a few minutes to an hour, the cellulose acetate membrane must first be deproteinized and stained in order to see the antigen–antibody complex. The total time involved using either gel media or cellulose acetate membranes is about the same. Since the eight-sample applicator has been successfully used in conjunction with agarose gel for the analysis of lipoproteins (Fig. 6), the system should equally and successfully work for cross-over electrophoresis in gels.

2. Phenotyping of polymorphic enzymes. While the unknown bloodstain extracts (supposedly human) are in their respective wells in the sample holder, the "individualizing" factors (*7*) can now be determined. It is important to establish what the probability is that the bloodstain came from a particular individual. This is done most simply by electrophoresis of the genetically controlled polymorphic enzymes and proteins. Examples of applications of the equipment described here for phenotyping of polymorphic enzyme systems was published by this author elsewhere (*2*,*3*).

RESULTS AND DISCUSSION

Figures 3, 4, 5, 6, and 7 illustrate the utilization of the electrophoretic accessories described.

Protein Electrophoresis

Figure 3 is a cellulose acetate electrophoretogram of a commonly used clinical procedure for fractionating plasma proteins. The row of eight plasmas from eight different individuals to the left is identical with that on the right. These samples were applied to the same membrane in units of eight. It is clear from the picture that the resolution of some seven protein bands (an eighth band, a prealbumin, is visible on the original membrane) differs little between corresponding samples in the two positions on the membrane from plasmas of the same individual. The band

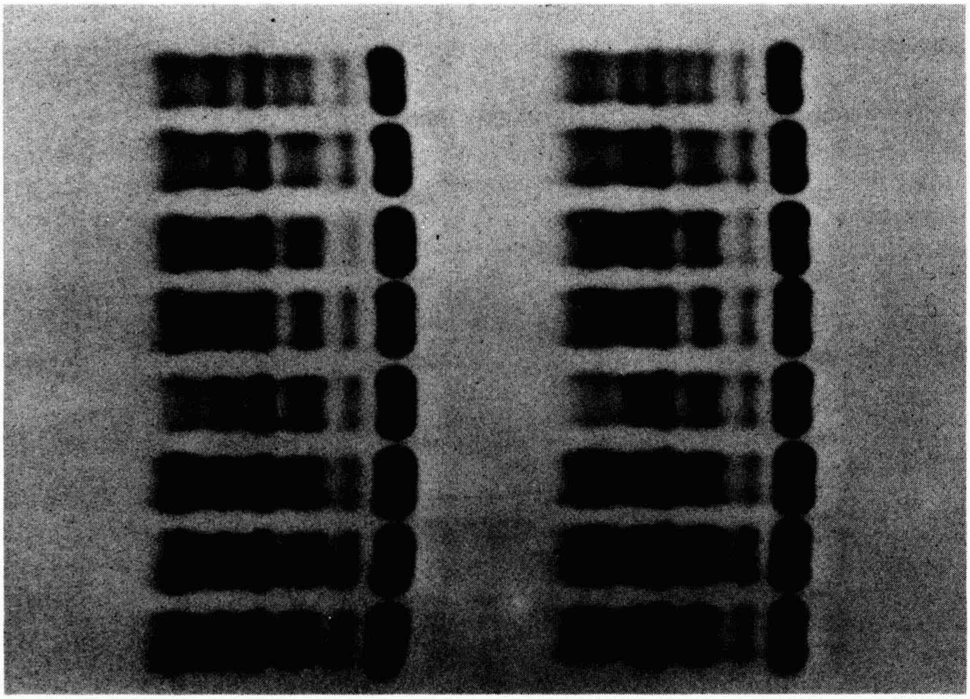

FIG. 3. An electrophoretogram of 16 plasma proteins on a single cellulose acetate membrane. The index hole seen in the membrane at the lower left corner indicates position of sample No. 1. The cathode is to the left. The row of samples from eight different individuals on the cathodic side is identical to that on the anodic side. The resulting resolution of the individual zones was not appreciably affected.

separation is quite adequate for good densitometric quantification. It is obvious, therefore, that 16 different samples can be electrophoresed and quantified using a single membrane. The general procedural details for buffers, staining, and clearing were described earlier (*6*). In this electrophoretogram, the electrophoresis time was 20 min at 250 volts. The lower left perforation in the cellulose acetate membranes indicates the position of sample No. 1. In this manner, all the other samples are identified by their position. In all the electrophoretograms shown, the cathode is on the left and the anode on the right.

Hemoglobin Electrophoresis

Figure 4 shows a hemoglobin electrophoretogram of 24 red blood cell hemolysates run simultaneously. These samples were stained with Ponceau S containing 5% sulfosalicylic acid and show no abnormal hemoglobin or genetic hemoglobin variants. This procedure is especially useful as a screening method in search of hemoglobinopathies, such as

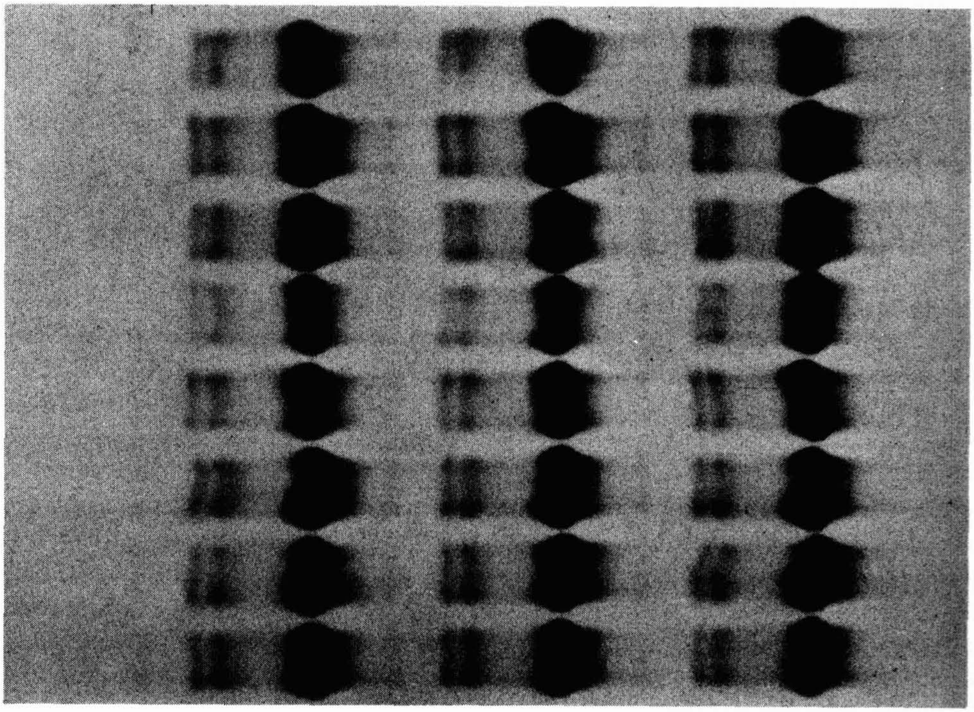

FIG. 4. An electrophoretogram of 24 saline washed and defatted red blood cell hemolysates. Hemoglobin A (the predominant one in human blood) moving towards the anode is seen as a heavy zone in three rows. The other zones are nonhemoglobin proteins from the red blood cells stained by the nonspecific Ponceau S dye. For specific hemoglobin staining see text.

thalasemias (*1*) and sickle trait. If specific staining is desired for hemoglobin, then *O*-dianisidine (*5*) or benzidine should be used, according to the following procedure.

Staining Procedure for Specific Determination of Hemoglobins

1. Electrophorese with any desired buffer.
2. Following electrophoresis, immerse cellulose acetate membrane in a 5% solution of sulfosalicylic acid (or 7.5% trichloroacetic acid) for 3–5 min.
3. Rinse membrane in 5% acetic acid.
4. Stain in a *fresh* solution consisting of:
    (a) 10 ml 3% acetic acid.
    (b) 5 ml 0.2% *O*-dianisidine solution (in methanol). (Benzidine can be substituted for *O*-dianisidine.)
    (c) 1 ml 3% hydrogen peroxide.
    (d) 1 ml 1% sodium nitroferricyanide.
5. Stain in above solution only for as long as needed to clearly visualize the hemoglobin bands. Do not overstain.
6. Rinse well in several changes of 5% acetic acid and follow with a water wash.
7. Dry between clean blotters under pressure and place in a clear plastic envelope.

Isoenzymes of Lactic Acid Dehydrogenase (LDH)

In Fig. 5 an electrophoretogram is shown of 16 samples of LDH. On the lefthand side, eight plasmas were applied simultaneously. Each plasma specimen applied was about 0.25 μl. On the righthand side, the same plasma specimens were placed onto the cellulose acetate membrane as two successive applications of eight samples each, or about 0.5 μl volumes. This was done to show that it is possible to repeat sample application without distorting the resolution. The plasma samples in positions 1, 2, 7, and 8 are from four different humans, and the plasmas in positions 3, 4, 5, and 6 are from subhuman primates. It is clearly visible that each plasma, whether 0.25 μl or 0.5 μl, was completely fractionated into the five typical isoenzymes. The fastest (anodic) mobility is shown by LDH-1 and the slowest mobility, almost stationary, by LDH-5. Since the eight-sample applicator leaves no application mark on cellulose acetate membranes, there is no fear of interfering artifacts.

The buffer system was prepared as follows:

1. 4.77 g Trizma base (Sigma No. T-1503) was weighed and dissolved in about 500 ml distilled water, then adjusted to pH 8.6 with Trizma-HCl (Sigma No. T-3253) by adding it as crystals. It was then brought up to 1 l volume with distilled water. This buffer is 0.037 *M*.

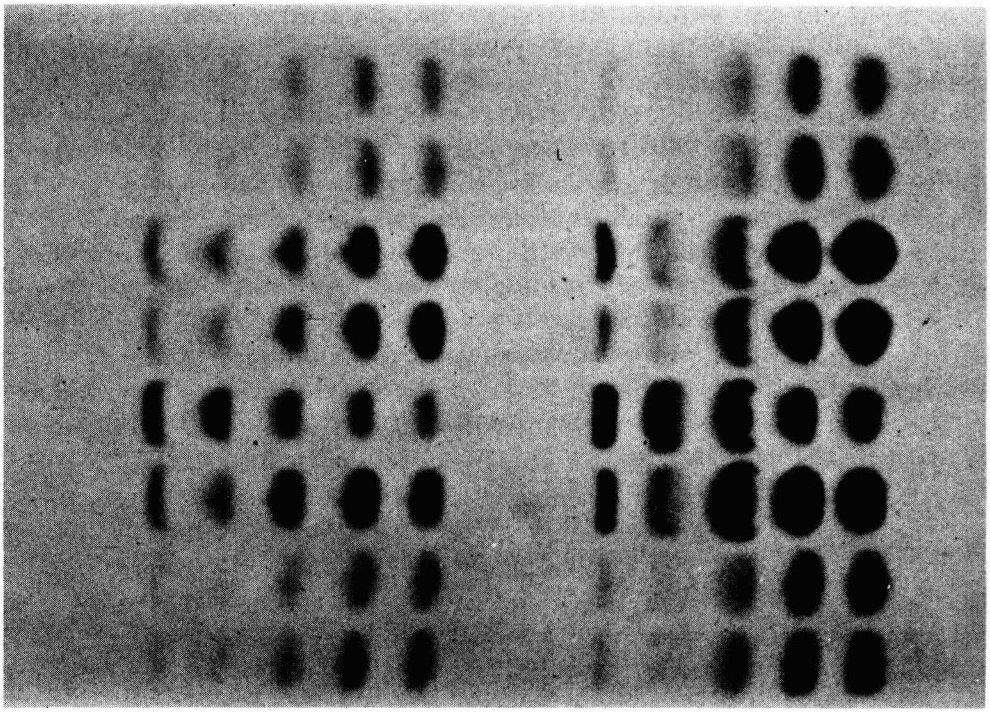

FIG. 5. An electrophoretogram of 16 plasma specimens of the five isoenzymes of lactic acid dehydrogenase in a single cellulose acetate membrane. The plasmas in both rows are identical in origin and in arrangement. The plasma sample volume in the left row is 0.25 μl per sample and in the right row 0.5 μl per sample (applied in two successive applications). Samples in positions 1, 2, 7, and 8 are from different humans and those in positions 3, 4, 5, and 6 from four different subhuman primates (pig-tailed monkeys). The separation of isoenzymes was completed in 20 min of electrophoresis. The density of zones and resolution in the 0.25 μl samples is adequate for good densitometric quantification. If total LDH enzyme is also determined, the densitometric measurements of the individual isoenzymes can be expressed in absolute terms.

2. 2.76 g diethylbarbituric acid and 15.40 g sodium diethylbarbiturate were weighed and dissolved in 1 l distilled water. This buffer was 0.075 *M* and pH 8.6.

3. Equal volumes of buffers 1 and 2 were mixed; the resultant mixed buffer had an ionic strength of 0.056 *M*.

Electrophoresis was performed at 250 volts constant voltage for 20 min.

For the color development it was convenient to use a kit available from Dade (Division American Hospital Supply Corp., Miami, Florida 33152). The color reagents, consisting of tetrazolium dye, phenazine methosulfate, and NAD, were mixed with a lactate salt in a pyrophosphate buffer. A 2% noble agar is dissolved separately. When the temperature of the agar was reduced to 55°C, the color reagent was mixed with the agar and quickly poured out onto a disposable square plastic petri dish (Lab-Tek Products No. 4021, Division Miles Laboratories Inc., Westmont, Illinois). The gel and contents solidified readily. At the completion of electrophoresis, the cellulose acetate membrane was lifted from the bridge and placed face side down onto the gel reaction mixture without allowing entrapment of air bubbles. The technical details for this kind of procedure were described and discussed by Grunbaum in the determination of the genetic variants of phosphoglucomutase (*2*).

Since proper color development time is usually a function of the isoenzyme concentration, no fixed time can be assigned. The investigator must watch the color development, and when he feels that there is sufficient intensity for measurement of either quality or quantity, the cellulose acetate membrane should be removed and washed in plenty of tap water, followed by a rinse in 5–10% acetic acid and again in water. If the membrane is allowed to react for too long with the substrate, the intensity of the bands will increase, but the background will also stain and thus decrease contrast.

Lipoprotein Electrophoresis in Agarose Gel

Lipoproteins have been successfully fractionated and stained on cellulose acetate membranes (*4*). Occasionally, however, the staining can be very messy and unpredictable. The difficulty may be due to variations in the quality of cellulose acetate between manufacturers and may also depend on the kind of additives impregnated to enhance the wetting properties. At this time, the best reproducible results are obtained in lipoprotein electrophoresis when performed on agarose gel. Figure 6 shows a lipoprotein electrophoretogram of 16 plasmas applied with the eight-sample applicator in two discrete positions along the gels. The plasma origin and arrangement was identical with that described for LDH in Fig. 5. The agarose gel is a product of Marine Colloids, Inc., and was rehydrated from a dry state into about a 1-mm thickness. First, the dry agarose was placed in distilled water where it swelled up to its ultimate thickness in about 60 min. Subsequently it was placed in an equilibration buffer for 15 min prior to electrophoresis. This equilibration buffer was 0.05 *M* barbital to which 1% bovine serum albumin and 0.3% EDTA were added. After 30 min electrophoresis at 150 volts (constant voltage) in a 0.05 *M* barbital buffer, the proteins in the gel were fixed for 30 min in a 1 : 1 solution of 95% ethanol and 5% acetic acid. The agarose gel (which is supported on a Mylar sheet) was then dehydrated for 15 min at 75°C and placed for 10 min into a solution of Sudan Black B stain containing Triton X-100 and sodium hydroxide.

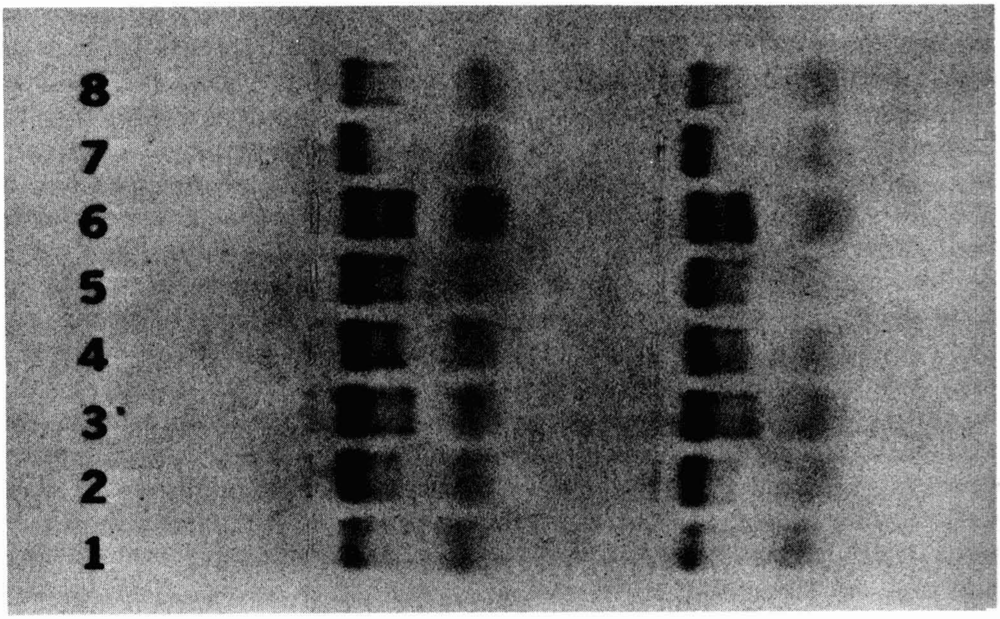

FIG. 6. A rehydratable agarose gel electrophoretogram of 16 plasma proteins placed as two applications of eight specimens each. The sample origins and arrangement are identical with that shown in Fig. 5. The anode is to the right. The origin of sample application is to the left of the intensely stained beta lipoprotein fractions. A faint mark resembling the shape and size of the eight-sample applicator tips forms an unavoidable artifact when used with gels.

The stained gel was then rinsed for 3–5 min in the fixing-rinsing solution of 1 : 1 95% ethanol and 5% acetic acid.

An artifact caused by the application tips can be noted in Fig. 6. Such artifacts are unavoidable when gels are used. No interference, however, occurred since the lipoprotein fractions have moved away adequately from the origin. The three major fractions of lipoproteins, namely the alpha, pre-beta, and beta are sufficiently separated for either typing or quantification. The anodic alpha lipoprotein fraction has the fastest mobility.

It is of interest to note that the subhuman primates (the pig-tailed monkeys) exhibit considerably higher intensities (concentrations, supposedly) for an equal sample volume than that from humans in both LDH isoenzymes and lipoprotein fractions.

Beckman Instruments, Inc. provided the rehydratable agarose gel, the buffers, and a kit for the staining method (Beckman Instruments Inc., Part No. 655338). The Beckman Company has similar kits for the isoenzymes of LDH and alkaline phosphatase using rehydratable agarose gel. A rigid type disposable plastic eight-sample applicator is also available from Beckman. However, the application of samples with the Beckman gel applicator is very costly in terms of money, time, and sample size. The Beckman eight-sample plastic applicator must be overfilled with about 10–20 times the amount it will actually transfer onto the gel. It is impossible to assess the actual sample volume placed in the gel. The eight-sample applicator must then be discarded, thus creating sanitary and pollution problems.

The flexible eight-sample applicator described in this paper requires only a rinse of the tips in a 1% solution of Tween 20 (Sigma Chemical Co. No. P-1379) and blotting on filter paper between two successive applications of sample.

While use of agarose gel for lipoprotein fractionation has some merit at present, its use for LDH and alkaline phosphatase isoenzymes is inferior compared to cellulose acetate. The Beckman kits and methods for LDH and alkaline phosphatase on agarose gel were compared side by side with cellulose acetate as the supporting medium, using the electrophoretic accessories described above. In the case of LDH isoenzymes, the separations were good on agarose gel, but the method required a running time of about 3–4 hr, compared to less than 60 min on cellulose acetate. With alkaline phosphatase isoenzymes, the cellulose acetate was found to be far superior. Pathologic serums on cellulose acetate yielded as many as six bands compared to three on agarose gel from the same serum sample. The advantage of agarose gel is its transparency; it can be scanned photometrically. On the other hand, cellulose acetate has a higher sensitivity, but it is more difficult to scan because it is optically dense. It should be more important to a clinical chemist to visualize a pathologic isoenzyme rather than measure its relative concentration. Data comparing cellulose acetate and agarose gel will appear in a separate publication.

Cross-over Electrophoresis

The technique of cross-over electrophoresis, also called electroendosmophoresis, is also becoming an indispensible clinical procedure for screening people to find carriers of passive or active disease agents. An example of such clinical application is the testing of blood for the Australia antigen (hepatitis-associated antigen). Theoretically, any protein molecule for which a monospecific antiserum can be produced will form a precipitin when reacted by the method of cross-over electrophoresis. The success of this method depends to a very large extent on the medium in which it is done and on the geometry of the applied samples. This was taken into consideration in the design of the eight-sample applicator, the sample holder and the cell cover.

In Fig. 7 is an illustration of the capability of performing cross-over electrophoresis on cellulose acetate membranes in a fast, reproducible

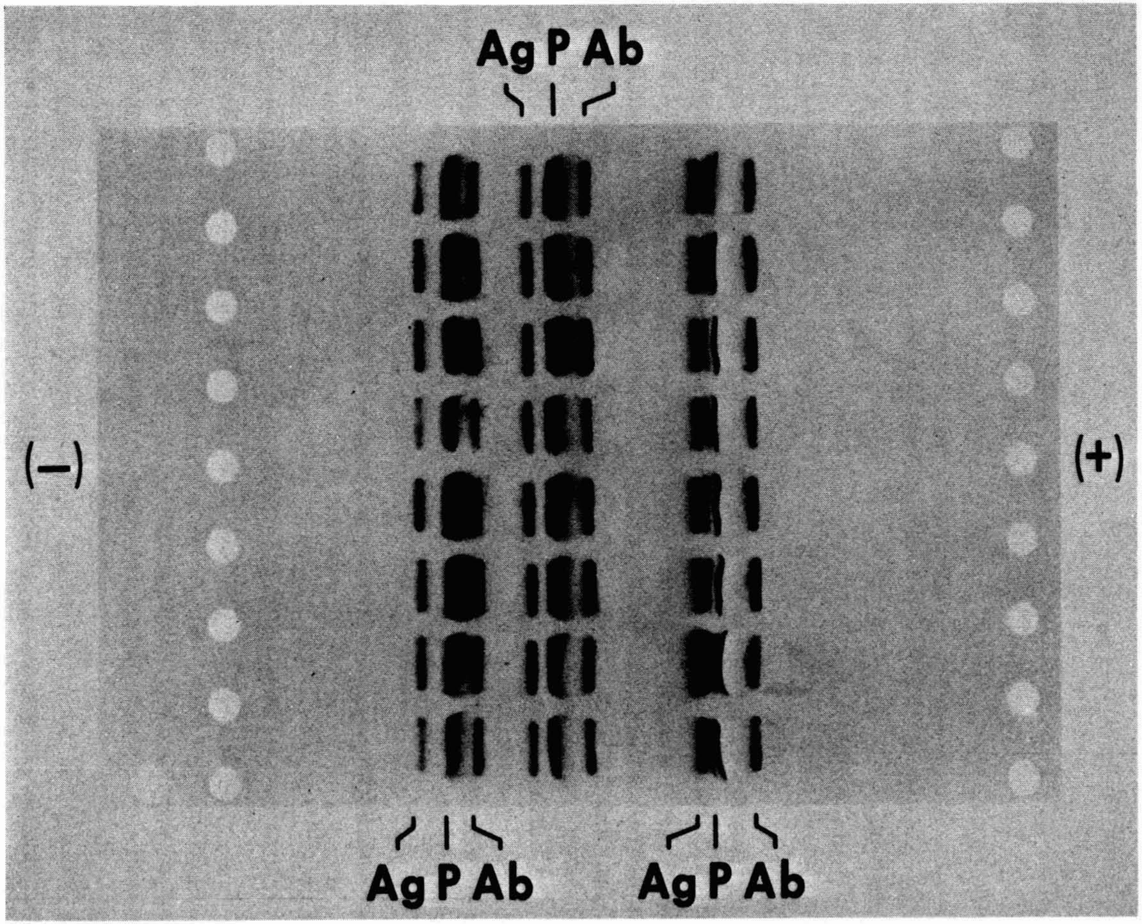

FIG. 7. An illustration of cross-over electrophoresis of 24 specimens on cellulose acetate membranes. Here the eight-sample applicator was utilized to transfer in rapid succession three rows of eight each of sera (antigens) and three rows of eight each of antisera. Ag, antigen; Ab, antiserum (or antibody); P, precipitin zone. For details see text.

manner. Shown in Fig. 7 is a single cellulose acetate membrane used for the cross-over electrophoresis. Twenty-four serums (antigens) were placed on the cathodic side and 24 antiserums placed in juxtaposition on the anodic side. In the center between them are seen the precipitin bands. Only six out of the 10 indexing slots were used in this experiment. All the samples were applied in units of eight in very rapid succession.

The particular cell cover with 10 predetermined positions and a one- to eight-sample application system permits considerable flexibility in placing the antigen and antibodies with varying distances between them. For instance, a number of antigens can be placed in a staggered position on the membrane relative to a straight line of samples of antisera, or vice-versa. Many combinations are possible in order to find the most efficient juxtaposition for a given system.

The cross-over electrophoresis shown in Fig. 7 was performed in Tris–glycine buffer of pH 8.4 (21.8 g glycine and 4.5 g hydroxymethyl aminomethane). In this buffer it was possible to use 500 volts (constant voltage) without overheating. After 5 min electrophoresis, the membrane with the bridge holder was placed in a bath of 0.9% saline as a deproteinizing solution and washed for 20–30 min with several changes of saline. The precipitin bands were then made visible by staining in Ponceau S.

SUMMARY

An electrophoresis sample application system was developed which provides for fast, reliable, and economical microanalyses to measure both quality and quantity of many blood proteins. The system consists of a flexible eight-sample applicator with a well integrated complimentary 32-sample holder and cell cover. The system permits simultaneous pickup and transfer of one to eight samples and the concurrent electrophoresis of up to 24 specimens on a single cellulose acetate membrane or an agarose gel supporting medium. Examples and illustrations of the quality of analytical results for the fractionation of plasma proteins, hemoglobins, lactic acid dehydrogenase isoenzymes, lipoproteins, and cross-over electrophoresis are described. The resolution of constituents is adequate for comparative analyses in the forensic science laboratory and for quantification in most medical clinical analyses.

ACKNOWLEDGMENT

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A Specific Microcrystalline Test for Indolamine Derivatives*

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INTRODUCTION

With the exception of certain phenethylamines and some other unsaturated compounds isolated from *Myristica* sp., the majority of psychomimetic compounds are indolamines. A number of such compounds have been the subject of abuse within the last decade, and legislation has been enacted to control their use and possession.

Various techniques have been employed for the analysis and identification of these materials. In general, the techniques used are those which the forensic chemist has found to be applicable to the examination of other classes of controlled substances, and include color tests, thin-layer chromatography, and infrared spectroscopy.

Although microcrystalline tests have been described for indolamine derivatives (*1*,*2*), this approach has not found the utility that such tests hold for the identification of barbiturates and common narcotics. This reflects, in part, the rather low sensitivity of previously described microcrystalline tests to LSD, the principal indolamine compound of abuse.

MATERIALS AND METHODS

In the case of undiluted material, approximately 1 mg is deposited on a clean microscope slide. A drop of 10% HCl is added, which dissolves most indolamine salts; into this is drawn a drop of 15% ethylenediamine in H_2O. An amorphous precipitate forms immediately, followed by the formation of characteristic crystals within 1 min. The crystals are best observed between crossed polars at a magnification of 100–150×.

With mixtures containing only a few per cent of indolamine derivative, the presence of common excipient materials tends to prevent crystallization, and the procedure becomes more involved. Purification can be effected by means of solvent–solvent extraction or by thin-layer chromatography. If thin-layer chromatography is routinely used for the iden-

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* Contribution No. 175 from the Criminalistics/Forensic Science Program, University of California, Berkeley.

tification of indolamines, the microcrystalline test may be conducted subsequently as an adjunctive method of analysis.

In the solvent–solvent extraction, the suspected material from half a tablet is ground to a powder in a 10 × 75-mm tube using a glass rod. To this powder, or an equivalent volume of powder taken directly from a capsule, is added 1 ml of 2% KOH and the tube is shaken until the mixture attains uniform consistency. The free base is then extracted with two 1-ml portions of chloroform. The chloroform extract is removed, placed in a small beaker, and 3 drops of *n*-heptane is added (*3*). The solution is then evaporated until only the heptane remains. This yields a concentrated solution of the material which can be spotted onto a clean microscope slide and reacted as described above or purified further by thin-layer chromatography.

In particularly intractable cases, it is necessary to further purify the material prior to microcrystalline testing. This is accomplished using a 10-cm 250-μm thick Silica Gel G plate which is spotted with the heptane solution described above and developed with $CHCl_3:CH_3OH$ (9:1). The indolamine may be detected with long wavelength ultraviolet, scraped from the plate, extracted with two 1-ml portions of the $CHCl_3:CH_3OH$ solution, evaporated to a small volume, spotted onto a microscope slide with a disposable micropipette and reacted with the ethylenediamine reagent.

RESULTS AND DISCUSSION

Characteristic crystals, as illustrated in Figs. 1–11, were obtained with the following indolamine compounds: 1. bufotenine, 2. *N*,*N*-diethyltryptamine (DET), 3. gramine, 4. harmine, 5. harmaline, 6. lysergic acid, 7. lysergic acid diethylamide (LSD), 8. α-methyltryptamine, 9. psilocin, 10. psilocybin, 11. tryptamine.

The following compounds were tested with the ethylenediamine reagent with negative results, the compounds giving an oil, an amorphous precipiatate, or not reacting at all: 1. dihydroergocrystine (no ppt.), 2. *N*,*N*-dimethyltryptamine (DMT) (oil), 3. ergocryptine (no ppt.), 4. ergotamine (no ppt.), 5. ergotaminine (amorphous ppt.), 6. 10-hydroxy lysergic acid diethylamine (no ppt.), 7. ibogaine (amorphous ppt.), 8. indole (oil), 9. N-isopropyl,N-methyltryptamine (amorphous ppt.), 10. *iso*-lysergic acid diethylamide (no ppt.), 11. lysergic acid ethanolamide (oil), 12. methylergonovine (oil).

The sensitivity of the test was determined to be on the order of 50 μg for LSD. It was observed, however, that the presence of *iso*-LSD produces a total inhibiting effect when the percentage of this isomer exceeds 20%. With respect to specificity, it appears that the ethylenediamine reagent yields unique crystals with those compounds that do react.

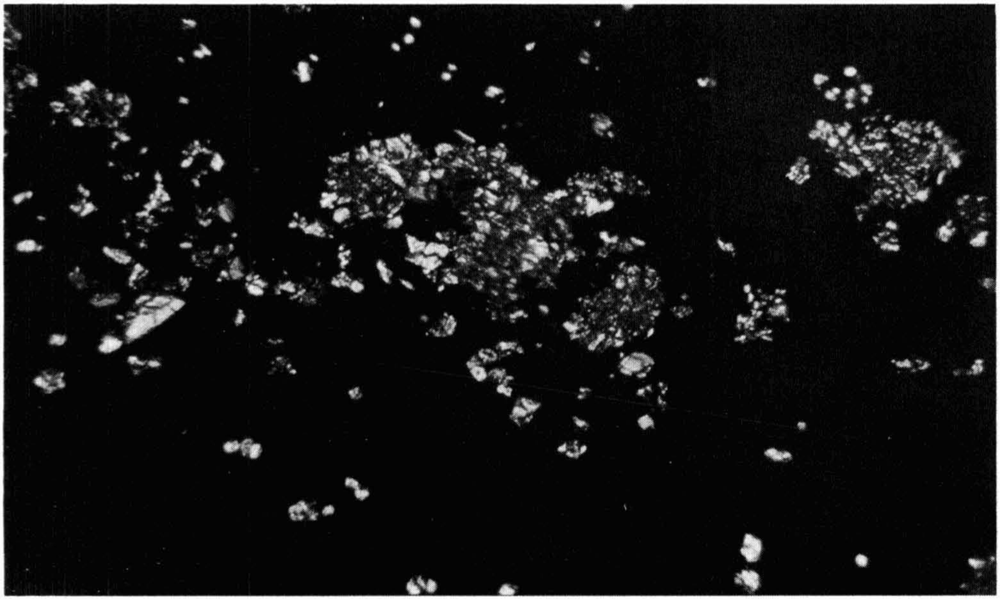

FIG. 1. Bufotenine in 15% ethylenediamine.

FIG. 2. N,N-Diethyltryptamine in 15% ethylenediamine.

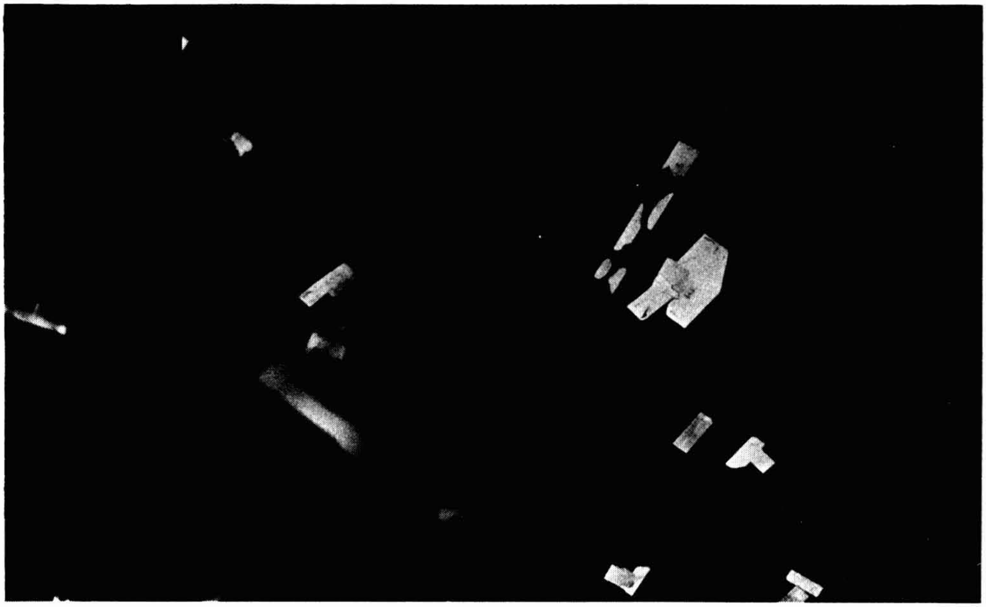

FIG. 3. Gramine in 15% ethylenediamine.

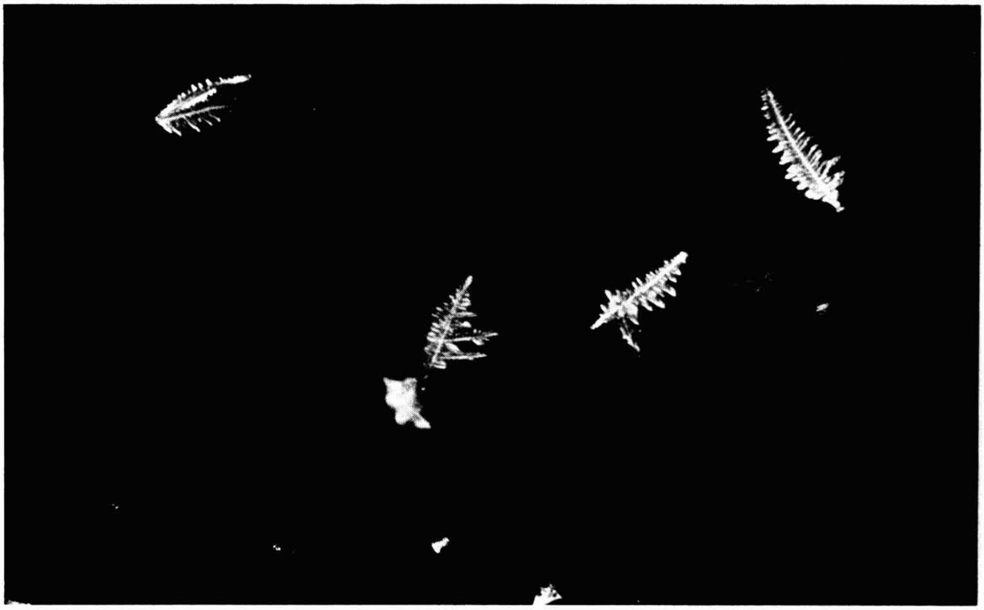

FIG. 4. Harmaline in 15% ethylenediamine.

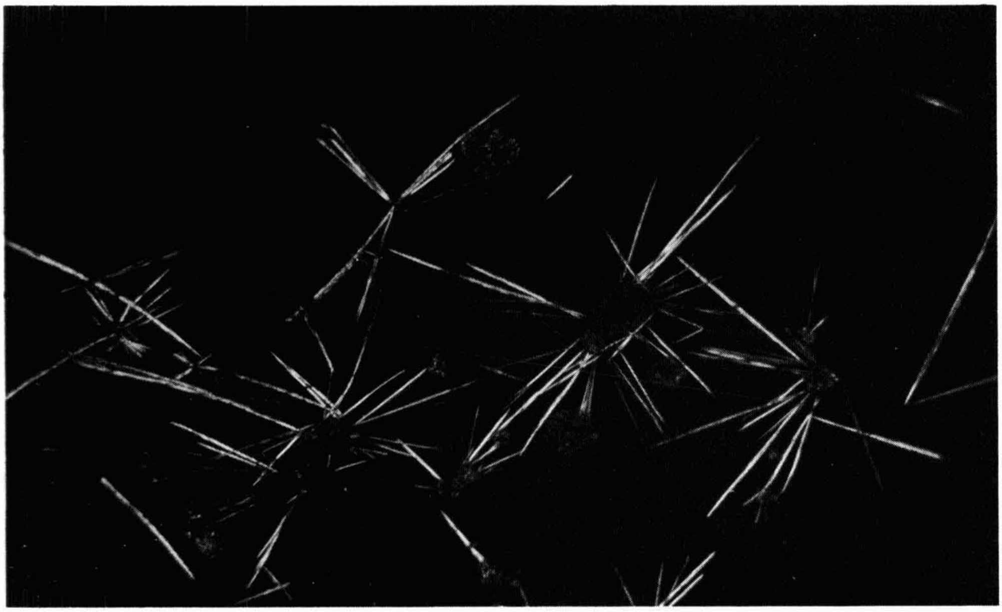

FIG. 5. Harmine in 15% ethylenediamine.

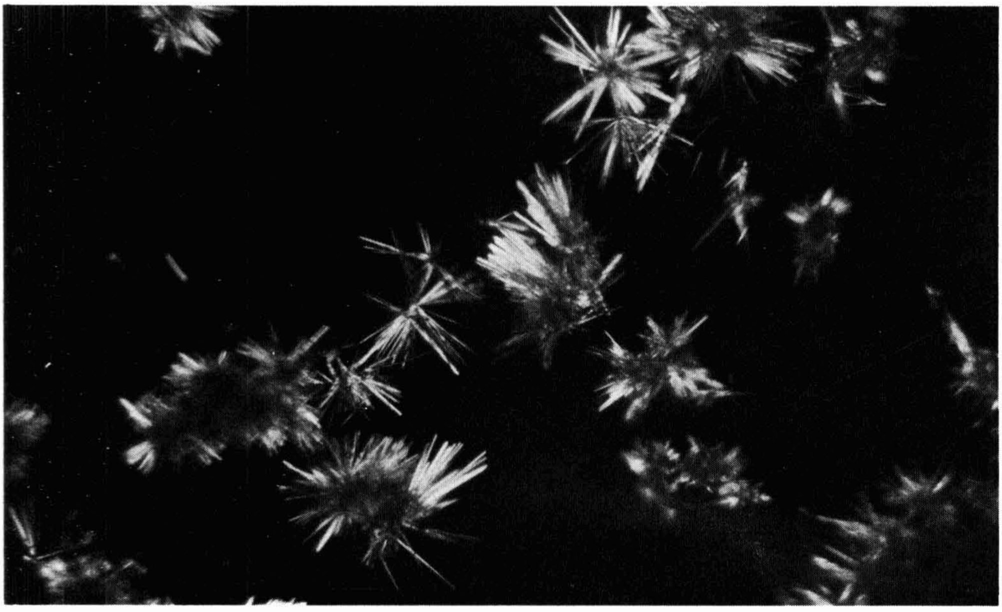

FIG. 6. Lysergic acid in 15% ethylenediamine.

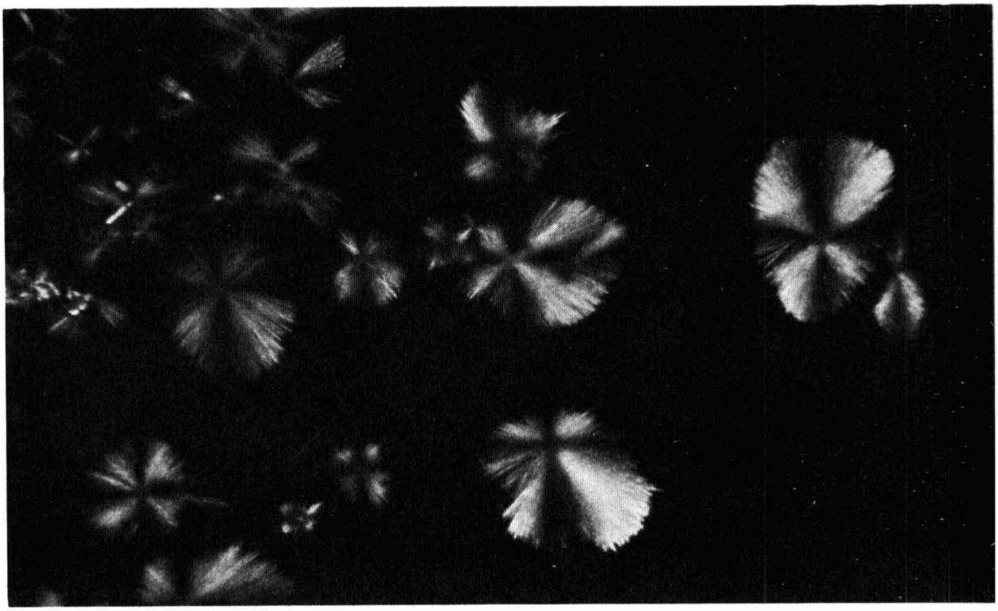

FIG. 7. Lysergic acid diethylamide in 15% ethylenediamine.

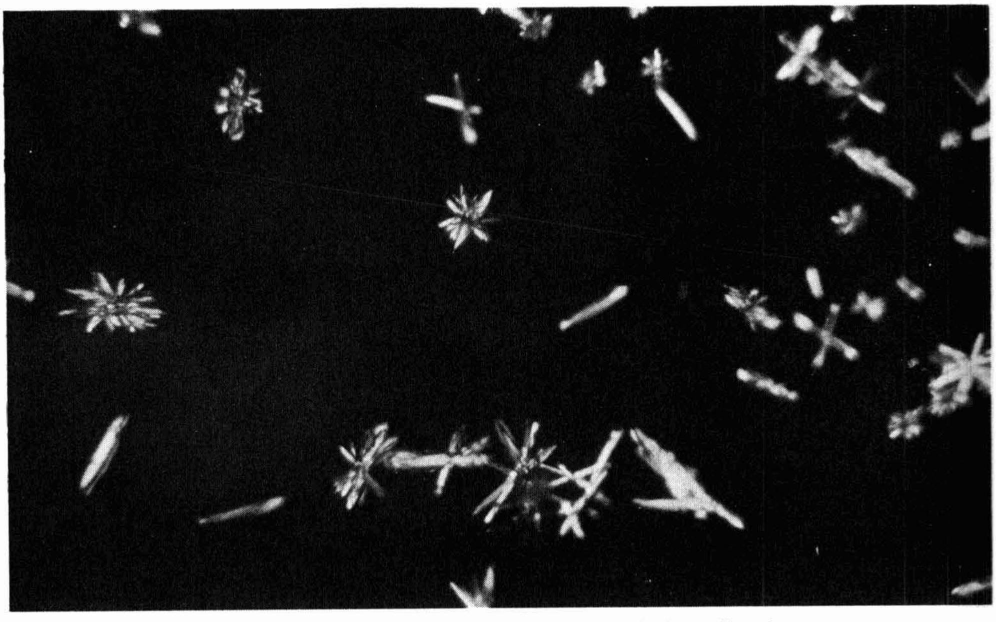

FIG. 8. α-Methyltryptamine in 15% ethylenediamine.

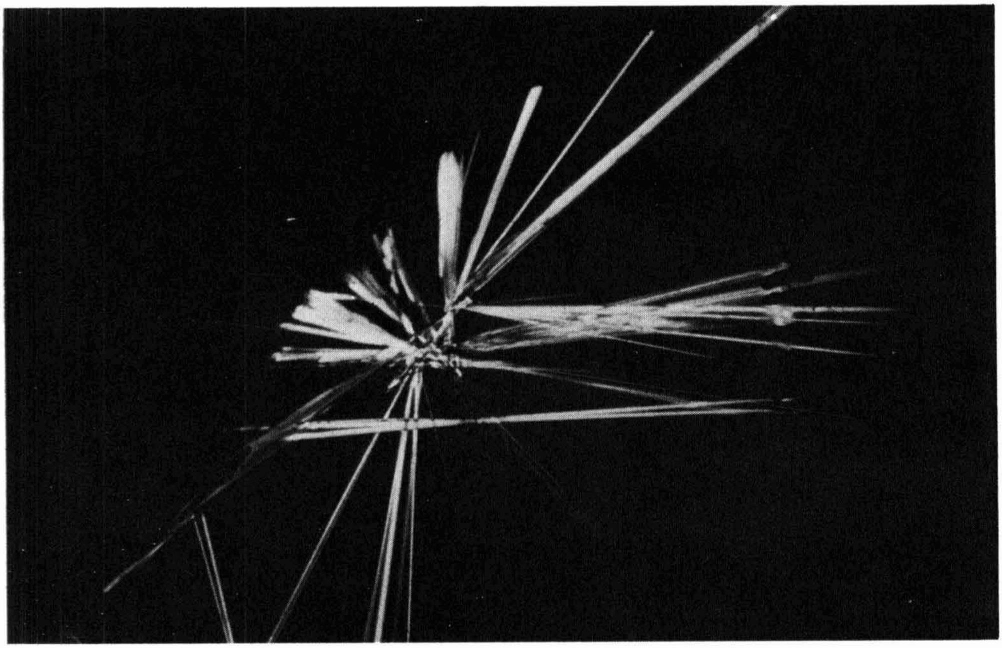

FIG. 9. Psilocin in 15% ethylenediamine.

FIG. 10. Psilocybin in 15% ethylenediamine.

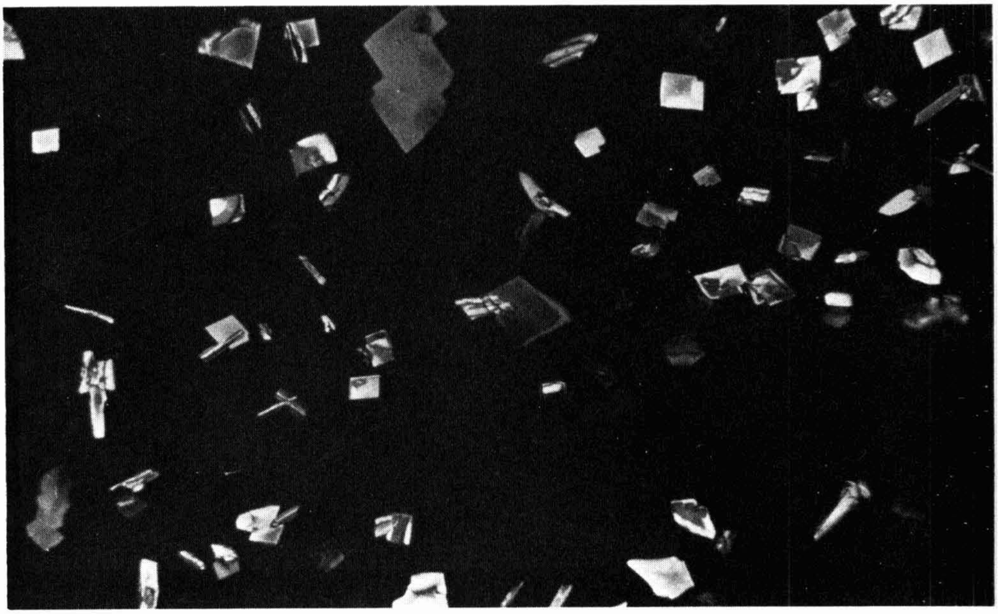

FIG. 11. Tryptamine in 15% ethylenediamine.

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The Nephelometric Determination of Selenium with 2,3-Diaminonaphthalene

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INTRODUCTION

Selenium is a cumulative toxic substance; thus its determination is of interest to the health sciences (*2*). The selective reaction of 2,3-diaminonaphthalene with Se(IV) to form the fluorescent, colored product naptho-[2,3-d]-2-selena-1,3-diazole (DANSe) is widely used and has been employed for the fluorometric, spectrophotometric, gravimetric, and liquid chromatographic method for the determination of this element (*5*). The fluorometric and spectrophotometric methods are applicable, in conventional use, to a maximum concentration of 0.1 ppm and 4 ppm, respectively. The gravimetric procedure, in conventional practice, is applicable to a minimum concentration of about 100 ppm. In the concentration range between the maximum of the spectrophotometric procedure and the minimum of the gravimetric procedure, very fine, small solid particles of DANSe are formed that limit the applicability of the optical procedures, while an insufficient amount of precipitate is formed for an accurate conventional gravimetric determination. However, these small particles lend themselves readily to a nephelometric measurement of the solution formed by adding 2,3-diaminonaphthalene (DAN) to the sample. The method is applicable to colored solutions that would interfere in a spectrophotometric procedure and can circumvent the need for a separation step, such as an ion-exchange separation (*4*). The method is as highly selective as the gravimetric procedure previously described (*4*) and requires a minimum of operator time.

MATERIALS AND METHODS

2,3-Diaminonaphthalene solution (J. T. Baker) was prepared weekly by dissolving 0.500 g in 100 ml of 0.2 *N* HCl, and stored in the dark at 5°C. Standard Se(IV) solution (I) was prepared by dissolving 265.2 mg of H_2SeO_3 in 100 ml of H_2O. The Hach Model 2424 Clinical Nephelometer was standardized with the latex standards furnished with the instrument. Conditioning reagent was prepared by adding to 25 ml of glycerol, 1.45 g of KCl and diluting to 100 ml with water. Masking agent consisted of a solution 0.05 *M* in EDTA and 0.05 *M* in NaF.

EXPERIMENTAL PROCEDURE

Transfer the solution of the selenium containing sample into a 100-ml volumetric flask. Add 10 ml of masking agent, add 25 ml of conditioning reagent, adjust to pH 2 by adding 0.2 *M* HCl, and dilute to volume. Transfer to a 250-ml beaker and add approximately a sixfold mole excess of DAN to the anticipated selenium concentration. Stir for 30 min and then measure the turbidity in nephelometer units.

RESULTS AND DISCUSSION

A plot of the instrument reading vs. time is approximately hyperbolic in shape (Fig. 1). Although the reaction is not complete until after 50 min, slightly shorter stirring times can be used, and straight line calibration curves will result. When a 30-min stirring time was used, a slope of 17 instrument units per 10 ppm Se was obtained. With a stirring time greater than 50 min, a slope of 23 was obtained (Fig. 2). In both cases, the lines extrapolated to an X intercept of 1 mg Se, indicating, that at least 10 ppm of Se are required for appreciable solid formation. With such lengthy stirring times, the stirring conditions need not be perfectly reproducible. It is important, though, that the reaction will have proceeded to the same extent in each determination, as the reproducibility of the extent of the reaction should be greater than the precision of the nephelometer (30 min is sufficient).

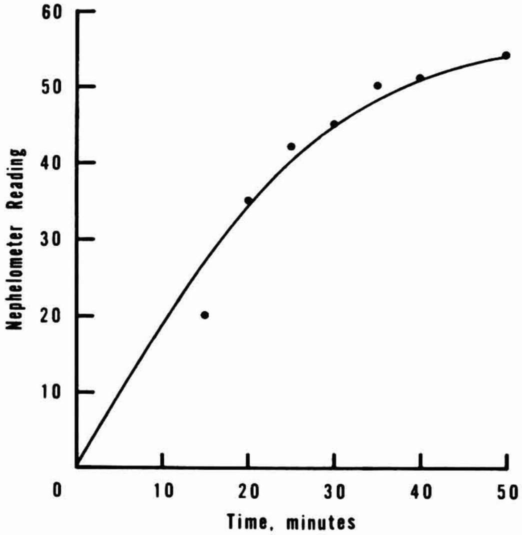

FIG. 1. Extent of precipitation as a function of time.

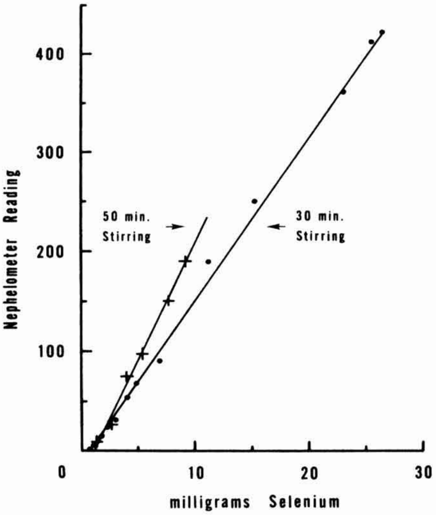

FIG. 2. Calibration curves for selenium analysis at two stirring times.

It is important that the stirring be uniform and at a rapid rate throughout the mixture without causing splattering. It was observed that if a volume greater than 150 ml is used in a 250-ml beaker, the top portion of the liquid was not stirred as fast as the bottom. Thus, stirring conditions were not equivalent throughout the system and the degree of precipitate formation can be a function of the size and speed of the stirring bar employed. Accordingly, some care need be considered in keeping equivalent experimental conditions. In practice, the time of stirring is not too critical, and reproducible results were obtained when several magnetic stirrers were employed to permit the analysis of several samples concurrently.

The procedure is generally free of interfering ions; the reaction is essentially specific for selenium. Nitrite interference is removed by boiling the sample with hydrogen peroxide to form the nitrate ion; the interference of small amounts of strong oxidizing agents can be minimized by adding hydroxylamine to the sample. Care must be taken to prevent the addition of a large excess of DAN to the sample to prevent a precipitation of DAN, or DAN salts such as the 2,3-diaminonaphthalene sulfate, which have a limited solubility under the reaction conditions.

Glycerol is added to the mixture as a partial suspending agent and to promote a uniform particle growth extending the method for a large concentration range. The KCl is added as a pH buffer (*1*) and to maintain the ionic strength of the solution. The optimum of pH 2 was determined by previous kinetic studies (*3*). Table 1 reports the results for the analysis of selenium in a number of mixtures using 30 min stirring. The relative SD was 3.0%.

TABLE 1
EFFECT OF FOREIGN IONS ON THE DETERMINATION OF SELENIUM

Mixture[a]	Se(IV) found (mg)
Al(III), Zn(II)	9.40
Cu(II), Ca(II)	8.93
Cd(II), Mn(II), Ni(II)	8.80
Ca(II), Mg(II), Ba(II)	9.30
Mn(II), Ni(II), Sr(II)	9.55
Fe(III), Cr(III)	9.30
Na(I), Li(I), K(I)	9.30
Br^-	9.30
F^-	8.80
SO_4^{2-}	9.55
NH_4^+	8.93
NO_3^-	9.40

[a] Each sample contained 9.12 mg Se and was 0.05 *M* in each foreign ion and the masking agents.

SUMMARY

A nephelometric determination of selenium has been developed in the concentration range from 10 to 300 ppm based on the reaction of Se(IV) with 2,3-diaminonaphthalene. The method is inexpensive, simple, and requires little operator time or skill.

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Spectrophotometric Study of Drug Interferences in an Aqueous 17-Ketosteroid Reaction[1]

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INTRODUCTION

The Kraushaar modification (*7*) of the Epstein technique (*3*) for the determination of 17-ketosteroids (17KS) by the Zimmermann reaction is different from others that have been described in that the chromogen that is forming is associated with the precipitate of a surfactant, Hyamine 1622 (H1622). This was a quantitative colorimetric technique derived from concepts developed for the chromatography of 17KS (*4*) wherein uniform staining was made possible by a surfactant precipitation phenomenon. The H1622 is not soluble at the level of alkalinity used in the color development step and comes out of solution as an oil carrying with it the forming chromogen. The end product, the Zimmermann chromogen, is removed from an equilibrium reaction by what appears to be either a postprecipitation or an adsorption phenomenon. In effect, it then acts as if it were also a precipitate, and the reaction appears to go rapidly to completion. Therefore, by using the precipitation device, a higher molar absorptivity is generated than would appear when the same reaction takes place in a monophasic system of lower alkalinity. Another factor of the H1622 system is that the final reaction contains only a small volume of methanol, differing from other common procedures for the determination of 17KS which make use of and/or feel the need of much higher ratios of organic solvent (*1,2,5*). The final solutions of some procedures are in fact virtually all organic in makeup for even the alkali is prepared as an alcoholic solution (*1*).

Recently, two instances in which drugs were shown to interfere with the determination of urinary 17KS have been described (*6,9*). The drugs were Cephalothin (Lilly), a commonly used broad spectrum antibiotic of

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[1] Supported in part by a Grant-in-Aid by the Detroit General Hospital Research Corporation.

the cephalosporin classification, and Tegretol (Geigy), an antiepileptic drug. It seemed reasonable to test the effect of each drug in the H1622 procedure because of procedural differences between the aqueous detergent reaction and the more common organic solvent systems in which the interferences were first noted (*6,9*). The following description of the testing of these drugs for their potential interference in this aqueous 17KS reaction included several spectral studies: (1) Zimmermann reactivity, (2) interaction of the drugs and 17KS in the color reaction, (3) extraction of the compounds after hot acid hydrolysis, and (4) extraction of the Zimmermann chromogen after reaction for the purpose of color purification.

MATERIALS AND METHODS

Reagents

Hyamine 1622 solutions. Prepare 2.5% (w/v) and 5% (w/v) aqueous solutions from the solid reagent.

Metadinitrobenzene solution. Add 1 g of purified *m*-dinitrobenzene (MDB) to 100 ml of 5% Hyamine 1622, warm at 37° for 60 min, filter when cool and store in an amber bottle.

Potassium hydroxide, 10 N. Store in a polyethylene bottle in the refrigerator.

Stock dehydroisoandrosterone solution. Prepare a methanolic solution to contain 1 mg/ml of dehydroisoandrosterone.

Working dehydroisoandrosterone (DHI) solutions. Prepare standards by diluting the stock standard with methanol to obtain 20–90 μg/ml in 10 μg/ml increments.

Sodium hydroxide wash solution, 10%. Store in a polyethylene bottle.

Distilled H_2O, ether saturated. Keep this solution refrigerated.

Methanol, specially purified for 17KS. Use the same lot of this commercially available grade for any run of standards, samples, and blanks.

Procedure (Urine)

This is essentially the procedure of Kraushaar *et al.* (*7*). Pipette 5 ml of urine into a screw-capped 50 ml tube which has a polyethylene insert in the cap. Add 1.5 ml of concentrated HCl and heat the mixture for 10 min in a boiling water bath. Cool, add 20 ml of cold ether and shake the tube vigorously for 1 min. Discard the lower aqueous layer. Wash the ether layer first with 5 ml of 10% NaOH and then with 10 ml of cold, H_2O-saturated ether. After removal of the lower phase, centrifuge the tube briefly to separate any residual entrained aqueous phase. Carefully evaporate to dryness two 4-ml aliquots of the ether layer in 6-in. tubes. These constitute the sample and sample blank. To the sample blank add

0.5 ml of methanol to dissolve the residue followed by 0.2 ml of 5.0% H1622 and 1.0 ml of 10 *N* KOH. Repeat the procedure with the sample tube, but substitute the MDB solution for the 5% H1622. To a third tube containing no residue, the reagent blank, add the same reagents to 0.5 ml of the same methanol that was added to the sample residue. Let the solutions stand at room temperature for 10 min during which time an oily precipitate forms. If extraction of the final color is needed, it must be carried out at this point by shaking the precipitated chromogen with several ml of ether for 10–15 min, centrifuging to separate the phases and then measuring the colored ether layer against a similarly treated reagent blank. If extraction is not to be carried out, add 1.5 ml of 2.5% H-1622 solution to dissolve the precipitated H1622 and chromogen. Read the sample versus the sample blank and the reagent blank versus H_2O. Subtract the reagent blank reading from the sample reading to obtain the final corrected reading. Standards prepared by substituting dilutions of the stock standard in methanol for the sample need only be corrected for the reagent blank.

RESULTS

Cephalothin is reported to interfere in the Zimmermann reaction (*6*), but it can be proven that this finding is questionable because no evidence of such a problem could be shown in the following described studies while using this aqueous 17KS modification. First, an experiment was carried out to determine whether Cephalothin is Zimmermann reactive. Fig. 1 contains the spectra of several concentrations of Cephalothin,

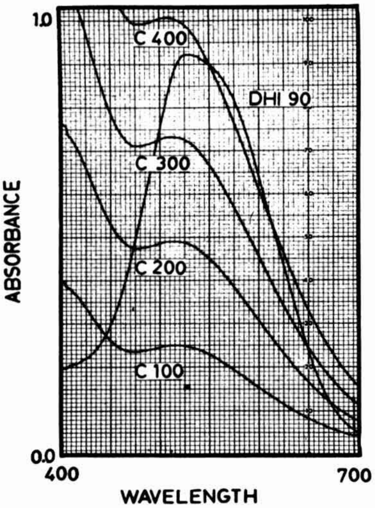

FIG. 1. Spectra of Zimmermann reaction for several concentrations of Cephalothin (C) at 100–400 μg/ml and a dehydroisoandrosterone (DHI) at 90 μg/ml.

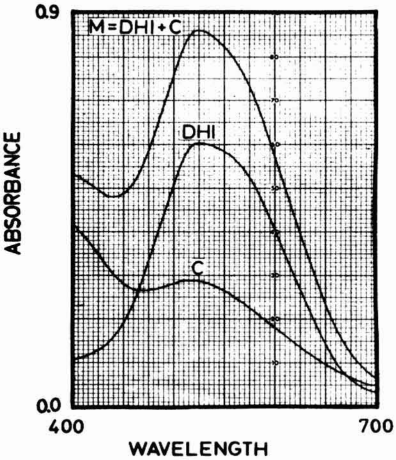

FIG. 2. Zimmermann reaction spectra for Cephalothin (C), dehydroisoandrosterone (DHI) and a mixture of the two compounds (M = DHI + C).

along with a comparison spectrum of DHI obtained by introducing each directly as methanolic solutions into the H1622-MDB-KOH color reaction. These graphic data show that Cephalothin is an obvious Zimmermann chromogen capable of reacting in this medium to generate a spectrum which overlaps that of DHI. In order to prove that DHI and Cephalothin react independently with the aqueous Zimmermann reagent, DHI, Cephalothin and a mixture of the two were reacted and then scanned. The resultant spectra are shown in Fig. 2. When the spectra of DHI and then Cephalothin were automatically subtracted from the spectrum of the mixture (M = DHI + C) using a double beam recording spectrophotometer, the resultant spectra generated were those of the pure compounds. These are graphed in Fig. 3.

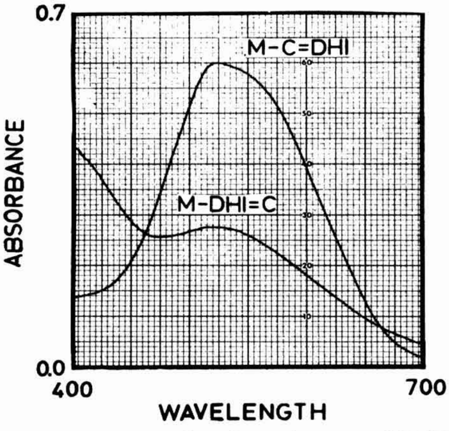

FIG. 3. Difference spectrophotometry for the mixture of DHI plus the drug spectrum minus both the dehydroisoandrosterone spectrum (M − DHI = C) and the Cephalothin spectrum (M − C = DHI) to regenerate the individual spectra.

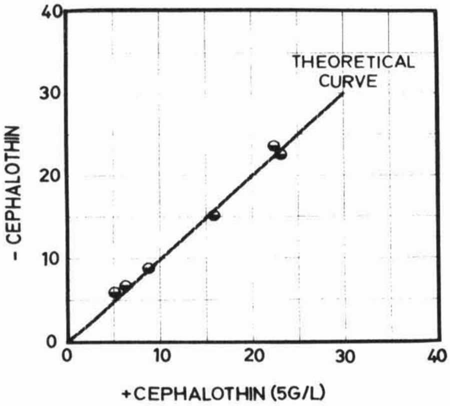

FIG. 4. Recoveries of 17KS ± the presence of Cephalothin along with the theoretical curve representing no interference by the drug.

A second experiment was then carried out to determine whether or not Cephalothin which is now established as a Zimmermann chromogen is ether extractable after acid hydrolysis using the conditions defined under Procedure. Figure 4 shows the results obtained from several representative urines bridging low to relatively high values of 17KS. Cephalothin was added to a portion of each urine specimen and the latter were then carried through the complete procedure including acid hydrolysis, extraction with ether, alkali and ether-saturated H_2O washes, evaporation to a residue and finally the color reaction with its precipitating surfactant phenomenon. The graph of values obtained with uncontaminated portions of the urines plotted against the results obtained with contaminated urines approaches a theoretical curve. This creates an obvious and definite discrepancy in findings and interpretations between the present work and the previously described investigation by King (*6*) in which Cephalothin was described and demonstrated to be a major systemic interference.

One further study was undertaken in proof of this contention. Duplicate solutions of Cephalothin in H_2O were treated as urines and taken through the entire 17KS procedure. Another duplicate set was treated with HCl, but not heated as they would have been in the conventional 17KS determinations. In the heated system, one residue of washed ether extract of each pair was dissolved in methanol and the other in a methanolic solution of DHI. No color from Cephalothin appeared in the DHI-free residue after reaction with H1622-MDB-KOH reagent, while the other residue showed an undistorted spectrum of the DHI added (Fig. 5). In the unheated system, a very small positive error occurred in the residue containing DHI, and its unheated blank showed an equal small positive absorbance above the heated reagent blank. The interference of Cephalothin is obviously insignificant for the system in which 17KS esters are hydrolyzed by heating them in an HCl medium. How-

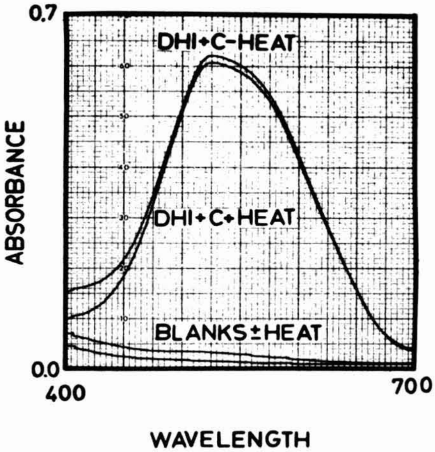

FIG. 5. Spectra obtained with paired residues of Cephalothin solutions processed ± the procedural step and ± the addition of dehydroisoandrosterone to each pair of residues.

ever, if Cephalothin is present, the finding shown for the unheated system might represent a small concern if hydrolysis of esters is accomplished by the use of enzymes, although the difference between blank and sample is identical for both the heated and unheated circumstances. This finding was not pursued further in the present study because hot acid hydrolysis was the method used for the rest of the investigation.

Tegretol, an antiepileptic drug, was also shown to interfere in a 17KS determination in which a mathematical calculation (*8*) recommended by England's Medical Research Council (M.R.C.) on Clinical Endocrinology was used to make the final correction for irrelevant absorption because this drug also reacted with the alkaline MDB reagent (*9*). The purpose of what follows is to show that Tegretol is a primary interference only when the mathematical corrections are used. The correction formula is:

$$\text{Corrected } A_{520\,nm} = \frac{\text{Observed } A_{520\,nm} - 0.6A_{430\,nm}}{0.73}.$$

The spectra of several concentrations of Tegretol and one of DHI for comparison are shown in Fig. 6. If one considers direct measurement, positive errors from the feet of the Tegretol spectra are present at the peak maximum for DHI. However, if measurements were made near 550 nm, there would be no interference and only a small percentage loss in total absorbance would result. Since the reaction as described is more sensitive than other commonly used 17KS reactions (*1,2,5*), this loss in sensitivity can be tolerated easily. In order to study the total effect of Tegretol in the aqueous Zimmermann reaction, interference studies were

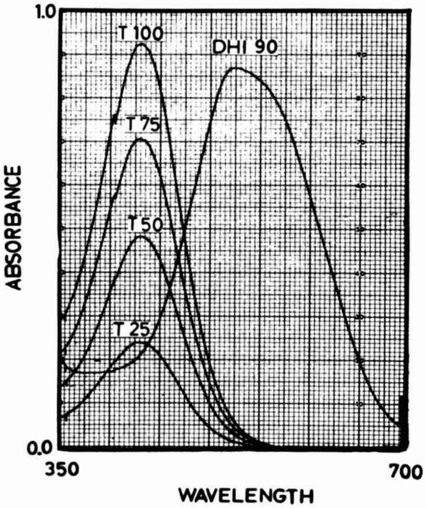

FIG. 6. Examples of Zimmermann reaction spectra for several concentrations of Tegretol (T) at 25–100 μg/ml and dehyroisoandrosterone (DHI) at 90 μg/ml.

carried out which were similar to the ones for Cephalothin (Figs. 2,3,5). Tegretol, DHI and a mixture of the two at the same concentrations were each reacted in the H1622-MDB-KOH medium and the resulting spectra were graphed as shown in Fig. 7. The spectra are additive in nature as demonstrated by the spectra shown in Fig. 8. When DHI and Tegretol reaction spectra were each subtracted by double beam spectrophotometry from the spectrum of the mixture, the regenerated spectra

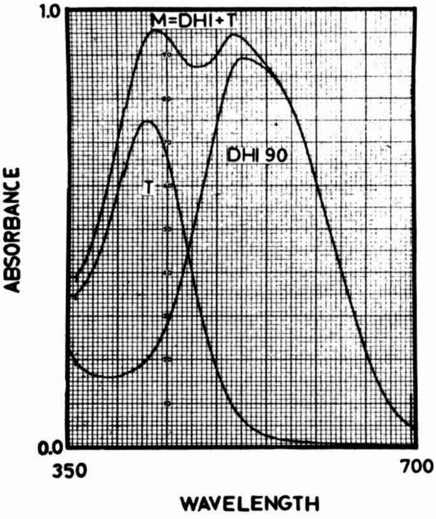

FIG. 7. Zimmermann reaction spectra for Tegretol (T), dehydroisoandrosterone (DHI) and a mixture of the two compounds (M = DHI + T).

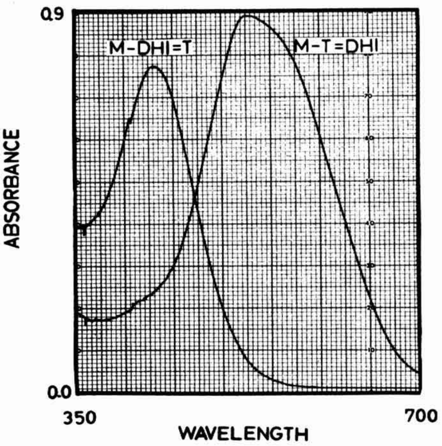

FIG. 8. Difference spectrophotometry for the mixture of DHI plus the drug spectrum minus both the dehydroisoandrosterone spectrum (M − DHI = T) and the Tegretol spectrum (M − T = DHI) to regenerate the individual spectra.

obtained were the same as those that would have resulted if the individual compounds had been individually reacted with the H1622-MDB-KOH reagents.

Figure 9 shows the results of an experiment designed to prove that the drugs Tegretol and Cephalothin do indeed undergo reaction with MDB in the described system and that the spectra obtained are Zimmermann

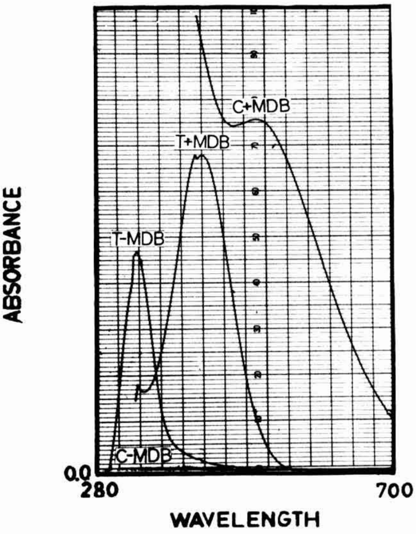

FIG. 9. Zimmermann reaction spectra plus MDB for Cephalothin (C + MDB) and Tegretol (T + MDB) and the same system minus MDB for the same respective reactions.

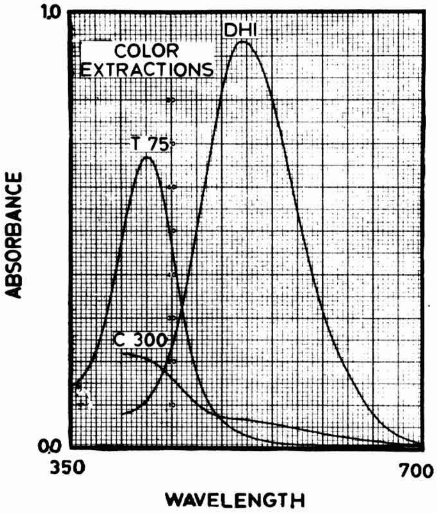

FIG. 10. Extraction of the Zimmermann chromogens of dehydroisoandrosterone (DHI), Tegretol (T) and Cephalothin (C).

chromogens and not just side reactions of the H1622-KOH portion of the color reagent. Curves (C + MDB) and (T + MDB) show the spectra obtained when Cephalothin and Tegretol are reacted with the 17KS color reagent and scanned against their reagent blanks between 700–280 nm. Curves (C-MDB) and (T-MDB) are derived from the same solutions subjected to the same reaction medium except that the MDB was left out. It is clear from the spectra shown that the reaction is with the MDB in this medium.

Figure 10 shows the results of another experiment carried out to determine whether extraction of the final color could be helpful in interference problems by eliminating irrelevant absorption. Dehydroisoandrosterone, Cephalothin, and Tegretol were individually reacted and the H1622 precipitated chromogens were then extracted with ether to obtain the following results. The extraction of DHI was quantitative but its spectrum was narrower than when the Zimmermann chromogen was in aqueous media (see previous figures). The extracted Tegretol spectrum was the same as the aqueous reaction spectrum and its Zimmermann chromogen was quantitatively extractable. However, the product of the Zimmerman reaction with Cephalothin, was almost quantitatively nonextractable as can be seen, with only a small percentage going into the ether phase. So one might infer that extraction of the final color of the 17KS Zimmermann chromogen might or might not leave behind the reaction product of an interfering compound, and that fact would have to be predetermined before any general use of such a separatory phase could be recommended as a purification device.

DISCUSSION

The inference drawn from Figs. 1–3 is that Cephalothin reacts independently of the DHI present, there is no interaction, and summation spectra are truly additive. The experimental results lead to the inevitable conclusion that Cephalothin is capable of reacting with the H1622-MDB-KOH reagent, but that, fortunately, it is not extractable in the procedure described here. Therefore, it is not a substance of concern for such procedures. In addition, if any acid-hydrolyzed products form which do extract, they do not undergo an H1622-MDB-KOH reaction and therefore are also not a problem. The total findings then remain contradictory to the one that has been reported previously (*6*). The only time a reaction was found for Cephalothin was when, as described, the drug was introduced directly into the reaction system as shown in Figs. 1–3.

Investigation of the effect of Tegretol (as with Cephalothin) again reinforces the concept that both reactions are taking place independently with no interference in reactivity evident (Figs. 6–8). The amount of interference obtained at peak maximum for DHI is small as can be seen from the spectrum of the mixture. Obviously, peak maximum readings with such small errors due to interfering drugs yield reasonably accurate information. However, if one considered mathematical correction as recommended by the M.R.C. (*8*), there would be an obvious spectral distortion with a urine sample owing to the presence of the Zimmermann chromogen. In this case one would subtract an absorbance at 430 nm for the Tegretol in the contaminated sample, whereas such an absorbance would not have existed for the standard, and yet the standard would also have been subjected to the same corrective action. Cephalothin and Tegretol are just two examples of possible interferences in the measurement of 17KS by use of a modified Zimmermann reaction. Although they both were shown to react with MDB under specified conditions their effects on actual analysis differ. A number of compounds which do not contain the acetone-like configuration of a 17KS are undoubtedly able to undergo a Zimmermann reaction to form an atypical color. A list of such compounds would include drugs such as Tegretol which does not have a methylene carbonyl reaction site in its structure, a reaction site often cited as necessary for formation of a Zimmermann chromogen (*1*). Some consideration should be given to the potential reactivity of structures other than methylene carbonyl in the Zimmermann reaction and further studies are in progress to elucidate that point.

Finally, extraction of the 17KS Zimmermann chromogen as a purification device should be examined quite critically (Fig. 10). Based on the extraction results described above for Cephalothin and Tegretol, the following conclusion seems likely. It is quite probable that a compound which is organic solvent extractable and results in a Zimmermann chromogen will yield to extraction of that chromogen, whereas the chromogen of an unextractable compount will resist extraction. Without preferential extraction characteristics, the dangers involved in extraction would then appear obvious and the need to extract dubious.

SUMMARY

A spectrophotometric study of two reported drug interferences in an aqueous Zimmermann reaction for 17-ketosteroids has been described. One drug, Tegretol, did not interfere spectrally with the 17-ketosteroid determination because its Zimmermann reaction spectrum was displaced far enough toward the ultraviolet so there was no significant superimposition of its spectrum with that of 17-ketosteroids. The other drug, Cephalothin, proved to be no problem analytically, because, even though it was Zimmermann-reactive, it was unextractable from acid hydrolyzed urines and therefore could not interfere in the color-forming step.

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Determination of Hexafluoropropan-2,2-Diol in its Binary Mixtures with Water

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INTRODUCTION

The characteristic properties of fluoroalcohols (*18,20,23*) make them useful solvents for a variety of compounds. The perfluorinated diol hexafluoropropan-2,2-diol (HFPD) and, in particular, HFPD-water mixtures have been used as solvents for polyalkylene oxides (*15,20*), polyamides (*20*), polyacrylonitrile (*20*), polyvinyl alcohol (*20*), polyesters (*20*), peptides (*2,8,14*), polypeptides (*3–6,13,16,17,25*), and proteins (*16,24*).

In some HFPD-containing solvent mixtures and, particularly in the HFPD-water system (HFPD·nH_2O), a strong interaction takes place between the cosolvents (*20,26*). As a result, the solvation ability of the components in the mixture towards a solute is extensively altered. The phenomenon is expressed, among others, by the various solvent-induced structural changes of polypeptides occurring in such solvent systems (*3–5,9,16,17,25*). In HFPD·nH_2O these changes occur predominantly in the range $0.5 < n < 10$. Therefore, a convenient method for determining the HFPD content in these solvent mixtures was desirable.

Perfluorinated primary alcohols exhibit higher acidities than their hydrocarbon analogs (*18,20,23*), and this is more so for secondary or tertiary derivatives. Still higher acidities are exhibited by fluorinated diols and gem-diols, and some representative pK_a values are listed in Table 1. The relatively high acidity of HFPD (see Table 1) allows its quantitative determination by potentiometric titration (*20,26*). The procedure requires, however, the appropriate instrumental setup. In the present communication we describe a visual titration procedure, by which HFPD is titrated in ethanol or in dimethylformamide (DMF), using sodium methoxide as titrant and thymol blue as indicator.

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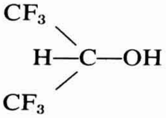
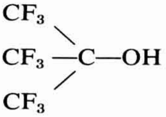
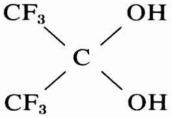
TABLE 1
ACID IONIZATION CONSTANTS OF ALCOHOLS IN WATER AT 25°C

Alcohol	pK_a	Reference
$CH_3—OH$	15.5	(*7*)
$CF_3—CH_2—OH$	12.4	(*21*)
	9.4	(*26*)
	5.4	(*12*)
	6.65	(*26*)

MATERIALS

Hexafluoropropan-2,2-diol: water binary mixtures were prepared by bubbling gaseous hexafluoroacetone into water (*3,11,15,20*). The detailed procedure was reported previously (*15,25*).

The sodium methoxide solution (0.1 *N* in benzene:methanol, 8 : 2 v/v) was prepared from metallic sodium and standardized against benzoic acid, as described elsewhere (*22*).

Dimethylformamide (Fluka, Switzerland or Merck, Germany) and ethanol (Fluka, Switzerland), used as solvents for the titrations, were of analytical grade and were used without further purification.

Thymol blue (0.2% solution in ethanol) was used as indicator.

The carbonate-free 1 *N* KOH solution, required for the potentiometric titrations, was prepared according to Albert and Serjeant (*1*) and standardized against potassium hydrogen phthalate.

METHODS

The visual titration procedure is essentially the one previously reported for determination of organic acids (*22*). The $HFPD\cdot nH_2O$ sample (about 150 mg, containing 0.5–0.75 mmoles HFPD) is diluted with DMF or ethanol (5 ml), and the titration is carried out to the yellow-to-blue color change of the indicator. A blank titration (i.e., titration of the solvent itself) was always performed under identical conditions.

The potentiometric titrations were carried out with 1 *N* KOH in aqueous solutions, using a titration setup (Radiometer, Copenhagen) consisting of: (a) TYPE TTTlc titrator, (b) type SBR2c titrigraph recorder,

(c) type TTA31 titration assembly equipped with a 25-ml jacketed titration vessel and with a 0.5-ml syringe type buret B101, (d) type G202B glass electrode, and (e) type K401 calomel reference electrode, connected to the titration vessel by a saturated KCl–agar bridge in polyethylene capillary tubing. The samples (about 75 mg, containing 0.2–0.4 mmoles HFPD) were diluted with carbonate-free water (2 ml), and titrated at 25°C, under a stream of CO_2-free nitrogen. Prior to the titration, about 30 μl of 1 *N* HCl were added in order to fully protonate the organic acid, and thus allowing the complete titration curve of HFPD ($pK_a = 6.65$, see Ref. *26*) to be obtained by the back-titration with the base.

In the following, the composition of the HFPD-water mixtures is expressed as the water-to-HFPD molar ratio (n), or in terms of mole fraction of HFPD (X_{HFPD}).

RESULTS AND DISCUSSION

The results of HFPD determinations, as obtained by various procedures, are summarized in Table 2. It can be seen that good agreement is reached between the results obtained by potentiometric titrations and by the visual titrations in DMF. Somewhat higher values are obtained for X_{HFPD} when the visual titration was carried out in ethanol. In the latter solvent, also the reproducibility of the titrations was not as good as in the former, due to the fact that the color change of the indicator is less sharp.

The small amount of water present in the titration mixture seems not to interfere with the visual titrations. In the HFPD-water samples considered, the water content reached up to about 35% (by weight). However, due to the dilution with the nonaqueous solvent, the water content of the titration mixtures was 1% or less, which is within the limits recommended by Deal and Wyld (*10*).

TABLE 2
HFPD CONTENT OF HFPD: WATER MIXTURES

	Mole fraction of HFPD, X_{HFPD}		
Sample	By potentiometric titration	By visual titration in DMF	By visual titration in ethanol
1540	0.47	0.45	0.50
1585	0.40	0.40	0.44
1654	0.36	0.39	0.40
1809	0.20	0.19	0.22
1854	0.165	0.165	0.17

Attempts have been made to carry out the visual titrations in water, using 0.1 *N* aqueous NaOH or 0.2 *N* KOH in methanol:water (80:20 v/v) as titrants, and thymol blue or phenolphtalein as indicators. Although such titrations are feasible, no sharp end points were obtained with the available indicators. Under these conditions the more acidic perfluoro-*tert*-butanol could, however, be titrated (*12*).

It can thus be concluded that the determination of HFPD in HFPD-water mixtures can conveniently be performed in DMF, the end point being marked by the yellow-to-blue color change of the indicator.

SUMMARY

A visual titration method for the determination of hexafluoropropan-2,2-diol (HFPD) in HFPD-water mixtures is suggested. The procedure involves the titration of the weakly acidic HFPD with sodium methoxide, using dimethylformamide or ethanol as solvent and thymol blue as indicator. The results are in good agreement with the ones obtained by potentiometric titration in water.

ACKNOWLEDGMENTS

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MICROCHEMICAL JOURNAL **20**, 539–543 (1975)

Investigation of the Mechanism of Visualization of Fatty Acids with New Fuchsine in TLC by Means of IR Spectroscopy

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INTRODUCTION

Visualization of the chromatographically separated substances is an important analytical problem. In our previous papers we showed the inactive visualizing properties of new fuchsine (*1*) and characterized the process of visualization of fatty acids with the help of new fuchsine from the physical chemistry point of view (*2*).

Now we attempt to present the infrared spectroscopic results, indicating a possible mechanism of visualization of fatty acids with new fuchsine on thin layer and proving the results shown previously (*2*).

EXPERIMENTAL AND DISCUSSION

Assuming the difference between the adsorption efficiency of new fuchsine on silica gel G and on the silica gel G–fatty acid system (*2*), the surface areas of standard silica gel G samples and those of silica gel G impregnated with fatty acids were determined.

The samples were prepared with the use of 5% impregnating substance per weight of the whole system. The measurements were performed according to the method of thermal desorption. The results are shown in Table 1.

These results prove that impregnation of silica gel with fatty acid evokes the decrease of surface areas. Therefore, the increase in adsorption of fuchsine dye must depend on the properties of adsorbent, the silica gel G–fatty acid system, and on the properties of adsorbed dye.

The IR spectroscopic measurements were performed for the silica gel G–fatty acid system and for the mixture of silica gel G and fatty acid as a reference. The measurements were done applying the technique of KBr discs, using the Unicam SP-1200 spectrophotometer. The optimum recording conditions were determined experimentally. Because of the complicated spectrum of silica gel itself in the range of 400–1300 cm^{-1},

TABLE 1
THE SURFACE AREA VALUES OF SILICA GEL G AND SILICA GEL G IMPREGNATED WITH FATTY ACIDS

Substance	Surface area values [m^2/g]
Silica gel G	246.6
Silica gel G impregnated with arachidic acid	120.2
Silica gel G impregnated with capric acid	169.7

our interpretation dealt with the 1200–4000 cm^{-1} region, where most of the absorption bands of arachidic acid on silica gel occur. The 1720, 2853, and 2926 cm^{-1} absorption bands of the acid were taken into consideration. These bands correspond with the stretching vibrations of carbonyl in carboxylic group and symmetrical and asymmetrical C-H vibrations imposed on the stretching O-H vibrations of carboxyl (Fig. 1).

The low intensity of the a/m bands was evoked with the low amount of the acid in sample – 5% of acid per weight of the system. One could not increase this amount of acid because the complementary investigations showed that any higher amount than this used for impregnation of silica gel gives a mixture of the silica gel G–fatty acid system and of fatty acid itself. Because of the shift of the acidic absorption bands in the case of silica gel G–fatty acid system of about 10 cm^{-1} towards the higher wave number values, compared with the samples of pure ara-

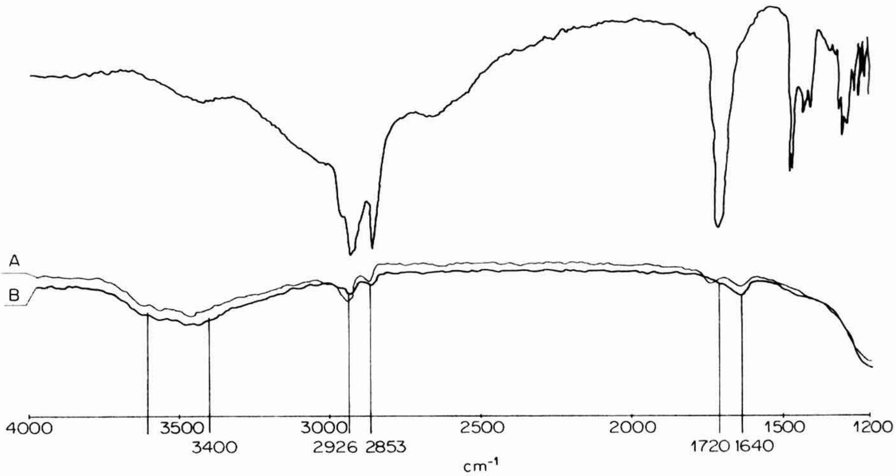

FIG. 1. (A) The IR spectrum of arachidic acid, silica gel G impregnated with arachidic acid and (B) mixture of silica gel G with arachidic acid.

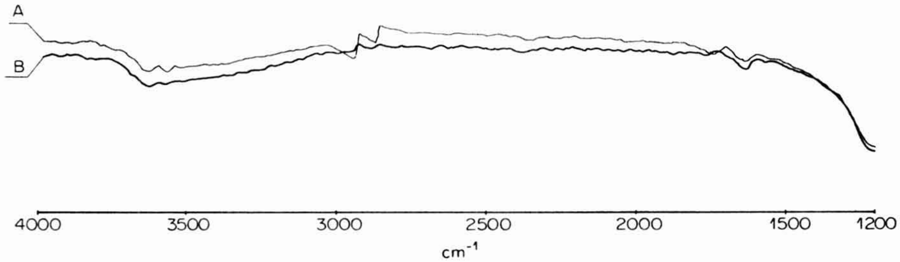

FIG. 2. (A) The IR spectrum of silica gel G impregnated with arachidic acid and (B) mixture of silica gel G with arachidic acid with a compensation with arachidic acid.

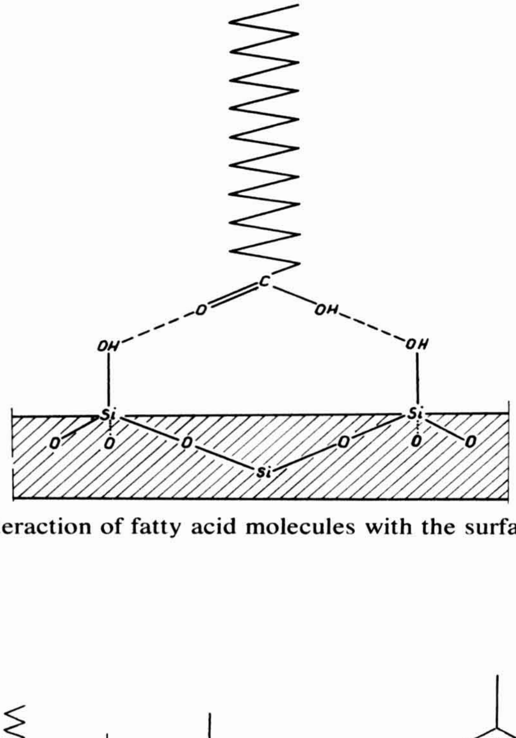

FIG. 3. The interaction of fatty acid molecules with the surface of silica gel.

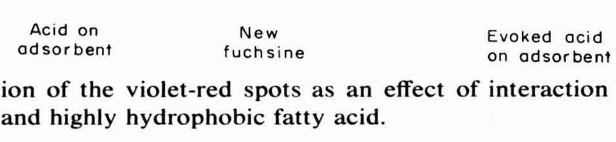

FIG. 4. Formation of the violet-red spots as an effect of interaction between the new fuchsine solution and highly hydrophobic fatty acid.

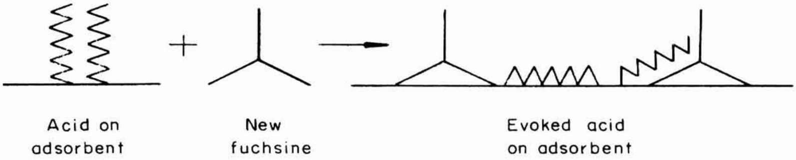

FIG. 5. Formation of the white spots as an effect of interaction between the new fuchsine solution and low hydrophobic fatty acid.

chidic acid, one applied the stoichiometric compensation with the sample of pure arachidic acid. The IR spectrum of silica gel G impregnated with arachidic acid and this of the mixture of silica gel with arachidic acid, using the arachidic acid compensation are shown in Fig. 2.

In the case of mixture between silica gel G and fatty acid, the compensation of spectrum is complete, and, with the silica gel impregnated with fatty acid, one could not get the compensation of the bands. The shift of the absorption bands in the case of impregnated silica gel and the impossibility to compensate these bands proves that the silica gel G impregnated with arachidic acid forms a determined system, which is different from the simple mixture of silica gel with acid. This system is a convenient phase for the absorption of triphenylmethane dye—new fuchsine. We attempted to give a schematic view of this system in Fig. 3.

Fatty acid is connected through its carboxylic group with the OH groups present in silica gel. The hydrocarbon chain of the acid remains at a certain angle to the silica gel surface. The efficient adsorption of new fuchsine on the a/m system can be explained by the molecular structure of this dye.

During visualization of the chromatogram with the new fuchsine solution, molecules of the dye are fixed on the highly hydrophobic molecules of the acid, which is adsorbed on silica gel. On the other hand, in the case of low hydrophobic acids, the spraying of the chromatogram with the water solution of dye affects the partial dissolution of acid on the silica gel surface and, consequently, of the dye (Figs. 4 and 5).

According to what was stated before, the highly hydrophobic acids give the violet-red spots, when visualized with new fuchsine, while the low hydrophobic acids dissolved with the water solution of the dye give white spots on the chromatograms. On the basis of these visualizing effects, the possibility of distinguishing between the high and low hydrophobic fatty acids exists.

On the basis of our previous papers (*1,2*) and the presented investigations, the proposed mechanism of visualization of fatty acids with new fuchsine seems to be fully proved.

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Decimilligram-Scale Determination of Sulfur in Organic Compounds

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INTRODUCTION

Over the past few years several authors have elaborated methods for the microdetermination of sulfur in organic compounds based on the use of chromotropic acid derivatives, as chromatic indicators in the titration of sulfate ions with barium salts (*1–7*). Comparative studies of such indicators have shown that the best results are obtained using dimethylsulfonazo III or 4,5-dihydroxy-3,6-bis(*p*-methyl-*o*-sulfophenylazo)-2,7-naphthalenedisulfonic acid (*3,9*).

We now report a method for the decimilligram-scale determination of sulfur in organic compounds, which is based on the combustion of the sample in a Schoniger flask (*8*) followed by titration of the sulfate ions with barium perchlorate and dimethylsulfonazo III.

With the appropriate variations it was also possible to obtain a more rapid titration with a net end-point at equivalence, thus overcoming the difficulties of the method described previously by some authors (*10*). This was done by using an absorption solution containing 0.1 milliequivalents of potassium ions, added as hydroxide, and carrying out the titration at a pH about 3, obtained by adding 0.5 N HNO_3 up to the end-point of bromophenol blue.

In all probability, the effect is due to variation in the ionic strength of the medium, which leads to an increase in the rate of formation of the barium sulfate precipitate and to the absence of color due to the initial formation of the complex before reaching the equivalence point.

MATERIALS AND METHODS

Reagents and Equipment

A. Thomas filter paper for flask combustion. 0.1 N solution of potassium hydroxide. 30% hydrogen peroxide. Acetone. 0.05% solution of bromophenol blue in ethyl alcohol. 0.5 N HNO_3. 0.1% aqueous solution of dimethylsulfonazo III. 0.01 M titrated solution of barium perchlorate.

TABLE 1
SULFUR ANALYTICAL RESULTS

Compound	Sample weight (mg)	S (%)		
		Calculated	Found	Δ
Potassium	0.6543	18.39	18.43	+0.04
	0.7657		18.52	+0.13
	0.9517		18.32	−0.07
Sulfamic acid	0.7158	33.03	32.79	−0.24
	0.8091		32.82	−0.21
	0.8289		32.79	−0.24
Diphenyl sulfone	0.7524	14.66	14.77	+0.11
	0.9222		14.67	+0.01
	0.9568		14.77	+0.11
Sulfanilic acid	0.5439	18.48	18.74	+0.26
	0.7123		18.42	−0.06
	0.7366		18.36	−0.12
Sulfanilamide	0.6447	18.63	18.68	+0.05
	0.7500		18.53	−0.10
	0.9320		18.39	−0.24
Phenylthiourea	0.4964	21.07	21.09	+0.02
	0.6843		20.98	−0.09
	0.7854		20.84	−0.23
Sulfonal	0.6727	28.09	28.15	+0.06
	0.7765		28.07	−0.02
	0.9783		28.27	+0.18
S-benzylisothiouronium chloride	0.6622	15.82	15.86	+0.04
	0.8977		15.82	0.00
	0.9066		16.00	+0.18
Cysteine hydrochloride monohydrate	0.5810	18.21	17.96	−0.25
	0.8210		18.24	+0.03
	0.9856		18.33	+0.12
Acetylthiocholine iodide	0.6558	11.08	11.30	+0.22
	0.7451		11.27	+0.19
	0.9997		11.27	+0.19
8-hydroxy-7-iodo-5-quinolinesulfonic acid	0.7728	9.13	8.99	−0.14
	0.9105		9.16	+0.03
	0.9812		9.14	+0.01

200 ml Schöniger combustion flask. 1 ml Metrohm automatic microburet.

Procedure

From 0.5 to 1 mg of substance are weighed on a Mettler UM7 ultramicrobalance and then enclosed in a paper container for flask com*bustion of dimensions* 2 × 2 cm. Combustion is performed in a 200 ml Schöniger flask, employing as absorption solution 1 ml of 0.1 N KOH, 3 ml of water and 3 drops of 30% hydrogen peroxide. The flask is shaken for 10 min and then left to stand for 20 min. The stopper, platinum boat, and walls of the flask are washed with 6 ml of acetone; 3 drops of bromophenol blue solution added; and the solution taken to the end-point with 0.5 *N* HNO_3. After addition of a drop of dimethylsulfonazo III solution, the solution is titrated under magnetic stirring with 0.01 *M* barium perchlorate. The color change at the end-point is from violet to persistent blue-green.

RESULTS AND DISCUSSION

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

The method allows rapid titration to be performed and there is no reversibility in the color of the indicator before the equivalence point.

SUMMARY

A simple and rapid method for the decimilligram-scale determination of sulfur in organic compounds is described. The procedure utilizes the Schöniger flask method for the combustion and the sulfate-ion titration, with 0.01 *M* barium perchlorate solution and dimethylsulfonazo III as indicator. The results obtained are exact and precise.

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The Application of Thin-Layer and Gas Chromatography to Identification of the Oxidation Products of *n*-Alkylaromatic Hydrocarbons

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INTRODUCTION

In order to find what changes occur during the oxidation reaction of organic compounds and what is the yield of this reaction, it is necessary to carry out a separation and identification of obtained products. In our investigations, the oxidation reaction of *n*-alkylaromatic hydrocarbons in the presence of cobalt(II) stearate proceeds mainly to appriopriate *n*-alkylaromatic ketones (*4*).

The data obtained could be the subject of theoretical considerations as well as of practical utilization attempts (*2*).

The purpose of this work was a qualitative identification of oxidation products of some *n*-alkylaromatic hydrocarbons by means of thin-layer and gas chromatography.

EXPERIMENTAL

The oxidation of selected *n*-alkylaromatic hydrocarbons was carried out in the previously described way (*4*), using the oxygen from air and cobalt(II) stearate as a catalyst. After 8 hr of oxidation, the reaction mixtures were analyzed by means of thin-layer and gas chromatography. We looked for such a TLC mobile phase, which would enable the separation of all the oxidation products from our hydrocarbons. The mobile phases used until recently helped to separate the oxidation products coming from the particular hydrocarbon (*1*,*3*). The mobile phase which proved to be specially useful was composed of *n*-hexane, carbon tetrachloride, diethyl ether, and acetone in a volume ratio of 4:2:1:0.2. Applying this mobile phase we developed the firm precoated glass plates (0.25-mm thick silica-gel 60-F_{254} layer; E. Marck, G. F. R), which were previously activated at 110°C for 30 min. In every case the developed amounts of reaction mixtures were 4 μl. After developing the plates and evaporating the mobile phase, the chromatograms were evoked with a 10% solution of ammonium molybdate in 30% sulphuric acid. The obtained chromatograms are shown in Fig. 1.

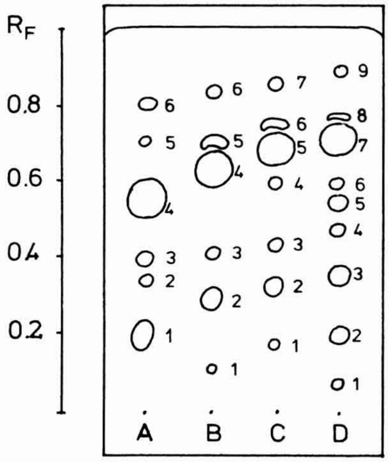

FIG. 1. Separation of the oxidation products from *n*-alkylaromatic hydrocarbons by means of thin-layer chromatography: (a) for ethylbenzene, (b) for *n*-propylbenzene, (c) for *n*-butylbenzene, (d) for *n*-pentylbenzene.

The chromatogram shown in Fig. 1 gives no evidence of one of the main oxidation products, benzoic acid. Because of trouble obtaining a good chromatographic trace for this compound, benzoic acid was previously isolated from the reaction mixtures with the aqueous solution of sodium hydroxide (*1*).

The qualitative identification of the oxidation products was carried out by comparison of their R_f values with the R_f values of standard samples. Data given in Table 1 represent the mean R_f values calculated from ten different analyses.

The qualitative identification performed on thin-layer was confirmed by the results received by means of gas chromatography. The gas chromatograms were run on the Varian Aerograph (U.S.A.) type 2868 chromatograph connected with the type 485 integrator. The measurements were performed both isothermally and with the programmed change of column temperature.

Analysis conditions: 6-ft long glass column, inner ϕ 2 mm, filled with the 3% ethylenesuccinate–phenylsilicone copolymer (EGSP-Z) on Gas-Chrom Q, 100/120 mesh (Applied Science Laboratories Inc., U.S.A.); the carrier-gas: argonium, flow rate 30 ml/min; FID; the temperature of injection chamber 240°C.

The isothermal separation was performed at 140°C. Using this temperature of thermostate and all the a/m conditions, we managed to separate the products of oxidation of *n*-alkylbenzenes and also to identify some components of the mixtures, by means of the inner standard technique. The main products of oxidation of *n*-alkylbenzenes are (apart from benzoic acid) the corresponding alkylaromatic ketones and α-

TABLE 1

THE R_f VALUES FOR THE SEPARATED OXIDATION PRODUCTS FROM *n*-ALKYLBENZENES

Oxidation product	R_f	Oxidation product	R_f
A. Ethylbenzene		B. *n*-Propylbenzene	
1. 1-Phenyl-1-ethanol	0.20	1. Unidentified compound	0.12
2. Phenol	0.34	2. 1-Phenyl-1-propanol	0.28
3. Ethylbenzene hydroperoxide	0.38	3. n-Propylbenzene hydroperoxide	0.41
4. Acetophenone	0.54	4. Propiophenone	0.63
5. 1-Phenyl-1-ethanol benzoate	0.65	5. 1-Phenyl-1-propanol benzoate	0.68
6. Bis (1-phenyl-1-ethyl) ether	0.81	6. Unidentified compound	0.84
C. *n*-Butylbenzene		D. *n*-Pentylbenzene	
1. Unidentified compound	0.16	1. Unidentified compound	0.08
2. 1-Phenyl-1-butanol	0.32	2. Unidentified compound	0.21
3. n-Butylbenzene hydroperoxide	0.45	3. 1-Phenyl-1-pentanol	0.36
4. 1-Phenyl-1-butanol propionate	0.60	4. n-Pentylbenzene hydroperoxide	0.49
5. Butyrophenone	0.71	5. Acetophenone	0.53
6. 1-Phenyl-1-butanol benzoate	0.74	6. 1-Phenyl-1-pentanol n-butyrate	0.58
7. Unidentified compound	0.86	7. Valerophenone	0.74
		8. 1-Phenyl-1-pentanol benzoate	0.77
		9. Unidentified compound	0.89

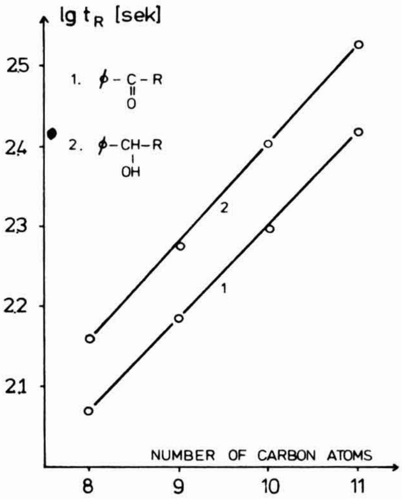

FIG. 2. Dependence of the logarithm of the retention time t_R from the number of carbon atoms for the main oxidation products.

phenylalkyl alcohols. The additional proof of the presence of these compounds in the reaction mixtures is the straight-line dependence of their retention data from number of carbon atoms in a molecule. Along with the isothermal separation of the oxidation products, we performed the analyses with the temperature of the thermostat programmed. The range of changes was 100°–200°C, and the rate of temperature increase was 12°C/min. The other working conditions remained without change. Measurements with the programmed increase of temperature enabled the complete separation of the components of oxidation mixtures. The qualitative identification was also carried out with the help of inner standard technique. The obtained chromatograms are shown in Fig. 3.

The presented separations of the oxidation products (Fig. 3) were received for the 0.6 μl samples of mixtures, using the sensitivity of 8×10^{-10} and 2×10^{-10}. These parameters of measurements and the applied program of temperature changes enabled separation and detection even of the trace products of oxidation. Nevertheless, we did not succeed in performing the complete qualitative identification of all the components of the examined mixtures.

DISCUSSION

On the basis of the presented chromatographic investigations, the partial identification of the oxidation products for the selected n-alkylaromatic hydrocarbons was given. In presence of cobalt(II) stearate the following main products are obtained in the course of oxidation of n-alkylbenzenes: (a) for ethylbenzene: acetophenone and 1-phenyl-1-ethanol; (b) for *n*-propylbenzene: propiophenone, 1-phenyl-1-propanol,

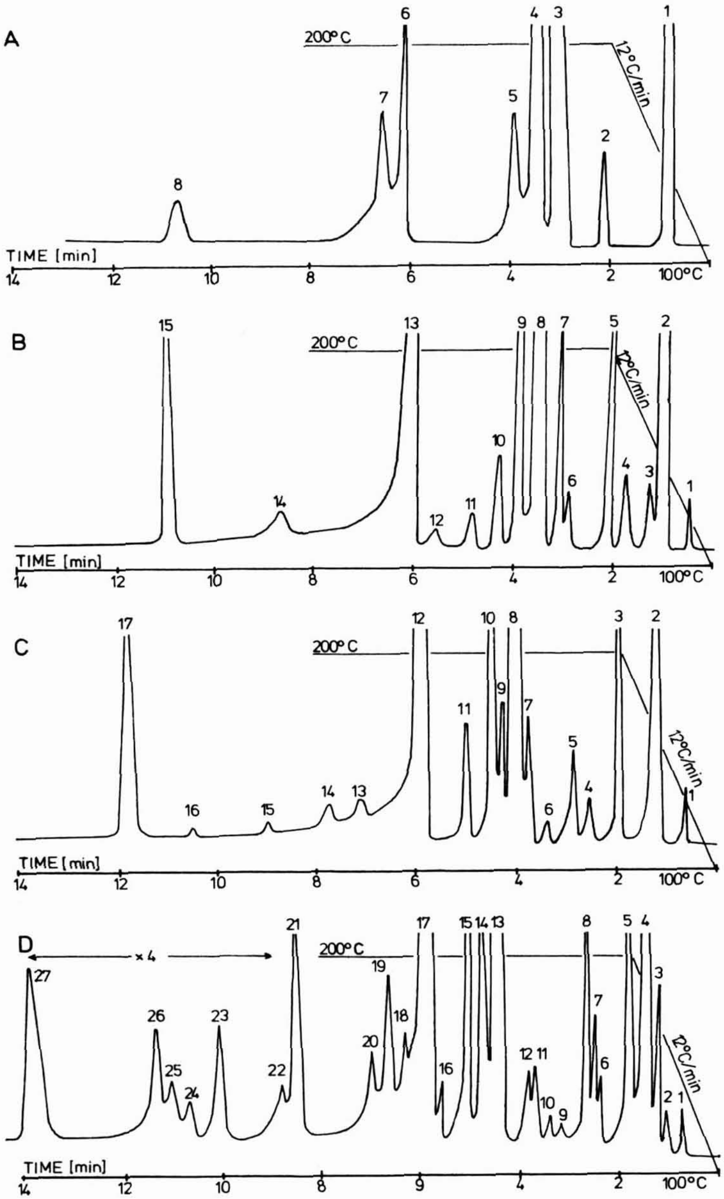

FIG. 3. Chromatograms of the oxidized *n*-alkylbenzenes. (A) Ethylbenzene: 1. unoxidized hydrocarbon; 2. benzaldehyde; 3. acetophenone; 4. 1-phenyl-1-ethanol; 5. phenol; 6. benzoic acid; 7. bis-(-1-phenyl-1-ethyl) ether; 8. 1-phenyl-1-ethanol benzoate. (B) *n*-Propylbenzene: 1. acetic acid; 2. unoxidized hydrocarbon; 5. benzaldehyde; 6. acetophenone; 8. propiophenone; 9. 1-phenyl-1-propanol; 10. 1-phenyl-1-propanol acetate; 13. benzoic acid; 15. 1-phenyl-1-propanol benzoate; the other products were not identified. (C) *n*-Butylbenzene: 1. propionic acid; 2. unoxidized hydrocarbon; 3. benzaldehyde; 5. acetophenone; 6. phenol; 8. butyrophenone; 10. 1-phenyl-1-butanol, 11. 1-phenyl-1-butanol propionate; 12. benzoic acid; 17. 1-phenyl-1-butanol benzoate; the other products were not identified. (D) *n*-Pentylbenzene: 1. propionic acid; 3. *n*-butyric acid; 4. unoxidized hydrocarbon; 5. benzaldehyde; 8. acetophenone; 9. 1-phenyl-1-ethanol; 10. phenol; 12. benzyl alcohol; 13. valerophenone; 15. 1-phenyl-1-pentanol; 16. 1-phenyl-1-pentanol propionate; 17. benzoic acid; 19. 1-phenyl-1-pentanol *n*-butyrate; 27. 1-phenyl-1-pentanol benzoate; the other products were not identified.

and benzoic acid; (c) for *n*-butylbenzene: butyrophenone, 1-phenyl-1-butanol, and benzoic acid; (d) for *n*-pentylbenzene: valerophenone, 1-phenyl-1-pentanol, and benzoic acid.

The a/m compounds were identified by thin-layer and gas chromatography. With the help of gas chromatography, we managed to identify the major portion of the oxidation products of the investigated *n*-alkylbenzenes. The identification was carried out by comparison of the R_f values, retention times t_R and log t_R of the substances formed during oxidation with the corresponding data of the standard compounds. Thin-layer chromatography can be used exclusively for identification of the main products of oxidation. The application of gas chromatography helps to the deeper understanding of the oxidation of *n*-alkylbenzenes, to determine the yields of the formed products and to trace the mechanism of reactions.

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MICROCHEMICAL JOURNAL **20**, 553–557 (1975)

BOOK REVIEWS

Analytical Profiles of Drug Substances, Vol. 3. Edited by KLAUS FLOREY. Academic Press, New York, 1973. ix + 581 pp. $19.50.

The third volume of "Analytical Profiles of Drug Substances," presents comprehensive analytical information about important drug substances. As in previous volumes, this profile includes physical and chemical properties of drug substances as well as synthesis, methods of analysis, pathways of physical and biological degradation and metabolism, solubility, pH and pK values, spectra and spectrophotometric constants, and stability data.

Drug compounds mentioned in volume 3 are: Acetaminophen, Alpha-Tocopheryl Acetate, Amitriptyline Hydrochloride, Digitoxin, Diphenhydramine Hydrochloride, Echothiophate Iodide, Ethynodiol Diacetate, Fludrocortisone Acetate, Flurozepam Hydrochloride, Iodipamide, Methodone Hydrochloride, Oxazepam, Phenazopyridine Hydrochloride, Phenylephrine Hydrochloride, Tolbutamide, Trimethaphan Camsylate, and Tropicamide.

Although the drug substances described in this book are defined as to identity, purity, strength, and quality in the official compendia (United States Pharmacopeia and National Formulary), these profiles provide important supplemental information that contributes to the better understanding of drug substances. The information presented in this volume is a collection of data scattered throughout the literature and files of pharmaceutical laboratories. The profiles provide information from important instrumentation including Infrared, Nuclear Magnetic Resonances, Mass Spectroscopy, Differential Scanning Calorimetry, Thermogravimetric Analysis, and Gas–Liquid Chromatography. The profiles are sufficiently thorough and provide an authoritative source of information concerning the properties of drug substances as well as up-to-date references. Also included is a cumulative index.

This book provides a valuable and convenient reference and should be welcomed by research pharmaceutical chemists and research pharmacists.

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Biopolymers. By A. G. WALTON and J. BLACKWELL, Academic Press, New York, 1973. x + 604 pp. $35.00.

This is another volume in the highly successful "Molecular Biology" series published by Academic Press.

Two closely related volumes in the series have been recently reviewed in this Journal {Conformation of Fibrous Proteins [**19**, 337 (1974)] and Conformational Properties of Macromolecules |**20**, 113 (1975)|}.

The present volume, unlike the two other recently reviewed ones attempts to provide an overall approach to the study of biopolymers. That is, it attempts coverage of nearly all significant methods presently utilized in the study of the structure of biopolymers. This approach has some advantages and some drawbacks. The advantages are primarily pedagogical in that this volume could serve as a textbook for a course in Physical Biochemistry (or Biophysical Chemistry). Such courses have been offered by most graduate departments of biochemistry (or even chemistry) and the standard texts for these courses (such as Tanford's Physical Chemistry of Macromolecules or Martin's Biophysical Chemistry or Edsall and Wyman's Biophysical Chemistry) are all badly outdated so far as the literature coverage or even a multitude of novel methods are concerned.

The volume contains the following chapters:

1. Structural units of biopolymers (mainly concerned with nomenclature); 2. Conformation (concerned with a description of the conformational conventions in polypeptides, polynucleotides, polysaccharides as well as semiempirical potential energy maps such as those advanced by Liquori, Scheraga, Ramachandran and others); 3. Structure determination by X-ray diffraction with emphases on the identification of the type of ordering present (i.e., helical, etc.); 4. Morphology of synthetic biopolymers (e.g., electron micrographs); 5. Infrared and Raman spectroscopy—this is a particularly valuable chapter since the application of Raman spectroscopy to studies of polypeptide (and enzyme) and polynucleotide structural studies is very recent indeed. 6. Electronic spectroscopy (including absorption, optical rotation, circular dichroism) applied to biopolymer structural studies, especially the important applications to nucleotide conformation and interactions. 7. Physical properties of biopolymers (by Stephen H. Carr) in dilute solutions—encompassing most "classical" methods of molecular weight and shape determination: osmometry, light scattering, small angle X-ray, viscometry, sedimentation, flow birefringence, molecular weight distribution and the applications of all the above to conformations and conformational transitions. This is a 46 page long chapter and it treats very briefly a substantial portion of Tanford's book. 8. The interaction of electric and magnetic fields with biopolymers—primarily nuclear magnetic resonance and briefly dielectric dispersion. The remaining chapters (9. on polypeptides; 10. on fibrous proteins and biopolymer models; 11. on polysaccharides; 12. on nucleic acids and polynucleotides; and 13. on globular proteins) survey the application of the various methods to the conformational and structural studies on some specific biopolymers.

Now for the drawbacks. First of all, the volume is relatively expensive and this reviewer finds it difficult to require students to spend this much money for a text. Secondly, due to the very extensive coverage, the literature quoted is most often terminated with 1971. Thirdly, perhaps a corollary of the second problem, some important methods are left out (e.g., electron spin resonance, fluorescence) and the applications quoted are out of date in mid-1974 already (the time of writing of this review).

Again, and finally, the book is valuable as a teaching aid and as such is highly commendable. The researcher in any of the areas will still have to survey the literature in his area. Purchase of this volume by libraries can be highly recommended.

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Organic Reactive Intermediates. Edited by S. P. McMANUS, Academic Press, New York, 1973. x + 539 pp. $39.50.

The editor states in the preface: "It is the purpose of "Organic Reactive Intermediates" to present a modern treatment of all the major intermediates for those who have a basic course in organic chemistry. The chapters were written with advanced undergraduates and graduate students as the intended audience since budding chemists at that level of training would need and appreciate a more thorough introduction to reactive intermediates than the scattered accounts presently available." This goal is met very admirably.

The topics covered are divided into chapters entitled free radicals, carbenes, nitrenes, carbonium ions, carbanions, radical ions, and arynes. Each chapter is written by experts in the field. The authors have been very well chosen. The chapters provide sufficient background material, explanations, and critical evaluation so that a novice in the field could readily comprehend developments up to the present time, in the areas discussed.

The book could serve very well as a text for graduate students in a course in mechanistic organic chemistry or as an introduction to a research worker who desires information in the fields covered. The literature is covered very extensively up to early 1972. Regrettably, the very high price [$39.50] will probably prevent this book from receiving the wide distribution it deserves.

IRVIN ROTHBERG *Department of Chemistry, Rutgers University, Newark, New Jersey 07102*

Advances in Biochemical Engineering, Volume 3. Edited by T. K. GHOSE, A. FIECHTER, AND N. BLAKEBROUGH. Springer-Verlag, New York, 1974. 290 pp., $29.00.

At first glance, there would appear to be few common areas of interest between the fields of biology and engineering. However, in the production of drugs and processes such as air sterilization, oxygen transfer in large-scale fermentation systems and continuous microbial culturing, knowledge from both scientific fields is essential. The purpose of this series of volumes on *Advances in Biochemical Engineering,* of which this is the third volume, is to break down existing communication barriers between biologists and engineers, and thereby promote useful research in the area of biochemical engineering. Although the text contains no introduction explaining how the articles included in the book were selected, the authors represent five countries including Great Britain, Canada, CSSR, Federal Republic of Germany, and Switzerland.

The volume begins with a brief summary of a seminar on topics of fermentation microbiology held in Switzerland, in 1972. The remainder of the book presents seven papers prepared for this volume on various aspects of biochemical engineering. The titles of the seven papers are: "Genetic problems of the biosynthesis of tetracycline antibiotics," "Some aspects of basic genetic research on fungi and their practical implications," "Microbial oxidation of methane and methanol," "Modeling and stimulation in biochemical engineering," "Transient and oscillatory states of continuous culture," "The significance of microbial film in fermenters," and "Present state and perspectives of biochemical engineering." Each article is well-referenced; however, relatively few illustrations are used (varying widely in quality). The book does not include an index.

The articles included in this volume are well-written and do fulfill the stated purpose of this series. The presentation suffers from the lack of an introduction or other device to tie the articles together under the concept of biochemical engineering. In addition, although this is a book designed for a limited group of scientists, the price does seem exceptionally high, and this factor will probably further limit the number of scientists who will wish to purchase a copy.

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Encyclopedia of Industrial Chemical Analysis. Vol. 20. Edited by F. D. SNELL AND L. S. ETTRE. Wiley (Interscience), New York, 1974. xl + 679 pp. $50.00 ($35.00 by subscription).

Ten or more years ago, this reviewer had the opportunity to discuss with Foster Dee Snell the underlying philosophy and overall plan for his proposed *Encyclopedia of Industrial Analysis*. In retrospect, Dr. Snell had the vision to see how the work could be structured to serve a spectrum of uses for analytical chemists and chemical analysts and how it could be shepherded to reality. "What is worth doing at all is worth doing well" was the maxim of Lord Chesterfield. With Volume 20, this unique work is complete. It was worth doing. It has been done well. Dr. Snell, the executive editors, C. L. Hinton (Volumes 1–7) and L. S. Ettre (Volumes 8–20), and the associated editorial staff are to be complimented on their perseverance.

The work had over 350 contributors, largely drawn from U. S. companies. This reviewer might be charged with bias towards the work (he contributed six articles to it). Yet, in his honest opinion any industrial library should have this encyclopedia, if it has among its chemistry-oriented clientele, individuals and groups involved in quality control, in-process analysis, specifications and standards, or analytical support to research. The work can often be the first sourcebook to examine when an unfamiliar compound or material must be either specified, assessed, or controlled.

Volume 20 presents a 679-page subject index to Volumes 4–19. (Volume 3 included a subject index to Volumes 1–3, which consider general analytical techniques.) Volume 20 also lists, with pagination, all articles in Volumes 1–19 and delineates the almost 300 contributors to Volumes 4–19 by their professional association and the title of their article.

A comprehensive subject index can be viewed as the most important part of an encyclopedia; it must bring unity to diversity. Criteria for judging a subject index include adequacy of depth, extent of cross-references, and consistency of indexing practices. The index to this work certainly meets the criterion of depth: about 116 lines of entries per page and 679 pages! Both the frequency and aptness of cross-references are excellent. Consistency in indexing practices is a challenge to the indexer, especially where the text varies widely in subject, nomenclature, and terminology. For this encyclopedia, the indexters have usually preferred to retain each key term essentially as it appears in the text and have often not elected to *gather* (in the vernacular of the indexing art) identical or closely related terms under a single rational heading. As a consequence, for example, references to the diethyldithiocarbamate extraction of metals are scattered under "diethylammonium diethyldithiocarbamate," "diethyldithiocarbamate," and "sodium diethyldithiocarbamate." Similarly, references to 8-quinolinol (the preferred *Chemical Abstracts* name) appears partially under "8-hydroxyquinoline" and "oxine." Admittedly, in many situations of this type, cross-references have been provided. The nomenclature for organic compounds in the index is as satisfactory as can be expected considering that the individual articles were, unfortunately, not styled to a single set of practices. Occasionally, the *alphabetic* arrangement of simple substituents (multiplying prefixes being disregarded), which is the practice of *Chemical Abstracts* and most U. S. authorities, has not been followed. But such criticism of the indexing employs a magnifying glass rather than normal vision.

Supplementary volumes to update and to extend this work may be appropriate and the appearance of a second edition over a period of years can earnestly be hoped for. For the *Microchemical Journal,* this reviewer has critiqued Volumes 8–20 and other reviewers have considered some earlier volumes. The criticism, thereby fractionally distilled, is largely constructive and should assist the editors in rethinking the philosophy and practices for a new edition and in defining a program of revision.

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Chemistry of Viruses. Ed. 2. By C. A. KNIGHT. Springer-Verlag, New York, 1975. x + 325 pp. $17.80.

The aim of the first (1963) edition of *Chemistry of Viruses* was to review some major principles and techniques of chemical virology in a concise manner and to accompany the review with a compilation of pertinent references. The aim of the present edition is to present a concise, integrated account of chemical virology which embodies the enormous amount of data published since the first edition, and to relate the chemical features of viruses to their biological activity.

Beginning with a brief introductory chapter outlining some events leading to the chemical era of virology there follows a survey of the various purification techniques which are being used. Enough details are presented to guide the reader.

A long chapter dealing with the composition of viruses discusses the major components (proteins, nucleic acids, lipids, and carbohydrates) in terms of preparation, analysis, and functions of each. The bulk of the material is concerned with the first two components.

In reviewing the morphology of viruses there is included a long tabulation of the size and shape of groups of animal, bacterial, insect, and plant viruses, as well as short discussions of some of the typical shapes encountered.

The action of chemical and physical agents on viruses is surveyed. Both inactivation and mutation are examined plus a discussion of the viral genes.

A final chapter deals with the reproduction of viruses both within the cell and extracellularly.

As in the first edition there is a compilation of reference books and a long list of textual references plus an index.

The publisher advocates this book as a suitable text for advanced undergraduate and graduate students and as a convenient reference. The reviewer concurs.

BILL ELPERN, *9 Surrey Way,*
White Plains, New York 10607

MICROCHEMICAL JOURNAL **20**, 558–559 (1975)

Author Index for Volume 20

Microchemical Journal

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Editor: Al Steyermark
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NUMBER 1, MARCH 1975

NUMBER 2, JUNE 1975

NUMBER 3, SEPTEMBER 1975

NUMBER 4, DECEMBER 1975

Theoretical Chemistry: a series of monographs No. 4
Series editors D. P. Craig and R. McWeeny

MOLECULAR COLLISION THEORY

M. S. Child
Department of Theoretical Chemistry
University of Oxford, England

September 1974, x+302 pp., £8.50/$22.00
0.12.172650.9

This book contains an introduction to molecular collision theory for those seeking an interpretation of experimental results. Emphasis is given to the validity and relevance of available theoretical techniques, rather than to the formal structure of the theory. The argument starts from a quantum mechanical standpoint, but the reader is quickly led to appreciate the importance currently given to semi-classical methods.
Molecular Collision Theory will serve primarily as a reference book, but one which also consolidates recent advances in the literature, and should prove of value to chemists and physicists with a grounding in bound state quantum mechanics.

Contents

Introduction.
Classical scattering by a central force.
Quantum scattering by a central force.
Elastic scattering phase shifts.
Semi-classical models.
General theory of inelastic collisions.
Quantum inelastic transition probabilities.
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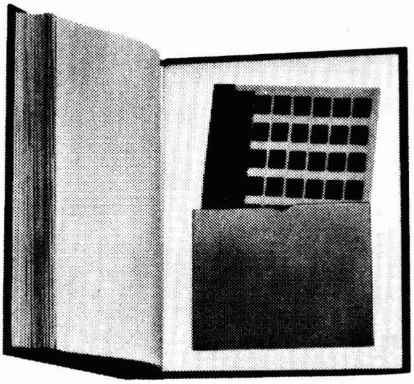

INFORMATION FOR AUTHORS

The MICROCHEMICAL JOURNAL will invite research papers on all phases of chemistry, including clinical and biochemical, which involve small-scale manipulation. This will include both organic and inorganic work on preparation, purification, separation, detection, determination, trace analysis, and all types of instrumentation.

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Units of weights, measures, etc. when used in conjunction with numerals should be abbreviated and unpunctuated (e.g., 10%, 50 ml, 3 g, 8 cm, 5 μg, 3 μl).

Names of compounds should agree with those presently in use by Chemical Abstracts.

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All figures and illustrations are to be submitted in such form as to permit photographic reproduction without retouching or redrawing. This includes the lettering which is reproduced as part of the photoengraving and is not set in type. (Lettering should be done with a lettering device, such as a Leroy). Line drawings should be carefully drafted with black India ink on white drawing paper or blue drafting cloth, no larger than 8.5 × 11.5 inches (21 × 27.5 cm). The lettering should be large enough so that when reduced by two-thirds it will still be legible. High quality glossy prints are acceptable. Xerox prints are not acceptable.

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Footnotes should be designated in text by superscript numbers and listed on a separate sheet; in tables, by superscript letters and placed at bottom of page containing table.

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Pattern of styling, punctuation, and capitalization after these examples, noting the particular style for journal articles, books, and edited (multi-author) books.

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Quality Control in the Pharmaceutical Industry

Edited by MURRAY S. COOPER
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