ISSN 0026-265X

This Number Completes Volume 25
Volume 25, Number 4, December 1980

Microchemical Journal

devoted to the application of microtechniques in all branches of science

Editor: Al Steyermark

Published under the auspices of the American Microchemical Society by

ACADEMIC PRESS
New York London Toronto Sydney San Francisco

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Published quarterly by Academic Press, Inc.,
111 Fifth Avenue, New York, N.Y. 10003

1980: Volume 25. Price: $73.00 U.S.A.; $86.00 outside U.S.A.
1981: Volume 26. Price: $79.00 U.S.A.; $93.00 outside U.S.A.
All prices include postage and handling.

Information concerning personal subscription rates may be obtained by writing to the Publisher. All correspondence and subscription orders should be addressed to the office of the Publishers at 111 Fifth Avenue, New York, N.Y. 10003. Send notices of change of address to the office of the Publishers at least 6 to 8 weeks in advance. Please include both old and new addresses.
Second class postage paid at New York, N.Y., and at additional mailing offices.

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Briefs

Spectrophotometric Determination of Anions by Reextraction through the Exchange of Ligands: The Determination of Traces of Oxalate Using Ferric Indol-2-carboxylate in Isoamyl Alcohol. M. ROMÁN CEBA, J. A. MUÑOZ LEYVA, AND F. VINAGRE JARA, *Department of Analytical Chemistry, Faculty of Sciences, The University Extremadura, Badajoz, Spain.*

The new method is based on the decoloration produced in organic solutions of colored complexes when mixed with aqueous solutions of complexing anions.

Microchem. J. **25**, 443–457 (1980).

Spectrophotometric Determination of Vanadium(V) with Oxine in Isoamyl Alcohol. PILAR BERMEJO-BARRERA, ADELA BERMEJO-BARRERA, AND F. BERMEJO-MARTINEZ, *Department of Analytical Chemistry, Faculty of Chemistry, University of Santiago de Compostela, Santiago de Compostela, Spain.*

A new method employing the use of oxine (8-hydroxyquinoline) is described and applied to the determination of vanadium in foods.

Microchem. J. **25**, 458–464 (1980).

Determination of Platinum with 4-*S*-Benzyl-1-*p*-chlorophenyl-5-phenyl-2,4-isodithiobiuret (BPPTB). B. K. DESHMUKH AND R. B. KHARAT, *Department of Chemistry, Nagpur University, Nagpur 440010, India.*

Microchem. J. **25**, 465–470 (1980).

Preparation of Some Carbon-14-Labeled Amino Acids by Radiophotosynthesis Using Sugar Beet Plant. M. F. BARAKAT, A. A. EL-GHARBAWY, AND A. N. FARAG, *Nuclear Chemistry Department, Nuclear Research Center, Atomic Energy Authority, Cairo, Egypt*

The apparatus and procedure are described for preparing a number of labeled amino acids.

Microchem. J. **25**, 471–484 (1980).

The Cleavage Mechanism of 1,4-Dithiaspiro(4.4)nonane by Gas-Phase Pyrolysis. M. MOHAMMADI AND J. D. WILLETT, *Department of Chemistry, University of Idaho, Moscow, Idaho 83843.*

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Ionic Strength and Buffer Capacity of Wide-Range Buffers for Polarography. CRAWFORD JORDAN, *Department of Analytical Chemistry, The Queen's University, Belfast BT9 5AG, Northern Ireland.*

A procedure for the calculation of the ionic strength and buffer capacity of a series of buffers prepared by the partial neutralization of a mixture of mono- and polybasic acids is outlined and data tabulated for a mixture 0.02 *M* in each of CH_3COOH, H_3PO_4, and H_3BO_3. The useful polarographic range of each buffer solution is also given.

Microchem. J. **25**, 492–499 (1980).

A New Continuous Flow Micromethod for Determination of Creatinine in Serum or Plasma. E. D. RYAN AND W. H. C. WALKER, *Department of Pathology, McMaster University Medical Center, Hamilton, Ontario L8S 4J9, Canada.*

Reported is the development of an assay in which the major barrier to achieving increased sensitivity in a continuous flow system, i.e., the dialysis step, is eliminated. Only 7.5 μl of plasma is required for the assay.

Microchem. J. **25**, 500–506 (1980).

Extractive Separation of Platinum from Macroamounts of Palladium Using Triphenylphosphine Oxide and Its Successive Spectrophotometric Determination by the Stannous Chloride Method. M. MOJSKI AND K. KALINOWSKI, *Department of Analytical Chemistry, Warsaw Technical University, Noakowskiego 3, 00-664 Warsaw, Poland.*

Trialkylphosphine oxides extract chloride complexes of platinum more effectively than those of palladium. Of the oxides studied, triphenylphosphine oxide is best.

Microchem. J. **25**, 507–513 (1980).

Luminol-Dependent Chemiluminescence Analysis of Human Platelets. KNOX VAN DYKE,* CYNTHIA VAN DYKE,* DAVID PENDEN,* GEORGE JONES,† VINCENT CASTRANOVA,† ERIC BRESTEL,‡ AND MICHAEL RINGROSE,§ *Departments of *Pharmacology and Toxicology and of †Physiology and ‡Medicine–Allergy Section, West Virginia University Medical Center, Morgantown, West Virginia 26506; †(ALOSH, NIOSH, CDC, AND HEW), Morgantown, West Virginia 26506; and §Analytical Luminescence Laboratory, West Lake Village, California 91361.*

A simple, sensitive, and reproducible assay system to measure the chemiluminescence produced by injecting arachidonic acid into a preparation of human platelets containing luminol is described.

Microchem. J. **25**, 514–523 (1980).

Spectrophotometric Determination of Mercury with 5-(6-Methyl-2-pyridyl)methylene-2-thiohydantoin. F. BARRAGAN DE LA ROSA, M. T. MONTAÑA GONZALEZ, AND J. L GÓMEZ ARIZA, *Department of Analytical Chemistry, Faculty of Science and Pharmacy, University of Seville, Seville, Spain.*

A method for the spectrophotometric determination of mercury with 5-(6-methyl-2-pyridyl)methylene-2-thiohydantoin is described, the influence of different experimental parameters on the formation of the complex is studied, and optimum conditions for the determination are established.

Microchem. J. **25**, 524–530 (1980).

Determination of Cadmium and Copper in Galvanic Coatings by Means of Atomic Absorption. A. BUDNIOK, *Institute of Physics and Chemistry of Metals, Silesian University, 12 Bankowa Street, 40-007 Katowice, Poland.*

An atomic absorption method was adjusted to determine copper and cadmium in a bath in the presence of ethylenediamine and in the Cu-Cd alloy coatings obtained from that bath.

Microchem. J. **25**, 531–534 (1980).

A Sensitive Reaction for Dilute Cholesterol Determinations. J. D. ARTISS* AND B. ZAK,† **Department of Medical Biochemistry, St. Joseph's Hospital, London, Ontario N6A 4V2, Canada and †Department of Pathology, Wayne State University School of Medicine, and Detroit General Hospital, Detroit, Michigan 48201.*

A sensitive reaction for the peroxidase-coupled sequence of the determination of a dilute total cholesterol mixture of free and esterified forms is described. Substitution of a chlorinated auxochrome of phenol made water soluble by sulfonation through a synthetic procedure created a severalfold enhancement factor which magnified considerably the sensitivity of one equilibrium reaction over the other.

Microchem. J. **25**, 535–542 (1980).

The Determination of Trace Amounts of Chlorophenols by High-Performance Liquid Chromatography. ZLATA IVANOV AND R. J. MAGEE, *Department of Inorganic and Analytical Chemistry, La Trobe University, Bundoora, Melbourne, Victoria, 3083 Australia.*

A method is given for the separation and determination of eight chlorophenols using high-performance liquid chromatography. After extraction from aqueous solution by means of diethyl ether the compounds are taken up in methanol–petroleum ether and injected. Separation of all eight is achieved in 25 min.

Microchem. J. **25**, 543–547 (1980).

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Spectrophotometric Determination of Molybdenum with Lobeline. K. NYTKO AND H. SIKORSKA-TOMICKA, *Department of Chemistry, Białystok Technical University, Wiejska 45 a, Bialystok, Poland.*

Molybdenum is reduced with hydrazine Mo(V) and complexed with thiocyanate and lobeline.

Microchem. J. **25**, 548–550 (1980).

Study of the Electrochemical Oxidation of Phenothiazine Derivatives in Acetonitrile Medium: The Effect of the Structure on the Voltammetric Behavior. N. ŠULCOVÁ, I. NĚMEC, K. WAISSER,* AND H. L. KIES,† *Department of Analytical Chemistry, Faculty of Natural Sciences, Charles University, Albertov 2030, 128 40 Prague 2, Czechoslovakia, *Pharmaceutical Faculty, Charles University, Hradec Kralove, Czechoslovakia, and †Laboratory for Analytical Chemistry, University of Technology, Delft, The Netherlands.*

The electrochemical oxidation of phenothiazine and its derivatives substituted in the 2 and 10 positions was studied.

Microchem. J. **25**, 551–566 (1980).

Microanalysis of Catecholamines in Human Plasma by High-Performance Liquid Chromatography with Amperometric Detection as Compared with a Radioenzymatic Method. STIG ALLENMARK, LISBETH HEDMAN,* AND ANITA SÖDERBERG,† *Clinical Research Centre, Departments of *Surgery and †Medicine, Linköping University Hospital, S-581 85 Linköping, Sweden.*

A high-performance liquid chromatographic procedure is described for the determination of epinephrine, norepinephrine, and dopamine in human plasma.

Microchem. J. **25**, 567–575 (1980).

Spectrofluorometric Study of 1,4-Diaminoanthraquinone–Calcium Complex in Aqueous Sulfuric Acid Mediums: Spectrofluorometric Determination of Calcium. M. ROMÁN CEBA, A. FERNÁNDEZ-GUTIÉRREZ, AND F. CÁRDENAS, *Department of Analytical Chemistry, Faculty of Science of Badajoz, University of Extremadura, Badajoz, Spain.*

A study was made of the complex in acid mediums and a new method for the determination of trace amounts of calcium was proposed.

Microchem. J. **25**, 576–588 (1980).

MICROCHEMICAL JOURNAL 25, 443–457 (1980)

Spectrophotometric Determination of Anions by Reextraction through the Exchange of Ligands: The Determination of Traces of Oxalate Using Ferric Indol-2-carboxylate in Isoamyl Alcohol

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Received December 18, 1979

INTRODUCTION

The photometric determinations of anions are generally scarce in number, and, therefore, any new contributions in this area are of importance.

In the References there are few adequate procedures for determining oxalates in concentrations of the parts per million range. Among the most common methods, those which stand out are the colorimetric and spectrophotometric methods. Most of these are indirect and generally based on the decrease of the absorbance of colored complexes by the action of $C_2O_4H_2$. Some examples are: the use of orange-colored peroxivanadate, previously formed by the reaction between VO_3NH_4 and H_2O_2 (*8*); decoloration of Fe(III) complexes with salicylic acid (*3*); with 3-hydroxy-1-*p*-sulfonato-phenyl-3-phenyltriazine (*7*); 1-(2-carboxy-4-sulfonato-phenyl)-3-hydroxy-3-phenyltriazine (*6*); Sc(III) complex with Bordeaux C monochromic acid and Zr(IV) with 2-carboxybenzenazo-3,4-dihydroxybenzene (*15*); and the most commonly used method which used 4-(2-pyridylazo)resorcinol complex with UO_2(III) to determine from 0.4 to 4 ppm of oxalate (*1*).

In this study a new technique for determining anions has been developed and has been termed "reextraction through the exchange of ligands." This process occurs when organic solutions of a colored metalic complex are mixed with diluted aqueous solutions of anions. In this way the exchange of ligands takes place. The new complex passes to the aqueous phase while the initial ligand remains in the organic phase. The possibility of determining anions is based on the decoloration which occurs in the organic phase, and which is found to be in proportion to the anion concentration in the aqueous phase.

0026-265X/80/040443-15$01.00/0

Some reactions of cation exchange through reextraction are known. In this case the reaction that occurs is:

$$n\ M_{II}A_{m(o)} + m\ M^{n+}_{I(a)} \rightleftharpoons m\ M_{I}A_{n(o)} + n\ M^{m+}_{II(a)}.$$

These reactions are important for the photometric determination of cations since they increase selectivity. Thus, in determining copper by extraction photometry, as diethyldithiocarbamate in Cl_3CH, Hg(II), Ag(I), Au(III), Pt(IV), Os(IV), Pd(II), Sb(III), Te(IV), Tl(III) and Bi(III) interfere. Nevertheless, if lead diethyldithiocarbamate is used as reagent, instead of sodium salt, only Ag(I), Hg(II), Tl(III), and Bi(III) (*9*) interfere. Also, cations that form colorless complexes can be determined by exchanging the cation for another that forms a colored complex. Thus, Cd(II) can be determined as diethyldithiocarbamate by mixing its organic solutions with aqueous solution of Cu(II). The intensity of color in the organic solution of copper diethyldithiocarbamate is proportional to the concentration of Cu(II) (*13*). Another method of determining cations is to measure the decoloration produced in the organic solutions of colored complexes, as in the case of determining Hg(II) by using organic solutions of copper diethyldithiocarbamate (*5*).

Exchange reactions have been used widely in determining cations (*16*). In determining anions, however, only the determination of xanthate and sodium diethyldithiocarbamate have been described. For these cases an excess of either Ni(II) or Zn(II) solution is added to the aqueous solution and the complexes are extracted. The extract is separated and titrated with an aqueous solution of mercuric acetate (*2*). Although anions are being determined, the exchange of cations, as in the previous example, is used.

The reaction of the ligand exchange can be written in the form:

$$L_nM_{(o)} + n\ XH_{(a)} \rightleftharpoons X_nM_{(a)} + n\ LH_{(o)},$$

where $L_nM_{(o)}$ is the colored complex in the organic phase which is used as reagent, XH is the anion to be determined, $X_nM_{(a)}$ is the newly formed complex, and $LH_{(o)}$ is the initial ligand.

In the exchange of anions, as compared with the reactions of cation exchange, the newly formed complex, particularly if there is decoloration, remains in the aqueous phase. Also, once the reaction of exchange has taken place, the initial ligand is liberated in the organic phase. For this reason, it is convenient to use organic solutions of complex which are free of reagent, so that the reaction of exchange will be more quantitative.

The constant of the reaction of ligand exchange is:

$$K_i = \frac{(X_nM)_a(LH)^n_0}{(L_nM)_0(XH)^n_a},$$

which can be included as a function of the extraction constant of the complex $L_nM(K_{ex})$ and of the formation constant of the new complex (K_{X_nM}). Thus, the equation:

$$K_{ex} = \frac{(L_nM)_0(H^+)_a^n}{(LH)_0^n\,(M)_a} = \frac{K_{L_nM} \cdot P_{L_nM}}{P_{LH}^n} \cdot K_{LH}^n.$$

After the appropriate substitutions, the result is:

$$K_i = \frac{K_{X_nM}}{K_{ex}} = \frac{K_{X_nM} \cdot P_{LH}^n}{K_{L_nM} \cdot P_{L_nM} \cdot K_{LH}^n},$$

where P_i are distribution constants and K_{LH} is the constant of acidity of the initial ligand. From this we can deduce that the reaction of ligand exchange will improve by high values of the formation constant of the new complex and of the distribution constant of the initial ligand, and by low values of the formation constant of the complex used as reagent, of its distribution constant, and of the acidity constant of the initial bond. The above expression brings out the potential selectivity of this technique, as there are many factors which may be varied to obtain different values for the constant of exchange.

This study proposes the determination of oxalate by the reextraction of anion exchange. This is the first time this technique has been used to determine anions. The colored metallic complex used as reagent was ferric indole-2-carboxylate dissolved in isoamyl alcohol, analyzed in an earlier study (*11*).

To carry out the spectrophotometric determination of oxalate, the necessary experimental conditions for the use of ferric indol-2-carboxylate as reagent were established, as well as the most favorable conditions for its use.

MATERIALS AND METHODS

Apparatus. The apparatuses used were: Unicam SP 8000 and Beckman 25 spectrophotometers, equipped with 1.0-cm glass or quartz cells; Crison digital model 74 pH meter, with glass–calomel electrodes; Selecta Bibromatic agitator 384.

Solutions. The solutions used were: acid indole-2-carboxylic solution, Aldrich, r.a., in isoamyl alcohol at 0.2%; standardized solutions of sodium oxalate; standardized solutions of iron(III); all other products used (solutions of cations, anions, solvents) are of analytical-reagent grade.

Recommended procedure for the determination of oxalate. Considering the experiments made, the following procedure is proposed for determining traces of oxalate: Fifty milliliters of aqueous solution containing between 50 and 300 μg of oxalate is placed in a 100-ml separating funnel. The

pH is set between 2.5 and 3.5 and the solutions are shaken with 10 ml of indole-2-carboxylate–iron(III) solution in isoamyl alcohol, obtained as previously indicated. The phases are then separated, the organic phase is dried with anhydrous Na_2SO_4, and the absorbance is measured at 400 nm against isoamyl alcohol. From the absorbance measurement the decoloration which takes place is determined by the difference with the original absorbance of the organic solution. The unknown concentration is deduced by comparison with a graph established for known concentrations of oxalate (from 1 to 6 ppm).

RESULTS

Preparation of the Solutions of the Complex Iron(III) Indole-2-carboxylate in Isoamyl Alcohol

The solution of the complex in organic phase should be free of reagent. If not, the results of the exchange reactions would be altered, as can be deduced theoretically and has been proven experimentally. Since organic solutions of the complex with an excess of reagent are used at the start, the first procedure must be to remove this excess. This is done by establishing a graph of the reagent in the organic phase to monitor the efficiency of the elimination process. In this way the pH zone in which the reagent passes from the organic to the aqueous phase can be established as well as the pH zone at which the complex is reextracted to the aqueous phase. This gives the pH interval of use for organic solutions of the complex used as reagent in the exchange reaction, and also other variables.

Obeyance of the Lambert–Beer law was also studied for this reagent. From the data obtained, it is calculated that the molar extinction coefficient at 294 nm is 17,600 $M^{-1} \cdot cm^{-1}$.

To discover the influence of the pH in the extraction of the indole-2-carboxylic acid from isoamyl alcohol to water, a series of samples were prepared. The extraction was found to begin at pH 4 and that at pH 6 more than 90% had been extracted.

Then, by preparing samples at pH 5.0, 5.5, and 7.7, and by varying the agitation time for each series, it was found that 1 min of agitation is sufficient to obtain the complete extraction of indole-2-carboxylic acid from isoamyl alcohol to water. Likewise, working at pH 6.0, the number of extractions necessary to free the organic phase of reagent was studied. The results show that after two extractions the concentration of indole-2-carboxylic acid is 10^{-5} M. That is, the organic phase becomes free of reagent after two extractions.

To establish the pH zone in which the complex remains in organic phase, a solution of the complex was prepared in the organic phase with an excess of reagent. In this way, the organic solution, diluted suffi-

ciently, had an absorbance of 0.801 at 400 nm, against an organic solution of indole-2-carboxylic acid at 0.2%. The preparation of samples consists of taking 10-ml portions of the solution of complex and shaking them for 5 min with 50 ml of aqueous solution at different pH values. Then the organic phase is dried with anhydrous Na_2SO_4 and its absorbance is measured at 400 nm against a blank solution of indole-2-carboxylic acid at 0.2% in isoamyl alcohol. These results, along with those of the extraction of indole-2-carboxylic acid from isoamyl alcohol to water, are presented in Fig. 1.

From these data, the complex is found to reextract, at pH zones below 2.2 and above 6.2, and not in the interval between 2.3 and 6.2. Therefore, it is within this zone that organic solutions can be used in reactions of exchange.

It can also be observed that the pH zone between 5.6 and 6.2 is ideal for the reextraction of the reagent without extracting the ferric complex. This pH zone is obtained with an adequate buffer solution of acetic–acetate.

The results of the previous experiments establish the operating conditions for the preparation of organic solutions of ferric indole-2-carboxylate. The procedure is as follows.

(1) To obtain an organic solution of the ferric complex: place between 200 and 1000 μg of Fe(III) in a 100-ml separating funnel, the pH is set at between 3.45 and 3.75 (with HCl or NaOH), distilled water is added to 50 ml, and the complex is extracted with 20 ml of indole-2-carboxylic acid

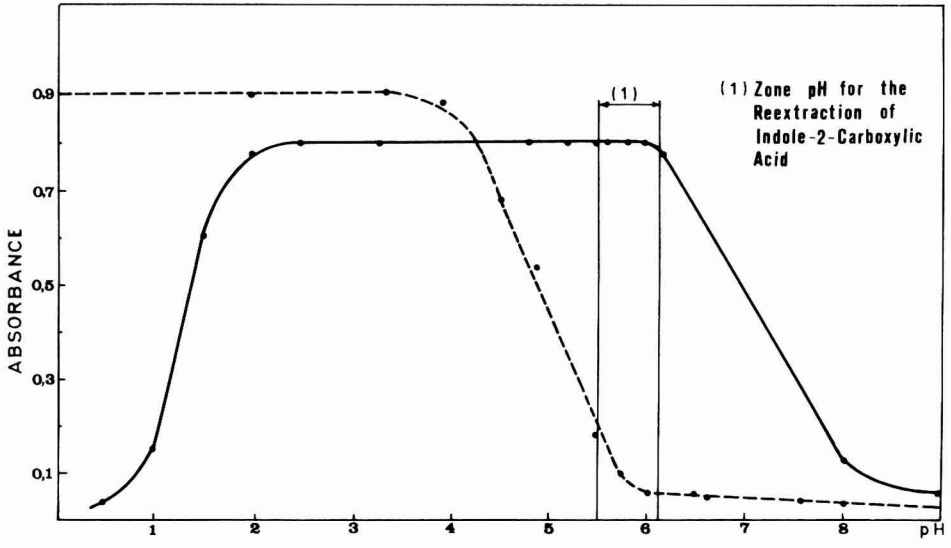

FIG. 1. pH zones for the extraction of indole-2-carboxylic acid and indole-2-carboxylate–iron(III) from isoamyl alcohol to water.

solution at 0.2% in isoamyl alcohol, shaking for 5 min. The phases are separated, ignoring the aqueous phase.

(2) Remove the excess of reagent in the organic phase by shaking with aqueous solutions at pH 5.8. The ratio of aqueous phase/organic phase should be 5, and the agitation time 1 min. The process is repeated a second time.

(3) Obtain an adequate absorbance of the organic solution by drying with anhydrous Na_2SO_4 and then diluting with isoamyl alcohol. The resulting organic solution, with an absorbance near 0.9 at 400 nm will be used as reagent in the following reactions.

The stability of these solutions is an important characteristic. The results found in Table 1 show that the solutions prepared in this manner are stable during the first 6 hr.

Reactions of Exchange

In studying the reactions of exchange, the organic solutions of the ferric complex were of a concentration with an absorbance between 0.75 and 0.95 at 400 nm. The technique used consists of mixing 10 ml of organic solution of the complex with 50 ml of aqueous solution of the ion in question, with pH between 2.3 and 6.2. After shaking, the organic phase is dried, as before, and the absorbance is measured at 400 nm against isoamyl alcohol. The results obtained show that there is observable decoloration when the aqueous solution contains traces (2–5 ppm) of Mo(VI), F^-, PO_4^{3-}, $C_2O_4^{2-}$, $C_4O_6H_4^{2-}$, $C_6O_7H_5^{3-}$, or AEDT and greater concentrations (50 ppm) of Pt(IV) or $S_2O_3^{2-}$ and CN^- (100 ppm).

Results from these experiments formed the basis of the quantitative study of decoloration caused by oxalate ion and aided in the development of the new technique for the spectrophotometric determination of anions.

SPECTROPHOTOMETRIC DETERMINATION OF OXALATE BY THE USE OF FERRIC INDOLE-2-CARBOXYLATE SOLUTIONS IN ISOAMYL ALCOHOL

For the photometric determination of oxalate, based on this decoloration, it is necessary to study several variables, such as the influence of the pH of the oxalate solution, the agitation time, etc. The study of these variables is discussed below.

Influence of the pH

To discover how the pH modifies the intensity of decoloration produced by the oxalate ion, a series of samples were prepared with 3 ppm of $C_2O_4^{2-}$, 5 ml of buffer solution CH_3COO^- or HCl/KCl to obtain different pH values (always in the reagents zone of use), and distilled water to 50 ml. Then the solutions were shaken for 15 min with 10 ml of indole-2-

TABLE 1
STABILITY OF THE ORGANIC SOLUTIONS OF INDOLE-2-CARBOXYLATE-Fe(III)

Time (hr)	Absorbance at 400 nm	Time (hr)	Absorbance at 400 nm
1/4	0.811	6	0.801
1/2	0.809	8	0.797
1	0.806	10	0.748
2	0.803	24	0.521
5	0.807	32	0.489

carboxylate–iron(III) solution in isoamyl alcohol, free of indole-2-carboxylic acid and prepared as indicated. This solution has an absorbance of 0.789 at 400 nm against isoamyl alcohol. When the phases have separated the organic phase is dried with anhydrous Na_2SO_4 and its absorbance measured at 400 nm against isoamyl alcohol. The results are shown in Fig. 2. Here, the maximum decoloration is shown to be produced in the pH range 2.5 to 3.5. This range is obtained with the solution HCl/KCl.

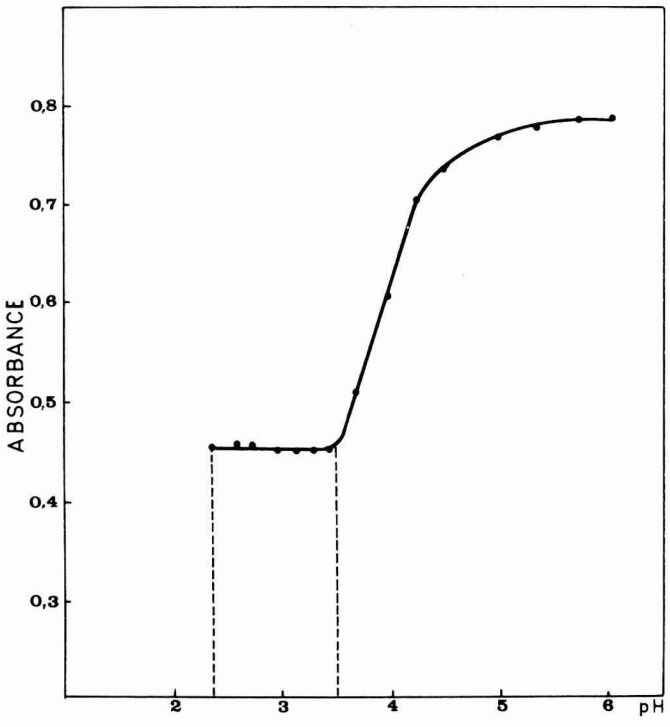

FIG. 2. Influence of the pH in the reaction exchange.

TABLE 2
INFLUENCE OF THE AGITATION TIME

Time, min	1	5	10	15	20	30
Absorbance at 400 nm	0.538	0.483	0.458	0.454	0.455	0.457
Decoloration	0.267	0.322	0.347	0.351	0.350	0.348

Influence of the Agitation Time

The study of agitation time was carried out by preparing several samples with 50 ml of aqueous solution with 3 ppm of $C_2O_4^{2-}$ at pH 3.0. These are shaken for varying time periods with 10 ml of the ferric complex solution in isoamyl alcohol (absorbance is 0.805 at 400 nm). When the phases separate, the organic phase is dried as usual and the absorbance measured at 400 nm against isoamyl alcohol. The results, given in Table 2, show that 10 min of agitation is sufficient to obtain maximum decoloration. Nevertheless, to be safe, 15 min can be considered the ideal agitation time.

Influence of the Ratio of Volumes of Aqueous Phase/Organic Phase

To study this variable samples were prepared with a fixed amount of $C_2O_4^{2-}$ (200 μg), all at pH 3. The volumes of aqueous phase varied between 10 and 70 ml. These were shaken with 10 ml of organic solution of the ferric complex used as reagent (absorbance is 0.805 at 400 nm).

Proceeding as in the previous experiments, the results presented in Table 3 were obtained. It can be seen that as the ratio of volumes of aqueous phase/organic phase is increased, there is less decoloration. The ratio of 5 was selected for convenience and because previous studies employed this ratio.

TABLE 3
INFLUENCE OF THE RATIO OF VOLUMES OF AQUEOUS PHASE/ORGANIC PHASE

Volume aqueous phase/ volume organic phase	Absorbance at 400 nm	Decoloration
1	0.319	0.486
2	0.326	0.479
3	0.331	0.474
4	0.337	0.468
5	0.343	0.462
6	0.356	0.449
7	0.365	0.440

Influence of the Initial Coloration of the Organic Solutions in the Amount of Decoloration

All of the above experiments were made with organic solutions of ferric indole-2-carboxylate that were 3.9×10^{-5} *M* (that is, their absorbance is 0.805 at 400 nm). It is necessary to check whether decoloration is always the same for the same oxalate concentrations and that it does not depend on the initial coloration. If this were the case, it would not be necessary to control precisely the absorbance of the initial coloration. This factor was studied by preparing several samples in which the organic phase presented an absorbance of 0.954, 0.891, 0.832, 0.799, and 0.746. The aqueous phase contained 3, 4, or 5 ppm of $C_2O_4^{2-}$. The operating conditions were those already established. The results obtained are found in Table 4. The data show that the decoloration depends only upon the concentration of $C_2O_4^{2-}$. The initial absorbance has an upper and lower limit. The upper limit is due to the fact that the absorbance of the initial coloration must obey the Lambert–Beer law established for Fe(III) with the use of indole-2-carboxylic acid. The lower limit is a practical one, that is, to determine concentrations with large quantities of $C_2O_4^{2-}$ it is necessary that the decoloration they produce be less than the absorbance of the initial coloration.

Influence of the Concentration of Oxalate

To test whether different concentrations of oxalate produce different decoloration and to discover whether or not the ratio between the two is linear, samples were prepared with aqueous solutions with varying concentrations of oxalate (from 1 to 6 ppm).

Under the same working conditions, these were shaken with 10-ml portions of organic solution of ferric indole-2-carboxylate (absorbance was 0.906 at 400 nm). Then, as in previous experiments, the absorbance at 400 nm was measured. The results are represented in Fig. 3, where it can be pointed out that the decoloration varies linearly on the concentration of

TABLE 4
INFLUENCE OF THE INITIAL COLORATION OF THE ORGANIC SOLUTIONS IN THE AMOUNT OF DECOLORATION

Initial absorbance (at 400 nm)	0.954	0.891	0.832	0.799	0.746
Decoloration for 3 ppm of oxalate	0.344	0.343	0.346	0.346	0.345
Decoloration for 5 ppm of oxalate	0.564	0.570	0.564	0.568	0.567

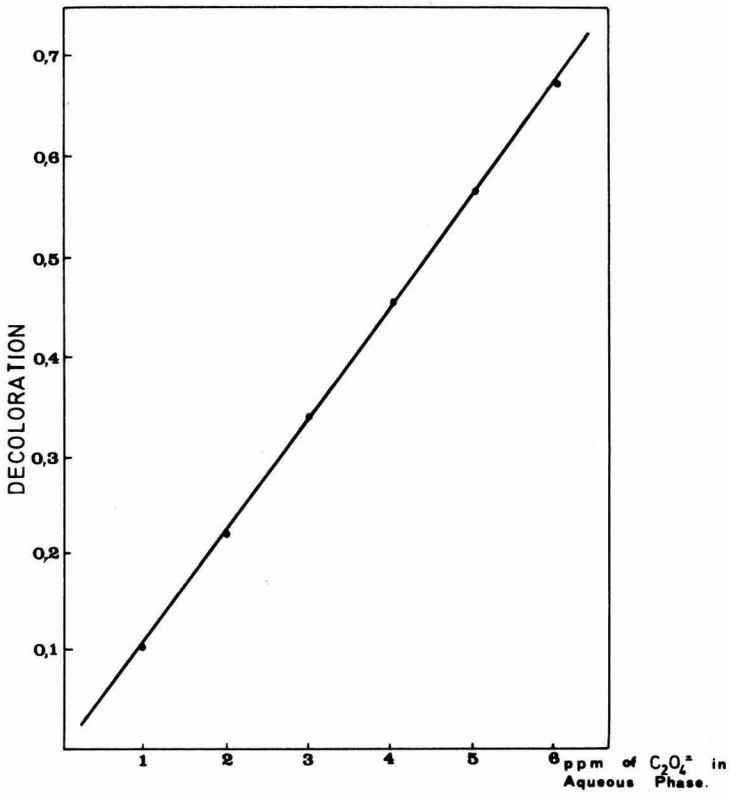

FIG. 3. Influence of the concentration of oxalate.

oxalate in the aqueous phase. From previous results the apparent molar extinction coefficient was calculated to be 10,180 $M^{-1} \cdot cm^{-1}$.

Reliability and Accuracy of the Method

To determine the interval of concentration of oxalate in which the error is minimum, the Ringbom graph (*10*) was constructed from the data obtained in the previous experiment. This representation indicates that the zone of minimum error is that between 2 and 5 ppm of oxalate.

To establish the reproducibility of the method, three series of 11 samples were prepared under independent conditions. Each sample contained a different concentration of oxalate (1.5, 3, and 5 ppm). After operating under the conditions established, the absorbance was measured at 400 nm to determine the amount of decoloration. From the results obtained and setting the confidence level at 95%, the statistical values were calculated and are presented in Table 5. The values calculated for the percentage of errors are quite acceptable considering that they are for an indirect method and also considering variables that influence the determination.

TABLE 5
RELIABILITY OF THE METHOD

Concentration of oxalate (ppm)	Standard deviation	Relative error (%)
1.5	3.57×10^{-3}	±1.44
3.0	4.84×10^{-3}	±0.96
5.0	9.58×10^{-3}	±1.14

Interferences and Their Removal

Study of the influence of other ions was carried out by preparing various samples, all with 3 ppm of oxalate and varying concentrations of the ion to be studied. The determination of oxalate was made following the recommended procedure. The results obtained are summarized in Table 6. The maximum amount tolerable was fixed at that which produces an error below 2% in the determination.

To eliminate interferences of some cations use has been made of the ionic exchange in an acidic medium, using Zerolit 225 NA resin, strongly acid. For the elimination of anions (CN^-, $S_2O_3^{2-}$, NO_2^-, AEDT, PO_4^{3-}, $C_4H_4O_6^{2-}$, and $C_6O_7H_5^{3-}$) the addition of Ag(I) or Cd(II) have been used up to the maximum amount tolerable. The results are found in Table 7.

Nature of the Exchange Reaction

As indicated above, the exchange reaction of bonds can be expressed in the following form:

$$L_nM_{(0)} + n\ XH_{(a)} \rightleftharpoons X_nM_{(a)} + n\ LH_{(0)},$$

TABLE 6
INTERFERENCES OF FOREIGN IONS ON THE DETERMINATION OF OXALATE

Maximum amount tolerable (ratio ion/oxalate, w/w)	Ion added
100	Ag(I), As(III), Cd(II), Li(I), Na(I), K(I), Cs(I), Br^-, Rb(I), I^-, Cl^-, SCN^-, SO_3^{2-}, IO_3^-, ClO_3^-, NO_3^-, $B_4O_7^{2-}$
15	W(VI), Ba(II), CN^-
10	$S_2O_3^{2-}$
6	Pb(II), V(V), Ce(IV), Mn(II).
1	Pt(IV), Cr(III), Zn(II), Al(III), Sr(II), and NO_2^-. Equal amount to the oxalate, of the ions: Cu(II), Mo(VI), Sb(III), Ni(II), Au(III), Bi(III), UO_2(II), Th(IV), Ca(II), Mg(II), F^-, AEDT, PO_4^{3-}, $C_6O_7H_5^{3-}$, and $C_4H_4O_6^{2-}$ cause errors between 9 and 91%.

TABLE 7
ELIMINATION OF INTERFERENCE

Maximum amount tolerable (ratio ion/oxalate, w/w)	Ion added
<40	Ca(II), Sr(II), Mg(II), Zn(II), Pb(II), CN^-, and $S_2O_3^{2-}$
<10	Cu(II), Ni(II), Al(III), Cr(III), Sb(III), Bi(III), UO_2(II), Th(IV), and Ce(IV)

In this section the specific reaction of ferric indole-2-carboxylate and oxalate is discussed. For this discussion, it is necessary to know that oxalate–Fe(III) complex is formed under these conditions. From the results obtained in the study of the influence of the oxalate concentration, we have calculated the amount of ferric indole-2-carboxylate complex destroyed and therefore the concentration of Fe(III) with indole-2-carboxylic acid (*1*). The results obtained show that the molar ratio of $C_2O_4^{2-}$/Fe(III) is close to 2 in all cases. Thus, it can be deduced that the causing complex of the decoloration is $(C_2O_4)_2Fe^-$.

With the above result, together with the other known data, and considering the pH of the oxalate solution, the most probable exchange reaction can be written:

$$L_3Fe_{(0)} + 2\ C_2O_4H^-_{(a)} + H^+_{(a)} = (C_2O_4)_2Fe^-_{(a)} + 3\ LH_{(0)},$$

which corresponds to the constant of exchange:

$$K_i = \frac{(C_2O_4)_2Fe^-_{(a)}\ (LH)^3_{(0)}}{(L_3Fe)_{(0)}(C_2O_4H^-)^2_{(a)}(H^+)_{(a)}}, = 10^{-4.1}$$

On the other hand, we know that:

$$K_{ex} = \frac{(L_3Fe)_{(0)}(H^+)^3_{(a)}}{(Fe^{3+})_{(a)}(LH)^3_{(0)}} = 6.0 \times 10^{-3},$$

where K_{ex} is the constant of extraction of ferric indole-2-carboxylate and whose value was determined previously (*14*). Also, we know that (*4*):

$$K_2 = \frac{((C_2O_4)_2Fe^-)}{(C_2O_4^{2-})^2(Fe^{3+})} = 10^{16.2}, K_{A_2} = \frac{(C_2O_4^{2-})\ (H^+)}{(C_2O_4H^-)} = 10^{-4.1}$$

the following expression is obtained:

$$K_i = \frac{K_2}{K_{ex}}(K_{A_2})^2,$$

in which a value for the constant of exchange of 1.67×10^{10} is obtained by substitution.

DISCUSSION

This study proves that the decoloration of organic solutions of colored complexes by agitation with aqueous solutions containing certain ions, by means of the "reextraction through the exchange of ligands" technique, leads to the establishment of methods to determine anions in aqueous solution.

The background for this technique is found in the exchange reactions of cations described in the References, but has been applied for the first time in this study. The basis of this method is different from other techniques for determining anions by means of extraction. It is based on the positive effect that certain sizable anions exercise upon the extraction of sizable cations. A good example is the determination of ClO_4^- through the use of the copper complex of the azine of the 6-methyl-picolinealdehyde (*12*). Thus, in the reextraction through the exchange of ligands, the anion to be determined passes to the internal coordination sphere of the cation. In the case of determining ClO_4^- the anion remains in the external coordination sphere of the metallic ion and does not destroy the initial complex.

From the results obtained in this paper we can expect the following advantages for the method of reextraction through the exchange of ligands:

(a) This method of determining anions, although indirect, is of importance as there are few methods described in this area. Because it is based on the formation of complexes, we feel that it is not applicable to anions with little or no capacity for complexation, even the most favorable complexes and solvents. In any event, the mechanism of the decoloration could be expanded by the formation of precipitates or by means of redox reactions, which would widen the range of applicability to include all anions.

(b) The sensibility which can be reached is variable, although it is possible to increase it by using other colored complexes of greater molar absorptivity or a more favorable ratio of volumes of phases.

(c) The selectivity improves with respect to the decoloration methods in a homogeneous medium. As demonstrated, the constant of exchange (the indirect form of measuring the possibility and quantity of decoloration) depends not only on the observable constants of formation of the complexes, but also, among others, upon the constant of extraction of the complex used as reagent. All this depends on the solvents used as well as other factors. Proof of this greater selectivity lies in the fact that in determining oxalate as proposed in this study, anions which could interfere in a homogeneous medium (such as I^-, SCN^-, or CN^-) do not interfere, at least within the fixed limits.

SUMMARY

In this paper an indirect spectrophotometric method is proposed to determine oxalate, by a new technique called "reextraction through the exchange of ligands." This new method is based on the decoloration produced in organic solutions of colored complexes when mixed with aqueous solutions of complexing anions.

The established procedure for the preparation of solutions of ferric indole-2-carboxylate in isoamyl alcohol is given, as well as a discussion of the variables which influence the reextraction through the exchange of ligands.

Finally, the technique was applied in determining traces of oxalate in an aqueous solution. The decoloration varies linearly with the oxalate concentration between 1 and 6 ppm. The error and reproducibility of the method were calculated. Also studied were the value of the constant of exchange and the interferences caused by various ions.

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MICROCHEMICAL JOURNAL 25, 458–464 (1980)

Spectrophotometric Determination of Vanadium(V) with Oxine in Isoamyl Alcohol

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Received February 10, 1980

INTRODUCTION

The study of vanadium content in food, plants, animals, and the environment has recently attained remarkable importance, which has permitted the compilation of data of great interest on the biogeochemistry of vanadium. The essentiality of vanadium as a trace element for animals was discovered in 1971 by Schwarz and others (*12*).

The vanadium content in plants and animals is related to the vanadium content of the physical environment in which the biosphere is immersed.

The functions of vanadium in biochemistry and its participation in the drama of evolution seem to concur with the present problems, actual or assumed, associated with environmental pollution plans.

Among the various methods used for trace determination of vanadium, the photometric method using 8-hydroxyquinoline (oxine) as chromogenic reagent stands out for its simplicity, availability, and sensitivity.

In other work (*3*) the sensitivity of the colorimetric determination of vanadium(V) with oxine using the extraction with isoamyl alcohol was reported, and disposing of spectrophotometric methods, in the present work, the determination of vanadium with oxine is improved and the new procedure is applied to vanadium determination in biological samples.

MATERIALS AND METHODS

Reagents

Standard solution of vanadium (V). Dissolve 0.2296 g of ammonium metavanadate in water to 1 liter (1 ml contains 100 μg of vanadium).

8-Hydroxyquinoline (oxine) solution, 0.5%. Dissolve 0.5 g of 8-hydroxyquinoline (oxine) in 100 ml of isoamyl alcohol (bp 132°C).

8-Hydroxyquinoline (oxine) solution, 0.0099 M. Dissolve an excess amount of oxine in isoamyl alcohol.

Sulfuric acid. Concentrated (d = 1.84).

Sulfuric acid solution. 0.005 *M*.

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Apparatus

Spectrophotometer. Bausch & Lomb "Spectronic 700," equipped with a glass cell, 10-mm optical path.

Centrifuge. 3000 rpm.

Absorption Spectra

The spectral characteristics of the solutions of vanadium(V)–oxine complex in isoamyl alcohol were studied in the range 370–800 nm. In a 25-ml volumetric flask solutions containing 4 μg of vanadium(V)/ml were prepared, adjusted to acid, basic, and neutral pH, and then transferred to a 100-ml separatory funnel. Five milliliters of 0.5% oxine was added; the solution was shaken vigorously and the organic layer was allowed to separate. The organic layer was transferred to a spectrophotometer cell to measure absorbance, using isoamyl alcohol as a blank. The system under investigation showed a broad range of absorption maxima between 450 and 490 nm (Fig. 1).

Various authors (*1*, *8*, *9*) had improved the absorbance of solutions of the vanadium–oxine complex by extraction with benzene in the presence of various anions, principally the perchlorate. We have attempted to extend that method to the extraction of vanadium–oxinate in isoamyl alcohol in the presence of sodium perchlorate and verified that it did not produce variations in the absorbance.

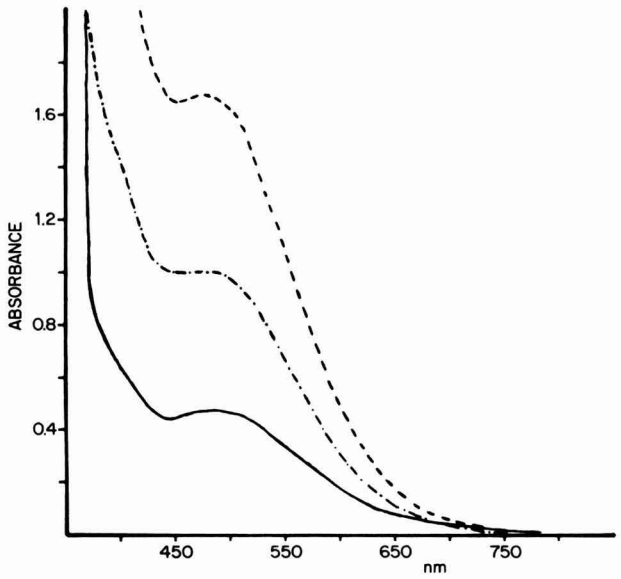

FIG. 1. Absorption spectra of the vanadium(V)–oxine complex in isoamyl alcohol. Concentration: 4 μg vanadium/ml. pH 9.5, ——; pH 6.5, · – · – ·; pH 3.5, – – –.

Effect of Acidity

To meet the optimal acidity reported in (*7*), we prepared solutions containing 10 μg of vanadium(V)/ml and various concentrations of sulfuric acid (between 1.6 and 8 $\times$ 10^{-5} *M*) in a 25-ml volumetric flask. Subsequently the solution was treated employing the same procedure used for determination of spectral characteristics. The organic layer was separated and the absorbance was measured in the spectrophotometer at 475 nm. Other experiments with various concentrations of sodium hydroxide were performed, but complex formation was not observed. From the results obtained we deduced the optimal acidity to be 0.004 *M*.

Effect of Amount of Reagent

Samples containing a fixed amount of vanadium(V) were developed and measured by the same procedure used for determination of spectral characteristics, except that the amount of 8-hydroxyquinoline added was varied successively in order to obtain optimal experimental conditions. The addition of 5 ml of a 0.5% oxine solution was determined optimal.

Effects of Time and Temperature

The effects of time and temperature on the stability of the system were also studied. At optimal conditions of other variables the absorbance of the vanadium–oxine complex in isoamyl alcohol is stable with time and temperature.

Beer's Law, Optimal Range, Molar Absorptivity, Sensitivity and Minimum Photometric Error

The relationship between the absorbed radiant energy and the complex concentration was studied under the previously established conditions. Several standard solutions of vanadium(V) were prepared in a 25-ml volumetric flask, to which was added a sufficient amount of sulfuric acid to obtain an acidity of 0.004 *M;* the solutions were transferred to a separatory funnel. Then 5 ml of 0.5% oxine was added, the mixture was shaken vigorously, and the organic layer was allowed to separate. Centrifugation is performed if necessary. The absorbance of the solutions was measured at 475 nm; the results were linear on the calibration graph over the range 0.5–4.0 μg vanadium/ml. Ringbom's (*10*) optimal working range is, at 475 nm, 0.75–3.50 μg vanadium(V)/ml (Fig. 2). The molar absorptivity was $1.03 \times 10^4\ M^{-1}\ cm^{-1}$. The sensitivity of the reagent as defined by Sandell (*11*) was 0.005 μg vanadium(V)/cm^2 using a 10-mm cell. The minimum photometric error (*2*) was 2.82.

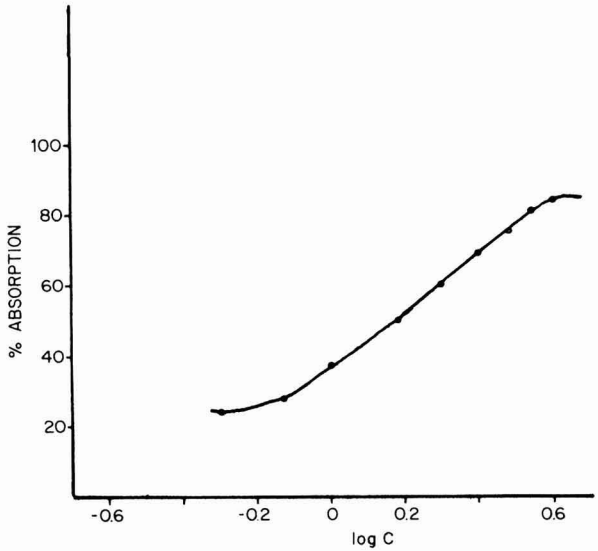

FIG. 2. Ringbom's optimal working range.

Reproducibility and Precision

To carry out these studies four series of solutions of different concentration were prepared following the above-described procedure. The precision data were obtained using a previously described statistical technique (*4*) and the following values were calculated. For concentration: 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 μg vanadium(V)/ml; for standard deviation: 0.0057, 0.0048, 0.0067, 0.0081, 0.0103, 0.0079, 0.0063, and 0.0094; for relative error: 3.83, 1.70, 1.62, 1.46, 1.46, 0.95, 0.63, and 0.84, respectively.

Effect of Diverse Ions

The interference due to various diverse ions in the direct spectrophotometric determination of vanadium(V) with oxine was studied. Vanadium, 2 μg/ml, was determined in the presence of the following ions: K^+, Tl^+ (1 mg/ml); Na^+, Mg^{2+} (0.5 mg/ml); Ba^{2+}, Co^{2+} (0.2 mg/ml); Cr^{3+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+} (0.05 mg/ml); Al^{3+} (0.01 mg/ml); Li^+ (0.004 mg/ml); Cu^{2+}, Bi^{3+} (0.002 mg/ml); Ce^{4+}, Ni^{2+} (0.001 mg/ml); and Fe^{3+} (0.00005 mg/ml); and the following anions: SO_4^{2-}, BrO_3^- (1 mg/ml); NO_3^-, Br^-, PO_4^{3-} (0.5 mg/ml); I^-, Cl^-, SCN^-, $C_2H_3O_2^-$ (0.2 mg/ml); MoO_4^{2-} (0.004 mg/ml); CrO_4^{2-}, $Cr_2O_7^{2-}$, $C_2O_4^{2-}$ (0.002 mg/ml); and WO_4^{2-} (0.001 mg/ml).

Determination of the Stoichiometry of the Complex

The nature of the vanadium–oxine complex in isoamyl alcohol was studied utilizing Job's continuous variations method (*6*) and Yoe and

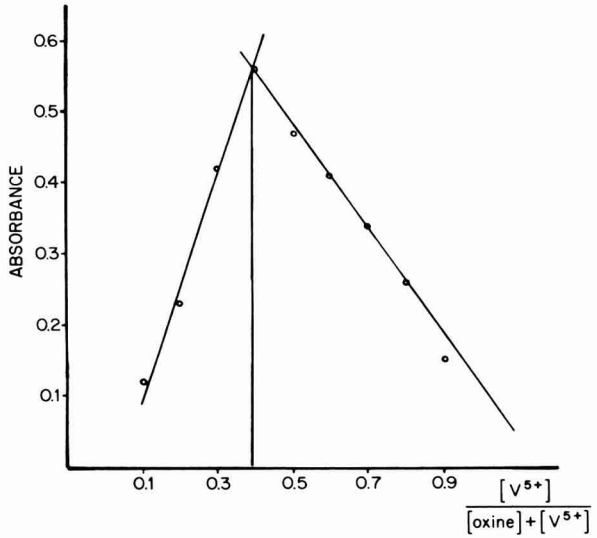

FIG. 3. Nature of the vanadium(V)–oxine complex: Job's (*6*) method.

Jones' molar ratio method (*13*) under the conditions previously mentioned. The concentrations of vanadium and oxine were 0.0098 and 0.0099 *M*, respectively. The results presented in Figs. 3 and 4 show that the ratio of vanadium to oxine is 1:2.

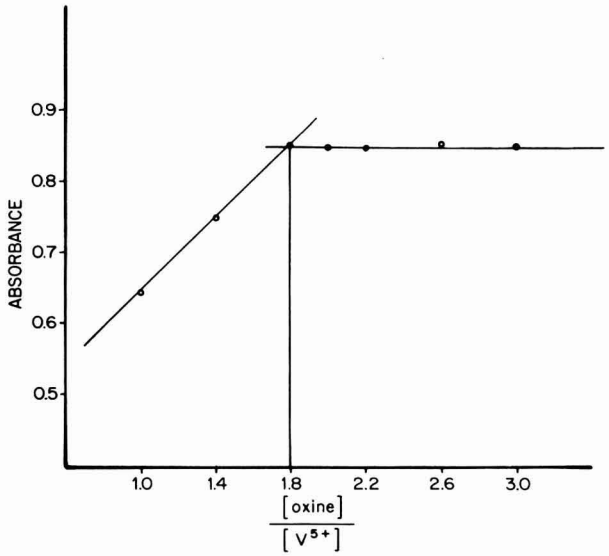

FIG. 4. Nature of the vanadium(V)–oxine complex: Yoe and Jones' method.

TABLE 1
DETERMINATION OF VANADIUM IN FOODS

Sample	Vanadium added (ppm)	Vanadium found (ppm)	Error	Percentage recovery
Potatoes	0.00	2.49	—	—
	1.00	3.57	0.08	102.29
	1.50	3.88	0.11	97.24
	2.00	4.60	0.11	102.45
Pilchard	0.00	0.40	—	—
	1.00	1.50	0.10	107.14
	2.00	2.50	0.10	104.17
	3.00	3.60	0.20	105.88

Application to Determination in Foods

The proposed method has been applied to the determination of vanadium in foods. For organic material decomposition the moist method proposed by Bermejo (*3*) for fish samples was used; good results have been obtained in the analysis of potatoes. In all cases 50-g samples dried at 110°C are used. The percentage recovery obtained using the method is reported in Table 1 and shows that the method proposed is acceptable for the determination of vanadium in foods.

SUMMARY

A new method for the spectrophotometric determination of vanadium(V) with 8-hydroxyquinoline in isoamyl alcohol is described. The spectra show broad absorption maxima between 450 and 490 nm. The effects of amount of reagent, acidity, time, and temperature were also studied. The optimal interval of Beer's law application is 0.75–3.50 μg vanadium/ml. The nature of the complex in solutions, as well as the interference, reproducibility, and precision of the method, was investigated. The method has been applied to the determination of vanadium in foods.

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MICROCHEMICAL JOURNAL 25, 465–470 (1980)

Determination of Platinum with 4-*S*-Benzyl-1-*p*-chlorophenyl-5-phenyl-2,4-isodithiobiuret (BPPTB)

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Received July 25, 1978

INTRODUCTION

The complexes of platinum with thio-organic reagents are widely studied (*3*,*6*) and employed for determination of the metal. Although 4-*S*-benzyl-1-*p*-Cl-phenyl-5-phenyl-2,4-isodithiobiuret (BPPTB) having anionic thio and basic imino groups is an analytically valuable reagent for palladium (*4*), it was not studied as a reagent for platinum. In the current course of studies on platinum metals in this laboratory, it was found that BPPTB is a sensitive and selective reagent for the fast extraction and spectrophotometric determination of platinum. It was observed that quantitative determination of platinum can be achieved in the presence of large number of ions.

EXPERIMENTAL METHODS

4-*S*-Benzyl-1-*p*-chlorophenyl-5-phenyl-2, 4-isodithiobiuret (BPPTB) was synthesized and purified as reported earlier (*4*). The fresh solutions of BPPTB in ethanol-free chloroform (*10*) was utilized for extraction studies. The solutions of hexachloroplatinic acid (Johnson Matthey, London) and stannous chloride (Hanawa, Japan) in hydrochloric acid (Analar R) were utilized. The platinum was standardized by the stannous chloride method (*8*). The stock solution of sodium chloride (E. Merck, GR) of 5.0 *M* was prepared in double-distilled water. The solutions of required strength were made by appropriate dilution of stock solutions. The easily available analytical grade chemicals were used for the preparation of their solutions in water.

Beckman DU 2 spectrophotometer with matched 10-mm silica cells have been employed for absorptiometric studies.

Procedure. An aliquot containing less than 200 μg platinum at 4.0 *M* hydrochloric acid in the presence of 0.1 *M* stannous chloride was equilibrated for 5 min with 10 ml BPPTB solution in chloroform. The absorbance of the organic phase was measured at 407 nm against similarly equilibrated reagent solution as a blank. The quantity of platinum extracted was determined from a calibration curve.

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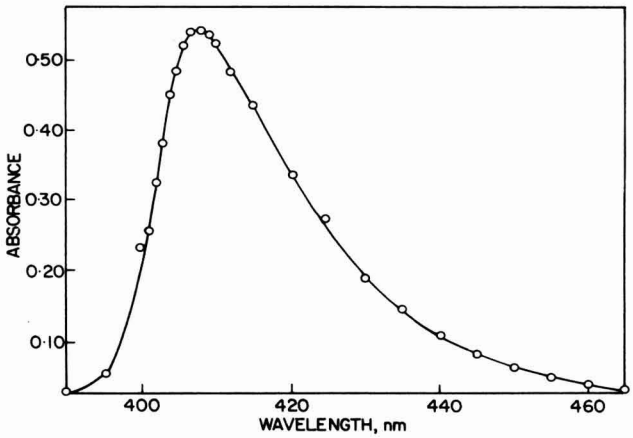

FIG. 1. Absorption spectra of Pt(II)–BPPTB complex in chloroform.

RESULTS AND DISCUSSION

Absorption Spectra of Extractable Species

The absorption spectrum of platinum(II)–BPPTB complex in chloroform (Pt = 3.78×10^{-5} *M*) is shown in Fig. 1. The spectra were scanned against the similarly processed reagent as a blank. The colored complex shows a strong and sharp peak of absorbance at 407 nm and hence all the absorbance measurements were carried out at this wavelength. The molar absorptivity of the complex at 407 nm is 1.42×10^4 cm^2 mol^{-1}, calculated on the basis of platinum content (2.24×10^{-5} *M*) with the absorbance of 0.320 + 0.005 when 10-mm cells were used. No evidence for photodecomposition was observed.

Extraction as a Function of Acidity

The acid dependence of the extraction was investigated by preparing a series of solutions at different acidities (1.0 – 8.0 *M*) containing 43.76 μg of platinum and 0.1 *M* of stannous chloride solution at the total volume of 10 ml. The solutions were extracted with 10 ml chloroform solution of BPPTB for 5 min. The absorbances were measured at 407 nm against the similarly processed reagent as a blank. A maximum absorbance was observed at 4.0 *M* hydrochloric acid corresponding to maximum extraction of 97.08%. The absorbance decreases above 4.0 *M* hydrochloric acid which may be due to the decomposition of the reagent with concentrated acid.

Effect of BPPTB Concentration

The effect of BPPTB concentration on extraction of platinum was in-

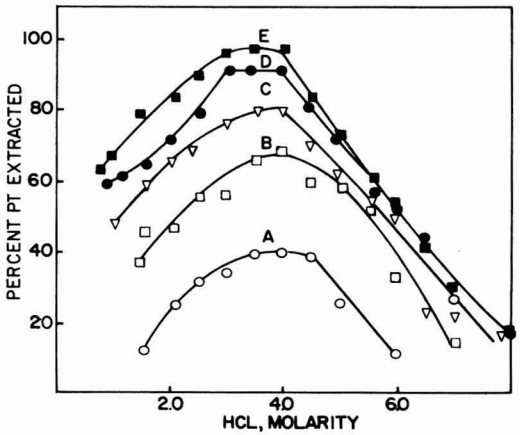

FIG. 2. Extraction of Pt(II) as a function of concentration of hydrochloric acid and BPPTB

vestigated by varying the reagent concentration in the range of 1.0×10^{-4} to 3.0×10^{-3} *M* BPPTB on 43.76 μg platinum at different acidities which revealed that for maximum extraction (97.08%) hundred times excess of BPPTB is needed. (Fig. 2).

Effect of Ionic Strength

The distribution of platinum was studied at various concentrations of sodium chloride (1.0–5.0 *M*) and at different acidities (1.0–4.0 *M*). It was observed that the extent of extraction remains the same up to 4.0 *M* NaCl. However, extraction decreases at above 4.0 *M* NaCl.

Effect of Stannous Chloride Concentration

The stannous chloride concentration has no effect on the color density of organic phase above 2.0 *M* hydrochloric acid concentration.

Effect of Solvents

Among the various nonaqueous solvents tried, viz. isoamyl alcohol, isobutanol, benzene, carbon tetrachloride, chloroform, etc., chloroform was found to be most efficient.

Time of Agitation

Pt–BPPTB system was equilibrated for times varying from 0.5–30 min. The extraction was quantitative with 2 min of agitation and hence a time of 5 min was arbitarily chosen for entire extraction studies.

Beer's law Sensitivity and Color Stability

The varying amounts of platinum ranging from 1.0 to 20.0 μg per ml were taken and extracted at 4.0 *M* hydrochloric acid with 0.001 *M* BPPTB in chloroform. It was observed that the system obeys Beer's law

TABLE 1
EFFECT OF DIVERSE IONS ON EXTRACTION OF PLATINUM[a]

Ion	Added as	Tolerance limit (μg)
Cd^{3+}	$3CdSO_4 \cdot 8H_2O$	22,500
Cr^{3+}	$Cr(NO_3)_3 \cdot 9H_2O$	2,600
Co^{2+}	$CoSO_4 \cdot 7H_2O$	29,500
Cu^{2+}	$CuSO_4 \cdot 5H_2O$	600
Au^{3+}	$HAuCl_4$	None
Fe^{2+}	$FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$	250
Fe^{3+}	$(NH_4)_2SO_4 \cdot Fe_2(SO_4)_3 \cdot 24H_2O$	600
Hg^{2+}	$HgCl_2$	4,000
Ni^{2+}	$NiSO_4 \cdot 6H_2O$	8,600
Os^{8+}	OsO_4	100
Pd^{2+}	$Pd(ClO_4)_2$	None
Rh^{3+}	$RhCl_3 \cdot 3H_2O$	100
Ru^{3+}	$RuCl_3 \cdot 3H_2O$	50
Th^{4+}	$Th(NO_3)_4$	1,100
Zn^{2+}	$ZnSO_4 \cdot 7H_2O$	32,700
Persulfate	$K_2S_2O_8$	38,400
Sulfate	Na_2SO_4	19,200
Sulfite	Na_2SO_3	400
Phosphate	$NH_4H_2PO_4$	80
Thiocyanate	NH_4SCN	600
Borate	H_3BO_3	11,600
Oxalate	$H_2C_2O_42H_2O$	17,600
Acetate	$NaOOCCH_4 \cdot 2H_2O$	3,900
Formate	$HCOOH$	20,000
Citrate	$C_6H_8O_7 \cdot H_2O$	37,800
Tartarate	$C_4H_6O_6$	29,400
Meleate	$C_4H_2O_3$	19,200
L-Glutamate	$C_5H_9O_4N$	43,500
Lactate	$C_3H_6O_3$	35,200
Malonate	$C_3H_4O_4$	40,800
DL-Maliate	$C_4H_6O_5$	53,200
Mandelate	$C_8H_8O_3$	75,000
Ethylenediamine-tetraacetate	Na_2EDTA	65,200
Nitrilotriacetate	$C_6H_9O_6N$	10,000
Sulfosalicylate	$C_7H_7O_6S \cdot 2H_2O$	100,000
Thiomalate	$C_4H_6O_4S$	4,200
Hydroxylamine	$NH_2OH \cdot HCl$	30,600
Ethylenediamine	$C_2H_8N_2$	18,000
o-Phenonthyoline	$C_{12}H_8N_2$	750
Glycinate	$C_2H_5O_2N$	30,000
Hydrazine	$NH_2NH_2 \cdot H_2SO$	52,000
Diethyldithio-carbamate	$C_5H_{10}NS_2^-Na^+$	150
Dithizone	$C_{13}H_{12}N_4S$	500
Acetylacetone	$C_5H_8O_2$	19,000
Dimethylglyoxime	$C_2H_6N_2O_2$	1,150

[a] Pt = 43.76 μg; [BPPTB] = 1.0×10^{-3} *M*; $SnCl_2$ = 0.2 *M*; $[H^+]$ = 4.0 *M*.

in the range of 2.0–18.0 μg Pt per ml of chloroform. The working range for spectrophotometric studies is 2.0–14.0 μg Pt per ml of chloroform according to the Ringbom curve. Sandell's sensitivity (*8*) corresponds to 0.013 μg Pt cm^{-2}. Color of the extractable complex is stable for 8 hr.

Effect of Foreign Ions

Many foreign ions were tested for interferences. The tolerance limit was set at an amount of foreign ion needed to cause ± 5% error in the recovery of platinum. The tolerance limits are given in Table 1. The tolerance of Cd(II), Co(II), and Zn(II) is many fold whereas that of Os(VIII), and Rh(III) is twofold. The strong interfering ions include Au(III), Pd(II), thiosulfate, and thiogalate (TGA). The high tolerance to many anions suggests the high stability of extractable species.

Precision and Accuracy

The absorbance obtained from 15 different determinations of 43.76 μg Pt was 0.320 ± 0.005. The relative mean deviation and standard deviation have been found to be ± 0.94 and ± 2.13%, respectively.

The total operation requires 30 min. Thus, the method is simple, rapid, and applicable at trace level and affords a clear cut separation of platinum from large numbers of ions.

Nature of the Extractable Species

The stoichiometric studies of Pt:BPPTB system showed that the extractable, neutral complex is 1:2 which is supported by Job's method and slope ratio method as applied to liquid–liquid extraction studies (*2, 4, 8*).

Equilibrium Constants

The equilibrium constants k_1, k_2, β_2, β_{ext}, and β_{abs} were determined by different methods (*1, 7, 9*). The values obtained are incorporated in Table 2.

It seems from Table 2 that the stability constants by different methods agree well with each other. The value for log P_M comes out to be 1.52.

TABLE 2
STABILITY CONSTANTS FOR THE Pt(IV)–BPPTB SYSTEM AT 30 ± 1°C

Primary parameters	Yatsimirskii's method	Leden's method
log k_1	4.51	4.82
log k_2	4.27	4.06
log β_2	8.78	8.88
log β_{ext}	34.38	34.48
log β_{abs}	32.86	32.96

SUMMARY

Platinum gets extracted in chloroform as 1:2 (Pt:BPPTB) complex from 4.0 *M* hydrochloric acid. It exhibits a peak of absorption at 407 nm ($\epsilon = 1.42 \times 10^4$) and can be extracted in the presence of a large number of diverse ions. The equilibrium constants were calculated by various methods.

ACKNOWLEDGMENTS

Authors are grateful to the Council of Scientific and Industrical Research, New Delhi, for award of a Junior Research Fellowship to one of them (B. K. Deshmukh). They are also thankful to Prof. K. N. Munshi, Head, Department of Chemistry, Nagpur University, for providing necessary facilities.

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MICROCHEMICAL JOURNAL 25, 471–484 (1980)

Preparation of Some Carbon-14-Labeled Amino Acids by Radiophotosynthesis Using Sugar Beet Plant

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Received March 6, 1979

INTRODUCTION

Carbon-14-labeled amino acids or their simple derivatives find extensive applications in chemistry, biology, and medicine. The wide use of these materials stimulated various investigations involving the methods of their preparation. Each of the reported methods has its advantages and also its limitations. Thus, although chemical synthesis methods offer the best control over yield and position of label, they generally suffer from lengthy and multistage operations that are normally associated with losses and many inconveniences (*1, 10, 13, 19, 20, 24, 28, 32*). Isotopic exchange methods, being the most successful in labeling molecules containing labile atoms, were found to be of little value in labeling amino acids. For example, in a trial to label alanine, no measurable exchange reaction has been observed between alanine and [^{14}C]methyl iodide, sodium [^{14}C]carbonate, or [^{14}C]acetic anhydride (*26*). Labeling of amino acids by recoil carbon-14 atoms has been studied only to a limited extent and it was generally observed that the final products were always contaminated with a large number of labeled pyproducts (*7*).

Apart from these methods, photosynthesis when carried out in the presence of radioactive carbon dioxide seems to be a method particularly suitable for preparing naturally occurring chemicals labeled with isotopic carbon. Although the mechanistic aspect of the process has been dealt with in a large number of publications, the use of photosynthesis as a preparative means for carbon-14-labeled amino acids has been treated only to a limited extent in spite of the fact that in photosynthetic systems extremely high molecular specific activities could be acheived due to multiple labeling. It has been shown by some authors that among the earliest products formed in two types of unicellular algae, namely *Chlorella pyrenoidosa* and *scenedesmus* D-3, was a group of amino acids, the highest content of isotopic carbon was found in glutamic acid, alanine, serine, and arginine in the first and glutamic acid, leucine, alanine, valine, glycine, and β-alanine in the second (*29*). Other authors showed that about 30% of

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0026-265X/80/040471-14$01.00/0

radioactive carbon taken up photosynthetically were fixed into amino acids in *C. pyrenoidosa* (*27*). Amounts of amino acids ranging from 11 mg for valine to 270 mg for aspartic acid were obtained by photosynthesis in *C. pyrenoidosa* (*25*). Many of the reported studies were devoted to the effect of the prevailing experimental conditions during photosynthesis on the yield of the amino acids obtained. Thus, it has been reported that amino acids are much more rapidly labeled in algae if it were photosynthesizing in light until the moment of radiocarbon addition (*8, 21, 29*). Moreover, a marked increase in the activity incorporated into amino acids has been observed when blue light was used with the major part being in alanine and aspartic acid (*30, 31*). The effect of nitrogen nutrition has also been studied and it has been shown that nitrogen nutrition increased most of all the incorporation of activity into amino acids and to a lesser degree into organic acids (*17*). On the other hand, nitrogen deficiency caused a reduction in the overall rates of carbon dioxide assimilation (*23*). The age of the plant used in photosynthesis was found to play an important role in the ultimate yield of the labeled chemicals obtained. Thus, in young leaves the metabolism is generally directed toward an intensive formation of amino acids and proteins while in mature leaves carbohydrate synthesis is more intensive (*3*). The extent of watering has also its effect. Thus, isotopic carbon was found to accumulate in amino acids and soluble carbohydrates in plants cultivated in water-deficient conditions (*18*).

In the present work, radiophotosynthesis has been applied as a means for preparing some carbon-14-labeled amino acids using sugar beet whole plant and radioactive carbon dioxide. The effect of some experimental conditions on the amino acids yield in sugar beet has been studied in cold runs and after choosing the appropriate conditions preparative photosynthesis experiments were carried out using radioactive carbon dioxide. From the results obtained it can be inferred that a number of carbon-14-labeled amino acids could be separated mainly from the leaves and to a lesser extent from the root of sugar beet plant via the photosynthetic route using $^{14}CO_2$.

EXPERIMENTAL

Sugar beet (*Beta vulgaris*) variety poly K-44 was used in the present work being one of the best photosynthetic terrestial plants. Barium [^{14}C]carbonate was obtained from the Zentral Institut fur Kernforschung DDR.

Preliminary experiments. The effect of the different growing conditions, as for example soil type, nitrogen nutrition, and also plant age on the amino acids yield in sugar beet has been studies. Thus, 10 sugar beet seeds were sown in pots (30 cm in diameter) each containing the same weight of the used soils, namely sandy or clay loamy soils. Sixty percent

of total water-holding capacity of the used soil was maintained throughout the growing period. After 1 month, the germinated seeds were thinned to one plant in each pot and at this stage the nitrogen nutrition was added. The normal nutrition level, denoted by 1 N, involved the addition of 1.44 g of $(NH_4)_2SO_4$ (*12*). A higher nitrogen nutrition level, denoted by 2 N, involved the addition of double the above-mentioned amount of ammonium sulfate while in the 0 N level no nitrogen nutrition was added. It is worth mentioning that all experiments were designed in a complete random blocks of 15 replicates.

From plants grown in different nitrogen nutrition levels periodic samples were taken every month, for the next 4 months from the date of nitrogen nutrition addition, to be chemically treated in order to determine the amino acid content in sugar beet as affected by the plant age.

Photosynthesis experiments. After choosing the appropriate growing conditions, photosynthesis experiments were carried out using a specially designed simple photosynthesis chamber that is schematically shown in Fig. 1. The process was started by introducing the plant to be used and the appropriate amount of inactive or radioactive barium carbonate into the chamber which was then tightly sealed using an adhesive material. The system was slightly evacuated and an amount of lactic acid was introduced to destroy the barium carbonate in the chamber. The inside pressure was then equalized with the atmospheric pressure. The whole assembly was kept in daylight, out of reach of direct sun rays, for a certain time interval after which the chamber was opened and the plants were taken for subsequent chemical treatment. Closed chamber photosynthesis ex-

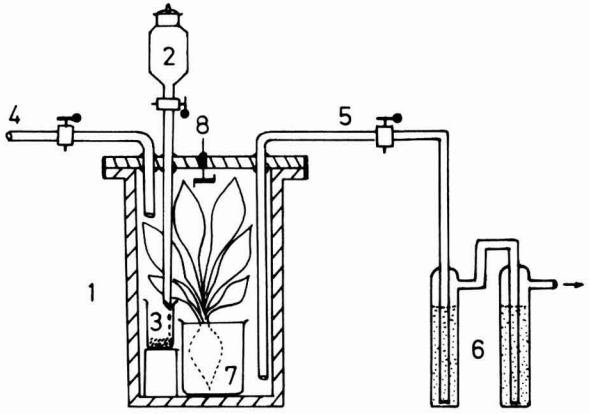

FIG. 1. Schematic representation of the laboratory photosynthesis chamber. (1) 16 L tight glass tank (40 × 20 × 20 cm). (2) Lactic acid delivery funnel. (3) $Ba^{14}CO_3$ destruction vessel. (4) Inlet tube. (5) Outlet tube. (6) Scrubbers charged with 3 *N* NaOH solution. (7) Sugar beet plant contained in a 1-liter beaker. (8) Ventilator.

periments were conducted using different carbon dioxide concentrations in the chamber for different time periods and under different humidity conditions in order to investigate the effect of these variables on the ultimate amino acid yield in the sugar beet. Based on the results obtained the radiophotosynthesis experiments were planned. In radioactive runs the residual radioactive carbon dioxide in the chamber at the end of the process was collected by sweeping the chamber with argon and passing the effluent stream in a series of scrubbers containing 3 *N* sodium hydroxide solution.

Chemical treatment of the plant material after photosynthesis. After photosynthesis, the plants were cleaned, separated into leaves and root, and then dried in an electric oven kept at 80°C until a constant weight was attained. The dry material was then thoroughly ground using an electric blender. Three grams of the dry plant material, of either leaves or root, was extracted by boiling under reflux for 10 min with 50 ml absolute ethanol. This was followed by grinding the mixture, in a porcelain mortar, and filtration by centrifugation. Further extractions of the solid material were carried out using boiling 80% aqueous ethanol. After centrifugation, the solid material was kept for further treatment and the clear alcoholic extract was treated to separate the amino acids whereby an equal volume of chloroform–water mixture (1:1) was added in a separatory funnel and the contents were thoroughly shaken. The aqueous layer was separated and passed at a rate of 3 ml/min through a cation exchange column packed with Amberlite IR-120 that has been previously activated by washing with dilute HCl, water, and finally aqueous 80% ethanol. After washing the column with 15 ml 80% aqueous ethanol, the adsorbed amino acids were eluted by successive addition of 40 ml 0.4 *N* NH_4OH in 80% aqueous ethanol, 15 ml ethanol, 15 ml water, 15 ml aqueous NH_4OH, and finally 15 ml water. The collected eluate was concentrated to about 1 ml by heating at 70°C under reduced pressure, then quantitatively transferred and completed to the mark in a small volumetric flask using 10% aqueous isopropyl alcohol to which were added few drops of dilute HCl until the solution became slightly acidic. This solution was analyzed by paper chromatography to determine the amino acid content.

The protein-bound amino acids were obtained by hydrolysis of the solid material remaining after alcoholic extraction of the original dry plant material with 6 *N* HCl in an air bath kept at 110°C for 24 hr. The hydrolysate, containing the protein-bound amino acids, was separated by centrifugation, decolorized by passing through an activated charcoal column, and after removing the acid by gentle evaporation on a water bath with occasional addition of water, the resultant solution was finally dissolved in 19% aqueous isopropyl alcohol and passed through an Amberlite IR-120

cation exchange resin column to separate the amino acids as described before.

In another experiment, the raw dry plant material was directly subjected to acid hydrolysis followed by separation of the amino acids from the hydrolysate obtained according to the same procedure described before.

Analysis of the amino acid solutions. Two-dimensional paper chromatography has been applied in the analysis of the amino acids separated from the alcoholic plant extracts. The materials and adopted techniques have been described in detail in a previous publication (*5*). The amino acids were identified on the chromatograms by their position relative to the position of a certain standard acid. The identity was confirmed by adding an amount of the acid to be identified to the unknown sample and observing the subsequent change in the spot area of the given acid on the chromatogram. For further confirmation, authentic mixtures were analyzed under the same experimental conditions.

The quantitative determination of the amino acids separated by two-dimensional paper chromatography was carried out by the method described in detail in a previous publication (*5*). This method is extremely simple and nondestructive. The chromatograms were further used for the radioactivity measurement of individual spots after the concentration of the individual amino acids in each spot was first determined.

Radioactivity measurements. The radioactivity of $Ba^{14}CO_3$ and all solid samples has been determined in the present work by counting thin films using a Philips low-voltage halogen-quenched Geiger–Muller tube having a window thickness of 2.5–3.5 mg/cm^2 and all values obtained were corrected for self-absorption (*6*). The radioactivity of all liquid samples has been determined after drying aliquots in small aluminum plates. The efficiency of the counter in such measurements has been determined using a standard $Na_2{}^{14}CO_3$ solution. The same counter has been also calibrated for the direct measurement of the radioactivity of amino acids separated by paper sheet chromatography and was successfully used for that purpose. When the spot activity was too low, spots from several chromatograms were collected and extracted for 45 min, using a microextracting device. The extracts were concentrated, quantitatively transferred, and evaporated to dryness on aluminum plates. After counting the samples, the data obtained were corrected using the appropriate efficiency factor.

RESULTS AND DISCUSSION

The conversion of electromagnetic energy of light into chemical energy in photosynthesis is a process which leads to the formation of many chemicals in green plants and certain algae. In the development of a

photosynthetic method as a means for preparing isotope-labeled compounds, many problems are usually encountered. The most important of these are the selection of the organism to be used, the existence of an isolation method of the required components, the feasibility of efficiently purifying the final product, and measuring its specific activity.

In the present work, the sugar beet plant has been used, being one of the best photosynthetic terrestrial plants. It was important, before using the plant in the radiophotosynthesis experiments, to investigate the different growing conditions that would affect the final amino acid content in it. In this respect several factors have been investigated, namely, soil type, plant age, nitrogen nutrition, and humidity. The results obtained are given in Table 1. From these results it could be observed that the free amino acid content in the leaves was markedly higher than their content in the root. The two types of soil used gave the same types of amino acids in sugar beet plant but their content when clayey soil was used was slightly higher than when sandy soil was used.

The results of further experiments carried out to investigate the effect of nitrogen nutrition and age of the plant on the amino acid yield are given in Table 2. From these results it is possible to observe that on using the normal nitrogen nutrition level (1 N) the amino acid yield was higher than when no nitrogen nutrition was used. Increasing the nutrition level to twice the normal level resulted in a marked decrease in the amino acid yield in the leaves. This is in agreement with the previously reported conclusions that the presence of higher amounts of nitrogen nutrition can hinder the primary plant growth (*15*) which is obviously reflected on the

TABLE 1
AMINO ACID CONTENT IN SUGAR BEET GROWN IN DIFFERENT TYPES OF SOILS

	Weight (μg/g dry plant material)			
	Clay loamy earth		Sand	
	Leaves	Root	Leaves	Root
Alanine	1550	260	1240	610
γ-Aminobutyric acid	900	450	480	1080
Aspargine	540	—	410	—
Aspartic acid	790	—	690	—
Glutamic acid	700	—	550	—
Glycine	540	100	560	260
Norleucine	600	170	380	320
Serino	580	—	—	—
Valine	690	160	600	260

Note. No nitrogen nutrition used.

TABLE 2
TOTAL AMINO ACID CONTENT IN SUGAR BEET AT DIFFERENT GROWING INTERVALS AND WITH DIFFERENT NITROGEN NUTRITION LEVELS

Nitrogen nutrition level	Growing interval (months)	Total amino acids content (μg/g dry plant material)	
		Leaves	Root
0 N	2	10,040	1,260
	3	5,240	2,810
	4	1,900	1,730
	5	1,520	2,590
1 N	2	12,080	1,130
	3	4,160	3,020
	4	1,150	1,410
	5	1,650	3,870
2 N	2	11,090	4,510
	3	3,240	1,760
	4	930	1,210
	5	1,220	4,110

ultimate amino acid yield. The results in Table 2 also show that at the time of the first sample, i.e., 1 month after adding the nitrogen nutrition and 2 months after growing the plant, all amino acids were present in their maximum yields in the leaves. After that, the amino acid content in the leaves sharply decreased at the time of the second sample and then gradually decreased during the following 2 months. This is in agreement with the well-known fact that during the active growth period, i.e., during the increase in the area and weight of the plant leaves, an intensive increase in the amount of total and protein nitrogen occurs and as the leaf growth slows down the rate of decay of proteins starts to be greater than the rate of protein formation. In the root it is possible to observe a certain tendency of the amino acid content to increase slightly with age.

The results of closed chamber photosynthesis experiments conducted for varying periods of time and at different carbon dioxide concentrations are given in Table 3. It is possible to observe a slight increase in the amino acid yield as the carbon dioxide concentration in the chamber was increased. In fact photosynthetic inhibitions have been observed in aerial plants at carbon dioxide levels as high as 20% (*14*) and as low as 2.5% (*4*). In other studies it has been reported that changing the carbon dioxide concentrations from 1 to 0.3% did not lead to any appreciable changes in the intensity of photosynthesis (*33*). The results obtained when using a drying agent in the chamber during photosynthesis, shown also in Table 3, were difficult to interpret, and further experiments were carried out without the use of any drying agent.

TABLE 3
TOTAL AMINO ACID CONTENT DETERMINED IN SUGAR BEET AFTER CLOSED CHAMBER PHOTOSYNTHESIS EXPERIMENTS[a]

mmol CO_2 used	Duration of photosynthesis (hr)	Total amino acids content (μg/g dry plant material)	
		Leaves	Root
3.3	2	10,450	5,440
	4	9,460	4,460
	8	8,910	5,870
	8[b]	11,550	6,530
6.6	2	9,930	5,930
	4	8,400	5,030
	8	11,810	6,350
	8[b]	10,830	4,390
Blank (atmospheric photosynthesis)	8	8,930	5,740

[a] Plant age 2 months; 2 N nitrogen nutrition level.
[b] Experiment carried out in presence of a drying agent in the chamber.

Table 4 shows the conditions used in the radioactive experiments. In these experiments a 2-month-old sugar beet plant grown in clay loamy soil and containing the normal nitrogen nutrition level, has been used. The carbon dioxide concentration level in the chamber did not exceed 1%. The gross activity found in the resultant dried plant material before and after alcoholic extraction and also the activity found in the amino acid solution obtained after purifying the alcoholic extract are also given in Table 4. It could be observed that as the carbon dioxide concentration in the chamber was reduced, the percentage of absorbed carbon dioxide gradually increased. This probably shows that the plant used has a certain capacity for absorbing the carbon dioxide used.

From the data given in Table 4 it is possible to observe that 60–80% of the total activity absorbed in the plant was found in the leaves while 40–20% was found in the root. About two-thirds of the total activity entering the plant, in the leaves or root, was found in the alcoholic extracts in all radioactive experiments. The alcoholic extracts contained amino acids, sugars, lipids, pigments, etc. The complementary part of the activity entering the plant was found in the solid residue remaining after extraction of the original dry material with alcohol. The total activity found in the free amino acids, after being separated from the alcoholic extract by column chromatography, amounted to 5% and 1.5% of the total activity entering the plant in the leaves and root, respectively.

TABLE 4

EXPERIMENTAL CONDITIONS AND RADIOACTIVITY DISTRIBUTION IN DIFFERENT FRACTIONS AND SOLUTIONS AFTER CLOSED CHAMBER RADIOPHOTOSYNTHESIS EXPERIMENTS

Experiment No.	$Ba^{14}CO_3$ used		Photosynthesis duration	Absorbed activity in plant		Percentage of the absorbed activity found							
						In dry material of		In alcoholic extract of		In solid remains after alcoholic extraction of		In amino acid solution after purification of the alcoholic extract of	
	m*M*	μCi	hr	μCi	Percentage	Leaves	Root	Leaves	Root	Leaves	Root	Leaves	Root
1	6.6	123.7	4	77.1	62.3	72	28	49	27	23	1	5.1	1.5
2	6.6	123.7	8	56.3	45.5	81	19	45	12	36	7	6.3	0.6
3	3.3	61.8	8	46.9	75.9	63	37	35	34	28	3	5.4	1.9
4	3.3	61.8	8	43.3	69.9	80	20	49	18	31	2	4.6	2.5
5	3.1	265.6	8	258.2	97.2	75[a]	25[b]	46	23	29	2	4.8	1.2

[a] Total weight of dry leaves 15.1 g.

[b] Total weight of dry root 7.3 g.

Results of the detailed paper chromatographic analysis of the pure amino acids solution, obtained in experiment No. 5, are given in Table 5. It could be observed that alanine was obtained in an abnormally high radiochemical yield. γ-Aminobutyric acids, aspargine, aspartic acid, and glutamic acid were obtained in relatively higher specific activities than the remaining acids found in the leaves. In the root, serine and valine were obtained in relatively low yields and consequently acquired high specific activities. It is important to point out that the total activity of the amino acids determined after paper chromatography was found to represent only about 50% of the corresponding activity determined before analysis. This could be attributed to the effect of ninhydrin used in staining the chromatograms. Thus, it is well known that the application of ninhydrin elicites the oxidative deamination of the α-amino group in the amino acid molecule liberating ammonia, carbon dioxide, and the corresponding aldehyde (*16*). The release of carbon dioxide and the loss of the volatile aldehyde could explain the observed loss in radioactivity (*9*). Consequently, a correction factor of 2 could be safely used to calculate the actual specific activity values cited in Table 5.

The solid remaining after the alcoholic extraction of dry plant materials yielded after being hydrolyzed for 24 hr, a group of amino acids labeled with carbon-14 as indicated in Table 5. While the radioactivity values found in these amino acids were slightly higher than the values found in the free amino acids, the specific activities of the former acids were much lower than the specific activities of the free found amino acids due to the greater concentration of the protein-bound amino acids liberated upon hydrolysis. When the hydrolysis time was increased to 45 hr the amino acid yield was found to decrease. Actually, it has been reported before that prolonged heating during protein hydrolysis enhances the further destruction of the formed amino acids (*31*).

Based on the above-mentioned results it is possible to conclude that after radiophotosynthesis the ^{14}C-labeled amino acids are mainly found in the leaves rather than in the root of sugar beet. Small amounts of high specific activity amino acids could be directly separated by alcoholic extraction of the dry raw material whereas relatively larger amounts of lower specific activity amino acids could be obtained from the hydrolysate of the material remaining after the alcoholic extraction step.

Alternatively, when the initial dry raw material was directly subjected to acid hydrolysis followed by separation, analysis, and radiometry of the products, the overall amino acids yield was markedly lower and the specific activities of the isolated acids were slightly higher than those separated from the hydrolysate of the solid material remaining after alcoholic extraction, as could be observed from the data given in Table 6. The observed loss in the amino acids in this case could be attributed to the

TABLE 5

CONTENT, RADIOACTIVITY, AND SPECIFIC ACTIVITY OF CARBON-14-LABELED AMINO ACIDS FOUND AFTER CHEMICAL TREATMENT OF THE ORIGINAL DRY PLANT MATERIAL (OF EXPT. 5)

	Amino acids found in the alcoholic extract of dry plant material						Amino acids separated from the hydrolysate of the solid remains after alcoholic extraction					
	A[a]		B[b]		C		A[a]		B[b]			
	L	R	L	R	L	R	L	R	L	R	L	R
Alanine	0.10	0.10	1.40	0.19	21.2	6.3	0.52	0.06	0.39	0.015	1.16	0.85
γ-Aminobutyric acid	0.07	0.09	0.35	0.10	9.1	4.2	—	0.05	—	0.02	—	0.65
Aspargine	0.02	0.03	0.06	0.01	7.6	2.1	—	—	—	—	—	—
Aspartic acid	0.06	0.08	0.27	0.04	10.3	2.6	0.65	0.12	0.58	0.03	2.03	1.04
Glutamic acid	0.05	0.07	0.30	0.04	16.0	3.1	0.60	0.13	0.70	0.04	2.96	1.74
Glycine	0.03	0.01	0.14	0.01	6.1	2.4	0.44	0.07	0.34	0.015	1.00	0.57
Lysine	—	—	—	—	—	—	0.73	0.09	0.26	0.01	0.88	0.70
Norleucine	0.06	0.06	0.07	0.02	2.7	2.0	0.39	0.06	0.30	0.01	1.74	0.74
Serine	0.03	0.02	0.05	0.08	3.0	13.3	0.28	0.05	0.27	0.01	1.75	0.97
Valine	0.05	0.02	0.14	0.05	5.5	10.4	0.52	0.095	0.17	0.007	0.66	0.32

Note. A: Percentage of total dry weight. B: Percentage of total activity absorbed by plant. C: Specific activity, μCi/mmol. L: leaves, R: root.

[a] Total weight of dry leaves 15.1 g and of dry root 7.3 g.

[b] Total activity absorbed by plant 258.2 μCi.

TABLE 6
CONTENT, RADIOACTIVITY, AND SPECIFIC ACTIVITY OF CARBON-14-LABELED AMINO ACIDS SEPARATED FROM THE HYDROLYSATE OF THE ORIGINAL DRY PLANT MATERIAL (OF EXPT. 5)

	A[a]		B[b]		C	
	L	R	L	R	L	R
Alanine	0.28	0.19	0.74	0.09	3.94	1.56
γ-Aminobutyric acid	—	0.15	—	0.17	—	3.34
Aspargine	—	—	—	—	—	—
Aspartic acid	0.30	0.32	0.38	0.09	2.85	1.33
Glutamic acid	0.39	0.28	0.75	0.21	4.84	3.91
Glycine	0.28	0.17	0.26	0.06	1.21	0.98
Lysine	—	0.20	—	0.015	—	0.39
Norleucine	0.19	0.18	0.36	0.10	4.30	2.50
Serine	—	0.10	—	0.03	—	1.04
Valine	0.22	0.17	0.26	0.04	2.45	0.94

Note. A: Percentage of total dry weight. B: Percentage of total activity absorbed by plant. C: Specific activity μCi/mmol. L: leaves, R: root.

[a] Total weight of dry leaves is 15.1 g and of dry root 7.3 g.

[b] Total activity absorbed by plant 258.2 μCi.

presence of carbohydrates in the hydrolyzed material which normally leads to negligible-to-large losses of amino acids during hydrolysis, particularly when mineral acids are used in hydrolysis (*2*). It is therefore recommended to isolate the labeled amino acids formed in sugar beet by the two-step process.

It is important to note that it is possible to increase the specific activity of the final products by simply increasing the specific activity of the carbonate used. Actually, this is not unfeasible since in the type of experiments conducted, where the plants are exposed to radioactive carbon dioxide for relatively short time periods (a few hours), it is generally believed that if the activity of carbon dioxide in the gaseous mixture was significantly increased there should be no appreciable change in the intensity of photosynthesis (*34*). Some authors have reported that increasing the activity of radiocarbon, up to 10 mCi, did not induce marked changes in the prevailing physiological processes (*11, 22*). On the other hand, prolonged exposure to radioisotopes (for days and weeks) very probably leads to some changes in the physiological processes in the plant (*34*).

SUMMARY

Radiophotosynthesis has been applied as a means for preparing a number of carbon-14-labeled amino acids using sugar beet plants grown under controlled environmental condi-

tions. The apparatus used, conditions of operation, isolation of products, and their analysis and radiometry have been discussed in detail. From the results obtained, it can be inferred that a number of labeled amino acids could be separated from the leaves of sugar beet including [^{14}C]alanine, γ-[^{14}C]aminobutyric acid, [^{14}C]glutamic acid, [^{14}C]aspartic acid, [^{14}C]serine, [^{14}C]valine, and [^{14}C]aspargine with specific activities of several microcuries per millimole. The specific activities of the products could be significantly increased by increasing the specific activity of the radioactive carbonate initially used.

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MICROCHEMICAL JOURNAL 25, 485–491 (1980)

The Cleavage Mechanism of 1,4-Dithiaspiro(4.4)nonane by Gas-Phase Pyrolysis

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Received August 29, 1979

INTRODUCTION

Comparison of the photochemical and electron-impact-induced fragmentations indicated that pyrolysis might be the preferred means of examining the importance of ground-state *d*-orbital interactions as a determinant of the reaction pathway. Thermal decompositions are less energetic than photochemical or electron-impact-induced decompositions. Therefore, opportunities for observation of *d*-orbital interaction should be greater. Ideally one would like to correlate this information with the results of theoretical molecular orbital calculations on this system. Exact calculations which could, in principle, assess the importance of *d*-orbital interaction in the ground state of these molecules have yet to be performed.

In a previous paper (*9*) the results for pyrolysis compounds of some cyclic dithioketals were discussed and it was suggested that to some extent the 3*d*-orbital interaction is involved and affects the consistency of the pyrolysis products.

The present work was invoked because further study of the mechanism of cleavage was necessary to elucidate the role of the 3*d*-orbital interaction of sulfurs in cyclic dithioketals.

EXPERIMENTAL

The compound 1,4-dithiaspiro(4.4)nonane was synthesized and pyrolyzed at 600, 700, and 800°C as described in Ref. (*9*). The yields as a function of pyrolysis temperatures are given in Table 1.

RESULTS AND DISCUSSION

The thioketone (**7**) forms at 600°C from the dithioketal (**I**) and is presumed to arise via the diradical (**43**). Evidence for the initial formation of thiocyclopentanone (**7**) is based on the immediate formation of a pink color when a sample of the mercaptale is pyrolyzed (*1, 6, 7*). The cleavage of (**I**) to (**7**) has been observed in studies of the photochemical (*13*) and electron-impact-induced fragmentation (*8*) of (**I**). The thioketone (**7**) is reported (*13*) to be pink in color, with a maximum in the visible spectrum

0026-265X/80/040485-07$01.00/0

TABLE 1

ABSOLUTE PERCENTAGE YIELD OF PYROLYSIS PRODUCTS OF 1,4-DITHIASPIRO(4,4)NONANE(I) IN ORDER OF GC RETENTION TIME

Temperature °C	Product No.[a]													
	H_2S	C_2H_4	CS_2	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
600	1.0	2.0	0.9	0.17	0.87	—	0.77	—	0.05	4.84	1.02	—	—	0.2
700	4.0	10.0	1.2	10.0	5.57	2.1	5.22	—	—	—	—	—	—	—
800	2.5	5.3	1.4	12.6	9.5	2.2	6.8	0.63	—	—	—	0.5	0.2	0.35

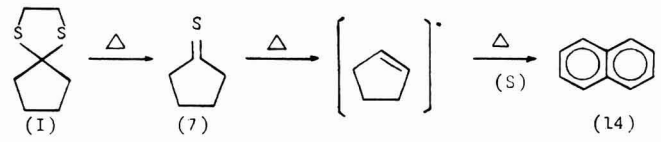
	Product No.													
	(12)	(13)	(14)	(15)	(I)	(16)	(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)
600	1.17	—	—	—	41.31	0.05	0.05	0.1	—	—	—	0.1	0.1	0.15
700	0.65	—	6.7	6.67	17.4	—	—	—	v.s.[b]	v.s.	3.4	—	—	—
800	—	0.35	10.25	0.85	—	—	—	—	0.53	1.47	3.2	—	—	—

[a] *Name of compound (Product No.):* Benzene (1); thiophene (2); toluene (3); 2-methylthiophene (4); 3-methylthiophene (5) cyclopentylmercaptane (6); thiocyclopentanone (7); thiacyclohexane (8); cyclooctatetraene (9); 2-vinylthiophene (10); indene (11); 2-methyl-3-vinylthiophene (12); 1-methyl-1H-indene (13); naphthalene (14); thianaphthene (15) *trans*-2,5-dithiabicyclo(4.3.0)nonane (16); *cis*-2,5-dithiabicyclo(4.3.0)nonane (17); 1,6-dehydro-2,5-dithiabicylo(4.3.0)nonane (18); biphenyl (19); 2-naphthalenethiol (20); acenaphthylene (21); $C_{10}H_{12}S$ (22); $C_{10}H_8S_2$ (23); dicyclopentyl disulfide (24).

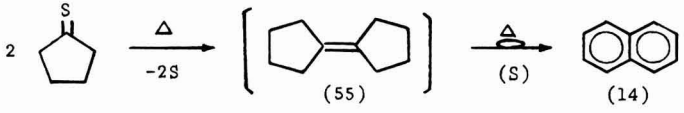
[b] Very small.

at 495 nm (ϵ = 10). The visible spectrum of crude pyrolysate shows a weak maximum at 495 nm. This is considered to be fair evidence for the intermediacy of the thioketone (7) at higher pyrolysis temperatures. Further, when the pyrolysate (600°C) was assayed by GC/MS, the peak corresponding to (7) had a mass spectrum identical to that of authentic thioketone. When the same peak was collected from GC and stored overnight at room temperature, the mass spectrum then obtained indicated that the thioketone had dimerized to (45).

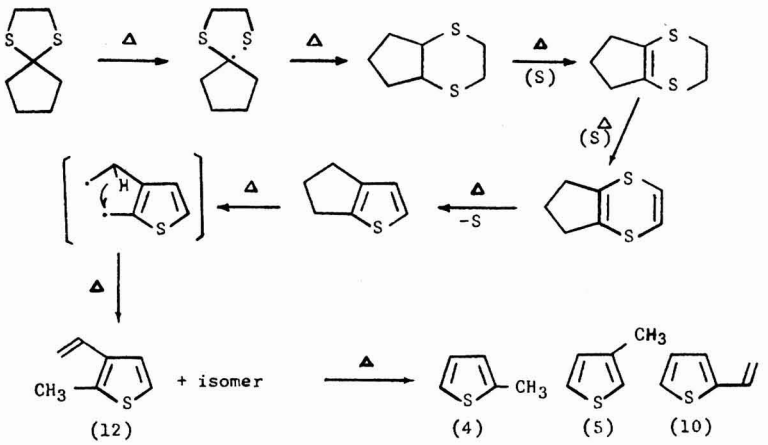
The naphthalene isolated from the higher-temperature pyrolysis could arise via loss of a sulfhydryl radical from the thioketone (7) in the following manner:



The intermediacy of thioketone (7) is established in the pyrolysis of similar spirodithioketals (*2, 5*). The cyclopentene radical was suggested (*8*) as an intermediate on the basis of the mass spectral fragmentation pattern of the starting material (I). The cyclopentene radical is the base peak at 70 eV. Romovacek and his co-workers (*10*) have investigated the thermal decomposition of cyclopentane over quartz glass and have obtained aromatic compounds. When the thioketone (7) was pyrolyzed, naphthlene was obtained as a major product (12.55% absolute yield). However, naphthalene can also arise after rearrangement of intermediate (55) which in turn arises from the desulfurization of the thiocarbonyl group:

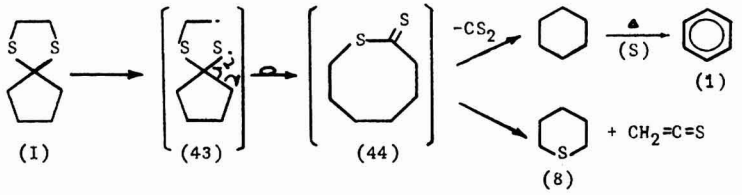


The majority of the thiophene derivatives most likely result from the pyrolytic degradation of 2-methyl-3-vinylthiophene. Thiophene (2) and 2-methylthiophene (4) could be obtained from thiocyclopentanone (7). Pyrolysis of thioketone (7), under identical conditions, gave thiophene (2) and 2-methylthiophene (4). Compound (12) appears to be an intermediate, as its yield decreases with increasing temperature.

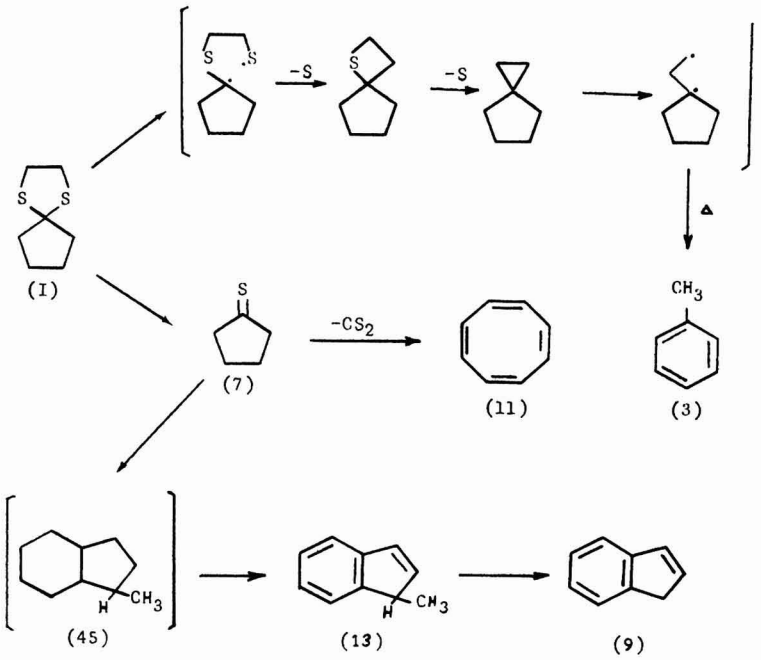


As shown in the above diagram, the above argument is substantiated by the presence of the rearrangement products (16), (17), and (18) from the pyrolysis at 600°C.

The cyclic dithioester (44) is a possible intermediate in the formation of benzene (1) and thiacyclohexane (8). The dehydrogenation of cyclohexane in the presence of sulfur and heat to the more stable benzene is known (*4*). Cyclohexane was identified (by GC/MS) in one of the pyrolyses of (I) at lower temperature in a very low yield. This mechanism of benzene formation is also substantiated by both carbon disulfide and thiacyclohexane.

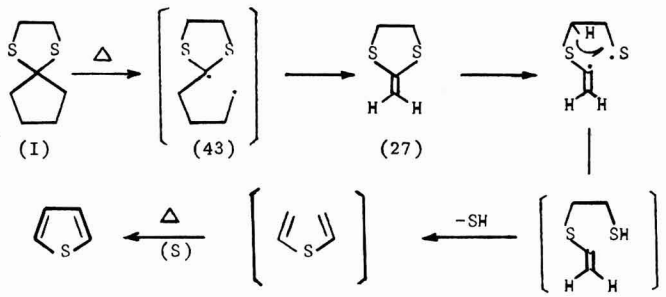


The following is a possible route to compounds (3), (9), (11) and (13).

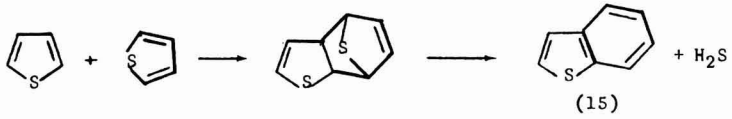


To substantiate the intermediacy of thioketone (7), it was pyrolyzed at 800°C; the presence of all three compounds (9), (11), and (13) was established by GC/MS and their respective retention times by GC. The intermediate of (45) arises by loss of sulfur from thioketone (7), followed by dimerization and dehydrogenation. This was followed by loss of a methyl group and abstraction of a hydrogen radical to give indene (9). Indeed, pyrolysis of the *cis*-8,9-dihydro-1-indenyl cation gives indene (11) (*11*). In our case dehydrogenation of the saturated intermediate (45) by sulfur is extremely likely.

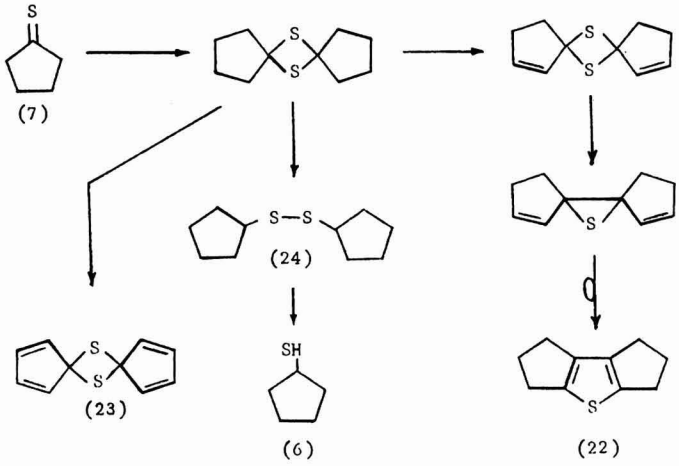
As mentioned earlier when thiocyclopentane (7) is pyrolyzed at 800°C, a small amount of thiophene is formed. This probably is not the sole route to the production of thiophene. Considering the yields as a function of temperature and what is known about the electron-impact-induced fragmentation (*8*) of the starting material (I), the following is a reasonable speculation concerning the formation of thiophene.



Compound (27), with molecular ion *m/e* 118 and P + 1 and P + 2 peaks in accord with sulfur atoms, was detected in the pyrolysis of 1,4-dithiaspiro(4.5)decane. Wynberg and Bantjes (*14*) pyrolyzed thiophene at 800–850°C for an unspecified length of time and found benzothiophene, which they explained as arising from the Diels–Alder addition of thiophene to itself.



About the only possible pathway for the formation of compounds (22), (23), and disulfide (24) is by dimerization of thioketone (7), and this has been seen in the photolysis of (I).



The fate of the ethylene fragment of the 1,3-dithiolane ring of (I) is still uncertain. It is possible that ethylene formed in the pyrolysis arises from this portion of the molecule. However, this is only speculative. The possible formation of ethylene sulfide from this fragment was examined in detail, but its presence was never detected. There is a small amount of

white polymer that does not dissolve in any organic solvent which is formed in the liquid nitrogen trap during pyrolysis of (I). This would be expected if the enethiol ($CH_2=CH-SH$) was formed. This material has been prepared at low temperatures and is known to polymerize readily at room temperatures (*12*). Formation of the white polymer has been seen during PVC measurements for molecular weight determination of ethylene.

SUMMARY

The initial product formed in the pyrolysis of 1,4-dithiaspiro(4.4)nonane (I) is the thioketone (7). During pyrolysis at 600°C, this material has been isolated in absolute yields as high as 4.85%. The increase in yield of benzene (1), toluene (3), and naphthalene (14) as the temperature is increased from 600 to 800°C could be anticipated from dehydrogenation under pyrolysis conditions.

The other products isolated, with the exception of the rearrangement products and toluene, appear to arise via further pyrolytic reaction of thioketone (7). These saturated products are dehydrogenated by sulfur to generate the aromatic compounds.

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MICROCHEMICAL JOURNAL 25, 492–499 (1980)

Ionic Strength and Buffer Capacity of Wide-Range Buffers for Polarography

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Received March 29, 1980

INTRODUCTION

In studies of the polarographic behavior of organic compounds, it is important to determine the effect of pH on the polarographic half-wave potential (*10*). This effect can be very sensitive to the ionic strength of the test solution. The diffusion currents and the slope of the polarographic wave can also be affected by the ionic strength of the solution, though to a lesser degree. As a consequence, it is essential to use a buffer system of constant ionic strength during pH studies. This is also true when making investigations in other areas such as spectrophotometry (e.g., in the determination of pK values) and reaction kinetics where the ionic strength can strongly influence the measurement.

Many sources describe the preparation of a wide variety of buffer systems, some of which are of, or can be easily adjusted to, a constant ionic strength (*1, 7, 9, 12*). However, the systems are often unsatisfactory for polarographic work for one reason or another, e.g., they may contain a component which is electroactive at the potential(s) of interest or they may not span a wide enough pH range so that more than one buffer system is required. According to Meites (*11*), the latter can lead to variations of the double-layer effects which may well obscure the effect of pH. Furthermore, such tables often give no indication of the buffering capacity of the buffer solutions, particularly with the more complex systems such as those proposed by Prideaux (*14*),[1] Prideaux and Ward (*15*), and Britton and Robinson (*2, 3*) which involve a mixture of mono- and polybasic acids. It is essential that the buffer solution has sufficient capacity to maintain a fixed pH at the electrode surface during the electrode reaction. For a given buffer system, the buffer capacity itself varies, often abruptly, with the pH of

[1] Some authors refer to these as Britton and Robinson buffers when in fact they were first proposed and calibrated by Prideaux (*14*) in 1916. The confusion seems to have arisen from a paper published 15 years later by Britton and Robinson (*3*) in which these authors remeasured and tabulated the pH values of these buffers at closer intervals than did Prideaux.

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the solution. Tables of buffer systems ought therefore to contain values of the buffer capacity but seldom do. A brief outline of the calculations involved for the preparation of Prideaux buffers suitable for polarographic studies prefaces a table detailing the composition of such buffers. By gathering all the necessary equations in one place, other workers may find it easier to evaluate the ionic strength and buffer capacity of those systems already published in the literature and adjust the composition accordingly in order to ensure that the buffers are adequate for the purpose in hand.

MATERIALS AND METHODS

Reagents and Apparatus

Analytical grade reagents were used throughout. pH measurements were made with a standard Pye pH meter using a shielded glass–saturated calomel, electrode pair.

Polarograms were recorded with a three-electrode potentiostated circuit at a scan rate of 2 $mV \cdot sec^{-1}$ using a Tacussel PRG 5 polarograph coupled to a Hewlett–Packard 7035B X-Y recorder. The outflow velocity of the dropping mercury electrode was 0.625 mg sec^{-1} at a mercury pressure (h_{Hg}) of 50 cm. The drop-life was controlled mechanically at 2.0 sec. A damping setting of 3 on an 11-point scale was used with the low pass filter set on MAX. In the differential pulse mode, a pulse amplitude of 50 mV (cathodic) with a pulse width of 48 msec and a sampling window of 8 msec was chosen to optimize the recordings.

All polarograms were run on 20-cm^3 samples of buffer deaerated with analytical grade nitrogen gas and thermostated at 20°C in a Tacussel CPRA polarographic cell.

Method of Calculation

(*a*) *Ionic strength, I.* Consider a tribasic acid H_3A with successive acid dissociation constants K_1, K_2, and K_3, respectively, and whose analytical concentration is c, expressed in moles per cubic decimeter. Ignoring the hydrogen ions, we can write

$$H_3A \overset{K_1}{\rightleftharpoons} H_2A^- \overset{K_2}{\rightleftharpoons} HA^{2-} \overset{K_3}{\rightleftharpoons} A^{3-}.$$

The fraction (f) of each species present in solution is given by the following expressions (*5*) where [ ] represents the concentration of the species in moles per cubic decimeter.

$$f_{H_3A} = \frac{[H_3A]}{c} = \left\{ 1 + \frac{K_1}{[H^+]} + \frac{K_1K_2}{[H^+]^2} + \frac{K_1K_2K_3}{[H^+]^3} \right\}^{-1},$$

$$f_{H_2A^-} = \frac{[H_2A^-]}{c} = f_{H_3A} \cdot \frac{K_1}{[H^+]},$$

$$f_{HA}{}^{2-} = \frac{[HA^{2-}]}{c} = f_{H_3A} \cdot \frac{K_1K_2}{[H^+]^2},$$

$$f_A{}^{3-} = \frac{[A^{3-}]}{c} = f_{H_3A} \cdot \frac{K_1K_2K_3}{[H^+]^3},$$

$$f_{H_3A} + f_{H_2A^-} + f_{HA}{}^{2-} + f_A{}^{3-} = 1.$$

Equivalent expressions can be written for di- and monobasic acids. Thus, for a dibasic acid we have

$$H_2A \overset{K_1}{\rightleftharpoons} HA^- \overset{K_2}{\rightleftharpoons} A^{2-},$$

$$f_{H_2A} = \frac{[H_2A]}{c} = \left\{ 1 + \frac{K_1}{[H^+]} + \frac{K_1K_2}{[H^+]^2} \right\}^{-1},$$

$$f_{HA^-} = \frac{[HA^-]}{c} = f_{H_2A} \cdot \frac{K_1}{[H^+]},$$

$$f_A{}^{2-} = \frac{[A^{2-}]}{c} = f_{H_2A} \cdot \frac{K_1K_2}{[H^+]^2},$$

$$f_{H_2A} + f_{HA^-} + f_A{}^{2-} = 1,$$

and, for a monobasic acid, we have

$$HA \overset{K_1}{\rightleftharpoons} A^-,$$

$$f_{HA} = \frac{[HA]}{c} = \left\{ 1 + \frac{K_1}{[H^+]} \right\}^{-1},$$

$$f_{A^-} = \frac{[A^-]}{c} = f_{HA} \cdot \frac{K_1}{[H^+]},$$

$$f_{HA} + f_{A^-} = 1.$$

The contribution from the acid's anionic forms to the overall ionic strength is then given, for each acid type, by

$$I_{\text{tribasic}} = (c/2)\,(9f_A{}^{3-} + 4f_{HA}{}^{2-} + f_{HA^-}),$$

$$I_{\text{dibasic}} = (c/2)\,(4f_A{}^{2-} + f_{HA^-}),$$

$$I_{\text{monobasic}} = (c/2)\,(f_{A^-}).$$

The contribution from the H^+ and OH^- ions in the solution is simply

$$\frac{1}{2}\left\{[H^+] + \frac{K_w}{[H^+]}\right\}, \quad (1)$$

where K_w is the ionic product of water. The contribution from (1) is negligible between pH 3 and 11. Allowance for the cations (other than H^+) from any acid salt used (or prepared indirectly by adding an alkali to an acid or acid mixture) must be made as appropriate. For a mixture of acids (or bases) values of the ionic strength are additive.

(*b*) *Buffer capacity, β.* The buffer capacity is defined as the number of equivalents of strong acid or base needed to cause 1 liter of the buffer to undergo a pH change of 1.0 unit. The term was introduced by Van Slyke (*16*) who showed that, for a monobasic acid of analytical concentration c (mol.dm^{-3}),

$$\beta = 2.303\left\{\frac{cK_1[H^+]}{(K_1 + [H^+])^2} + [H^+] + \frac{K_w}{[H^+]}\right\}, \quad (2)$$

and, for a polybasic acid whose successive acid dissociation constants $K_1, K_2, \ldots, K_i, \ldots, K_n$ are several orders of magnitude different,

$$\beta = 2.303\left\{\sum_{i=1}^{i=n} \frac{cK_i[H^+]}{(K_i + [H^+])^2} + [H^+] + \frac{K_w}{[H^+]}\right\}. \quad (3)$$

If the K_i values are not sufficiently different, the actual buffer capacity will be greater than that calculated by Eq. (3) (*4*, *6*). As with values of the ionic strength, β values for a mixture of acids are additive (*6*) and the contribution made by the H^+ and OH^- ions to the overall buffer capacity, viz., 2.303 ($[H^+] + (K_w/[H^+])$) is negligible between pH 3 and 11. Consequently, between pH 3 and 11 at fixed ionic strength and hence fixed K_i values, the contribution of an individual acid to the overall buffer capacity will be directly proportional to the analytical concentration of the acid.

RESULTS

For voltametry in general, and polarography in particular, a mixture of acetic, phosphoric, and boric acids, partially neutralized with a potassium hydroxide solution, provides a useful system for a study of the effect of pH over most of the pH range. Table 1 summarizes the composition of such buffers together with their ionic strength and the amount of potassium chloride (or other suitable 1:1 electrolyte) to be added to adjust the ionic strength to 0.5. Other ionic strengths can be attained by calculating the amount of KCl, etc., to be added on the

TABLE 1
COMPOSITION, INITIAL IONIC STRENGTH (I'), AND FINAL BUFFER CAPACITY (β) OF SOME PRIDEAUX BUFFERS

pH	Volume of 1 M KOH to be added[a] (cm^3)	I'	Volume of 2 M KCl to give $I = 0.5$[b] (cm^3)	β	Polarographic range (−V vs SCE)	
					$i_{dpp} \leq 25$ nA[c]	$i_{dpp} \leq 100$ nA[d]
					0.10 V to:	0 V to:
2.0	0.5	0.013	24.35	0.035	1.23	1.31
2.2	1.0	0.014	24.30	0.026		
2.6	1.5	0.017	24.15	0.014	1.24	1.31
3.0	1.8	0.019	24.05	0.007		
3.3	2.0	0.020	24.00	0.005	1.28	1.35
3.7	2.2	0.022	23.90	0.006		
4.1	2.5	0.025	23.75	0.009	1.33	1.39
4.4	2.8	0.028	23.60	0.011		
4.7	3.0	0.030	23.50	0.012	1.37	1.43
5.0	3.5	0.035	23.25	0.010	1.40	1.46
5.3	3.7	0.038	23.10	0.008		
5.7	4.0	0.042	22.90	0.007	1.45	1.52
6.1	4.2	0.047	22.65	0.009		
6.4	4.5	0.052	22.40	0.011	1.47	1.55
6.8	5.0	0.061	21.95	0.012		
7.2	5.5	0.070	21.50	0.009	1.50	1.60
7.6	5.8	0.076	21.20	0.006		
8.0	6.0	0.079	21.05	0.006	1.50	1.66
8.4	6.3	0.083	20.85	0.008		
8.7	6.5	0.085	20.75	0.010	1.52	1.64
9.1	7.0	0.090	20.50	0.012		
9.6	7.5	0.096	20.20	0.009	1.51	1.75
10.0	7.8	0.099	20.05	0.006		
10.4	8.0	0.103	19.85	0.005	1.52	1.77
10.8	8.2	0.109	19.55	0.008		
11.2	8.5	0.118	19.10	0.014	1.52	0.04−1.78
11.6	9.0	0.133	18.35	0.021		
12.0	10.0	0.152	17.40	0.032	1.52	0.06−1.78

[a,b] Volume of 1 M KOH and 2 M KCl to be added to 25.0 cm^3 of a stock solution which is 0.08 M in CH_3COOH, H_3PO_4, and H_3BO_3, followed by dilution of the mixture to 100.0 cm^3, to give a buffer of the desired pH at a final ionic strength of 0.5 M.

[c] Equivalent to i_{Tast} of 5 (anodic) to 60 nA (cathodic); drop-life, 2 sec at $h_{Hg} = 50$ cm.

[d] Equivalent to i_{Tast} of 50 (anodic) to 100 nA (cathodic); drop-life, 2 sec at $h_{Hg} = 50$ cm.

basis of the ionic strength of the buffer system alone. The buffer capacity of each mixture at $I = 0.5$ is also given. Higher buffer capacities can be obtained, if desired, simply by increasing the concentration of the stock acid mixture and adding proportionately more KOH to maintain the same pH. Between pH 3 and 11, at fixed I, β is directly proportional to c. Thus, to triple the tabulated value of β at pH 8.0 one would add 25.0 cm^3 of a stock solution 0.24 M in each of CH_3COOH, H_3PO_4, and H_3BO_3 to 18.0 cm^3 of 1 M KOH, add 13.1 cm^3 of 2 M KCl to bring I to 0.5, and dilute the mixture to 100.0 cm^3 with deionized water. The buffer capacity of this solution would then be 0.0165.

Changes in the value of I will affect the values of both β and the

pH slightly. Allowance for the effect of I on the values of the thermodynamic dissociation constants, K_i, was made in the calculations involved in preparing Table 1, as described by Perrin and Dempsey (*13*). The correction terms given there are based on the Davies equation (*8*) and apply up to $I = 0.5$. Data in Table 1 are quoted to the nearest 0.1 pH unit only. Intermediate pH values can be readily interpolated from the data. Of course, the pH of all solutions prepared should be checked before use by using an appropriate pH meter.

These buffers have been used in the author's laboratory in a study, using differential pulse polarography (dpp), of the rate of attainment of tautomeric equilibrium in some bis(phenylhydrazones). The useful polarographic ranges quoted in Table 1 were arbitrarily set as the potentials between which the differential pulse current of the aqueous buffer as supporting electrolyte was $\leq$25 and $\leq$100 nA, respectively.[2]

DISCUSSION

These buffer solutions are ideally suited for polarographic work over the whole pH range on solutions containing depolarizer concentrations up to 1×10^{-3} M, as a typical example shows. Consider the polarography of 20 cm^3 of a 1×10^{-3} M solution of an organic compound, the recording of the polarographic wave taking 5 min to achieve. A typical waveheight would be ≤ 10 μA. If the electrode reaction is represented by

$$O + mH^+ + ne^- \rightarrow R, \tag{4}$$

where O and R represent the depolarizer in the oxidized and reduced forms, respectively, and m and n are, respectively, the number of hydrogen ions and electrons involved in the electrode reaction, then the charge consumed during polarography will be 3×10^{-3}C and the number of moles of O consumed will be

$$\frac{3 \times 10^{-3}}{96{,}500n} = \frac{3.11 \times 10^{-8}}{n} \text{ mol.}$$

Hence the number of moles of H^+ consumed from 20 cm^3 of solution will be $3.11 \times 10^{-8} \times (m/n)$ mol. corresponding to $1.56 \times 10^{-6} \times (m/n)$ mol. H^+ from 1 liter of solution. Thus, the buffer capacity must be at least

$$\beta = \frac{1.56 \times 10^{-6}}{\Delta \text{pH}} \times m/n ,$$

[2] The addition of up to 0.005% (w/v) of Triton X-100 to these buffers gives differential pulse currents $\leq$25 nA around -0.4 and -1.7 V vs SCE.

where ΔpH is the change in pH that can be tolerated without causing an error in the half-wave potential measurement or waveheight. Setting ΔpH to equal 0.01 implies $\beta \geq 1.56 \times 10^{-4} \times (m/n)$. For

$$\frac{m}{n} \leq 4, \beta \geq 0.006.$$

This buffer capacity is easily achieved by a monobasic acid with $c = 0.02\ M$ over a pH range of p$K \pm 0.7$. Alternatively, as illustrated by the data in Table 1, this buffer capacity can be maintained over the whole pH range by using a mixture of mono- or polybasic acids whose individual analytical concentrations are 0.02 M and whose pK values, at the desired ionic strength, differ by less than 2.6. For such a solution, it can be shown that diffusion of H^+ ions or of proton donors (other than water) to the diffusion layer surrounding the mercury drop in polarographic work provides sufficient protons, at all pH values, to permit reduction of most depolarizers according to Eq. (4) for depolarizer concentrations up to $1 \times 10^{-3}\ M$ ($m/n \leq 4$).

SUMMARY

A procedure for the calculation of the ionic strength and buffer capacity of a series of buffers prepared by the partial neutralization of a mixture of mono- and polybasic acids is outlined and data tabulated for a mixture 0.02 M in each of CH_3COOH, H_3PO_4, and H_3BO_3. The useful polarographic range of each buffer solution is also given. Illustrative calculations have also been made to show that, at any given pH value, the minimum buffer capacity required for polarographic reduction of up to $1 \times 10^{-3}\ M$ solutions of an organic compound, and which involves hydrogen ions, is 0.006.

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MICROCHEMICAL JOURNAL 25, 500–506 (1980)

A New Continuous Flow Micromethod for Determination of Creatinine in Serum or Plasma

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Received April 24, 1980

INTRODUCTION

It is generally accepted that the serum creatinine is a useful index of renal function because it is relatively little affected by diet, metabolic rate, or urine volume (*3*). Accordingly, most clinical laboratories offer this test, where creatinine is determined by some modification of the original Jaffe alkaline picrate procedure (*4*). Unfortunately, adequate analytical sensitivity and specificity have been difficult to achieve with this method because of the low molar absorption coefficient of the creatinine–picrate complex (*1*) and because several other plasma constituents react with picrate to give interfering colored complexes (*2*).

We report here the development of an assay in which the major barrier to achieving increased sensitivity in a continuous flow system, i.e., the dialysis step, is eliminated. The specificity of the assay is enhanced by removal of the plasma proteins by prior trichloroacetic acid (TCA) precipitation and by controlling the pH during color development with a phosphate buffer system. Only 7.5 μl of plasma is required for this assay.

MATERIALS

Equipment. The analytical train was composed of Technicon Auto-Analyzer II components—sampler, pump, and colorimeter. The sampler was modified to hold Eppendorf microcentrifuge tubes by drilling ¼-in.-diameter holes in the Lucite cover. A Rikadenki recorder with a 20-mV span was used. Interference filters (510 nm) were used in the colorimeter. A 20-μl SMI positive displacement pipet and a Brinkman dispenser set at 1.0 ml were used to deliver plasma and 10% (w/v) TCA, respectively.

Reagents. The reagents used were: 0.66 *N* trichloroacetic acid (TCA), 100 g/liter water; 1.0 *N* NaOH, 40 g/liter, plus 5 ml Triton X-405/liter; 0.03 *N* picric acid, half saturated solution; 1.08 *N* NaH_2PO_4, 149 g $NaH_2PO_4 \cdot H_2O$/liter. All chemicals were of reagent grade. Solutions were made up in deionized water and were filtered once.

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Creatinine, glucose, and acetoacetate, together with the sodium or lithium salts of β-hydroxybutyrate, lactate, pyruvate, and ascorbate, were obtained from Sigma Chemicals, St. Louis, Missouri.

PROCEDURE

Sample deproteinization. Twenty microliters of serum or plasma was pipetted into Eppendorf microcentrifuge tubes followed by 1.0 ml of 10% TCA. The tubes were capped, vortexed for 1 min and centrifuged for 5 min. After centrifugation, the tubes were inserted into the modified AA II sampler and the supernatant sampled directly for analysis as described below.

Continuous flow manifold. Samples were analyzed at the rate of 60 per hour on the manifold shown in Fig. 1. A unique feature of this manifold is that the sample stream is debubbled prior to its addition to the NaOH stream. The debubbler was constructed by combining an A10 and an A6 as shown in Fig. 2. The debubbling space was adjusted (by raising or lowering the upper connector) to the minimum size necessary to prevent any bubbles entering the NaOH stream.

RESULTS AND DISCUSSION

The method has a stable baseline and well-formed sample peaks, without any detectable carry-over between samples. Of a range of wetting agents tested only Triton X-405 was found to give satisfactory results. Other wetting agents were found to precipitate from the 1.0 *N* NaOH solution on standing for several days at room temperature, resulting in increasing baseline noise.

It was necessary to debubble the TCA (sample) stream because the interruptions, introduced as the probe traveled between the sample and wash pot, caused fluctuation in the final NaOH concentration between

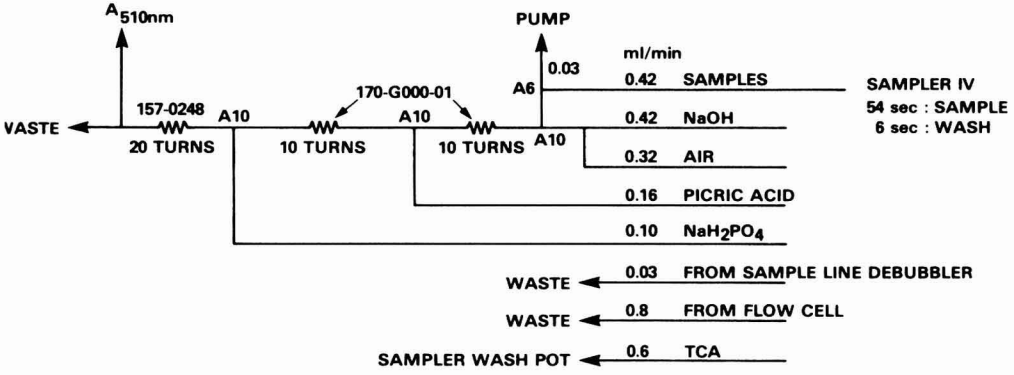

FIG. 1. Flow diagram for the present method.

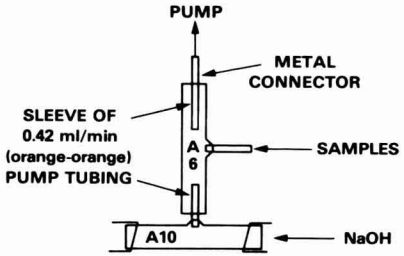

FIG. 2. Details of sample line debubbler.

518 and 201 m*M* (Table 1). The color of the alkaline picrate reagent is pH dependent and, even in the presence of the phosphate buffer, this fluctuation caused unacceptable peaks on either side of the sample peak.

Linearity and range. Figure 3 shows a linear response up to at least 500 mg creatinine/liter. Both aqueous and protein based creatinine standards were run, giving identical results. The method can thus be calibrated with either type of standard.

Recovery of added creatinine. Fifty microliters of aqueous standards of concentration 0, 100, 200, 800, and 1600 mg/liter was added to 1-ml aliquots of pooled plasma and analyzed. For the five pools tested, recoveries ranged between 110 and 84%. The results of a representative pool are given in Table 2.

Interfering substances. It is well established that the Jaffee reaction is not specific for creatinine (*2*). Several commonly occurring plasma constituents such as protein, glucose, ketone bodies, and ascorbate may influence the color development and invalidate the assay. In this method, protein is removed by precipitation and thus cannot interfere. Some other potential interfering substances were investigated and comparison made between the standard AA II creatinine method and the present micromethod, in the presence and absence of the phosphate buffer component.

Potential interfering substances were tested in aqueous solution and after addition to a pooled plasma. The apparent creatinine results obtained are given in Table 3 and show that the proposed micromethod

TABLE 1
CONCENTRATION OF REACTANTS IN BUFFERED JAFFE MICROMETHOD

Reagent	Flow rate (ml/min)	Dilution factor	Concentration (m*M*)
0.66 *N* TCA	0.39	2.08	317
1.0 *N* NaOH	0.42	1.93	518
0.03 *N* Picrate	0.16	6.06	5
1.08 *N* NaH_2PO_4	0.10	10.7	101

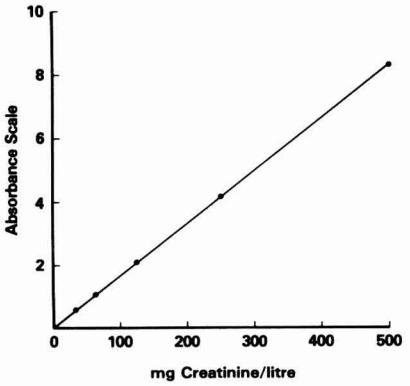

FIG. 3. Creatinine calibration curve using the proposed micromethod.

shares with the standard AutoAnalyzer method (with dialysis) freedom from interference by the compounds tested.

In the absence of the phosphate buffer the micromethod shows a marked nonspecificity, especially with glucose and acetoacetate. In the presence of the phosphate buffer the pH of the stream emerging from the colorimeter was 11.2. When water was substituted for the phosphate buffer the pH was 13.5. This enhanced specificity obtained by lowering the pH has also been found by Yatzidis (*7, 8*) who measured "true" creatinine by a manual Jaffe procedure buffered to pH 11.5.

It will be noted from Fig. 1 that the phosphate solution is added about 2 min after the picric acid. The initial unbuffered reaction at pH 13.5 was found to be necessary for adequate color development. Subsequent lowering of the pH to 11.2 either reversed or prevented the interference by noncreatinine chromogens without reversing the creatinine–picrate reaction.

Between-run precision. Long-term precision of the method was determined by analysis of Ortho QC pools each day over a 2-week period and the results are given in Table 4.

TABLE 2
RECOVERY OF CREATININE ADDED TO PLASMA POOL

Creatinine added (mg/l)	Creatinine measured (mg/l)	Creatinine recovered (mg/l)	Percentage recovered
0	12.5	0	0
4.76	17.5	5.0	105
9.52	22.5	10.0	105
38.0	48.0	35.5	93
76.1	84.0	71.5	93

TABLE 3
EFFECT OF POTENTIAL INTERFERING SUBSTANCES ON CREATININE VALUES: COMPARISON OF AUTOANALYZER II METHOD WITH PRESENT MICROMETHOD ± BUFFER COMPONENT

Interfering substance (mg/l)	Medium	Apparent creatinine (mg/l) AAII	Present method +buffer	Present method −buffer
—	Plasma	9	9	10
Glucose				
4760	Plasma	8.5	9	12
9090	Plasma	9	10	13
9090	Water	0	1	5
Acetoacetate				
122	Plasma	9	10	13
236	Plasma	10	9	14
236	Water	0	0	6
β-Hydroxybutyrate				
143	Plasma	9	10	12
273	Plasma	9	9	11
273	Water	0	0	3
Lactate				
24	Plasma	9	9	11
45	Plasma	8	9	11
45	Water	0	0	2
Pyruvate				
100	Plasma	9	9	9
1000	Plasma	10	9	11
1000	Water	1	0	2
Ascorbate				
38	Plasma	9	9	12
73	Plasma	9	9	14
73	Water	0	0	4

TABLE 4
BETWEEN-BATCH PRECISION

QC Pool	*n*	Creatinine (mg/l) Mean	±1 SD	CV%
Normal	14	7.3	0.9	12.3
High	14	68.4	0.7	1.0

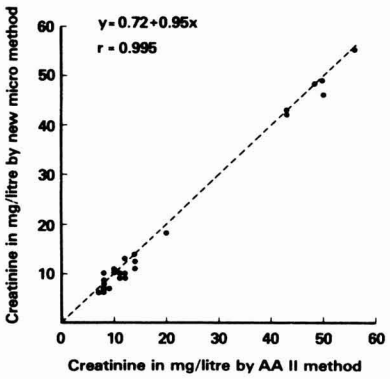

FIG. 4. Correlation between serum creatinine results by standard AutoAnalyzer II method and the present method.

Comparison with Technicon AAII method. As the proposed micromethod was designed to provide a microalternative to the Technicon AAII creatinine method, this latter was the comparative method chosen. Seventy patient samples were analyzed using both methods under routine working conditions and the results are shown in Fig. 4. The data were analyzed by the method of Wu *et al.* (*6*). There is no significant difference between the two methods and the micromethod presented here is a satisfactory alternative to the Technicon AAII method.

An initial manual protein precipitation step may be considered an undesirable drawback. It is, however, readily accomplished with current high-precision dispensing devices at a rate of 10 samples per minute and with a precision of 0.5% (1 CV). The resultant supernate is compatible with other automated micro analyses often requiring less than 1 μl plasma per test (*5*) so that sodium, potassium, chloride, urea, glucose, and phosphate may be measured together with creatinine on a single 20-μl plasma sample.

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MICROCHEMICAL JOURNAL 25, 507–513 (1980)

Extractive Separation of Platinum from Macroamounts of Palladium Using Triphenylphosphine Oxide and Its Successive Spectrophotometric Determination by the Stannous Chloride Method

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Received May 6, 1980

INTRODUCTION

Separation of microamounts of a platinum element from macroamounts of another element of this group presents serious difficulties. Because of similar chemical properties of platinum metals only few micro–macro separation methods have been developed for this group of elements (*1, 2, 5*).

There are many effective methods of extractive separation of similar amounts of platinum(IV) and palladium(II) or trace amounts of palladium(II) from macroamounts of platinum(IV). Of the above methods those based on the extraction of complexes of palladium(II) with oximes of various structure are especially useful. For a reverse case, i.e., separation of trace amounts of platinum(IV) from macroamounts of palladium, there are no effective extractive methods so far. Extraction of platinum(IV) from hydrochloric or hydrobromic acid media with tributylphosphate (*3, 4*) makes possible good separation from similar quantities of palladium.

In a previous work (*7*) we have pointed that platinum(IV) is more effectively extracted with trioctylphosphine oxide (TOPO) than palladium(II), particularly at higher hydrochloric acid concentrations in the aqueous phase.

On the basis of these findings we have undertaken the present study aimed at evaluation of the suitability of TOPO and other phosphine oxides (e.g., tributylphosphine oxide (TBPO) and triphenylphosphine oxide (TPPO)) for separation of microamounts of platinum from macroamounts of palladium.

MATERIALS AND METHODS

Reagents

Tri-*n*-butylphosphine oxide (Merck), tri-*n*-octylphosphine oxide, and triphenylphosphine oxide (BDH), reagent grade, were crystallized twice

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from hot ethanol. Solutions (0.1 *M*) of the reagents in dichloroethane were used for extraction.

Palladium(II) solution of a concentration of 50 mg/ml was prepared by dissolving palladium chloride (Pfaltz and Bauer) in 7 *M* hydrochloric acid. The solution was standarized gravimetrically with dimethylglyoxime.

Standard solution of platinum(IV) in 4 *M* hydrochloric acid of a concentration of 1 mg/ml was prepared by dissolving the pure metal in aqua regia. The solution was evaporated with concentrated hydrochloric acid several times and the residue was dissolved in 4 *M* hydrochloric acid.

Other reagents used throughout this work were of analytical reagent grade.

Apparatus

Spectrophotometric measurements were carried out with the aid of a VSU-2P spectrophotometer and a SPECORD UV/VIS recording spectrophotometer (Carl Zeiss, Jena) using 1- and 5-cm quartz cells.

Determination of the Distribution Ratios

The distributions ratios of platinum(IV) and palladium(II) in the extraction with phosphin oxides were determined as described in a previous work (*7*). Determination of platinum concentration in organic phases was carried out also with the aid of the stanneous chloride procedure, described in this work.

Determination of Platinum in Palladium Samples

A 0.2-g sample of palladium was dissolved in 10 ml of aqua regia. The solution was evaporated to dryness, the residue was dissolved in 5 ml of conc. HCl and evaporated once more. The residue was then dissolved in 20 ml 7.5 *M* HCl and gold was separated from the obtained solution by single extraction with 20 ml of 0.1 *M* TOPO solution in chloroform. Platinum was then extracted from the aqueous phase by shaking with three 10-ml portions of 0.1 *M* TPPO solution in dichloroethane for 10 min. The organic phases were combined and palladium was back-extracted with 10 ml of 7.5 *M* HCl.

The organic phase containing platinum was shaken with 10 ml of 0.1% solution of $SnCl_2$ in 4 *M* HCl for 5 min. The absorbance of the color solution in dichloroethane was measured at 400 nm against water as reference and the content of platinum was calculated from a standard curve.

When the content of platinum in the sample did not exceed 0.01% the dichloroethane extract was slowly evaporated to dryness and the organic residue mineralized in a mixture of concentrated hydrochloric and nitric acids (3 + 1). The residue was evaporated to dryness with 2 ml of conc. HCl and dissolved in 10 ml of 7.5 *M* HCl. Platinum was then extracted

from the obtained solution with two 5-ml portions of 0.1 *M* TPPO in dichloroethane and determined in the organic phase with $SnCl_2$ as described above.

RESULTS AND DISCUSSION

Extraction of Chloride Complexes of Pt(IV) and Pd(II) with Phosphine Oxides

The distribution ratios of platinum and palladium in the extraction with TBPO, TOPO, and TPPO in dependence on the hydrochloric acid concentration in the aqueous phase were determined. 1,2-Dichloroethane was used as a diluent because it makes the organic phase heavier than the aqueous one thereby making easier the phase separation and does not interact with phosphine oxides. In these experiments the initial concentrations of platinum and palladium were equal and amounted to 1×10^{-3} *M*. The obtained results, presented in Fig. 1, indicate considerable difference in the extraction yields of platinum(IV) and palladium(II) as well as different courses of the dependence on acid concentration for the individual extractants.

The possibility of extractive separation of metals can be evaluated on the basis of the separation factor, i.e., the ratio of respective distribution ratios under the same conditions. The separation factors in the extraction of platinum and palladium with phosphine oxides in dependence on hydrochloric acid concentration in the aqueous phase are shown in Fig. 2. The highest separation factors of platinum and palladium are attained in the case of extraction with TPPO in the HCl concentration range 6–9 *M*

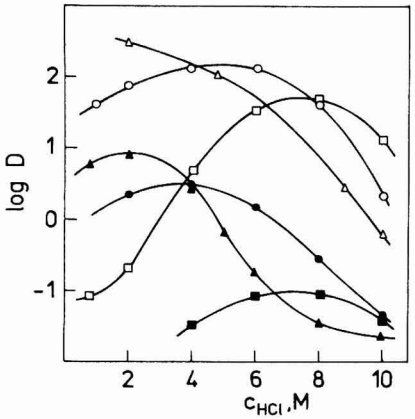

FIG. 1. The effect of hydrochloric acid concentration on the extraction of platinum (open symbols) and palladium (closed symbols) with 0.1 *M* solutions TBPO (△, ▲), TOPO (○, ●), and TPPO (□, ■) in dichloroethane.

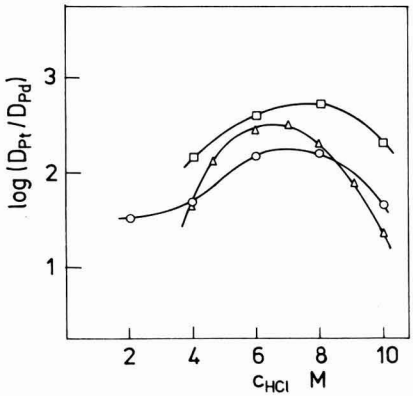

FIG. 2. The effect of hydrochloric acid concentration on the separation factors of platinum and palladium in the extraction with TBPO (△), TOPO (○), and TPPO (□).

(D_{Pt}/D_{Pd} exceeds 400). In this acid concentrations range extraction of platinum(IV) is sufficiently high.

The extraction of platinum and palladium from aqua regia solution with 0.1 *M* TBPO, TOPO, and TPPO solutions in dichloroethane has also examined. Palladium and platinum solutions in aqua regia, or these solutions diluted (2−10 times) with water, were used as aqueous phase. It has been found that palladium is practically not extracted from these solutions ($D < 0.01$). The distribution ratio of platinum does not exceed 1.3. This partial extraction of platinum makes direct use of aqua regia solutions in the extractive separation of platinum and palladium with phosphine oxides impossible.

Separation of Microgram Amounts of Platinum from Macroamounts of Palladium

On the basis of the separation factors the extraction with triphenylphosphine oxide from 7.5 *M* HCl solutions has been chosen. The effect of palladium concentration in the aqueous phase on the distribution ratio of platinum(IV) was determined. The concentration of platinum was constant and amounted to 20 μg/ml while the concentration of palladium varied in the range 1−50 mg/ml. The obtained results, shown in Fig. 3, indicate that the distribution ratio of platinum unfavorably changes with increasing palladium concentration in the aqueous phase. While at a palladium concentration of 1 mg/ml the distribution ratio amounts to approx. 30 it decreases to 4.9 and 0.9 at palladium concentrations of 10 and 50 mg/ml, respectively. As has been found previously the distribution ratio of palladium in the extraction with TPPO is small and it decreases too with increasing concentration of palladium but to a lower extent than in the case of platinum.

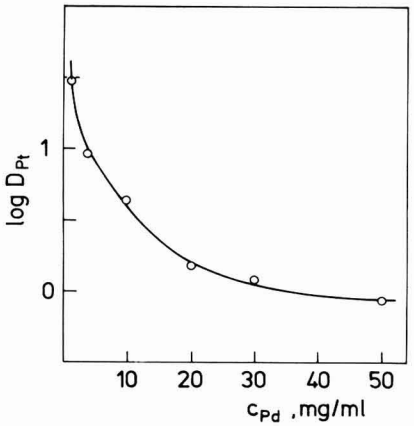

FIG. 3. The effect of palladium concentration on the extraction of platinum with TPPO in dichloroethane: c_{Pt} = 20 μg/ml, c_{HCl} = 7.5 *M*, c_{TPPO} = 0.1 *M*.

It has been found that at a palladium concentration of 10 mg/ml the changes in platinum concentration in the range 2–200 μg/ml do not affect the distribution ratio of platinum in the extraction with TPPO.

On the basis of the results presented in Fig. 3 it can be assumed that palladium concentration in the extraction of platinum with TPPO should not exceed 10 mg/ml. At this concentration of the macrocomponent a trifold extraction with 0.1 *M* TPPO solution in dichloroethane results in separation of 96% of platinum.

Despite the small distribution ratio of palladium under the chosen conditions at a palladium concentration of 10 mg/ml the organic phase after extraction contains microgram amounts of palladium. By washing with 7.5 *M* hydrochloric acid it is possible to back-extract palladium only partly and it remains in the organic phase in amounts 0.5–2 μg/ml.

Spectrophotometric Determination of Platinum after Extraction with TOPO

Extraction of platinum(IV) with TPPO can be directly combined with its spectrophotometric determination by the stannous chloride method. When shaking the TPPO extract with a solution of stannous chloride in hydrochloric acid, platinum (contained in the extract) forms a color complex, which makes a basis of the known spectrophotometric method of the determination of this metal (*6*). Under these conditions of the determination of platinum with stannous chloride the molar absorptivity $\epsilon = 1.1 \times 10^4$ at $\lambda_{max} = 400$ nm.

Palladium interferes with the determination of platinum according to this procedure when present in the TPPO extract in amounts 1.5 times higher than those of platinum. Due to the fact that palladium cannot be

quantitatively back-extracted with 7.5 *M* hydrochloric acid the platinum determination by this simple procedure is possible only for platinum concentrations higher than 0.01%.

Smaller platinum concentrations in palladium can be determined after evaporation and mineralization of the organic phase, followed by dissolution of the residue in 7.5 *M* HCl and extraction of platinum with TPPO. This procedure ensures almost complete separation of platinum and palladium and also enhances the sensitivity owing to reduction of the volume of the solution used for the determination.

Traces of palladium can be removed also from the solution (0.1−0.3 *M* HCl, obtained after mineralization) by extraction or precipitation with dimethylglyoxime. The determination of platinum with $SnCl_2$ can be carried out in the aqueous solution.

Gold interferes with the determination of platinum when its concentrations in the initial sample solution is three times higher than that of platinum. This interference can be removed by preliminary extraction with TOPO solution in chloroform (*7*). Other noble metals do not interfere with the determination of platinum when present in 20-fold excess, and common metals even at a 100-fold excess.

The above procedure was applied to the analysis of palladium for platinum content. At the same time its was carried out the analysis of solutions these samples, to which known amounts of platinum had been added. The results of the determination, summarized in Table 1, indicate that the obtained precision and accuracy of the determination are satisfactory.

SUMMARY

Trialkylphosphine oxides extract more effectively chloride complexes of platinum than of palladium(II). Of the examined tributylphosphine (TBPO), trioctylphosphine (TOPO), and triphenylphosphine (TPPO) oxides the latter one makes possible best separation of these metals.

TABLE 1
DETERMINATION OF PLATINUM IN PALLADIUM SAMPLES

	Pd found[a] (%)	Standard deviation (%)	Pd added (%)	Total Pd found (%)
Sample 1	4.9×10^{-2}	2.8×10^{-3}	2.0×10^{-2}	7.2×10^{-2}
				7.0×10^{-2}
Sample 2	2.8×10^{-2}	1.1×10^{-3}	1.0×10^{-2}	3.7×10^{-2}
				3.7×10^{-2}
Sample 3	7.3×10^{-3}	8.2×10^{-4}	2.0×10^{-3}	9.8×10^{-3}
				9.0×10^{-3}

[a] The average of five determinations.

The extraction of platinum with TPPO from solutions containing platinum and palladium unfavorably decreases with increasing palladium concentration. Using 0.1 *M* TPPO solution in dichloroethane, at HCl concentration 7.5 *M*, it is possible to separate 2–200 μg Pt/ml at a palladium concentration not higher than 10 mg/ml.

Separation of platinum from macroamounts of palladium has been combined with spectrophotometric determination of platinum by means of stannous chloride. The method has been applied to the analysis of palladium for platinum content.

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MICROCHEMICAL JOURNAL 25, 514–523 (1980)

Luminol-Dependent Chemiluminescence Analysis of Human Platelets

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Received March 28, 1980

INTRODUCTION

Recently Mills *et al.* (*6*) detected chemiluminescence (CL) in human platelets and platelet particulate fractions. The CL was ascribed mostly to the synthesis of prostaglandins because several criteria were met including: (i) a dependency on oxygen, (ii) activation by arachidonate, (iii) parallel production of CL and malondialdehyde production and, (iv) inhibition by aspirin. This study was thoroughly done and even included screening of 15 different precursor fatty acids. The reproducibility of the system with many platelet donors was excellent.

A difficulty with the original study was that fairly large quantities of blood were required to do extensive studies. We decided to modify the original system by using luminol-dependent chemiluminescence so that as little as 1–10 ml of blood could be utilized. Conditions in which platelet numbers were severely depressed would still be practical using such a system.

We made several interesting new observations using the luminol-dependent system. Malmsten *et al.* (*4*) have shown that platelets are somewhat defective in the production of PGE_1 and $PGF_2\alpha$. Selected drug studies using compounds developed by Higgs and associates (*3*) and Egan *et al.* (*1*) produced the possibility that lipoxygenase (*10*), cyclooxygenase (*5*), and peroxidases (*8*) may be involved with the CL production from arachidonate in human platelets. This platelet CL may be an effective screening system for anti-inflammatory drugs because platelets are intricately involved with the inflammatory response.

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MATERIALS AND METHODS

Preparation of platelets. Blood was drawn from normal donors (no aspirin for 14 days) into dextrose–citrate buffer (pH 6.5) in a ratio of 9 parts blood to 1 part anticoagulant. A solution of 6% dextran–1% gelatin (DG) was added in a ratio of 3 parts DG to 10 parts blood. The blood was drawn into plastic syringes which were inverted (standing on plunger). The syringes were fit with a bent needle (16 gauge) and incubated at 37°C for 1 hr. The platelet-rich plasma was centrifuged at 200 *g* for 20 min at 15°C to pellet erythrocytes and leukocytes for removal. Platelets were prepared by adding 10% EDTA (pH 7.4) in a ratio of 1 part EDTA to 9 parts platelet-rich plasma. The mixture was centrifuged at 1800 *g* for 10 min at 15°C. The platelets were resuspended via 20-gauge needle and syringe in balanced Hepes solution (pH 7.4). Balanced Hepes solution contains 5 m*M* KCl, 5.5 m*M* glucose, 145 m*M* NaCl, 10 m*M* Hepes, 1 m*M* $CaCl_2$ with the pH adjusted to 7.4. The platelets were counted and sized by Coulter counter (Model B). Final counts vary from 1 to 5 $\times$ 10^8 platelets per ml. If kept at room temperature, the platelets were fully active for at least 24 hr. Examination of the platelet preparation by microscope indicated very minimal contamination by other cell types.

Preparation of sodium arachidonate. The sodium salt of arachidonic acid was prepared according to Mills *et al.* (*6*). However, the final resuspension was made in deoxygenated balanced Hepes, pH 7.4 at 0.16 mmol/ml. Arachidonic acid (99% pure) was purchased from Sigma Chemical Company, St. Louis, Missouri. Once prepared, the unstable sodium arachidonate was stored under liquid nitrogen.

Preparation of luminol. Luminol (10 mg) was added to a 100-ml volumetric flask and dissolved in 1 ml dimethyl sulfoxide (DMSO). Physiological saline was added to make a 100-ml volume. Luminol was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin.

Preparation of drugs and control. Sulindac sulfide (Clinoril sulfide) and indomethacin were donated by Merck Sharp & Dohme, West Point, Pennsylvania; BW 755C was donated by Burroughs–Wellcome, Research Triangle Park, North Carolina. The drugs were dissolved in 1 ml DMSO and diluted with Hepes (no Ca^{2+}, Mg^{2+}), pH 7.4, to a 100-ml volume at appropriate concentrations. The control is prepared similarly without the addition of drug.

Chemiluminescence (CL) assay. CL assay was accomplished using plastic vessels in a darkened room with red light. Twenty-milliliter plastic LSC vials were filled with the following ingredients: (i) 1 ml platelets, 1–5 $\times$ 10^8, (ii) 1 ml drug or control, (iii) 1 ml luminol, and (iv) 2 ml Hepes (complete), pH 7.4. The reaction vial was counted (without cap) to measure background light (0.1-min counts).

The reaction was initiated (with the vial sitting on the LSC elevator) by injecting 200 μl of arachidonate salt (8 μmol) with a Teflon constant-rate CR 700-200 Hamilton gas chromatography syringe. The vial was quickly swirled and counted immediately. Counts per 0.1 min were recorded for several minutes. Each vial must be assessed individually. For a review of CL assay see Trush *et al.* (*9*).

Settings of the liquid scintillation counter (LSC). The following settings were used on an Ambient Packard Model 2002 LSC fitted with bialkali phototubes:

Front of instrument	Back of instrument
Gain, 100%	Coincidence of phototubes, off
Window, A−B	Input selection, 1 + 2
Channel A, 25	
Channel B, 1000	

These settings were essentially wide open for the gathering of light and the coincidence between photomultiplier tubes was shut off so that single-photon events could be recorded.

Possible chemiluminescence reactions with luminol. Possible reaction pathways which can interact with luminol to produce CL are seen in Fig. 1. The light so produced is blue in aqueous solution (425 nm). The wavelength is detected maximally in an LSC with bialkali phototubes set in an out-of-coincidence mode. Free radicals produced on the surface of the cyclooxygenase, lipoxygenase, or the peroxidase are possible candidates for the CL reaction with luminol. Veldink *et al.* (*10*) have discussed the role of superoxide with lipoxygenase and they feel that O_2^- could be bound to the Fe^{3+} in the nonheme iron of the enzyme. We have found that

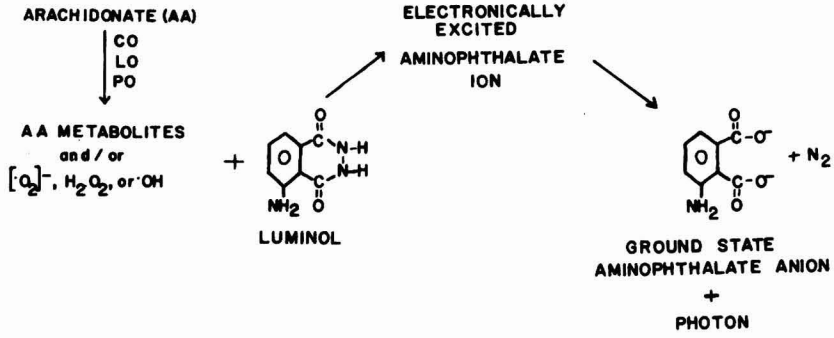
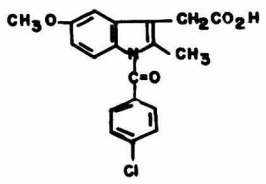
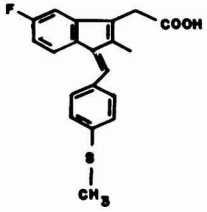


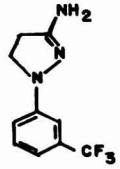
FIG. 1. Reaction pathways which could interact producing chemiluminescence (CL). CO, LO, and PO stand for the cyclooxygenase, lipoxygenase, and peroxidase, respectively. All of these enzymes and their substrates are known to produce the blue light (425 mm) from luminol. See Fig. 7 for the arachidonate metabolites.



INDOMETHACIN



SULINDAC SULFIDE



BW755C

FIG. 2. Drugs that inhibit either cyclooxygenase, lipoxygenase, and/or peroxidases. BW755C inhibits cyclooxygenase and lipoxygenase and possibly the peroxidases in natural sequence. Sulindac sulfide (Clinoril sulfide) and indomethacin inhibit cyclooxygenase and possibly peroxidases (PO) in sequence although the inhibition of PO may be accomplished by indomethacin and sulindac causing stimulation under some conditions.

microgram quantities of superoxide dismutase (SOD) inhibit (unpublished observations) this luminol CL 80 to 90%. Certain iron-containing peroxidases (e.g., horseradish peroxidase) are easily measured using luminol (*8*).

Structures of drugs. The structure of the drugs used in this work is shown in Fig. 2.

Preincubation of platelets with drugs. Platelets are preincubated with drugs for 20 min at 22°C (room temperature) before assay with arachidonate.

Controls. Control tubes with a complete assay system with one factor omitted were determined. The omissions include: arachidonic acid (AA), luminol, and platelets; in each case a negligible amount of chemiluminescence was displayed.

RESULTS AND DISCUSSION

In Fig. 3 is depicted the variation in the CL response from platelets drawn from the same person's blood on the same day and used each day over a 2-day period. Sterile technique, at room temperature, was used to

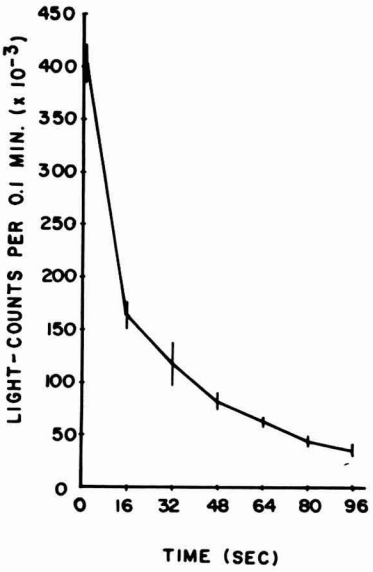


FIG. 3. Repetitive CL response from arachidonate (8 μmol) and platelets (1.0×10^8). The response were measured over a 2-day period from same day cells of a single individual's blood. Six separate assays were done and the range of the values are depicted.

avoid bacterial contamination. Eight micromoles of arachidonic acid (sodium salt) initiates the CL response. It is known that AA causes the platelets to aggregate (*9*) but it is unclear if aggregation and CL are related. Maybe the AA initiates the pertubation of the membrane, which allows contact with the enzymes from the platelets which metabolize the AA. Note that the CL response does not measure the initial burst, but appears only to decay from the initial measurement. This may be related to the 16-sec delay in counting the sample caused by mechanical and electronic hesitation of the liquid scintillation counter. Mills *et al.* (*6*) reported a peak CL response but they used no luminol and their analysis was made on a different LSC. Also, they used no spring-loaded injection device.

In Fig. 4 are shown the effects of the various drugs that disturb arachidonate-induced metabolism in platelets and the CL response. Drugs have been preincubated with the platelets for 20 min before the addition of AA. These drugs are all inhibitory to varying extents in this individual. BW755C at 10^{-4} *M* inhibits the CL completely, while aspirin, indomethacin, and sulindac sulfide at 10^{-4} *M* inhibit CL from 35 to 65%. In some individuals indomethacin and sulindac sulfide do not inhibit the CL. The reasons for this are unclear at present.

In Fig. 5 these inhibitory effects of drugs can be seen more clearly in the

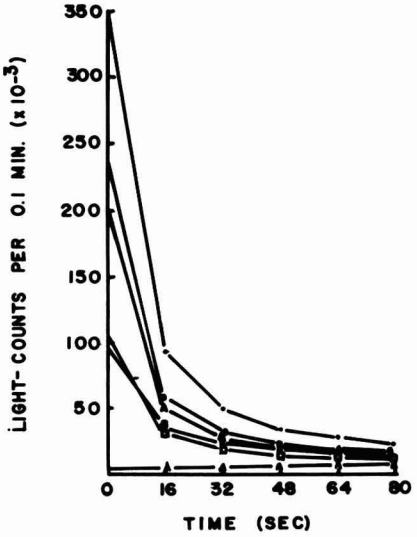


FIG. 4. The CL response is from arachidonate (8 μmol). Platelets (1.0×10^8) were preincubated for 20 min at 22°C. The symbols used are: (•) control, (□) 10^{-4} *M*, sulindac sulfide, (S) 2×10^{-5} *M*, sulindac sulfide, (○), 1×10^{-4} *M*, indomethacin, (△) 10^{-4} *M*, BW755C, (▲) 1×10^{-4} *M*, aspirin. This assay depicts a typical result of three assays.

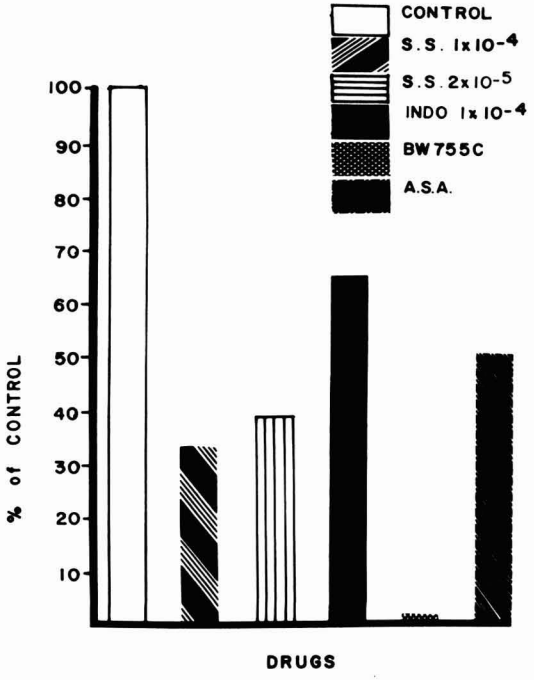


FIG. 5. Bar graph of the effect of the various drugs on CL response based on percentage control.

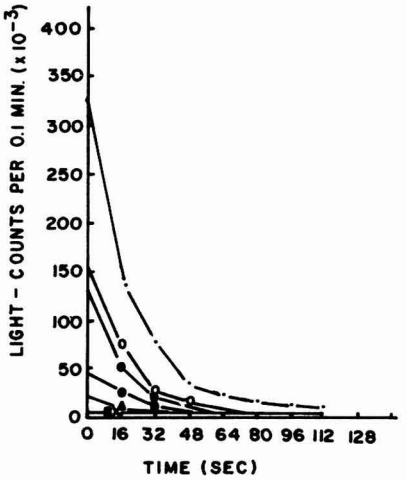


FIG. 6. Dose–response effect of BW755C on CL of arachidonate (16 μmol) and platelets (1×10^8). The symbols are: (•) control, (○) 3.2×10^{-8} *M*, (⊙) 1.6×10^{-7} *M*; (●) 8×10^{-7} *M*; (▲) 4×10^{-6} *M*; (X) 2×10^{-5} *M*; (■) 1×10^{-4} *M*.

form of a bar graph which compares the integrated area under the curves to the control at 100%.

In Fig. 6 a dose response of BW755C indicates that doses of the drug from 1×10^{-4} to 2×10^{-5} *M* completely inhibit CL produced in platelets from injection of AA. At a dose of 3.2×10^{-8} *M* the drug is about 50% inhibitory to the CL response. Trypan blue exclusion (TBE) in human granulocytes indicated that BW755C at high doses was not cytotoxic.

It is known that the human platelet metabolizes arachidonic acid (sodium salt) via two separate pathways (*2*). The first pathway is via cyclooxygenase, and the second is via lipoxygenase (see Fig. 7). However, as Samuelsson *et al.* (*2*) has shown, the production of prostaglandins in platelets is limited. Prostaglandins E_2 and $F_2\alpha$ are produced in very small amounts. Instead, HHT and PHD and malondialdehyde are produced from prostaglandin G_2. The second pathway (lipoxygenase) produces hydroperoxides which are reduced to the corresponding hydroxylated fatty acids, HETE compounds. It has been demonstrated by Vane and co-workers (*3*) that BW755C inhibits both pathways. Therefore, such an inhibition that blocks all the arachidonate-based metabolism should and does block all the CL (assuming the two are linked). The partial linkage has been shown by the parallel measurements of malondialdehyde and CL by Mills *et al.* (*6*). The fact that the cyclooxygenase (CO) inhibitors (e.g., aspirin, sulindac sulfide, and indomethacin) are only partially inhibitory of CL could indicate that the second pathway, lipoxygenase, is responsible for the CL which is not inhibited. Clearly lipoxygenase can

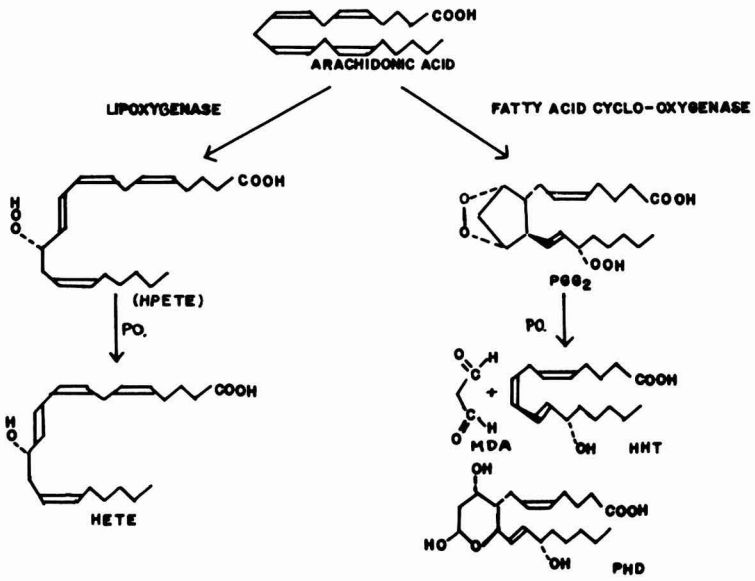


FIG. 7. The platelet metabolism of arachidonate as developed by Samuelsson *et al.* (*5*). Note the PO depicts peroxidases.

produce chemiluminescence as noted by Veldink *et al.* (*10*). When the authentic HETE and HPETE metabolites are more available, the correlations between lipoxygenase CL and these products can be made.

This assay can be performed with small amounts of blood, as little as 1 to 10 ml. This test could be valuable in neonates or those patients who are thrombocytopenic (decreased blood platelets).

This assay system may be valuable in screening certain anti-inflammatory drugs because platelets are so important in the inflammatory response. The effect of aspirin and BW755C are clear in patients not

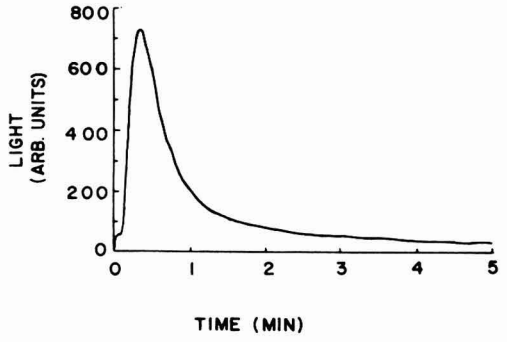


FIG. 8. Kinetic response of arachidonate-stimulated human platelets. Conditions to be noted in Addendum.

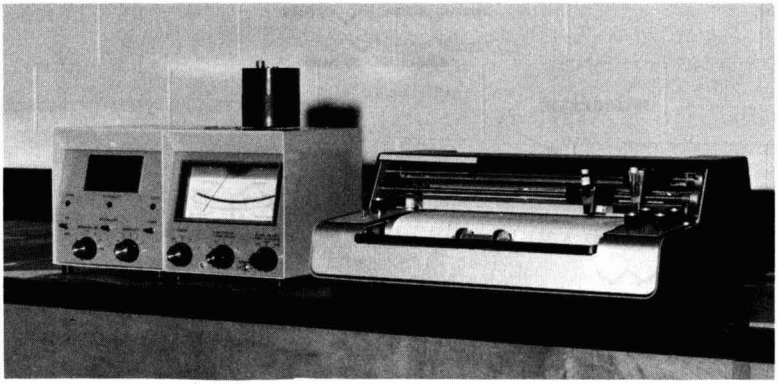


FIG. 9. Monolight 301 photometer with integrator and Houston recorder.

taking aspirin for 14 days. BW755C is inhibitory even at small doses (10^{-8} *M*) in all patients but the effects of aspirin appear to depend on the patients prior history of aspirin usage (*6*). Details of these studies will be the subject of a future manuscript.

ADDENDUM

Owing to the mechanical and electrical delays of the liquid scintillation counter, a complete kinetic response of chemiluminescence (CL) in the system described above cannot be obtained. However, an alternative approach to this problem is more successful. If the less expensive Monolight 301 photometer is used as detector, the entire CL kinetic response from arachidonate-stimulated human platelets in the presence of luminol can be seen in Fig. 8. The volumes of reactants (in microliters) are as follows:

platelets	400 at 10^8/ml
luminol	400
control solution	400
arachidonate	200

In Fig. 9 is depicted the Monolight 301 photometer with integrator and recorder. This photometer is equipped with a "fat head" which accommodates larger tubes (1.2 cm wide × 4.5 cm high) than the standard model.

SUMMARY

We describe a simple, sensitive, and reproducible assay system to measure the chemiluminescence (CL) produced by injecting arachidonic acid (AA) into a preparation of human platelets containing luminol.

The CL appears to result from the metabolism of the AA by enzymes in human platelets, namely, cyclooxygenase, lipoxygenase, and possibly peroxidases. It is believed that when

the AA is injected, free radicals and/or oxidizing agents are formed that react with the luminol producing an excited state and emitting blue light (425 nm).

The enzymes can be inhibited by drugs to varying degrees. BW755C inhibits all CL at micromolar doses and it is known to inhibit both lipoxygenase and cyclooxygenase. Aspirin, indomethacin, and sulindac sulfide inhibit only cyclooxygenase and inhibit 35–65% of the light from an individual. This assay system can be used to screen certain drugs that are effective in inflammatory diseases. It could be used to determine whether the drugs would be effective in a given individual and also whether drugs have a long-term toxic effect *in vivo* on platelets. Further the assay is practical with a few milliters of blood.

ACKNOWLEDGMENTS

We thank Dr. T. Y. Shen and co-workers at Merck Sharp & Dohme for supplying indomethacin and sulindac sulfide (Chlinoril) and Dr. John Vane at Burroughs–Wellcome for supplying the BW755C.

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MICROCHEMICAL JOURNAL 25, 524–530 (1980)

Spectrophotometric Determination of Mercury with 5-(6-Methyl-2-pyridyl)methylene-2-thiohydantoin

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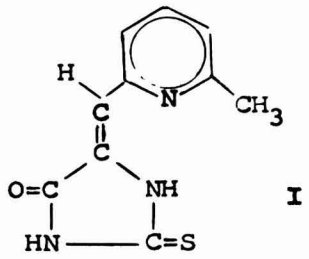
Received March 29, 1980

INTRODUCTION

Pyridine derivatives of 2-thiohydantoin may be used as possible analytical reagents for some heavy metals. A preliminary study of their reactionability was undertaken initially (*4*), and now a broad survey of the metal ion interactions of reagents has been carried out. The present paper deals with the photometric determination of mercury with 5-(6-methyl-2-pyridyl)methylene-2-thiohydantoin I. Without a doubt, dithizone is the best reagent for the spectrophotometric determination of mercury and other reagents: di-β-naphthylthiocarbazone (*8–10*), diphenylcarbazide (*3*), *p*-dimethylaminobenzilidenerhodanine (*2, 6, 7*) have been progressively substituted by dithizone. Nevertheless, the dithizone procedure is cumbersome (extraction with nonaqueous solvents, use of masking agents, etc.) and time-consuming, and it would be interesting to propose new mercury reagents which could eliminate this difficulty, although their sensibility might be lesser.



I

MATERIALS AND METHODS

Apparatus. Unicam SP800 and Unicam SP600 spectrophotometers equipped with 1.0-cm glass or quartz cells, Phillips PW9408 digital pH meter with glass-calomel electrodes, and a Perkin–Elmer 460 atomic absorption spectrophotometer were used.

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Reagents. Salts and solvents of analytical grade purity or better were used throughout.

5-(6-Methyl-2-pyridyl)methylene-2-thiohydantoin. The reagent was prepared by a modification of Wheeler and Hoffman's method. 2-Thiohydantoin (0.014 mol) was dissolved in 20 ml of 0.4 *M* sodium hydroxide and a solution of the pyrydinic aldehyde (0.014 mol) in 20 ml of ethanol was added; 5 ml of glacial acetic acid was slowly added to the mixture and the product began to appear rapidly. The mixture was heated for 5 min at 60°C, and then allowed to stand overnight at 2°C. The crystalline product was filtered off under suction, drained well, dried in a desiccator, and recrystallized from ethanol, mp 245–247°C.

Mercury(II) stock solution, 2741 $\mu g \cdot ml^{-1}$. Dissolve 4.435 g of $HgNO_3$ in 10 ml of concentrated nitric acid and dilute to 1000 ml with distilled water. This solution is standardized with EDTA by back titration with Zn(II) standard solution and black Eriocrom T as indicator. Dilute standard mercury(II) solutions are prepared by diluting this stock solution with the necessary volume of water.

Recommended procedure. Place mercury solution (125 μg) in a 25-ml standard flask. Add 5 ml of 0.1% reagent solution in ethanol, 10 ml of ethanol, 3 ml of 0.1 *N* nitric acid, 2.5 ml of 0.5 *M* potassium nitrate and dilute to the mark with distilled water. Measure the absorbance, in 1-cm cells, at 444 nm against a reagent blank.

RESULTS

Absorption spectra. Figure 1 shows the absorption spectra of the reagent and its mercury(II) complex at pH 2.5. The absorption maximum of the spectrum of the complex occurs at 430 nm. Because the blank absorbs at the absorption-maximum-complex proximity, further measures

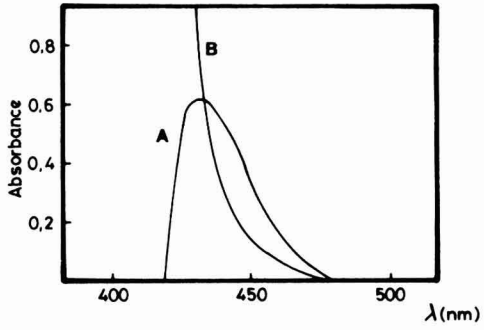

FIG. 1. Absorption spectra: A, mercury–5-(6-methyl-2-pyridyl)methylene-2-thiohydantoin complex containing 5 ppm of mercury(II) measured against reagent blank; and B, reagent blank measured against water.

will be carried out to 444 nm to avoid the influence of blank on the reproducibility of the method.

Influence of pH. The influence of the pH on the absorbance at 444 nm was examined over a pH range from 1.0 to 13.0 (Fig. 2). A maximum and constant absorbance was obtained between pH 2.1 and 2.8. Therefore, the pH was adjusted to about 2.5.

Salt and reagent concentration. The effect of the amount of KNO_3 on the absorbance was examined by varying the molar concentration of this salt (from 0.02 to 0.1), the amount of mercury being kept constant. The results showed that the absorbance of the complex was constant for all tests. The influence of reagent concentration was studied between 7.5- and 52.5-fold molar excess of the reagent. The absorbance of the complex was constant up to a molar concentration of reagent 9.0×10^{-4}; above this value the absorbance decreases. This is probably caused by the higher absorption of the blank when the concentration of reagent was increased.

Effect of time. The time necessary for the complete formation of the 5-(6-methyl-2-pyridyl)methylene-2-thiohydantoin and mercury complex was examined. When a 38-fold molar excess of reagent was added, about 10 min was sufficient for complete reaction at room temperature; and the absorbance of the complex did not change for at least 2 hr. Therefore, the measurement of the absorbance was carried out at least 15 min after having added the reagent.

Nature of the complex. Attempts to determine the composition of the complex in aqueous solution were made by continuous-variation and molar-ratio methods (Fig. 3), which revealed that mercury(II) forms two complexes, at pH 2.5, of molar ratios 1:1 and 1:2 (metal–ligand). This is in agreement with the observation of Borissova *et al.* (*1*) that *p*-dimethyl-

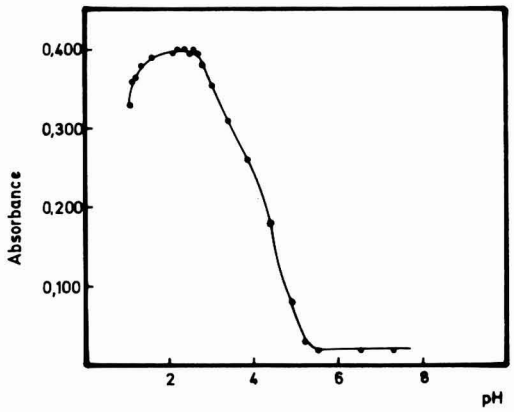

FIG. 2. Effect of pH on absorbance measured at 444 nm.

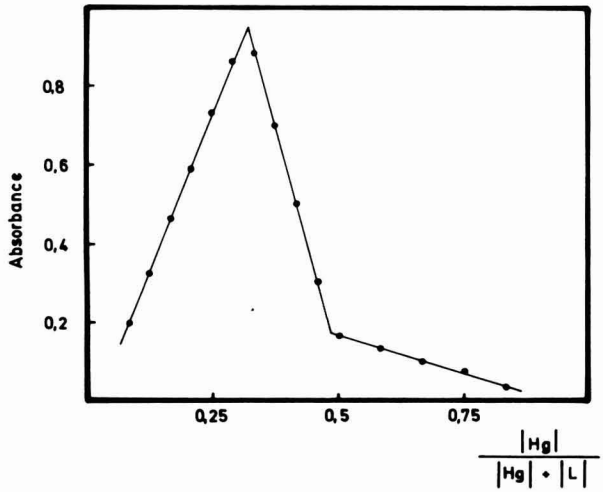

FIG. 3. Composition of mercury–reagent complexes by the continuous-variation method.

aminobenzylidenerhodanine (which is structurally related to 2-thiohydantoin derivatives) forms 1:1 and 1:2 complexes with Ag(I) and Pd(II).

As described above, the formation of mercury complex was seriously influenced by pH value. The optimum pH range was located between 2.1 and 2.8. Analogous behavior was observed for 5-(2-pyridyl)methylene-2-thiohydantoin (*4*); contrary to this, non-pyridine derivatives of 2-thiohydantoin (*5*) react well with Hg(II) in acetic acetate medium but react poorly at low pH value. In order to explain this reaction selectivity in relation to pH values, we suppose that a hydrogen bonding occurs, in pyridine derivatives, between the

$$-\overset{(1)}{\underset{|}{N}}-H$$

group of the thiohydantoin and the N atom of the pyridine ring. This bond prevents the reaction with mercury in acetic acetate medium. At a low pH value the protonation of the pyridine ring would destroy the hydrogen bonding and the reaction with mercury would be possible. We cannot support this assumption with experimental evidence, and it should only be considered as an hypothesis.

Calibration graph. The solution of the complex obeys Beer's law over a range of concentration to 2–9 ppm of mercury. The sensibility of the determination, expressed in terms of molar absorptivity at 444 nm, was 15,800 $M^{-1} \cdot cm^{-1}$.

The reproducibility of the determination under optimum conditions was established by carrying out 11 determinations on 125 μg of mercury, the amount included in the minimum error interval (Ringbom's graph). The

set of absorbance values obtained was used to evaluate the precision of the method, which, expressed in terms of relative standard deviation was found to be 0.2%.

Interferences. The effect of several possible interferences on the determination of 125 μg of mercury is shown in Table 1. It is remarkable that ions, such as Pb(II), W(VI), Cd(II), As(III), Se(IV), Ni(II), and Zn(II), do not interfere when they are present in 100 ppm concentration level. Other ions can be tolerated in amounts equal to or twice that of mercury (thus Pt(IV), Cu(II), Pd(II), Mo(VI)).

Application to the determintion of mercury in dental amalgam. Ash Globe amalgam for dental use was analyzed in order to check the validity of the method. This sample contained copper amounting to half the mercury, because this concentration level was below the interference one for this ion (Table 1). The use of a masking agent or other means to eliminate possible interference from copper was unnecessary.

The preparation of the sample solution is as follows. Transfer a suitable mass (0.044 to 0.053 g) of the amalgam into a 100-ml beaker and add 10 ml of concentrated nitric acid. Heat gently until all the metal is in solution; then evaporate almost to dryness. Dissolve the residue in 5 ml of concen-

TABLE 1
EFFECT OF DIVERSE IONS ON THE DETERMINATION OF 5 ppm OF Hg(II)

Ion added	Amount of ion added/ppm	Amount of mercury recovered/ppm	Recovery (%)
Pt(IV)	10	5.0	100
Au(III)	5	9.4	188
Ag(I)	5	6.6	132
Pb(II)	100	5.0	100
Cu(II)	10	5.0	100
W(VI)	100	5.0	100
Cd(II)	100	5.0	100
Bi(III)	5	5.6	112
As(III)	100	5.0	100
Sn(II)	5	3.0	60
Mo(VI)	5	5.0	100
Se(IV)	100	4.4	88
Fe(III)	5	4.0	80
Pd(II)	5	5.0	100
Co(II)	5	4.5	90
Ni(II)	100	4.4	88
Zn(II)	100	5.0	100
Mn(II)	5	5.0	100
Al(III)	100	5.0	100
Cr(III)	5	3.0	64
Cr(VI)	5	4.3	86

TABLE 2
DETERMINATION OF MERCURY(II) IN DENTAL AMALGAM

Sample	Mass of sample/g	Mercury content (%)		Relative error (%)
		aas[a]	Found[b]	
Ash Globe copper amalgam	0.035	70.0	74.2	+6.0
	0.044	id.	70.4	+0.5
	0.053	id.	70.1	+0.1
	0.062	id.	67.5	−3.5

[a] Atomic-absorption spectrometry.
[b] Average of six determinations.

trated nitric acid and then transfer the solution completely in a 250-ml volumetric flask and dilute with distilled water to the mark. Transfer 1.0 ml of the solution, with a pipet, into a 25-ml calibrated flask and determine the mercury as described under Recommended Procedure.

The results obtained are given in Table 2. It can be seen that the experimental results are in close agreement with atomic-absorption spectrometry ones, when the mass of sample taken is between 0.044 and 0.053 g. At this interval the mean of relative error is below 1%.

SUMMARY

A method is described for the direct spectrophotometric determination of mercury(II) with 5-(6-methyl-2-pyridyl)methylene-2-thiohydantoin. The influence of the different experimental parameters on the formation of the complex were studied and optimum conditions for the determination of mercury were established. The precision of the procedure, expressed in terms of relative standard deviation, was 0.2%. Two different complexes (1:2 and 1:1 cation−reagent stoichiometries) have been detected. The method has been tested on copper amalgam for dental use.

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Determination of Cadmium and Copper in Galvanic Coatings by Means of Atomic Absorption

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Received March 25, 1980

INTRODUCTION

The possibility of applying atomic absorption to the analysis of galvanic baths and coatings is both broad and limited. Atomic absorption can, with significant ease, determine a vast number of elements in a simple and fairly rapid way and, in certain cases, can also produce unsatisfactory sensitivity because of chemical interactions among elements or even organic compounds simultaneously occurring in galvanic baths and coatings. Measurements were made in solutions containing various salts in high concentrations and in the presence of superfluous amounts of acids, which are used to transfer alloys into solution. Mutual chemical interactions and also the means used to reduce or at least to diminish them are extremely important in the determination of different elements in various kinds of alloys.

The usual method of transferring copper alloys into solution is application of hydrochloric acid followed by addition of an oxidant (e.g., HNO_3, H_2O_2). The oxidant suppresses the anionic influence (*4*) when determining copper and the accompanying elements.

The determination of copper proceeds rather easily and few chemical interactions are observed in the air–acetylene flame. Similarly no chemical interaction in the cadmium determination is observed (*4*). Nevertheless determination of these elements in galvanic baths demands preparation of a new analytical procedure, because composition of the baths is not well known. Normally such a determination is carried out according to a standard obtained in a method of additions simultaneously present as a matrix (a background), when determining a given element.

In our previous paper (*2*) a possibility was described to electrolytically obtain a Cu–Cd alloy from an ethylenediamino electrolyte. It was also pointed out that the Cu–Cd alloys show high microdurability and resist rubbing (*1*, *3*). Thus a necessity arises for the analytical control of the amounts of copper and cadmium in galvanic baths as well as in the electrolytically obtained Cu–Cd coatings.

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An attempt was made to establish a new analytical method to determine the levels of cadmium and copper in the alloy Cu–Cd coatings and in an ethylenediamino bath for electrolytical preparation of this alloy using the atomic absorption method.

EXPERIMENTAL

Apparatus and Working Conditions

The determination of copper and cadmium was performed using the Perkin–Elmer Model 603 atomic absorption spectrophotometer. The work was accomplished using a lamp with a hollow cathode and applying parameters recommended by the manufacturers (Table 1). Analyses were run in the acetylene–air flame.

Standards

Copper. Metallic copper (1.0000 g, spectrographically standardized by Johnson–Mathay) was dissolved in a minimum volume of nitric acid (1:1, v/v) and the sample was made 1 dm^3 by adding 1% nitric acid. From that solution several standards were prepared (200 cm^3 each), containing, respectively, 1.0, 2.0, 5.0, and 10 $\mu g/cm^3$ copper.

Cadmium. Metallic cadmium (1.0000 g, spectrographically standardized by Johnson–Mathay) was dissolved in a minimum volume of hydrochloric acid (1:1, v/v) and then the sample was made 1 dm^3 by adding 1% hydrochloric acid. From that solution several standards were prepared (200 cm^3 each), containing, respectively, 1.0, 1.5, 2.0, and 5.0 $\mu g/cm^3$ cadmium.

Preparation of Samples for Analysis

The galvanic bath. To prepare samples for copper and cadmium determination in the ethylenediamino bath used for electrolytical preparation of the Cu–Cd alloy, 1 cm^3 of the bath solution and 1 cm^3 of concentrated H_2SO_4 were added and diluted with distilled water up to 100 cm^3. Sulfuric acid was added to decompose ethylenediamino complexes of copper and

TABLE 1
WORKING PARAMETERS

Element	Wavelength (nm)	Slit setting (nm)	Lamp current (mA)	Straight-line range ($\mu g\ cm^{-3}$)
Cu	324.8	4 0.7	15	5
Cd	228.8	4 0.7	4	2

cadmium. The 2- to 5-cm^3 samples of the solution were taken and after diluting to the volume of 200 cm^3, the determination of copper and cadmium was performed.

Galvanic Cu–Cd coatings. The Cu–Cd alloys were obtained in the electrode potential range of −425 to −825 mV measured toward SCE and using the No. IV electrolyte, composition of which presented in our previous work (*2*). Coatings were placed on silver monocrystals and their weights were determined each time. The mean thickness of the obtained coatings was 2–3 μm. Known quantities of the Cu–Cd coatings were dissolved in a minimum volume of nitric acid (1:3, v/v) and distilled water was added to a volume of 25 cm^3. Determination of cadmium was performed directly from that solution, and with those samples containing more cadmium the basic solution was dissolved in such a way that determinations were performed in the straight-line range of the standard curve. For the determination of copper in the basic solution a similar analytical method was performed.

Measurements

After adjusting the measuring equipment to zero using distilled water and introducing standard values into the microprocessor, standardizing of the apparatus was done for two standards from the straight-line range from the determination of a given element. The correctness of the standard curve was controlled through determination of the concentration of a given element in various standard solutions. The determination of elements was performed in the examined samples; three separate determinations were performed each time.

DISCUSSION AND CONCLUSIONS

On the basis of the performed investigations and the obtained results it was stated that the proposed working procedure of determination of copper and cadmium from galvanic baths and the alloy Cu–Cd coatings enabled analytical control of work with ethylenediamino baths as well as the evaluation of the cadmium percentage in the coatings (Table 2). With satisfactory reproducibility, it was determined that the cadmium level was not lower than 0.5%.

Application of sulfuric acid for decomposition of ethylenediamino complexes of copper and cadmium helped to limit the influence of eventual chemical interactions on the determination of these elements. Furthermore, no mutual influence of copper and cadmium on the absorbance values of the determined elements in a solution matrix was established.

The completed chemical analysis of the Cu–Cd coatings proved that the cadmium level in coatings increased with an increase of the electrode potential.

TABLE 2
RESULTS OF THE DETERMINATION OF THE Cd AND Cu LEVELS IN THE BATH AND THE ALLOY COATINGS

No.	Cu–Cd coating composition (% per weight)			Bath composition ($g\ dm^{-3}$)	
	E (mV)	Cu	Cd		
1	−425	98.02 ± 0.08	1.90 ± 0.06	Cu	Prepared
2	−475	99.41 ± 0.08	0.50 ± 0.07		15.25
3	−525	98.47 ± 0.08	1.52 ± 0.06		Determined
4	−575	97.71 ± 0.07	2.24 ± 0.05		15.21 ± 0.07
5	−625	88.62 ± 0.09	11.35 ± 0.07	Cd	Prepared
6	−675	80.29 ± 0.08	19.62 ± 0.05		11.24
7	−775	74.02 ± 0.07	25.92 ± 0.06		Determined
					11.18 ± 0.05

SUMMARY

A demand arose to establish an analytical method to determine copper and cadmium in galvanic baths and coatings, induced with the possibility of application of an ethylenediamino bath for electrolytical preparation of Cu–Cd coatings. An atomic absorption method was adjusted to determine copper and cadmium in a bath in the presence of ethylenediamine and in Cu–Cd coatings. Using the new method, it was established that cadmium levels in coatings cannot be lower than 0.5%.

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MICROCHEMICAL JOURNAL 25, 535–542 (1980)

A Sensitive Reaction for Dilute Cholesterol Determinations[1]

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Received June 12, 1980

INTRODUCTION

In a recent study a determination for cerebrospinal fluid cholesterol was described in which a dry reagent was weighed out and a sample of dilute cholesterol solution such as cerebrospinal fluid was used as the solvent to dissolve the reagent (*5*). This novel reverse concentration technique allowed a sequence of required reactions to take place which culminated in the final colored product, a quinone imine complex, that was the spectral measuring form. Although this technique resulted in a 100-fold concentration of cholesterol when compared to the recommended procedure for serum, a remaining difficulty with the determination involved the very low concentrations which one would encounter in the normal range (*6*) in cerebrospinal fluid (CSF) and the low molar absorptivity (6000 liters/mol-cm) of the colored product used to measure absorbances obtained in that normal range of cholesterol. The substitute determinant for cholesterol in this enzyme reagent system was hydrogen peroxide, and it was reacted in a horseradish peroxidase (EC 1.11.1.7) catalyzed coupling of 4-aminoantipyrene-phenol substrate used as the hydrogen donor system to form the colored complex representing the final equilibrium of the reaction. Since the normal range mean for CSF is well below 1 mg/dl (0.0259 mmol/liter) and the important free cholesterol determination is approximately 30% of the total cholesterol, a more sensitive substitute for the 4-aminoantipyrene-phenol reagent was sought. A similar reaction system adapted from a serum glucose determination (*1–3*) has been used in the determination of a choline-containing phospholipid such as lecithin in which hydrogen peroxide was generated by choline oxidase (*Arthrobacter globiformis*, EC number not assigned) from the choline formed by the action of phospholipase (EC 3.1.4.4) from cabbage on the lecithin. In that circumstance, a chlorinated and sulfonated phenol was substituted

[1] Supported in part by a grant-in-aid from the Detroit General Hospital Research Corporation. Materials generously prepared for us by Sandra Leach, Technical Specialist of Abbott Laboratories, Diagnostics Division, South Pasadena, Calif.

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for phenol. Its colored product with 4-aminoantipyrene in the peroxidase coupled step was several times as sensitive as that obtained with phenol (*7*) in this version (*1–3*) of the Trinder reaction. Therefore, it was decided to apply the reaction to the determination of total cholesterol at the levels expected to be encountered in CSF and also for serum high density lipoprotein cholesterols. The present report will describe the characteristics of the reaction as it applies to CSF total cholesterol. The use of the CSF sample as the sole diluent and the source of all of the water needed for the reaction (*5*) would still be followed here. However, the primary intent of the present study is to characterize the phenolic substitution in the reaction itself for use with extremely low concentrations of cholesterol, that is with 1–20 mg/liter (0.00259–0.05180 mmol/liter). That intent will be accomplished here by the use of known materials with and without the presence of known concentrations of several potential interfering compounds. The inclusion or omission of the cholesterol esterase from the reagent will determine whether total cholesterol or free cholesterol is to be measured. One limitation will obviously be the reliability of measurement for the lower signals obtained from the free cholesterol content of the fluids.

MATERIALS AND METHODS

Reagents. The bulk of the reagents required to carry out this study were supplied premixed by Abbott Laboratories, Diagnostics Division (820 Mission St., So. Pasadena, Calif.). This premixed material was supplied to us in a dry weighable form to contain cholesterol oxidase, EC 1.1.3.6, 167 IU/liter, cholesterol ester hydrolase, EC 3.1.1.13,117 IU/liter, horseradish peroxidase, EC 1.11.1.7, 27667 IU/liter, sodium cholate, 3 mmol/liter, 4-aminoantipyrene (4-AAP), 0.8 mmol/liter, Na_2HPO_4, 50 mmol/liter, NaH_2PO_4, 50 mmol/liter, and Carbowax-6000, 0.2 mmol/liter. When the material was weighed and dissolved with the described volume of H_2O, the pH at 25°C was 6.70 ± 0.20. This material may also contain inert ingredients as either fillers and/or enzyme stabilizers which presumably have no effect on the reaction characteristics of the reagent.

In order to conduct this study, the reagent was prepared at double strength to contain 80 mg of reagent material per milliliter of water. Sufficient 4-AAP and sodium 2-hydroxy-3,5-dichlorobenzenesulfonate (HDCBS) were added to the reagent to bring their respective concentrations to 4.8 and 18 mmol/liter. The HDCBS was sulfonated in a manner similar to that of Trinder (*3*) and was isolated as the sodium salt as previously described (*1, 2, 4*).

Procedure. As a convenience for the experiments to be described, equal portions of double-strength standard and double-strength reagent were mixed together. Therefore 250 μl of reagent and 250 μl of standard

were added and mixed. After incubation at room temperature for 15 min, the absorbance was measured against a reagent blank at 510 nm.

Alternatively, if the dry preweighed material were available already containing the appropriate amounts of 4-AAP and HDCBS, then 500 μl of sample could have been used as the solvent reagent as previously described (*5*).

RESULTS AND DISCUSSION

Some measure of the comparative sensitivity of the reaction can be derived from Fig. 1 where phenol is a reactant in one case, and the chlorinated–sulfonated derivative is the reactant under identical concentration circumstances in the other. It seems rather obvious from the difference in slope characteristics that the chloride derivative has desirable auxochromic properties. All other factors being equal, the halogenated analog system is obviously a much more suitable one (*1–3*) for the deter-

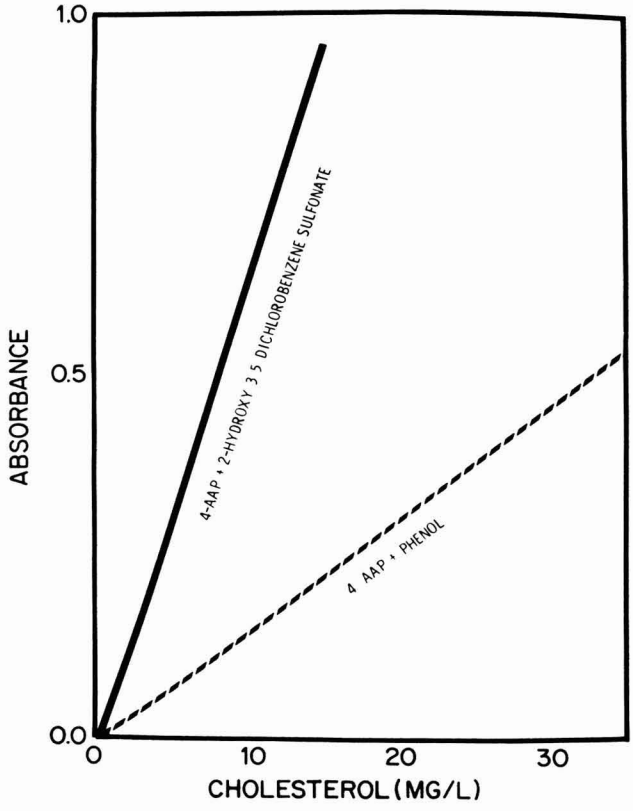

FIG. 1. Comparative sensitivity of the reaction is shown by calibration slopes. Identical concentrations of cholesterol are present in both reactions.

mination of low concentrations of cholesterol than is the original phenol system (*7*). In the absence of interfering factors which could be the protein concentration or the cations present in the sample itself, factors which are yet to be established, the increase in sensitivity over the previously proposed technique makes this reaction appear a reasonable sensitizing modification.

Because this reversed concentrating technique requires that the sample size be 100 times that normally used for serum, that is, 0.5–1.0 ml rather than 0.005–0.01 ml, it seemed necessary to rule out the effect of high concentrations of divalent cations present. A replication study on the effect of having 30 mg/liter calcium (0.75 mmol/liter) and 30 mg/liter magnesium (1.233 mmol/liter) in the cholesterol reaction was carried out and compared to a similar replication study in which no calcium and magnesium were added. Ten samples without calcium or magnesium added at a constant concentration of cholesterol showed a mean absorbance of 0.615, a range of 0.611–0.619 and a standard deviation of ±0.002. When calcium and magnesium were included, the mean absorbance for 10 replications was a similar mean of 0.614, with a range of 0.610–0.617 and a standard deviation of 0.001. It was concluded from these findings that the concentrations of calcium and magnesium one could normally encounter in cerebrospinal fluid would be insignificant as interfering compounds.

A similar consideration of protein concentration as a factor in the large sample size used requires that the effect of concentration of the protein on the reaction be known. To learn about this potential effect of human serum albumin on the cholesterol reaction a study was designed to test whether or where interference would occur. Normally, the total protein content of CSF is between 0.15 and 0.45 g/liter. Several standards varying in cholesterol concentration between 2.5 and 30 mg/liter (0.0065–0.0777 mmol/liter) with all standards containing 1 g/liter of human serum albumin were reacted as described under procedure and the results are shown as curve C in Fig. 2. A linear response was obtained across the entire range tested but only the critical concentration range absorbances between 0 and 10 mg/liter (0–0.0259 mmol/liter) are shown as curve C. The absorbances obtained in this case are, within experimental error, identical to those expected had the albumin been left out of the reaction mixture.

To complete the picture, a second experiment was carried out in which the cholesterol concentration was kept constant at 10 mg/liter (0.0259 mmol/liter) while the protein concentration was varied across 0–10 g/liter. The results are shown graphically as curve A of Fig. 2. No variation occurred until 2.5 g/liter was exceeded and at 5.0 g/liter an absorbance 98% of the expected plateau was obtained. However, when 10 g/liter of human serum albumin was present only 95% of the expected value was obtained. Since both are extremely high CSF protein values while the

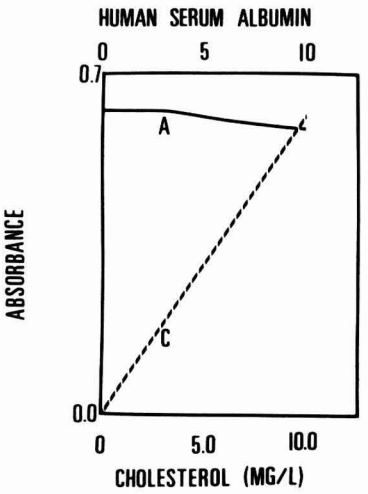

FIG. 2. Curve C shows absorbances obtained using a constant human serum albumin concentration (1 g/liter) with a series of cholesterol standards of 0–10 mg/liter, (0–0.0259 mmol/liter). Curve A shows absorbances obtained with variable albumin concentrations (0–10 g/liter) and a constant cholesterol concentration of 10 mg/liter (0.0259) mmol/liter).

error is relatively small, it would seem that the procedure would be functional in both normal as well as pathological circumstances. When one also considers the high absorbance signals one can achieve with even normal CSF specimens which certainly contain very low concentrations of cholesterol at but a few micrograms of cholesterol in the aliquot of test sample used, then the procedure seems quite attractive analytically.

An experiment to show how the interference of bilirubin can be demonstrated for the peroxidase–hydrogen peroxide-coupled step involving the oxidative reaction on 4-AAP and HDCBS is shown in Figs. 3 and 4. The concentration of cholesterol tested was a normal value for serum total cholesterol or a moderately high CSF cholesterol. The interference to be demonstrated was expected to be more striking than it would be if phenol were present in place of its auxochrome derivative, HDCBS, because of the marked differences between the molar absorptivities of the chromogens formed on using the two phenolic compounds and the peak maximum of the conjugate which is displaced bathochromically to 510 nm instead of 500 nm as with phenol itself.

In Fig. 3, the potential effect derived from this interfering molecule can be demonstrated. Curves A and B are the spectra for cholesterol and bilirubin when each is put into the cholesterol reagent solution. Cholesterol is reactive here and generates the chromogen involving HDCBS and 4-AAP while the bilirubin spectrum is derived entirely from its natural chromophoric character because no oxidase action occurs here and no

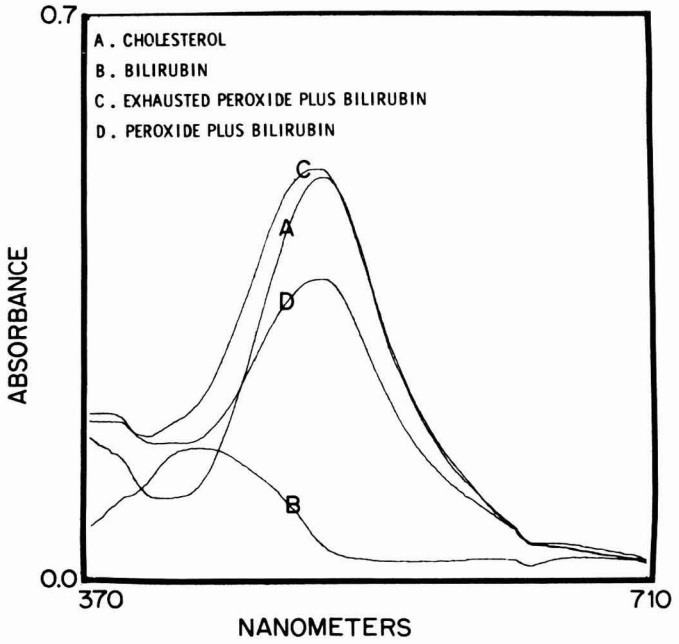

FIG. 3. Spectra A and B are those spectra obtained with individually reacted cholesterol and bilirubin (in albumin) solutions. Spectrum C is that of a mixture where H_2O_2 was exhausted before bilirubin was added. Spectrum D is the result obtained with a mixture of bilirubin and cholesterol reacted at the same time.

peroxide is generated for the action of the peroxidase-coupled indicator reaction. If one now adds the same concentration of bilirubin to the hydrogen peroxide-exhausted cholesterol reaction, spectrum C is obtained. This is an ideal and true summation effect in which a small increase in the absorbance of the hypsochromically shifted peak is shown. However, if bilirubin were present in the sample at the same time that oxidase action produces hydrogen peroxide, then an entirely different spectral picture would present. This is shown as spectrum D where the peak maximum value is severely diminished due to the competitive action of bilirubin substituting in part as a hydrogen donor for the HDCBS and 4-AAP mixture. This is a complicated summation spectrum because it may include the oxidatively coupled reaction product of 4-AAP and HDCBS, some residual bilirubin and also whatever compound is formed from the remaining bilirubin which has been oxidized.

An interesting additional bit of information can also be derived from the difference spectra of Fig. 4. If curve B is subtracted spectrophotometrically from curve C, the summation spectrum of Fig. 3, then spectrum E is obtained and this is a correct spectrum for what should be obtained for

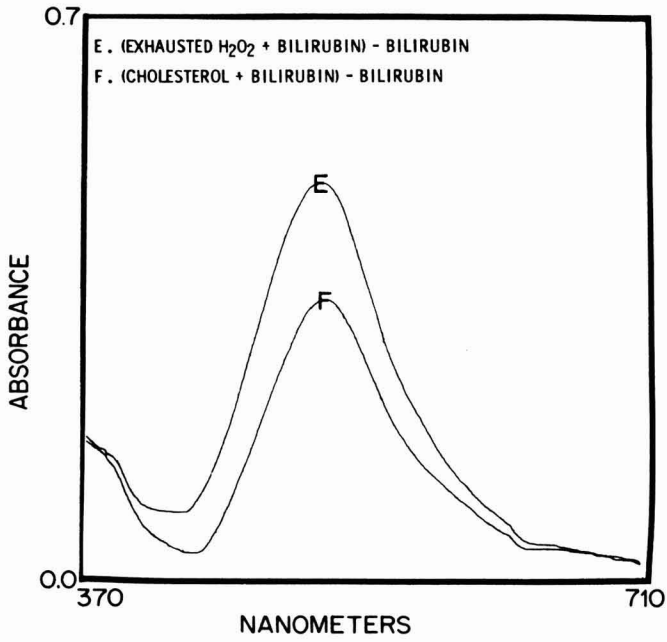

FIG. 4. Spectrum E is the residuum obtained when a bilirubin spectrum is subtracted spectrophotometrically from spectrum C while spectrum F results when spectrum B is subtracted from spectrum D.

cholesterol. In fact it virtually superimposes spectrum A of Fig. 3. But if curve B is subtracted from an interaction spectrum represented by spectrum D then the lower reading spectrum of curve F is obtained. If one considers the true value to be spectrum E, then spectrum F falls short by a considerable quantity, in this case, 30%. In the relatively rare case that bilirubin is present, it is recommended that an equivalent volume of CSF be extracted of its cholesterol and that the evaporated residue of the extract be treated with an appropriate volume of the weighed-out reagent dissolved in distilled water according to the weight of reagent used. It may help to add a small volume of dissolving solution such as isopropanol to the residue before adding the reagent.

SUMMARY

A sensitive reaction for the peroxidase-coupled sequence of the determination of a dilute total cholesterol mixture of free and esterified forms is described. Substitution of a chlorinated auxochrome of phenol made water soluble by sulfonation through a synthetic procedure previously described created a severalfold enhancement factor which magnified considerably the sensitivity of one equilibrium reaction over the other. This enabled the determination of dilute cholesterol solutions to be carried out with high absorbance signals across the range of 0.0–10.0 mg/liter (0–0.0259 mmol/liter) of cholesterol, a range which includes

and exceeds the normal values one would encounter in dilute solutions such as cerebrospinal fluid. Important potential interference factors including increased protein concentrations and calcium and magnesium were considered and studied. The formidable interacting compound, bilirubin, which is competitive with the 4-aminoantipyrene and sodium 2-hydroxy-3,5-dichlorobenzenesulfonate mixture in the peroxidase sequence was also studied, its interference characteristics were determined and an alternative methodology suggested for obviating this perturbing effect. It is believed that the simple substitution of the auxochrome derivative is a useful contribution not only here but in studies presently ongoing involving needed sensitivity for cholesterol fractions which are considerably lower than the serum total cholesterol concentrations most commonly determined.

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MICROCHEMICAL JOURNAL 25, 543–547 (1980)

The Determination of Trace Amounts of Chlorophenols by High-Performance Liquid Chromatography

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Received April 5, 1980

INTRODUCTION

Chlorophenols are widely used in industry and agriculture. Pentachlorophenol, for example, has extensive use as a herbicide, fungicide, and insecticide, particularly for the preservation of wood and wood products and for slime control. Considerable amounts of chlorophenols are said to be discharged, as industrial effluent, into water systems (*5*). Because of the toxicity of these substances, which resist degradation and tend to persist in the environment, such discharges can be hazardous and create serious public health and water quality problems (*4*), even in small amounts. Pentachlorophenol, for example, is fatal to the more sensitive species of fish in concentrations as low as 0.2 ppm (*8*). A number of methods have been developed for the determination of chlorophenols, but all of them possess some disadvantages. The colorimetric method is not selective, as it measures total phenols; thin-layer chromatography is difficult to quantitate, and gas–liquid chromatography, although a very sensitive method is time-consuming, because of the concentration and derivation steps involved. They may be determined directly by gas–liquid chromatography with electron capture detection, using specially prepared columns (*10*), but difficulties arise due to the suitability of the packing material. Gee *et al.* (*7*) and Parr *et al.* (*12*) determined trace amounts of chlorophenols in chicken flesh and wood shavings, respectively: the chlorophenols were converted to the corresponding ethyl esters. However, the ethyl esters were not resolved completely from interfering coextractants. Procedures involving acylation (*2, 3, 14, 16*) have encountered similar problems. During the past decade, much attention has been focused on gas chromatographic methods and many derivatization methods have been reported in the literature for the chlorophenols (*1, 6, 9, 11, 15*).

The aim of the present work was to develop a method for the separation of chlorophenols, without the necessity for derivatization. In this paper, we report the application of high-performance liquid chromatography

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(HPLC) to the direct analysis of a range of chlorophenols in water. The method is simple, rapid, reproducible, and quantitative.

EXPERIMENTAL METHODS

Chemicals and apparatus. Pentachlorophenol, 2,3,5,6-tetrachlorophenol, 2,3,4,5-tetrachlorophenol, 2,4,5-trichlorophenol, 2,3,4-trichlorophenol, 2,3,5-trichlorophenol, and 3,4,5-trichlorophenol were obtained from the Aldrich Chemical Company.

2,4,6-Trichlorophenol was obtained from T.C.I. Kasei. All were recrystallized before use. All other reagents used were of A.R. grade. Petroleum spirit (40–60°C), specially purified for HPLC, was obtained from Ajax Chemicals.

The HPLC apparatus was a Hitachi Differential Refractometer Model 110/10 and was used with a reciprocating pump from Accurex (Model 110/A) (Melbourne, Australia). The sample injection valve was supplied by Sidamp, fitted with a 20-μl loop. The analytical column was 15 cm long, made from a seamless, stainless-steel tube 6.35 mm o.d. × 4.6 mm i.d. obtained from Chromatographic Systems (Melbourne, Australia). A precolumn was also used. This was 5 cm long, made from a seamless, stainless-steel tube 6.35 mm o.d. × 4.6 mm i.d., obtained from Chromatographic Systems.

Preliminary investigations. Investigations were carried out to determine the type of column to achieve optimal separation, and the most suitable mobile phase. Conditions were examined for eight chlorophenols: 2,4,6-tri-, 2,3,5,6-tetra-, penta-, 2,3,5-tri-, 2,4,5-tri-, 2,3,4,5-tetra-, 2,3,4-tri-, and 3,4,5-trichlorophenol. A nitrile column was found to be very suitable. Initial work on mobile phases began with the use of *n*-hexane containing varying amounts of methanol. The best mixture of this composition was one containing 1% methanol. Ultimately, however, it was found that petroleum spirit (40–60°C) containing 1% methanol was at least as effective as the *n*-hexane–methanol mixture. For the extraction of the chlorophenols from aqueous solution, methylene chloride was found to be suitable and was used initially. However, this was later replaced by diethyl ether, which facilitated more rapid evaporation.

Recoveries for all the chlorophenols investigated were greater than 90%, except for pentachlorophenol (>80%) and 2,3,5-trichlorophenol (~70%).

Optimal conditions for the separation of the chlorophenols may be summarized as follows:

Column	15 cm S 5 CN; SS: i.d., 4.6 mm, o.d., 6.35 mm; wavelength (λ) = 215 nm
Solvent	1% methanol in petroleum spirit (40–60°C)

Flow rate	1 ml/min
Injection size	20 μl
Temperature	ambient
Chart speed	5 mm/min
Injection time	20 sec

Procedure. Aliquots (50 ml) of samples of water containing 1 μg/ml of the eight chlorophenols were extracted five times with 50 ml of diethyl ether (with smaller volumes of extractant there is a tendency to emulsion formation), the extracts combined and dried over anhydrous sodium sulphate. The organic solvent was carefully evaporated to dryness using a rotary evaporator at low temperature and the mixture of chlorophenols was taken up in 50 ml of 1% methanol–petroleum spirit. A 20-μl aliquot was injected directly onto the column. Complete separation of the chlorophenols was achieved in about 25 min. Retention times for each of the chlorophenols are shown in Table 1. A typical separation of the eight is shown in Fig. 1.

Calibration curve. A calibration curve was prepared for each chlorophenol. A straight line relationship was found to exist for concentrations in the range 0.1 to 1 ppm. The smallest amount detectable using the procedure was 0.02 μg/ml.

Unknowns. A number of unknowns containing 0.1–10 ppm of chlorophenol was analyzed by one of us (Z.I.) using the following procedure. A solution containing all eight chlorophenols was injected onto the column and their retention times noted. An unknown mixture was then injected onto the column and after separation their retention times were checked against the standards. By this means the chlorophenols present in the mixture and their concentration were determined. Retention times for two of the unknown mixtures are shown in Table 2.

TABLE 1
RETENTION TIMES FOR CHLOROPHENOLS

Compound	Retention time (min)
Pentachlorophenol	11.9
2,3,5,6-Tetrachlorophenol	10.9
2,3,4,5-Tetrachlorophenol	17.2
2,3,5-Trichlorophenol	14.9
2,4,5-Trichlorophenol	16.2
2,4,6-Trichlorophenol	10.1
2,3,4-Trichlorophenol	18.1
3,4,5-Trichlorophenol	24.9

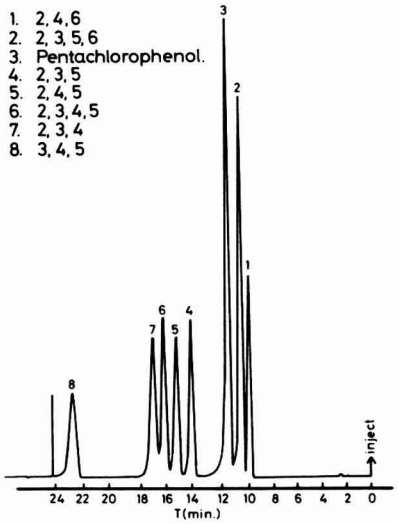

FIG. 1. The separation of chlorophenols by HPLC.

DISCUSSION

While separation and determination of the chlorophenols is very satisfactory, the extraction of pentachlorophenol and 2,3,5-trichlorophenol from aqueous samples was not as efficient as that for the other chlorophenols. The extraction process may be replaced by steam distillation which, if slower, is very successful: recently Realini and Burce (*13*) have claimed that excellent results for the extraction may be achieved by acidifying the aqueous samples and extracting with methylene chloride. These authors used a reverse phase column and acetonitrile as eluent to separate the following phenols: phenol, *p*-nitrophenol, *o*-dichlorophenol, 2,4-dinitrophenol, *o*-nitrophenol, 2,4-dimethylphenol, 4-chloro-*m*-cresol, 2,4-dinitrophenol, 4,6-dinitro-*o*-cresol, 2,4,6-trichlorophenol, pentachlorophenol.

TABLE 2
RETENTION TIMES FOR THE UNKNOWN MIXTURES

Mixture 1	Retention time (min)	Mixture 2	Retention time (min)
2,3,5,6-Tetrachlorophenol	10.9	2,3,5,6-Tetrachlorophenol	10.9
Pentachlorophenol	11.8	2,4,6-Trichlorophenol	10.0
2,4,6-Trichlorophenol	10.0	2,3,5-Trichlorophenol	14.8
		2,4,5-Trichlorophenol	16.2

SUMMARY

A method is given for the separation and determination of eight chlorophenols using HPLC. The chlorophenols after extraction from aqueous solution by means of diethyl ether are taken up in a 1% methanol–petroleum spirit mixture and injected onto the column. Separation of a mixture of all eight chlorophenols can be achieved in 25 min and a linear relationship exists for each chlorophenol for concentrations in the range 0.1–1.0 ppm.

ACKNOWLEDGMENTS

Special thanks to Mr. D. Gunew for his help and guidance throughout this work and to Dr. Markovec for the use of the HPLC equipment.

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Spectrophotometric Determination of Molybdenum with Lobeline

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Received May 6, 1980

INTRODUCTION

The spectrophotometric determination of molybdenum by the thiocyanate has been frequently investigated in several modifications (*4, 6, 7*). This method is not, however, sufficiently selective which makes it especially difficult to determine molybdenum in the presence of rhenium.

In this paper a spectrophotometric method for the determination of a molybdenum complex with lobeline is described. Using this method it is possible to determine molybdenum in the presence of a 500-fold greater quantity of rhenium.

EXPERIMENTAL

Reagents

Molybdenum solution (1 mg/ml). Sodium molybdate dehydrate (2.522 g) was dissolved in 1 liter of water. The solution was standardized by the oxine method (*4*). Working solutions of molybdenum were prepared by dilution.

Lobeline solution (0.1 M). Lobeline hydrochloride (0.2739 g) was dissolved by heating in 100 ml of water.

Ammonium thiocyanate solution (5 M). Dry salt (38.06 g) was dissolved in water to give a 5 *M* solution.

Solutions of various elements. Standard stock solutions of other elements (10 mg/ml) were prepared by dissolving their salts in water.

Procedure

A 5-ml aliquot of the 1 *M* HCl sample solution containing max 100 μg of molybdenum (VI) with solid hydrazine sulfate was boiled in a 100-ml cover beaker for 2 min. After cooling, the solution was transferred to a separatory funnel containing 4 ml of ammonium thiocyanate solution, 6.5 ml of 10 *M* HCl, 5 ml of 0.1 *M* lobeline, and sufficient water to make up the final volume. Four extractions were then made using 5 ml of $CHCl_3$ for each one. The combined chloroform phases were filtered into a 25-ml measuring flask and the absorbance at 465 nm was measured against the reagent blank using a Specol Zeiss spectrophotometer and 1-cm cells.

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Preparation of the solid complex. The extracted complex was precipitated by the addition of 50 ml of 0.01 *M* molybdenum (V) solution of 30 ml 10 *M* HCl and 10 ml of 5 *M* NH_4SCN to the concentrated solution of lobeline in chloroform. The red precipitate obtained was dried over calcium chloride and analyzed. The analytical data were consistent with the formula $MoO/NCS/_5/Hlobeline/_2$.

Calcd: 17.22% Mo; 28.13% SCN; 54.64% lobeline. Found: 17.09% Mo; 27.89% SCN; 53.96% lobeline.

Analysis of carbon, hydrogen, nitrogen, and sulfur were carried out by the standard microanalytical procedures. Molybdenum was determined after decomposition of the complex.

RESULTS AND DISCUSSION

Molybdenum forms a complex with thiocyanate and lobeline in a hydrochloric acid solution which can be extracted with chloroform as an orange solution. This complex possesses maximum absorbance at 465 nm (Fig. 1). The optimum conditions giving maximum absorbance are 0.3–0.8 *M*/liter ammonium thiocyanate, 1–5 *M*/liter hydrochloric acid, and more than 0.03 *M*/liter lobeline in the aqueous phase. Four extractions of 5 ml $CHCl_3$ each give complete extraction of the colored species. Least-squares analysis of the linear part of the calibration curve gives the equation $A = 0.143x + 0.040$ (*5*). The molar absorptivity with a standard deviation is $\epsilon = 1.38 \times 10^4$ mol$^{-1}\cdot$cm$^{-1}\cdot$liter, $s_\epsilon = 0.01 \times 10^4$. The value and standard deviation of the reagent blank absorbance is $\bar{A}_0 = 0.040$, $s_0 = 0.005$. The limit determination is from $x = 0.13$ to 4 μg/ml.

Determination of the Composition

The ratio of molybdenum to the thiocyanate and lobeline ligand was determined by the Bent French (*2*) method. The results obtained show that the molar ratio of Mo:NCS:lobeline was 1:5:2.

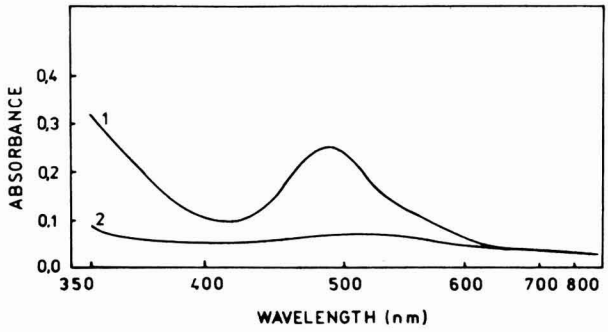

FIG. 1. Absorption spectra of Mo–lobeline complex. Chloroform extract: 1, $C_{Mo} = 1.7 \times 10^{-5}$ *M*, $C_{NH_4SCN} = 0.5$ *M*, $C_{Lob} = 0.04$ *M*; 2, reagent blank.

The ir spectra of the complex gave a strong band at 2065 cm^{-1}/ $\nu C-N$/, a weak band at 480 cm^{-1} (σ N−C−S), and a medium double band between 980 and 960 cm^{-1} (Mo−O) which indicates that molybdenum is bonded through nitrogen (*3*) and that the complex contains Mo=O group (*1*). The absence of the double bands between 2000 and 2150 cm^{-1} in the ir spectra of the chloroform extracts shows that all the thiocyanate ions are bonded through molybdenum.

The Influence of Diverse Ions

It was found that in this method that fluoride, chloride, sulfate, acetate, oxalate, and tartrate did not interfere in 10,000-fold quantities with respect to molybdenum. Ammonium, potassium, sodium, magnesium, calcium, barium, aluminium, and nickel(II) were tolerated in 1000-fold quantities. Tin(II), lead(II), antimony(V), bismuth(III), chromium(II), citrate, and perchlorate could be present in 250-fold quantities and vanadium(V), palladium(II), cerium(IV), niobium(V), and iron(III), in 100-fold quantities. Copper(II), titanium(IV), cobalt(II), and tungsten(VI) must be separated. When the procedure described above is used, up to 500-fold quantities of rhenium do not interfere with the determinations. The criterion for the significance of the interference of foreign ions was taken to be ±2% relative error.

SUMMARY

Lobeline hydrochloride has been tested as a reagent for molybdenum(V). Molybdenum(VI) is reduced with hydrazine sulfate in hydrochloric acid solution to Mo(V) only and complexed with thiocyanate and lobeline which is extracted with chloroform. In the paper experimental conditions for the formation of the ion pair of lobeline with molybdenum thiocyanate are described and the composition of this complex is given.

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Study of the Electrochemical Oxidation of Phenothiazine Derivatives in Acetonitrile Medium: The Effect of the Structure on the Voltammetric Behavior

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Received May 6, 1980

INTRODUCTION

In the past much attention has been paid to the study of phenothiazine derivatives, largely because of their pharmacological interest. Recently the structure of phenothiazine derivatives and their oxidation products as well as the mechanism of the oxidation process have been closely examined (*8, 10, 13*).

As the electrochemical behavior of phenothiazine and its substitution products is rather complicated in aqueous medium the use of nonaqueous media is preferable. In this connection reference should be made to Billon's investigations (*2, 3*). This author studied the oxidation of phenothiazine and a number of its methyl derivatives in acetonitrile (AN), acidified with perchloric acid. EPR spectroscopy was used for the detection of radical cations. Tacussel and Fombon (*12*) investigated the kinetics of this process by means of the ring-disc electrode. Further the more recent publication in this field by Cauquis *et al.* (*5*) should be mentioned.

The present publication is particularly centered on the effect of substituents in the 2 and 10 position on the electrochemical behavior in AN, so far not published in literature. A more subsidiary aim was looking for possibilities with respect to the electrochemical determination of this class of medicines. In addition to the study of these derivatives, we also studied structurally more complicated phenothiazine pharmaceuticals. The results obtained do not lie within the framework of this work and will be published later.

EXPERIMENTAL

Materials

Acetonitrile (AN). Reagent grade AN (Merck or Mallinckrodt) was purified by a method proposed by Walter and Ramaleye (*14*). Its purity

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was controlled by uv spectrofotometry. Its water content was below $3 \times 10^{-3}\%$, estimated by K. Fischer titration.

Sodium perchlorate. This was prepared by the method of Biedermann (*1*), dried at 150°C for 24 hr and stored *in vacuo* over P_2O_5.

Perchloric acid 70%. The commercial product analytical grade (C. Erba) was used as such.

Silver nitrate. The reagent grade salt was dried at 120°C and stored over P_2O_5.

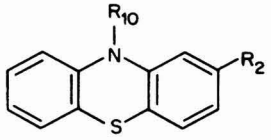
Tetrabutylammonium hydroxide. The aqueous 40% solution, analytical grade (BDH) was used as such.

Phenothiazine and a number of derivatives were commercially available (Aldrich). Both substitution products in the −10 position were synthetized at the Pharmaceutical faculty, Charles University. The purity of the derivatives was controlled by measuring their melting points and the content of the substances by titration with $HClO_4$ in anhydrous acetic acid (*4*). The structure of both synthetized products has been established by means of ir spectroscopy. A list of the substances studied has been presented in Table 1.

Instrumentation and Procedure

A Radiometer PO 4 was used for recording the dc voltammograms. Cyclic voltammetry (CV) was performed with the Universal modular polarograph E-310 from Brucker. The voltammograms displayed on the

TABLE 1
SURVEY OF THE STUDIED PHENOTHIAZINE DERIVATIVES



Compound	R_2	R_{10}
Phenothiazine	−H	−H
Group I		
2-Methoxyphenothiazine	$-OCH_3$	−H
2-Chlorophenothiazine	−Cl	−H
2-Acetylphenothiazine	$-COCH_3$	−H
2-Trifluoromethylphenothiazine	$-CF_3$	−H
Group II		
10-Isobutylphenothiazine	−H	$-CH_2CH\langle^{CH_3}_{CH_3}$
10-Propylphenothiazine	−H	$-CH_2CH_2CH_3$

screen of the osciloscope, Hewlett–Packard 1201 A, were photographed with the Polaroid camera 198 A from the same manufacturer.

A rotating platinum disc electrode (RDE) served as indicating electrode. The manufacture of the RDE used for dc measurements has been described elsewhere (*11*). For the cyclic measurements a Tacussel electrode was used.

The reference electrode was a silver sheet, dipping into a solution, being 0.01 *M* $AgNO_3$ and 1.0 *M* $NaClO_4$ in AN.

The auxiliary electrode was a spiralized platinum wire; a similar electrode was used as a generating electrode in coulometric experiments.

The electrolytic cell has been described previously (*9*). Its working compartment was filled with 0.1 *M* $NaClO_4$ in AN, to which was added the substance under investigation. The intermediate compartment contained a solution of 0.5 *M* $NaClO_4$ in AN. The resistance of the system was measured using a CDM conductoscope from Radiometer. Potentiometric coulometry enabled the determination of the number of electrons exchanged.

The diffusion coefficients were determined on the basis of Levich's equation (*11*) from the slope of the limiting diffusion current dependence on various rates of rotation of the electrode. The viscosity of a 0.1 *M* $NaClO_4$ solution in AN has to be known.

The polarographic vessel was thermostated during measurements.

The data were treated statistically. The standard deviation is given by the relationship.

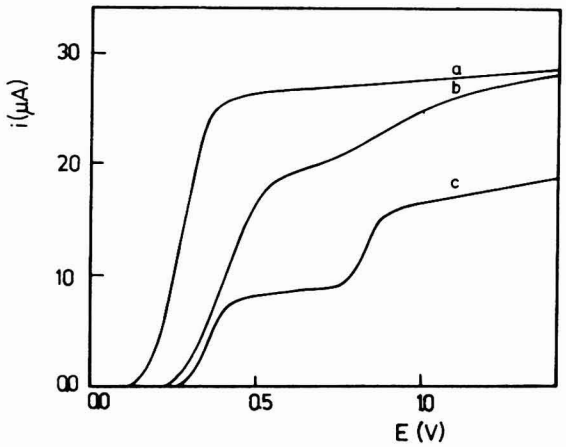


FIG. 1. Diffusion current recording of the anodic oxidation of 2-chlorophenothiazine ($c = 1 \times 10^{-4} M$) in medium: (a) 0.1 *M* $NaClO_4$ in anhydrous AN; (b) 0.1 *M* $NaClO_4$ in AN with $c_{HClO_4} = 1 \times 10^{-3} M$; (c) 0.1 *M* $NaClO_4$ in AN with $c_{HClO_4} = 1 M$. t = 20°C, electrode area (A) is 0.11 cm^2, 1226 rpm.

TABLE 2

THE EFFECT OF $HClO_4$ IN CH_3CN ON THE ANODIC BEHAVIOR OF PHENOTHIAZINE AND ON THE GROUP I PHENOTHIAZINE DERIVATIVES

c_{HClO_4} (M)	i_d^1 ($\times 10^6$A)	$E_{1/2}^1$ (V)	i_d^2 ($\times 10^6$A)	$E_{1/2}^2$ (V)	k_1^{-1a} (mV)	k_2^{-1} (mV)	i_d^1 ($\times 10^6$A)	$E_{1/2}^1$ (V)	i_d^2 ($\times 10^6$A)	$E_{1/2}^2$ (V)	k_1^{-1} (mV)	k_2^{-1} (mV)
	Phenothiazine $c = 9.8 \times 10^5 M$						2-Methoxyphenothiazine $c = 9.8 \times 10^5 M$					
—	23.20	0.24	—	—	60	—	26.40	0.18	—	—	55	—
0.02	12.05	0.29	10.40	0.67	60	63	12.20	0.21	9.60	0.72	58	59
0.11	11.55	0.29	10.00	0.74	58	63	11.60	0.21	9.70	0.74	57	57
0.54	9.60	0.29	9.30	0.77	59	59	8.80	0.21	8.40	0.75	60	61
1.04	7.20	0.29	7.32	0.79	59	59	6.40	0.20	6.80	0.76	60	58
1.49	5.76	0.29	5.76	0.82	57	55	4.20	0.20	5.20	0.77	61	61
$s_{ci_d}(\times 10^6)$	0.24		0.29				0.28		0.20			
$s_{cE_{1/2}}$		0.00		0.03				0.00		0.01		
	2-Chlorophenothiazine $c = 1.10 \times 10^5 M$						2-Acetylphenothiazine $c = 1.10 \times 10^5 M$					
—	25.40	0.27	—	—	56	—	23.35	0.28	—	—	59	—
0.02	12.40	0.36	10.00	0.72	56	60	11.40	0.38	8.80	0.77	58	62
0.11	12.10	0.36	10.00	0.75	58	59	11.00	0.38	8.70	0.82	58	60
0.54	10.00	0.35	8.40	0.81	60	56	9.20	0.37	7.60	0.83	59	58
1.04	8.00	0.34	6.80	0.82	57	56	7.20	0.37	6.20	0.84	59	58
1.49	6.60	0.33	5.70	0.83	56	56	5.40	0.37	5.20	0.85	60	57
$s_{ci_d}(\times 10^6)$	0.24		0.20				0.04		0.10			
$s_{cE_{1/2}}$		0.00		0.02				0.00		0.02		

2-Trifluoromethylphenothiazine $c = 9.8 \times 10^5 M$						
—	22.1	0.33	—	—	53	—
0.02	10.1	0.41	8.88	0.70	56	70
0.11	10.1	0.42	8.50	0.74	58	62
0.54	9.0	0.42	7.38	0.82	59	56
1.04	7.3	0.41	6.06	0.84	59	58
1.49	5.9	0.40	5.04	0.85	56	56
$s_{ci_d}(\times 10^6)$	0.2		0.10			
$s_{cE^{1/2}}$		0.01		0.03		

Note. Electrode area (A) is 0.11 cm², $t = 20 \pm 0.1$°C, 1226 rpm.

[a] k is the slope of the semilogarithmic analysis.

$$s_x = [(\sum x_i^2 - (\sum x_i)^2/n)/(n - 1)]^{1/2}.$$

The standard deviation of the linear regresion is given by the relationship:

$$s_{x,y} = [\sum (y_i - Y_i)^2/(n - 2)]^{1/2}.$$

RESULTS

As it has been observed that the derivatives in the 2 position closely follow the behavior of phenothiazine; they are treated together as group I. The substances with a substituent in the 10 position behave distinctly otherwise, and have been classified as group II.

Group I

In anhydrous AN the representatives of this group produce a single dc wave (Fig. 1, curve a). The calibration curves are linear within the concentration range $(4-19) \times 10^{-5}$ *M*. The relative standard deviation of their slopes is 0.4×10^{-6} A. The half-wave potential values can be considered to be independent on the concentration, the standard deviation from the mean value amounting ca. 0.01 V. The semilogarithmic analysis of the wave gives evidence for assuming a one-electron exchange (Table 2). However, the potentiostatic coulometric experiment incontestably yields $n = 2$. This apparent discrepancy will be discussed later.

Addition of water in very low concentrations has no influence, but larger amounts result in a decrease of the wave height and a negative shift of the half-wave potential. Of the substances investigated 2-methoxyphenothiazine appeared the most sensitive to the presence of water; interference occurs already above a water content above 0.01% (v/v). This limit was a little higher for the other substances.

The combined influence of water and acid has also been examined. It was found that for acid concentrations above 5×10^{-3} *M* wave splitting occurs. This effect has been studied at concentrations up to 1.49 *M* $HClO_4$ (Table 2, Figs. 1b, c, 2, and 3). Proportionality between wave height and concentration remains maintained. For the acid range investigated, the half-wave potential values, especially that of the first wave, are almost invariable when changing the acid concentration. The position of the first step is slightly shifted to the positive if compared with the voltammograms recorded in water and acid-free media. In acidic AN medium the further presence of water produces a decrease in the values of the limiting diffusion current for both waves and a decrease in the half-wave potential value. The values of the half-wave potential in the first oxidation step remain constant after the initial decrease.

Potentiostatic coulometry proves the removal of one electron in the first step for acid concentrations between 0.1 and 1.0 *M;* the slope of the semilogarithmic plot is in agreement herewith.

The reversibility was studied by means of CV. In the concentration

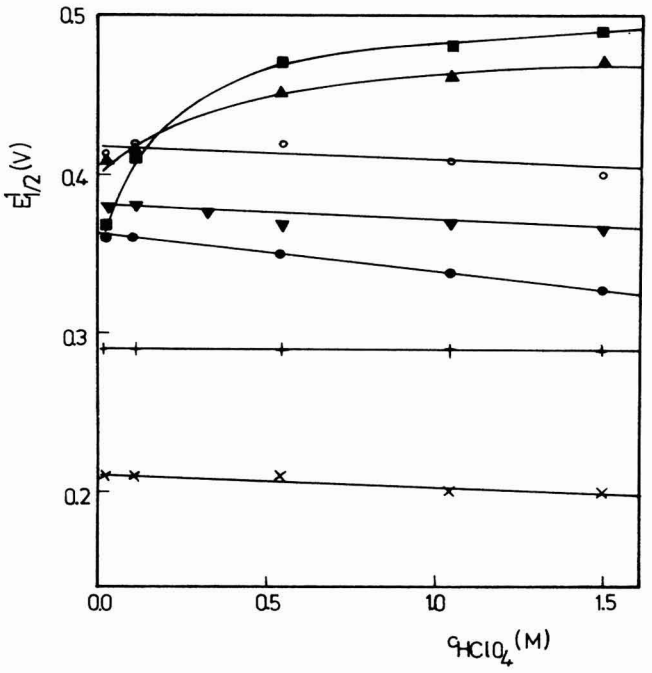


FIG. 2. Dependence of the half-wave potential values of the first step in the oxidation, $E^1_{1/2}$ (values are taken from Tables 2 and 4), on the concentration of $HClO_4$ in an AN solution of 0.1 *M* $NaClO_4$ at 20 ± 0.1°C. +, phenothiazine; ×, 2-methoxyphenothiazine; ●, 2-chlorophenothiazine, ▼, 2-acetylphenothiazine; ○, 2-trifluoromethylphenothiazine; ▲, 10-propylphenothiazine; ■, 10-isobutylphenothiazine.

range between 0.02 and 0.5 *M* $HClO_4$, the first oxidation step is reversible and the second one quasi-reversible. It appeared that at 0.02 *M* $HClO_4$ the ratio of the peak currents, i_{pA}/i_{pC}, approaches one for the first step (Table 3); for the second step this ratio exceeds one. In the concentration range 0.7–1.3 *M* $HClO_4$ both oxidation steps are reversible. Figure 4 shows a typical voltammogram.

Addition of tetrabutylammonium hydroxide makes the solution almost unsuitable for analytical applications. Above a concentration of 10^{-4} *M* poor reproduceability of the recordings has been observed. Moreover the first wave height decreases in favor of the second one. The half-wave potential of the first step practically does not change whereas that of the second step decreases with increasing alkalinity.

Group II

Unlike the substances of group I, these derivatives show a double wave in anhydrous AN. Reversibility of the first step and irreversibility of the second one have been assessed by means of CV (Fig. 5). It could be

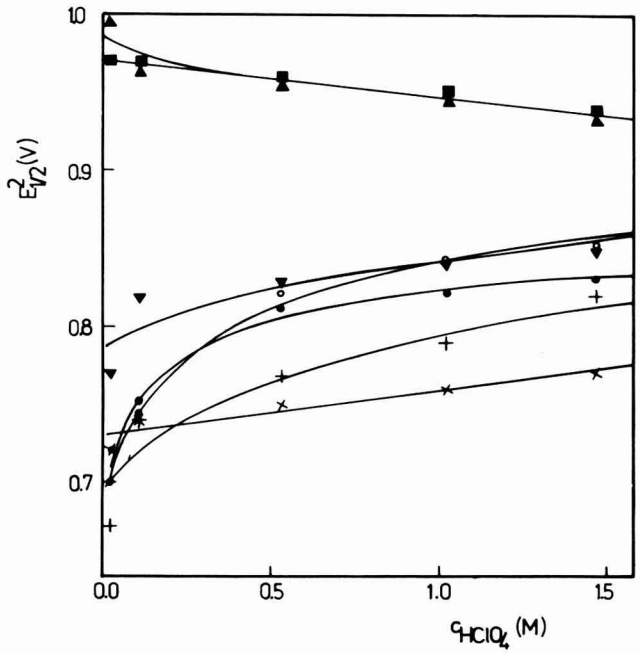


FIG. 3. Dependence of the values of the half-wave potentials of the second step in the oxidation, $E^2_{1/2}$ (values are taken from Tables 2 and 4), on the concentration of $HClO_4$ in an AN solution of 0.1 *M* $NaClO_4$ at 20 ± 0.1°C. (See legend to Fig. 2 for symbol identification.)

established that a single electron is involved in the first oxidation step. Linearity of the calibration curve has been proved for the concentration range $(6-20) \times 10^{-5}$ *M* (the standard deviation of its slope is 0.3×10^{-6} A). In the given concentration range the half-wave potentials of the first wave do not change with variation of the concentration.

The presence of water in concentrations over 1% (v/v) decreases the quality of the records such that evalution of the voltammograms is questionable. Below a concentration of 1% of water a third wave has been observed in the case of 10-isobutylphenothiazine.

A third wave also arises on addition of perchloric acid. The first step remains reversible (Fig. 6), the second and third steps are totally irreversible (Fig. 7). By potentiostatic coulometry it has been established that the first step requires the removal of one electron; the same information is obtained from semilogarithmic analysis of the dc voltammogram (Table 4).

Addition of tetrabutylammonium hydroxide lowers the first wave, while the shape of the second wave becomes irregular; a third wave is observed for 10-isobutylphenothiazine. If the base concentration exceeds 3×10^{-3} *M*, the first wave vanishes and at the same time the resulting voltammogram has become worthless for evaluation.

TABLE 3
THE EXPERIMENTAL AND CALCULATED VALUES OBTAINED BY THE CV METHOD

v^a (V sec^{-1})	E_{pa} (V)	E_{pc} (V)	ΔE_p (V)	i_{pa} ($\times 10^{-6}$A)	i_{pc} ($\times 10^{-6}$A)	i_{pa}/i_{pc}	(A M^{-1} $v^{-1/2}$ sec$^{1/2}$)	
							$i_{pa}/(c\ v^{1/2})$	$i_{pc}/(c\ v^{1/2})$
				Phenothiazine				
0.46	0.328	0.251	0.077	49.4	48.5	1.02	0.162	0.159
0.63	0.330	0.250	0.080	57.6	57.6	1.00	0.161	0.161
10.00	0.356	0.225	0.131	230.5	230.0	1.00	0.162	0.162
				2-Methoxyphenothiazine				
0.46	0.275	0.197	0.078	46.9	47.3	0.99	0.153	0.155
0.83	0.267	0.188	0.079	62.3	63.1	0.99	0.152	0.153
1.25	0.269	0.184	0.085	76.2	76.2	1.00	0.151	0.151
12.50	0.300	0.163	0.137	246.6	248.8	0.99	0.155	0.156
				2-Chlorophenothiazine				
0.63	0.416	0.340	0.076	64.3	64.2	1.00	0.180	0.180
1.00	0.419	0.338	0.081	81.0	79.0	1.03	0.176	0.180
2.50	0.425	0.334	0.091	129.9	125.0	1.04	0.180	0.176
				2-Acetylphenothiazine				
0.43	0.419	0.347	0.072	49.3	48.5	1.02	0.166	0.163
0.63	0.419	0.344	0.075	57.9	57.9	1.00	0.162	0.162
1.00	0.425	0.344	0.081	74.2	72.3	1.03	0.165	0.161
10.00	0.441	0.328	0.117	230.5	234.4	0.98	0.162	0.165

Note. t = 22°C, A = 0.13 cm^2, c_{HClO_4} = 2 × $10^{-2}M$, derivative concentration: c = 4.5 × $10^{-4}M$.
[a] v is dE/dt.

Diffusion Coefficients

Once the concentration of the electroactive substance, the electrode area, the rotation rate, and the kinematic viscosity are known, the Levich relationship (*11*) allows calculation of the diffusion coefficient from the limiting diffusion current. Repeating the experiment at other rotation rates, a mean value of the diffusion coefficient can be calculated (Table 5).

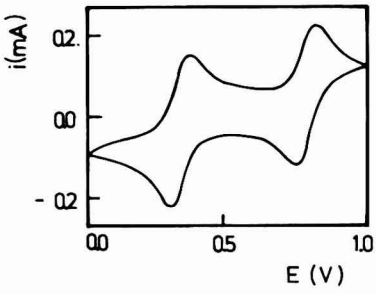


FIG. 4. Cyclic voltammogram of the anodic oxidation of 2-chlorophenothiazine (c = 1.6 × $10^{-3}M$) in 0.1 M $NaClO_4$ in AN, where c_{HClO_4} = 0.9 M, the rate of potential scan v = 0.91 V sec^{-1}, t = 20°C, A = 0.288 cm^2.

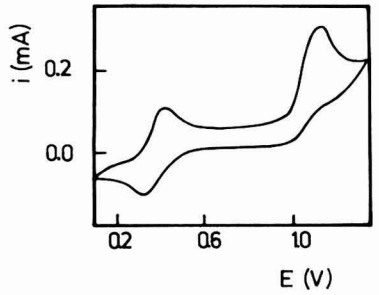


FIG. 5. Cyclic voltammogram of the anodic oxidation of 10-propylphenothiazine ($c = 7.8 \times 10^{-4}$ *M*) in an AN solution of 0.1 *M* $NaClO_4$ at $t = 20°C$, $A = 0.288$ cm², $v = 0.92$ V sec^{-1}.

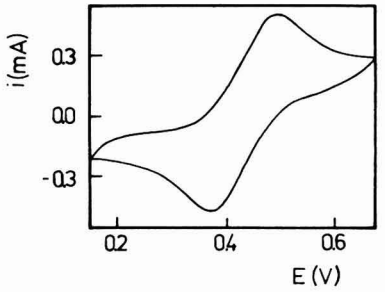


FIG. 6. Cyclic voltammogram of the first oxidation step of 10-isobutylphenothiazine ($c = 8 \times 10^{-4}$ *M*) in an AN solution of 0.1 *M* $NaClO_4$ at 20°C, $A = 0.288$ cm², $v = 0.2$ V sec^{-1}.

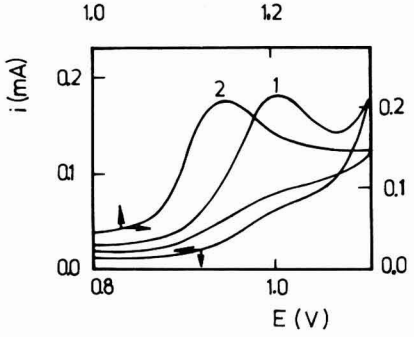


FIG. 7. Cyclic voltammogram of the anodic oxidation 10-isobutylphenothiazine ($c = 8 \times 10^{-4}$*M*) in 0.1 *M* $NaClO_4$ in AN medium, where $c_{HClO_4} = 0.1$ *M*, $v = 3.67$ V sec^{-1}. (1) Second oxidation step; (2) third oxidation step.

TABLE 4

THE EFFECT OF $HClO_4$ IN AN AN SOLUTION OF 0.1 *M* $NaClO_4$ ON THE ANODIC BEHAVIOR OF THE GROUP II DERIVATIVES

	10-Propylphenothiazine $c = 8.0 \times 10^5 M$							10-Isobutylphenothiazine $c = 10.4 \times 10^5 M$						
c_{HClO_4} (*M*)	i_d^1 (×10⁶A)	$E_{1/2}^1$ (V)	i_d^2 (×10⁶A)	$E_{1/2}^2$ (V)	i_d^3 (×10⁶A)	$E_{1/2}^3$ (V)	k_1^{-1}[a] (mV)	i_d^1 (×10⁶A)	$E_{1/2}^1$ (V)	i_d^2 (×10⁶A)	$E_{1/2}^2$ (V)	i_d^3 (×10⁶A)	$E_{1/2}^3$ (V)	k_1^{-1} (mV)
—	8.3	0.36	14.1	1.04	—	—	55	11.6	0.40	19.2	1.03	—	—	58
0.02	7.4	0.37	5.3	0.97	1.1	1.14	58	10.4	0.41	8.0	0.99	3.2	1.10	57
0.11	6.4	0.41	5.6	0.97	1.6	1.19	57	9.4	0.41	7.5	0.97	3.5	1.20	57
0.54	4.7	0.47	5.3	0.96	1.9	1.22	60	7.8	0.45	7.3	0.96	3.2	1.25	57
1.04	3.6	0.48	4.7	0.95	2.0	1.23	60	5.8	0.46	6.2	0.95	2.9	1.25	56
1.49	2.4	0.49	4.0	0.94	2.0	1.21	60	4.4	0.47	5.5	0.94	2.7	1.24	60
$s_{c i_d}$ (×10⁶)	0.5		0.2		0.3			0.3		0.2		0.1		
$s_{c E_{1/2}}$		0.03		0.00		0.03			0.01		0.01		0.05	

Note. $A = 0.11$ cm², $t = 20 \pm 0.1$°C, 1226 rpm.

[a] k is slope of the semilogarithmic analysis.

TABLE 5
DIFFUSION COEFFICIENTS OF PHENOTHIAZINE AND ITS DERIVATIVES IN AN AN SOLUTION OF 0.1 *M* $NaClO_4$ AT 20.0°C

Compound	D^a ($\times 10^5$ cm^2sec^{-1})
Phenothiazine	1.58
2-Methoxyphenothiazine	1.98
2-Chlorophenothiazine	1.82
2-Acetylphenothiazine	1.53
2-Trifluoromethylphenothiazine	1.52
10-Propylphenothiazine	1.31
10-Isobutylphenothiazine	1.51

Note. The kinematic viscosity value of 0.1 *M* $NaClO_4$ in AN is $4.86 \times 10^{-3} cm^2sec^{-1}$ at 20.0°C.

[a] $s_D = 0.2 \times 10^5 cm^2sec^{-1}$.

DISCUSSION

The oxidation of phenothiazine is assumed to proceed in two reversible steps, according to the reaction scheme

$$\mathrm{Ph} \underset{+e}{\overset{-e}{\rightleftharpoons}} \mathrm{Ph}^{\cdot+} \underset{+e}{\overset{-e}{\rightleftharpoons}} \mathrm{Ph}^{2+}$$

Ph stands for phenothiazine, $Ph^{\cdot+}$ is the radical cation, and Ph^{2+}, the dication). However, a single step is observed in anhydrous AN of the group I compounds. Most surprising in the results reported is the number of electrons removed during oxidation of derivatives of group I in anhydrous AN. Determination along two independent pathways gives values that differ by a factor of 2. In acidified AN solutions both methods yield identical results, but in this medium the anodic wave has been split up already.

The oxidation apparently occurs in two single-electron steps; in anhydrous AN the derivatives are more readily oxidized (mainly in the second step) and the processes coalesce. The fact that separations of these processes exist at a low concentration of acid can be explained by the difference in the solvation ability of AN and water (the perchloric acid being an aqueous solution). The low solvation of protons in AN in distinction from water is evident from the high value of the transfer activity coefficient $\gamma_{t(H^+)_1H_2O \rightarrow AN} \sim 10^6$. AN solvates especially anions less strongly than water and thus traces of water in AN medium lead to an increase of the dissociation constant of perchloric acid (for illustration (*6*): the dissociation constant of a solution of anhydrous $HClO_4$ in AN is 8.3×10^{-3}, while that of an equally concentrated solution of 70% $HClO_4$ in AN is 1.46×10^{-2}).

The oxidation of derivatives of group II in anhydrous AN proceeds through a two-step mechanism. The first step is reversible, corresponding to the exchange of a single electron. The second step is irreversible. The oxidation occurs in three steps in acidic AN medium; the first step is reversible and the second and third are irreversible.

The studied derivatives yield the first reversible wave in acidic AN medium which corresponds to exchange of a single electron and is proportional to the concentration of electroactive substance. The value of the half-wave potential is independent of the concentration of studied substance. The experimental dc voltammetric results can be used for the analytical determination of the concentration of studied substances. The character of the voltammetric curves and the half-wave potential values characterize the purity of studied phenothiazine pharmaceuticals and the differences in the half-wave potential values can be employed for simultaneous determination of these phenothiazine derivatives. The presence of acid leads to a linear decrease in the limiting diffusion current values and for analytical purposes it is thus preferable to work in a medium of anhydrous AN or in media where the acid concentration is 0.02−0.1 *M*.

In addition to the analytical research and the study of the reversibility of electrode reactions, the effect of substituents on the mechanism of the electrochemical oxidation was studied. It is clear from the comparison of the half-wave potential values for two groups of derivatives that derivatives of group I are more readily oxidized than derivatives of group II. In AN medium where the acid concentration is 0.02−1.49 *M*, potential values for the first step decrease or remain constant and the half-wave potential values for the second step increase with increasing acid concentration for group I. An opposite situation is found for the derivatives of group II. The difference in behavior can be explained by the effect of the substituent on the reaction center. In the following discussion we will assume that only electron influences are operative (resonance or mesomeric effect M and induction effect I). Substituents of group I have a −I and −M or +M effect. Billon assumed that oxidation occured at the nitrogen atom (*2*, *3*). It is, however, possible to determine whether the oxidation occurs at the sulfur or nitrogen atom by correlation of the half-wave potential values and Hammet constant. If the oxidation occurs at

TABLE 6
THE HAMMET CONSTANT VALUES FOR THE SUBSTITUENTS OF THE GROUP I DERIVATIVES (*7*)

R_2	$-OCH_3$	$-H$	$-Cl$	$-COCH_3$	$-CF_3$	$-CH_3$
σ_p	−0.268	0.000	0.227	0.516	0.551	−0.170
σ_m	0.115	0.000	0.373	0.306	0.415	−0.069

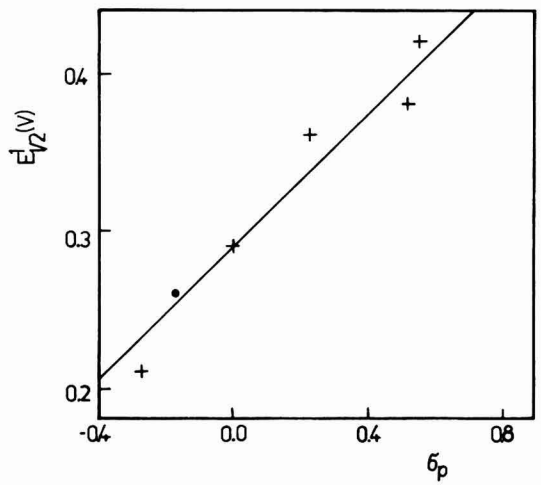


FIG. 8. Dependence of the value of the half-wave potential of the first oxidation step $E^1_{1/2}$ on the Hammet constant σ_p, $c_{HClO_4} = 0.11\ M$. +, Experimental values; ●, value taken from literature for 2-methylphenothiazine (*3*).

the sulfur atom, then the half-wave potential values should be correlated with the Hammet constants σ_p. A similar correlation with the σ_m constants should hold for oxidation at the nitrogen atom. The values of constants σ_p and σ_m for substituents of group I are listed in Table 6. Figure 8 graphically depicts the dependence of the half-wave potential in the first oxidation step on σ_p. Table 7 gives statistical treatment of the data. It follows from the graphical dependence and statistical data that oxidation probably occurs at the sulfur atom with derivatives of group I. The different behavior of the derivatives of group II (the highest values of the half-wave potential values and three oxidation steps in acidic AN

TABLE 7
STATISTICAL TREATMENT OF THE HALF-WAVE POTENTIAL CORRELATIONS WITH THE HAMMET CONSTANTS

c_{HClO_4} (*M*)	$E^1_{1/2}$ vs σ			
	k_p	s_p	k_m	s_m
—	0.95	0.02	0.76	0.04
0.02	0.98	0.02	0.81	0.06
0.11	0.98	0.02	0.81	0.07
0.54	0.97	0.02	0.80	0.06
1.04	0.98	0.02	0.76	0.06
1.49	0.98	0.02	0.74	0.06

Note. s_p or s_m are the standard deviations of the plot of $E^1_{1/2}$ vs σ_p or σ_m, k_p or k_m are the correlation coefficients of the same plot.

medium) cannot, however, be interpreted in terms of the classical concepts of induction and mesomeric effects of the alkyl group. This deviation is apparently caused by a difference in the solvation of the radical cation formed or by a greater deplanarization of the molecules of the derivatives.

Lastly, as a general conclusion for all compounds, it can be ascertained that voltammetry is a suitable quantitative method for phenothiazine derivatives. These results of our study can also be used for chemical study of the oxidation mechanism and for determining the electron donor properties of phenothiazines used in pharmacy.

SUMMARY

The electrochemical oxidation of phenothiazine and its derivatives substituted in the 2 and 10 positions was studied. The voltammetric behavior of the studied substances was compared. An attempt was made to quantitatively characterize the effect of the substituents. The reversibility of electrode reaction was studied by CV method.

Values of diffusion coefficients of phenothiazine derivatives in anhydrous acetonitrile medium were estimated; these values have not yet been published.

ACKNOWLEDGMENTS

Authors N.Š. and I.N. wish to thank the Dutch Ministry of Education for providing grants to enable this investigation and the University of Technology in Delft for providing facilities.

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MICROCHEMICAL JOURNAL 25, 567–575 (1980)

Microanalysis of Catecholamines in Human Plasma by High-Performance Liquid Chromatography with Amperometric Detection as Compared with a Radioenzymatic Method

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Received April 23, 1980

INTRODUCTION

In recent years many attempts have been made to find a reliable analytical method for the determination of catecholamines in human plasma or serum. Due to their extremely low endogenous concentrations, however, many difficulties are associated with the methods available, and the results obtained have to be interpreted with great care. At present, research activities are concentrated upon two quite different analytical principles, i.e., the radioenzymatic procedure (*5, 10, 11, 13*) in which the catechols are radiolabeled by enzymatic 3-*O*-methylation and separated by thin-layer chromatography, vs the utilization of liquid chromatography for separation combined with a highly sensitive and selective detector system (*7, 9, 12*), a method that must always be preceded by preconcentration on alumina. Despite its inherent extreme sensitivity, the radioenzymatic procedure, due to its complexity, appears less attractive from the analytical point of view than a straightforward liquid chromatographic method with no need for derivatization.

In recent reports (*1, 6, 8*) chromatographic systems based on high-performance cation exchange with amperometric detection and some preliminary results concerning their application to the analysis of catecholamines in human plasma have been described. This paper describes a complete analytical method and its application to direct studies of plasma catecholamine concentrations under normal and pathological conditions.

METHODS

Materials

The catecholamine standards were obtained from Sigma Chemical Company (St. Louis, Mo.) and other chemicals were of analytical grade

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0026-265X/80/040567-09$01.00/0

qualities from Merck (Darmstadt, GFR). The water used throughout was obtained by double distillation (the second time from alkaline permanganate) of deionized water. The alumina was prepared by hydrochloric acid treatment of neutral alumina (Macherey-Nagel, Düren, GFR) according to the procedure given by Anton and Sayre (*2*).

All glassware used in the extraction process was siliconized by pretreatment of neutral alumina (Macherey-Nagel, Düren, GFR) according to Erlenmayer flasks were used for the adsorption process and cylindrical glass-filter funnels for the washing and liberation steps. For the injection into the chromatograph a 250-μl Hamilton LC-syringe (Bonaduz, Switzerland) with stainless-steel needle was used.

Chromatography

Instrumentation. The equipment consisted of an Altex Model 100 constant-flow solvent pump, a Rheodyne Model 7120 injection valve provided with a 100-μl loop and a Model 7302 2-μm column inlet filter, a Lichroma 200 × 4.6 mm i.d. stainless-steel column, slurry-packed with 5 μ Nucleosil SA (Macherey-Nagel) an electrochemical detector (Bioanalytical Systems Inc., Lafayette, Ind.) and a Linear Model 264 potentiometric recorder (Irvine, Calif.). The electrochemical detector was composed of a Plexiglas cell containing a graphite paste anode (CPS) and with a 50 μ PTFE spacer defining the total cell volume (3.5 μl), a reference electrode compartment with an Ag/AgCl electrode and a Model LC-4 operational amplifier capable of converting 0.1 nA to 1 V. The column was separated from the pump by means of a 10 m × 1/16 in. o.d. PTFE coil and the detector potential was maintained at 0.65 V vs the Ag/AgCl reference electrode. The injector, column and detector parts were all contained within a carefully grounded Faraday cage.

The system was further improved at a later stage in this investigation by the introduction of a pulse dampening device (Touzart-Matignon, Vitry sur Seine, France) placed between the pump outlet and the Teflon coil.

Column packing. The column was prepared by the upward slurry packing technique described by Bristow *et al.* (*4*). The cation-exchange material was slurried in chloroform, ultrasonicated, and forced into the column tubing at an average flow rate of ca. 15 ml/min. This was followed by a successive replacement with acetone and water before sealing of the column. In this way high efficiency was obtained (ca. 9000 plates/200 mm).

Chromatographic conditions. The mobile phase was a citrate-acetate buffer of pH 5.2, kept at ca. 50°C and prepared as described previously (*1*). The flow rate used was 0.60 ml/min throughout. The LC-4 unit was always operated with a time constant of 2.0 sec (position B) giving a signal response fast enough to ensure no sacrifice in resolution under the condi-

tions used. In order to obtain a constant background current the system was allowed to stabilize for at least 3 hr before any injections were made.

Detector electrode. The cavity of the detector cell was carefully packed with silicone oil-based graphite paste (CPS) and the surface polished as smooth as possible. This operation was conveniently followed by inspection of the surface in a stereo microscope at 50× magnification.

Procedure for Plasma Extraction

Blood (10–20 ml) was collected in heparinized Vacutainer tubes which were kept on ice until centrifugation. After centrifugation at ca. 2000*g* for 10 min the plasma was removed and the samples stored at −70°C, if not processed immediately. Frozen plasma samples were thawed at room temperature and, if turbid, centrifuged at 16,000*g* for 15 min and the clear supernatant was withdrawn for further treatment. A solution of sodium bisulfite (75 μl 2.5 m*M* $Na_2S_2O_5$/ml plasma) was added as an antioxidant and 3,4-dihydroxybenzylamine (DHBA) was added as an internal standard (0.5 μl 10 μ*M* solution, i.e., 5 pmol,/ml plasma) with a microliter syringe. To 6.0 ml of this spiked plasma was then added 2.25 ml of 1 *M* Tris–HCl buffer of pH 8.6 containing 20 mg EDTA/ml, 4 ml of water, and finally 50 mg of acid-washed alumina. The solution was stirred for 15 min, the alumina filtered off into 100 × 10-mm i.d. glass-filter funnels with PFTE stoppers by gentle pressure application and washed four times with water (2-ml portions). After the last washing, desorption of the material was effected by shaking with 500 μl of 0.5 *M* perchloric acid for 15 min. One hundred microliters were then injected immediately into the chromatograph.

Calibration and Recovery

The detector response curves for the compounds of interest were generated by injection of serial dilutions (to 1:100) of stock solutions (1 μ*M*). Recoveries of the internal standard were determined by separate injections of 6 pmol of DHBA into the chromatograph and evaluation of DHBA peak height ratios by comparison with the sample chromatograms. The recoveries of the other catecholamines were checked by carrying out the extraction procedure with the use of radioisotopically labeled compounds.

Radioenzymatic Procedure

A commercially available kit (Upjohn Co., Kalamazoo, Mich.) was used throughout. The procedure is based on the method originally described by Engelman and Portnoy (*5*) and later modified by others (*10, 11, 13*).

RESULTS AND DISCUSSION

Plasma Extraction

The procedure given in the experimental part is to an essential degree based upon the original method by Anton and Sayre (*2, 3*). However, the extraction of plasma is associated with some problems with respect to optimization of the recovery and the exclusion of undesired chromatographically unretained material. We have generally found that centrifugation of the plasma just prior to carrying out the extraction procedure is essential when dealing with turbid samples. In these cases this yields a considerable increase of recovery together with a marked reduction of front peak tailing in the chromatogram. Plasma volumes of 3 to 6 ml were found to be suitable with regard to the amount of alumina used for extraction, in order to obtain as high a recovery as possible with a minimum quantity of PCA for liberation.

Our earlier results (*1*) have shown that no significant differences in total recoveries of the catecholamines during one extraction procedure are obtained. This was further confirmed in this study and therefore the recovery of the internal standard was considered to be equal to the recovery of the endogenous compounds.

Chromatography

The column performance, as well as the recovery obtained after extraction of a mixture of catecholamine standards, is shown in Fig. 1. The recoveries are lower in this particular case than that generally found for the extraction of DHBA from DHBA-spiked plasma. The results pertaining to the recoveries, detector response values and retention parameters obtained, are collected in Table 1. The detector response linearity has been evaluated in a previous study (*1*).

The limit of detection defined as the amount injected at $S/N = 2$, was typically found to be 40, 80, and 120 fmol, respectively, for NE, E, and DA. The interassay coefficient of variation of the method is ca. 15% ($n = 5$) at plasma catecholamine levels around 1 n*M*.

In order to obtain these limits of detection, one has to consider the factors of importance not only to achieve a high detector response but also a low background current and noise. For a given column efficiency and solvent system these factors are all associated with the performance of the electrochemical cell. Because the electrode potential at which the current generated in the electrooxidation reaches a plateau value is dependent upon the reversibility of the reaction, which, in turn, is dependent upon the nature of the carbon paste, the buffer pH, etc., the choice of the operating electrode potential must always be a compromise.

In our system we found that even if a slight increase in detector re-

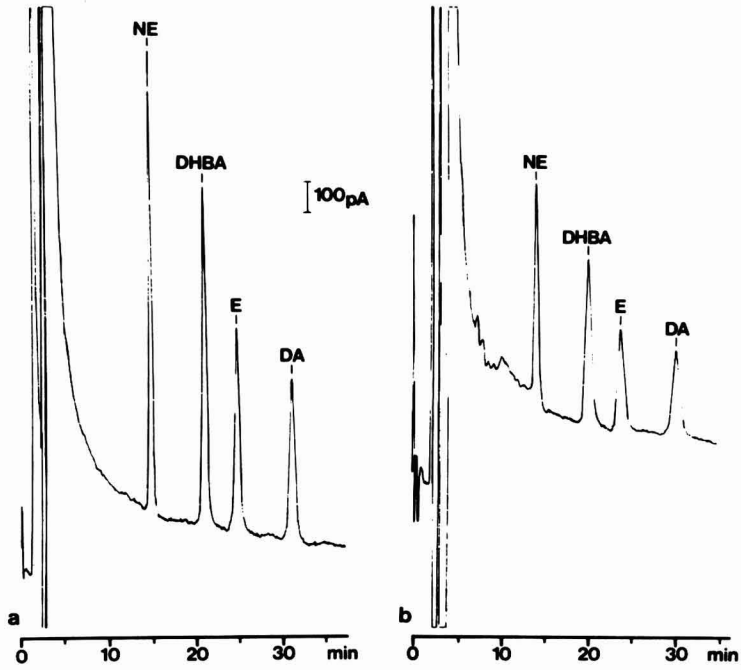

FIG. 1. Chromatograms obtained from (a) injection of a mixture of norepinephrine (NE), dihydroxybenzylamine (DHBA) epinephrine (E), and dopamine (DA), 5 pmol of each; (b) injection of a PCA extract obtained after the alumina adsorption procedure applied to the same mixture, yielding theoretically (at 100% recovery) an identical chromatogram as in (a). Full scale, 2 nA.

sponse was obtained above +0.65 V, this fact did not improve the signal-to-noise ratio because of the competing increase in background current and noise.

A factor of great importance is the coulometric yield which is improved

TABLE 1
CAPACITY FACTORS (k'), DETECTOR RESPONSE VALUES, AND RECOVERIES FOR THE CATECHOLAMINES INVESTIGATED

Compound	k'	Detector response		Extraction recovery (%)
		pA pmol^{-1}	Normalized factor f_{CA}	
NE	5.5	314	1.36	47
DHBA	8.3	230	1	49
E	10.0	139	0.60	49
DA	12.8	110	0.48	51

by an increase in the ratio between the area of the electrode surface and the cell volume. Consequently, the detector response will increase with a decrease in spacer thickness of the electrochemical flow cell, provided the flow rate is low enough. However, technical difficulties limit the use of ultrathin spacers.

Calculation

All calculations are based upon a reasonable approximation, which consists of equating all intraassay extraction recoveries. According to the experimental conditions described, the plasma catecholamine concentration, C_{CA} (nmol/liter), may be calculated by the expression

$$C_{CA} = \frac{5 \cdot h_{CA}}{f_{CA} \cdot h_{IS}},$$

where h_{CA}/h_{IS} denotes the peak height ratio between the endogenous catecholamine and the DHBA internal standard and f_{CA} represents the normalized detector response factor.

Application to the Analysis of Plasma

Because of the low detection limits obtained, it is usually possible to quantitate all three catecholamines even at the low concentrations in normal human plasma. A typical chromatogram is shown in Fig. 2a. Naturally, concentration data are evaluated with higher precision from the chromatograms when the plasma catecholamine levels are somewhat elevated, which may be the case under a variety of conditions, pathological or not. One example is illustrated in Fig. 2b which shows a chromatogram of extracted plasma from a patient with a severe burn injury. Some concentration data obtained are collected in Table 2.

In Fig. 3 the correlations between the HPLC (y-axis) and the radioenzymatic (x-axis) methods for norepinephrine (a) and epinephrine (b), respectively, are shown. It is of interest to note that the regression line in both cases gives a small positive y-axis intercept and a slope of 1.0 ± 0.17. The correlation coefficients were 0.92 and 0.95, respectively. These data points correspond to plasma samples obtained from hyperthyroid patients subjected to an insuline tolerance test, a fact which explains the elevated epinephrine values.

The data presented in this paper are collected from a systematic investigation in progress in our laboratory. This study, together with a more thorough investigation pertaining to the clinical significance of the method and its application to other areas of medical interest, will be published later.

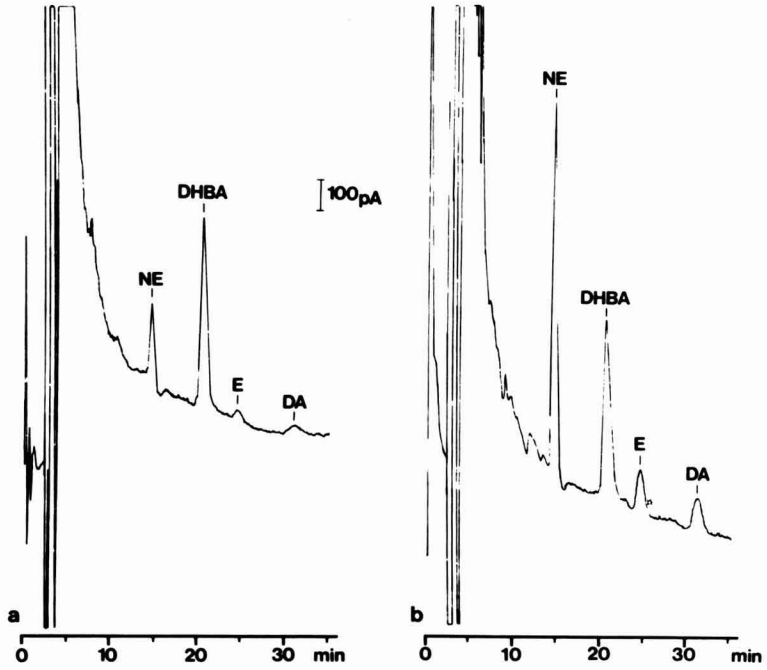

FIG. 2. Chromatograms obtained from alumina extraction of 6 ml of human plasma. (a) From a healthy individual, (b) from a patient with a severe burn injury. Full scale, 2 nA.

SUMMARY

A high-performance liquid chromatographic procedure is described for the quantitative determination of epinephrine, norepinephrine, and dopamine in human plasma. The method, which is based on adsorption of the catecholamines to alumina and, after liberation, separation on a microparticulate bonded strong cation-exchange resin and amperometric detection, has been optimized to give complete baseline separation of the substances of interest.

TABLE 2
PLASMA CATECHOLAMINE CONCENTRATIONS OBTAINED FROM HEALTHY AND INJURED SUBJECTS

Subject No.	Condition	Concentration (n*M*)			Recovery (%)	Note
		NE	E	DA		
I	Healthy	1.4	0.4	0.4	82	Fig. 2a
II		1.6	<0.3	<0.3	90	
III	Burn	7.8	1.4	1.4	87	Fig. 2b
IV	injured	10.1	0.6	1.6	89	

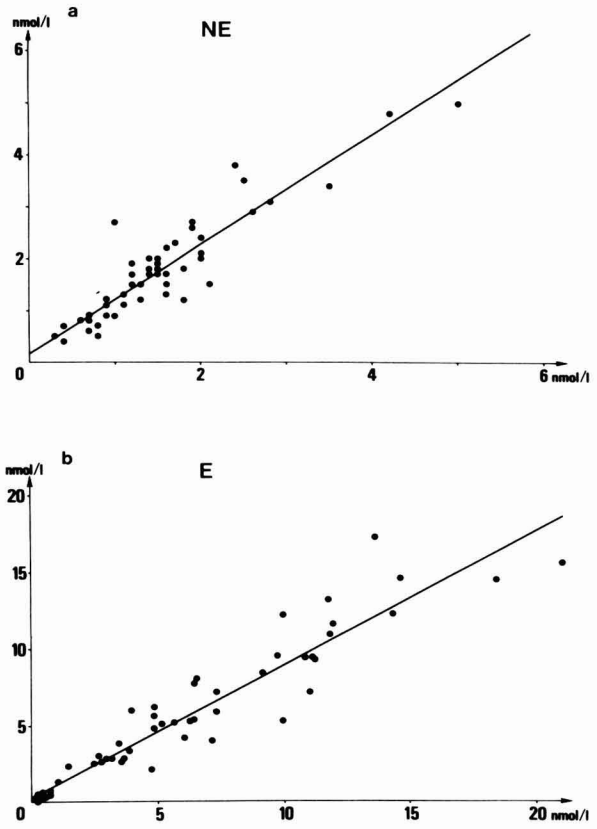

FIG. 3. Illustration of the correlation between the two methods compared. (a) Norepinephrine, (b) epinephrine.

Dihydroxybenzylamine, a nonendogenous catecholamine, is used as the internal standard. The detection limit is about 0.1 pmol for dopamine. Analysis of data obtained for norepinephrine and epinephrine from a total of 59 plasma samples showed a good correlation to the corresponding values obtained with a radioenzymatic method. Some results from normal and pathological conditions are compared.

ACKNOWLEDGMENTS

We are grateful to Professor S.-O. Liljedahl, Dr. B. E. Karlberg, and Dr. O. R. Nilsson for making plasma samples from different groups of patients available to us. We are also indebted to Drs. H. Hallman and B. Hamberger (Department of Histology, Karolinska Institutet, S-104 01 Stockholm) and Drs. L.-E. Edholm and C. Hansson (Department of Technical Analytical Chemistry, Chemical Centre, Box 740, S-220 07 Lund) for valuable discussions.

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MICROCHEMICAL JOURNAL 25, 576–588 (1980)

Spectrofluorometric Study of 1,4-Diaminoanthraquinone–Calcium Complex in Aqueous Sulfuric Acid Mediums: Spectrofluorometric Determination of Calcium

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Received May 15, 1980

INTRODUCTION

It is well known that hydroxy and amino derivates of anthraquinone are found among those organic reagents able to form fluorescent metallic chelates. These reagents possess a rigid molecular structure and therefore the high fluorescence shown by some of their chelates cannot be attributed to the appearance of rigid structures after the chelating process. In these cases the principal cause is the change in absorption, due precisely to the formation of the complex (*5*).

Concretely the reagent 1,4-diaminoanthraquinone shows an appreciable selectivity in ethanolic medium (*1*) not found in a sulfuric medium in which an appreciable increase of reactionability is observed. This different behavior of the reagent, depending on the medium can be justified by the existence of intramolecular hydrogen bonds (*3, 4*) which are not formed, or are considerably inhibited in a sulfuric medium. Thus, 1,4-diaminoanthraquinone like other anthraquinone compounds, reacts with boric acid in a concentrated H_2SO_4 medium giving rise to a dark violet coloration (*2*). This reaction is the base for a spectrophotometric determination of traces of boron, operating in concentrated H_2SO_4 medium, and within oxidants and F^- ion interfere (*6*).

In a high-content H_2SO_4 medium, the 1,4-diaminoanthraquinone reacts fluorescently with Ca and Sr ions, showing values of sensitivity 7 and 6, respectively, expressed in pD. The complexes showed a fluoroscence of orange tone when excited with a radiation of 365-nm wavelength while the reagent subjected to equal conditions did not show significant fluorescence (*7*). We haven't found bibliographic data about reactions of 1,4-diaminoanthraquinone in a sulfuric medium.

For the spectrofluorometric determination of Ca, the reagent most rec-

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ommended are calceine, 8-hydroxyquinoline, 8-quinolylhydrazone (*9*), and 1,5-bis(dicarboxymethylaminomethyl)-2,6-dihydroxynaphtalene (*10*). This work describes the reaction between the Ca ion and 1,4-diaminoanthraquinone in aqueous sulfuric mediums, and a new spectrofluorometric method for the determination of Ca is established. This method presents a similar or better sensibility than the above methods and allows the determination of Ca in the presence of 2-fold Mg, 4-fold Sr, 6-fold Ba, and 50-fold Cl^-, PO_4^{3-}, oxalates, and EDTA.

In a former publication we have described the spectrophotometric study of this reaction (*8*).

MATERIAL AND METHODS

Reagents

All the reagents used were of analytical quality. A solution of Ca (1.072 g/liter) was prepared from $Ca(NO_3)_2 \cdot 4H_2O$ by dissolution in deionized water and standardized with the complexometric method. Reagent solution (2×10^{-3} *M*) in a concentrated sulfuric medium was prepared 1,4-diaminoanthraquinone Schuchart R. A. Other dissolutions obtained by exact diluting of the former solutions were also used.

Apparatus

Instruments used for the experiment were: spectrofluorimeter Perkin–Elmer MPF-43, the source of exciting light was provided by a xenon lamp (XBD, Osram-150W), photomultiplicator R-508, registrator recorder 056, and fluorescence intensity indicator. Thermostate equipment and 1.0-cm quartz cells were used. Also used for the qualitative tests was a Mineralight S-68 to provide 245- and 365-nm wavelengths.

Calibration of the spectrofluorimeter. Adjusting the apparatus to the following parameters and values: Sensitivity $C = 1$ and $F = 1$, Slit (nm) 5 for excitation and 3 for emission, temperature 23°C; wavelength of excitation (λ_{ex}) 480 nm, and wavelength of emission (λ_{em}) 580 nm; the indicator showed a reading of 44 units for the standard stick of fluorescence N°.6, equivalent to dissolution 3×10^{-4} *M* of rhodamine B. All the spectrums which are presented in this paper have not been corrected and therefore must be understood as apparent spectrums.

Recommended Procedure for Determination of Ca(II) Traces

According to the study carried out, we consider the following operating method recommendable: In a 10-ml flask, introduce the problem sample with a Ca content such that the final dissolution contains between 150–400 ppb. If necessary deionized water should be added to complete a final volume of 3 ml. Then, 6 ml of dissolution 1,4-diaminoanthraquinone (3.3×10^{-4} *M*) in concentrated sulfuric is added, diluting to final volume

with water. The fluorescence intensity of the dissolution is registered at 580 nm of emission exciting at 410 nm and using as a blank a reagent solution, prepared in the same way but without Ca.

The standard line is established with Ca dissolutions whose concentrations are included between 150 and 400 ppb, treated by the same procedure as the problem sample. We will take care that the samples are at a temperature comprised between 20–36°C when we carry out the measures.

RESULTS AND DISCUSSION

Spectrofluorometric Study of the Complex 1,4-Diaminoanthraquinone–Ca

Former experiences of qualitative nature have shown that the concentration of water present in the medium, just as the order of addition of the reagents, considerably influences the fluorescence intensity of the dissolution of complex. Quantitatively the maximum fluorescence was observed when the concentration of water was approximately 30%, and when the order of addition followed was: cation, reagent, deionized water, and finally H_2SO_4. Under these conditions, consequently, the emission and excitation spectrums of the complex and the reagent were established.

Emission and excitation spectrums. Using a dissolution whose final volume was 10 ml and whose content was 0.5 ml of Ca dissolution (2×10^{-3} *M*), 1 ml of reagent solution (2×10^{-3} *M*), 2.5 ml of H_2O, and concentrated sulfuric up to the final volume (10 ml) the emission and excitation spectrums of the complex were registered. The corresponding spectrums for the reagent, analogously prepared and registered, also were obtained. These spectrums are shown in Fig. 1. It can be observed how the complex shows a maximum of excitation at wavelength 410 nm and a maximum of emission at 580 nm, with a considerable Stokes effect. This must be interpreted as a total absence of overlapping with a difference between the maximums of 170 nm. The spectrums of reagent registered at the wavelength maxims of excitation and emission for the complex show maximums remarkably lower and placed at different wavelengths.

Influence of the water content of the medium on emission and excitation spectrums. For this study, we operated with concentrations which were: 1 and 2×10^{-4} in Ca and reagent, respectively, and containing, in each case, variable quantities of water to achieve the desired concentrations. Figures 2 and 3 show how emission and excitation spectrums of the complex 1,4-diaminoanthraquinone–Ca, vary in function of the H_2O concentration and Fig. 4 shows what corresponds to the emission of the reagent under the same operating conditions.

The excitation spectrum of the complex shows a well-defined maximum

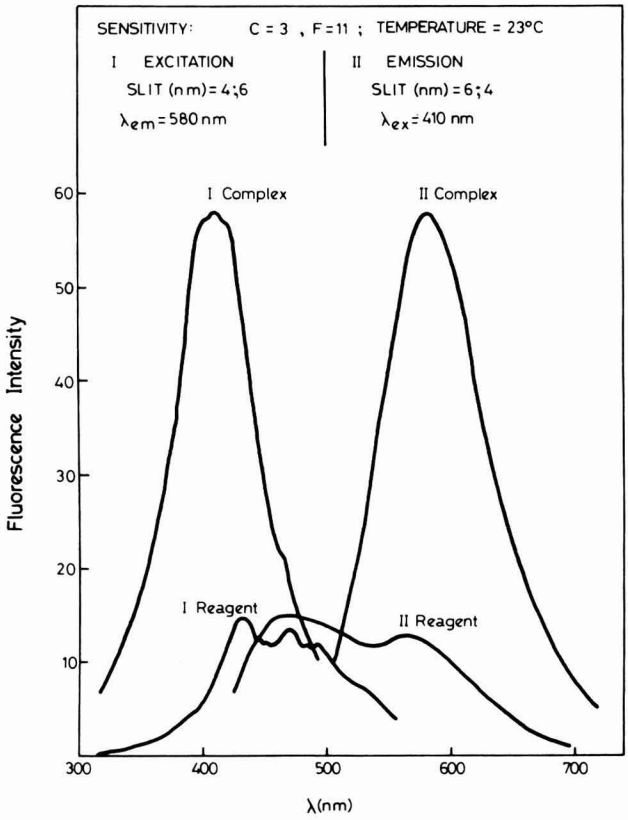

FIG. 1. Emission and excitation spectrums of the complex and reagent.

for a wavelength of 410 nm, practically within the whole interval of the H_2O concentrations studied, increasing its magnitude up to a 30% of H_2O content, and progressively decreasing with higher contents. On the other hand, H_2O concentrations, low as well as high, provoke the appearance of a second maximum for 470, possibly caused by the free reagent, since under these conditions the formation of complex is minimum.

The emission spectrum of the complex shows a maximum for a wavelength 580 nm when the medium H_2O content is found within the range 20–70%. For a 10% content there is practically no formation of fluorescent complex, and for the order of 90% content, the maximum of emission suffers a batocromic shifting, setting at 610 nm with a lesser magnitude.

The emission spectrum of the reagent shows two maximums of low sensibility at 470 and 570 nm, noting that the magnitude of the first becomes lower as the H_2O content of the medium is increased. The opposite

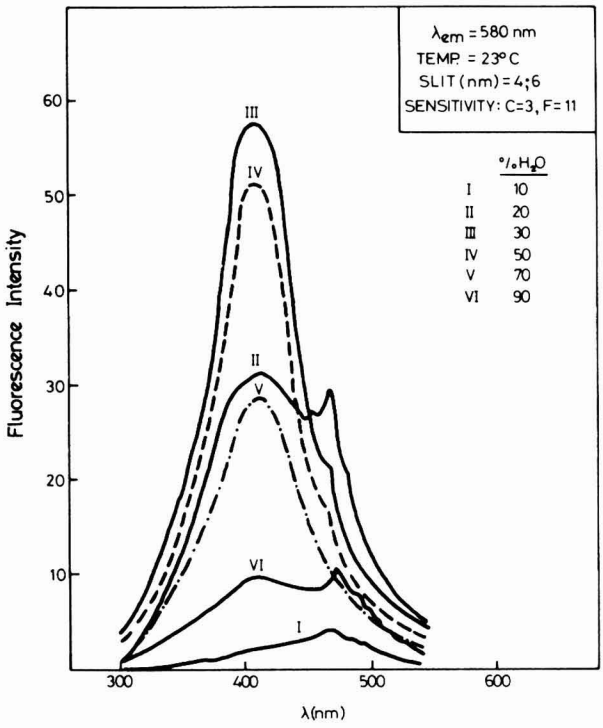

FIG. 2. Excitation spectrums of the complex in function with the H_2O concentration.

occurs with the second maximum. When the water content becomes 90%, the first maximum disappears, and the second one suffers a batocromic shifting to 610 nm. From this it can be infered that emission spectrums of both reagent and complex become similar, as the H_2O concentrations are increased. This indicates that the complex is not formed.

As suitable wavelengths for the measures, 410 nm for excitation and 580 nm for emission were fixed.

Relation: Fluorescence intensity–H_2O concentration. Operating under the above-mentioned conditions, the differences of fluorescence intensity between complex and reagent for a series of samples with a variable H_2O content were obtained. In Fig. 5, the obtained results are represented and it shows that the optimum range for the formation of fluorescent complex is 30–45%; concentrations outside of this range show progressively lower intensities, until reaching null values, because the complex is not formed. As a suitable H_2O concentration, 40% has been chosen, because it is a central value in the optimum interval, and makes it possible to work with sulfuric mixtures of greater H_2O content.

Stability of the complex and effect of temperature. Solutions of com-

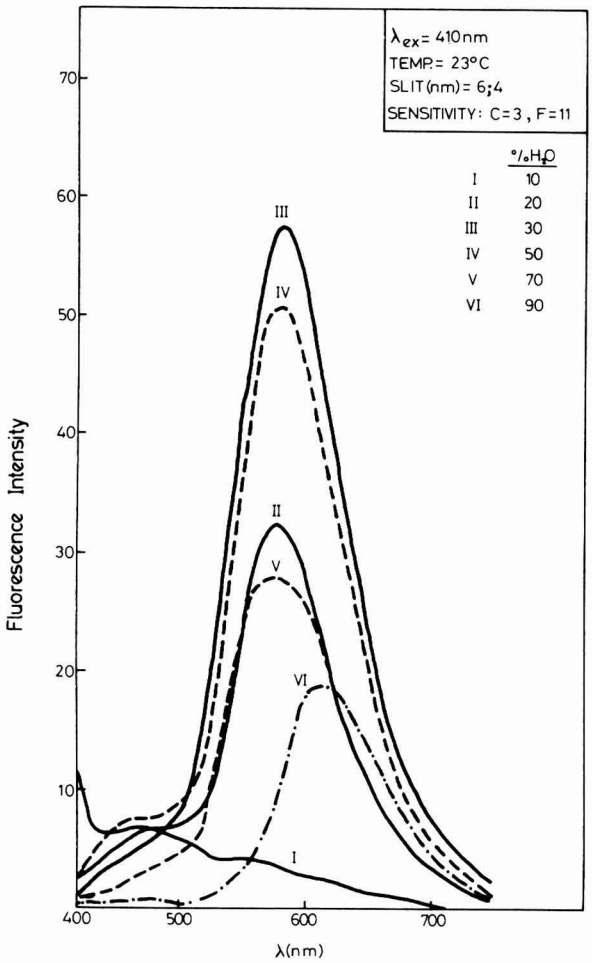

FIG. 3. Emission spectrums of the complex in function with the H_2O concentration.

plex and 1,4-diaminoanthraquinone both prepared with 40% of water and with equal concentrations of reagents as formerly mentioned, showed a stable fluorescence intensity from the time of preparation of the sample, and remaining stable for at least 4 hr.

As can be seen in Fig. 6, the fluorescence intensity of both solutions (complex and reagent), progressively decrease as the temperature rises. However, the difference between both remain practically constant in the following ranges: 20–35 and 65–90°C, though in the latter case the sensibility is lower. It points out that, it is possible to carry out the measures without thermostating, since the room temperature is found within the first indicated range.

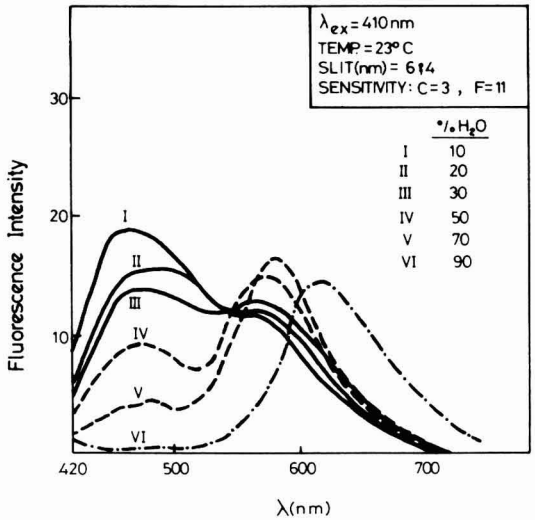

FIG. 4. Emission spectrums of the 1,4-diaminoanthraquinone in function with the H_2O concentration.

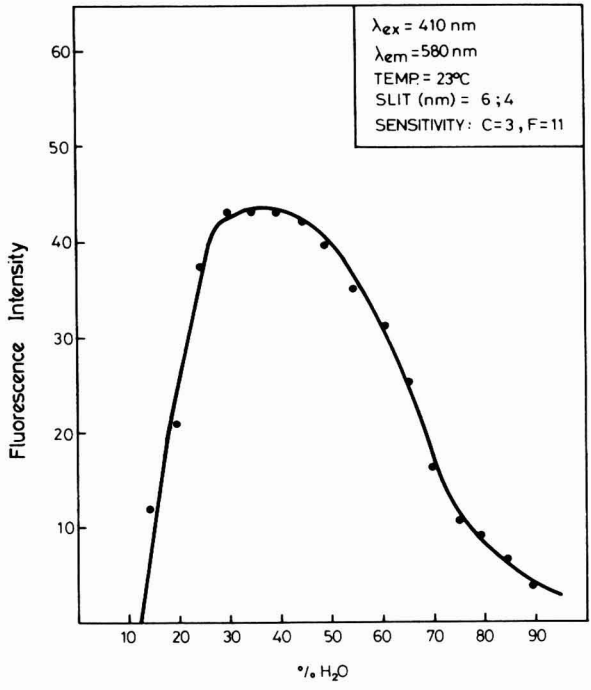

FIG. 5. Relation: fluorescence intensity $-H_2O$ concentration.

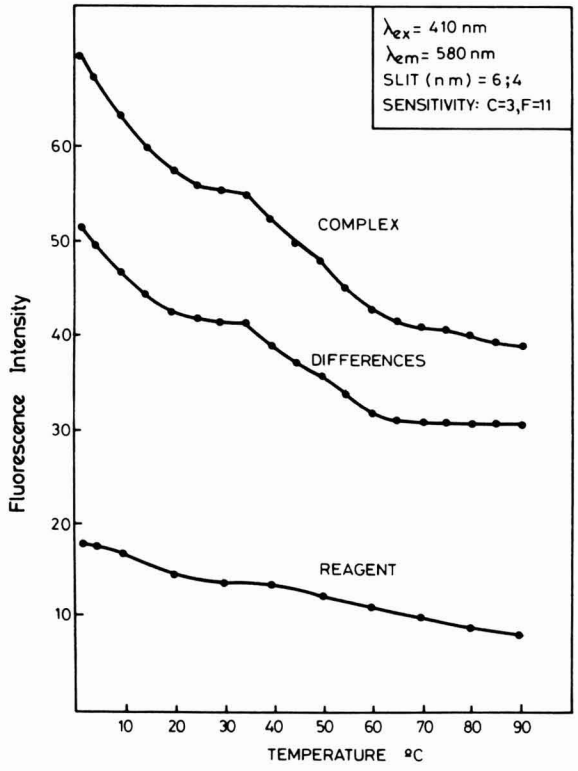

FIG. 6. Temperature influence in the fluorescence intensity.

Order of addition of reagents. As it was formerly pointed out, the qualitative tests indicated that the order of addition had a considerable influence on the fluorescence of the sample. More precise experiences carried out on this question showed that not only the order of addition influenced the formation of the fluorescent complex, but also the initial proportion between the aqueous solution of Ca and the reagent's sulfuric solution. As a consequence of this study, it is possible to assert that the order and proportion of reagents that should be fixed for reaching a higher fluorescence intensity is: 30% of aqueous solution of Ca, 60% of solution, with adequate concentration, of 1,4-diaminoanthraquinone in concentrated H_2SO_4, and 10% of H_2O up to the final volume.

Effect of the concentration of reagent. This effect was studied preparing a series of solutions whose concentration in Ca was 10^{-4} *M*, and contained variable quantities of reagent, and with a final water proportion of 40%. The fluorometric measures were carried out against a blank reagent. As can be observed (Fig. 7) the fluorescence intensity rises, as is

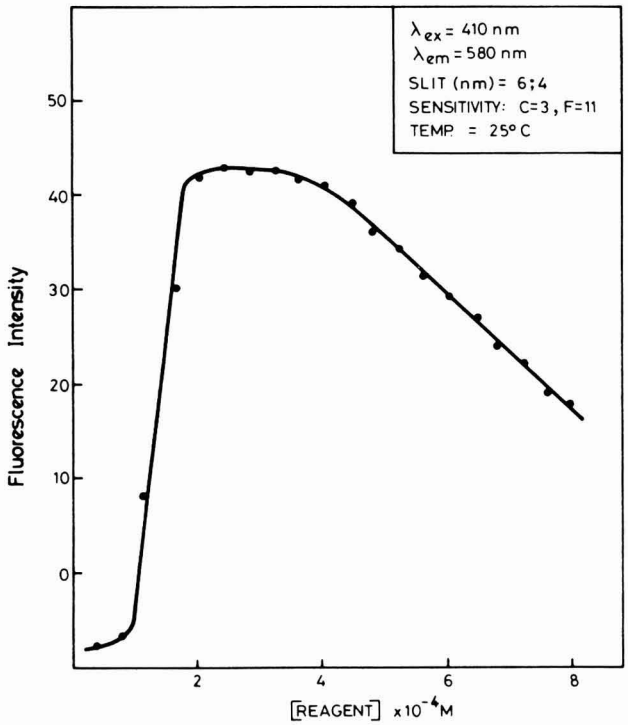

FIG. 7. Effect of the concentration of the reagent.

logical, at the same rate as the concentration of reagent is increased, up to a value of 2×10^{-4} *M*. It then remains constant up to a value of 4×10^{-4} *M* later decreasing for greater concentrations. That is an inversion, or reversion of fluorescence per concentration is observed. For concentration of reagent lesser than 10^{-4} *M*, the fluorescence intensity of the complex is less than that of the reagent. As optimum concentration of reagent, 2×10^{-4} *M* can be used.

Effect of the concentration of cation. Stoichiometry. Modifying now the quantities of Ca(II) present at the dissolution and keeping the concentration of reagent constant at 2×10^{-4} *M* (6 ml of its dissolution 3.3×10^{-4} *M* in a final volume at 10 ml), the fluorescence against a blank reagent was measured. In figure 8, the obtained results are assembled, and from them it can be deduced that the intensity rapidly increases, as does the concentration of Ca up to values of 10^{-4} *M* (Relation reagent-cation 2:1). From this point, the intensity also rapidly decreases up to values of Ca concentration of 2×10^{-4} *M* (Relation reagent-cation 1:1). For higher concentrations, the fluorescence keeps decreasing but with a different slope. The maximum concentration that fulfils lineality is 0.6×10^{-4} *M*.

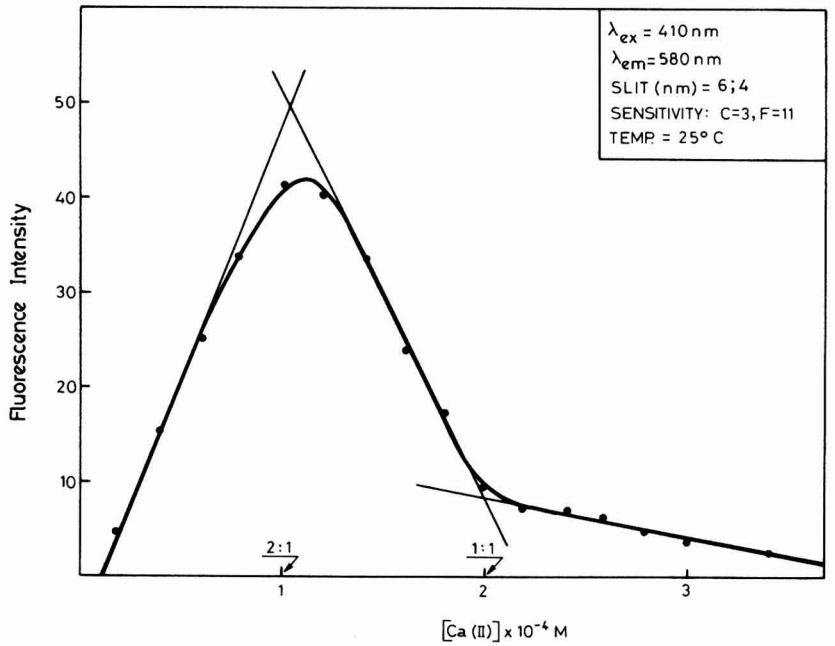

FIG. 8. Effect of the concentration of the cation.

The two perfectly defined changes of the slope that can be seen in Fig. 8 point out, according to the stoichiometry determination method of Yoe and Jones (*8*), the existence of two complexes whose stoichiometries (2:1 and 1:1) are defined by the cutting points of the tangents to each one of the parts of the graphic representation.

These results were confirmed by the method of the continuous variations (Fig. 9) using dissolutions of a total and constant concentration of 1,4-diaminoanthraquinone and Ca equal to 4×10^{-4} *M*. In this case the existence of the two complexes mentioned above can be observed under our operating conditions. The complex of stoichiometry 2:1 (reagent-cation), seems to be responsible for the fluorescence, and it is probably the one that appears in those conditions in which an excess of reagent exist. The complex of stoichiometry 1:1 is not itself fluorescent, but it is formed with a cation excess, and that can explain that the fluorescence progressively disappears as the concentration of Ca is increased.

Spectrofluorometric Determination of Ca(II) Traces

Relation: Fluorescence intensity–Ca(II) concentration. Under those conditions formerly considered as optimum, the existence of a lineal relation between the intensity of fluorescence and the concentrations of Ca

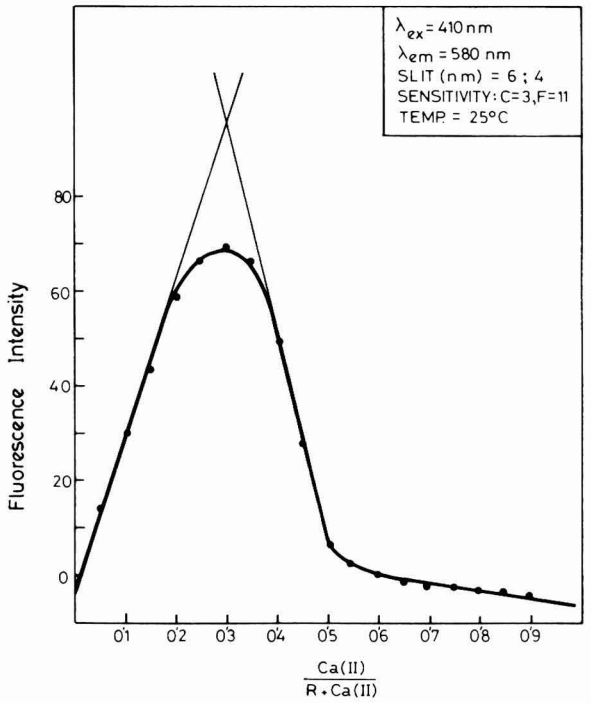

FIG. 9. Stoichiometry of the complex by the continuous variation method.

has been studied. Table 1 shows how this relation is satisfactorily fulfilled in the interval comprised between 150 and 400 ppb.

Reproducibility of the results. Working under the same conditions as in the operating method, with two series of 10 samples containing 200 and 300 ppb, respectively, and applying the statistic calculation of errors (95% confidence level) to the obtained results, the values found for the typical deviation, the medium deviation, and the relative errors on the average values were ±6.10, ±1.93 and ±1.71, respectively.

TABLE 1
RELATION FLUORESCENCE INTENSITY–Ca(II) CONCENTRATION

Ca (ppb)	150	200	250	300	350	400
Fluorescence intensity	7.0	25.0	43.5	61.0	78.5	95.5
λ_{ex} = 410 nm λ_{em} = 580 nm		Slit (nm) = 6, 4 Temperature = 25°C			Sensitivity C = 3 F = 11	

TABLE 2
EFFECTS OF FOREIGN IONS ON THE DETERMINATION OF 300 ppb OF Ca(II)

Maximum tolerance foreign ion/Ca(II) (w/w)	Ion added
1	Pb(II), Cu(II), Sb(V), W(VI)
2	Mg, Hg(II), Al(III), Ni(II), Co(II), Ce(IV), Ti(IV), Zr(IV)
4	Sr, Cd, V(V), F^-.
6	Ba, Ag(I), I^-, BrO_3^-
10	As(V), Br^-, ClO_3^-, IO_3^-
50	Li, Na, K, Rb(I), Cs, NO_3^-, PO_4^{3-}, Cl^-, oxalate, EDTA

Note. The limiting value of the concentration of foreign ions was taken as that which caused an error of not more than 2.5% in the fluorescence intensity.

Interference of foreign ions. The maximum permissible foreign ions/Ca weight ratios, for the determination of Ca are shown on Table 2.

SUMMARY

The spectrofluorometric study was made of the complex 1,4-diaminoanthraquinone-Ca in aqueous sulfuric mediums [$\lambda_{max,ex}$ = 410 nm; $\lambda_{max,em}$ = 580 nm; 50% H_2O; stable for at least 4 hr; range temperature optimum = 20–35°C; $[R]_{optimum} = 2 \times 10^{-4}$ *M;* stoichiometry 2:1 (fluorescent complex) and 1:1 (no fluorescent complex)]. A new method for the spectrofluorometric determination of Ca traces is proposed for concentrations between 150 and 400 ppb. The relative error and the interferences of the method have been investigated.

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MICROCHEMICAL JOURNAL 25, 589–594 (1980)

Spectrophotometric Extractive Determination of Iodide as Tris(2-(phenyliminomethyl)pyridine) Fe(II) Iodide

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Received June 2, 1980

INTRODUCTION

Methods for the quantitative determination of iodide generally fall into four categories: (a) formation of insoluble salts (gravimetric methods and precipitation titrimetry); (b) complex or slightly ionized compound formation (mercurimetric determination, photometry such as I_2Pd, etc.); (c) methods based on the reductor character of iodide either chemically (redox titrimetry) or electrochemically (amperometry, polarography, coulometry, etc.); (d) other methods such as photometry, X-ray fluorescence, indirect flame photometry, radiochemical methods, chromatographic techniques, etc.

The present determination of iodide is based on solvent extraction. Tris(2-(phenyliminomethyl)pyridine) Fe(II) sulfate (analogous to ferroin (*7*)) is not extracted from aqueous solution into organic liquids. In the presence of several anions it is extracted into organic solvents, by the formation of mixed complexes (*7*) like CN^- or ionic pairs, I^-, ClO_3^-, SO_3^{2-}, ClO_4^-, $B(C_6H_5)_4^-$, etc.

This type of extraction, by the formation of ionic pairs has been used for the determination of ClO_4^-, PF_6^-, and AsF_6^- with ferroin (*1, 2, 8*) or CN^- with tris(2-(phenyliminomethyl)pyridine) Fe (*1*).

The iodide is extracted as tris(2-(phenyliminomethyl)pyridine) Fe (II) iodide into nitrobenzene and the amount extracted is proportional to the amount of iodide present. Iodide is determined by measuring the absorbance of the extract spectrophotometrically.

EXPERIMENTAL, RESULTS, AND DISCUSSION

Apparatus. A Beckman ACTA III spectrophotometer with 1-cm cells was used.

Reagents. (a) Potassium iodide standard solution, 1 g/liter (1000 g/liter of I^-).

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0026-265X/80/040589-06$01.00/0

(b) Tris(2-(phenyliminomethyl)pyridine) Fe(II) solution, 10^{-2} *M*. 2-(Phenyliminomethyl)pyridine, 0.547 g, previously synthesized by us (*6*) and 0.392 g of Mohr's salt were mixed together and leveled off to 100 ml with water. The color is deep purple and the reagent remains stable for 1 week.

(c) Acetate buffer, pH 6.

(d) Nitrobenzene and other chemicals employed were reagent grade.

Study of the extraction system. The iodide ion is extracted into several solvents in the presence of the ferrous complex of 2-(phenyliminomethyl)pyridine, showing purple in chloroform, isoamilic alcohol, and nitrobenzene.

Nitrobenzene was chosen for the present work because it has proved to be more suitable for extraction, since the organic and aqueous layers settle out more rapidly after shaking. The absorption spectrum of the extract shows maximum at 580 nm and shoulders at 526 nm. In water the maximum appears at 570 nm.

Influence of pH. Solutions of the 10^{-3} *M* reagent and of 5×10^{-4} *M* I^- were made. pH was changed by the addition of several buffer solutions. After extraction with an equal volume of nitrobenzene (10 ml) lasting 1 min, the absorbance is read at 580 nm. Figure 1 shows that maximum extraction appears between 4.5 and 7.

The origin of such dependence is to be found in the strong hydrolysis that the ferrous complex suffers in acid and basic solutions (*4*, *5*). The pH dependence in the ferrous complex formation in aqueous solutions, studied by us (*7*), is analogous to what we find here (maximum between 5 and 7). This dependence is found whenever anions of weak basic charac-

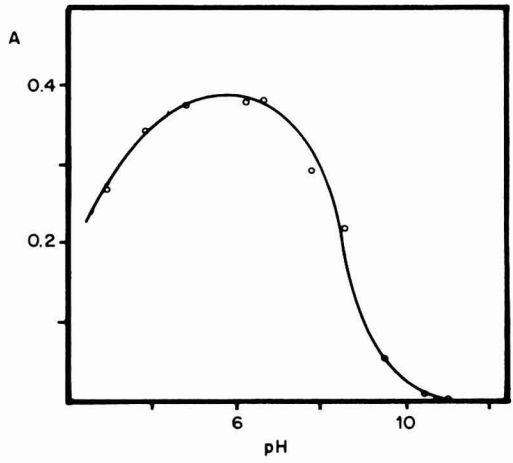


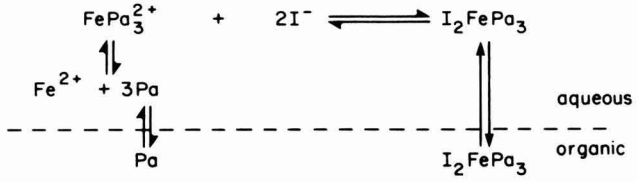
FIG. 1. Influence of pH on the extraction of I^-–ferrous complex system.

ter (I^-, ClO_3^-, ClO_4^-, etc.) are extracted with that reagent, but the extraction reaches its maximum at basic pH if the anions show basic character (CN^-).

AcH/Ac^-, pH 6, was chosen as a suitable buffer, since it has been shown that others, such as $PO_4H_2^-/PO_4H^{2-}$, reduce the extraction. The influence of ionic strength adjusted with AcH/Ac^- buffer in different concentrations is studied, demonstrating that the extraction increases till an ionic strength of 0.1, and decreases from 0.75 ionic strength. We work with a buffer that gives an ionic strength of 0.1.

Extraction kinetics. Operating as above, but at pH 6 and extracting over different time periods, we obtained the results plotted in Fig. 2. The extraction is extremely quick, the maximum extraction is obtained within 1 sec, as always occurs with ion association extractions; nevertheless there is a competitive reaction that reduces the extraction as the phase contact time increases, which is shown in the curve form.

The experiments carried out show that this is due to the favorable extraction into nitrobenzene of 2-(phenyliminomethyl)pyridine which is quantitatively extracted at pH work (R = 99%). This can be represented in a simplified form:



This process is observed if the reagent is extracted into nitrobenzene without iodide, because the charged complex is not extracted, but the intense violet color tends to disappear if the contact time is adequate, demonstrating the presence of Schiff base in the organic layer.

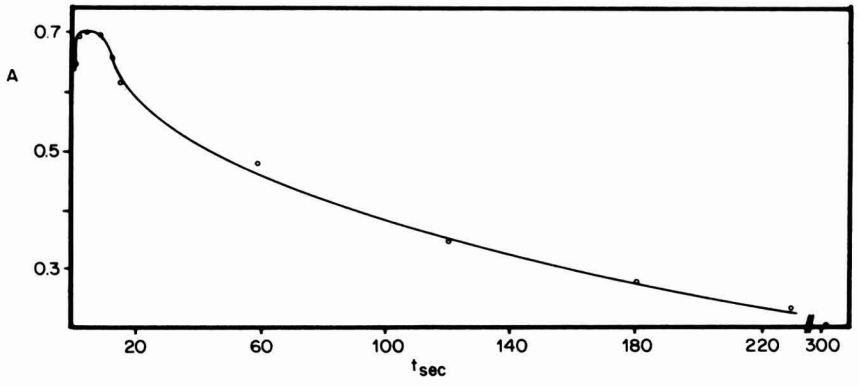


FIG. 2. Extraction kinetics.

This agrees with what also occurs in chloroform where the reagent decomposes on coming into contact with it, although more slowly, which can be accounted for by the more favorable distribution ratio in the case of nitrobenzene. In practice we used 30 sec as the extraction time because it is more easily repeated than shorter times.

Stoichiometry. The Bent and French method (*3*) is applied to an iodide concentration constant. One series of dissolutions into 10-ml volumetric flasks were made by 1.8×10^{-4} *M* of iodide and $1-8 \times 10^{-3}$ *M* of reagent, also containing 1 ml of acetate buffer. After extraction and measurement we deduced that the molar ratio $I^-/FePa_3^{2+}$ is 2:1 (exactly 1.95), which indicates the formation and extraction of an ionic association.

Total extraction. A series of solutions prepared as above is extracted five times with 1-ml portions of nitrobenzene, after combining the nitrobenzene extracts in a 10-ml volumetric flask and diluting to full volume with nitrobenzene.

If we compare the standard line obtained with the plotted line established by extracting only once with an equal final volume of nitrobenzene we find less slope for the first than the second ($\epsilon = 7200$ $1cm^{-1}mol^{-1}$) for total extraction and $\epsilon = 10700$ $1cm^{-1}mol^{-1}$ for one extraction only). We ascribe this to the continuous reduction of the reagent concentration when it is extracted with nitrobenzene, as mentioned above. This fact makes it impossible to calculate the distribution ratio for that extraction system by spectrophotometric methods; although we may suppose that it is very favourable to the organic phase.

OPTIMAL CONDITIONS FOR THE EXTRACTION OF THE IONIC ASSOCIATION

Stability. The stability of nitrobenzene extracts is maintained for 90 min; over longer periods it disminishes slowly.

Influence of the reagent concentration. An increase in the extraction is found as the reagent/iodide ratio increases, maximum extraction appears from ratio 20:1 on. This higher ratio may be related with the reagent decomposition by Schiff base extraction in organic layer.

Standard line. The results obtained show that there is a linear relation between 2 and 40 ppm. Negative deviation is found at higher concentrations. The optimum concentration range, evaluated by the Ringbom method, is between 5 and 30 ppm of iodide.

Recommended procedure. The sample solution containing between 5 and 30 ppm I^- when diluted to the mark is placed in a 10-ml volumetric flask, 2 ml of buffer solution AcH/Ac^-, pH 6, and 5 ml 10^{-2} *M* reagent are added and diluted with deionized water. It is then extracted with 5 ml of nitrobenzene over 30 sec. After settling out the layers exactly 2.00 ml of nitrobenzene layer are pipetted, and 1 ml of nitrobenzene is added to

TABLE 1
TOLERANCE OF FOREIGN IONS

$BO_3H_2^-$	380	Cl^-	700	CN^-	2	NO_2^-	700
F^-	700	ClO_3^-	30	$Fe(CN)_6^{4-}$	10	SO_4^{2-}	700
BrO_3^-	250	SO_3^{2-}	20	Ftalate	50	NO_3^-	20
$C_2O_4^{2-}$	700	$S_2O_3^{2-}$	50	IO_4^-	30	ClO_4^-	2
IO_3^-	700	$PO_4H_2^-$	100	$B(C_6H_5)_4^-$	3	Br^-	500
CrO_4^{2-}	40	SCN^-	3				

suppress the slight turbidity. The absorbance is measured at 580 nm against nitrobenzene.

Reproducibility of results. Working on one series of 10 samples of 20 ppm in the manner described above, a relative standard deviation of 2.5% was found.

Interference of anions. A series of anions was checked for possible interference following the above procedure. Table 1 shows the tolerance (the concentration of ion that causes an error of 5%) in the determination of 20 ppm of I^-.

We would emphasize the interference produced by several anions, such as SCN^-, CN^-, ClO_4^-, $B(C_6H_5)_4^-$, which are strongly extracted. The interference of oxidants that can coexist with iodide in a neutral solution was checked, the noninterference of BrO_3^-, IO_3^-, NO_2^-, etc., being specially noticeable. We indicate also the noninterference of Cl^- and the slight interference of Br^- in the determination, since the extraction is controlled by, among other factors, anionic atomic volume.

CONCLUSIONS

The extraction of iodide in the presence of the cationic complex tris(2(phenyliminomethyl)pyridide) Fe(II) into nitrobenzene provided a suitable method for the determination of iodide. We emphasize that as the determination is carried out in a neutral solution the presence of oxydants that can coexist in the above solution is permissible, since they are mostly not extracted. On the other hand our method allows the determination of I^- in the presence of Cl^- and of moderate amounts of Br^-.

SUMMARY

The iodide:tris(2-(phenyliminomethyl)pyridine) Fe(II) extraction system into nitrobenzene has been studied. The same method is proposed for the determination of iodide. The interference of foreign ions is also determined.

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BOOK REVIEWS

Analysis with Ion-Selective Electrodes, By J. VESELÝ, D. WEISS, AND K. ŠTULÍK. Halsted Press, New York, 1978. 245 pp., $40.00

This book, translated into English by M. Štulíková, is a part of the Ellis Horwood Series in Analytical Chemistry, edited by R. A. Chalmers and Mary Masson. It is a comprehensive, up-to-date review of the theory and analytical applications of ion-selective electrodes.

The first chapter adequately covers the theoretical aspects of ion-selective electrodes. Such concepts as the electrode potentials at the different types of membranes, the selectivity coefficient, and the calibration of these electrodes are discussed along with the appropriate mathematical expressions.

The second chapter extensively deals with the instrumentation involved in making analytical measurements. Beginning with the construction of the various ion-selective electrodes, the authors go on to discuss the choice of the reference electrode and alternative measuring devices. The importance of the effects of the liquid junction potential and temperature on the potential measurement is emphasized.

The third chapter discusses the various experimental techniques involved in making analytical measurements. These techniques include sample preparation, analyte-addition and subtraction methods, potentiometric titrations, graphical and numerical methods of calculation, and automated measurements. A brief discussion of the applications of ion-selective electrode measurements in biochemistry, biology, and medicine is also included in this chapter.

The fourth chapter gives a detailed summary of the applications of ion-selective electrodes. The determinations of fluorine, chlorine, bromine, iodine, carbon, sulfur, nitrogen, phosphorus, and boron compounds, as well as such metal ions as lithium, sodium, potassium, magnesium, calcium, silver, mercury, copper, lead, and cadmium, are extensively discussed. Various determinations of other inorganic and organic compounds are included.

In addition to 1253 references, useful conversion tables and constants are provided in the book's appendix. This book is recommended for both inorganic and organic analysts in teaching, research, and industry, and may offer some insight into more widespread application in the fields of environmental, biological, medical, and clinical chemistry.

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New Applications of Lasers to Chemistry. Edited by G. M. HIEFTJE. American Chemical Society, Washington, D.C., 1978. x + 244 pp., $23.50.

"New Applications of Lasers to Chemistry," the American Chemical Society Symposium 85, collects and compiles some 12 papers presented at a Symposium which was organized under the auspices of the ACS Division of Analytical Chemistry at the 175th Meeting of the American Chemical Society, Anaheim, California, March 14–15, 1978. All papers were addressed by invited chemists, physicists, and engineers who represent academic

laboratories, industrial organizations, and government agencies. The subject materials ranged from new developments in laser technology and measurement systems to the use of lasers in the detection of single atoms and molecules. The topics are generally categorized as the four main subject areas: high-resolution spectroscopy, high-sensitivity analysis, time-resolved or kinetic spectroscopy, and new techniques in laser Raman spectrometry. These divisions represent, to a large extent, areas of chemistry in which the laser has had greatest impact recently.

The analytical methodology of selective excitation of probe ion luminescence (SEPIL) contributed by J. C. Wright *et al.* appears in the first paper. These include the developments of trace methods for lanthanide ion analysis (La^{3+} itself is the analyte) and the methods for other ions where the analyte ion must be associated with La^{3+}. As have been examined and demonstrated on a number of different chemical systems, the analytically useful lines can be found in 15 of the elements as well as La^{3+} ions. It is believed that the method can be extended to include a majority (if not all) of the ions in the periodic table although considerable more work is required to be done.

In Chap. 2, J. F. Butler *et al.* review and discuss the new applications of tunable diode laser spectroscopy in several areas relating specifically to chemical analysis and measurement, including nonlinear spectroscopy and photochemistry. Particular interest is placed on the illustration of the applications of laser-induced IR spectroscopy to a wide variety of chemical systems. The following paper reports a complete study of a two-photon excited molecular fluorescence spectroscopy carried out by M. J. Wirth and F. E. Lytle. This is a promising method for chemical analysis with a diversity of applications.

Theoretical considerations and experimental illustrations of laser-excited luminescence spectrometry are presented in Chap. 4 by J. D. Winefordner. Pseudocontinuum laser-excited atomic fluorescence (both flame and furnace) spectrometry, narrow line laser-excited both molecular luminescence and luminescence spectrometry, and multiple photon laser-excited luminescence spectrometry are of the primary interest and concern in this paper. These possess tremendous analytical potential of great sensitivity and low detection limits and great selectivity. Then, G. J. Diebold and R. N. Zare provide the evidence of using a high-sensitivity laser fluorimeter incorporated with high-pressure liquid chromatography (HPLC) for detection of aflatoxin B_1 in contaminated corn at ultratrace *ppb* levels in Chap. 5.

Chapter 6 by J. C. Travis *et al.* deals with the laser-enhanced ionization for trace metal analysis in flames. This is a sensitive and selective method. It has a multielement potential and the capability of avoiding traditional spectral interferences.

Chapter 7 by B. R. Ware focuses on the discussion of the application of laser Dopper Velocimetry to detection of electrophoresis, a technique so called "electrophoretic light scattering (ELC)," or a spectroscopically detected electrophoresis. This new technique combines the advantages of speed, accuracy, resolution, and objectivity and will be applied to solving a number of important problems in biology and surface chemistry in the near future.

New laser-based methods for the measurement of transient chemical events are reviewed by G. M. Hieftje *et al.* in Chap. 8. A fluctuation analysis spectroscopic technique for luminescence life time measurement serves as a good example to illustrate their utility.

The ninth paper contributed by S. P. Perone *et al.* is devoted to the description of laser applications in photoelectrochemistry. It reports the results of their studies of laser-induced photoemission processes and laser-induced photolysis. Instrumentation and methodology necessary for examining laser-stimulated photoelectrochemistry have been established in the authors' laboratories.

The 10th paper by B. S. Hudson considers coherent anti-Stokes Raman scattering (CARS) spectroscopy. CARS has recently become an interesting technique because of developments

in high-power tunable lasers. It is the application of a nonlinear optical phenomenon known as three-wave mixing to obtain Raman spectral information. The most important advantages of CARS are its ability to completely reject fluorescence or other isotropic sample luminescence and its potential for very high resolution Raman spectroscopy of gases.

Spectroscopy by inverse Raman scattering (SIRS) is one of many new techniques in molecular spectroscopy made possible by the development of high-power lasers. SIRS offers many unique advantages in the study of molecules, such as the rejection of fluorescence in Raman spectra, the ease for obtaining quantitative information, the high speed at which a Raman spectrum can be recorded, the high spectral resolution achievable, and the simplicity in performing polarization studies. Theoretical considerations, experimental methodology, and practical applications are extensively discussed in Chap. 11 by E. S. Yeung.

The last paper by W. H. Woodruff and S. Farquharson reviews the new development of time-resolved resonance Raman spectroscopy (TR^3) and related vidicon Raman spectroscopy. A range of commonly encountered types of resonant and nonresonant Raman samples are investigated and demonstrated. In addition, a systematic comparison of the capabilities of the vidicon Raman spectrograph and the conventional scanning spectrometer are presented.

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Analytical Profiles of Drug Substances, Vol. 6. Edited by KLAUS FLOREY. Academic Press, New York, 1977. ix + 600 pp., $27.50; and **Analytical Profiles of Drug Substances**, Vol. 7. Edited by KLAUS FLOREY. Academic Press, New York, 1978. ix + 504 pp., $24.00.

The Pharmaceutical Analysis and Control Section of the Academy of Pharmaceutical Sciences has brought about publication of Vols. 6 and 7 of "Analytical Profiles of Drug Substances." This series provides important analytical information about physical and chemical properties, synthesis, methods of analysis, pathways of physical and biological degradation and metabolism, solubility, pH and pK values, spectra and spectrometric constants, and stability data. 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 profiles provide information from important instrumentation including fluorescence, ultraviolet, infrared, nuclear magnetic resonance, mass spectroscopy, differential scanning calorimetry, thermogravimetric analysis, optical rotation, X-ray diffraction, and gas–liquid chromatography.

Drug compounds described in Vol. 6 are: Amphotericin B, Betamethasone Dipropionate, Clonazepam, Cyclizine, Diperodon, Ergotamine Tatrate, Fenoprofen Calcium, Isoniazid, Kananmycin Sulfate, Ketamine, Minocycline, Nystatin, Proparacaine Hydrochloride, Propylthiouracil, Sodium Nitroprusside, Sulfamerazine, Triamcinolone, and Hexacetonide.

Drug compounds described in Vol. 7 are: Alopurinol, Amoxicillin, Chlorpheniramine, Maleate, Dihydroergotoxine Methonesulfonate, Diphenoxylate Hydrochloride, Droperidol, Epinephrine, Ethambutol Hydrochloride, Fluoxymesterone, Hextidine, Hydroflumethiazide, Hydroxyzine Dihydrochloride, 6-Mercaptopurine, Phenobarbital, Sulfamethazine, Thiostrepton, Trimethoprim, and Tubocurarine Chloride.

The concept of analytical profiles is important not only for compendial drugs but also in the industrial research laboratories. Analytical profiles are being prepared and updated to

provide easy reference for the analysis or identification of a chemical substance or product origin. The book provides sufficient detail to give meaningful references and includes a cumulative index and up-to-date bibliographical reference.

As with the preceding volumes, this book provides a wealth of important information and should be welcomed by pharmaceutical chemists and research pharmacists. The book is well organized with information easily obtained and clearly understandable and provides an excellent and quick reference.

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Handbook of Microscopy (Manuel de Microscopie) (in French). By MARCEL LOCQUIN AND MAURICE LANGERON. Masson, Paris, 1978. xii + 352 pp., 152 figures, 160 F.

Microscopy has always been one of the tools used by the microchemist, even when the frequency of its application varied considerably with time and area of special interest. The microchemist should thus be informed about the progress in the methodology in the area. Even when the area of electron microscopy is rather well covered by monographs, optical spectroscopy is rarely dealt with. The review in Pt. 3A of Vol. 1 of "Physical Methods in Chemistry" (edited by A. Weissberger and B. W. Rossiter) published in 1972 seems to be the last attempt to cover this field. The reviewed volume might thus be found useful by those who have command of French. In particular, the first chapter dealing with instruments and instrumental techniques (pp. 1–108) will be found of interest by the chemist. The remainder of the book, dealing with methods of handling biological samples and dyes and their use and applications in histology, cytology, and other areas of biology, is directed more to workers in biological fields. It seems that particulary for instructors in histology and cytology as well as for laboratory supervisors in these areas the book could be very useful. The chemist can obtain from the first chapter systematic and rather detailed information on function, construction, and usage of modern microscopes, including microscopy in uv, use of phase contrast, polarization microscopes, and fluorescence. Attention is paid also to the recording of images, including stereoscopic measurements and micromanipulation, particularly well documented by figures. Some information is offered also on microelectrophoresis, micromethods involving heat, microrefractometry, and radiographic methods. The presentation is systematic, brief, and to the point; experimental details are given in full. The book will be found to be a source of useful information.

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Non-Isothermal Reaction Analysis. By E. KOCH. Academic Press, New York, 1978. xx + 607 pp. $62.50.

The considerations and the results in this book are based on fundamental reaction kinetics as well as on conditions of thermal analysis.

Chapter 1 deals with the concept of reaction analysis, temperature as a parameter, problems, and the present states and aspects of research in reaction analysis.

Chapter 2 deals with the reaction and its temperature dependence. Included are the

accompanying phenomena, the elementary reaction, rate constant, and its dependence on temperature, low temperature investigations, etc.

Chapter 3 presents the nonisothermal reaction kinetics and includes discussions of reactivity function, constant heating rate, nonconstant heating rate, etc.

Chapter 4 treats nonisothermal reaction kinetics such as peculiarities, different reaction mechanisms, and interaction of the fundamental kinetics types.

Chapter 5 discusses differential thermal analysis as a universal method of reaction analysis while the remaining four chapters treat the subjects of foundations and applications of differential thermal analysis in solutions and other methods of reaction analysis.

The book is rich in figures, tables, references, and methematical derivations. It will be found to be of considerable value and interest to those dealing with differential thermal analysis and reaction rates, but is too involved for the beginner.

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Cannabinoid Analysis in Physiological Fluids. Edited by J. A. VINSON. American Chemical Society, Washington, D.C., 1979. 242 pp. $25.00.

Drugs containing tetrahydrocannabinol (THC) are the most widely used of the illicit drugs, with an estimated 300 million people presently users of these drugs worldwide. The extremely low levels of THC present in the body fluids of those using this drug makes it very difficult to measure, a matter of great concern to law enforcement officials and forensic scientists. In recent years several procedures have been developed to measure THC in body fluids at extremely low levels. This book presents papers on these methods originally given at a symposium sponsored by the Division of Analytical Chemistry of the American Chemical Society. It is Vol. 98 of the ACS Symposium Series, edited by Robert Gould.

The volume includes twelve papers and a detailed subject index. The first paper presents a survey of metabolic transformations of tetrahydrocannabinol. The remaining eleven papers discuss a variety of methods for measuring THC in body fluids including: gas–liquid chromatography, high-performance liquid chromatography, thin-layer chromatography, mass spectrophotometry (MS), GC/MS procedures, and radioimmunoassay methods.

The papers present detailed methodology which can be immediately used for analysis. Those involved in the measurement of THC in body fluids will find this book to be a valuable addition to their libraries.

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A Handbook of Decomposition Methods in Analytical Chemistry. By RUDOLF BOCK. Translated and revised by IAIN L. MARR. Wiley, New York, 1979. xii + 444 pp. $54.95.

This book surveys the methods required to decompose a sample. It treats the solvent systems required for the "dissolution" of a material (dissolving it in a liquid system) and the steps to be taken if this fails—the "opening out" of the sample. "Opening out" refers

primarily to the high-temperature decompositions as are achieved by the use of fluxes. If you need to know how to decompose, for example, tungsten carbide, ores, lead glass, titanium alloys, clays, ferroberyllium, coal, cement, oil shale, boron nitride, etc., as well as simpler materials such as calcium carbonate or brass alloys, your source for information is at hand. The treatment of alloys and inorganic related materials is excellent and easy to find. If you need a quick solution on decomposing "Teflon," "nylon," neoprene, or paint you may be disappointed. It probably is in the book, but you will have difficulty finding the information. The decomposition of organic and biological material is covered. However, compared to the treatment of the inorganic material, it is quite scant. This is also reflected in the index cross-referencing. Finding a treatment to decompose inorganic material is excellent. There are a large number of literature citations; including the current literature. The book is most recommended to anyone as a first source in decomposing any sample, particularly inorganic material. If it only did an equivalent job for organic material, the book would be recommended for every chemist's book shelf.

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Table of Molecular Weights. A Companion Volume to the Merck Index, 9th ed. Edited by MARTHA WINDHOLZ, SUSAN BUDAVARI, MARGARET NOETHER FERTIG, AND GEORG ALBERS-SCHÖNBERG. Merck, Rahway, New Jersey, 1978. vii + 257 pp. + APP-6.

The book opens with a table of the atomic masses of most abundant isotopes. This is followed by a table (257 pages) which gives the molecular weights (to the sixth decimal), the empirical formulas, and the compound names and monograph numbers of all compounds listed in the monograph. For example, for a molecular weight of 75.032028 and an empirical formula of $C_2H_5NO_2$, it lists ethyl nitrite, glycine, methyl carbamate, and nitroethane, along with the monograph number of each compound. The last molecular weight shown is 3053.648265 for porcine secretin, whose empirical formula is $C_{130}H_{220}N_{44}O_{41}$ and whose monograph number is 7173.

The appendix (6 pages) shows the atomic masses and abundances of naturally occurring isotopes. It lists the atomic number, element, mass number, mass of each isotope to the sixth decimal, and interim isotopic compositions for average properties in percentages.

This book will be invaluable to the mass spectroscopist as well as the student of advanced organic chemistry utilizing mass spectral data. Certainly no library can afford to be without one.

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MICROCHEMICAL JOURNAL 25, 601–602 (1980)

Author Index for Volume 25

Microchemical Journal

devoted to the application of microtechniques in all branches of science

Editor: Al Steyermark

Volume 25

1980

Published under the auspices of the American Microchemical Society by

ACADEMIC PRESS

New York London Toronto Sydney San Francisco

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NUMBER 1, MARCH 1980

NUMBER 2, JUNE 1980

NUMBER 3, SEPTEMBER 1980

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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.

Original papers only will be considered. Manuscripts are accepted for review with the understanding that the same work has not been and will not be published nor is presently submitted elsewhere, and that all persons listed as authors have given their approval for the submission of the paper; further, that any person cited as a source of personal communications has approved of the paper; further, that any person cited as a source of personal communications has approved such citation. Written authorization may be required at the Editor's discretion. Articles and any other material published in the *Microchemical Journal* represent the opinions of the author(s) and should not be construed to reflect the opinions of the Editor(s) and the Publisher.

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1. Ames, S. R., Swanson, W. J., and Harris, P. L. Biochemical studies on vitamin A. XIV. Biopotencies of geometric isomers of vitamin A acetate in the rat. *J. Amer. Chem. Soc.* 77, 4134–4136 (1955).
2. Pease, D. C., "Histological Techniques for Electron Microscopy," 274 pp. Academic Press, New York, 1960.
3. Stern, K. C., Electrophoresis and ionophoresis. *In* "Physical Techniques in Biological Research" (G. Oster, ed.), Vol. 2, pp. 61–121. Academic Press, New York, 1956.

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