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Briefs

Biamperometric End-Point Detection with Quinhydrone Electrodes in Coulometric Neutralization Titrations in Nonaqueous Media. F. F. GAÁL, D. LJ. KUZMIĆ, AND R. I. HORVAT, *Institute of Chemistry, Faculty of Sciences, University of Novi Sad, Veljka Vlahovica 2, 21000 Novi Sad, Yugoslavia.*

Some new possibilities for the continuous titration of bases, alone and in mixtures, as well as of mineral acids, alone and in mixtures, were investigated in nonaqueous media.

Microchem. J. **23**, 417–425 (1978).

An Evaluation of Acid Digestion Methods for Amniotic Fluid Phospholipids. HUGH Y. YEE, *The Department of Pathology, Hutzel Hospital, 4707 St. Antoine, Detroit, Michigan 48201.*

Five methods of acid digestion of amniotic fluid phospholipids for inorganic phosphate release were evaluated at a temperature of 225°C and a heating time of 30 min. All methods were found to be equivalent.

Microchem. J. **23**, 426–432 (1978).

Indirect Amplification Method for Determining Mercury by Direct-Current Polarography. Application to Organomercury Compounds. S. W. BISHARA, S. A. RAHIM, AND F. M. EL-SAMMAN, *Department of Chemistry, College of Science, Mosul University, Mosul, Iraq.*

An amplification method for the determination of 0.5–70 ppm of Hg (II) is described. The Hg(II) is reacted with an excess of KI and the excess is oxidized with bromine and measured polarographically as iodate with a sixfold amplification.

Microchem. J. **23**, 433–444 (1978).

A Colorimetric Method for the Determination of Carboxylic Acids. MAMDOUH Y. KAMEL AND ZEINAB A. EL-AWAMRY, *Laboratory of Biochemistry, National Research Centre, Dokki, Cairo, Egypt A. R. E.*

The color formed with bromocresol green follows Beer's law and is stable for 24 hr. A linear relationship between the pK_1 of different acids and the calculated molar extinction coefficients of the color formed at 440 nm is demonstrated.

Microchem. J. **23**, 445–452 (1978).

3-Methyl-4-amino-5-mercapto-1,2,4-triazole as a Ring-Colorimetric Reagent for the Selective Microevaluation of Au(III), Tl(I), and Ag(I). K. N. JOHRI AND SHASHI SAXENA, *Department of Chemistry, University of Delhi, Delhi-110007, India.*

3-Methyl-4-amino-5-mercapto-1,2,4-triazole has been studied for its microanalytical uses. Selective evaluation of the three metals has been carried out, incorporating masking and solvent extraction techniques in conjunction with ring colorimetry.

Microchem. J. **23**, 453–459 (1978)

A Semi-Automated and Manual Method for Determining the Total Phospholipid Phosphorus in Amniotic Fluid. HUGH Y. YEE AND BOBETTE JACKSON, *The Department of Pathology, Hutzel Hospital, 4707 St. Antoine, Detroit, Michigan 48201.*

The method described requires 1 ml of sample. Perchloric acid containing a trace of molybdate is used to convert to inorganic phosphate. Stannous chloride–hydrazine sulfate is used as the reducing agent for phosphate color development.

Microchem. J. **23**, 460–465 (1978).

Evaluation of Toluene as Replacement for Benzene in Tetrabutylammonium Hydroxide Titrant. WALTER SELIG, *Lawrence Livermore Laboratory, University of California, Livermore, California 94550.*

Toluene has been evaluated as a replacement for benzene in tetrabutylammonium hydroxide titrant and the precision obtained with the titrants is identical. In view of the greater toxicity of benzene, toluene is recommended as a replacement.

Microchem. J. **23**, 466–468 (1978).

Dilution Method in X-Ray Fluorescence Spectrometry. B. W. BUDESINSKY, *Phelps Dodge Corporation, Morenci, Arizona 85540.*

The Lachance–Traill method was modified to a dilution method that retains all the advantages of the original method plus it enables the determination of as many elements as one pleases and is far simpler mathematically. The method has been tested with the determination of copper, molybdenum, zinc, and arsenic in copper intermediates.

Microchem. J. **23**, 469–472 (1978).

Separation of Vanillylmandelic (VMA) and Homovanillic (HVA) Acids from Urine for Assay by a Two Resin Column System. ELLEN C. CALVARY, ROBERT L. MURRAY, AND SAMUEL NATELSON, *Department of Biochemistry of the Michael Reese Hospital and Medical Center, Chicago, Illinois 60616.*

Urine is passed over a cation exchange column to remove catechol amines and amino acids. The HVA and VMA are then adsorbed on an anion exchange column. The VMA can be determined by oxidation to vanillin directly.

Microchem. J. **23**, 473–480 (1978).

Monothiourea-3-nitrophthalic Acid as a New Reagent for Spectrophotometric Analyses. I. Determination of Palladium(II) Ions. ZBIGNIEW GREGOROWICZ AND EWA BOBROWSKA, *Instytut Chemii Analitycznej i Ogólnej, Politechniki Ślaskiej, 44 100 Gliwice, Pstrowskiego 2, Poland.*

The reaction between the reagent and the metal ion was studied as a possible means of analysis and an extraction-spectrophotometric method was developed.

Microchem. J. **23**, 517–520 (1978).

Monothiourea-3-nitrophthalic Acid as a New Reagent for Spectrophotometric Analyses. II. Determination of Ruthenium(III) Ions. ZBIGNIEW GREGOROWICZ AND EWA BOBROWSKA, *Instytut Chemii Analitycznej i Ogólnej, Politechniki Ślaskiej, 44 100 Gliwice, Pstrowskiego 2, Poland.*

The reaction between the new reagent and the metal was studied and a spectrophotometric method was developed.

Microchem. J. **23**, 521–524 (1978).

Spectrophotometric Determination of Germanium by Phenylfluorone. S. K. TOBIA, M. F. EL-SHAHAT, AND E. A. SAAD, *Chemistry Department, Faculty of Science, Ain Shams University, Abbassia, Cairo, Egypt, A.R.E.*

Study was made of the reaction between germanium ions and phenylfluorone which resulted in a spectrophotometric determination of that element. The sensitivity of the method was found to be 2.55×10^{-3} mg/cm^3.

Microchem. J. **23**, 525–529 (1978).

Zinc and Cadmium Complexes of 2,2′-Dipyridyl-2-pyridylhydrazone as Visual Acid–Base Indicators. H. ALEXAKI-TZIVANIDOU AND G. KOUNENIS, *Laboratory of Physiology, Veterinary Faculty, University of Thessaloniki, Thessaloniki, Greece.*

The complexes of the reagent with zinc and cadmium are suggested as very useful indicators in visual acid–base titrations in aqueous solution and give sharp end points.

Microchem. J. **23**, 530–536 (1978).

Monothiourea-3-nitrophthalic Acid as a New Reagent for Spectrophotometric Analyses. I. Determination of Palladium(II) Ions. ZBIGNIEW GREGOROWICZ AND EWA BOBROWSKA, *Instytut Chemii Analitycznej i Ogólnej, Politechniki Šlaşkiej, 44 100 Gliwice, Pstrowskiego 2, Poland.*

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Microchem. J. **23**, 517–520 (1978).

Monothiourea-3-nitrophthalic Acid as a New Reagent for Spectrophotometric Analyses. II. Determination of Ruthenium(III) Ions. ZBIGNIEW GREGOROWICZ AND EWA BOBROWSKA, *Instytut Chemii Analitycznej i Ogólnej, Politechniki Šląskiej, 44 100 Gliwice, Pstrowskiego 2, Poland.*

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Microchem. J. **23**, 530–536 (1978).

Determination of Stability Constant of Copper(II) with Thiosemicarbazide by Use of a Cupric Ion-Selective Electrode. MARCO TADDIA, *"G. Ciamician" Chemical Institute of the University, Via Selmi 2, 40126 Bologna, Italy.*

The stability constant of copper(II)-thiosemicarbazide complex was determined potentiometrically by means of a cupric ion-selective electrode and corresponded well with the values given in the literature measured spectrophotometrically.

Microchem. J. **23**, 537–540 (1978).

An Improved Kinetic Determination for Creatinine Using the Abbott ABA-100. R. J. LAPLANTE, R. J. THIBERT, AND H. S. ASSELSTINE, *Department of Chemistry, University of Windsor, Windsor, Ontario, N9B 3P4, Canada, and Department of Pathology, Metropolitan General Hospital, Windsor, Ontario, N8W 1L9, Canada.*

An improved method for the kinetic determination of creatinine on the Abbott ABA-100 is described. By simplex optimization of the picrate and hydroxide reaction concentration a 35% increase in reaction rate, with respect to the Abbott methodology, was obtained. The new method correlated more closely with the Technician AutoAnalyzer Method (N-30) than did the latter method.

Microchem. J. **23**, 541–551 (1978).

A Fast, Simple Colorimetric Determination of Total Urinary Estrogen. R. J. LAPLANTE, R. J. THIBERT, AND H. S. ASSELSTINE, *Department of Chemistry, University of Windsor, Windsor, Ontario, N9B 3P4, Canada and Department of Pathology, Metropolitan General Hospital, Windsor, Ontario, N8W 1L9, Canada.*

A relatively rapid simplified colorimetric method which uses an enzymatic hydrolysis for the determination of total urinary estrogen is described. All reagents and the enzyme preparation are prepared within the laboratory. A comparative study of the hydrolytic ability of β-glucuronidase from *E. coli* and *Helix pomatia* indicated the latter to be the enzyme of choice for the proposed methodology.

Microchem. J. **23**, 552–565 (1978).

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Biamperometric End-Point Detection with Quinhydrone Electrodes in Coulometric Neutralization Titrations in Nonaqueous Media

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INTRODUCTION

The end-point determination in coulometric neutralization titrations is most frequently performed by application of various instrumental methods. For this purpose in our laboratories the following methods were developed: visual (*12, 14*), catalytic thermometric (*12–14*), manometric (*11*), potentiometric at a small constant current (*1–3, 15*), and biamperometric (*6, 7, 16, 17*). In the last two methods quinhydrone electrodes with a platinum as metal phase were often applied (*1, 2, 11, 15, 16*); the use of palladium and gold for this purpose is less frequent (*11, 13*). The less noble metals such as antimony (*17*), bismuth (*6*), tin (*7*), and tungsten (*10*) proved to be less suitable for the end-point determination of these titrations.

Mineral acids, in contrast to organic ones, were not often determined by coulometric titrations. Applying the potentiometric or the visual end-point determination, sulfuric acid was coulometrically titrated as a two-base acid in t-butanol (*5*). Perchloric acid was determined in various nonaqueous solvents, such as dimethylsulfoxide (*4*), isopropanol (*9*), and acetic acid–acetic anhydride mixture (*10*).

This paper deals with the possibilities of the application of continuous coulometric titration of both mineral acids and organic bases, alone and in some mixtures. The end-point determination is biamperometric using the quinhydrone electrodes with platinum, palladium, and gold as the metal phase.

MATERIALS AND METHOD

Reagents. The substance to be determined, organic bases and mineral acids, were either pharmaceutical products or p. a. chemicals. They were used without any additional purification.

Acetic anhydride, the solvent of organic bases, was purified as follows: Having been refluxed at least 1 hr, the solution of 1 liter of anhydride with 2 ml of concentrated sulfuric acid was distilled under atmospheric pressure, the distillate being taken at 136°C.

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In determinations of mineral acids, particular solutions were made in anhydrous acetic acid which was prepared as described earlier (*18*).

Sodium perchlorate, 0.1 *M* in acetic anhydride served as a conductivity salt for all titrations of bases. To improve the solubility of sodium acetate and theobromine in pure acetic anhydride, 5 and 10% of glacial acetic acid, respectively, were added to it.

Solutions of mineral acids were prepared in anhydrous acetic acid which was 0.15 *M* in sodium perchlorate.

The corresponding solutions of the supporting electrolyte, as mentioned previously, served as catholytes in determinations of organic bases, and as anolytes in mineral acid titrations.

The quinhydrone added to saturation to each probe in biamperometric coulometric titrations of bases was substituted for hydroquinone in corresponding catalytic thermometric determinations. As each of them is an anode depolarizer, they served as the source of hydrogen ions. In the same time, the quinhydrone, together with metal phase (Pt, Pd, Au), represented the biamperometric indicator electrode system. Hydroquinone, in the case of catalytic thermometric titrations, served as a component of the indicator reaction (*14*).

In photometric determinations of acids a 0.5% solution of malachite green and a 0.02% solution of sudan III, both prepared in glacial acetic acid, were used.

All mentioned solutions of bases were standardized by the catalytic thermometric method (*12–14*), and the acid solutions were standardized by photometric titration in the presence of both indicators alone.

Apparatus. The device for coulometric titrations and the apparatus for catalytic thermometric and photometric end-point determination were prepared as already described (*6, 8, 13*). It was observed that the end-point determination in photometric titrations was more accurate if it was performed from the maxima in titration curves, i.e., from minima in corresponding derivative titration curves. The latter ones were determined by use of an *RC* circuit ($R=9500\ \Omega$, $C=1000\ \mu F$).

In biamperometric titrations the usual apparatus was employed (*6*). Metal phases of the quinhydrone electrodes (0.5-cm^2 area) of platinum, palladium, and gold were prepared in our laboratory. Electrode pretreatment consisted of mild heating in an ethanol flame before each titration (*3, 15*).

Titration curves were continuously registered by the Servogor recorder, Type RE 511.

Procedure. Take an aliquot of 2.00 ml of the base solution (0.6–1.4 mg of base) or 4.00 ml of the acid solution (2 mg of acid). Dilute it to 8.0 ml with the supporting electrolyte in the anodic compartment of the coulometric titration cell when bases are to be determined, or in cathodic

one when titrations of acids are to be performed. In photometric titrations of acids the final dilution is 12.0 ml. To the cathodic (or anodic) compartment of the cell add the catholyte (or anolyte) so that the levels of the solution in both compartments are equal.

Polarize the metal phase of the quinhydrone electrode with the preselected voltage and dip it into the solution.

Due to the influence of the generator circuit, at the moment it is switched on, on the indicator system, the pen recorder charts a vertical line which enables us to determine the start of the titration very precisely.

RESULTS AND DISCUSSION

To find optimum electroanalytical conditions for biamperometric determinations of the particular system under investigation, the titrations

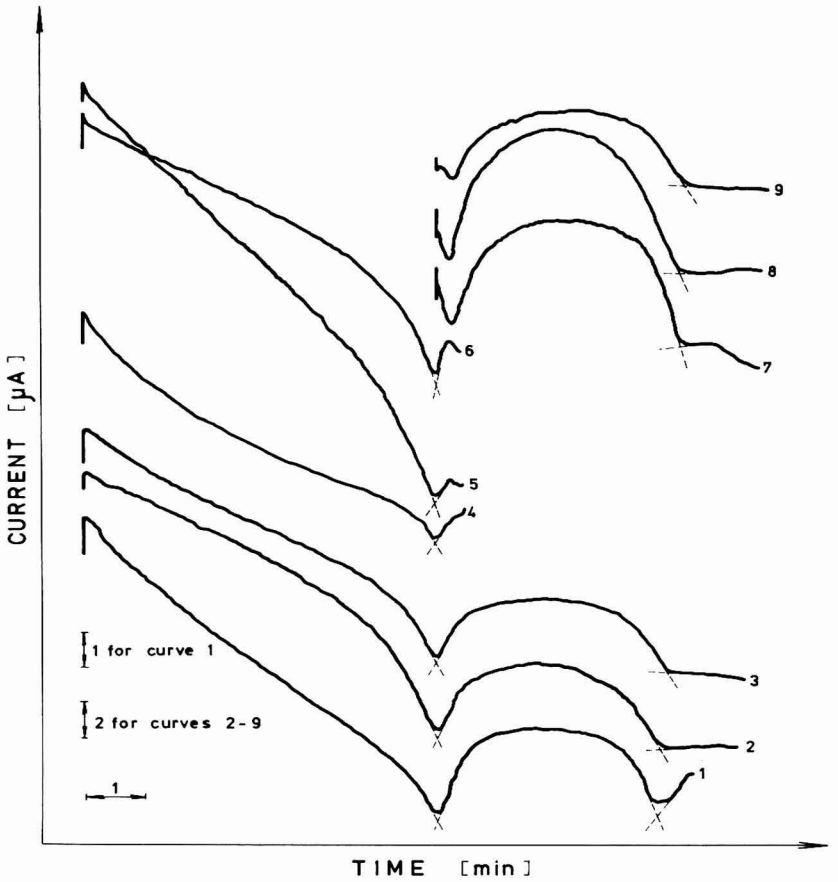

FIG. 1. Biamperometric titration curves of the strong base, triethylamine (4, 5, 6), the weak base, caffeine (7, 8, 9), and their mixture (1, 2, 3) obtained with the quinhydrone electrodes with metal phases: Pt (3, 6, 9), Pd (2, 5, 8), and Au (1, 4, 7). Generating current: 2.50 mA.

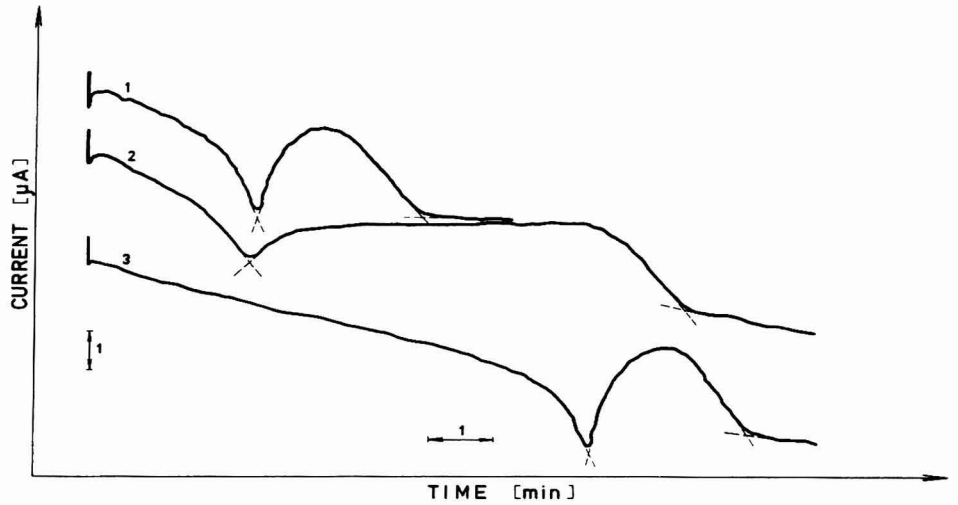

FIG. 2. Biamperometric titration curves of aminopyrine (1), mixture of aminopyrine and caffeine (2), and mixture of aminopyrine and triethylamine (3) obtained with the quinhydrone electrodes with metal phase Pt (1, 2, 3). Generating current: 2.50 mA.

were performed in the presence of quinhydrone electrodes polarized with various voltages. The polarization voltages of the electrodes which enable the registration of biamperometric titration curves with the most convenient shape are 100 mV for platinum and palladium, and 200 mV for gold as metal phase in determinations of bases; in titrations of acids the voltages are 150 mV for platinum, 200 mV for palladium, and 300 mV for gold metal phase.

Base Titration

Typical biamperometric titration curves are bases of different strengths, triethylamine and caffeine, are shown in Fig. 1 (curves 4–9). As can be seen, at the equivalence point a sharp minimum in the curve appears, so that the end point of the titration can be easily determined. In the same figure the titration curves of the mixture of the two bases are also given. It is seen that both components of the mixture can be determined due to well-separated, sharp minima corresponding to the equivalence points of titrations of the stronger base (triethylamine, first minimum) and the weaker one (caffeine, second minimum).

Since the supporting electrolyte has minimal basic properties, it is necessary to correct the results of particular titrations of bases. For this purpose the same volume of electrolyte as that in the probe was titrated. The only change in the experimental conditions was that the generating current and the chart rate were one half of those used in the base titration. The correction term thus obtained was subtracted from the result of titration of a base alone. It was established that in the titrations of base

TABLE I

RESULTS OF COULOMETRIC TITRATIONS OF BASE MIXTURES WITH BIAMPEROMETRIC TITRATION END-POINT DETERMINATION

Substance titrated		Catalytic thermometric titration			Biamperometric titration								
					Electrodes								
					Pt–Pt			Pd–Pd			Au–Au		
		No. of titrations	Microequivalents found	Average deviation (%)	No. of titrations	Microequivalents found	Average deviation (%)	No. of titrations	Microequivalents found	Average deviation (%)	No. of titrations	Microequivalents found	Average deviation (%)
Caffeine	Alone in a	6	6.37	0.8	6	6.35	0.5	6	6.35	0.4	6	6.19	1.0
	mixture with triethylamine	—	—	—	7	6.32	1.0	6	6.24	1.2	6	6.37	1.0
Triethylamine	Alone in a	8	8.99	0.9	6	9.07	0.1	6	9.04	0.2	6	9.07	0.4
	mixture with caffeine	—	—	—	7	9.04	0.9	6	8.91	0.6	6	8.94	0.9
Pyridine	Alone in a	6	8.94	0.5	6	8.76	1.0	6	8.65	0.3	6	8.68	0.8
	mixture with theobromine	—	—	—	7	8.94	1.1	6	8.96	0.8	6	8.94	0.6
Sodium acetate	Alone in a	6	9.79	0.3	6	9.58	0.5	6	9.61	0.5	6	9.64	0.5
	mixture with theobromine	—	—	—	8	9.64	0.8	7	9.64	0.2	8	9.74	0.9
Theobromine	Alone in a	7	7.20	0.8	7	7.33	0.6	6	7.44	1.1	6	7.31	0.6
	mixture with pyridine in a mixture with Na acetate	—	—	—	6	7.49	0.8	6	7.38	1.0	6	7.64	0.6
		—	—	—	6	7.41	1.2	6	7.38	0.9	6	7.44	1.4

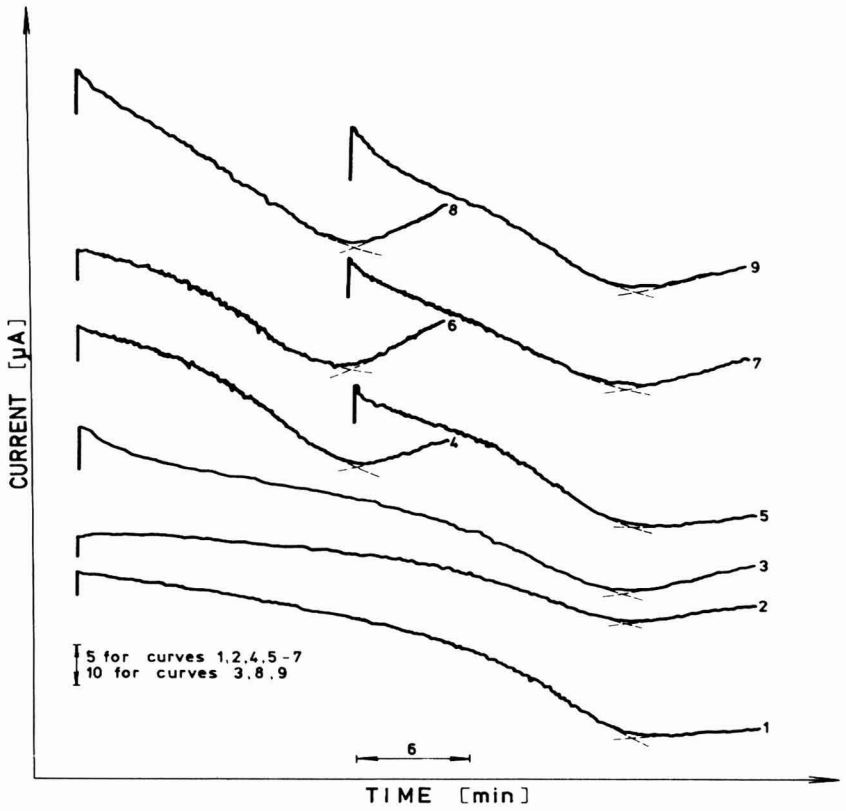

FIG. 3. Biamperometric titration curves of perchloric acid (4, 6, 8), sulfuric acid (5, 7, 9), and of their mixture (1, 2, 3) obtained with quinhydrone electrodes with metal phases Pt (1, 4, 5), Pd (2, 6, 7), and Au (3, 8, 9). Generating current: 2.50 mA.

mixtures the supporting electrolyte was titrated with the weaker component; therefore the correction term has to be subtracted from the result of its determination.

The reproducibility and accuracy of biamperometric determinations of bases, alone and in mixtures, using the quinhydrone electrodes, can be seen in Table 1. The average deviation is less than 1.4%. The agreement between the results of this method and those of the catalytic thermometric titration is satisfactory; the average error is less than ±2%.

It was of interest to investigate the possibilities for application of the biamperometric method to the determination of diacid bases like aminopyrine. The typical titration curve is shown in Fig. 2, curve 1. As can be seen, two well-marked minima are obtained in the curve, corresponding to each equivalent of the base. From the biamperometric titration curves of aminopyrine in the mixture with caffeine, i.e., triethylamine (the same figure, curves 2 and 3, respectively, it can be observed that caffeine

TABLE 2

RESULTS OF COULOMETRIC TITRATIONS OF MINERAL ACIDS WITH BIAMPEROMETRIC TITRATION END-POINT DETERMINATION

Acid titrated	Photometric titration						Biamperometric titration								
	Indicator						Electrodes								
	Sudan III			Malachite green			Pt–Pt			Pd–Pd			Au–Au		
	No. of titrations	Micro-equivalents found	Average deviation (%)	No. of. titrations	Micro-equivalents found	Average deviation (%)	No. of titrations	Micro-equivalents found	Average deviation (%)	No. of titrations	Micro-equivalents found	Average deviation (%)	No. of titrations	Micro-equivalents found	Average deviation (%)
Perchloric	6	20.95	0.2	6	20.98	0.4	7	20.79	0.7	7	20.82	0.8	6	20.75	0.5
Sulfuric	7	21.39	0.3	6	21.74	0.5	8	21.10	0.7	6	21.13	0.9	8	21.09	0.6
Hydrochloric	6	46.57	0.7	6	46.86	0.8	—	—	—	—	—	—	—	—	—
Perchloric + sulfuric	6	43.11	0.2	6	43.34	0.3	7	43.54	0.3	7	43.51	0.3	6	43.11	0.3
Perchloric + hydrochloric	6	65.20	0.5	6	65.74	0.4	—	—	—	—	—	—	—	—	—

is titrated with the second equivalent of aminopyrine, while triethylamine, as a strong base, is titrated with the first equivalent. In this simple way it is possible to get information about the content of a basic admixture in aminopyrine.

Acid Titration

In Fig. 3 are given the biamperometric titration curves of perchloric and sulfuric acid, alone and in their mixture, obtained by various pairs of indicator electrodes. At the equivalence point a current minimum appears, from which the end point can be determined in a simple way. If sulfuric acid is titrated, only the signals which correspond to the first equivalent can be registered. When acid mixtures were titrated only the total acidity of the system could be determined. Hydrochloric acid could not be determined biamperometrically, but only by photometric titration.

Some of the results of biamperometric determinations of acids, alone and in mixture, given in Table 2, show that the average deviation is less than $\pm 0.9\%$, and the average error is less than $\pm 1.2\%$.

SUMMARY

Some new possibilities for continuous coulometric titration of bases, alone and in mixture, as well as of mineral acids, alone and in mixture, were investigated with acetic anhydride–anhydrous acetic acid as solvents. The titration end point was determined biamperometrically. It was established that quinhydrone electrodes with platinum, palladium, and gold as the metal phase can be equally satisfactorily applied to the end-point determination of both the titrations of bases or acids alone, and of their two-component mixtures. Amounts of 6–10 μequiv. of bases, i.e., 20–47 μequiv. of mineral acids, were determined with the maximal average deviation of 1.4%. Results of biamperometric determinations of bases are compared to those of catalytic thermometric determinations, while those of acids are compared with the results of photometric titrations. The agreement between the methods is satisfactory.

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An Evaluation of Acid Digestion Methods for Amniotic Fluid Phospholipids

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INTRODUCTION

Investigations have shown that there is a continuity between the fetal lung and maternal amniotic fluid, so that the phospholipids present in the fetal lung tissue would very likely be found in the amniotic fluid (*9*). Fortunately, this has proven to be the case for human amniotic fluid, and its analysis provides valuable information for determining fetal lung maturity. Thus, the estimation of the phospholipid concentration of amniotic fluid has become increasingly important as a diagnostic test (*2–4, 8*). The most widely used and accepted of these measurements are the total phospholipid phosphorus (TTP) (*8*) and the lecithin/sphingomyelin ratio (L/S) (*4*). Several phospholipids which have been identified and/or isolated are: phosphatidyl choline (PC; lecithin), phosphatidyl glycerol (PG), phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE), lysophatidyl choline (LPC; lysolecithin), sphingomyelin (S), and the cardiolipins (*5*).

The quantitative analysis of phospholipids consist of extraction, release of inorganic phosphate from an organic matrix, and the measurement of the released phosphate. Our work with the TPP, used to assess fetal lung maturity, has necessitated the development of a rapid and reliable method to quantitate all of the phospholipids found in amniotic fluid. A simple means of phosphate release that could be reproduced easily by most clinical laboratories was considered essential. The most common approach has been to employ wet acid digestion. Perchloric and/or sulfuric acid used together or individually with various catalysts such as selenious acid or vanadium pentoxide are by far the most popular (*1*). An investigation was made to define the optimum conditions which would be the most practical for the clinical laboratory to accomplish acid digestions.

MATERIALS AND METHOD

Reagents

Phospholipids. Phosphatidyl glycerol, sphingomyelin, and phosphatidyl choline (synthetic dipalmitoyl lecithin) were obtained from

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Sigma Chemical Co., St. Louis, Mo. Phosphatidyl ethanolamine, phosphatidyl serine, and phosphatidyl inositol were from P-L Biochemicals, Inc., Milwaukee, Wis. Lysophosphatidyl choline was from Supelco, Bellefonte, Pa. All of the phospholipids were dissolved in chloroform to give a concentration of 20 mg/100 ml.

Stock phosphate standard (0.1 mg/ml P).

Aqueous phosphate standards. Prepare standards of 0.05, 0.1, 0.2, 0.3, and 0.5 mg/100 ml P.

Phosphate standard (0.1 mg/100 ml P in 1.2 mol/liter sulfuric acid).

Molybdate reagent. Dissolve 3 g of ammonium molybdate tetrahydrate into water. Add 6.6 ml of sulfuric acid and mix. Dilute to 100 ml.

Reducing solution. Dissolve 0.3 g of hydrazine sulfate and 0.03 g of stannous chloride dihydrate into water. Add 2.8 ml of sulfuric acid and mix. Dilute to 100 ml.

Method A reagent. 70% perchloric acid.

Method B reagent. Add 0.12 ml of molybdate reagent to 100 ml of 70% perchloric acid and mix.

Method C reagent. Sulfuric acid; 5 mol/liter. 30% hydrogen peroxide. Urea solution; dissolve 0.5 g of urea per 10 ml of water.

Method D reagent. Carefully with cooling, add 55 ml of perchloric acid to 45 ml of sulfuric acid.

Method E reagent. Add 4 ml of water to 40 ml of sulfuric acid. Dissolve 0.2 g of vanadium pentoxide into 5 ml of 1 mol/liter of sodium hydroxide. Add 0.5 ml of the vanadium pentoxide solution to the diluted sulfuric acid. Add 4 ml of 70% perchloric acid and mix.

Method

One-milliliter aliquots of the phospholipids solutions (20 mg/100 ml in chloroform) were evaporated to dryness using an air stream in 55°C water bath. Aqueous phosphate standards (1 ml) were pipeted into a tube and the digestant reagent was added along with a boiling granule. Amniotic fluid pools (1 ml) were extracted with 4 ml of a 2:1 chloroform–methanol solution. The solvent extract was separated and evaporated to dryness at 55°C using an air stream.

The residues or solutions were digested with the appropriate digestant reagent for a timed interval, generally 30 min or otherwise indicated, at a temperature of 225°C using a heating block (Hycel). After cooling, the solutions were diluted with the designated volume of water as indicated in the following directions:

Method A. Add 0.6 ml of digestant reagent to the residue. After digestion, cool, and dilute with 2 ml of water. The dilution factor is 2.5 ×.

Method B. Same directions as for A.

Method C. Add 0.5 ml of the 5 mol/liter of sulfuric acid to the res-

idue. After digesting at the desired time interval, remove the tube from the block, let cool, and add one drop of 30% hydrogen peroxide. Heat the tube for 10 min at 225°C. Again remove the tube and let cool. Add one drop of urea solution. Heat at 225°C for 10 min. Remove the tube, let it cool, and add 2 ml of water. The dilution factor is 2.1×.

Method D. Add 0.35 ml of digestant reagent to the residue. After digestion, cool and add 2.9 ml of water. The dilution factor is 3×.

Method E. Add 0.18 ml of digestant reagent. After digestion, cool and dilute with 2.4 ml of water. The dilution factor is 2.5×.

Color development is carried out by pipeting 0.5 ml of the diluted digest into a clean tube. For a standard, 0.5 ml of the 0.1 mg/100 ml P standard in 1.2 mol/liter of sulfuric acid is used. As a blank, pipet 0.5 ml of 1.2 mol/liter of sulfuric acid into a tube. Add 2 ml of molybdate reagent to all tubes and mix. Add 1 ml of the reducing solution to all tubes and mix. Let stand 5 min and measure the absorbance of all tubes against the blank at 660 nm within 15 min. Use the appropriate dilution factor for the calculation of the sample concentration.

RESULTS AND DISCUSSION

Prior to our investigation, two constraints were made for the methods selected. These were: (a) the decomposition was to be carried out at a maximum temperature of 225°C, so that a relatively inexpensive heating block could be utilized, and (b) the final acid concentration of about 2.5 mol/liter H^+ was to be obtained by diluting with no more than 3 ml of water that would provide the proper pH for a modified phosphate method which employed stannous chloride–hydrazine (*11*). Five methods of phosphate release were evaluated for their effectiveness for the phospholipids found in amniotic fluid. Method A used perchloric acid (*7*); method B used perchloric acid containing molybdate; C used sulfuric acid with 30% hydrogen peroxide and 5% urea (*10*); D used a mixture of 55 parts perchloric and 45 parts sulfuric acids; and E used a mixture of sulfuric acid, perchloric acid, and vanadium pentoxide (*6*).

The time of heating at 225°C for decomposition of the organic matrix was determined by measuring recoveries of standards containing 20 mg/100 ml of phosphatidyl choline and sphingomyelin with methods A–E. The selection of these phospholipids was based upon the fact that PC accounts for 65% and S for 15% of the total phospholipid content of amniotic fluid. The most difficult phospholipid to hydrolyze is sphingomyelin (*6*), so that the time required for a high recovery of it will be a controlling factor. Tables 1 and 2 show the data obtained. Examination of the data will show that all methods are suitable for re-

TABLE 1
RECOVERY OF SPHINGOMYELIN

	Time of heating (min)				
	10	15	30	45	60
Method	Percentage recovery				
A	76.3	81.8	82.3	92.7	98.1
B	75.8	81.8	90.8	104.1	101.2
C	100.2	101.2	102.2	99.5	106.3
D	86.4	87.2	88.6	91.5	91.5
E	89.6	92.7	98.1	98.1	100.5

leasing inorganic phosphate from amniotic fluid phospholipids. Methods C and E could be used with heating times of 10 or 15 min. All methods were satisfactory with a heating time of 30 min. Longer heating times of up to 60 min did not appear to significantly affect recoveries, so that under our conditions the heating time is not critical between 30 and 60 min. However, adherence to a selected time is advisable to minimize any variations. Also, the longer heating times will give a slightly greater loss of acid that may cause problems in maintaining the appropriate pH for the color reaction. We have selected a heating time of 30 min, because it is the shortest time which will yield good recoveries for all methods of digestion. Obviously, the choice of method and the time of heating will be based upon the user's preference. Factors which may influence a decision are: (a) the convenience of preparation of the acid mixture, (b) the number of additional manipulations, (c) the number of additional reagents, and (d) the phosphate color reaction used.

With the time and temperature of acid digestion established, the recovery of various phospholipids was used to assess the efficiency of phosphate release. This data is shown in Table 3. Method A using

TABLE 2
RECOVERY OF PHOSPHATIDYL CHOLINE (LECITHIN)

	Time of heating (min)				
	10	15	30	45	60
Method	Percentage recovery				
A	89.7	91.0	92.2	93.9	105.0
B	89.7	93.9	93.9	101.8	106.2
C	102.0	102.0	102.0	109.5	109.5
D	84.4	97.3	107.6	110.7	110.7
E	98.6	105.0	103.4	101.8	102.0

TABLE 3
RECOVERY OF THE PHOSPHOLIPIDS USING VARIOUS METHODS OF INORGANIC PHOSPHATE RELEASE

Phospholipid	Method A	B	C	D	E
	Percentage recovery				
Phosphatidyl glycerol	91.1	92.4	98.2	102.8	100.7
Phosphatidyl ethanolamine	91.3	93.8	106.0	99.2	103.7
Phosphatidyl serine	94.8	94.2	97.6	98.5	98.7
Phosphatidyl inositol	101.9	103.9	106.4	106.3	110.0
Phosphatidyl choline	99.3	98.2	102.0	107.3	103.4
Sphingomyelin	84.5	89.4	96.9	89.9	98.1
Lysophosphatidyl choline	83.0	88.8	85.2	87.2	98.4

perchloric acid alone appears to give slightly lower recoveries than the other methods. The best recoveries were obtained with method E. This may be due to the catalytic action of vanadium pentoxide that shortens the time necessary for complete phosphate release. Method C also gave high recoveries and like E may be used with shorter heating times (see Tables 1 and 2). A disadvantage of method E is the preparation of the digestion mixture. Method C requires two additional reagents as well as extra manipulations, although method C does not involve the use of perchloric acid. Since many laboratories consider the use of perchloric as hazardous, they may prefer to use C. Our own preference is for method B, as it requires minimal preparation of the acid digestion mixture and needs no extra manipulations or reagents for digestion.

TABLE 4
COMPARISON OF METHODS USING AMNIOTIC FLUID

Sample number	Method A	B	C	D	E
	Concentration found (mg/100 ml P)				
Pool 1	0.35	0.36	0.37	0.30	0.30
2	0.58	0.63	0.64	0.63	0.60
3	0.15	0.18	0.16	0.17	0.15
4	0.08	0.09	0.10	0.08	0.09
5	0.21	0.23	0.22	0.23	0.22
6	0.12	0.13	0.13	0.12	0.13
7	0.38	0.41	0.40	0.42	0.43
8	0.53	0.51	0.57	0.56	0.56
9	0.38	0.38	0.41	0.41	0.39
10	0.25	0.25	0.28	0.25	0.27

TABLE 5
RECOVERY OF AQUEOUS PHOSPHATE STANDARDS

	Method				
	A	B	C	D	E
Concentration of standard (mg/100 ml P)					
Taken	Found				
0.05	0.05	0.05	0.05	0.05	0.06
0.10	0.10	0.11	0.11	0.11	0.13
0.20	0.18	0.20	0.19	0.18	0.22
0.30	0.29	0.29	0.29	0.27	0.31
0.50	0.46	0.48	0.48	0.45	0.50

Ten amniotic fluid pools were digested by each method and assayed. These data are given in Table 4. Method C gave slightly higher results for the majority of fluids, although all methods yielded quite comparable results. Further evidence that all methods of digestion are equivalent was given by taking several concentrations of aqueous phosphate standards through the entire procedure. Table 5 shows the data obtained. Similar results were obtained for all methods, and they were considered to be quantitative.

SUMMARY

Five methods of acid digestion of amniotic fluid phospholipids for inorganic phosphate release were evaluated at a temperature of 225°C and a heating time of 30 min. The methods used the following digestants: (A (perchloric acid), B (perchloric acid containing molybdate), C (sulfuric acid, 30% hydrogen peroxide, 5% urea), D (sulfuric–perchloric acids), and E (sulfuric–perchloric acids with vanadium pentoxide). After digestion and dilution with water, a hydrogen ion concentration of about 2.5 mol/liter was obtained that permitted the use of stannous chloride–hydrazine sulfate as the reducing agent for phosphate color development. Recoveries of the different amniotic fluid phospholipids and aqueous phosphate standards were quantitative for all methods. A comparative study with amniotic fluids showed similar results for all methods. Since all methods were found to be equivalent under the specified conditions described, the choice was dependent upon the user's preference.

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Indirect Amplification Method for Determining Mercury by Direct-Current Polarography. Application to Organomercury Compounds

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INTRODUCTION

Various polarographic techniques have been reported for the determination of mercury, e.g., alternating-current (ac) polarography (*13*), inversion oscillographic polarography (2), direct-current (dc) polarography (*7, 17–19, 22, 24*), and amperometry (*12, 23*).

However, there are only a few publications dealing with the microdetermination of mercury in organomercurials in spite of the biological and industrial importance of these compounds. Organic Hg(II) has been determined by titration with NH_4SCN following decomposition in a Kjeldahl flask (*9*), in a closed flask (*16*), or in dry combustion (*21*). Korshun and Chumachenko (*15*) advocated heating with potassium metal in a microbomb; after dissolving the elemental mercury in HNO_3 and addition of excess NaCl, the unreacted Cl^- was titrated with $Hg(NO_3)_2$. Gouverneur and Hoedeman (*8*) recommended direct titration of Hg(II) with sodium diethyldithiocarbamate following closed flask combustion. Johnson *et al.* (*10*) measured the absorbance of the mercuric dithizonate complex. Amperometric titration of Hg(II) with (ethylenedinitrilo)tetraacetic acid (*20*) or with bis(2-hydroxyethyl)-dithiocarbamate (*5*) has been described. Wasilewska (*24*) determined mercury in pharmaceuticals by closed flask combustion and polarographic measurement of Pb^{2+} released from lead diethyldithiocarbamate.

The increasing popularity of amplification reactions is not unexpected since they increase the sensitivity and improve the accuracy of the determination. The present paper describes a method based upon the reaction between Hg(II) and a known excess of I^-; the compound formed is removed by extraction, and the excess I^- is oxidized to IO_3^- which is measured polarographically. Iodate ion undergoes reduction at the

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dropping mercury electrode producing a single wave which corresponds to direct reduction to I^- as shown by the equation (*14*):

$$IO_3^- + 6\ H^+ + 6\ e^- \rightarrow I^- + 3\ H_2O.$$

Thus, I^- can be determined after conversion into another species (IO_3^-) which yields a wave six times greater than that of I^- with a one-electron transfer, i.e., I^- is determined after sixfold amplification. Further amplification, 36-fold, is possible: Following the above steps, the IO_3^- solution is acidified, treated with I^- to liberate iodine which is extracted into benzene, followed by back extraction using aqueous sodium hydrogen sulfite (*6*) to reduce iodine into I^-; the latter is then oxidized by bromine water to yield IO_3^-. Thus six IO_3^- are obtained for every I^- originally present.

Another amplification method involves the use of periodate (*25*). Periodate oxidizes I^- according to the equation:

$$I^- + 3\ IO_4^- \rightarrow 4\ IO_3^-.$$

Subsequent polarographic reduction of the IO_3^- formed requires 24 electrons and, therefore, allows 24-fold amplification for the determination of I^-.

EXPERIMENTAL

Apparatus

A Cambridge polarograph with accessories was used. The polarograph was connected to an automatic desk-top recorder (Jasco RC-200) with 250-mm chart. A universal U-shaped Kalousek cell was used as the electrolytic vessel; the anode was a saturated calomel electrode (SCE). The ir drop in the cell was negligible. The capillary used had a drop time of 3–5 sec under an open head of 60 cm of mercury. The dropping electrode, the polarographic cell, and the reagents were kept in an air thermostat at 25°C.

Reagents and Materials

All reagents used were of A.R. grade quality except where otherwise stated.

Standard mercuric solutions. Stock solutions were prepared by dissolving 78.824 mg of $Hg(NO_3)_2 \cdot H_2O$ or 68.989 mg of $HgSO_4$ in 10 ml of 1.5 *N* HNO_3 and adding water to 500 ml; 1 ml contains 92.3 or 93.3 μg of mercury, respectively. Another stock solution, prepared by dissolving 1328.824 mg of the nitrate salt in 10 ml of 1.5 *N* HNO_3 and making up to 500 ml with water so that 1 ml contains 1.556 mg of mercury, served to establish the method for organomercurial compounds. These solutions were standardized by titrating known weights of sodium chloride against the mercuric solution using diphenylcarbazone as indicator (*3*).

Potassium iodide, two solutions. (i) One solution was prepared so that 1 ml contained 0.1 mg of iodide and was used with the 6-, 24-, and 36-fold amplification methods. (ii) Another solution was prepared so that 1 ml contained 2 mg of iodide to be used for organomercurial compounds. The solutions were prepared fresh every 2 weeks.

Potassium iodate, two standard solutions. (i) One solution was prepared by dissolving 170 mg in 1 liter of water (0.1 mg of I^-/ml) and was used for constructing curve a in Fig. 1. (ii) The other solution was prepared by dissolving 1000 mg in 1 liter of water (0.6 mg of I^-/ml) and was used for constructing curves b, c, and d in Fig. 1. The straight-line calibration curves pass through the origin and therefore satisfy the Ilkovic equation. The calibration graphs should be checked for every batch of KIO_3 solutions.

Potassium periodate. This was prepared fresh by dissolving 350 mg in 20 ml of hot water, adding 0.6 ml of saturated borax solution, and making up to 100 ml with water. It is kept in an amber bottle.

Other materials.

Bromine water	Saturated aqueous solution.
Formic acid	80% aqueous solution.
Ammonium chloride	10% solution.
Zinc acetate	10% solution.
Sodium hydrogen sulfite	0.5% solution.
Gelatin	1% solution, prepared every week.
Urea	solid.

O_2-Free nitrogen gas was obtained by passing the stream of gas through the purification train consisting of three bubblers containing pyrogallol followed by one bubbler containing 1 *N* HCl and one empty bubbler.

Procedures

Sixfold amplification method. In a ground-stoppered, conically shaped 50-ml separatory funnel with a maximum body diameter of 29 mm and 20-mm-long stems, introduce an aliquot (not more than 8 ml) of the sample solution containing 45–250 μg of Hg(II) in HNO_3 followed by exactly 4 ml of potassium iodide solution introduced from a buret. The solution has a pH value between 2 and 3 (if necessary, add HNO_3 or KOH to adjust the pH value within this range using a pH meter or universal indicator). Add water to give a total volume of 12 ml. Introduce 6 ml of diethyl ether and shake. Allow for complete separation. Quantitatively transfer the aqueous layer, containing the unconsumed iodide, into a 100-ml conical flask. Add 1 ml of bromine water and stopper the flask. Stir the solution for 10 min. Remove the stopper, destroy the excess bromine by adding 0.2 ml of formic acid solution and stirring for 5 min. Transfer quantitatively to a 50-ml volumetric flask. Add 2 ml of 4.5 *N* potassium hydroxide solution, followed by 1.2 ml of gelatin solution. Add water up to the mark. Mix

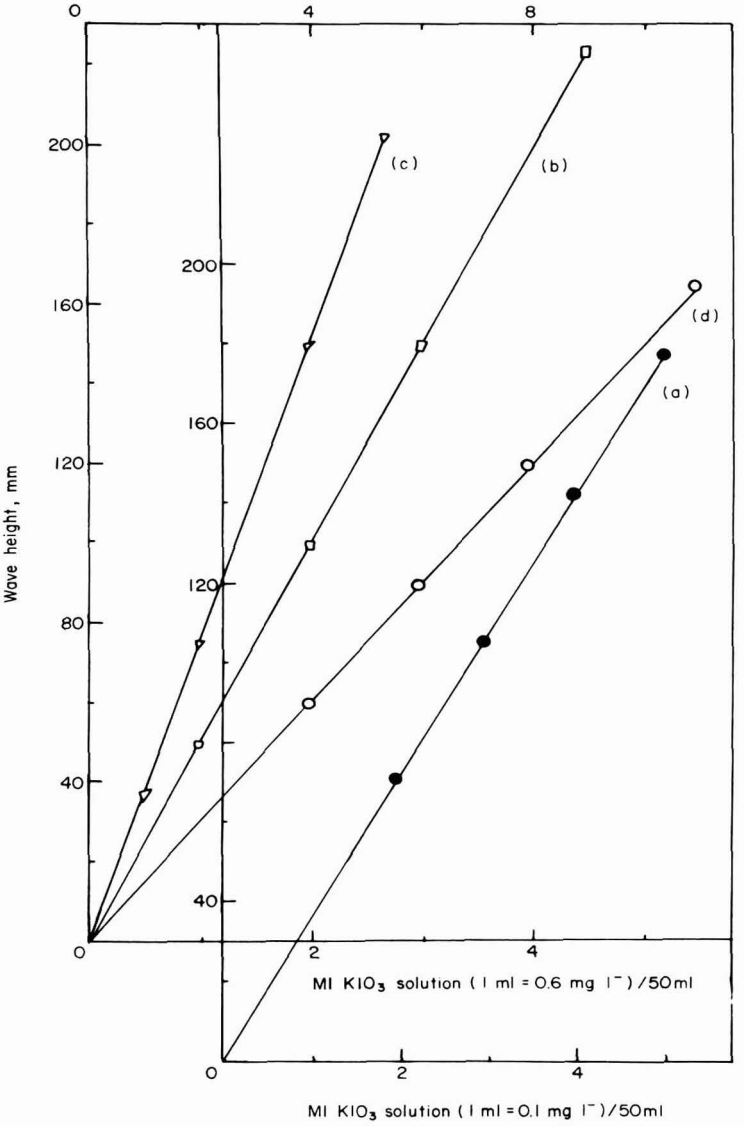

FIG. 1. Calibration curves for potassium iodate solutions. Sensitivity: (a) 1.75×10^{-8}, (b) 7.5×10^{-8}, (c) 5×10^{-8}, and (d) 2.5×10^{-7} A/mm (upper abscissa).

well, transfer an aliquot (8–10 ml) to the polarographic cell, and bubble nitrogen gas for 3 min. Record the iodate wave starting at −0.7 V vs SCE and using a sensitivity of 1.75×10^{-8} A/mm.

Following the same steps, record the iodate wave for a blank solution using the same amount of iodide (4 ml) but in the absence of mercury. Compare the height of each of the blank and sample waves with curve a in

Fig. 1 to obtain the amount of iodide present before and after reaction with Hg(II).

36-Fold amplification method. In a 50-ml separatory funnel, introduce a suitable volume (less than 8 ml) of the sample solution containing 25–250 μg of Hg(II) in HNO_3 followed by exactly 4 ml of potassium iodide solution. The pH value of the solution lies between 2 and 3 (if necessary, add KOH or HNO_3 to adjust the pH value within this range using a pH meter or universal indicator). Add water so that the total volume is 12 ml. Introduce diethyl ether (6 ml) and proceed as for the sixfold amplification method including the removal of the excess bromine. To the iodate solution, add 2 ml of 2 *N* sulfuric acid solution and 200 mg of potassium iodide. Stopper the flask and leave in a dark place for 5 min at room temperature (about 25°C). Transfer the solution quantitatively to a 100-ml separatory funnel. Extract the iodine using two 30-ml portions of benzene. Collect the extracts in a 100-ml separatory funnel. Add 10 ml of water and shake to remove traces of iodide. Discard the aqueous layer and shake the iodine solution with 0.4 ml of sodium hydrogen sulfite solution and 10 ml of water to reduce iodine into iodide. Transfer the aqueous layer, containing iodide, to a 100-ml conical flask. Add 2 ml of bromine water and stir for 10 min. Introduce 0.5 ml of formic acid solution and stir for 5 min. Transfer quantitatively to a 50-ml volumetric flask; add 2 ml of 4.5 *N* potassium hydroxide solution, 1.2 ml of gelatin solution, and water up to the mark. After bubbling nitrogen through the solutions, polarograph an aliquot (8–10 ml) starting from −0.7 V vs SCE and using a sensitivity of 7.5×10^{-8} A/mm.

Following the same steps, record the iodate wave of a blank solution using the same amount of iodide (4 ml) but no mercury. Compare the height of each of the blank and sample waves with curve b in Fig. 1 to obtain the amount of iodide present before and after reaction with Hg(II).

24-Fold amplification method. To an aliquot (not more than 8 ml) of the sample solution containing 35–200 μg of Hg (II) in HNO_3, placed in a 50-ml separatory funnel, add quantitatively 4 ml of potassium iodide solution. The solution now has a pH value between 2 and 3 (if necessary, add KOH or HNO_3 to adjust the pH value within this range using a pH meter or universal indicator). Add water until the total volume is 12 ml. Introduce 6 ml of diethyl ether, shake, and quantitatively transfer the aqueous layer into a 100-ml conical flask. Oxidize the residual iodide by adding 4 ml of the periodate solution; stopper and allow to stand for 5–10 min at room temperature (25°C) and then immerse the flask in a boiling water bath for 30 min. Cool to room temperature, add 2 ml of 10% NH_4Cl followed by 2 ml of 25% ammonia solution. Heat just to boiling and precipitate the excess periodate by adding 2 ml of 10% zinc acetate solution. Leave the mixture undisturbed for 30 min at room temperature.

Filter off the zinc periodate using Whatman No. 42 filter paper and wash the precipitate with three 5-ml portions of distilled water. Receive the iodate-containing filtrate and washings in a 50-ml volumetric flask. Add 2.5 ml of 2 *N* sulfuric acid solution followed by 1 ml of gelatin solution and water up to the mark. Shake and transfer 8–10 ml of the solution to the polarographic vessel. Bubble a stream of nitrogen gas through the solution and record the iodate wave starting at +0.15 V vs SCE and using a sensitivity of 5×10^{-8} A/mm. Under identical conditions, record the iodate wave of a blank solution using the same amount of iodide (4 ml) in the absence of mercury. Compare the height of each of the blank and sample waves with curve c in Fig. 1 to obtain the amount of iodide present before and after reaction with Hg(II).

Analysis of organomercurial compounds after sixfold amplification. Weigh exactly 3–7 mg of the organic sample. Use a platinum gauze for holding the weighed sample placed in a filter paper container. Charge a 250-ml Erlenmeyer flask with 6 ml of 1 *N* HNO_3 and flush with oxygen gas for ca. 60 sec. Ignite the fuse and quickly insert the stopper. Invert the flask and hold it tightly together with the stopper. After complete combustion, shake intermittently for 10 min. Rinse the platinum gauze with distilled water and then introduce 2 ml of bromine water. Heat the solution on a hot plate until colorless, and then add 1.5 g of urea. Concentrate until the volume is 2–3 ml. Cool to room temperature, and transfer quantitatively to a 50-ml separatory funnel using 10 ml of water. Add exactly 3 ml of potassium iodide solution and adjust the pH value at 2–3 by adding two to three drops of 1 *N* nitric acid solution. Add 8 ml of diethyl ether and extract. Quantitatively transfer the aqueous layer into a 100-ml conical flask. Add 2 ml of bromine water. Stopper and stir for 10 min. Introduce 0.5 ml of formic acid solution and stir for 5 min. Transfer quantitatively to a 50-ml volumetric flask. Add 3 ml of 4.5 *N* potassium hydroxide solution, 2.5 ml of gelatin solution, and complete with water. Transfer an aliquot (8–10 ml) to the polarographic cell. After bubbling nitrogen through the solution for 3 min, record the iodate wave starting at −0.7 V vs SCE and using a sensitivity of 2.5×10^{-7} A/mm.

Carry out a blank run using 13 ml of water, 3 ml of potassium iodide solution, two to three drops of 1 *N* nitric acid solution, 8 ml of diethyl ether, and continue as for the sample run. Compare the height of each of the blank and sample waves with curve d in Fig. 1 to obtain the amount of iodide present before and after reaction with Hg(II).

Calculate the weight of mercury using the formula,

$$\text{weight of mercury (mg)} = \frac{\text{atomic wt of Hg} \times W}{2 \times \text{atomic wt of I}} = 0.79 \times W.$$

where W = weight (milligrams) of iodide reacted with Hg(II) as calculated

from the corresponding calibration graph. For the 24- and 36-fold amplification methods, divide the weight of mercury obtained by 4 and 6, respectively.

RESULTS AND DISCUSSION

The present method depends on using a known excess of iodide to form quantitatively HgI_2. This has been proven experimentally as follows. To 2 ml of potassium iodide solution (4 mg of I^-) placed in a 50-ml separatory funnel, add 1 ml of mercuric ion solution (1.556 mg of Hg in 0.03 *N* HNO_3) and 9 ml of water (pH = 2.5–3). Use a pipet with rubber filler to add 6 ml of diethyl ether, shake, separate the two layers, and then oxidize the residual iodide in the aqueous layer (placed in a 100-ml conical flask) by adding 2 ml of bromine water and stirring. Add formic acid solution (0.5 ml) and stir. Add to the resulting iodate solution 2 ml of 2 *N* H_2SO_4 followed by about 0.2 g of potassium iodide. Stopper the flask and leave for 10 min. Titrate the liberated iodine against 0.02 *N* standardized thiosulfate solution to the starch end point; 4.96 ml of thiosulfate solution was consumed. A blank run using the same amount of iodide, but no mercury, consumed 9.48 ml of the thiosulfate solution. The difference between the two titers shows that 1.912 mg of iodide had reacted with the 1.556 mg of mercury or that 2 Eq of iodide combine with 1 Eq of mercuric ion. The fact that the compound could be extracted in diethyl ether seems to indicate that the species is HgI_2 and not $(HgI_4)^{2-}$ since doubly charged complexes are not extracted by diethyl ether (*4*). To attain complete extraction, the pH value of the aqueous layer should be between 2 and 3, and the ether:water volume ratio, 1:2. If the pH value is outside the range of 2–3, add KOH or HNO_3 as required using a pH meter or universal indicator.

Removal of the excess periodate in the 24-fold amplification method has been tried by adding molybdate solution (*1*) at pH3, but the wave of molybdenum interfered with that of iodate. Zinc acetate removed the excess periodate, as insoluble zinc periodate, from an alkaline medium (*11*). Recording of the iodate wave without filtration was not feasible since zinc produces a wave in the alkaline medium used, which interferes with that of iodate. After the separation of zinc periodate, acidification with sulfuric acid eliminated the interference due to zinc.

Although dc polarography is useful at the ca. 10^{-5} *M* level for Hg(II), the proposed amplification procedure enables analysis of a solution as dilute as 2.5×10^{-6} *M* (0.5 ppm) Hg(II) solution with reasonable accuracy. For about 50 determinations (Table 1), the relative error did not exceed 3.4%. For 45, 140, and 230 μg of Hg, the sixfold method gave percentage standard deviations of 1.16, 0.85, and 0.64, respectively; for 25, 150, and 250 μg of Hg, the 36-fold method gave percentage standard deviations of

TABLE 1

MICRODETERMINATION OF MERCURY BY AMPLIFICATION REACTIONS

Salt	6-Fold amplification				24-Fold amplification				36-Fold amplification			
	Hg(II) solution (ml)	Hg (μg)		Relative error (%)	Hg(II) solution (ml)	Hg (μg)		Relative error (%)	Hg(II) solution (ml)	Hg (μg)		Relative error (%)
		Taken	Found			Taken	Found			Taken	Found	
$Hg(NO_3)_2 \cdot H_2O$												
	0.5	46.2	46.4	+0.43	0.4	36.5	35.7	−2.19	0.4	36.5	35.7	−2.19
			45.5	−1.52			36.8	+0.82			36.3	−0.55
			46.0	−0.43								
	1.0	92.3	92.3	00	1.0	91.3	90.5	−0.88	1.0	91.3	90.6	−0.77
			91.5	−0.87			91.0	−0.33			90.2	−1.20
	1.5	138.5	137.9	−0.43	1.7	155.2	156.5	+0.84	1.7	155.2	155.0	−0.13
			137.0	−1.08			154.3	−0.58			154.3	−0.58
			138.9	+0.29								
	2.0	184.6	184.9	+0.16	2.3	210.0	207.8	−1.05	2.0	182.6	180.5	−1.15
			183.1	−0.81			211.1	+0.52			182.5	−0.06
			185.8	+0.65								
	2.5	230.8	229.5	−0.56					2.8	255.6	252.6	−1.17
			230.1	−0.30							255.5	−0.04

$HgSO_4$									
	0.5	46.7	47.1	+0.86		0.3	26.5	26.8	+1.13
			46.2	−1.07				26.0	−1.89
			46.4	−0.64					
	1.0	93.3	92.6	−0.75		0.5	44.1	42.6	−3.40
			93.6	+0.32				43.3	−1.81
			94.1	+0.86					
	1.7	158.6	157.6	−0.63		1.0	88.3	88.4	+0.11
			158.8	+0.13				88.1	−0.23
								88.6	+0.34
	2.5	233.3	232.2	−0.47		2.0	176.6	176.3	−0.17
			233.4	+0.04				175.9	−0.40
			231.3	−0.86					
			SD	0.88	1.20				1.07

2.20, 0.60, and 0.97, respectively; the 24-fold method gave percentage standard deviations of 2.36, 1.02, and 1.17 for 35, 150, and 210 μg of Hg, respectively.

One determination requires 30, 65, and 85 min using the 6-, 36-, and 24-fold methods, respectively. Metal ions which form ether-soluble iodides (e.g., Sb^{3+}, Cd^{2+}, Sn^{2+}), or water-insoluble iodides (e.g., Ag^{+}, Pb^{2+}, Cu^{+}) are expected to interfere; similarly would those which yield polarographic waves from KOH (e.g., Zn^{2+}) or H_2SO_4 supporting electrolyte (e.g., Mo^{6+}, Cu^{2+}, Bi^{3+}) with half-wave potentials close to that of iodate.

The sixfold amplification method has been used to fit the analysis of organomercurial compounds following closed flask combustion. The results obtained for three organic compounds are given in Table 2; the average percentage error for 10 determinations amounts to ± 0.40. One determination requires 45 min including weighing of sample. The compounds analyzed contain, besides carbon and hydrogen, oxygen, bromine, and sodium.

Polarography is a nondestructive tool of analysis. Therefore, following polarographic recording of iodate, mercury can be redetermined indirectly using any other method suitable for estimation of the residual iodide present as iodate.

SUMMARY

An amplification method for the determination of 0.5–70 ppm (2.5×10^{-6} to 3.5×10^{-4} *M*) of Hg(II) is described. Hg(II) is reacted with a slight excess of KI, and the excess iodide is oxidized by bromine water and measured polarographically as iodate with sixfold amplification. Alternatively, the iodate formed is reacted to liberate iodine which is then reduced to iodide, and again oxidized to yield six iodate ions for every iodide ion originally present.

TABLE 2
MICRODETERMINATION OF MERCURY IN ORGANOMERCURIAL COMPOUNDS

Compound	Sample (mg)	Hg (%) Calcd	Found	Error	SD (%)
Phenylmercuric acetate	4.682	59.57	60.04	+0.47	0.68
	3.690		59.08	−0.49	
Fluorescein–mercuric acetate	3.000	47.22	47.09	−0.13	0.64
	3.325		47.21	−0.01	
	4.173		46.33	−0.89	
Mercurochrome	6.884	26.72	25.73	−0.99	0.59
	5.427		26.12	−0.60	
	3.180		26.74	+0.02	
	4.903		26.50	−0.22	
	4.542		26.88	+0.16	

polarographic reduction requires 36 electrons. Oxidation of the excess iodide with periodate yields four iodate ions for every iodide ion and therefore allows 24-fold amplification.

Microdetermination of mercury in organomercurials is achieved using the sixfold method following closed flask combustion; the average percentage error for 10 determinations is ±0.40 and the time required for one sample run is 45 min.

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A Colorimetric Method for the Determination of Carboxylic Acids

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INTRODUCTION

pH indicators have been satisfactorily used for quantitative and chromatographic location of organic acids on paper chromatograms. Alkaline thymol blue reagent was used for the quantitative determination of the ammonium salts of organic acids (*6*). Thymol blue and phenol red were also used for the quantitative determination of enzymatically liberated acetic acid by the action of acetylcholine esterase on acetylcholine (*1*, *3*, *4*). For the location of organic acids on paper chromatograms, bromocresol green was recommended as a spraying reagent (*7*, *8*). In this paper bromocresol green reagent is used for the quantitative determination of organic acids in solution. The principle is applied successfully for the microdetermination of both volatile and nonvolatile organic acids.

EXPERIMENTAL

Reagents. Bromocresol green reagent (0.54 m*M*) of analytical grade was prepared by dissolving 38 mg of the dye in 100 ml of absolute ethanol, and the final pH was adjusted to 9.3 with 0.1 *N* NaoH solution (carbonate free).

Standard solutions of 0.001 *M* malic, citric, succinic, oxalic, tartaric, fumaric, α-ketoglutaric, gallic, propionic, and capric acids were prepared.

All chemicals were of analytical grade

Apparatus. Spectrophotometric measurements were performed on a Carl Zeis spectrophotometer Model M_4 Q_{11}. A steam distillation apparatus (*5*) was used for the separation of volatile fatty acids.

Organism. Penicillium oxalicum was maintained and subcultured on Czapek–Dox's medium. It was grown on liquid Czapek–Dox's medium for 4 days in shaking cultures at 28°C.

Chromatography of organic acids. A standard mixture of malic, succinic, oxalic, citric, tartaric, fumaric, and α-ketoglutaric acids was

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chromatographed on Dowex 1-X8 (200–400 mesh) formate from a column (*2*). Organic acids were eluted from the column by a formic acid stepwise gradient ranging from 0.0 to 12 *M*. For quantitative determination, samples of a known volume (0.1–0.2 ml) of each fraction were placed in test tubes and dried in a vacuum oven at 40°C. A control sample from each gradient was treated similarly. To the dry residue absolute ethanol was added and evaporated. The last step was repeated at least three times for complete assurance of formic acid removal. The residue was constituted in a known volume of carbon-dioxide-free water for organic acid determination. Organic acids in Dowex 1-X8 eluates were identified by ascending paper chromatography. The spotted Whatman No. 3 chromatograms were developed by the following solvent systems: I, ethanol–ammonia–water (80:10:10); II, *n*-butanol–acetic acid–water (20:10:10); III, *n*-butanol–formic acid–water (40:10:50).

Bromocresol green was used as a spray reagent for location of organic acid spots on the air-dried chromatograms.

RESULTS AND DISCUSSION

The absorption spectra of 36 μM bromocresol green solution in alkaline and 1.0 and 0.066 m*M* acid solutions are shown in Fig. 1. In alkaline

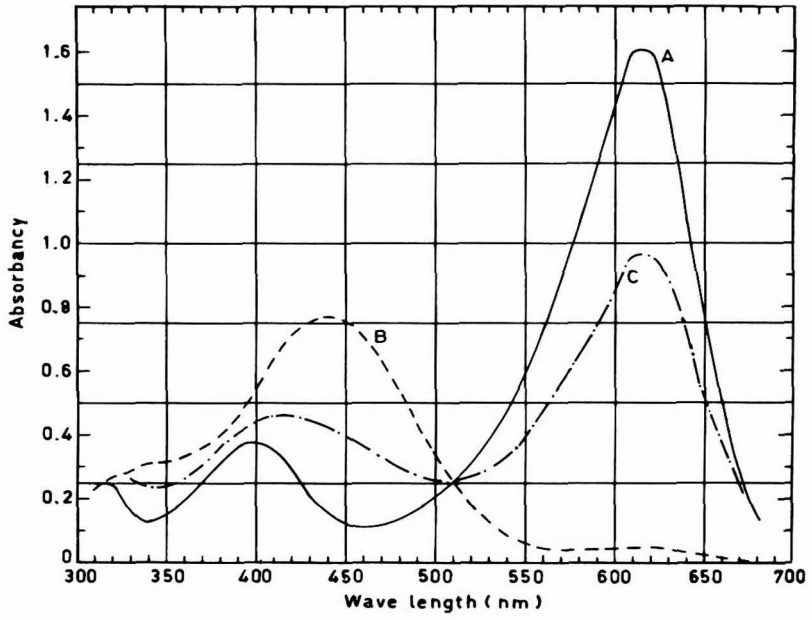

FIG. 1. Absorption spectra of 36 μM bromocresol green reagent (A) at alkaline pH, (B) in the presence of 1.0 m*M* malic acid, and (C) in the presence of 0.066 m*M* malic acid.

solution two maxima at 620 and 400 nm and two minima at 460 and 340 nm are observed.

The absorbancy increases linearly with the increase of bromocresol green concentration in both acid and alkaline solutions at 440 and 620 nm, respectively. At a 9 μM dye concentration absorbancies of 0.02 at the minimum of 620 nm in acid solution and 0.06 at the minimum of 440 nm in alkaline solution are recorded (Fig. 2). The magnitude of this absorbancy is not significantly increased with increases of the dye concentration up to 20 μM. Therefore, one may anticipate that the changes in absorbancy at 440 and 620 nm, respectively, are directly proportional to the concentration of the test acid. However, a blank of the dye is routinely measured at 440 and 620 nm.

Standard curves of malic acid in the presence of 36 and 90 μM bromocresol green at 620 and 440 nm are shown in Fig. 3. The formed color follows Beer's Lambert law. However, the change in absorbancy is not linear for amounts greater than 0.25 and 0.4 μmol of malic acid at low and high bromocresol green concentrations, respectively. Thus for high concentrations of organic acids a 90 μM dye concentration is recommended, and the change in absorbancy can be followed at 440 nm. At low acid concentrations, a 36 μM dye concentration is used and the colour is read at either 620 or 440 nm. The formed color is stable, and no change in absorbancy can be noticed after 24 hr.

Data shown in Table 1 show the dependence of the molar extinction coefficient ϵ on the dissocation constants and the pK_1 of the tested organic acids. Figure 4 shows the linear relationship between the pK_1 of different carboxylic acids and the calculated molar extinction coefficients of the

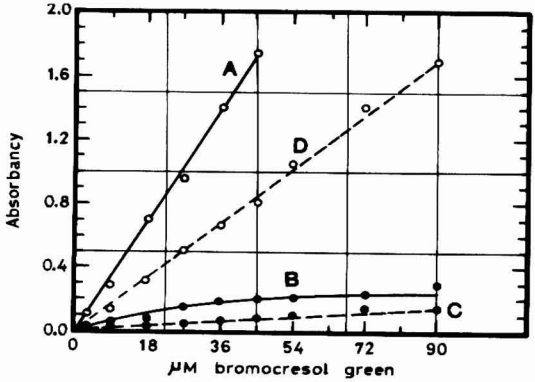

FIG. 2. Changes in absorbancy at 440 and 620 nm at different bromocresol green concentrations. (A) In alkaline pH at 620 nm, (B) in alkaline pH at 440 nm, (C) in the presence of 1.0 mM malic acid at 620 nm, and (D) in the presence of 1.0 mM malic acid at 440 nm.

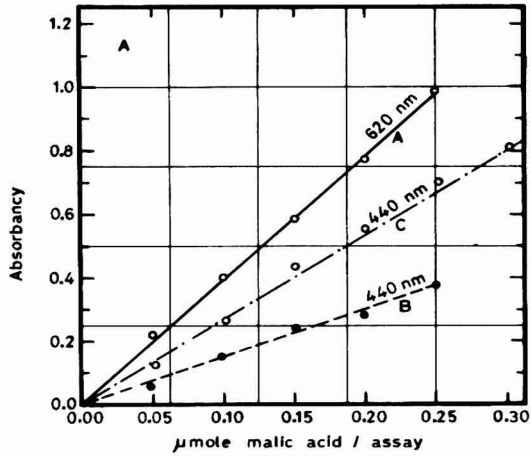

FIG. 3. Standard curves of malic acid (A) using 36 μM bromocresol green at 620 nm, (B) using 36 μM bromocresol green at 440 nm, and (C) using 90 μM bromocresol green at 440 nm. Reaction volume used is 3.0 ml.

TABLE 1

MOLAR EXTINCTION COEFFICIENTS FOR DIFFERENT CARBOXYLIC ACIDS

Acid	Step	K^a	$pK_1{}^a$	ϵ at 440 nm^b (±SE)
Oxalic acid	1	5.9×10^{-2}	1.23	1155
	2	6.4×10^{-5}		± 5.56
Succinic acid	1	6.39×10^{-5}	4.16	687
	2	2.47×10^{-6}		± 7.4
Fumaric acid	1	9.3×10^{-4}	3.03	1225
	2	3.6×10^{-5}		± 7.08
Malic acid	1	3.9×10^{-4}	3.4	868.2
	2	7.8×10^{-6}		± 10.2
α-Tartaric acid	1	1.04×10^{-3}	2.98	1285.2
	2	4.55×10^{-5}		± 7.36
Citric acid (18°C)	1	7.10×10^{-5}	3.14	1057
	2	1.68×10^{-5}		± 7.93
	3	6.4 ± 10^{-6}		
Gallic acid		3.9×10^{-5}	4.41	630.8
				± 10.64
Propionic acid		1.34×10^{-5}	4.87	269
				± 4.48
Capric acid		1.28×10^{-5}	4.89	308
				± 5.83

[a] Dissociation constants and pK_1 values are taken from Ref. (9).

[b] Dye concentration, 90 μM.

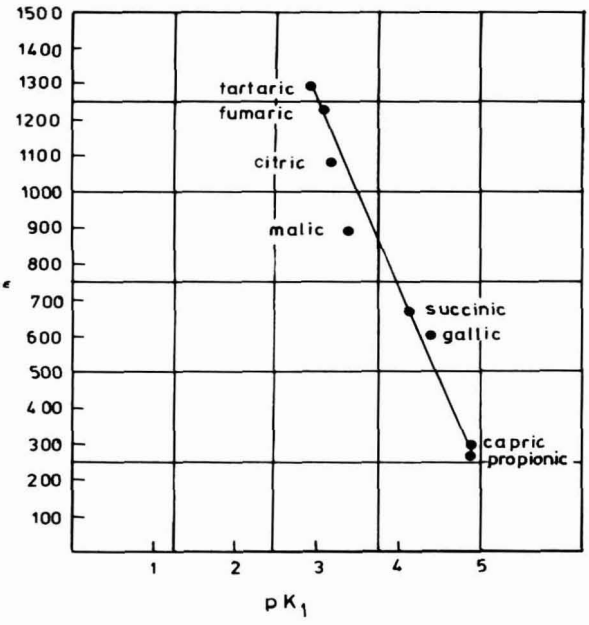

FIG. 4. A plot relating the pK_1 of different carboxylic acids to the molar extinction coefficient ϵ at 440 nm using 90 μM bromocresol green reagent.

color formed at 440 nm. The linearity holds for carboxylic acids with pK_1 values higher than 2. However, no spectral shift in the recorded spectra of the acids–dye mixtures is noticed. Therefore, one may suggest that the absorbancy change at 440 nm is dependent on the degree of ionization of organic acids rather than on a direct interaction of the dye with the test organic acid.

This method can be applied successfully for quantitative measurement

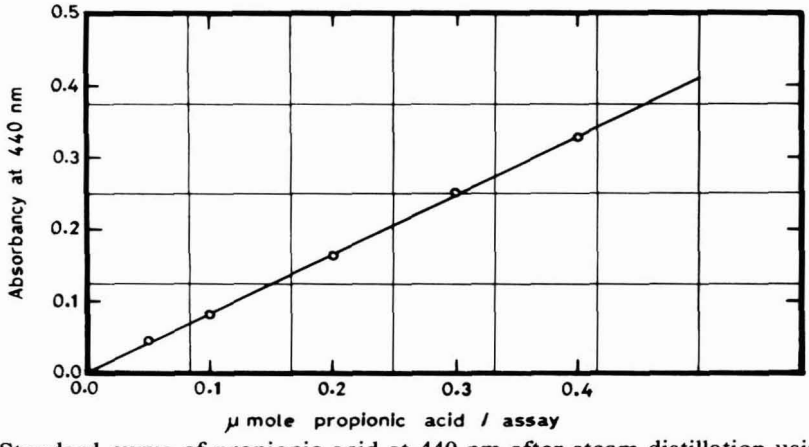

FIG. 5. Standard curve of propionic acid at 440 nm after steam distillation using 36 μM bromocresol green. Reaction volume, 3.0 ml.

TABLE 2
COMPARISON BETWEEN TITRIMETRIC AND BROMOCRESOL GREEN METHODS FOR VOLATILE FATTY ACIDS DETERMINATION

Method	Propionic acid[a] (μmol)	
	Zero time	After 3 days fermentation
Titrimetric (using 0.01 *M* NaoH)	1590	1037
Bromocresol green	1620	1108

[a] Steam distillates of *P. oxalicum* culture medium supplemented with 120 mg/100 ml of propionic acid.

of the rate of disappearance of volatile fatty acids added to *P. oxalicum* culture media. The concentration of propionic acid is calculated from a previously established standard curve (Fig. 5). For comparison propionic acid concentration in the steam distillate is determined both colorimetrically and titrimetrically. Data shown in Table 2. indicate that both methods are comparable.

A mixture of six standard organic acids (50 μmol of each) is chromatographed on Dowex l-X8 formate-form column (1.4 × 1 cm) as previously

TABLE 3
R_f VALUES OF STANDARD ORGANIC ACIDS AFTER ELUTION FROM DOWEX I-X8 FORMATE COLUMN

Organic acid	R_f		
	Solvent system[a]		
	I	II	III
Succinic acid	0.36	0.79	0.77
F_1	0.36	0.80	0.79
Aalic acid	0.30	0.63	0.55
F_2	0.29	0.64	0.55
Oxalic acid	Tailing	0.54	0.43
Tartaric acid	0.25	0.49	0.32
Citric acid	0.13	0.56	0.49
F_3	0.13, 0.26	0.49, 0.54	0.31, 0.42, 0.49
Fumaric acid	0.43	0.86	0.89
F_4	0.44	0.85	0.90
α-Ketoglutaric	0.43	0.67	0.66
F_5	0.42	0.69	0.66

[a] I, ethanol–ammonia–water (80:10:10); II, *n*-butanol–acetic acid–water (20:10:10); III. *n*-butanol–formic acid–water (40:10:50).

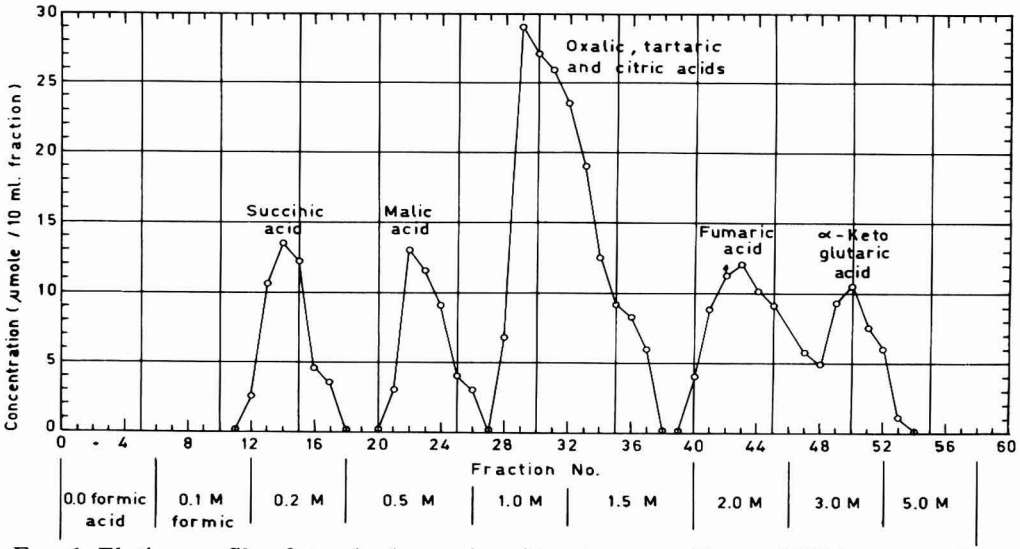

FIG. 6. Elution profile of standard organic acids mixture on Dowex l-X8 formate column.

described in the experimental section (Fig. 6). The elution sequence of the acids from the column is confirmed by paper chromatography (Table 3). The recovery percentage of the applied standard organic acids are: for succinic, 93%; malic, 87%; oxalic, tartaric, and citric, 111%; and fumaric and α-ketoglutaric, 105%. The method is reproducable and can be used for the determination of organic acids after their separation on Dowex l-X8 columns.

SUMMARY

A new colorimetric method for the determination of carboxylic acids using bromocresol green reagent is established. The formed color follows Beer's Lambert law and is stable for 24 hr. The method is successfully applied for measuring the disappearance of volatile fatty acids in *P. oxalicum* culture media. The method is reproducable and is used also for the quantitative determination of carboxylic acids in Dowex l-X8 eluates. A linear relationship between the pK_1 of different carboxylic acids and the calculated molar extinction coefficients of the color formed at 440 nm is demonstrated.

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MICROCHEMICAL JOURNAL 23, 453–459 (1978)

3-Methyl-4-amino-5-mercapto-1,2,4-triazole as a Ring-Colorimetric Reagent for the Selective Microevaluation of Au(III), Ti(I), and Ag(I)

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INTRODUCTION

3-Methyl-4-amino-5-mercapto-1,2,4-triazole (MAMT) has a great potential for applications in microanalytical chemistry. In our earlier publication (*2*) we reported the analytical applicability of MAMT as a chromogenic reagent for the visualization of the chromatographed species and subsequently for the colorometric evaluation of Ru(III), Rh(III), Pd(II), Pt(IV), and Au(III) by ring colorimetry. The present investigations involve the systematic study of its reactions with various metal ions, with a view of their applications in "ring colorimetry." The metal ions studied for this purpose are Li(I), Na(I), K(I), Rb(I), Cs(I), Be(II), Mg(II), Ca(II), Sr(II), Ba(II), Al(III), Tl(I), Ge(IV), Sn(II), Sb(III), As(V), Bi(III), Se(IV), Te(IV), Cr(III), Cr(VI), Fe(II, III), Co(II), Ni(II), Mn(II), Pt(IV), Pd(II), Ru(III), Rh(III), Os(VIII), U(VI), Th(IV), V(V), Zr(IV), Ce(IV), Cu(II), Cd(II), Hg(II), Ag(I), Zn(II), Mo(VI), and W(VI). Of these, only a few metal ions give positive tests which are summarized in Table 1.

The growing importance of trace analysis has given the Weiszring oven (*10*) a preeminent position in this field. This technique has acquired special importance when economy of time, economy of materials, ease of handling, and sufficiently good results become the necessities. Work in this laboratory has continued in the search for selective micromethods for the estimation of metal ions, adopting the ring oven technique (*3–6*) and other techniques in conjunction with ring colorimetry (*7*). In this technique, a single drop of a test solution is applied to the center of circular filter paper placed on an electrically heated metallic ring. One or more of the components of a test sample are fixed (precipitated) with one or more suitable reagents while others are washed out to the ring zone. For the semiquantitative analysis, after development with specific reagents, the rings are compared with the rings forming the standard scale. The standard scale in a particular case is prepared by developing known microamounts of metal ions in the ring with the same reagent.

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TABLE 1
REACTIONS OF MAMT WITH METAL IONS

Metal ions	Temperature and pH	Test	Solubility or extractability	Masking effect of EDTA
Pt(IV)	High temp	Bright red color	Inextractable in common organic solvents	No effect
Pd(II)	Room temp	Orange ppt soluble in acetone	Inextractable in common organic solvents	Reaction is completely masked
Ru(III)	High temp	Pink mauve color	Inextractable in common organic solvent	Reaction is completely masked
Rh(III)	High temp	Yellow color	Inextractable in common organic solvent	Reaction is completely masked
Au(III)	Room temp	Brown ppt	Soluble in acetone	No effect
	High temp	White ppt	Soluble in acetone	No effect

Ag(I)	Room temp	White ppt (insensitive to light)	Highly insoluble	No effect
Hg(II)	Room temp	White ppt	Soluble in acetone and hot water	EDTA masks the reaction and dissolves the reaction product
Cu(II)	Room temp (neutral)	Brown ppt (not quantitative precipitation)	—	—
	In ammoniacal medium	White ppt (quantitative)	Highly insoluble	Presence of EDTA masks the reaction, but once the complex is formed it is stable toward EDTA
Bi(III)	Room temp neutral pH	Pale yellow color		
	In ammoniacal medium	Dirty white ppt	Highly insoluble	Soluble in EDTA
Tl(I)	Room temp	White shining crystals	Insoluble	Soluble in EDTA
	Ammoniacal medium			

In the present communication various studies have been framed to study its utility for the selective separation and evaluation of Au(III) Tl(I) and Ag(I). Various separations and estimations have been accomplished with MAMT, incorporating masking and solvent extraction techniques in conjunction with ring colorimetry.

EXPERIMENTAL METHODS

Materials

Instrument. The Weisz Ring Oven from National Appliance Co., Portland Oregon, was used for the estimations. The standard solution was applied to the filter paper with a Hamilton microsyringe.

Filter paper. Circles of Whatman filter paper No. 40 of 55mm diameter was used.

MAMT solution. A 1% solution of MAMT in 50% aqueous acetone was used. The reagent was prepared by the method reported by Potts and Huseby (*9*). Thiocarbohydrazide needed for the above preparation was prepared by the method of Audrieth *et al.* (*1*)

Standard Au(III), Ag(I), and Tl(I) solutions. The 1 mg/ml solutions of Au(III), Ag(I), and Tl(I) were prepared as stock solutions and, after suitable dilution, were used to work out the standard scale.

Chromogenic reagent. Potassium thiocarbonate (PTC) 1 *M* was used as stock solution and, after proper dilution, was used as spray reagent.

Masking agent. A 1% aqueous solution of disodium salt of ethylenediamine tetraacetic acid (EDTA) in ammoniacal medium was used.

Metal ion solution. The 1 mg/ml solutions of various metal ions were prepared by dissolving their analytical grade salts in 1 *M* HCl, while those of Bi(III), Sn(II), Sb(III), and Cd(II) were prepared by dissolving the corresponding salts in 4 *M* HCl.

Procedure

Selective separation and evaluation of Au(III). A circle of Whatman No. 40 filter paper (55-mm diameter) was placed on the ring oven set at 100–110°C. A known volume of the standard solution of Au(III) ions containing a number of diverse ions was transferred to the marked center of the filter paper circle and one drop each of masking agent and MAMT solution was applied, respectively, one by one after drying. The filter paper was then dried and the diverse ions were washed to the ring zone with double-distilled water. The paper was again dried and the inner zone containing gold along with silver was removed by punching the disk containing it. The small filter paper disk (6-mm diameter) was placed in the middle of another round filter paper (55-mm diameter) moistened with a drop of acetone and the fil-

ter paper carrying the disk was placed on the ring zone. The Au(III) complex was selectively transferred from the disk (6-mm diameter) to the filter paper circle (55-mm diameter) and collected in the ring zone by washing with acetone. The filter paper was dried and the ring was compared with "standard scale rings" to obtain the concentration of gold ions.

Selective separation and evaluation of Tl(I). A known amount of Tl(I) along with a number of diverse ions was applied to the center of the filter paper placed on the ring oven. A drop of a 1% solution of MAMT in 50% aqueous acetone was applied and the spot was subjected to ammonia fumes. Prior to ammonia treatment, a drop of tartaric acid was also introduced so as to prevent the formation of metal hydroxides. The diverse ions were then washed to the ring zone, first with water and then with acetone. The disk containing Tl(I) along with Ag(I) and Cu(II) was punched out and the Tl(I) was selectively transferred to another filter paper circle by washing with EDTA. Tl(I) was then washed to the ring zone with water and visualized by spraying with PTC as a chromogenic reagent. The rings so obtained were compared with the standard scale rings to compute the amount of Tl(I) in the rings.

Selective separation and evaluation of Ag(I). A known amount of Ag(I) solution containing a number of diverse ions was transferred to the filter paper circle placed on the ring oven, and one drop each of masking agent and MAMT solution was applied one after the other after drying. The diverse ions were then washed out to the ring zone with water and acetone, respectively. The filter paper was then dried and the disk containing Ag(I) was punched out and exposed to ammonia fumes so as to oxidize sulfide into sulfate. The soluble sulfate formed was transferred from the disk to another filter paper circle and washed to the ring zone with water. The filter paper was then dried and sprayed with PTC, the resulting ring was compared with the "standard scale rings," and the concentration of silver ions in the ring was computed.

The results of the estimations of Ag(I), Au(III), and Tl(I) have been summarized in Table 2.

RESULTS AND DISCUSSION

The results show that MAMT acts as a good selective fixing agent and also as a sensitive chromogenic reagent in the case of Au(III). In all the separations use has been made of the masking action of EDTA on the MAMT complexation. When MAMT is applied to metal ions in the presence of ammoniacal EDTA, only Ag(I) and Au(III) are precipitated. The Au(III) complex being soluble in acetone is washed to the ring zone, thereby affording the separation of the two. After the

TABLE 2
RESULTS OF ESTIMATIONS OF Au(III), Tl(I), AND Ag(I) WITH MAMT

Estimation of Au(III)	
Chromogenic reagent	MAMT
Fixing agent	MAMT
Color of the ring	Brown
Amount of Au(III) taken (mg/ml)	1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0
Amount of Au(III) found (mg/ml)	1.05, 2.09, 3.0, 3.87, 4.95, 6.15, 6.75
Difference (%)	+5.0, +4.5, 0.0, −3.2, −1.0, +2.5, −3.5
Estimation of Tl(I)	
Fixing agent	MAMT
Chromogenic reagent	PTC
Color of the ring	Brown red
Amount of Tl(I) taken (mg/ml)	0.80, 1.6, 2.4, 3.2, 4.0, 4.8, 5.6
Amount of Tl(I) found (mg/ml)	0.78, 1.66, 2.3, 3.4, 3.8, 4.7, 5.75
Difference (%)	−2.5, +3.7, −4.1, +6.2, −5.0, −2.0, +2.7
Estimation of Ag(I)	
Fixing agent	MAMT
Chromogenic reagent	PTC
Color of the ring	Black
Amount of Ag(I) taken (mg/ml)	0.25, 0.5, 1.0, 3.0, 5.0, 7.0, 8.0
Amount of Ag(I) found (mg/ml)	0.25, 0.51, 0.99, 2.90, 5.25, 7.25, 7.0
Difference (%)	0.0, +2.0, −1.0, −3.3, +5.0, +3.6, −5.0

Ag(I) complex is fixed, it is converted into sulfide by the addition of PTC. This is followed by the oxidation of the sulfide with bromine vapor so as to bring silver in soluble form as sulfate. The sulfate is then washed with distilled water to the ring zone and again developed with PTC to give a dark brown ring.

In the separation of Tl(I) use has been made of the solubility of its MAMT complex in EDTA. Hg(II) and Au(III) complexes being soluble in acetone are first washed out to the ring zone and thus removed. Tl(I) is then transferred to another filter paper and washed to the ring zone with the help of EDTA. It is then estimated using PTC as a chromogenic reagent.

As MAMT forms insoluble white complexes with Tl(I) and Ag(I) it is used only as a fixing agent and not as a spray reagent. For this purpose PTC has been used as chromogenic reagent. In the case of Au(III), the complex being colored, MAMT itself has been used as a chromogenic reagent.

The various cations taken in varying amounts up to 25 μg have been studied and those found not to interfere are: NH_4^+, Li(I), Na(I), K(I), Rb(I), Cs(I), Cu(II), Hg(I, II), Cd(II), Sn(II, IV), Mg(II), Sr(II), Ba(II), Ca(II), Be(II), Pb(II), Fe(II, III), Ni(II), Co(II), Zn(II), Mn(II),

Cr(III, VI), Ga(III), In(III), Sb(III, V), As(III, V), Se(IV), Te(IV), Au(III), Pt(IV), Pd(II), Ru(III), Rh(III), Os(VIII), Ir(III), V(V), Ge(IV), Mo(VI), U(VI), W(VI).

Among the various anions studied, Cl^-, Br^-, I^-, NO_2^-, $NO^-{}_3$, SO_3^{2-}, SO_4^{2-}, CH_3COO^-, CO_3^{2-}, $C_2O_4^{2-}$, and $C_2H_4O_6^{2-}$ do not interfere. However, large amounts of $[Fe(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$ do interfere.

Ag(I), Au(III), and Tl(I) do not interfere among themselves. Only Bi(III) interfered in the estimation of Tl(I) as its complex is also soluble in EDTA like that of Tl(I).

SUMMARY

MAMT has been studied for its microanalytical uses. Selective microevaluation of Au(III), Ag(I), and Tl(I) have been carried out with MAMT, incorporating masking and solvent extraction techniques in conjunction with ring colorimetry. MAMT has also been used as a chromogenic reagent for Au(III), while PTC had to be used for Ag(I) and Tl(I) as their MAMT complexes are white.

ACKNOWLEDGMENT

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MICROCHEMICAL JOURNAL 23, 460–465 (1978)

A Semi-Automated and Manual Method for Determining the Total Phospholipid Phosphorus in Amniotic Fluid

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INTRODUCTION

If induction of labor is being considered during pregnancy, the assessment of the status of the fetal pulmonary maturity is of immense value. The primary concern of the clinician is to minimize the possibility of the neonate developing Respiratory Distress Syndrome (RDS). In 1969, Nelson (*5*) suggested that the amniotic fluid phospholipid pattern might be associated with RDS. Later, he advocated the determination of lecithin phosphorus and the total phospholipid phosphorus (TPP) as a means of measuring fetal pulmonary maturity (*6*). Scandinavian investigators (*1*) have followed this concept successfully.

Gluck *et al.* (*3*) have described a relatively rapid method for estimating the lecithin/sphingomyelin ratio (L/S) using thin layer chromatography that has gained considerable usage. This determination of the L/S ratio has proven to be of great value for monitoring fetal pulmonary maturity.

Although these two approaches for measuring amniotic fluid phospholipids are similar in concept, they differ in their execution. Our work with the TPP has demonstrated that it can be a valuable diagnostic test which could serve as an alternative or supplementary test to the L/S ratio.

In view of the importance of measuring the total phospholipid phosphorus and/or the various phospholipid fractions, a simple method for decomposing the phospholipid to release inorganic phosphate as well as its subsequent quantification would be useful. Of the several methods for phosphate release that were examined (*9*), the one employing perchloric acid containing molybdate was preferred. This method was adapted for measuring the TPP content of amniotic fluid. The color development and measurement could be carried out manually or with the AutoAnalyzer at an analysis rate of 40/hr. The sensitivity and speed of analysis with the AutoAnalyzer allows the assay of phospholipids to be accomplished with relative facility, especially if large numbers of samples are to be done.

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MATERIALS AND METHOD

Reagents

Boiling granules, 10 mesh. Hengar Company, Philadelphia, Pa.

Chloroform–methanol, 2:1 (v/v).

Molybdate solution. Dissolve 30 g of ammonium molybdate tetrahydrate into water. Add 66 ml of sulfuric acid and mix. Dilute to 1 liter. Prepare only 100 ml for the manual method.

Reducing solution. Dissolve 3 g of hydrazine sulfate and 0.3 g of stannous chloride dihydrate into water. Add 28 ml of sulfuric acid and mix. Dilute to 1 liter. Add 0.2 ml of Aerosol-22 per liter for automated analysis. Prepare only 100 ml for the manual method.

Perchloric acid containing molybdate. Add 0.12 ml of the molybdate solution without Aerosol-22 to 100 ml of 70% perchloric acid and mix.

Sulfuric acid (1.2 mol/liter). Add 0.2 ml of Aerosol-22 per liter for automated analysis.

Phosphate stock standard (0.1 mg/ml P).

Phosphate standards in 1.2 mol/liter sulfuric acid. Prepare a standard of 0.1 mg/100 ml P concentration for manual measurements. For automated analysis, prepare standards of 0.05, 0.1, 0.2, 0.3, and 0.5 mg/100 ml P.

Procedure

Sample handling. Centrifuge the specimen at 2000 rpm for 10 min only if the amniotic fluid contains hemolyzed red blood cells or meconium, otherwise proceed directly to the extraction. A notation must be made indicating that the results may not be valid due to the presence of such materials, and, if possible, another sample should be submitted for assay.

Storage. If the specimen cannot be analyzed immediately, centrifuge it at 2000 rpm for 10 min, separate the supernatant, and freeze. Preferably, the sample should be extracted and the separated solvent extract stored under refrigeration.

Extraction. Pipet 1 ml of a well mixed *uncentrifuged* amniotic fluid sample into a 15-ml glass stopper centrifuge tube. Add 4 ml of a 2:1 chloroform–methanol mixture. Vortex for 30 sec. Centrifuge the tube to separate the phases. Aspirate off the fluid and any precipitated material. Transfer the entire organic phase to a clean 16 × 100-mm test tube. Evaporate the contents to dryness in a 55°C water bath with the aid of an air stream.

Digestion. Add 0.6 ml of the perchloric acid–molybdate digestion mixture to the dried residue. Add a boiling granule to the tube. Place the tube into the heating block (Hycel Thermal Block) which is at 225°C. Heat the tube for 30 min. Remove and let cool. Add 2 ml of water.

Color development and measurement, manual. Pipet 0.5 ml of 1.2 mol/liter of sulfuric acid into a tube as a blank. Pipet 0.5 ml of the 0.1 mg/100

ml P standard into another tube. Transfer 0.5 ml of the diluted digest into a tube. Add 2 ml of the molybdate reagent to all tubes and mix. Add 1 ml of reducing solution to all tubes and mix. Let stand 5 min. Measure the absorbances of all tubes within 15 min against the blank at 660 nm.

Color development and measurement, automated. Place the manifold (Fig. 1) on the pump of an AutoAnalyzer system consisting of a sampler II, pump II, colorimeter, and recorder. Place the 660-nm filters into position with a No. 1 aperture. Sample at a rate of 40/hr 1:1 (45-sec sample:45-sec wash). Use the 1.2 mol/liter of sulfuric acid in the wash cups between samples. Depending upon the usage, approximately every 2 to 3 weeks, pump 2 mol/liter of sodium hydroxide through the entire system for 10 min followed by water for 20 min.

RESULTS AND DISCUSSION

The main purpose of this communication is to describe a relatively simple, rapid method for quantitating the total phospholipids in amniotic fluid. Although phospholipids may be measured directly without acid digestion (*7, 8*), the varying chromogenicities with the color reagent renders this approach less than ideal. The usual analytical steps taken for the measurement of phospholipids are extraction, inorganic phosphate release, and determination of the inorganic phosphate.

Extraction

The extraction of the phospholipids from amniotic fluid is carried out by using a 2:1 (v/v) chloroform–methanol solvent mixture as described by Folch *et al.* (*2*) with a solvent to sample ratio of 4:1. It was not necessary to wash the solvent extract with water to remove any inorganic phosphate. This was confirmed by extracting 1 ml of 10 mg/100 ml P standard and taking the extract through the entire procedure. Values of 0.03 mg/100 ml or less were obtained consistently. With lower concentrations of phosphate, contamination was generally undetectable.

Inorganic Phosphate Release

A study of methods of inorganic phosphate release by acid digestion (*9*) showed that at a heating time of 30 min and a temperature of 225°C, a variety of methods were entirely suitable with a color reaction utilizing stannous chloride–hydrazine sulfate under strongly acidic conditions. Our preference was for the digestant of perchloric acid containing a catalytic amount of molybdate. Sphingomyelin, which is the most resistant phospholipid toward hydrolysis, gave slightly lower recoveries of about 82% with perchloric acid alone under the conditions described by Kraml (*4*). The addition of a catalytic amount of molybdate as well as extending the heating time to 30 min raises the recovery of sphingomyelin to an analytically acceptable value of 90%. In practical terms, the differ-

ence is not overly significant, since sphingomyelin is not a major phospholipid component of serum or amniotic fluid comprising but 18 and 10%, respectively. For amniotic fluid, this conclusion is borne out by the data presented for the assay of pooled samples. No significant differences were found using the five methods of phosphate release (*9*).

Color Development

The reduction of the phosphomolybdate complex by stannous chloride–hydrazine sulfate was chosen because high sensitivity was obtained under the strong acid conditions. A final concentration of about 2 mol/liter of hydrogen ion was needed with the 3% molybdate and 0.03% stannous chloride–0.3% hydrazine sulfate reagents. This acid concentration was obtained after digestion by simply diluting with the appropriate volume of water. Neutralization with alkali or evaporation of the acid were not necessary. Another advantage was the extremely rapid reducing action of the stannous chloride–hydrazine sulfate, so that heating is not required. This is particularly important for automating the color development.

Automated Color Development

The color development and measurement were adapted for use with the AutoAnalyzer at an analysis rate of 40/hr (sample:wash ratio of 1:1) (Fig. 1). A sample size of 0.6 ml of diluted digest was required. Carryover for a

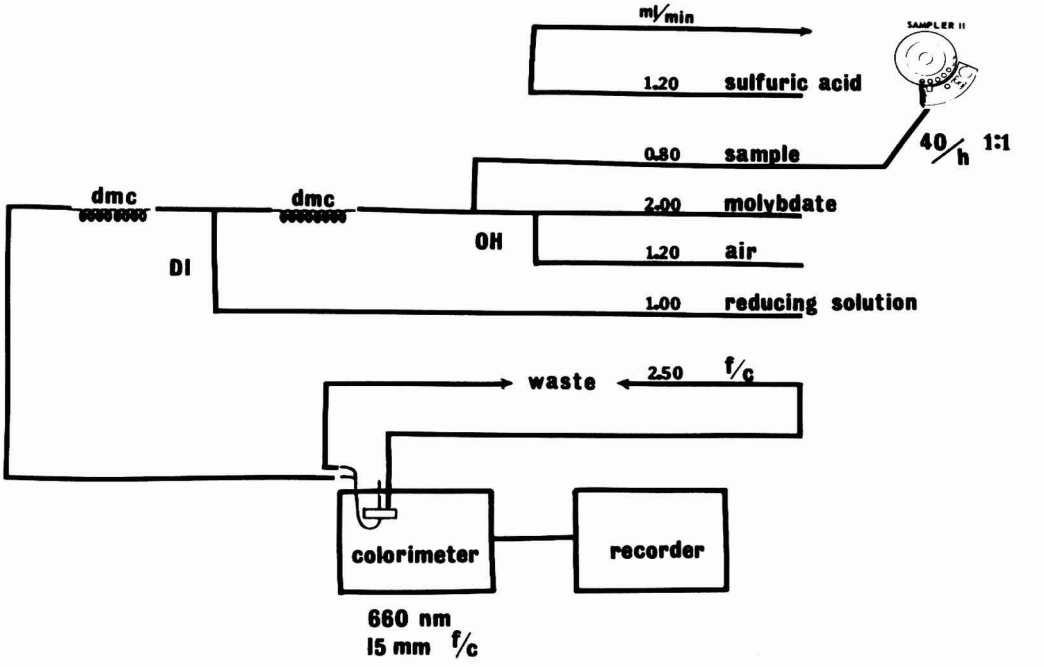

FIG. 1. Semi-automated phospholipid.

TABLE 1
RECOVERY OF PHOSPHOLIPIDS ADDED TO AMNIOTIC FLUID

Phospholipid[a]	Found (mg/100 ml P)	Added (mg/100 ml P)	Recovery (%)
Phosphatidyl choline	0.198	0.197	100.5
Phosphatidyl ethanolamine	0.212	0.208	101.8
Phosphatidyl glycerol	0.195	0.200	97.5
Phosphatidyl inositol	0.196	0.181	108.3
Phosphatidyl serine	0.193	0.197	98.0
Lysophosphatidyl choline	0.285	0.305	93.4
Sphingomyelin	0.195	0.207	94.2

[a] One milliliter of a 5 mg/100 ml in chloroform of each phospholipid was added to an amniotic fluid extract containing 0.23 mg/100 ml P.

0.05 mg/100 mg P standard following either one of 0.2 or 0.5 mg/100 ml P concentration was less than 1%. The wavelength selected for measurement was 660 nm. Linearity was found for concentrations of 0.05 to 1.0 mg/100 ml P.

Recoveries

One milliliter of a chloroform solution of each of the phospholipids at a concentration of 5 mg/100 ml was added to an amniotic fluid extract. After evaporation of the solvents and digestion, recoveries were made and results are given in Table 1. All results were greater than 93%, and they were considered to be quantitative.

Precision

Triplicate analysis of an amniotic fluid pool was done by the manual and semi-automated procedure for 5 days. Results are shown in Table 2. A coefficient of variation of 6 to 7% may be obtained by either method with an SD of ± 0.02. For clinical purposes, either method is suitable.

TABLE 2
PRECISION OF METHOD

	Day				
	1	2	3	4	5
			(mg/100 ml P)		
Semi-automated	0.27	0.24	0.28	0.28	0.28
	0.26	0.26	0.28	0.28	0.31
	0.27	0.30	0.28	0.29	0.28
	$\bar{x}$ = 0.277		SD = 0.017	CV = 6.1%	
Manual	0.24	0.31	0.26	0.27	0.31
	0.28	0.27	0.28	0.29	0.29
	0.28	0.28	0.29	0.29	0.26
	$\bar{x}$ = 0.280		SD = 0.019	CV = 6.8%	

SUMMARY

A rapid method for estimating the total phospholipid content of amniotic fluid has been described that requires 1 ml of sample. Perchloric acid containing a trace of molybdate is the acid digestant for inorganic phosphate release. After digestion at 225°C for 30 min, dilution with water yields a solution with a hydrogen ion concentration of about 2.5 mol/liter that allows the use of stannous chloride–hydrazine sulfate as the reducing agent for phosphate color development. The color development and measurement may be carried out manually or with use of the AutoAnalyzer at an analysis rate of 40/hr. A coefficient of variation of about 7% and a standard deviation of 0.02 will be obtained with either the manual or semi-automated procedure.

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Evaluation of Toluene as Replacement for Benzene in Tetrabutylammonium Hydroxide Titrant[1,2]

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INTRODUCTION

The most widely used titrant for acids in nonaqueous solutions is probably tetrabutylammonium hydroxide (Bu_4NOH). This titrant is usually prepared in benzene:methanol solution, in ratios of 10:1 or 8:2 (*3–5*). It can conveniently be prepared by dilution of commercially available Bu_4NOH in methanol.

The revised N.I.O.S.H. recommendation limits the exposure of workers to benzene to no more than 1 ppm (*6, 7*). For toluene, however, the 8-hr time-weighted average of 200 ppm is acceptable (*1*). We have, therefore, compared toluene and benzene as the major solvents in the preparation of approximately 0.05 *N* Bu_4NOH, the titrant used in our Laboratory for the microtitration of organic acids.

EXPERIMENTAL METHODS

The titrants were approximately 0.05 *N* Bu_4NOH, prepared from a 25% solution in methanol (Eastman Organic Chemicals) by dilution with reagent grade benzene or toluene. Benzoic acid was reagent ACS grade. Dimethylsulfoxide (DMSO) was "Baker Analyzed" reagent, purified by stirring a minimum of 2 days over a mixed resin bed (Fisher Scientific Co., Rexyn 300) (*2*).

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[2] "This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Department of Energy, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately-owned rights."

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The titration system was controlled by a Tektronix 4051 graphics system, as previously described (*9*). Emfs were monitored by a glass indicator electrode and a ceramic fiber-junction calomel reference electrode (Beckman No. 39402) in which the salt bridge was a saturated solution of tetramethylammonium chloride in methanol.

Stirring was provided by a magnetic stirrer. The stirring motor was separated from the titration vessel by a water-cooling plate and an aluminum plate connected to ground.

Titration endpoints were calculated according to Savitsky and Golay (*8*). A convolute was used for a third-order second derivation using 25 points. The zero crossing was found by linear interpolation near the sign change.

RESULTS

A stock solution of benzoic acid in DMSO was used for all experiments. Replicate titrations for aliquots containing 12.03 mg of benzoic acid were done with the titrant prepared with benzene and the one prepared with toluene. A representative titration curve with the toluene-containing titrant is presented in Fig. 1. The results are summarized in Table 1 which shows that the standard deviations for the two titrants were almost identical. The mean endpoint break was slightly lower for the toluene-containing titrant, 337 vs 363 mV for the benzene-containing titrant. This difference is approximately 7%. The solvent blanks for both titrants were approximately 3.5×10^{-3} mmol of base.

Based on these results we recommend replacement of benzene in Bu_4NOH titrants by toluene to decrease the hazards in working with toxic materials.

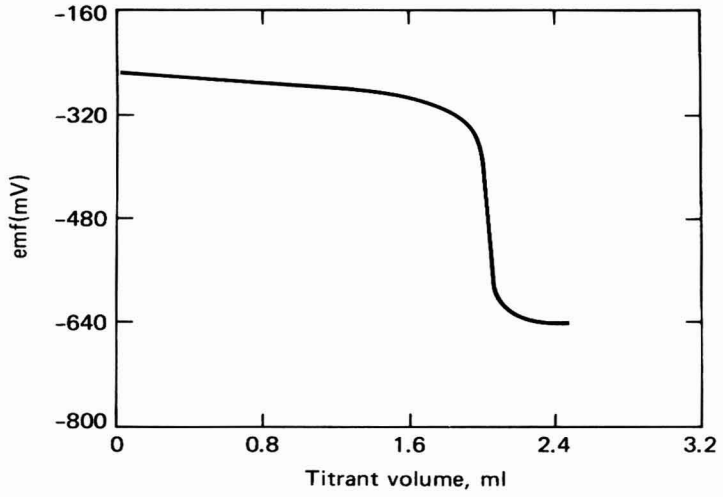

FIG. 1. Titration of 12.03 mg of benzoic acid in DMSO vs 0.05 *N* Bu_4NOH (with toluene).

TABLE 1
COMPARISON OF TOLUENE AND BENZENE IN 0.05 *N* Bu_4NOH TITRANTS

	Benzene	Toluene
Mean normality	0.050770	0.050165
Standard deviation	0.000106	0.000105
Number of replicates	4	4
Mean endpoint break (mV)	363	337

SUMMARY

Toluene has been evaluated as a replacement for benzene in tetrabutylammonium hydroxide titrant. The precision obtained with the titrants is identical. The toluene-containing titrant yields slightly smaller endpoint breaks. In view of the greater toxicity of benzene, toluene is recommended as a replacement in tetrabutylammonium hydroxide titrants.

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Dilution Method in X-Ray Fluorescence Spectrometry

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INTRODUCTION

During the last 15 years the efficiency of dilution methods in ultraviolet and visible spectrometry has come up (*2*, *3*). Even in quantitative x-ray fluorescence spectrometry several modifications of dilution method are described (*1*). Those modifications usually contain a relatively complicated theory and are of limited application. In this paper we describe a theoretically simple method that is sufficiently universal.

MATERIALS AND METHODS

Reagents. Metallic copper, molybdenum, and zinc of 99.9% purity had been the products of Ventron Corporation (Beverly, Mass.). The other chemicals had been Baker's analyzed reagents. Cupric and zinc acetate, ammonium molybdate, and arsenic acid had been used for preparation of 0.1% (w/v) aqueous solutions of corresponding metals. Other materials had been usual copper intermediates of daily production.

Instruments. X-Ray measurements had been made with a Universal Norelco Corporation (Mount Vernon, N.Y.) vacuum spectrometer with a chromium target tube.

Procedure. The following standard methods had been used: electrolytic gravimetry for copper, α-benzoin oxime gravimetry for molybdenum, barium sulfate gravimetry for sulfur (*5*), atomic absorption spectrometry for zinc (*7*), and silver dithiocarbamate colorimetry for arsenic (*8*).

For x-ray fluorescence spectrometry, about 1 g of a particular copper intermediate had been dissolved by boiling in 20 ml 68% nitric acid and 10 ml 49% hydrofluoric acid in a 250-ml Teflon beaker. The acids had been evaporated and the residue dissolved in 5% (w/v) nitric acid so that the total volume had been exactly 25 ml. Exactly 4 ml of that solution had been placed into a 7-ml plastic cup, covered with Spex-Film (Spex Industries, Metuchen, N.J.), and measured. The second solution had been prepared by dilution of 2 ml of the above solution with 2 ml of 5% (w/v) nitric acid directly in plastic cup. The rest of the procedure had been the same as above. The standard solutions had been prepared as 0.1% (w/v) primary solutions of the corresponding metals in 5% (w/v) nitric acid in

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TABLE 1
INSTRUMENT SETTINGS FOR INDIVIDUAL ELEMENTS[a]

Parameter	Element			
	As	Cu	Mo	Zn
Pulse-height analyzer base line (V)	10	0	28	0
Pulse-height analyzer window (V)	60	70	64	80
Spectrometer angle (2 θ°)	33.95	44.95	20.49	41.70

[a] X-Ray tube voltage, 46 kV; current, 18 mA; lithium fluoride crystal, air path medium, coarse collimation, scintillation detector, 0.8 kV; linear amplifier attenuation 5 and 50 sec time had been used throughout.

the same way. The parameters of measurement for individual elements are listed in Table 1.

THEORY

It is known (*4*) that the general formula of relative fluorescence intensity can be described with sufficient accuracy by the semiempirical Lachance–Traill (*6*) equation

$$c/R = 1 + \alpha(1 - c - c_m) + \alpha_m c_m \tag{1}$$

where c is the concentration of the given element, R is the relative fluorescence intensity, and α is the average correction constant of the sample matrix in the medium of concentration c_m and correction constant α_m used for dissolution. There is $R = \Delta I/\Delta I_1$ where ΔI and ΔI_1 are the fluorescence intensities of the particular element corrected by the corresponding background for the common and 100% sample ($c = 1$), respectively.

If we dilute the sample k-times with the same medium we get instead of Eq. (1)

$$c/(kR') = 1 + \alpha(1 - c - c_m)/k + \alpha_m c'_m \tag{2}$$

where $R' = \Delta I'/\Delta I_1$. The new concentration of medium c'_m must obey the basic condition of the Lachance–Traill equation that the total concentration of the system components must be unity

$$c/k + (1 - c - c_m)/k + c'_m = 1 \tag{3}$$

so that

$$c'_m = 1 - (1 - c_m)/k \cdot \tag{4}$$

Combination of Eqs. (1), (2), and (4) with elimination of α and c_m results in

$$c = (k - 1)(1 + \alpha_m)RR'/(R - R') \tag{5}$$

and after introduction of fluorescence intensities

$$c = (k - 1)[(1 + \alpha_m)/\Delta I_1]\Delta I \Delta I'/(\Delta I - \Delta I'). \quad (6)$$

Certainly we can perform the measurement and the k_s–times dilution with another solution containing the concerned element in the known concentration c_s. We will get another Eq· (6) with the subscripts s but with the same value of $[(1+ \alpha_m)/\Delta I_1]$ that can be calculated. Then we calculate c of unknown sample by means of Eq. (6). Obviously it may be $k = k_s$ or not. Knowing the value of ΔI_1 we can also calculate the value of α_m.

RESULTS AND DISCUSSION

The values of α_m (as other common Lachance–Traill correction constants) can be either positive (corresponding to the partial absorption of fluorescence) or negative (corresponding to the enhancement of fluorescence). Equation (6) shows that the values of α_m should not be too different from zero (>10) since they multiply the error of fluorescence intensity measurement. Table 2 contains the values of α_m for some common media. It is not advisable to use concentrated acids or bases since they can destroy the Spex-Film.

Examples of determination of some elements in intermediates of copper production are shown in Table 3. The values of k and k_s should not be too high since they also multiply the error of fluorescence measurement. In our opinion, their optimum value is in the range of 1.5 to 3.0. With the proper selection of the medium and the constants k and k_s the relative error of determination does not exceed 5%.

The described method has the following advantages: (a) It enables the determination of as many elements as one pleases (including just one element only). (b) It performs the necessary correction of interelement effects. (c) Common chemicals can be used as standards that can be either

TABLE 2
VALUES α_m OF SOME COMMON MEDIA

	Element			
	As	Cu	Mo	Zn
Medium	α_m			
Water	4.3388	−0.7171	1.9243	7.1140
1% aq. HCl	4.8331	−0.7863	2.0544	8.0550
1% aq. HNO_3	4.1894	−0.7622	1.7594	6.8847
1% aq. HF	4.2017	−0.7713	1.7825	6.9055
1% aq. H_2SO_4	4.4208	−0.7451	2.4252	7.1783
1% aq. NH_3	3.9572	−0.7099	2.1440	6.6032
1.8 aq. diNa-EDTA	4.0769	−0.6975	2.4801	6.9280

TABLE 3
DETERMINATION OF ELEMENTS (IN %)

Material	Element	Dilution method[a]	Standard method
Copper concentrate	Cu	24.3 ± 0.82	24.21
	Mo	0.22 ± 0.013	0.21
	Zn	1.25 ± 0.025	1.26
Reverberatory matte	Cu	41.3 ± 0.95	41.15
Convertor slag	Cu	4.52 ± 0.21	4.49
Convertor dust	Zn	12.1 ± 0.32	12.23
	As	0.25 ± 0.011	0.25

[a] Average of five determinations.

primary or secondary standards (no similarity between the sample and standard matrix is necessary). (d) The calculation of results can be performed even by means of a simple pocket calculator.

SUMMARY

The Lachance–Traill method was modified to a dilution method that retains all the advantages of the original method plus it enables the determination of as many elements as one pleases and is far simpler mathematically. The method had been tested with determination of copper, molybdenum, zinc, and arsenic in copper intermediates.

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Separation of Vanillylmandelic (VMA) and Homovanillic (HVA) Acids from Urine for Assay by a Two Resin Column System

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INTRODUCTION

In analyzing urine for vanillylmandelic acid (3 methoxy-4-hydroxy mandelic acid, VMA) and homovanillic acid (3 methoxy-4-hydroxy phenyl acetic acid, HVA) the general procedure is to extract the acidified urine into an organic solvent. The HVA and VMA may then be reextracted into water with alkali before being assayed chemically or by gas chromatography (*1–4*). Some reports suggest the use of a column to adsorb and then elute the VMA and HVA (*5–10*). In all of these cases, interference from hippuric acid and other components, often present in substantial amounts in the urine, is experienced in both the chemical and gas chromatographic procedures. In addition, extraction procedures are slow and processing of large numbers of specimens presents serious problems.

A procedure is described herein whereby all of the amino acids, catechol amines, and other amino compounds are first removed from the diluted urine by means of a cation exchange resin. This leaves an eluate containing the organic acids, free of amino groups. The acids are then fixed on an anion exchange resin, washing out other contaminants from the column. Finally, the HVA and VMA are eluted. They can then be assayed chemically or by gas chromatography after extraction.

MATERIALS AND METHODS

Reagents

Maleate buffer, pH 2.3. Dissolve 23 g maleic acid in 500 ml H_2O. Adjust pH to 2.3 with 5 mol/liter NaOH. Make to 1000 ml with H_2O. This solution is 0.2 mol/liter. Dilute 10-fold to make the 0.02 mol/liter maleate buffer.

Acetate buffer, pH 6. Add 5.0 ml gl. acetic acid to 500 ml H_2O. Titrate with 5 mol/liter NaOH solution to pH 6. Add H_2O to make to 1 liter.

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Metaperiodate solution, 5%. Dissolve 5 g sodium metaperiodate ($NaIO_4$) and make to 100 ml with H_2O.

Metabisulfite solution, 20%. Dissolve 20 g sodium metabisulfite ($Na_2S_2O_5$) and make to 100 ml with H_2O.

$NaCl-K_2CO_3$ eluting solution. Add 138 g of anhydrous K_2CO_3 and 175 g NaCl to 500 ml of water and stir to dissolve. Make up to 1 liter with water.

VMA standard solutions. Dissolve 100 mg vanillylmandelic acid (D,L-4-hydroxy-3-methoxy mandelic acid, Sigma Chemical Co., St. Louis, Mo.) in 0.01 mol/liter HCl solution and make to 100 ml with H_2O. This solution contains 1 mg/ml. Make 0.5 ml of this solution to 100 ml with the $NaCl-K_2CO_3$ eluting solution for the 5 μg/ml standard.

Methylating reagent. Trimethyl anilinium hydroxide, 0.5 mol/liter in methanol (South Western Analytical Chemical Co., P.O. Box 485, Austin, Texas).

Internal standard. 4-Methoxy benzophenone (Aldrich Chemical Co., Milwaukee, Wis.).

Homovanillic acid standard. 4-Hydroxy-3-methoxy-phenyl acetic acid (Sigma Chemical Co.), 10 μg/ml in $NaCl-K_2CO_3$ solution.

n-Pentanone-2. *n*-Pentanone-2, 300 ml (Aldrich Chemical Co., Milwaukee, Wis.) is freed of organic acids by extraction with an equal volume of 1% NaOH in a separatory funnel. Wash successively with 300 ml of H_2O twice, 100 ml 0.1 mol/liter HCl and finally 100 ml of H_2O twice. Dry over anhydrous $CaSO_4$, preferably overnight. Filter into a distilling flask and distill. Discard the first forerunnings (about 10%) and collect a fraction boiling within 2°C at approximately 100°C. If all the water has not been removed by the $CaSO_4$, the forerunnings will be turbid. This is discarded.

Cation exchange column. To 100 g cation exchange resin, AG 50W-X12, 100 to 200 mesh, hydrogen form (Bio-Rad Lab., Richmond, Cal.) add 1 liter of 2.5 mol/liter NaOH and stir for 30 min. Allow to settle and decant. Add 1 liter of water, mix, and allow to settle and decant. Repeat this washing once more. Filter on a Buchner funnel and wash with water until the washings are at pH 7.0 or lower.

Transfer the resin back to the beaker and add 1000 ml of a 1 mol/liter HCl solution and stir for 30 min. Allow to settle and decant the supernatant and fine particles. Add 1000 ml of H_2O, stir, allow to settle and decant. Repeat the washing once more. Filter on a Buchner funnel (Whatman No. 42 filter paper) and wash with 1 liter of H_2O. Transfer the resin back to the beaker and stir with 1 liter of 0.2 mol/liter malate buffer pH 2.3 for 20 min. The pH will now be 2.3. Adjust with 0.1 mol/liter NaOH solution if necessary. Filter on the Buchner funnel and wash with 500 ml of 0.2 mol/liter malate buffer pH 2.3. Transfer to a glass container and keep under maleate buffer, 0.2 mol/liter, pH 2.3. Stable indefinitely, at room temperature.

Anion exchange resin. Mix 100 g anion exchange resin, AG 2 -X8, 100 to 200 mesh, chloride form (Bio-Rad) with 1 liter of 1 mol/liter HCl solution in a 2-liter beaker. Stir for 20 min. Allow to settle and decant the supernatant. Add 1 liter of H_2O, stir, allow to settle and decant the supernatant. Repeat this process once more. Filter with a Buchner funnel and wash with 1 liter of H_2O. Transfer the residue back to the beaker and add 1 liter of 1 mol/liter NaOH solution and stir for 20 min. Allow to settle and decant the supernatant, discarding the fine particles. Add 1 liter of water, stir, allow to settle and decant the supernatant. Repeat this process once more. Transfer the resin to the Buchner funnel, filter, and wash well with water until the washings are at pH 7.0 or less. Transfer the resin back to the beaker and add 1 liter of 0.1 mol/liter acetate buffer pH 6. While stirring, titrate with 2 mol/liter acetic acid until the pH reaches 6.0 and remains there for at least 15 min. Filter on the Buchner funnel and wash with 500 ml of acetate buffer, 0.1 mol/liter, pH 6.0. Transfer to a glass bottle and keep under acetate buffer pH 6.0. Stable indefinitely at room temperature. *Note, used resin may be reconstituted by treating as for the unused resin.*

Preparation of the Columns

The columns were glass, 15 cm in length and 8 mm bore. They were fitted with a stopcock at the bottom and a 50-ml reservoir at the top. Place a glass wool plug at the bottom of the column. Aspirate some buffer solution into the column through the open stopcock. This is done to displace the air from the glass wool plug. Remove any air bubbles from the glass wool plug by pressing gently with a glass rod. Do not pack the plug too tightly. Add the resin with a pipet in 2-ml aliquots allowing to settle between additions, until a height of 7 cm is reached. Drain the column until the buffer extends approximately 0.5 cm above the resin. Do not allow the column to run dry. This introduces air bubbles and slows up the flow through the column. The columns are ready for use.

For the cation exchange column, which is used first (column No. 1) the cation exchange resin is washed in with 0.2 mol/liter maleate buffer, pH 2.3. For the anion exchange resin (column No. 2) the resin is washed in with 0.1 mol/liter acetate buffer pH 6.0.

Procedure

VMA assay. Collect 24-hr urine samples with 10 ml of 6 mol/liter HCl. Metanephrine and catechol amine concentrations can be determined on the same specimens.

To 10 ml of filtered urine add 1 ml of 0.2 mol/liter maleate buffer, pH 2.3. Add 20 ml of H_2O and adjust the pH to 2.3 with 0.5 mol/liter NaOH or HCl solution of necessary. Add this solution to the prepared cation exchange resin, column No. 1. Adjust the flow rate so as not to exceed 2 ml/min. Follow the solution with 10 ml of 0.02 mol/liter maleate buffer and allow to

drain. Adjust the pH of the collected combined solutions to 6.0 with 1 mol/liter NaOH solution. Pour this solution on to the anion exchange column No. 2. Allow to flow through at 1 ml/min. Wash the column with 30 ml of water. Discard the collected solutions.

Elute the column with 10 ml of the $NaCl-K_2CO_3$ solution. When the level of the eluting solution reaches the upper level of the resin add an additional 5 ml of the $NaCl-K_2CO_3$ eluting solution until a total of 15 ml has been collected, as measured in a graduated cylinder used for the collection. This solution is stable for several days in the refrigerator and may be stored to continue with the color development later.

To each of two test tubes, one marked blank, the other marked unknown, add 2 ml of the eluate. To the blank add 0.3 ml of H_2O. To the unknown tube add 0.3 ml of the sodium metaperiodate solution and mix. Incubate for 30 min at 37°C. Add 0.3 ml of the sodium metabisulfite solution to each tube and mix. Set the spectrophotometer to read zero absorbance with water and read the absorbance of the blank and unknown at 345 and 400 nm. Alternatively, a tracing is made on the recording spectrophotometer and the difference in absorbance from 400 to 345 nm is measured.

For the standard, add 2 ml of the 5 μg/ml VMA standard solution to each of two test tubes. Add water to the tube marked blank and metaperiodate solution to the tube marked standard, as for the unknown and unknown blank. Proceed as for the unknown.

The absorbances measured at 345 nm are substracted from that measured at 400 nm to obtain the net absorbance for the vanillin generated. The amount of VMA excreted is calculated as follows,

$$\frac{\text{absorbance unknown}}{\text{absorbance standard}} \times 7.5 = \mu\text{g VMA/ml}$$

$$\mu\text{g VMA/ml} \times \frac{\text{24 hr vol}}{1000} = \text{VMA (mg)/24 hr}$$

$$\frac{\text{VMA (mg)/24 hr}}{\text{creatinine (g)/24 hr}} = \mu\text{g VMA/g creatinine}$$

HVA assay. Transfer 5 ml of the $NaCl-K_2CO_3$ eluate to a 50-ml centrifuge tube with glass stopper. Adjust pH to 1.0 with 6 *N* HCl (approx. 2 ml) and add 1 g NaCl to the tube. Add 4 ml of pentanone-2 and shake vigorously until the NaCl dissolves. Centrifuge and transfer a 3-ml aliquot of the pentanone-2 layer to a calibrated 12-ml centrifuge tube and evaporate under nitrogen at 50°C to 0.5 ml. Add 200 μl (20 μg) of the internal standard solution and evaporate to dryness. Dissolve the residue in 50 μl of methylating reagent and inject 2 μl into the gas chromatography column, (3% OV-1. on gas chrom Q, 100/120 mesh, 6 ft × 4 mm, glass).

The injection port temperature is 350°C, column temp. 160°C, detector temp. 300°C, gas flow, helium, 40 ml/min. Flame; air 350 ml/min, hydrogen 40 ml/min, Hewlett–Packard, Model 5711. The concentration of HVA and VMA is determined by comparison to the internal standard.

RESULTS

The coefficient of variation as measured on replicate samples on different urines was ±4.5% by the resin column procedure as compared to ±6% by the extraction procedure. Table 1 records the results obtained on seven 24-hr urine samples submitted to the routine laboratory which were analyzed by both procedures. It is to be noted that both procedures were able to detect the high abnormal value found in one patient. The normal range given for VMA in human urine is 1.5 to 7.0 mg/g of creatinine. A similar range was noted for the resin procedure.

Adding known amounts (4 μg/ml) of VMA to urines which had been previously analyzed and then analyzing them again resulted in a mean recovery of 108% with a coefficient of variation of ±4.7%. A modestly high recovery is obtained by this procedure. This is difficult to explain except by assuming that some degradation of the resin columns takes place adding to the values obtained when the oxidation of the homovanillic acid to vanillin is carried out.

If ethyl acetate were used to extract the VMA and HVA from the $NaCl-K_2CO_3$ eluate of the anion exchange column, recoveries ranging from 50 to 75% were obtained. When 2-pentanone was used for extraction, recoveries rose markedly to the figures shown above.

Addition of 50 μg of hippuric acid per ml of urine for assay did not interfere with the procedure nor could the hippuric acid be observed on the gas chromatograph. By the extraction procedure, a huge peak was noted for hippuric acid. This was also observed in many urines when the extraction procedure was used followed by gas chromatography. This is so because many of our foods contain benzoic acid as a preservative.

Addition of 50 μg/ml of metanephrine or vanillin did not produce any

TABLE 1

COMPARISON OF RESULTS OBTAINED ON THE SAME SPECIMEN BY THE TWO COLUMN RESIN METHOD AND THE SIMPLE EXTRACTION PROCEDURE

	Specimen number						
Method	1	2	3	4	5	6	7
By extraction (mg/g creatinine)	2.2	9.7	0.80	2.8	0.40	29.3	1.86
By column (mg/g creatinine)	8.5	6.4	2.4	3.1	0.44	22.4	3.5

absorbance by the resin column procedure but interfered when the extraction procedure was used.

Experimentation with various internal standards resulted in the selection of 4-methoxy benzophenone as a practical standard since it comes off the column in an area free of substances being assayed. This can be seen in Fig. 1, where HVA and VMA were added, as a recovery in urine, in the method described.

DISCUSSION

In carrying out the assay of vanillylmandelic acid (VMA), the acid is oxidized to form vanillin. The vanillin absorbance is then measured at 345 nm. Many urines contain substances with substantial absorbance, especially due to bilirubin, in this area of the ultraviolet spectrum. In these cases, the absorbance rises continuously into the ultraviolet range. This is corrected by plotting a tracing on the recording spectrophotometer encompassing the range from 400 to 345 nm. The existence of a peak at 345 nm vanillin can then be ascertained and its height estimated from its background. Where a recording spectrophotometer is not available, this can be estimated by subtracting the absorbance at 400 nm from that at 345 nm. Table 1 demonstrates that this approach will detect the presence of abnormal amounts of VMA for clinical purposes.

A major problem, in applying gas chromatography to urine extracts, is the interference from contaminants which yield peaks on the chromatogram at the site where VMA and HVA are being assayed. The procedure described herein minimizes this interference (Fig. 1). Another major problem was the fact that poor recoveries ranging from 50 to 75% were obtained at first with the resin column procedure. Study of various stages of the procedure indicated that the material was being lost during the final extraction with ethyl acetate, of the $NaCl-K_2CO_3$ eluate from the anion exchange column. A search was instituted for a more effective extractant. Methyl, propyl ketone (*n*-pentanone-2) turned out to be ideal. Although it boils at 100°C, it has a very high vapor pressure even at room temperature and can readily be evaporated at low temperatures in a stream of nitrogen. The solvent cannot be purchased in a high degree of purity and a simple method for its purification is described.

The gas chromatograph can be used for simultaneous assay for HVA and VMA. However, sensitivity for VMA is low. Since the chemical procedure applied on the solution obtained from the columns can be readily applied on large numbers of specimens simultaneously, it is preferred for the routine laboratory.

At first, trimethoxy benzoic acid was used as an internal standard for gas chromatography. This comes off the column between VMA and HVA and was not satisfactory. The 4-methoxy benzophenone appears, remote from the area where cathechol amine metabolites emerge from the col-

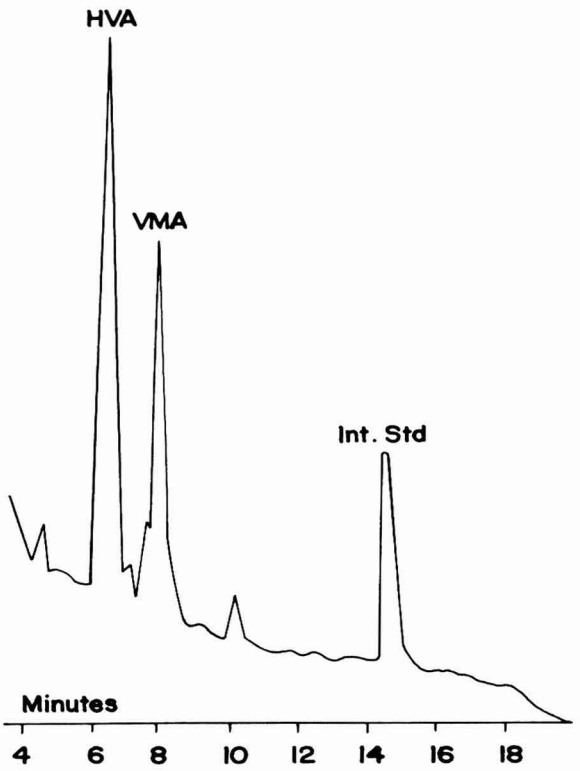

FIG. 1. Gas chromatogram of a urine extract to which has been added 40 μg of HVA and VMA and 20 μg of the internal standard. This results in 1.6 μg of HVA and VMA and 0.8 μg of internal standard being injected.

umn, and seems to have advantages over other internal standards which have been recommended.

Some urines show a high osmotic pressure and high salt concentration. With these urines adsorption to a column is interfered with and recoveries are low. This is readily corrected by first diluting the urine with twice its volume of water, and this is recommended in the procedure.

The objective of this publication is to demonstrate a general procedure for the isolation of the catechol amine metabolites VMA and HVA free of major contaminants so that they can be conveniently assayed. For gas chromatography, columns and derivatizing reagents, other than those we have used, may serve the purpose better.

SUMMARY

A procedure is described for separating vanillylmandelic acid (VMA) and homovanillic acid (HVA) from urine so that they can be assayed by chemical means or gas chromatography. The process comprises passing a threefold diluted urine over cation exchange column (AG 50W–X12) to remove catecol amines and amino acids. The VMA and HVA is then

adsorbed from the diluted urine to an anion exchange column (AG 2–X8). After washing with water, the HVA and VMA are eluted with an $NaCl-K_2CO_3$ solution. The VMA can be determined by oxidation to vanillin directly. By extraction with *n*-pentanone-2, after acidification and evaporation to dryness, the residue may be redissolved for processing or treated with a methylating reagent for gas chromatography. For the latter purpose, 4-methoxy benzophenone is recommended as the internal standard.

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Microdetermination of Silicon in Blood, Serum, Urine, and Milk Using Furnace Atomic Absorption Spectrometry

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INTRODUCTION

Silicon makes up 25.7% of the earth's crust, by weight, and is the second most abundant element, being exceeded by oxygen. Silicon is not found free in nature, but occurs chiefly as oxides, and as silicates. It is important in plant and animal life (*6*, *7*). Diatoms in both fresh and salt waters extract silica from water to build up their cell walls.

Allison (*1*) has suggested that lysosomes may be involved in malignant transformations brought about by silica and that enzymes released from lysosomes damage chromosomes, with a mutation leading to malignancy. In 1972, Carlisle (*4*) found that silicon-deficient chicks exhibited retarded skeletal development and skull deformations. He has shown by electron probe microanalysis that 0.5% silicon can be found in the rather narrow region of active calcification, that is, in the area of growth in young bone (*5*). In the same year, Schwarz and Milne (*14*) found that silicon-deficient rats showed slow weight gain and skull deformations. He further concluded that silicon is essential. Silicon has been reported to have an integrating function with an enzyme, lipoprotein-lipase (*9*).

Markkanen and Kalliomaki (*13*) reported an average of 0.27 ppm silicon in human serum; Aumonier and Quilichini (*2*) reported 0.61 to 1.4 ppm silicon in animal blood serum; Datskii *et al.* (*8*) reported 11.3 ppm silicon in urine, and Zatolokin (*16*) reported 85 mg% silicon in the ash content of urine; Dilanyan and Aslanyan found 0.22 ppm silicon in buffalo milk (*10*).

The determination of silicon in human blood and biological fluids has been described by several workers using colorimetry (*2*, *13*) or emission spectroscopy (*13*). Most methods involve time-consuming sample treatment, with consequent risk of contamination. Furnace atomic absorption spectroscopy offers a sensitive and precise method for the determination

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of traces of silicon in microsamples. A detection limit of 9×10^{-10} g is possible, with a precision of 2% at the 2 ppm level using a 10-μl sample (*12*).

The objectives of the present study were to develop a simple and rapid means of determining silicon quantitatively in microliter volumes of biological fluids with minimum sample handling and to test whether a difference exists in silicon levels in human whole blood and serum.

METHOD

Apparatus

(a) Atomic absorption spectrometer. Perkin–Elmer Model 403 in conjunction with graphite tube atomizer HGA-2000; nitrogen purging rate 1.54 liters/min.

(b) Recorder. Beckman Electroscan TM30P, Model 1002 at full scale expansion 10 mV at chart speed of 0.6 in./min.

(c) Silicon hollow cathode lamp. Perkin–Elmer Intensitron operated at 18 mA, wavelength at 251.6 nm, spectral slit width of 2Å.

(d) Deuterium arc background corrector. Perkin–Elmer.

(e) Micropipet (10 μl). Sherwood Medical Industries, P. O. Box 14522, St. Louis, MO 63178.

(f) Natelson capillary tubes. Sherwood Medical Industries, 250 μl volume and 150 mm length.

(g) Microcentrifuge. Beckman microfuge.

Reagents

(Deionized distilled water used in cleaning dilution and making standard solutions.)

Sodium silicate ($Na_2SiO_3 \cdot 9H_2O$). Fisher Scientific Co. 1000 ppm aqueous solution. Working standards of 20, 10, 6, 4, 2, 1, and 0.5 ppm were prepared by dilution of 1000 ppm solution. All solutions were stored in polyethylene bottles or vials.

Sample Collection

For blood and serum, samples were collected from five healthy individuals by the finger puncture technique. Their hands were cleaned and then rinsed with deionized water, air dried, and sterilized with rubbing alcohol. Two 100- to 150-μl blood samples from each individual were collected in separate Natelson capillary tubes. Heparinized tubes were used for blood collection, and plain tubes were used for serum. The samples were immediately transferred into separate clean polyethylene vials in which serum was obtained by microcentrifugation with a Beckman microfuge. The samples were analyzed on the same day. For urine, samples were collected in a clean 2-liter polyethylene bottle on a 24-hr basis.

Six samples were collected on different days from one healthy individual. Duplicate samples of ca. 10 ml were capped in 10-ml polyethylene vials and kept frozen until analysis. For milk, four 1-pt cartons of Carnation homogenzied vitamin D milk were purchased and the milk was stored in 10-ml polyethylene vials and kept cold until analysis.

All vials had been cleaned and rinsed at least three times with deionized water and air dried. Vials had been checked for silicon contamination; none was found.

Furnace Atomic Absorption Determinations

Fifty-microliter samples were diluted 1:1 for all runs except urine, which was diluted 1:7. A method of standard additions was employed. For example, in blood analysis 50-μl of whole blood was mixed with 50 μl of deionized water or appropriate standards. Duplicate samples of 10 μl of the mixture were pipetted separately into the center of the furnace for analysis. Their respective peak heights were measured and a graph was made to determine the silicon concentration in blood. The x intercept is multiplied by 2 because of 1:1 dilution. The sequential drying, ashing, and atomizing were performed automatically (except the ashing phase of blood analysis) under preset conditions. For blood and serum, the cycle was 30 sec drying at 100°C, 70 sec ashing from 250 to 1250°C (manual temperature programming: 20 sec at 200°C, 20 sec at 500°C, 10 sec at 750°C, 10 sec at 1000°C, and 10 sec at 1250°C), 15 sec atomizing at 2550°C; for urine 30 sec at 100°C, 30 sec at 1250°C, and 15 sec at 2550°C; and for milk and aqueous standards 20 sec at 100°C, 20 sec at 1250°C, and 15 sec at 2550°C. A standard additions curve was plotted with peak height in mm vs ppm Si added.

RESULTS AND DISCUSSIONS

Temperature Settings

Initial experiments were concerned with establishing the characteristics of the graphite tube atomizer for silicon determination in the four different types of sample. Interference from the sample matrix was the primary concern. Blood and serum, because of their complex matrices, proved to be the most difficult to deal with. Modification of the heating cycle was necessary. The longer the drying and ashing phases, the less were interferences, and the better were the sensitivities and precision. Longer ashing times, as well as increased temperatures, led to a total decomposition of organic compounds into their elements (*15*). In the present studies, both blood and serum required manual gradual elevation of the temperature from 100 to 1250°C prior to the atomizing phase. The manufacturer's manual quotes the temperature settings as approximately ±50°C at high temperature range.

A temperature setting 100°C was chosen for drying the samples. By varying the temperature of the ashing phase from 1200 to 1600°C at 100°C intervals, 1400°C was found to be the temperature beyond which the silicon signal of a 10μl standard solution (4 ppm) would diminish (*12*). A lower temperature was selected and was found to be satisfactory in eliminating most of the interferences without risking loss of silicon through volitalization.

The initial 100 sec of the cycle was devoted to sample drying and ashing. Blood tends to foam at around 300°C, which can create erroneous signals. To minimize this, the described cycle was adopted for 10-μl (1:1) blood and serum samples.

The single most important factor in governing the sensitivity was the atomizing temperature (*12*). There is a linear relationship between signal peak height and atomizing temperature range of 2500 to 2700°C, and this was observed also using blood samples. A temperature of 2550°C was selected to atomize all the samples. A better sensitivity would be obtained at a higher temperature, but considering that the life span of the tube would be shortened at higher temperatures, the lower temperature was chosen and found to be satisfactory. An atomization cycle of 15 sec was selected.

Biological Fluid Analysis

For urine and milk analysis, a simpler cycle was adopted. For urine, because of nonlinearity of calibration curves around 15 ppm, dilution was 1:7. Although a suitable secondary line could be used to correct the nonlinearity (*12*), this procedure was not preferred for sequential analysis of different sample materials.

Since blood (along with serum) was the most difficult to work with because of its matrix interference and relatively low silicon concentrations, it was studied more extensively. Fifty microliters of 1:1 blood was used at one point. The analysis time was longer than with smaller volumes, in addition to more interference and poorer precision. Ten microliters of 1:1 blood was found to require less times and yet produced 0.005 to 0.020 absorbance units.

Iron at 500 ppm was tested for possible absorption at the silicon line. No appreciable absorption was observed. Blood normally contains about 500 ppm Fe.

Sensitivity and Precision

By varying the lamp current, spectral slit width, and nitrogen gas flow rate, sensitivity and precision were relatively unchanged, although peak height was slightly decreased with increased nitrogen flow rate (*12*). The background corrector was necessary to avoid background interference.

The sensitivity ranged from 1.3 ng with aqueous solutions to 2.8 ng with

milk. Sample matrices like milk, blood, and serum somewhat suppressed the analytical silicon signals and these values varied slightly from one matrix to another, necessitating the use of standard additions calibration. The sensitivity is readily increased by elevating the atomizing temperature. The instrument manufacturer quotes 1.2×10^{-10} g at 2700°C with aqueous solutions.

The relative standard deviation with 10 μl of 2 ppm Si standard solution was 2.0%. With 10-μl (1:1) blood samples, it was between 1.0 and 3.1% for quantities of silicon varying from 0.70 to 2.70 ppm. For 10 μl of (1:1) serum (1.0 ppm Si), (1:7) urine (2.3 ppm Si), and (1:1) milk (1.7 ppm) relative standard deviations were 1.3, 1.6, and 3.4%, respectively. Three to four runs were made to determine the precision in all cases.

The precision depended critically on the stability of the atomizing temperature and the reproducible positioning of the sample in the furnace. As the furnace aged, minute graphite particles accumulated and formed a layer between the furnace tube surface and the metal housing chamber (cones were not used to support the tube.) The layer led to a change in the electrical resistance and minor variations in electrical contact, especially during the atomizing phase. Apparent temperatures which had been observed to fluctuate ±25°C in this phase tend to drift down as tubes age. Lowering of the temperature by 25°C from 2550°C causes the signal peak height to decrease 14% (*12*). This is well above the present precision value. When apparent temperature variation occurred, the furnace tube was either cleaned or replaced and the furnace chamber cleaned with tissue.

Impurities

All samples and standard solutions were kept in polyethylene bottles or vials which had been cleaned with 20% nitric acid. The containers were checked for possible contamination. Deionized water was analyzed before and after storage for 4 months. No silicon was detected at the 0.5 ng/50 μl level. A 0.5 ppm silicon standard solution was analyzed for change in concentration resulting from absorption or desorption of silicon between the solution and the container wall. The concentration remained unchanged for more than 2 months.

An attempt was made to perform wet digestion of samples in the furnace tube using concd HNO_3, concd H_2SO_4, or H_2O_2 (all reagent grade). All were found to contain significant amounts of Si, on the order of parts per million. Further uses of other reagents were not pursued, although ultrapure reagents may possibly be used. The 0.5 ppm silicon solution was stored for 2 months without any observable changes in concentration. Deionized water was checked for possible silicon content and on occasion was found to have a quantity up to 0.039 ppm, but usually none was detected.

TABLE 1
ANALYTICAL RESULTS OF SILICON DETERMINATION IN HUMAN BLOOD, SERUM, AND URINE, AND COW MILK[a]

Sample	Blood	Serum	Ratio blood:serum	Urine[b]	Milk[c]
A	1.34	0.60	2.23	5.92	1.33, 1.36
B	3.04	1.24	2.45	18.4	1.50, 1.58
C	0.88	0.34	2.59	10.0	1.98, 1.62
D	1.06	0.60	1.77	13.8	2.50, —
E	1.14	1.08	1.06	22.0	— —
F	—	—	—	6.80	— —
Mean	1.49	0.77	2.02	12.8	1.70
Literature values	4.0	0.273	—	11.3	0.215 (buffalo)
	5.0	1.3–3.0	—	15.9	
				85.0 mg% ash	

[a] Units in parts per million (wt/vol).
[b] From one healthy individual.
[c] Duplicate samples.

Results of Analyses

The results for the analyses of samples are listed in Table 1 and compared with other reported values. Silicon from the five healthy individuals appears to be generally more concentrated in blood than in serum by a factor of about two. Higher values indicated in the literature (*13, 2, 11, 3*) could be due to contamination in the sample preparation. While the drinking water in the subject's area has about 5 ppm Si, the urine has a range of 5.92 to 22.0 ppm, implying significant silicon comes from food intake. The literature reported 11.3 ppm for healthy individuals and 15.9 ppm for silicotic patients (*1, 16*). Regular homogenized milk contains between 1.33 and 2.50 ppm Si. The literature value is 0.215 ppm in buffalo milk (*10*).

SUMMARY

A simple, direct microanalytical method for quantitative determination of silicon in human whole blood, serum, urine, and milk by furnace atomic absorption technique has been developed. The method employs standard additions and combines the inherent specificity and simplicity of atomic absorption analysis with the greatly increased sensitivity possible with a heated graphite tube atomizer for the determination of silicon in microliter samples. The sensitivity of the method is 1.3 ng. The method is suitable for the direct analysis of silicon with no sample preparation other than dilution with deionized water, thereby minimizing contamination due to sample preparation. The relative standard deviation for 10 μl of blood (1:1), serum (1:1), urine (1:7), and milk (1:1) was 3.45% or less.

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A Simplified Method for the Amplification of Iodine

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INTRODUCTION

Present day demands on analytical chemistry include the necessity of detecting and determining smaller amounts and at lower concentrations than ever before. Among the approaches to meet these challenges is chemical multiplication or amplification. The terminology is not yet completely settled. The history of the approach, a great number of examples, as well as attempts to clarify classification are found in papers by Belcher and coworkers (*1*, *2*).

One example, pertaining to the determination of iodide has been known for a long time. The reaction sequence is as follows

$$1/2\ I_2 \text{ or } I^- \xrightarrow{\text{oxidant}} IO_3^-$$

$$IO_3^- + 5\ I^- + 6\ H^+ \rightarrow 3\ I_2 + 3\ H_2O$$

The amplification factor is six. As to what oxidant is used and how its excess is rendered inactive differs according to the various procedures published, but bromine and formic acid, respectively, seem at the present time the preferred choices.

There are several reasons why this particular reaction sequence has very early become a favorite. The reactions proceed stoichiometrically and without unwanted side reactions when the iodine has been separated from other material. This separation is most easily effected by extraction of the iodine into an organic solvent. The iodine may either be present initially in elemental form or be brought to it by reduction or oxidation. Neither of these is difficult. Iodine or iodide reacts readily with a large number of entities and thus the possibility is offered of indirect determinations and amplifications. Most notable in this respect is the microanalytical determination of alkoxyl groups (*6*).

The amplification sequence cannot be applied to the iodine in the organic phase. A reextraction into an aqueous medium is necessary. The elemental iodine obtained after amplification may then be determined by

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titration, photometry, or any other means; or else it may be again extracted into an organic solvent to start a second cycle. With two amplifications the factor becomes 36. Cyclic amplification, therefore, seems to be a choice method to obtain, in whatever finish is employed, a very large signal output for a very small amount of substance as original input. There is, of course, a fallacy in this reasoning. Not only the original amount of sought-for substance is amplified, but also each and every error. Of special importance in this respect are the impurities that pertain to the reagent blank. These impurities are brought in with each cycle and are amplified in the next one. As a consequence there is a practical limit to the number of repetitions. The situation is somewhat unfortunate, because the smaller the original amount of sought-for substance the more cycles are desirable but, then, the larger is the effect of impurities. For lower trace levels, therefore, the number of repetition is commonly not more than two or three.

In trace analysis reducing the number of steps and reagents, or at least decreasing the amount of reagent needed, are important tools not only to improve accuracy and precision, but also to lower the limit of detection or determination. Then the possibilities of losses and contaminations are greatly reduced. This basic idea was clearly stated and made the guiding principle in an investigation by Flaschka and Myers (*3*) directed toward the improvement of sample openings for trace analyses. When dealing with amplification the principle becomes even more important. In order to apply it to the present case of amplifying iodine, attention was focused on a procedure presented long ago by Spitzy and co-workers (*5*), as it seemed that a significant amplification should be possible. These researches used the following sequence.

Iodine, either present as such or obtained from iodide by oxidation, is extracted into carbon tetrachloride. Then the iodine is extracted into an alkaline aqueous phase to yield hypoidite. Upon acidification of the solution and addition of bromine, iodate is formed, which after reduction of the excess bromine with formic acid is allowed to amplify by a factor of six by reactions with iodide. The iodine resulting can be extracted into carbon tetrachloride and thus the next cycle is started. The method works well as long as the initial amounts of iodine are not too small and the cycling is not repeated too often. With the term "too small" is meant that the iodine of the sample is within a "reasonable" proportion to that introduced by the reagents, or, in other words, that the blank is small in comparison to the amount of analyte. The impurity iodine stems mostly from the bromine and the sodium hydroxide. Bromine is not too difficult to be freed from iodine impurities (up to a certain degree, of course) and as a matter of fact special grade bromine "low in iodine" is commercially available for application to the amplification reaction. The task of getting

the iodide out of the sodium hydroxide is a much more difficult one. Thus, if the reextraction of the iodine into a sodium hydroxide solution could be avoided the procedure would eliminate one step, an advantage per se, but also would exclude the reagent responsible for the largest contribution of extraneous iodide.

A possibility of operating toward this goal seems to be as follows. The iodine would be extracted from the sample solution into an appropriate organic solvent and the organic solution treated with bromine water. Thereby, the iodine should be oxidized to iodate which is insoluble in the organic phase and should move into the aqueous phase. At the same time the bromine should move from the aqueous phase to the organic one. After removing and rejecting the organic phase, the aqueous phase would be treated with iodide, and the iodine formed would either be determined or again extracted into an organic solvent, treated with bromine water, and the next cycle started. Preliminary experiments showed that the first assumption of the iodate being insoluble in the organic phase and moving to the aqueous one was correct. The bromine, however, did not distribute itself so favorably as to be completely removed from the water phase. For complete removal more than one extraction with organic solvent was necessary. It therefore seemed simpler to resort to the old approach of destroying bromine with formic acid. The latter reagent is the least offensive one in the whole cycle when it comes to iodine impurities, as it does not contain any significant amount of this element.

In the new approach with the alkaline solution not existing, the formic acid has benefits beyond reactions with the bromine. It establishes the correct pH necessary for this reaction but also provides the protons necessary for the reaction between iodate and iodide. Thus addition of buffer is unnecessary, eliminating another source of possible contamination.

In addition to the elimination of the reextraction into a sodium hydroxide solution only one change has been made in comparison to the Spitzy procedure, the carbon tetrachloride has been replaced by chloroform which is less objectionable with regard to odor and health considerations.

The question still to be answered is what kind of finish should be applied to the solution containing the x-times amplified iodine. Contrary to situations in other analytical cases the problem of matrix is of no concern here. Consequently, the selection depends greatly on the final amount or concentration of iodine present, and for a given case on personal preference and available instrumentation.

There is at first the possibility of extracting the iodine one final time from the aqueous phase into chloroform and subjecting this violet solution to a photometric measurement. The absorptivity of iodine is high in com-

parison to that of other inorganic species but not high enough to allow serious consideration of direct photometry after having gone through all the efforts of amplification.

The "dead stop" titration with thiosulfate is well established and applicable to a concentration range from traces to quite high levels and off hand may seem, and probably is, the most general approach. In the particular instance here, however, the thiosulfate titration using starch as the indicator was selected. Such a titration can be effected with quite dilute solution (0.001 *F* and even somewhat lower) and gives acceptable results because of the exquisitely well-discernible end point. In case the blue color due to the iodine–starch reaction is too weak for consideration of a visual end point, the solution can at once be subjected to a photometric titration.

The photometric titration was greatly facilitated by the particular instruments recently developed in the authors' laboratory (*4*), but, of course, any photometer or phototitrator can serve as well.

With these theoretical and practical considerations at hand, experimentation was initiated that finally resulted in the procedure presented below.

CHEMICALS AND EQUIPMENT

Chemicals

Distilled water, passed through a mixed-bed deionizer column, was used exclusively. All acid, base, and buffer solutions were prepared from reagent grade chemicals. The potassium iodate was primary standard grade. The potassium iodide was J. T. Baker "Analyzed Reagent" grade.

All organic chemicals and solvents were reagent grade or better, with the exception of the chloroform (see below).

Spectrophotometer

The optoelectronic photometer previously described (*4*) was used here. The light source was a Hewlett–Packard 5082-4658 with a peak emission at 635 nm, the detector a Fairchild FPT-120 phototransistor.

Glassware

Common laboratory glassware such as beakers and flasks was used as needed. The titration vessel used was a 50-ml erlenmeyer flask. Class A volumetric glassware was used where needed and without further calibration. The buret was a Kimax No. 4834 with platinum micro tip.

Reagents and Solutions

Potassium iodate reference solution. Add 0.01686 g KIO_3 to 100 ml water in a 2-liter flask. Shake until all solid is dissolved and then make to mark with water; 1 ml of this solution, upon acidification and the addition of iodide, will yield exactly 30 μg iodine.

Potassium iodide solution, 0.1 F. Add about 2 g KI to 100 ml water. Slow oxidation of iodide to iodine can occur; thus the solution should be prepared as needed.

Sodium thiosulfate stock solution, 0.01 F. Dissolve 1.6 g $Na_2S_2O_3$ in 1 liter of water. Allow to age for at least 1 day before preparing working titrants.

Sodium thiosulfate titrant solution, 0.001 F. Dilute the stock solution 1:10 with water and standardize against the iodate solution after adding KI and dilute HCl. Prepare the titrant anew everyday.

Bromine water. Add 3 ml of bromine to about 25 ml water and stopper tightly.

Formic acid. Concentrated reagent grade formic acid directly from the container.

Starch solution. Add 3 g starch to about 3 ml boiled water, and make into a paste. Dissolve the paste in about 400 ml boiled water. The solution is stable for 1 or 2 days.

Chloroform. Use spectral grade material and for removal of the last ethanol traces shake a portion with an equal volume of water and discard the aqueous phase.

PROCEDURAL DETAILS

Sample Treatment

If the iodine in the sample is present totally in elemental form, the procedure can be started immediately. If part or all is present as iodide add dilute potassium permanganate dropwise until a slight, pink color persists. If higher oxidation states of iodine are present add a reductant like sulfurous acid and then proceed with the liberation of iodine through addition of permanganate.

Procedure

(1) Select a volume of sample so that the concentration after amplification is at least 0.5 μg/ml iodine in the solution to be titrated. Place the sample into a separatory funnel and shake with two 5-ml portions of chloroform. Drain the chloroform layers into a 60-ml separatory funnel.

(2) To the chloroform solution add 5 ml water, 1 ml bromine water, and shake for 5 min.

(3) Allow the phases to separate and discard the chloroform. Withdraw the aqueous phase into a 50-ml erlenmeyer flask, and rinse the inside of the funnel tip with some water. Add 4 ml formic acid, and mix well.

(4) Add 5 ml of the iodide solution, mix well, and then add 2 ml of the starch indicator solution.

(5) Align the flask in the light path of the photometer and titrate with thiosulate solution.

(6) Carry 10 ml of pure chloroform through the procedure and titrate to determine the blank.

(7) Plot the results of both titrations and determine the end point in the usual manner.

RESULTS AND DISCUSSION

To test the assumption on which the new procedure is based, a solution containing a known amount of iodate was shaken with chloroform, the organic layer drained and discarded, and after addition of acid and iodide to the aqueous phase the iodine titrated with thiosulfate. The correct amount of titrant was consumed indicating that iodate is insoluble in chloroform.

Next some bromine was shaken with chloroform, the organic layer discarded, and to the aqueous layer added some potassium iodide, acid, and starch. A deep blue color developed indicating that the removal of bromine was not complete. Several extractions were needed to achieve this goal. Upon repetition of the experiment, a small amount of formic acid was added before the iodide and starch; no blue color formed, indicating that in pure aqueous solution, the formic acid–bromine reaction finds the correct conditions and that adding the usual buffer is not necessary.

The photometric titration was tested as to reproducibility and lower limits. Known amounts of the iodate reference solution were treated with

TABLE 1
PHOTOMETRIC TITRATION OF IODINE TO ESTABLISH THE TITER OF A THIOSULFATE SOLUTION

ml IO_3^- reference solution	μg Iodine	ml $S_2O_3^{2-}$ consumed	Titer (μg/ml)
0.825	25	0.275	90.9
		0.277	90.2
		0.275	90.9
		0.276	90.6
		0.273	91.6
		0.275	90.0
		0.275	90.9
1.65	55	0.549	91.1
		0.548	91.2
		0.550	90.9
		0.551	90.7
		0.550	90.9
		0.549	91.1
		$\bar{x}$ =	90.9
		s =	±0.033

TABLE 2
PHOTOMETRIC TITRATION OF IODINE AFTER SIXFOLD AMPLIFICATION

μl IO_3^- reference solution	Iodine		ml $S_2O_3^{2-}$		
	μg given	μg after sixfold amplification	Consumed	Calculated	Δ%
100.0	3.0	18	0.200	0.198	+1.01
			0.195	0.198	−1.52
			0.195	0.198	−1.52
			0.200	0.198	−1.01
			0.205	0.198	+3.54

acid and potassium iodide, starch added, and the photometric titration effected. Results of such titrations on two levels are presented in Table 1. The high precision is remarkable, especially in the light of the reported known difficulties that occur in the titration of iodine with thiosulfate when the starch is added at the beginning of the titration. It seems that these difficulties do not occur when very small amounts of iodine are involved.

Next known amounts of iodine were carried through the procedure described above. When attempting to titrate the resulting very small amounts of iodine, unexpected difficulties with the blue color occurred and at levels where the titration without amplification had presented no problems. The occurrences were traced to alcohol impurities in the chloroform. After shaking the chloroform with water the results become normal. No explanation for this phenomenon can be given. When using the treated chloroform the results were quite satisfactory as can be seen from Table 2.

With the equipment and chemicals used as described, an error exceeding 5 to 10% became evident with original amounts of 85 ng iodine, that is, 0.5 μg of iodine to be titrated after amplification. In part the blank is responsible as it amounts to about 0.05 μg. Experiments were performed in which the appropriate amount of sodium hydroxide [according to Spitzy (*5*)] were added and neutralized with hydrochloric acid. The blank jumped by a factor of 10 to 15. In addition the fluctuations of these blanks were quite pronounced.

With the elimination of the sodium hydroxide the blanks fluctuated only between 0.05 and 0.07μg. The main sources of impurities are the bromine and the potassium iodide. By using higher grade bromine and probably subjecting the potassium iodide solution to a chloroform extraction prior to use, the blank could be further reduced. However, then a photometric blank titration would become impossible because not enough points

TABLE 3
PHOTOMETRIC TITRATION OF IODINE AFTER 36-FOLD AMPLIFICATION

μl IO_3^- reference solution	Iodine		ml $S_2O_3^{2-}$		
	μg given	μg after 36-fold amplification	Consumed	Calculated	Δ%
25.0	0.75	27	0.295	0.297	−0.67
50.0	1.5	54	0.600	0.594	+1.01
75.0	2.25	81	0.890	0.891	−0.11
100.0	3.0	108	1.185	1.190	−0.42
200.0	6.0	216	2.385	2.38	+0.21

would be obtained to plot a useful curve, even if going to the difficult task of decreasing the thiosulfate concentration below 0.001 F or employing finer subdivided microburets.

It should be realized that the existing blank consumes only about 0.005 ml while involving an absorbance change of about 0.4 absorbance units when using a beaker providing a path length of approximately 10 cm.

For a very small blank the following, more complicated, way of determining it could possibly be employed.

Take a certain known volume of iodine, say A ml. Titrate this solution then take $2A$ ml and titrate again. The amount of thiosulfate corresponding to A ml iodine shall be denoted as B and the volume due to the blank as X. Then the result of the first titration will be $B + X = R$ ml. That of the second titration will be $2B + X = R'$ ml. From these two equations $X = 2R - R'$. It is necessary to hold R very low in order to avoid that the hopefully minute blank X does not become the small difference between two large numbers. This approach of evaluating the blank was, however, not followed in detail. Also, no attempts were used to improve the quality of the reagents as mentioned above. The blank of only 0.05 μg with rather common reagents and equipment was deemed acceptable and the main

TABLE 4
RESULTS OF THE ANALYSIS OF "UNKNOWNS"

ng Iodine			
Given	Found	Difference	Percentage
111	114	+3	+2.7
143	145	+2	+1.4
188	190	+2	+1.1
297	301	+4	+1.3
512	518	+6	+1.3

point has been proven, namely, that the results are significantly better when the sodium hydroxide is eliminated.

Test were made as to the feasibility of cycling. The results of two cycles are shown in Table 3.

Table 4 shows results of "unknowns." These were solutions the contents of which were disclosed to the experimenter only after performance of the determinations. The amounts of iodine involved are in the nanogram region and although very close to the limit of determination of the methods the errors are not exceedingly large.

SUMMARY

The sample solution is treated so that all iodine is present in the elemental state. This iodine is extracted into chloroform and thereby separated with very high selectivity from almost any matrix. Until now, in order to apply amplification via oxidation to iodate and reaction with iodide, a reextraction into a sodium hydroxide solution was necessary. In the new procedure the organic phase is shaken with bromine water. Thereby, the iodate formed moves completely into the water phase while the bromine accumulates in the chloroform. Remaining bromine in the water is destroyed with some formic acid. No buffer is needed, because the acid establishes the correct conditions for this reaction and also that between iodate and iodide. The iodine formed in sixfold amount can now be titrated visually or photometrically with thiosulfate or subjected to a second amplification cycle. The new procedure eliminates the reextraction, and the addition of some reagents especially sodium hydroxide which is the main contributor of extraneous iodine. Thus, the blank is reduced by a factor of 10 or more and is also more constant. Iodine at lower levels (< 1 μg/ml) can be determined and with higher reliability.

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A Semi-Automated and Manual Method for Serum Phospholipids

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INTRODUCTION

The role of the various phospholipids in the human body has been delineated in great detail. Their measurement in blood plasma, amniotic fluid, and tissues has proven to be invaluable for diagnostic purposes. Generally, the determination of phospholipids in biological materials can be divided into three parts which include separation by either extraction or precipitation, release of inorganic phosphate, and the quantitation of the phosphate. A great many methods for accomplishing phospholipid analysis are available (*1*).

One of the more popular methods for serum phospholipids was described by Kraml (*2*) who used perchloric acid as the phosphate-releasing agent at a temperature of 200 to 225°C and heating for 15 min. My investigations have indicated that sphingomyelin is only hydrolyzed about 80% under these conditions (*3*). The addition of a catalytic amount of molybdate to the perchloric acid as well as extending the time of heating to 30 min will increase the phosphate release to 90%. Similarly, the addition of vanadium pentoxide as a catalyst to a mixture of sulfuric and perchloric acids (*4*) markedly accelerates the breakdown of sphingomyelin, so that it may be 100% recovered with a heating time of 10 min at 225°C. I have adapted the use of this latter digestion mixture for the measurement of serum phospholipids. The color development and measurement can be carried out manually or with use of the AutoAnalyzer at an analysis rate of 60/hr with a carryover of 1.5%.

MATERIALS AND METHOD

Instrumentation. An AutoAnalyzer system consisting of a sampler II, pump II, colorimeter, and recorder was used for the automated color development and measurement. A Turner Model 350 spectrophotometer was used for manual measurements. A Hycel Thermal Heating Block with a temperature control setting of 225°C was used for the acid digestion process.

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Reagents

Phospholipids. Sphingomyelin and phosphatidyl choline (synthetic dipalmitoyl lecithin) were obtained from Sigma Chemical Co., St. Louis, Mo. Phosphatidyl ethanolamine was from P-L Biochemicals, Inc., Milwaukee, Wis. Lysophosphatidyl choline was from Supelco, Bellefonte, Pa. All of the phospholipids were dissolved in chloroform to give a concentration of 10 mg/100 ml.

Ethanol–ethyl ether, 3:1 (v/v).

Molybdate solution. Dissolve 30 g of ammonium molybdate tetrahydrate into water. Add 66 ml of sulfuric acid and mix. Dilute to 1 liter. Prepare only 100 ml for the manual method.

Reducing solution. Dissolve 3 g of hydrazine sulfate and 0.3 g of stannous chloride dihydrate into water. Add 28 ml of sulfuric acid and mix. Dilute to 1 liter. Add 0.2 ml of Aerosol-22 per liter for automated analysis. Prepare only 100 ml for the manual method.

Sulfuric acid (1.2 mol/liter). Add 0.2 ml of Aerosol-22 per liter for automated analysis.

Phosphate stock standard (0.1 mg/ml P).

Phosphate standards in 1.2 mol/liter sulfuric acid. Prepare a standard of 0.1 mg/100 ml P concentration for manual measurements. For automated analysis, prepare standards of 0.05, 0.1, 0.2, 0.3, and 0.5 mg/100 ml P.

Acid digestion mixture. Add 8 ml of water to 80 ml of concentrated sulfuric acid. Dissolve 0.2 g of vanadium pentoxide into 5 ml of 1 mol/liter sodium hydroxide. Add 1 ml of the vanadium pentoxide solution to the diluted sulfuric acid. Add 8 ml of 70% perchloric acid and mix.

Procedure

Extraction. With vortexing, pipet 0.075 ml of serum into a tube containing 2 ml of an ethanol–ethyl ether mixture (3:1 v/v). Centrifuge at 2500 rpm to pack the precipitate. Carefully decant all of the solvent into a clean 16 × 100-mm test tube. Evaporate the contents to dryness in a 55°C water bath with the aid of an air stream. If possible, concentrate the residue at the bottom of the tube.

Acid digestion. Add 0.18 ml of the acid digestion mixture to all tubes containing residues of serum extractions and to an empty tube for a blank. Heat all tubes for 10 min at 225°C. After 3 min of heating, swirl the acid in the tubes to rinse down any carbon on the sides of the tube to ensure complete digestion. When finished, remove the tubes and let cool. Add 2.4 ml of water to all tubes and mix. The final volume is 2.5 ml.

Color development and measurement, manual. Pipet 0.5 ml of the 0.1 mg/100 ml P standard into a tube. Transfer 0.5 ml of all the diluted digest into tubes. Add 2 ml of the molybdate reagent to all tubes and mix. Add 1 ml of the reducing solution to all tubes and mix. Let stand 5 min. Measure the absorbances of all tubes within 15 min against the blank at 660 nm.

Color development and measurement, automated. Place the manifold (Fig. 1) on the pump of the AutoAnalyzer. Place the 660-nm filters into position with a No. 1 aperture. Sample at a rate of 60/hr 2:1 (40-sec sample:20-sec wash). Use the 1.2 mol/liter sulfuric acid in the wash cups between groups of samples. Depending upon the usage, approximately every 2 to 3 weeks, pump 2 mol/liter sodium hydroxide through the entire system for 10 min followed by water for 20 min.

Calculations. For automated analysis, subtract a blank value of 0.005 mg/100 ml P from each concentration value found. Multiply the corrected concentration by a dilution factor of 33.33. To obtain the phosphatidyl choline (lecithin) concentration, multiply by an additional conversion factor of 25. For manual analysis, multiply by a dilution factor of 66.67.

RESULTS AND DISCUSSION

Digestion

Previously, it was demonstrated that five different acid digestion mixtures were suitable for quantitative inorganic phosphate release from the phospholipids found in amniotic fluid (*3*). Similar phospholipids are present in serum with phosphatidyl ethanolamine, phosphatidyl choline, lysophosphatidyl choline, and sphingomyelin accounting for 95% of the total (*5*), so that any of the digestion mixtures described earlier can be used. However, in contrast to amniotic fluid, serum samples will yield a larger amount of extractable organic compounds, and for complete diges-

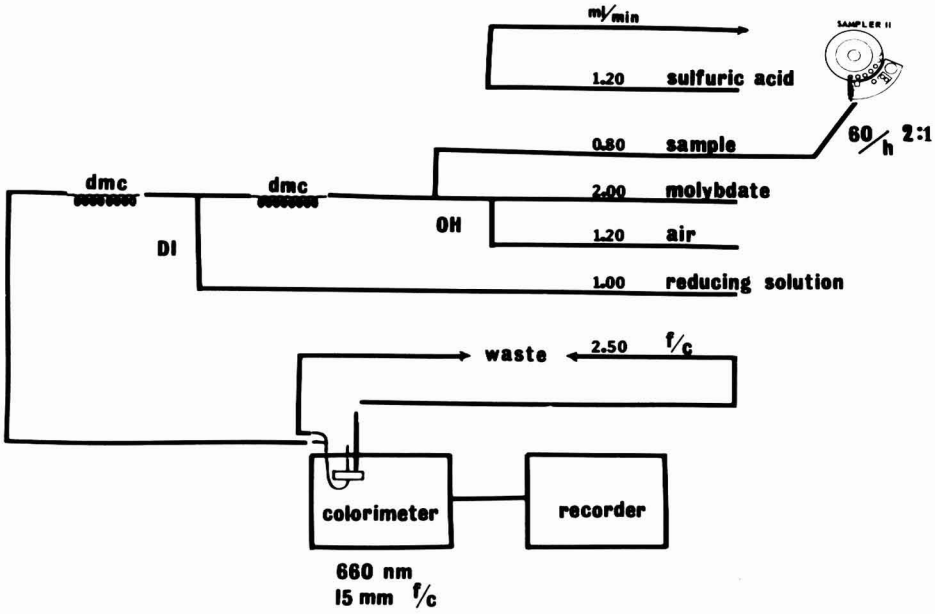

FIG. 1. Semi-automated phospholipid.

tion of all of the phospholipids with a relatively short heating time, a stronger oxidizing digestion mixture is required. The best digestants appear to be sulfuric acid–perchloric acid containing vanadium pentoxide and sulfuric acid used with 30% hydrogen peroxide (*3*). I have selected the sulfuric acid–perchloric acid–vanadium pentoxide mixture because of the short heating time needed for a quantitative recovery of sphingomyelin. Figures 2 and 3 show that a minimum time of 5 min at a temperature of 200°C were sufficient conditions. Because of possible differences in heating blocks, temperature controls, and heat transfer characteristics, I recommend heating at 225°C for 10 min to allow a wide margin of error. For those laboratories which prefer not to use perchloric acid as part of the digestant, the digestion procedure with sulfuric acid, 30% hydrogen

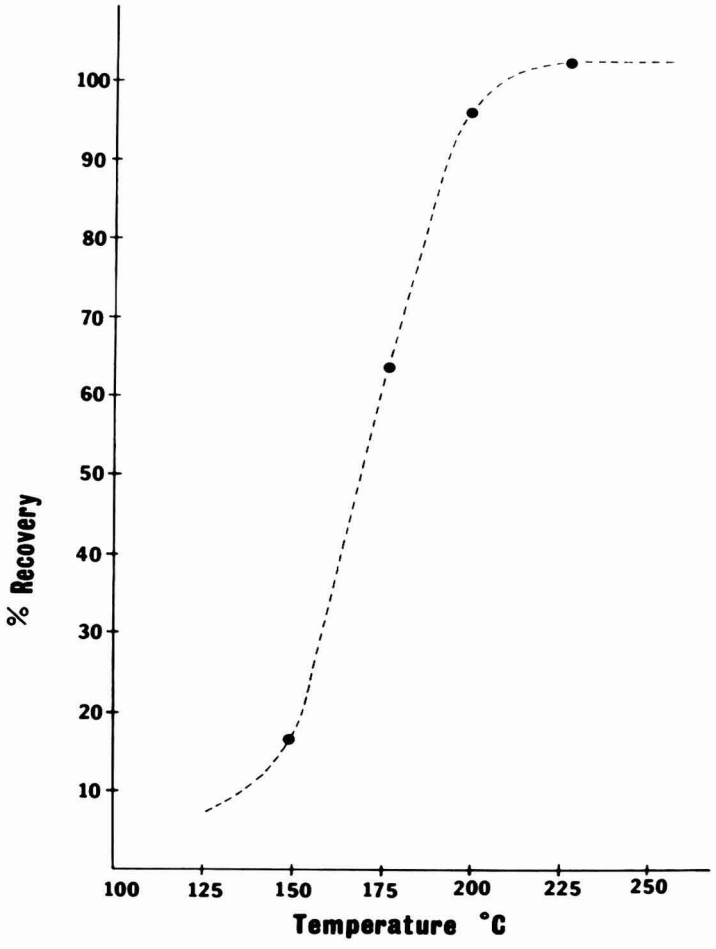

FIGURE 2

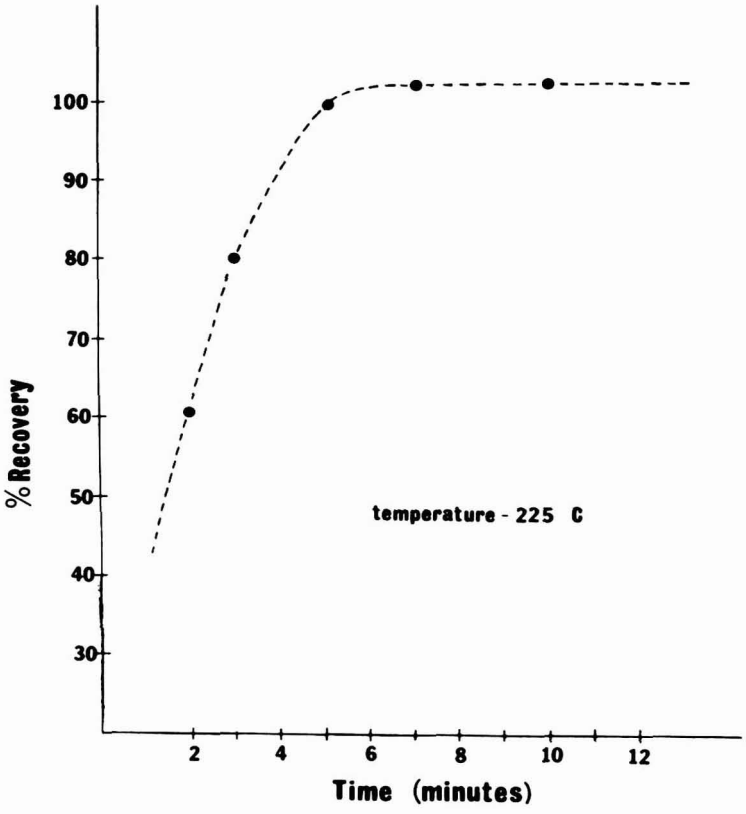

FIGURE 3

peroxide, and 5% urea (*7*) is outstanding, although additional manipulations and time are necessary.

Color Development, Manual

Color development is accomplished with 3% w/v ammonium molybdate and 0.03% w/v stannous chloride–0.3% w/v hydrazine sulfate reagents. This reducing reagent was selected because high sensitivity was obtained under the strongly acidic conditions used (*8*). The final H^+ concentration was about 2 mol/liter. The acid concentration of the diluted digest was approximately 2.1 mol/liter for the digestant described, although this can range from 2.1 to 2.9 mol/liter depending upon the acid digestion mixture. Since only 0.5 ml of the diluted digest is required for color development, the H^+ concentration of the aliquot may vary somewhat without greatly affecting the final concentration of acid.

The stability of the color is about 25 min, so that measurements should be made within a 15-min period following color development. In time, a

yellow chromogen forms that changes the blue color to one of blue–green.

Color Development, Automated

For automated color development and measurement, an AutoAnalyzer was used (Fig. 1). A sample volume of about 0.5 ml was required at an analysis rate of 60/hr (sample:wash ratio of 2:1). Carryover was 0.8% for a 0.05 mg/100 ml P standard following one of 0.2 mg/100 ml and 1.4% following a 0.5 one. At a slower analysis rate of 50/hr (1:1), the carryover is lowered to 0.8%.

The wavelength selected for manual or automated measurement was 660 nm. Linearity was obtained for concentrations of 0.05 to 1.0 mg/100 ml P.

Recovery and Precision

The recovery of the four main phospholipids found in serum is shown in Table 1. Recoveries ranged from 90.1 to 106.4%, and they were considered to be quantitative.

Replicate samples were assayed for 5 days to yield a coefficient of variation of 7%.

Comparative Methods

Four commercial control materials were assayed for total phospholipids. These results are given in Table 2. The data show that values within an acceptable range were obtained with the proposed method. These values were compared to those given by the manufacturers using the Boehringer Mannheim method (BMC).

Thirteen serum samples were assayed for total phospholipids by the method of Kraml (*2*), the proposed method, and one using sulfuric acid–hydrogen peroxide as the digestant. These results are given in Table 3. Assuming that the sulfuric acid–hydrogen peroxide method yields a 100% recovery, the average recovery for the proposed method was 100.2% with a range of 91.0 to 117.5%. The method of Kraml gave an average recovery

TABLE 1
RECOVERY OF SERUM PHOSPHOLIPIDS[a]

Material	Taken	Found	Recovery (%)
Phosphatidyl choline	0.393	0.418	106.4
Phosphatidyl ethanolamine	0.416	0.375	90.1
Sphingomyelin	0.413	0.383	92.7
Lysophosphatidyl choline	0.610	0.595	97.5

[a] A 1-ml sample of each phospholipid containing 10 mg/100 ml in chloroform was taken and added to a serum extract containing 215 mg/100 ml of phospholipid expressed as lecithin.

TABLE 2
COMPARISON OF METHODS USING COMMERCIAL CONTROL MATERIAL

Material	Manufacturer method and value given	Value found (n = 4)
	mg/100 ml as lecithin	
Validate lot 0704084	177 ± 35 (BMC)	162 (158–168)
Validate A lot 0075014	126 ± 15 (BMC)	133 (128–138)
Validate El lot 4B134	373 ± 53 (BMC)	380 (363–392)
Ortho Normal lot 8P015	222 ± 30 (BMC)	198 (190–208)

of 91.5% with a range of 79.8 to 111.4%. Based upon the recoveries of the phospholipids found in serum with the different methods of digestion (*3*) and an estimated serum composition of 4% phosphatidyl ethanolamine, 69% phosphatidyl choline, 18% sphingomyelin, 8% lysophosphatidyl choline, and 1% miscellaneous phospholipids (*5, 9, 10*), the calculated recovery for perchloric acid digestion was 90% and for the proposed method 98%. The average recovery found for the proposed method and that of Kraml agreed quite well with the calculated value, although the

TABLE 3
COMPARISON OF METHODS FOR SERUM PHOSPHOLIPIDS

Sample number	Method		
	Sulfuric acid–H_2O_2	Kraml ($HClO_4$)	Proposed
	Concentration mg/100 ml as lecithin		
1	223	183	219
2	205	170	206
3	217	182	216
4	198	197	200
5	186	158	179
6	221	205	201
7	214	187	213
8	233	198	220
9	351	280	342
10	263	293	262
11	154	162	159
12	214	179	224
13	160	178	188

latter method gave a majority of its recoveries in the 80% range. From this evidence, the proposed method appears to give results which are about 10% higher than those found using perchloric acid for digestion.

SUMMARY

A rapid method for determining the total phospholipids in serum has been described. The residue from the extraction of 0.075 ml of serum with 3:1 v/v ethanol–ether was digested with an acid mixture of sulfuric acid, perchloric acid, and a catalytic amount of vanadium pentoxide at 225°C for 10 min. Color development and measurement at 660 nm may be done manually or with use of an AutoAnalyzer at an analysis rate of 60/hr. Comparative analysis of four commercial control materials showed excellent agreement. A comparison of the total phospholipid concentration of 13 serum specimens analyzed using digestion with perchloric acid, sulfuric acid–30% hydrogen peroxide, and sulfuric acid–perchloric acid–vanadium pentoxide indicated that the latter two means of digestion are interchangeable and yield average recoveries of about 10% higher than those from the perchloric acid. The proposed method had a coefficient of variation of 7%, and gave quantitative recoveries of the serum phospholipids.

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MICROCHEMICAL JOURNAL 23, 505–516 (1978)

A Simple Prototype Procedure for Cerebrospinal Fluid Cholesterol Determinations[1]

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INTRODUCTION

Several disease states have been considered as contributing to the concentration of cerebrospinal fluid (CSF) cholesterol as a reflection of decomposition in the central nervous system (*16*). This is intriguing from the analyst's viewpoint because the lipid levels in CSF are quite low in comparison to serum where they are approximately 500 times higher. Therefore, measurements in CSF are obviously much more difficult to carry out.

The use of enzymes as reagents for the treatment of lipids such as cholesterol or triglycerides in order to generate sensitive chromophores or fluorophores for measurement could provide a convenient means by which cholesterol might be quantified in CSF providing that the concentration of cholesterol could be increased in the final step to that approximating the level of a more concentrated serum specimen after it is processed. Extraction of a large sample followed by separation of the organic phase for evaporation is the most frequently used technique for increasing the concentration of the constituent to be measured (*5*) and this could be applied to CSF. Spectrophotometric detection techniques for cholesterol in CSF have included color reactions to produce concentrated chromophores (*8, 17*), or fluorescence reactions to generate somewhat less concentrated fluorophores (*14*) but enzyme reagents for hydrogen peroxide production as an indirect measure of CSF cholesterol have not yet been applied to the problem.

The primary purpose of the present investigation is to establish a gen-

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eral concept for a procedure in which a naturally dilute sample represents the entire diluent volume required to bring a constituent to a suitable concentration level for an effective determination. Although no claim is made here for a final product in terms of a procedure, it is believed that the described approach is based on a valid concept. As a specific application of this concept, we will demonstrate that it would be quite simple to measure a very low concentration of cholesterol as one might encounter it in CSF without resorting to a tedious preliminary step such as extraction that would otherwise be required in order to maintain good Ringbom characteristics (*2*). Also, an example of a naturally occurring interference, such as bilirubin, will be used as a model to show how the use of the sample as sole diluent for reagents could magnify the effect of an interfering substance. Bilirubin is a known interactant of the quantification scheme (*9, 10*), and would have to be either removed, masked, or corrected for. Therefore, an alternative procedure will be suggested that could serve to eliminate the interference, but unfortunately it increases the number of procedural steps. Fortunately, however, at the same time it would also provide a means for further concentration of the cholesterol in the dilute sample to be investigated. This added step, as will be shown, could provide strong absorbances as an extensive factor (*4*) for concentrations that do not normally exceed 0.5 mg/dl or 0.013 mmol/liter. Free cholesterol could also be determined in a nonhydrolyzing extraction process (or direct process) merely by eliminating the cholesterol ester hydrolase of the reagent. In addition, the demand of specially prepared reagent tubes would provide the opportunity for the laboratory and the manufacturer to enter into a useful association, since, in this instance, the manufacturer has almost sole access to the instruments of preparation for providing small weights of mixed reagents in a readily accessible single tube in which the reaction is initiated through mere solution of the reagents by the sample; this represents a truly one-step procedure for handling up to spectrophotometry.

MATERIALS AND METHODS

Single piece cholesterol reagent. Various compounds were required for the one-piece reagent used in the processes of saponification to prepare free cholesterol, oxidation at the 3-beta-hydroxide position to generate the secondary product, hydrogen peroxide, and finally generation of a chromophore with 4-aminoantipyrene and phenol as the hydrogen donor by using the peroxide along with peroxidase. These reactants, including enzymes, were weighed out in bulk quantities, mixed thoroughly, and then weighed into individual tubes to provide the proper environment for reaction of a single 1-ml CSF sample. This reagent, specially prepared by Abbott Laboratories, Diagnostics Division (Pasadena, Calif.), contained

the following ingredients: cholesterol ester hydrolase, 66 IU/liter, cholesterol oxidase, 117 IU/liter, peroxidase, 67000 IU/liter, sodium cholate, 3 mmol/liter, 4-aminoantipyrene, 0.8 mmol/liter, phenol, 14 mmol/liter, Na_2HPO_4, 50 mmol/liter, NaH_2PO_4, 50 mmol/liter, and carbowax-6000, 0.2 mmol/liter. When this mixture was diluted with water in the manner prescribed by the manufacturer the $pH_{25°}$ was 6.70 ± 0.10. Other ingredients, such as fillers and stabilizers, are also incorporated into the material but in unknown quantities.

This reagent can be used in several ways. First, a dilute sample itself can be used to dissolve the weighed out solids and initiate the reactions with no other liquid required for the measurement. This is the method we propose should be used for CSF cholesterol. Second, in the conventional way, the reagent is first dissolved with water to which a much smaller volume of a more concentrated sample such as serum is added to generate the reactions. In the third way, a calibration device used for convenience in our studies, the material is dissolved with half the volume of water required for the conventional way. This provides a double-strength reagent to which a double-strength aqueous standard can be added to bring both solutions to their correct volumes and concentrations. We do not propose this last method as one of choice, but merely used it to conserve our specially prepared single-tube material for some of the described studies. Also, in this latter instance, advantage can be made of larger quantities of dried preweighed reagents which at present are more easily and cheaply obtainable. Besides calibration, this manner of using dried reagent can be utilized for abnormal CSF samples where the cholesterol concentration may be higher (*17*). All three ways are described in detail under procedure.

PROCEDURE

Normally, using commercially prepared reagent, one dissolves the given amount of reagent with an appropriate amount of water and then adds 0.01 ml of serum to a 1-ml aliquot to initiate the reactions. That would not provide enough cholesterol to measure when CSF is the sample. Therefore, 1-ml of CSF sample is pipetted onto the solid reagents in the specially prepared tube and vortexed well to dissolve the reactants. Ten minutes after solution, the color generated at room temperature is read at 500 nm against a reagent blank. However, for accurate calibration, as will be seen from the studies to be described, the best calibrator would be one which contained a similar reaction matrix to CSF.

An alternative procedure for handling the conventional reagent, which normally is supplied in either 3, 6, 12, 21, or 60 ml reconstitution sizes, is to dissolve it in half the stated volume and to then use an aqueous standard of double strength with which the double-strength reagent is mixed

on a 1:1 basis. For example, if 1 ml of 400 mg/dl aqueous standard is mixed with 1 ml of double-strength reagent, a solution corresponding to a 200 mg/dl standard for the described procedure would result. As previously stated, higher concentration samples can be treated in this manner for a dilution effect and conservation of the sample. The aqueous cholesterol standards which we found most useful were from Pierce Chemical Co. (Rockford, IL), and New England Reagent Laboratory, Inc. (Riverside, RI). They could be diluted with deionized water without precipitation of cholesterol, a step that eliminated high detergent concentration, a factor that has been reported to be inhibitory to the reactions involving cholesterol esters (*3*).

Another alternative procedure could be used for determination of CSF cholesterol when one is faced with a sample in which an interference such as bilirubin might be present. In this circumstance, 2 ml of CSF can be extracted using a modification of the Abell procedure and the total extract evaporated to dryness (*1*, *12*). Ten microliters of isopropanol can then be added to solubilize the residue followed by reaction with 0.5 ml of liquid reagent. The process, if one chooses to use the total extraction fluid, serves to concentrate the cholesterol by a factor of four over the direct procedure which could be useful when the concentration of cholesterol is well below 1 mg/dl of cerebrospinal fluid or in the range of 0.026 mmol/liter. But, even an aliquot of the extraction fluid would serve to increase the concentration of cholesterol in the final reaction solution over that of the proposed direct procedure.

RESULTS AND DISCUSSION

The calibration characteristics shown by the spectra in Fig. 1 include the three procedural approaches that were described. On the left are the spectra of 0 to 5 mg/dl (0.0–0.1295 mmol/liter) cholesterol for the proposed direct technique. In the center is the scale expanded approach (0.25 absorbance full scale), providing better readability at lower absorbance values using concentrations of 0 to 1 mg/dl (0 to 0.259 mmol/liter) cholesterol. On the right are the calibration spectra at the same low levels of cholesterol as the center using the extraction and concentration technique described as an alternative under procedure. Versatility and procedural sensitivity are obviously attainable with the proposed extraction technique, which should detect actual concentrations in CSF as low as 1 μg/ml (0.00259 mmol/liter). However, direct solution of reagents by the sample should suffice as a procedure for most of the measurements required.

Using the described technique, an important fact can be determined concerning the ability to measure cholesterol. That is, the expected normal concentrations measured of cholesterol in CSF will be similar to those

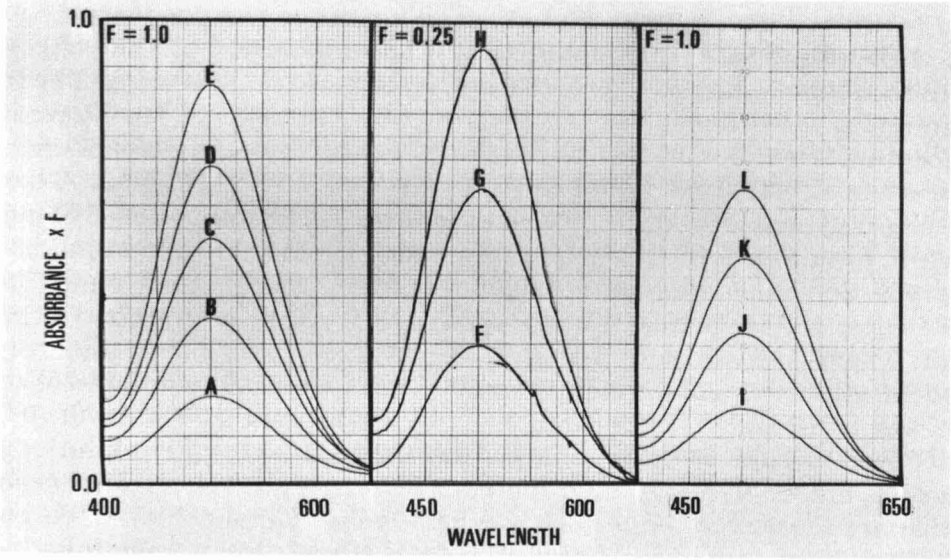

FIG. 1. Representative calibration curves for (left) 1 to 5 mg/dl (0.026 to 0.13 mmol/liter) described direct procedure, (center) 0.25 to 0.75 mg/dl for scale expanded readability, and (right) 0.25 to 1.0 mg/dl for extraction–concentration process.

of processed serums in the determination of high density lipoprotein (HDL) cholesterol, covering the range of 0.0026 to 0.0260 mmol/liter (*7*). But these concentrations do not result in high absorbance signals. Therefore, the substitution of fluorescence for colorimetry by use of a substrate such as homovanillic acid (*6*) or *p*-hydroxphenylacetic acid (*15*) to replace 4-aminoantipyrene plus phenol should increase the sensitivity of the proposed prototype procedure considerably and allow smaller samples to be used. Such an approach is presently under investigation along with a study of a histochemical technique for the lipids in electrophoretically separated lipoproteins. The ability to concentrate the cholesterol of the sample itself several times further as described under procedure remains an available option to the user even though extraction would be required.

On the surface, normal CSF does not appear to contain any serious problems for the determination of cholesterol other than the concentration of cholesterol, which according to literature reports (*8, 16, 17*) is quite low. However, several factors including bilirubin levels were studied in order to see what the effect might be if some variation in the concentration of otherwise normal CSF constituents took place. For example, the increase in protein concentration to rather high levels in pathological circumstances is not an uncommon phenomenon. Since one may encounter somewhat higher concentrations of protein from CSF than is usually

found with processed, thus diluted serum owing to the concentrating effect of the proposed technique, it becomes mandatory to test the affect of protein on the reactions. This factor was studied in several ways. The first is graphically described by the spectral data of Fig. 2. An increasing concentration of protein was added to a constant concentration of cholesterol while a fixed time period for color formation was followed. A bending occurred at a concentration corresponding to approximately 300 mg/dl of CSF protein. Here the graph shows the peak value plots indicating where a decreased response began for 5.0 mg/dl (0.1295 mmol/liter) cholesterol under the reaction conditions described. In a second experiment, which is graphically described by the spectra of Fig. 3, the protein concentration was kept constant at 100 mg/dl while the cholesterol content was continually varied until a bending occurred at 9.0 mg/dl (0.220 mmol/liter). At this point one might infer that an exhaustive limit of the reagents, and/or a Beer's law problem had occurred. The spectral results of this are illustrated along with the peak value plots in Fig. 4. To complete the picture, an increasing volume of a dilute serum, containing both protein and cholesterol, was added to a fixed volume of total cholesterol reagent and the reaction was allowed to procede for a specified time interval of 10 min before each spectrum was obtained. A linearly increasing response was obtained until the concentration of 250 mg/dl of protein and 8 mg/dl (0.181 mmol/liter) of cholesterol in the mixture was reached. The graph shows the spectral peak value plots representing the resultant cholesterol (right) and protein effects (left) found in this manner.

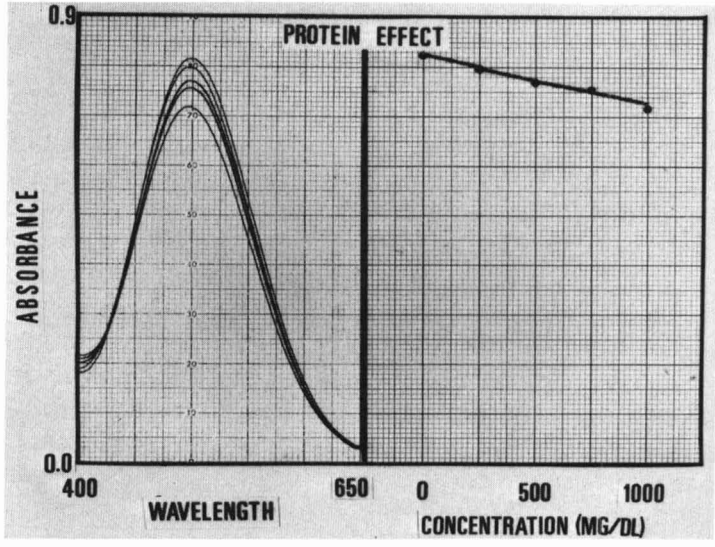

FIG. 2. Protein effect at a constant cholesterol concentration showing reaction repression. Peak values from spectra at left plotted on the right.

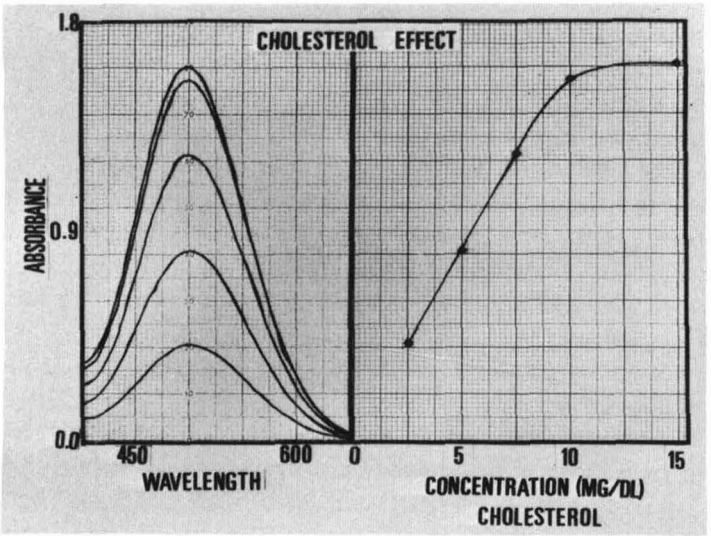

FIG. 3. Cholesterol effect at a constant protein concentration showing exhaustive limits with peak values from spectra on left plotted on the right.

A test for the stability of color indicated that the final concentration of color generated using free cholesterol standards occurred quickly, within 3 to 4 min, plateaued for a very short period, and then began to gradually fade. When excess protein was present, the fading was parallel in its effect, but reaction repression occurred. When a serum was treated in the same manner, using the same lot of reagent, a somewhat different rise curve was obtained. Here the peak (not shown in the figure) was obtained at 9 to 10 min, a quite different time interval, and again the plateau was followed by fading, although the fading was not as rapid. The latter fact could be indicative of the cholesterol ester hydrolase action which slows up the entire process of color formation in the reaction sequence. It may very well be true that the fading phenomenon is really a lot problem and that this should be tested for all lots purchased. These curves are shown in Fig. 5. Several distinct observations can be made from this last simple experiment: The rise curves to reaction equilibrium representing reaction as a function of time are different for standard and sample and thus rate measuring methodology would be obliged to consider this phenomenon. The time of reading for an equilibrium measurement would need to be judiciously chosen and rigidly adhered to because of the more rapid fading of the indicator color of the standard, a fading that was repeated using three conventional lots as well as the lot of the specially prepared solid reagent mixture. The choice of calibrator must be of one that is similar in makeup to a CSF, or else the variation in rate of fade, if this occurs with all lots, could cause a small error of 2 to 4%.

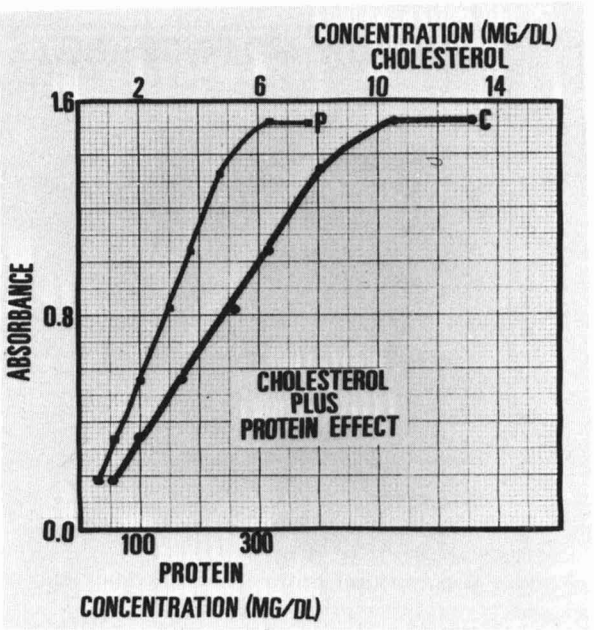

FIG. 4. Combined cholesterol (right) and protein (left) effects created by increasing the concentrations of both constituents simultaneously and sequentially.

The proposed direct technique used to concentrate the cholesterol in CSF might be described as a process of reverse concentration. Instead of concentrating the desired constituent of the sample with all of the potential problems associated with such a procedure, the reagents are concentrated instead, that is, used as solids, so that the sample, in effect, becomes the entire diluting agent of the procedure. Obviously, other problems could be encountered in the form of interferences which might be avoided in partial separatory processes involving a step such as extraction. Reverse concentration therefore may have the adverse effect of magnifying an interference such as bilirubin that could be present in xanthochromic spinal fluid up to a level of at least 800 μg/dl (*13*). Using the direct technique described, each 100 μg of bilirubin per dl of CSF would act as 10 mg of bilirubin per dl of serum. Therefore, it would not be difficult to encounter some aberrant results altered by the competitive interaction of bilirubin with the peroxidase associated substrate of the contemplated reaction. As a pictorial model of what could occur, the results of an experiment to demonstrate the effect of bilirubin on the direct determination of CSF cholesterol are shown in Fig. 6. Here we can see the spectra for cholesterol (0.08 mmol/liter) reacted with the reagents shown as curve C, bilirubin alone and unreacted (500 μg/dl) since no peroxide would be generated here, shown as curve B, and then the same

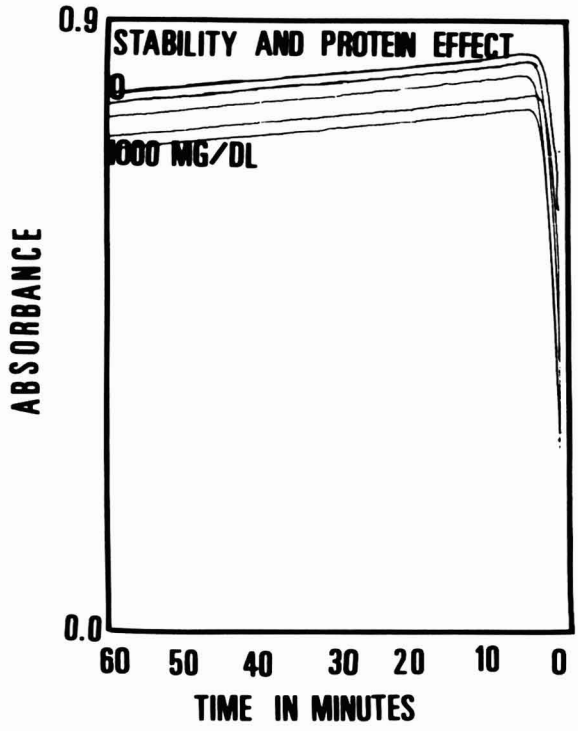

FIG. 5. Stability study of the rise curves of cholesterol formation at varying protein concentrations showing reaction repression and a fading effect.

concentration of cholesterol to which the bilirubin was added, designated as the composite C + B curve after the peroxide generated due to the cholesterol reaction was exhausted by the peroxidase coupled reaction. Keeping the latter composite in mind, since it contains a true summation effect, a fourth composite curve, in which bilirubin and cholesterol were present together before reacting with reagents, is shown as the actual C + B curve. The successful competitive reaction of bilirubin in substituting as a hydrogen donor for the intended substrate of 4-aminoantipyrene and phenol acted on by peroxide–peroxidase is striking. When one considers that some residual bilirubin absorbance occurs at 500 nm, or that some conversion product of bilirubin could have formed which may also absorb at 500 nm, the significance of the interference becomes more manifest (*9*). For, since it is the product of the hydrogen peroxide–peroxidase action on 4-aminoantipyrene and phenol which one intends to measure, the residual absorbance observed here represents a chance compensating error. This would not occur at a higher wavelength (*11*) where bilirubin did not absorb, unless the oxidized bilirubin product showed absorption there as another compensating error, or if the competitor as hydrogen donor or its product

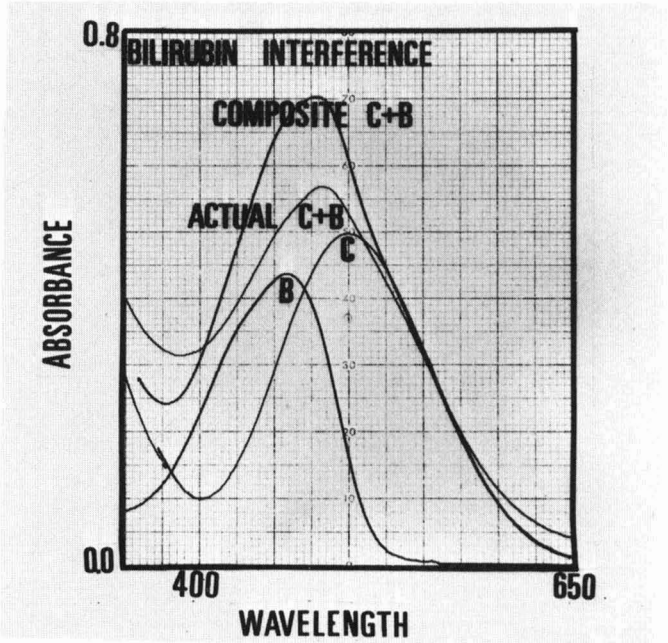

FIG. 6. Bilirubin study in which bilirubin (B) and cholesterol (C) spectra are summed shown as the composite C + B curve and compared to the simultaneous reaction involving the competitive interaction of bilirubin, shown as the actual C + B curve.

had no color itself at 500 nm. This measurement of a chance mixture rather than the intended product suggested a potential need for elongation of the procedure by an extraction step in order to eliminate the compensating error. Furthermore, the conventional suggestion that a serum blank be used to correct for bilirubin could not rescue the measurement inasmuch as an unreacted blank would not be truly representative of the interactions at play within the sample. The result of such a correction would be a low value for the sample. Fortunately, encountering bilirubin in CSF may not be as common as with blood serum. Additionally if ignored, the result is still reasonable owing to the compensatory phenomenon. But, it still would be relatively simple to extract cholesterol from CSF and then react it with a one piece reagent following the removal of the extraction solvent even though the added step may be routinely less attractive. This alternative approach can also be used when there is a need to concentrate the color for greater determinative reliability.

The data in Table 1 were generated as a measure of the ability to accurately reproduce known values by the described direct technique. Several low to high concentrations of cholesterol were determined by simply dissolving the solid reagent with the liquid samples. From the

TABLE 1
RECOVERY OF ABSOLUTE MICROCENTRATIONS OF CHOLESTEROL

Number	Range 0.3 to 0.6 mg/dl[a]		Range 1.5 to 4.0 mg/dl[b]	
	Cholesterol present	Cholesterol found	Cholesterol Present	Cholesterol found
1	0.30	0.28	1.50	1.39
2	0.30	0.30	1.50	1.42
3	0.30	0.31	1.50	1.44
4	0.30	0.29	1.50	1.42
5	0.30	0.28	1.50	1.42
6	0.60	0.58	3.0	2.96
7	0.60	0.58	3.0	2.95
8	0.60	0.58	3.0	2.96
9	0.60	0.59	3.0	2.98
10	0.60	0.58	3.0	2.98
11	0.90	0.90	4.0	3.91
12	0.90	0.90	4.0	3.94
13	0.90	0.89	4.0	3.96
14	0.90	0.89	4.0	3.99
15	0.90	0.87	4.0	4.00

[a] 0.0078 to 0.0155 mmol/liter (1 mg/dl = 0.0259 mmol/liter).
[b] 0.039 to 0.1036 mmol/liter.

results shown, one can infer that the concept on which the direct procedure is based is a workable one for the low concentrations of cholesterol that might be encountered in CSF or other biological fluids.

SUMMARY

A preliminary procedure for CSF cholesterol is described which can be considered as a prototype method for the special case in which a biological sample is so dilute with respect to all of its constituents that it can be used as the sole diluent for the reactants used to measure the desired constituent in the specimen. Since it would be comparatively simple to generate a fluorophore in place of the chromophore and thereby increase the sensitivity of the procedure, one could easily alter the limits of the determination and/or the size of the sample needed. This is contemplated as a technique which should result from the prototype procedure described. Avenues for the determination of other constituents such as triglycerides or biuret reactive compounds may possibly be accomplished by using a similar determinative approach. Again, in the specific case of cholesterol one may have to resort to detection devices other than colorimetric in order to achieve strong signals for measurement. Fluorescence appears to be a best first approach to attempt in these circumstances for a final procedure based on the suggested prototype.

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Monothiourea-3-nitrophthalic Acid as a New Reagent for Spectrophotometric Analyses

I. Determination of Palladium(II) Ions

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In our previous papers we have described the synthesis and properties of new derivatives of thiourea and dicarboxylic acids anhydrides (*2, 3*). In the present work the results of the examination of the reaction between one of them, i.e., monothiourea-3-nitrophthalic acid (MT3NF), and palladium ions will be discussed.

MT3NF acid reacts with palladium ions in hydrochloric media to form a yellow, water-soluble complex. Because of the good solubility of the products, this reaction was mainly examined spectrophotometrically.

The series of samples were prepared by using a fixed concentration of Pd(II) ions and HCl ($C_{PD} = 2.25 \times 10^{-5}$ *M*; $C_{HCl} = 0.5$ *M*) and various molar excess of MT3NF acid, from one- to fivefold over palladium. The absorbance was measured within the uv and visible region in a 1-cm cell against 0.5 *M* HCl solution.

The results proved that the maximum absorbance of the reagents are partially on the maximum absorbance of the products. The wavelength of the maximum absorbance of a palladium complex with MT3NF acid was found by measuring the absorbance against the reagent blank. Two wave maxima occurred at 245 and 305 mm.

The optimum concentration of HCl and the period of contact of the reagents were determined for samples consisting of a fixed palladium concentration $C = 2.25 \times 10^{-5}$ *M* and a fivefold molar excess of ligand.

The necessary HCl concentration was obtained by adding adequate volumes of HCl or NaOH solutions. In order to avoid opacity every sample contained 20% v/v of ethanol.

Over the range 0.2 to 2.2 *M* HCl there is no difference between the obtained curves, and the absorbance was stable for a period of 24 hr.

The increase of temperature up to 85°C does not change these spectra. When the HCl concentration was less than 0.2 *M* and both

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the temperature and the period of contact of the reagents increased, a displacement of λ_{max} to longer wavelengths had been observed.

This decrease of stability of palladium complexes with MT3NF acid might be due to the formation of hydroxycomplexes of palladium in the applied conditions just mentioned.

The Composition of the Complexes

The composition of the formed complexes was found by means of the molar excess method.

All the samples contained the same concentration of palladium ($C_{Pd} = 2.25 \times 10^{-5}$ *M*), HCl ($C = 0.5$ *M*), and ethanol ($C = 20\%$ v/v). The absorbance was measured against 0.5 *M* HCl solution in 20% ethanol. Because of the superposition of the maximum absorbance of reagents and of the products, two different wavelengths were chosen, viz. 335 and 320 nm, at which the absorbance of ligand was negligible, in order to determine the molar ratio of Pd to MT3NF acid. The shape of the obtained curve indicated that this molar ratio of Pd:MT3NF=1:4.

When the concentration of Pd(II) and MT3NF acid was greater by about 100 times, and equimolar amounts of reagents were used, an orange precipitate would form. The good solubility of this precipitate in ligand excess confirmed the progressive formation of palladium complexes.

The Determination of the Charge Value

The sorption of the yellow soluble complex of palladium, formed in the presence of a large excess of ligand, on ion-exchange resins has proved that the obtained product is positively charged. The value of this charge was determined by means of the ion-exchange method. The cation-exchange resin Dowex 50W × 12, 200 to 400 mesh was used to solve this problem (*1*).

All samples consisted of the same concentration of Pd(II) ($C = 2.8 \times 10^{-5}$ *M*), HCl ($C = 0.5$ *M*), and MT3NF ($C = 1.25 \times 10^{-4}$ *M*) and different concentrations of Na^+, added as $NaClO_4$. The range of the Na^+ concentration was 0.25 to 2.5 *M*, its total volume amounting to 20 cm^3.

Resin (0.1 g), previously saturated with the reagent solution, was added to prepare the samples. The mixtures were shaken continuously for 24 hr, then absorbance was measured at $\lambda = 306$ nm against the reagent blank.

The charge value was calculated from the slope of the straight line of log K_d vs log[Na^+], where K_d was the distribution coefficient of the palladium complex between the cation exchanger and the solution. The value found was +1 (Fig. 1).

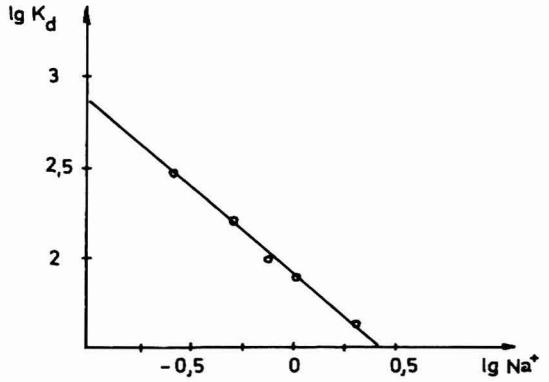

FIG. 1. Determination of the charge value of palladium complex with MT3NF acid by means of ion-exchange method.

The result indicates that a mixed cation complex of palladium with MT3NF acid, including a halogen anion, may be obtained. Palladium ions, as we know from the literature, form similar products with some thiourea derivatives (*4*).

DETERMINATION OF PALLADIUM

Based on the investigations described above, we have used MT3NF acid for the determination of microgram amounts of palladium. A straight-line dependence between absorbance and the concentration of palladium was obtained over the range 0.3 to 3 μg of Pd/cm^3. The molar absorptivity was $3.3 \times 10^4\ M^{-1}\ cm^{-1}$ liter.

In spite of the high sensitivity of this method, its selectivity is poor, as the maximum absorbance of the palladium complex occurs in the same range as the absorbance of a number of the inorganic salts of heavy metals, though these metals do not react with MT3NF acid.

When the extraction with organic solvents was applied an increase of selectivity was observed. The optimum composition of the organic phase was a 2:1 mixture of chloroform and *n*-amyl alcohol.

Palladium was transferred from the 0.5 *M* HCl medium to the organic phase by extraction with MT3NF acid solution in the mixture of organic solvents described above. In these conditions the complex had a molar absorptivity of $2.9 \times 10^4\ M^{-1}\ cm^{-1}$ liter.

Procedure

A sample containing less than 30 μg of Pd was placed into a separatory funnel, and an adequate volume of HCl was added in order to obtain a 0.5 *M* concentration. The volume of 5 cm^3 5×10^{-4} *M* MT3NF in a 2:1 mixture of $CHCl_3$ and *n*-amyl alcohol was put in,

after which the samples were shaken for a period of 2 min. After a twofold extraction, both organic phases were mixed and the absorbance was measured at $\lambda = 306$ nm against the reagent blank. Beer's law is obeyed over the range 0.1 to 3 μg of Pd/cm^3. Molar absorptivity is 2.9×10^4 liter mol^{-1} cm^{-1}, with average standard deviation of 560 ($n = 25$).

A 100-fold molar excess of Os(IV), Co(II), Hg(II), a 50-fold molar excess of Ru(III), and a 25-fold molar excess of Pt(IV), V(V), Fe(III), and Ag(I) do not interfere.

The sensitivity of the described method is four times greater than the thiourea method, due to the substitution of hydrogen with a large organic radical.

SUMMARY

The results of the examination of the reaction between a new reagent, MT3NF acid, and palladium(II) ions, as well as the composition of the formed complexes have been discussed. The new extraction-spectrophotometric method for the determination of palladium(II) ions by using MT3NF acid was presented. Beer's law is obeyed over the range 0.1 to 3 μg of Pd/cm^3. Molar absorptivity is 2.9×10^4 liter mol^{-1} cm^{-1} at the absorption maximum of 306 nm. The influence of different ions has been described.

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MICROCHEMICAL JOURNAL 23, 521–524 (1978)

Monothiourea-3-nitrophthalic Acid as a New Reagent for Spectrophotometric Analyses

II. Determination of Ruthenium(III) Ions

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INTRODUCTION

The synthesis and properties of monothiourea-3-nitrophthalic acid (MT3NF) were described by the authors in previous papers (*1*, *2*). In the present paper the investigation results of the reaction between MT3NF acid and ruthenium(III) ions as well as the application of this new reagent in the ruthenium analysis are presented.

Spectrophotometric Examination of the Reaction

The MT3NF acid reacts with Ru(III) ions in a hydrochloric medium at 85°C to form a blue-colored complex soluble in water–ethanol mixtures. The optimum acid strength for color development is 6 to 7 *M*. If the acid concentration is below 4 *M*, the color does not develop. As these products are soluble, this reaction was examined spectrophotometrically.

The maximum absorbance wavelength, as well as the absorbance of the solutions, depend on the HCl concentration, the period of heating of the reagents, the molar excess of ligand over ruthenium, and the quantity of ethanol in the medium.

The influence of HCl concentration and the period of the heating of the reagents were examined in the presence of a great excess of ligand.

The mixture of compounds was heated in a water bath at 85°C. All the spectra were characterized by one maximum absorbance at 598 nm. The following optimum conditions have been found: acid (HCl) concentration, $\times$ 7 *M*; period of heating, 15 min. In order to avoid opacity of the solutions in the presence of such an excess of ligand, 40% (v/v) ethanol was added to each sample.

Based on these results all the following samples were prepared according to the conditions described above.

Influence of the Excess of Ligand

The investigation of the influence of the excess of ligand on the absorbance and λ_{max} has shown that if an equimolar or nearly equimolar excess

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of MT3NF acid was used, a red- (not blue-) colored product would be formed. Its λ_{max} moved toward the longer wavelengths due to the increase of the excess of ligand.

To form a blue-colored product, a greater than 100-fold molar excess of MT3NF acid was necessary. Such an excess of ligand, as well as the color change of the complex from blue to red during the dilution of its samples, indicates that the blue product easily dissociates.

Taking into account all the properties mentioned above, electrophoresis and ion exchange methods were used to discover the composition of the formed complexes.

The Composition of the Formed Complexes

Electrophoresis method. The following conditions were applied in the electrophoresis method:

	Red complex	Blue complex
Concentration of Ru(III)	5.17×10^{-4} *M*	1.93×10^{-4} *M*
Concentration of MT3NF	5.17×10^{-4} *M*	3.30×10^{-2} *M*
Electrolyte	0.1 *M* Na_2SO_4 in 30% ethanol	
Voltage	300 V	
Current	10–40 mA	

Ion exchange method. The ruthenium complexes were sorbed on two kinds of ion exchange resins: strong acid cation exchanger Dowex 50W × 2 and strong basic anion exchange Dowex 1 × 2.

The 0.1-g amounts of the respective resins were placed in the complex solution for a period of 24 hr. Before use, the resins were saturated with solutions of reagents, also for 24 hr.

The absorbance change was measured spectrophotometrically at λ = 548 and 598 nm.

The data obtained by means of the electrophoresis method agreed well with the results of the ion-exchange method. The red-colored complex moved toward the anode and was sorbed on the anion exchanger, whereas the blue-colored one was not attracted by any electrode and was not sorbed at all.

This result might indicate that there are two products which are successively formed during the reaction between Ru ions and MT3NF acid: the first, negatively charged and the second uncharged.

The molar ratio of Ru to MT3NF might be respectively 1:1 and 1:2. If the reaction runs according to the scheme, proposed previously for the reaction between thiourea and its *N*-substituted derivatives (*3*), the formation of these products would be possible:

$$RuCl_5^{2-} + MT3NF \rightarrow RuCl_4MT3NF^- + Cl^-$$
$$RuCl_4MT3NF^- + MT3NF \rightarrow RuCl_3MT3NF_2^0 + Cl^-$$

Pilipenko and co-workers have proved that Ru(III) forms a $RuCl_5^{2-}$ complex in this HCl medium (*4*).

DETERMINATION OF RUTHENIUM

The reaction described above was applied in order to develop a spectrophotometric method for the determination of the ruthenium ions.

Procedure

Different amounts of Ru(III) less than 24 $\mu g/cm^3$ were placed in an Erlenmayer flask and 5 cm^3 concn HCl, 5 cm^3 of ethanol, saturated with gas, HCl, 1 cm^3 9% MT3NF acid were added successively. A blue color was developed by heating in a water bath at 85°C for a period of 15 min. After cooling, the solutions were transferred into a 25-cm^3 volumetric flask and adjusted with such a mixture of HCl concn and ethanol to abtain 7 *M* HCl and 40% ethanol concentration at the final volume.

The absorbance was measured in a 1-cm cell at 598 nm against 7 *M* HCl solution. Beer's law is obeyed over the range 2 to 24 μg of Ru/cm^3. The molar absorptivity was 3.9×10^3 liter mol^{-1} cm^{-1}, with the average standard deviation of 35 ($n = 30$).

It is possible to determine ruthenium in the presence of a 10-fold molar excess of Pt(IV), Pd(II), Ir(IV), and Rh(III), 5-fold molar excess of Os(IV), and a 10-fold smaller amount of Fe(III), Hg(II), Cu(II), and Ag(I).

A blue-colored ruthenium complex with MT3NF acid can be extracted to the organic phase, which consists of a 3:2 mixture of chloroform and *n*-amyl alcohol.

In order to decrease the mixing of both these phases during the extraction, the aqueous phase, containing 20% of ethanol, was used.

The extraction method was applicable over the same range, 2 to 24 μg of Ru/cm^3, but its selectivity increased in comparison to the determination of ruthenium in water solution. The presence of a 10-fold molar excess of Os(IV), Pt(IV), Pd(II), Ir(IV), Rh(III), Cu(II), Ag(I) and a 10-times smaller amount of Fe(III) and Hg(II) do not interfere.

The described method does not require any previous distillation of the octavalent oxide of ruthenium. Its sensitivity is of the same range as the thiourea method, but its selectivity is better, for the extraction can be used.

SUMMARY

The reaction between ruthenium(III) ions and MT3NF acid, i.e., the optimum conditions and composition of the formed complexes, have been described. The application of MT3NF

acid—a new thiourea derivative—for the spectrophotometric determination of ruthenium (III) ions is presented. Beer's law is obeyed over the range 2 to 24 μg of Ru/cm^3. Molar absorptivity is 2.9×10^3 liter mol^{-1} cm^{-1} at the absorption maximum of 598 nm. The influence of different ions is described.

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Spectrophotometric Determination of Germanium by Phenylfluorone

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INTRODUCTION

Germanium among other elements is of great practical and industrial use. It is found in relatively very low concentrations in soil or mineral water. Thus for the determination of micro amounts, spectrographic (*12*), X-ray fluorescence (*1*), polarographic (*2*), and spectrophotometric methods have been proposed. The spectrophotometric methods mostly depend on complex formation between germanium and organic reagents such as alizarin complexan (*8*), quinalizarin and diphenylguanidine (*4*), methyl violet and gallic acid, and pyrogallol or catechol (*9*). Other methods are based on the production of molybdenum yellow or the reduced form molybdenum blue (*3, 5*).

Although phenylfluorone has also been used in the determination of germanium colorimetrically (*6, 7, 11*), no detailed study of its reaction conditions has been made. Therefore the aim of the present study is to find the optimum conditions under which a stable colored complex is formed, and to pay maximum contributions to the work for development of its colorimetric method.

EXPERIMENTAL

Apparatus

All measurements corresponding to absorptions were made by a Perkin–Elmer 4000A recording spectrophotometer or by a manual spectrophotometer of type MOM 204 using 1-cm silica cells at room temperature (≃25°C).

PH measurements were made by a Prolabo TS4N pH meter.

Reagents and Solutions

All the chemicals used were of analytical or very pure grade.

Phenylfluorone solution (32 mg ph. fl./100 ml). The phenylfluorone (2,6,7-trihydro-9-phenyl 3-isoxanthone) is slightly soluble in alcohol, but its solubility can be increased by adding a small volume of dilute HCl. Thus a stock solution was prepared by dissolving 0.0320 g of

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phenylfluorone (B.D.H.) in 85 ml of absolute ethanol and 5 ml of 2.5 *M* HCl, warmed, cooled, and diluted to 100 ml with ethanol.

Germanium solution (10 mg of Ge/ml). Stock solution was prepared by dissolving 1.4408 g of GeO_2 (B.D.H. Laboratory reagent) in a small amount of hot double-distilled water acidified with 12 ml of 2.5 *M* HCl and then diluted to 100 ml with double-distilled water. It was standardized gravimetrically.

Procedure

Transfer an aliquot containing 10 to 120 mg of germanium in a 25-ml volumetric flask and add 5 ml of cyclohexanol, 10 ml of phenylfluorone, and double-distilled water. Mix well and leave for 20 min to let the maximum color develop. Measure the absorbance at 525 nm versus blank (containing all reagents except germanium).

RESULTS AND DISCUSSION

Absorption Spectra

To a 5-ml aliquot of reagent added 5 ml of cyclohexanol, diluted to a 25-ml volume with double-distilled water. Its absorbance was measured against a solution (containing all the reagents except phenylfluorone) using a 1-cm cell. The curve recorded is as shown in Fig. 1(b).

Curve a in Fig. 1 shows the absorption spectrum of the orange-colored solution produced by mixing 2.5 ml of germanium solution (2.5×10^{-4} *M*)

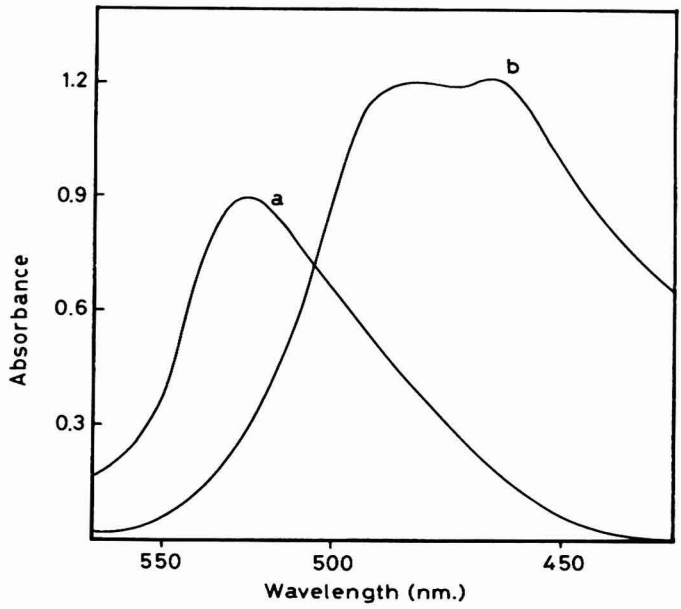

FIG. 1. Absorption spectra of (a) germanium–phenylfluoronate complex; (b) phenylfluorone.

with 5 ml of phenylfluorone and 5 ml of cyclohexanol diluted to a 25-ml volume. Absorptions were made 30 min after the addition of reagents.

Phenylfluorone exhibits two maxima, at 465 and 485 nm, while the complex formed exhibits a red shift at 525 nm.

Owing to the slight absorbance of reagents at 525 nm it is necessary to use blank solution containing all reagents except germanium.

Factors Affecting Color Stability

Effect of time. Preliminary experiments indicate that solutions of concentration higher than 2.5×10^{-4} *M* tend to precipitate after a short time, while very dilute solutions require a long time to produce measurable color. Suitable concentrations were found to be in the range 10 to 120 mg of Ge/25 ml. The orange-colored complex formed by mixing germanium and phenylfluorone solution, became stable after 30 min of standing at room temperature. During this period, the peak characteristics of reagents at 465 and 485 nm shift gradually toward the complex peak characteristics at 525 nm as shown in Fig. 2.

Effect of pH. To study the effect of pH on the reagent and complex formed, the experimental work was made at different pH scales. The pH of a test solution containing 45 mg of Ge was adjusted by the addition of HCl/NaOH (pH should be adjusted after maximum color development). pH of the blank (all reagents except Ge) was adjusted the same as that of the test solution before its measurement.

At pH 2.6 the spectrum of phenylfluorone exhibits two absorption bands at 465 and 485 nm. By increasing the pH gradually the former band became weaker while the latter became stronger and gave an isosbestic point at 470 nm.

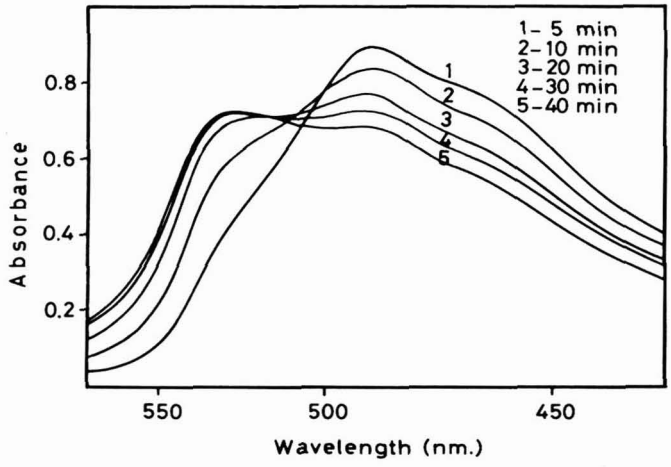

FIG. 2. Absorption spectra of the complex at different times using water blank.

At pH higher than 6.5, the peak at 515 nm becomes predominant.

At pH range 2.5 to 3.4 the complex gives only one peak at 525 nm and its height decreases as pH increases.

At pH greater than 4.2 a red shift occurs with low intensity which is probably due to hydroxo-complex formation.

Effect of HCl concentration. A study was made to investigate the effect of increasing amounts of HCl on fixed amounts of germanium, phenylfluorone, and alcohols in the test solution. The intensity of color decreases with the increase of HCl concentration from 0.2 *N* (optimum concentration) to 0.8 *N* (no formation of complex, which may be due to suppression of ionization of the reagent at such low pH values).

Effect of reagent concentration. In order to investigate the effect of phenylfluorone concentration on the fixed quantities of germanium and alcohol, varying amounts of phenylfluorone were added to the solution containing 50 mg of germanium and 5 ml of cyclohexanol. It was observed that absorbance reaches a maximum when 2 ml of 0.032% phenylfluorone was added, and it decreases by decreasing the amount of phenylfluorone. By increasing the amount of phenylfluorone up to 10 ml, the intensity of color becomes constant. This behavior can be explained on the basis that germanium forms a complex with phenylfluorone at a certain concentration, below which it is partially dissociated and above which it becomes stable.

Effect of foreign ions. Table 1 lists the ions tested. Varying amounts of foreign ions were added to a fixed amount of germanium, and the color

TABLE 1
VALIDITY OF BEER'S LAW[a]

Ion	Concentration multiple to germanium	Ion	Concentration multiple to germanium
Sn^{2+}	1	Na^{+}, K^{+}, Li^{+}	Very High
Sn^{4+}	1	Cu^{2+}	1000
As^{3+}	50	Ba^{2+}, Ca^{2+}, Mg^{2+}	1000
AsO_4^{3-}	100	Zn^{2+}, Cd^{2+}, Hg^{2+}	1000
Sb^{3+}	1	Al^{3+}, PO_4^{3-}	1000
Bi^{3+}	10	Cr^{3+}, Mn^{2+}	1000
Ti^{4+}	2	Fe^{2+}, Co^{2+}, Ni^{2+}	1000
Ga^{3+}	1	Fe^{3+}	10
MoO_4^{2-}	1		

[a] A standard series of solution containing 10 to 120 mg of germanium was tested according to the recommended procedure. The absorbance was drawn against the concentration of germanium and a straight line passing through the origin was obtained, i.e., Beer's Law is obeyed. The relative standard deviation was 2%. The sensitivity of the method according to Sandell (*10*) for ($A = 0.001$) is 2.55×10^{-3} mg/cm^3. The molar absorbitivity based on Ge is 1.175×10^3.

was developed according to the recommended procedure. A 2% relative error in the determination of germanium was considered. Table 1 indicates the permissible quantity of foreign ions in the test solution.

SUMMARY

Spectrophotometric studies on the reaction between germanium ions and phenylfluorone were discussed including the reaction condition to develop a spectrophotometric method for the determination of germanium. The absorbance was measured at 525 nm after 20 min from the moment of adding the reagent. Beer's Law is obeyed, the relative standard deviation is 2%, the senstivity of the method is 2.55×10^{-3} mg/cm^3, and the molar absorbtivity based on germanium is 1.75×10^3.

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Zinc and Cadmium Complexes of 2,2′-Dipyridyl-2-pyridylhydrazone as Visual Acid–Base Indicators

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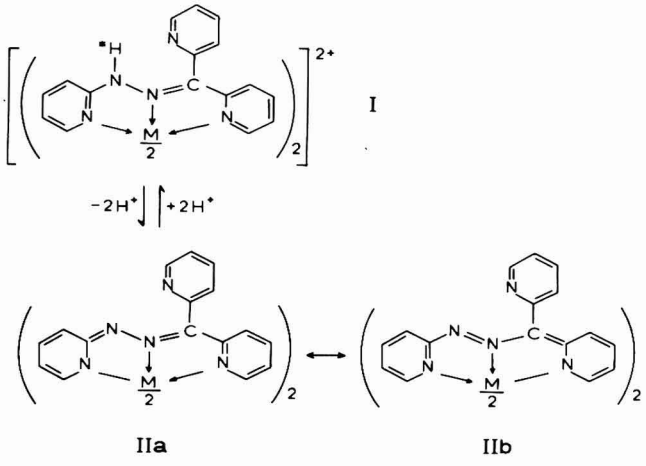
INTRODUCTION

The analytical properties of a nitrogen-containing heterocyclic hydrazone, 2,2′-dipyridyl-2-pyridylhydrazone (DPPH), have been reported recently (*1–4*).

The cationic bis complexes of DPPH, as well as of some similar substituted hydrazones, with certain divalent metals, have two acidic protons (marked with an asterisk in I) which can dissociate with alkalis to give intensely colored uncharged complexes (*1, 5–8*).



I

IIa IIb

During the course of the spectrophotometric investigation of DPPH as analytical reagent it was found that the DPPH bis complexes with zinc and cadmium exhibit distinct color changes with pH. It was therefore thought that these compounds should be useful as acid–base indicators.

This paper is concerned with the pH intervals of the DPPH bis complexes with zinc and cadmium and the application of these complexes as visual indicators in acid–base titrations.

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EXPERIMENTAL

Apparatus

The absorbance measurements were made with a Unicam SP 800 recording spectrophotometer, using 10-mm matched quartz cells. The pH of the solutions was measured with an L. Pusle ACL-112 pH meter, using a combination electrode.

Reagents

The purest materials readily available were used throughout this work and the ethanol was glass distilled. All solutions were prepared in glass-redistilled water, free from carbon dioxide.

Stock zinc(II) solution ($10^{-2}M$). This solution was prepared by dissolving zinc powder (E. Merck, Darmstadt) in diluted hydrochloric acid. More diluted solutions were prepared by proper dilution.

Stock cadmium(II) solution (10^{-2} M). This solution was prepared by dissolving metallic cadmium (E. Merck, Darmstadt) in diluted nitric acid. More diluted solutions were prepared by proper dilution.

2,2'-Dipyridyl-2-pyridylhydrazone (DPPH) ethanolic solution (10^{-2} M). The reagent was synthesized as previously reported (*2*). The 10^{-2} M ethanolic solution of DPPH is stable for at least 2 years if kept in a dark bottle.

Preparation of the zinc—DPPH bis complex (ZnIn). The compound was prepared as previously described (*1*). A warm ethanolic solution of DPPH (0.275 g in 25 ml) and a warm aqueous solution of zinc(II) chloride (50 ml $10^{-2}M$) were mixed. The pH of the solution was brought to about 11 with a 4 *N* sodium hydroxide solution. After cooling, the orange bis complex was pecipitated and recrystallized from aqueous ethanol.

Preparation of the cadmium-DPPH bis complex (CdIn). The compound was prepared with the above mentioned procedure, using a 10^{-2} *M* cadmium nitrate solution. The orange cadmium—DPPH bis complex was precipitated immediately after bringing the pH to about 11.

Indicator solutions. (a) Ethanolic solution of ZnIn, 0.1%. (b) Ethanolic solution of CdIn, 0.1%.

The pH control. The following mixtures were used for adjusting pH: acetic acid—sodium acetate, for pH below 6; boric acid—borax, for pH 6.5 to 9.0; borax—sodium hydroxide, for pH 9.5 to 11.0. For pH values above 11 sodium hydroxide was used.

The ionic strength. For spectrophotometric studies the ionic strength was kept constant at 0.1 with sodium perchlorate.

Titration solutions. For titrations freshly prepared 0.1 *N* solutions of sodium hydroxide, hydrochloric acid, oxalic acid, and acetic acid, as well as a 0.02 *N* solution of benzoic acid, were used.

The Indicator Properties

Variation of absorbance with pH. The absorption spectra in the visible of the zinc–DPPH (ZnIn) and the cadmium–DPPH (CdIn) bis complexes in aqueous solution were scanned at different pH values between 5 and 13 at 0.1 ionic strength. A selective number of them is illustrated in Figs. 1 and 2. The absorption maximum appeared at 442 and 444 nm, respectively, and there was no shift in maximum absorbance even when changing the metal-to-DPPH molar ratio.

The color-change interval of the indicator and the apparent indicator constant. A plot of maximum absorption against pH (Figs. 3 and 4) indicates the pH range of the indicator. For both indicators full color development is achieved in an acceptable pH interval of about 3 pH units.

From the pH-absorbance curve (Figs. 3 and 4) the pH value corresponding to one-half color development, e.g., the apparent indicator constant, was obtained graphically. Its value came out to be 8.5 for ZnIn and 9.5 for CdIn.

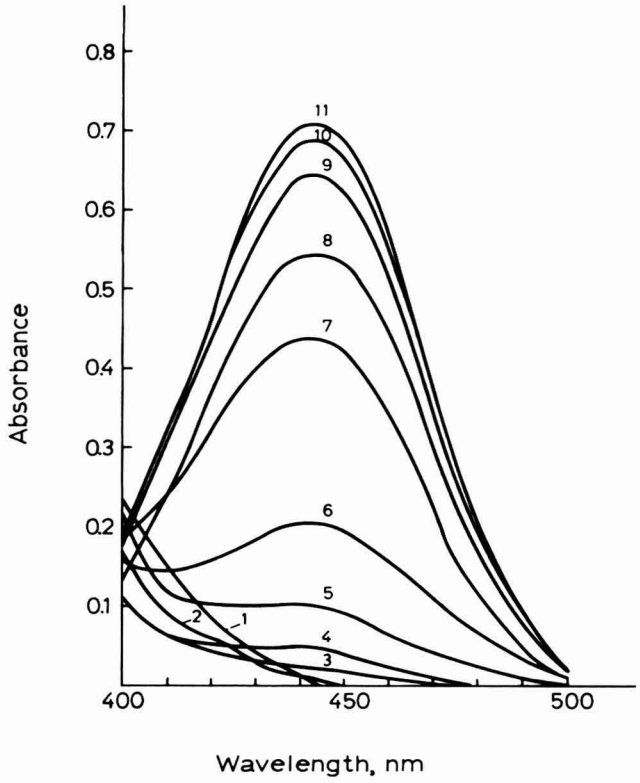

FIG. 1. Absorption spectra of 1.5×10^{-5} *M* ZnIn at different pH values. $\mu = 0.1$. pH: 1 = 5.55; 2 = 5.90; 3 = 6.62; 4 = 6.98; 5 = 7.40; 6 = 7.89; 7 = 8.87; 8 = 9.39; 9 = 9.85; 10 = 10.39; 11 = 10.95, 11.63 and 12.29.

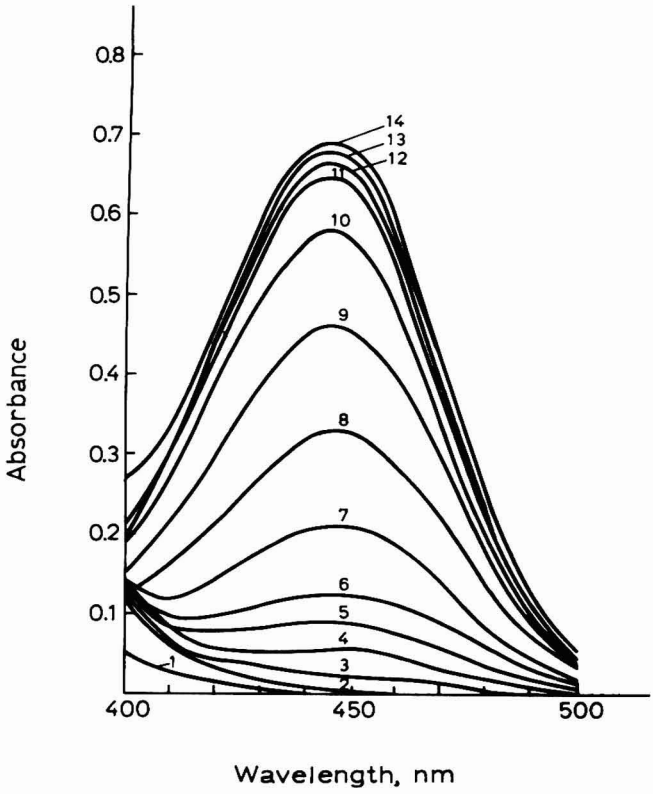

FIG. 2. Absorption spectra of 1.5×10^{-5} *M* CdIn at different pH values $\mu = 0.1$. pH: 1 = 6.74; 2 = 7.03; 3 = 7.60; 4 = 8.06; 5 = 8.31; 6 = 8.54; 7 = 8.96; 8 = 9.45; 9 = 9.95; 10 = 10.50; 11 = 10.95; 12 = 11.42; 13 = 11.96; 14 = 12.49 and 12.97.

Stability of the indicator solution. Freshly prepared 0.1% ethanolic solutions of the indicators were stored in different ways and analyzed by spectrophotometry. The spectra in the visible were taken before and after storage. If stored in the dark, both solutions were stable for at least 3 months. For storage in daylight, a decrease in optical density was observed 24 hr after preparation for the ZnIn solution and about a week for the CdIn one. Consequently, the indicator solutions are sufficiently stable if stored in the dark.

Acid—Base Titrations

Titration procedure. To a sample of acid solution indicator was added and titration was performed against a 0.1 *N* standard sodium hydroxide solution. At the end point the color of the solution turned from colorless to yellow, the yellow tint being permanent for at least 15 sec.

Recommended quantity of indicator. Tests were carried out to solutions of about 60 ml at the end point, using different quantities of indi-

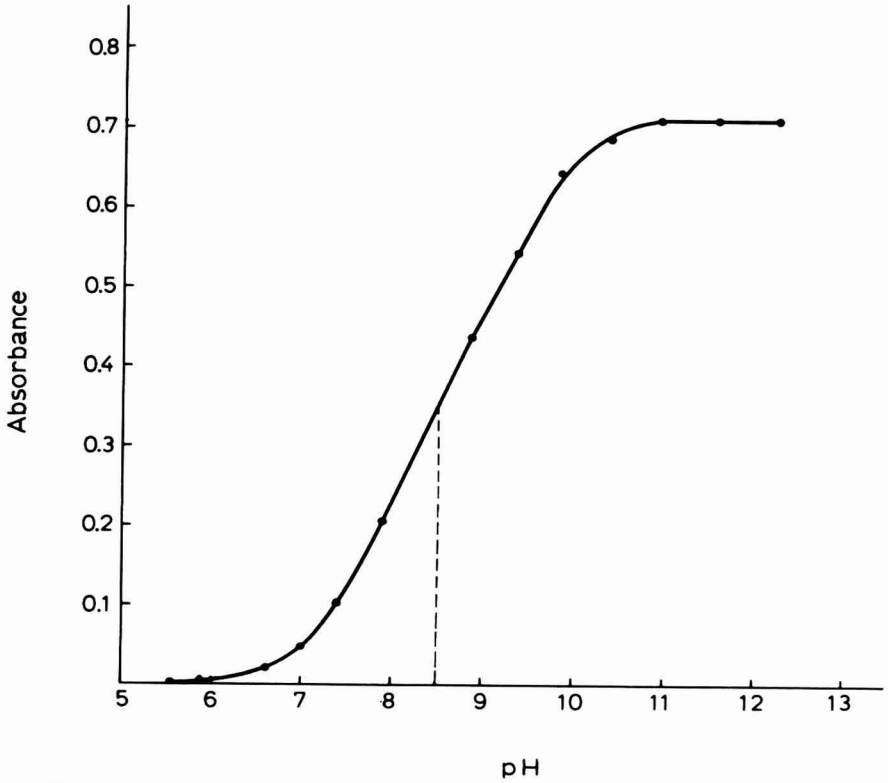

FIG. 3. The pH range of ZnIn. pH absorbance curve taken at 442 nm. Concentration of ZnIn = 1.5×10^{-5} *M*.

cator. For both indicators it was found that 7 to 8 drops of a 0.1% ethanolic solution were recommended to give sharp end points in visual acid–base titrations.

RESULTS AND DISCUSSION

Titrations of strong (hydrochloric) or weak (acetic, oxalic, benzoic) acids were made with a strong alkali (sodium hydroxide), using the recommended quantities of indicator. Five titrations were performed in each case. For both indicators the change of color at the end point was sharp and could be judged visually with great ease. The precision obtained was very good, the maximum deviation from the mean not excedding 0.2%.

Reverse titrations were carried out, too, using hydrochloric or oxalic acid as the titrant, in order to evaluate the ability of the indicator color disappearance. At the end point the yellow color of the solution disappeared sharply and the solution became colorless. The results were compared with those obtained by direct titrations. No differences were observed in precision.

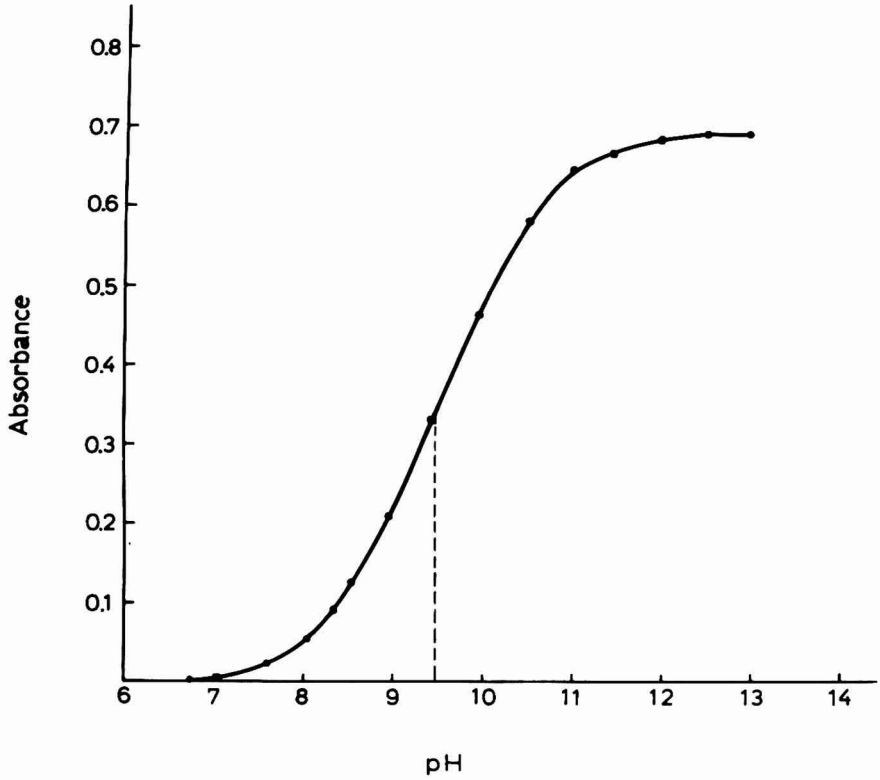

FIG. 4. The pH range of CdIn. pH absorbance curve taken at 444 nm. Concentration of CdIn = 1.5×10^{-5} *M*.

The error of titration was in general less than 0.8%.

For the sake of comparison the above mentioned direct or reverse titrations were all repeated with phenolphthalein as indicator. The results with phenolphthalein were found to be in close agreement with those obtained with ZnIn or CdIn.

SUMMARY

The DPPH complexes of zinc and cadmium are suggested as very useful indicators in visual acid—base titrations in aqueous solution. The pH range of the indicators was determined and the apparent indicator constants were found to be 8.5 and 9.5, respectively. The indicators give sharp end point and have proved to be similar in behavior to phenolphthalein.

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Determination of Stability Constant of Copper(II) with Thiosemicarbazide by Use of a Cupric Ion-Selective Electrode

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INTRODUCTION

Copper(II) complexes with thiosemicarbazide (TSC) were first prepared by Jensen *et al.* (*10*). More recently (*3*), their spectrochemical and magnetic properties were discussed and the heats of formation were measured (*1*). Few equilibrium data for the $Cu(TSC)_2^{2+}$ complex were found in the literature (*1*, *3*, *8*). Campbell *et al.* (*3*) and Ajayi *et al.* (*1*) both employed spectrophotometric methods to measure the stability constant. Complex formation studies by use of ion-selective electrodes (ISE) were carried out successfully over some copper and cadmium amino acid systems (*5*, *6*). The purpose of this investigation was to extend this technique to the $Cu(TSC)_2^{2+}$ complex by employing a cupric ion-selective electrode. The particular interest toward the ligand TSC was justified since it offers a possibility of masking for Cu(II), also in a very acid media (*7*, *8*).

EXPERIMENTAL

Apparatus. An Orion Model 94-29A solid-state cupric ISE referred to a saturated calomel reference electrode (SCE) with a potassium nitrate bridge was used for measurement of pCu in solution. The pH was measured with an Ingold 401 glass electrode. The emf and pH measurements were recorded with a Tacussel ISIS 20000 mV/pH meter. All measurements were made at 25 ± 0.1°C.

Reagents. Thiosemicarbazide and other reagents were Merck p.a. grade. Potassium nitrate was used to keep the ionic strength at 0.1. Redistilled water was always used.

Procedure. The calibration of the ISE was performed in the region of pCu = 2 to 6 by normal dilution technique from 0.1 *M* cupric nitrate, previously standardized with EDTA. The ionic strength in all solutions was made up to 0.1. In the region of pCu = 7 to 12 the ISE was calibrated by employing Cu(II) buffers prepared with nitrilotriacetic acid (NTA), the pH being adjusted to 4.75 as described in detail by Hansen *et al.* (*4*).

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Preconditioning in 10^{-3} M nitrilotriacetate solution and regular polishing of the electrode surface were necessary for obtaining a ± 1 mV reproducibility in potentials measurements. The time needed for the potential to reach equilibrium after immersion of the electrode in a stirred solution at pCu = 5 to 12 was found to be higher than 1 hr. The determination of the stability constant of $Cu(TSC)_2^{2+}$ complex was carried out by mixing cupric nitrate and TSC solutions to obtain a $C^\circ_{Cu} = 5.10^{-5}$ M and a $C^\circ_L = 1.10^{-2}$ M at $\mu = 0.1$. The pH was gradually varied by small additions of perchloric acid or sodium hydroxide and the pCu was measured.

RESULTS AND DISCUSSION

The method used was described by Hansen *et al.* (5). In the present study their general formula:

$$\log K_{CuL_2} = p\text{Cu} + 2 \log \alpha_{L(H)} - \log \frac{(C^\circ_L - 2C^\circ_{Cu})^2}{C^\circ_{Cu}}$$

was employed without corrections as the conditions for its validity were satisfied. The pCu values were obtained from the calibration plot shown in Fig. 1. The acidity constant of TSC ($pK_a = 1.743$) was taken from the

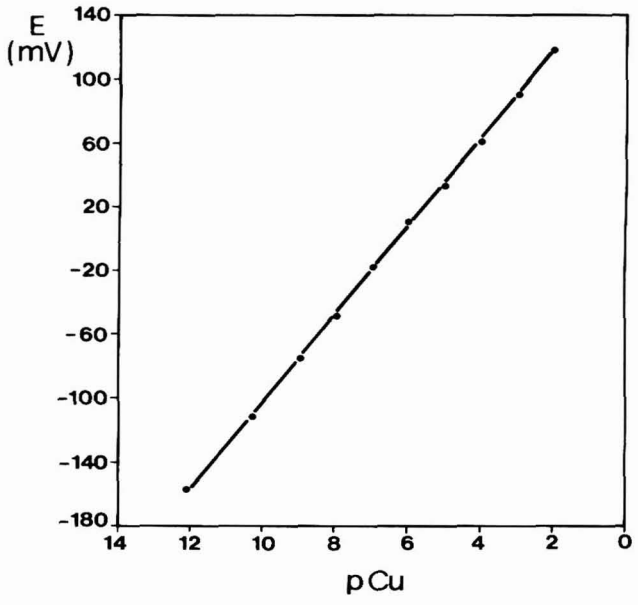

FIG. 1. Calibration curve for the Orion cupric ISE. For conditions see text.

TABLE 1
pH AND pCu VALUES FOR THE DETERMINATION OF THE STABILITY CONSTANT OF THE 1:2 COMPLEX OF Cu(II) AND TSC[a]

pH	$\log\alpha_{L(H)}$	E (mV)	pCu	$\log K_{CuL_2}$
2.18	0.135	−132	11.15	11.1
2.24	0.120	−133	11.20	11.1
2.48	0.073	−137	11.30	11.2
2.68	0.048	−138	11.35	11.2
2.81	0.036	−138	11.35	11.1
2.95	0.026	−141	11.45	11.2
3.01	0.023	−138	11.35	11.1
3.23	0.014	−142	11.50	11.2
$\log K_{CuL_2}$ = 11.2 (mean value)				

[a] $C^{\circ}_{Cu} = 5.10^{-5}\ M$; $C^{\circ}_{L} = 1.10^{-2}\ M$; μ = 0.1 (KNO_3); t = 25°C.

literature (*2*). Table 1 shows recorded pH and pCu values. The upper pH limit is imposed because of the limited stability of the $Cu(TSC)_2^{2+}$ complex at pH > 5, as previously reported from other authors (*9*). It was noted that the complex which is formed at low pH undergoes partial self-reduction slowly as the pH is raised to 5 to 6. This reduction to $Cu(TSC)_2^{+}$ was considered responsible for the anomalous increase in potential of the ISE observed at pH > 5 in the course of the present investigation. The logarithm of the stability constant was found $\log K_{CuL_2} = 11.2$ (as mean value). As shown in Table 2, it agrees well with previous results obtained from other authors by different methods.

SUMMARY

The stability constant of copper(II)—thiosemicarbazide complex has been determined potentiometrically by means of a cupric ion-selective electrode. Value of $\log K_{CuL_2}$ obtained in 0.1 M KNO_3 at 25°C corresponded well with the values given in the literature measured spectrophotometrically.

TABLE 2
LOGARITHMIC VALUES OF STABILITY CONSTANT OF Cu(II)-TSC COMPLEX

Reference	Method	$\log K_{CuL_2}$	μ	°C
Campbell and Grzeskowiak (*3*)	Spectrophotometric	12.9	—	25
Ajayi and Goddard (*1*)	Spectrophotometric	11.6	0.1	30
Ringbom (*8*)	—	11.2	—	—
This work	Potentiometric	11.2	0.1	25

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MICROCHEMICAL JOURNAL 23, 541–551 (1978)

An Improved Kinetic Determination for Creatinine Using the Abbott ABA-100

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INTRODUCTION

Because serum creatinine levels are relatively unaffected by diet, metabolic rate, or urine volume they are a better index of renal function than is the blood urea level. Therefore, serial determinations of serum creatinine are used frequently in evaluating the progress of renal disease (*10*). The possibility of the determination of creatinine in the presence of protein by a reaction rate principle, using the Jaffé reaction (*12*) has been examined by Bartels and Bohmer (*2*) and by Cook (*8*). Parasitic chromogens apparently have a negligible effect on the resulting absorbance during the time periods used for monitoring the reaction rate. In general their reaction rates are either much slower than that of creatinine, for example glucose (*4*), or they are much faster, as in the case of acetoacetate (*15*).

The kinetic method as described by Abbott (*1*) for use on their ABA-100 is based on the measurement of the rate of color development between 30 and 120 sec after adding alkaline picrate to the serum sample. During this time interval the rate of development of the creatinine–picrate chromogen (i.e., ΔA) yields essentially a linear response with respect to creatinine concentration.

Initially this method suffered from a low sensitivity of response as well as a poor correlation with the automated method (*7*) in use at the time. By optimizing the reaction concentration of picrate and hydroxide we have increased the reaction response by 35% with respect to the Abbott method. In addition the new method correlates more closely with the automated method. Protein was found to have a negative effect upon the reaction rate necessitating preparation of the standards in a protein base.

MATERIALS AND METHODS

Equipment. An Abbott ABA-100 Bichromatic Analyzer (Abbott Laboratories, South Pasedena, Calif.) was used.

Reagents. Bovine serum albumin (BSA) was obtained from Sigma Chemical Company, St. Louis, Mo., creatinine from J. T. Baker Chemical Company, Phillipsburg, N.J., and saturated picric acid (11.75

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g/liter) from Harleco, Gibbstown, N.Y. The only other reagents required were 0.35 and 2.5 *M* sodium hydroxide and 0.01 *N* HCl.

Creatinine standards; aqueous standards. A 100 mg% creatinine stock standard was prepared in 0.01 *N* HCl. Using this stock standard 1 to 20 mg/dl working standards were prepared by diluting the appropriate stock aliquot with 0.01 *N* HCl.

Bovine serum albumin standards. A 100 mg% stock solution was prepared as above using 4.5% BSA in 0.01 *N* HCl as diluent in lieu of 0.01 *N* HCl. The above creatinine working standards were similarly prepared by the dilution of the appropriate stock aliquot with the 4.5% BSA solution. BSA diluent was used as the reagent blank for the protein-containing standards.

Color Reagent. The color reagent was prepared just prior to use and was discarded after completion of the sample run. The color reagent for the Abbott method was prepared as follows: To 1.1 ml of 2.5 *M* sodium hydroxide 5.5 ml of a saturated picric acid solution was added and diluted to 20 ml with distilled water.

Color reagent for the proposed method was prepared as follows: To 15 ml of saturated picric acid solution 20 ml of a 0.35 *M* sodium hydroxide solution was added.

PROCEDURE

The instrument parameters are set as per the Abbott ABA-100 operations manual (*1*). To avoid a short draw of sample 100 μl of sample was placed in the appropriate sample cup. To minimize sample concentration due to sample evaporation, a drop of purified silicone oil (Sera-Seal. Abbott Cat. No. 8073-03) was applied to the surface of each sample. Only after the sample carousel was loaded and the instrumental parameters established was the color reagent prepared.

The total absorbance change (i.e., ΔA_T) was obtained as follows:

$$\Delta A_T = \Delta A_1 + \Delta A_2 + \Delta A_3 \tag{1}$$

where

$$\Delta A_1 = A_{60} - A_{30}$$

$$\Delta A_2 = A_{90} - A_{60}$$

$$\Delta A_3 = A_{120} - A_{90}$$

where the subscripted value represents the time in seconds at which the respective absorbance reading was read. The corrected total absorbance change (ΔA_{corr}) was equal to

$$\Delta A_{corr} = \Delta A_T - \Delta A_B \tag{2}$$

where ΔA_B = total absorbance change obtained from the reagent blank.

RESULTS

Protein Interference

Both aqueous and protein-based creatinine standards were run using the Abbott color reagent (*1*). The negative effect due to the presence of protein is indicated in Fig. 1. This negative effect however did not appear to affect the linearity of response of the protein-based standards which was linear to 20 mg% creatinine. The data suggest the effect is of an relative nature. Because of this protein effect all further tests were performed using protein-based standards.

Optimization of Picrate and Hydroxide Concentration

A survey of the literature yielded a number of picrate–hydroxide reaction concentrations considered to be optimal by the various authors on their respective instruments, none of which was an Abbott ABA-100 (*2–5, 9, 11, 14–16, 18*). A comparison of these concentrations with that proposed by Abbott resulted in marked variations in the reaction responses of the former methods with respect to the latter. Several of the

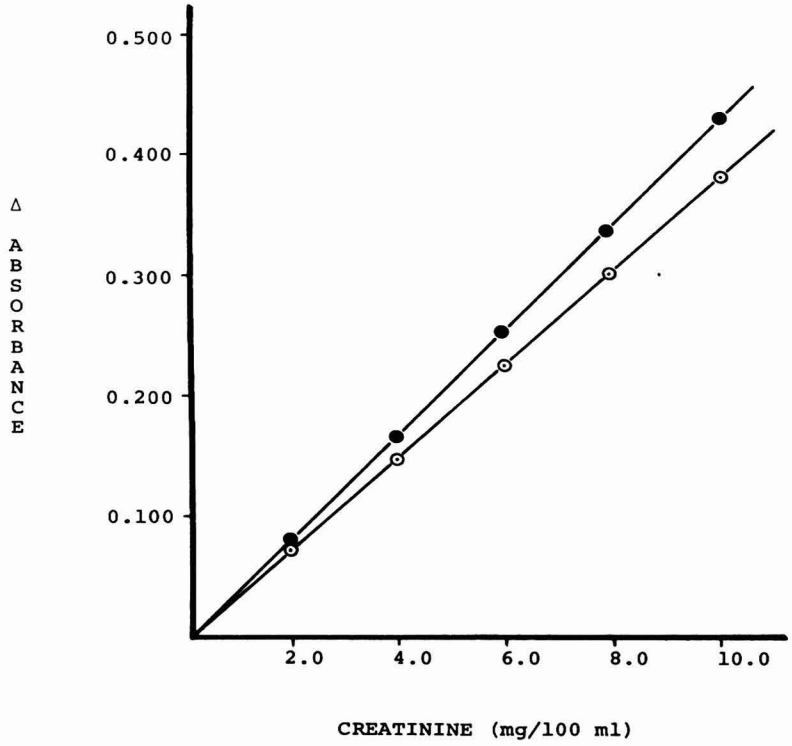

FIG. 1. The negative effect of protein upon the reaction response of (●) aqueous creatinine standards and (○) creatinine standards in 4.5% BSA.

reaction concentrations were found to produce an increased reaction response (*2, 3, 11, 15*), however their within-run as well as between-run reproducibility was poor, and thus they were of little use in optimizing the reaction response. Therefore a more systematic approach was used, in which the concentration of either picrate or hyroxide was held constant while the other was varied over a wide concentration range.

Variation in Hydroxide Concentration

To ensure that the picrate concentration would not be rate limiting (*6*), it was fixed at approximately 43 m*M* and the concentration of hydroxide was varied as follows: 80, 160, 240, 320, and 420 m*M*, respectively. As indicated in Fig. 2 the reaction response increased with increasing hydroxide concentration up to a concentration of about 240 m*M* hydroxide. Beyond this concentration the reaction response decreased markedly with increasing hydroxide, which was in agreement with the findings of Cook (*9*). The optimal reaction concentration of hydroxide

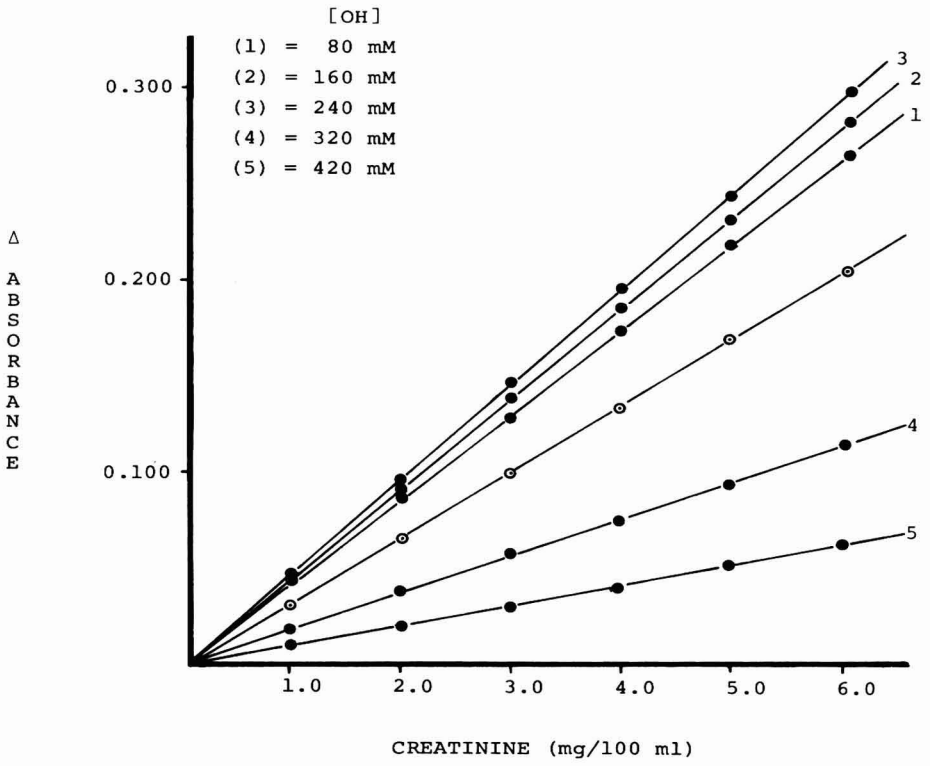

FIG. 2. The effect altering the hydroxide concentration has upon the reaction rate when picrate concentration is fixed at 43 m*M*; (●) Abbott concentrations (picrate = 13 m*M*, OH = 130 m*M*).

appeared therefore to fall in the range of 160 to 240 m*M* which agreed with the findings of others (*2, 3, 5, 8, 14*). A simplex optimization (*13*) of the data suggested the optimal hydroxide concentration to be in the region of 210 m*M*.

Variation in Picrate Concentration

The reaction concentration of hydroxide was fixed at 210 m*M* and the concentration of picrate was varied from 1.7 to 65 m*M*. As indicated in Fig. 3 the reaction response increased with increasing picrate concentration up to 43 m*M* beyond which a decreased response was observed. This decreased response was largely due to an instability of the color reagent as well as a severe blanking problem.

Though picrate concentrations of 43 and 32 m*M* gave the highest reaction response these concentrations were not considered to be optimal for the following reasons. Because of the instability of the resulting color

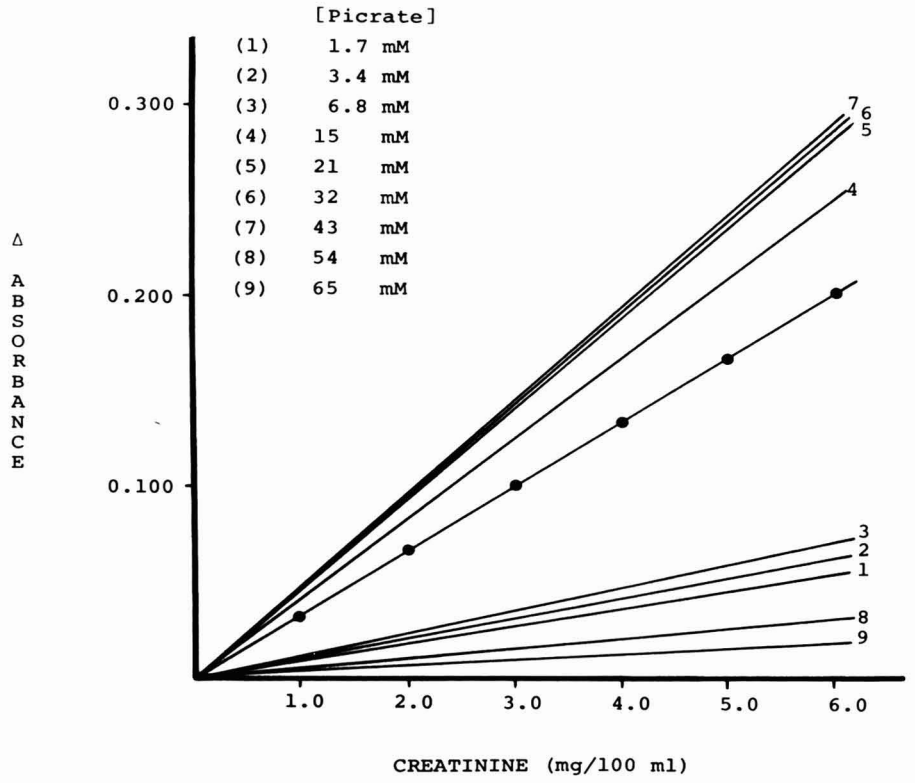

FIG. 3. The effect altering picrate concentration has upon the reaction rate, when hydroxide concentration is fixed at 210 m*M*; (●) Abbott concentration (picrate = 13 m*M*, OH = 130 m*M*).

reagent a gradual but continuous decrease in the reaction response was obtained as consecutive samples were analyzed. Under this condition in order to maintain an acceptable level of within- and between-run precision as well as overall test accuracy it was necessary to run a large number of standards interspersed with the test samples. This situation did not exist with the picrate concentration of 21 m*M*. In addition the color intensity of the blank at these concentrations was at least twice that at the 21 m*M* concentration. Since the maximization of precision calls for the magnitude of color developed by the blank to be minimal, these two concentrations were considered to be less satisfactory than the 21 m*M* concentration. Since the latter concentration gave essentially the same reaction response as the former concentrations without any of their shortcomings it was

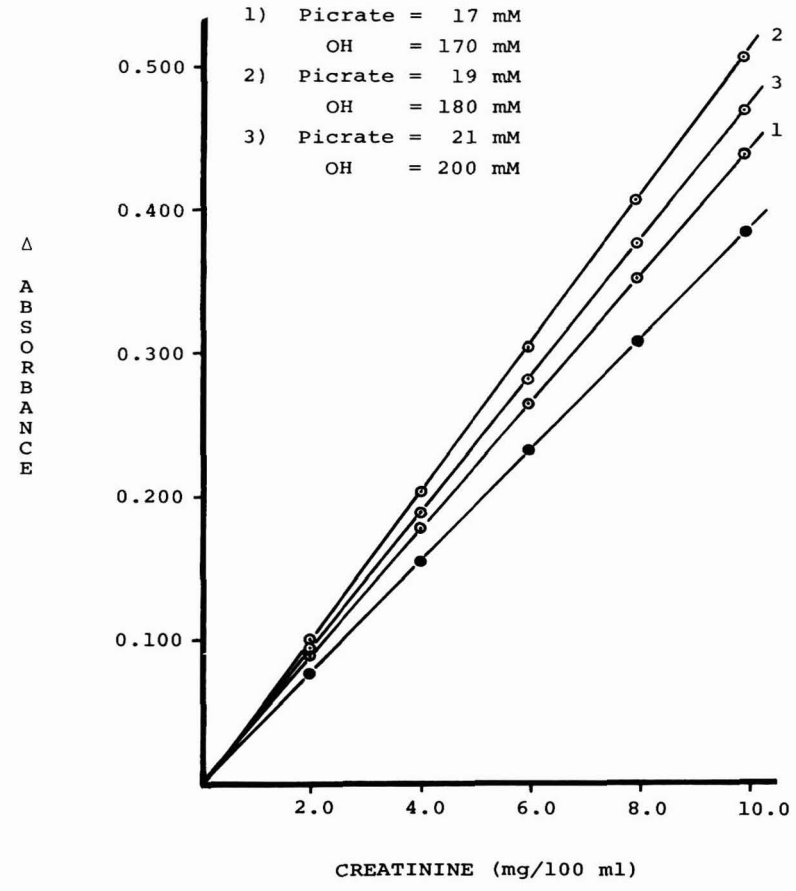

FIG. 4. The effect of slight variations of picrate and hydroxide concentrations about the simplex optima. (●) Abbott concentration.

selected as being essentially optimal. A simplex optimization of the data indicated the optimal picrate concentration to lie in the region of 19 m*M*.

The concentrations of both picrate and hydroxide were simultaneously varied slightly about the simplex concentration optima (Fig. 4). In all cases the experimental concentration resulted in an increase of 15 to 35% in the reaction response with respect to the reaction concentration recommended by Abbott. The most significant increase was obtained with a picrate–hydroxide concentration of 19 and 180 m*M*, respectively. This reaction concentration was therefore selected as being optimal and was used in all further testing. The reaction response was found to be linear to at least 20 mg% creatinine (Fig. 5).

Reproducibility Study

Both high and low level quality control sera were run over a period of 1 month using both the Abbott and the proposed kinetic method. The resulting data were compared to that obtained during the same period using an automated method (*7*).

As Table 1 indicates for the low level quality control both kinetic methodologies had significantly better coefficients of variation than the automated method. They did however give a higher mean value for the

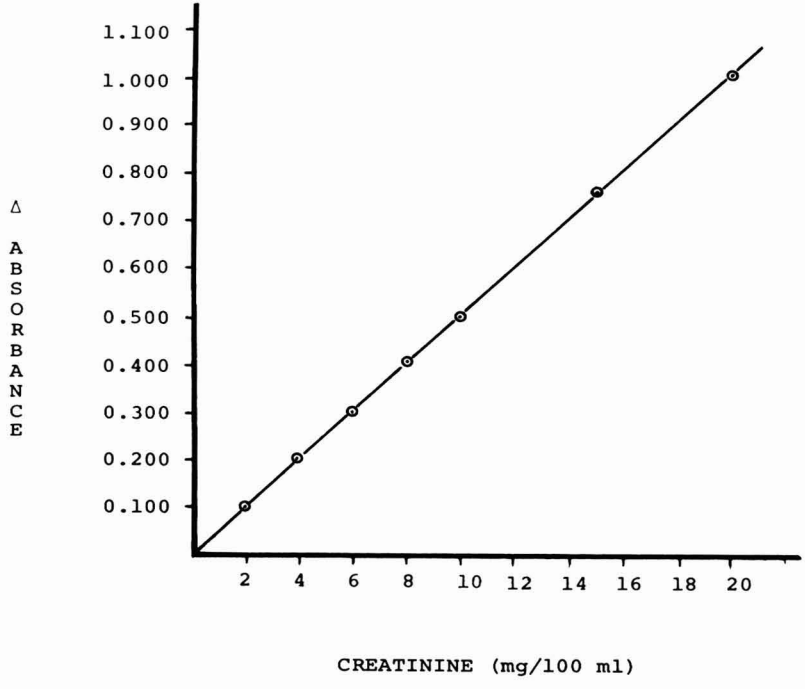

FIG. 5. Creatinine calibration curve using the proposed method.

TABLE 1
REPRODUCIBILITY OF KINETIC AND AUTOMATED METHODS

	Low level control			High level control		
	$\bar{X}$	SD	CV	$\bar{X}$	SD	CV
Auto-method	0.95	0.06	6.00	5.12	0.12	2.20
Proposed method	1.10	0.04	3.63	4.91	0.12	2.44
Abbott method	1.05	0.04	3.81	4.64	0.11	2.37

control sera than did the automated method. This finding was not observed however in actual specimens containing this level of creatinine suggesting that the observed positive bias may be an artifact of the control serum.

On the high level control both kinetic methods possessed a mean value negatively biased with respect to the automated method. However, the proposed method's mean was only 4% lower than the automated method compared to 9.5% lower for the Abbott method. The Abbott method was observed to possess a similar negative test bias on specimens containing levels of creatinine greater than 3.0 mg%.

Comparative Study

A total of 70 patient sera having creatinine concentrations in the range of 0.7 to 2.0 mg% were analyzed by each of the three methods. The automated method was considered to be the reference method in this comparative study (Table 2). This concentration range was selected because of the previously mentioned test bias possessed by the Abbott methodology at higher creatinine concentrations. The inclusion of data from specimens having higher creatinine concentrations significantly weighted the comparative data in favor of the proposed method.

TABLE 2
STATISTICAL COMPARISON OF DATA FROM KINETIC METHODS AND REFERENCE METHOD FOR SERUM SAMPLES CONTAINING 0.8 TO 2.0 mg% CREATININE

	Auto-method	Proposed method	Abbott method
$\bar{X}$	1.13	1.11	1.04
SD	0.29	0.28	0.31
Test bias		0.02	0.09
t test		1.58	5.70
F test		0.96	1.20
Slope		0.975	0.860
Intercept		0.050	0.245
Corr. coeff.		0.942	0.919

DISCUSSION

Protein has a twofold effect on the Jaffé reaction. The first is a positive colorimetric response due to the reaction between albumin and alkaline picrate. This results in the formation of a protein–picrate complex having spectral properties similar to the creatinine–picrate complex. The rate of this reaction, which is maximal in the first 30 sec of the reaction (i.e., fast acting interference), is proportional to the concentration of albumin in solution (*8*). Because of the time period over which the reaction is monitored, the contribution of this effect to the total absorbance change is negligible.

The second potential effect is due to the buffering capacity of the serum proteins which results in a decrease in the reaction rate (*14,15*). Because kinetic methodologies are very sensitive to pH change in the first minute or so of the reaction, the protein buffering effect can be very pronounced depending on the time period during which the reaction is monitored. This negative effect is especially pronounced when the reaction hydroxide concentration is 140 m*M* or less and /or when the serum dilution factor is 1:6 or less (*15*). The 12% decrease in total color change obtained with the protein-based standards was felt to be partially due to this buffering effect. The likely cause was the rather low hydroxide reaction concentration used in the Abbott method (i.e., 130 m*M*). In the proposed method the hydroxide reaction concentration was increased to 180 m*M* which resulted in a decrease in the negative effect due to protein from 12% to approximately 6%. Because protein still had an effect on the reaction rate its use in the preparation of the standards is recommended.

The picrate–hydroxide reaction concentration studies indicated that a marked fluctuation in the reaction rate may occur due to small changes in the actual reaction concentration of one or both of these reagents. The reaction response was found in general to be more sensitive to changes in the hydroxide concentration than in the concentration of picrate. Therefore, in order to maximize reproducibility it was necessary to maintain a very tight control on the preparation and handling of the color reagent and its constituents. The reproducibility data of low level creatinine samples indicated that both kinetic methods possessed lower percentage CVs than did the automated method. Both these methods, however, gave higher mean values than did the auto-method, suggesting the presence of a slight positive test bias with respect to the latter. These mean values did, however, fall within the accepted range for the control sera studied. This difference between mean values was not detected with the proposed method with actual serum samples containing the equivalent level of creatinine. This suggests that the difference may be an artifact of the control serum as has been reported by Weatherburn (*17*).

Both kinetic methods gave a lower mean value than did the automated

method for the high level control though all possessed essentially the same percentage CV. The mean value obtained with the proposed method however fell within the acceptable range for the control sera while the Abbott method value did not. The Abbott method was found to consistently give lower creatinine values than the other two methods with samples having creatinine levels of 3.0 mg% or more. This suggests the method may be suffering from a loss of accuracy at elevated creatinine levels, possibly due to an alteration in the reaction kinetics brought on by the low picrate concentration used in this method (*6*).

The comparative study data did not indicate an absolute test bias existed between the proposed method and the automated method. However, a t test value of 5.7 indicated such a bias did exist between the Abbott method and automated method. An F test indicated that neither kinetic method was more precise than the other with respect to the automated method under the study conditions. However, when the test results from samples containing higher levels of creatinine were included, the proposed method was shown to be more precise than the Abbott method. The inclusion of higher level samples in the comparative study resulted in the Abbott data being significantly less favorable than indicated in Table 2. Correlation studies (i.e., $y = 0.975x + 0.050$, $r = 0.942$) indicated the proposed method agreed more closely with the automated methodology than did the Abbott method (i.e., $y = 0.860x + 0.245$, $r = 0.919$).

In conclusion, we feel the use of protein-based standards, plus the optimization of the concentration of both picrate and hydroxide, offers not only a significant improvement in the resulting reaction response but also in the correlation of the test data with that obtained using an automated method. In addition the operational benefits of performing the determination on the ABA-100 are retained.

SUMMARY

An improved method for the kinetic determination of creatinine on the Abbott ABA-100 is described. By simplex optimization of the picrate and hydroxide reaction concentration a 35% increase in reaction rate, with respect to the Abbott methodology, was obtained. The new method correlated more closely with the Technician AutoAnalyzer Method (N-30) than did the latter method. The percentage CV for the improved method for high and low quality control sera was 2.4 and 3.6%, respectively. The presence of protein in the standards was found to cause a decrease in the reaction rate with respect to aqueous standards.

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A Fast, Simple Colorimetric Determination of Total Urinary Estrogen

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INTRODUCTION

Uniquely to man, in the last trimester of pregnancy urinary estrogens are excreted largely as estriol conjugates which account for 85 to 95% of the total urinary estrogens excreted (*5, 21*). The clinical value of serial estriol determinations during this period in the monitoring of the high risk fetus is well established (*7, 22, 30*). Low or rapidly falling urinary estriol having been found to have a high positive correlation with poor fetal outcome (*6, 13, 17, 29*) and low baby birth weights (*9, 16*).

The spectrophotometric quantification of urinary estriol is based on the production of a yellow–orange chromogen when free estriol reacts with sulfuric acid (*25*). Prior to this reaction the estriol conjugates must be hydrolyzed to free estriol. The method of choice in the past has been by means of an acid hydrolysis. However, this method has numerous shortcomings, the major one being its relative nonspecificity (*4, 8, 27, 31*). An alternate and much more specific means of hydrolyzing these conjugates is by means of an enzymatic system consisting of β-glucuronidase and aryl sulfatase (*11, 14, 32*).

With the development of serum estriol determinations by RIA, urinary estrogen analysis has fallen into disfavor with some clinicians. The preference of the former over the latter with respect to the clinical significance of the results is debatable, with several authors suggesting that both be done (*23, 24, 26*). We feel this gives a clearer picture of fetal well being, especially in cases where a reduced renal clearance is associated with a decreased estriol production, which could result in the serum level appearing to be within the normal range (*15*). One disadvantage possessed by the RIA methodologies is their cost factor, which makes them financially unfeasible in clinical situations where a large number of fetal viability tests are not regularly performed.

The purpose of this study was first to develop an inexpensive, simple accurate method for the determination of total urinary estriol using an enzymatic hydrolysis step. A secondary aim was to develop a method

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which allowed all the necessary reagents and the enzyme system to be prepared within the laboratory, with a minimal degree of technical difficulty. This latter aim not only results in a marked cost/test saving, but also allows smaller institutions to perform fetal viability tests without a large capital commitment to instrumentation and reagent kits.

MATERIALS AND METHODS

For the colorimetric measurements a Beckmann Model 35 uv/vis scanning spectrophotometer equipped with a thermostatically controlled cuvette holder was used (Beckmann Instruments Inc., Fullerton, Calif.).

Reagents

All inorganic and organic reagents were of analytical grade and were obtained from Fisher Scientific unless otherwise stated. The following were obtained from Sigma Chemical Co., St. Louis, Mo.: estriol-3β-D-glucuronide, sodium salt; estriol-16α-(β-D-glucuronide); β-D-glucuronide glucuronohydrolase (EC 3.2.131, β-glucuronidase), Type H-2, crude solution from *Helix pomatia*, activity approx. 100,000 Fishman units/ml at pH 4.55; Type V-A, soluble bacterial powder from *Escherichia coli*, activity approx. 80,000 Fishman units/g at pH 6.8 to 7.0; estriol (estra-1, 3,5(10)triene-3, 16α,17β-triol), B.D.H. Chemicals, Toronto, Ontario M8Z 1K5, Canada.

Dilute sulfuric acid (70% v/v).

Acetate buffer (0.1 M, pH 4.55). To 127.5 ml of 0.2 *M* acetic acid 122.5 ml of 0.2 *M* sodium acetate was added. This was diluted to 500 ml with deionized distilled water and the pH was adjusted to lie between 4.5 and 4.6. This reagent was refrigerated when not in use.

Phosphate buffer (0.075 M, pH 6.75). To 500 ml of 0.075 *M* potassium phosphate monobasic, 500 ml of 0.075 *M* sodium phosphate dibasic was added.

E. coli β-Glucuronidase

β-Glucuronidase of *E. coli* origin containing approximately 80,000 Fishman units/g was weighed out and dissolved in 0.075 *M* phosphate buffer to yield the following levels of activity; 100, 200, 400, 500, 1000, and 2000 Fishman units/ml, respectively.

Helix pomatia β-Glucuronidase

The crude *Helix pomatia* β-glucuronidase enzyme preparation was obtained from Sigma in 1-ml vials. The contents of one vial of crude enzyme was quantitatively transferred and diluted to 50 ml with 0.1 *M* acetate buffer. This preparation was stable for at least 1 month when kept refrigerated. This volume of working reagent was sufficient for 10 assays.

Preparation of Standards

A stock estriol standard (2×10^{-4} g/ml) was prepared in absolute ethanol. Using the appropriate aliquot of the stock standard, working standards were prepared containing 4 to 40 μg/ml estriol, respectively. The standards were kept refrigerated when not in use, and were stable for 6 months or more.

Sample Preparation

Because of the wide diurnal variation in urinary estriol excretion (i.e., $\pm$ 50%) 24-hr samples without any preservative were considered to be the minimal sample collection with 48-hr collections being optimal (*23, 24, 36*). In conjunction with this a creatinine determination was performed on each urine sample to ensure that a 24- or 48-hr collection had in fact been obtained.

Procedure

A urine blank such as a male urine sample or quality control urine having very low total estriol levels was run with each set of 24-hr urine samples. To 5.0 ml of the enzyme preparation, 1.0 ml of the urine sample was added and mixed several times by gentle inversion. The stoppered test tubes were placed in a 60 to 62°C water bath and incubated for exactly 35 min, from the time of addition of the urine sample. At the end of the incubation period the test tubes were removed and placed in a cold water bath (20 to 22°C) for several minutes. To each test tube approximately 1.0 g of sodium chloride was added using a precalibrated scoop or by means of premeasured capsules. Each test tube was then gently mixed until the sodium chloride had dissolved. Via a repipet, 10.0 ml of dichloromethane was then added and each test tube was gently mixed for 1 min. Excessively vigorous mixing resulted in the formation of a dense emulsion. Centrifugation was used to facilitate the separation of the two phases. The aqueous layer was removed by aspiration and discarded.

The reagent blank (i.e., ethanol), 5 and 10 μg/ml estriol standards were prepared by adding 4 ml of dichloromethane to 1 ml of the respective solution. To a 5.0-ml aliquot of the sample dichloromethane layer, reagent blank, and standard solutions, 90 mg of hydroquinone was added using a precalibrated scoop. Several anti-bumping glass chips were then added to each tube. These solutions were then taken carefully to dryness. To the dried residue 2.0 ml of 70% (v/v) sulfuric acid was added. The tightly stoppered test tubes were placed in a vigorously boiling water bath (98 to 100°C) for exactly 25 min. The tubes were mixed twice during the first 5 min of heating to ensure complete dissolution of the test residue and the hydroquinone. Upon completion of the heating period, the tubes were

allowed to cool and to each, 1.0 ml of distilled water was added. The stoppered tubes were then returned to the boiling water bath for exactly 15 min. The tubes were then removed to a cooling bath and the color developed was read on a scanning spectrophotometer from 575 to 450 nm. The samples were read against the urine control blank, while the standards were read against the reagent blank.

The maximal absorbance was corrected for the contribution of nonestrogen chromogens by means of the Allen correction (*1*).

$$A_{corr} = A_{514} - \frac{(A_{472} + A_{556})}{2}$$

where A = absorbance of test at the subscripted wavelength.

The concentration of estriol/24-hr urine is calculated as follows:

$$\text{mg estriol/24-hr urine} = \frac{A_{corr}\ \text{sample}}{A_{corr}\ \text{standard}} \times C \times U \times 2$$

where C = concentration of the appropriate standard and U = volume in ml of the 24-hr urine collection.

RESULTS AND DISCUSSION

The Spectrophotometric Linearity of the Kober Reaction

The Kober reaction was performed directly upon the appropriate level of estriol standard in the absence of both the enzymatic hydrolysis and the solvent extraction steps. The spectrophotometric linearity of this reaction with respect to free estriol concentration was found to be linear in the region of clinical significance (Fig. 1).

Sensitivity of the Kober Reaction to the Reaction Heating Parameters

Using a 10 μg/ml standard as above, the effect of variations in the duration of the initial heating period at 100°C on the Kober reaction were examined. The results (Fig. 2) indicated that when heating periods of greater than 20 min were used, small variations (± 30 sec) in the timing of the heating period did not significantly affect the final degree of color developed. However, to optimize assay precision it was necessary to maintain relatively accurate and consistent control of the timing of this initial heating period. A heating time of 25 min was ultimately chosen, as it ensured complete color development and secondly small errors in the timing of this heating period had virtually no effect upon the final result. The duration of this heating period was similar to that used by both Oakey *et al. (28)* and Crowley *et al. (10)*.

Unlike the time of heating, small fluctuations in the temperature of the heating bath were found to have a significant effect on the degree of color developed by the Kober reaction. A comparative study using heating bath

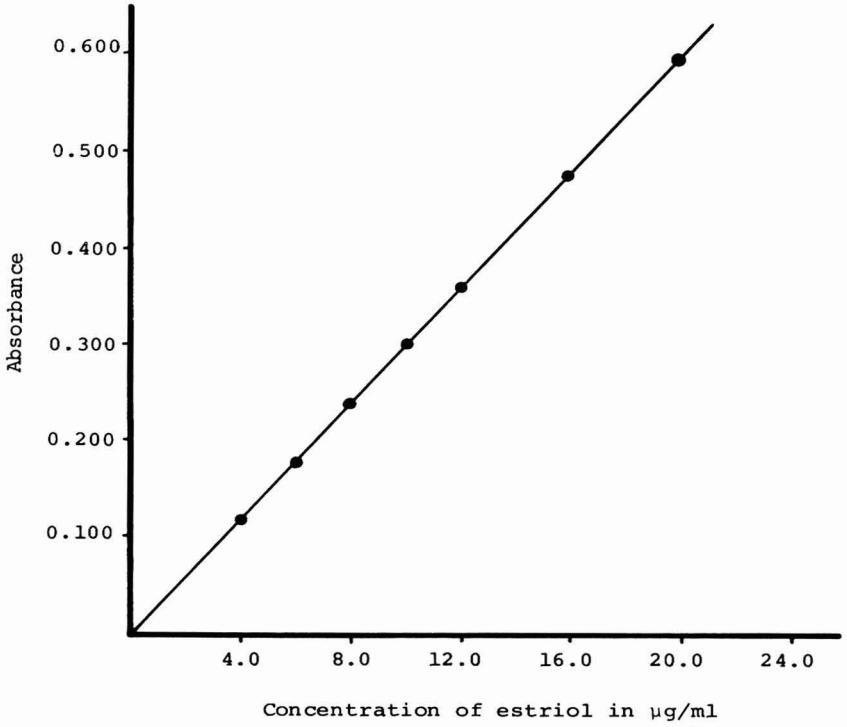

FIG. 1. Spectrophotometric linearity of the Kober reaction with respect to estriol concentration.

temperatures of 93 ± 3 and 98 ± 2°C, respectively, with a heating time of 25 min, showed that the former temperature produced on the average only 84% of the color developed at the latter bath temperature (Table 1). Thus to obtain maximal color development and test precision it was necessary to maintain a tight control on the bath temperature (i.e., 98 ± 2°C).

Solvent Extraction Efficiency

To determine the efficiency of the solvent extraction step, ethanolic estriol standards were added to blank urine samples such that the following levels of total estriol were obtained; 2.5, 5.0, and 10 µg/ml, respectively. The corresponding percentage recoveries were 98, 96, and 96%, respectively.

Enzyme Hydrolysis

It has been suggested by Dray *et al.* (*14*) that β-glucuronidase of *E. coli* origin has a higher hydrolytic activity than that of *Helix pomatia* with respect to the hydrolysis of urinary estriol glucuronides, and thus should be the preferred enzyme system for the hydrolysis of such glucuronides. However, a study by Graef *et al.* (*19*) has shown that there is no significant

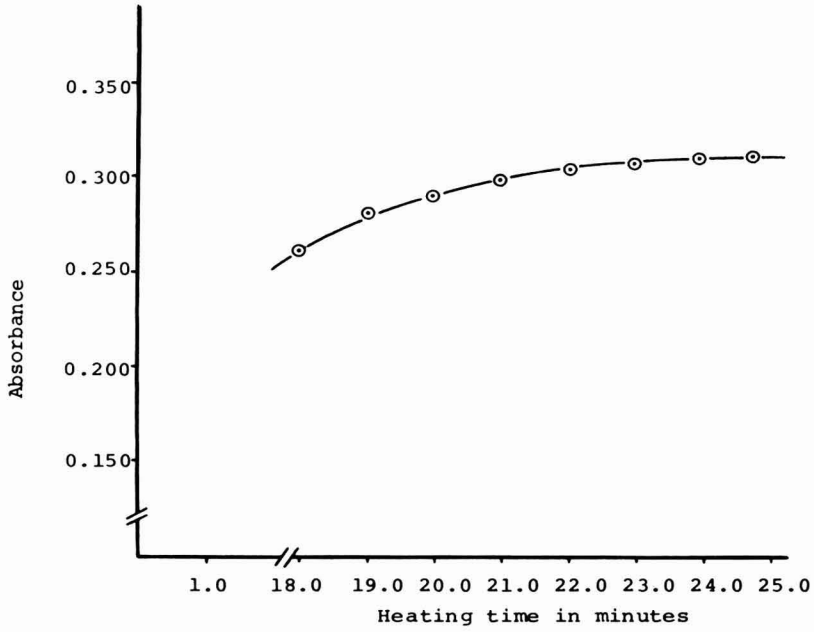

FIG. 2. The effect of variations in the time of heating at 100°C upon the Kober color reaction for a 10 μg/ml estriol standard.

difference in their ability to hydrolyze urinary estriol glucuronides. It was, therefore, decided to examine both enzyme systems to determine which was more applicable for use in the proposed method. The incubation temperature and pH of the respective enzyme preparations were; *E. coli* β-glucuronidase, pH = 6.75 at 37°C (*11*); *Helix pomatia* β-glucuronidase, pH = 4.55 at 60 to 62°C (*32*).

TABLE 1
COMPARATIVE STUDY OF THE EFFECT OF BATH TEMPERATURE ON THE LEVEL OF COLOR PRODUCED BY THE KOBER REACTION AT A HEATING TIME OF 25 min

Concentration of estriol (μg/ml)	Mean corrected OD		Percentage difference (*A*/*B* × 100)
	Bath temp. 93 ± 3°C (*A*)	Bath temp. 98 ± 2°C (*B*)	
3.0	0.076	0.088	86.4
5.0	0.127	0.145	87.6
6.0	0.156	0.182	85.7
8.0	0.201	0.243	82.7
10.0	0.247	0.306	80.7
20.0	0.508	0.622	81.7

Various activity levels of the respective enzyme were used to hydrolyze a control urine sample having a mean total estriol concentration of 14.0 μg/ml. To ensure complete conjugate hydrolysis, an enzyme incubation time of 45 min was used. Though the β-glucuronidase of *E. coli* origin was found to have a higher hydrolytic activity per unit of activity than the *Helix* enzyme (i.e., 2500 Fishman units versus 10,000 Fishman units), its use resulted in the apparent hydrolysis of only 90% of the total estriol conjugates hydrolyzed by the latter (Fig. 3).

Using 2500 Fishman units/test of the *E. coli* enzyme and 10,000 units/test of the *Helix* enzyme, the time of the enzyme incubation was varied from 5 to 35 min. As indicated by the plateauing of the hydrolysis curve (Fig. 4) the *E coli* glucuronidase achieved complete hydrolysis after only 20 min incubation compared to 30 min for the *Helix* enzyme. Again the use of the *E. coli* enzyme system resulted in the production of only 90% of the total estriol obtained using the *Helix* system. To ensure complete hydrolysis of the urinary estriol conjugates, especially for urine samples having less than 16.0 mg/liter estriol, incubation times of 25 and 35 min were selected as being optimal for the respective enzyme.

Actual patient urine samples were then subjected to enzymatic hy-

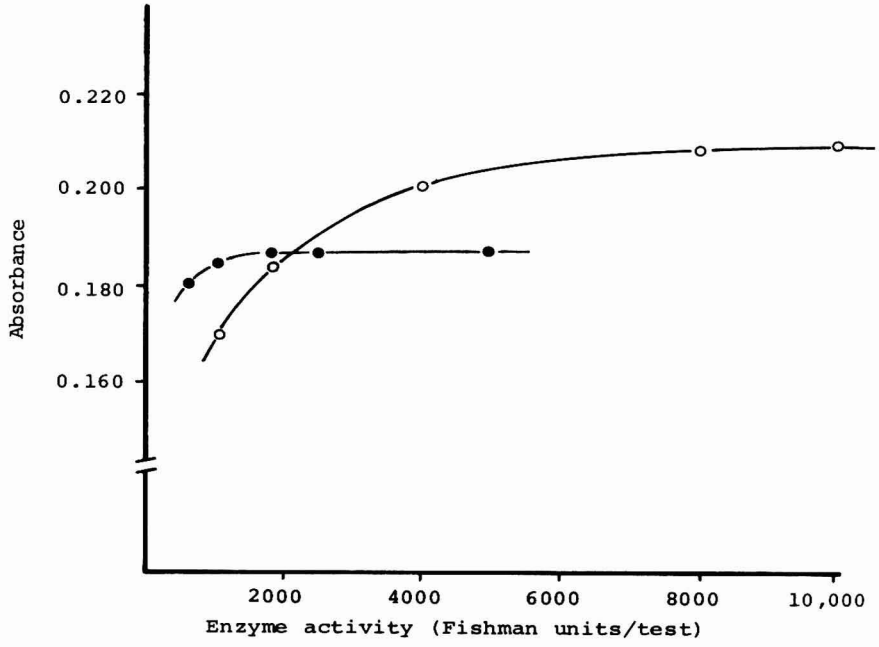

FIG. 3. Comparative study of the hydrolytic activity of β-glucuronidase of *Helix pomatia* (○) and *E. coli* (●) origin upon urinary estrogen conjugates, using an incubation time of 45 min.

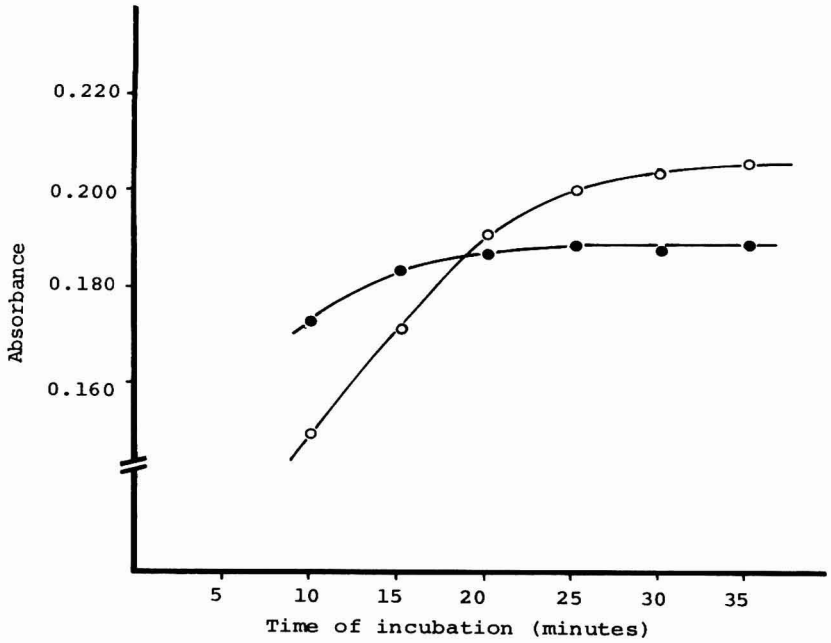

FIG. 4. Comparative study of the effect of variations in the time of incubation upon the degree of hydrolysis of urinary estrogen conjugates by β-glucuronidase of *Helix pomatia* (10,000 Fishman units) (○) and of *E. coli* (2500 Fishman units) (●) origin.

drolysis under the following conditions: *E. coli* enzyme, 2500 Fishman units/test for 25 min incubation, and *Helix pomatia* enzyme, 10,000 Fishman units/test for 35 min. As indicated in Table 2, the *E. coli* enzyme again resulted in the average production of only 90% of the total urinary estriol obtained with the *Helix pomatia* system. It is unlikely that this observed decrease in hydrolytic ability is due to an inhibition of the enzyme by the urine sample used (i.e., an artifact of the sample). The discrepancy is more likely due to the lack of aryl sulfatase activity in the *E. coli* preparation, due not only to the inhibitory effect of phosphate in the buffer system, but also the subsequent pH of the buffer (*18*). Therefore, the lower observed recovery was likely due to the incomplete hydrolysis of urinary estriol sulfate conjugates, which account normally for 5 to 12% of the total urinary estriol conjugates (*34*). This conclusion is in agreement with the findings of Nordström (*27*), who found a decrease of 5 to 13% in the total estriol recovered from urine when the enzymatic system was lacking in aryl sulfatase activity.

In summary, though the *E. coli* β-glucuronidase had a higher hydrolytic activity and thus required a shorter incubation time than did the *Helix pomatia* enzyme, it did possess several shortcomings which resulted in

TABLE 2
COMPARATIVE HYDROLYTIC ABILITY OF β-GLUCURONIDASE FROM *Helix pomatia* AND *E. coli* UPON URINARY ESTROGEN CONJUGATES

Sample numbers	Hydrolytic enzyme used (mg estriol/24-hr urine)		Percentage difference
	E. coli (A_1)	*Helix pomatia* (A_2)	($A_1/A_2 \times 100$)
1	17.3	19.2	90.1
2	19.7	21.7	90.8
3	17.1	19.6	86.7
4	19.4	22.1	87.8
5	19.5	21.3	91.5
6	27.1	29.0	93.5
7	27.7	28.6	96.8
8	16.5	18.1	91.1
9	19.1	22.0	85.0
10	17.5	19.6	89.2
Average	20.1	22.1	89.3

the acceptance of the latter enzyme as the basis of the hydrolytic media for this methodology. The primary shortcoming was the lack of any aryl sulfatase activity resulting in a decreased total free estriol recovery. Second, though less of the *E. coli* enzyme was required on a per test basis, it was significantly more expensive and much more labile than its *Helix pomatia* counterpart. Thus the following enzymatic hydrolysis parameters were selected as being optimal for the hydrolysis of urinary estriol glucuronides: *Helix pomatia* β-glucuronidase, activity 10,000 Fishman units/test incubated at 60 to 62°C for 35 min at a pH of 4.55.

Enzymatic Hydrolysis Efficiency

To study the combined efficiency of both the enzymatic hydrolysis and solvent extraction steps, estriol-3-glucuronide (E-3-G) and estriol-16-glucuronide (E-16-G) were added to a blank urine sample such that the following total estriol concentrations were obtained, 7.2 and 17.4 μg/ml, respectively. To simulate the percentage composition of a normal urine sample, these estriol conjugates were added in such a fashion that the final estriol concentration consisted of 70% E-16-G and 30% E-3-G (*26, 34*). These concentrations were for free estriol and thus assumed complete hydrolysis of the glucuronide conjugate. The percentage of free estriol recovered after enzymatic hydrolysis and the performance of the Kober reaction on the resulting dichloromethane extract fell in the range of 97% (±3%). The lower percentage recoveries were found with the higher ini-

tial concentration, while the 7.2 μg/ml sample was almost completely recovered.

The borderline of the area of concern is described by the curve obtained by plotting the following 24-hr total estriol concentration versus the respective gestational age: 4 mg at 28 weeks, 8 mg at 34 weeks, and 12 mg at 40 weeks (*12, 20*). Estriol levels falling below this boundary tend to be indicative of impending fetal distress or fetal death, while levels greater than 16 mg/24 hr are usually indicative of fetal well being (*22*). Since the low level of estriol in this study closely approximated a 12 mg total estriol/24-hr urine (assuming a total urine volume of 1500 ml), one could be confident of virtually complete conjugate hydrolysis and total recovery of the subsequent free estriol in urine samples falling on or below this borderline. Thus no problem existed in distinguishing borderline cases from nonborderline cases. This is of prime importance, as intervention by the attending physician should be performed only in cases of confirmed fetal distress, as the intervening process may be more detrimental to the fetus than if the pregnancy were allowed to continue upon its natural course.

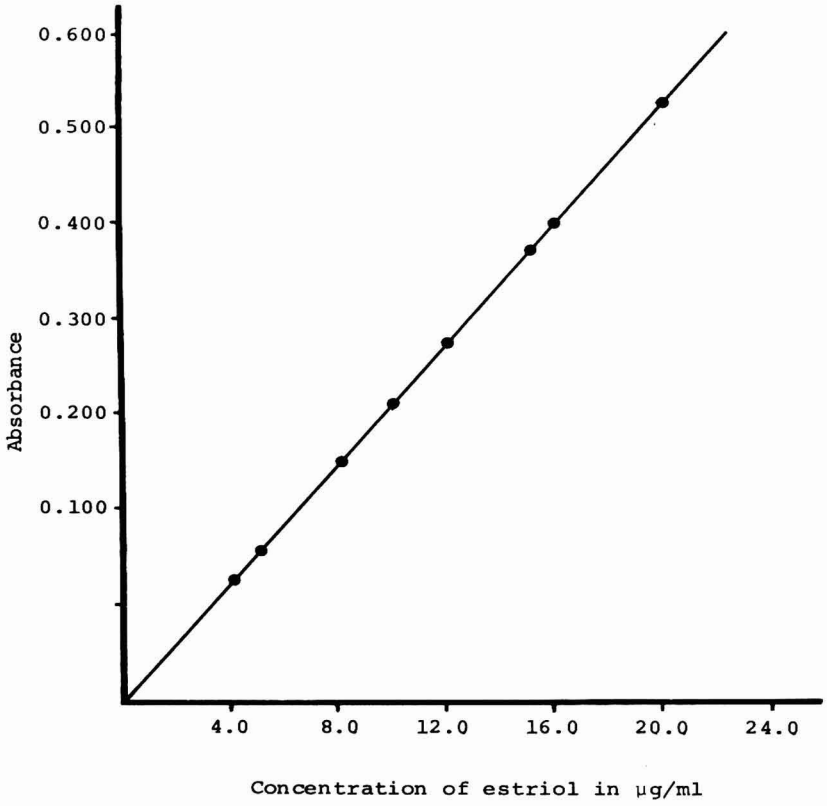

FIG. 5. Estriol calibration curve.

Linearity and Reproducibility of the Methodology

The linearity of response with respect to free estriol concentration for the proposed method is given in Fig. 5. The method's overall spectrophotometric response was found to be linear to at least 20 μg/ml estriol. To test the reproducibility of the proposed methodology two frozen urine samples (−40°C) having mean total estriol concentrations of 18.7 and 29.6 mg/24-hr urine, respectively, were analyzed for 6 consecutive weeks (Table 3). The respective coefficients of variation were 1.6 and 2.4%. The data also indicated the relative stability of the reagents which were from the same batch and were used for the complete 6-week period, except for the enzyme preparation which was prepared just prior to use.

Comparative Study

A comparative study was performed between the proposed enzymatic hydrolysis methodology and a methodology based on the Searle Diagnostics Tekit (*33*) which used an acid hydrolysis. All urine samples analyzed

TABLE 3
REPRODUCIBILITY STUDY USING FROZEN URINE SAMPLES HAVING MEAN TOTAL ESTRIOL CONCENTRATIONS OF 29.6 AND 18.7 mg/24-hr URINE

Week	Concentration of estriol (mg/24-hr urine)	
1	30.5	19.6
	29.9	19.4
	29.5	—
	29.5	19.0
2	29.1	18.7
	29.8	18.9
	29.8	18.9
	30.5	18.7
3	29.1	18.2
	29.8	18.7
	29.6	18.7
	29.4	19.2
4	29.1	19.0
	28.8	18.7
	—	18.7
5	29.8	18.0
	30.1	18.4
	29.5	18.2
	29.9	17.9
6	29.2	18.2
	29.7	18.2
Mean	29.6	18.7
SD	±0.47	±0.46
CV	1.59%	2.40%

TABLE 4
STATISTICAL DATA FROM A COMPARATIVE STUDY BETWEEN THE USE OF ACID HYDROLYSIS AND ENZYMATIC HYDROLYSIS PRIOR TO THE KOBER REACTION

Numbers of paired data	53.0
Mean estriol value ($\bar{X}$)	
Acid method ($\bar{X}_a$)	20.0
Enzymatic method ($\bar{X}_e$)	24.6
Test bias ($\bar{X}_a - \bar{X}_e$)	−4.6
Student's t test	5.98
Linear regression study, $Y = aX + b$	
Slope (a)	0.805
Intercept (b)	0.153
$Y = 0.805X + 0.153$	
Correlation coefficient (r)	0.944

were from cases in which the attending physician considered fetal viability to be in jeopardy. The accumulated data (Table 4) were examined using the method of comparative analysis as described by Barnett and Youden (*2*).

The comparative analysis of the data showed the existence of an absolute test bias of −4.6 between the two methods, as indicated by a t test value of 5.86. The use of the acid hydrolysis method resulted in a total urinary estriol level that was on the average only 80% of the value obtained using the enzymatic hydrolysis method. This finding was in agreement with the findings of both Brown (*4*) and Nordström (*27*). This decreased level in the total urinary estriol was likely caused by the nonspecific nature of acid hydrolysis upon the urinary conjugates resulting not only in their hydrolysis but also in their subsequent degradation. Methodologies using acid hydrolysis are also affected by both a high urinary glucose and urine density (*31, 37*), as well as various medications such as hexamethylene tetramine drugs (*35*), while enzymatic hydrolysis is not affected by any of these.

Therefore, the use of an enzymatic hydrolysis in the proposed method gives the method not only a high degree of hydrolytic specificity but also a high hydrolytic activity. This ensures essentially complete hydrolysis of the urinary estriol conjugates in a short period of time. Associated with this is a high level of test accuracy and precision, with minimal sample turn around time. Also because the test's total reagent complement is prepared in the laboratory not only is the resultant cost saving with respect to the method's commercial counterpart quite significant, but it allows in laboratory monitoring of reagent quality.

SUMMARY

A relatively rapid simplified colorimetric method which uses an enzymatic hydrolysis for the determination of total urinary estriol is described. All reagents and the enzyme prepara-

tion are prepared within the laboratory. Thus not only is reagent quality readily controlled but a marked cost saving is obtained with respect to the method's commercial counterparts. A comparative study of the hydrolytic ability of β-glucuronidase from *E. coli* and *Helix pomatia* indicated the latter to be the enzyme of choice for the proposed methodology. The proposed method was found to be linear to greater than 20 μg estriol/ml with coefficients of variation of 1.6 and 2.4% for 24-hr urinary estriol concentrations of 18.7 and 29.6 mg, respectively. A comparative study between the described method and a colorimetric method using an acid hydrolysis indicated the latter to yield only 80% of the total urinary estriol obtained with the former.

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BOOK REVIEWS

The Analysis of Rocket Propellants. By HUGH E. MALONE. Academic Press, New York, 1976. x + 148 pp., $14.25.

In the continuing search for the optimum propellant for specific rocket propellants, a wide variety of compounds have been or are now being used as fuels. While analytical procedures for some of these compounds are within the normal range of methods for an analytical laboratory, many are unusual and require special analysis, or else the combination with other compounds to form a propellant poses special problems. The purpose of this monograph is to present information about the compounds making up these propellants and describe methods for analysis for these compounds in the laboratory and in the field.

The book includes six chapters, a brief subject index, and an author index. Each chapter is subdivided into sections devoted to assays for a particular propellant component. The chapters include a number of excellent illustrations and each chapter is closed by a list of references to the current literature. Chapter 1 is an introduction to propellant analysis and a discussion of sampling procedures. Eleven different fuels are discussed in Chapter 2 with assays presented for each component element of the fuel. Chapter 3 discusses eight fuel mixtures and presents assays for each. Nine oxidizers are discussed in Chapter 4, from chlorine trifluoride to ozone. Chapters 5 and 6 present field methods for analysis of six propellant components and procedures for measuring six propellant components as contaminants in water.

This monograph is part of an international series of monographs under the series title "The Analysis of Organic Materials," edited by R. Belcher and D. M. W. Anderson. It is an excellent review of methods for analysis of rocket propellants and should be extremely valuable to those working in analytical chemistry laboratories or engaged in research in this specific field.

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

A Textbook of Pharmaceutical Analysis. 2nd ed. By KENNETH A. CONNORS. Wiley–Interscience, New York, 1975. x + 611 pp. $20.75.

This textbook is designed to be used by the student in pharmacy and the practicing pharmacist in order to understand the principles of most analyses. It is not a cookbook-type text on pharmaceutical analytical chemistry; neither is it a commentary on the official compendia (United States Pharmacopeia and National Formulary). As it turns out, this book is also very fruitful to have for everyone involved in pharmaceutical analysis in general. The book is arbitrarily divided into six parts: titrimetric analysis, physical and instrumental methods, separation techniques, elemental analysis, functional group analysis, followed by general topics. This sixth part deals with analytical toxicology and the analytical problem, which concerns sampling, statistics, the analytical literature, and calculations. The end of each chapter has a set of problems, for which the answers are given in the last section of the book.

Since pharmaceutical analysis concerns drugs which are mostly of organic chemical nature, the choice of organic functional group analysis as a separate part is very thoughtful. The first edition of this book was published in 1967. In the second edition the following topics have been added: ion-selective electrodes, optical rotatory dispersion and circular dichroism, nuclear magnetic resonance and electron spin resonance, mass spectrometry, high-pressure liquid

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chromatography, and analytical toxicology. The treatment of the subject matter and the examples given are very lucid. The presence of structural formulas contributes to make the book more readable and understandable for someone without a pharmaceutical background. The fact that additional current topics have been added to the second edition makes the book up to date, a sign that it is keeping up with the progress in the field. This book is certainly of value for everyone working in the field of pharmaceutical analysis.

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The Condensed Chemical Dictionary. 9th ed. Revised by GESSNER G. HAWLEY. Van Nostrand Reinhold Company, New York, 1977. xiii + 957 pp. $32.50.

This 9th edition of "The Condensed Chemical Dictionary" is (as were the previous volumes) more than an assembly of definitions or a glossary of terms. It is a dictionary in the full meaning of Noah Webster's description: "A work of reference in which the words of a language (or some part of it) are listed, usually with their meanings. . . ."[1] Being a dictionary, not an encyclopedia, no entry can be more than a condensation of essential information. The 9th follows very closely the form and intent of its predecessor volumes, continuing the opus which has held such a unique position in the technical library for more than half a century.

So why another revision? In a living science expanding as fast as chemistry, new words and/or expressions appear almost daily and former meanings take on new interpretations. For example, the 8th edition was definitely ecologically oriented whereas the 9th, with no deemphasis on environment, is unusually concerned with energy.

Thousands of industrial products are listed, and, as in previous editions, the information given is what the manufacturer has made available to the editor and must be considered in that light. Trademarked products are indicated by superior numbers which refer to the corporate name of the manufacturer. These names are listed numerically in Appendix I; the full names and addresses are given in alphabetical order in Appendix II.

This 9th revision fully lives up to the high reputation of this nearly indispensable series. The "old-timer" will by happy to add it to his personal library. To those just getting started, there can be no better advice than to do the same.

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

[1] "Webster's New International Dictionary of the English Language" (G&C Merriam Company, Springfield, Mass.), 1953, p. 724.

Botulism. By LOUIS D. S. SMITH, Charles C. Thomas, Springfield, Illinois, 1977. xiii + 236 pp. $18.75.

Although this is not a book for microchemists, many will be surprised to learn that botulism has not been eradicated in the United States. There were 21 confirmed outbreaks in 1974 and 14 in 1975. Obviously, modern canning processes have not eliminated this organism. When you consider that the *Clostridia* are one of Nature's most widely distributed organisms, perhaps the surprise should be that there is not more of it. Fortunately, rapid diagnosis and improved treatments have reduced the mortality from 23% in 1970–1973 to 11% in 1975.

As indicated above, the chemist will find this short monograph fascinating, but it has little to contribute to microtechniques.

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Electrochemical Stripping Analysis. By F. VYDRA, K. ŠTULÍK, AND E. JULÁKOVÁ. Halsted Press, John Wiley, New York, 1976. 283 pp. $40.00.

Electrochemical stripping analysis is a method of trace analysis that concentrates the substance to be determined electrolytically onto the measuring electrode and then transfers it back into the solution by the reverse electrolytic process. This stripping process is then monitored by a suitable electrochemical technique and, since the concentration of the substance to be determined is at a much higher concentration on the electrode than initially present in the solution, the sensitivity of the determination is greatly increased. The process has been used largely to determine readily reduced ions such as Cu, Cd, Pb, Zn, Ag, Hg, Fe, Ni, In, as well as less anticipated ions such as Ba, Sr, As, Se, Te, and organic materials such as dithizone, thiourea, cysteine, and 2-mercaptobenzothiazole. Under ideal conditions, concentrations as low as 10^{-10} M can be determined. The methods have been applied to the determination of water and atmospheric pollutants, semi-conductor materials, clinical specimens, foods, and geological samples, to list only a few applications. This book describes how it is done.

The Czechoslovakian authors, who are specialists in this field, have written an excellent, highly recommended survey in this addition to the Ellis Horwood series in analytical chemistry. Through the efforts of the translation editor, Julian Tyson, the book is most readable and the English cannot be faulted.

The work is largely practical. Chapter I introduces the electrochemical approach to trace analysis covering the principles of stripping methods, types of working electrodes, methods for monitoring the stripping process, and applications of the methods. Chapter II concentrates on the theory of the electrochemical process and includes such topics as the rate of charge-transfer reactions, mass transport rates, chemical kinetic rates, efficiency of the preconcentration process, theory of types of electrodes (such as the rotating disk electrode and hanging drop electrode), properties of amalgams, and properties of films. Chapter III treats primarily linear sweep polorography, coulometric determinations, and superimposed (ac, pulse, square wave) voltage methods thus covering the stripping process and monitoring methods. In Chapter IV, the experimental details and apparatus are covered such as the types of electrodes, vessels for the stripping determination, and instruments for the stripping process. Some information is also provided for the construction of the electrochemical instrumentation based on the use of operational amplifiers. Also covered is material on preliminary operations in stripping analysis: e.g., purity of reagents, sample preparation, preseparations, etc., choices of electrodes, and the evaluation of stripping curves. Chapter V covers practical applications starting with a section on the conditions for determination of elements by stripping analysis and examples of practical procedures. The index to the book is quite complete; a large number of literature citations are presented at the end of each chapter. Few references are included to the 1975 literature; most citations appear to be work done between 1965 and 1972, with appropriate citations of earlier work. Possibly the only fault in coverage could be the section on electronic instrumentation where, because of the rapid changes in technology, improved components and commercial instrumentation are available.

The work is highly recommended; the coverage is excellent.

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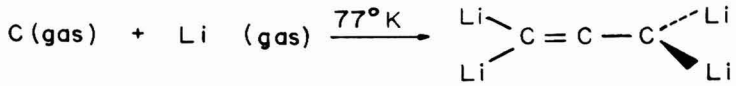
Cryochemistry. By MARTIN MOSKOVITZ AND GEOFFREY A. OZIN. Wiley, New York, 1976. 532 pp., $32.50.

Cryochemistry, as defined in this text, is a relatively recent science developing rapidly only during the last 10 years. Distinct from reactions normally carried out at low temperatures in the laboratory, cryochemistry is concerned with the reaction of unstable species such as atoms or radicals which may be generated at very high temperatures but trapped at very low temperatures. In this way synthesis of novel compounds and reaction mechanisms may be studied.

An example will serve to illustrate a typical technique used in cryochemistry. Elemental carbon is vaporized in an arc or furnace with a narrow outlet to permit the volatile carbon to escape (Knudsen cell). A surface is provided, cooled to a very low temperature (e.g., with liquid helium, 4.2°K). The carbon atoms are swept on to the cold surface with an excess of an inert gas, such as argon, at reduced pressure. The argon forms a solid inert matrix with a low vapor pressure at a temperature far below its boiling point. Along with the vaporized carbon (in our example) a reactant, such as lithium, is vaporized at a very high temperature in order to generate lithium atoms, this mixes and deposits on the super-cooled surface with the carbon vapor forming the new product.



Metals such as Mg, Al, B, Si, and Ge are readily vaporized in an oven along with some other organic or inorganic reactant, trapping the unstable reactant by sweeping onto the cold surface.

A radical may be trapped in an argon, neon, or krypton matrix and irradiated with monochromatic light so as to monitor changes. This is practicable since the inert gases are transparent to most of the UV, visible, and infrared light. Raman and Mossbauer spectra of deposited species may also be studied.

The text is a workbook which describes in detail the techniques used in instruments designed to carry out these studies. The characteristics of various substances used as matrices, methods of vaporization, vacuum formation, and obtaining the very low temperatures required are all explored. Methods for monitoring the weight of material being deposited are also presented. An interesting technique is to collect the deposit on a thin quartz wafer and measure the change in resonance frequency as a measure of the mass deposited. The techniques are applied to practically every element in the periodic table including isotopes. Preparative techniques are described for some compounds which could not be made otherwise. A list of chapters serves to outline the material in the text.

Techniques of Matrix Cryochemistry
Techniques of Preparative Cryochemistry
Organometallic and Organic Syntheses Using Main Group Elemental Vapors
Organometallic and Organic Syntheses Involving Transition Metal Vapors
Infrared and Raman Spectroscopic Studies of Alkali-Metal-Atom Matrix-Reaction Products
Matrix-Isolation Studies Involving Main Group Metals
Matrix Cryochemistry Using Transition Metal Atoms
Synthesis of Transition Metal Diatomic Molecules and Binuclear Complexes Using Metal Atom Cocondensation Techniques
Spectroscopic Identification and Characterization of Matrix-Isolated Atoms
Photochemistry in Low-Temperature Matrices

This book serves as an introduction to the chemist of the vast potential of this field. It should be of interest to chemists working in the fields of both inorganic and organic chemistry in synthesis, spectroscopy, surface chemistry, catalysis, and photochemistry. The material in the text should be included in the educational curriculum in both graduate and undergraduate courses in chemistry. The text should be available in the chemistry library for study. It is well written and clearly understandable. The authors should be congratulated on a job well done.

SAMUEL NATELSON,
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Modern Methods of Chemical Analysis. Second Edition. By ROBERT L. PECSOK, L. DONALD SHIELDS, THOMAS CAIRNS, AND IAN G. MCWILLIAM. Wiley, New York, 1976. xviii + 573 pp., $17.50.

This second edition has been reorganized to improve clarity and to save space for new topics. The authors feel that they have included enough material for a two-semester course covering most of the topics of the more traditional courses in quantitative analysis, qualitative organic analysis, and instrumental methods of analysis.

The book is divided into twenty-eight chapters, the first seven dealing with the general heading of "Phase Changes and Separations." Here, various phases of different types of chromatography are covered—analytical uses, extraction, types and theory, liquid column, plane, and gas.

The second section, "Electromagnetic Radiation," has eight chapters and treats electromagnetic radiation and its interaction, quantitative analysis by this means, general information regarding spectrometry, infrared, ultraviolet, flame emission and atomic absorption, X-ray, and nuclear magnetic resonance spectroscopy.

The third section, "Mass Spectrometry," has two chapters and treats mass spectrometry of organic compounds and spark source mass spectrometry. Interpretation of spectra, identification of unknowns, analysis of mixtures, qualitative identification of elements, and quantitative analysis are included.

The next section, "Electroanalytical Chemistry," has two chapters and deals with cells, potentials, pH, titrations, and electrodeposition.

The fifth section, "Acids, Bases and Their Salts and Complexes," has three chapters which include monoprotic systems, polyprotic systems, and metal ion complexes. Discussed are concepts, role of the solvent, relative strengths of acids and bases, equilibrium, titrations in aqueous and nonaqueous solvents, etc.

The next section, "Radiochemistry," devotes nineteen pages to such subjects as disintegration, interaction with matter, counting statistics, and some applications.

The seventh section, "Evaluation and Processing of Analytical Data," gives information on the statistical treatment of data and data processing. Significant figures, errors, indicating and recording devices, and the use of computers in connection with various types of spectrographic methods are treated.

The final section, "Automatic and Process Analyzers," contains three chapters and treats several automatic analyzers, process analyzers, calibration, and control. No great detail is given, but rather enough information to let the student be aware of the fact that these exist.

In general, there is a world of information presented in this text. It will be more valuable to the students who have had courses in organic chemistry and in analytical chemistry. This

reviewer feels that without the above courses, most students would be "lost." Much of the material also needs a knowledge of physical chemistry. In other words, this is an excellent book for a course on advanced analytical chemistry.

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Chemistry of Organic Fluorine Compounds. 2nd (Revised) Edition. A Laboratory Manual with Comprehensive Literature Coverage. By MILOŠ HUDLICKÝ. Ellis Horwood, Halsted Press, Division of Wiley, New York, 1976. xiv + 903 pp., $77.50.

Due to the very high cost, this text will not find its way into as many libraries, private and institutional, as should be. It is an excellent piece of work and contains an enormous amount of information in regard to the chemistry of organic fluorine compounds.

There are many sections dealing with such subjects as addition of fluorine, replacement of hydrogen by fluorine, replacement of halogens by fluorine, replacement of oxygen by fluorine, replacement of nitrogen by fluorine, properties of compounds, analysis and structure determination, practical applications, etc. In these sections are shown many reactions with some details for each. Included are 208 tables which contain such things as reaction products, conditions, yields, references, etc. In all, there are 3857 references given. Some spectral material is also presented. The last chapter gives 205 procedures (57 pages) for preparing compounds.

This reviewer is pleased to have a copy in his private library.

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ANNOUNCEMENTS

Clinical and Environmental Applications of Quantitative Thin-Layer Chromatography

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January 15–1979

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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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