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Editor-in-Chief: Al Steyermark

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Amperometric Titrations with Sodium Tetraphenylborate at the Drum-activated Carbon Electrode. JOHN T. STOCK AND DONALD R. BOWERMAN II, *Department of Chemistry, University of Connecticut, Storrs, Connecticut 06268.*

Drum-activated electrode systems were examined with the purpose of obtaining stable anodic currents of sodium tetraphenylborate.

Microchem. J. **17**, 253 (1972).

Colorimetric Determination of Gold(III) by Ascorbic Acid. M. H. HASHMI, A. SUBHAN, A. A. AYAZ, AND I. AHMAD, *Pakistan Council of Scientific and Industrial Research Laboratories, Lahore-16, West Pakistan.*

A method for the determination of gold(III) is reported, based on the color produced when this element reacts with ascorbic acid. The sensitivity of the color reaction is found to be 1 μg/ml.

Microchem. J. **17**, 258 (1972).

Application of Fast Grey RA to the Spectrophotometric Determination of Copper in Serum of Egyptian Camels. H. KHALIFA, M. T. FOAD, Y. L. AWAD, AND M. E. GEORGY, *Veterinary Research Laboratories, Dokki, Cairo, Egypt, U.A.R.*

The ortho–ortho hydroxy azo dye 15690-Solochromate Fast Grey RA, C.I. mordant black 15 (reddish navy-bluish black) has been used as a reagent for the successful estimation of copper in serum of camels.

Microchem. J. **17**, 266 (1972).

On the Mechanism of Detecting Aliphatic Monocarboxylic Acids by Means of New Fuchsin in Thin-Layer Chromatography. JÓZEF ŠLIWIOK AND BOŻENA KOCJAN, *Department of Organic Chemistry, Silesian University, Katowice, Poland.*

Continuation of the senior author's work using new fuchsin is described. This paper deals with the mechanism of detecting aliphatic monocarboxylic acids. The solvent system is composed of chloroform and acetone in the ratio of 96:4 by volume. Twenty-two micrograms of sample is employed.

Microchem. J. **17**, 273 (1972).

On the Spectrophotometric Measurement of Methylene Blue. WOLFGANG J. KIRSTEN AND VINOD J. PATEL, *Department of Chemistry, Royal Agricultural College of Sweden, S-750 07 Uppsala 7, Sweden.*

The association of methylene blue molecules in water solution can be eliminated by the addition of pyridine or dodecylbenzenesulfonate. The absorbance follows Beer's law up to at least 5.10^{-5} m solutions, absorbance 0.9 with 2 mm light path. The methylene blue solution obtained in the determination of sulfur according to the method of Johnson and Nishita–Gustafson [Johnson, C. M., and Nishita, H., *Anal. Chem.* **24**, 736–742 (1952); Gustafson, L., *Talanta* **4**, 227–235 (1960)] can be extracted with perchlorate and concentrated into a small volume of tetrachloroethane and octanol. Fractions of a microgram of sulfur may be determined by the method described.

Microchem. J. **17**, 277 (1972).

The Microdetermination of Stannous Tin With N-Bromosuccinimide. M. Z. BARAKAT AND SARWAT I. DOWEIDAR, *Biochemistry Department, Faculty of Medicine, Azhar University, Madina Nasr, Cairo, U.A.R.*

A new titrimetric method for the microdetermination of stannous tin, e.g., stannous chloride is proposed. The mechanism of the reaction in the presence of dilute hydrochloric acid is discussed. The determination of stannous tin is done on concentrations ranging from 10 mg to 100 μg.

Microchem. J. **17**, 285 (1972).

Accelerated Automated Microdetermination of Serum Calcium. E. S. BAGINSKI, S. S. MARIE, W. L. CLARK, J. A. SALANCY, AND B. ZAK, *Departments of Pathology, St. Joseph Mercy Hospital, Pontiac, Michigan, Holy Cross Hospital, and Wayne State University School of Medicine, Detroit, Michigan.*

A rapid procedure for the automated determination of serum calcium by direct determination in raw serum is described. Dimethyl sulfoxide is included in the reagents as a solvent to lower the dielectric constant of the medium and to help repress the ionization of the color reagent, cresolphthalein complexone. A discrete sample analyzer, the Beckman DSA 560 was used in the method.

Microchem. J. **17**, 293 (1972).

Methods for the Isolation and Characterization of Constituents of Natural Products. XVI. Quantitative Microdetermination of Diols as *Bis*-Esters of Pyruvic Acid 2,6-Dinitrophenylhydrazone: Separation from Monohydric Alcohol Derivatives and Resolution of an Homologous Series. D. P. SCHWARTZ, C. R. BREWINGTON, AND J. L. WEIHRAUCH, *Dairy Products Laboratory, Eastern Marketing and Nutrition Research Division, Agricultural Research Service, United States Department of Agriculture, Washington, D.C. 20250.*

A quantitative colorimetric method is described for the acylation of micro- and submicromole amounts of dihydric alcohols as *bis*-esters of pyruvic acid 2,6-dinitrophenylhydrazone. The *bis*-esters which are obtained exclusively, can be

quantitatively separated from a complex mixture of monohydric alcohol derivatives on an alumina column. Separation of an homologous series of terminal diol derivatives by thin-layer partition chromatography is also described.

Microchem. J. **17**, 302 (1972).

The Reaction Between Copper(II) and Cyanide Ions. RAM PARKASH AND JAROSLAV ZÝKA, *Department of Analytical Chemistry, Charles University, Prague, Czechoslovakia.*

The titration of cyanide with copper(II) involves simultaneous reduction of copper(II) and formation of cuprocyanides. The reduction is not complete in the reverse titration in solutions more concentrated than 0.03 *M* until the Cu^{2+}/CN^- molar ratio of 1:5 is reached. The two abrupt potential changes indicating the formation of $[Cu(CN)_4]^{3-}$ and $[Cu(CN)_2]^-$ have been utilized for the microdetermination of these ions. As little as 0.5 mg of cyanide and copper(II) can be estimated even in the presence of 80 times an excess of chloride and bromide. Iodide interferes.

Microchem. J. **17**, 309 (1972).

Microelectrophoresis in Thin-Layer Slab of Mixed Agarose Acrylamide Gel. HIDEBUMI HAZAMA, *Department of Neuropsychiatry, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan* AND HIDEYUKI UCHIMURA, *Hizen National Mental Hospital, Kanzaki, Saga 842–01, Japan.*

Microelectrophoresis in a thin-layer slab of mixed agarose acrylamide gel is reported. Thin slits are carved in the slabs and protein samples introduced under an oil chamber by means of a micromanipulator and a current of 1 mA (200 V) is applied for 15 minutes between two electrodes placed in two of the reservoirs. After electrophoresis, the gel slab is fixed and stained with Coomassie brilliant blue.

The method is applicable to protein samples in the picogram range.

Microchem. J. **17**, 318 (1972).

Technique for Automatic CHN Determination of Volatile Liquid Samples. CLARENCE I. KENNEDY, *Mead Johnson Research Center, Evansville, Indiana 47721.*

Glass capillary tubes are used successfully for the determination of CHN on volatile liquid samples in the automatic unit.

Microchem. J. **17**, 325 (1972).

Determination of Nanogram Amounts of Albumin by Radioimmunoassay. MARIE GAIZUTIS, AMADEO J. PESCE, AND JOHN E. LEWY, *Department of Biology, Illinois Institute of Technology, Renal Division, Department of Medicine and Section of Pediatric Nephrology, Michael Reese Hospital and Medical Center and the University of Chicago, Pritzker School of Medicine, Chicago, Illinois.*

A very sensitive radioimmunoassay is described for the measurement of albumin based on the fact that an antibody will adsorb to the walls of a polystyrene tube. Advantage is taken of the observation that when a mixture of labeled and unlabeled albumin reacts with a limited amount of antibody, the radioactivity adsorbed is a function of the two albumin concentrations. As little as 1 ng of rat albumin can be detected with a coefficient of variation of 12%.

Microchem. J. **17**, 327 (1972).

Coulometric Decimilligram Determination of Carbon and Hydrogen in Organic Compounds. KAN-ICHI NAKAMURA, KIKUSHIGE ONO, AND KATSURO KAWADA, *Central Research Laboratories, Sankyo, Co., Ltd., Tokyo, Japan.*

A coulometric method is applied to the simultaneous determination of carbon and hydrogen in decimilligram quantities of organic compounds. Samples are combusted in a stream of nitrogen using Co_3O_4 as the oxygen donor. The H_2O and CO_2 are swept into a Pt–P_2O_5 hygrometer and a pulse titration cell, respectively, where they are electrolyzed. From the electrolytic quantities required for the measurements of CO_2 and H_2O, the carbon and hydrogen amounts are determined. Results are shown for two methods, one requiring 15 minutes and the other requiring only 7 minutes.

Microchem. J. **17**, 338 (1972).

The Coelectrodeposition of Magnesium with Nickel and Determinaiton of Magnesium in Nickel Plate. JOHN T. STOCK AND VINCENT RICCI, *Department of Chemistry, University of Connecticut, Storrs, Connecticut 06268.*

Titration with EDTA is used to determine submilligram quantities of magnesium in electrodeposited nickel.

Microchem. J. **17**, 347 (1972).

Determination of Chromium by EDTA Titration. LASZLO SZEKERES, *Technological Institute, Budapest, Hungary.*

EDTA, acetic acid, and sodium acetate are added to a solution of chromium (III) ions. Formation of the violet colored chelate is completed after heating on a steam bath for 15–20 minutes. The excess EDTA is titrated with lead nitrate in the presence of potassium chromate and the end point is indicated by the formation of lead chromate. This procedure is applicable to the determination of metals that form stable chelates with EDTA at pH 5.

Microchem. J. **17**, 360 (1972).

MICROCHEMICAL JOURNAL **17**, 253–257 (1972)

Amperometric Titrations with Sodium Tetraphenylborate at the Drum-activated Carbon Electrode

JOHN T. STOCK AND DONALD R. BOWERMAN II

Department of Chemistry, University of Connecticut, Storrs, Connecticut 06268

Received January 26, 1972

In their study of the anodic voltammetry of sodium tetraphenylborate at a rotating pyrolytic graphite electrode, Stock and Pugliese (*9*) found that the current decayed rapidly with time. However, these workers were able to use sodium tetraphenylborate as a current-producing amperometric titrant by application of intermittent polarization. Recently, Stock (*7*) demonstrated that the drum-activated platinum electrode of Berge and Strübing (*1*) could be used to obtain essentially stable anodic currents of various organic compounds. The initial aim of the present work was to examine drum-activated electrode systems with a view to the obtaining of stable anodic currents of sodium tetraphenylborate.

MATERIALS AND METHODS

Equipment. The drum-activated platinum electrode (DAPE) was essentially the same as that used previously (*7*), but the drum speed was 170 rpm. Berge and Strübing (*2*) have described an assembly in which the platinum electrode is replaced by one of paraffin-impregnated carbon. In the present work with a drum-activated carbon electrode (DACE), the electrode proper was a "glassy carbon" cylinder approximately 3 mm in diameter and 10 mm long. This sealed with epoxy cement into the lower end of a length of 6 mm o.d. glass tubing so that the cylinder projected about 2 mm but was covered with cement. When this had hardened, careful abrasion was used to expose the circular cross-sectional plane of the cylinder. Drums used were of acrylic coated hardwood (*7*) and of lightly sandblasted glass (*8*). Current–voltage and current–time curves were obtained by use of a Leeds & Northrup Electrochemograph, which had a single scan rate of 0.2 V/minute. All potentials are with respect to the saturated calomel electrode (SCE).

Reagents and procedure. All runs were made in an acetate buffer

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of pH 4.6. This, the other reagents, and general procedures, were as used previously described (*7, 9*).

RESULTS

Anodic voltammetry of sodium tetraphenylborate (TPB). The peaked and poorly reproducible current–voltage curve of TPB obtained at a DAPE with a wood drum is shown at A in Fig. 1. This suggested that continuous electrode reactivation was not being achieved. The platinum electrode was cleaned, reinserted into its guide, and polarized at a constant potential of +0.70 V. The current decreased with time, the rate being quite rapid in the early stages of the run (Table 1). Mere replacement of the platinum electrode by one of "glassy carbon" did not bring about current stabilization. Initial experiments with a DACE in which the carbon electrode rode upon a lightly sandblasted glass drum were promising, but the residual current was unacceptably high. Quite by accident, if was found that preelectrolysis in a solution of corypalline (7-hydroxy-6-methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline) caused the residual current to sink to an acceptable level.

Approximately 0.5 m*M* corypalline in pH 4.6 acetate buffer was placed in the cell, then the potential was scanned to +0.80 V and

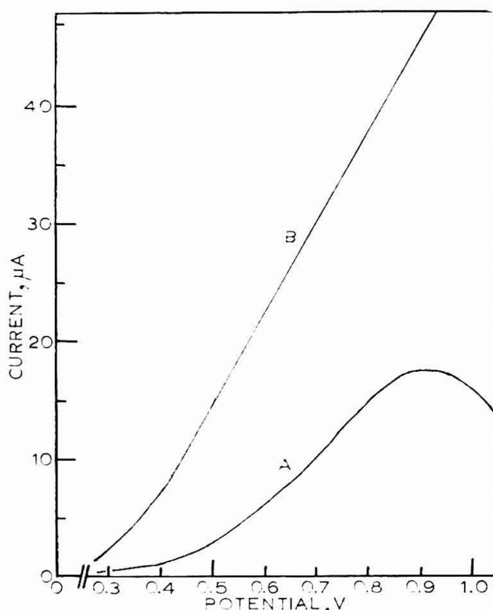

FIG. 1. Current–voltage curves of 3 m*M* TPB in pH 4.6 acetate buffer at (A), DAPE; (B), DACE.

TABLE 1

CURRENT (μA)–TIME RESPONSE AT E = +0.70 V OF ELECTRODE SYSTEMS IN 3 mM TPB

Time (min)[a]	:	0	0.5	1	3	5	10	15	20	25	30
Current, DAPE	:	10.8	9.2	8.8	7.8	6.0	5.2	4.0	5.0	5.4	5.2
Current, DACE	:	25.5	25.5	25.4	25.1	25.0	25.0	25.1	25.0	25.0	25.0

[a] Timing started 10 seconds after circuit closure.

left at this value for approximately 3 minutes. The potential was then scanned downwards until the current had fallen almost to zero. This sequence was repeated in the buffer solution alone. A typical current–voltage curve obtained after the DACE had been thus preconditioned is shown at B in Fig. 1. Although this wave, obtained in 3 mM TPB, showed no evidence of a region of limiting current, successive runs gave curves closely similar to the first. Current–time data at a potential of +0.70 V are given in Table 1 and demonstrate that satisfactory current stabilization had been achieved. Current–concentration plots at fixed potentials in the range 0.5–0.8 V were linear over the approximate concentration range 0.05–1 mM. At higher TPB concentrations, the slope of the plot decreased and became quite small for concentrations greater than approximately 1.5 mM.

Amperometric titrations. The amperometric titration of potassium ion with TPB was attempted by use of the DACE. The potential chosen, +0.55 V, was a value used successfully by Stock and Pugliese (*9*) in TPB titrations of potassium ion that were carried out by the intermittent polarization technique. A typical titration curve is shown at A in Fig. 2. The results, summarized in Table 2, indicate a standard deviation of approximately 0.2%.

Although corypalline does exhibit anode-deactivating properties, these are minimized at the DAPE (*7*). As expected, it was found that stable anodic currents of corypalline could also be obtained at the DACE. The results of titrations of corypalline listed in Table 2 are those obtained at the same potential as was used for potassium ion. This potential falls near the foot of the corypalline wave in the acetate buffer, so that the corypalline currents under the titration conditions were small. A typical titration curve is shown at B in Fig. 2.

DISCUSSION

Although there are some notable exceptions (*5*, *6*), amperometric titrations in which organic substances behave electroactively are usually carried out at a cathodic indicator electrode. Many organic substances

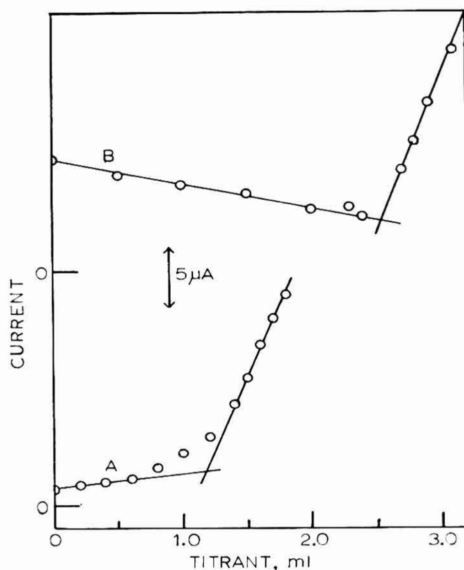

FIG. 2. Titration with ~0.1 M TPB of 50.0 ml of (A) 2.414 mM KCl; (B) ~5.3 mM TPB.

tend to form films on platinum or other solid anodes, so that these become deactivated. Steady currents, mandatory for successful amperometric titrimetry, are then unobtainable. It seems that the use of continuously-activated anodes may permit the development or improvement of titrations involving electrooxidizable compounds that deactivate the rotating platinum or other common voltammetric anodes.

Anodic amperometric titrimetry can be advantageous in cases where interferences prevent the use of the cathodic technique. For example, if the cathodic titration or mercury(II) in the presence of a large excess of silver ion is attempted, the massive current due to the reduction of silver ion would swamp all other currents. However, if the titration can be performed with a mercury-selective titrant that is electrooxidizable,

TABLE 2

AMPEROMETRIC TITRATIONS AT THE DACE WITH ~0.1 M TPB [a]

Titrand	Titers, ml				
2.414 mM KCl :	1.174	1.172	1.176	1.178	1.172
~5.3 mM Corypalline :	2.60	2.65	2.55	2.49	2.47
~5.9 mM Corypalline :	2.85	2.88	2.82	2.80	2.79

[a] Titrand volume, 50.0 ml = E = +0.55 V.

the difficulty might be circumvented. The anodic current should remain at or near zero until the mercury has been titrated, and should rise only when free titrant is present. This particular titration was developed by Khadeev and Bazarbaev (*4*), who used EDTA as titrant. This anodic EDTA technique has been much used by Russian workers (*6*). Khadeev (*3*) comments that the anodic current of EDTA is more stable at a tantalum electrode than at a platinum one.

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Colorimetric Determination of Gold (III) by Ascorbic Acid

M. H. HASHMI, A. SUBHAN, A. A. AYAZ, AND I. AHMAD

Pakistan Council of Scientific and Industrial Research Laboratories, Lahore, West Pakistan

Received January 28, 1972

INTRODUCTION

Many workers (*3, 9, 12*) have developed colorimetric procedures for the determination of gold (III); in particular, Beamish (*2*) has contributed much in this field. But the feasibility of these methods suffers from certain major drawbacks. Most of the colorimetric methods are carried out through the formation of a fine colloidal suspension of gold with different reagents, and suffer from the irreproducibility of the results.

During a systematic study of the action of organic reagents on inorganic substances it was found that ascorbic acid reacts with gold to produce color which obeys Beer's law. Reference to the literature indicates that this color reaction has never been reported previously (*5, 14, 15*). The behavior of the presence of other metal ions in the determination of gold has also been extensively studied. The visual limit of identification of gold is 1 μg/ml. The method is sensitive (*1, 6–8*) and simple.

EXPERIMENTAL

All the reagents used were of analytical grade or comparable purity.

Ascorbic acid. One hundred milligrams of ascorbic acid (E. Merck) was dissolved in distilled water to make up 100 ml. (Color Producing reagent).

Standard gold solution. Gold trichloride (E. Merck) crystals were dissolved in distilled water and a solution containing 200.00 μg of gold/ml was prepared.

APPARATUS

The absorbance measurements were made on a colorimeter, Unicam SP 1300, using glass cells having 1-cm optical path length with filter No. 3 in position.

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Pipettes graduated to 1 ml and accurate to ± 0.005 ml were used for manipulating the solution.

PROCEDURE

Spot Test. Take 1 ml of the test solution and adjust the pH to 5.5–9.0. Add 1 ml of ascorbic acid. The appearance of pink-violet or blue color will mark the presence of gold.

METHOD

Take 1 ml of the test solution containing 20–500 μg of gold and dilute to 5 ml; adjust the pH to 5.5–9.0 by citric acid, hydrochloric acid or sodium hydroxide.

Add 1 ml of the color producing reagent (ascorbic acid) and shake well. Dilute the solution to 10 ml and keep for 1 minute. Measure the absorbance of this solution using filter No. 3 and 1-cm glass cells.

A standard calibration curve was prepared by taking different amounts of gold solution and the value of the extinction coefficient for gold calculated. The amount of gold in an assay sample is directly calculated by inserting values in the following formula:

$$A = V \times d \times 58.447;$$

where A = amount of gold in μg in the test sample, d = optical density of the test solution, V = the volume of the test solution whose optical density is d.

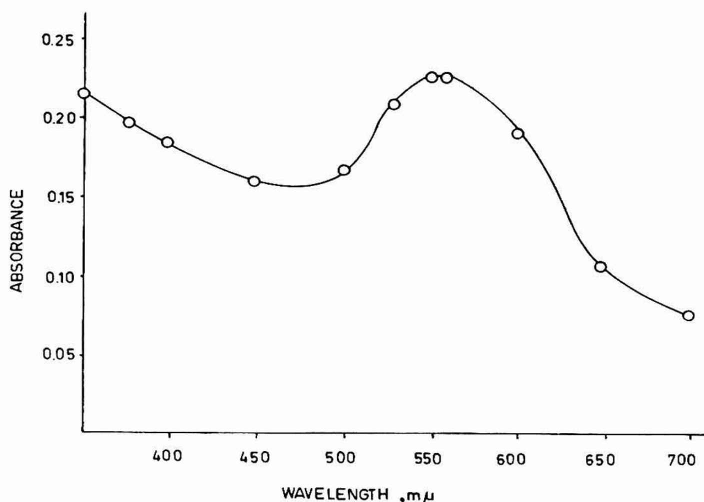

FIG. 1. Absorption characteristics of gold(III)-ascorbic acid complex.

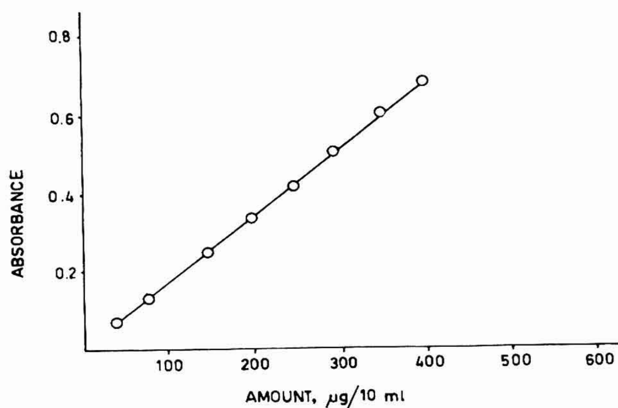

FIG. 2. Typical quantitative calibration curve of gold(III).

RESULTS AND DISCUSSION

The effects of pH, temperature and reagent concentration are given in Figs. 3, 4 and 5, respectively.

The effect of time on color development could not be determined due to the fast rate of reaction. However, complete color development takes place within 30 seconds after addition of the color producing reagent (ascorbic acid).

If a solution containing more than 500 μg/ml of gold is not diluted prior to the addition of the color producing reagent a fine colloidal suspension of gold will be formed.

It has been found that the color changes from pink to violet and blue with the change in pH or in the presence of other metal ions. How-

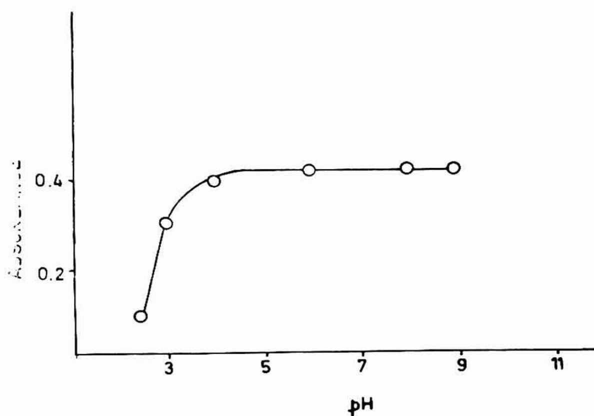

FIG. 3. Effect of pH on gold(III) complex formation.

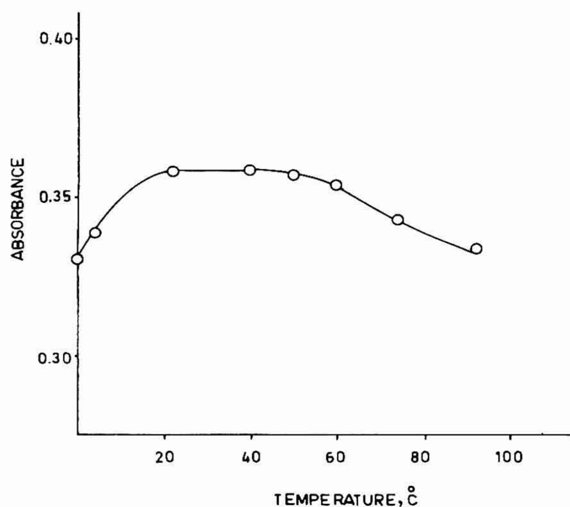

FIG. 4. Effect of temperature on gold(III) complex formation.

ever, the same absorbance value may be obtained in a fairly wide range of pH, i.e., 5.5–9.0.

With increasing temperature, the color intensity decreases slightly (*cf.* Fig. 4).

We have made a thorough survey of already existing methods for the estimation of gold and compared them with the present work. Plank (*10*) has determined gold with benzidine, but the method is not applicable where heavy or alkali metals are also present. Moreover, Beer's law is not obeyed in this case, rendering the method more difficult (*11*). Erdey and Rady (*4*) used ascorbic acid for the determination

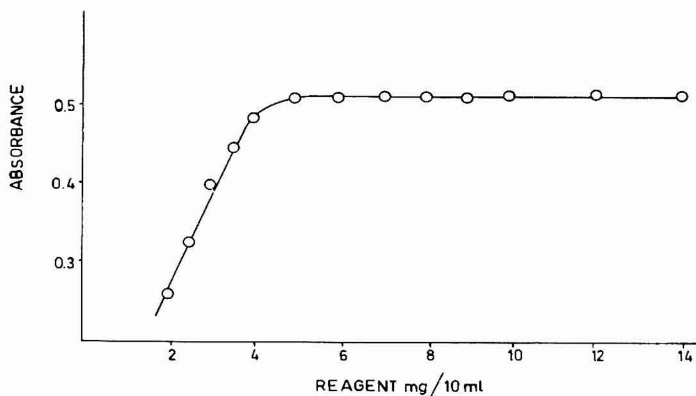

FIG. 5. Effect of reagent concentration on the color development.

TABLE 1

QUANTITATIVE ASSESSMENTS OF TOLERABLE AMOUNT OF DIFFERENT METAL IONS

Metal ion	Maximum amount not interfering in μg/10 ml	Remarks
Na^{+}	1000	
K^{+}	1000	
Ag^{+}	200	Color changes to blue
Hg^{+}	5	Ppt.
Pb^{2+}	10	Masking of Au^{3+} color
Ca^{2+}	100	Masking of Au^{3+} color
Co^{2+}	50	Masking of Au^{3+} color
WO_2^{2+}	1000	—
Ba^{2+}	1000	Color changes to blue
Cd^{2+}	200	Color changes to blue
Cu^{2+}	200	Color changes to blue
Zn^{2+}	200	Color changes to blue
Sr^{2+}	1000	Color changes to blue
Ni^{2+}	200	Color changes to blue
UO_2^{2+}	50	Color changes to blue
Be^{2+}	100	Color changes to blue
VO^{2+}	200	Color changes to blue
Mn^{2+}	10	Color changes to blue
Hg^{2+}	5	Ppt. and masking
Sn^{2+}	5	Color masking effect on gold color
Pd^{2+}	5	Color masking effect on gold color
Al^{3+}	20	Pptn. starts
La^{3+}	200	Color changes to blue
Fe^{3+}	1000	Color changes to blue
Ru^{3+}	5	Color masking effect on gold color
Ce^{3+}	10	Color masking effect on gold color
Bi^{3+}	50	Color changes to blue
Rh^{3+}	20	Color changes to greenish blue
Cr^{3+}	100	Color changes to greenish blue
Tl^{3+}	10	Color masking effect on gold color
In^{3+}	20	Masking effect on gold color
Y^{3+}	100	Blue color
Th^{4+}	50	Masking effect on gold color
Pb^{4+}	10	Masking effect on gold color
Zr^{4+}	150	Color changes to blue
Pt^{4+}	100	Color changes to blue
Sn^{4+}	5	Color changes to blue
Mo^{6+}	1000	—

TABLE 2

MAXIMUM TOLERABLE AMOUNTS OF SOME ORGANIC COMPOUNDS WHICH DO NOT INTERFERE IN THE DETERMINATION OF GOLD(III)

Compound	Maximum amount not interfering in μg/10 ml	Effect when amount is increased
Vitamins		
Folic acid	50	Color changes to blue black
Thiamine HCl	10	Color changes to blue black
Riboflavine	5	Color of the compound itself interferes
Choline chloride	200	Color changes to violet blue
Biotin	20	Color changes to blue black
Pyridoxine	20	Masking effect
Cyanocobalamine	250	—
Amino acids		
L-Aspartic acid	500	—
DL-Ornithine HCl	20	Color changes to blue black
DL-Serine	500	—
DL-Threonine	800	—
L-Asparagine	700	—
DL-Lysine HCl	30	Color changes to blue black
L-Hydroxyproline	700	—
d,l-Valine	500	—
L-Cysteine	5	Color changes to blue black
Phenylalanine	300	Color changes to blue black
Glycine	200	Color changes to blue black
L-Leucine	300	Masking effect
DL-Methionine	200	Color changes to blue black
Tyrosine	300	Color changes to violet blue
Glutamic acid	50	Color changes to violet blue
DL-Tryptophane	60	Color changes to violet blue
β-Alanine	50	Color changes to violet blue
Alkaloids		
Nicotine	2	Serious masking of color
Ephedrine HCl	50	Color changes to blue black
Strychnine HCl	50	—
Narcotine	100	Masking effect
Morphine	30	Color changes to pink red
Atropine	60	—
Quinine sulfate	70	—
Brucine	60	—
Papaverine	50	—
Cinchonine	150	—

TABLE 3

DETERMINATION OF GOLD FROM SYNTHETIC MIXTURES

Amount of Au^{3+} in $\mu g/10$ ml	Other metal ion	Amount in μg	Amount of Au^{3+} found	% recovery
200	Cu^{2+}	100	197.0	98.5
200	Co^{2+}	30	198.0	99.0
200	Ni^{2+}	100	198.0	99.0
200	Rh^{3+}	10	198.0	99.0
200	Cr^{3+}	100	199.0	99.5
200	Pt^{4+}	50	200.0	100.0

of gold by potentiometric redox titration. Another volumetric method using ascorbic acid for the determination of gold has been reported by Stathis and Gatos (*13*) but these methods are not as accurate and simple as the present one, which can be used in routine analysis. Copper and cobalt, in amounts of 200 and 50μg, respectively, do not interfere showing the superiority of this method over the one reported earlier.

Interference by other metal ions has also been checked and the maximum tolerable amounts which do not interfere in the determination of gold are given in Table 1. The effects of the presence of vitamins, amino acids and alkaloids have also been studied and the results are given in Table 2.

The extinction coefficient of gold(III) has been calculated to be 3.375×10^3 and a formula developed for the direct determination of gold(III) without the help of a calibration curve. The determination of gold from synthetic mixtures has been cited in Table 3.

SUMMARY

A very simple economical and accurate method for the determination of gold with ascorbic acid over a fairly wide range of pH has been developed. The effect of the presence of other metal ions on the determination of gold has been discussed. The sensitivity of this color reaction is 1 μg/ml. This reaction obeys Beer's law in the range 20–500 μg of gold.

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Application of Fast Grey RA to the Spectrophotometric Determination of Copper in Serum of Egyptian Camels

H. KHALIFA, M. T. FOAD, Y. L. AWAD, AND M. E. GEORGY
Veterinary Research Laboratories, Dokki, Cairo, Egypt, U.A.R.

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INTRODUCTION

Copper is essential for red cell maturation as it aids conversion of iron into hemoglobin and participates in the construction of cytochrome oxidase or supplements its action (*3*). Its deficiency interferes with synthesis of phospholipids (*6*). In serum, copper is found attached to alpha on beta globulin fraction (*5,8*). Its estimation is essential for enzymology, nutrition, and toxicology.

Colorimetric reagents used for copper determination include: sodiumdiethyldithiocarbamate for copper in serum, in a method involving deproteinization by heat (*4*) or by 2 N HCl (*7*); complete precipitation of protein and extraction of copper by trichloroacetic acid with the limitation of maximum color development of the color complex at alkaline pH 8–10; zincdibenzyldithiocarbamate for 0.02 to 2.0 ppm of copper in oils and fats in a method involving chloroform extraction (*1*); 1:1 neocoprine bathophenanthroline mixture for copper and iron in a somewhat complicated procedure involving chloroform extraction (*15*); and oxalydihydrazide acetaldehyde for copper in serum, urine, liver, brain, or kidney in a rapid wet ashing procedure.

Khalifa used 15690 Solochromate Fast Grey RA (C. I. mordant black 15; 2-(2-hydroxy-1-naphthylazo)-6-nitro-1-phenol-4-sulfonic acid, sodium salt) for the accurate spectrophotometric determination of metal ions (*9,11–13*), including copper(II) in the range of 0.025 to 10 ppm eliminating interference from iron up to 500-fold copper by reduction with ascorbic acid (*10*). With its acid-base properties investigated and the molecular structure of its complexes elucidated (*14*). FG is used for the determination of traces of copper in serum of Egyptian camels, which was the aim of the present investigation.

The present method provides for determining copper with the advantages of simplicity of technique, high sensitivity, development of the water-soluble colored complex in 0.01 *N* acid, suitable also for deproteinization and avoiding tedious separation of interfering elements.

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EXPERIMENTAL METHODS

The water used was always twice distilled in glass. The chemicals of the highest purity available were copper (II) sulfate; hydrochloric, trichloroacetic, and ascorbic acids; citrate and pyrophosphate of sodium. Fast Grey RA and sodium diethyldithiocarbamate reagents.

Solutions

The stock copper(II) solution (0.1 mg/ml) was prepared by accurate weighing and proper dilution. Lower dilutions were prepared from this stock solution. The trichloroacetic acid (TCA), sodium pyrophosphate, diethyldithiocarbamate (DEDTC) and FG reagents were 20, 7, 0.1, and 0.005 to 0.05% respectively.

Equipment

The centrifuge was sixteen 15-ml tubes, AHT type 5000 No. 1286. The spectrophotometer was Unicam Sp 600 with the blue sensitive photocell and 1-cm cells.

Procedures

I-A. To determine copper in animal serum: transfer a 2-ml sample (expected to contain 1–8 μg of copper) into a 15-ml centrifuge tube; add 2 ml of water; heat to a measured temperature of 90–95°C using boiling water until just opaque; add 2 ml of TCA acid; heat for 6 minutes with constant stirring at the same temperature; cool; centrifuge at 3500–4500 rpm for 20–30 minutes; decant the clear supernatant solution (extract 1).

B. To the residue in the same centrifuge tube add 1 ml of water, 1 ml of TCA acid, continue as in A (extract 2).

C. Repeat (B) (extract 3). Centrifuge, if necessary, the extracts 1, 2, and 3. Determine copper in the total of the three extracts [0.19584 *N* in TCA as in (D)].

D. Into a 25-ml flask, transfer (mixing after each addition) the copper-containing extracts (about 10 ml), about 10 ml of water, 2 ml of 0.2 *M* ascorbic acid, 3 ml of 0.05% FG, and water up to the mark. Measure the extinction against a blank containing the same acidity and reagent content at 555 μg. Compute the amount of copper from a standard curve prepared from standard solutions containing 1–8 μg of Cu/2 ml (50–400 μg of Cu/100 ml) using the same amounts of TCA acid and reagent.

Comparative procedure. For the sake of comparison, copper in 16 samples was determined using sodium diethyldithiocarbamate as recommended by Gubler *et al* (7) and the present reagent (procedure IA, B, C, and D).

Recovery procedure. For accurate confirmatory test of this method, add 2 ml of standard copper solution (2 μg of Cu) to a 2-ml serum sample. Determine the copper content following the above procedures. Calculate the percentage copper recovery.

Since FG is useful in weakly acid medium (5 ml 0.02 N HNO_3/10 ml, two additional sets of experiments were carried out following the above procedure using 0.02448 N TCA acid.

Experiments of the first set involved each, one extraction with the same amount of acid, while those of the second set involved three extractions as described before (procedures A, B, and C).

II-A. One-extraction procedure. Essentially proceed as in IA using a 5-ml sample (2.5–20 μg of Cu) and 4 ml of TCA acid as a whole for one extraction, dilute to 100 ml; into 10-ml measuring flask, pipet (mixing after each addition) 5 ml of the diluted extract (DE) followed by 2 ml of 0.2M ascorbic acid, 3 ml of 0.005% FG reagent; measure the extinction against a blank containing the same acidity and reagent content at 555 mμ, compute the amount of copper from a standard curve prepared from standard solutions containing 2.5–20 μg of Cu/5 ml (50 to 400 μg Cu/100 ml) using the same amounts of TCA acid and reagent.

Comparative procedure. For the sake of comparison copper in 17 serum samples was determined by both the diethyldithiocarbamate and the present procedure (IIA).

Recovery procedure. Add 5 ml of standard copper solution (10 μg of Cu) to a 5-ml serum sample; determine the copper content by following procedure IIA. Calculate the percentages of copper recovery.

II-B. Three-extraction procedure. Proceed essentially as under IA, B, and C but using a 5-ml serum sample expected to contain 2.5 to 20 μg of Cu, dilute the three extractions (DEs) and proceed as under (IIA), computing the amount of copper from a standard curve.

Comparative and recovery procedures were applied to 16 samples using the same three-extraction technique.

RESULTS AND DISCUSSION

Table 1 a lists the results of extinction measurements of 8 standard solutions covering the range 1 to 8 μg of Cu/25 ml, prepared from solutions containing amounts of copper equivalent to those expected in serum (50 to 400 μg/100 ml) with the sequence, 2 ml of 0.2 M ascorbic acid + 3 ml of 0.05% FG aqueous solution + water up to 25 ml. The corresponding extinction curve (not represented) was modified for optimum linearity using the straight-line equation $Y = ax + b$ where Y is extinction, a is slope regression = 0.015172, X is μg of Cu present

TABLE 1

EXTINCTION AND CORRECTED EXTINCTION VALUES OF STANDARD COPPER SOLUTIONS

X[a]		E[b]		Mean	Y[c]
		Ia. Using procedure I			
1	0.009	0.016	0.026	0.017	0.0154
2	0.020	0.022	0.032	0.025	0.0310
3	0.032	0.038	0.039	0.036	0.0460
4	0.064	0.054	0.067	0.063	0.0610
5	0.080	0.075	0.079	0.078	0.0760
6	0.085	0.083	0.095	0.088	0.0910
7	0.096	0.130	0.112	0.113	0.1070
8	0.112	0.148	0.126	0.128	0.1230
		Ib. Using procedure II-A and II-B			
X[d]	0.250	0.500	0.750	1.000	1.250
E_1[b]	0.007	0.0140	0.0200	0.0260	0.0300
E_2	0.009	0.170	0.0230	0.0290	0.0350
E_3	0.006	0.0130	0.0200	0.0270	0.0320
E_4	0.007	0.0140	0.0210	0.0260	0.0310
Mean	0.0073	0.0145	0.0210	0.0270	0.0320
Y[c]	0.0067	0.0137	0.0204	0.0272	0.0340

[a] X = μg of Cu/25 ml.
[b] E = extinction.
[c] Y = corrected extinction value.
[d] X = μg of Cu/10 ml.

in 25 ml, i.e., 2 ml of serum and b is the intersected part of the y axis = 0.000226.

By the aid of the present procedure (IA, B, C, and D) we analyzed 32 serum samples (Table 2, IIa) computing the copper/100 ml of serum either by aid of the standard curve or by applying the relation $X =$

TABLE 2

MICRODETERMINATION OF COPPER IN CAMEL SERUM WITH FG
Expressed as μg of Cu/100 ml of serum.

	IIa[a]	IIb	IIc
Nos.	32	17	16
Range	85.0–272.5	76.9–211.7	71.5–200.0
Mean	160.89	141.0	138.9
$S\pm$	9.68	7.67	11.35

[a] IIa, using procedure I; IIb, using procedure IIA (DE); IIc, using (DEs); S = standard error.

$[(Y - b)/a] \times 50$. The amounts of copper found covered the range 85 to 272 μg with a mean value of 160.89 ± 9.7 μg/100 ml. Results of determining copper in 16 serum samples (Table 3, IIIa) by the diethyldithiocarbamate and by the present method exhibited a significant difference amounting to 3.96 on the thumb rule. Table 4 a lists the results of the recovery test on 17 serum samples using FG procedure, exhibiting percentage recovery between 50.00 and 87.00 with a mean value of 72.01 ± 2.2. The above significant difference, as well as the low percentage recovery, may be attributed to the fact that the high acidity of 0.1958 *N* TCA acid leads to turbidity observed after 1 hour. It was therefore necessary to control conditions under which maximum accuracy could be achieved.

Hence we measured extinction of standards prepared with lower acid and FG concentrations with the sequence: 5 ml of DE (0.04 *N* in TCA acid and containing 0.250 to 1.250 μg of Cu) + 2 ml of 0.2 *M* ascorbic acid + 3 ml of 0.005% aqueous solution of FG). Corrected extinction values (Table 1, Ib) and the corresponding curve (not represented) were made using the same above straight-line equation where *a* amounted to 0.027287 and *b* to 0.00010525. The corresponding concentrations to the determined unknown extinction values were obtained (μg of Cu/100 ml of serum) by the aid of the relation $x = [(y - b)/a] \times 400$. We also determined copper in 17 camel serum samples (Table 2, IIb) where the amounts of copper ranged between 76.9 and 211.7 μg/100 ml of serum with the mean value of 141.0 + 7.67. For comparison we determined copper in the same 17 samples by the DEDTC method (Table 3, IIIb) where the values ranged between 100 and 172 with a mean level of 134.5 ± 5.6 μg of Cu/100 ml of serum. The difference between the two means is significant, amounting to 2.85 using the thumb rule. It was of interest to investigate the recovery percentage using one extraction. Table 4, IVb illustrates the range, mean, and standard error of recovery percentage which amounted to 94.21 ± 1.21%.

TABLE 3

MICRODETERMINATION OF COPPER IN CAMEL SERUM WITH DEDTC
Expressed as μg Cu/100 ml of serum.

	IIIa	IIIb	IIIc
Nos.	16	17	16
Range	70.4–147.6	100.00–172.0	100–172.0
Mean	112.45	134.55	134.5
S± [a]	8.1	5.6	5.6

[a] *S* = standard error.

TABLE 4

PERCENTAGE OF RECOVERED ADDED COPPER

	IVa	IVb	IVc
Nos.	17	17	17
Range	50.0–78.0	84.9–104.8	84.5–116.4
Mean	72.01	94.21	97.74
$S\pm$	2.2	1.21	1.79

[a] IVa, using procedure I; IVb, using procedure IIA; IVc, using procedure IIB.

The results of extinction measurements of standard solutions prepared following the sequence: 5 ml of DEs + 2 ml of 0.2 *M* ascorbic acid + 3 ml of 0.005% FG reagent, were essentially identical to those obtained using 5 ml of DE (Table 1b). Using the straight-line equation and the extinction values of unknowns, the corresponding concentrations were computed from the relation $x = [(y - b)/a] \times 400$. Copper in 17 camel serum samples (Table 2, IIc) was determined by the three-extraction technique using FG. Its content ranged between 71.5 and 200 μg with a mean value of 138.9 $\pm$ 11.35 μg of Cu/100 ml of serum. Comparison of results of the same 17 serum samples using DEDTC (Table 3, IIIc) showed a range between 100 and 172 with a mean value of 134.5 $\pm$ 1.59 μg of Cu/100 ml. The difference between the two mean values was insignificant, amounting to 1.59 using the thumb rule. Recovery percentage procedure applied to 17 serum samples gave results that ranged between 84.5 and 116.4 with a mean value 97.74 $\pm$ 1.79% as shown in Table 4, IVc.

It is thus established that FG can be successfully applied to the determination of copper in serum of camels using 20% TCA acid as the deproteinizing agent and as an extractant for this ion from the beta globulins using three extractions with 2, 1, and 1 ml for 5 ml of serum. No additives are required for neutralization or masking other constituents except for 2 ml of 0.2 *M* ascorbic acid for each determination. The less consumption of reagents, as well as the high reproducibility of results obtained by the present method (as shown by the complete concordance between results obtained by the one- and three-extraction techniques as applied to standards); and the direct production of color of a water-soluble complex recommends the use of Fast Grey as a colorimetric reagent for the accurate determination of copper in serum.

SUMMARY

The ortho–ortho hydroxy azo dye 15690–Solochromate Fast Grey RA, C.I. mordant black 15 (reddish navy-bluish black) has been used as a reagent for the successful estimation of copper in serum of camels. To determine copper, extract

5 ml of serum with 2, 1, and 1 ml of 20% trichloroacetic acid; dilute the combined extracts to 100 ml; to 5 ml of the diluted extracts add 2 ml of 0.2 *M* ascorbic acid and 3 ml 0.005% aqueous solution of the reagent; after complete development of the pink color of Cu–FG (15 minutes) measure the extinction at 555 mμ.

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On the Mechanism of Detecting Aliphatic Monocarboxylic Acids by Means of New Fuchsin in Thin-Layer Chromatography

JÓZEF ŠLIWIOK AND BOŻENA KOCJAN

Department of Or anic Chemistry, Silesian University, Katowice, Poland

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INTRODUCTION

Previous publicatior (*1, 3*) have described the visualizing properties of fuchsin dyes and the possibility of predicting the detectability of organic compounds in thin-layer chromatography by means of those dyes.

In this paper researches on the mechanism of detection of aliphatic monocarboxylic acids by means of new fuchsin have been presented.

EXPERIMENTAL AND DISCUSSION

The chromatographic procedure was as follows: The glass plates were covered with Kieselgel G, the thickness of a layer being 0.5 mm; then they were activated at 110° for 30 minutes. The mobile phase was composed of chloroform and acetone in a volume ratio of 96:4. On every chromatographic plate 22 μg of individual fatty acid was spotted. After the run, the chromatogram was developed with a 0.005% solution of new fuchsin in water. The colors of the chromatographic spots and the solubility of the mined acids in water is presented in Table 1.

As noted in our in estigations, the color of the chromatographic spots depends on the ydrophobic properties of the examined acids. The scheme in Fig. 1 illustrates this regularity.

The colors of the chromatographed spots of the acids when investigation result from the following two processes: The partial washing out of the visualized ubstance from the adsorbent and the fixing of the dye to that substance. If the process of washing out predominates, the obtained chromatographic spots are white. In the second case, i.e., when the dye fixation to the investigated substance is stronger, the spots obtained are violet-red. The color of the chromatographic spots is closely connected with the adsorption of the dye on the system

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TABLE 1

THE COLOR OF THE CHROMATOGRAPHIC SPOTS AND THE SOLUBILITY OF THE EXAMINED ACIDS IN WATER

Substance (acid)	Number of C atoms in molecule	Color of spot	Solubility (in g/100g H_2O) at 20° (2)
Caproic	6	white	0.968
Caprylic	8	white	0.068
Capric	10	white	0.015
Lauric	12	white	0.0055
Myristic	14	light violet	0.0020
Palmitic	16	violet-red	0.00072
Stearic	18	violet-red	0.00029
Arachidic	20	violet-red	—

chromatographic layer–aliphatic acid. The values of adsorption of new fuchsin on the a/m system in the case of the fattty acids investigated are presented in Fig. 2.

As shown in Fig. 2, the amount of dye adsorbed on the system silica gel–fatty acid differs for different acids. The higher the number of carbon atoms in the molecule of fatty acid, the greater the microgram amount of dye adsorbed on the system silica gel–fatty acid. In the case of the C_{14}–C_{20} acids, the adsorption is extremely high; the corresponding chromatographic spots are violet-red. The C_6–C_{12} acids give white chromatographic spots and in their case the adsorption is respectively lower. For the C_6 and C_8 acids the microgram amounts of new fuchsin adsorbed on the system silica gel–fatty acid are lower than those adsorbed on a pure layer.

On the basis of the experiments conducted one may conclude that the C_{14}–C_{20} acids occupy some special position on the adsorbent surface. Namely, their functional groups are adsorbed on the layer and the aliphatic chains are directed at some angle to the surface of

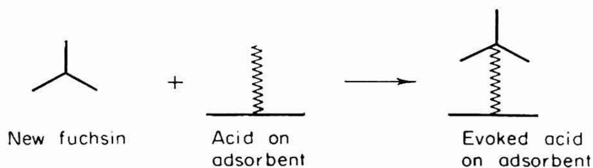


FIG. 1. The color of the chromatographic spots against the hydrophobic properties of the examined acids.

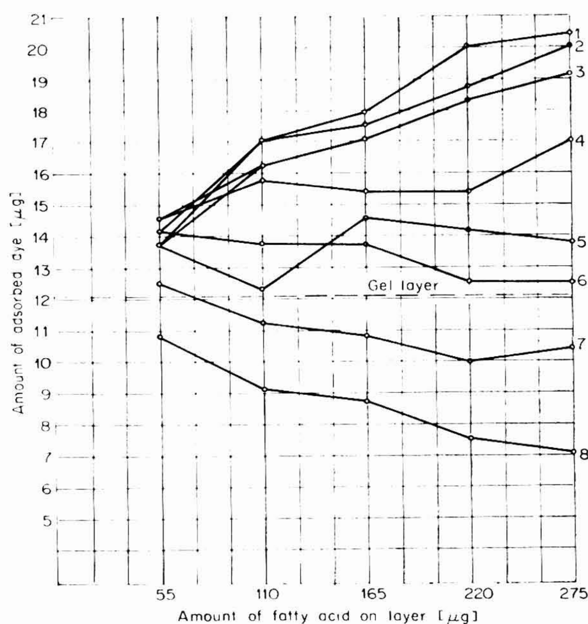
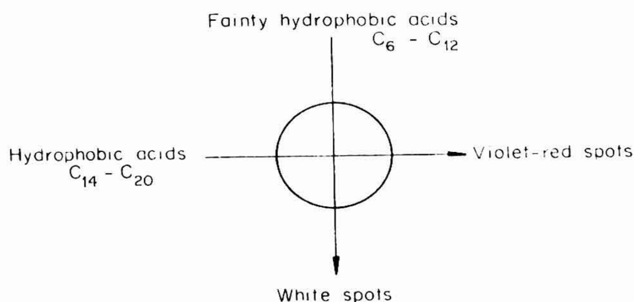


FIG. 2. The microgram values of new fuchsin adsorbed on the system adsorbent-fatty acid against the amounts of fatty acids: 1, Gel layer + arachidic acid; 2, gel layer + stearic acid; 3, gel layer + palmitic acid; 4, gel layer + myristic acid; 5, gel layer + lauric acid; 6, gel layer + capric acid; 7, gel layer + caprylic acid; 8, gel layer + caproic acid.

this layer. It gives an opportunity for the fuchsin dye to freely adsorb on the aliphatic hydrocarbon chains. The proposed scheme is as follows:



Because of the different adsorption values of new fuchsin on the system silica gel–fatty acid and pure silica gel (Fig. 2), the corresponding chromagraphic spots are violet-red.

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MICROCHEMICAL JOURNAL 17, 277–284 (1972)

On the Spectrophotometric Measurement of Methylene Blue

WOLFGANG J. KIRSTEN AND VINOD J. PATEL

Department of Chemistry, Royal Agricultural College of Sweden, S-750 07 Uppsala 7, Sweden

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INTRODUCTION

The spectrophotometric measurement of methylene blue is the final step in a number of analytical determinations, such as the determination of sulfide, sulfur, sulfate, based upon the methylene blue color reaction of sulfide. In other determinations methylene blue is reduced and the excess is measured. In many instances methylene blue is used for ion pair extraction and determination of different anions, and it is also used for the determination of the adsorptive activity of charcoal and other surface-active compounds.

MEASUREMENT IN AQUEOUS SOLUTION

In the commonly used St. Lorant (*1*) method for the determination of sulfate according to Johnson and Nishita (*2*) as modified by Gustafsson (*3*) the methylene blue is measured at 665 nm in a strongly acidic water solution. According to Lewis and Bigeleisen (*4*) the ion is not present in a homogenous state in such solutions, but the bridge nitrogen is partly ionized, which is indicated by the formation of a second absorption peak at 750 nm. This peak disappears completely when the solution is neutralized to pH 2, the pK_a value of HSO_4.

A second disadvantage of this measuring method is the association of methylene blue molecules in water solution, which causes a considerable deviation from Beer's law.

Večera (private communication) recommended the addition of alcohol to the methylene blue solution to eliminate the association, a procedure which is based upon an investigation of Rabinowitch and Epstein (*5*). This method is quite convenient, when not too diluted solutions are to be measured. It does not eliminate the association completely unless a rather large volume of alcohol is added. In our case a further dilution of the rather weak methylene blue solution was not desirable. Also we had to measure the absorption in cuvettes with

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a volume of 20 ml and with 20 cm light path. With the alcohol containing solutions the slightest change of temperature—such as caused by touching the cuvettes loosely with the fingers—caused the formation of schlieren, which obviated any accurate measurements in the long cuvettes. No such troubles were encountered with the ordinary water solutions.

Two other approaches were therefore made to solve the problem of association: It was hoped that an added compound with some structural resemblance with methylene blue might associate with the latter and thus separate the methylene blue molecules from each other. A large number of substances was tried, but only the addition of pyridine and a few of its homologues gave a sufficiently strong effect. Another approach was made to eliminate the association by ion pair formation. If the stability of an ion pair is sufficiently high already in water solution it could be expected that the ion pair formation would separate the methylene blue ions from each other. Many ion pair formations with methylene blue have been reported, and we tried many of them. Some of the complexes, such as those with fluoborate and perchlorate were not stable enough in water solution to eliminate the association, others, which were stable enough, were insoluble in water and precipitated. Only the tensides such as sodium dodecyl benzene sulfonate, Nansa, gave water-soluble ion pairs, which were stable enough to eliminate the association of methylene blue.

Reagents

Methylene blue, Merck Darmstadt 6040, was finely ground and mixed and samples of the mixture were weighed out and used for the determinations. All results were corrected for moisture content 19.7% and ash content 0.9%.

Sodium dodecyl benzene sulfonate, Nansa HS80/S, Albright and Wilson Ltd, Whitehaven, England. Active matter 80%, water 5% maximum, balance sodium sulfate. No corrections were made. All reported values are related to the commercial product.

Addition of Pyridine

Figure 1a shows the influence of the addition of pyridine upon the light absorption of a pure water solution of commercial methylene blue chloride. When 4 ml of pyridine were added to 100 ml of methylene blue solutions of different strengths the following molar absorbances were obtained:

4.83×10^{-5} M solution, 666 nm, 2 mm cuvette, absorbance 0.860, molar absorbance 89, 100.

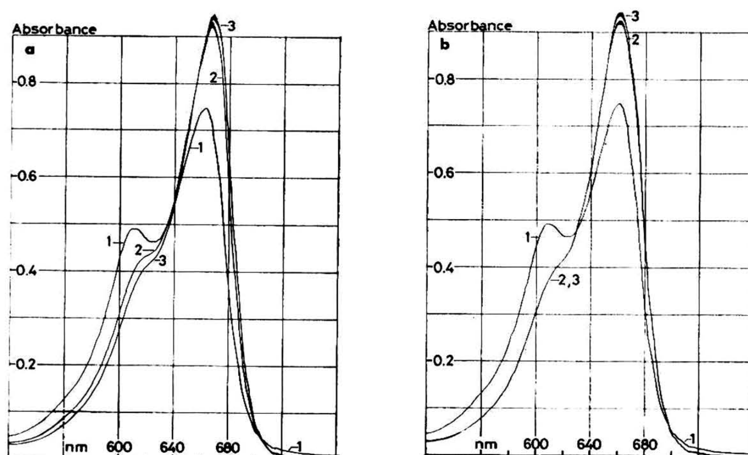

FIG. 1a. Influence of addition of pyridine to water solution of methylene blue. (1) No pyridine, (2) 4 ml, (3) 20 ml of pyridine in 100 ml of solution containing 15 mg of water-free methylene blue chloride/liter. Measurement in 2 mm cuvettes, Beckman DB. (b) Influence of addition of Nansa to water solution of methylene blue. (1) No Nansa, (2) 4 ml, (3) 20 ml of 10% Nansa in 100 ml of solution containing 15 mg of water-free methylene blue chloride/liter. Measurement in 2 mm cuvettes, Beckman DB.

9.65×10^{-7} M solution, 666 nm, 100 mm cuvette, absorbance 0.869, molar absorbance 90,000.

Addition of Nansa

The influence of the addition of Nansa solution upon the light absorption of a pure water solution of commercial methylene blue chloride is shown in Fig. 1b. When 4 ml of a 10% Nansa solution were added to 100 ml of methylene blue solutions of different strengths the following molar absorbances were obtained:

4.95×10^{-5} M solution, 662 nm, 2 mm cuvette, absorbance 0.882, molar absorbance 89,200.

9.89×10^{-7} M solution, 662 nm, 100 mm cuvette, absorbance 0.885, molar absorbance 89,500.

We tried also to measure the absorbance of the pure water solutions of methylene blue without pyridine or Nansa. We found it, however, impossible to obtain reliable measurements because of the strong adsorption of methylene blue upon the walls of all glass and quartz vessels used. As absorption values have been reported by several authors we made no further efforts to obtain our own results.

The fact that the molar absorbances with pyridine and with Nansa

and with strong and diluted solutions are equal within the experimental errors indicates that the association of the methylene blue molecules has been practically completely eliminated.

Addition of Pyridine and Nansa to the Sulfide Reagent Solution

In the sulfide determination procedure given by Johnsson and Nishita (*2*) as modified by Gustafsson (*3*) the methylene blue is obtained in a strongly acid solution. It appeared interesting to try the addition of pyridine and Nansa to this solution. A blank solution was, therefore, prepared and commercial methylene blue was added to it. The influence of the addition of pyridine resp Nansa is shown in Fig. 2a and b. The following molar absorbances were obtained with 4 ml of pyridine:

4.87×10^{-5} M solution, 665 nm, 2 mm cuvette, absorbance 0.683, molar absorbance 70,100.

9.74×10^{-7} M solution, 665 nm, 100 mm cuvette, absorbance 0.855, molar absorbance 87,800.

When 10 ml of pyridine were added instead of 4 ml the results were:

5.43×10^{-5} M solution, 667 nm, 2 mm cuvette, absorbance 0.938, molar absorbance 86,400.

10.86×10^{-7} M solution, 667 nm, 100 mm cuvette, absorbance 0.975, molar absorbance 89,800.

Four milliliters of a 7.5% solution of EDTA disodium salt had to be

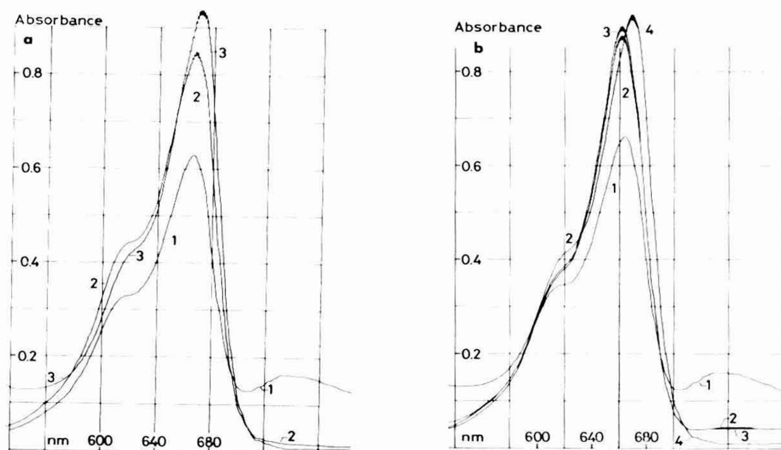

Fig. 2a. Influence of addition of pyridine to methylene blue in sulfide reagent solution. 1: no pyridine, 2: 4 ml, 3: 20 ml in 100 ml of solution. Conditions as in figure 1. (b) Influence of addition of Nansa in Nansa and pyridine to methylene blue in sulfide reagent solution. (1) No Nansa, (2) 4 ml, (3) 20 ml of 10% Nansa solution, (4) 20 ml of pyridine and 4 ml of Nansa, all in 100 ml of methylene blue solution. Conditions as in Fig. 1.

incorporated into 100 of the solutions with 10 ml of pyridine to avoid the precipitation of iron from the reagent.

Obviously the first thing that happens on addition of pyridine is a neutralization of the solution, which eliminates the ionization of the bridge nitrogen of methylene blue. However, even with 10 ml of pyridine the association of methylene blue is not completely eliminated.

When Nansa was added to the solutions precipitations occurred after a short time even when EDTA was incorporated and even when both Nansa, EDTA and pyridine were added. In the meantime a paper by Rees, Gyllenspetz, and Docherty (*6*) on the use of *N,N*-diethyl-*p*-phenylenediamine for sulfide determination appeared. This method appeared very promising, and the experiments to apply the Nansa procedure to the methylene blue sulfide determination method were, therefore, discontinued and the efforts were concentrated on the new method. Incorporation of 4 ml of pyridine into 100 ml of the methylene blue measuring solution has, however, been used in our routine work during about 1 year with good results.

Also in the new procedure (*6*) pyridine and Nansa were found to eliminate the association and improve the results considerably. A paper will be published soon.

MEASUREMENT IN ORGANIC SOLVENT

Extraction of methylene blue as an ion pair is widely used for the determination of many anions. Chloroform is usually used as the solvent and exhaustive extraction is used. Several authors recommend 1,1,2,2-tetrachloroethane.

In the determination of sulfide by the methylene blue method a rather large volume of weakly colored solution is obtained. It appeared possible to increase the sensitivity of the measurement by extracting the methylene blue into a small volume of organic solvent.

The boiling point of tetrachloroethane is 146.5°C compared with 61.2°C for chloroform, and its solubility in water is reported to be 0.2% by volume compared to 0.5% for chloroform. In qualitative experiments with extraction of methylene blue perchlorate it was found that tetrachloroethane gave a much more complete extraction than chloroform. Even when quite strong solutions of methylene blue were extracted, no traces of color could be detected in the aqueous phase. We decided, therefore, to try to work out a method for the measurement of methylene blue after sulfide determination based upon extraction of its perchlorate into a small, accurately measured volume of tetrachloroethane and measurement of the light absorption of an aliquot part of the organic phase.

We found that it was extremely difficult to obtain a clear organic layer after the extraction. Even after strong centrifugation a thin fog remained. When 5% by volume of 2-octanol was added this difficulty disappeared. The solubility of 2-octanol in water is 0.1% by volume and the boiling point is 178.5°C.

The following method was tried.

Reagents

Tetrachloroethane solution: Mix 95 ml of 1,1,2,2-tetrachloroethane with 5 ml of *n*-octanol-2.

Magnesium perchlorate solution: Dissolve 20 g of anhydrous magnesium perchlorate in water and dilute to 100 ml.

Procedure

Develop color in sulfide determination method according to Gustafsson (*3*) using 30 ml flasks, but do not make up the solution to the mark. Allow to stand for 1 hour. Add 2.00 ml of tetrachloroethane solution and 1.5 ml of magnesium perchlorate solution. Shake violently during 30 seconds. Allow phases to separate. Pour away most of the water phase and pour the rest of both phases into a centrifuge tube. Cool in fountain water or ice water for a short while and centrifuge during 1 minute at 3000 rpm. Measure the light absorbance of the organic phase at 660 nm, either directly in the centrifuge tube or after pipetting it into a cuvette.

RESULTS

In order to evaluate the extraction procedure a number of extractions of commercial methylene blue from sulfide blank solution were made. The results are shown in Fig. 3. The molar absorbance of a 5.6×10^{-6} M solution in the organic solvent, corrected for the solubility of the solvent in water was found to be about 80,000. The calibration curve does not follow Beer's law.

A series of analyses was then run. Unfortunately the organic solvent destroyed our ultramicrocuvettes, which were cemented with epoxy resin. The determination had, therefore, to be carried out with larger volumes, and the ultimate sensitivity of the method could not be reached. Between 30 and 230 μg of a standard substance—dimer dithioacetylacetone dissolved in paraffin containing 0.563% of sulfur—were weighed out and analyzed by tube combustion and subsequent hydrogenation (*7*). The sulfide was absorbed in 100 ml flasks and the color developed according to Gustafsson (*3*). The methylene blue was then extracted with 5 ml of the organic solvent and measured in 4 cm microcuvettes.

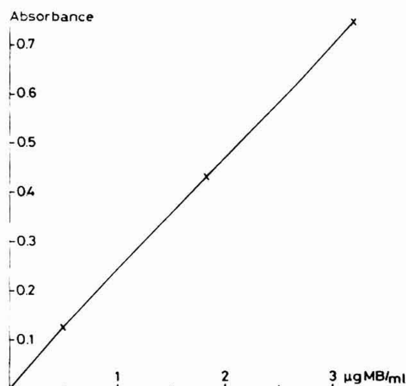

FIG. 3. Calibration curve: Commercial methylene blue extracted from blank solution from sulfide determination. 1 cm cuvette, 660 nm; solvent, 1,1,2,2-tetrachloroethane containing 5% of *n*-octanol-2.

The curve obtained is shown in Fig. 4. The blank point on the curve represents 4 blank determinations which gave the same results. The molar absorbance per atom of sulfur in the samples calculated from the curve and corrected for the solubility of the organic solvent is about 57,000.

The blank obtained in the method is low enough to allow much more sensitive measurements, and such measurements will be made as soon as we have obtained new ultramicrocuvettes.

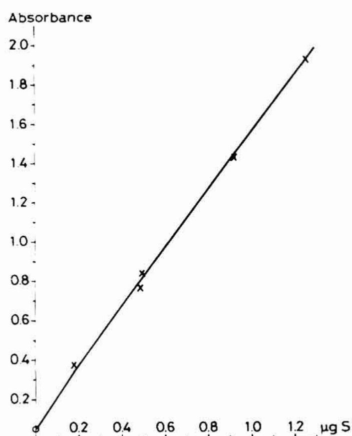

FIG. 4. Calibration curve: Sulfur determination by combustion and subsequent hydrogenation (*7*) and measurement of sulfide as methylene blue extracted into organic solvent. 4 cm cuvette, 660 nm. Blank point represents 4 runs.

SUMMARY

The association of methylene blue in water solution can be eliminated by the addition of pyridine or dodecylbenzenesulfonate. The absorbance follows then Beer's law up to at least 5×10^{-5} M solutions, absorbance 0.891 in a cuvette with 2 mm light path.

From the methylene blue solution obtained in the sulfur determination method according to Johnson and Nishita–Gustafsson [Johnson, C. M., and Nishita, H., *Anal. Chem.* **24**, 736–742 (1952); Gustafsson, L., *Talanta* **4**, 227–235 (1960)] the dye can be extracted as the ion pair with perchlorate and concentrated into a small volume of tetrachloroethane–octanol solvent, which makes it possible to determine fractions of micrograms of sulfur.

ACKNOWLEDGMENTS

The authors are indebted to Olle Pettersson and Malin Åkerblom for a part of the technical work with the extraction method. The work was made possible by grants from the Swedish Medical Research Council and the Swedish Natural Science Research Council.

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MICROCHEMICAL JOURNAL 17, 285–292 (1972)

The Microdetermination of Stannous Tin with N–Bromosuccinimide

M. Z. BARAKAT AND SARWAT I. DOWEIDAR

Biochemistry Department, Faculty of Medicine, Azhar University, Madina Nasr, Cairo, U.A.R.

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INTRODUCTION

The reducing properties and uses of stannous tin are remarkable. For example, stannous chloride is used in laboratories and in the industry wherever a high degree of purity is required (*2*). Hence the determination of stannous tin is of practical interest.

For quantitative determination the tin must be present in the stannous condition. Previous titrimetric methods with standard potassium iodate, chloramine T, or iodine solution recommend the determination on such amounts as 0.4 or 0.5 g of $Sn\ Cl_2,\ 2H_2O$; otherwise the error increases with lower concentrations of stannous tin (*6*). Furthermore, volumetric method of determining tin in food by reduction and iodimetric titration was rejected on the ground of the probable interference by ferrous ions (*7*). In addition, the effect of foreign substances is not discussed in titration with chloramine T (*4*).

The present work describes a method for the microdetermination of stannous tin in quantities as low as 100 μg, involving the use of standard *N*- bromosuccinimide.

MATERIALS AND METHODS

Equipment and Reagents.

1. Microburette of 5-ml capacity graduated to 0.01 ml.
2. Graduated pipettes of 1-, 2-, 5-, and 10-ml capacity.
3. Volumetric flasks of 50- and 100-ml capacity.
4. Standard *N*-bromosuccinimide solution, e.g., 0.1 and 0.01% (w/v), aqueous.
5. Methyl red solution 0.04% (w/v), alcoholic.
6. Potassium bromide solution 10% (w/v), aqueous.
7. Concentrated and dilute hydrochloric acid 10% (v/v).

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Reaction Between N-Bromosuccinimide and Stannous Chloride in Acid Medium

A 1.12825 g portion of stannous chloride $SnCl_2$, $2H_2O$ (0.005 mole) was dissolved in 30 ml of concentrated hydrochloric acid and diluted with 10 ml of distilled water. Then 0.89 g of *N*-bromosuccinimide (0.005 mole) was suspended in 10 ml of distilled water and gradually added with shaking to the stannous chloride solution.

The solution was then divided into two equal portions (25 ml). One portion was used to detect stannic tin and hydrogen bromide. Two recognized tests for stannic compounds (*5*) were positive as listed below.

1. A total of 15 ml of the solution was treated with hydrogen sulfide. A yellow precipitate of stannic sulfide was deposited. The precipitate was soluble in concentrated hydrochloric acid (distinction from arsenious sulfide) and also in colorless and yellow ammonium sulfide.
2. To 5 ml of the solution was added 5 ml of 5% mercuric chloride solution. No precipitate appeared (difference from stannous compounds).

The presence of hydrogen bromide was confirmed by treating 5 ml of the solution with 2 ml of dilute nitric acid and 3 ml of 10% silver nitrate solution. A yellowish white precipitate of silver bromide was formed.

The other portion of the solution (25 ml) was evaporated to dryness and the solid residue was recrystallized from hot water giving colorless crystals (mp$_3$ 189°C) which proved to be succinic acid by melting-point and mixed melting-point determinations with an authentic sample.

Validity of the Reaction for Quantitative Determination

Before applying the reaction between *N*-bromosuccinimide and stannous chloride in acid medium for determination, it was decided to verify its validity from a quantitative point of view.

An accurately measured volume, e.g., 5 ml of solution containing 0.22565 g (1 mmole) of stannous chloride, in 25 ml of concentrated hydrochloric acid per 100 ml, was placed in a 100-ml conical flask. An equal volume of 10% potassium bromide solution and 2 drops of methyl red indicator were added. A 0.178% (1 mmole) or 0.356% (2 mmole) *N*-bromosuccinimide solution (w/v), respectively, was run in dropwise from a microburette with thorough shaking until the red color of the indicator just disappeared and the titer used was noted. It was found that the reaction was stoichiometric in acid medium at room temperature; the results were:

Volume of stannous chloride solution (1 mmole/100 ml) taken (ml):

10 5 4 3 2 1

Titer of N-bromosuccinimide solution (1 mmole/100 ml) used (ml):

10.1 5 4.1 3 2 1

Titer of N-bromosuccinimide solution (2 mmole/100 ml) used (ml):

5.02 2.52 2 1.5 1 0.51

Procedure

To an accurately measured volume, e.g., 5 ml of stannous solution in a 100-ml conical flask, add an equal volume of 10% potassium bromide, 10 ml of dilute hydrochloric acid, and 2 drops of methyl red indicator. Titrate the mixture with a 0.1 or 0.01% (w/v) *N-bromosuccinimide* solution added gradually from a microburette with continuous shaking. When the red color of the indicator fades add another drop of the indicator and then add the N-bromosuccinimide solution until the red color of the indicator is just discharged. This is the end point, and record the volume of the titer. A blank experiment is done at the same time and the reading is subtracted from the titer before calculation. Calculate the stannous tin or stannous chloride content of the sample solution as follows:

$$\text{Stannous tin present (mg or } \mu\text{g)} = V \times C \times (118.7/178),$$

$$\text{Stannous chloride present (mg or } \mu\text{g)} = V \times C \times (225.65/178),$$

where V is the titer of N-bromosuccinimide in milliliters, and C is the concentration of the N-bromosuccinimide solution in milligrams or micrograms per milliliter.

Determination of Stannous Tin

A stock solution containing 100 mg of stannous tin was prepared by dissolving 0.1901 g of stannous chloride ($SnCl_2$, $2H_2O$) in 25 ml of concentrated hydrochloric acid and the volume was completed to 100 ml with distilled water in a volumetric flask.

The stock solution was also diluted 10 times with dilute hydrochloric acid in a volumetric flask. Various volumes of the 0.1 and 0.01% stannous tin solution were determined by the proposed method as if they were unknowns with 0.1% or 0.01% (w/v) N-bromosuccinimide solution, respectively. The results are shown in Table 1.

Comparative Analysis of Tin Sheets by the Proposed Method and Iodine Method

A 250-mg portion of tin sheet was dissolved in 30 ml of concentrated hydrochloric acid and completed to 100 ml with distilled water in a standard flask so that 1 mg of the solution was equivalent to 2.5 mg of tin sheet.

Various known volumes of the tin solution were titrated simultane-

TABLE 1

RECOVERY OF STANNOUS TIN BY THE PROPOSED METHOD

Vol. of solution taken (ml)	Stannous content (mg)	Titer of 0.1% (w/v) N-bromo-succinimide solution [a] (ml)	Stannous found (mg)	Error (%)
10	10	15.15	10.10	1.00
9	9	13.55	9.04	0.44
8	8	12.00	8.00	—
7	7	10.55	7.04	0.57
6	6	9.00	6.00	—
5	5	7.50	5.00	—
4	4	6.00	4.00	—
3	3	4.50	3.00	—
2	2	3.05	2.03	1.50
1	1	1.50	1.00	—
Dilution 1 × 10				
	(μg)	0.01% [b] (ml)	(μg)	
10	1000	14.80	987	1.30
9	900	13.40	894	0.67
8	800	11.90	794	0.75
7	700	10.40	694	0.86
6	600	9.00	600	—
5	500	7.40	494	1.20
4	400	6.00	400	—
3	300	4.50	300	—
2	200	3.00	200	—
1	100	1.50	100	—

[a] One ml of 0.1% *N*-bromosuccinimide ≡ 0.6669 mg of stannous tin.
[b] One ml of 0.01% *N*-bromosuccinimide ≡ 66.69 μg of stannous tin.

ously as if they were unknowns with 0.1% (w/v) *N*-bromosuccinimide solution and 0.01 *N* iodine, respectively, to determine the stannous tin content. The results are recorded in Table 2.

Interfering Substances

Ferrous ions do not interfere with the proposed method but cuprous ions interfere.

Application of the Proposed Method

The proposed method was applied for the determination of tin rods and tin foils available on the market for use in making soft solders and for tin plating giving reproducible results.

A 100-mg portion of the tin sample was dissolved in 25 ml of concentrated hydrochloric acid and completed with distilled water to 100 ml in a volumetric flask. Various known volumes were titrated according to the proposed method and then the percentage of tin in the sample was calculated. The determination of the same sample was done simultaneously by the iodine method. The results are listed in Table 3.

Furthermore, the determination of tin in canned food, e.g., tomato sauce was done by the proposed method giving satisfactory results.

The whole canned sample under investigation was weighed and then dried at 105 °C in an electric oven. The solid residue was digested with concentrated sulfuric acid (10 ml) and potassium sulfate (0.1 g) as in the ordinary Kjeldahl process for nitrogen with the formation of a soluble stannic sulfate. The cold solution was diluted to 50 ml with distilled water. The stannic solution was reduced to stannous tin by boiling with 2 *N* hydrochloric acid (50 ml) and nickel (2 g) in an atmosphere of CO_2. The nickel used was given a preliminary etching by boiling with 6 *N* HCl for 10 minutes. Then the solution was allowed to cool under CO_2 pressure. The solution was filtered and completed with distilled water to 100 ml in a volumetric flask. A known volume (e.g. 25 or 50 ml) of the stannous solution was determined by the proposed method and thus the amount of tin in the whole sample of tomato sauce was easily calculated and expressed in parts per million (ppm).

TABLE 2

COMPARATIVE ANALYSIS OF TIN SHEETS BY THE PROPOSED METHOD AND THE IODINE METHOD

Content (mg)	Sn^{2+} found by the proposed method (mg)	Found (%)	Sn^{2+} found by the iodine method [a] (mg)	Found (%)
25.0	21.81	87.24	21.72	86.88
12.5	10.87	86.96	10.86	86.88
10.0	8.74	87.40	8.67	86.70
7.5	6.50	86.67	6.53	87.07
5.0	4.33	86.60	4.33	86.60
2.5	2.17	86.80	2.17	86.80
Av		86.95		86.82

[a] One ml of 0.01 *N* iodine ≡ 0.5935 mg of stannous tin.

TABLE 3

COMPARATIVE ANALYSIS OF TIN RODS AND FOILS BY THE PROPOSED METHOD AND THE IODINE METHOD

Sample	Content (mg)	Sn^{2+} found by the proposed method (mg)	Found (%)	Sn^{2+} found by the iodine method (mg)	Found (%)
Tin rods	10	3.47	34.70	3.62	36.20
	5	1.73	34.60	1.78	35.60
	4	1.33	33.25	1.42	35.50
	3	1.00	33.33	1.07	35.67
	2	0.67	33.50	0.71	35.50
	1	0.33	33.00	0.36	36.00
Av			33.73		35.75
Tin foils	10	7.40	74.00	7.72	77.20
	5	3.67	73.40	3.86	77.20
	4	2.93	73.25	3.09	77.25
	3	2.20	73.33	2.31	77.00
	2	1.47	73.50	1.54	77.00
	1	0.73	73.00	0.77	77.00
Av			73.41		77.11

Five samples of canned tomato sauce available on the market for human consumption, each of which weighed 170 g, were analyzed for tin as mentioned above and showed such values as 104, 141, 145, 147, and 191 ppm, respectively.

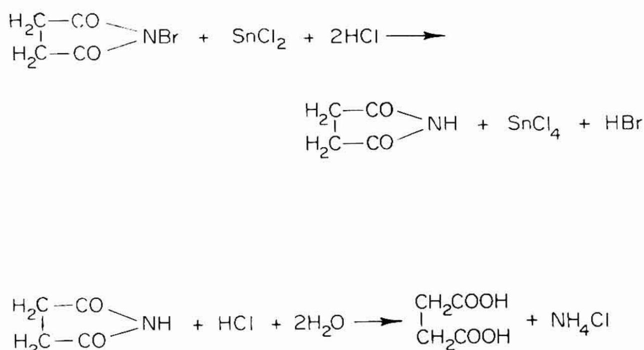
DISCUSSION

The iodimetric titration of stannous tin is subject to a variable error due to the presence of dissolved oxygen in the iodine solution. The error may be as high as 1% for 0.1 *N* iodine and 10 per cent for 0.01 *N* iodine (6).

The proposed method is based on the fact that *N*-bromosuccinimide oxidizes readily and quantitatively stannous tin, e.g., stannous chloride in the presence of dilute hydrochloric acid when methyl red is used as the indicator. The end point is reached when the red color of the indicator just disappears.

The mechanism of the oxidation of stannous tin by *N*-bromosuccinimide in the presence of dilute hydrochloric acid has been established

on the basis of the accumulating data and is shown by the following equations:



Previously the oxidizing action of *N*-bromosuccinimide was reported (*1*). The formation of stannic tin was confirmed by recognized tests and succinic acid was isolated. Also hydrogen bromide was identified.

The advanced method is simple, rapid, and sensitive to determine concentrations as low as 100 μg of stannous tin. The experimental error does not exceed $\pm 2\%$ (Table 1).

Comparative analysis of tin sheets, tin rods, and tin foils was simultaneously done by the proposed method and the commonly used iodine method. The average percentage content of tin sheets, i.e., 86.82%, is slightly lower when determined by the iodine method than by the proposed method, i.e., 86.95% (Table 2). Lower results obtained by the iodine method may be due to the presence of dissolved oxygen in the iodine solution (*6*).

The tin content of typical tin alloys such as plumber's solder is 33.3%; whereas that of beaning metals is 75% (*3*). It is obvious that the average content of tin rods is 33.73% when determined by the proposed method. This finding is lower than the average content of 35.75% obtained by the iodine method. Similarly the average content of tin foils is 77.11% by the iodine method which is higher than the average value of 73.41% obtained by the proposed method (Table 3).

Higher results of tin content obtained by the iodine method may be attributed to partial loss of iodine by volatilization particularly in hot weather (*1*).

Furthermore, the determination of tin in 5 samples of canned tomato sauce by the proposed method has shown that the tin content ranges from 104 to 191 ppm. Previously Buchanan (1908) examined many canned foods of all kinds and found that most of them contained less

than 286 ppm. He suggested the adoption of this value as a maximum limit for administration purposes (7).

SUMMARY

A new titrimetric method for the microdetermination of stannous tin, e.g., stannous chloride is proposed. The mechanism of the reaction in the presence of dilute hydrochloric acid is discussed. The determination of stannous tin is done on concentrations ranging from 10 mg to 100 μg. The experimental error does not exceed $\pm 2\%$. Comparative analysis of tin sheets, rods, and foils by the proposed method and the iodine method is reported. The method has been shown to be suitable for the determination of tin in canned foods.

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MICROCHEMICAL JOURNAL 17, 293–301 (1972)

Accelerated Automated Microdetermination of Serum Calcium [a]

E. S. BAGINSKI, S. S. MARIE, W. L. CLARK, J. A. SALANCY, AND B. ZAK

Departments of Pathology, St. Joseph Mercy Hospital, Pontiac, Michigan, Holy Cross Hospital, Detroit General Hospital and Wayne State University School of Medicine, Detroit, Michigan

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INTRODUCTION

It should be of value if automated on-stream spectrophotometric systems commonly used for clinical chemistry determinations (autoanalyzers) were capable of operating at greater speeds than at present without significant alteration in the characteristics of the mechanical part of the instrumentation. As an example of how this could be accomplished, a system was devised which used the determination of calcium with *o*-cresolphthalein complexone as a model for an accelerated nondialyzing single-channel system. The manifold of a single-channel procedure established to operate at the rate of 50 samples/hour was modified in such a way as to enable one to process 100 samples/hour. Some characteristics of this more rapid procedure were then evaluated for reproducibility, sample-to-sample interaction, recovery of known values and percentage of continuous draw which some call the steady state. In addition, the procedure was also adapted to the Beckman DSA 560, a discrete sample analyzer, which processed samples at the rate of 120 tests/hour. The latter system is similar in nature to a hand method because both handle samples by aliquots in a discrete manner.

REAGENTS

Cresolphthalein complexone solution. Transfer 100 mg of cresolphthalein complexone (CPC) into a 1 liter volumetric flask containing 360 ml of dimethyl sulfoxide. Shake the mixture until it is dissolved, then add 16 ml of concentrated HCl and 2.5 g of 8-hydroxyquinoline (8-HQ). When solution is complete, dilute to the mark with metal-free H_2O. Add ½ ml of Brij 35 solution/per liter of reagent.

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Diethylamine reagent. Transfer 38.0 ml of diethylamine into a 1-liter volumetric flask containing several hundred milliliters of metal-free H_2O. Add 0.5 g of KCN, shake to effect solution and dilute to the mark with metal-free H_2O.

Standards. Transfer 250 mg of dried and dessicated anhydrous $CaCO_3$ into a 1 dl volumetric flask containing some H_2O. Add concentrated HCl dropwise to effect solution, then dilute to the mark of the flask with metal-free H_2O. Alternatively, commercial atomic absorption standards similarly prepared can be used.

Calcium working standards. Dilute 0.0, 5.0, 10.0 and 15.0 ml of the calcium stock standard to 1 dl with metal-free H_2O.

PROCEDURE

Figure 1 shows the simple schematic of the manifold for calcium and the autoanalyzer modules used. Samples were aspirated at the rate of 100/hour at a 2:1 ratio of sample to wash. The sample was introduced into an air bubble segmented stream of acid CPC reagent and passed through a mixing coil. This solution was then reacted with

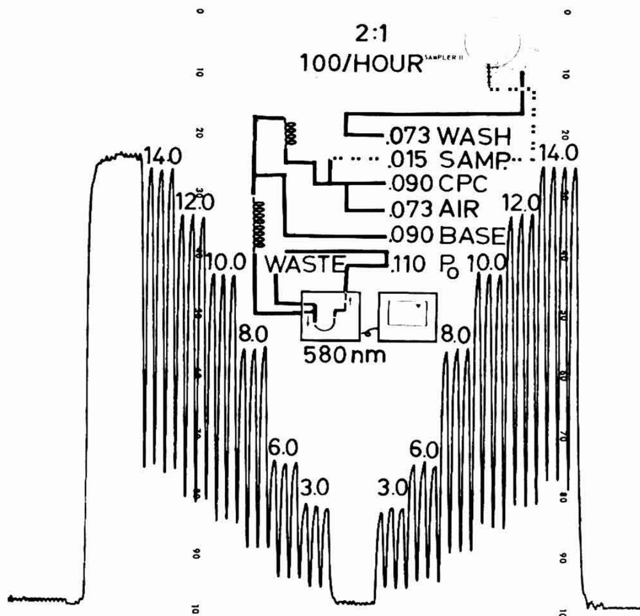

FIG. 1. Shows the manifold used for the automated system at 100 samples per hour. Replication of standards from high to low and low to high is also graphed with a steady state recording shown at upper left.

an alkaline buffer which caused the calcium to form a colored complex with the CPC. The presence of 8-HQ eliminated interference from magnesium while cyanide tied up trace metals such as copper, iron and zinc (*1–3*). The final stream was debubbled, passed through the colorimeter and the absorbance was determined at 580 nm before discarding it to waste. Dimethyl sulfoxide was included in the reagents to lower the dielectric constant of the medium and to aid in the repression of ionization of cresolphthalein complexone. The use of ethanol and methanol for a similar purpose in titrimetric procedures was previously described (5).

The procedure for the Beckman DSA 560 discrete sample analyzer is outlined in tabular form below for a transport rate of 120/hour.

	Sample pump		Reagent pump I		Reagent Pump II	
Pump capacity	50	250	500	500	500	500
Volume used	20	250	400	400	400	400
% Volume	40	100	80	80	80	80

The system used is virtually identical to the one described for the autoanalyzer with the exception that samples and reagents are handled and transported in discrete aliquots in a manner similar to a hand method rather than by injecting discrete samples intermittently into a continuous moving stream of reagent. Twenty microliters of sample was delivered by the first dual cylinder pump followed by a pump wash out of 250 μl of H_2O. CPC was added by the second pump with an equal volume of 400 μl of H_2O for this pump's washout. The same volume of base reagent was then added with its 400 μl of H_2O washout. The final color was measured at 575 nm against a reagent blank.

DISCUSSION

The theoretical aspect considered here was essentially a simple one. The optimized manifold characteristics of a continuous-flow spectrophotometric system can usually be established from a simple manual procedure especially when the reaction can be carried out directly on serum. If one enlarged all reagent lines and the sample line by a factor representing the ratio of faster to slower rates of determination, and in addition if one also sampled at a rate representing that ratio, i.e., 100/hour rather than 50/hour, then the actual sample size would be exactly the same at the two rates and the same amount of reagents would have been used per sample. Thus the reagents and the sample would be flowing twice as fast, but twice as many samples would be processed and the rate of determination would be doubled. If more time was needed for completion of reaction, a delay coil could be added before

the absorbance of the solution was measured by the photometer. The delay coil would lengthen the time required for sample reaction before the latter entered the colorimeter, but the rate of processing would still be determined by the rate at which the sample cam was set. However, an extra delay coil if used would represent part of the initial dead time before sample values are recorded, and it would not increase the dead time of the faster manifold over the slower manifold. In the described procedure, the dead time is approximately 3 minutes between drawing the sample to its complete recording. This represents more than a factor of two in dead time over the single channel dialysis system (*2–3*) with which it was compared.

The diameters of all manifold lines were increased so that the volumes of reagents and samples pumped were approximately double what they were when an optimized manifold was designed for a 50/hour rate. However, the time of sampling was changed from 48 to 24 seconds and the reagent washout time was halved to 12 seconds. The original procedure established at a 50/hour rate with a 2:1 cam, pumped the sample at 0.05 ml/minutes and reagents at 3.2 ml/minute for 48 seconds (24-second washout). This allowed approximately 0.04 ml of sample to be added to approximately 2.6 ml of reagents. When the sampling rate was doubled to 0.1 ml/minute, the reagent pumping rate to 5.8 ml/minute and the time of aspiration changed to 24 seconds (12-second washout), then approximately 0.04 ml was added to approximately 2.3 ml of reagents and the main change was the speed at which the liquids were traveling through the system. Although the ratios of sample to reagent in the two cases were not exactly the same, they were sufficiently close so that the results obtained were essentially the same. Lack of selection of tubes of all varied sizes makes it difficult to prepare a manifold which is exactly two times the size of the smaller manifold in terms of liquid drawn by each tube of the manifold. This fact, along with the change in response time of the recorder to the signals presented may account in part for apparent differences in sensitivity for the fast versus the slow manifold as well as the apparent difference in percentage of steady state. In this case the time of response to a given signal level when using the faster system is exactly half that of the slower system. Another factor to consider is that the volumes of the Tygon tubes are not precise and the values stated by the manufacturer are only approximate. Replication at 100/hour is described in the figure by triplicate determinations going from high to low and low to high values in the recordings.

Figure 2 represents the recordings obtained under several circumstances. The plateaus labeled CD represent 3 and 14 mg/dl for aqueous

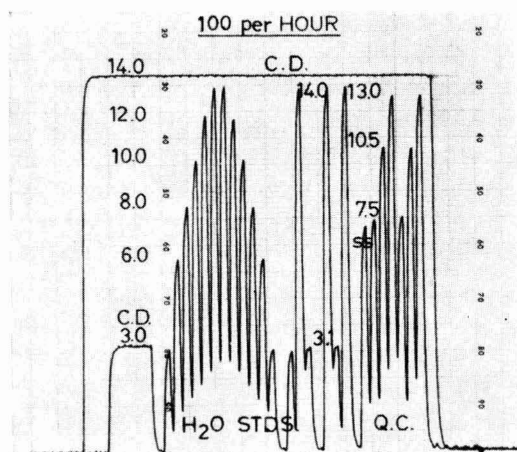

FIG. 2. Calibration and quality control standards with continuous drawings for 3.0 and 14.0 mg per dl. The minimal interaction of low and high standards is described in this graph.

standards continuously drawn to a constant absorbance. The 14 mg/dl CD reading of the 100/hour system was 0.545 while the peak obtained for 14 mg/dl was 0.530. This represents a figure of 97.5% of the continuous draw. The extent of interaction is seen to be small as a 3.0 mg/dl standard calculates at 3.1 mg/dl when it followed a 14.0 mg/dl standard. In similar manner the 14.0 mg/dl was not diluted by the 3.0 mg/dl standard immediately preceding it. In the quality controls (QC) shown at the right of the figure, a deliberately drawn short sample (SS) peaked a little lower (7.3 mg/dl) than the 7.5 mg/dl for the same QC sample adjacent to its right. The replication of the H_2O standards of the same figure ranging from 3.0 to 14.0 mg/dl while going up then down in concentration appears to be quite satisfactory.

Figure 3 shows the calibration characteristics of the 100/hour undialyzed system *vs.* the 50/hour undialyzed system. The width of the peaks has been halved and the apparent sensitivity decreased to a small extent. The continuous draw curves for both speeds are also shown. Obviously, except for the small decrease in sensitivity, there is really quite little to choose between them. The width of the peaks can be increased by changing the speed at which the recorder turns. The advantage of graphing a wider peak is for the occasional case of the accidentally drawn short sample (SS) shown in Fig. 2 which is usually graphically indicated by a more pointed peak than is obtained with the proper length of sample or standard. When all peaks are very sharp as shown here, the short sample is more difficult to identify since it

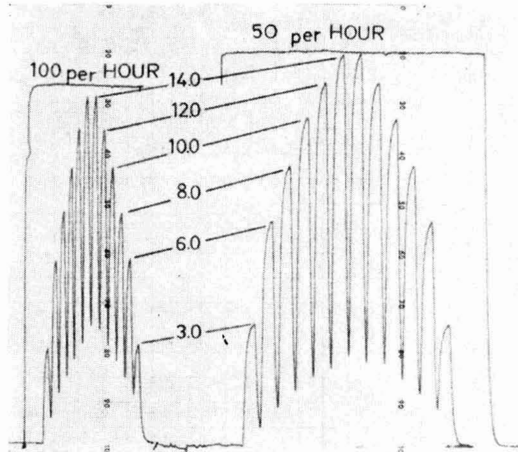

FIG. 3. Shows the calibration standards determined at 50 per hour(right) and 100 per hour(left) along with their steady state patterns graphed above them for the 14.0 mg per dl standard.

looks like the normal graphs of the run. However, since only 40 μl of sample are aspirated at the 100/hour rate, ensuring that the sample cup contains enough serum to obviate a short sample should be relatively easy.

Figure 4 shows the graphic results obtained with aqueous standards (3.0–14.0 mg/dl) and quality controls (7.5–13.0 mg/dl) repeated several times. Again a deliberately drawn short sample in the last

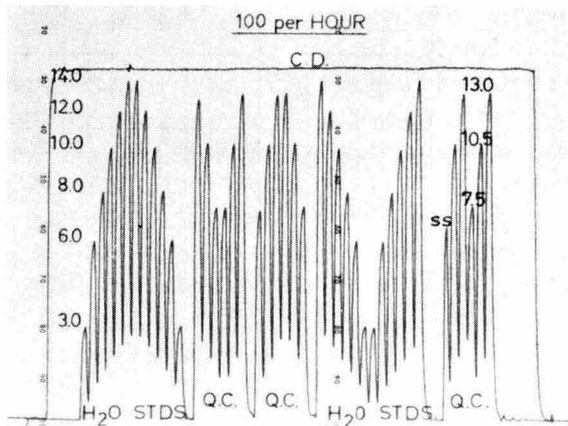

FIG. 4. Shows repetitive graphs for both Quality control standards and aqueous standards along with the steady state drawing of a 14.0 mg per dl aqueous standard.

quality control batch at the right of the graph shows a value of 6.7 rather than 7.5 mg/dl, an error of approximately 10%. The absorbances obtained for aqueous standards is the same as for proteinaceous standards. This simplifies calibration and eliminates the need to add protein to the standard. Recently, it was alleged that proteins were implicated as the cause for increased dialysis of calcium as a Donnan membrane effect (4). Elevated values as a function of increased dialysis whatever the mechanism, would not be a problem with the undialyzed system suggested here.

Table 1 shows the data obtained in a comparison between the proposed procedure carried out at 100 samples/hour, the conventionally used and accepted Technicon single channel dialysis procedure carried out at 40 samples/hour (*2–3*) and the calcium channel of a multiphasic system carried out at 60 samples/hour. The results indicate that the procedures are comparable for random samples tested over a period of several months. Therefore, the simpler, and much faster system is workable. Sensitivity of the undialyzed system as described seems a little lower than the single channel dialysis procedure and

TABLE 1

COMPARATIVE VALUES OBTAINED WITH SMA-12-60, AA-1-40 DIALYZED AND AA-1-100 UNDIALYZED

SMA-12-60	AA-1-40-D	AA-1-100-UD	SMA-12-60	AA-1-40-D	AA-1-100-UD
10.8	11.3	11.0	7.6	7.4	7.6
10.1	10.5	10.0	10.8	10.4	10.3
10.5	11.0	10.7	8.8	8.5	8.7
9.8	9.7	9.8	9.3	9.4	9.3
5.5	5.6	5.7	9.2	9.7	9.4
9.0	9.1	9.2	10.3	10.5	10.4
9.7	10.0	9.9	7.5	7.8	7.7
9.1	9.3	9.2	10.5	10.4	9.7
9.7	9.6	9.2	9.2	9.4	9.1
10.4	10.3	10.0	9.5	9.5	9.5
9.4	9.5	9.1	10.8	10.8	10.5
10.1	10.2	9.8	10.1	10.2	9.8
8.5	8.7	8.5	8.3	8.8	8.2
9.3	9.5	9.2	10.4	10.7	10.3
8.4	8.4	8.2	8.7	9.0	8.9
9.1	8.8	9.0	10.5	11.1	10.5
9.5	9.8	9.5	10.9	11.2	10.5
9.9	10.0	9.7	7.7	7.8	7.6
12.5	13.0	12.8	8.8	9.1	9.0
10.7	11.0	10.7	9.1	9.4	9.3

some of the probable reasons have been discussed. It is possible to increase the sensitivity by changes in the makeup of reagents such as decreasing the acidity of the CPC reagent. However, the prime purpose of the present study was to establish a prototype for increasing the speed of determination without loss in accuracy or increase of interaction between samples and all the data obtained indicate that this is possible.

Table 2 shows the comparative data obtained from the direct serum determinations on a Beckman DSA-560 at 120 samples/hour *vs.* the dialysis process of the SMA-12 at its rate of 60/hour. The results clearly indicate that the procedures are comparable. The DSA procedure at 120 samples/hour does not represent an accelerated system but it was included for several reasons. It is robotized discrete sample automation analogous to a manual procedure and it was not a previously available procedure on this analyzer before the dimethyl sulfoxide system was applied to it. The inference drawn from the comparison is that the robotized system used on serum directly and the on-stream

TABLE 2

DOUBLE BLIND VALUES[a] OBTAINED WITH DSA-560 VERSUS SMA-12-60

SMA-12-60	DSA-560[b]	SMA-12-60	DSA-560
8.6	8.5	7.5	7.7
8.9	8.7	8.9	9.1
8.5	8.7	8.8	9.2
8.9	9.1	9.0	9.6
10.1	10.4	9.3	9.5
11.3	11.6	9.4	9.4
9.2	9.6	8.7	9.0
7.2	6.8	10.7	11.0
9.3	9.5	9.5	9.9
9.8	10.4	9.5	9.4
9.7	10.0	9.4	9.8
9.7	9.9	10.0	10.1
9.2	9.3	9.4	9.9
9.5	9.6	10.6	10.8
9.6	9.7	8.3	8.4
9.0	9.1	9.6	9.6
9.1	9.1	7.8	7.9
9.1	9.2	9.0	8.7
9.8	10.3	8.1	8.3
9.6	9.6	10.5	10.4

[a] Milligrams per deciliter.

[b] DSA-560 was operated at rate of 120 samples/hour.

dialysis system used on a diffusate achieve near identical results. The ratio of speed of determination favors the DSA-560 by a factor of 2.

SUMMARY

A rapid procedure for the automated determination of serum calcium by direct determination in raw serum is described. In this case, a 50/hour direct serum determination was modified to operate at 100/hour, but the increased rate of processing did not affect accuracy, reproducibility of the determinations or sample to sample interaction. Dimethyl sulfoxide was included in the reagents as a solvent to lower the dielectric constant of the medium and to help repress the ionization of the color reagent, cresolphthalein complexone, thereby decreasing the blank. Potential interference from lipemia, jaundice or hemolysis was not covered in the study because the primary stated purpose here was to develop a model concept for accelerated on-stream automation. However, further studies along these lines are in progress. A discrete sample analyzer, the Beckman DSA 560 was also used for the determination and the same reagents were adequate. Acceleration of direct automated determinations such as described seems feasible within limits when using the concept of manifold lines enlarged in the ratio of desired faster to slower speeds. Recoveries indicate that the proposed accelerated model system is useful for the determination of serum calcium.

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Methods for the Isolation and Characterization of Constituents of Natural Products

XVI. Quantitative Microdetermination of Diols as *Bis*-Esters of Pyruvic Acid 2,6-Dinitrophenylhydrazone: Separation from Monohydric Alcohol Derivatives and Resolution of an Homologous Series

D. P. SCHWARTZ, C. R. BREWINGTON AND J. L. WEIHRAUCH

Dairy Products Laboratory [1]
Washington, D. C. 20250

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INTRODUCTION

In a recent report from this laboratory (*2*), a quantitative micro procedure was described for determining a wide variety of monohydric alcohols as colored esters of pyruvic acid 2,6-dinitrophenylhydrazone. This paper reports an extension of the method for quantitatively determining micro amounts of dihydric alcohols as *bis*-esters of pyruvic acid 2,6-dinitrophenylhydrazone. In addition, a procedure is given for separating the dihydric alcohol derivatives from the monohydric. A thin-layer partition chromatographic system is also described for separating an homologous series of the *bis* derivatives of terminal diols.

Although diols react quantitatively with several reagents (*1*, *4*) there is at present no simple procedure for quantitating nonvicinal diols in the presence of monohydric alcohols.

MATERIALS AND APPARATUS

The source of reagents and purification of benzene were the same as previously described (*2*) except for the following modifications. Pyruvic acid chloride 2,6-dinitrophenylhydrazone was recrystallized three times from pure, dry benzene (*2*). CaH_2 was pulverized in a mortar and stored in a desiccator. Acidic alumina (Woelm, Activity Grade I, Waters Associates, Framingham, MA) was partially deactivated by addition of 8% distilled water. The dihydric alcohols were obtained from the Aldrich Chemical Co., Milwaukee, WI, except for 1,4-dodecanediol and

[1] Eastern Marketing and Nutrition Research Division, Agricultural Research Service, U. S. Department of Agriculture.

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1,5-tetradecanediol which were prepared by $LiAlH_4$ reduction of the corresponding lactones. Methyl-*threo*-9,10-dihydroxystearate was kindly supplied by Dr. G. Maerker, ARS, EMN, USDA, Philadelphia, PA.

EXPERIMENTAL METHODS

Preparation of dihydric alcohol solutions. These were made up to contain between approximately 1.5–2.1 μmoles/ml. The solvents used for solution of the alcohols are listed in Table 1. Where mixed solvents were employed, the diol was dissolved first in the more polar solvent and diluted with the less polar solvent.

Preparation of reagent solutions. A benzene solution of pyruvic acid chloride 2,6-dinitrophenylhydrazone was prepared to contain 23.5 μmoles/ml and was stored over 10–20 mg CaH_2/50 ml solution. A benzene solution of triethylenediamine was made up to contain 37 μmoles/ml and was also stored over CaH_2 in a similar manner.

General assay procedure. Assays were conducted in 10-ml dry test tubes fitted with Teflon-lined screw caps. Using a single 0.5 ml graduated pipette, 0.00, 0.25, 0.50, 0.75, and 1.00 ml aliquots of the diol solution were pipetted into the tubes. This was followed by 0.6 ml (14.1 μmoles) of the acid chloride solution, permitting the solution to run down the side while rotating the tube. Benzene was added in the same manner to the appropriate tube to bring the volume to 1.6 ml. A few milligrams (microspatula tip) of CaH_2 were added, the tubes loosely stoppered and allowed to stand until gas evolution ceased. When dry, 0.6 ml (22.2 μmoles) of the triethylenediamine solution was added while carefully shaking the tube. The solution turned bright red upon addition of the base, became turbid and eventually faded to a lighter color.

Isolation and quantitation of derivatives. The reaction mixture can be analyzed immediately following addition of the base or within a few hours. The contents of the tube were transferred to a chromatographic column (approximately 1 cm i.d. × 17 cm) containing about 5 g of alumina which had been poured dry into the column. There is considerable gassing in the solution above the column bed due to the reaction of CaH_2 with water in the solvents and in the alumina of the surface of the column, but this does not affect the results. The column was washed with about 20 ml of benzene which will elute most monohydric alcohols.[2] The diol derivatives were then eluted with methylene chloride until all color below a slow-moving impurity band was removed. The solvent was evaporated on the steam bath under N_2, the

[2] For the known diols used in this study, no or only traces of monhydric alcohol derivatives were found. For unknowns with monohydric alcohols present the procedure described below should be used.

TABLE 1

THE DETERMINATION OF DIHYDRIC ALCOHOLS AS *Bis*-ESTERS OF PYRUVIC ACID 2,6-DINITROPHENYLHYDRAZONE

Compound	Solvent	Range investigated (μmoles)	No. of assays within range	Average yield %	Average deviation %
1,2-Cyclohexanediol [a]	20% methylene chloride in benzene	0.48–1.44	4	101.3	±2.0
2,5-Dimethyl-2,5-hexanediol		0.61–2.40	4	96.2	±1.6
Methyl-*threo*-9,10-dihydroxystearate		0.39–1.57	3	102.3	±0.2
1,4-Dodecanediol	methylene chloride	0.51–2.07	4	100.5	±0.6
2-Ethyl-1,3-hexanediol		0.49–1.95	4	97.5	±2.0
2,5-Hexanediol		0.45–1.80	4	97.6	±2.5
1,9-Nonanediol		0.49–1.97	4	100.0	±1.5
1,8-Octanediol		0.47–1.88	4	98.2	±1.3
1,5-Tetradecanediol		0.41–1.65	4	107.5	±0.2
1,6-Hexanediol		0.54–2.16	3	100.6	±0.4
1,4-Butanediol	5% 2-butanone in methylene chloride	0.61–1.84	3	101.8	±2.0

[a] Two spots on thin-layer plate.

derivative taken up in benzene (or in the case of short-chain diols, in methylene chloride) and read at 400 mμ against the blank. The concentration of derivative was calculated using E = 11,850. This value was experimentally determined on pure *bis* derivatives of the diols and is very close to twice the molar absorptivity determined for monohydric alcohol derivatives. (*2*).

Purity of derivatives. Each diol derivative prepared in the assay procedure was checked by thin-layer chromatography using the system described below. Authentic crystalline derivatives were spotted with the derivatives obtained in the assay.

Separation of an homologous series of terminal dihydric alcohol bis-derivatives by thin-layer partition. This was achieved on plates of Microcel T-38 coated with polyethylene glycol 400. The plates (8 × 10 in.) were prepared as described by Schwartz *et al.* (3). The derivatives were spotted from benzene solution (except 1,4-butanediol which was spotted from ethyl acetate) and the chromatogram developed with hexane: benzene (65:35) saturated with stationary phase.

Separation of diols from monohydric alcohol derivatives. A complex mixture of 26 monohydric derivatives was prepared in order to determine whether a mixture of dihydric alcohol derivatives could be separated from them. The following monohydric alcohol derivatives were used in a concentration of approximately 0.15 μmole each/ml of benzene: 2-methyl-2-nonanol, 4-methyl-1-penten-3-ol, dihydrolanosterol, 4-methyl-4-penten-2-ol, β-phenylethanol, lanosterol, 3-ethyl-3-hepten-1-ol, *cis*-9,10-epoxyoctadecan-1-ol, 2-methyl-1-penten-3-ol, 3-methyl-5-hexen-3-ol, 3-phenyl-1-propanol, L-menthol, 2-undecanol, farnesol, 3-methyl-2-hexanol, 4-methyl-3-pentanol, L-isopulegol, 3-decanol, cinnamyl alcohol, 2-ethyl-5-hexen-3-ol, citronellol, 2,2-dimethyl-3-ocranol, isobutyl alcohol, 2,2-dimethyl-1-propanol, and methanol. The dihydric alcohol mixture consisted of 1,8-octanediol, 1,5-tetradecanediol, methyl-*threo*-9,10-dihydroxystearate, 2-ethyl-1,3-hexanediol and 1,4-dodecanediol, each in a concentration of approximately 0.3 μmole/ml of solvent. Following derivatization in the manner described, the solution was quantitatively transferred to a chromatographic column (1.2 cm i.d. × 31 cm) containing 15 g of alumina which had been poured into the column about two-thirds filled with benzene. All monohydric alcohol derivatives were eluted within 50 ml of benzene; the dihydric alcohol derivatives were then eluted with methylene chloride.

RESULTS AND DISCUSSION

Table 1 lists 12 dihydric alcohols representing primary, nonvicinal secondary, tertiary, mixed primary and secondary, and vicinal sec-

ondary alcohols. Also given in Table 1 are the solvent systems used for dissolving them, the range (in micromoles) over which the compound was assayed, the number of determinations made within the range and the yield. The yield (based on 100% purity of the starting material) is the average of the yields obtained at the various concentrations assayed within the range.

As Table 1 clearly shows, the reagent acylates all of the diols investigated in quantitative or near-quantitative yield. The *bis* derivatives of all of the diols studied were exclusively formed. This was substantiated by the absence of any colored bands following the diol band on the alumina column and also by TLC. There was always a very small impurity band well above the diol band which moved very slowly with methylene chloride on the column and did not offer any problem in the collection of the diol band.

The separation of the mixture of 28 monohydric alcohol derivatives from the 5 dihydric alcohol derivatives was readily accomplished on the 15-g alumina column. Separation was clean-cut with about 5 cm separating the tail end of the monohydric alcohol band and the leading edge of the diol fraction as the former was leaving the column. A near quantitative yield of both the monohydric and dihydric alcohol fractions was obtained in two trials; with 0.46 and 0.92 μmoles of dihydric alcohol mixture and with 0.40 and 0.80 μmoles of monohydric alcohol mixture. The yields were 106 and 104% for the dihydric alcohols and 96 and 100% for the monohydric alcohols, respectively. Thin-layer partition chromatography of the diol derivatives showed it to be entirely free of spots other than the diol spots.

It should be pointed out that vicinal diols do not react quantitatively with the reagent under conditions established earlier (*2*) for acylation of monohydric alcohols. Increasing the concentration of triethylenediamine to the level specified in this report effectively remedies this situation. The increase in the concentration of base still gives a quantitative yield of monohydric alcohol derivatives and no adverse effect of the increase has been noted.

Figure 1 is a reproduction of a thin-layer partition plate showing the separation of an homologous series of terminal diol *bis* derivatives. Exposure of the plate to diethylamine vapor gives greyish-blue spots.

Interferences in the acylation of diols by the reagent are the same as for the acylation of monohydric alcohols. Interference by water, thiols, amines, and fatty acids has been discussed (*2*). Mehods are being perfected in this laboratory to effectively remove amines, thiols and fatty acids from mixtures containing alcohols.

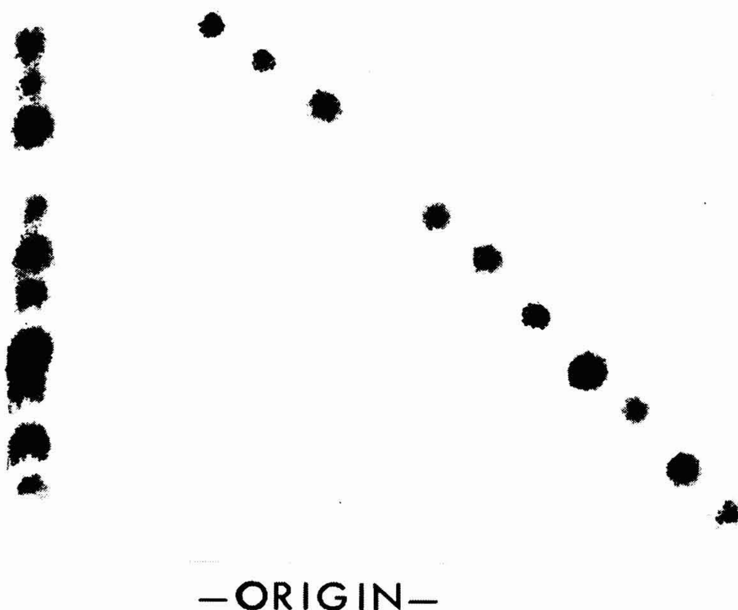

FIG. 1. Thin-layer partition chromatography of an homologous series of terminal diols as *bis*-esters of pyruvic acid 2,6-dinitrophenyl-hydrazone. Diagonally from top to bottom: C_{14}, C_{13}, C_{12}, C_{10}, C_{1}. Column on left is mixture; stationary phase is polyethylene glycol 400 on Microcel T-38; mobile phase is hexane:benzene (65:35) saturated with polyethylene glycol 400.

SUMMARY

A quantitative colorimetric method is described for the acylation of micro- and submicromole amounts of dihydric alcohols as *bis*-esters of pyruvic acid 2,6-dinitrophenylhydrazone. The *bis*-esters which are obtained exclusively, can be cleanly and quantitatively separated from a complex mixture of monohydric alcohol derivatives on an alumina column. Separation of an homologous series of terminal diol derivatives by thin-layer partition chromatography is also described.

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MICROCHEMICAL JOURNAL 17, 309–317 (1972)

The Reaction Between Copper(II) and Cyanide Ions

RAM PARKASH [1] AND JAROSLAV ZÝKA

Department of Analytical Chemistry, Charles University, Prague, Czechoslovakia

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INTRODUCTION

The reaction between copper(II) and cyanide ions has been the subject of much interest ever since 1827 when Gmelin (*5*) obtained tripotassium cuprocyanide. Bassett and Corbet (*1*) have reviewed the earlier work and confirmed the formation of some of the reported cuprocyanides by carrying out a phase rule study of the system $KCN–CuCN–H_2O$ at 25°C but observed that dipotassium cuprocyanide is not capable of existence at this temperature. However, its formation has been inferred by several other workers (*5*). Moles and Izaguirre (*7*) have studied the colorless, yellow and violet cupric cyanide complexes using physicochemical methods. Glasner and Asher (*4*) have formulated the water-soluble violet ion as $[Cu(CN)_4.HCN]^{2-}$. Duke and Courtney (*3*) have reported the formation of cyanogen and $[Cu(CN)_3]^{2-}$ during this reaction. Baxendale and Westcott (*2*) have observed that copper(II) and cyanide ions rapidly form the equilibrium concentration of the $[Cu(CN)_3]^-$ complex which subsequently reacts bimolecularly to give $[Cu(CN)_2]^-$ and cyanogen. A survey of literature reveals that copper(I) cyanide is the final product in this reaction but the data regarding the intermediates and the nature of the reaction is unsufficiently reliable and uniform (*5*). An attempt has, therefore, been made in the present investigations to study this reaction visually as well as potentiometrically to explain the course of the reaction and to work out a simple method for the volumetric determination of cyanide in the presence of Cl^- and Br^-.

MATERIALS AND METHODS

Apparatus

The potentiometric titrations were carried out on a commercially available potentiometer Multoscop V (Laboratorní přístroje n.p.,

[1] On leave from: Chemistry Department, Panjab University, Chandigarh, India.

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Czechoslovakia) and the pH meter Acidimeter EK (Dělba, Czechoslovakia) using a saturated calomel electrode as a reference electrode and a pure copper wire, graphite, glassy-carbon and bright platinum electrodes as the indicator electrode.

Spectrophotometric measurements were carried out with the Unicam SP-800 spectrophotometer using 1 cm quartz cuvettes. Water and copper(II) sulfate solution (0.02 *M*) were used as blank.

The stepwise measurement of the gas evolved during the titration was carried out in a closed system using an air thermostated graduated pipette connected to a small water reservoir.

Reagents

All the reagents used were of analytically pure grade. The solutions (0.5 *M*) of potassium cyanide and copper(II) sulfate were prepared by direct weighing and diluted to obtain the solutions of different molarities. Potassium cyanide solution was standardized by titrating with standard silver nitrate using fluorescein as an indicator. Copper(II) sulfate was standardized iodometrically against standard thiosulfate.

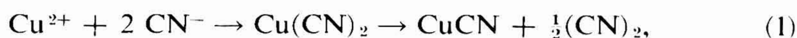
General Procedure

The visual titrations were carried out by adding copper(II) sulfate solution from a microburette to 10, 20 and 25 ml of potassium cyanide solution of same molarity and vice versa with and/or without adding 20 ml of Britton-Robinson universal buffers of pH 2.5, 3.2, 3.8, 7.7 and 11.0 or sodium acetate–hydrochloric acid buffers of pH 1.3 and 4.8. The potentiometric titrations were carried out at 20°C in the presence and absence of chloride, bromide and iodide ions. Every titration has been repeated at least three times. The changes in potential have been plotted as a function of CN^-/Cu^{2+}, molar ratio (Figs. 1 and 3) and some typical results presented in Table 1.

RESULTS AND DISCUSSION

Addition of Copper(II) to Cyanide

When copper(II) sulfate solution (0.5 *M*) is added to the cyanide solution (5–25 ml of 0.5 *M*), every drop forms a bright yellow precipitate of copper(II) cyanide which dissolves on slight shaking or standing forming complex cuprocyanides:



$$Cu^{2+} + 2\ CN^- \rightarrow Cu(CN)_2 \rightarrow CuCN + \tfrac{1}{2}(CN)_2, \quad (1)$$

$$CuCN + x\ CN^- \rightarrow Cu(CN)_{x+1}^{x-} \quad (2)$$

where x is at least greater than 3. The evolution of cyanogen gas is observed from the very beginning of the titration indicating thereby the

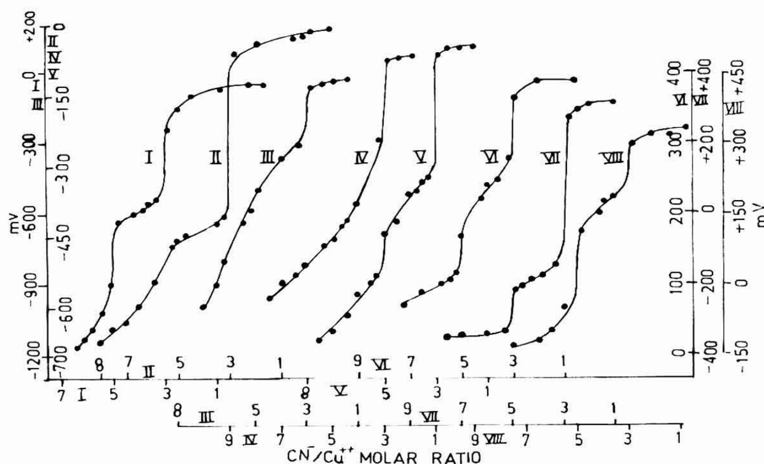
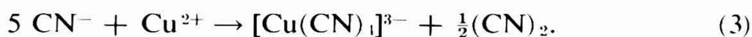


FIG. 1. Potentiometric titration of 0.5 *M* CN^- with 0.5 *M* Cu^{2+} without buffer (I) and with buffer of pH 3.8 (III), 0.05 *M* CN^- with 0.1 *M* Cu^{2+} at pH 3.2 (II) and pH 4.8 (IV) using copper wire electrode; 0.01 *M* CN^- with 0.01 *M*-Cu^{2+} using copper wire (V), graphite (VI), glassy-carbon (VII) and platinum (VIII) indicator electrodes.

reduction of copper(II) to copper(I) but the white precipitate of copper(I) cyanide does not separate out being soluble in excess cyanide. The reduction is quantitative due to the presence of cyanide ions in large excess and thus there is no absorption maximum corresponding to copper(II) in the spectra. It is contrary to the one observation reported earlier that if copper(II) is added to excess potassium cyanide, the reaction proceeds completely to copper(II) cyanide without any reduction (5). The successive additions of copper(II) gradually impart yellow color to the solution depending upon the concentrations of the reactants so much that there is no visible yellow coloration if the solutions are about or less than 0.1 *M*. Thus the concentration of the metal ions in weaker solutions is too low for the yellow color to be visualized.

The evolution of cyanogen gas continues as the titration proceeds further suggesting thereby that the copper(II) gets reduced as fast as it is added to potassium cyanide. There is an abrupt rise in potential at CN^-/Cu^{2+} molar ratio of 5:1 (Fig. 1). Thus all the copper(II) added has been reduced and $[Cu(CN)_4]^{3-}$ is quantitatively formed.



$$5\ CN^- + Cu^{2+} \rightarrow [Cu(CN)_4]^{3-} + \tfrac{1}{2}(CN)_2. \qquad (3)$$

It is, therefore, a process of simultaneous reduction and complex forma-

TABLE 1

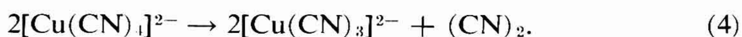
MICRODETERMINATION OF CYANIDE AND COPPER(II) IONS IN PRESENCE OF HALIDES

Indicator electrode	Halide[a]	Amount of CN^- (mg)			mV/0.1 ml of 0.01 *M* solution		Amount of Cu^{2+} (mg)			mV/0.1 ml of 0.01 *M* solution	
		Taken	Found[b]				Taken	Found			
			b	c	b	c		b	c	b	c
Copper wire	—	0.52	0.51	0.52	35	95	1.91	1.91	1.90	80	40
	Cl^-	3.90	3.89	3.92	60	55	3.81	3.82	3.81	50	55
	Br^-	2.60	2.60	2.61	35	60	3.18	3.17	3.17	45	30
Platinum wire	—	3.90	3.90	3.82	55	85	3.18	3.18	3.16	40	25
	Cl^-	3.90	3.89	3.98	80	35	6.35	6.37	6.35	35	45
	Br^-	2.60	2.67	2.59	50	40	9.53	9.55	9.50	30	25
Glassy-carbon	—	2.60	2.60	2.68	35	60	3.18	3.17	3.18	35	110
	Cl^-	3.90	3.91	3.88	30	47	3.18	3.19	3.17	80	65
	Br^-	3.90	3.90	3.90	20	30	3.18	3.17	3.17	40	40
Graphite	—	2.60	2.60	2.68	55	85	3.18	3.16	—	55	—
	Cl^-	3.90	3.98	3.88	30	40	4.76	4.75	—	38	—
	Br^-	3.90	3.92	3.89	15	65	6.35	6.37	—	25	—

[a] In presence of 80-fold excess of Cl^- and Br^- ions.
[b] b and c correspond to Eqs. (3) and (5), respectively.

tion. The solution at this stage is yellow to colorless depending upon the concentrations of the reactants and absorbs in the region of 200–250 nm with maxima at $\lambda = 205$ and 235 nm. It is in agreement with the spectrophotometric studies carried out by Baxendale and Westcott (*2*). It may be mentioned that although the color is yellow in concentrated solutions even up to 1:5 molar ratio of Cu^{2+}/CN^-, the compositions of the complex cyanides are different at different molar ratios as is expected and indicated by the difference in their absorption spectra.

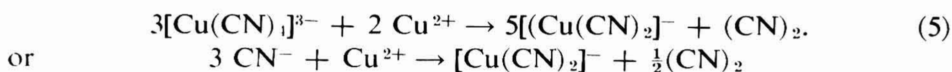
A purple to violet solution is obtained when one molecular proportion of copper(II) has been added for nearly four molecular proportions of the cyanide ions. The solution is colorless to faintly purple when the concentrations are less than 0.2 *M*. The appearance of purple red color was first reported in 1856 (*5*). Later on, Lallemand (6) mentioned that a wine red compound is formed on adding copper(II) salts to aqueous potassium cyanide. Moles and Izaguirre (*7*) have obtained a violet solution below 0°C and assigned it the formula $K_2Cu[Cu(CN)_4]_2$. Glasner and Asher (*4*) have also reported that the copper is most probably in the bivalent state because when the solutions were mixed in 1:4 molar ratio of Cu^{2+}/CN^-, no gas bubbles were seen and no pressure was exerted on the stopper of the flask. In the present investigations, however, the evolution of the gas has been detected more correctly. The $[Cu(CN)_4]^{2-}$ complex suggested by the above authors, is very unstable in view of the following redox reaction:



$$2[Cu(CN)_4]^{2-} \rightarrow 2[Cu(CN)_3]^{2-} + (CN)_2. \qquad (4)$$

Furthermore, it is unreasonable to assume that copper(I) in $[Cu(CN)_4]^{3-}$ formed [Eq. (3)] will get oxidized back to copper(II) as the titration proceeds although it is possible that all the copper(II) added may not be reduced instantaneously. The violet color of the solution may, therefore, be due to a mixture of cupric- and cuprocyanides.

As more and more copper(II) is added, the violet color starts fading and there is another sudden leap up in the potential at CN^-/Cu^{2+} molar ratio of 3:1 (Fig. 1) which may be attributed to the formation of potassium cuprocyanide:



$$3[Cu(CN)_4]^{3-} + 2\,Cu^{2+} \rightarrow 5[(Cu(CN)_2]^- + (CN)_2. \qquad (5)$$

or

$$3\,CN^- + Cu^{2+} \rightarrow [Cu(CN)_2]^- + \tfrac{1}{2}(CN)_2$$

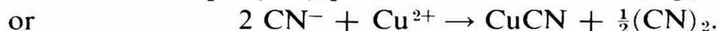
If the reactants are more than 0.1 *M* in concentration, a yellow precipitate and solution are obtained.

The formation of cyanogen continues until Cu^{2+}/CN^- molar ratio of 1:2 is reached when there is a thick white precipitate and the solution

is completely colorless indicating the quantitative formation of copper(I) cyanide:

$$2[Cu(CN)_2]^- + Cu^{2+} \rightarrow 3\ CuCN + \tfrac{1}{2}(CN)_2 \qquad (6)$$

or



$$2\ CN^- + Cu^{2+} \rightarrow CuCN + \tfrac{1}{2}(CN)_2.$$

However, there is surprisingly no jump in potential corresponding to this molar ratio though the potential remains almost constant on further additions of copper(II). The solution acquires bluish to greenish tinge indicating the presence of unreduced free copper(II) ions.

Similar results have been obtained when the titrations were carried out with different volumes (5–25 ml) of the solutions of different molarities. During these titrations, the pH dropped approximately from 10 to 2.5. The color changes are the same as mentioned above. However, when the cyanide ions are titrated with copper(II) keeping the pH between 1.3 to 2.5 using buffer solutions, there is neither any jump in potential nor a change in color except that the solution acquires bluish tinge when excess of copper(II) has been added. Thus there appears to be no reaction between these ions under these conditions. When the titrations are repeated at pH 3.2, 3.8 and 4.8, the jump in potential corresponds to Cu^{2+}/CN^- molar ratio of 1:3 [Fig. 1, Eq. (5)]. The reactions is, therefore, not only a function of pH and cyanide ion concentration but also influenced by the presence of foreign ions. When the pH is higher than 7, there is no indication of formation of any cyanide complex visually or potentiometrically. However, a bluish green precipitate, probably due to copper hydroxide, is formed.

Addition of Cyanide to Copper(II)

The dropwise addition of cyanide ions to copper(II) solution results in the formation of a yellow precipitate of copper(II) cyanide which subsequently dissolves yielding cyanogen and a curdy white precipitate of copper(I) cyanide. The evolution of cyanogen gas starts from the very beginning of the titration and continues even after 1:2 molar ratio of Cu^{2+}/CN^- has been crossed indicating that the reduction of copper(II) and, therefore, the formation of copper(I) cyanide is not quantitative due to the cyanide ions not being in large excess. Because of the presence of a mixture of copper(II) and copper(I), there is no abrupt change in potential corresponding to this molar ratio and the color in the solutions more concentrated than 0.1 *M*, changes from blue through green and greenish yellow to yellow.

As more and more of the cyanide ions are added, there is a sudden fall in the potential somewhere between 1:2 to 1:3 molar ratio of $Cu^{2+}/$

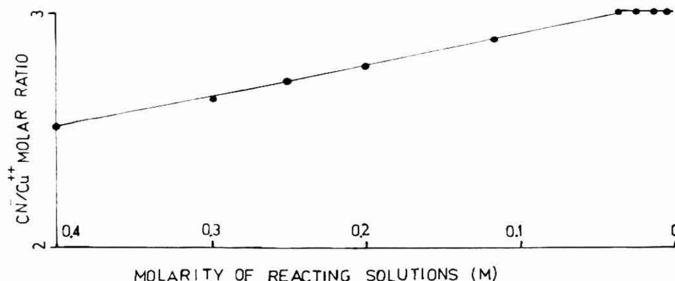


FIG. 2. Effect of molarity of reactants on reduction of copper(II).

CN^- (Fig. 2). In more dilute solutions, the molar ratio tends to approach 1:3. It is reasonable in view of the fact that very little cyanogen is evolved in such solutions near 1:3 molar ratio of Cu^{2+}/CN^-. Since cyanogen is being evolved even when the amount of cyanide ions added is greater than that required for the formation of copper(I) cyanide, it is safe to assume that the reduction is a slower process than the formation of cuprocyanide [Eq. (5)]. It is in keeping with the observation that the titration is relatively slow in the beginning. The solution acquires pinkish tinge and a yellow precipitate is formed in this molar range. However, there is no visible color when the solutions are less than 0.1 *M* in concentration. The evolution of cyanogen ceases at Cu^{2+}/CN^- molar ratio of 1:3 in solutions weaker than 0.03 *M* and in such cases, there is a sudden fall in potential at this molar ratio (Fig. 3) indicating the formation of cuprocyanide [Eq. (5)].

The intensity of the purple color observed above is concentration dependent and increases with further additions of cyanide ions. It becomes distinctly violet when about four molar proportions of cyanide

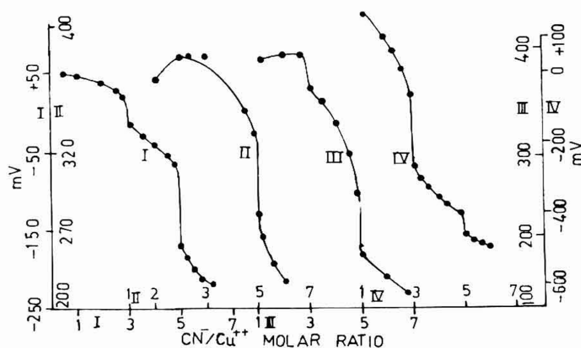
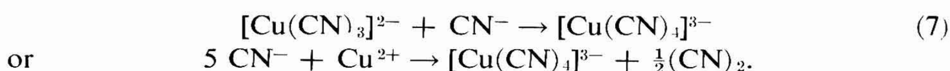


FIG. 3. Potentiometric titration of 0.01 *M* Cu^{2+} with 0.01 *M* CN^- using copper wire (I), graphite (II), platinum (III) and glassy carbon (IV) as indicator electrodes.

ions have been added per molar proportion of copper(II) as is the case in the reverse titration. There is still some evolution of cyanogen in more concentration solutions (0.5 *M*) lending further support to the above conclusion that copper(I) is definitely present in addition to a little copper(II) and this color should, therefore, not be attributed to copper(II) complex cyanide alone.

The reduction of copper(II) is complete before the ratio of Cu^{2+}/CN^- reaches a value of 1:5 when a sudden change in potential is noted once again (Fig. 3). The solution has absorption maxima at $\lambda = 205$ and 235 nm and may, therefore, be attributed to the formation of $[Cu(CN)_4]^{3-}$:



$$[Cu(CN)_3]^{2-} + CN^- \rightarrow [Cu(CN)_4]^{3-} \quad (7)$$

or

$$5\ CN^- + Cu^{2+} \rightarrow [Cu(CN)_4]^{3-} + \tfrac{1}{2}(CN)_2.$$

When potassium cyanide added is in fairly large excess (10–15 times), the solution acquires a stable pale color as is the case in the reverse titration. The absorption spectra in both the cases are also similar. Since there is no unreduced copper(II) present at this stage, the color is only due to the higher cuprocyanide complexes formed.

Microdetermination of Copper(II) and Cyanide Ions

These potentiometric titrations are simple, quantitative and suitable for the volumetric redox determinations of microquantities of the cyanide and copper(II) ions. As little as 0.5 mg of cyanide and copper(II) ions can be estimated (Table 1). That there are two abrupt changes in potential (Fig. 1 and 3) corresponding to the formation of $[Cu(CN)_4]^{3-}$ and $[Cu(CN)_2]^-$ as indicated by the Eqs. (3) and (5), respectively, is an added advantage. A copper wire, graphite, glassy-carbon or bright platinum can be used as an indicator electrode. Copper wire gives better results even that platinum electrode which, in addition, has to be polarized every now and then to get consistent results. Unlike other electrodes, graphite indicates the potential of both the cyanide and copper(II) solutions on the positive side of the millivolt scale. In the titration of cyanide with copper(II), both the potential changes are fairly good. However, in the reverse titration, the potential change corresponding to Eq. (3) is preferred to the one corresponding to Eq. (5) which is neither indicated by graphite electrode nor obtainable in solutions more concentrated than 0.03 *M* due to incomplete reduction of copper(II) by cyanide ions. Even 80-fold excess of chloride and bromide does not interfere with the titrations. In this respect, the titration of cyanide with copper(II) exceeds in its sensitivity and selectivity the argentometric determination. But the potentiometric

estimation of copper(II) with cyanide in the presence of iodide is not possible because of the formation of copper(I) iodide and iodine in the very beginning. In the reverse titration, a white precipitate of copper(I) cyanide is, no doubt, formed but the reaction is not quantitative because iodide also reacts to some extent with copper(II) liberating iodine which imparts yellowish tinge to the solution.

SUMMARY

The titration of cyanide with copper(II) involves simultaneous reduction of copper(II) and formation of cuprocyanides. However, the reduction is not complete in the reverse titration in solutions more concentrated than 0.03 M until Cu^{2+}/CN^- molar ratio of 1:5 is reached. The two abrupt potential changes indicating the formation of $[Cu(CN)_4]^{3-}$ and $[Cu(CN)_2]^-$, have been utilized for microdetermination of these ions. As little as 0.5 mg of cyanide and copper(II) can be estimated even in the presence of 80 times excess of chloride and bromide. Iodide, however, interferes.

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Microelectrophoresis in Thin-Layer Slab of Mixed Agarose Acrylamide Gel

HIDEBUMI HAZAMA

Department of Neuropsychiatry, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

AND

HIDEYUKI UCHIMURA

Hizen National Mental Hospital, Kanzaki, Saga 842-01, Japan

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INTRODUCTION

We have applied microdisc electrophoresis to fractionation of isoenzymes (*3*) and resolution of S-100 protein (*4*) using cell samples dissected from the central nervous system. Although the microdisc electrophoresis can be adapted to the measurement of protein in amounts as small as 10^{-9}g, (*2, 5, 8*) waste of protein sample is unavoidable to some extent at the filling of the capillaries. Accordingly in the case of dissected nerve cell samples more than 70 big cells such as Deiters' cells in the lateral vestibular nucleus were necessary for a column. Therefore, we attempted to develop a method for separating small volumes of protein samples with minimal waste during mounting samples on electrophoretic gels. In this paper a method of ultramicroelectrophoresis in a piece of thin-layer slab of mixed agarose acrylamide gel is reported.

MATERIALS AND METHODS

Preparation of the electrophoresis gel slabs. The following solutions were used in the preparation of the gels: (1) 1.2 ml of 1 N HCl and 0.915 g of Tris in 10 ml of water (pH 8.6); (2) 4 g of recrystallized acrylamide (*6*) and 0.086 g of recrystallized methylene bisacrylamide (*6*) in 20 ml of water; (3) 5 μl of TEMED (*N,N,N′,N′*-tetramethylenediamine) and 3 mg of ammonium persulfate in 10 ml of water; (4) agarose solution (0.6 g agarose dissolved in 40 ml of water in a water bath at 100°).

The four solutions were kept in an incubator at 60° before use and

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mixed together quickly. Consequently the mixed solution contained 5% acrylamide and 0.75% agarose. Triton-X 100 was added 1% in final concentration to the solution (3) for gels in an attempt to run brain samples. In order to obtain a gel of appropriate thickness a glass slide mold, 8 × 6 cm, framed by 5-mm wide scotch tape stuck over to 0.3 mm thick was used. The mixed solution was poured on the glass mold, and covered with a siliconized plastic plate without enclosing air bubbles. The polymerization reaction of the mixture was completed in a closed space formed the glass slide mold and the plastic plate within an hour at 15°. Then the cover was taken off and the gel attached to the glass mold was immersed in a viscous Tris–HCl buffer (pH 8.6) solution with added glucose (60 g glucose dissolved in 100 ml of the buffer), left at room temperature for 6 hours and stored in a refrigerator (4°) until use.

Before use, a piece of gel slab (50 × 5 × 0.3 mm) was obtained by cutting the gel with a razor blade and stripping it out of the glass mold with a forceps. Excess moisture was removed from the gel surface by repeated transfer onto a dried clean glass surface, and the gel slab was spread out on a glass slide (40 × 8 mm) parallel to the long side. Superfluous parts of the gel slab from both short sides of the glass slide were folded back over the other surface of the slide. Some thin slits about 100–500 μ long were made in the gel slab with a piece of razor blade for the application of protein sample (Fig. 1). The gel slab was immediately covered with liquid paraffin, and the glass slide was placed inverted to form the top of an oil chamber.

Application of protein samples. Protein samples were placed with a capillary on the lower surface of an extended 5-mm wide cover glass arranged parallel to the gel slab over the groove of the chamber with liquid paraffin and as hanging drops. By means of a de Fonbrune micromanipulator and a micropipette with a tip diameter under 10 μ the protein samples were transferred and introduced into the slits in the gel (Fig. 2). This procedure was carried out under a microscope equipped with phase contrast optics.

Electrophoresis. After completion of sample application, the glass slide was taken off from the oil chamber and a coverslip, 30 × 8 mm,

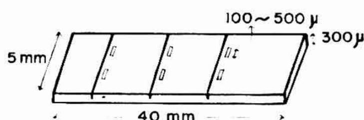

FIG. 1. A schematic picture of the gel slab. Arranged rectangles show the application points.

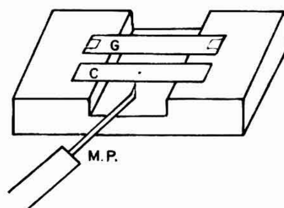

FIG. 2. A combined oil chamber for sample application. C, cover glass with a hanging drop of protein sample; G, gel slab spread out on a glass slide; M.P., micropipette connected with a micromanipulator and a microsyringe.

was gently placed over the gel slab. The glass slide with the gel was transferred to an electrophoretic apparatus (Fig. 3), which consisted of two platinum electrodes (0.5 mm in diameter and 10 cm in length), two reservoirs wherein each electrode was placed, and two filter paper bridges connecting the reservoirs and the gel. The parts of the gel folded back to the other surface of the glass slide were just placed on the filter paper bridges. The bridges of the both sides were layered by several pieces of filter paper. In order to obtain high resolution of proteins, the cathodal bridge was constructed thicker than the anodal bridge. As an electrophoretic buffer borate–NaOH buffer (12.5 g of boric acid and 1.33 g of sodium hydroxide in 1000 ml of distilled water, pH 8.2) was used.

After the system was prepared in the manner mentioned above, the separation was carried out for 15–20 minutes at 4° with a constant current of about 1 mA (200 V). As electrophoresis proceeded, there was a tendency for the current to fall down a little. As soon as elecrophoresis was completed, the coverslip was forced off and the gel slab was taken from the glass slide by a forceps. Coomassie brilliant blue R 250 (0.026%) (Kishida Chemicals Co. Ltd. Osaka) dissolved in

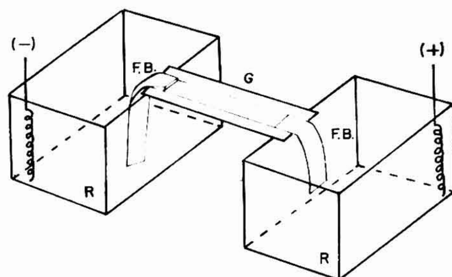

FIG. 3. Electrophoretic apparatus. F.B., filter paper bridge; G, gel slab covered with a cover glass; R, electrophoretic buffer reservoir.

methanol:glycerol:acetic acid:water (40:2.5:5.5:52, by vol) was used for fixing and staining. After 3 hours of staining, the gel slab was transferred into the destaining solution (methanol:glycerol:acetic acid:water 20:2.5:5.5:72, by vol) in order to elute excess dye. These gel slabs were air dried on a glass surface; this produces a permanently mounted specimen. Densitometer curves of protein fractions were obtained directly with dried specimens on a Joyce Loebl microdensitometer.

RESULTS AND DISCUSSION

Human serum separated according to the method mentioned was shown in Fig. 4. There found approximately 10 bands including albumin, postalbumin, transferrin, β-globulin, haptoglobulin and γ-globulin as well as those in disc electrophoresis.

When electrophoresis was run with continuous buffer system of ⅛ N HCl–Tris buffer (pH 8.6) instead of with discontinuous system of borate–NaOH buffer, electrophoretic patterns became less distinct (Fig. 5). Optimum migration distance between the albumin fraction and the application slit was about four times of slit length. Regarding the electric current, we also tried to carry out the separation with 90 μA (1500 V) for 2 hour at 4°. In this condition the separation was not satisfactory.

In case of human serum the volume applied in one of the slits of the gel slab was calculated 0.5 to 1 pl and the amount of containing

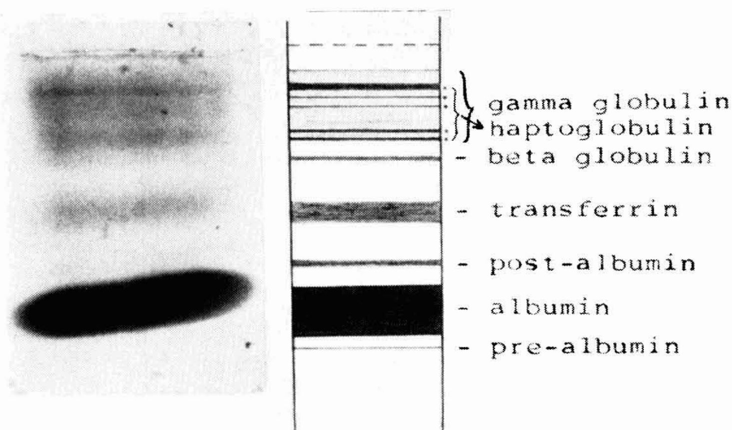

FIG. 4. Microelectrophoretogram of serum protein in amounts as small as a picogram in one picoliter of human serum. The separation was carried out in mixed agarose acrylamide gel slab with borate–NaOH buffer described in the text. The stained specimen was photographed under a stereomicroscope. Magnification ×100.

protein was in 10^{-11} g (10 pg) level. Microdisc electrophoresis has been applied to protein resolution in amounts as small as 10^{-9} g by Grossbach (*2*) and Neuhoff (*8*) and even lesser amounts by Hydén and associates (*5*). Recently Oken (*9*) reported the methods for quantitation of picogram quantities of serum albumin by ultramicrodisc electrophoresis and direct densitometry. The method here is of advantage for using protein samples in amounts as small as a picogram for electrophoresis with minimal waste of samples.

With regard to brain tissues the method could be applicable to protein fractionation with fewer than 10 big nerve cells if cell protein was solubilized up to a certain degree. We have found some difficulties in doing slab electrophoresis with brain tissue as compared with disc electrophoresis—in the latter case electrophoretic patterns are distinct and well reproducible and moreover concentration of the sample is a result of the large pore gel. These difficulties seem to be exaggerated with the microslab electrophoresis. In order to obtain good separation of brain protein according to this method, we attempted some trials but have not been completely successful. Freeze–dried rat brain slices (20 μ thick) prepared according to Lowry's method (*7*) were homogenized with 0.2 M ammonium bicarbonate–acetate buffer (pH 8.2) Triton-X 100 was added to 0.12% final concentration and the extract was used as the protein sample. The sample application to gel slits in the volatile buffer resulted in a sample concentration free from buffer electrolytes.

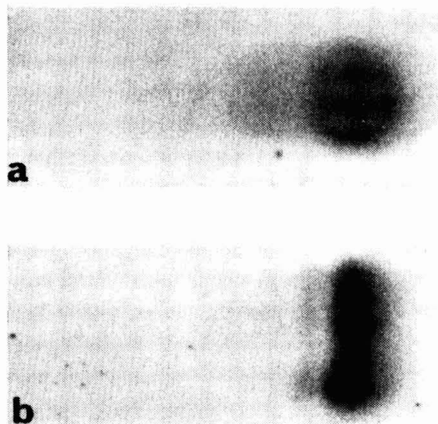

FIG. 5. Microelectrophoretogram of bovine serum albumin dissolved in distilled water (5 mg/ml). The following electrophoretic buffer was used; (a) continuous system of ⅛N HCl–Tris buffer (pH 8.6); (b) discontinuous system of borate–NaOH buffer (pH 8.2). Magnification ×75.

Consequently, the resolution of brain protein became much better; however, it still requires further examination.

Concerning RNA, Ringborg and coworkers (*10*) developed a new method for separating high molecular weight RNA by microslab electrophoresis. They used the method for analyses of microisolated RNA from cytoplasm, nuclear sap, nucleoli and chromosomes of salivary gland cells in *Chironomus tentans,* and for separations of 500–1000 pg of *Escherichia coli* RNA. Dan (*1*) presented a microelectrophoresis of hemoglobin contained in single erythrocytes in acrylamide gel polymerized with erythrocyte suspension. In this connection, microelectrophoresis of extracted proteins in thin-gel slabs have not been reported.

SUMMARY

Microelectrophoresis in a thin-layer slab of mixed agarose acrylamide gel is reported in this paper.

The gel slab (50 × 5 × 0.3 mm) composed of 5% acrylamide and 0.75% agarose and carved into thin slits about 100–500 μ long with a piece of razor blade was used. Into these slits protein samples were introduced under an oil chamber by means of a de Fonbrune micromanipulator; and a current of 1 mA (200 V) was applied for 15 minutes between two electrodes placed in two of the reservoirs. After electrophoresis the gel slab was fixed and stained with 0.026% Coomassie brilliant blue solution.

The method is of advantage when using protein samples in amounts as small as a picogram for electrophoretic resolution with minimal waste of small samples.

ACKNOWLEDGMENTS

We thank Miss Tsunemi Tanaka for her invaluable technical assistance.

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MICROCHEMICAL JOURNAL **17**, 325–326 (1972)

Technique for Automatic CHN Determination of Volatile Liquid Samples

CLARENCE I. KENNEDY

Mead Johnson Research Center, Evansville, Indiana 47721

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The technique described here permits rapid and accurate determinations of carbon, hydrogen, and nitrogen for volatile liquid samples using the Hewlett–Packard Model 185 Automatic CHN Analyzer and small glass capillaries sealed at either one end or both ends. The manufacturer's suggested use of aluminum capillary tubes has been found to be tedious and extremely time consuming. Furthermore, the aluminum tubes are fragile and easily fractured and are reactive toward liquid samples containing active halogens or acidic functions. Glass ampules (*1*) have been used in classical microanalytical techniques for weighing volatile liquid samples and have provided reliable and reproducible results.

EXPERIMENTAL

The glass capillary tubes are drawn and cut to approximately 1 mm o.d. and 10 mm in length, sealed at one end and fire polished on the other end to an opening of 0.5 mm diameter. Initially, the glass capillary is placed into an aluminum weighing boat and weighed. Having been weighed, the capillary is removed from the boat with forceps and held momentarily in a microflame which is directed just above the seal. The capillary is then immediately immersed at its open end into the liquid sample to be analyzed. After withdrawing the capillary tube from the sample and wiping off any excess sample, the capillary tube is centrifuged using a small hand centrifuge. The capillary tube with sample is then placed into the aluminum boat and reweighed. The correct weight range is attained by adding or removing sample using a stainless steel probe or smaller glass capillary. Having attained a suitable weight range, the capillary is positioned in the weighing boat so that its open end is directed toward the gas flow inlet. Finally, the capillary is covered with catalyst and analysis is completed according to standard instrument operating procedures. For the analysis of samples that boil lower than

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TABLE 1

CHN DETERMINATIONS OF VOLATILE LIQUIDS

Sample	% Calculated			% Found		
	C	H	N	C	H	N
1	69.63	11.04	9.02	69.74	10.97	8.99
2	67.09	11.96	9.78	66.98	11.99	9.81
3	65.07	11.70	10.84	64.98	11.55	10.94
4	55.14	10.41	16.08	55.03	10.69	16.38
5	54.94	9.99	10.68	55.02	9.84	10.86
6	46.71	3.92	21.79	46.86	3.92	22.06
7	32.00	6.71	18.66	31.92	6.91	18.78
8	37.48	12.58		37.37	12.29	

100°C, the capillary needs to be sealed at both ends. Having attained a suitable weight range for the sample, the capillary is held with a wet cloth while sealing the open end. On heating in the furnace of the analyzer, the liquid sample is volatilized within the sealed capillary causing the capillary tube to be fractured by the expanding gases which are then released into the helium gas flow.

RESULTS

Table 1 shows results obtained by this technique on several volatile liquids and low boiling oils, with boiling points in the range 65–150°C, atmospheric pressure. Sample 8 was Merck methyl alcohol, spectrophotometric grade. These same samples did not yield satisfactory results using the boat technique and the identical instrument.

The applicability of this technique can be extended to include hygroscopic samples and samples which rapidly pick up atmospheric carbon dioxide.

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MICROCHEMICAL JOURNAL 17, 327–337 (1972)

Determination of Nanogram Amounts of Albumin by Radioimmunoassay [1]

MARIE GAIZUTIS, AMADEO J. PESCE, [2] AND JOHN E. LEWY [3]

Department of Biology, Illinois Institute of Technology, Renal Division, Department of Medicine and Section of Pediatric Nephrology, Michael Reese Hospital and Medical Center and the University of Chicago, Pritzker School of Medicine, Chicago, Illinois

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The technique of nephron puncture with the micromanipulator (*1*) offers a direct means of sampling fluid for the measurement of albumin in the nephron in the healthy rat and in the experimental proteinuric state. Published methodology for the measurement of albumin is not sensitive enough for our purposes (*2*). The concentration of albumin in the tubular lumen of the nephron is estimated from 1 to 5 ng/100 ml (*4, 5*). The sample size obtained by nephron puncture in a relatively short period of time, 10 minutes, is about 100 nl. Hence, it is important than an assay method measure amounts of albumin on the order of 1 ng.

Several methods are reported for measuring albumin in the proximal tubule. One investigator used the Folin Lowry phenol reagent for tyrosine which is not specific for albumin (*5*). Immunoassays such as the micro-ouchterlony only gave a range of values within a factor of three (*3*). The microacrylamide gel system has been used, but it is not suitable for a large number of samples and requires an expensive microscopic scanner.

The method using the radioimmunoassay technique for quantitation of urinary albumin has been published (*2*), but it is sensitive only to 100 ng and thus unsuitable without modification. We decided to explore the radioimmunoassay method further in order to adapt it to

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[2] Established Investigator, American Heart Association.

[3] Present address: Division of Pediatric Nephrology, Cornell University Medical College, 1300 York Avenue, New York, NY.

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the measurement of albumin. In this method a known amount of radioactivity labeled protein and unlabeled protein is mixed with a limited amount of antibody. The amount of the labeled protein complexed with the antibody is a function of the ratio of the two concentrations. The complex is separated from the excess unreacted forms and assayed.

There are several approaches to the method of radioimmunoassay. In one procedure equilibrium between the antibody, labeled protein, and unlabeled protein is established in solution. Subsequently, ammonium sulfate selectively precipitates the complex allowing the unreacted materials to remain in solution. The precipitate and supernatant are then washed and counted (*6*). A more specific procedure is to prepare an antibody to the complex, precipitate the complex with this antibody and as before count the radioactive distribution between supernatant and precipitate (*7*).This has the advantage that the antibody to the complex is a more specific precipitant than the ammonium sulfate solution.

In our studies the salt fractionation procedure was unsuccessful since efficient conditions could not be developed for selective precipitation of the complex. The method using antibody to the complex lacked adequate sensitivity in our hands and was abandoned.

We then explored the use of a solid phase on which the antigen or antibody could be absorbed. We found that styrene resin was excellent for this purpose since it readily absorbed proteins from solution. We approached this technique in two ways. In one procedure we adsorbed albumin on the surface and then adsorbed antibody to the albumin (*8*), the idea being to form a homogeneous surface of only antibody to albumin for utilization in the reaction. We reasoned that while styrene would adsorb any protein indiscriminately, a surface coated with albumin would only adsorb its antibody. The concept was applicable but required at least 5 ng of albumin to be present for measurement. This seemingly minute amount was too large for our nephron puncture studies in the rat. We thus had to abandon this procedure.

The final procedure developed proved to be relatively simple. The antiserum was added to a test tube; the antibody and other serum proteins allowed to be adsorbed (*9*). The mixture of labeled and unlabeled albumin was then added. After allowing the albumin to be adsorbed, the supernatant was discarded and the radioactivity adsorbed to the tube read in the gamma counter. This remarkably simple procedure permitted the assay of as little as 1 ng of protein.

MATERIALS AND METHODS

Reagents

Buffers

Phosphate buffer, pH 7.0, 1.0 M. Dissolve 53.0 g of KH_2PO_4 and 106.25 g of K_2HPO_4 in 800 ml of water. Bring volume to 1 liter. Dilute to either 0.1 or 0.2 *M* buffer as needed.

Phosphate–Albumin buffer pH 7.0. Dilute 10 ml of 30% bovine serum albumin solution (Miles Laboratories, Pentex Division, 81013) with 290 ml of 0.1 *M* phosphate buffer, pH 7.

Phosphate buffered saline, pH 7.4. Dissolve 1.13 g of Na_2HPO_4, 0.258 g of KH_2PO_4 and 8.5 g of NaCl in 600 ml of water. Make up to 1 liter.

Barbital buffer, pH 8.7, 0.07 M. Dissolve 57.52 g of sodium barbital in 1 liter of water. Adjust the pH to 8.7 with 28 ml of 1 *N* HCl and dilute the solution to 4 liters.

Iodinating solutions

Sodium iodide ^{125}I solution. Obtained from New England Nuclear, Boston, MA, in polyethylene vials (polyvial) in quantities of 2 to 5 mCi.

Chloramine T solution. Dissolve 2.5 mg of chloramine T into 1 ml of 0.1 *M* phosphate buffer, pH 7.0. Use immediately after dissolving in the buffer.

Sodium metabisulfite solution. Dissolve 5 mg of $Na_2S_2O_5$ into 1 ml of 0.1 *M* phosphate buffer, pH 7.0.

Potassium iodide solution. Dissolve 16 mg of KI in 1 ml of 0.1 *M* phosphate buffer, pH 7.0.

Working Solutions

Rat albumin solution. Dissolve 10 mg of purified rat albumin (see below) in 2.5 ml of 0.1 *M* potassium phosphate buffer. This is the stock albumin solution. Dilute to 1 mg/ml for the working solution for labeling. The stock solution contains 4×10^6 μg/ml. Prepare from this working solutions containing 100, 200, 500, 1000, 2000, 5000, and 10,000 μg/ml by dilutions of 1/40,000, etc., into phosphate buffered albumin. These contain 1, 2, 5, 10, 20, 50 and 100 ng/10 μl.

Saline wash solution. 9 g of NaCl and 0.1 g of NaN_3 are made to 1 liter with water.

Immunizing Solutions

Rat albumin. Filter the stock rat albumin solution through a Millipore Swinex-13 0.45 μ filter (Millipore Corp., Bedford, MA) in order to

sterilize it. Mix 2.5 ml of the sterile protein solution with 2.5 ml of Freund's adjuvant (Bacto Adjuvant Complete Freund, Difico Labs, Detroit, MI). Inject intramuscularly into six 2–3 kg rabbits. Repeat at 2-week intervals and bleed the animal at 2 months. This is the anti-rat albumin antiserum.

Polystyrene tubes. 12 × 75 mm Falcon polystyrene tubes were purchased from Matheson Scientific Co. Counting tubes 16 × 125 mm were obtained from the same source.

Gamma Counting

Count the radioactivity of the sample while in the 12 × 75 mm polystyrene tubes by placing them inside 16 × 125 mm polystyrene tubes. Count the samples on a Nuclear Chicago Auto Gamma II with window settings of 2.00 for the window and 2.36 for the base. Count all assay samples so that more than 10,000 disintegrations are recorded for the labeled albumin added to each tube.

Isoelectric Focusing Equipment

Set up the electrofocusing column (LKB #8101 Ampholine electrofocusing equipment; LKB-Produkter AB S-161 25 Bromma 1, Sweden) as described by the manufacturer. Cool the column with running tap water.

Urine Collections from Healthy Rats

Sprague Dawley rats weighing between 150 and 200 g were placed in metabolic cages and allowed access to food and water *ad libitum*. The urines were collected under mineral oil containing thymol crystals. The urines were centrifuged and stored frozen until assayed.

Nephron Puncture Samples

The nephron puncture method as described by Windhager (*1*) was used. Approximately 100 nl of fluid was obtained and immediately accurately measured and transferred into the assay tube.

Preparation of Pure Rat Albumin

Prepare a density gradient of carrier ampholyte and sucrose by adding 1.8 ml of 8% ampholyte solution pH 3-10 (LKB 8141), to 40 ml of distilled water. Dissolve 28 g of sucrose in the solution. Then add 20 mg of rat albumin (Pentex Fraction V powder #82501). This forms the dense solution for the gradient. This is mixed in a gradient mixing device (LKB 8121) with a light solution which consists of 0.7 ml of the same ampholyte and 59 ml of distilled water. For the cathode solu-

tion at the top of the column dissolve 0.2 ml of monoethanolamine into 10 ml of distilled water. For the anode solution at the bottom of the column, dissolve 12 g of sucrose in 14 ml of water and add 0.2 ml of concentrated phosphoric acid. The isoelectric focusing column is allowed to equilibrate for 2 days under a current of 30 mA. The column is drained and separated into 60 fractions and the proteins are measured by absorbancy at 280 nm. The albumin fraction is isolated by dialysis against distilled water and lyophylized. The proteins resolve into a single major component as illustrated in Fig. 1 and the albumin isolated gives a single strong band in an immunoelectrophoresis system when tested against rabbit anti-rat whole serum.

PREPARATION OF SEPHADEX GEL COLUMN FOR SEPARATION OF UNREACTED ^{125}I

Suspend 10 g of Sephadex G-25 coarse in 500 ml of 0.1 *M* potassium phosphate buffer, pH 7.0. Allow it to swell overnight. Suspend the gel in a 500 ml graduated cylinder, allow it to settle for 10–15 minutes and pour off the fine particles which do not settle. Add enough buffer to bring the volume to 500 ml. Resuspend the gel, allow it to settle and decant the fine material. Repeat until the supernatant is clear. Prepare a column of 1.2 × 45 cm. When the gel has settled to about 5 cm from the top, allow the buffer to drain so that it is even with the gel. Place 2 ml of phosphate buffered BSA on the column and wash through with 50 ml of 0.1 *M* potassium phosphate buffer pH 7.0. The column is ready for

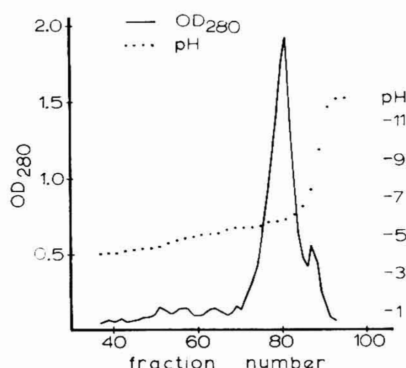

FIG. 1. Isoelectric focusing pattern of rat albumin. OD_{280} is the absorbancy of the fractions at 280 nm. Dotted line represents pH of each fraction. Material was focused for two days in an ampholyte which makes a gradient from pH 3 to 10. The major albumin fraction was dialyzed and concentrated for use in the radioimmunoassay.

use. The albumin is added to saturate sites which might adsorb labeled albumin.

LABELING OF RAT ALBUMIN

Add 50 μl of 0.2 *M* potassium phosphate buffer to the polyvial containing 2–5 mCi of sodium iodide. Add 50 μl of rat albumin solution (200 μg). Add 20 μl of chloramine T and mix by aspirating the solution up and down in the pipette. Allow the reaction to go for 1 minute, then add 20 μl of sodium metabisulfite to destroy excess chloramine T. Add 50 μl of potassium iodide as carrier. Transfer all of the solution into 0.5 ml of phosphate albumin buffer. Separate the radioactive albumin from the free radioiodine by placing the entire solution on the Sephadex G-25 column and elute with phosphate buffer. The radioactive albumin solution is followed with a hand Geiger counter and is eluted in a volume of about 3 ml. Test the albumin solution for free radioactive iodine by taking 1 μl and adding it to 0.5 ml of phosphate albumin buffer followed by 0.5 ml of 10% trichloracetic acid. Collect the precipitate by centrifugation and count the supernatant and precipitating fraction. Fractions with highest radioactivity are pooled, and prepared for use in assay system by diluting 1/5000 in phosphate albumin buffer.

PROCEDURE

Incubate 0.2 ml of diluted rabbit anti-rat albumin antiserum (1:500 in 0.07 *M* barbital buffer pH 8.7) in the polystyrene tube overnight in the cold. Using a Pasteur pipet remove the unreacted antibody and wash the tube three times (by aspiration) with 0.5 ml saline wash solution. To the empty tube which has antibody attached to the walls add 0.2 ml of the phosphate buffered albumin solution, followed by 10 μl of the unknown sample and 0.1 ml of ^{125}I albumin. Allow the albumin to come to equilibrium with the antibody by placing the covered tubes in the cold overnight. Count the tubes with the solution in them by placing them inside the larger plastic tubes in a Nuclear Chicago Autogamma II counter. This is the total amount of radioactive iodine in the tube. After counting, aspirate the unreacted albumin solution and wash three times with the saline solution. Take the empty tube and recount in the gamma counter. This gives the amount of radioactivity bound to the tube. The percentage of radioactivity bound is given from the formula

$$\frac{\text{cpm of washed tube}}{\text{cpm of unwashed tube}} \times 100 = \%\ \text{bound.}$$

A standard curve is made by substituting 10 μl of the dilutions of the standard protein solution for the unknown. These dilutions contain 100

200, 500, 1000, 2000, 5000 and 10,000 ng/ml. The percentage of bound ^{125}I albumin is plotted versus the ng/ml of albumin solution on semilogarithmic paper (Fig. 2). One reads the value of the concentration of albumin in the unknown sample from the standard curve. The activity of the antiserum is variable, as would be expected. To determine the optimal dilution incubate as in the procedure, overnight, successive dilutions of antibody over the range of 1:25 to 1:51,200 in the polystyrene tubes. After rinsing and washing the ^{125}I albumin is added, incubated overnight, the tubes are rinsed, washed and counted. A typical curve for such an antibody dilution experiment is shown in Fig. 3. The maximum amount of ^{125}I albumin bound is 60% and this decreases as the antibody is diluted. The dilutions in the range of 1/800 to 1/2000 are tested for usefulness. This is the range where the binding is decreasing because both the antibody bound to the tube and the labeled albumin are near equivalence.

RESULTS

When ^{125}I labeled albumin is added to the antibody coated tube, the amount bound is a function of the logarithm of the albumin concentration. This is shown in Fig. 3. Not all the ^{125}I albumin is bound under this range of conditions.

To establish the validity of the method for the measurement of albumin in rat urine, both recovery and dilution experiments were done. In the recovery experiments the amount of albumin in a sample of rat urine was found to be 75 μg/ml. In ten different samples 50 μg/ml of rat

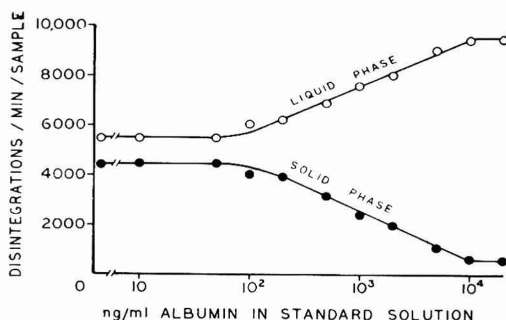

FIG. 2. Standard curve of the radioimmunoassay, plotted as disintegrations per minute per sample versus logarithm of the unlabelled albumin concentration. The tubes were coated with a 1/500 dilution of antiserum and after washing and rinsing were incubated with 1 ng (10,000 dpm) of ^{125}I albumin. The solid phase (the tubes) and the supernatant liquid phase were counted. When increasing amounts of albumin the ^{125}I was displaced from the tube into the liquid phase.

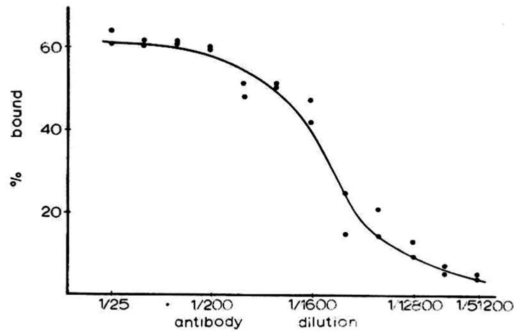

FIG. 3. Titration of antibody by dilution. Antibody was diluted in barbital buffer and incubated overnight as in the procedure. After washing and rinsing the coated tube was incubated with 1 ng of ^{125}I albumin. The tube was then rinsed, washed and counted. The percentage of albumin bound to the tube was plotted versus antibody dilution. The region most suitable for assay is that between dilutions of 1/800 to 1/2000. More concentrated antibody will yield less sensitivity. Less concentrated antibody will yield low accuracy.

albumin were added. The average amount recovered was 60 ± 10 μg/ml. Similarly, 10 samples were enriched with 165 μg/ml. The amount recovered was 145 ± 20 μg/ml (see Table 1).

For the dilution experiment a rat urine was diluted geometrically by factors of two. The results observed are compared to the standard curve (Fig. 4). Since these lines were superimposed on each other, only one line is drawn.

The assay system was used to measure the albumin excretion of normal and male and female rats. The average excretion of the males was 0.096 μg/minute with a range of 0.0087 to 0.295 μg/minute. The average excretion of the females was 0.086 μg/minute with a range of 0.013–0.61 μg/minute. The distribution curve of albumin excretion followed the logarithmic distribution frequency curve (Table 2).

Using the nephron puncture technique the tubular albumin concentration along the proximal tubule of the nephron was measured. From

TABLE 1

MEAN OF 10 RECOVERY VALUES ON RAT ALBUMIN AT TWO DIFFERENT LEVELS

μg/ml Initially (mean)	μg/ml Added	Mean μg/ml found	μg/ml Recovered	% Recovered
75	50	135 ± 10	60 ± 10	120 ± 20
75	165	220 ± 20	145 ± 20	88 ± 12

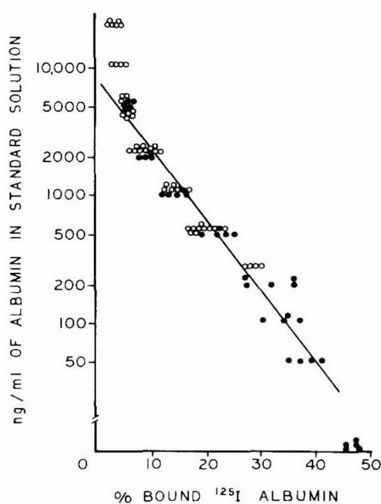

FIG. 4. Correlation between concentration found in the urine and the standard curve. The standard curve (closed circles) is plotted as % of ^{125}I albumin bound versus logarithm of the unlabeled albumin concentration. The open circles represent dilutions of rat urine added to a parallel set of assay tubes. The curve of these dilutions is superimposable over the standard curve.

5 rats 47 nephron puncture samples were obtained. Of these 28 were in the range of greater than 1 mg%. The mean value and standard deviation for albumin in the fluid was 2.9 ± 0.6 mg%.

DISCUSSION

The problem of measuring albumin in nanogram quantities in the presence of the many possible interfering substances in urine essentially limits the ivestigator to immunochemical techniques. In the present study, using the method of nephron puncture, samples of 100 nl volume containing about 1–2 ng of albumin, dictated that the method be done

TABLE 2

ALBUMIN EXCRETION INTO THE URINE OF NORMAL RATS

	Female	Male
Mean excretion (μg/min)	0.0859	0.9591
95% confidence limits (μg/min)	0.07268–0.1017	0.01471–0.6249
Coefficient of variation	36.55	41.45
Number of animals	28	41
Range of values	0.013–0.61	0.0087–0.295

on as small a volume as possible. Colorimetric and fluorometric procedures are not feasible in this range.

The assay developed here followed the displacement pattern expected from an equilibrium reaction between limiting binding antibody, constant labled albumin, and increasing concentration of unlabeled albumin (Figs. 2 and 4). From Fig. 2 it can be seen that when radioactive ^{125}I albumin (about 1 ng or 10,000 dpm) is placed in an antibody coated tube about 45% is bound to the surface, the remainder is in the solvent phase. When unlabeled albumin in concentrations up to 100 ng/ml is added, there is no displacement of the radioactive material. Displacement does not occur until a concentration of unlabeled albumin which exceeds 100 ng/ml is added. From 100 to 10,000 ng/ml is then the range over which practical measurement is possible.

As can be seen from Figs. 2 and 4 there is a semilogarithmic relationship between the concentration of albumin and either the amount of radioactivity bound to the tube or in the solution phase. Increasing the concentration of albumin significantly above 10^4 ng/ml results in no accurately measurable change in either ^{125}I displacement from the tube or increase of radioactivity in the solution phase.

The reproducibility of the assay was tested on 20 successive standard curves each with 5 replicates. The average coefficient of variation over the range of 1 to 50 ng was ± 12%.

From the recovery experiments (Table 1) it is obvious that substances normally found in rat urine do not interfere with the radioimmunoassay measurements. In addition, the dilution experiments (Fig. 4) clearly demonstrate that the assay is specific for albumin. Fragments or degradation products would give results not superimposable with the standard curve.

The value of 2.9 mg% for the albumin concentration in the proximal tubule is 1/7 that reported using the Folin-Lowry procedure (5). Obviously the higher results with this reagent is due to interfering substances. The data obtained with the microacrylamide gel system are comparable to those presented here.

This technique has made possible the measurement of the reabsorption of albumin by the nephron and to estimate the amount filtered into the nephron. The amount filtered, 2.9 mg%, is 1/1000 of the amount present in plasma. From the measurement of glomerular filtration rate and alubmin concentration in the proximal tubule it was calculated that about 26 μg/minute of albumin was filtered. Since the albumin excreted in these animals was 1.4 μg/minute it was clear that the great bulk of the albumin was reabsorbed by the nephron.

SUMMARY

A sensitive radioimmunoassay for the measurement of albumin was developed using the fact that an antibody will absorb to the walls of a polystyrene tube. Advantage was taken of the observation that when a mixture of labeled and unlabeled albumin reacts with a limited amount of antibody the radioactivity adsorbed is a function of the two albumin concentrations. The method was found to be sensitive enough to detect as little as 1 ng of rat albumin with a coefficient of variation of 12%.

Rabbit anti-rat albumin is incubated in the plastic tube. The antibody is adsorbed to the tube. After washing the unknown sample is added along with a measured amount of ^{125}I labeled rat albumin to react with the adsorbed antibody and establish an equilibrium. The adsorption of radioactive albumin is a function of the albumin concentration in the unknown. After washing, the adsorbed radioactivity is counted and compared to a standard curve prepared by utilizing known concentrations of albumin.

Using this method the average albumin excretion rate of male and female rats was measured and found to follow a log normal distribution with a mean excretion on the order of 0.1 μg/minute.

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MICROCHEMICAL JOURNAL **17**, 338–346 (1972)

Coulometric Decimilligram Determination of Carbon and Hydrogen in Organic Compounds [1]

KAN-ICHI NAKAMURA, KIKUSHIGE ONO, AND KATSURO KAWADA

Central Research Laboratories, Sankyo Co., Ltd., Tokyo, Japan

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INTRODUCTION

Coulometric applications have contributed greatly to advances in elemental analysis in that decimilligram analysis is possible and the automation of analytical processes is highly promising.

A major contribution was made by Keidel (*5*) who constructed a Pt–P_2O_5 electrolytic cell for the determination of moisture present in gases and liquids. Olson (*14*), Haber (*4*) and Salzer (*16*) applied a Keidel cell to the measurement of the water produced from combustion of samples and succeeded in the simultaneous milligram determination of carbon and hydrogen in organic compounds.

Barendrecht (*2*), Anisimova (*1*) and Chumachenko (*3*) developed modified types of Keidel cell for the microdetermination of hydrogen in organic compounds. In 1967, we constructed various types of Pt–P_2O_5 electrolytic cells for application to the determination of carbon and hydrogen (*8*), hydrogen and nitrogen (*9*) and oxygen (*10*) in organic compounds. We found that these dispersion type cells had no such defects as pointed out by some workers (*1, 4*) wherein the Keidel cell had a tendency to give positive errors which could be ascribed to small amounts of water produced by the recombination of oxygen and hydrogen generated by the electrolysis of the water absorbed on the hygrometer.

On the other hand Oelsen (*13*) first attempted the determination of carbon in steel and organic compounds by the coulometric titration of CO_2. Martin (*6*) succeeded in the microdetermination of carbon and hydrogen in organic compounds by the pulse coulometric titration of CO_2. The present authors applied this pulse coulometric technique to the milli- and decimilligram determination of carbon in organic compounds (*11*).

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Reduction (*7*) has been recognized as the most suitable method for the complete elimination of nitrogen oxides produced in the combustion process. Nevertheless, we applied a tube packed with manganese dioxide for the absorption of nitrogen oxides in a previous study (*11*) and found that manganese oxides varied considerably in activity. The electrolytic quantity of the pulse wave employed previously (*6, 11*) was too large for a precise determination of carbon in decimilligram amounts of organic compounds.

Based on these results, we applied the reduction method to the combustion of samples and tested a simultaneous decimilligram determination of carbon and hydrogen in organic compounds by combining a pulse titration cell and a Pt–P_2O_5 hygrometer.

MATERIALS AND METHODS

APPARATUS

The whole flow system of the apparatus is shown in Fig. 1, which is divided into the following three sections: (a) combustion system; (b) an electronic system for the determination of H_2O; and (c) for the determination of CO_2. All the joints used in the apparatus were glass ball joints (12 mm diam), lubricated with silicone grease.

(a) *Combustion system.* A sample was burned instantaneously in a nitrogen stream with the aid of oxygen donor, Co_3O_4. Nitrogen gas was fed from a cylinder through a pressure reducing gauge, and the flow rate was adjusted to 20 ml/minute or 40 ml/minute with a needle valve.

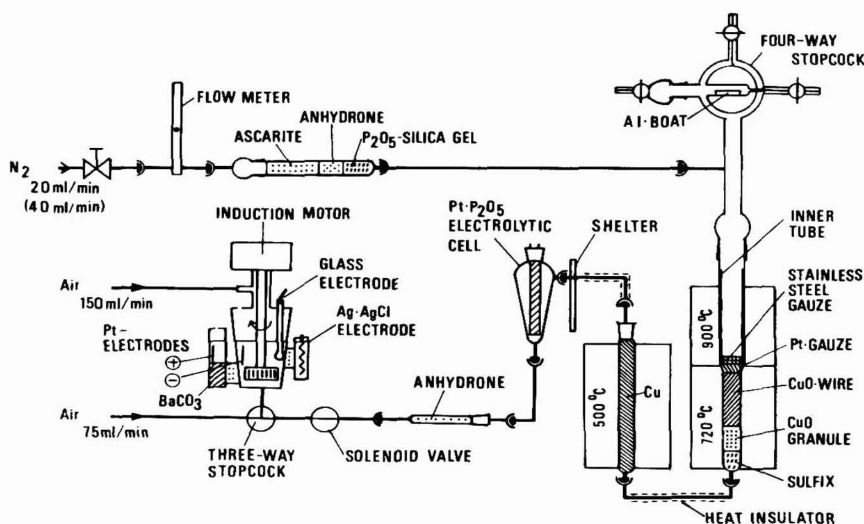

FIG. 1. Schematic diagram of apparatus.

The transport gas was purified by passing through an Ascarite, Anhydrone and P_2O_5–silica gel charged tube before entering the combustion tube.

A quartz inner tube (18 mm o.d.) equipped with a stainless steel gauze at the lower end was inserted in the upper section (19 mm i.d.) of the vertical quartz combustion tube heated at 900°C. A sample boat was dropped into the inner tube from a sample-cock (*12*). In the lower part (12 mm i.d.) of the combustion tube heated at 720°C were packed platinum gauze, copper oxide wires, copper oxide granules and Sulfix (Co_3O_4–Ag mixtures).

The reduction tube (12 mm i.d.) was also a vertical quartz tube charged with reduced copper granules. The copper layer was kept at 500°C in order to reduce any nitrogen oxides produced from samples to molecular nitrogen.

(b) *H_2O measurement system.* The water derived from hydrogen in a sample was absorbed in a Pt–P_2O_5 cell (hygrometer) (*9*). Two platinum wires of 0.27 mm o.d. and 320 cm in length were wound bifilary close to one another on a Teflon rod, 10 mm o.d. and 70 mm in length. This sensing element was coated with a solution of phosphoric acid in acetone. The weight of the coated phosphoric acid was about 95 mg after evaporation of acetone.

The sensing element was mounted in a glass mantle, then the coated phosphoric acid was converted to phosphorus pentoxide by electrolysis. The cell coated with phosphorus pentoxide acted as both water absorbent and electrolyte. The electrolysis was carried out at 30 V from a transistorized power supply. A variable resistance of 1 kohm was connected in series with the cell. The limiting current of 100.0 mA was adjusted by the resistance when the cell potential was zero.

An electronic digital integrator, Hewlett Packard 3370A, was employed for the measurement of the electrolytic current subtracting the basic current. The integration of the electrolytic current was started and terminated automatically by the auto-programming system of the integrator. A recorder, Hitachi QPD 73, was used for recording the maximum electrolytic current.

(c) *CO_2 measurement system.* A pulse titrator, Coulomatic C(*11*) of Kokusai Electric Co., Ltd., was employed. The carbon dioxide derived from the combustion of a sample was passed through the hygrometer, then it was led into a titration cell via a solenoid valve and diluted with CO_2-free air at a flow rate of 75 ml/minute which was introduced from the other mouth of the three-way stopcock. The diluted CO_2 was absorbed into a 5% barium perchlorate solution, cathode electrolyte,

which had been electrolyzed to pH 10 prior to the analysis. When the pH value decreased from the present value with the absorption of CO_2, the gate circuit of the pulse generator was activated and pulse electrolysis generation of OH^- ion was undertaken for the neutralization of CO_2. If the pH of the cathode electrolyte decreased by 0.2 pH units below the end point by a too rapid absorption of CO_2, the meter relay of the pH measuring circuit was activated and the combustion gas flow was turned off by a solenoid valve. Generally the pH value of the electrolyte decreased more than 0.2 pH units due to the CO_2 present in the dead space of the solenoid valve and in the tube between the three-way stopcock and the cell. When by electrolysis, the pH was restored to within 0.2 pH units below the end point, the solenoid valve was opened and the combustion gas flow was led into the cell again. In this way the CO_2 was titrated within a narrow pH range. This sequence was repeated until the content of CO_2 in the combustion gas flow decreased. When the pH value of the electrolyte reached the end point the pulse electrolysis was stopped and the pulses required for the titration was recorded on a pulse counter. The total number of pulses corresponded to the carbon content in a sample.

The interval of pulse generation reached a maximum of 12.5/second (50 Hz) and decreased to 0.5–1/second near the end point. The CO_2-free air flow of 150 ml/minute was applied from the mantle of the stirring rod to expel the air entering the cathode from the narrow gap between the stirring rod and the lid of the cell.

Reagents for the titration cell were as follows:

Cathode solution (absorbent):	
barium perchlorate	5 g
water	100 ml
isopropanol	2 ml
Anode solution:	
barium perchlorate	20 g
water	100 ml
Reference cell solution:	
5% barium perchlorate solution	100 ml
NaCl	2 g

PROCEDURE

A sample (0.3–0.8 mg) weighed in an aluminum boat by a Cahn electrobalance G, was covered with finely powdered Co_3O_4 (300 mg) as an oxygen donor. The boat was introduced into the sample-cock

which was rotated to drop the boat into the inner section of the vertical combustion tube.

Prior to each determination the hygrometer was dried by electrolysis until the electrolytic current fell to 2.0 or 4.0 mA. The counts of the integrator (mV second), peak-area, was correlated with the weight of hydrogen. The maximum electrolytic current (mV), peak-height, was also correlated with the hydrogen content when the carrier gas flow rate was set at 20 ml/minute. By analyzing sucrose (0.3–0.7 mg) as a standard compound, straight calibration curves, hydrogen weight vs. peak-height and peak-area were obtained. The blank value determination was unnecessary for hydrogen analysis.

The hydrogen content was calculated from the following equation in both methods:

$$H\% = \frac{\text{Weight of hydrogen } (\mu g)}{\text{Sample weight } (\mu g)} \times 100.$$

The CO_2 determination was carried out in the pulse titration cell at the same time as the H_2O analysis. One pulse corresponded to about 0.13 μg of carbon by analyzing sucrose as a standard compound. The precise value of the factor must be measured prior to the analyses of samples. The blank value for carbon was determined by running the whole cycle for the carbon determination without a sample.

The carbon content was calculated from the following equation:

$$C\% = \text{factor} \times \frac{\text{No. of pulse (found} - \text{blank)}}{\text{Sample weight } (\mu g)} \times 100$$

Two methods were applied to the simultaneous determination of carbon and hydrogen in the decimilligram samples of organic compounds:

Method (A). The analysis time was less than 15 minutes. The carrier gas flow rate was regulated at 20 ml/minute. The basic current of the hygrometer was fixed at 2.0 mA by the programming system of the integrator. Both the peak-height and peak-area methods were used for the hydrogen determination.

Method (B). The analysis time was less than 7 minutes. The carrier gas flow rate was regulated at 40 ml/minute and the basic current was fixed at 4.0 mA by the integrator. Only the peak-area method was found to be practical for the hydrogen determination.

RESULTS AND DISCUSSION

For a comparison of these two methods, together with the peak-area and peak-height methods, 11 different samples of sucrose (0.4–0.7 mg) were analyzed. The standard deviations ($\sigma\%$) were:

	Carbon	Hydrogen	
		Peak-area	Peak-height
Method (A)	0.15	0.22	0.26
Method (B)	0.30	0.28	—

Analytical results of some other standard compounds obtained by method (A) are shown in Table 1 and those by method (B) are shown in Table 2.

It is clear from these results that method (A) is more precise than method (B) both in the carbon and hydrogen determination, and that the peak area method shows higher precision than the peak-height method for the hydrogen determination.

By applying a very small pulse wave for the CO_2 titration, the drift of the end point potential became negligibly small so that the reset of the end point potential was unnecessary as long as the temperature of the laboratory was kept constant by air conditioning. The factors measured daily were varied from 0.13 ± 0.004 μg of carbon. The blank value of carbon determination was about 10 μg of carbon for each run.

The blank-free method can now be employed for the hydrogen determination by using the extra pure and well-dried oxygen donor, Co_3O_4 powder. However, the calibration curves for hydrogen should be determined every day prior to the analyses of samples. These calibration curves obtained were straight lines under 100 μg of hydrogen.

A new and simple technique was applied to the simultaneous determination of carbon and hydrogen in the decimilligram range for organic compounds by the combination of a Pt–P_2O_5 hygrometer and a pulse titration cell. The continuous run of analysis was made successfully. Automation of this analysis is highly promising by the introduction of an autosampler (15).

SUMMARY

A coulometric method was applied to the simultaneous determination of carbon and hydrogen in the decimilligram amount of organic compounds.

Samples were burned in a nitrogen stream with a rapid combustion system using Co_3O_4 as an oxygn donor. The H_2O and CO_2 produced were swept into a Pt–P_2O_5 hygrometer and a pulse titration cell, respectively, where they were electrolyzed. From the electrolytic quantities required for the measurement of CO_2 and H_2O, the carbon and hydrogen content was determined. The analysis time required was 15 minutes. Satisfactory analytical results were obtained for the analysis of several standard organic compounds. A more rapid method was also developed which required 7 minutes for a sample.

TABLE 1

RESULT OF DUPLICATE ANALYSIS ON STANDARD COMPOUNDS

Compounds	Sample weight (μg)	Carbon (%)			Hydrogen (%) (peak-area method)			(peak-height method)	
		Theory	Found	Diff.	Theory	Found	Diff.	Found	Diff.
Acetanilide	620.8	71.09	70.87	−0.22	6.71	6.59	−0.12	6.72	+0.01
	485.5		70.66	−0.43		6.84	+0.13	7.55	+0.84
Benzoic acid	381.1	68.85	68.92	+0.07	4.95	4.62	−0.33	4.30	−0.65
	551.5		68.83	−0.02		4.70	−0.25	4.79	−0.16
p-Bromoacetanilide	601.6	44.89	44.84	−0.05	3.77	3.89	+0.12	3.66	−0.11
	607.3		44.72	−0.17		3.42	−0.35	3.21	−0.56
Cholesterol	308.2	83.87	84.11	+0.24	11.99	12.09	+0.10	13.27	+1.28
	456.5		83.75	−0.12		12.22	+0.23	11.28	−0.71
2,4-Dichlorophenoxy	414.1	43.47	43.58	+0.11	2.74	2.61	−0.13	2.54	−0.20
acetic acid	666.7		43.05	−0.42		2.58	−0.16	2.43	−0.31
m-Dinitrobenzene	576.0	42.87	42.83	−0.04	2.40	2.33	−0.07	2.37	−0.03
	322.3		42.66	−0.21		2.48	+0.08	2.98	+0.58
o-Iodobenzoic acid	750.6	33.90	33.82	−0.08	2.03	1.70	−0.33	1.74	−0.29
	461.6		34.10	+0.20		1.93	−0.10	1.99	−0.04
Nicotinic acid	480.5	58.54	58.69	+0.15	4.09	4.14	+0.05	4.22	+0.13
	493.0		58.61	+0.07		3.94	−0.15	3.83	−0.26
Sulfathiazole	643.3	42.34	42.48	+0.14	3.55	3.45	−0.10	2.90	−0.65
	886.4		42.38	+0.04		3.52	−0.03	3.37	−0.18
Sulfonal	338.6	36.82	36.87	+0.05	7.06	6.53	−0.53	6.79	−0.27
	683.8		37.21	+0.39		6.89	−0.17	7.65	+0.59

TABLE 2

RESULT OF DUPLICATE ANALYSIS ON STANDARD COMPOUNDS

Compounds	Sample weight (μg)	Carbon (%)			Hydrogen (%)		
		Theory	Found	Diff.	Theory	Found	Diff.
Acetanilide	426.0	71.09	70.70	−0.39	6.71	7.41	+0.70
	424.6		71.58	+0.49		6.71	Nil
Benzoic acid	588.2	68.85	68.59	−0.26	4.95	4.11	−0.84
	670.9		68.73	−0.12		3.94	−1.01
p-Bromoacetanilide	592.8	44.89	45.21	+0.32	3.77	3.96	+0.19
	577.9		44.85	−0.04		3.56	−0.21
Cholesterol	383.3	83.87	83.92	+0.05	11.99	12.39	+0.40
	552.1		83.75	−0.12		11.99	Nil
2,4-Dichlorophenoxy acetic acid	516.1	43.47	43.62	+0.15	2.74	2.81	+0.07
	592.9		43.67	+0.20		2.61	−0.13
m-Dinitrobenzene	573.4	42.87	42.83	−0.04	2.40	2.15	−0.25
	673.5		42.86	−0.01		2.12	−0.28
o-Iodobenzoic acid	490.0	33.90	33.99	+0.09	2.03	2.27	+0.24
	554.9		34.03	+0.13		2.04	+0.01
Nicotinic acid	474.8	58.54	58.58	+0.04	4.09	3.85	−0.24
	611.9		58.52	−0.02		3.46	−0.63
Sulfathiazole	524.0	42.34	42.01	−0.33	3.55	3.59	+0.04
	399.0		42.26	−0.08		3.60	+0.05
Sulfonal	384.1	36.82	36.69	−0.13	7.06	6.56	−0.50
	585.0		37.23	+0.41		7.03	−0.03

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The Coelectrodeposition of Magnesium with Nickel and Determination of Magnesium in Nickel Plate

JOHN T. STOCK AND VINCENT RICCI

Department of Chemistry, University of Connecticut, Storrs, Connecticut 06268

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INTRODUCTION

In view of the great difference in the standard potentials of the Ni^{2+}/Ni and the Mg^{2+}/Mg systems, it is at first sight surprising to find textbook references to the coelectrodeposition of nickel and magnesium (*11, 18, 20*). These references appear to spring from claims first made 70 years ago. The addition of magnesium salts to nickel plating baths had been patented before the end of the 19th century (*1, 15*). Apparently, Coehn and his student, Siemens, were the first to examine nickel deposits thus obtained for the presence of magnesium. In his preliminary report of 1902, Coehn (*7*) gave only sketchy experimental details, but claimed to have made deposits containing up to 10% of magnesium. Two years later, Siemens (*22*) published a fuller account that contradicted the earlier report in some respects. In particular, Siemens concluded that it was not possible to deposit nickel–magnesium alloys containing more than 3% of magnesium.

In 1911, Engemann (*8*) noted that "one could obtain especially hard nickel plate, according to the finding of Coehn and Siemens," and that "0.2 to 0.4% magnesium passed over into the nickel." However, Thompson (*25*) who reexamined the matter in 1925, was unable to detect *any* magnesium in the deposits! Ten years later, Harr (*14*) made deposits under conditions presumed to be those used by Siemens to obtain a reported magnesium content of 1.31%. Harr found the magnesium content to be only 0.03%. Bulakh (*6*) likewise found only small amounts of magnesium in deposits made by him. He concluded that if deposits containing much magnesium can be made, then special conditions which were not described by Coehn (*7*) or Engemann (*8*) are necessary.

In 1959, Geneidy, Koehler and Machu (*10*) described the effect of magnesium sulfate in nickel plating baths that contained boric acid.

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These workers found only from 0.011 to 0.14% of magnesium in the deposits. The present work was initiated with a view to examining the conflicting reports and, if possible, finding the "special conditions" needed to yield nickel deposits that contain sizable amounts of magnesium.

MATERIALS AND METHODS

Equipment. Except in a few cases where a larger vessel was used, the cell shown in Fig. 1 was a 100-ml beaker. The 40 mm high × 25 mm wide (nominal one-side area 0.1 dm^2) plane 33-gauge 95% platinum–5% ruthenium cathode A was welded to a 20-gauge supporting wire. A 25-mm wide strip of 33-gage nickel sheet formed anode B. This strip, initially 240 mm long, was clamped 12 to 13 mm distant from, and parallel to, the cathode, so that a length of 40mm was immersed in the solution. The anode was inspected after each run and, if necessary, the partially dissolved end was sheared off. Saturated calomel electrode (SCE) C, Fisher Microprobe combination electrode system D, and thermometer E were used to monitor the cathode potential, solution bulk pH, and solution temperature, respectively. Electrodepositions were performed with a Harrison Laboratories Model 895A regulated power supply.

Reagents. Solutions were prepared from analytical grade reagents and stored in polyethylene bottles. The distilled or deionized water was repeatedly checked for absence of magnesium. Stock magnesium solution

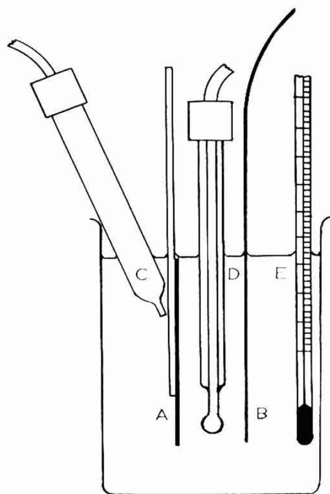

FIG. 1. Electrodeposition cell.

(~ 0.100 mg/ml) was made from the metal by dissolution in hydrochloric acid (1 + 1 v/v), evaporation nearly to dryness, dilution with water, and standardization with 0.0100 *M* EDTA. The pH 10 buffer solution contained 68 g of ammonium chloride and 570 ml of ammonia (sp gr 0.90) per liter.

Preparation of plating solutions. Each plating solution was prepared by the direct weighing into the cell of nickel sulfate ($NiSO_4 \cdot 6H_2O$), magnesium sulfate ($MgSO_4 \cdot 7H_2O$) or magnesium chloride ($MgCl_2 \cdot 6H_2O$), and water. The initial volume of the solution was always 100 ± 0.5 ml and the initial concentrations were 2.0 *M* in magnesium (as sulfate or chloride, as specified) and 0.5 or 1.0 *M* in nickel sulfate.

Electrodepositions. Before each run, the carefully cleaned cathode was weighed. The submersible portion of the anode was rubbed with emery cloth, rinsed with acetone, and wiped. Most of the depositions were carried out at 90 ± 1 °C. When the solution had reached this temperature, the hotplate, which was placed on a jack, was raised so that the electrodes and thermometer were correctly immersed in the solution, then the power supply and the timer were switched on. Except at high currents, very little adjustment of the power supply was needed after the run had proceeded for 2 to 3 minutes. The cell was raised progressively to compensate for fall in the liquid level due to evaporation.

The run was terminated by removing the Microprobe, SCE, and thermometer and then cutting down the current to 0.01 A. After lowering the cell until A and B dipped into the solution to the extent of 2 mm only, the cathode was thoroughly rinsed with a jet of water as the cell was finally lowered away from A and B. The cathode was rinsed with acetone, dried at 105°C for 5 minutes, cooled and reweighed.

Dissolution of the deposit. The reweighed cathode was placed in a 150-ml beaker that contained 100 ml of nitric acid (1 + 1 v/v). Stripping was carried out under gently boiling conditions and was always complete in 30 minutes. The cathode was then rinsed into the beaker and withdrawn. After the solution had been evaporated to approximately 5 ml, 10 ml of hydrochloric acid (1 + 1 v/v) were added and the liquid was carefully evaporated just to dryness. This evaporation was repeated after the addition of a further 10 ml of hydrochloric acid. The residue was taken up in 10 to 20 ml of water and either titrated in the same beaker or (for deposits containing 0.22 g or more of nickel) made up to 50.0 ml, so that aliquots could be titrated.

Titration of magnesium. The procedure was adapted from that used by Langford (*16*) for the determination of magnesium in nickel plating solutions. To 10 to 20 ml of solution containing less than 0.22 g of

nickel, solid potassium cyanide was added to slight excess of that required to redissolve the greenish-blue precipitate. After the addition of 1.5 ml of pH 10 buffer and 1 drop of Eriochrome Black T (1% w/v in triethanolamine), the solution was titrated with 0.0100 *M*. EDTA. The titer was corrected by use of a blank solution that had been carried through all of the workup stages. This correction rarely exceeded 1 drop of titrant (= 0.01 *mg* of magnesium).

RESULTS

The recovery of magnesium was examined by the addition of standard magnesium solution to a nickel solution that had been prepared by dissolution of nickel obtained by deposition from a magnesium-free solution. Results are summarized in Table 1.

A total of 49 depositions of nickel from magnesium-containing solutions was made. The deposits were examined, then dissolved up so that the magnesium could be titrated. Table 2 gives typical data obtained during one of these runs.

Table 3 summarizes the deposition conditions in a series of 23 runs that were made in 2.0 *M* magnesium sulfate–0.50 *M* nickel sulfate, and the results obtained.

In view of the high percentage of magnesium in deposit No. 22, it was hoped that deposits having both satisfactory appearance and high magnesium content might be obtained by decreasing the current density or by increasing the concentration of nickel sulfate. Deposit No. 23, obtained at low current density, certainly had an excellent appearance, but contained very little magnesium. Six 40-minute runs (Nos. 24 to 29) at room temperature (25 to 29°C) were made with a current of 0.1 A and in 2.0 *M* magnesium sulfate–1.0 *M* nickel sulfate. Deposits that contained zero, 0.02, and 0.08%, respectively, of magnesium were smooth and white. The others contained 0.05, 0.06, and 0.10%, respectively, of magnesium, and exhibited vertical black streaks. Depositions under various other conditions were tried, but the plate nearly always

TABLE 1

RECOVERY OF MAGNESIUM FROM NICKEL SOLUTIONS

Nickel (mg)	Magnesium (mg)	Magnesium found (mg)[a]
202	0.39	0.41, 0.40, 0.39, 0.39
210	0.19	0.21, 0.21, 0.21, 0.20
210, 202	0.00	0.01, 0.01

[a] Not corrected for blank.

contained less than 0.1% of magnesium. In general, deposits prepared from all-sulfate (i.e., chloride-free) solutions were either silvery or flawed by black spots or streaks, presumably caused by oxides of nickel, i.e., by "burning." Deposits that were dull or dark but did not exhibit blackening were obtained in all cases when chloride ion was present in an acidic plating bath. Typical examples, deposits Nos. 31 and 32, are shown in Fig. 2.

In another series of experiments, sodium acetate was added in an attempt to hold the pH in the region of 5. In one case (deposit No. 34; Fig. 2), the deposit contained 0.39% of magnesium. This result could not be repeated. A similar run (deposit No. 35; Fig. 2) was made in chloride-containing medium. No magnesium was found in this deposit.

The final set of experiments involved brief deposition from unstirred sulfate solutions that contained both 1% of sodium acetate and a trace of gelatin. The results, summarized in Table 4, indicate that deposits containing more than 0.1% of magnesium can be expected in about four cases out of five. The results at the end of Table 4 illustrate the marked effect of stirring on the magnesium content of the deposit.

DISCUSSION

Determination of magnesium. Disturbing features of the work of Siemens (*21, 22*) are (i) lack of details of the analytical procedures (ii) absence of replicate depositions or of analyses and (iii) absence of information concerning reagent blanks. It is therefore impossible to assess either the precision or the accuracy of Siemens' results.

Apparently, Siemens (*21*) rejected the difficult sulfide separation of nickel in favor of the following. After dissolution of the deposit in

TABLE 2

DEPOSITION FROM 2.0 *M* $MgSO_4$–0.50 *M* $NiSO_4$ AT 90° C AND AT 1.0 A (10 A/dm^2)

Time (minutes)	Cathode potential (mV)	Bulk pH	Deposit mass and appearance[a]	Magnesium found (%) [b]
0	—	5.1	1.003 g	0.11, 0.13,
1	830	—	silver-white,	0.13, 0.14
15	822	—	black stain	
20	822	1.3	at bottom	
45	805	—		
60	790	—		

[a] At termination of run.

[b] Four aliquots of the deposit solution were titrated.

nitric acid, barium (presumably as chloride) was added to test for occluded sulfate. The small amount of iron (contained in the nickel salt used by Siemens) was precipitated by boiling with ammonia and an ammonia salt, then nickel was removed by electrodeposition. After

TABLE 3

SUMMARY OF DEPOSITIONS FROM 2.0 *M* $MgSO_4$–0.50 *M* $NiSO_4$ AT 90° C

Deposit number	Duration (minutes)	Current (A)	Mass (g)	Deposit appearance	Magnesium (%)
1	60	1.0	1.003	silver-white, black stain at bottom	0.13
2	60	1.0	1.031	as No. 1	0.07
3	60	1.0	0.997	Silvery, no black stain	0.19
4 [a]	60	1.0	0.970	as No. 3	0.01
5	60	1.0	1.013	Silver-white, slight blackness on lower edges	0.24
6	60	1.0	1.032	Blackness along bottom edge	0.51
7	12	1.0	0.212	Some blackness at bottom	0.40
8	10	1.0	0.184	Vertical black patches	0.35
9	12	1.0	0.216	Silver-white, only slight backness at bottom edge	0.28
10	12	1.0	0.215	as No. 8	0.41
11	65	0.9	0.985	Silver-white, very slight blackness	0.29
12	65	0.9	0.973	as No. 11	0.22
13 [d]	65	0.9	0.944	as No. 11	0.23
14 [d]	65	0.9	0.978	no blackness	0.11
15	10	0.9	0.162	as No. 8	0.49
16 [a]	8	0.9	0.132	as No. 14	0.01
17	10	0.9	0.159	green inclusions; black on washing	0.40
18	8	0.9	0.124	as No. 14	0.00
19	85	0.7	1.019	as No. 3	0.10
20	85	0.7	0.988	as No. 3	0.07
21	118	0.5	0.975	as No. 3	0.02
22 [b, d]	90	0.1	0.062	nearly all black	2.8
23 [c, d]	480	0.01	0.039	as No. 3	0.05

[a] Solution stirred magnetically.
[b] Temperature, 18° C.
[c] Temperature, 12° C.
[d] See Fig. 2.

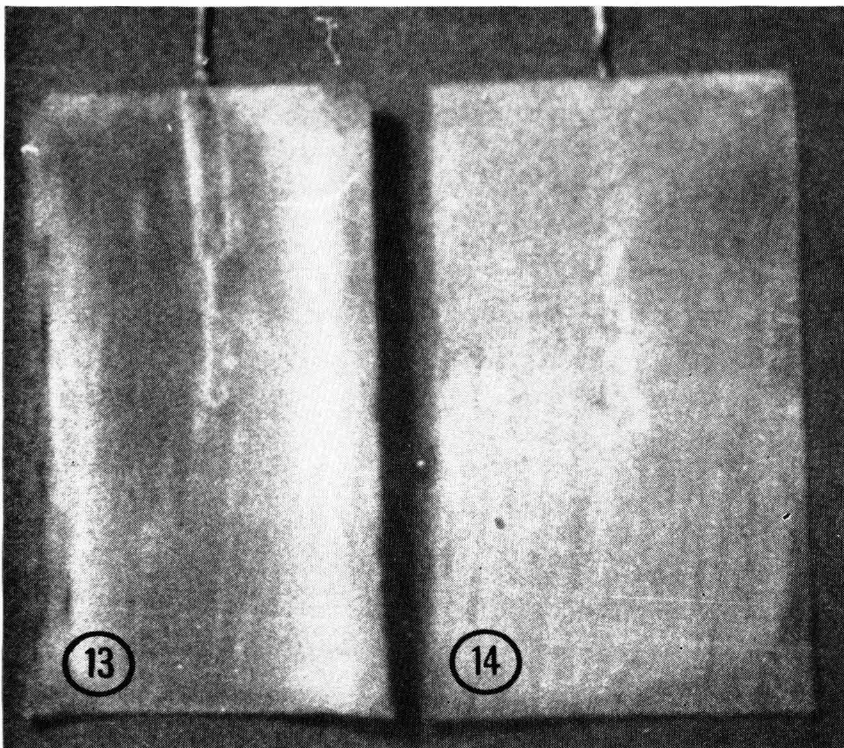

FIG. 2. Deposits obtained from magnesium–nickel solutions. Nos. 13, 14, 22, and 23, see Table 3; Nos. 31 and 32, from 2.0 *M* $MgCl_2$–0.50 *M* $NiSO_4$, 0.9 A, 90°C, 10 minutes; Nos. 34 and 35, see Table 4.

precipitation of excess barium, magnesium was determined gravimetrically. Siemens sometimes had difficulty with the pyrophosphate method and it appears that he determined magnesium as the sulfate merely by evaporating the solution. The comments concerning removing iron, testing for sulfate, and determining magnesium as the sulfate occur in Siemen's dissertation (*21*), but not in his paper (*22*). Thompson (*25*) stated that he verified the accuracy of the method, but he refers only to Siemens' *paper* (*22*), and may have determined magnesium as the pyrophosphate. Engemann (*8*) did not give the method that he used for the determination of magnesium.

It is significant that the modern evidence that the coelectrodeposition of magnesium with nickel is slight is based upon well-established analytical methods such as colorimetry (*14*) and arc spectrography (*10, 14*). The simple titrimetric EDTA method used in the present work has been shown to be adequate for the search for significant amounts of magne-

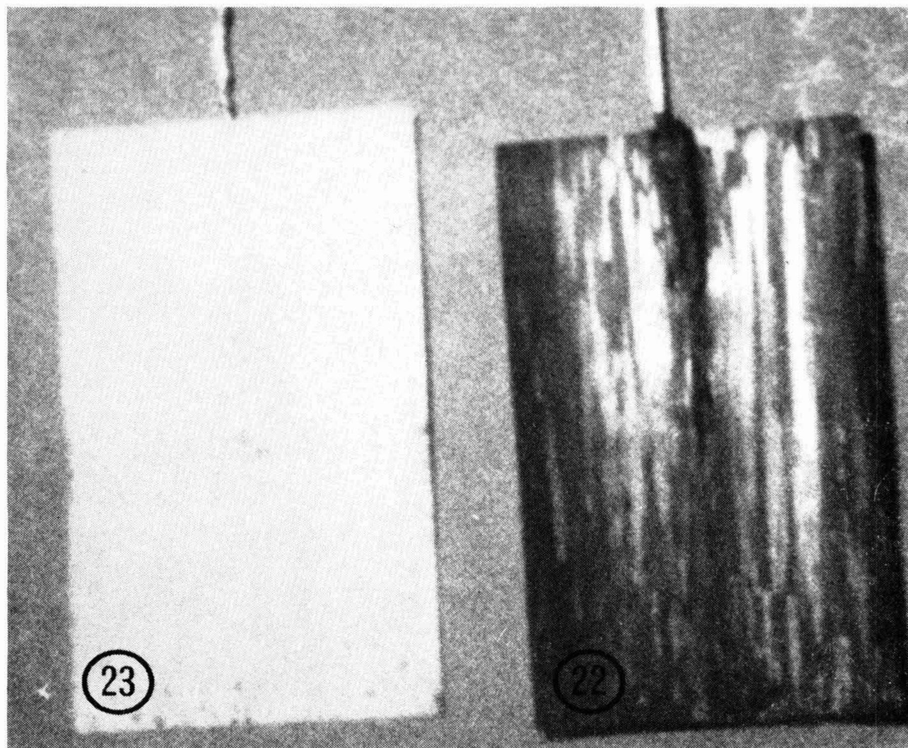

FIG. 2.

sium. Sulfate and sodium ions, likely to be present in plating baths and found by Langford (*16*) to reduce end point sharpness, were absent from the solutions to be titrated.

Factors that influence magnesium codeposition. Siemens (*21, 22*) prepared his plating bath solutions by mixing 50 ml of 8 *N* (i.e., 4 *M*) magnesium sulfate with an equal volume of a specified nickel sulfate solution. This has misled some later workers (*4, 14*), who assumed that Siemens used a magnesium ion concentration as high as 8 *N*. The room-temperature solubility of magnesium sulfate is only 2 *M* (*13*), so presumably Siemens used a hot solution of this compound.

The present work leaves no doubt that significant amounts of magnesium can occur in nickel that is deposited from 2 *M* magnesium sulfate–0.5 *M* nickel sulfate. The highest magnesium percentage, 2.8, was obtained in an unrepeated run at 18°C (deposit No. 22). However, the deposit was black and useless as an example of nickel plating. Percentages in the range 0.3 to 0.5% were found occasionally, but values of less than 0.3% were the general rule. Siemens claimed to have ob-

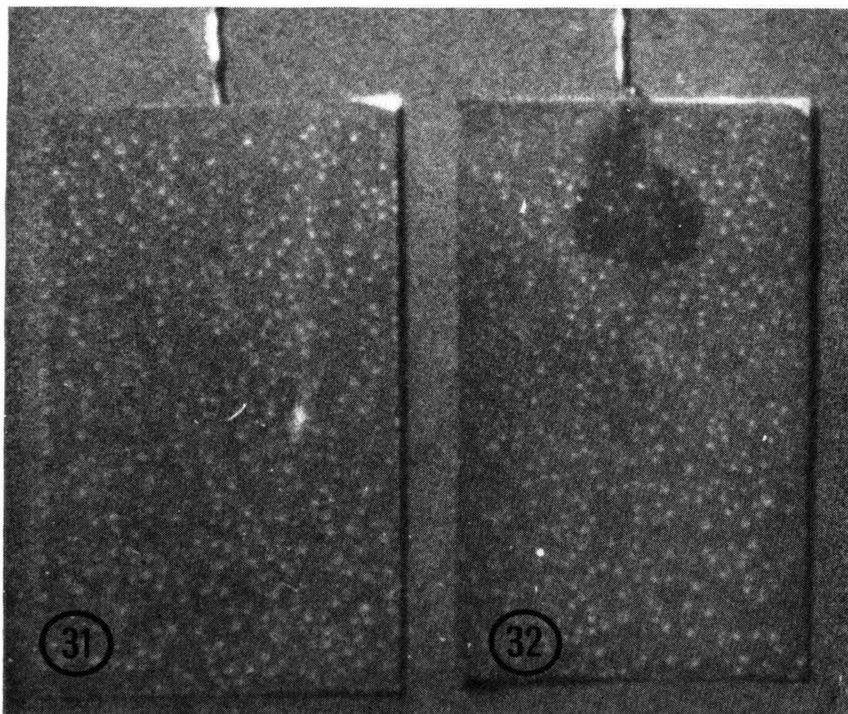

FIG. 2.

tained compact white or bright deposits that contained upwards of 1% of magnesium.

The principal findings of the present work can be summarized as follows.

(i) Blackening often occurs when a nickel deposit contains more than about 0.2% of magnesium. However, deposits that contain 0.1% or less of magnesium can exhibit blackening, especially if the electrodeposition is brief or is carried out at room temperature.

(ii) Deposits from stirred solutions contain much less magnesium than deposits obtained without intentional stirring. The effect of stirring appears to have been ignored by Siemens (*21, 22*).

(iii) Deposits made from unstirred solutions under essentially identical conditions may contain widely differing amounts of magnesium.

(iv) Approximately 0.2-g deposits obtained in short runs often exhibit both blackening and a quite high magnesium content.

(v) The presence of gelatin favors deposits of quite high magnesium content that are fair to satisfactory in appearance.

Several workers (*2, 3, 5, 9, 12, 19, 23*) have shown that during the

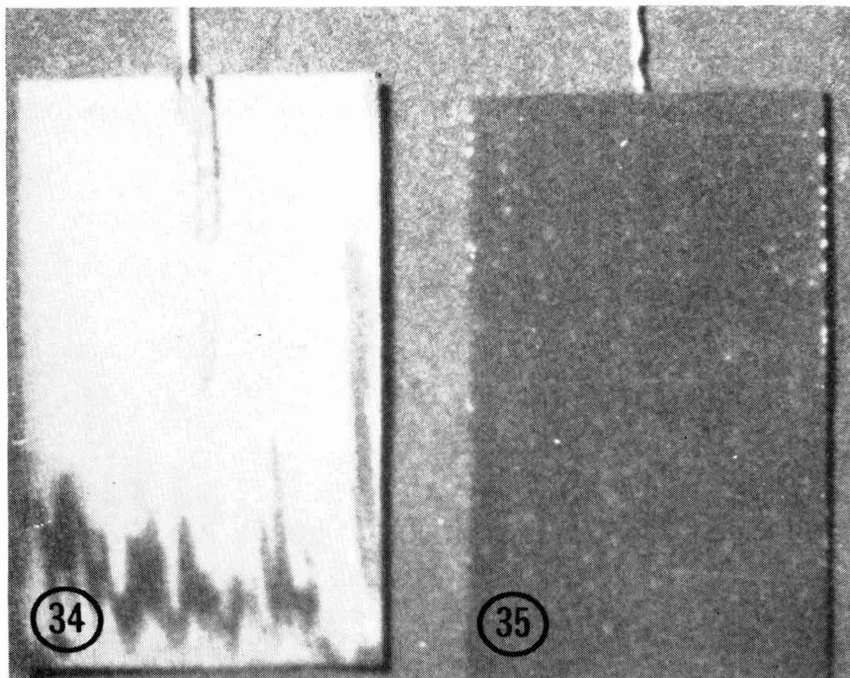

Fig. 2.

electrodeposition of nickel, the composition of the layer of solution immediately adjacent to the cathode may differ considerably from the bulk composition of the solution. For example, the pH of the cathode layer may be as much as 3 units greater than the bulk pH (*5*). Because the solubility product of magnesium hydroxide is only approximately 10^{-11}, this compound will tend to separate if the pH becomes high. Bulakh (*6*) has suggested that the depositions reported by Coehn (*7*) may have been performed under conditions where the formation of colloidal magnesium hydroxide was possible. Occlusion of this compound in the deposit might then occur. Matulis and Slizys (*17*) used dark-field microscopy to show that in their nickel deposition experiments, a cloud of nickel hydroxide appeared around the cathode a few seconds after the beginning of the electrolysis. Impurities in electrodeposited nickel have been attributed to their codeposition with the nickel hydroxide that is formed in the cathode layer (*24*).

It is therefore reasonable to assume that magnesium does not enter the plating as magnesium metal. Entry as magnesium hydroxide, either alone or combined with nickel hydroxide, seems much more likely. This neither precludes nor involves eventual reduction to elemental magne-

TABLE 4

DEPOSITIONS FROM 2.0 *M* $MgSO_4$–0.50 *M* $NiSO_4$ IN THE PRESENCE OF CH_3 COONa AND GELATIN [a–f]

Deposit number	Bulk pH Initial	Bulk pH Final	Deposit mass ([g]) and appearance	Magnesium, %
34 [a, f]	5.5	4.5	0.158. As #7	0.39
35 [a, b, f]	5.0	5.1	0.168. Dull grey, no blackness. As #31	0.00
36 [a, c, g]	5.5	—	0.164. As #11	0.03
37	5.5	—	0.161. As #11	0.31
38	5.6	4.3	0.134. Some black patches	0.37
39	5.7	4.3	0.136. As #38	0.30
40	5.8 [d]	4.2 [d]	0.140. As #14	0.11
41	5.7 [d]	4.2 [d]	0.142. As #11	0.21
42	5.7 [d]	4.2 [d]	0.159. As #14	0.28
43	5.6 [d]	4.2 [d]	0.166. As #11	0.47
44	5.5 [d]	4.1 [d]	0.127. As #11	0.30
45	5.5 [d]	4.1 [d]	0.158. As #14	0.14
46 [e]	5.5 [d]	4.1 [d]	0.151. As #14	0.00
47 [e]	5.5 [d]	4.1 [d]	0.135. As #14	0.00
48 [e]	5.5 [d]	4.1 [d]	0.131. As #14	0.00
49 [e]	5.5 [d]	4.1 [d]	0.152. As #14	0.00

[a–f] Temperature, 90° C. Current, 0.9 A; Time, 10 minutes except where noted.

[a] No gelatin added. Deposits 37 through 39 were obtained after the addition of 100 mg of gelatin. Five mg of gelatin were used in all other runs.

[b] $MgCl_2$ used in place of $MgSO_4$.

[c] Time, 12 min.

[d] Measured at 27° C.

[e] Solution magnetically stirred during deposition.

[f] See Fig. 2.

[g] Current, 0.8A.

sium. Small changes in conditions may greatly affect colloidal phenomena, so that poorly reproducible magnesium percentages may be expected. Magnesium contamination that resulted merely from the simple reduction of a soluble magnesium species should not be particularly sensitive to stirring. The act of stirring should reduce the thickness of the cathode layer in which the pH can differ markedly from the bulk pH. This, in turn, should exert a profound influence upon inclusion phenomena that follow from the precipitation or colloid formation of metal hydroxides. The poorly-appearing high-magnesium deposits that were sometimes obtained in the short-run experiments suggest that contamination of the deposit occurs quite early in the run. Continued deposition, with or without further contamination, may cause the "bad spots" to become covered over by plating.

SUMMARY

Titration with EDTA has been used to determine submilligram quantities of magnesium in electrodeposited nickel. Factors that govern the coelectrodeposition of magnesium with nickel have been examined. Although deposits of satisfactory appearance containing up to approximately 0.4% of magnesium can be prepared, the percentage of magnesium becomes very small if the solution is vigorously stirred during the electrolysis.

ACKNOWLEDGMENT

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Determination of Chromium by EDTA Titration

LÁSZLÓ SZEKERES [1]

Technological Institute, Budapest, Hungary

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The determination of chromium by direct titration with EDTA cannot be accomplished since formation of the chromium(III)–EDTA chelate in aqueous solution is slow even at reflux temperature. On the other hand, after formation of the strongly violet-colored chromium(III)–EDTA chelate is completed at pH 5, the chromium(III) content can be measured by back titrating the excess of EDTA with an appropriate metal salt solution. Due to the great stability of this chelate the back titration can be carried out in a wide range of pH; however, the intense color limits the suitability of this method to the measurement of a few milligrams of chromium only, when indicator dyes are used to signal the end point.

Different metal salt solutions in the presence of various chelatometric indicators have been suggested for the back titration of excess EDTA in the presence of the colored chromium(III)–EDTA chelate: Fe(III) measuring solution with Binschedler's green, or Tiron, or sulfosalicylic acid has been used (*3, 8, 13*); Kinnunen and Wennerstrand employed Mn(II) salt solution and Eriochrom Black T or xylenol orange as indicators (*6, 7*). Furthermore the use of Ni(II) measuring solution in the presence of murexide indicator (*14*), copper(II) salt solution and calcein indicator (*11, 15*), bismuth(III) measuring solution and xylenol orange, or methylthymol blue as indicators (*4, 9*) have been recommended by various authors.

In order to overcome the difficulty caused by the intense color of chromium(III)–EDTA chelate amperometric end point indication has been applied (*2*). Colorimetric (*1*) and spectrophotometric (*12*) methods have also been described for the determination of chromium(III). The latter two methods utilize the intense color of the Cr(III)–EDTA chelate.

Szekeres *et al.* (*10*) utilized the reaction of Cr(III) and MnO_4 for the determination of chromium. The manganese content of the MnO_2 pre-

[1] Present address: 1322 Stockbridge, Kalamazoo, Michigan 49001.

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TABLE 1

DETERMINATION OF CHROMIUM VIA EDTA TITRATION

No.	Present (mg) Cr	Found (mg) Cr	Difference (mg)	Difference (%)
1	8.817	8.694	−0.12	−1.3
2	8.817	8.694	−0.12	−1.3
3	17.634	17.696	+0.06	+0.34
4	17.634	17.696	−0.04	−0.22
5	22.042	22.353	+0.31	+1.4
6	44.084	44.699	+0.61	+1.4
7	44.084	44.491	+0.41	+0.9
8	44.084	44.075	−0.01	−0.02

cipitate formed in this quantitative reaction was measured, after reduction with ascorbic acid, by chelatometric methods.

A new method of end point indication in the back titration of excess EDTA and the use of $Pb(NO_3)_2$ measuring solution comprises the subject of this paper. The method is based on the nearly complete insolubility of $PbCrO_4$ precipitate. A small amount of K_2CrO_4 is added to the solution containing Cr(III)–EDTA chelate, and the excess of EDTA is back titrated immediately with $Pb(NO_3)_2$ solution. The CrO_4^{2-} ions and EDTA do not react, but the first drop of excess Pb(II) causes turbulence of the solution by precipitating yellow $PbCrO_4$. In addition to the determination of Cr(III) this principle of end point indication by $PbCrO_4$ precipitate can be applied to the measuring of any metal ion that gives a stable EDTA chelate at pH 5.

In our experiments solutions of known Cr(III) content were prepared from K_2CrO_4 or $K_2Cr_2O_7$ solutions of known titer, by heating the latter with EtOH in the presence of H_2SO_4. The pH of the chromium(III) solutions obtained in this manner was adjusted to 5 by addition of sodium acetate solution and acetic acid and then EDTA was added. Thus it follows that CrO_4^{2-} or $Cr_2O_7^{2-}$ ions can also be determined by this method after quantitative conversion to Cr(III) as described above.

EXPERIMENTAL

1. *Determination of chromate and biochromate.* An approximately 0.2% solution was prepared from the chromate sample to be measured; 1–50 ml of the above solution was refluxed with 10–15 ml of 1 N H_2SO_4 and 10–15 ml of EtOH for 10–15 minutes. EDTA solution (5–50 ml of 0.02 M) was added to the green solution obtained above, and then the procedure described below for the determination of chromium(III) was followed.

TABLE 2

DETERMINATION OF CHROMIUM VIA EDTA TITRATION

No.	Present (mg) Cr	Found (mg) Cr	Average (mg)	Difference (mg)	Difference (%)	Standard deviation
9	4.408	4.555		+0.14	+3.18	
10	4.408	4.399		−0.01	−0.22	
11	4.408	4.399		−0.01	−0.22	
12	4.408	4.399		−0.01	−0.22	
13	4.408	4.451		+0.04	+0.88	
14	4.408	4.451		+0.04	+0.88	
15	4.408	4.430		+0.02	+0.44	
16	4.408	4.409		0.00	0.00	
17	4.408	4.388	4.4312	−0.02	−0.44	±0.05 mg
18	1.763	1.840		+0.08	+4.53	
19	1.763	1.778		+0.01	+0.56	
20	1.763	1.820		+0.06	+3.40	
21	1.763	1.768		0.00	0.00	
22	1.763	1.778		0.00	0.00	
23	1.763	1.778		0.00	0.00	
24	1.763	1.757		−0.01	−0.56	
25	1.763	1.861	1.7975	+0.10	+5.67	±0.035 mg
26	0.8817	0.899		+0.02	+1.13	
27	0.8817	0.899		+0.02	+1.13	
28	0.8817	0.859		−0.02	−1.12	
29	0.8817	0.848		−0.03	−1.70	
30	0.8817	0.859		−0.02	−1.13	
31	0.8817	0.910		+0.02	+1.13	
32	0.8817	0.900		+0.02	+1.13	
33	0.8817	0.879		0.00	0.00	
34	0.8817	0.848	0.87788	−0.03	−1.70	±0.023 mg

2. *Determination of chromium(III).* Solutions containing 0.5–1 mg Cr(III)/ml were used at best in these measurements. To 1–50 ml of Cr(III) solution was added 10–15 ml of 1 M acetic acid, 5–50 ml of 0.02 M EDTA and 10–15 ml of 1 M sodium acetate solution, and the mixture was heated on a steam bath for 15–20 minutes and then cooled to room temperature. The solution was diluted to 100–200 ml and 2–3 ml of 5% K_2CrO_4 was added. The orange solution was then titrated with 0.02 M $Pb(NO_3)_2$ solution until precipitate appeared. The titration was finished slowly, thus allowing the small excess of EDTA still present in the final stages of the titration to dissolve the $PbCrO_4$ precipitate. The end point is indicated by turbulence due to irreversible precipitation of $PbCrO_4$. Results of typical experiments are shown in Tables 1 and 2.

SUMMARY

EDTA, acetic acid and sodium acetate are added to a solution of chromium(III) ions. Formation of violet chromium(III)–EDTA chelate is completed after heating on a steam bath for 15–20 minutes. The excess of EDTA is titrated with $Pb(NO_3)_2$ solution in the presence of K_2CrO_4. The end point is indicated accurately by precipitation of $PbCrO_4$. The use of K_2CrO_4 as an indicator is applicable to the determination of any metal ion that forms a stable chelate with EDTA at pH 5.

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Book Reviews

Pesticides Identification at the Residue Level. Edited by FRANCIS J. BIROS. Advances in Chemistry Series 104, American Chemical Society, Washington, D.C., 1971. ix + 182 pp. $8.50.

This monograph reports the papers presented in a symposium sponsored by the Division of Pesticide Chemistry of the ACS at the Joint Conference of the Chemical Society of Canada and the American Chemical Society, held in Toronto, Canada, in May 1970. Microchemists will indeed be interested in this book; the title is deceptive, for it is really a monograph on microchemical techniques for the analysis of organic compounds. It covers a large amount of information, with an outstanding listing of references at the end of each chapter to current literature; the index is quite comprehensive. Readers of the book will find that it has a wealth of practical ideas and suggestions for avoiding both pitfalls in procedure and misinterpretation of analytical results. It is not a "cookbook" of directions for pesticide identification but a survey of the general techniques of microanalysis.

Major areas discussed in the book include the philosophical aspects of microanalysis, instrumental techniques, microchemical methods and biological assay methods; most detail is placed on the instrumental techniques.

The titles of the 11 chapters follow: 1. Possible Limits of Ultramicro Analysis; 2. Chemical Derivatization Techniques for Confirmation of Organochlorine Residue Identity; 3. A Review of Enzymatic Techniques Used for Pestcide Residue Analysis; 4. Flame Detectors for Residue Analysis by GLC; 5. Gas Chromatographic Measurement and Identification of Pesticide Residues with Electron Capture, Microcoulometric, and Electrical Conductivity Detectors; 6. Infrared Microtechniques Useful for Identification of Pesticides at the Microgram Level; 7. Ultraviolet Spectrophotometry in Residue Analysis; Spectra–Structure Correlations; 8. Past, Present, and Future Application of Paper and Thin-Layer Chromatography for Determining Pesticide Residues; 9. Applications of Combined Gas Chromatography–Mass Spectrometry to Pesticide Residue Identifications; 10. The Identification of Pesticides at Residue Concentrations; 11. Analysis of Pesticide Residues: Immunological Techniques.

All of the chapters are excellent; microchemists, however, will probably be particularly interested in the following chapters; Chapter 1, which surveys the problem of ultimate sensitivity at ultramicroanalysis, and particularly relates to the advantage of combining mass spectrometry in conjunction with gas chromatography for unambiguous identification; Chapter 4, for a discussion and comparison of flame ionization, flame photometric and alkali flame detectors in gas chromatography; and Chapter 5 for a comparison of electron capture, microcoulometric and electrical conductivity detectors. Chapter 6 is extremely useful on the extension of IR techniques to microgram quantities of material. It presents a survey of the techniques for this work and gives certain comparisons on the results obtained by different methods such as the multiple internal reflectance technique and the micro-KBr-pellet method. Chapter 7 has an outstanding general coverage

on the relationship of chemical structure and ultraviolet absorption. Chapter 8 performs a corresponding function for thin layer and paper chromatography. The book is highly recommended.

PETER F. LOTT, *Department of Chemistry, University of Missouri-Kansas City, Kansas City, Missouri 64110*

Analytical Metabolic Chemistry of Drugs (Medicinal Research Series, Vol. 4). By JEAN L. HIRTZ, English translation (from the French), edited by EDWARD R. GARRETT. Marcel Dekker, Inc., New York, 1971. xvii + 395 pp. $24.50.

This excellent book, an "Americanized" English translation of the French edition which appeared in 1968, purports to be a first monograph (Medicinal Research Series, Vol. 4) on the analytical chemistry of drug metabolism. However, it is not a treatise on drug metabolism (it considers the metabolic reaction only when it is necessary for the understanding of the analytical methods), nor is it a specialized treatise on analytical chemistry. Thus, instead of beginning with and passing through a fundamental illustration of the theories and disciplines of analytical chemistry, the book emphasizes particularly the practical aspects of analytical procedures for drug metabolites in the whole text. It comprehensively surveys the relevant literature and outlines the physicochemical methods, instrumental methods, and analytical techniques that have been employed to identify and determine the metabolic products of drugs. Besides, the book also clearly analyzes and critically discusses how the various analytical methods can be selectively and effectively applied to separate, purify, identify, characterize, and quantitatively determine the minute amounts of metabolites and the unchanged parent drugs in the complex biological systems (such as blood plasma, tissue, urine, and the other biological fluids, etc.).

The text of this book constitutes 20 chapters. Each chapter discusses a class of drugs on the basis of their chemical similarities or relationships and ignores the difference in their activity or pharmacological utility. A well-written section of "Introduction" serves as a concise guideline for using the book. The extensive bibliography contains an alphabetical listing of 1044 references which cover the literature from 1883 to January 1, 1967. An author index which contains underlined numerals referring to the pages in the text on which the author's name appears and Roman numerals indicating the reference numbers in the bibliography section on which the reference has been contributed by the corresponding author is provided. A subject index involves all empirical formula names of drugs is also available for easy access to the textual discussion.

In the 20 chapters of the text, approximately 350 drugs and 650 metabolites, classified into 20 categories, have been examined. The discussion of these categories thus covers the materials: phenolic acids and derivatives; amines; aminophenols and catecholamines; phenothiazines; dibenzazepines and benzodiazepines; carbamates; anilides; barbiturates; derivatives of urea, guanidine, and others; sulfonamides; imides; hydrazides and hydrazines; heterocycles with one nitrogen; heterocycles with two nitrogens; heterocycles with more than two nitrogens; heterocycles not containing nitrogen; alkaloids antibiotics; glycosides; and miscellaneous drugs (included are sulfur-containing drugs, metal-containing drugs, halogenated drugs, and miscellaneous compounds) etc. Throughout

the book the treatment of subject matter is comprehensive and logical, and places an emphasis on the special techniques for separation of the complex mixture of metabolites. This sort of effort is very useful, since the elimination of the influence of the interfering species in the biological mixtures is the vital part of the analytical operation. Four typical figures showing the complete procedures for separation of the complicated urinary metabolites (that is, adrenaline and metadrenaline, chlorphenesine carbamate, thalidomide, and isoniazid metabolites, etc.) are given for a quick suitable reference. Almost all modern analytical techniques are applied, including various forms of chromatography (such as thin-layer, paper, column, gas-liquid chromatography), different types of spectrophotometry (uv and ir), spectrometry (X-ray and NMR), radioactive isotope labeling, electrophoresis, solvent extractions, and separations. In addition, the following information: 355 different structural formulas of drugs; values of R_f (for indication of the sensitivity of chromatographic separations), t_r (retention time of glc), electrophoretic mobility, λ_{max} (of UV spectrum), and percentage of total radioactivity, etc. for a great number of drugs and their metabolites tabulating in 70 tables; is also presented. This is the wonderful format that Dr. Hirtz, presents his comprehensively surveyed and well-compiled materials in this "Analytical Metabolic Chemistry of Drugs."

Typographically the book is excellent, the translation is clear and lucid, and the book is easily and pleasantly readable. Paper, printing, and cloth binding are of good quality. Both author and translator are to be sincerely congratulated on their great success of having done such excellent jobs. Although this book is intended primarily for the analyst, owing to the appraisal of the modern drug action, it will also be an invaluable reference source book for drug synthesizers and dosage designers. This book is highly recommended for all those engaged in the study of drug metabolism—bioanalytical chemists, biochemists, biopharmaceutical scientists, hospital pharmacists, pharmacologists, toxicologists, and others as a most useful reference book. However, because of its relatively high price the volume would appear rather expensive for placing on the individual desk as a private ownership, but it should be unconditionally added to the library shelves.

GEORGE W. C. HUNG, *Materials Science Toxicology Laboratories, The University of Tennessee, Medical Units, Memphis, Tennessee 38103*

Modern Practice of Liquid Chromatography. Edited by J. J. KIRKLAND. Wiley-Interscience, New York, 1971. ix + 454 pp. $14.95.

This book is a compendium of writings on the various techniques of high-speed liquid chromatography. Contributing articles were provided by a group of authors representing a spectrum of disciplines within the physical and life sciences. Each person is an expert in the field of chromatography.

The book, which is based on a course held in 1970 under the auspices of the Chromatography Forum of Wilmington, Delaware, is divided into three broad categories: fundamentals, practical aspects and applications. The initial chapter, entitled, "The Relationship of Theory to Practice in High-Speed Liquid Chromatography," describes the fundamental mathematical equations which govern liquid chromatography. Anyone with a limited mathematical background easily can understand the contents of this lucid presentation.

The next few chapters contain detailed descriptions of the basic equipment which is required to perform high-speed liquid chromatography. (Several com-

mercially available systems also are described.) Each of the components of the system including pumps, sample introduction devices, columns, column packings, ovens, fraction collectors and detectors is treated in a logical, stepwise manner. A section on data acquisition also is included.

Some obvious laboratory saftey hazards in the handling of solvents and toxic compounds are described. Guidelines are suggested for proper containment of these materials.

Also included within the category of fundamentals is an in-depth treatment of the physicochemical properties of solvents and of how they influence the separation of compounds.

The next major category contains articles dealing with the practical aspects of liquid chromatography. The first chapter in this section is written by the editor of the book. It is clearly and concisely written. All physical and chemical factors which affect column separations, including column supports, column dimensions, and column preparation are treated in detail.

Other chapters on the practical aspects of liquid chromatography contain dissertations on adsorption, gel permeation, and ion-exchange chromatography. Some general applications for each of these techniques are included.

The final category of the book consists of three chapters on the application of high-speed liquid chromatography to separation problems. The first describes general mechanisms which control separations in adsorption, partition, gel permeation and ion-exchange chromatography. The next-to-last chapter contains specific chromatographic procedures for fractionation of a wide variety of classes of compounds ranging from analgesics, pesticides, and antibiotics through alkaloids.

The final chapter provides some applications of high-speed liquid chromatography in biomedical research. Specific procedures are furnished for the separation of mixtures of various kinds of nucleic acids. Also included is a brief discussion of an exciting new application of this technique to the separation and identification of the ultraviolet absorbing constituents of human urine.

An appendix of mathematical symbols is listed at the end of the book.

The references in each chapter were up-to-date.

The editor has "attempted to foster presentations which emphasize the teaching aspects." He has been eminently successful in achieving this goal. Anyone who has a limited knowledge of the subject of high-speed liquid chromatography should be able to use this analytical technique after reading the book. It will have appeal to scientists in all disciplines of research.

In summary, each of the articles in the book was well written and the material was well integrated. Experimentalists who are contemplating the use of liquid chromatography should seriously consider procuring the book. All libraries should own this outstanding treatise.

IRWIN L. SHAPIRO, *J. T. Baker Chemical Company, Phillipsburg, New Jersey 08865*

Crystal Growth in Gels. By HEINZ K. HENISCH. Pennsylvania State University Press, University Park, Pa., 1970. 111 pp. $6.95.

Growing large single crystals in gels is not new but it is so simple. No elaborate equipment is necessary. Aside from their value in research, the formation of such specimens is an attention-catching demonstration for high school students and college freshmen. It is also a relaxing hobby. This past decade

has seen a renaissance of the art and Dr. Henisch's book presents clear and precise directions for its practice along with an admirable review of past work.

There are five chapters, the first summarizing the history of the techique and giving basic growth procedures. These include specific directions for the preparation of hydrogels. Commercial waterglass, or (preferably) reagent grade sodium metasilicate, or agar, gelatin, or any other gel, can be used. Much depends on the type of chemical reaction producing the crystal. The next two chapters present, respectively, lucid, comprehensive discussions of gel structure and properties; and of growth mechanisms and characteristics. The fourth is concerned with nucleation; and the last chapter discusses some problems that have been solved and others that have not. The illustrations, of which there are many, can only be described as beautiful. The index is adequate and the bibliography contains 182 references.

This book is highly recommended for anyone interested in crystals whether for research, as a personal hobby that can be pursued in the kitchen (retirees take note) or as demonstration to interest young people in the beauty of science. For this latter purpose, it can be used either as a supplement to Holden and Young's "Crystals and Crystal Growing" [reviewed in *Microchemical Journal* **5**, 632, 1961] or as a simpler alternative.

David B. Sabine, *484 Hawthorne Avenue, Yonkers, N.Y. 10705*

Advances in Optical and Electron Microscopy. Vol. 4. Edited by R. Barer and V. E. Cosslett. Academic Press, London, 1971. xi + 424 pp. $21.50, £7.50.

The seven articles of this volume provide an impressive demonstration of the complexity, sophistication, and power of modern microscopy. While intricate electronics are not a surprising requirement for the electron microscopy laboratory, this book shows that attainment of the full capabilities of the optical microscope also requires complicated and expensive instrumentation. These two branches of microscopy are not always separate and distinct, and advances in one branch frequently nourish and improve the other.

The first article, "The Optical Transfer Theory of the Electron Microscope," is a compact mathematical treatment of the theory of image formation in electron microscopy. In spite of some errors in numerical examples (a wrong sign and an inaccurate calculation on page 7), the article appears to be authoritative and thorough.

There is a pair of articles on "Image Processing for Electron Microscopy." The first of these, "I. Enhancement Procedures," shows how results may be improved by the use of scanner equipment and a digital computer. Examples are given of moon photographs and of electron micrographs of catalase, and these show the improvements obtained as the computer applies various corrections. The companion article ("II. A Digital System") treats the specific details of a digital processing system, with the facilities of the Jet Propulsion Laboratory serving as a model.

Seven authors have collaborated to produce a 101-page chapter, "Mirror Electron Microscopy." The "mirror" in this technique consists of an electrostatic field used to deflect an electron beam near the surface of a specimen. The electron trajectories are highly sensitive to variations in the specimen surface,

and an image of the surface can be constructed. This article could be a sufficiently complete monograph itself, and it covers the mathematical background of its subject, the design of the instrument, and some results and applications.

Another long article (98 pages) is on "Energy Analysing and Energy Selecting Electron Microscopes." These instruments produce the electron microscopy analog of color photographs. The electron beam loses energy by plasmon excitation, by single electron interactions, or by phonon excitations. The fractional energy loss is typically of the order of 0.1%, but information about the processes involved may be derived from an energy loss spectrum. In the energy analyzing electron microscope, a line in the image is subjected to energy analysis. The energy selecting microscope first disperses the electrons into various energy components, and an image of the specimen is formed from one component. The article discusses the principles, the instrumentation, and the applications of each method.

The shortest chapter in the book (23 pages) is on "The Quantimet Image Analysing Computer," which uses an image plane scan and a television system to analyze microscopic images. The Quantimet, which was designed for metallurgical applications, may be used on either electron or optical microscopes. It provides speed, flexibility, and convenience, at the expense of some resolution and discrimination.

The final article, "Photomicrography and its Automation," describes methods by which instrumentation may be used to obviate the limits of visual observation and permit direct photography up to 3000×. Completely automatic equipment determines and controls the exposure time, takes the photograph, and advances the film.

The writing in this volume occasionally lapses into such constructions as the doubly superlative "most predominant." Generally, however, the presentations are clear, the illustrations are of excellent quality, and the proofreading has been carefully done ("electrostic" is one that was missed). The articles were written by experts, and they are for experts, but they may all be recommended for the enlightenment and enjoyment of all optical and electron microscopists.

DONALD E. SANDS, *Department of Chemistry, University of Kentucky, Lexington, Kentucky 40506*

Crystals and the Polarising Microscope. 4th ed. By N. H. HARTSHORNE and A. Stuart. American Elsevier, New York, 1970. xi + 614 pp. $29.50.

This book, in its 4th edition, is an advanced work describing crystals, crystalline forms, stereographic projection, the morphology of crystals (including the 32 crystal classes or point groups with excellent illustrations), and the optical properties of crystals. A complete description of the polarising microscope, the methods of sample illumination, the mounting of crystals for microscopic observation are well presented. The methods of microscopic examinations include the use of parallel and convergent light, the measurement of sizes of crystals, the determination of isotropism, and the measurement of extinction angles. Special methods, such as phase contrast illumination, are described to determine the refractive indices of crystals. Hot and cold stages and their applications to microscopic examination of materials are discussed in a separate chapter. The last three

chapters are on chemical and industrial applications, liquid crystals, and polymers and biological materials, respectively.

The appendices tabulate the major manufacturers of good polarising microscopes, some optical-crystallographic studies on *classes* of organic compounds (with references), and some sources of optical-crystallographic data. An author index and a very good subject index round out the volume. The book represents a comprehensive work, through the 4th edition, to the serious worker in microscopy. It is very well written; the print is large and clear. Because of its price, and because of its limited appeal, copies of this text will not be found in everyone's library; but, this text is strongly recommended for those whose work involves microscopy, and as a reference text for industrial and school libraries.

ROGER A. LALANCETTE, *Rutgers University, Department of Chemistry, Newark, New Jersey 07102*

Organic Electronic Spectral Data. Vol. VII. Edited by JOHN P. PHILLIPS, JOSEPH C. DACONS, AND R. G. RICE. Wiley, New York, 1971. XV + 1318 pp. $40.00.

The data for Volume VII of this series, covering the years 1964–1965, were assembled by a page by page search of approximately 100 journals.

The presentation of uv data is tabular. The first column consists of molecular formulas and names of the organic compounds; the sequence and nomenclature both are in accordance with Chemical Abstract practice. The second column designates the solvent and pH. Wavelength values are given in the third column for maxima, shoulders, and inflections together with corresponding molar absorptivities. References are coded in the fourth column, and a complete list of references is given at the end of the volume.

This series is accepted as a prime source for uv data. Once again the gratitude of all chemists to the 50 or so dedicated contributors is acknowledged.

R. M. SILVERSTEIN, *State University College of Forestry, at Syracuse University, Syracuse, New York 13210*

Encyclopedia of Industrial Chemical Analysis. Volume 14. Edited by FOSTER DEE SNELL and LESLIE S. ETTRE. Wiley (Interscience), New York, 1971, xiv + 616 pp. Each volume $45.00 ($35.00 by subscription).

This reviewer has now assessed some six volumes of this encyclopedia for *Microchemical Journal*. This compendium continues a "must buy" for chemistry-oriented libraries and especially those in industry.

Volume 14 continues the treatment of individual products and groups of products from gold (and its alloys and compounds) through iodine (and its compounds). Intermediate topics include graphite and related carbons, gypsum and gypsum products, hafnium and zirconium (and their alloys and compounds), halohydrins, hormones, hydrazine (and alkyl derivatives), hydrides, hydrocarbon-resins, hydrocarbons, hydrochloric acid, hydrogen, hydrogen chloride, hydrogen cyanide, hydrogen peroxide, hydroxylamines and their salts, imines, indigoid dyes, indium (and its alloys and compounds), and insulin.

The 50-page article on hafnium and zirconium (placed under 'H' by reversal

of the conventional order of the two metals) provides a well-balanced treatment of the determination of these elements and the analysis of the metals, alloys, and compounds; some 112 papers and monographs are cited. The 26-page article on halohydrins includes information and procedures for their general assessment based on the halogen function (neutron activation, argentometric titration after hydrolysis, and quartz tube combustion and mercurimetric titration of absorbed chloride) and the hydroxyl function (infrared, paper chromatography, derivatization, and acetylation); additionally specific methods are considered for some six commercial chlorohydrins.

The excellent 52-page article on hormones, which is supplemented by a 39-page article in Volume 10 on estrogens and progestogens, written by S. Ahuja of CIBA-Geigy Corporation presents in concise fashion a tremendous amount of information on the analytical chemistry of these physiologically important substances. R. C. Vollmar in drafting his 56-page article on hydrocarbons must have faced agonizing decisions as to what could be treated in the available space and the difficulty of providing a fully organized presentation when related information is presented elsewhere in earlier articles on natural gas, gasoline, and liquified petroleum gases.

This volume, as previous ones, has an abundance of tables, many presenting physical properties and specifications for key chemicals, and of figures and illustrations. Infrared spectral curves, largely taken from the files of Sadtler Laboratories, are provided in generous number and in the present volume to the extent of over 33.

This volume accomplishes what it is expected to do; this encyclopedia is accomplishing what it set out to do.

A. J. BARNARD, JR., *J. T. Baker Chemical Co., Phillipsburg, New Jersey 08865*

Cyclic AMP. By G. A. ROBISON, R. W. BUTCHER, AND E. W. SUTHERLAND, Academic Press, New York, 1971. xi+531 pp. $17.50.

This monograph is an account of the discovery, chemical and biochemical properties and numerous physiological functions of the "second hormonal messenger" molecule, cyclic adenosine-3′, 5′-monophosphate, cyclic AMP. The current interest and continuing importance of the topic was underlined recently with the awarding of the Nobel Prize in Medicine for 1971 to Professor Sutherland for his discovery and far-reaching contributions to the understanding of the incredibly complex functions of this molecule.

The second messenger concept implies that "the first messenger carries the required information to the cell, and the second messenger transfers this information to the cell's internal machinery" (Sutherland). This volume describes both the action of the large variety of hormones (first messengers) which, in response to some outside stimuli, can activate the enzyme adenyl cyclase responsible for synthesizing cyclic AMP from ATP, and the results of the created level of cyclic AMP translated into physiological responses.

Those actively pursuing research in any of the areas related to the function of cyclic AMP will find this volume indispensable (references dated 1972 appear in abundance).

Among other topics can be found the chemistry and derivatization of cyclic AMP (by Professor Th. Posternak); the formation and metabolism of cyclic AMP

(employing the two enzyme systems adenyl cyclase and phosphodiesterase, respectively); the effect of glucagon and insulin as well as of a host of other hormones on cyclic AMP levels; the relationship of cyclic AMP levels to the mediation of the effects of catecholamines (including the mediation of the lipolytic action of catecholamines); the role of cyclic AMP in lower organisms. There is also a chapter summarizing the possible role of other cyclic nucleotides (especially cyclic GMP) as second messengers (contributed by Dr. J. G. Hardman). Finally, there is an excellent Appendix with a detailed description of the assay systems (particularly the one employing dog liver phosphorylase activation by cyclic AMP) available for the detection of cyclic AMP levels. It is to the credit of the researchers in this field that cyclic AMP concentrations in the nona and picomolar ranges now can be routinely determined.

This reviewer finds it most admirable that the book also serves an important instructional function in pointing out the areas of research in this field which need further exploration.

FRANK JORDAN, *Rutgers, the State University, Newark, New Jersey 07102*

Thermomicroscopy in the Analysis of Pharmaceuticals. By MARIA KUHNERT-BRANDSTÄTTER. International Series of Monographs in Analytical Chemistry. Volume 45. Pergamon, London/New York, 1971. 409 pp. $28.50.

This book, while dedicated to the identification and characterization of organic compounds of pharmaceutical interest, also satisfies the long felt need for an up-to-date, English language guide to the well-known Kofler techniques. The book is very well written and amply illustrated with line drawings and photomicrographs, many of them in color. The tables are clear, uncrowded, and arranged in order of increasing melting temperature. An alphabetical listing is also included.

Of the first 18 chapters, which discuss the techniques used in thermomicroscopy, the section on polymorphism is of particular value. It is stated that of the barbiturates used medicinally, about 70% are polymorphic; phenobarbitol alone being able to exist in 11 different modifications. Likewise, many of the steroid hormones and sulfonamides can also exhibit polymorphism depending on their previous history. The detection of pseudopolymorphism and solvation is also discussed.

A new addition to the usual Kofler type information is the use of characteristic infrared and ultraviolet absorption maxima and minima.

The tables, listing over 1,000 substances, include the name of the compound, its formula weight, melting temperature, eutectic temperature when mixed with reference compounds, the refractive index data, its ultraviolet maxima and minima, and pertinent remarks on the behavior upon melting, recrystallization, etc.

A chapter on the use of the Kofler Hot Bench, together with identification tables to accompany its use is included.

This book is a "must" for any student of microscopy or pharmacy, and for anyone interested in the characterization of pharmaceutical materials.

HOWARD J. FRANCIS, JR., *Pennwalt Corporation, King of Prussia, Pennsylvania 19406*

Announcement

International Symposium on Microchemical Techniques—1973

"Progress and Projections for Microchemistry," will be the general theme for the International Symposium on Microchemical Techniques—1973. The symposium will be held at The Pennsylvania State University, University Park, Pennsylvania, on 26 August to 31 August 1973 and will be conducted by The American Microchemical Society.

The scientific program will consist of sessions dedicated to topics of current interest, general papers, discussion groups, practical demonstrations, an equipment exhibit, and will also include a number of instructional workshop sessions. Special sessions will be included on such topics as:

Automated Elemental Analyzers—Ten Years Later
Computers in Elemental Analysis
Organic Elemental Analysis: New Methods and Equipment
Environmental Microanalysis: New Sensors and Techniques
Microelectrodes
Forensic Analysis: Narcotics and Drugs of Abuse
Organic Functional Group Analysis: New Directions
Electroanalytical Advances, including Ion Selective Electrodes
Microscale Separations: Advances in Techniques and Methods
Standards and Standardization for Microchemistry and Microanalysis
Trace Analysis: Advances in Organic and Inorganic Analysis
New Techniques in Microchemistry

Persons interested in presenting a paper under any of the above topics, or a paper on the general topic of microchemistry, should submit their paper to:

Mr. Howard J. Francis, Jr.
Pennwalt Corporation
900 First Avenue
King of Prussia, Pennsylvania 19406, U.S.A.

Included in the program of scientific presentations will be classroom workshops on the topics of:

Applications of Ion Selective Electrodes
Theory and Applications of Thermal Methods of Analysis

A number of semitechnical and social events are planned, an intro-

ductory evening mixer, a banquet, a social evening, an evening demonstration of gadgets, and an evening session on "The Art of Presenting a Paper."

Ladies and families are welcome to attend. A separate program is planned for the ladies as well as for the children.

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A later announcement will be made concerning the program details.

Inhibition and Destruction of the Microbial Cell

edited by

W. B. Hugo

Department of Pharmacy
University of Nottingham, England

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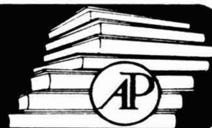

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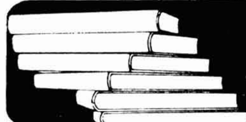

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