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Briefs

A Micro Procedure for the Estimation of the Protein Content of Mucus Glycoproteins. MARGARET A. CARROLL, ALEX POST, LEE J. GRIGGS, AND JOHN E. ZAREMBO, *Smith Kline and French Laboratories, 1500 Spring Garden Street, Philadelphia, Pennsylvania 19101.*

The method, which uses samples in the range of 0.5 mg (10–250 μg of protein), employs a modification of the conventional amino acid analysis using the unresolved neutral and acidic acid peak to estimate the total protein content. Hexosamines, sialic acid, and amide amino acids do not interfere.

Microchem. J. **24**, 137–142 (1979).

Analytical Applications of Picolinealdehyde Salicyloylhydrazone. II. Extraction and Spectrophotometric Determination of Vanadium(V). M. GALLEGO AND M. GARCIA-VARGAS, *Department of Sevilla, Spain* AND M. VALCARCEL, *Department of Analytical Chemistry, Faculty of Sciences, University of Cordoba, Spain.*

Picolinealdehyde salicyloylhydrazone reacts with vanadium(V) to form a yellow 1:1 complex which can be extracted and used for the spectrophotometric determination of trace amounts of vanadium.

Microchem. J. **24**, 143–149 (1979).

Graphical and Visualizing Method of Identification of Selected Groups of Aliphatic Compounds in Thin-Layer Chromatography. JÓZEF ŚLIWIOK AND HALINA WIRA, *Institute of Chemistry, Silesian University, Katowice, Poland.*

A new method of group identification is described for aliphatic compounds based on constant ΔR_M and directional coefficient "a" values [the "a" values being tangents of the angle between the course of the $R_M = f(n_c)$ function and the "x" axis].

Microchem. J. **24**, 150–157 (1979).

Results of Computerization and Automation of a Perkin–Elmer 240 Carbon–Hydrogen–Nitrogen Analyzer. W. R. BRAMSTEDT AND D. E. HARRINGTON, *Diamond Shamrock Corporation, P.O. Box 348, Painesville, Ohio 44077.*

The results obtained indicate that a CHN system can be automated and computerized for increased flexibility while maintaining a high degree of accuracy and precision.

Microchem. J. **24**, 158–167 (1979).

Microdetermination of Carbon in Organic Compounds by Oxygen Flask Combustion with Atomic Absorption Spectrophotometric, Gravimetric, and Titrimetric Finishes. ALFY B. SAKLA AND A. M. SHALABY, *Microanalytical Centre, Faculty of Science, Cairo University, Giza, Egypt, A.R.E.*

Results presented indicate a simple and accurate method. The five modifications all gave approximately the same results.

Microchem. J. **24,** 168–172 (1979).

A Rapid Method for Simultaneous Estimation of 5-Hydroxy-3-indole Acetic Acid (5HIAA) and 5-Hydroxytryptamine (5HT) in Rat Brain. O. DOUAY AND P. KAMOUN, *Laboratoire de Biochimie, Hôpital Necker–Enfants Malades, 75730 Paris Cedex 15, France.*

A method is described to separate 5-hydroxy-3-indole acetic acid from 5-hydroxyindole derivatives using Dowex WX 12 resins. These compounds were assayed, and 5-hydroxytryptamine was calculated as the difference between the total 5-hdyroxyindoles and the 5HIAA.

Microchem. J. **24,** 173–178 (1979).

Periodatometric Determination of Organotrithiocarbonates and Mercaptans (via Trithiocarbonate Formation). BALBIR CHAND VERMA AND SWATANTAR KUMAR, *Department of Chemistry, Himachal Pradesh University, Simla-171005, India.*

A method is described for the direct visual and potentiometric determination of organotrithiocarbonates in the presence of potassium iodide, using potassium periodate as an oxidizing reagent. Mercaptans may also be determined by first converting them to the corresponding organotrithiocarbonate by reaction with carbon disulfide and alkali.

Microchem. J. **22,** 179–183 (1978).

A Method for Recording X-ray Diffraction Patterns of Milligram Quantities of Particulates. RONALD LEE FOSTER AND PETER F. LOTT, *Department of Chemistry, University of Missouri-Kansas City, Kansas City, Missouri 64110.*

A method for recording X-ray diffraction patterns on milligram quantities of particulates is described. The resulting patterns are similar to patterns obtained by standard methods. Application of this method to analysis of airborne particulates in the vicinity of a lead smelting operation has demonstrated the practical use of the method. Samples weighing 1.1 mg produced useful patterns.

Microchem. J. **24,** 184–191 (1979).

BRIEFS

The Rapid Determination of Microgram Amounts of Total Collagen in Pathological Lesions. PAUL BAILY, TERENCE A. KILROE-SMITH, HELEN B. RÖLLIN, AND BERTIE GOLDSTEIN, *National Research Institute for Occupational Diseases of the South African Research Council, P.O. Box 4788, Johannesburg 2000, South Africa.*

A rapid and convenient method for the determination of collagen in tissue is presented. The simple and reproducible method for hydrolysis allows many specimens to be processed simultaneously, and the rapid spectrophotometric determination of hydroxyproline, the presumptive index for collagen, ensures accurate and precise results.

Microchem. J. **24**, 192–198 (1979).

Precipitation of Copper(II) by Formation of a Complex with 2,4-Dioxo-4-(4-hydroxy-6-methyl-2-pyrone-3-yl) Butyric Acid Ethyl Ester. V. DREVENKAR AND B. ŠTENGL, *Institute for Medical Research and Occupational Health, M. Pijade 158, 41001 Zagreb, Yugoslavia,* AND M. J. HERAK AND Z. ŠTEFANAC, *Laboratory of Analytical Chemistry, Faculty of Science, The University, Strossmayerov trg 14, 41000 Zagreb, Yugoslavia.*

The metal is readily precipitated in ethanolic medium. The metal to ligand ratio in the crystalline species was found to be 1:2. On the basis of the spectroscopic data collected so far, the side of coordination could not be identified.

Microchem. J. **24**, 199–211 (1979).

Selective Microdetermination of Phosphate Based on Volhard's Titration. SALAH SHAHINE AND SAMIR EL-MEDANY, *Faculty of Engineering, Ain Shams University, Abbasia, Cairo, Egypt.*

Phosphate is precipitated as silver phosphate, which is filtered off then dissolved in nitric acid, and the silver ions are determined by Volhard's titration.

Microchem. J. **24**, 212–216 (1979).

Analytical Uses of Phenylhydrazines and Phenylhydrazones. I. A. G. ASUERO, *Department of Analytical Chemistry, Faculty of Sciences and Pharmacy, The University of Seville, Seville-4, Spain.*

A review of the inorganic analytical uses of phenylhydrazines and phenylhydrazones is given. They have found use in qualitative and quantitative analysis (gravimetric), as indicators, and in spectrophotometric and catalytic procedures.

Microchem. J. **24**, 217–233 (1979).

BRIEFS

Pyridine-2-aldehyde *p*-Nitrophenylhydrazone as an Indicator for Colorimetric pH Measurements. J. CARRILLO AND M. GUZMÁN. *Department of Analytical Chemistry, Faculty of Sciences, University of Sevilla, Sevilla, Spain.*

Pyridine-2-aldehyde *p*-nitrophenylhydrazone is suggested as an indicator for colorimetric pH measurements in the high-alkaline range. Absorption spectra and indicator constant have been determined. Color change interval and other indicator characteristics have been established. A visually distinguishable transition and a good stability of color are obtained.

Microchem. J. **24**, 234–238 (1979).

A Procedure for the Direct Determination of Micromolar Quantities of Lecithin Employing Enzymes as Reagents. J. D. ARTISS,*,† T. F. DRAISEY,*,† R. J. THIBERT,*,† AND K. E. TAYLOR,* **Department of Chemistry, University of Windsor, Windsor, Ontario, N9B 3P4, Canada and †Departments of Pathology, Salvation Army Grace Hospital, and Windsor Western Hospital Centre, Windsor, Ontario, Canada.*

The procedure, which utilizes enzymes as reagents, is relatively quick, simple, and inexpensive and involves no extractions.

The synthesis of sodium 2-hydroxy-3, 5-dichlorobenzenesulfonate is described. It has proven to be very convenient for routine use in a peroxidase-catalyzed coupling to 4-aminoantipyrine.

Microchem. J. **24**, 239–258 (1979).

MICROCHEMICAL JOURNAL 24, 137–142 (1979)

A Micro Procedure for the Estimation of the Protein Content of Mucus Glycoproteins

MARGARET A. CARROLL, ALEX POST, LEE J. GRIGGS, AND JOHN E. ZAREMBO

Smith Kline and French Laboratories, 1500 Spring Garden Street, Philadelphia, Pennsylvania 19101

Received October 20, 1978

INTRODUCTION

Methods frequently used for the estimation of protein include the Lowry colorimetric assay (*5*), which is based primarily on the number of tyrosine and tryptophan residues of the protein; fluorometric assay (*2*), which is dependent on the free amino groups of the protein; and total nitrogen content (*4*) using a factor of 6.25 for converting nitrogen to protein. Gottschalk (*3*) recommends using the sum of the amino and imino acid residues determined by complete amino acid analysis as the most reliable assessment of the total protein content of glycoproteins.

The determination of protein by amino acid analysis on our samples gave a satisfactory material balance at a high cost of sample and instrument time. Other methods were not feasible due to the lack of a comparable reference standard or the presence of interfering components. We were able to adapt our amino acid procedure to provide an estimate of protein content on 0.5 mg of material within 90 min of instrument time. The method has been applied to unfractionated mucus glycoproteins and column fractions containing from 2 to 50% protein. The chromatographic system employed is based on the Moore and Stein procedure (*6*) for basic amino acids.

MATERIALS AND METHODS

Materials. A protein hydrolysate calibration mixture of 19 amino acids was diluted with 0.1 *N* hydrochloric acid to contain 0.02 μmol/ml of each amino acid (0.01 μmol/ml cystine). An internal standard, α-amino-β-guanidinopropionic acid, was added at a concentration of 0.3 μmol/ml. All the amino acids were A Grade, from Calbiochem, La Jolla, Calif. The instrument was calibrated with 500 μl of the diluted stock solution equivalent to 0.185 μmol of total amino acids and 0.150 μmol of the internal standard. The buffer used to elute the amino acids was pH 5.28, 0.35 *N* sodium citrate buffer (*6*).

Sample preparations. Each glycoprotein sample (0.5 mg), accurately weighed, was hydrolyzed for 24 hr at 110°C in 6 *N* hydrochloric acid.

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The samples were dried on an Evapo-mix[1] and the residue was dissolved in 1 ml of 0.1 *N* hydrochloric acid containing 0.5 μmol of internal standard.

Estimation of protein content. Loadings of 50–500 μl of each sample were eluted from a 10 × 0.9-cm column (packed with cation-exchange resin, MR-200[2]) of an amino acid analyzer[3] with sodium citrate buffer at a flow rate of 90 ml/hr. The effluent was reacted with ninhydrin solution in a boiling water bath and the absorbance was measured at 570 nm. Eight samples were analyzed sequentially.

The protein estimation was based on the comparison of the area of the unresolved neutral and acidic amino acid peak (Fig. 1) of the unknown to the standard. For the purpose of calculation, the peak is assumed to be Gaussian. The percentage protein is calculated by the equations used for amino acid analysis.

$$\frac{\text{Area}_{IS} \times 0.185}{\text{Area}_{Std} \times 0.150} = F. \tag{1}$$

$$\frac{\text{Area}_{S} \times \mu\text{mol}_{IS} \times F \times \text{Avg. residue MW}}{\text{Mg}_{S} \times \text{Area}_{IS} \times 10} = \%\ \text{protein}. \tag{2}$$

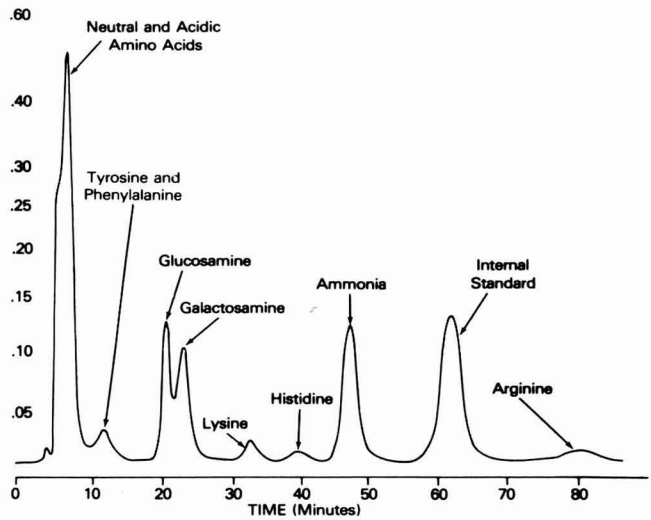

FIG. 1. Chromatogram of a hydrolyzed sample of glycoprotein (CTM). Load, 0.10 mg; internal standard, 0.10 μmol; protein, 33.0%.

[1] Buchler Instruments, Fort Lee, N.J.
[2] Mark Instrument Co., Villanova, Pa.
[3] Model 9000 Biolyzer, Phoenix Precision Instrument Co., formerly Philadelphia, Pa.

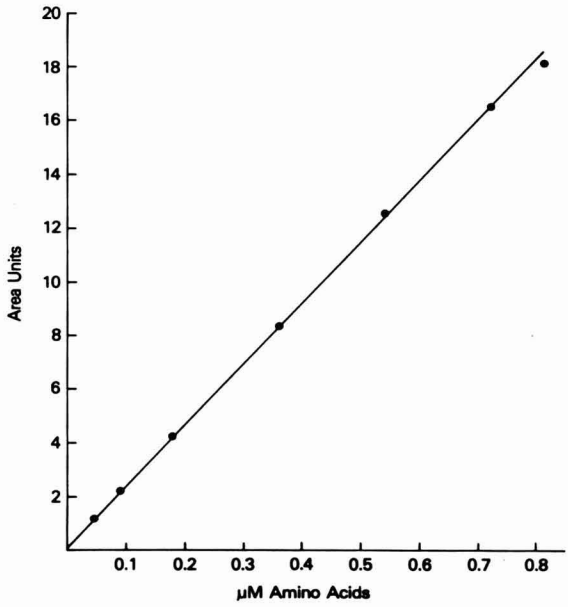

FIG. 2. Linearity of response. Correlation coefficient, 0.9998.

DISCUSSION

This laboratory required a rapid method for the estimation of protein in order to complete the material balance of the glycoprotein samples under study. As less than 1-mg samples of variable protein content were available for assay, a complete amino acid analysis was not feasible if the total protein content was low. In addition, the time required on our instrument (about 4.5 hr) for a complete analysis also precluded its use for a large number of samples. Determination of the total nitrogen content and conversion to its protein equivalent were not valid since the glycoproteins in

TABLE 1
PRECISION OF THE PROTEIN ESTIMATION[a]

Determination (%)
(1) 20.2
(2) 20.8
(3) 20.7
(4) 21.3
(5) 20.7
$\bar{X}$ = 20.7 ± 0.4
RSD (1σ) = 1.9%

[a] Sample: canine tracheal mucin.

question contain the nitrogen-contributing hexosamine and sialic acid residues. Since no related standard reference protein was available, we could not expect to obtain a reasonable estimate of the protein content by either of the aforementioned colorimetric or fluorometric procedures. A comparison with a reference bovine serum albumin would not yield a representative estimation of protein content. Thus, we evaluated and modified the Moore–Stein procedure to provide an alternate method of analysis.

The protein estimation is based on the area of the unresolved neutral and acidic amino acid peak eluted from a "basic" column of the amino acid analyzer. The colorimetric response of this peak is linear and follows Beer's law from 0.045 to 0.81 μmol of total amino acids (Fig. 2). The area of the combined peak is appreciable for samples containing small amounts of protein and can be measured with good precision (Table 1). Hexosamines, which are known to be present in these samples, and ammonia, which is produced in the hydrolysis of proteins, do not interfere. These compounds elute later from the column.

The standard used in this modified procedure is a synthetic mixture of neutral, acidic, and basic amino acids typically used to calibrate an amino acid analyzer. Since the color yield of most of the amino acids found in hydrolyzed protein ranges from 0.94 to 1.10 relative to that of leucine as unity (*1*), variations in amino acid composition between the standard and unknown would not greatly affect the estimation of protein content. Two amino acids found in glycoproteins, proline and cystine, are exceptions, having color yields of 0.19 and 0.55, respectively (*1*). As these two amino acids are present only in small amounts in the glycoprotein under study, they would not be expected to cause significant variations in total color

TABLE 2
A COMPARISON OF PROTEIN ANALYSIS[a] BY SEVERAL METHODS

Sample	Percentage protein			
	Proposed procedure	Amino acid analyzer	Lowry	Total nitrogen ×6.25
CSM[b]	41.2	38.2	42.4	—
CTM[c]	20.7	18.3	—	—
CTM column fraction	6.9	7.6	—	—
CTM column fraction	12.2	13.8	—	—
CTM	21.8	18.1	—	—
Pentex albumin	99.5	—	102.6	96.8
Bovine serum albumin	97.0	—	—	95.1

[a] Single determinations.
[b] Canine submaxillary mucin.
[c] Canine tracheal mucin.

TABLE 3
THE EFFECT OF CARBOHYDRATE ON THE DETERMINATION OF PROTEIN

Theory		Found			
% Protein[a]	% Carbohydrate[b]	Protein estimation	Δ	Lowry	Δ
100.0	0.0	100.9	+0.9	104.0	+4.0
90.3	9.7	89.2	−1.1	92.5	+1.8
80.5	19.5	81.9	+1.4	83.6	+3.1
70.7	29.3	71.7	+1.0	73.0	+2.3
60.6	39.4	61.1	+0.5	60.9	+0.3
50.8	48.2	51.4	+0.6	52.3	+1.5
40.8	59.2	41.3	+0.5	44.8	+4.0
30.7	69.3	29.9	−0.8	33.0	+2.3
20.1	79.9	19.1	−1.0	22.1	+2.0
10.0	90.0	12.3	+2.3	12.0	+2.0
0	100.0	0.96	+0.96	0.70	+0.70

[a] Bovine serum albumin.
[b] Galactose:fucose (4:1).

yield. The composition of the standard can be modified for other proteins, such as collagens, which contain high levels of proline and hydroxyproline.

The calculation is based on a comparison of the peak area of the unresolved neutral and acidic amino acids of the unknown to the peak area of the standard mixture. As tyrosine, phenylalanine, and the resolved basic amino acids are separated and do not contribute to this peak area, their estimated micromolar contributions to the total protein content in the sample are included by ratioing the total micromoles of the standard on the basis of the 19 acidic, neutral, and basic amino acids present to that of the sample weight. A comparison of values obtained from various methods of analysis is listed in Table 2. The protein estimation provided valuable data for a material balance of glycoprotein samples with a saving of both time and material.

High levels of carbohydrate had no significant effect on the protein estimation (Table 3). A mixture of carbohydrates consisting of the neutral sugars found in glycoproteins was added in varying proportions to bovine serum albumin. The samples were assayed by the proposed procedure and by the conventional Lowry method. The data indicated that carbohydrate does not interfere with either method.

SUMMARY

A sensitive automated procedure for the estimation of the protein content of glycoproteins has been developed using 0.5-mg samples (10−250 μg of protein). The method employs a modification of the conventional amino acid analysis using the unresolved neutral and acidic amino acid peak to estimate the total protein content. Hexosamines, sialic acid, and amide amino acids do not interfere.

REFERENCES

1. Blackburn, S., "Amino Acid Determination," pp. 72–74. Marcel Dekker, New York, 1968.
2. Böhlen, P., Stein, S., Dairman, W., and Udenfriend, S., Fluorometric assay of protein in the nanogram range. *Arch. Biochem. Biophys.* **155,** 213–220 (1973).
3. Gottschalk, A., "Glycoproteins," Vol. 5, pp. 143–144. Elsevier, Amsterdam–London–New York, 1966.
4. Hashimoto, Y., and Pigman, W., A comparison of the composition of mucins and blood group substances. *Ann. N.Y. Acad. Sci.* **93,** 541–554 (1962).
5. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275 (1951).
6. Moore, S., Spackman, D. H., and Stein, W. H., Chromatography of amino acids on sulfonated polystyrene resins. *Anal. Chem.* **30,** 1185–1190 (1958).

MICROCHEMICAL JOURNAL 24, 143–149 (1979)

Analytical Applications of Picolinealdehyde Salicyloylhydrazone

II. Extraction and Spectrophotometric Determination of Vanadium(V)

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Received October 10, 1978

INTRODUCTION

The characteristics and analytical possibilities of picolinealdehyde salicyloylhydrazone (SHPA), and the spectrophotometric determinations of small amounts of nickel and zinc with this reagent, have been reported (*4*).

In this paper, the properties of vanadium(V) complex with the above reagent are described, and an extraction and spectrophotometric method for the determination of traces of vanadium has also been developed. The determination of small amounts of vanadium in two materials is described.

MATERIALS AND METHODS

Equipment

A Unicam SP 8000 spectrophotometer was used for recording spectra in the ultraviolet and visible regions of the spectrum, and a Coleman 55 (digital) instrument was used for measurements at fixed wavelengths. Quartz or glass cells (10-mm path length) were used. A Philips PW 9408 pH meter, with a glass and calomel electrode pair, was used for pH measurements.

Chemicals and Solutions

Reagents. A stock solution of picolinealdehyde salicyloylhydrazone was prepared by dissolving 0.075 g of recrystallized reagent in 3 ml of

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dimethylformamide and diluting to 100 ml with chlorobenzene. This solution was stable for several days. A reagent solution of concentration 0.1% in ethanol was also used. Standard vanadium(V) solution, 5.295 g·liter^{-1} of vanadium(V), was prepared by dissolving vanadium(V) pentoxide in 1 *M* sodium hydroxide solution, and was standardized by using a gravimetric method with cupferron (*3*). Buffer solution, pH 2.7, was prepared by dissolving 84.4 g chloroacetic acid and 24.1 g sodium hydroxide in distilled water and diluting the mixture to 1 liter. Dowex 50 × 8 resin, sodium form, and Dowex 1 × 8 resin, chloride form, were used. All other solvents and reagents were of analytical grade.

Procedure

Aqueous medium. In a 25-ml standard flask, containing up to 44 μg of vanadium, add 7.5 ml of 0.1% ethanolic reagent solution, 5 ml of buffer solution, and 2.5 ml of 0.1 *M* potassium chloride solution, and dilute to the mark with distilled water. Measure the absorbance at 400 nm against a reagent blank prepared in a similar manner.

Extraction. To 10 to 100 ml of sample solution, containing up to 20 μg of vanadium, in a separating funnel, add 2.5 ml of buffer solution and extract the mixture with one 10.0-ml volume of reagent solution in chlorobenzene shaking for 2.5 min. Allow the phases to separate, and transfer the lower (organic) layer into a 10-ml flask containing anhydrous sodium sulfate. Measure the absorbance at 425 nm against a reagent blank (prepared in a similar way) or at 430 nm against distilled water.

The calibration graph is prepared by using standard solutions containing from 0.04 to 0.40 ml of a 50.0 ppm solution of vanadium(V) (0.2 to 2.0 ppm in 10 ml of chlorobenzene phase) treated in the same way.

RESULTS AND DISCUSSION

Formation and Study of Vanadium(V) Complex

Absorption spectra. Addition of a solution of SHPA to a solution of vanadium(V) ions produced a yellow complex: its absorption spectra remains stable for 6 hr, at pH 2.7 (Fig. 1). At other pH values, the absorbance varies with time.

Influence of pH. This is shown in Fig. 2, by means of the absorbance at 400 nm after a 30-min reaction time. The optimum pH range is 2.5–4.0.

Stoicheiometry. The stoichiometry of the vanadium(V) complex was evaluated by the continuous variation method (*5*), and was found to be 1:1. The metal:ligand ratio for vanadium(IV) complex with SHPA, at pH 2.7, was 1:3 (Fig. 3).

From the data obtained by Job's method (*5*) the stability constant was calculated to be 4.3×10^4. The vanadium(V)–SHPA system is moderately dissociated in aqueous-ethanolic solution.

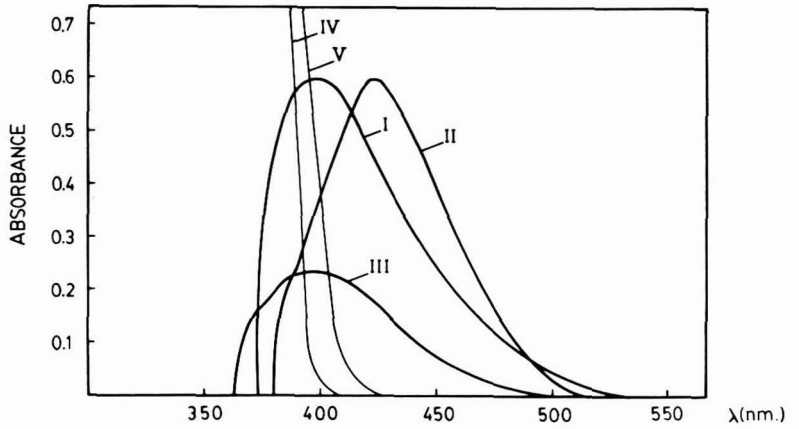

FIG. 1. Absorption spectra of vanadium complexes with SHPA at pH 2.7. Concentration of vanadium, 1.4 ppm. (I) Vanadium(V) complex in aqueous-ethanolic solution; (II) vanadium(V) complex extracted into chlorobenzene; (III) vanadium(IV) complex in aqueous-ethanolic solution; (IV) reagent blank in aqueous media; and (V) reagent blank in chlorobenzene.

Oxidation state of vanadium and charge of the complex. From experimental evidence it was concluded that the reagent forms the yellow complex with vanadium(V). The vanadium complex was not retained on either a cationic or an anionic ion-exchange resin, indicating it was uncharged. The reagent itself, at pH 2.7, was retained on a cationic resin, but not on

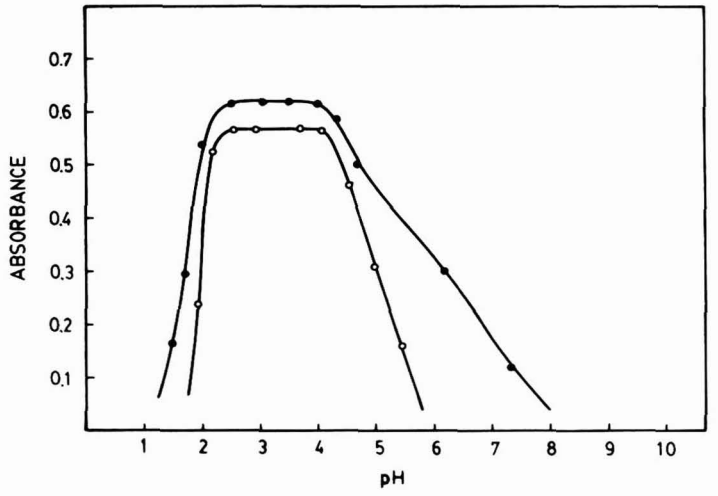

FIG. 2. Influence of pH on the formation of V(V)–SHPA complex: (○) in aqueous media (λ_{max} = 400 nm, C_V = 1.3 ppm); (●) extracted into chlorobenzene (λ_{max} = 425 nm, C_V = 1.4 ppm).

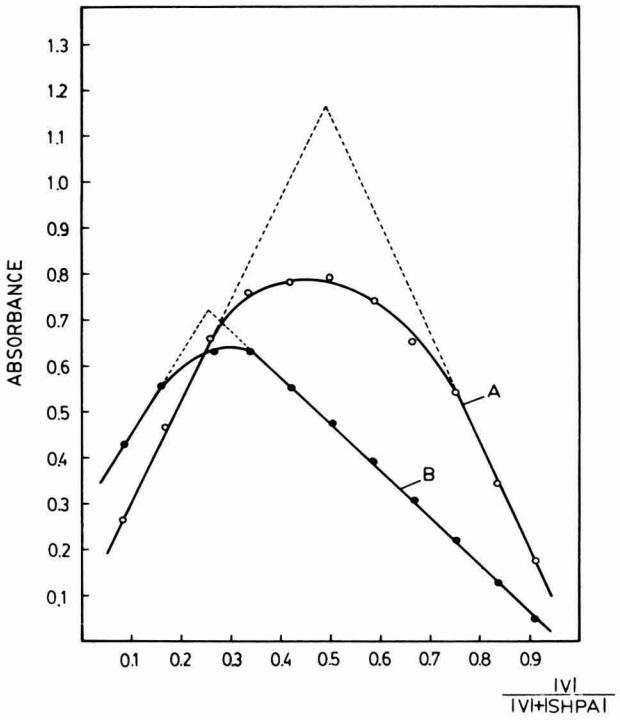

FIG. 3. Stoichiometry of vanadium(V) and vanadium(IV) complexes of picolinealdehyde salicyloylhydrazone, in aqueous media at pH 2.7 (continuous variation): (A) vanadium(V) complex (5.7×10^{-4} *M*) and (B) vanadium(IV) complex (6.4×10^{-4} *M*).

an anionic resin, indicating it was positively charged. This effect was attributed to the protonation of the pyridine nitrogen atom (*4*).

From the results obtained it may be supposed that SHPA acts as a tridentate ligand when it reacts with vanadium(V); the coordination occurs by bonding from the first two nitrogen atoms of salicyloylhydrazide residue, from the oxygen atom of the carbonyl group, and from the pyridine nitrogen atom so that five-membered chelate rings are produced. This fact explains the great stability of the yellow complex into chlorobenzene.

Extraction of the complex. When a solution of SHPA reagent in an organic solvent is shaken with a weakly acidic aqueous solution of vanadium(V), the yellow complex is formed immediately in the organic phase and it remains stable for at least 48 hr. When higher alcohols and methyl isobutyl ketone were used the resulting complex is unstable; of the other solvents tried, chlorobenzene proved to be the best as the complex shows a small bathochromic shift (Fig. 1). The optimum pH range for extraction of the complex is 2.5–4.0 (Fig. 2). One 2.5-min extraction with 10 ml of

TABLE 1
COMPARISON WITH EXISTING METHODS

Compound	Optimal pH	λ_{max} (nm)	ϵ^a ($\times 10^3$)	Metal/ ligand	Reference
Hydrazinium hydrazinecarbodithioate	4–6	460	1.1	1:2	(*1*)
Anthranilic acid isopropylidenehydrazide	H_2SO_4	525	5.1	1:2	(*2*)
Nicotinic acid hydrazide	1.8–2.2	420	0.75	1:1	(*6*)
Picolinealdehyde salicyloylhydrazone	2.5–4.0	425	21.6	1:1	—

[a] $L \cdot mol^{-1}\ cm^{-1}$.

the 0.075% solution of SHPA in chlorobenzene is necessary for the complete extraction of vanadium(V) from aqueous solution.

Spectrophotometric Determination of Vanadium

Direct. The optimum concentration range, evaluated by Ringbom's method (*7*), is 1.12–1.50 ppm of vanadium. The molar absorptivity at 400 nm is 2.17×10^4 litre$\cdot mol^{-1}\ cm^{-1}$. The relative error ($P = 0.05$) of the method is 0.2%.

TABLE 2
TOLERANCE LIMITS OF EXTRACTIVE-DETERMINATION OF 5 μg/10 ml OF VANADIUM(V) WITH SHPA

Amount tolerated[a] (μg/10 ml)	Ions added[b]
10000	Ca(II), Ba(II), Sr(II), Mg(II), Pb(II), Se(IV), alkaline metal ions, PO_4^{3-}, SO_4^{2-}, SCN^-, NO_3^-, $B_4O_7^{2-}$, ClO_4^-, Cl^-, and I^-
7500	Cr(III), Al(III), and Tl(I)
5000	As(III and V) and tartrate
1500	Cd(II) and Mo(VI)
1000	$S_2O_3^{2-}$ and MnO_4^-
750	La(III), CN^-, and citrate
200	U(VI)

[a] The limiting value of the concentration of foreign ions was taken as that which caused an error of not more than 2% in the absorbance.

[b] Cations were added in the form of chlorides, nitrates, or acetates to a maximum of 1000 ppm; anions were added in the form of sodium or potassium salts.

TABLE 3
ELIMINATION OF INTERFERENCES IN THE EXTRACTIVE-DETERMINATION OF 5 μg/10 ml OF VANADIUM WITH SHPA BY ADDING OF MASKING AGENTS

Foreign ion	Amount tolerated (μg/10 ml)		Masking agent
	Without masking agent	With masking agent	
Sb(III)–Hg(II)	50	2000	Tartrate; 5 mg
Ag(I)	100	1500	Cl^-; 1 ml of 2 *M* solution
Cr(VI)	25	1000[a]	As^{3+}; 1000 ppm
La(III)–Sn(II)	750–50	1000	Tartrate; 5 mg
Hg(I)	—	1000	Cl^-; 1 ml of 2 *M* solution
W(VI)–Zr(IV)	50–15	500	Tartrate; 5 mg
Th(IV)	50	250	SO_4^{2-}; 1 ml of 0.5 *M* solution
In(III)	50	250	SCN^-; 1 ml of 0.2 *M* solution
Be(II)	15	250	Tartrate; 5 mg
Pd(II)	—	250	CN^-; 3 ml of 0.01 *M* solution
Zn(II)	50	200	CN^-; 3 ml of 0.01 *M* solution
Ti(IV)	50	200	Tartrate; 5 mg
Mn(II)	50	100	CN^-; 3 ml of 0.01 *M* solution
Bi(III)	25	100	S^{2-}; 3 ml of 0.001 *M* solution
Fe(II)–Fe(III)	—	50	Tartrate; 5 mg
Ce(IV)	15	50	PO_4^{3-}; 1 ml of 0.1 *M* solution
Cu(II)	15	50	S^{2-}; 3 ml of 0.001 *M* solution
Ni(II)–Co(II)	15–5	20	CN^-; 3 ml of 0.01 *M* solution
S^{2-}	100	1000	Pb^{2+}; 1000 ppm
$C_2O_4^{2-}$	15	1000[a]	As^{5+}; 1000 ppm
F^-	100	1000	Al^{3+}; 1000 ppm

[a] Heating gently before extraction.

Mercury(II), calcium, barium, strontium, magnesium, alkaline metal ions, thiosulfate, tartrate, thiocyanate, phosphate, borate, nitrate, and perchlorate did not interfere at a concentration 100 times that of the vanadium. Lead, thallium(I), cadmium, molybdenum(VI), lanthanum, and aluminium did not interfere at a concentration 25 times that of the vanadium.

Extraction. Beer's law is obeyed between 0.25 and 2.0 ppm of vanadium and the molar absorptivities at 425 and 430 nm are 2.16×10^4 and 2.15×10^4 litre·mol^{-1} cm^{-1}, respectively. The Sandell sensitivity (*8*) is 0.0024 μg·cm^{-2} at 425 nm. Therefore, the method is more sensitive for vanadium than many existing methods. The method has also been advantageously compared with the other methods previously reported that use related ligands (Table 1). Ringbom's graph shows that 0.3 to 1.75 ppm of vanadium(V) is the minimum range of error. The relative error ($P = 0.05$) is 0.4% at 425 and 430 nm.

For the determination of 5 μg of vanadium by this method, the foreign ions can be tolerated at the levels given in Tables 2 and 3. The good results obtained by masking them with sodium tartrate were due to the fact the metal–tartrate complexes were not extracted.

Applications

The method has been satisfactorily applied to the determination of vanadium in two standard samples (Bureau of Analized Samples Ltd.). Iron was separated by extraction with ether in the analysis of steel. The results obtained were the means of five determinations in both instances.

High-speed steel 64b had the following certificate composition: carbon 0.9, vanadium, 1.99, chromium 4.55, molybdenum 4.95, and tungsten 7.05%. The vanadium content found was 1.97 ± 0.02%.

Lead–vanadium concentrates 70aG had the following certificate composition: lead 65.4 and vanadium(V) pentoxide 3.20%. The vanadium found was 3.204 ± 0.003% (percentage as V_2O_5).

SUMMARY

Picolinealdehyde salicyloylhydrazone reacts with vanadium(V) to produce a yellow 1:1 complex (λ_{max} = 400 nm, $\epsilon = 2.17 \times 10^4$ liters·mol^{-1} cm^{-1}) in aqueous ethanolic solution. The yellow complex can be extracted into chlorobenzene (λ_{max} = 425 nm, $\epsilon = 2.16 \times 10^4$ liters·mol^{-1} cm^{-1}) and used for the spectrophotometric determination of trace amounts of vanadium. Interferences have been investigated. The method has been applied to the determination of vanadium in steel and in lead concentrates.

REFERENCES

1. Byr'ko, V. M., Busev, A. I., Tikhonova, T. I., Baibakoba, N. V., and Shepel', L. I., Some analytical properties of hydrazinium hydrazinecarbodithioate. Complex formation with iron(II) or vanadium(V). *Zh. Anal. Khim.* **30**, 1885–1891 (1975).
2. Dolgorev, A. V., and Karpova, O. I., Anthranilic acid isopropylidenehydrazide as a spectrophotometric reagent for vanadium. *Zav. Lab.* **40**, 771–773 (1974).
3. Erdey, L., "Gravimetric Analysis," Part II p. 562. Pergamon Press, New York, 1965.
4. Gallego, M., Garcia-Vargas, M., Pino, F , and Valcarcel, M., Analytical applications of picolinealdehyde salicyloylhydrazone. I. Spectrophotometric determination of nickel and zinc. *Microchem. J.* **23**, 353–359 (1978).
5. Job, P., Formation and stability of inorganic complexes in solution. *Ann. Chim.* **9**, 113–203 (1928).
6. Krych, Z., and Lipiec, T., Spectrophotometric determination of small amounts of vanadium(V) with nicotinic acid hydrazide. *Chemia Anal.* **12**, 535–538 (1967).
7. Ringbom, A., Accuracy of colorimetric determination. I. *Z. Anal. Chem.* **115**, 332–343 (1939).
8. Sandell, E. B., "Colorimetric Determination of Traces of Metals." Interscience, New York, 1959.

MICROCHEMICAL JOURNAL 24, 150–157 (1979)

Graphical and Visualizing Method of Identification of Selected Groups of Aliphatic Compounds in Thin-Layer Chromatography

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INTRODUCTION

In our previous paper (*3*) we described a method of group identification of chemical compounds giving various homologous series, based upon the constant ΔR_M difference. The constant ΔR_M value was the consequence of the parallel course of two functions $R_{M_X} = f(n_c)$ and $R_{M_Y} = f(n_c)$, determined for two different mobile phases.

The results presented in this paper deal with the group identification of the following homologous series of organic substances: higher fatty acids and their ethyl esters, amides of higher fatty acids, higher aliphatic amines, ethylalkyl ketones, and dicarboxylic acids.

Identification of the investigated groups of substances took advantage of the constant ΔR_M differences and of the directional coefficient "a" [tangents of the angle between the course of the $R_{M_{X,Y}} = f(n_c)$ function and the "x" axis], obtained for the different $R_M = f(n_c)$ functions.

Simultaneously another method of identification of the examined compounds was established, based upon the different visualizing effects obtained with a wide range of applied visualizing agents.

EXPERIMENTAL

Development of the investigated homologous series was accomplished under varying chromatographic conditions.

Higher fatty acids (R-COOH, n_c = 10–20 carbon atoms per molecule) and their ethyl esters were analyzed under the following chromatographic conditions.

In the stationary phase, glass plates were covered with a 0.25-mm-thick layer of Kieselguhr and impregnated with the 15% solution of paraffin oil in benzene. Then 2% solutions of the examined acids and esters were prepared in CCl_4 and spotted on the plates in the amounts of 5 μg/2 μl. Samples were developed in the following mobile phases: (A) acetic acid + ethyl acetate + paraffin oil + water, 4:4:1:1 (v/v/v/v), and (B) acetic acid + ehtyl acetate + paraffin oil, 3:4:3 (v/v/v).

Saturation of the chromatographic chamber took 1 hr, and the height of

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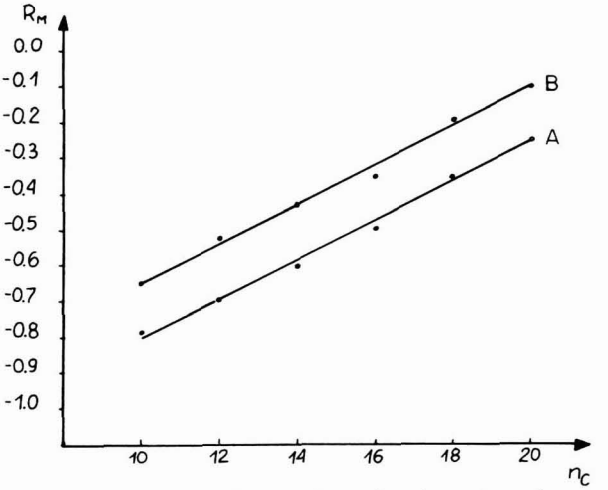

FIG. 1. The R_M coefficient values vs the number of carbon atoms in a molecule for higher fatty acids (A, and B refer to the applied mobile phases; see text).

developing was 13 cm. After developing the glass plates were dried at room temperature for 24 hr and visualized with suitable agents (Table 2).

Amides (R-$CONH_2$, n_c = 10−18 carbon atoms per molecule) were developed on ready-made glass plates covered with Kieselgel 60, Art. 5721, impregnated with a 5% solution of paraffin oil in benzene. The investigated amides were placed on the plates in form of 2% chloroform solutions in amounts of 5 μg/2 μl and developed in the following mobile phases: (C) diethylamine + benzene, 1:2 (v/v), and (D) diethylamine + benzene, 1:4 (v/v).

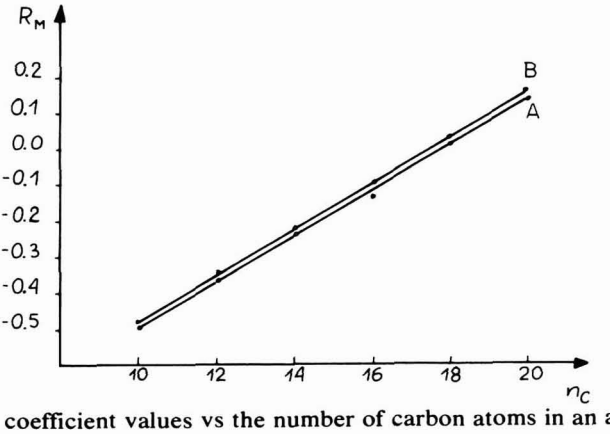

FIG. 2. The R_M coefficient values vs the number of carbon atoms in an acidic radical for ethyl esters of higher fatty acids (A and B as in Fig. 1.).

Saturation of the chromatographic chamber took 1 hr, and the height of developing was 13 cm. After developing the glass plates were dried in open air for 24 hr and visualized with a 0.001% water solution of methylene violet.

In the case of amines (R-NH_2, n_c = 8–20 carbon atoms per molecule) development was carried out using glass plates covered with 0.25-mm-thick Kieselguhr layers and impregnated with a 15% solution of paraffin oil in benzene. Amines were spotted on chromatographic glass plates in amounts of 0.15 μg/5 μl from 2% methanol solutions, and then developed in the following mobile phases: (E) methyl ethyl ketone + water + paraffin oil, 94:4:2 (v/v/v), and (F) methyl ethyl ketone + water + paraffin oil + formamide, 94:2:2:2 (v/v/v/v).

Saturation of a chromatographic chamber took 1 hr, and the height of developing was 13 cm. Chromatograms, which were dried after developing in open air for 24 hr, were then visualized with a ninhydrin solution (*1*).

Conditions for the chromatographic development and separation of dicarboxylic acids ($R<^{COOH}_{COOH}$, where R = radical including n_c = 1, 2, 4, 5, 7, and 8 carbon atoms) were as follows:

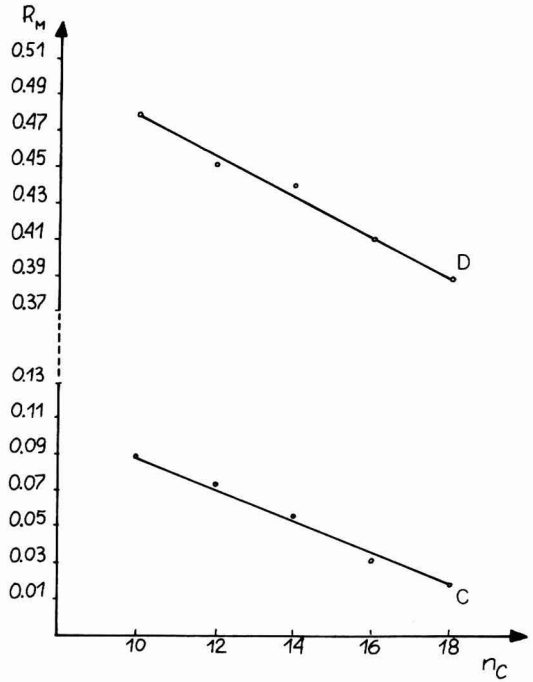

FIG. 3. The R_M coefficient values vs the number of carbon atoms in a molecule for amides of higher fatty acids (C and D refer to the applied mobile phases; see text).

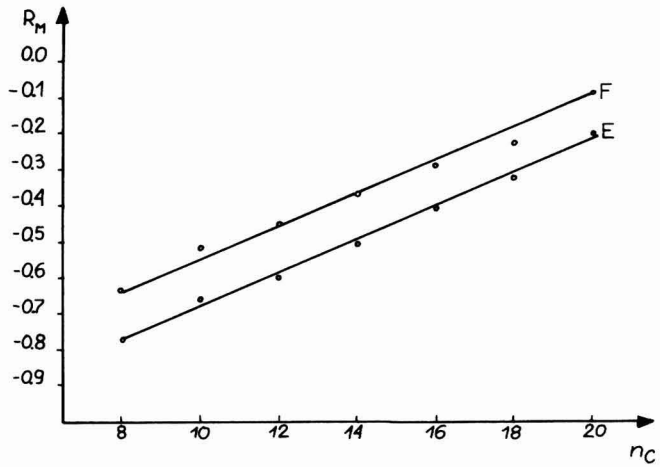

FIG. 4. The R_M coefficient values vs the number of carbon atoms in a molecule for higher aliphatic amines (E and F refer to the applied mobile phases; see text).

In the stationary phase ready-made chromatographic glass plates covered with Kieselgel 60, Art. 5721 (E. Merck, West Germany), were activated for 30 min at 120°C. The examined acids were placed on plates in amounts of 0.1 μg/5 μl from 2% methanol solutions and then developed in the following mobile phases: (G) methanol + ethyl ether + water, 7:1:2 (v/v/v), and (H) methanol + ethyl ether + water = 8:1:1 (v/v/v).

Saturation of the chromatographic chamber took 1 hr, and the height of developing was 13 cm. After developing and drying, chromatograms were sprayed with a ready-made solution of bromocresol green (Bromkresolgrün, 0.05% Sprühreagenz, Art. 1998, E. Merck, West Germany).

Developing of ketones ($R\text{-}\overset{\overset{O}{\|}}{C}\text{-}CH_2\text{-}CH_3$, where R = radical including n_c = 8,9,10,11, 13, and 15 carbon atoms) was performed in the following way.

In the stationary phase, chromatographic glass plates covered with a 0.25-mm-thick layer of Kieselguhr and impregnated with (I) a 10% or (J) a 20% solution of paraffin oil in benzene were used. The examined ketones were analyzed in form of the 2,4-dinitrophenylhydrazine derivatives (*4*).

Then 2% solutions of 2,4-dinitrophenylhydrazones derived from the investigated ketones were prepared, using ethyl acetate as a solvent, and 0.1 μg/5 μl amounts of these samples were placed on chromatographic glass plates. The mobile phase was methanol. Due to the intense yellow color of 2,4-dinitrophenylhydrazones derived from the examined ketones, no visualizing agents were needed in this case.

On the basis of the performed measurements R_f values were calculated for each homologous series in addition to the R_M values.

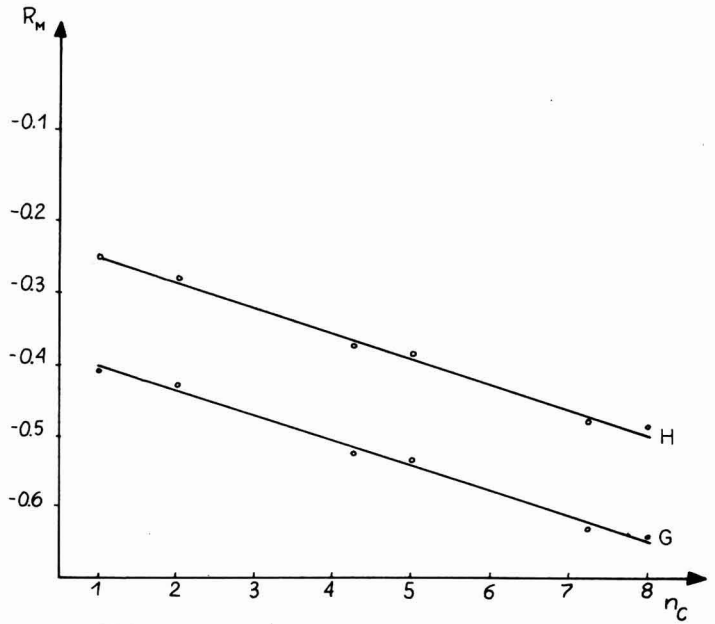

FIG. 5. The R_M coefficient values vs the number of carbon atoms in the acidic radical for dicarboxylic acids (G and H refer to the applied mobile phases; see text).

Taking advantage of the obtained R_M values, the following functional dependences, $R_{M_X} = f(n_c)$ and $R_{M_Y} = f(n_c)$ (with X and Y, the corresponding mobile phases, and n_c, the number of carbon atoms in a molecule), were established for each group of examined aliphatic compounds.

The straight-line and parallel courses of the $R_{M_X} = f(n_c)$ and $R_{M_Y} = f(n_c)$ functions were obtained in each case, as well as the constant $\Delta R = R_{M_X} - R_{M_Y}$ values. In addition the directional coefficient "a" values were calculated for each of the discussed functions.

The ΔR_M and "a" values enabled graphical group identification of the discussed homologous series.

Results of the performed investigations are given in Figs. 1–6 and in Table 1.

Simultaneously, selected visualizing agents were applied, giving different effects for the various examined groups of aliphatic compounds. This allowed further identification of the investigated groups of substances.

The results of visualizing effects are given in Table 2.

DISCUSSION

From the data given in Figs. 1 and 2, and on the basis of the constant $\Delta R_{M_{A,B}}$ and "a" values shown in Table 1, one can assume that there is a possibility of differentiating between acids and esters.

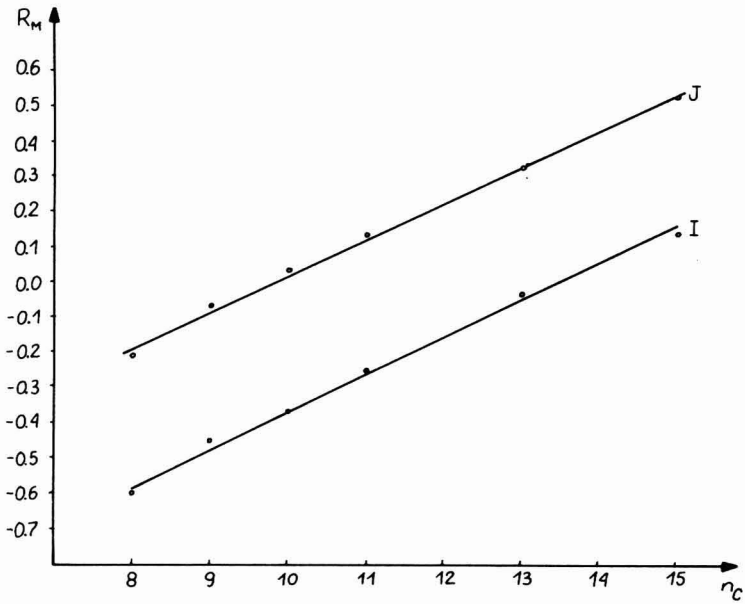

FIG. 6. The R_M coefficient values vs the number of carbon atoms in the alkyl radical R for ketones (I and J refer to the applied mobile phases; see text).

Figures 3–6 and the data from Table 1 support our assumption that with each group of aliphatic compounds which form homologous series a possibility exists for establishing a graphical method of group identification, employing thin-layer chromatography.

The visualizing effects of the selected agents with the examined substances allows determination of their group identity. A complex approach toward the constant ΔR_M difference values and the directional coefficient "*a*", which was closely connected with the course of the $R_M = f(n_c)$ functions, as well as toward the observed visualizing effects, allowed

TABLE 1
ΔR_M AND DIRECTIONAL COEFFICIENT "*a*" VALUES WITH THE EXAMINED GROUPS OF ALIPHATIC COMPOUNDS

No.	Examined groups of compounds	Applied phases	ΔR_M	*a*
1	Acids	A,B	0.14	0.133
2	Esters	A,B	0.00	0.066
3	Amides	C,D	0.38	0.01
4	Amines	E,F	0.148	0.043
5	Dicarboxylic acids	G,H	0.14	0.032
6	Ketones	I,J	0.385	0.99

TABLE 2
VISUALIZING EFFECTS WITH THE EXAMINED GROUPS OF ALIPHATIC COMPOUNDS

Visualizing agent	Examined groups					
	Acids[a]	Esters[a]	Amides[a]	Amines[a]	Dicarboxylic acids[b]	Ketones[a]
Sodium 2,4-dichloroindophenolate (*1*)	Red	—	—	Navy blue	—	—
Bromocresole green	Yellow	—	—	—	Yellow	—
2% H_2O solution of $FeCl_3$ + 1% H_2O solution of K_3 Fe $(CN)_6$ in a ratio of 1:1 (v/v)	White	—	—	—	—	—
2,7-Dichlorfluoresceine (Art. 9677, Merck, W. Germany)	Yellow	—	—	Dark pink	—	—
Rhodamine B (Art. 7596, 0.1% Sprühreagenz, Merck, W. Germany)	Blue	—	—	—	—	—
Ninhydrine (*1*)	—	—	—	Pink	—	—
Diphenylcarbazone (*1*)	White	—	—	—	—	—
Tymolphthaleine (*2*)	Pink	—	—	—	—	—
Methylene violet	—	—	—	Pink	—	—
2,4-Dinitrophenylhydrazine (*1*)	—	—	—	—	—	Yellow
Vaniline in H_2SO_4 (*1*)	—	—	Yellow	—	—	—
5% Solution of ammonium molybdate in 20% H_2SO_4	—	—	White	—	—	—

[a] Partition chromatography.
[b] Adsorption chromatography.

establishment of a new methodical approach, concerning chromatographic group identification of organic compounds, giving different homologous series.

SUMMARY

A new method of group identification was established for selected aliphatic compounds

giving homologous series, based upon constant ΔR_M and directional coefficient "a" values (the "a" values being tangents of the angle between the course of the $R_M = f(n_c)$ function and the "x" axis).

The results presented concern identification of higher fatty acids and their ethyl esters, amides of higher fatty acids, higher aliphatic amines, ethylalkyl ketones, and dicarboxylic acids.

Simultaneously, another method of identifying groups of aliphatic compounds was established, taking advantage of differentiated visualizing effects.

REFERENCES

1. "Anfärbereagenzien für Dünnschicht- und Papier-Chromatographie," E. Merck, Darmstadt, 1970.
2. Sliwiok, J., and Kwapniewski, Z., Detection of higher fatty acids in thin-layer chromatography, *Microchem. J.* **9**, 237–240 (1965).
3. Sliwiok, J., and Wira, H., The group identification of organic compounds by means of thin-layer chromatography. *Microchem. J.* **22**, 135–143 (1977).
4. Vogel, A. J., "A Text-book of Practical Organic Chemistry." Longmans, Green and Co., London, 1956.

MICROCHEMICAL JOURNAL 24, 158–167 (1979)

Results of Computerization and Automation of a Perkin–Elmer 240 Carbon–Hydrogen–Nitrogen Analyzer[1]

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Carbon, hydrogen, and nitrogen analysis has been a routine determination for many years, but has been very time-consuming for analysts to carry out. The advent of automated C, H, and N analyzers greatly improved the productivity of this determination (*1, 2, 6, 11, 18*). The recent availability of autoloading carrousels for C, H, and N analyzers has provided an opportunity to further improve the efficiency of this analytical technique (*7–9, 16*).

Our initial efforts to computerize data acquisition and calculations with the Perkin–Elmer 240 resulted in a major time savings (*12*). However, it was still necessary for analysts to be present to insert and inject samples every 6 or 7 min. Originally, much of the dead time between samples was occupied with sample weighing, but with the new autozeroing electronic microbalances this time has been minimized. Acquisition of the autoloading system provided the possibility of freeing the analyst from routine duties associated with the P–E 240 system and provided larger blocks of time to do other determinations.

CHN ANALYTICAL SYSTEM

To minimize hardware costs and take advantage of existing hardware and software, we integrated the autoloading carrousel into an existing C, H, and N analytical system as shown in Fig. 1. The basic programming wheel of the P–E 240 remains the central timing mechanism for the system. The recorder is automatically turned on when data acquisition is about to begin. This triggers a relay which sets up a timing sequence in the PDP-15/76 computer. The computer is in another location and is connected by coaxial cables to the P–E 240 thermal conductivity detectors. The program wheel also alerts the autoloader when it is ready to start a new cycle. A teletype is used for data entry and for preparation of reports. An ME-22 electronic microbalance is used off-line, and the data are en-

[1] This paper was presented in part at the Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, No. 112, 1978.

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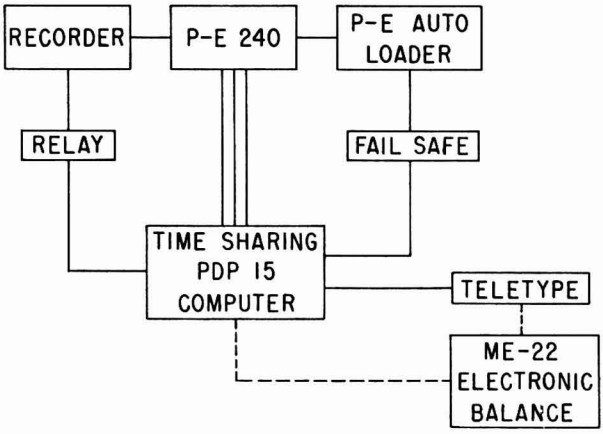

FIG. 1. Block diagram of CHN system.

tered via the teletype. We are investigating the possibility of interfacing the balance directly to the PDP-15/76.

A fail-safe device provides two functions for the system. If the computer is not available, a signal is sent to turn off the autoloader. Second, if the program indicates that a standard is out of the preset range, it will signal the fail safe device to turn off the autoloader. With these two safeguards, the analytical system can be shut off if the data cannot be processed or verified.

By building this C, H, and N analytical system up in a modular manner, we were able to incorporate many benefits of using a time-sharing computer (*14*) as well as minimize the cost of installation. While there are drawbacks in using this large computer system, such as the fact that we were dependent upon it being up and running, there are a number of overwhelming benefits that can be gained. We have 90 storage spaces in the computer which can be used to generate permanent hard-copy records, which are illustrated in Fig. 2. Second, the system can generate analytical reports which are in a format for sending directly to the initial sample submitter, as shown in Fig. 3. The large computer gives us the flexibility of monitoring the current tube file which indicates which samples are loaded in the autoloader carrousel. Access to C, H, and N data in the computer is possible through a teletype network which includes 24 terminals connected in the satelite system. This satelite system allows a number of users to retrieve information from our current file and improves the speed of communication of analytical results. The large computer also has auxiliary programs which calculate percentage composition and empirical formulas from the experimental results.

The analytical report contains the date and time that the analysis was

Blank values:	N = 56.000	C = 117.00	H = 165.000
Sensitivity values:	N = 7.353	C = 21.254	H = 59.755

Total weight	N weight	C weight	H weight	N %	C %	H %
3 J1		HN				
2901.	587.604	1504.784	145.911	20.255	51.871	5.030
4 S-1866 3201-001 9440-109 Name I-24						
2422	573.545	1072.635	72.524	23.681	44.287	2.994
5 S-1865 3201-001 9975-56-27 Name I-24						
2844.	687.970	1499.854	156.230	24.190	52.737	5.493
6 J1		HN				
2342.	471.014	1214.647	117.445	20.112	51.864	5.015
7 S-1865 3201-001 9975-56-27 Name I-24						
2896.	703.243	1534.015	160.537	24.283	52.970	5.543
9 J1		AA				
2575.	268.240	1831.026	172.713	10.417	71.108	6.707
10 S-1867 3202-001 9900-118-39 Name I-24						
2557.	310.267	1316.103	176.281	12.134	51.471	6.894
11 S-1869 1202-001 9754-91-13 Name I-25						
2860.	205.965	1429.787	115.410	7.202	49.993	4.035
12 J1		AA				
2210.	234.824	1573.274	150.673	10.626	71.189	6.818
13 S-1868 1202-001 9909-110-32 Name I-25						
2787.	159.725	2062.608	202.929	5.731	74.008	7.281
14 S-1871 630		10058-4-24 Name I-25				
2906.	287.465	1447.960	234.632	9.892	49.827	8.074
15 S-1870 630		10058-7-30 Name I-25				
2750.	386.560	1303.249	199.688	14.057	47.391	7.261
16 S-1871 630		10058-4-24 Name I-25				

FIG. 2. Summary: carbon–hydrogen–nitrogen analysis. Date, 1/25/78; time, 16:19.

14 Sample number S-1880 1202-001
Sample name 10027-56-18 Name 2-1
Sample weight = 2718 μg

Element	Sensitivity	Weight %
N	7.368	0.4
C	21.274	57.5
H	59.762	4.2

FIG. 3. Carbon–hydrogen–nitrogen analysis. Date, 2/1/78; time, 14:8:5.

completed. The sample I.D. number, project number, name of the sample submitter, and the date that he submitted the sample are included. The actual sample weight obtained from the microbalance is recorded, and after the thermal conductivity detector results are read by the computer, final results are calculated for percentage carbon, hydrogen, and nitrogen. This report not only minimizes the amount of time the analyst must expend for calculations and recopying results, it also has some additional information which is useful for running and managing the laboratory. By comparing the date run and the date submitted, we can calculate average turn-around times.

The summary report contains several types of data useful to the analyst. It contains all of the data from the analytical report plus the current blank values and the weights of the carbon, hydrogen, and nitrogen determined from the thermal conductivity detectors. In addition to this information, there are indications of which standard was used for that particular series and how frequently the standards were run during the series. Copies of the summary are made for the analyst's notebook and the permanent file.

RESULTS

This analytical system has the possibility of providing a number of time-saving advantages. It not only freed analysts to do other work by allowing the instrument to run unattended, it also provided an opportunity for using semitrained analysts to weigh samples and load the autocarrousel. We evaluated the precision and the accuracy of the system to ensure that the trade-offs in time saving did not detrimentally affect the validity of the analytical results.

Initial precision studies on acetanilide are indicated in Table 1. Theoreticals, means, and standard deviations are reported for percentage carbon, hydrogen, and nitrogen. The standard deviation on the percentage carbon was higher than the expected ±0.3%. The accuracy of the system was slightly skewed, as the mean values obtained were 0.2% low for carbon, as shown in Table 1. But we wondered whether this was a consistent bias or a random error. As indicated in Fig. 4, the results obtained appeared to be fairly random. The solid lines indicated around each of the percentage

TABLE 1
ACETANILIDE—INITIAL PRECISION, FEBRUARY–JUNE 1977 (N = 43)

	% C	% H	% N
Theoretical	71.1	6.7	10.4
Mean	70.9	6.7	10.5
SD	0.46	0.11	0.18

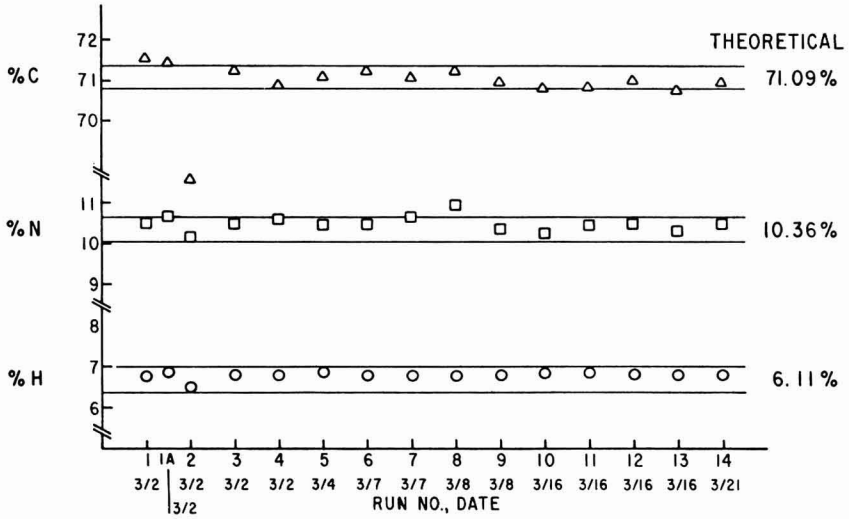

FIG. 4. Acetanilide, CHN results.

carbon, nitrogen, and hydrogen values indicate a spread of ±0.3% from the theoretical values.

To separate the effects of long-term instrument stability from the short-term instrument performance, 10 samples of acetanilide were run in a 1-day period, as shown in Table 2. The mean values were very close to the theoretical values. Results were improved over the initial long-term precision study. Standard deviation of ±0.13 for percentage carbon is well

TABLE 2
RESULTS OF SHORT-TERM PRECISION STUDY (6/8/77)

Trial no.	% C	% H	% N
1	71.25	6.79	10.40
2	71.33	6.85	10.54
3	71.14	6.83	10.45
4	70.96	6.81	10.55
5	71.08	6.83	10.51
6	71.06	6.83	10.51
7	71.01	6.81	10.48
8	71.28	6.85	10.53
9	71.05	6.82	10.44
10	70.97	6.82	10.53
Theoretical	71.09	6.71	10.36
Mean	71.11	6.82	10.49
SD	0.13	0.02	0.05

TABLE 3
LITERATURE SURVEY OF STANDARD DEVIATION FOR CHN

Reference	Carbon	Hydrogen	Nitrogen
10	0.19	0.08	0.08
19	0.17	0.10	0.14
13	0.12	0.08	0.09
13	0.10	0.05	0.06
3	0.15	0.05	0.17
14	0.18	0.18	0.10
5	0.12	0.06	

within the manufacturer's specifications and well within what most organic chemists would feel is satisfactory.

At the time, we were reporting two or three decimal values for each of the carbon, hydrogen, and nitrogen results. Even if we could obtain ±0.3%, we should only be reporting one decimal place based on the previous precision results. After instituting reporting one decimal, there were a number of inquiries whether the new C, H, and N system was as accurate as our previous semiautomated instruments or as reliable as other scientifically accepted C, H, and N analytical systems.

A literature survey found six articles that reported standard deviations for standard compounds. These are shown in Table 3. These standard deviations data for carbon, hydrogen, and nitrogen show that only one decimal place is justified in reporting the results for any C, H, and N analyses. Most of the results for carbon indicate that ±0.3% at the one σ level can be obtained. Instrumental limitations affecting precision have been reviewed by other authors (*4*, *19*).

During the review of operating procedures, a number of crucial instrumental components were identified that affect long-term stability. Two of these components were the O rings in the sample transfer block, which develop minute cuts caused by the alumina wool used for packing samples in the platinum capsules. Also, it was found necessary to establish a stringent maintenance schedule. These operational changes improved the long-term precision and accuracy, as shown by the 16 acetanilide standards from the permanent file summarized in Table 4. The mean values

TABLE 4
ACETANILIDE—LONG-TERM PRECISION, JANUARY 1978 (N = 16)

	% C	% H	% N
Theoretical	71.1	6.7	10.4
Mean	71.1	6.7	10.4
SD	0.12	0.06	0.16

TABLE 5
CYCLOHEXANONE-2,4-DINTROPHENYLHYDRAZONE (N = 10)

	% C	% H	% N
Theoretical	51.8	5.1	20.1
Mean	51.8	5.1	20.1
SD	0.08	0.04	0.12

are identical with the theoretical values, and the standard deviations are within the ±0.3% limits set for good operational practices and procedures.

A number of problem areas deserve special attention. To optimize turn-around times, it is essential that we minimize the number of specialty type compounds that have to be put into the system in 1 day's operation. Specialty areas include the high hydrogen- or nitrogen-containing compounds, liquid samples, and hydroscopic solids (*17*). To date, we have been unsuccessful in defining a single algorithm to establish one standard that would cover all of these cases. Therefore, we have had to institute special days to run each of these categories.

When we are running high nitrogen-containing compounds, we standardize on cyclohexanone-2,4-dinitrophenylhydrazone. The results in Table 5 show excellent accuracy and precision on this standard. To optimize use of the carrousel, we have found that the best operating proce-

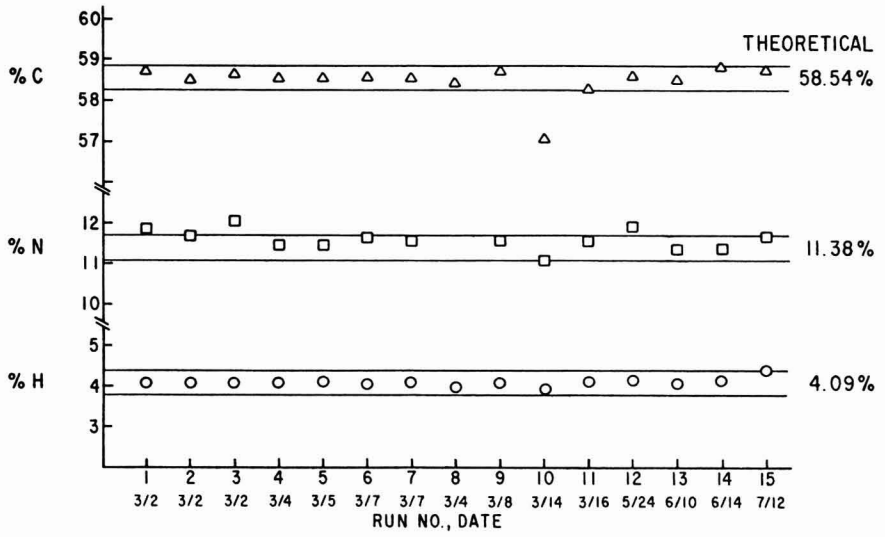

FIG. 5. Accuracy distribution plot of NBS standard nicotinic acid.

TABLE 6
BLIND STANDARDS—NICOTINIC ACID

	% C	% H	% N
Theoretical	58.5	4.1	11.4
Mean	58.5	4.2	11.6
SD	0.4	0.3	0.5
N	7	7	7

dure is to make our specialty runs early each morning and then switch over in the afternoon to our routine acetanilide standard and run the majority of the routine compounds.

While all of these previous results indicate that the precision of our measurements is very good, we went to some extremes to evaluate the accuracy. An accuracy distribution plot of NBS standard nicotinic acid is shown in Fig. 5. The theoretical values are shown on the right-hand side. The lines indicate what the spread would be to obtain ±0.3% accuracy, and the run number and date that data were obtained are shown on the *X* axis. The samples of nicotinic acid were all run as samples, and the distribution is fairly random.

As a cross-check on our standardization procedure, a number of nicotinic acid samples were submitted blind to the analyst. In Table 6, the theoretical and mean values are in close agreement, but the standard deviation is slightly higher than that obtained for acetanilide.

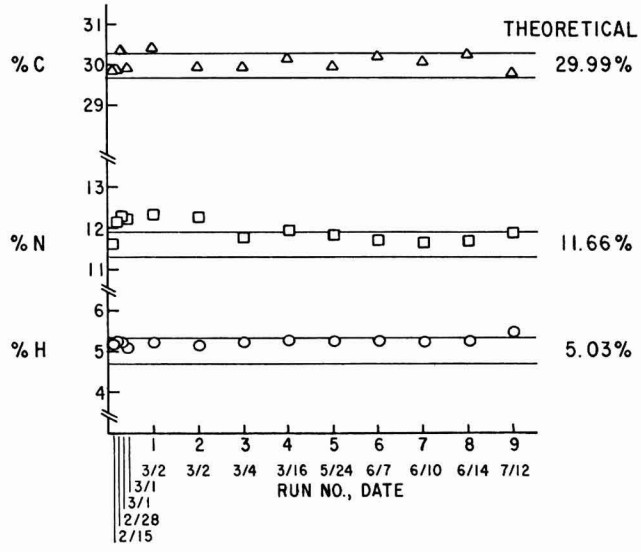

FIG. 6. Accuracy distribution plot of cystine.

TABLE 7
ACCURACY STUDY OF NBS COMPOUNDS

	% C	% H
	Triphenyl phosphate (N = 11)	
Theoretical	66.3	4.6
Mean	66.3	4.6
SD	0.19	0.07
	Bromobenzoic acid (N = 10)	
Theoretical	41.8	2.6
Mean	42.0	2.5
SD	0.20	0.04
	Fluorobenzoic acid (N = 8)	
Theoretical	60.0	3.6
Mean	60.1	3.6
SD	0.28	0.05
	Chlorobenzoic acid (N = 12)	
Theoretical	53.7	3.2
Mean	53.9	3.2
SD	0.26	0.02

Another NBS standard used for accuracy and precision study was cystine. As shown in Fig. 6, during the early stages of using the instrument, the precision was poor. As operating conditions were refined, the accuracy fell within the ±0.3% limits expected from the instrument.

We also were interested in characterizing the results when halogens and other elements were present, as shown in Table 7. In the case of chlorobenzoic acid, theoreticals and means again agreed fairly well and the standard deviation is very close to what we expect for routine determinations. Twelve samples were run, as indicated by $N=12$.

For triphenyl phosphate, the accuracy obtained from comparing theoreticals and means is very good and the standard deviation is very acceptable. We also characterized fluorobenzoic acid and bromobenzoic acid.

SUMMARY

These results indicate that a C, H, and N system can be automated and computerized for increased flexibility while maintaining a high degree of accuracy and precision. There are a number of significant advantages which can be realized in terms of hard-copy summaries, report formats that can be torn off and sent out, and tube files that can be used to summarize which samples are currently being run. There are some drawbacks in the fact that specialty runs must be made for high nitrogen-containing compounds, liquids, and hydroscopic mate-

rials. However, the time saving is substantial, and the analyst has blocks of time to run other determinations. Satisfactory turn-around times can be maintained. This indicates that the automation and computerization are well worth the efforts.

REFERENCES

1. Buis, W. J., and Rotscheid, G. J., Automatisering in de organische elementanalyse I. Koolstof-waterstof bepaling. *Chem. Tech. (Amsterdam)* **29**, 285–288 (1974).
2. Celler, Z., Automatyzacja analizy elementarnej i kierunki jej rozwoju. *Przem. Chem.* **53**, 534–540 (1974).
3. Childs, C. E., and Henner, E. B., A direct comparison of the Pregl, Dumas, Perkin–Elmer, and Hewlett–Packard (F&M) carbon–hydrogen–nitrogen procedures. *Microchem. J.* **15**, 590–597 (1970).
4. Clerc, J. T., and Simon, W., Limitations of a self-intergrating method for the simultaneous C, H, and N determination by thermal conductivity measurement. *Microchem. J.* **7**, 422–436 (1963).
5. Condon, R. D., A new automatic organic elemental microanalyzer. *Microchem. J.* **10**, 408–26 (1966).
6. Ebel, S., Uber ein automatisiertes, rechnergesteuertes System in der Mikroelementaranalyse zur simultanen CHN-Bestimmung. *Z. Anal. Chem.* **262**, 349–352 (1972).
7. Ellison, M., Aids and a modification carried out to a Carlo Erbe CHN automatic analyzer. *Chem. Ind. (London)* **19**, 787–788 (1974).
8. Fraisse, D., Automatic microanalyzers. *Microchem. J.* **21**, 170–177 (1976).
9. Fraisse, D., and Schmitt, N., Automatic microanalyzers. *Microchem. J.* **22**, 109–130 (1977).
10. Graham, J. H., Improved instrumental carbon, hydrogen, and nitrogen analysis with electronic integration. *Microchem. J.* **13**, 327–345 (1968).
11. Gustin, G. M., Automatic C–H–N analyzer case history. *Progr. Anal. Chem.* **5**, 319–336 (1973).
12. Harrington, D. E., and Bramstedt, W. R. On-line data acquisition system for Perkin–Elmer 240 carbon, hydrogen, and nitrogen analyzer. *Microchem. J.* **21**, 60–69 (1976).
13. Hozumi, K., Tsuji, O., and Kushima, H., An automatic microdetermination of carbon, hydrogen, and nitrogen in organic compounds using data printing system. *Microchem. J.* **15**, 481–497 (1970).
14. Merz, W., Use of data-processing in modern rapid methods of elemental analysis. *Talanta* **21**, 481–499 (1974).
15. Miyahara, K., and Takaoka, T., Ultramicrodetermination of nitrogen in organic compounds. *Microchem. J.* **22**, 7–14 (1977).
16. Perkin–Elmer Corporation, "Model 240 Elemental Analyzer," (1976).
17. Roemer, F. G., van Schaik, J. W., Brunt, K., and Griepink, B., On the automatic sub-milligram determination of carbon and hydrogen, VIII. *Z. Anal. Chem.* **273**, 109–111 (1975).
18. Salzer, F., Physiochemical measuring methods for end-point determination in organic elemental microanalysis. *Microchem. J.* **16**, 145–164 (1971).
19. Wachberger, E., Dirscherl, A., and Pulver, K., Automatic procedure for the CHN analysis of organic substances in the milligram range. *Microchem. J.* **16**, 318–328 (1971).

MICROCHEMICAL JOURNAL 24, 168–172 (1979)

Microdetermination of Carbon in Organic Compounds by Oxygen Flask Combustion with Atomic Absorption Spectrophotometric, Gravimetric, and Titrimetric Finishes

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INTRODUCTION

Few methods have been reported in the literature for the determination of carbon in organic compounds by oxygen flask combustion. Juvet and Chiu (*5*) placed the sample on glass-wool and ignited it electrically in an atmosphere of oxygen. The evolved carbon dioxide was absorbed in an aqueous sodium hydroxide solution and was determined acidimetrically. Cheng and Smullin (*1*) used a small porcelain boat as a carrier for the organic compound, then ignited it in the presence of oxygen. The carbon dioxide formed was precipitated as barium carbonate which, in turn, was dissolved in an excess of acid and, from the back titration of the acid, the carbon content was estimated. Later, Gutbier and Ihn (*3*) gave a modification of the combustion apparatus of Cheng and Smullin. The sample was covered with fine sand and a platinum wire gauze, and then was burned in an oxygen atmosphere. The carbon dioxide formed was absorbed in a standard barium hydroxide, alkaline strontium nitrate, or sodium hydroxide solution. The excess base was back titrated with a standard hydrochloric acid using (α-naphtholphthalein), disodium dihydrogenethylene diamine tetra-acetate (EDTA) using (methyl thymol blue/phenolphthalein), or hydrochloric acid using (thymol blue, methyl red/methylene blue). In the present work the organic compound was weighed in a tin foil boat which was wrapped in an aluminium foil, then decomposed by an electric oxygen-filled flask (Fig. 1) using calcium hydroxide solution as an absorbent. After combustion the percentage of carbon was determined by five methods for one experiment (Table 1):

(i) Excess calcium ions in the supernatant solution was determined by atomic absorption spectrophotometry at 422.7 nm (*4*) (Method I), or (ii) titrimetrically by EDTA (*6a*) using Eriochrome Black T (Method II). (iii) The formed calcium carbonate was determined gravimetrically (*6b*) (Method III), or (iv) was dissolved in hydrochloric acid (2 *N*) and then

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TABLE 1

MICRODETERMINATION OF CARBON IN ORGANIC COMPOUNDS BY OXYGEN FLASK COMBUSTION WITH ATOMIC ABSORPTION SPECTROPHOTOMETRIC, GRAVIMETRIC, AND TITRIMETRIC FINISHES

Compound	Weight (mg)	Calc.	Carbon (%) found; method:					Error (%)				
			I	II	III	IV	V	I	II	III	IV	V
Benzoic acid	2.354	68.84	68.71	68.95	69.00	68.80	69.00	−0.13	+0.11	+0.16	−0.04	+0.16
	2.430		68.65	69.00	68.75	68.60	68.50	−0.19	+0.16	−0.19	−0.24	−0.34
	3.110		68.59	68.70	68.60	68.70	69.10	−0.25	−0.14	−0.24	−0.14	+0.26
Acetanilide	3.216	71.09	71.00	71.12	71.00	70.95	71.05	−0.09	+0.03	−0.09	−0.14	−0.04
	3.510		71.12	71.20	70.91	71.10	70.81	+0.03	+0.11	−0.18	+0.01	−0.28
	2.020		70.80	71.25	71.21	71.00	71.20	−0.29	+0.16	+0.12	−0.09	+0.11
l-Chloro-2-4-dinitrobenzene	2.090	35.54	35.44	35.56	35.35	35.40	35.38	−0.10	−0.02	−0.19	−0.14	−0.26
	2.845		35.71	35.63	35.71	35.51	35.61	+0.17	+0.09	+0.17	−0.03	+0.07
	1.987		35.55	35.50	35.62	35.55	35.34	+0.01	−0.04	+0.08	+0.01	−0.20
4-Bromobenzoic acid	3.025	41.82	41.65	41.73	41.61	41.56	41.80	−0.17	−0.09	−0.21	−0.26	−0.02
	3.659		41.68	41.90	41.99	41.81	41.91	−0.14	+0.08	+0.17	−0.01	+0.09
	2.987		41.88	41.70	41.75	41.67	41.85	+0.06	−0.12	−0.07	−0.15	+0.03
2-Iodobenzoic acid	3.155	33.89	33.99	34.10	33.75	33.74	33.81	+0.10	+0.21	−0.14	−0.15	−0.08
	2.703		33.71	33.90	33.67	33.61	33.71	−0.18	+0.01	−0.22	−0.28	−0.18
	2.543		33.73	33.70	34.00	33.90	33.99	−0.16	−0.19	+0.11	+0.01	+0.10

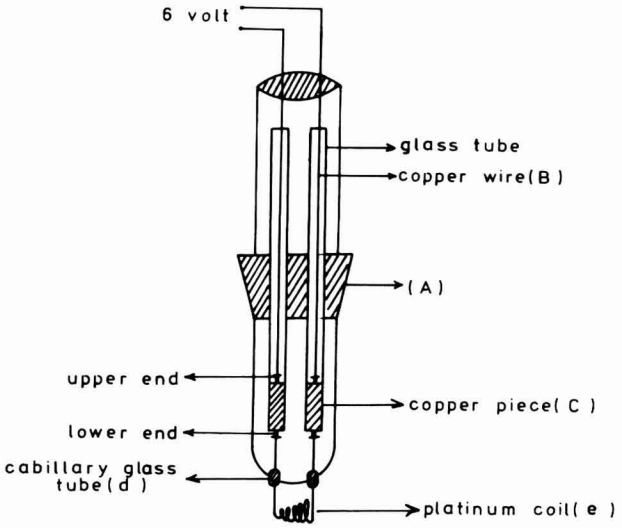

FIG. 1. The upper part of the combustion flask.

determined by atomic adsorption spectrophotometry (Method IV) or (v) titrimetrically with EDTA using Eriochrome Black T (Method V).

EXPERIMENTAL METHODS

Apparatus

Atomic absorption spectrophotometer, Unicam SP 1900.

Electric oxygen flask. It is made of a 250-ml flat-bottom flask, with a specially designed ground-glass stopper equipped with an electrically heated device for combustion of the sample. The arrangement of the upper part of the combustion flask is shown in Fig. 1: (A) is the stopper made of ₹ 29/32 inner grind; (B) indicates the copper leads; the upper ends are connected to a 6.0-V transformer, and the lower ends are welded to two copper screws through two copper pieces (C). The two ends are connected with the platinum coil by two screws in the lower ends.

The platinum coil is 25.0 cm in length and about 0.6 mm in diameter and consists of five spirals (e). The two ends of the coil are fused into two short length capillary glass tubes (d) sealed to the base of the stopper, also connected to the two screws in the lower ends of the two copper pieces.

Reagents

All reagents used are of M.A.R. grade except where otherwise mentioned.

Calcium carbonate, BDH.

Concentrated hydrochloric acid (sp. gr. 1.18).

Calcium hydroxide solution: 2.5 g in 1 l, filtered and kept in a stoppered flask.

Disodium dihydrogenethylene diamine tetra-acetate (EDTA), 0.01 *M* solution in distilled water.

Eriochrome Black T indicator: 0.2 g in triethanol amine (15 ml) and absolute ethanol (5 ml).

Ammonia–ammonium chloride buffer solution, pH 10: Concentrated ammonia (142 ml, sp. gr. 0.88–0.9) plus 17.5 g ammonium chloride, dissolved and diluted to 250 ml with distilled water.

Magnesium complex of EDTA. EDTA (3.01 g) and hydrated magnesium sulfate (1.99 g) are dissolved in water (75 ml), and neutralized with sodium hydroxide (phenolphthalein just reddened), made up to 100 ml with distilled water.

General Procedure for Flask Combustion

The sample is weighed in a tin foil boat (1–5 mg) which is wrapped in an aluminum foil and then inserted in the platinum coil. Combustion products are absorbed in 25.0 ml of calcium hydroxide solution. Combustion is done in the normal manner.

Finishing Procedure

Method I. The calcium ions concentration is measured at 422.7 nm with an atomic absorption spectrophotometer.

Method II. Titration with the standard EDTA solution in the normal manner.

Method III. The filtered calcium carbonate precipitate is dried to constant weight by heating for 3 hr at 150°C and weighed as $CaCO_3$.

Method IV. The filtered off calcium carbonate is dissolved in 2 *N* hydrochloric acid and the calcium ions concentration was determined with an atomic absorption spectrophotometer.

Method V. The dissolved carbonate is titrated with EDTA using Eriochrome Black T as indicator.

RESULTS AND DISCUSSION

The oxygen flask combustion is suitable for the quantitative conversion of the carbon content of organic compounds to carbon dioxide. The use of a tin foil boat wrapped in aluminium foil proved to be sufficient for complete combustion. Calcium hydroxide solution was found to be an excellent absorbent for carbon dioxide to give calcium carbonate quantitatively.

Application of this method gives a simple and accurate method for determination of carbon in the different combinations of organic compounds containing carbon, hydrogen, nitrogen, oxygen, chlorine, bromine, and iodine. Results were obtained using gravimetric, volumet-

ric, and atomic absorption spectrophotometric finishes. Application of this method for determination of carbon in organic compounds containing sulfur gave good results; this may be attributed to the partial solubility of calcium sulfate in acid medium (HCl, HNO_3, H_2SO_4, H_3PO_4, HCOOH, and $Cl-CH_2-COOH$) (*2*).

SUMMARY

A simple and accurate method is presented for the determination of the carbon content of organic compounds by electric oxygen flask combustion. Five results are obtained for one experiment by atomic absorption spectrophotometric, gravimetric, and titrimetric finishes.

REFERENCES

1. Cheng, F. W., and Smullin, C. F., An apparatus for the flask combustion of organic compounds adaptable to carbon determination. *Microchem. J.* **4**, 213–215 (1960).

2. Comey and Hanhn, "A Dictionary of Chemical Solubilities Inorganic," p. 946. Macmillan, New York, 1921.

3. Gutbier, G., and Ihn, W., Volumetric microdetermination of carbon in organic compounds after combustion in an oxygen filled flask. *Mikrochim. Acta* 24–33 (1966).

4. Ramirez-Munoz, J., "Atomic Absorption Spectroscopy," p. 401. Elsevier, Amsterdam, London, New York, 1968.

5. Juvet, R. S., and Chiu, J., Determination of carbon in organic substances by an oxygen flask method. *Anal. Chem.* **32**, 130–131 (1960).

6. Vogel, A. I., "A Text-Book of Quantitative Inorganic Analysis," 3rd ed. (a) p. 436, (b) p. 476. Longmans, Green, New York, 1961.

MICROCHEMICAL JOURNAL 24, 173–178 (1979)

A Rapid Method for Simultaneous Estimation of 5-Hydroxy-3-Indole Acetic Acid (5HIAA) and 5-Hydroxytryptamine (5HT) in Rat Brain

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Investigations of 5HT metabolism and its turnover in the central nervous system require a measure of the neurotransmitter 5HT and its degradation product 5HIAA in the same extract. Most routinely available 5HT and 5HIAA determination techniques require numerous manipulations and take a lot of time. A batch procedure is used here to separate 5-hydroxyindole derivates. When isolated, 5-hydroxyindoles were assayed by the Maickel and Miller procedure (*7*), which is based on the fluorescence given by a condensation product formed in strong acid medium between 5-hydroxyindoles and *o*-phthaldialdehyde. Its adaptation to the determination of 5HT and 5HIAA in brain tissue after rapid isolation of 5HIAA is now described.

METHODS

Reagents

All reagents were prepared with desalted and then twice-distilled water. Methanol, L-cysteine hydrochloride monohydrate, and iron-free hydrochloric acid ($< 3 \times 10^{-5}\%$) were purchased from Prolabo, Paris. Serotonin creatinine sulfate, 5 hydroxyindolacetic acid, 5 hydroxytryptophan (5HTP), *o*-phthaldialdehyde, and ethylenediaminetetraacetic acid disodium salt were purchased from Sigma Chemical Co., L(+)-ascorbic acid from Merck Laboratories, and Dowex 50 WX-12 from Bio-Rad Laboratories.

(1) The methanol extraction solution contains 1.7 mmol/liter L-ascorbic acid and 2.7 mmol/liter Na_2-EDTA. These compounds were added to avoid heavy metal interferences in fluorescence reactions and 5HT oxydation as described by Anden and Magnusson (*1*).

(2) Dowex 50 WX-12 (100–200 mesh) in hydrogen form was washed repeatedly with 0.1 mol/liter HCl. Particles which did not settle were removed by aspiration. Resins were then rinsed with distilled water to remove the acid, 0.1 mol/liter NaOH was then added, and resins were finally rinsed with distilled water until obtention of a neutral eluate. A

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resin suspension in a water–methanol mixture (60%, v/v) is then prepared.

(3) L-Ascorbic acid solution, 1.7 mmol/liter.

(4) Cysteine hydrochloride solution, 4.2 mmol/liter; 0.5 ml/liter Brij 35 was added to it.

(5) *o*-Phthaldialdehyde reagent, 0.6 mmol/liter, was prepared in concentrated HCl (12.2 mol/liter).

(6) Standard solutions. (a) Stock solution: The concentration of the stock solution of each standard compound was 1 mmol/liter. The 5HT solution was prepared with 0.01 mol/liter HCl; the 5HIAA solution was prepared in twice-distilled water. These solutions could be stored for 1 month at −80°C. (b) Dilute standard solutions: Just prior to assay, a 1000-fold dilution of each stock solution was made in a mixture of methanol extraction solution. To 200 μl of this mixture was added L-ascorbic acid solution up to a final volume of 2 ml.

Tissue Preparation

Male Wistar rats weighing about 200 g were housed four to a cage with free access to water but not to food for 18 hr before their sacrifice. Rats were always killed in the morning between 10 and 11 AM to account for nycthemeral variations observed by Dixit and Buckley (*4*). The animals were guillotined and their brains were quickly removed from the skull, then rinsed with chilled physiological saline and dissected to eliminate cerebellum, olfactory, and spinal bulbs. Then brains were blotted dry and weighed. These samples were frozen and stored at −80°C until assayed. In some experiments rats were treated intraperitoneally with reserpine (5 mg/kg) or with pargyline (75 mg/kg). Rats were killed, respectively, 16 hr and 2 hr after injections. Rats brains weighing about 1.5 g were homogenized in chilled methanol extracting solution up to a final volume of 5 ml. All of these operations as well as the storage before assay were carried out in ground ice and in subdued light. Homogenized samples were transferred in a 6.5-ml tube and centrifuged for 10 min at 4°C and 4000 rpm (2500*g*).

Batch Procedure

For total 5-hydroxyindole determinations, 100 or 200 μl of the supernatants was diluted to 2 ml with 1.7 mmol/liter L-ascorbic acid solution and this solution was directly used for the fluorometric automated method.

For 5HIAA determinations, 200 μl of the supernatants was added to 200 μl of Dowex suspension in 2.2-ml stoppered cups (Technicon Corp.). The resulting mixture was shaken vigorously, and 1.6 ml of 1.7 mmol/liter L-ascorbic acid solution was added to it. Dowex beads were sedimented for 5 min. These cups were then transferred to the Technicon sampler where the supernatant was directly pumped for fluorometric assays.

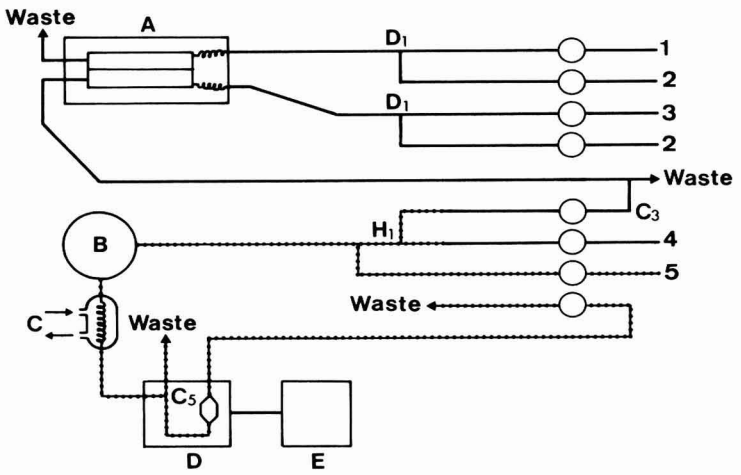

FIG. 1. Flow system for fluorimetric measurements (A) Dialyzer, 37°C (Technicon), with mixing coil 14 turns and cuprophane membrane; (B) double-coil heating bath, 96°C; (C) cooling coil; (D) ratio fluorometer (Farran) with excitation filters 350 nm, fluorescence 450 nm. Internal diameters of tubing are (in inches): (1) sample, 0.065; (2) air, 0.035; (3) L-cysteine chlorhydrate reagent, 0.065; (4) air, 0.030; (5) *o*-phthaldialdehyde reagent, 0.065. Glass fittings are from Technicon Corp. All manifold proportioning tubes are polyvinyl chloride. All lines carrying *o*-phthaldialdehyde reagent (broken line) are of Acidflex tubing.

Fluorometric Determinations

Manifold and flow-rate conditions are described in Fig. 1. Practical conditions were previously described (*5*, *11*).

RESULTS AND DISCUSSION

The results obtained for dilute standard solutions of 5HT and 5HIAA are given in Table 1. Tissue extracts obtained from several rat brains (as described in Methods) are mixed, then 15 assays on this mixed tissue extract are performed to determine the reproducibility of the method. 5-Hydroxyindoles (expressed as 5HT), 5HIAA, and calculated 5HT in the

TABLE 1
FLUORESCENCE IN ARBITRARY UNITS (MEAN ± SD)
OF 5HT AND 5 HIAA SOLUTIONS IN METHANOL EXTRACTION SOLVENT

Concentration (nmol/ml)	5HT	5HIAA
2.0	141 ± 2 (5)[a]	122 ± 2 (5)
1.5	111 ± 3 (5)	96 ± 1 (3)
1.0	78 ± 2 (5)	65 ± 1 (4)
0.5	33 ± 1 (5)	33 ± 1 (5)

[a] Number of determinations.

extraction solvent are, respectively, 1.33 ± 0.02, 0.54 ± 0.04, and 0.90 ± 0.05 nmol/ml (mean ± SD). The reproducibility of assays as measured by the variation coefficient is, respectively, 1.5, 7.4, and 5.6%. The separation of 5-hydroxyindoles is checked by adding known quantities (ranging from 0.5 to 2.0 nmol) of 5HT and 5HIAA to 1 ml of aqueous solutions (eight determinations) or cerebral extracts (eight determinations) and processing such samples through the method. The results obtained indicate that 5HT produces a slight interference, 4.4 ± 1.7% (mean ± SE), in 5HIAA determination. The interference of added 5HIAA in 5HT assays is 4.4 ± 1.0%. The same results indicate that the recovery of added 5HT is 97.8 ± 0.9% and that of 5HIAA is 96.6 ± 0.5% (mean ± SE).

The sensitivity of the method allows us to measure 50 pmol of 5HT and 50 pmol of 5HIAA in cerebral extracts. Therefore the cerebral concentrations of 5HT and 5HIAA can be determined on only 60 mg of cerebral tissue in rat.

Storage of brains at −80°C for 10 months decreases the 5HT concentration (43% in a typical run) without significant variations of the 5HIAA concentration (Table 2). The storage of methanolic extracts is possible for 5 days (Fig. 2), but after this delay cerebral extracts become dark. Blanks were performed as described by Peuler and Passon (*9*). If the cerebral extracts are used in the first week after their preparation, the fluorescence observed with a condensing reagent free of *o*-phthaldialdehyde is very low. Thus the 5-hydroxyindoles measured may well be overestimated by only about 0.08 nmol/ml of the extract and the 5HIAA by about 0.15 nmol/g wet weight (mean of five experiments).

The results obtained on rat brains by our method are compared to some already published (Table 3). No significant differences are observed with the results of any authors except those of Curzon and Green (*3*). An experiment is also carried out on rats with intraperitoneal injections of reserpine and pargyline, respectively, at a dose of 5 and 32 mg/kg. The

TABLE 2
EFFECT OF STORAGE ON THE RECOVERY OF 5HT AND 5HIAA IN BRAIN (MEAN ± SD)

	nmol/g wet weight	
	5HT	5HIAA
A[a]	1.49 ± 0.21	2.27 ± 0.54
B	2.61 ± 0.38	2.63 ± 0.43
	$P < 0.001$	NS

[a] (A) Six brains obtained from adult rats were stored at −80°C for 10 months before being assayed. (B) Five brains obtained from adult rats were assayed less than 3 hr after they were removed.

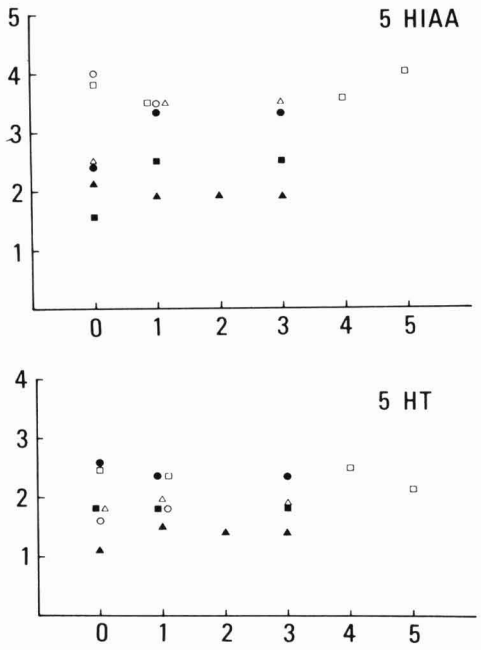

FIG. 2. Effect of storage (at −80°C) on the values of 5HT and 5HIAA in methanol extracts obtained from rat brain. Abscissa, days of storage; ordinate, nmol/g wet weight. Each symbol refers to an animal.

TABLE 3
CONCENTRATIONS OF 5HT AND 5HIAA IN WHOLE BRAIN OF RAT (nmol/g WET WEIGHT)[a]

Authors		5HT	5HIAA
Curzon and Green (*3*)	(a)[b]	3.78 ± 0.02 (10)[c]	1.88 ± 0.02 (4)
Scapagnini *et al.* (*10*)	(a)	3.48 ± 0.18 (ns)	1.89 ± 0.09 (ns)
Fischer and Aprison (*6*)	(a)	2.25 ± 0.24 (6)	1.40 ± 0.14 (6)
Maruyama and Takemori (*8*)	(a)	2.96 ± 0.17 (4)	0.94 ± 0.03 (4)
	(b)	7.84 ± 0.09 (4)	0.30 ± 0.05 (4)
Peuler and Passon (*9*)	(a)	2.42 ± 0.07 (10)	
	(e)	0.97 ± 0.04 (5)	
	(c)	3.92 ± 0.07 (5)	
Douay and Kamoun	(a)	2.61 ± 0.17 (5)	2.63 ± 0.19 (5)
(present study)	(d)	1.07 ± 0.17 (5)	3.19 ± 0.19 (5)
	(b)	4.04 ± 0.68 (5)	2.02 ± 0.14 (5)

[a] Mean ± SE. Some results were calculated from published results.

[b] (a) Controls; (b) 75 mg/kg pargyline 2 hr before sacrifice; (c) 32 mg/kg pargyline 4 hr before sacrifice; (d) 10 mg/kg reserpine 16 hr before sacrifice; (e) 10 mg/kg reserpine 4 hr before sacrifice.

[c] Number of experiments.

animals are killed, respectively, 16 hr and 4 hr after these injections. Then 5HIAA and 5HT are determined on cerebral extracts as described in Methods. As expected, a consistent increase after pargyline and a significant decrease after reserpine are observed for cerebral 5HT concentrations and opposite variations are pointed out for 5HIAA. The interference of 5-hydroxytryptophan (5HTP) is also studied by its addition to aqueous solutions and cerebral extracts. The 5HTP binds to the ion-exchange resin and so interferes in 5HT determinations when its concentration is higher than 0.3 nmol/ml.

The method described here is rather simple and can be used to determine quickly 5HIAA and 5HT concentrations in rat brains, but it cannot be applied to the assay of these products when high amounts of 5HTP are present, for example, if an inhibitor of 5HTP decarboxylase such as R04-4602 could be injected (*2*).

SUMMARY

The authors describe a method to separate 5-hydroxy-3-indole acetic acid (5HIAA) from 5-hydroxyindole derivatives by means of a batch procedure using Dowex 50 WX 12 resins. These compounds were assayed by the Maickel and Miller procedure, and 5-hydroxytryptamine (5HT) was then calculated as the difference between total 5-hydroxyindoles and 5HIAA. This procedure allows a rapid assay of 5HT and 5HIAA in about 60 mg of rat cerebral tissue, but it may not be used when large amounts of 5-hydroxytryptophan are present.

REFERENCES

1. Anden, N. E., and Magnusson, T., An improved method for the fluorimetric determination of 5-hydroxytryptamine in tissues. *Acta Physiol. Scand.* **69**, 87–94 (1967).
2. Colmenares, J. L., Wurtman, R. J., and Fernstrom, J. D., Effects of ingestion of a carbohydrate-fat meal on the levels and synthesis of 5-hydroxyindoles in various regions of the rat central nervous system. *J. Neurochem.* **25**, 825–829, (1975).
3. Curzon, G., and Green, A. R., Effects of hydrocortisone on rat brain 5-hydroxytryptamine. *Life Sci.* **7**, 657–663 (1968).
4. Dixit, B. N., and Buckley, J. P., Circadian changes in brain 5-hydroxytryptamine and plasma corticosterone in the rat. *Life Sci.* **6**, 755–758 (1967).
5. Douay, O., and Kamoun, P., Microdosage automatique de la sérotonine sur sang capillaire. *Ann. Biol. Clin.* **34**, 47–51 (1976).
6. Fischer, C. A., and Aprison, M. H., Determination of nanomole levels of 5-hydroxytryptophan, 5-hydroxytryptamine and 5-hydroxyindolacetic acid in the same sample. *Anal. Biochem.* **46**, 67–84 (1972).
7. Maicker, R. P., and Miller, F. P., Fluorescent products formed by reaction of indole derivatives and *o*-phthaldialdehyde. *Anal. Chem.* **38**, 1937–1938 (1966).
8. Maruyama, Y., and Takemori, A. E., New gas chromatographic method for the analysis of 5-hydroxytryptamine and 5-hydroxyindolacetic acid in the brain. *Biochem. Pharmacol.* **20**, 1833–1841 (1971).
9. Peuler, J. D., and Passon, P. G., An automated assay for brain serotonin. *Anal. Biochem.* **52**, 574–583 (1973).
10. Scapagnini, U., Vandenbroeck, R., and De Schaepdryver, A., Simultaneous estimations of 5-hydroxytryptamine and 5-hydroxyindol-3-acetic acid in rat brain. *Biochem. Pharmacol.* **18**, 938–940 (1968).
11. Welch, K. M. A., and Meyer, J. S., Estimation of levels of serotonin and 5-hydroxyindoles in whole blood by an automatical procedure. *J. Neurochem.* **19**, 1079–1087 (1972).

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Periodatometric Determination of Organotrithiocarbonates and Mercaptans (via Trithiocarbonate Formation)

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Organotrithiocarbonates may be considered as salts of the corresponding organotrithiocarbonic acids (I) and are

$$RS-\underset{\underset{S}{\|}}{C}-SH$$

(I)

characterized by the presence of $-\underset{\underset{S}{\|}}{C}-S^-$ group. Because of their applications in analysis, industry, medicine, and agriculture, the determination of these compounds is of great interest and scope. This communication reports the use of potassium periodate for the first time for the determination of organotrithiocarbonates which have been titrated both visually and potentiometrically in bicarbonate medium in the presence of potassium iodide. Organotrithiocarbonates are smoothly, rapidly, and quantitatively oxidized to the corresponding bis(alkyl/arylmercapto thiocarbonyl) disulfides under the specified conditions.

$$2\ RS-\underset{\underset{S}{\|}}{C}-S^- + IO_4^- + H_2O \rightarrow RS-\underset{\underset{S}{\|}}{C}-S-S-\underset{\underset{S}{\|}}{C}-SR + IO_3^- + 2\ OH^-$$

(R = alkyl or aryl group)

The method has been extended to the determination of mercaptans after their quantitative transformation to the corresponding organotrithiocarbonates through reaction with carbon disulfide in the presence of alkali.

$$RSH + CS_2 + KOH \rightarrow RS-\underset{\underset{S}{\|}}{C}-SK + H_2O$$

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The resulting trithiocarbonates are titrated with potassium periodate in the manner described above. The proposed methods for the determination of organotrithiocarbonates and mercaptans are simple, accurate, reliable, and widely applicable.

MATERIALS AND METHODS

Reagents

Acetonitrile. Acetonitrile was distilled twice from phosphorus pentoxide (5 g/liter).

Potassium periodate. Potassium periodate, 0.02 *N* in water, was prepared by dissolving a little more than the calculated amount of the compound (BDH) in water. The solution was standardized iodometrically in acid medium.

Organotrithiocarbonates. Potassium salts of ethyl, isopropyl, *n*-propyl, isobutyl, *n*-butyl, *n*-amyl, and benzyl trithiocarbonic acids were prepared (2) by shaking an equimolar mixture of corresponding mercaptan, potassium hydroxide, and carbon disulfide at a temperature below 10°C. The compounds were stored in a vacuum desiccator. The purity of the compounds was checked by nonaqueous redox titration (3).

Mercaptan. Ethyl, isopropyl, *n*-propyl, isobutyl, *n*-butyl, *n*-amyl, and benzyl mercaptans, all commercial grade, were distilled before use.

Phenolphthalein. The phenolphthalein used was a 0.1% (w/v) solution in acetonitrile.

Amylose. Amylose was a 1% aqueous solution.

Carbon disulfide. Carbon disulfide, Riedel German, was used as such.

All other reagents used in this investigation were of guaranteed quality.

Apparatus

Potentiometric titrations were performed with Toshniwal titration potentiometer (Type CLO6A), using a saturated calomel reference electrode and a bright platinum wire indicator electrode.

A microburet of 10-ml capacity, graduated in 0.01-ml divisions, was used.

Procedure

Determination of organotrithiocarbonates. Aliquots of the solution in water of organotrithiocarbonates were taken in titration vessels, and the volume made to 50 to 60 ml with water in each case. The solution was mixed with 5 g of sodium bicarbonate, 1 ml of 10% potassium iodide, and 1 ml of amylose solution and then cooled to room temperature (~20°C). Each solution was titrated visually and potentiometrically with standard (0.02 *N*) potassium periodate solution run from a microburet. In visual titrations, the end point was detected by the appearance of blue color. A

sharp jump in potential was observed at the equivalence point in potentiometric titrations.

From the volume of periodate solution used corresponding to the end point in visual and potentiometric titrations, the amount of each trithiocarbonate was calculated. Results are given in Table 1.

Determination of mercaptans. Aliquots of solution in acetonitrile of each mercaptan were taken in glass-stoppered titration flasks and mixed with 1 to 2 ml of carbon disulfide, 2 ml of potassium hydroxide, (~1 *N*) and 7 to 10 ml of acetonitrile. The flask was stoppered, swirled to mix the reactants and set aside for 5 min to ensure completion of the reaction. The resulting yellow-colored solution (indicating the formation of organotrithiocarbonates) was mixed with 40 ml of water and neutralized with acetic acid (0.1 *N*) using phenolphthalein as indicator. Each solution was then mixed with about 5 g of sodium bicarbonate, 1 ml of 10% potassium iodide, and 1 ml of amylose solution and titrated both visually and potentiometrically with standard periodate in the manner described above.

From the volume of standard periodate used corresponding to the end

TABLE 1

DETERMINATION OF ORGANOTRITHIOCARBONATES AND MERCAPTANS (VIA TRITHIOCARBONATE FORMATION) WITH POTASSIUM PERIODATE

Compound	Amount found[a] (mg)		Amount found[b] (mg)	
	Visual method	Potentiometric method	Visual method	Potentiometric method
Organotrithiocarbonates (potassium salts)				
Ethyl	9.96 ± 0.04	10.02 ± 0.03	40.12 ± 0.08	40.09 ± 0.06
Isopropyl	9.99 ± 0.04	10.00 ± 0.03	39.90 ± 0.08	39.92 ± 0.06
n-Propyl	10.00 ± 0.04	9.99 ± 0.03	40.05 ± 0.08	40.04 ± 0.06
Isobutyl	10.02 ± 0.04	10.01 ± 0.03	40.10 ± 0.08	40.06 ± 0.06
n-Butyl	9.98 ± 0.04	9.99 ± 0.03	40.12 ± 0.08	40.08 ± 0.06
n-Amyl	10.01 ± 0.04	10.00 ± 0.03	39.91 ± 0.08	39.94 ± 0.06
Benzyl	10.03 ± 0.04	10.01 ± 0.03	39.86 ± 0.08	39.92 ± 0.05
Mercaptans				
Ethyl	8.03 ± 0.03	8.02 ± 0.02	20.08 ± 0.05	20.07 ± 0.04
Isopropyl	7.99 ± 0.03	8.00 ± 0.03	19.94 ± 0.05	19.95 ± 0.05
n-Propyl	8.00 ± 0.02	7.99 ± 0.03	20.06 ± 0.05	20.04 ± 0.05
Isobutyl	7.98 ± 0.03	7.98 ± 0.02	20.07 ± 0.05	20.05 ± 0.05
n-Butyl	8.02 ± 0.03	8.00 ± 0.03	19.93 ± 0.05	19.95 ± 0.05
n-Amyl	8.02 ± 0.03	8.01 ± 0.03	19.95 ± 0.05	20.03 ± 0.05
Benzyl	8.03 ± 0.03	8.02 ± 0.03	20.07 ± 0.06	20.04 ± 0.04

[a] Amount of each organotrithiocarbonate taken, 10 mg; amount of each mercaptan taken, 8 mg. Results are means ± SD of 10 determinations.

[b] Amount of each organotrithiocarbonate taken, 40 mg; amount of each mercaptan taken, 20 mg. Results are means ± SD of 10 determinations.

point in visual and potentiometric titrations, the amount of resulting trithiocarbonate and consequently the amount of mercaptan was found out. Results are recorded in Table 1.

RESULTS AND DISCUSSION

The results recorded in Table 1 show that organotrithiocarbonates (potassium salts of ethyl, isopropyl, *n*-propyl, isobutyl, *n*-butyl, *n*-amyl, and benzyl trithiocarbonic acids) can be successfully titrated with potassium periodate in sodium bicarbonate medium in the presence of potassium iodide both visually (using amylose as indicator) and potentiometrically. In potentiometric titrations, the potentials attain stable values immediately on addition of each installment of the oxidant. A sharp jump in potential on the order of 50 to 110 mV per 0.05 ml of 0.02 *N* oxidant solution was observed at the equivalence point in potentiometric titrations. The overall standard deviations calculated from the pooled data of all the visual and potentiometric titrations performed with 10 mg of each compound were 0.04 and 0.03, respectively. The values for 40-mg samples were 0.08 and 0.06, respectively.

That mercaptans react with carbon disulfide in the presence of alkali to form organotrithiocarbonates has been made the basis for the determination of mercaptans. The resulting trithiocarbonates are titrated with standard periodate as usual. The excess of carbon disulfide does not interfere in these titrations. Ethyl, isopropyl, *n*-propyl, isobutyl, *n*-butyl, *n*-amyl, and benzyl mercaptans have been successfully determined. The overall standard deviations calculated from the pooled data of all the visual and potentiometric titrations performed with 8 mg of each mercaptan are 0.03 and 0.03, respectively. The values for 20-mg samples were 0.05 and 0.05, respectively.

In bicarbonate medium, potassium periodate, like other oxidants, forms iodine through reaction with potassium iodide:

$$IO_4^- + 2\ I^- + H_2O \rightarrow IO_3^- + I_2 + 2\ OH^-$$

In all titrations where iodine (formed *in situ* during the titration) acts as the real oxidant, the appearance and disappearance of iodine is visually detected in the course of titrations, and with the first drop of oxidant in excess the liberated iodine colors the solution yellow or forms the well-known blue color with starch. When trithiocarbonates were titrated with chloramine-T or *N*-bromosuccinimide in bicarbonate medium in the presence of potassium iodide, the above reactions were in fact observed (unpublished work). But on using periodate as the oxidant under similar conditions, iodine was formed only at the end point. It may therefore be inferred that the oxidation of trithiocarbonates (by the proposed method) is brought about by periodate and not by iodine (expected to be formed

during the titration). In the absence of potassium iodide the oxidation of trithiocarbonates, as indicated by potentiometric titrations, does not appear to proceed smoothly and quantitatively. From these observations, it may be concluded that potassium iodide acts as a catalyst and an indicator. This is further supported by earlier workers (1) who found the reaction between arsenite and periodate to proceed quantitatively only in the presence of potassium iodide in bicarbonate medium.

The effect of some typical compounds was studied in the determination of organotrithiocarbonates with potassium periodate by the recommended procedure in the usual way. No interference was found from alkyl isothiocyanates, isocyanates, carbon disulfide, and urea even when present in up to fivefold amounts. Xanthates, dithiocarbamates, and thioureas, however, interfere. Alcohols and amines interfere in the determination of mercaptans.

The methods described for the determination of organotrithiocarbonates and mercaptans are simple, accurate, reliable, and widely applicable.

SUMMARY

A method has been described for the direct visual and potentiometric determination of organotrithiocarbonates in bicarbonate medium in the presence of potassium iodide, using potassium periodate as an oxidimetric reagent. The method was extended to the determination of mercaptans after their quantitative transformation to the corresponding organotrithiocarbonates through reaction with carbon disulfide in the presence of alkali. The resulting trithiocarbonates are titrated with periodate in the manner described herein. The excess of carbon disulfide does not interfere in these determinations. Organotrithiocarbonates are smoothly, rapidly, and quantitatively oxidized to the corresponding bis(alkyl/aryl mercapto thiocarbonyl) disulfides with the oxidant under the specified conditions.

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REFERENCES

1. Berka, A., Vulterin, J., and Zyka, J., "Newer Redox Titrants," 1st Ed., p. 67. Pergamon Press, New York, 1965.
2. Reid, E. E., "Organic Chemistry of Bivalent Sulphur," Vol. 4, pp. 178, 247. Chemical Publishing Co., Inc., New York, 1962.
3. Verma, B. C., and Kumar, S., Determination of organotrithiocarbonates with iodine and iodine halides in non-aqueous media. *Talanta* **23**, 612–613 (1976).

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A Method for Recording X-ray Diffraction Patterns of Milligram Quantities of Particulates

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INTRODUCTION

X-ray diffraction has long been the favored tool for compound identification. The relatively low reflection efficiencies of most crystallites produces a lower detection limit of about 1% and have resulted in the need for gram weight samples in standard window mounts (*2*). In a recent study of "Hi-Vol" air filter samples (*1*), the removable particulate weights were too low to provide adequate samples by traditional methods, and this necessitated developing a sample preparation technique by which milligram amounts of material would provide an "infinitely" thick specimen of suitable surface geometry for a diffraction pattern.

MATERIALS AND METHODS

For the 5- to 15-mg particulate range an Atomic Accessories stainless-steel isotope filtering apparatus was used.

For the 1- to 5-mg particulate weight range a similar apparatus was machined from a cylinder of aluminum (Fig. 1). The deposition area was cut as a rectangular opening 10 mm long by 9 mm wide. The interior cup was polished to reduce adhesion loss of particulates. The filtering base is a standard 30-mm, medium porosity fritted disk filter tube. This is joined to the filtering cup by a screw-locking joint clamp. To prevent suction bypass of the active filtering center, the edge of the sintered filtering disk is coated with epoxy cement, and the base of the filter cup is coated with a thin film of silicone grease. The system is clamped and allowed to dry, producing a glassy smooth surface over all but the center surface of the filtering disk. This limits the passage of solvent or air to the center where the rectangular opening is located.

A Philips-Norelco XRG-3000 X-ray diffractometer system using a proportional counter was employed. The source was a standard focus, copper-target X-ray tube and nickel filter to render the radiation monochromatic. The target potential and current density varied with the sample. Pulse height discrimination was set on the 111 reflection plane of lead metal at the strongest reflection angle of 31.27°2θ.

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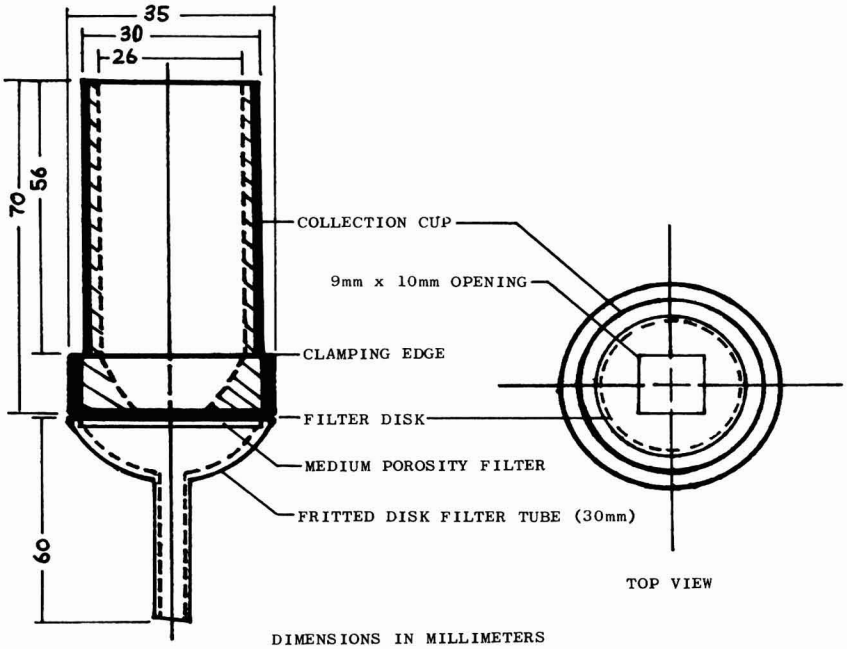

FIG. 1. Micro sample filtering apparatus.

The solvent wash used was an approximately 5% Duco cement in acetone solution.

Schleicher and Schuell No. 596, 24-mm paper filters were used to mount the samples.

PROCEDURE

The filter paper is mounted between the collection cup and filtration base. The clamp is applied and tightened in the use of the micro filter. About 5 ml of the wash solution is placed in the collection cup. The "Hi-Vol" or other gross filter is hand held directly over the collection cup (a section of the filter may be cut and treated if the total filter is too large to handle efficiently), and the spray from a plastic wash bottle containing the Duco cement acetone solution is directed against the filter so as to wash the particulates to a corner which drains directly into the collection cup. About 100 ml of wash will normally remove the available particulates. If the wash level approaches the volume of the cup, apply suction to lower the level but do not drain dry. When washing is complete suction is applied. As the solvent is removed a uniform deposit of particulates is laid down over the exposed area of the filter. When the filter is dry, the clamp is removed, followed by the removal of the collection cup. Suction is maintained to hold the filter to the filtration base, from which it can be easily removed when the suction is discontinued.

The prepared disk can be mounted on a standard aluminum diffraction slide by "Scotch"-taping the edges, or can be held in place with a suitably designed frame and clamp (Fig. 2). This holder is ideal for rapid sample changes, allows for precise centering of the deposit, and will not alter the weight of the disk, as is the case with the use of tape. The frame was made by cutting down a standard mount leaving only the window. This window when placed over a positioned filter will hold it in place with equal pressure. It, in turn, is secured with a plastic clip made from a section of the slip-on spine of inexpensive protective report binders such as the EZC report cover manufactured by the National Blank Book Co., Inc. The disk surface should be flat to insure proper interaction geometry. Any variation will cause shifts in the observed 2θ angle. The paper filter will occasionally warp slightly during drying. The resulting shifts, however, should be within about $0.2°2\theta$, and not interfere with identification. After collection of the diffraction data the disk is easily removed and stored for future reference or study.

RESULTS AND DISCUSSION

An investigation of the beam irradiation coverage of the specimen showed that for the angles scanned, 19° to $60°2\theta$, the area irradiated was about 9 × 10 mm. Trials indicated that as little as 5 mg of material provided infinite sample thickness with noise levels comparable to specimens prepared by traditional methods and that useful data could be obtained for samples of high reflectivity weighing as little as 1 mg.

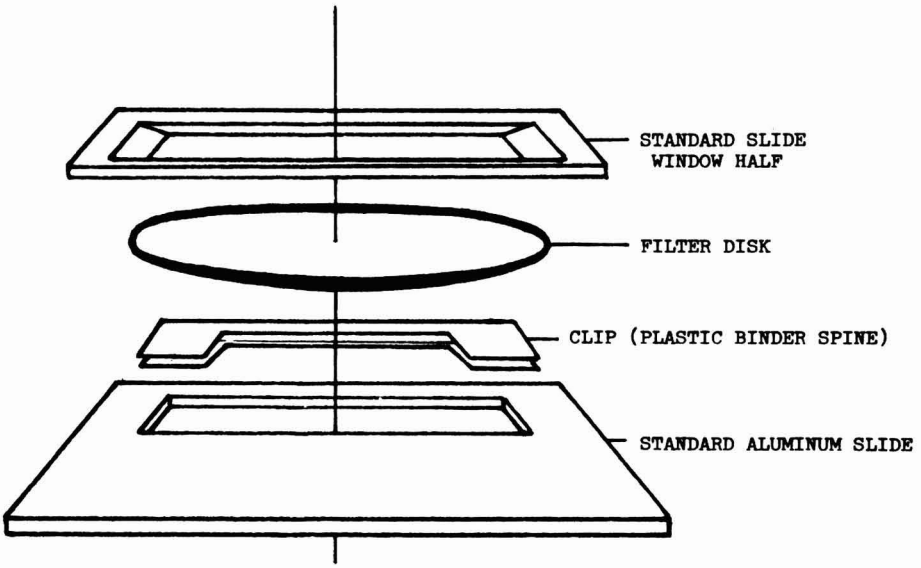

FIG. 2. Filter holder and clamp.

Samples of lead ore concentrate (Galena and matrix minerals) were prepared by the three methods and subjected to diffraction analysis. The superimposed results shown in Fig. 3 indicate the essentially identical nature of the three patterns.

The lowest trace employs the standard window mount (1.5-g sample). This pattern was run at 35 kV and 20 mA with a full-scale sensitivity of 1000 counts, a time constant of 2 sec, a scan rate of 1°2θ/min, and a chart speed of 30 in./hr. The PbS is clearly indicated by the three major peaks. Matrix compounds of $CaCO_3$, dolomite, ZnS, etc., are also easily observed.

Next, a filter disk sample of 7.1-mg weight was run at the same conditions using the Atomic Accessories isotope filter apparatus. The peak intensities remain nearly identical. Although a shift of about 0.1°2θ is observed, the patterns are obviously identical with the exception of the appearance of the filter paper amorphous scatter broad peak from 21° to 24°2θ. The low intensity of the filter paper peak indicates that the sample is approaching infinite thickness. A comparison with the blank filter pattern clearly illustrates this (Fig. 4).

A sample of lead ore concentrate of 1.1-mg weight was prepared using the micro filter apparatus. The resulting pattern was obtained at 35 kV and 8 mA at a full-scale range of 100 counts, and a time constant of 10 sec to reduce the noise. The scan rate was reduced to 0.25°2θ/min and the chart speed was correspondingly reduced to 7.5 in./hr. The resulting pattern compares very closely with both the standard pattern and the previous filter pattern. Peak intensities remain nearly the same and shifting is not significant. While the amorphous filter scatter is obviously much greater for this sample, the scatter does not significantly interfere with the pattern.

Application of this method is illustrated by the pattern in Fig. 5. This sample was provided by ASARCO, Inc., from a lead smelting operation located in Glover, Mo. Transferable particulates from the filter resulted in a prepared disk sample weight of 3.3 mg. Analysis at 35 kV and 15 mA with the prescribed scale and time constants, scan rate, and recorder rate produced a pattern that clearly shows quartz, $CaCO_3$, dolomite, and other normal background constituents. Compared with the pattern produced by 1.1 mg of the lead ore concentrate, the conspicuous absence of PbS justifies the conclusion that this compound and other smelting associated compounds are not detectable in this sample.

This technique should be valuable in air pollution and water pollution particulate studies where sample weights are low due to the short sampling periods needed to isolate incidents.

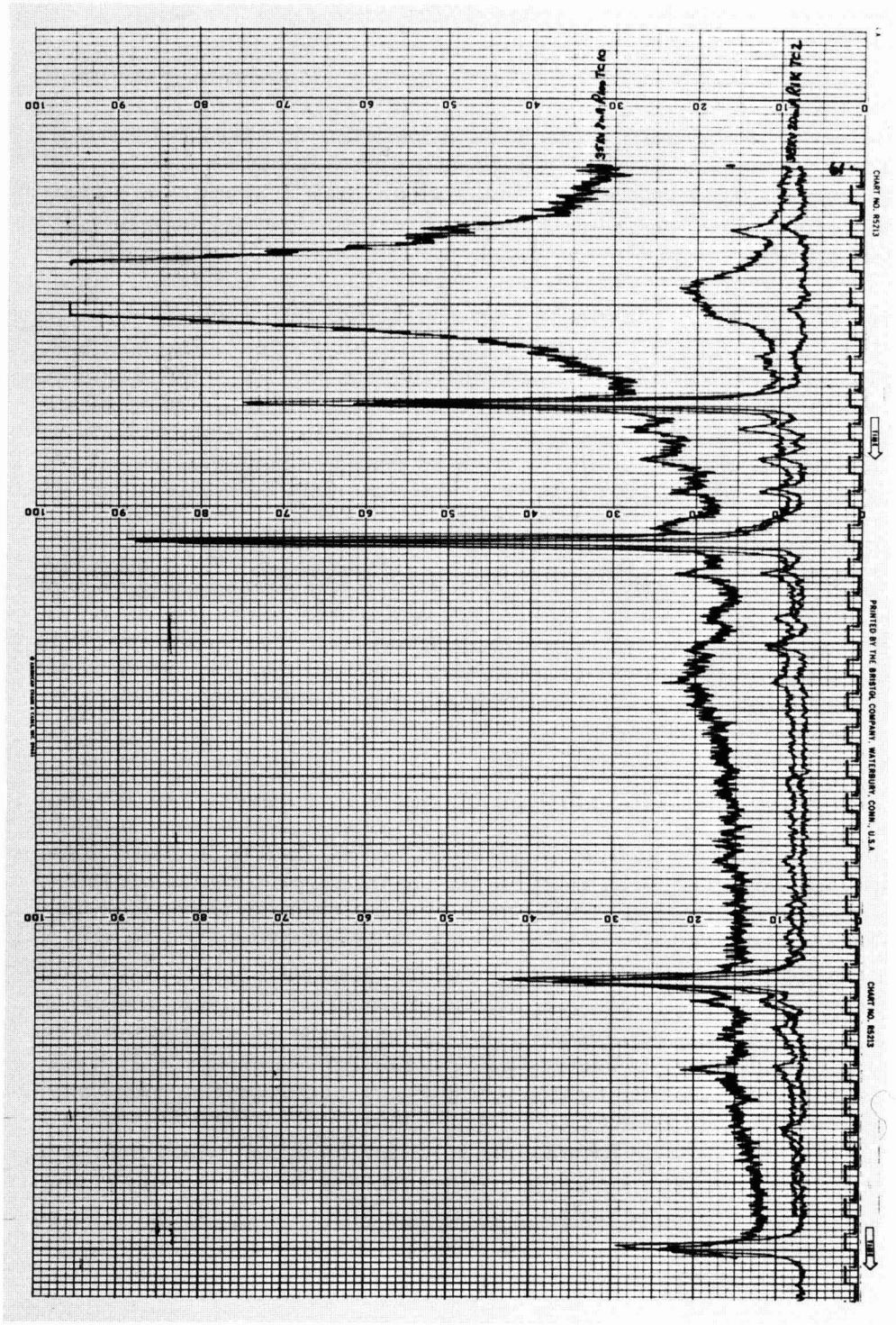

FIG. 3. Diffraction pattern comparison for different preparation methods.

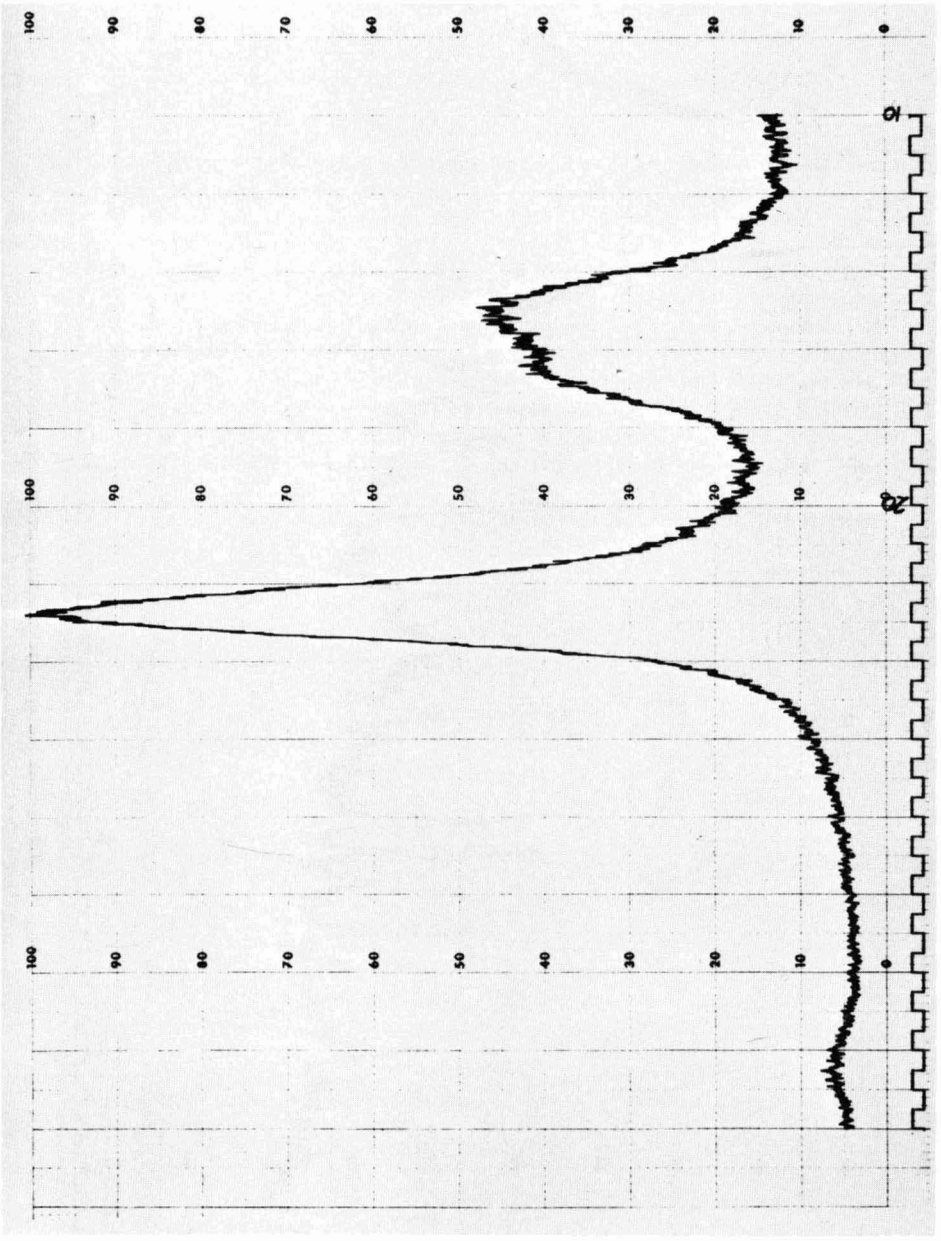

FIG. 4. Diffraction pattern for filter paper disk.

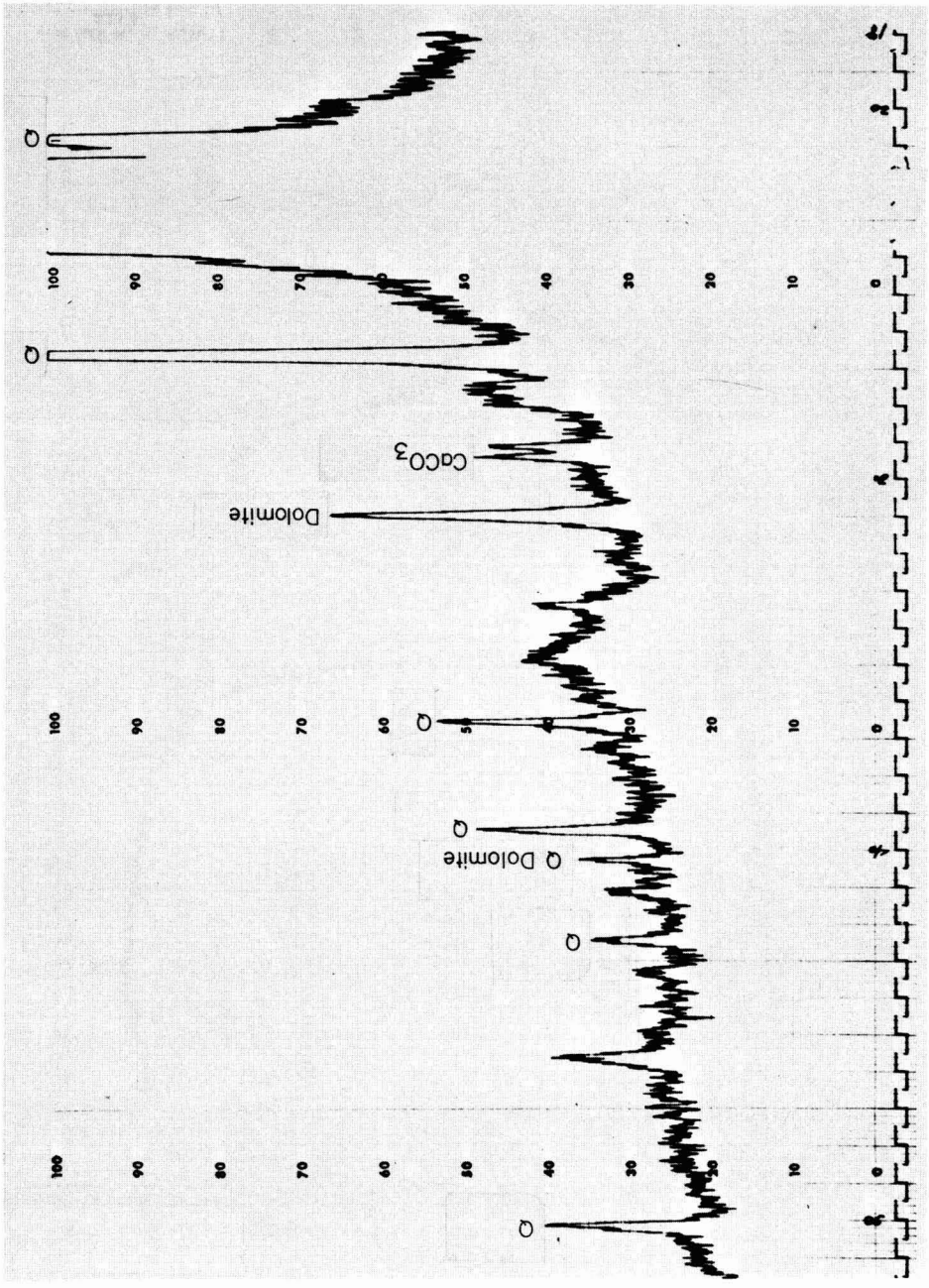

FIG. 5. Diffraction pattern for smelting operation particulates.

SUMMARY

A method for recording X-ray diffraction patterns of milligram quantities of particulates has been developed. The resulting diffraction patterns are similar to patterns obtained by standard methods. Application of this method to analysis of airborne particulates in the vicinity of a lead smelting operation has demonstrated the practical use of the method. Sample sizes as low as 1.1 mg have produced useful diffraction patterns.

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REFERENCES

1. Foster, R. L., and Lott, P. F., The X-ray identification and semi-quantification of toxic lead compounds emitted into air by smelting operations. *In* "Proceedings of the 8th IMR Symposium," National Bureau of Standards Special Publication 464, Methods and Standards for Environmental Measurement, pp. 351–366. U.S. Dept. of Commerce, U.S. Government Printing Office, Washington, D.C., 1977.
2. Klug, H. P., and Alexander, L. E., "X-ray Diffraction Procedures for Polycrystalline and Amorphous Materials," 2nd ed. Wiley, New York, 1974.

MICROCHEMICAL JOURNAL 24, 192–198 (1979)

The Rapid Determination of Microgram Amounts of Total Collagen in Pathological Lesions

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INTRODUCTION

A rapid method for quantitative collagen determination in pathological lesions is needed, particularly to elucidate the nature of fibrous connective tissue lesions, for example, in silicosis and other pneumoconioses. The development of an efficient and rapid method for the determination of hydroxyproline (the presumptive index for the quantitative assessment of collagen) is germane to studies involving collagen. In a previous study (*2*) we proposed a convenient method for determining microgram amounts of collagen metabolites. But this method took 3 days in all, including a 24-hr acid hydrolysis of the tissue and a further 20-hr incubation time for the Bergman and Loxley (*4*) spectrophotometric method for hydroxyproline. The present study reports a method which takes 8 hr, by combining a new digestion technique outlined by Gałasiński *et al.* (*7*) with a subsequent rapid determination of the resultant hydroxyproline using the methods previously proposed by Baily (*1*, *3*).

MATERIALS AND METHODS

Reagents

All chemicals used were of analytical reagent grade. Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) was recrystallized twice from ethyl alcohol according to the directions of Grunbaum and Glick (*8*). A working solution of hydrogen peroxide (2%, w/v) was prepared fresh daily by diluting 30% (w/v) hydrogen peroxide (BDH Chemicals, England) with glass double-distilled water.

Apparatus

The digestion apparatus recommended in the previous study (*2*) was used for the present work. Instead of the Beckman Model C colorimeter used previously (*1*), a Zeiss PMQII spectrophotometer with 1-cm-light path quartz cells was employed. For the absorption spectra of chromophores from hydroxyproline, a Unicam SP-800 recording ultraviolet spectrophotometer was used.

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Procedure

Rat lungs were perfused with normal saline solution, until relatively free from blood. They were then dried for at least 12 hr in an oven at 110°C and powdered. The dry powdered tissue was defatted (*5*) by vigorous stirring with a mixture of chloroform–methanol (2:1, v/v) at room temperature. The extractions were repeated three times in all, and the defatted tissue was dried again and ground to a fine powder once more.

Hydrolysis of Tissue

To test the validity of the new method for tissue hydrolysis proposed by Gałasiński *et al.* (*7*), the method was compared with the two methods previously employed by Baily (*1*, *2*). Originally, dried, defatted tissue was hydrolyzed with a large excess of 6 *N* HCl (*1*) under reflux for 24 hr. The large excess of acid ensured a minimal destruction of amino acids, and after completion of the digestion, the excess acid was removed under vacuum on a Büchi Rotavapor-R rotating evaporator. In the second method (*2*), 10 mg of dried, defatted tissue was hydrolyzed with 1 ml 6 *N* HCl in the Rotaflo hydrolysis tube under partial vacuum at 110°C for 24 hr. The third method, by Gałasiński *et al.* (*7*), recommends that proteins be hydrolyzed with 72% perchloric at 100°C for 2 hr. In the method finally adopted for this study, 5 mg of dried, defatted tissue was hydrolyzed with 0.25 ml 72% perchloric acid in a Rotaflo hydrolysis tube under partial vacuum at 110°C for 4 hr.

Analytical Method

After digestion by the above three methods, any humin formed was completely removed by filtering the hydrolysates under suction through Whatman GF/A glass-fiber filter papers supported on porosity 4 sintered glass funnels. The solutions were then transferred to 20-ml volumetric flasks, the pH was adjusted to between pH 3 and pH 4.5 with 6 *N* NaOH, and the solutions were finally made up to volume with glass double-distilled water. A 1-ml aliquot of each sample was treated according to the procedure outlined by Baily (*1*) with the exception that the citrate buffer was replaced by double-distilled water and mixing took place on a high-speed Whirlimixer for 2.5 min. After the addition of 2 *N* H_2SO_4 and 5% Ehrlich's reagent, the solution was again mixed for exactly 1 min on the Whirlimixer and then placed in a water bath for 30 min at 80°C to develop the color. Absorbances were read at 555 nm on the Zeiss PMQII spectrophotometer using 1-cm-light path quartz cells.

RESULTS

Figure 1 depicts the standard graphs for hydroxyproline before and after hydrolysis with 72% perchloric acid. Table 1 shows the effect of varying

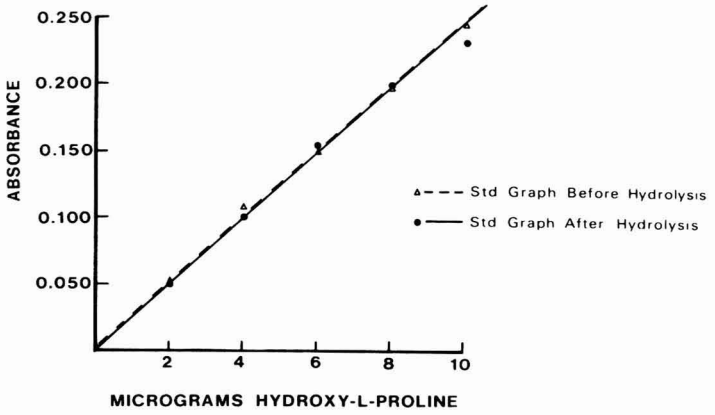

FIG. 1. Standard graph for hydroxy-L-proline before and after hydrolysis with 72% perchloric acid.

TABLE 1
HYDROLYSIS OF LUNG TISSUE

Weight of tissue (mg)	Volume of 72% perchloric acid (ml)	Time of hydrolysis (hr)	% Hydroxyproline[a]
10	0.25	2	1.41 ± 0.13
10	0.25	4	1.79 ± 0.12
5	0.25	2	1.64 ± 0.14
5	0.25	4	2.0 ± 0.13

[a] Dry, fat-free basis ± standard deviation. Each result represents the mean of five separate hydrolyses with duplicate spectrophotometric analyses.

TABLE 2
COMPARISON OF HYDROLYSIS TECHNIQUES ON LUNG TISSUE

Method	Acid used	Volume of acid (ml)	Weight of tissue (mg)	Time of hydrolysis (hr)	% Hydroxy-proline[a]
Baily (*1*)	6 *N* HCl	100	20	24	2.0 ± 0.12
Baily *et al.* (*2*)	6 *N* HCl	1	10	24	2.15 ± 0.15
Gałasiński *et al.* (*7*)	72% Perchloric acid	0.25	5	4	2.0 ± 0.13

[a] Dry, fat-free basis ± standard deviation. Each result represents the mean of five separate hydrolyses with duplicate spectrophotometric analyses.

TABLE 3
PERCENTAGE RECOVERY OF HYDROXYPROLINE ADDED TO LUNG TISSUE AFTER HYDROLYSIS WITH 72% PERCHLORIC ACID

Determination	Equivalent weight of tissue taken (μg)	Amount of hydroxy-proline added (μg)	Total hydroxy-proline found (μg)	% Recovery of added hydroxy-proline	% Recovery ± SD
1	50	—	1.16	—	—
	50	—	1.08	—	—
	50	—	0.96	—	—
2	50	2.00	3.20	106.7	
	50	2.00	3.00	100.0	100.0 ± 6.7
	50	2.00	2.80	93.3	
3	50	4.00	5.24	105.0	
	50	4.00	4.92	98.4	100.6 ± 3.8
	50	4.00	4.92	98.4	

hydrolysis times on different quantities of lung tissue while keeping the volume of perchloric acid constant. The resultant percentage hydroxyproline is the mean of 10 determinations, consisting of five hydrolyses followed by duplicate spectrophotometric analysis on tissue specimen. Table 2 compares the results of the two hydrolysis techniques used previously by Baily (*1*, *2*) with the perchloric acid hydrolysis method of Gałasiński *et al.* (*7*). Again, as above, the percentage hydroxyproline found in each case represents the mean of 10 determinations. Table 3 shows the percentage recovery of hydroxyproline added to lung tissue

TABLE 4
COMPARISON OF PRESENT STUDY AND BERGMAN/LOXLEY (*4*) SPECTROPHOTOMETRIC METHODS FOR HYDROXYPROLINE USING IDENTICAL SPECIMENS

	Present study		Bergman/Loxley (*4*)	
Determinations	% Hydroxy-proline	Mean % hydroxy-proline ± SD	% Hydroxy-proline	Mean % hydroxy-proline ± SD
1	1.91		1.84	
2	1.99		2.06	
3	1.86	1.99 ± 0.11	1.89	1.95 ± 0.09
4	2.06		1.99	
5	2.12		1.95	

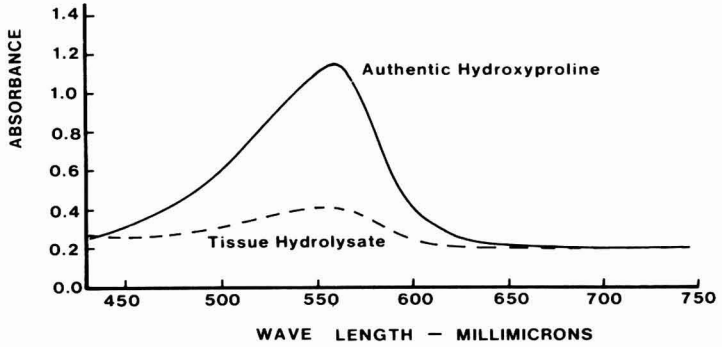

FIG. 2. Absorption spectra of chromophores from 50 μg of authentic hydroxyproline and an aliquot from 500 μg of hydrolyzed lung tissue run against a blank. A curve identical to that shown by the tissue hydrolysate was obtained with an equivalent amount of authentic hydroxyproline.

after hydrolysis with 72% perchloric acid. In both cases the recovery of added hydroxyproline was 100%. Table 4 gives a comparison of the respective spectrophotometric methods proposed by Baily (*1*) and Bergman/Loxley (*4*) on identical tissue specimens. Figure 2 depicts the absorption spectra of chromophores from 50μg of authentic hydroxyproline and an aliquot of 500 μg of hydrolyzed lung tissue. In both cases the maxima of the peaks were at a wavelength of 555 nm.

DISCUSSION

The original method of McFarlane and Guest (*10*) was adapted by Neuman and Logan (*12*) to determine hydroxyproline in hydrolysates containing 0 to 15 μg of hydroxyproline/ml. Grunbaum and Glick (*8*) found that a concentration of hydrogen peroxide lower than that used by Neuman and Logan (*12*) resulted in a more intense color and reduced the interference, due to an amount of tyrosine equal to that of hydroxyproline, to less than 1%. Miyada and Tappel (*11*) showed that the optimum temperature for developing the *p*-dimethylaminobenzaldehyde color was 80°C for 30 min. These modifications were combined by Baily (*1*) and, with more optimal concentrations of reagents, gave improved and reproducible development of color. Previously, the incomplete removal of hydrogen peroxide, which quenched the chromophore reaction, gave rise to the difficulties encountered in this determination. But in the present method, the high-speed agitation with the Whirlimixer followed by heating the mixture at 80°C for 5 min overcomes this difficulty. In well-trained, competent hands, the method presents no problems and is much more rapid than the chloramine-T method introduced by Stegeman (*14*) and subsequently used by Bergman and Loxley (*4*). In addition, the

tedious extraction technique employed by Prockop and Udenfriend (*13*) is obviated.

Wierbicki and Deatherage (*15*) found an approximate loss of 6% hydroxyproline under the conditions of hydrolysis outlined by them, but Dahl and Persson (*6*) could not confirm this loss, nor has any loss of hydroxyproline been found (Fig. 1) under the conditions used in this study. Again, with the methods employed by Dahl and Persson (*6*), they found that chloramine-T method gave consistently higher results for hydroxyproline (on the average, 8.1%) than did the method using hydrogen peroxide, but a comparison of the two methods in this study (Table 4) shows that the difference between them is not statistically significant ($P > 0.05$).

Gałasiński *et al.* (*7*) recommended a hydrolysis time of 2 hr, but in this study 5 mg of dried, defatted lung tissue required a 4-hr hydrolysis period for a maximum yield of hydroxyproline (Table 1). It is important to use no more than 5 mg of tissue for the hydrolysis and to keep the time for complete hydrolysis to 4 hr. Increasing the amount of both acid and tissue while keeping the ratio constant gave low yields of hydroxyproline (Table 1). A comparison of the hydrolysis method in this study with the two techniques previously employed (*1*, *2*) showed that the difference in results obtained for hydroxyproline (Table 2) by the three methods is not statistically significant ($P > 0.05$).

Le Roy (*9*) recommended that it was desirable for studies employing tissue hydroxyproline measurements to include spectral or other evidence of the absence of interfering chromophore formation. The present study shows that the absorption spectra (Fig. 2) obtained from both authentic hydroxyproline and tissue hydrolysate peak at 555 nm, thus demonstrating freedom from interfering chromophores.

SUMMARY

A rapid and convenient method for the determination of collagen in tissue is presented. The simple and reproducible method for hydrolysis allows many specimens to be processed simultaneously, and the rapid spectrophotometric determination of hydroxyproline, the presumptive index for collagen, ensures accurate and precise results.

REFERENCES

1. Baily, P., The biochemistry of lungs in relation to silicosis. I. The amino acid content of normal guinea pig lung tissue. *Arch. Biochem. Biophys.* **87**, 144–148 (1960).
2. Baily, P., Goldstein, B., Kilroe-Smith, T. A., and Soskolne, C. L., The determination of microgram amounts of collagen metabolities in the urine of primates inhaling silica dust. *Microchem. J.* **21**, 478–484 (1976).
3. Baily, P., Kilroe-Smith, T. A., and Harington, J. S., Biochemistry of lungs in relation to silicosis. II. The effect of quartz dust on the protein amino acids of guinea pig lung tissue. *Arch. Environ. Health* **8**, 547–554 (1964).
4. Bergman, I., and Loxley R., Lung tissue hydrolysates: Studies of the optimum condi-

tions for the spectrophotometric determination of hydroxyproline. *Analyst* **94**, 575–584 (1969).

5. Bligh, E. G., and Dyer, W. J., A rapid method of total lipid extraction and purification. *Canad. J. Biochem. Physiol.* **37** 911–917 (1959).
6. Dahl, O., and Persson, K., Hydroxyproline, methodological studies of analysis. *Acta Chem. Scand.* **17**, 2499–2503 (1963).
7. Gałasiński, W., Gadek, A., Ratkiewicz, A., and Rzeczycki, W., A convenient modification of the method for hydroxyproline determination in proteins. *Anal. Biochem.* **85**, 550–555 (1978).
8. Grunbaum, B. W., and Glick, D., Studies in histochemistry. XLV. Determination of hydroxyproline in microgram amounts of tissue. *Arch. Biochem. Biophys.* **65**, 260–267 (1956).
9. Le Roy, E. C., The technique and significance of hydroxyproline measurement in man. *Advan. Clin. Chem.* **10**, 213–253 (1967).
10. McFarlane, W. D., and Guest, G. H., A new colorimetric method for the determination of hydroxyproline and its application to protein hydrolyzates. *Canad. J. Res.* **B17**, 139–142 (1939).
11. Miyada, D. S., and Tappel, A. L., Colorimetric determination of hydroxyproline. *Anal. Chem.* **28**, 909–910 (1956).
12. Neuman, E., and Logan, M. A., The determination of hydroxyproline., *J. Biol. Chem.* **184**, 299–306 (1950).
13. Prockop, D.J., and Udenfriend, S., A specific method for the analysis of hydroxyproline in tissues and urine. *Anal. Biochem.* **1**, 228–239 (1960).
14. Stegemann, H., Mikrobestimmung von Hydroxyprolin mit Chloramin-T und p-dimethylaminobenzaldehyd. *Z. Physiol. Chem.* **311**, 41–45 (1958).
15. Wierbicki, E., and Deatherage, F. E., Hydroxyproline as an index of connective tissue in muscle. *J. Agr. Food Chem.* **2**, 878–882 (1954).

MICROCHEMICAL JOURNAL 24, 199–211 (1979)

Precipitation of Copper(II) by Formation of a Complex with 2,4-Dioxo-4-(4-hydroxy-6-methyl-2-pyrone-3-yl) Butyric Acid Ethyl Ester

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INTRODUCTION

Previous investigations have shown that 2,4-dioxo-4-(4-hydroxy-6-methyl-2-pyrone-3-yl) butyric acid ethyl ester (ligand) forms chelate complexes with numerous cations (*2*, *3*, *5*). In some cases the outstanding properties of the complexes make possible the application of the ligand as a specific analytical reagent. Thus beryllium and aluminium form fluorescent complexes, and a procedure was found for a specific spectrofluorometric determination of beryllium without interference of a series of diverse cations except aluminium (*2*). The ligand was also successfully applied as reagent in a trace analysis of metals by the ring oven technique (*3*). In the present paper the precipitation of copper(II) complexes with the ligand was systematically pursued and the possibility of taking it as a basis for analytical procedure is discussed.

EXPERIMENTAL

Materials

Absolute ethanol and deionized water were used throughout the experiments. Buffer stands for Sörensen's phosphate buffer, pH 7.00.

The inorganic metal salts used were of p.a. purity grade and produced by Kemika (Zagreb, Yugoslavia), except for the thorium nitrate and beryllium chloride, produced by Merck (Darmstadt, G.F.R.) and Fluka (Buchs, Switzerland), respectively.

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Apparatus

The uv/vis spectrophotometer Varian Techtron Series 635 D was used throughout the experiments. The ir spectra were taken in KBr pellets with a Perkin–Elmer Model 257 spectrophotometer. The ^{1}H-NMR spectrum was recorded on a Varian A-60 spectrometer in $CDCl_3$ with TMS as internal standard, and the mass spectra on the Varian MAT CH7 CEC 21-110 C. For X-ray diffraction analysis of isolated solid copper complex, the Debey–Scherrer method using Cu K(α) irradiation with a Straumanis camera was applied.

Preparation of Ligand

The ligand synthesized by condensation of 3-acetyl-4-hydroxy-6-methyl-2-pyrone (dehydroacetic acid) with diethyloxalate in the presence of sodium ethoxide as catalyst was kindly donated by the authors (*6*). The product was purified by recrystallization from ethanol and dried at room temperature.

Infrared spectrum: 1740 cm^{-1}, s; 1710 cm^{-1}, vs; 1660 cm^{-1}, m; 1615 cm^{-1}, m; 1585 cm^{-1}, vs; 1525 cm^{-1}, vs; 1340 cm^{-1}, s; 1250 cm^{-1}, vs (broad); 995 cm^{-1}, s.

^{1}H-NMR spectrum (δ values): 1.39 ppm, triplet (3H); 2.35 ppm, singlet (3H); 4.38 ppm, quartet (>2H); 5.99 ppm, singlet (1H); 7.82 ppm, singlet (<1H); 13.0 ppm, broad (<1H); 15.22 ppm, singlet (1H).

Electron impact mass spectrum: m/e 268 (4%); m/e 195 (72%); m/e 153 (100%); m/e 85 (19%); m/e 69 (23%); m/e 43 (33%).

Preparation of Metal Complexes

Copper(II) complex crystallized after addition of the ethanolic ligand solution to the aqueous metal salt solution. Recrystallization was achieved from ethanol yielding dark green tiny crystals, slightly soluble in cold ethanol.

$(C_{12}H_{11}O_7)_2Cu$ (597.98). Calcd: 48.20% C, 3.71% H, 10.62% Cu. Found: 48.65% C, 3.34% H, 10.54% Cu.

Ultraviolet spectrum: 390 nm (ϵ 2720); 278 nm; 235 nm.

Infrared spectrum: 3570 cm^{-1}, m; 1720 cm^{-1}, s; 1650 cm^{-1}, s; 1560 cm^{-1}, vs; 1550 cm^{-1}, vs; 1515 cm^{-1}, s; 1490 cm^{-1}, s; 1435 cm^{-1}, sh; 1415 cm^{-1}, vs; 1375 cm^{-1}, s; 1350 cm^{-1}, s; 1325 cm^{-1}, s; 1265 cm^{-1}, vs; 1215 cm^{-1}, s; 1170 cm^{-1}, m; 1150 cm^{-1}, sh; 1060 cm^{-1}, w; 1010 cm^{-1}, s; 965 cm^{-1}, m; 850 cm^{-1}, m; 800 cm^{-1}, m; 640 cm^{-1}, m.

Electron impact mass spectrum: m/e 268 (21%); m/e 250 (13%); m/e 226 (27%); m/e 195 (100%); m/e 177 (22%); m/e 153 (100%); m/e 111 (20%); m/e 93 (19%); m/e 85 (52%); m/e 69 (45%); m/e 43 (67%).

Field desorption mass spectrum: m/e 328 (very weak); m/e 268 (intense).

A roentgenogram is presented in Table 1.

TABLE 1
ROENTGENOGRAM OF THE MONONUCLEAR Cu(II) COMPLEX WITH THE LIGAND

d (Å)	I	d (Å)	I	d (Å)	I	d (Å)	I
9.57	100	3.97	7	3.01	15	2.49	3
8.67	30	3.87	15	2.97	2	2.46 B	3
6.76	15	3.84	10	2.92	2	2.36 B	2
5.63	20	3.65	75	2.88	2	2.25	7
5.53	15	3.52	25	2.86	2	2.21	2
5.37	30	3.45	2	2.81	7	2.19	5
4.90	20	3.37	10	2.77	2	2.09	2
4.75	20	3.33	40	2.71	5	2.04	7
4.25	20	3.22	10	2.67	2	1.98	7
4.15	5	3.17	5	2.64	5		

By mixing metal salt and ligand solutions prepared in Sörensen's buffer, pH 7.00, the 2:2 copper(II) complex has been obtained as a yellowish green fine precipitate, soluble in ethanol.

$(C_{12}H_{10}O_7)_2Cu_2 \cdot 4\ H_2O$ (731.56). Calcd: 39.40% C, 3.86% H, 17.37% Cu. Found: 39.11% C, 4.33% H, 16.11% Cu.

Ultraviolet spectrum: 375 nm (ϵ 3150); 268 nm; 224 nm.

Infrared spectrum: 3570 cm^{-1}, m; 1715 cm^{-1}, s; 1650 cm^{-1}, s; 1555 cm^{-1}, vs; 1505 cm^{-1}, s; 1485 cm^{-1}, s; 1430 cm^{-1}, sh; 1415 cm^{-1}, vs; 1370 cm^{-1}, s; 1345 cm^{-1}, s; 1320 cm^{-1}, s; 1260 cm^{-1}, vs; 1210 cm^{-1}, s; 1160 cm^{-1}, w; 1145 cm^{-1}, w; 1050 cm^{-1}, s; 1010 cm^{-1}, s; 985 cm^{-1}, m; 960 cm^{-1}, m; 850 cm^{-1}, w; 795 cm^{-1}, w; 750 cm^{-1}, w; 645 cm^{-1}, w.

Metal to Ligand Ratio of Complexes in Solution

The uv spectra of the ligand and its metal complexes were taken in predominantly ethanolic solutions (because of low solubility in water). The solution of the ligand was prepared in acetic acid or chloroform. The metal to ligand molar ratio in solution was determined spectrophotometrically by the modified continuous variation method of Job. The samples contained 1 ml of aqueous solution of the cation investigated, 2 ml of the ligand dissolved in glacial acetic acid, and 7 ml of ethanol.

Precipitation of copper(II) with ligand in aqueous solution. The stock 2×10^{-2} *M* ligand solution was prepared shortly before starting the experiments by dissolving 0.134 g of the substance in buffer. All other solutions were prepared by dilution with buffer. Stock solutions of $CuSO_4$, $Cu(NO_3)_2$, $FeCl_3$, $FeSO_4$, $Co(NO_3)_2$, $CoSO_4$, $NiSO_4$, $Ni(NO_3)_2$, $ZnSO_4$, $Al_2(SO_4)_3$, $Th(NO_3)_4$, and NaCl were prepared by dissolution with water. The experiments were carried out at room temperature.

Precipitation diagrams of copper(II) with ligand. The samples were prepared in 10-ml volumetric flasks or tubes with ground stoppers. To

each flask or tube, an aliquot volume of $CuSO_4$ or $Cu(NO_3)_2$ solution of a definite concentration was added with a pipet, followed by water to obtain a volume of 5 ml. Different concentrations of ligand were added, and the flasks were filled to the mark with buffer and mixed thoroughly. The precipitate formation was observed visually. The observations obtained after 20–24 hr were taken for the precipitation diagrams.

Effect of other ions on the precipitation boundaries of copper(II) with ligand. The effect of a series of ions, Co(II), Fe(II), Fe(III), Ni(II), Zn(II), Al(III), Th(IV), and Cl^-, on the precipitation boundaries of copper(II) with ligand was tested with samples prepared in the same way as described before except that the 5 ml aqueous solution additionally contained increasing concentrations of the ion tested.

Precipitation diagrams of nickel(II) with ligand. Preparation of the samples and experimental conditions were analogous to those for the precipitation of copper(II) with ligand.

Precipitation of ^{64}Cu(II) with ligand. Optimal conditions for the quantitative precipitation of copper and characteristics of the copper(II)–ligand complex were studied in a series of experiments performed with a copper(II) solution prepared with the isotope ^{64}Cu (half-life, 12.8 hr). Precisely 12.7 mg of an irradiated copper foliage of 99.999% purity was dissolved with a few drops of concentrated nitric acid and diluted with water to a definite volume. The copper foliage was previously irradiated over a period of 9 hr in the TRIGA reactor (Institute "Jožef Štefan," Ljubljana) by a neutron flux of 10^{13} n cm sec^{-1}.

Time dependence of copper(II) precipitation with ligand. A series of seven samples was prepared simultaneously, each of them containing 1 ml of 1.0884×10^{-3} *M* $^{64}Cu^{2+}$ solution, 4.5 ml of buffer, and 0.5 ml of a 10^{-2} *M* ligand solution in buffer. The volumes of the samples were filled up to 10 ml with water. At the same time, for each sample a corresponding standard was prepared containing all ingredients except the ligand. The activity of the supernatant was measured at definite time intervals from the instant of ligand addition and after a complete separation of the precipitate was achieved by centrifugation for 10 min at 10,000*g*.

The radioactivity of the liquid phase was compared with the activity of the relevant standard. A NaI(Tl) well-type scintillation counter coupled to a present-time EKCO-Electronics scaler was used for all measurements.

Precipitation of copper(II) in different copper(II) concentrations and different copper(II) to ligand ratios. Solubility products. A series of samples was prepared containing: *a* ml of 2×10^{-3} *M* $^{64}Cu(NO_3)_2$, $(5 - a)$ ml of water, *b* ml of 2×10^{-2} *M* (or 1×10^{-2} or 1×10^{-3} *M*) solution of ligand in buffer, and $(5 - b)$ ml of the buffer solution. Corresponding standards were prepared simultaneously containing 5 ml of buffer omitting the ligand. After 20–24 hr the samples were submitted to centrifugation at

10,000*g*. The percentage of precipitated copper(II) was calculated from the ratio of the values obtained by measuring the activity of 1 or 2 ml of sample supernatant and the activity of the supernatant of the corresponding standard.

Spectrophotometric determination of copper(II) precipitated with ligand. To a series of graduated 10-ml tubes with ground stoppers, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 3.3, and 4.0 ml of an aqueous 1×10^{-3} *M* $Cu(NO_3)_2$ solution were added, followed by water to form a volume of 5 ml. After the addition of 0.8 ml of a 10^{-2} *M* ligand solution in buffer, the volume was filled up to 10 ml with buffer. The precipitate was removed after 20–24 hr by centrifugation during 10 min at 955*g*. The clear supernatant was decanted completely and the precipitate dissolved on a Vortex mixer with 10 ml of ethanol. The absorbance of the solution obtained by dilution with ethanol at a 1:4 volume ratio was measured at 374 nm against ethanol.

The effect of the pH value of the medium and a series of cations on the spectrophotometric determination of copper(II) precipitated with ligand. The effect of cations was investigated with aqueous solutions of NH_4NO_3, $BeCl_2$, $Co(NO_3)_2$, $Ni(NO_3)_2$, $ZnSO_4$, $FeSO_4$, $Al_2(SO_4)_3$, $Th(NO_3)_4$, and NH_4VO_3. Because of the low solubility of $Ce(SO_4)_2$, several drops of H_2SO_4 conc. should be added, causing the pH of the solution to drop to 0.41. Increasing concentrations of each of the cations listed above, along with 1.5 ml of 1×10^{-3} *M* $Cu(NO_3)_2$, were present in 5 ml of water, thus the precipitation procedure could be pursued as described previously.

At higher concentrations Co(II), Be(II), Ce(IV), and Th(IV) hydroxides were precipitated simultaneously with the copper(II)–ligand complex. As these hydroxides were not soluble in ethanol, they could be separated by centrifugation of the ethanolic solution of the copper(II) complex, which was subsequently diluted with ethanol for spectrophotometric measurement. The corresponding blanks, containing all ingredients except $Cu(NO_3)_2$, did not contain any precipitate except the hydroxides.

RESULTS AND DISCUSSION

Copper(II) Complexes with the Ligand

The ligand, an unsymmetrical triketone, can take triketo, two different monoenol, and bisenol forms in their tautomeric equilibrium (Fig. 1). The presence of a triketo tautomer was not evident in the spectra showing the characteristics of the enolized dehydroacetic acid group (*4*). This evidence eliminates one of the monoenol forms too.

Chemical shifts of the signals in the ^{1}H-NMR spectrum are characteristic of the bisenol form. By a comparison of peak areas, it could be proved that in $CHCl_3$ solution about 80% of the bisenol and 20% of the monoenol tautomer were present with the signal of methylene protons between two

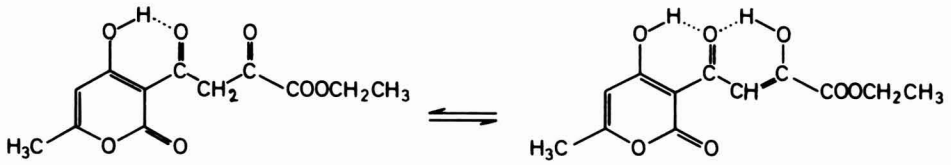

FIG. 1. Monoenol and bisenol forms of the ligand as evidenced by the ^{1}H-NMR spectrum.

carbonyl groups hidden in the quartet of ethoxy methylene protons. This is consistent with the general assumption that the equilibrium moves to favor the bisenol as the bulk of substituents on the triketo system is increased (*1*).

The ir spectrum of the solid state shows a strong absorption band at 1710 cm^{-1} attributable to the ethoxy carbonyl group of the bisenol form. This band probably overlaps with the carbonyl of β-diketone and the ethoxy carbonyl of the monoenol tautomer with 2-pyrone, $\nu C = O$ at 1740 cm^{-1}.

In solution and in the solid state, the ligand takes monoenol and bisenol form in tautomeric equilibrium and behaves as bidentate ligand to form metal chelate complexes.

Metal to ligand ratios (1:1) in a predominantly ethanolic medium and (2:3) in an aqueous buffer solution were obtained. The apparent stability constants were determined by the method of Likussar and Boltz (7) and were found to be log K_f = 3.700 and 16.991, respectively. As with other 1,3,5-triketones, copper(II) reacts with the ligand to give mononuclear 1:2 (metal/ligand) and binuclear 2:2 complexes. When an aqueous cupric nitrate solution is added to an ethanolic solution of the ligand, the mononuclear complex is formed. The binuclear complex is obtained by precipitation with an aqueous buffer solution of the ligand, as was described previously for nickel(II) (*5*).

The elemental analyses of the mononuclear and binuclear complexes agree with the calculated values.

In the case of the mononuclear copper(II) complex, the question arises as to the site of coordination of the ligand.

The ir spectrum shows absorption bands resembling those of copper(II) complex with 2-acetoacetyl phenol at 1570 and 1515 cm^{-1}, with the proposed coordination site at the acetoacetyl group (*10*). Molecular ion is not present in the electron impact mass spectrum with 70 eV energy and no fragments contain the metal. The spectrum is very similar to that of ligand itself like in copper acetylacetonates (*9*). By in-beam experiments and under ionization conditions similar to those of chemical ionization, it was

not possible to ionize the complex without disintegration. However, fragments with m/e values up to 556 were registered. The field desorption mass spectrum does not show molecular ion either. A very small signal at m/e 328 corresponds to 1:1 (copper/ligand) complex, and a significant one at m/e 268 to molecular ion of the ligand itself.

In mass spectra of the binuclear complex taken under various conditions, molecular ion is not present either.

The solubility of the complexes is too low for a molecular weight determination by vapor pressure lowering osmometry. The crystalline form is not suitable for X-ray structure determination. Unequivocal proof for the structure of the copper(II) complexes with the ligand remains to be found.

Precipitation of Copper(II) with the Ligand

The copper(II) complexes with the ligand readily precipitate from ethanolic and aqueous solutions. In the latter case the precipitation is observed even in 2.5×10^{-5} mol dm^{-3} copper(II) solution (Fig. 2a). In the series copper, cobalt, iron(II), iron(III), zinc, aluminium, thorium, and nickel, only in the case of nickel has a precipitate been noticed in solutions containing at least 6×10^{-4} mol dm^{-3} of the metal (Fig. 2b). A systematical pursuit of copper and nickel precipitation in pure solutions as well as copper precipitation in the presence of different concentrations of other cations is summarized in the form of precipitation diagrams in Figs. 2a–h. Although the complexes of cations investigated except nickel do not precipitate, their presence effects the precipitation boundaries of copper(II) complexes.

Radiometric Measurements with ^{64}Cu Isotope

The dependence of the amount of precipitated copper(II) on the time elapsed from the addition of the ligand in buffer solution was pursued radiometrically with ^{64}Cu isotope.

The maximum was reached after 16 hr (Fig. 3) with a 90% yield of the precipitated copper from a 1×10^{-4} mol dm^{-3} solution.

The solubility product of 2:2 (copper/ligand) complex was found to be $1.09 \cdot 10^{-9}$ (Fig. 4).

The lower limit of copper(II) precipitation in 99% yield corresponded to that given for the precipitation with dehydroacetic acid (*8*), i.e. 4×10^{-4} mol dm^{-3}. The excess of ligand optimal for the precipitation with the highest yield is 5- to 6-fold, and should be increased to 10-fold at copper concentrations lower than 6×10^{-5} mol dm^{-3}.

Too small or too big an amount of the added ligand causes losses in precipitation, a circumstance that is evident also from the precipitation diagrams (Fig. 2).

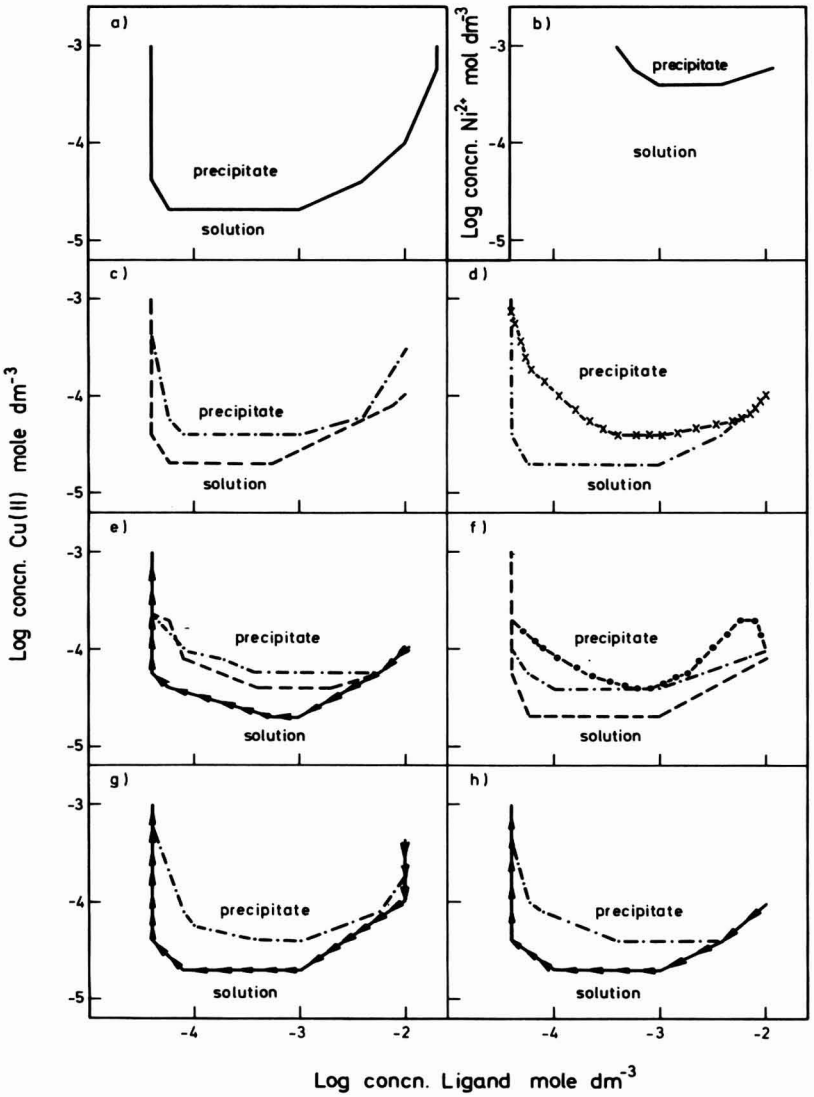

FIG. 2. Precipitation diagrams obtained 24 hr after preparation of the following systems: (a) Cu(II)–ligand; (b) Ni(II)–ligand; (c) Cu(II)–Ni(II)–ligand; (d) Cu(II)–Co(II)–ligand; (e) Cu(II)–Fe(II)–ligand; (f) Cu(II)–Zn(II)–ligand; (g) Cu(II)–Al(III)–ligand; (h) Cu(II)–Th(IV)–ligand. (←←←←) 10^{-5} mol dm^{-3}; (-----) 5×10^{-5} mol dm^{-3}; (–·–·–·–·–) 10^{-4} mol dm^{-3}; (– ●– ●– ●–) 5×10^{-4} mol dm^{-3}; (–x–x–x–) 10^{-3} mol dm^{-3}.

Therefore the precipitation in samples with an unknown copper(II) content should be carried out with gradually increasing amounts of the ligand. The precipitation yield is diminished to 86% by successive decreases in

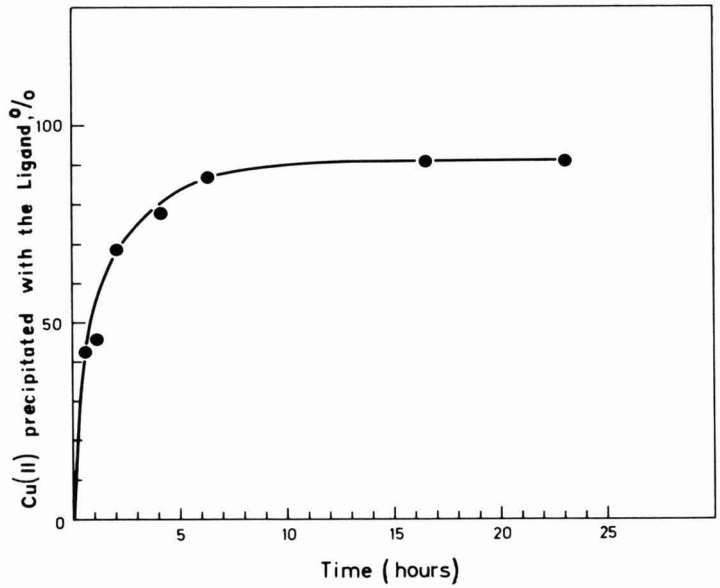

FIG. 3. Dependence of the precipitated copper(II) on the time elapsed after addition of the ligand in buffer solution. Copper(II) concentration, 1×10^{-4} mol dm^{-3}; ligand concentration, 5×10^{-4} mol dm^{-3}.

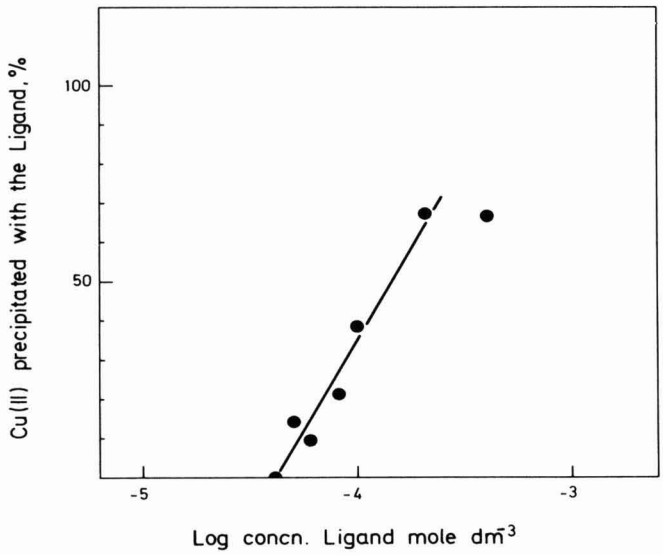

FIG. 4. Solubility product of 2:2 (copper/ligand) complex obtained radiometrically: $(2.5 \times 10^{-5}) \cdot (4.36 \times 10^{-5}) = 1.09 \times 10^{-9}$.

the copper(II) concentration down to 4×10^{-5} mol dm^{-3}, being only 60% at the detection limit of 2×10^{-5} mol dm^{-3} copper(II) present.

Determination of Copper(II) by Precipitation with the Ligand

Instead of a gravimetric procedure, the precipitate isolated by centrifugation after 20–24 hr and decantation of the supernatant, is dissolved in a given volume of ethanol and the copper determined by absorbance reading at 374 nm. A correction for solubility loss of the precipitate is made to reduce the systematic error by constructing a calibration diagram with known amounts of copper (Fig. 5). For the tested range, 2×10^{-5}–4×10^{-4} mol dm^{-3}, of copper concentrations, the validity of Beer's law has been confirmed, with 7×10^{-6} mol dm^{-3} as a constant magnitude of the systematic error negative in sign. The optimal pH range for the determination of copper is found to be 4.5–6.5 (Fig. 6).

The interferences of a number of cations in copper determination by this procedure are shown in Fig. 7. Increased results are obtained for copper (1.5×10^{-4} mol dm^{-3}) if nickel is present in concentrations $>10^{-6}$ mol dm^{-3}. Diminished copper values are obtained if aluminium, zinc, iron(II), thorium(IV), or vanadium(V) is present in concentrations $>10^{-5}$ mol dm^{-3}.

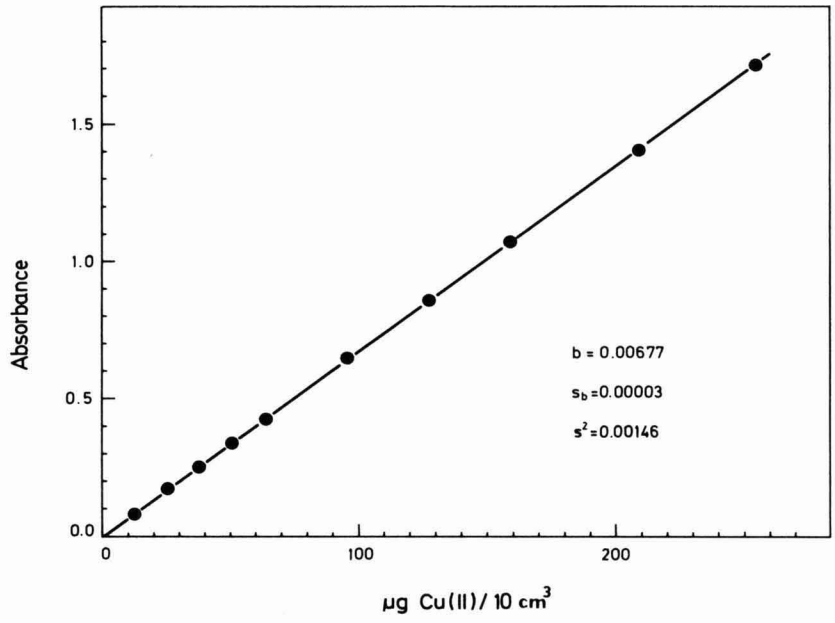

FIG. 5. Calibration diagram for copper(II) determination by precipitation with the ligand.

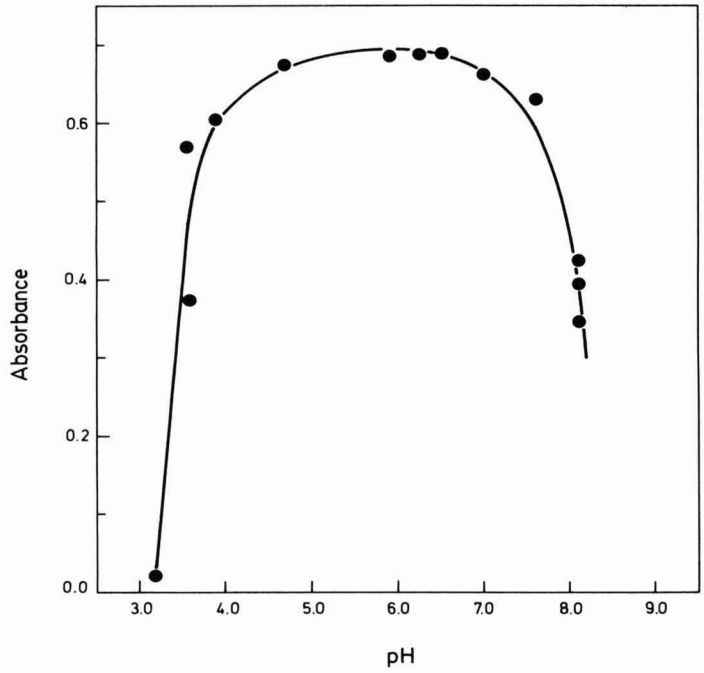

FIG. 6. Effect of pH on the results of copper(II) determination by precipitation with the ligand.

SUMMARY

Mononuclear copper(II) complex with 2,4-dioxo-4-(4-hydroxy-6-methyl-2-pyrone-3-yl) butyric acid ethyl ester is readily precipitated in ethanolic medium. The metal to ligand ratio in the crystalline species was found to be 1:2. On the basis of the spectroscopic data collected so far, the site of coordination could not be identified. The detection limit of the precipitation of the binuclear complex in aqueous buffer, pH 7.00, solution is at a 2×10^{-5} mol dm^{-3} copper(II) concentration. By radiometric measurements with ^{64}Cu isotope, the time neccessary for a quantitative precipitation, the amount of copper(II) in the precipitate and in the solution, the amount of ligand needed for the optimal precipitation yield, and the solubility product of the complex were determined.

The precipitate separated from the supernatant can be dissolved in ethanol and copper(II) determined by absorbance measurement at 374 nm. The sensitivity of this procedure lies at the detection limit of the complex precipitation. The calibration diagram, a straight line (b = 0.00677; s_b = 0.00003; s^2 = 0.00146), confirms the validity of Beer's law in the range of 2×10^{-5}–4×10^{-4} mol dm^{-3} copper(II) concentrations, with a systematic error of 7×10^{-6} mol dm^{-3} arising from solubility loss of the precipitate remaining constant.

Concentrations exceeding 10^{-6} mol dm^{-3} of nickel(II) cause too high values and those exceeding 10^{-5} mol dm^{-3} of aluminium, zinc, iron(II), thorium(IV), or vanadium(V) too low values in copper determinations.

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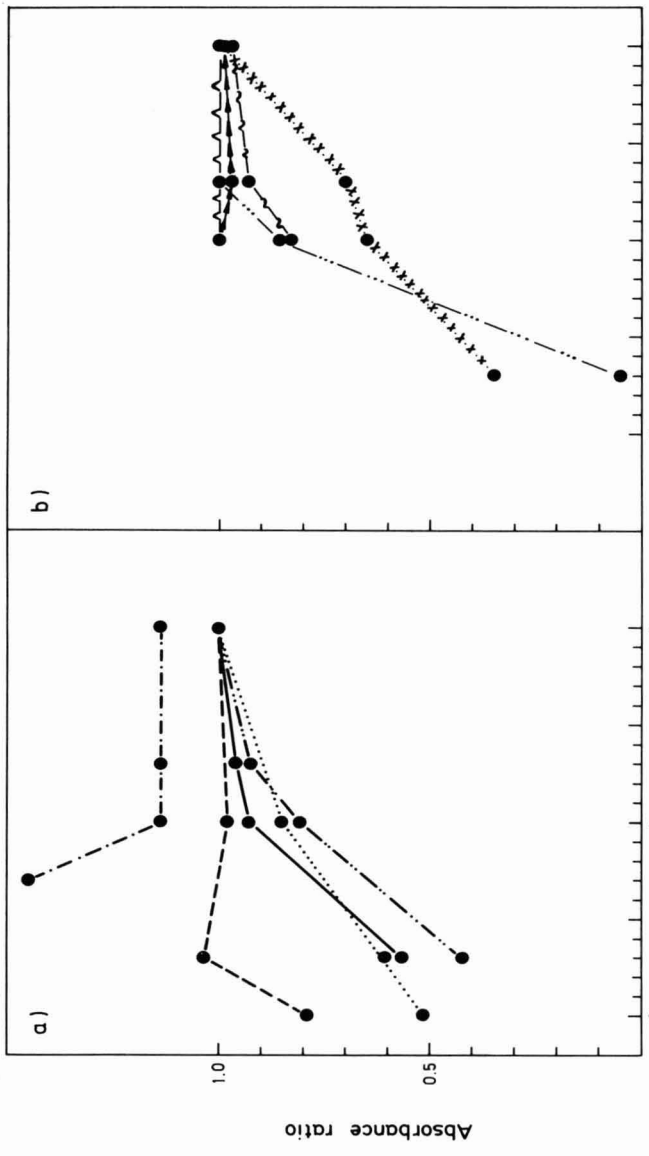

FIG. 7. Interferences of various cations on the results of copper(II) determination by precipitation with the ligand. (a) (— · — · — · —) Ni^{2+}; (— — — —)Cu^{2+}; (— · — · — · —) Fe^{2+}; (——) Zn^{2+}; (· · · · · · · · · · · · ·) Be^{2+}. (b) (→→→) NH_4^+; (∿∿∿) Al^{3+}; (— · · · — · · · —) Ce^{4+}; (× · × · × · × · ×) Th^{4+}; (∿∿∿) V^{5+}. Absorbance ratio = $A_{(Cu + cation)}/A_{Cu}$.

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REFERENCES

1. Casellato, U., and Vigato, P. A., Transition metal complexes with binucleating ligands. *Coord. Chem. Rev.* **23**, 31–117 (1977).
2. Drevenkar, V., Štefanac, Z., and Brbot, A., A new selective reagent for the spectrofluorometric determination of beryllium. *Microchem. J.* **21**, 402–410 (1976).
3. Gertner, A., and Pavišić, D., 2,4-Dioxo-4-(4-hydroxy-6-methyl-2-pyron-3-yl) butyric acid ethyl ester: Reagent for identifying and estimating metal ions in ring oven analysis. *Microchem. J.* **23**, 336–340 (1978).
4. Gren, R., Dehydracetsäure. *Deut. Apoth. Ztg.* **111**, 219–224 (1971).
5. Laćan, M., Malazogu, S., Džanić, H., and Sušnik-Rybarski, I., Die Metallchelate von symetrischen 1,5-Diaryl-1,3,5-pentantrions und Äthylester 2,4-Dioxo-4-(4-hydroxy-6-methyl-2H-piran-2-on-3-yl) Buttersäure mit Cu(II), Ni(II) und Co(II). *Bull. Soc. Chem. Technol. Sarajevo (Yugoslavia)* **21/22**, 35–37 (1973–1974).
6. Laćan, M., Sušnik-Rybarski, I., Brbot, A., Katušin, B., and Drenovački, V., submitted for publication.
7. Likussar, I. W., and Boltz, D. F., Theory of continuous variations plots and a new method for spectrophotometric determination of extraction and formation constants. *Anal. Chem.* **43**, 1265–1272 (1971).
8. Manku, G. S., and Sarpal, P. D., Dehydroacetic acid as a reagent for the separation and gravimetric determination of copper(II), aluminium and beryllium. *Talanta* **18**, 1079–1082 (1971).
9. Sasaki, S., Itagaki, Y., Kurokawa, T., Nakanishi, K., and Kasahara, A., Mass spectrometric studies of the metal acetylacetonates. *Bull. Chem. Soc. Japan* **40**, 76–80 (1967).
10. Taguchi, Y., Sagara, F., Kobayashi, H., and Ueno, K., Metal chelates of some β,δ-triketone homologues. Copper(II) chelates of 2,6-diacetyl-4-methylphenol, 2,2′-dihydroxybenzophenone, 2-acetoacetylphenol and 2-acetyl-1, 8-dihydroxy naphthalene. *Bull. Chem. Soc. Japan* **43**, 2470–2474 (1970).

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Selective Microdetermination of Phosphate Based on Volhard's Titration

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INTRODUCTION

Silver nitrate reacts with the phosphate ion in neutral or slightly alkaline solutions to form a sparingly soluble precipitate of silver phosphate, Ag_3PO_4 ($K_{sp} = 1.3 \times 10^{-20}$). De Sousa (*2*) made use of this reaction for the complexometric determination of phosphate. The phosphate is precipitated as the silver salt, which after being filtered and washed is dissolved in potassium nickelocyanide solution, and the demasked nickel is titrated with EDTA solution. However, this method is of limited applicability since it lacks specificity. Chloride, bromide, iodide, and sulfate (the latter in high concentrations) react similarly and must be absent.

In the present method, however, microamounts of phosphate can selectively be determined in the presence of large excesses of the above anions. The phosphate is precipitated as silver phosphate from homogeneous solutions to avoid coprecipitation with other anions. The precipitate is filtered off, washed, and treated with dilute nitric acid to dissolve silver phosphate; any silver halide or sulfate that may have formed remains unaffected on the filter. The silver ions set free in the acid are then estimated by Volhard's titration. The whole procedure takes about 30 min.

EXPERIMENTAL

Apparatus and Reagents

Grade A microburettes (5-ml capacity) and grade A 2-ml pipets were used throughout.

All chemicals used were of analytical grade. Potassium dihydrogen phosphate (Analar BDH; minimum assay, 99.5–100.5%) was recrystallized twice from conductivity water for use as a standard.

Cadmium ammonium phosphate monohydrate, $CdNH_4PO_4 \cdot H_2O$, magnesium ammonium phosphate hexahydrate, $MgNH_4PO_4 \cdot 6H_2O$, manganese ammonium phosphate monohydrate, $MnNH_4PO_4 \cdot H_2O$, and zinc ammonium phosphate, $ZnNH_4PO_4$, were prepared according to the standard gravimetric methods (*3*). Calcium hydrogen phosphate dihydrate was prepared according to Bailar (*1*). These salts were used as samples for analysis.

Ammoniacal silver nitrate solution was freshly prepared (every day) by

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mixing 6 ml of 30% (w/v) silver nitrate solution with 8 ml of 7 *N* ammonia solution, adding more ammonia if necessary to dissolve any persisting precipitate, and filtering.

Procedure

Transfer an aliquot of the slightly acidic test solution (1–2 ml) containing 2–6 mg of phosphate into a 10-ml filter beaker, then add 2 drops of 2 *N* nitric acid, 3 drops of 3 *M* ammonium nitrate solution, and 2 ml of the ammoniacal silver nitrate solution. Heat gently on a mild hot plate until the pH reaches 6.5–7 (pH paper). Allow to cool to room temperature, filter with suction, and wash with 5 ml of saturated silver phosphate solution, then with two 2-ml portions of cold distilled water. Rinse the beaker with three 2-ml portions of 1 *N* nitric acid, allowing each portion to percolate through the filter disk to dissolve the precipitate, and collecting the solution in a 50-ml Büchner flask. Add 5 drops of ferric alum indicator and titrate with 0.05 *N* potassium thiocyanate.

$$1\ \text{L}\ N\ \text{KSCN} \equiv \frac{1}{3}\ PO_4^{3-}.$$

Notes

(1) The test solution must be made slightly acidic before analysis and boiled gently for 2–3 min to decompose any carbonate, sulfite, or sulfide that may coexist.

(2) If the sample solution contains cations such as Ca^{2+}, Ba^{2+}, Mg^{2+}, Al^{3+}, etc., that form insoluble phosphates in basic medium, they must be removed before analysis by passing the solution through a cation-exchange column (0.8 × 6 cm) in the hydrogen form (Amberlite IR-120).

RESULTS AND DISCUSSION

General

Before establishing the procedure outlined above, the phosphate was precipitated by the addition of silver nitrate in the conventional manner. Good results were obtained in the absence of halides, but very low results were obtained in their presence, as shown in Table 1. This is clearly due to the occlusion of some silver phosphate precipitate in the silver halide precipitate which prevents the dissolution of the former in dilute nitric acid. The extent of occlusion depends on the relative solubilities of the precipitates, the relative concentrations of ions, and the rate of addition of the precipitant. The effect of relative solubilities can be studied qualitatively from Table 1. Thus, among the halides, the iodide caused the smallest extent of occlusion. This is because silver iodide is much less soluble than silver phosphate. When silver nitrate is added gradually, most of the

TABLE 1
EFFECT OF HALIDES ON PHOSPHATE DETERMINATION BY CONVENTIONAL PRECIPITATION (SAMPLE TAKEN, 2 ml 0.0100 *M* KH_2PO_4)

Halide ion	Concentration (*M*)	Added as	PO_4^{3-} found (*M*)	% Error
—	—	—	0.0998	−0.2
—	—	—	0.0998	−0.2
Cl^-	0.01	NaCl	0.0079	−21
	0.02		0.0076	−24
Br^-	0.01	KBr	0.0081	−19
	0.02		0.0083	−17
I^-	0.01	KI	0.0091	−9
	0.02		0.0093	−7

silver iodide will be precipitated before silver phosphate starts to form. However, when the difference between the solubilities of the silver halide and silver phosphate is not too large, the two precipitates will form, more or less, simultaneously. This increases occlusion, as in the case of chloride and bromide.

Trials to minimize occlusion by slow and dropwise addition of silver nitrate solution and by digestion of the precipitate did not improve the results to a measurable extent. A prior precipitation of silver halides in acidic medium followed by neutralization to precipitate silver phosphate gave much better results, but the precision was still unsatisfactory.

Using the technique of precipitation from homogeneous solutions, however, no interferences were observed from the halides. In this technique, the precipitant, (Ag^+), is slowly and homogeneously generated in the solution by gradual decomposition of silver ammine complex.

$$[Ag(NH_3)_2]^+ \xrightarrow{\text{heat}} Ag^+ + 2\,NH_3.$$

Under this condition, the different precipitates are formed sequentially in the order of increasing solubility. The final product is thus a mixture of precipitates and not a mixed precipitate.

Validity of the Method

To study the validity of the procedure, synthetic mixtures of known phosphate concentrations and containing different amounts of foreign anions were analyzed. The results obtained were satisfactory (Table 2), the maximum error being 1% and the standard deviation 0.61%.

Analysis of Inorganic Phosphates

As a test of the applicability of the method, representative inorganic

TABLE 2
DETERMINATION OF PHOSPHATE BY THE NEW METHOD; EFFECT OF FOREIGN ANIONS (SAMPLE, 2 ml xM KH_2PO_4 + ADDED SALT)

Foreign anion	Concentration (*M*)	Added as	Phosphate concentration (*M*)		% Error
			Taken	Found	
—	—	—	0.0100	0.0101	+1
			0.0200	0.0200	0.0
			0.0250	0.0250	0.0
Cl^-	0.1	NaCl	0.0100	0.0999	−1
	0.2		0.0200	0.0201	+0.5
	0.2		0.0250	0.0250	0.0
Br^-	0.1	KBr	0.0100	0.0100	0.0
	0.2		0.0200	0.0200	0.0
	0.2		0.0250	0.0249	−0.4
I^-	0.1	KI	0.0100	0.0999	−1
	0.2		0.0200	0.0200	0.0
	0.2		0.0250	0.0252	+0.8
SO_4^{2-}	0.1	K_2SO_4	0.0100	0.0100	0.0
	0.2		0.0200	0.0198	−1
	0.2		0.0250	0.0251	+0.4

Standard deviation from true value = $\sqrt{\sum(\%E)^2/(n-1)} = 0.61\%$.

TABLE 3
ANALYSIS OF INORGANIC PHOSPHATES

Sample	Taken (mg)	mg of PO_4^{3-}		Recovery (%)	Mean recovery (%)
		Calc.	Found		
$CaHPO_4 \cdot 2H_2O$	6.78	3.74	3.70	98.93	
	7.54	4.16	4.20	100.96	99.81
	7.65	4.22	4.20	99.53	
$CdNH_4PO_4 \cdot H_2O$	10.32	4.03	3.98	98.76	
	10.88	4.24	4.22	99.53	99.17
	9.98	3.89	3.86	99.23	
$MgNH_4PO_4 \cdot 6H_2O$	10.52	4.07	4.04	99.26	
	11.24	4.35	4.36	100.23	100.09
	10.06	3.89	3.92	100.77	
$MnNH_4PO_4 \cdot H_2O$	7.92	4.04	4.00	99.01	
	8.18	4.18	4.16	99.52	99.11
	8.24	4.21	4.16	98.81	
$ZnNH_4PO_4$	7.82	4.16	4.16	100.00	
	7.45	3.97	4.00	100.76	100.43
	7.18	3.82	3.84	100.52	

phosphates were analyzed. The test samples were dissolved in dilute nitric acid and freed from cations by ion exchange before determination. The method gave satisfactory results for all samples; the mean recoveries ranged between 99.1 and 100.4% (Table 3).

SUMMARY

A selective method for the microdetermination of phosphate has been developed. The phosphate is precipitated from homogeneous solutions as silver phosphate, which after being filtered and washed is dissolved in dilute nitric acid, and the silver ions set free are determined by Volhard's titration. Whereas the halide ions interfere if the conventional precipitation is adopted, they do not interfere if precipitation is conducted from homogeneous solutions. The method is simple and accurate to a maximum error of ±1%.

REFERENCES

1. Bailar, J. C., "Inorganic Syntheses," Vol. IV, p. 20. McGraw–Hill, New York, 1953.
2. DeSousa, A., Microdetermination of phosphate with EDTA. *Microchem. J.* **18,** 137–141 (1973).
3. Vogel, A. I., "A Text Book of Quantitative Inorganic Analysis," 3rd printing, pp. 477, 493, 532, and 536. Longmans, London, 1962.

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Analytical Uses of Phenylhydrazines and Phenylhydrazones. I.[1]

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INTRODUCTION

One of E. Fischer's memorial contributions to organic chemistry was the introduction in 1884 of the use of phenylhydrazine, which formed with numerous sugars derivatives in the form of beautiful crystals with very limited solubility (*23*). He had already discovered in 1874—ten years before—working at the University of Strassbourg (as it seems accidentally), the first hydrazine base, precisely, phenylhydrazine. However, E. Fischer demonstrated its relationship with hydrazobenzene and with a sulfonic acid described by Strecker.

Phenylhydrazine is a yellowish to red–brown liquid soluble in ethyl alcohol, ethyl ether, and water. It boils at 241°C with slight decomposition and has a specific gravity of 1.098 (*55*). Phenylhydrazine and derivatives, mainly *p*-nitro-and 2,4-dinitrophenylhydrazine, have been used for a long time for the characterization of carbonilic compounds for measures of their melting points (*2, 68, 71*), being also a means of purifying the carbonilic compounds by isolation from the reaction mixture and later regenerating them in an acidic medium.

Phenylhydrazine is highly toxic, and E. Fischer suffered its insidious effects. Recently several papers have been published dealing with the toxicological aspects of phenylhydrazine on animals. Thus it induces hemolysis during amphibian metamorphosis (*26*) and on behavior of regenerating marrow stroma (79). Phenylhydrazine induces anemia on the appearance of adult hemoglobin in *Rana catesbiana* tadpoles (*53*), in rats (*85*), and in chinook salmon (*74*). It also induces depletion of cardiac norepinephrine (*77*). Production of erythroid cells has been observed in phenylhydrazine-treated *Rana catesbiana* tadpoles (*20*). The interaction of phenylhydrazine with amphibian erythrocytes has been studied, a model system being suggested for the study of erythropoiesis (*27*). The absence of lipid peroxidation in human red cells exposed to acetylphenylhydrazine has been noted (*87*).

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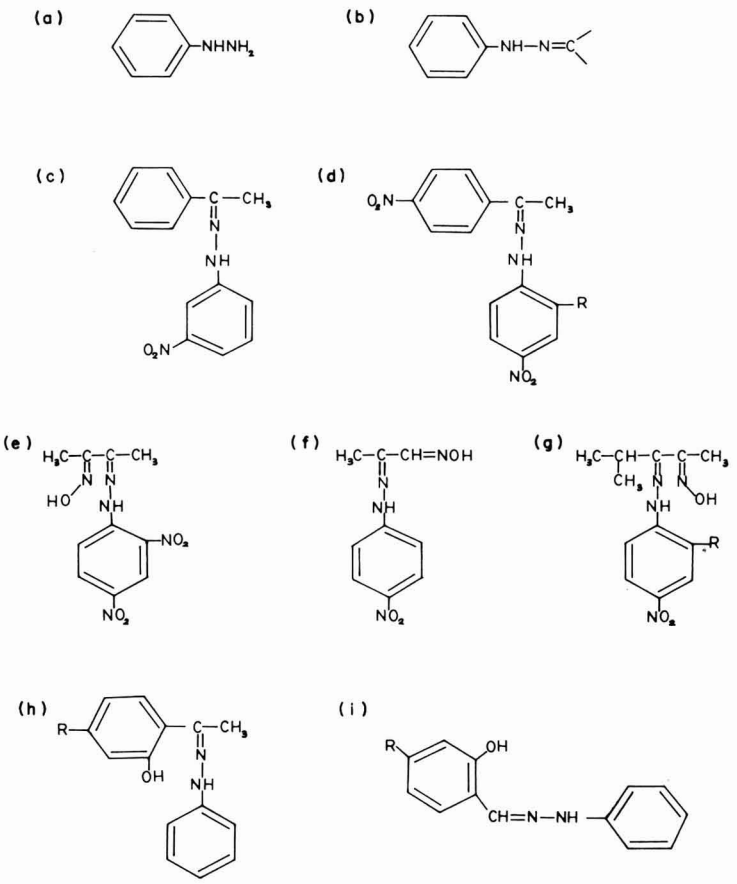
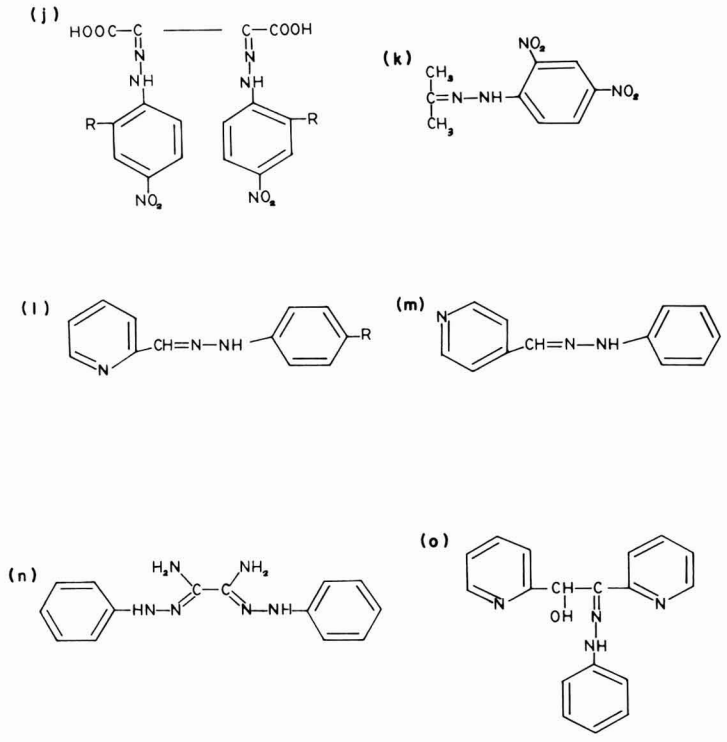


SCHEME I. (a) Phenylhydrazine; (b) phenylhydrazone; (c) *m*-nitrophenylhydrazone acetophenone; (d) *p*-nitro- (R = H) and 2,4-dinitrophenylhydrazone of 4′-nitroacetophenone; (e) diacetylmonoxime 2,4-dinitrophenylhydrazone; (f) pyruvaldoxime *p*-nitrophenylhydrazone; (g) *p*-nitro (R = H) and 2,4-dinitrophenylhydrazone (R = $-NO_2$) of 4-methylpentane-2,3-dione-2-oxime; (h) *o*-hydroxyacetophenone phenylhydrazone (R = H) and resacetophenone phenylhydrazone (R = OH); (i) salicylaldehyde phenylhydrazone (R = OH) and 2,4-dihydroxybenzaldehyde phenylhydrazone (R = OH); (j) *p*-nitro (R = H) and 2,4-dinitrophenylosazone (R = NO_2) of dihydroxytartaric acid; (k) acetone 2,4-dinitrophenylhydrazone; (l) picolinealdehyde phenylhydrazone (R = H) and picolinealdehyde *p*-nitrophenylhydrazone; (m) isonicotinealdehyde phenylhydrazone; (n) bis(phenylhydrazone) of oxamide; (o) pyridoin phenylhydrazone.

The herbicidal activity of various halogenated phenylhydrazones and related azocompounds (*60*), and the acaricidal (*59*), insecticidal (*58*), and antibacterial (*63*) activities of certain kinds of phenylhydrazones have also been the subject of studies. Aryl sulfonyl hydrazones posses antineoplastic activity (*1*).



SCHEME I—*Continued.*

In general, phenylhydrazones have been little used as reagents in inorganic analysis. Nevertheless, at the beginning of the century Cazeneuve had already obtained with a good yield the carbazide of phenylhydrazine (*13, 14*), pointing out that it would be a very sensitive reagent for copper, mercuric salts, and chromic acid. By means of this reaction, guaiacol carbonate can be distinguished from the other guaiacol preparations (all used for medicinal purposes) (*22*). By the action of urea on *p*-nitrophenylhydrazine is obtained the dinitrodiphenylcarbazide which gives a blue color with cadmium hydroxide (*31*).

Likewise, phenylhydrazine is an intermediate in the synthesis of dithizon (diphenylthiocarbazone) (*8, 24*), perhaps the most widely used analytical reagent (*34*) in spite of its inconvenience. It is of interest to note that it was E. Fischer who first synthesized diphenylthiocarbazone in 1878, recognizing its acidic nature and even describing the formation and color of its zinc salt, but it remained for H. Fischer to initiate the investigations of this remarkable substance in 1925 (*25, 76*).

Among the classical reagents for metals (*76*), no phenylhydrazone reagent is found. Phenylhydrazines and phenylhydrazones have not been

mentioned in Welcher's book (*86*), in spectrophotometric data (*33*), or in reviews, e.g., Ueno (*80*). Sandell's book (*70*) or more recent publications, e.g., Marczenko (*54*), cite only the use of phenylhydrazine as reductor in the molybdenum assay. Marczenko's book also cites the use of diacetylmonoxime *p*-nitrophenylhydrazone as a reagent for cobalt.

Phenylhydrazines and phenylhydrazones have found use mainly in qualitative and gravimetric analysis, as indicators of high alkalinity, and in spectrophotometric and catalytic procedures. The analytical aspects of hydrazones have been previously reported by Katyal (*39*), but he paided little attention to the use of phenylhydrazones. So the present paper constitutes a good supplement to that paper which concerns the analytical uses of phenylhydrazones. The organic analytical applications of phenylhydrazines and phenylhydrazones will be the subject of a future report. For the structures of various phenylhydrazines and phenylhydrazones, see Scheme I.

QUALITATIVE ANALYSIS

As early as 1907, Escot had obtained a violet coloration by reaction of phenylhydrazine acetate with a gold solution in an acidic medium (*21, 55*), but in the later literature more references concerning this coloration are not found.

In hot mineral acid solution phenylhydrazine in excess gives a red-colored product with molybdate (*57, 75*). The sensitivity of the reaction is fairly high, $25 \cdot 10^{-6}$. In the absence of H_2SO_4, a red–brown precipitate is formed. Tungsten gives no color, vanadium gives a green color, iodates under the same conditions release iodine, persulfates form a yellow coloration, and permanganates are decolorated; perchlorates and perborates do not react. The test is specific for molybdates and, according to Montignie (*57*), depends on the fact that the molybdates oxidize part of the phenylhydrazine into a diazonium salt, which reacts with the excess of phenylhydrazine and molybdate to give an azo compound. An inverse reaction may be used to test for phenylhydrazine with a sensitivity of $6 \cdot 10^{-5}$ (*57*). The direct reaction has been in spot tests for molybdenum (*22*).

The reaction of phenylhydrazine with molybdate has been applied to the determination of molybdenum in rocks. The average content of Mo in volcanic rocks is $1.5 \cdot 10^{-5}$. A method is described which is suitable for the determination of this small quantity of material (*32*).

A test was made on the determination of Mo by measuring the red color produced by the action of a solution of phenylhydrazine in dilute sulfuric acid (*29*). This test is only about one-fourth as delicate as the test which uses the red color produced with KSCN, but the color developed is less sensitive with respect to temperature changes.

A description has been given of observations of the reaction of 14

phenylhydrazone derivatives with cations of alkaline earth metals and, in certain cases, with Ni(II) and Cu(II). The color and shape of the precipitates and the sensitivity of the reaction in alkaline and neutral medium were given, a hypothesis being suggested concerning the structure of the colored compounds with Mg in an alkaline medium. If 1 drop of 0.05% *m*-nitrophenylhydrazone acetophenone and 1 ml of 0.05 *N* NaOH are mixed with 5 μg of Mg, a rose–violet precipitate is formed (*15*).

2,4-Dinitrophenylhydraxone of diacetylmonoxime (*3*), *p*-nitrophenylhydrazone of pyruvaldoxime (*4*), and *p*-nitro- and 2,4-dinitrophenylhydrazones of 4-methylpentane-2,3-dione-2-oxime (*5*) form complexes with cobalt in an ammoniacal medium, providing a selective and sensitive test for cobalt (Table 1). All of these later investigations have been carried out by Anand *et al.*

GRAVIMETRIC ANALYSIS

Phenylhydrazine precipitates to aluminium in the hydroxide form, to chromium, zirconium, and tungsten (*89*).

Phenylhydrazine sulfinic acid has been employed for the gravimetric determination of Te in the presence of several metals. The reagent precipitates Te quantitatively from strong hydrochloric acid solutions (25–75%); 5–100 mg of Te can be estimated rapidly by this procedure (*35*).

Phenylhydrazones containing the donor grouping -OH have been used

TABLE 1
COMPLEXES OF COBALT WITH PHENYLHYDRAZONES

Reagent	Color	Limit of detection	Limit of dilution	Interferences[a]	Ref.
2,4-Dinitrophenylhydrazone of diacetylmonoxime	Red	0.1 μg	$1:5 \cdot 10^5$	Cu, Ni	(*3*)
p-Nitrophenylhydrazone of pyruvaldoxime	Pink	0.1 μg	$1:2 \cdot 10^6$	Cu, Ni	(*4*)
p-Nitrophenylhydrazone of 4-methylpentane-2,3-dione-2-oxime		2 μg	$1:5 \cdot 10^5$	Cu, Ni, Ag	(*5*)
2,4-Dinitrophenylhydrazone of 4-methylpentane-2,3-dione-2-oxime		2 μg	$1:5 \cdot 10^5$	Cu, Ni, Ag	(*5*)

[a] Large amounts of Ni and Cu interfere, owing to the deep color of their amine complexes. Interference from Ni and Cu was prevented by the addition of KCN. Centrifugation was necessary if metals that formed insoluble hydroxides were present. High concentrations of NH_4^+ and CN^- inhibited complex formation.

in gravimetry. An ethanolic solution of the resacetaphenone phenylhydrazone quantitatively precipitates cooper from ammoniacal solutions without rigid control of the experimental conditions. Up to 32 mg of Cu can be accurately determined in the presence of, e.g., 40 mg of cadmium (*81*).

Of eight phenylhydrazone derivatives of phenolic aldehydes and ketones, salicylaldehyde phenylhydrazone was found to be the best gravimetric reagent for copper(II). The reagent was used dissolved in ethanol–water (10% of ethanol), the precipitate being ignited to the oxide before weighing. Twenty-five to 60 mg of Cu(II) could be determined with an error of 0.04 or 0.08% in the presence of 100 mg of Cd (*82*).

Likewise, of several hydroxy derivatives of phenylhydrazones tested as gravimetric reagents for palladium, *o*-hydroxyacetophenone phenylhydrazone was found to be the best. It is possible to determine 15 to 47 mg of Pd. The palladium is weighed as metal (*83*).

INDICATORS

Phenylhydrazine is a weak base ($K_b = 1.62 \cdot 10^{-9}$ at 25°C), in contrast with hydrazine, due to the introduction of the benzene ring poor in electrons, which decreases the density of electrons on the hydrazine group. Introducing an acidic group into the benzene ring of phenylhydrazine further reduces the basic strength.

Thus, *p*-nitrophenylhydrazine is a very weak base, amphoteric in character, because it contains both acidic and basic groups (*9*).

$$\underset{\text{Colorless}}{O_2N-C_6H_4-NH-NH_3^+} \overset{H^+}{\underset{OH^-}{\rightleftharpoons}} \underset{\text{Greenish yellow}}{H_2N-C_6H_4-NH-NH_2} \overset{H^+}{\underset{OH^-}{\rightleftharpoons}} \underset{\text{Orange (sharp color change)}}{O_2N-C_6H_4-NH-NH^-}$$

2,4-Dinitrophenylhydrazine changes from yellow to brown at pH 11.0 to 11.6. Ethanolic solutions of *p*-nitrophenylhydrazine and 2,4-dinitrophenylhydrazine have been recommended for use in the titration of certain weak acids (1 mequiv) with 0.1 *N* NaOH in a medium of acetone or ethanol. Both indicators tend to be unstable under alkaline conditions (*46*).

Spectrophotometric parameters for the uv and visible regions, and dissociation constants for acyl derivatives of 2,4-dinitrophenylhydrazine were described (*7*). These compounds were found to be suitable as indicators for the titration of weak acids in the dissociation constant range of 10^{-4} to 10^{-6}. Other compounds related to phenylhydrazine have been used as indicators (*47, 50, 52*), the effect of the medium on the indicator con-

stants being determined (*48*) and the developments in indicator research reviewed (*51*).

Although the number of papers dealing with the use of phenylhydrazones is not high, more than 50 phenylhydrazone derivatives have been tested as indicators.

A number of nitrophenylhydrazones were prepared to determine their possible use as indicators and the influence of the nitro group on color. Of the compounds studied, the 3- and 4-nitrophenylhydrazones of 2,4-dihydroxybenzaldehyde were the most suitable for use as indicators (*69*). The presence of the nitro group in the hydrazine–benzene ring intensified the colors of the original carbonyl compounds; when the number of nitro groups predominated in the benzaldehyde–benzene ring, the color was darker.

Chugreeva (*16*) suggested the use of *p*-nitrophenyl- and 2,4-dinitrophenylosazones of dihydroxytartaric acid and 2,4-dinitrophenylhydrazone of acetone, which behave as color indicators at pH 11.5–13.5 and may be used in solutions with high concentration of salts, ethanol, and proteins in the temperature range from 0 to 80°C. The possible use of some compounds of the hydrazone type as indicators for the determination of NaOH in mixtures with inorganic and organic compounds was tested. Among the indicators studied, the *p*-nitro- and 2,4-dinitrophenylosazones of dihydroxitartaric acid can be used particularly for the accurate determination of free NaOH in mixtures with alkali-metal carbonates, aqueous ammonia, KCN, sodium phenoxide, sulfacetamide, and sulfapyridine sodium (*17*).

Absorption spectra were reported for benzaldehyde phenylhydrazone and analogs with substituents on the aryl component of the hydrazine portion. All spectra were reported in acid and alkaline media; all showed color changes in the pH range 9–13, generally from yellow to violet. The strongest indicator color changes were found among derivatives of *p*-nitrophenylhydrazones, while the *m* isomers are not effective indicators. Nitration of the aldehyde portion of the hydrazones in the *o* or *m* position relative to -CHO shifted the color change toward a more acidic region. *p*-Cl or *m* or *o*-OH groups had little effect on indictor properties (*69*).

Poirrier blue C4B, Tropaeoline O, and nitramine are used commonly as indicator for alkali, but they have some disadvantages, for example, indefinite color changes and instability of colors. Some *p*-nitrophenylhydrazones have proved to be better than those in their stability and readily observable color changes. The changes with pH of the absorption spectra have been studied, determining the useful pH ranges and stability of the colors of the *p*-nitrophenylhydrazones derived from crotonaldehyde and acraldehyde (*36*), furfuraldehyde and some furfuraldehyde derivatives (*38*), benzaldehyde and 4-nitro-, 4-hydroxy-, 2,4-dihydroxy-, and

4-dimethylaminobenzaldehydes among others (*37*), and the *p*-nitro- and 2,4-dinitrophenylhydrazones derived from 4′-nitroacetophenone (*49*).

The *p*-nitrophenylhydrazone of benzaldehyde was also studied with respect to the effects of the color change of various substituents in the benzaldehyde ring. General characteristics of *p*-nitrophenylhydrazones (Table 2) are: color change, yellow–red; pH range, 10–14; pK_a, 12–12.5; maintenance of stable coloration, more than 1 hr.

The potassium salt of benzaldehyde *p*-nitrophenylhydrazone (*62*) has also been the subject of study. Likewise, the phenylhydrazones of pyridine-2-aldehyde and pyridine-4-aldehyde, when used as indicators for the titration of a base with an acid, both yield a change from colorless to yellow. Sharp end points were obtained in the titration of, e.g., aqueous 0.1 *M* ammonia with HCl, and the compounds are suitable for use in spectrophotometric titrations (*84*). Absorption spectra of these two compounds and their dissociation constants in aqueous methanol and aqueous ethanol were calculated.

p-Nitrophenylhydrazone of pyridine-2-aldehyde has been recently proposed as an alkali indicator (*11*).

The acid–base properties of hydrazine derivatives are not bound to the presence of nitro groups; change can also be caused by other electrophilic

TABLE 2
CHARACTERISTICS OF SOME PHENYLHYDRAZONE INDICATORS

Reagent	Color change	pH range[a]	pK_a	Ref.
Crotonaldehyde *p*-nitrophenylhydrazone	Yellow–red	10.4–12.4		(*36*)
Acraldehyde *p*-nitrophenylhydrazone	Yellow–red	10.6–12.4		(*36*)
2,4-Dinitrophenylhydrazine	Yellow–brown	11.0–12.5	12.1	(*9*, *46*)
Benzaldehyde *p*-nitrophenylhydrazone	Yellow–red	11–12		(*62*)
Furfurylidene *p*-nitrophenylhydrazone	Yellow–red	10.8–11.8	12.2	(*38*)
5-Nitro-2-furfurylidene *p*-nitrophenylhydrazone	Yellow–blue	10.8–12.2	11.6	(*38*)
5-Methyl-2-furfurylidene *p*-nitrophenylhydrazone	Yellow–red	10.8–12.0	12.0	(*38*)
Picolinealdehyde phenylhydrazone	Colorless–yellow		5.17	(*84*)

[a] Visual.

substituents, e.g., sulfonic acid groups. The electrophilic groups reduce the electron density of the molecule and make possible the release of a proton, the degree of dissociation being higher the more electrophilic substituents are contained in the ring (*9*). If the proton is released by the nitrogen atom next to the phenyl group in an alkaline medium, it gives rise to a quinoidal structure. The color change of *p*-nitrobenzhydrazide in alkaline medium is due to the formation of a quinoidal structure (*9*).

SPECTROPHOTOMETRY

By means of the phenylhydrazine–molybdate reaction, contents as low as 0.05% of molybdenum can be determined (*29*). The absorbance of the red solution is measured at 520 nm, and an isopropanol–chloroform mixture is used as reaction medium.

The phenylhydrazine–molybdate reaction has also been applied to the determination of molybdenum in steels by measuring the absorbance at 525 nm in homogeneous medium (*6*). For quantitative results, Mo must be present as MoO_4^{2-}. In steels, Fe(III), Cr(III), Co(II), V(V), and W(VI) may interfere. Fe(III) interferes most in low-Mo steels but can be reduced to Fe(II) without appreciable reduction of Mo by a special technique in the Jones reductor. Zarovskif *et al.* have also used the same reaction for the determination of Mo in steels (*90*).

A study of the conditions for the formation of the red complex of phenylhydrazine with molybdate acid and its properties has been reported (*10*). MoO_4^{2-} oxidizes $PhNHNH_2$ to form PhN:N·OH and Mo_2O_5. By rearrangement, PhNHNO is formed and condensed with the excess of $PhNHNH_2$ to PhNHN:NNHPh. The four N atoms of PhNHN:NNHPh are coordinatively bound to Mo_2O_5 to form a red complex. The formation of the complex is considerably influenced by the pH and the temperature. The necessity of an excess of $PhNHNH_2$ is attributed to the simultaneous formation of the analytically inactive antiform of PhN:NOH. The effects of oxidants and reductants were discussed (*10*). The red complex can be extracted into butanol (*28*).

Phenylhydrazine also acts as a reductor in the colored method of phosphomolybdate (*89*).

p-Nitrophenylhydrazone of diacetylmonoxime (cobaltone I) forms in ammoniacal medium a red complex with cobalt ion. The excess of reagent is separated with ethyl ether, and the absorbance is measured at 520 nm against a reagent blank. Common anions do not interfere; Fe, Cr, V, and Mn are removed by precipitation with aqueous ammonia, while interference by Ni, Cu, Ag, and Pd is greatly reduced by the addition of KCN (*19*).

At pH 8–9 cobalt forms a negative charged complex with ethyl ester 2-hydroxyphenylhydrazone dioxobutyrate (A) which can be extracted

into benzene as the ion associated with rhodamine 3 S (B). Optimum reagent concentrations for the determination of 0.6–6.0 μg Co/ml include $5 \cdot 10^{-2}$ mol of (A) and $2 \cdot 10^{-4}$ mol of (B). The absorbance is measured at 565 nm. The sensitivity of the determination is 0.08 μg Co/ml. Zn(II), Cu(II), and Mn(II), at a 20-fold excess, do not interfere (*56*).

A method has been developed for obtaining diphenylhydrazoneoxalatediamine from dithiooxalatediamide and phenylhydrazine base (*44*). The mentioned hydrazone has been suggested as a reagent for determining chlorine, Hg(II), and Fe(III); it can be determined by means of ferricyanide.

Sensitive indirect methods for the determination of selenium take advantage of the oxidation by selenium(IV) of phenylhydrazine-*p*-sulfonic acid. The diazonium salt formed in the reaction of selenium with 4-hydrazinobenzenesulfonic acid is coupled with 4-aminonaphthalene-1-sulfonate sodique, and the extinction of the product is measured at 520 nm. By means of the appropriate procedure (*67*), microamounts of selenium in high-purity metallic germanium and in germanium dioxide can be determined. No interference is caused by 1000-fold amounts of alkali or alkaline earth metals, Al, B, Ga, In, Te(IV), Co, Ni, Mn, Cd, CN^-, CO_3^{2-}, F^-, SO_4^{2-}, SiO_3^{2-}, oxalate, but oxidizing or reducing agents must be removed.

The diazonium oxidation product can be coupled with 1-napthylamine to form an azo dye, which has an absorption maximum at 420 nm. The effects of acidity, reagent concentration, and rates of oxidation for both oxidation and coupling have been studied. A number of ions interfere, e.g., those that reduce H_2SeO_3 and those that oxidize the reagent. Solvent extraction of the cupferron complexes of Fe and Cu is used to eliminate the interference of these elements. The sensitivity according to Sandell's expression is 0.002 μg of Se/cm^2, although a value of 0.01 μg of Se/cm^2 is considered to be more practical (*42*).

Pyridoin phenylhydrazone has been recommended as an extractive reagent for the spectrophotometric determination of copper as copper(I) ($\epsilon = 2.1 \cdot 10^4$ l mol^{-1} cm^{-1} at 450 nm). The molecule possesses the cuproine grouping, but the method suffers from the interference of numerous anions even at microgram amounts. Likewise, the blank absorption is very elevated (*72*). Pyridoin phenylhydrazone in excess, at pH 4.7, forms with palladium an orange 2:1 complex (ϵ_{max} = 450 nm; = $2.41 \cdot 10^4$ l mol^{-1} cm^{-1}) which has been used for the photometric estimation of palladium. The method is compared with other spectrophotometric procedures used for determining palladium (*73*).

Picolinealdehyde *p*-nitrophenylhydrazone is another example of a phenylhydrazone reagent used in the spectrophotometric determination of palladium (*12*).

Phenylhydrazine dithiocarbamate has also been applied as a spectrophotometric reagent. Bivalent Fe reacts with a dithiocarbamoylhydrazinium salt to form a violet complex in buffer solution, which has an absorption maximum at 570 nm. This reaction may be used for the determination of Fe(II) in the concentration range 0.01 to 5 μg/ml (*61*).

CATALYTIC METHODS

Catalytic methods are generally much more sensitve than spectrophotometric methods based on stoichiometric reactions (*88*). Arylhydrazines are known to oxidize and couple with aromatic compounds to produce intensively colored azo dyestuff (*65*). It has been previously mentioned that the oxidative coupling reaction of phenylhydrazine-*p*-sulfonic acid with α-naphthylamine has been used for the photometric determination of microamounts of selenium. It was found by Tanaka and Awata (*78*) that the oxidation of phenylhydrazine-*p*-sulfonic acid by chlorate is catalyzed by vanadium, and thus the catalytic determination of submicrogram amounts of vanadium can be performed.

Phenylhydrazine-*p*-sulfonic acid was selected from some arylhydrazines because it is the most stable and soluble in aqueous solution and because its oxidation product is more stable than the other diazo derivatives. α-Naphthylamine was the coupling component selected among several compounds.

Vanadium is extracted from the sample solution at pH 4.5 into $CHCl_3$ as its complex with 8-hydroxyquinoline, and is reextracted into an aqueous NH_3–NH_4Cl buffer solution of pH 10. The V in an aliquot of the extract is used to catalyze the oxidation, at pH 2.5, of 4-hydrazinobenzenesulfonic acid by ClO_3^- to 4-diazobenzenesulfonic acid, which is then coupled with 1-naphthylamine. The resulting dyestuff is determined spectrophotometrically at 530 nm ($\epsilon = 4.37 \cdot 10^4$ l mol^{-1} cm^{-1}). From 0.02 to 1 ppm of V can be determined. The extraction procedure makes the method highly selective: Any Fe in the original solution is masked with 1,2-diaminecyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; Ag, Sn(IV), Bi(III), and Sb(III) interfere. Christian has adapted the method to the determination of vanadium in blood and urine (*18*).

The oxidation of 4-hydrazinobenzenesulfonic acid by ClO_3^- is also catalyzed by Se(IV), and the same reaction described above is valid for determining submicrogram amounts of selenium(IV). The method is simple and there are few interferences (*40*). The sensitivity of the method is about 20 times as high as that of the stoichiometric method proposed by Kirbright and Yoe (*42*) and provides an effective molar absorptivity of $7.2 \cdot 10^5$ for selenium (525 nm). The following ions caused no interference in the determination of 1 μg of Se(IV): 100 mg of SO_4^{2-} or Cl^-; 50 mg of Na or

NH_4^+; 30 mg of NO_3^-; 20 mg of K; 10 mg of Ca or Ba; 1 mg of Mg, Ni, Cd, Zn, Pb, Mn(II), or As(V); 10 μg of Sn(II), Sn(IV), Ce(III), Co(II), ClO_4^-, or SO_3^{2-}. Tellurium(IV), 1 mg, and selenium(VI), 10 μg, do not interfere with the proposed method of determination of selenium(IV). Ions with oxidizing power give rise to a positive interference. Iron(III) and iron(II) form violet precipitates with 1-napthylamine. Copper(II), Ce(IV), V(V), and Cr(VI) act as catalysts in the color development. Nitrite (1 mg) forms an orange product. The interfering metals can be easily removed by the extraction of oxinates.

If 1-napthylamine is replaced by *m*-phenylenediamine, the sensitivity and reproducibility of the results are greatly improved. Its forms a yellow azo dyestuff, *p*-(2,4-diaminophenylazo)benzenesulfonic acid (ϵ_{max} = 454 nm). The reaction proceeds catalytically with a minute amount of selenium(IV). As little as 10^{-7} mol selenium(IV) can be determined easily. Optimal conditions are: pH range, 0.8–1.0; reaction time, 60 min at 50°C. Beer's law is obeyed over the range 0–2.0 μg of Se(IV)/25 ml of the working solution. The effective molar absorptivity is $1.2 \cdot 10^6$ for Se(IV). The sensitivity of the proposed method can be increased further by extraction of the dyestuff into organic solvents in the presence of zephiramine (ϵ = $2.4 \cdot 10^6$ l mol^{-1} cm^{-1}) (*41*).

Copper(II), Ce(IV), Mo(VI), and I^- interfered in amounts of 100 μg. Further, 10 μg of Fe(III), V(V), or Cr(VI) causes a positive interference; these ions seem to catalyze the color development. The interference of Cu(II) can be suppressed by the addition of EDTA. The interferences of V(V) and Fe(III) can be eliminated by extraction with oxine. As the coexistence of Se(IV) and I^- in actual samples is rare, the iodide interference should not cause problems. Tellurium(IV) causes a negative interference which cannot be avoided.

MISCELLANEOUS

o-Aminobenzaldehyde phenylhydrazone (Nitrin) is a specific reagent for nitrous acid and its salts (nitrites), giving a violet–red color due to diazotation; this then hydrolyzes into the antidiazonium hydroxide which is yellow. The method is suitable for nitrite determination in water, milk, serum, alc. serum of blood, and extracts of sausage or other meats (*64*). The detection of nitrites in drinking water or in urine (indirect determination of *E. coli* infection) without interference of foreign ions can be performed in the form of a layer test (*28*); reddish–violet ring between the layers, yellowish–brown color on mixing the layers. The very stable yellowish–brown color developing in the second reaction phase is taken for the colorimetric determination.

Diazoted amines give colored compounds with ammonia. Sensitive test papers have been prepared with the aid of various diazoted amines,

among them the diazonium of phenylhydrazine, by means of which it was found possible to detect a small fraction of 1 mg of ammonia in 1 liter of air (*43*).

Recently bivalent Cu (1 to 16 μg) has been determined by amperometric titration with ethanolic resacetophenone phenylhydrazone in 0.15 *M* sodium acetate containing gelatine as supporting electrolyte; the applied potential is −0.4 V versus the s.c.e., and the mixture is deaerated with H before titration. The error is 0.06 μg. There is no interference from a 10-fold amount (relative to Cu) of Ni, Zn, or Cd; Fe(II) interferes, but an amount equal to that of Cu can be tolerated if NaF is added as a masking agent. The method was used in analyzing nickel silver (*66*).

SUMMARY

A review of the inorganic analytical uses of phenylhydrazines and phenylhydrazones is given. Phenylhydrazines and phenylhydrazones have found use in qualitative and gravimetric analysis, as indicators of high alkalinity, and in spectrophotometric and catalytic procedures.

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REFERENCES

1. Agraval, K. C., Booth, B. A., Sartorelli, A. C., and Broom, A. D., Antineoplastic activity of aryl sulfonyl hydrazones (meeting abstr.). *Abstr. Pap. ACS* **170,** 47 (1975).
2. Allen, C. F. H., and Richmond, J. H., Limitations of 2,4-dinitrophenylhydrazine as a reagent for carbonyl groups. *J. Org. Chem.* **2,** 222–226 (1937).
3. Anand, V. D., and Deshmukh, G. S., 2,4-Dinitrophenylhydrazone of diacetylmonoxime: A new sensitive reagent for cobalt. *Naturwissenschaften* **46,** 648 (1959).
4. Anand, V. D., New selective reagent for cobalt: The *p*-nitrophenylhydrazone of pyruvaldoxime. *Chem. Anal.* **50,** 44–45 (1961).
5. Anand, V. D., Deshmukh, G. S., and Kumar, M. B., New reagents for cobalt. *J. Prakt. Chem.* **22,** 98–100 (1963).
6. Ayres, G. H., and Tuffy, B. L., Spectrophotometric determination of molybdenum with phenylhydrazine hydrochloride. *Anal. Chem.* **23,** 304–308 (1951).
7. Berkovic, A., Sidoova, E., and Mayer, P., Use of acyl derivatives of 2,4-dinitrophenylhydrazone as acid-base indicators. *Chem. Zvesti* **24,** 38–45 (1970).
8. Billman, J. H., and Cleland, E. S., An improved method for the preparation of dithizone (diphenylthiocarbazone). *J. Amer. Chem. Soc.* **65,** 1300–1301 (1943).
9. Bishop, E., "Indicators." Pergamon Press, London, 1972.
10. Bozsai, J., Photometric determination of molybdenum with phenylhydrazine. I. New investigations on the phenylhydrazine–molybdenum complex. *Talanta* **10,** 543–548 (1963).
11. Carrillo, J., and Guzman, M., *p*-Nitrofenilhidrazona del picolinaldehido indicador colorimétrico del pH. *Anal. Quim.* (in press).
12. Carrillo, J., and Guzman, M., Determination of palladium with picolinealdehyde *p*-nitrophenylhydrazone. *Anal. Quim.* (in press).
13. Cazeneuve, M. P., Extrait des procés-verbaux des seances, Seance du vendredi 24 Fevrier 1899. *Bull. Soc. Chim. France* **21,** 258 (1899).

14. Cazeneuve, M. P., Sur la diphenylcarbazide reactif tres sensible de quelques composés metalliques, cuivre, mercure, fer au maximum, acide chromique. *Bull. Soc. Chim. France* **23**, 701–706 (1900).
15. Chugreeva, N. V., Hydrazones as analytical reagents. *Uchenye Zaplyki Saratov. Univ.* **71**, 233–237 (1959).
16. Chugreeva, N. V., Some nitrophenylosazones and nitrophenylhydrazones as indicators at high pH values. *Zhur. Anal. Khim.* **15**, 391–393 (1960).
17. Chugreeva, N. V., Use of some nitrophenylosazones and hydrazones in the determination of free sodium hydroxide by direct titration. *Izvest. Vysihikh. Ucheb. Zavedenii Khim. i. Khim. Tekhnol.* **1**, 16–19 (1961).
18. Christian, G. D., Catalytic determination of vanadium in blood and urine. *Anal. Lett.* **4**, 187–196 (1971).
19. Deshmukh, G. S., Anand, V. D., and Pandey, C. M., Spectrophotometric determination of cobalt with Cobaltone I (*p*-nitrophenylhydrazone of diacetylmonoxime). *Z. Anal. Chem.* **182**, 170–177 (1961).
20. DeWitt, W., Price, R. P., Vankin, G. L., and Brandt, E. M., Production of erythroid cells in phenylhydrazine-treated *Rana catesbiana*. *Develop. Biol.* **27**, 27–37 (1972)
21. Escot, E. P., *Ann. Chim. Anal. Chim. Appl.* **19**, 90 (1907).
22. Feigl, F., and Anger, V., "Spot Tests in Organic Analysis." Elsevier, Amsterdam, 1968.
23. Fieser, G. F., and Fieser, M., "Advanced Organic Chemistry." Reinhold, New York, 1962.
24. Fischer, E., *Ann.* **190**, 114 (1978); **212**, 316 (1882).
25. Fischer, H., Compounds of diphenylthiocarbazone with metals and their use in analysis. *Wiss. Veröff. Siemens-Konz.* **4**, 158–170 (1925).
26. Flores, G., and Frieden, E., Hemolytic effect of phenylhydrazine during amphibian metamorphosis. *Develop. Biol.* **27**, 406–418 (1972).
27. Flores, G., Interaction of phenylhydrazine with amphibian erythrocytes. Model system for the study of erythropoiesis. *Diss. Abstr. Int. B* **32**, 6195 (1972).
28. Fries, J., and Getrost, H., "Organische Reagenzien für die Spurenanalyse," p. 249. E. Merck, Darmstadt, 1975.
29. Goldstein, E. M., Colorimetric determination of molybdenum in tungsten metals and compounds. *Chem. Anal.* **45**, 47–49 (1956).
30. Hauptmann, H., and Balconi, M., Determination of molybdenum in manganese minerals. *Z. Anorg. Allgem. Chem.* **214**, 380–384 (1933).
31. Heller, K., and Krumholz, P., Systematic spot-analysis. *Mikrochemie* **7**, 213–222 (1929).
32. Hevesy, G. von, and Hobbie, R., Determination of molybdenum and tungsten in rocks. *Z. Anorg. Allgem. Chem.* **212**, 133–144 (1933).
33. I.U.P.A.C., "Tables of Spectrophotometric Absorption Data of Compounds Used for the Colorimetric Determination of Elements." Butterworths, London, 1963.
34. Iwantscheff, G., "Das Dithizon und seine Anwendug in der Mikround Spureanalyse." Verlag Chemie, GmbH, Weinheim, 1957.
35. Jain, S. K., Pandey, D. C., Joseph, K. C., and Satyanarayana, K., Phenylhydrazin sulfinic acid—A new gravimetric reagent for tellurium. *J. Indian Chem. Soc.* **54**, 405–406 (1977).
36. Kambe, M., Hasegawa, Y., and Shindo, E., New pH indicators for the alcali region. *Japan Anal.* **12**, 63–64 (1963).
37. Kambe, M., Hasegawa, Y., and Shindo, E., *p*-Nitrophenylhydrazones as indicators for the pH range 11 to 14. *Japan Anal.* **13**, 1218–1223 (1964).
38. Kambe, M., Shindo, E., and Morito, M., *p*-Nitrophenylhydrazones of 2-furaldehyde as indicators in the alkaline region. *Japan Anal.* **16**, 1015–1028 (1967).

39. Katyal M., and Dutt, Y., Analytical applications of hydrazones. *Talanta* **22**, 151–166 (1975).
40. Kawashima, T., Nakano, S., and Tanaka, M., Catalytic determination of submicrogram amounts of selenium(IV) by means of the oxidative coupling reaction of phenylhydrazine *p*-sulfonic acid with 1-naphthylamine. *Anal. Chim. Acta* **49**, 443–447 (1970).
41. Kawashima, T., Kai, S., and Takashima, S., Catalytic determination of ultratrace amounts of selenium(IV). *Anal. Chim. Acta* **89**, 71–82 (1977).
42. Kirkbright, G. F., and Yoe, J. M., New spectrophotometric method for the determination of microgram amounts of selenium. *Anal. Chem.* **35**, 808–811 (1963).
43. Korenman, I. M., Detection of ammonia in the air. *Z. Anal. Chem.* **90**, 115 (1932).
44. Koshkin, N. V., The use of diphenylhydrazoneoxalate diamide in analytical chemistry. *Zh. Anal. Khim.* **20**, 534–539 (1965).
45. Kuritsyna, R. M., and Chugreeva, N. V., Indicator properties of nitrophenylhydrazones of benzaldehyde and its derivatives. *Zh. Obshch. Khim.* **36**, 1927–1930 (1966).
46. Legradi, L., New acid-base indicators. I. Phenylhydrazine derivatives as acid-base indicators. *Magy. Kem. Foly.* **71**, 302–304 (1965).
47. Legradi, L., New acid-base indicators. II. 2-Acetyl-1(4-nitrophenyl)hydrazine *Magy. Kem. Foly.* **72**, 19–20 (1966).
48. Legradi, L., New acid-base indicators. IV. Effect of the medium on the indicator constants. *Magy. Kem. Foly.* **73**, 32–39 (1967).
49. Legradi, L., New acid-base indicators. V. 4′-Nitroacetophenone 4-nitrophenylhydrazone and 4′-nitroacetophenone 2,4-dinitrophenylhydrazone. *Magy. Kem. Foly.* **73**, 389–392 (1967); *Acta Chim. Hung.* **58**, 1–7 (1968).
50. Legradi, L., New acid-base indicators. VII. *N*-(2-Nitrophenyl)acetohydrazide. *Magy. Kem. Foly.* **77**, 80–82 (1971).
51. Legradi, L., Organic analysis and indicator research. *Magy. Kem. Lap.* **26**, 348–355 (1971).
52. Legradi, L., Developments in indicators. *Talanta* **19**, 1470–1474 (1972).
53. Maniatis, G. M., and Ingram, V. M., Effect of phenylhydrazine-induced anemia on appearance of adult hemoglobin in *Rana catesbiana* tadpoles. *Develop. Biol.* **27**, 580–583 (1972).
54. Marczenko, Z., "Spectrophotometric Determination of Elements." Ellis Horwood, New York, 1977.
55. Mellan, I., "Organic Reagents in Inorganic Analysis." Blakistan, Philadelphia, 1941.
56. Mittsel, Yu. A., and Vasil'ev, A. V., Photometric determination of cobalt with ethyl ester of dioxobutyric acid 2-hydroxyphenylhydrazone and rhodamine 3 S. *Zh. Anal. Khim.* **29**, 196–197 (1974).
57. Montignie, E., Un reactif tres sensible de l'ion molybdate. *Bull Soc. Chim. France* **47**, 128 (1930).
58. Moon, M. W., Insecticidal glyoxyloylhalide phenylhydrazone. Ger. Offen. 2, 156, 058 (Cl. CO7 dc, AO1n), 22 June 1972; U.S. Appl. 93, 496, 27 November 1970.
59. Moon, M. W., Gemrich, E. G., and Kaugars, G., Acaricidal activity of thioketal adducts of pyruvoyl chloride phenylhydrazones and related compounds. *J. Agr. Food Chem.* **20**, 888–891 (1972).
60. Moon, M. W., Friedman, A. R., and Steinhards, A., Herbicidal activity of phenylhydrazones and related azo-compounds. *J. Agr. Food Chem.* **20**, 1187–1190 (1972).
61. Musil, A., and Haas, W., Determination of bivalent and tervalent iron with a dithiocarbamoylhydrazinium salt by colorimetric or spectrophotometric means. *Mikrochim. Acta*, 481–487 (1959).
62. O'Connor, Rosenbrook, W., and Anderson, G., A new indicator for pH 11 to 12. *Anal. Chem.* **33**, 1282 (1961).
63. Pecca, J. G., Dobrecky, J., and Albonico, S. M., Synthesis and antibacterial activity of 5-nitro 2-furaldehyde phenylhydrazones. *J. Pharm. Sci.* **60**, 650 (1971).

64. Pfeiffer, H., *o*-Aminobenzalphenylhydrazone, a specific reagent for nitrous acid and its salts (nitrites). *Lebensm. Untersuch. Forsch.* **88**, 287–290 (1948); *Münch. Med. Wschr.* **92**, 1315 (1950).
65. Postowsky, J. J., Lugowkin, B. P., and Mandryk, G. T., Über die Reaktion des Selendioxyds mit einigen Aryl-Hydrazinen. *Ber.* **69**, 1913–1916 (1936).
66. Reddy, Y. K., Rao, S. B., and Raju, N. A., Amperometric determination of copper with resacetophenone phenylhydrazone. *Proc. Indian Acad. Sci. A* **84**, 71–78 (1976).
67. Rigin, V. I., Mel'nichenko, N. N., and Yanitskii, V. K., (Spectrophotometric) Determination of selenium in high-purity metallic germanium and germanium dioxide (by reaction with 4-hydrazinobenzenesulfonic acid). *Zav. Lab.* **33**, 1370–1372 (1967).
68. Roduta, F. L., and Quibilan, G., Condensation reactions of 2,4-dinitrophenylhydrazine. *Rev. Filipina Med. Farm.* **27**, 123–130 (1936).
69. Roduta, F. L., and Cabreros, J. C., Isomeric nitrophenylhydrazones. *Univ. Philippines Nat. Appl. Sci. Bull.* **7**, 341–348 (1940).
70. Sandell, E. B., "Colorimetric Determination of Traces of Metals," 3rd ed. Interscience, New York, London, 1959.
71. Shiriner, R. L., Fuson, R. C., and Curtin, D. Y., "The Systematic Identification of Organic Compounds. A Laboratory Manual." John Wiley, New York, 1966.
72. Silva, M., and Valcarcel, M., Selective determination of copper with pyridoin phenylhydrazone as chromogenic extraction reagent. *Mikrochim. Acta* **2**, 121–129 (1977).
73. Silva, M., Pino, F., and Valcarcel, M., Determinacion de trazas de paladio con la fenilhidrazona de la piridoina. *Anal. Quim.* **74**, 65–69 (1978).
74. Smith, C. E., McLain, L. R., and Taugg, N. S., Phenylhydrazine-induced anemia in chinook salmon. *Tox. Appl.* **84**, 20 (1973).
75. Spiegel, L., and Maas, Th. A., Ein empfindlicher Nachweis für Molybdänverbindungen. *Ber.* **36**, 512–515 (1903).
76. Stephen, W. I., Historical survey of the uses of organic compounds as reagents in analytical chemistry. *Analyst* **102**, 793–804 (1973).
77. Swann, J. W., Knupp, C. L., and Contrera, J. F., Phenylhydrazine induced depletion of cardiac norepinephrine. *Fed. Proc.* **31** A 522 (1972).
78. Tanaka, M., and Awata, N., Catalytic determination of submicrogram amounts of vanadium by means of the oxidative coupling reaction of phenylhydrazine-*p*-sulfonic acid with -naphthlamine. *Anal. Chim. Acta* **39**, 485–490 (1967).
79. Tavassoli, M., Maniatis, A., and Crosby, W. H., Effects of phenylhydrazine-induced hemolysis on behavior of reenerating marrow stroma. *Brit. J. Haematol.* **23**, 701–707 (1972).
80. Ueno, K., "Organic Reagents" (T. S. West, ed.), pp. 43–69. Butterworths, London.
81. Umapathy, P., and Raju, N. A., Determination of copper with resacetophenone phenylhydrazone. *Indian J. Chem.* **1**, 272–273 (1963).
82. Umapathy, P., and Raju, N. A., Phenylhydrazones as analytical reagents for copper. *Indian J. Chem.* **2**, 248–249 (1964).
83. Umapathy, P., Venkatareddy, D., and Raju, N. A., 2'-Hydroxyacetophenone phenylhydrazone as a gravimetric reagent for palladium. *Indian J. Chem.* **3**, 471 (1965).
84. Vasilikiotis, G. S., and Stratis, J., Phenylhydrazones of pyridine-2-aldehyde (picolinaldehyde) and pyridine-4-aldehyde (isonicotinaldehyde) as new acid-base indicators. *Anal. Chim. Acta* **75**, 227–230 (1975).
85. Walter, H., Miller, A., Krob, E. J., and Ascher, G. S., Membrane surface properties of reticulocytes from rats rendered severely anemic with phenylhydrazine as determined by partition in aqueous phase sistems. *Exp. Cell Res.* **73**, 145–153 (1972).
86. Welcher, F. J., "Organic Analytical Reagents." Van Nostrand, New York, 1947.
87. Winterbo, C. C., and Carrell, R. W., Absence of lipid peroxidation in human red-cells exposed to acetylphenylhydrazine. *Brit. J. Haematol.* **23**, 499 (1972).

88. Yatsimirkii, K. B., "Kinetic Methods of Analysis." Pergamon, Oxford, 1966.
89. Yoe, H., "Reactivos Orgánicos para el Análisis Inorgánico." Ediciones Núcleo, Barcelona, 1942.
90. Zarouskif, F. G., and Gavvilova, E. F., *Zavodskaya Lab.* **23,** 143 (1953); ref. *Z. Anal. Chem.* **158,** 64 (1957).

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Pyridine-2-Aldehyde *p*-Nitrophenylhydrazone as an Indicator for Colorimetric pH Measurements

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INTRODUCTION

For pH change detection in the high-alkaline range, a colorimetric method may advantageously be used. With this purpose, a few indicators have been proposed.

For the pH range 10.08–12.22, alizarine yellow GG, nitramine, and tropaeoline O are suggested (*8*). However, the color of these indicators is rather pale and they have indefinite color changes. Chugreeva (*1*) suggested indicators like *p*-nitrophenyl and 2,4-dinitrophenyl osazones of dihydroxytartaric acid, 2,4-dinitrophenylhydrazone of pyruvic acid, and 2,4-dinitrophenylhydrazone of acetone for the pH range 11.5–13.5. Konopik and Leberl (*4, 5*) found that certain azo dyes and oxazin derivatives are well suited for pH determination in the strong alkaline medium.

The present paper deals with the spectrophotometric study and application possibilities of colorimetric pH measurements of the new reagent pyridine-2-aldehyde *p*-nitrophenylhydrazone (PANPH). In previous work, benzaldehyde *p*-nitrophenylhydrazone (*6*) was reported for this aim. PANPH combines a sharp color change, due to the electrophilic substituent of the phenyl group, with a greater stability.

MATERIALS AND METHODS

Reagents

Pyridine-2-aldehyde *p*-nitrophenylhydrazone was synthesized by refluxing equimolar quantities of *p*-nitrophenylhydrazine with pyridine-2-aldehyde in ethanolic solutions. The crude product, yellow crystals, was separated and recrystallized from ethanol (mp, 257–260°C). Found: 59.6% C, 4.3% H, 23.4% N. Calculated for $C_{12}N_4H_{10}O_2$: 59.5% C, 4.1% H, 23.1% N. A 0.05% (w/v) ethanolic solution of PANPH was used.

Buffer solutions of constant ionic strength were prepared by using appropriate mixtures of sodium borate, hydrochloric acid, sodium hydroxide, and potassium chloride (*7*).

All solvents and reagents were of analytical grade.

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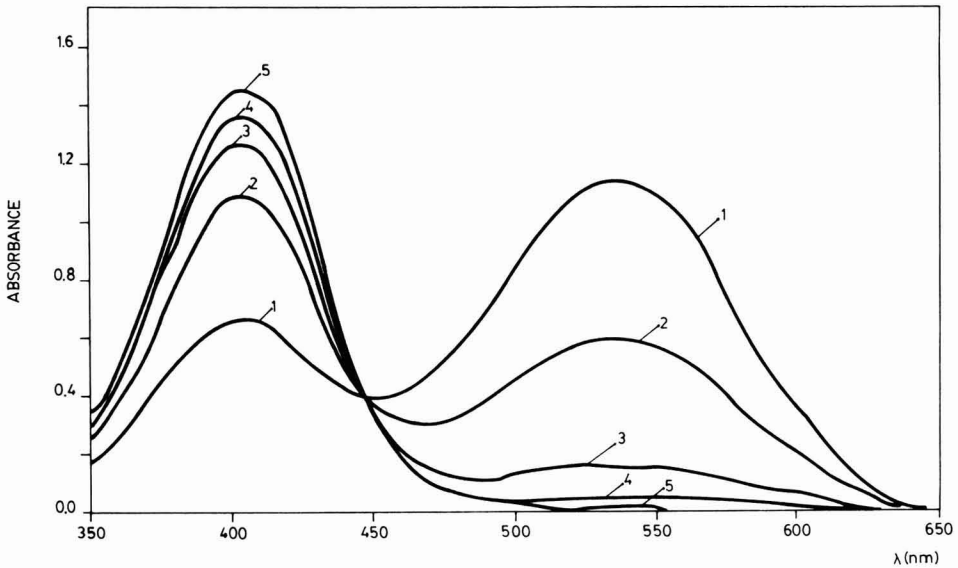


FIG. 1. Visible absorbance spectra of PANPH in aqueous–ethanolic (3–2) solutions at various pH values ($4 \cdot 10^{-5}$ *M*): (1) pH 12.85; (2) 12.30; (3) 12.00; (4) 10.80; (5) 10.20; (6) 9.10.

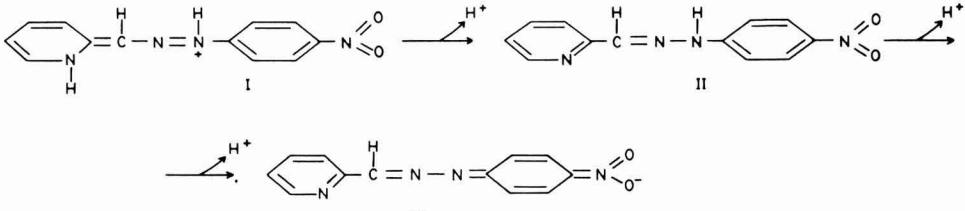
Apparatus

Pye–Unicam SP 8000 and Perkin–Elmer Coleman 55 spectrophotometers with 1.0-cm glass cells, a Metrohm E1009 photometric titrator with a 4.0-cm glass cell, and a Phillips PW9408 digital pH meter, with a combined glass–calomel electrode, were used.

RESULTS

Absorption Spectra and Indicator Constant

Absorption spectra of PANPH at different pH values are shown in Fig. 1. These spectra clearly demonstrate the change in absorbance and, therefore, intensity of color with change in pH. The isosbestic point was found at 450 nm. As the pH is raised, deprotonation of pyridine nitrogen occurs first, then hydracinic hydrogen is deprotonated and structural readjustment takes place (see formulas I–III). This is associated with a decrease



SCHEME 1

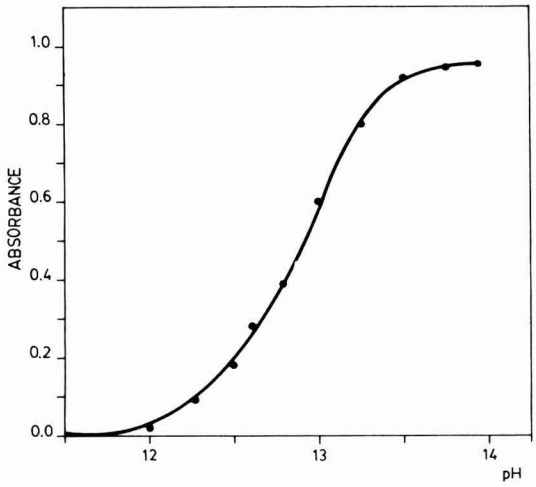


FIG. 2. Absorbance plotted against pH for PANPH solutions at 545 nm.

in the height of the absorption peak at ca. 405 nm and an increase in the height of the peak at ca. 545 nm. A plot of absorbance at this absorbance peak against pH gives a measure of the change in red–violet color intensity with change in pH (Fig. 2). From the resulting curve, the pH value corresponding to one-half color development, i.e., the apparent indicator constant, may be obtained ($pK_i^{ap} = 12.9$).

From data resulting from Fig. 2, the inflexion point that pH = pK_i was calculated graphically (*2*) (Fig. 3). The obtained value, $pK_i = 12.8 \pm 0.1$ [20°C, ethanol–water (2–3), ionic strength 0.01], was assigned to removal of the hydrogen from nitrogen by base; production of red–violet color does not appear to involve reaction of the nitro group itself with base.

Color Change Interval

The determination of the transition interval was achieved by processing a buffer series, according to the transition interval to be expected, in 10 test tubes of equal color and diameter. Into every test tube 10 ml of buffer and 0.1 ml of indicator solutions were pipetted. It was found that the transition interval (yellow to red–violet) lies between 12.1 and 13.1 pH values.

Reversibility and Stability of Indicator Solutions

The reversibility of the proposed indicator was tested by successive drop additions of 0.1 *M* hydrochloric acid and sodium hydroxide solutions, resulting in the solutions changing from red–violet to yellow and back to red–violet again.

Indicator solutions were stored for some weeks and exposed to diffuse daylight. Absorption spectra recorded periodically showed the solutions

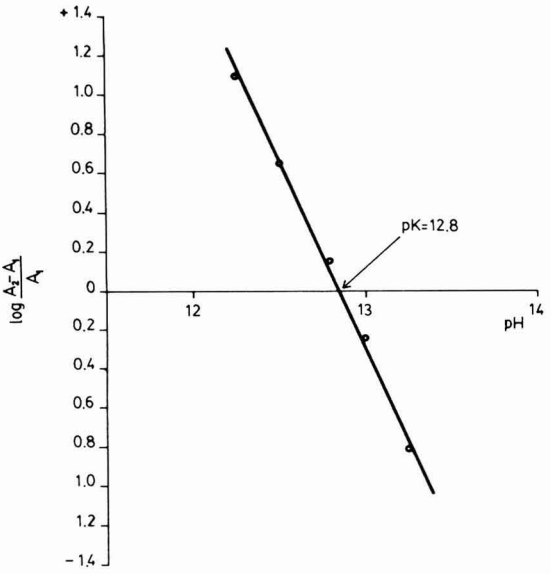


FIG. 3. pH−log $(A_2 - A_1) / A_1$ plot for determining pK_i.

to be stable. Red−violet dilute solutions of PANPH, at pH 13, remain stable for an hour.

DISCUSSION

In the acid and neutral range of pH, suitable colorimetric indicators are available. But in the alkaline range, especially in the high-alkaline range, they are not excellent indicators because of a wide interval of color change, slight stability, and lack of color brightness. For that reason, it was attempted to research some new organic groups capable of operating as alkaline range indicators for colorimetric pH measurements. Nitrophenylhydrazones, because of their special structure, proved adaptable for this purpose. However, some of these compounds show a certain objection. Thus, benzaldehyde nitrophenylhydrazone displays only slight stability (less than 30 min) and the red color of alkaline solutions gradually fades with time.

Alterations in electronic density around the hydracinic hydrogen, from the different radicals insertion, occur in color change indicator modification and stability disturbance (*3*). The pyridine ring brings in remarkable improvements of the indicator properties of PANPH, such as a greater stability of red−violet color, a sharp change of transition color, and a remarkable shift of maximum wavelength from 405 to 545 nm.

SUMMARY

Pyridine-2-aldehyde *p*-nitrophenylhydrazone is suggested as an indicator for colorimetric pH measurements in the high-alkaline range. Absorption spectra and indicator constant have been determined. Color change interval and other indicator characteristics have been established. A visually distinguishable transition and a good stability of color are obtained.

REFERENCES

1. Chugreeva, N. V., Some nitrophenylosazones and nitrophenylhydrazones as indicators at high pH values. *Zh. Anal. Khim.* **15**, 391–393 (1960).
2. Hildebrand, G. P., and Reilley, C. N., Indicator for complexometric titration of calcium in presence of magnesium. *Anal. Chem.* **29**, 258–264 (1957).
3. Kambe, M., Hasegawa, Y., and Shindo, E., *p*-Nitrophenylhydrazone as a basic indicator. *Japan Anal.* **13**, 1218–1223 (1964).
4. Konopik, N., and Leberl, O., Colorimetric determination of pH in the range 10 to 15. *Monatsh* **79**, 586–595 (1948).
5. Konopik, N., and Leberl, O., Colormetric determination of pH in the range 10 to 15. II. Indicator constants and salt errors. *Monatsh* **80**, 420–429 (1949).
6. O'Connor, R., Rosenbrook, W., Jr., and Anderson, G., A new indicator for pH 11 to 12. *Anal. Chem.* **33**, 1282 (1961).
7. Perrin, D. D., and Dempsey, B., "Buffers for pH and Metal Ion Control." Chapman and Hall Laboratory Manuals, London, 1974.
8. Simon, R. H. M., High alkaline colorimetric pH determination. *Anal. Chem.* **24**, 1215–1216 (1952).

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A Procedure for the Direct Determination of Micromolar Quantities of Lecithin Employing Enzymes as Reagents[1]

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INTRODUCTION

L-α-Phosphatidylcholine, dipalmitoyl (lecithin), concentrations in amniotic fluid are widely accepted to be indicative of fetal lung maturity (*8, 11, 14*) and are consequently of importance in the diagnosis of respiratory distress syndrome.

Until recently, the methods commonly employed to assess lecithin concentrations have involved either thin-layer (*4, 6, 8, 11, 24*) or gas–liquid (*8, 14, 24, 27*) chromatography. These procedures would appear to be either tedious, time-consuming and consequently expensive, or semiquantitative. Recently, a procedure appeared (*1*) which utilizes enzymes as reagents; however, it too appears to be quite lengthy. All of the above-mentioned procedures involve at least one extraction with organic solvents.

It was our intention to develop a relatively quick, simple, inexpensive, nonextraction technique, which employs enzymes as reagents, for the determination of micromolar concentrations of lecithin. Although clinical studies have, as yet, not been performed it is hoped that this procedure will prove to be applicable to biological specimens and in particular to amniotic fluid.

This assay (Fig. 1) involves the enzymatic hydrolysis of lecithin by phospholipase D [phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4] to generate phosphatidic acid and choline. The choline generated is subsequently oxidized, with formation of hydrogen peroxide, to betaine by choline oxidase (*Arthrobacter globiformis*, EC number not assigned). A red dye is produced by the peroxidase [hydrogen-peroxide oxidoreduc-

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$$\text{Lecithin} \xrightarrow{\text{PL-D}} \text{Phosphatidic acid} + \text{Choline}$$

$$\text{Choline} + O_2 \xrightarrow{\text{COD}} \text{Betaine aldehyde} + H_2O_2$$

$$\text{Betaine aldehyde} + O_2 + H_2O \xrightarrow{\text{COD}} \text{Betaine} + H_2O_2$$

$$H_2O_2 + \text{DCBS} + \text{4-AAP} \xrightarrow{\text{POD}} \text{Red dye}$$

FIG. 1. Shown is the assay reaction sequence. Phospholipase D (PL-D) hydrolyzes lecithin to generate choline. Choline oxidase (COD) oxidizes choline to the corresponding acid to generate two equivalents of hydrogen peroxide. The red dye is produced by the peroxidase-catalyzed coupling of DCBS and 4-AAP in the presence of H_2O_2.

tase, EC 1.11.1.7]-catalyzed coupling of sodium 2-hydroxy-3, 5-dichlorobenzenesulfonate (DCBS) to 4-aminoantipyrine (4-AAP) with hydrogen peroxide.

MATERIALS AND METHODS

Reagents

Stock hydrogen peroxide. Add 10 μl of 30% hydrogen peroxide to 1 liter of H_2O. Note that the volume of H_2O_2 used will vary with the shelf age of 30% solution.

Stock calcium chloride solution. Prepare this solution to be 550 m*M* in water. This corresponds to a final hydrolysis reaction mixture concentration of 55 m*M*.

Tris–HCl buffer for the comparison of phenol and DCBS. To 5 ml of Tris–HCl buffer, 400 m*M*, pH 8.0, add 2.55 mg phenol, 33 U peroxidase (POD), and 14 U choline oxidase (COD).

Phospholipase D solution. Prepare this solution to contain 1.1 U phospholipase D (PL-D) per 50 μl of β,β-dimethylglutarate (DMG) buffer, 10 m*M*, pH 7.0, with 0.1% bovine serum albumin. This reagent is stable for at least 8 hr at room temperature.

Pooled amniotic fluid. Clear frozen amniotic fluid specimens were brought to room temperature, mixed, pooled, and centrifuged for 10 min in a bench-top centrifuge. The central clear portion was collected by Pasteur pipet and used as required.

Working acetate buffer. Prepare this solution to contain 0.3% (w/v) Triton X-100, 6 m*M* SDS, and 24 m*M* 4-AAP in 200 m*M* acetate buffer, pH 5.5. This reagent is stable for at least 2 weeks when stored in a brown bottle at room temperature. If stored at 4°C the SDS may come out of solution but will redissolve upon gentle mixing with warming.

Working Tris–HCl buffer. Prepare this solution to contain 0.2% (w/v) Triton X-100 and 18 m*M* DCBS in 400 m*M* Tris–HCl buffer, pH 8.0. This reagent was found to be stable for several weeks when stored in a brown bottle at room temperature. Prior to use, add 1.4 U COD and 3 U POD per assay (500 μl of reagent). This enzyme solution is stable for at least 8 hr at room temperature.

Lecithin standard. Prepare this solution to contain 90 μM L-α-lecithin (β-γ-dipalmitoyl) (Calbiochem, LaJolla, CA 92037 synthetic) in 0.5% (w/v) Triton X-100. Several hours of constant stirring was necessary to effect solution of this material. Serial dilutions were prepared in 0.5% (w/v) Triton X-100.

Chlorine standard. Prepare fresh solutions to contain 600 μM choline chloride in 0.5 % (w/v) Triton X-100. Suitable dilutions were prepared in the same solvent.

Distilled deionized water was used to prepare all solutions. All chemicals were analytical grade.

Choline oxidase (*Arthrobacter globiformis*) was purchased from Toyo Jozo Co., Ltd., Matsuda-Yaesu-Dori Bldg., 1-10-7 Hacchubori, Chuo-Ku, Tokyo, Japan.

Phospholipase D (cabbage) was purchased from Sigma Chemical Co., P.O. Box 14508, St. Louis, Missouri 63178, U.S.A.

Peroxidase (horseradish) was purchased from bmc Diagnostics/ Biochemicals Ltd., 1475 Begin, St. Laurent, Quebec H4R 1V8, Canada.

The enzyme activities quoted are those of the various suppliers. Unit definitions are as follows: COD, one unit is that amount of enzyme which oxidizes 1.0 μmol of choline to betaine aldehyde per minute at 37°C at pH 8.0; PL-D, one unit is that amount of enzyme which will liberate 1.0 μmol of choline from L-α-phosphatidyl choline per hour, at 25°C at pH 5.6; POD, unit definition is not totally clear but appears to be in terms of the amount of enzyme which catalyzes the oxidation of 1 μmol of guaiacol by H_2O_2 per minute at 25°C at pH 7.0.

Methods

2,4-Dichlorophenol was sulfonated in a manner similar to that of Barham and Trinder (*2*). A procedure similar to that of Beech (*3*) was used to isolate the sodium salt of the sulfonic acid. Five grams of 2,4-dichlorophenol was placed in a 50-ml roundbottom flask equipped with a reflux condenser and was warmed to melting with the flask submerged in a hot water bath. Sulfuric acid (8 ml) was added through the condenser, the water bath was brought to a boil, and the solution was stirred constantly for 36 hr. Upon cooling, approximately 8 ml of water was used to transfer the reaction mixture to a 250-ml beaker. This solution was slowly neutralized with the addition of Na_2CO_3. The product was filtered under

reduced pressure and recrystallized from water overnight. A second recrystallization gave a final yield of 53%. As an alternative to neutralization of the sulfuric acid reaction mixture, the sodium salt may be obtained quite quickly upon addition of 10 g of NaCl. This does, however, lower the yield. Nuclear magnetic resonance spectra were obtained on a JEOLCO 60 HL spectrometer in deuterium oxide and are reported in parts per million from sodium trimethylsilylpropanesulfonate as internal standard. Microanalysis was performed by Galbraith Laboratories, Inc., Knoxville, Tennessee 37921.

The product upon drying appears as off-white flakes: mp > 200°C; NMR 7.57 (d, J = 2.5 Hz, 1H), 7.83 (d, J = 2.5 Hz, 1H). *Anal.* Calcd. for $C_6H_3Cl_2SO_4Na$: C, 27.19; H, 1.13; Cl, 26.75; S, 12.09. Found: C, 27.26; H, 1.19; Cl, 26.67; S, 11.75.

The optimum ratio of 4-AAP to DCBS was studied by calculating the ratios of similar reagents used in previous procedures (*2, 12, 13, 23*) and then applying these ratios to our system. Serial dilutions of these ratios were then added to the Tris–HCl buffer. Hydrogen peroxide was used as a substrate when PL-D and COD were not included in the reagents.

In order to obtain a suitable activity of POD, the concentration of H_2O_2 was adjusted until an absorbance of 0.8 was obtained upon completion of the color reaction. The color reaction was initiated upon addition of 1 μl of H_2O_2 to 1 ml of Tris–HCl buffer POD mixture.

Due to the lability of COD the activity of each purchase was checked according to the manufacturer's procedure (*25*). Fifty microliters of enzyme solution, 10 mM Tris–HCl–2 mM EDTA–1% KCl buffer (pH 8.0) containing approximately 0.1 U/ml COD, was mixed with 3 ml of 100 mM Tris–HCl buffer (pH 8.0) containing 0.01% (w/v) 4-AAP, 0.02% (w/v) phenol, 2.1% (w/v) choline chloride, and 300 U/ml POD. This mixture was quickly added to a cuvette, and the increase in absorption was followed for 7 min at 500 nm. The cell compartment was maintained at 37°C. The change in absorbance between 2 and 7 min (ΔA) is used to calculate the activity according to Eq. (1):

$$\text{Activity (U/mg)} = \frac{\Delta A \times 2.03}{\text{mg COD/ml enzyme solution}} \quad (1)$$

All spectral scans and time vs color development studies were performed in a Beckman Acta MVI. Single wavelength readings were performed with a Beckman Model 35, equipped with a return sipper. In those instances where color development was monitored the temperature of the cell compartment of the Acta MVI was maintained with a circulating water bath.

Unless otherwise stated the assay protocol is as follows: mix 50 μl of stock calcium chloride with 100 μl of working acetate buffer, add and mix 300 μl of sample. The hydrolysis reaction is initiated with the addition of 50 μl of PL-D solution. The hydrolysis reaction mixture is allowed to sit at room temperature for 15 min at which time 500 μl of working Tris–HCl buffer is added and mixed. This mixture is incubated for 12 min at 37°C. The absorbance is measured in 1-cm curvettes at 510 nm.

Blanking was performed as follows: the instrument was blanked with a reagent blank containing all of the reagents except PL-D in the DMG buffer. A PL-D blank was prepared by using 0.5% Triton X-100 as a sample. A sample blank was prepared in a manner similar to that of the reagent blank (DMG buffer without PL-D). The absorbance of the PL-D blank was subtracted from all tests and standards while the absorbance of the sample blanks was subtracted from the readings of their respective samples.

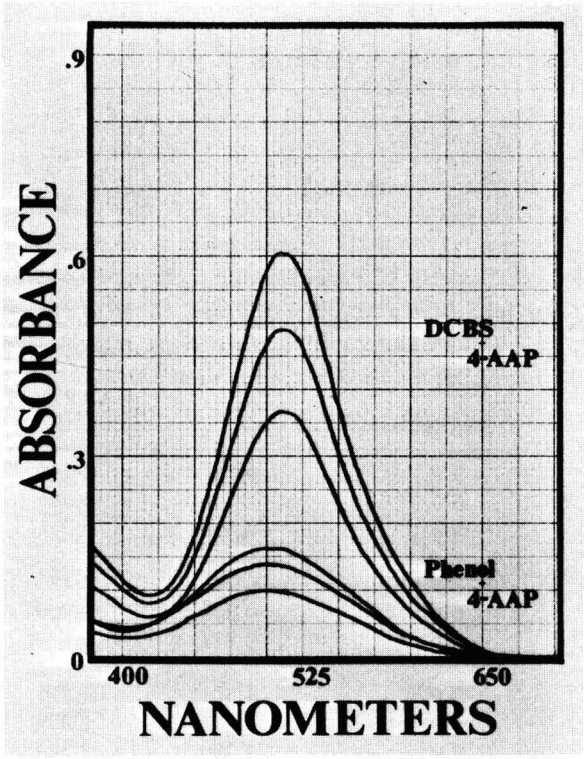

FIG. 2. The upper three spectra represent those generated with 50, 40, and 30 μM lecithin standards when DCBS and 4-AAP are used to produce the chromogen. The lower three curves are the same standards, however, phenol has been substituted for the DCBS.

RESULTS AND DISCUSSION

Previous procedures for the determination of PL-D activity (*13*) and for the determination of serum choline-containing phospholipids (*23*) employ phenol and 4-AAP to form a quinoneimine dye when coupled with H_2O_2. This chromogen, however, does not afford the sensitivity required in the present assay. Barham and Trinder (*2*) proposed the use of the sulfonic acid derivative of 2,4-dichlorophenol for use in peroxidase-coupled blood glucose determinations. However, they did not isolate this material but rather used it as a crude reaction mixture containing considerable amounts of unreacted sulfuric acid. We found this unreacted acid to have detrimental effects on the buffering capacity of the Tris–HCl buffer which we employ. Consequently we isolated and characterized the sulfonated dichlorophenol as its sodium salt.

Figure 2 shows spectral scans of 30, 40, and 50 μM lecithin standards with phenol and DCBS included in the reagents. Barham and Trinder (*2*) reported that use of the crude sulfonated dichlorophenol increased the sensitivity of their system fourfold over the corresponding phenol reaction. In our system purified DCBS increased the sensitivity by 3.7 times over the phenol reaction when measured at the absorbance maximum of 510 nm.

Table 1 contains the comparative data of various ratios of 4-AAP to DCBS. This evaluation was carried out according to the standard procedure except that H_2O_2 was used directly as a substrate in the absence of

TABLE 1
THE EFFECTS OF VARYING THE RATIOS OF 4-AAP TO DCBS[a]

μl H_2O_2/l H_2O[b]	DCBS (mM)	Absorbance (510 nm)		
		4-AAP (mM)		
		1.2	1.8	2.4
4	3.0	0.251	0.258	0.264
	6.0		0.264	0.269
	9.0	0.268	0.271	0.270
12	3.0	0.781	0.791	0.777
	6.0	0.800	0.800	0.803
	9.0	0.811	0.810	0.800
20	3.0	1.301	1.321	1.311
	6.0	1.347	1.359	1.350
	9.0	1.300	1.345	1.365

[a] Concentrations are expressed in terms of millimoles per liter of reaction mixture, 4-AAP = 4-aminoantipyrine; DCBS = 2-hydroxy-3,5-dicholorobenzenesulfonate.

[b] Stock H_2O_2 is approximately 30%.

TABLE 2
THE EFFECTS OF VARIOUS CONCENTRATIONS OF SODIUM DODECYL SULFATE

Sample	Absorbance at 510 nm					
	Sodium dodecyl sulfate (m*M*)[a]					
	0	1	3	5	7	9
29.8 μM Lecithin	0.197	0.282	0.451	0.476	0.468	0.454
Am. fl. bl.[b]	0.200	0.200	0.196	0.193	0.185	0.186
Am. fl.	0.448	0.468	0.540	0.545	0.535	0.527
Am. fl.-Am. fl. bl.	0.248	0.268	0.344	0.352	0.350	0.341

[a] Concentration is expressed in terms of millimoles per liter of working acetate buffer.
[b] Am. fl., amniotic fluid; Am. fl. bl., amniotic fluid blank.

lecithin, PL-D, and COD. Since it is difficult to standardize this reagent, suitable dilutions were employed to stimulate the range of absorbances which would be most likely encountered. It was decided from this data that a millimolar ratio of 2.4:9.0, 4-AAP:DCBS would be used. There is, however, very little difference among any of the ratios involving DCBS concentrations of 6.0 and 9.0 m*M* and 4-AAP concentrations of 1.8 and 2.4 m*M*.

It was found that if 0.056 U/assay POD was used with H_2O_2 as substrate, 12 μl 30% H_2O_2/liter H_2O, and at a 4-AAP to DCBS ratio of 1.2 to 3.0 (Table 1), full color development required about 2.5 min. In order to ensure that this step of the reaction sequence is not rate-limiting the activity of POD was increased to 3 U/assay. Calculations suggest that under similar conditions the reaction would be complete in less than 3 sec.

Sodium dodecyl sulfate (SDS) is a known activator of PL-D (*5, 7, 20, 22, 26*). A study of the optimum concentration of this surfactant, under otherwise standard conditions, is presented in Table 2 and Fig. 3. It is

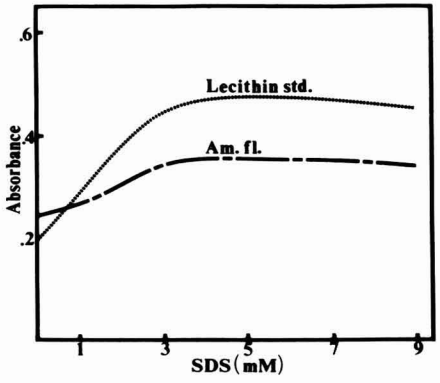

FIG. 3. Shown are the effects of various concentrations of sodium dodecyl sulfate (SDS) on the reactions with a 30μM lecithin standard and a pooled amniotic fluid (Am. fl.).

TABLE 3
THE EFFECTS OF CALCIUM CONCENTRATION ON THE HYDROLYSIS REACTION WITH A 30 μM LECITHIN STANDARD

Concentration of calcium (mM)[a]	Absorbance at 510 nm
0	0
20	0.290
40	0.338
60	0.344
80	0.322
100	0.310
120	0.314

[a] Concentration is expressed in terms of millimoles per liter of hydrolysis reaction mixture.

apparent that the optimum concentration of SDS, for the hydrolysis reaction, is between 5 and 7 mM in the acetate buffer for both the lecithin standard and amniotic fluid. Consequently 6 mM SDS was chosen.

Calcium is also a known activator of PL-D (*5, 7, 16, 22, 26*). Table 3 and Fig. 4 demonstrate the effects of calcium (as calcium chloride), under otherwise standard conditions, on a 30 μM lecithin standard. From this data it would appear that the optimal concentration of calcium ion in the hydrolysis reaction system is between 40 and 60 mM. However, the difference over the range of 40 to 120 mM is no more than ± 10%. As problems with precipitation, possibly due to SDS, arose at higher levels of calcium ion, a concentration of 55 mM in the lecithin hydrolysis mixture was chosen for the assay. Dawson and Hemington (*7*) have reported that the order of addition of reagents is important, as little hydrolysis occurs when PL-D is added to reagent containing SDS in the absence of Ca^{2+}. Presumably denaturation of the enzyme occurs under such circumstances.

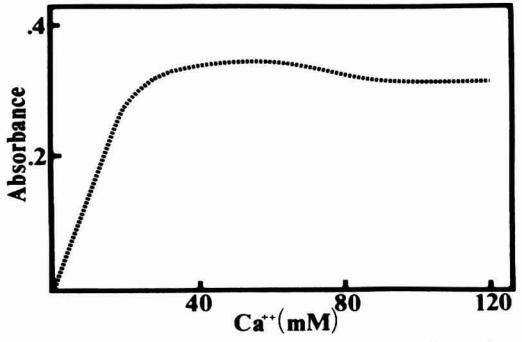

FIG. 4. Shown are the effects of various concentrations of calcium on the reactions with a 30 μM lecithin standard.

TABLE 4
THE EFFECTS OF ADDITION OF TRITON X-100 (W/V) TO THE ACETATE BUFFER

	Absorbance at 510 nm		
	Percentage of Triton X-100		
Sample	0.3	0.6	1.5
20 μM Lecithin	0.229	0.231	0.175
30 μM Lecithin	0.348	0.342	0.264
40 μM Lecithin	0.458	0.445	0.349
Amniotic fluid (pooled)	0.307	0.277	0.228

Triton X-100 has been reported to inhibit PL-D activity (22). However, it was found to be necessary in order to prevent turbidity upon addition of calcium to the acetate buffer containing SDS as well as upon addition of amniotic fluid to the buffer–calcium mixture. Table 4 and Fig. 5 illustrate the effects of various amounts of this surfactant when added to the acetate buffer under otherwise standard conditions. At none of the levels studied was turbidity visible upon addition of either calcium or amniotic fluid. Considering the apparent inhibition of the reaction by the Triton X-100, the lowest concentration studied, 0.3% (w/v) was selected for routine use. Turbidity also occurred upon addition of the Tris–HCl buffer reagent in the final step of the standard protocol. Since Triton X-100 prevented this problem in the hydrolysis reaction, and it had been used previously (23) it was added to this buffer as well. It was found that 0.2% (w/v) Triton X-100 in the Tris–HCl reagent was sufficient to prevent turbidity upon addition to the hydrolysis mixture.

In their assay for the determination of PL-D (cabbage) activity, Imamura and Hariuti (*13*) incorporated ethylenediaminetetraacetic acid (EDTA) in the Tris–HCl buffer to stop the PL-D reaction. It was found,

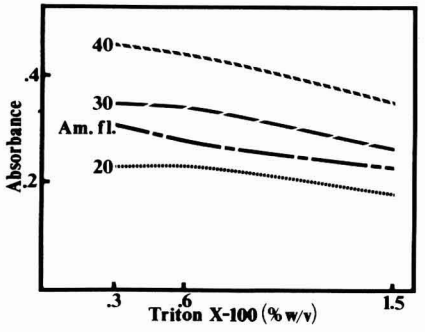

FIG. 5. Shown are the effects of various concentrations of Triton X-100 added to the working acetate buffer. Lecithin standards: number next to curves refer to micromolar concentrations of lecithin; pooled amniotic fluid: curve labeled Am. fl.

TABLE 5
THE BLANK REACTION WITH VARIOUS ACTIVITIES OF PL-D[a]

Units per assay	Absorbance at 510 nm		
	PL-D with buffers[b]	Reagent blank[c]	PL-D blank
0.9	0.018	0.067	0.147
1.6	0.027	0.067	0.179
2.4	0.048	0.067	0.208

[a] The specific activity of the enzyme was 34 U/mg of protein (16 U/mg of solid material).

[b] These buffers contain no 4-AAP or DCBS.

[c] This is the average of four readings against a buffer blank.

however, in our system that a concentration of EDTA sufficient to complex all calcium present caused precipitation to occur. In addition, it is probable that under our conditions the hydrolysis reaction has reached completion before addition of the Tris–HCl reagent. For these reasons, EDTA was not incorporated into the present assay system.

It is convenient to make the incubation for the lecithin hydrolysis as brief as possible. However, problems arise in that the PL-D contains a contaminant, supposedly lecithin and/or choline, which gives a considerable blank reaction (Table 5). Table 5 also suggests that there is a background absorbance due to the PL-D even in the absence of the color reaction materials. This background absorbance may be due to slight turbidity caused by impurities in the enzyme preparation.

The data in Table 6, a study of the effects of time and PL-D activity on lecithin hydrolysis, suggest that if the hydrolysis step is allowed 15 min then an activity of 1 U PL-D/assay is sufficient, for lecithin concentrations of 30 μM. Thus the blank reaction is kept relatively small. It may be seen from Fig. 6, which presents the data of Table 6 graphically, that of those PL-D activities studied, the difference in time for complete hydrolysis of the standard varies by only 5 min.

TABLE 6
THE HYDROLYSIS REACTION RATES OF VARIOUS PL-D ACTIVITIES

Units of PL-D per assay	Absorbance at 510 nm					
	2 min	5 min	10 min	15 min	20 min	30 min
0.9	0.140	0.236	0.305	0.331		
1.6		0.262	0.311	0.324	0.339	
2.4		0.304[a]	0.320	0.329	0.326	0.334

[a] Sample taken at 6 min.

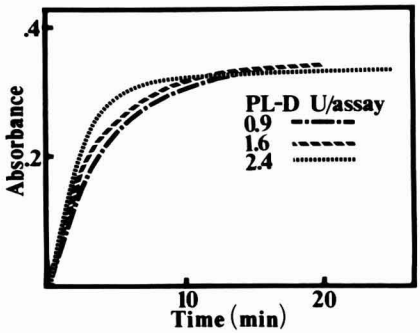

FIG. 6. Shown are the effects of various PL-D activities on the rate of lecithin hydrolysis.

Table 7 and Fig. 7 present a study of hydrolysis rate over a range of 10–60 μM lecithin when 1.0 U PL-D/assay is employed. It would appear that over this range 15 min is sufficient to effect constant color production.

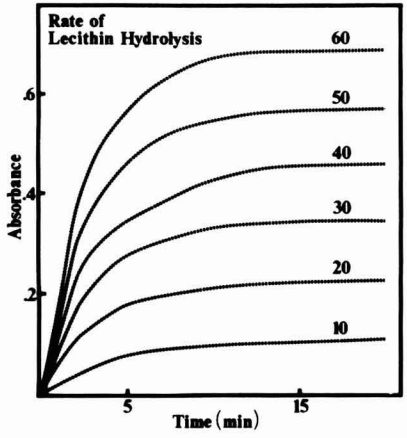

FIG. 7. Shown are the rates of hydrolysis of six lecithin standards when 1.0 U/assay PL-D is used.

TABLE 7
RATE OF LECITHIN HYDROLYSIS OVER A RANGE OF CONCENTRATIONS

	Absorbance at 510 nm				
Lecithin (μM)	2 min	5 min	11 min	15 min	20 min
10	0.039	0.078	0.098	0.100	0.110
20	0.110	0.181	0.214	0.222	0.227
30	0.171	0.276	0.333	0.344	0.344
40	0.237	0.347	0.443	0.447	0.461
50	0.312	0.461	0.550	0.567	0.579
60	0.376	0.569	0.675	0.679	0.693

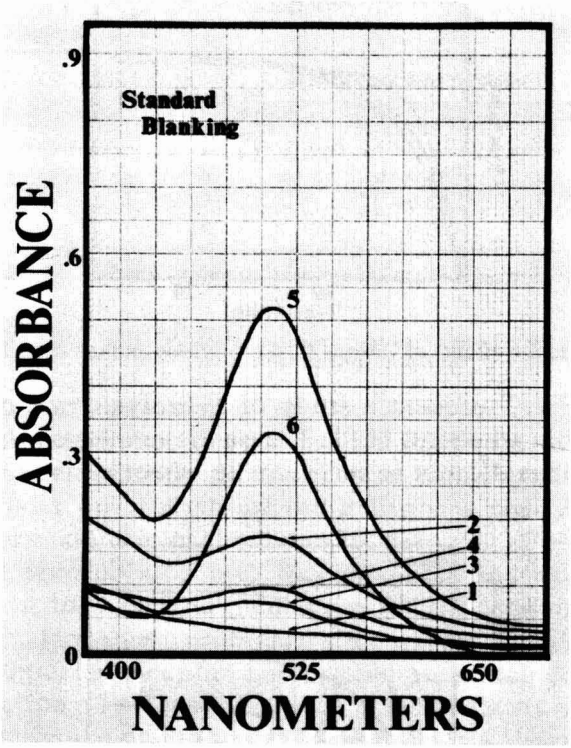

FIG. 8. Shown are the spectra of: the background absorbance of PL-D (1), the PL-D blank reaction (2), the reagent blank in the absence of PL-D (3), the PL-D blank scanned against the reagent blank (4), a 30 μM lecithin standard scanned against a reagent blank (5), and the same standard scanned against the PL-D blank (6).

Figure 8 consists of a series of spectra which are intended to show the necessity of proper blanking standards. The first, spectrum 1, demonstrates the background absorbance of PL-D in the reagent system in the absence of COD, POD, DCBS, and 4-AAP. Spectrum 2 is characteristic of the PL-D blank reaction in which some color was produced upon addition of PL-D to the reagent system in the absence of the substrate. It may be seen that spectra 1 plus 3, the reagent blank, does not generate spectrum 2. This difference is indicative of the color produced by impurities in the PL-D preparation. Spectrum 4 was produced by scanning the PL-D blank against the reagent blank and thus represents the spectrum due to the PL-D background absorbance and impurities. Spectrum 5 was generated by scanning a 30 μM standard against a reagent blank. Spectrum 6 is the same standard but scanned against the PL-D blank. A considerable difference may be noted in this instance.

Figure 9 illustrates the necessary blanking procedure for amniotic fluid samples. Spectrum 1 is indicative of a pooled amniotic fluid blank reaction

scanned against the buffer. Spectrum 2 is the same pooled fluid carried through the proposed procedure and scanned against a reagent blank. Spectrum 3 was generated by scanning this fluid against the PL-D blank. Spectrum 4 is a 30 μM standard scanned against the PL-D blank. Spectrum 5 is the pooled amniotic sample scanned against its blank. It is noteworthy that all of these spectra have similar shapes and that therefore if an interference was present in this particular pooled fluid then it did not alter the spectra resulting from the generated red dye.

The most appropriate blanking system was chosen from the results of Figs. 8 and 9. It was decided that the instrument should be blanked with a reagent blank, and thus the inherent color of the reagent is subtracted from all subsequent readings. A PL-D blank, prepared with 0.5%. Triton X-100 as sample, is measured and its value is subtracted from all standards and samples to which PL-D has been added. In addition a sample blank, to which no PL-D has been added, is subtracted from its corresponding test sample.

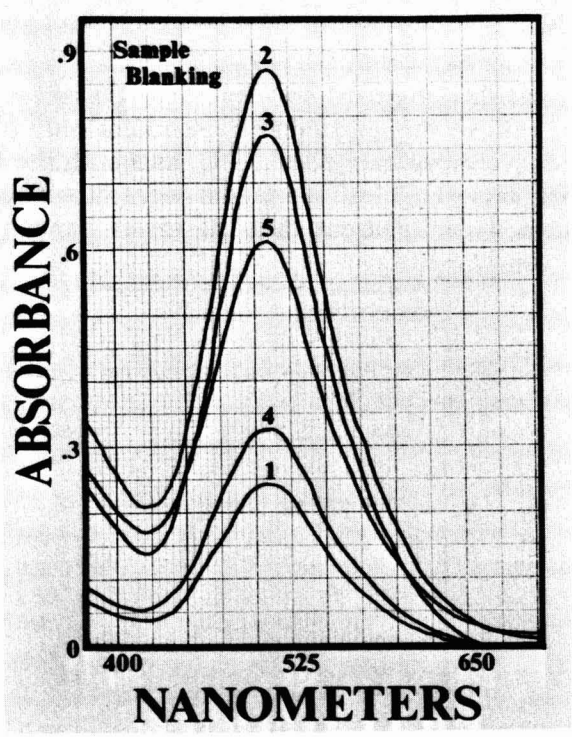

FIG. 9. Shown are the spectra of: the blank reaction of pooled amniotic fluid (1), the same amniotic fluid carried through the assay (2), the amniotic fluid scanned against a PL-D blank (3), a 30 μM lecithin standard scanned against a PL-D blank (4), and the amniotic fluid scanned against the amniotic fluid blank (5).

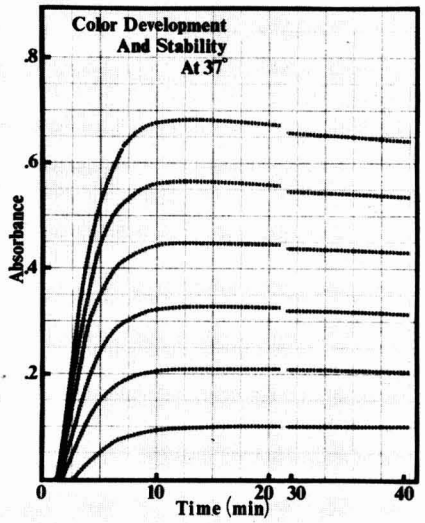

FIG. 10. Shown is the color development and stability, at 37°C, of a range (10–60 μM) of lecithin standards.

The rate of color development and stability of a series of standards is presented in Fig. 10. In this particular experiment only the cuvettes and cell compartment were prewarmed to 37°C, and both the hydrolysis mixture and working Tris–HCl buffer reagent were combined and mixed at room temperature. It is apparent that the lower concentrations have a

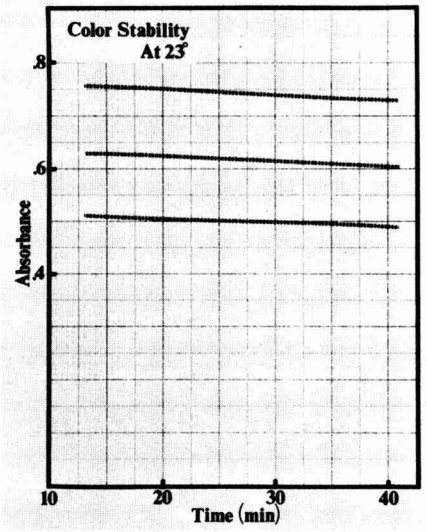

FIG. 11. Shown is the color stability of a 40, 50, and 60 μM lecithin standard at room temperature.

longer lag phase than do the 40–60 μM standards. It is also apparent that, with the exception of the 10 μM standard which took 15 min, all other standards had reached full color development between 10 and 12 min following addition to the 37°C cuvette. It should be pointed out that the 40, 50, and 60 μM standards decreased by 2.8, 3.9, and 4.6%, respectively, at 25 min from full color development. It was decided to determine if this decrease was because the reaction was held at 37°C, rather than being cooled to room temperature, before measurement.

Figure 11 illustrates the decrease in absorbance, at 510 nm, of the 40, 50, and 60 μM standards, at room temperature, following a 12-min color development at 37°C. After 25 min the decrease in absorbance was 3.2, 3.5, and 3.9%, respectively, for the 40, 50, and 60 μM standards. Following color development it is not apparent that there is any difference in color stability whether the mixture is held at 37°C or cooled to room temperature.

The within-run reproducibility of the assay was checked (Table 8) with a 30 μM standard as well as with fresh amniotic fluid. The coefficient of

TABLE 8
WITHIN-RUN REPRODUCIBILITY OF THE ASSAY

Absorbance at 510 nm	
30 μM Standard	Amniotic fluid
0.338	0.258
0.335	0.254
0.338	0.248
0.337	0.258
0.343	0.261
0.337	0.249
0.336	0.250
0.335	0.251
0.335	0.271
0.335	0.255
0.341	0.253
0.334	0.254
0.336	0.256
0.337	0.255
0.333	0.256
0.341	0.256
0.339	0.257
0.331	0.257
0.336	0.259
	0.260
Mean 0.337	0.256
SD ±0.003	±0.005

TABLE 9
STANDARD CURVE FOR END POINT DETERMINATIONS

Lecithin (μM)	Absorbance at 510 nm
10	0.107
20	0.219
30	0.344
40	0.456
50	0.571
60	0.703
r	0.9993
m	0.1169
b	−0.008

variation for the standard was ± 0.87% while that for the fluid was somewhat greater, ± 1.95%. This increased error probably is not due to the assay but rather the sample. The sample was centrifuged for no more than 5 min at about 3000 rpm in a desk-top centrifuge, as Lindback and Frantz (*13*) pointed out that prolonged centrifugation at high speed decreases the lecithin content of the supernatant. However, this type of treatment of the sample leaves particles suspended in the fluid. It is our belief that it is this particulate matter which causes the increased variability. The variability is not due to optical interference by the particles, as they dissolve upon addition to the reaction mixture. It is considered, however, that this problem is not unique to the assay, as the problem lies not with the assay but rather with the sample preparation.

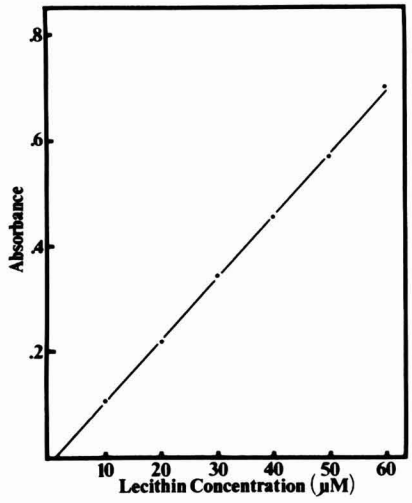

FIG. 12. Shown is a typical standard curve for end point determination at six concentrations of lecithin. Each standard was measured only once.

TABLE 10
STANDARD CURVES FOR RATE DETERMINATIONS

Lecithin (μM)	Change in absorbance per minute
10	0.055
20	0.127
30	0.175
40	0.245
50	0.301
60	0.372
r	0.9985
m	0.006
b	−0.003

A typical standard curve is presented in Fig. 12, the data for which is in Table 9. The best fit line was obtained through regression analysis.

The assay was designed and optimized for end point determinations; however, rate determinations were attempted (Table 10, Fig. 13). A minor problem arose in that the different substrate concentrations had different lag phases (Fig. 10). This might be overcome simply by increasing the COD activity per assay. As might be expected, since the assay is not designed for rate determination, the coefficient of variation is somewhat worse at 0.9985 than for the end point determination.

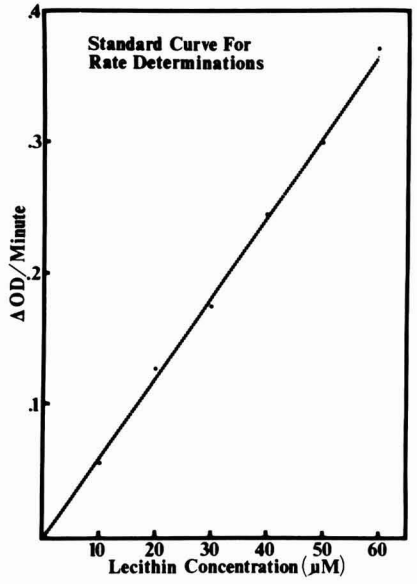

FIG. 13. Shown is a standard curve for rate determinations at six concentrations of lecithin. Each standard was measured only once.

TABLE 11
ABSORBANCES OF CHOLINE CHLORIDE STANDARDS

Choline chloride (μM)	Absorbance at 510 nm					Average
30	0.427	0.426	0.424	0.425	0.420	0.424
40	0.558	0.563	0.563	0.563	0.564	0.562

It was quite difficult to determine the completeness of PL-D hydrolysis and consequent overall efficiency of the enzymatic assay. Attempts to perform thin-layer chromatography of the hydrolysis mixture proved futile. It was believed that the relatively high concentration of Triton X-100 was the cause of the problem. Standard addition was considered, but discounted on the grounds that if it were matrix effects (i.e., the presence of Triton X-100) which we were looking for then it would be quite difficult to add standard to a sample without affecting the reaction matrix. It was decided to compare the absorbances produced by lecithin standards with those produced by choline chloride. Table 11 lists the data obtained from 30 and 40 μM choline chloride standards (in 0.5% Triton X-100). When compared to the lecithin standard curve (Fig. 12, Table 9), prepared at the same time it was seen that these standards produce 20–21% more color than the corresponding lecithin standards. This would seem to suggest that only about 80% of the lecithin is hydrolyzed by PL-D. This is an interesting point; however, considering Figs. 6 and 7, it would be of little consequence as the extent of the hydrolysis would appear to be constant.

Interferences other than sphingomyelin were not studied. A 53 μM sphingomyelin standard, prepared in 0.5% Triton X-100, showed no reaction under the described reaction conditions. Long *et al.* (*20*) have reported that PL-D will hydrolyze lysolecithin. This has not been studied under the proposed reaction conditions as it probably has little bearing on the clinical significance of the test. The choline oxidase used is reported to be very specific toward choline and betaine aldehyde (*12*). If these materials are present endogenously in the sample they will be corrected for by the sample blank. This assay is, no doubt, subject to many of the same interferences as other peroxidase-coupled assays (*9, 10, 17, 18, 19, 21*).

The buffers used are basically the same as those used by Imamura and Horiuti (*13*), for their determination of PL-D (cabbage) activity, with the one exception being that we chose not to use their 10 mM and 1 M Tris–HCl buffers. In order to reduce the number of pipettings, we used one 400 mM Tris–HCl buffer which is approximately equal to the final concentration of the two buffers.

SUMMARY

A procedure has been proposed for the determination of micromolar concentrations of lecithin. This procedure, which utilizes enzymes as reagents, is relatively quick, simple, and inexpensive, and involves no extractions. Clinical studies of the applicability of this procedure for the determination of lecithin concentrations in amniotic fluid have, as yet, not been concluded.

The synthesis of sodium 2-hydroxy-3, 5-dichlorobenzenesulfonate was described. This material in purified form has proven to be very convenient for routine use in a peroxidase-catalyzed coupling to 4-aminoantipyrine.

REFERENCES

1. Anoahar, S. G., Garry, P. J., and Stadefer, J. C., An enzymatic method for determination of amniotic fluid lecithin. *Clin. Chem.* **24**, 1044 (1978).
2. Barham, D., and Trinder, P., An improved colour reagent for the determination of blood glucose by the oxidase system. *Analyst* **97**, 142–145 (1972).
3. Beech, W. F., Preparation of certain nuclear-substituted 2-aminophenol-sulphonic acids. *J. Chem. Soc.*, 212–216 (1948).
4. Blass, K. G., Thibert, R. J., and Draisey, T. F., Miniature thin-layer chromatographic determination of the lecithin/sphingomyelin ratio in amniotic fluid. J. *Chromatogr.* **89**, 197–200 (1974).
5. Chen, J., and Barton, P. G., Studies of dialkyl ether phospholipids. II Requirements for the liquid–crystalline substrate for hydrolysis by cabbage leaf phospholipase D. *Canad. J. Biochem.* **49**, 1362–1375 (1971).
6. Coch, E., Meyer, J. S., Goldman, G., and Kessler, G., A modified procedure for evaluation of the lecithin/sphingomyelin ratio in amniotic fluid. *Clin Chem.* **19**, 967–972 (1973).
7. Dawson, R. M. C., and Hemington, N., Some properties of purified phospholipase D and especially the effect of amphipathic substances. *Biochem. J.* **102**, 76–86 (1967).
8. Getchell, G., Kingsley, G. R., and Schaffert, R. R., Direct automated determination of glucose by a glucose oxidase–peroxidase system. *Clin. Chem.* **10**, 540–548 (1964).
9. Gluck, L., Kulovich, M. V., Borer, R. C., Brenner, P. H., Anderson, G. G., and Spellacy, W. N., Diagnosis of respiratory distress syndrome by amniocentesis. *Amer. J. Obstet. Gynecol.* **109**, 440–445 (1971).
10. Gochman, N., Ryan, W. T., Sterling, R. E., and Widdouson, G. M., Interlaboratory comparison of enzymatic methods for serum glucose determination. *Clin. Chem.* **21**, 356–361 (1975).
11. Harding, P., Possmayer, F., Milne, K., Jaco, N. T., and Walters, J. H., Amniotic fluid phospholipids and fetal maturity. *Amer. J. Obstet. Gynecol.* **115**, 298–306 (1973).
12. Ihuta, S., Imamura, S., Misahi, H., Purification and characterization of choline oxidase from *Arthrobacter globiformis*. *J. Biochem. (Tokyo)* **82**, 1741–1749 (1977).
13. Imamura, S., and Horiuti, Y., Enzymatic determination of phospholipase D activity with choline oxidase. *J. Biochem. (Tokyo)* **83**, 677–680 (1978).
14. Ip, M. P. C., Draisey, T. F., Thibert, R. J., Gagneja, G. L., and Jasey, G. M., Fetal lung maturity, as assessed by gas–liquid chromatographic determination of phospholipid palmitic acid in amniotic fluid. *Clin. Chem.* **23**, 35–40 (1977).
15. Lindbach, T., and Frantz, T., Effect of centrifugation on amniotic fluid phospholipid recovery. *Acta Obstet. Gynecol. Scand.* **54**, 101–103 (1975).
16. Long, C., Odavic, R., and Sargent, E. J., The action of cabbage-leaf phospholipase D upon lysolecithin. *Biochem. J.* **102**, 216–220 (1967).

17. Nelson, D. R., and Huggins, A. K., Interference of 5-hydroxytryptamine in the assay of glucose by glucose oxidase:peroxidase:chromogen based methods. *Anal. Biochem.* **59**, 46–53 (1974).
18. Perlstein, M. T., Thibert, R. J., Watkins, R., and Zak, B., Spectrophotometric study of bilirubin and hemoglobin interactions in several hydrogen peroxide generating procedures. *Microchem. J.* **23**, 13–27 (1978).
19. Perlstein, M. T., Thibert, R. J., and Zak, B., Bilirubin and hemoglobin interferences in direct colorimetric cholesterol reactions using enzyme reagents. *Microchem. J.* **22**, 403–419 (1977).
20. Quarles, R. H., and Dawson, R. M. C., A shift in optimum pH of phospholipase D produced by activating long-chain anions. *Biochem. J.* **112**, 795–799 (1969).
21. Richmond, W., Cholesterol oxidase in assay of total and free cholesterol in serum. *Clin. Chem.* **22**, 1579–1588 (1976).
22. Roughan, R. G., and Stack, C. R., Is phospholipase D really an enzyme? A comparison of in situ and in vitro activities. *Biochim. Biophys. Acta* **431**, 86–95 (1976).
23. Takayama, M., Itoh, S., Nagasaki, T., and Tanimizu, I., A new enzymatic method for the determination of serum choline-containing phospholipids. *Clin. Chim. Acta* **79**, 93–98 (1977).
24. Thom, H., Dinwiddle, R., Fisher, P. M., Russell, G., and Sutherland, H. W. Palmitic acid concentrations and lecithin/sphingomyelin ratios in amniotic fluid. *Clin. Chim. Acta* **62**, 143–147 (1975).
25. Toyo Jozo Co. Ltd., Product information sheet, Enzyme T-05, Choline oxidase from *Arthrobacter globiformis*.
26. Tzur, R., and Shapiro, B., Purification of phospholipase D from peanuts. *Biochim. Biophys. Acta* **280**, 290–296 (1972).
27. Warren, C., Allen, J. T., Halton, J. B., Assessment of foetal lung maturity by amniotic fluid fatty acid analysis. *Clin. Chim. Acta* **44**, 457–459 (1973).

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BOOK REVIEWS

Introduction to Semimicro Qualitative Analysis. 5th ed. By C. H. SORUM AND J. J. LAGOWSKI. Prentice–Hall, Englewood Cliffs, N.J., 1977. xi + 310 pp., $8.95.

The "Sorum" is a time-proved qualitative text, and that another edition has become necessary is a pleasant sign that pure and basic chemistry is still (or should it be *Again*) appreciated as a more appropriate beginning of college study than orbital theory and the Schrödinger equation. What kind of scheme for analysis is best has been the point of discussion since qualitative analytical chemistry came into existence. Thus, there is no point in quibbling about this or that test employed in the experimental section, as long as it works reasonably well in practice, and, most of all, serves to teach some actual happenings and phenomena.

The theoretical part of the book, according to the Preface, has been completely rewritten. Here some golden opportunities have been missed. True, the level of pre-college education of students is on a downhill trend, but still it seems far-fetched to show (Tables 3, 4) that the writing of negative exponents means zeros after the decimal point and to have a nice pyramid of H^+ and OH^- concentrations with numbers containing up to fourteen such zeros. The pyramid is especially eye-catching in view of the fact that a few pages later complex compounds are explained by bond and crystal-field theory. This explanation, probably too short for creating a real appreciation in the beginner's mind, is disproportionately long in comparison to the mere twenty lines dealing with the nomenclature of the complexes.

More seriously, the authors have neglected to adopt the (1951!) IUPAC convention for signs in half-cell reactions. Since almost all books for subsequent courses follow this convention the student will be burdened with unncessary relearning. The nonexistent compound NH_4OH glares at the reader in bold print on pages 126 and 127. And good old ammonium hydroxide is not even necessary, because the acid–base concepts explained some pages earlier are on a post-Arrhenius basis.

Another unfortunate point is that no explanation, or apparently even a mention is found of important terms such as *reagent* and *test* and their difference (Feigl!) or *selective* and *specific* (confused even by people who should know better) and other basic concepts. But the book will serve the purpose, as it has in the past, and do so better than many of those freshman texts that have the work "Chemistry," for whatever obscure reason, on the title page.

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Trace Elements in Human Hair. VLADO VALKOVIĆ. Garland STPM Press, New York, 1977. 175 pp. $19.00

The analysis of trace elements in human hair provides a convenient means for assessing the biochemical and environmental history of the individual whose hair is sampled. For this reason, there is a growing interest in this type of analysis by investigators from a wide range of scientific disciplines. In this volume, Dr. Valković summarizes available information

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about human hair including growth, structure, composition, and a variety of methods for the analysis of trace elements in hair.

The monograph includes seven chapters, a list of references, an author index, and a subject index. Chapters 1–3 introduce the topic of the book and review information on hair growth and structure. Chapters 4–6 review studies of trace elements found in hair and relate these to a variety of biochemical and environmental courses. The last chapter discusses methods for analysis of trace elements in hair including sections on atomic absorption, neutron activation, X-ray emission spectroscopy, and mass spectrometry. Although some detailed procedures are given, no attempt is made to present detailed procedures for all methods presented.

This is a useful review of information on trace elements in human hair. It gives a good description of the advantages and disadvantages of human hair as an indicator of the biochemical or environmental history of the individual. Those interested in the use of human hair analysis will find a review of this book to be of value.

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Intermolecular Interactions and Biomolecular Organization. By A. J. HOPFINGER. Wiley–Interscience, New York, 1977. xvii + 395 pp. $26.00.

This volume is a very ambitious undertaking by Hopfinger, who is also the author of the earlier text *Conformational Properties of Macromolecules,* published in 1973.

Both experimental and theoretical approaches are discussed as applied to a wide range of problems. Topics discussed include the following:

Chapter 1. Background.

Chapter 2. "Conformation of Small Biologically Active Molecules in Solution and Crystals"—including acetylcholine, phenethylamine, amino acids, and cyclic peptides.

Chapter 3. "Intermolecular Interaction and Drug Action"—the Hansch method.

Chapter 4. "Interaction of Solvent Molecules with Polypeptides"—including IR, Raman, and NMR.

Chapter 5. "Interaction of Water with Proteins."

Chapter 6. "Drug Molecule–Protein Interactions"—with examples including glutamate dehydrogenase, CTP synthetase, aromatic hydrocarbon with Histone IV.

Chapter 7. "Hydration Properties of DNA."

Chapter 8. "DNA Intercalation Processes"—with actinomycin D, ethidium bromide, aminoacridines, and anticancer drugs.

Chapter 9. "Complexes of DNA with Synthetic Basic Polypeptides: Nucleoprotamine and Nucleohistone Models."

Chapter 10. "Glycosaminoglycan and Amylose Structural Organization"—the former including hyaluronic acid, chondroitin sulfates, dermatan sulfates, and heparin and keratin sulfates.

Chapter 11. "Interaction of Polypeptides and Collagen with Glucosaminoglycans and Proteoglycans."

Chapter 12. "Interaction of Ions with Biologic Macromolecules"—mainly with proteins and polypeptides.

Chapter 13. "Biomolecular Aggregation and Association"—of lipids, poly-L-lysine-chondroitin-6-sulfate, nucleoside bases, polypeptides, nucleohistones, and polyelectrolyte complexes.

Chapter 14. "Theories of Intermolecular Interactions."

Chapter 15. "Ultrastructural Organization"—of myosin, fibril structures of collagen aggregates, protein–protein interactions, and protein–nucleic–acid interactions.

Chapter 16. "Some Generalizations and Speculations."

While this book is very clearly written, most experimental descriptions are rather sketchy. Yet, the author supplies up-to-date referencing to all these important topics. It is in this sense that the text serves a good purpose. In addition, it allows the reader to sit back and survey these issues—to absorb what has been learned and most importantly to detect the many, many gaps in our knowledge of this most difficult of biochemical problems. Mainly as a survey of these topics, the text should be a useful library acquisition.

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A History of Analytical Chemistry. Edited by HERBERT A. LAITINEN AND GALEN W. EWING. The Division of Analytical Chemistry of the American Chemical Society, Washington, D.C., 1977. viii + 358 pp.

The analyst used to be "low man on the totem pole." As this book says, "Quite generally, professors in chemistry looked . . . down on analytical chemists." The same was true in industry—anybody could be an analyst, just use so much from this bottle, take that much from another, etc. Now all that has changed. At long last, analytical chemistry has come into its own as a discipline and a legitimate area for original research.

The slow progress from the servant class to equality is told and told well in "A History of Analytical Chemistry." It is not possible to review this book in detail. There is too much in it, as is indicated by the chapter headings: Chemical Methods of Analysis, Analytical Spectroscopy, Electroanalytical Chemistry, Analytical Separations, and Instrumentation (with from 5 to 10 subdivisions in each chapter). The tremendous progress that has made our world what it is would not have come about without those pioneers and their followers who stayed in the analytical labs and worked out structures and methods for the determination of the flood of new and unfamiliar chemicals pouring out of the research labs.

The only criticism this reviewer has is that more emphasis could have been placed on microchemistry. The contributions of those who toiled harder and harder with less and less, especially in biochemistry, deserve more recognition. Society owes them much not only for comfortable living but also for life itself. The late Rev. Francis Power, S. J. of Fordham University, once remarked that microchemistry (which is more than just chemistry on a small scale) was an art as well as a science. Those who have designed new instruments and developed new methods of analysis have done much to shift the emphasis from the former to the latter.

The book is profusely illustrated with drawings and photographs of instruments and portraits of notables, many of whom, still living, will be known to readers. There is also a comprehensive subject and author index. More than 50 workers contributed to the writing of this book, yet it reads as smoothly as if it had only one author—a magnificent editorial job.

It belongs in all libraries, particularly those of practicing chemists. History helps understanding and therefore performance.

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Organic Functional Group Analysis by Gas Chromatography. By T. S. MA AND ATHANASIOS S. LADAS. Academic Press, New York, 1976. x + 173 pp.

This book covers the subjects of the general aspects, integral parts of the gas chromatograph, sampling, coupling of functional group reactions, determinations of oxygen functions, sulfur functions, unsaturation, active hydrogen, C-methyl, etc.

The book is well written and has numerous illustrations that should be very helpful to those working in the field of gas chromatography.

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Toxicology of Trace Elements. Edited by ROBERT A. GOYER AND MYRON A. MEHLMAN. Hemisphere, John Wiley & Sons, New York, 1977. xiv + 303 pp. $24.50.

Drs. Goyer and Mehlman have assembled an excellent book on the subject of toxicology of trace elements. Some 10 internationally recognized researchers and teachers in chemistry, biochemistry, pathology, and medicine have contributed to this volume to interpret and summarize recent literature regarding the biochemistry, pathology, and potential toxicology of trace elements known to have adverse health effects on humans.

The chapters were authored by experts in a particular metal and detail information on sources of contamination, metabolism, epidemiology studies, and toxicological effects on experimental animals and humans. The metals discussed under these categories include mercury, lead, arsenic, copper, nickel, vanadium, selenium, and tellurium.

Of particular interest to industrial toxicologists and environmental health scientists are the last two chapters of the book, entitled "Nutrient Interactions with Toxic Elements" and "Metal Carcinogenesis." The former chapter discusses interactions of lead, cadmium, and mercury with such essential nutrients as calcium, iron, and vitamin D, and special consideration is given to the interactions that appear to have the greatest health implications for humans. The latter chapter reviews epidemiological studies and those of experimental carcinogenesis, as well as possible mechanisms of carcinogenesis for arsenic, beryllium, cadmium, chromium, cobalt, iron, lead, zinc, and nickel.

In the next edition, it would be desirable to see this excellent volume further expanded into the discussion of genotoxic effects, about which more data are beginning to emerge. For example, consider the use of short-term bioassay tests for the mutagenicity of metals. Compounds of beryllium, cadmium, chromium(II), cobalt, copper, lead, manganese, and nickel are believed to be carcinogens and/or mutagens on the basis of a screening test wherein they were found to interfere with the ability of molecules of DNA to correctly select the bases that they need for proper replication.[1] Many other short-term bioassay tests have been developed recently and are being developed.

I highly recommend this volume to analytical toxicologists, chemists, graduate students in inorganic chemistry, biochemistry, and medicine as well as public and environmental health scientists because it is a well-written text of definite value to anyone interested in potential metal toxicities for humans.

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[1] M. A. Sirover and L. A. Loeb, Infidelity of DNA synthesis in vitro: Screening for potential metal mutagens or carcinogens. *Science* **194**, 1434 (1976).

Serodiagnosis of Mycotic Disease. By DAN PALMER, LEON KAUFMAN, WILLIAM KAPLAN, AND JOSEPH K. CAVALLARO. Charles C Thomas, Springfield, Ill., 1978. xiv + 191 pp. $21.50.

The pathogenic fungi are important causes of human morbidity and death. In the U.S. alone, millions of persons have been infected with aspergillosis, blastomycosis, candidiasis, coccidioidomycosis, cryptococcosis, or histoplasmosis, and hundreds die annually from these diseases. There is a compelling need for rapid and specific indirect (serological) diagnostic tests for the etiologic agents of these illnesses. This urgency arises from the inherent slowness or failure of conventional isolation and identification procedures as well as the need to objectively monitor the effect of chemotherapy. Increasingly, mycoserological tests are alerting clinicians to the presence of fungal disease and to the specific nature of the agent.

The present manual is the first to be devoted exclusively to these procedures. It will serve to increase the use of serology and raise performance standards in diagnostic centers throughout the world. This manual should certainly be available, at the bench, in all clinical and epidemiological laboratories.

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MICROCHEMICAL JOURNAL 24, 264 (1979)

Announcement

J. Heyrovský Memorial Congress on Polarography

The J. Heyrovský Memorial Congress on Polarography will take place in Prague, Czechoslovakia, August 25 to 29, 1980 (not August 18 to 22 as originally announced). The Congress will honor the 90th anniversary of the birth of Professor Jaroslav Heyrovský, Nobel Prize Laureate in Chemistry 1959, and the 30th anniversary of the Polarographic Institute, which is now part of the J. Heyrovský Institute of Physical Chemistry and Electrochemistry of the Czechoslovak Academy of Sciences. The aim of the Congress is to survey the present state of polarography, related methods in the fields of basic research, contemporary instrumental techniques, analytical chemistry, and applications in industry, biology, medicine, and environmental science.

The Congress will proceed in two parallel sections. Five plenary and twelve section lectures will outline the main topics. Thematically related groups of problems of current interest will be considered in five microsymposia and four panel discussions. A practically unlimited number of original contributions will be accepted in the form of graphical communications, called posters. There are plans for a social program in addition to the scientific program. English is recommended as the working language. Final applications for participation must be received before November 30, 1979. For further information, write to:

Secretariat of the J. Heyrovský Memorial Congress
on Polarography
Vlašská 9, 118 40 Praha 1, Malá Strana
Czechoslovakia