Volume 21, Number 1, March 1976

Microchemical Journal

devoted to the application of microtechniques in all branches of science

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CONTENTS

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Briefs

Extraction and Spectrophotometric Determination of Iron (III) with 4-Benzoyl-2, 4-dihydro-5-methyl-2-phenyl-^{3}H-pyrazol-3-one. G. NAGESWARA RAO, *Chemistry Department, Indian Institute of Technology, New Delhi-110029, Indian;* AND H. C. ARORA, *Chemical Laboratory, Atomic Minerals Division, West Block No. VII, P. K. Puram, New Delhi-110022, India.*

A method for solvent extraction and spectrophotometric determination of iron (III) from aqueous mineral acid media is described. The method is free from interferences of most of the metals that are usually associated with iron (III).

Microchem. J. **21**, 1–4 (1976).

An Ultramicromethod for the Determinations of Phosphatase Activity in Serum or Plasma. C. V. ABRAHAM, *Lynchburg Training School and Hospital, Lynchburg, Virginia 24505; and* H. W. GERARDE, *Fairleigh Dickinson University, Teaneck, New Jersey 07666.*

A method is described for the determination of alkaline and acid phosphatase using dicyclohexylammonium salt of *p*-nitrophenylphosphate as substrate. The ammonium salt shares the advantages of the sodium salt, but is more stable.

Microchem. J. **21**, 5–13 (1976).

An Ultramicromethod for the Determination of Blood Glucose Using Modified *o*-Toluidine Reagent. C. V. ABRAHAM, *Lynchburg Training School and Hospital, Lynchburg, Virginia 24505; and* H. W. GERARDE, *Fairleigh Dickinson University, Teaneck, New Jersey 07666.*

A rapid method for the determination of glucose on ultramicro samples of plasma and serum is described. Protein precipitation does not seem to be necessary, and thus, has been eliminated from the method. Once the reaction is terminated by placing in cold water, the absorbance of unknown and known may be read at any time within 1 hr.

Microchem. J. **21**, 14–20 (1976).

A Semiquantitative Micromethod for the Determination of Barbiturates and Doriden. C. V. ABRAHAM, *Lynchburg Training School and Hospital, Lynchburg, Virginia 24505; and* H. W. GERARDE, *Fairleigh Dickinson University, Teaneck, New Jersey 07666.*

A semiquantitative thin-layer chromatographic method is described for the determinations from blood.

Microchem. J. **21**, 21–26 (1976).

The Microdetection of Organoparticulates from Diazonium Compounds. J. D. B. SMITH AND D. C. PHILLIPS, *Westinghouse Research Laboratories, Pittsburgh, Pennsylvania 15235.*

An ion chamber and a condensation nuclei detector were used to detect aerosols and particulates in microquantities. The lower limits of the ion chamber and condensation nuclei monitors are 2×10^{-10} g/l and 10 particles/cm^3, respectively. Of the 14 diazonium compounds evaluated, 11 were found to exhibit particulation behavior below 190°C.

Microchem. J. **21**, 27–37 (1976).

The Oxidation of Benzidine, *o,o'*-Tolidine, and *o,o'*-Dianisidine by Manganese Dioxide. A. BERKA, M., KOŘÍNKOVÁ, AND J. BAREK, *Department of Analytical Chemistry, Charles University, Albertov 2030, 128 40 Prague 2, Czechoslovakia.*

Oxidation of the compounds was carried out in acidic medium. They are oxidized to the corresponding quinonediimines, and the reaction can be utilized for the indirect titrimetric determination of the compounds, based either on ferrometric titration of unconsumed manganese dioxide or on ascorbinometric titration of the quinonediimine formed.

Microchem. J. **21**, 38–44 (1976).

Spectrophotometric Study of both the Zimmermann Reaction and the Application of a Corrective Measure for Irrelevant Absorption. CAROLYN S. FELDKAMP,[1] EMANUAL EPSTEIN,[2] ROGER J. THIBERT,[1,3] AND BENNIE ZAK.[1] Departments of Clinical Pathology at Wayne State University and Detroit General Hospital, Detroit, Michigan,[1] William Beaumont Hospital, Royal Oak, Michigan,[2] and the Department of Chemistry, University of Windsor, Windsor, Ontario, Canada.[3]

A spectrophotometric study of the Zimmermann reaction carried out in several solvent matrices is described. The advantages of an aqueous system over those containing pyridine are detailed. Purification devices used commonly in 17-ketosteroid procedures have been shown to be not always effective if the actions and reactions of the interferences mimic those of the 17-ketosteroids. Study of mathematical corrections for irrelevant absorption indicate the concept is not always easily applicable to the determination.

Microchem. J. **21**, 45–59 (1976).

On-Line Data Acquisition System for Perkin–Elmer 240 Carbon, Hydrogen, and Nitrogen Analyzer. D. E. HARRINGTON AND W. R. BRAMSTEDT, *Diamond Shamrock Corporation, Painesville, Ohio 44077.*

A Perkin—Elmer Model 240 CHN Analyzer was adapted for on-line data acquisition with a remote computer. The precision of the results obtained compare favorably to results calculated manually, and provides a 38% improvement in efficiency.

Microchem. J. **21**, 60–69 (1976).

An Efficient, Inexpensive Multisleeve Liquid Scintillation Counting System. MICHAEL A. TRUSH AND KNOX VAN DYKE, *Department of Pharmacology, West Virginia University Medical Center, Morgantown, West Virginia 26506.*

A method is reported which conserves greatly on the cost of counting radioactive samples for liquid scintillation counting. This is accomplished by the use of several glass sleeves, which are much less expensive than the conventional units.

Microchem. J. **21**, 70–74 (1976).

Microdetermination of Carbon and Hydrogen in Organic Compounds Using Flushed-Oxygen Combustion Tube. YOSHIKO BABA, *Tokyo College of Pharmacy, Uenosakuragi, Taito-ku, Tokyo 110, Japan.*

A rapid method of microanalysis for carbon and hydrogen in organic compounds using an empty combustion tube fused with two nozzles for flushing oxygen and employing reduced copper for the reduction of oxides of nitrogen is described. Samples of the order of 1 mg were used.

Microchem. J. **21**, 75–81 (1976).

Indirect Determination of Picogram Amounts of Germanium in Pure Water by Flameless Atomic Absorption (Mo) Spectroscopy. V. ROZENBLUM, *Department of Chemistry, Technion-Israel Institute of Technology, Haifa, Israel.*

The method involves the conversion of the germanium into yellow 12-germanomolybdic acid, extraction into 2-ethylhexanol, the decomposition of the heteropoly-compound with ammonium hydroxide, and the back extraction of the liberated molybdenum into aqueous solution for flameless AA determination.

Microchem. J. **21**, 82–84 (1976).

A Rapid Measure of Primary Antigen Binding Capacity of Antiserum. ROSS A. CAPUTO, DONALD D. BARNHART, AND RONALD W. TREICK, *Department of Microbiology, Miami University, Oxford, Ohio 45056.*

A procedure for measuring primary antigen binding capacity of antiserum is described. Only 2 min is required to determine the amount of Σ-DNP-lysine bound by specific antiserum. Possible applications of the technique to radioimmune assays are discussed.

Microchem. J. **21**, 85–91 (1976).

Semimicrodetermination of Hydroxy and Amino Compounds Using Pyromellitic Dianhydride. WALTER SELIG, *Lawrence Livermore Laboratory, University of California 94550.*

The PMDA method for determining hydroxyl and primary and secondary amino groups was applied on the semimicro scale. A sample containing 0.4 to 0.6 mEq of amine or hydroxyl was reacted with 25 ml of 0.04 *M* PMDA in a routine analysis. The uncomsumed PMDA was titrated with 0.08 *N* base. For colorless clear samples, the phenolphthalein endpoint was used; for colored or turbid samples, a potentiometric titration is recommended.

Microchem. J. **21**, 92–97 (1976).

Spectrophotometric Determination of Antimony by Extraction of Reduced Molybdoantimonylphosphoric Acid with Butyl Acetate. J. GOING AND J. THOMPSON, *Department of Chemistry, The University of Wisconsin-Milwaukee, Milwaukee, Wisconsin 53201.*

Butyl acetate quantitatively extracts reduced molybdoantimonylphosphoric acid. The range of application is 5–1000 ppb antimonyl.

Microchem. J. **21**, 98–105 (1976).

MICROCHEMICAL JOURNAL 21, 1–4 (1976)

Extraction and Spectrophotometric Determination of Iron (III) with 4-Benzoyl-2, 4-dihydro-5-methyl-2-phenyl-^{3}H-pyrazol-3-one

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Received July 27, 1974

Acyl pyrazolones have been introduced by Jensen *(4,5)* as versatile chelating agents in the solvent extraction of metals. The reagent 4-benzoyl-2, 4 dihydro-5-methyl-2-phenyl-^{3}H-Pyrazol-3-one (BMPP) has proved to be the most useful among the 4-acyl pyrazolones and solvent extraction of over 40 metals has been investigated employing this reagent. In spite of the considerable amount of published work on solvent extraction using BMPP, no attempt has so far been made to use it as colorimetric reagent in the same fashion as thenoyl trifluoroacetone or oxine *(3)*. Results of our study on the colorimetric determination of iron (III) are presented in this paper, and we presume this is the first time that BMPP is utilized as extraction-cum-colorimetric agent. Extraction of iron (III) from nitric, hydrochloric, and sulphuric acid media using BMPP in benzene has been investigated by Chmutova and Kochetkova *(1,2)*. Narayan, Ivanova, and Peshkova *(7)* reported the stability constants of the complexes formed in the extraction of iron (III) from $NaClO_4-HClO_4$, or $Na_2SO_4-H_2SO_4$ media with BMPP in chloroform as solvent.

EXPERIMENTAL

Stock solution of iron (III) was obtained by dissolving suitable amounts of reagent grade ferric alum in 1 *N* sulphuric acid and its exact strength was determined by titration with standard permanganate or dichromate after reduction. Stock solution was diluted suitably to obtain iron (III) solution containing 100 μg/ml. BMPP was prepared according to the method of Jensen; solutions of requisite concentration of BMPP were prepared by dissolving the reagent in benzene.

Absorption spectra were obtained employing Bausch Lomb spectronic 20 spectrophotometer. A wrist action shaker was used and experiments were performed in a thermostat maintained at 30 ± 1°C.

GENERAL PROCEDURE

For solvent extraction, suitable volumes (0.5 to 5.0 ml) or iron (III) solution were added to requisite amount of nitric acid. The volume was made up to 10 ml maintaining the nitric acid concentrations in the aqueous phase at 7 *N*. A solution of 10 ml of 0.1 *M* BMPP in benzene was added to the iron (III) solution. The mixture was shaken for 5 min. The organic phase was separated from the aqueous phase after transferring the mixture to a separating funnel. The absorbance of the organic phase was determined after diluting it to 25 ml with benzene against reagent blank prepared under the same conditions.

RESULTS AND DISCUSSION

Chmutova and Kochetkova *(1,2)* reported complete and rapid extraction of iron (III) from 7 *N* nitric acid employing 0.1 *M* BMPP in benzene. In the presence of lower nitric acid concentration, quantitative extraction occurred only after long phase contact time. During the extraction from sulphuric and hydrochloric acid solutions, it was found that quantitative extraction occurs from 1 to 9 *N* H_2SO_4 and 1−2 *N* HCl after long phase contact while rapid extraction occurred from 11 *N* HCl medium. For the calculation of distribution coefficients, activity of [^{59}Fe]isotope was determined in aqueous and organic phases. However, no attempt was made to develop a spectrophotometric method for the determination of iron. Also, in the present work, the optimum nitric acid concentration for rapid and complete extraction of iron (III) was found to be 7 *N*. The reagent BMPP itself absorbs quite strongly below 400 nm and the best wavelength for measuring the bright red iron (III) complex was found to be 500 nm. Beer's Law was tested by taking different initial amounts of iron (III) in the aqueous phase (50 to 300 μg) and the results are given in Table 1. Molar extinction coefficient of the iron complex was 4950 ± 150 at the wavelength 500 nm. The absorption spectrum of the complex shows a maximum at 460 nm and the absorbance decreases continuously after 460 nm becoming negligible at 630 nm.

Experiments conducted in hydrochloric acid and sulphuric acid media indicated the optimum acid concentration for the rapid quantitative ex-

TABLE 1
VARIATION OF ABSORBANCE WITH CONCENTRATION OF IRON (III)

Iron (III) μg	50	100	150	200	250
Absorbance	0.175	0.355	0.520	0.690	0.870

traction to be 1.0 and 7.2 *N*, respectively. Beer's law was found to be valid in both media for the same range of iron concentration as in nitric acid medium. The colour of the complex was quite stable for a period of at least 24 hr.

NATURE OF THE COMPLEX SPECIES

The complex of iron (III) with BMPP was prepared by the usual methods adopted in the preparation of iron (III) β-diketonates. Analytical results indicated that 3 mole of the ligand are attached to each mole of the metal. The reagent exists in both keto and enol forms (Fig. 1), and the enolic form of the ligand forms the complex with iron (III). Variation of the distribution coefficient with ligand concentration in extraction studies also indicated that 3 mole of ligand are attached to each mole of the metal. Thus, it is certain that the species extracted is of the type $Fe(A)_3$ where HA represents the ligand and A^- its anion.

INTERFERENCES

Interference of copper (II), nickel (II), aluminum (III), calcium (II), cobalt (II), mercury (II), uranium (VI), chromium (III), sodium (I), silver (I), and manganese (II) was studied by adding them to the aqueous phase containing iron (III) (100 μg). Barring chromium (III), none of them interfered at 10 mg level in the estimation of 100 μg of iron (III). The salts used were $CuSO_4 \cdot 5H_2O$, $AlCl_3 \cdot 6H_2O$, $Ni(CH_3COO)_2 \cdot 4H_2O$, $CaCl_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $UO_2(NO_3)_2 \cdot 6H_2O$, $HgCl_2$, $CrCl_3 \cdot 6H_2O$, $NaNO_3$, $AgNO_3$, and $MnCl_2 \cdot 4H_2O$.

To evaluate the accuracy of the method, known amounts of iron (III) were subjected to the general procedure. Results obtained from the ab-

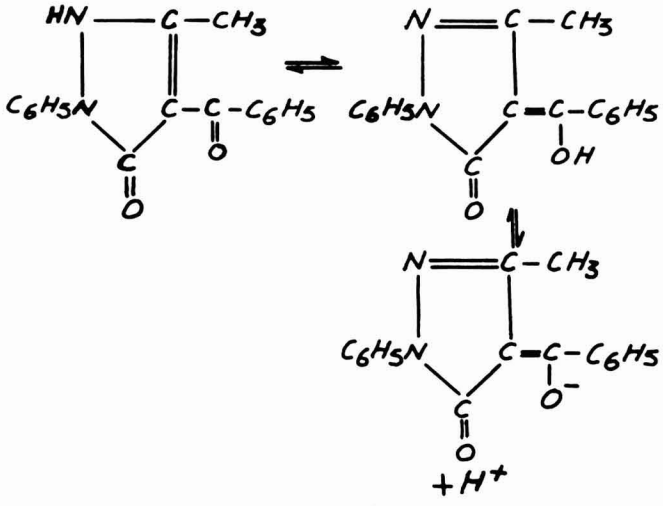

FIG. 1. Keto and enol forms of BMPP.

sorbance measurements agreed with the amounts taken within 2% in the 25 to 300 μg range.

Thus, the proposed method is rapid, fairly sensitive, and free from interferences of most of the metal ions which iron (III) is likely to be associated with in alloys, ores, and industrial materials. It is superior to the method employing thenoyltrifluoroacetone proposed by Khopkar and De *(6)*, since interferences are much less. Compared to the method of Testa *(8)*, which employs 0.5 *M* thenoyltrifluoroacetone, this method uses a less expensive and equally efficient reagent at a lower concentration (0.1 *M*), while retaining all the advantages of that method.

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MICROCHEMICAL JOURNAL 21, 5–13 (1976)

An Ultramicromethod for the Determinations of Phosphatase Activity in Serum or Plasma

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Received April 30, 1975

INTRODUCTION

The alkaline and acid phosphatase (orthosphoric monoester phosphohydrolase EC 3.1.3.1 and EC 3.1.3.2, respectively) are enzymes that catalyze the hydrolysis of phosphate ester at alkaline and acid pH. Measuring the activity of this enzyme in clinical laboratories has been recognized as a routine test to assist in interpreting a pathological condition. Numerous substrates have been proposed for alkaline phosphatase determination, and of these, *p*-nitrophenylphosphate, phenolphthalein monophosphate, 1-napthyl phosphate, and thymolphthalein monophosphate are commonly used.

The purpose of this paper is to describe an ultramicromethod for the determination of alkaline and acid phosphatase using the dicyclohexylammonium salt of *p*-nitrophenylphosphate as substrate. This has several advantages over procedures currently used in the clinical laboratory. It is stable at room temperature and not adversely affected by light. It remained white at room temperature for 1 yr in our laboratory, and the manufacturer claims at least 2 yr stability at room temperature.

The method is an adaptation of the Bessey–Lowery (*2*) procedure which uses *p*-nitrophenylphosphate as the substrate and chromogen. The procedure to be described has been incorporated into the Unopette System (Becton, Dickinson and Company, Rutherford, New Jersey), which makes it possible to have all of the reagents for a single analysis premeasured and stable at room temperature for an indefinite time.

MATERIALS AND METHODS

Substrate

Dicyclohexylammonium salt (300 μg) of *p*-nitrophenylphosphate (Calbiochem (No. 487611), Los Angeles, California) is stored in the overflow

[1] Deceased.

chamber of a 20 μl Unopette capillary pipette (Becton, Dickinson and Company, Rutherford, New Jersey). The dry substrate is obtained by adding 25 μl of an aqueous solution containing 300 μg of substrate to the overflow chamber of a 20 μl Unopette capillary assembly and drying the preparation at 37°C overnight. This quantity of substrate is used for each test of acid or alkaline phosphatase.

Serum Sample

Fasting blood samples were obtained by venipuncture from healthy laboratory workers who served as controls, and from patients hospitalized in a general hospital. After a clot was formed, the serum was separated by centrifugation and its activity measured within 24 hr.

Buffer

A. Alkaline Phosphatase. Several buffers including glycine, carbonate, 2-amino-2-methyl-1, 3 propanediol, 2-amino-2-methyl-1-propanol, diethanol amine and ethylaminoethanol were investigated. Ammonium buffer was selected for the standardization of the procedure. This was prepared by mixing 85 ml of 0.15 *M* ammonium hydroxide with 15 ml of 0.15 *M* ammonium chloride and adjusting the pH to 10. One ml of this buffer was premeasured in a Unopette reservoir.

B. Acid Phosphatase. Several buffers including citrate, maleate, succinate, and acetate were investigated. None appeared to increase enzyme activity more significantly than the others. Citrate was selected because it is widely used, and according to Anagnostopoulos *(1)*, enhances prostatic acid phosphatase. The citrate buffer was prepared as follows: A solution of 18.907 g of citric acid was dissolved in 180 ml of 1 *N* sodium hydroxide, 100 ml of 0.1 *N* hydrochloric acid was added to this, and the pH adjusted to 4.8. The final volume was made up to 1000 ml with distilled water. One ml of this buffer solution was premeasured in a Unopette reservoir.

Sodium Hydroxide

Sodium hydroxide (1.9 ml of 1.5 N) was premeasured in a Unopette reservoir.

Enzyme Control

Enzatrol (Warner–Chilcott, Morris Plains, New Jersey) was used for the enzyme control. The dried serum was reconstituted according to the instruction of the manufacturer.

Enzyme Activity Analysis

The substrate was incubated with a known amount of enzyme at 37°C for 30 min. The assays were done with 1 ml of reaction mixture. At the end of the incubation period an alkaline reagent was added to stop the enzymatic reaction and to develop the color of the hydrolyzed substrate.

PROCEDURE

A. *Alkaline Phosphatase*

20 μl of serum is collected in the phosphatase Unopak capillary and the excess serum is removed from the outside of the capillary pipette by carefully wiping with gauze. The Unopette reservoir containing the alkaline phosphatase buffer is squeezed slightly and the capillary holder is fitted into the reservoir. By alternately releasing and squeezing the walls of the reservoir, the buffer is forced through the capillary into the overflow chamber of the Unopak. By repeating the procedure several times the substrate is dissolved and quantitatively transferred to the diluent. Serum, dissolved substrate, and diluent are well mixed by inverting the reservoir several times. The buffer substrate is incubated at 37°C for 30 min. The reaction is then stopped by adding 1.9 ml of sodium hydroxide to the reaction mixture. This is accomplished by fitting a capillary holder onto the sodium hydroxide reservoir and transferring the sodium hydroxide solution to the reservoir containing the buffer substrate. The yellow color due to the *p*-nitrophenyl radical is measured in a spectrophotometer at 400 nm against a blank set at zero absorbance. After the reaction is stopped by adding the sodium hydroxide, the absorbance can be read at any time within a period of 6 hr.

B. *Blank*

Rinse out a phosphatase Unopak into a reservoir of buffer and incubate at 37°C for 30 min. Transfer the sodium hydroxide to the buffer substrate mixture and mix by inversion. Add 20 μl of serum or control to the same reservoir and set the absorbance at zero at 400 nm.

Calculations. IU phosphatase activity $= \frac{A_u}{A_k} X$ phosphatase concentration of known (Enzatrol). A_u = absorbance of unknown, A_k = absorbance of known.

If the alkaline phosphatase value is above 250 IU, the test should be repeated using a smaller volume of sample (10 μl) or 20 μl of diluted sample of serum.

C. *Acid Phosphatase*

The procedure is the same as the alkaline phosphatase except that the pH of the buffer used is 4.8 and the incubation period is 60 instead of 30 min for the test and the blank.

If the acid phosphatase value is above 50 IU, the test should be repeated using a smaller volume of sample (10 μl) or 20 μl of a diluted sample of serum.

EXPERIMENTAL

1. *Influence of Magnesium Ion on Phosphatase Activity*

It is known that Mg++ is required for phosphatase activity. We found

that Mg++ added to the buffers had no effect on the reaction rate. It appears that the quantity of Mg++ present in serum or plasma is sufficient to catalyze maximal phosphatase activity. This confirms the findings of Neumann and Van Vreedendall *(7)*.

2. *Stability of Substrate at Elevated Temperatures*

The stability of the dry substrate at elevated temperatures was determined by placing phosphatase Unopaks in an oven at 137°F. The phosphatase Unopaks were removed from the oven at various time intervals and the substrate dissolved in 1 ml of buffer. The extent of hydrolysis of the substrate was determined by adding sodium hydroxide to the buffer and measuring the absorbance of the alkaline buffer substrate solution at 400 nm. The stability of the sodium salt of *p*-nitrophenylphosphate was determined in the same manner.

The results of this experiment are summarized in Fig. 1. The dicyclohexylammonium salt of *p*-nitrophenylphosphate did not hydrolyze to the yellow chromogen, *p*-nitrophenylphosphate on continuous exposure to 137°F for 67 days. The sodium salt of *p*-nitrophenylphosphate under the same conditions underwent rapid hydrolysis.

It is conceivable that the substrate (dicyclohexylammonium salt) could have undergone nonhydrolytic changes on exposure to elevated temperature, which render it useless as a phosphatase substrate. To check this, we measured the alkaline phosphatase activity of Enzatrol using the substrate kept up to 67 days at 137°F. The results of this test are shown in Table 1.

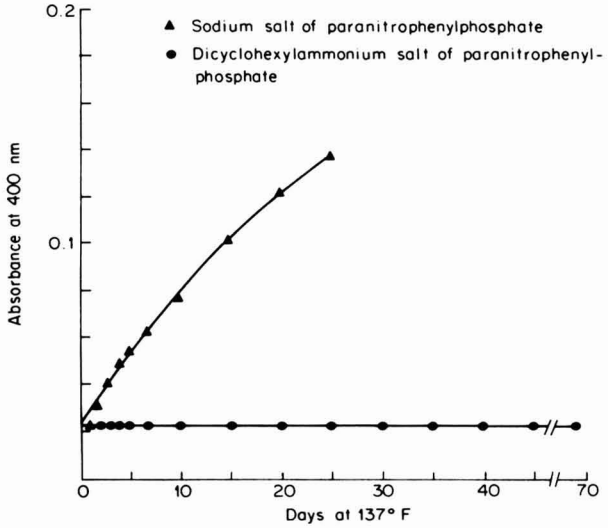

FIG. 1. Stability of substrate at 137°F.

TABLE 1
STABILITY OF THE SUBSTRATE

	Days							
	1	6	11	18	30	40	50	67
IU of alkaline phosphatase activity	82	86	82	84	86	82	84	82

Relationship Between Phosphatase Activity and Rate of Hydrolysis of Substrate

Increasing micro volumes of Enzatrol of known phosphatase activity were added to 1.0 ml of substrate. When less than 20 μl of serum was used volume of substrate was maintained by adding physiological saline to serum and when more than 20 μl of serum was added the buffer substrate volume was reduced accordingly. However, the substrate concentration was maintained at 300 μg for every test. The phosphatase activity was measured as described in the previous paragraphs. Figure 2 shows that the rate of hydrolysis of the substrate is proportional to the amount of alkaline phosphatase activity up to 240 IU.

A similar experiment was conducted with acid phosphatase. Figure 3 shows that the rate of hydrolysis of the substrate is proportional to the amount of acid phosphatase present up to 50 IU, which is about five times higher than the normal value.

Relationship of Time on the Degree of Hydrolysis of the Substrate by Phosphatase

This experiment was conducted using 20 μl of Enzatrol having 25 and 80 IU of acid phosphatase and alkaline phosphatase activity, respectively. Figure 4 shows that the rate of hydrolysis of the substrate by alkaline phosphatase in Enzatrol is linear with time. A 20 μl serum sample having normal alkaline phosphatase activity gives a good absorbance reading above the blank in a 30-min incubation period at 37°C.

A similar experiment was conducted on acid phosphatase in Enzatrol. Figure 5 shows that the rate of hydrolysis of the substrate by serum acid phosphatase is linear with time. The rate of hydrolysis of the substrate by acid phosphatase is slower than alkaline phosphatase. For this reason, the test for acid phosphatase is run for 60 min at 37°C to obtain sufficient chromogen to give an adequate absorbance measurement.

Precision and Accuracy

Table 2 shows the reproducibility of alkaline phosphatase procedure. A normal serum sample was analyzed 11 times. As seen from the table, the standard deviation is 1.253 IU, and the coefficient of variation is 1.8%.

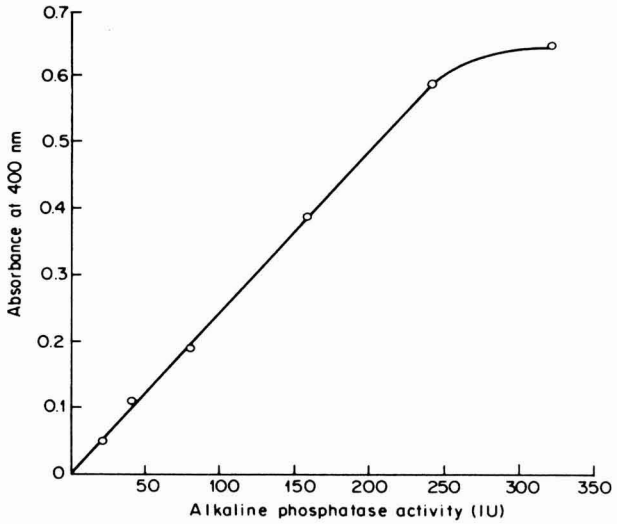

FIG. 2. Rate of hydrolysis of the substrate with increasing alkaline phosphatase concentration.

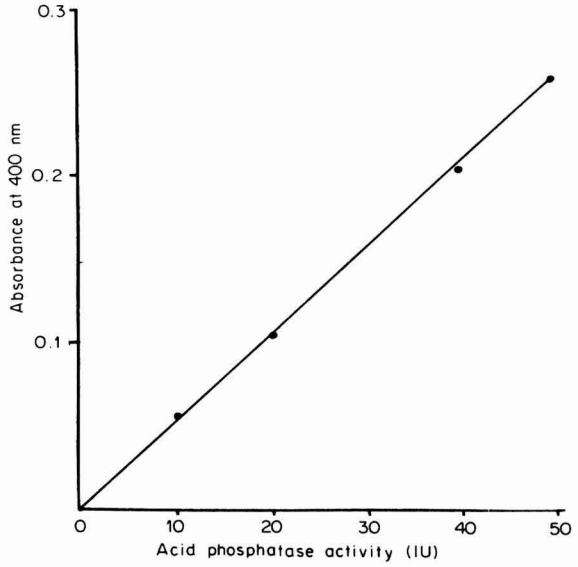

FIG. 3. Rate of hydrolysis of the substrate with increasing acid phosphatase concentration.

The accuracy of the proposed procedure was determined by comparing the values obtained on 127 clinical samples with the Auto-Analyzer (SMA-12), which used sodium salt of *p*-nitrophenylphosphate as substrate. The data obtained are summarized in Fig. 6

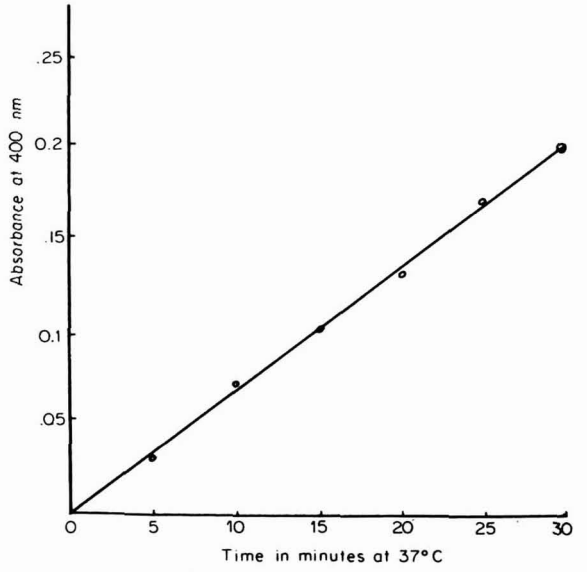

FIG. 4. Rate of hydrolysis of the substrate (alkaline phosphatase).

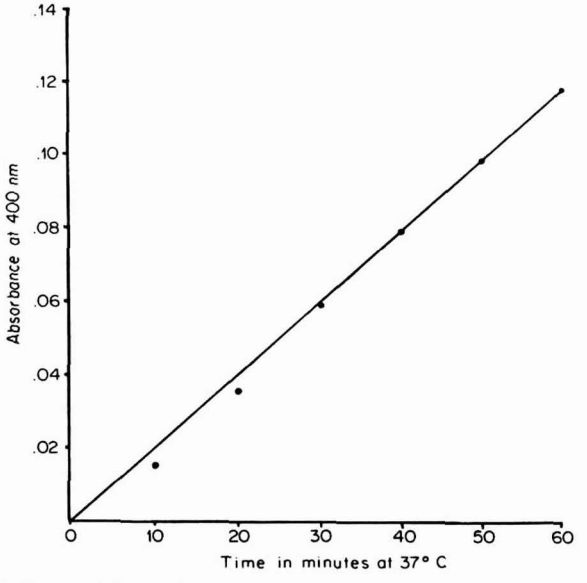

FIG. 5. Rate of hydrolysis of the substrate (acid phosphatase).

RESULT AND DISCUSSION

A simple ultramicromethod for the determination of alkaline phosphatase in serum is presented. The sera of laboratory workers as well as patients admitted to the general hospital were run. This was done

TABLE 2
REPRODUCIBILITY OF THE METHOD

Number	x	d	d^2
1	65.0	1.77	3.1329
2	66.5	0.27	0.0729
3	66.5	0.27	0.0729
4	68.5	1.73	2.9929
5	66.5	0.27	0.0729
6	66.5	0.27	0.0729
7	68.5	1.73	2.9929
8	65.0	1.77	3.1329
9	68.5	1.73	2.9929
10	66.5	0.27	0.0729
11	66.5	0.27	0.0729
Total	$(\Sigma x) = 734.5$		$\Sigma d^2 = 15.6719$
Average	$(x) = 66.7$		$\frac{\Sigma d^2}{10} = 1.56719$
SD = 1.253			CV = 1.8%

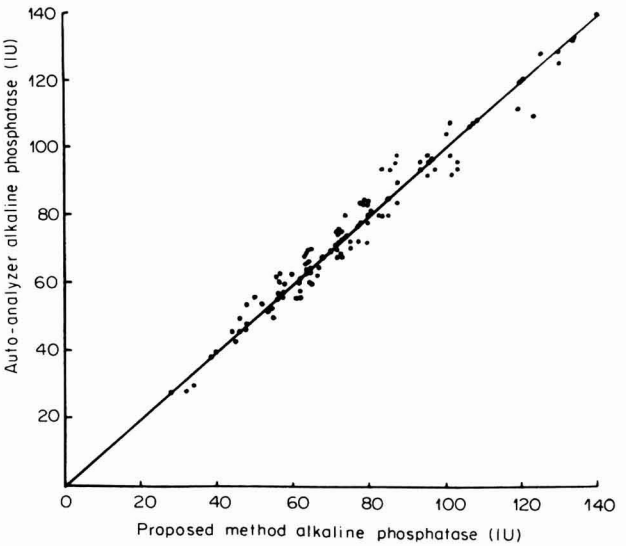

FIG. 6. Correlation of values obtained with the Unopette procedure and the Auto-Analyzer.

primarily to have the values evaluated for normal as well as abnormal sera.

Figures 1 and 2 show the linearity of the proposed method. For alkaline phosphatase it is linear up to 240 IU. This is a three-fold range above the upper limits of normal in human sera or plasma. For acid phosphatase it is

linear up to 50 IU. This is a four-fold range above the upper limits of normal.

The accuracy of the proposed procedure was determined by comparing the values obtained on 127 serum samples analyzed with the Auto-analyzer values. The Auto-Analyzer was Technicon SMA-12/60 and substrate was sodium salt of *p*-nitrophenylphosphate. The data obtained are summarized in Fig. 6.

The outstanding advantage of the method described is the stability of the substrate at ambient temperatures. Additional practical advantages inherent in the Unopette System are the premeasurement of all of the reagents and the complete disposability of the micro pipettes and containers used for the reagents. Although micromethods have a reputation for being inaccurate, particularly in the hands of unexperienced personnel, the self-filling, self-measuring feature of the Unopette capillaries, taken together with precise premeasurement of reagents, make it possible for medical technologists to conduct this test with good precision and accuracy. Simplicity and speed are also added advantages in the method described.

SUMMARY

An ultramicromethod has been described for the determination of alkaline and acid phosphatase using dicyclohexylammonium salt of *p*-nitrophenylphosphate as substrate. The ammonium salt shares the advantages of the sodium salt, but it is more stable, which results in more stable working reagent. This has been incorporated into the Unopette System. All of the premeasured, individually packaged reagents are stable at room temperature for at least 1 year. The method has been validated by comparing the results obtained with clinical samples analyzed with the Auto-Analyzer.

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MICROCHEMICAL JOURNAL 21, 14–20 (1976)

An Ultramicromethod for the Determination of Blood Glucose Using Modified *o*-Toluidine Reagent

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INTRODUCTION

The purpose of this paper is to describe an ultramicromethod for the determination of blood glucose. The method to be described does not require any special training or knowledge that cannot be learned in a few minutes. The procedures used are not exotic, and no special expensive equipment is required. More than 100 determinations have been run in our laboratory since the procedure was developed.

The method is based on the well-known *ortho*-toluidine method of Dubowski *(1, 2, 5, 6, 9)*. *Ortho*-toluidine reacts with the aldehyde group of glucose at an elevated temperature in an acetic acid, ethylene glycol solution, to form a colored equilibrium mixture of glycosylamine and the corresponding Schiff base. The blue–green colored end product has an absorption maximum at 630 nm.

The procedure has been incorporated into the Unopette System (Becton, Dickinson and Company, Rutherford, New Jersey) *(3, 4)*, which consists of a disposable precision glass capillary fitted into a plastic adapter that fits into a detachable plastic reservoir. For this determination, the plastic reservoir is prefilled with a measured volume of modified orthotoluidine reagent (Fig. 1). The serum specimen that is collected into a precision disposable capillary (Fig. 2) is added directly to the modified *ortho*-toluidine reagent in the reservoir. The reservoir is then incubated at a higher temperature for color development.

MATERIALS

1. *Modified Ortho-toluidine Reagent.* One gram of thio urea was dissolved in 250 ml acetic acid; 50 ml of *ortho*-toluidine was added to this and

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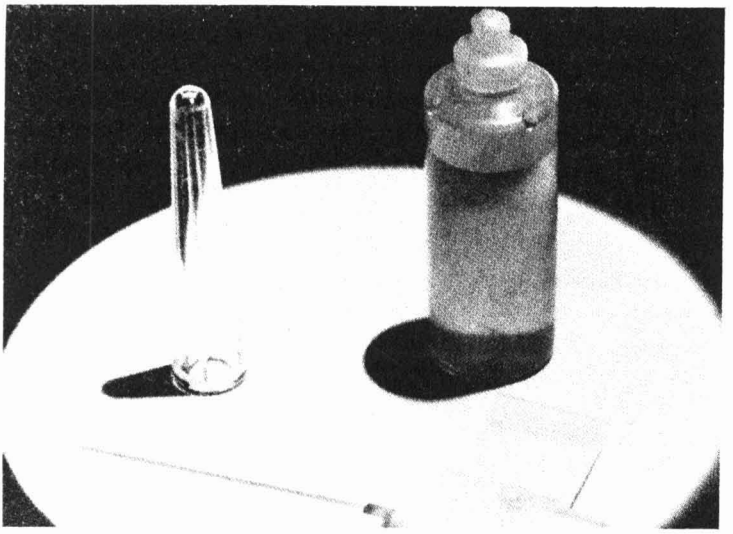

FIG. 1. A 20-μl capillary (shield removed) and Unopette reservoir containing modified *ortho*-toluidine reagent.

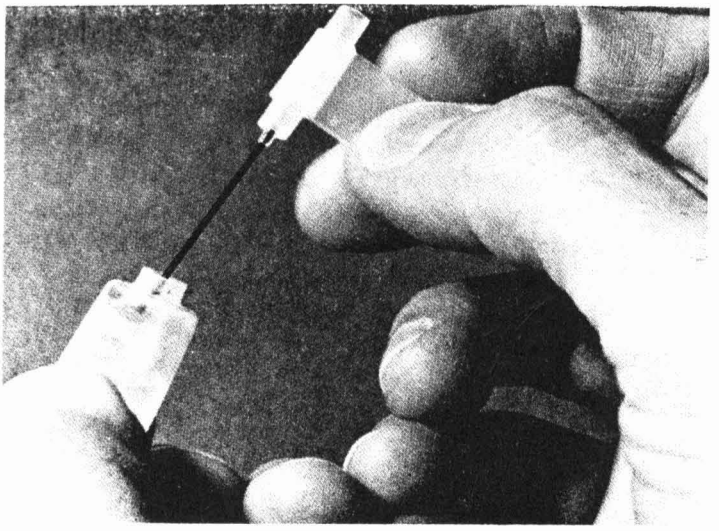

FIG. 2. Adding serum specimen to modified *ortho*-toluidine reagent.

the final volume was made to 1000 ml with propylene glycol. One-half gram of sulfamic acid was added to this and mixed for 2 hr to dissolve. Then, 3 ml of this was added to the unopette reservoir and stored in a brown bottle.

2. *Glucose standard, 100 mg/100 ml.* Weigh out 1 g of glucose and trans-

fer to a 1-l volumetric flask. Add distilled water almost to volume and dissolve the glucose by shaking. Saturate the solution with benzoic acid (preservative) and then bring up to volume with distilled water. Glucose working standards of different concentration are prepared by using different quantities of this standard solution (80 μl = 400 mg/100 ml; 40 μl = 200 mg/100 ml; 20μl = 100 mg/100 ml; and 10 μl = 50 mg/100 ml). See Fig. 3 for standard curve.

3. *Control.* Versatol containing 84.2 mg glucose/100 ml was used as a control in these studies.

4. *Pipettes.* Unopette capillary pipettes (40, 20, and 10 μl resp.) are used to measure the serum, plasma, control, and standard samples.

METHODS

1. *Collection of Blood Samples.* Heparinized or plain microhematocrit capillary tubes may be used to collect blood samples from finger punctures. When these tubes are centrifuged, sufficient serum or plasma is obtained with a Unopette pipette (after breaking the tube at the cell/serum or plasma interface) to make one glucose determination.

When using venous blood, vacutainer tubes were used and the serum or plasma separated after centrifugation.

2. *Glucose Analysis.* We added 20 μl of unknown (serum or plasma) and known (standard or control) directly to the glucose reagent in two separate reservoirs using the 20 μl Unopette capillary. Attach the Unopette capillary shield to the reservoir and place all tubes in boiling water bath for 10 min. Incubate one reservoir with glucose reagent without any serum or standard as blank with every batch. After 10 min, remove reser-

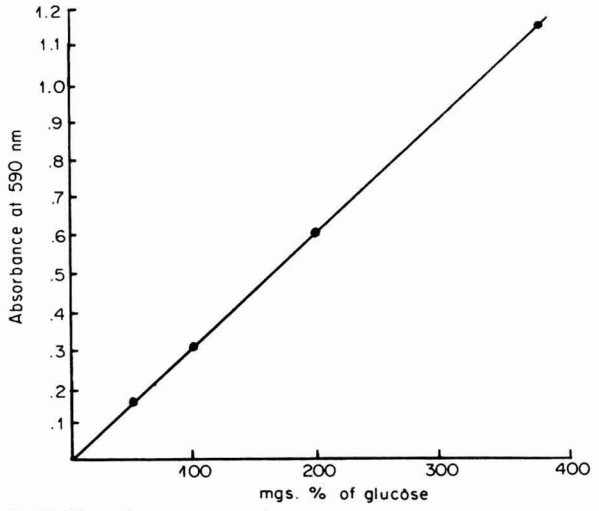

FIG. 3. Calibration curve using aqueous glucose standard.

voir and place in cold or iced water. Mix all reservoirs well and use the blank to zero the spectrophotometer set at 590 nm. (see Fig. 4). Measure the absorbance of standard, unknown, and controls within 1 hr after removal from boiling water bath at 590 nm.

3. *Preparation of Standard Curve.* The standard curve was prepared from the 100 mg/100 ml glucose standard. This standard was treated exactly as explained in the analysis. By using the 10, 20, 40, and 80 μl of this solution, a standard curve was obtained having a concentration of 50, 100, 200, and 400 mg/100 ml. This is shown in Fig. 3.

4. *Calculations.*

(a) mg glucose/100 ml plasma (or serum) $= \frac{AU}{AS} \times$ concn S.

where AU = absorbance of unknown and AS = absorbance of standard.

(b) These concentrations also can be read from the standard curve.

The accuracy of the proposed procedure was determined by comparing the values obtained on 103 samples with the original *ortho*-toluidine reagent. The data obtained are summarized in Fig. 5.

RESULTS AND DISCUSSION

Protein precipitation is eliminated in this procedure. To determine whether the presence of protein interferes with the reaction, a 100 mg/100 ml aqueous glucose standard was used to determine the glucose concentration of a known protein containing control (Versatol). The glucose concentration of the Versatol as determined by the Unopette method, (83.8 mg/100 ml) did not significantly vary from that established by the manufacturer, (84.2 mg glucose/100 ml).

To determine color stability the glucose concentration of a serum sample with 109 mg% was determined as described and the absorbance was read at various times after the reaction was stopped (Table 1), and the values were read from the standard curve. There was some progressive

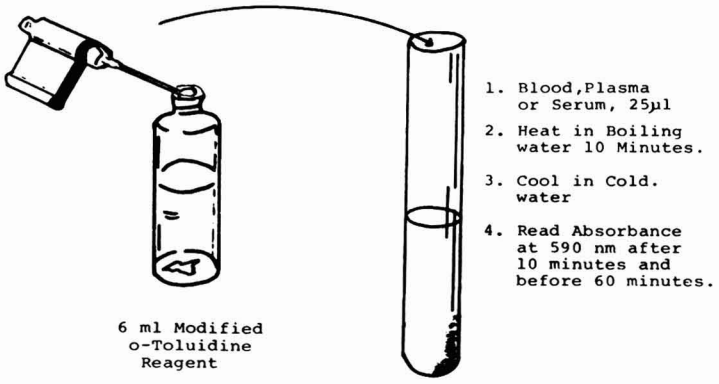

FIG. 4. Flow diagram of the Unopette procedure.

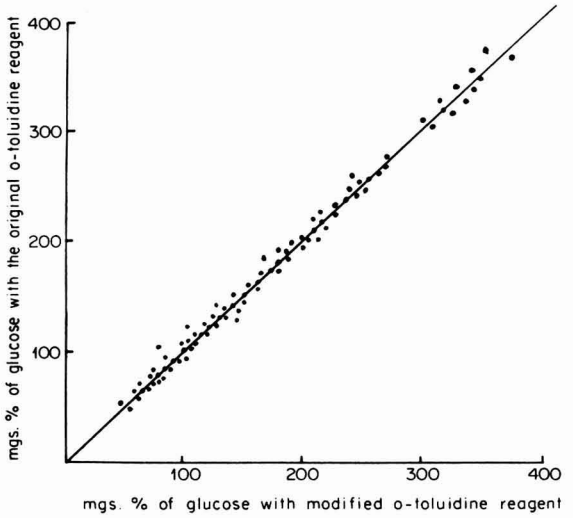

FIG. 5. Correlation of values obtained using the modified *ortho*-toluidine reagent and the regular *ortho*-toluidine reagent.

TABLE 1
BLOOD SUGAR CONCENTRATION OF A SINGLE SAMPLE CALCULATED USING THE STANDARD CURVE[a]

Time (min)	Glucose value
5	109
15	105
30	110
45	104
60	108
90	102
120	85
180	65

[a] At different time intervals after completion of test.

deterioration in color with time. However, if a standard was run with the sample and the value calculated from this standard, this color deterioration had no effect on the value (Table 2). This is because the color deterioration occurred to the same extent in both standard and unknown.

To evaluate the stability of the modified reagent preparation, prepackaged reagent was allowed to stand on a refrigerator shelf in a brown bottle for 6 months, and then used to analyze an unknown simultaneously with freshly prepared reagent. The analyses were performed by the

TABLE 2
BLOOD SUGAR CONCENTRATION OF A SINGLE SAMPLE CALCULATED USING A STANDARD RUN[a]

Time (min)	Glucose value
5	110
15	106
30	108
45	108
60	104
90	113
120	109
180	111

[a] With the sample at different time intervals after completion of the test.

method described using a 100 mg/100 ml aqueous glucose standard. The glucose concentration of the unknown with 6-month old prepackaged reagent was 86.6 mg/100 ml. When determined using freshly prepared reagent, the value was 88.6 mg/100 ml.

Dextran *(10)* is known to cause high absorbance reading by causing turbidity. Adding 25 μl of 0.7% solution of dextran to the Unopette reservoir and repeating the analytical procedure as explained earlier did not make any appreciable change in glucose value. Similarly, high bilirubin *(8)* concentration in serum is known to cause high values by forming a green oxidation product. We added 20 μl of 10 mg/100 ml bilirubin standard and repeated the procedure. On comparison with blanks that were run with this experiment, no appreciable change in optical density was noticed.

In the original *ortho*-toluidine method, the acetic acid fumes that came out were a problem for the technicians in the laboratory. With the proposed *ortho*-toluidine reagent, this problem was eliminated by using the propylene glycol that has a high boiling point and low vapor pressure.

The micromethod is not only a convenience for the technician in the saving of time for the analysis and the cleaning of glassware that follows, but it also spares the patient a venipuncture and the removal of several milliliters of blood when only 20 μl are needed. Individuals are much more willing to give a drop of blood from the fingertip or ear lobe than to give 5 ml of blood from a vein. This has made it possible to obtain blood samples to carry out the many blood sugar determinations in our laboratory since the method was first adopted. The venipuncture is also more expensive since it requires a sterile syringe or a vacutainer tube and needle. Repeated venipuncture is to be avoided because scarring and thickening of the vein at the puncture site make subsequent venipuncture increasingly difficult.

SUMMARY

A rapid method for the determination of glucose on ultramicro samples of plasma and serum has been described. Protein precipitation does not seem to be necessary, and thus, has been eliminated from the method. Once the reaction is terminated by placing in cold water, the absorbance of unknown and known may be read at any time within 1 hr. Accurate glucose determinations are obtainable when the reagents are stored at refrigerated temperatures for 6 months. Prepackaging of the reagents in individual test units of the Unopette System streamlines this method by reducing technicians' time and eliminating reagent waste. Ultramicrosampling is easy and accurate with the Unopette System, which makes it possible to use small samples of blood (a desirable quality in pediatrics and when performing glucose tolerance tests).

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MICROCHEMICAL JOURNAL 21, 21–26 (1976)

A Semiquantitative Micromethod for the Determination of Barbiturates and Doriden

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INTRODUCTION

The acute cases of overdose involving different drugs has been steadily increasing in recent years. Barbiturates are still the most commonly encountered in these overdoses. For monitoring these patients, simple and rapid semiquantitative screening procedures are needed. This is particularly important to small medical centers that cannot afford expensive equipment and a specially trained staff.

Gas chromatographic *(2,4)*, colorimetric *(3)* and ultraviolet spectrophotometric *(5)* methods are available for barbiturate determination. However, they all have their limitations. Gas chromatography requires special instrumentation, trained operators and is more suited for routine analysis. The colorimetric procedure is good for indicating whether a toxic amount of a barbiturate has been ingested. However, it lacks specificity and numerous interfering substances give false positive results. The ultraviolet methods require different scan at pH 10 and 13. Furthermore, ultraviolet spectrophotometry and gas chromatography are expensive. Thin-layer chromatography is a rapid technique that is suitable for the preliminary detection, identification, and semiquantitation of barbiturates.

The purpose of this paper is to describe a micromethod for the determination of barbiturates and Doriden (USV Pharmaceutical Corporation, Tuckahoe, N. Y. 10707) in blood. The method involves extraction of blood with chloroform and the estimation of barbiturates and Doriden in the extract semiquantitatively, using thin-layer chromatography. This method is simple, rapid and requires minimum laboratory space. In addition, the amount of blood required is only 50 μl so that the blood can be collected by a finger stick. the time required to complete the assay is less

[1] Deceased.

than ½ hr. This method is capable of producing semiquantitative results by visually comparing the unknown with the known graded series of the standard.

MATERIALS AND METHOD

1. Chromatogram Sheet. Eastman Kodak thin-layer chromatography sheet (silica gel without fluorescent indicator) 20 × 20 cm is used. These sheets are cut into small sheets of 10 × 6⅔ cm.
2. Screw-cap glass vials with Teflon-lined caps.
3. Syringe barrel filter. Take the 2.5 ml syringe barrel (B-D plastic pak) and insert two filter paper discs ¼-in. diameter (A. H. Thomas & Co.) into the bottom of the syringe.
4. Developing solvent. 2% ethanol in chloroform.
5. Detecting solution. (Unocaps, Becton–Dickinson Company, Rutherford, N. J. 07666) saturated aqueous solution of mercurous nitrate.
6. Extracting solution: chloroform.
7. Developing chamber.
8. Unopette capillary pipettes, 5 and 50 μl.

PROCEDURE

Extraction

Transfer approximately 1.0 ml of chloroform to one of the screw-cap vials. Add 50 μl of blood to the chloroform in the glass vial and agitate the tube by gentle shaking on a vortex mixer for a few minutes. Remove the chloroform layer into a syringe barrel filter with a pasteur pipet using a second screw-cap vial as a receiver for the filtrate. Add another 1.0 ml of chloroform to the first bottle and repeat the extraction and filtration procedure adding the second chloroform filtrate to the first portion of chloroform.

Concentration

Evaporate the pooled extracts in a water bath at 95°C. When the volume is reduced to almost dryness, remove the tube from the bath, wash down the walls with 0.5 ml chloroform, and evaporate to dryness. Cool to room temperature and save the sample until ready to run the thin-layer chromatography.

Preparation of Standards

The standards are prepared on the basis of the blood level *(1)* in comatose patients. The standards are above and below this level. On a single paper three drugs (phenobarbital, secobarbital, and Doriden) with three different concentrations are added. The concentration of the standards are given in Table 1.

Preparation of Paper

The Eastman chromatogram sheet (No. 6061, 20 × 20 cm) is cut to 6⅔

TABLE 1
CONCENTRATION OF RECOMMENDED STANDARDS

Standard	Doriden	Secobarbital	Phenobarbital
1	1.5	2	4
2	3.0	4	8
3	4.5	6	12

× 10 cm. Points for the application of the sample are marked with a pencil along the 6⅔-cm length, 1 cm from the edge and 1.5 cm apart. One sample and three standards can be spotted. Reconstitute the residue in the screw-cap bottle from the concentration step with 50 μl of chloroform. Rotate the bottle in a horizontal position to dissolve the residue. Using a 5-μl Unopette capillary pipette transfer the entire volume of chloroform from the bottle to the chromatogram paper marked "sample." The chloroform is added in small increments and allowed to evaporate between additions in order to keep the area of the spot to a minimum of approximately 2 mm in diameter.

Developing Chamber

For the development of the chromatogram, a 600-ml pyrex beaker can be used. When a pyrex beaker is used, it should be closed with Saran Wrap. A 9 × 11-cm round bottle with a screw cap also can be used. We work with both the bottle and the beaker. Both of them serve the purpose very well. The developing chamber must be clean. Twenty ml of developing solution is placed in the chamber and kept covered for approximately 5 min before putting the chromatogram in for development. This will saturate the developing chamber atmosphere with the developing solution vapor. This helps to migrate the solvent faster on the thin-layer chromatography sheet.

Development of Chromatogram

Place the chromatogram paper in the chamber with the bottom immersed in the solution. Cover the jar and allow the solvent front to rise 7 to 8 cm from the line of application. It takes approximately 10 min. Remove the chromatogram from the chamber, mark the solvent front with pencil, and allow it to dry in the air. The paper is ready for visualization.

Visualization

The mercurous nitrate is in the thin glass tube in a large Unopette reservoir (Unocap) containing deionized water. To prepare a saturated solution, crush the tube by squeezing the side of the reservoir and shake well for a few minutes. Perforate the diaphragm of the reservoir with a Unopette capillary shield and attach the adapter to the reservoir opening.

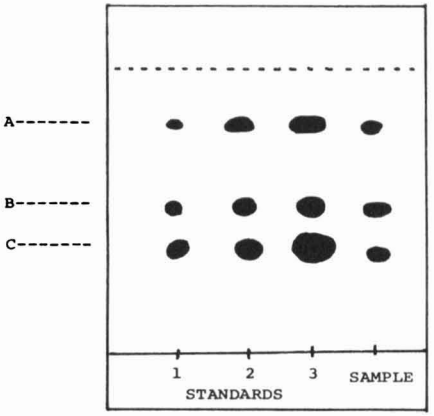
Squirt the mercurous nitrate solution on the chromatogram surface to wet it uniformly and allow the excess of the solution to drain off. The aqueous mercurous nitrate solution is prepared just before use.

The barbiturates and Doriden appear as gray spots as shown in Fig. 1. A positive result is confirmed by checking when the spot due to the sample travels exactly the same distance as the standards. A semiquantitative value can be obtained by comparing the area and the intensity of color of the sample spot with the standards. The Rf values calculated using the migration of the solvent and the sample provides further confirmation and identity of barbiturates and Doriden.

RESULTS

We checked the efficiency of our method in two ways: (1) By adding known amounts of barbiturates and Doriden to the blood and proceeding exactly as described above. We got a good recovery from the blood (Table 2). (2) We also checked the efficiency of our method by giving known amounts of barbiturates and Doriden to rats. The barbiturates, by intraperitoneal injection and Doriden by stomach feeding. The blood was collected by cardiac puncture and analyzed as explained above.

Figure 1 shows the results from a thin-layer chromatography examination of a blood sample taken 2 hr after ingestion of phenobarbital, secobarbital, and Doriden to the rat. The results are calculated by using the



A - DORIDEN

1. 1.5 mg% 2. 3 mg% 3. 4.5 mg%

B - SECOBARBITAL

1. 2 mg% 2. 4 mg% 3. 6 mg%

C - PHENOBARBITAL

1. 4 mg% 2. 8 mg% 3. 12 mg%

FIG. 1. Chromatogram for standard and blood extract.

standard on the chromatogram. The calculated blood concentration is given in Table 3.

DISCUSSION

The method that we described above is simple. We can do the analysis with as little as 50 μl of blood. This makes the test possible to do with blood collected by finger stick. The time required to do the estimation is approximately ½ hr. The procedure is so simple that any laboratory technician can be trained in a couple of hours.

The use of separating funnel and the filtration of the chloroform extract is achieved by using the syringe barrel filter. This filter is disposable. It saves time in cleaning the separating funnel and reduces the chance of contamination. The Eastman chromatogram paper (No. 6061) can be used without any activation. The solvent migrates very rapidly and the separation is very good. Four different barbiturates (phenobarbital, pentobarbital, secobarbital, and amobarbital) and Doriden are put on the same chromatogram sheet. Doriden separated very well from the barbiturates, and the barbiturates were separated enough to read even though they touched each other at high concentration.

TABLE 2
RECOVERY STUDY, BARBITURATES AND DORIDEN ADDED TO BLOOD

Drug	Amount added (mg%)	Semiquantitative results (mg%)
Phenobarbital	5	↓ 8 but ↑ 4
	10	↓12 but ↑ 8
	15	↑12
Secobarbital	1	↓ 2
	4	Approx 4
	8	↑ 6
Doriden	1	↓ 1
	2	↓ 3 but ↑ 1
	6	↑ 4.5

TABLE 3
RECOVERY STUDY, BARBITURATES AND DORIDEN GIVEN TO RATS

Drug	Concentration (mg%)
Phenobarbital	↓4
Secobarbital	↑4 but ↓6
Doriden	↑1.5 but ↓3

The outstanding advantages of this procedure are its simplicity, inexpensiveness, and suitability for pediatric cases.

SUMMARY

A semiquantitative thin-layer chromatographic method is described for barbiturates and Doriden determinations from blood using micro volumes.

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MICROCHEMICAL JOURNAL 21, 27–37 (1976)

The Microdetection of Organoparticulates from Diazonium Compounds

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INTRODUCTION

The thermal decomposition of organic compounds is amenable to study, using a new instrumental technique based on thermoparticulate analysis (TPA). Thermoparticulate analysis was originally used for the detection of condensation nuclei evolved from polymeric materials undergoing degradation when subjected to programmed heating *(2)*. TPA has been successfully adapted to follow the thermal decomposition of organic compounds; the new technique is termed organic particulate analysis (OPA) and enables a new physical property, namely, the temperature at which particulates are emitted from heated organic substances, to be measured. This new technique has been applied by us to study various diazonium compound decompositions; these types of organic compounds are noted for having tendencies to decompose at relatively low temperatures (i.e., < = below 200°C). Organic particulate analysis has revealed that some of these organic compounds are very strong sources of particulate emission: This particulate detection is possible because of the extreme sensitivity of the instrumentation used in this new technique.

INSTRUMENTATION

Two extremely sensitive analytical instruments are now commercially available for the detection of gas-borne particulate matter. (Both instruments marketed by Environment One Corp., 2773 Balltown Rd., Schnectady, N. Y. 12309.) One instrument, an ion chamber detector, utilizes the change in current output of an ion chamber as a function of particulate concentration, whereas the other instrument, a condensation nuclei detector, utilizes the principle of a cloud chamber in which water is condensed upon submicroscopic particles to produce visible, micron sized droplets. Both instruments were used in our studies and have been found to give identical results.

2.1. *Mode of Operation of Ion Chamber Detector (5)*

Submicron particles can be detected by their influence on the output current of an ion chamber arranged to collect the ions produced by a low level radiation source in the gas stream containing the particles. In the

absence of particles, almost all the ions are collected and this results in a maximum output current of a magnitude determined by the strength of the radiation source and the ionization properties of the gas stream. When particles are present in the ionized gas stream, some ion-particle combinations take place. Because the particles are much larger than the ions, the mobility of the resultant charged particles is less, and only a few of the species are collected in the ion chamber. The result is a decrease in the output current of the ion chamber; this decrease being a function of the particle concentration and particle size. Concentrations as low as 2×10^{-10} g/l can be detected.

2.2. *Mode of Operation of Condensation Nuclei Monitor (3, 4)*

In a condensation nuclei monitor, water vapor is caused to condense on particulate matter and the opacity of the vapor is then measured and related by the electrical signal to particle concentration.

A constant-flow gas sample is periodically diverted into a humidifier, where its relative humidity is raised to 100% with water. The sample then passes through a rotating valve into a cloud chamber, where it is expanded adiabatically, causing the sample to cool and the relative humidity to rise to a supersaturation of 400%. The cloud attentuates a light beam that is focused on a solid state light sensitive element. As the light value is decreased, an electrical pulse is created that is amplified and rectified into a dc signal proportional to the condensation nuclei concentration in the sample. After expansion, the cloud chamber is pressurized to the original atmospheric condition and then flushed with a new supply of gas. The instrument provides a continuous measurement of gas-borne particle concentrations in a size range down to 0.001 μm. The concentration range of the instrument is from 10 to 10^7 particles/cm^3 with a response time on the order of 1 to 2 sec.

EXPERIMENTAL TECHNIQUE

3.1 *Sample Preparation*

Fourteen different diazonium compounds were investigated in this work: These were in the form of chlorozincate, chlorostannate, tetrafluoroborate, or hexafluorophosphate salts. The compositions of the compounds are shown in Table 1, along with their commercial identifications. With the exception of *p*-chlorobenzenediazonium hexafluorophosphate, which was obtained from Ozark–Mahoning Company, all these materials were from General Aniline and Film Corporation.

The organic compounds under investigation were incorporated into a special air-drying epoxy polymer matrix, which serves to prevent the compound from producing "dusting" during testing in the gas stream; this "dusting" effect could produce false particulate signals on the instrument (i.e., the particulates are not derived from decomposition of the organic

TABLE 1
THE DIAZONIUM COMPOUNDS EVALUATED

Chemical name	Commercial designation
p-Chlorobenzenediazonium hexafluorophosphate	Phosfluorogen "A"
3-Chloro-4-diethylaminobenzenediazonium chlorozincate	Diazo 485-M
p-Diethylaminobenzenediazonium chlorozincate	Diazo 517-P
p-Diethylaminobenzenediazonium fluoroborate	Diazo 511-F
4-Morpholino-2,5-diethoxybenzenediazonium chlorozincate	Diazo 785-P
4-Diethylamino-2-methyldiazonium chlorozincate	Diazo 575-F
p-Amino-*N*-benzyl-*N*-ethylbenzenediazonium chlorostannate	Diazo 453-M
p-Dimethylaminobenzenediazonium chlorozincate	Diazo 605-P
4-Diethylamino-2-ethoxybenzenediazonium chlorozincate	Diazo 545-F
4-Ethylamino-3-methylbenzenediazonium chlorozincate	Diazo 695-F
p-Amino-*N*-ethyl-*N*-hydroxyethylbenzenediazonium chlorozincate	Diazo 665-P
p-Morpholinobenzenediazonium chlorozincate	Diazo 725-P
p-Amino-*N*-benzyl-*N*-ethylbenzenediazonium chlorozincate	Diazo 455-M
p-Diethylaminobenzenediazonium chlorozincate [Low $ZnCl_2$ salt form of Diazo 517-P]	Diazo 515-F

compounds). The special polymeric epoxy material has been shown to particulate at temperatures well above (i.e., above 200°C) that shown by the organic compounds under investigation. This ensured the minimum of interference from particulates derived from the epoxy matrix with those originating from decomposition of the organic compounds.

The most convenient method of sample preparation was to incorporate the diazonium compound into an air-drying, styrenated, alkyd-modified epoxy varnish using the following composition: diazonium compound, 100 parts; epoxy varnish, 100 parts; cobalt naphthenate solution, 1.0 part; and lead naphthenate solution, 0.25 part.

The epoxy varnish (B-276) was obtained from Westinghouse Industrial Materials Division and contained 50% by weight of toluene solvent. The cobalt naphthenate solution (6% in "Nuodex") and lead naphthenate (24% lead w/w) were added as "driers" for the epoxy varnish. It was found convenient to add these "driers" prior to the addition of the diazonium compound to ensure uniform mixing of the naphthenate solutions with the epoxy varnish.

The diazonium-epoxy mixtures were then brushed onto thin section aluminum strips (1 × 3 in.) and allowed to air-dry before being placed in an oven at 60°C for 4 hr to remove the last traces of solvent.

The diazonium-epoxy matrix prepared in this fashion was found to be well bonded to the aluminum metal surface with no indication of dusting or loosening of the material within the matrix.

Small portions were then cut off from these samples (usually measuring 1 × 1/4 in. and weighing ~0.5 g), and the organic particulation analyses were carried out as described in the next section.

3.2 *Measurement of "Organoparticulation Temperature"*

Figure 1 shows typical apparatus used to study the thermal decomposition of the organic compounds. Accurate temperature measurements were made through a Chromel–Alumel thermocouple attached to a stainless steel boat that rested directly on a strip heater. The entire assembly was mounted on insulating stand-off pedestals within a stainless steel tube (2 in. o.d.). A phase controlled temperature regulator and programer, connected through a sealed endplate to the boat, acted as a temperature control on the heater. The output of the thermocouple and an Ion Chamber Detector was monitored on a two-pen potentiostatic recorder. Hydrogen, at a constant flow rate of 6 l/min, was passed over the samples contained in the boat. A 6°C/min heating rate was maintained in each experiment.

Two temperatures were read from the charts; the *threshold* temperature, which corresponded to the onset of organoparticulation (as shown by an initial fall-off in amplified ion current), and the temperature that signified a 50% decrease in the ion current (usually 0.8–0.4 mA). These values enabled an "organoparticulation temperature range" to be determined for each sample (i.e., OPTR).

3.3. *Characterization of Particulates by Mass Spectrometry*

The total vapor pyrolyzate was collected on Porapak R (a modified divinyl-benzene-type absorbent obtainable from Waters Associates, Inc.). The analytical instrumentation consisted of the Perkin–Elmer Model 270 gas chromatograph/mass spectrometer. In this analysis, the

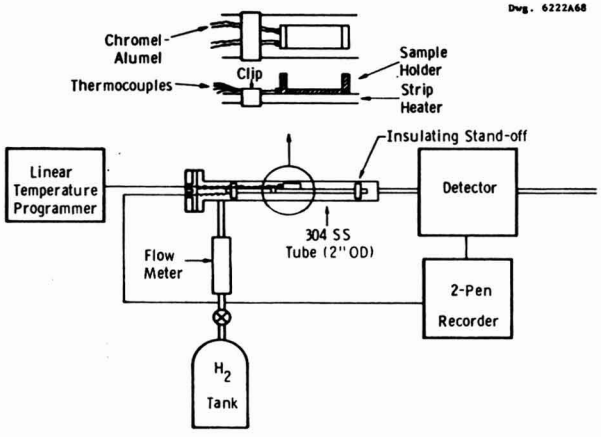


FIG. 1. System for determining particular temperatures

gas-chromatographic mode was not used. The trapped pyrolyzate was flushed off the adsorbent at 180°C onto a helium stream into the mass spectrometer through the self-contained helium separator. Mass spectra were taken when the total ion monitor of the Model 270 indicated that sample was reaching the ion source.

RESULTS

4,1. *Organic Particiulation Temperature Data*

Of the fourteen diazonium compounds evaluated by this technique, 11 were found to emit particulates at temperatures below 190°C. Although some of these were considered weak particulate emitters [i.e., the amplified ion current value did not drop beneath the 50% stage], there were others which gave very strong signals.

Figure 2 is an example of one of the strong particulate emitting diazonium compounds (i.e., *p*-chlorobenzenediazonium hexafluorophosphate). It is noted that the amplified ion current value begins to drop very rapidly at 132°C, reaches the 50% stage at 140°C, and is off-scale at 145°C.

In Tables 2 and 3 are summarized all of the organic particulation data obtained with the 14 diazonium compounds. For comparative purposes,

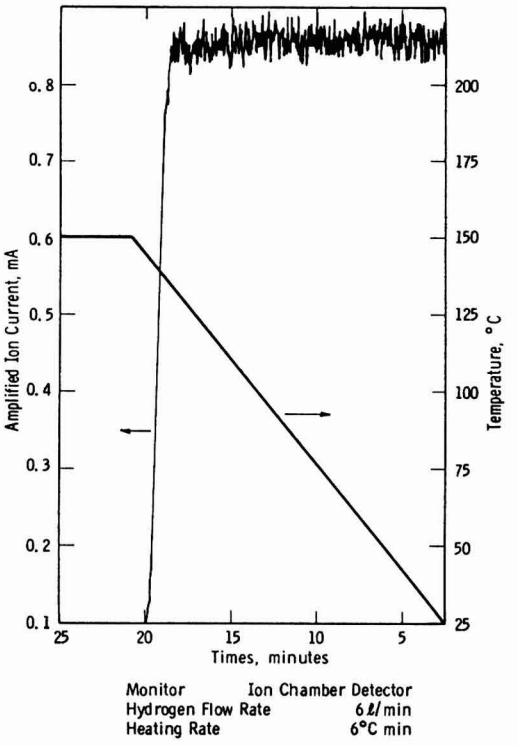
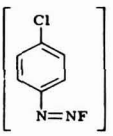
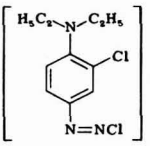
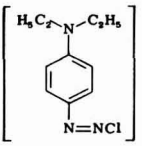
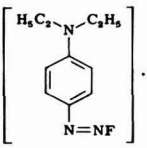
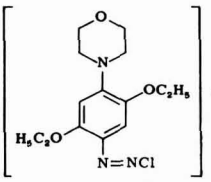
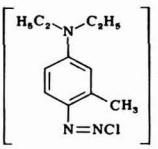
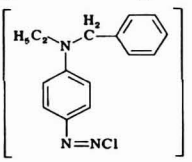


FIG. 2. Organoparticulation pattern for *p*-chlorobenzenediazonium hexafluorophosphate.

TABLE 2

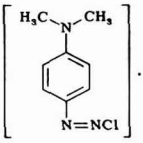
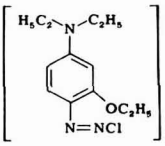
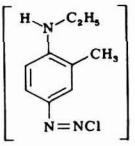
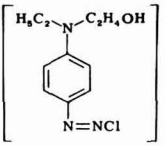
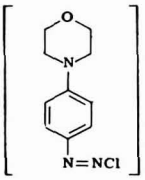
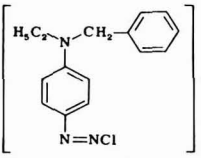
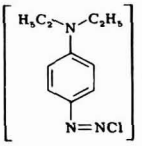
ORGANIC PARTICULATION DATA FOR THE DIAZONIUM COMPOUNDS

Test sample[a] no.	Diazonium compound commercial designation	Diazonium compound chemical composition	Organic particulation temperature range (°C (OPTR))	Literature decomposition temperature, (°C)
SC-3/8	Phosfluorogen "A"		132–140	150–152
SC-10/6	Diazo 485-M		150[b]	113–115
SC-11/6	Diazo 517-P		>190	117–119
SC-12/6	Diazo 511-F		147–149 (with a weak signal at 130)	108–110
SC-13/6	Diazo 785-P		182–187 (with a weak signal at 127)	120–130
SC-14/6	Diazo 575-F		159[b]	120–125
SC-44/6	Diazo 453-M		155–160	160–162

[a] Sample prepared as a 66% (w/w) diazonium compound in a cured epoxy matrix.

[b] Weak signals obtained; not strong enough to reach the "50%" stage on the ion chamber detector.

TABLE 3
ORGANIC PARTICULATION DATA FOR THE DIAZONIUM COMPOUNDS

Test sample[a] no.	Diazonium compound commercial designation	Diazonium compound chemical composition	Organic particulation temperature range (°C (OPTR))	Literature decomposition temperature (°C)
SC-45/6	Diazo 605-P	 · ½ $ZnCl_2$	>190	145–150
SC-46/6	Diazo 545-F	 · ½ $ZnCl_2$	184–196	140–147
SC-47/6	Diazo 695-F	 · ½ $ZnCl_2$	100[b]	125–129
SC-228/6	Diazo 665-P	 · ½ $ZnCl_2$	153[b]	140–144
SC-229/6	Diazo 725-P	 · ½ $ZnCl_2$	153[b]	130–160
SC-230/6	Diazo 455-M	 · ½ $ZnCl_2$	159–160	135–138
SC-231/6	Diazo 515-F [lower $ZnCl_2$ salt form of Diazo 517-P]	 · ½ $ZnCl_2$	>190	138–142

[a] Sample prepared as a 66% (w/w) diazonium compound in a cured epoxy matrix.
[b] Weak signals obtained; not strong enough to reach the "50%" stage on the ion chamber detector.

the decomposition temperatures for each of these compounds are also listed. These values were obtained from literature supplied by GAF and Ozark–Mahoning.

Examination of these data indicates that there is no obvious correlation between the organic particulation temperature range values and the thermal decomposition temperatures for these compounds. Only in these instances do the OPTR values appear to fall within a 10°C range of the decomposition temperature values (e.g., for samples SC-3/8, SC-44/6, and SC-229/6). Another interesting feature shown by the data in Tables 2 and 3 is the appearance of a weak particulate signal at temperatures beneath the main particulation signal for some of the test samples (e.g., SC-12/6 and SC-13/6).

4.2 *Mass Spectral Data on Particulates*

The effluent collected from one of the strong diazonium particulate emitting compounds (i.e., sample SC-44/6, Diazo 453-M) was analyzed by mass spectrometry. The mass-spectral pattern, shown in Fig. 3, is very complex and exhibits evidence of molecular fragments showing mass doublets due to the presence of one chlorine atom in the fragment. These doublets occur primarily at m/e 140, 142; 155, 157; 168, 170; 230, 232; and 245, 247.

The extreme complexity of this spectrum makes it difficult to identify the exact nature of the particulates arising from Diazo 453-M. However, it may be of significance that chlorine atoms are detectable in the fragments; the suggestion is that, during the thermal decomposition of Diazo 453-M, the liberated chloride ion might migrate and become substituted at some other location within the molecule leading to stabilization of particulate species (in the vapor phase).

DISCUSSION

In terms of organic particulation emission it would appear that the diazonium compounds can be broken into 4 main groups:

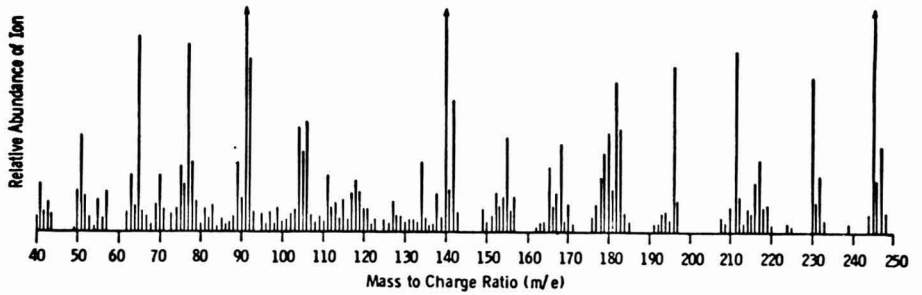
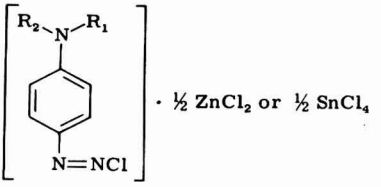


FIG. 3. Mass spectra of particulate samples from *p*-N-benzyl-N-ethylbenzenediazonium chlorostannate.

Group I has the general formula

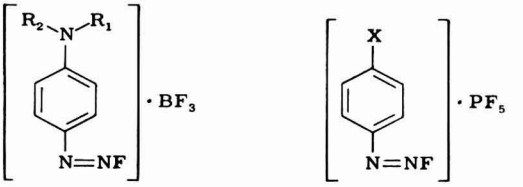


where R_1 and R_2 are C_2H_5 or CH_3 groups. These compounds appear to give no significant particulation below 190°C as demonstrated by Diazo 515-F, 517-P, and 605-P.

Group II has the same general formula as Group I, but $R_1$and R_2 are aralkyl (benzyl) or heterocyclic (morpholino) groups.

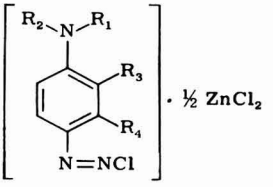
With this group, strong signals are detectable below 190°C (e.g., Diazo 785-P, 455-M, and 725-P). Diazo 665-P, in which R_2 is C_2H_4OH, may also be included in this group: although in this case, only weak particulation is detectable below 190°C.

Group III compounds could be comprised of the diazonium salts of strong Lewis Acids such as BF_3 and PF_5. These compounds have the general structural formulas:



where R_1 and R_2 are alkyl groups and X is a halide. These compounds give very strong particulation emission below 190°C. Examples would be Phosfluorogen "A" and Diazo 511-F.

Group IV compounds have the general structural formula:



where R_1 and R_2 are H, C_2H_5, and R_3 and R_4 are Cl, CH_3, or OC_2H_5. Examples of this group would be Diazo 695-F, 575-F, 545-F, and 485-M. These compounds give detectable particulation signals below 190°C. In general, these signals are of a weak nature and are not as strong as those from the compounds in Groups II and III. Diazo 785-P, which was included in Group II, could also be placed in this group of compounds. However, the salient factor in this compound's composition is probably the morpholino group substitution.

Although further studies with other types of diazonium compounds would be required before any general conclusions can be reached regarding the mechanisms leading to particulate formation with these materials, there is some indication from more basic studies carried out in this laboratory, that a certain *critical minimum* particulate size is necessary to produce an observable signal on the ion chamber and condensation nuclei detectors. This critical size appears to be of the order of 25Å. Since this size is much larger than the anticipated molecular fragments arising from the group of diazonium compounds studied here, (e.g., the largest organic fragment from SC-44/6 would be of the order of 10 Å) it would appear that vapor phase association of the molecular fragments would have to occur to give the critical particulate size.

Such vapor phase association would be most likely to occur through H-bonding (from amine fragments) or through halide atoms such as F or Cl. It is interesting to note, in this respect, that the groups of compounds that give the strongest particulation signals, i.e., Groups II and III, contain not only larger molecular fragments but also *substituent polar* functional groups (e.g., amino and hydroxy) and extremely polar gaseous by-products (BF_3 and PF_5): the situation here would be ideal for vapor phase association of the diazonium compound molecular fragments.

In a recent paper by Choa and Zwolinski *(1)* on the thermodynamic properties of acetic acid in the vapor phase, evidence has been found for the existence of H-bonded aggregates or polymeric species of acetic acid in the vapor state. The molecular fragments from some of these diazonium compounds may be behaving in a similar fashion.

Further mass spectral and also SEM studies are presently being carried out with the particulates derived from these diazonium compounds and these data may serve to shed further light on the exact nature of these species. The results obtained from these studies and also those found with other classes of particulate emitting organic compounds will be the subjects of future publications in this area.

SUMMARY

The thermal decompositions of a series of diazonium compounds have been investigated using the new technique of organic particulate analyses (OPA). This extremely sensitive technique makes possible the detection of particulates emitted from the diazonium compounds during thermal decomposition. The submicron particulates derived in this fashion are readily detectable by their influence on the output current of an ion chamber detector or by their effect on the cloud chamber of a condensation nuclei monitor.

Of the 14 diazonium compounds evaluated, 11 were found to exhibit particulation behavior below 190°C. In some cases, very strong particulation was detectable. No apparent correlation between the organic particulation temperature range (OPTR) and their literature thermal decomposition temperatures was evident. Efforts were made to characterize the nature of the particulates using mass spectrometry but this was hampered by the extreme complexities of the spectral patterns.

In terms of their abilities to produce particulates, the diazonium compounds can be placed

in four main groups reflecting their chemical composition, molecular size, and degree of substitution. Vapor phase association of the molecular fragments formed during thermal decomposition might be occurring to produce the required particulate size detectable by the present instrumentation.

ACKNOWLEDGMENT

The authors would like to record their appreciation to S. Anderson for carrying out the thermoparticulation measurements.

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The Oxidation of Benzidine, *o,o'*-Tolidine and *o,o'*-Dianisidine by Manganese Dioxide

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INTRODUCTION

This study of the redox reactions of benzidine, *o,o'*-tolidine and *o,o'*-dianisidine was carried out for two reasons. First, it is supposed that the oxidation mechanism of these substances is related to their carcinogenity *(8)* and, second, it seems that the oxidimetric methods for the determination of these substances *(3, 7)* have some advantages over common diazotization methods *(4)*.

During the oxidation of these substances various changes occur in the nitrogen oxidation state and the aromatic rings can even be destroyed, depending on the reagent used and the reaction conditions. Ceric sulfate *(7)* oxidizes these substances rapidly and quantitatively to the corresponding quinonediimines, which are then very slowly oxidized further by the reagent *(3)*. On the other hand, the quinonediimines formed are oxidized by cobaltic acetate *(5)* so rapidly that the two-electron oxidation step practically cannot be observed.

In the framework of a systematic study of the oxidation of organic substances by manganese in various valence states, attention has so far been paid to the oxidation of benzidine, *o,o'*-tolidine and *o,o'*-dianisidine by compounds of tervalent *(3)* and heptavalent *(6)* manganese. While compounds of tervalent manganese *(3)* oxidize the studied substances with exchange of two electrons to the corresponding quinonediimines, further oxidation of which is virtually negligible, the use of permanganate leads to rapid oxidation of the temporarily formed quinonediimines with basic decomposition of their aromatic system *(6)*.

In this paper, attention is paid to the oxidation of benzidine, *o,o'*-tolidine, and *o,o'*-dianisidine by manganese dioxide in order to compare action of the latter with that of tervalent and heptavalent manganese.

EXPERIMENTAL REAGENTS

Potassium permanganate, 0.01 *N* (equivalent = mol/5). Manganese (II) sulfate, 0.5 *M*. Hydrochloric acid, 1 *N*. Sulfuric acid, 4 *N*. Sodium pyrophosphate, saturated solution. Benzidine, *o,o'*-tolidine, and *o,o'*-

dianisidine, 0.005 *M* solutions in 0.02 *M* hydrochloric acid, were prepared by dissolving the accurately weighed substances in 400 ml of 0.05 *M* hydrochloric acid and diluting with distilled water to 1l. Ascorbic acid, 0.01 *N* solution, was prepared by dissolving 0.9 g of the substances in 1 l of distilled water and its titer was determined daily using bichromate *(2)*. Ferrous sulfate, 0.01 *N* solution in 0.5 *N* sulfuric acid. The solution titer was determined daily with bichromate. *p*-Dimethylaminobenzaldehyde, 1% solution, was prepared by dissolving 1 g of the substance in 5 ml of concentrated hydrochloric acid and diluting with ethanol to 100 ml. For thin-layer chromatography, commercial "Silufol UV 254" plates (Kavalier, Votice) and a benzene–ethanol (4:1) solvent system were employed. All chemicals used were of p.a. purity.

APPARATUS

Potentiometric titrations were performed on a TTT 1 titrator (Radiometer, Copenhagen) using a platinum indicator and saturated calomel reference electrode. Spectrophotometric measurements were carried out using a Unicam SP 800 spectrophotometer (Unicam Instruments Ltd., England) in 1-cm quartz cuvettes. IR spectra were obtained on a UR 10 spectrophotometer (Carl Zeiss, Jena).

PROCEDURE

Study of Stoichiometry

The dependence of the number of electrons exchanged on time. To 10.00 ml of 0.01 *N* potassium permanganate was added 0.5 ml of 0.5 *M* manganese (II) sulfate; hydrated manganese dioxide was formed, to which 15 ml of 1 *N* hydrochloric acid and 5.00 ml of a 0.005 *M* solution of an appropriate amine were added. The reaction mixture was stirred with an electromagnetic stirrer for time *t*, the unreacted manganese dioxide was then filtered off on an S 4 frit, thoroughly washed with seven 10-ml portions of distilled water, and then dissolved on the frit in 50 ml of a fresh mixture of saturated solution of sodium pyrophosphate, 4 *N* sulfuric acid, and 0.5 *M* manganese (II) sulfate (6:1:2). The solution of the pyrophosphate complex of tervalent manganese thus formed was then titrated potentiometrically with 0.01 *N* ferrous sulfate *(1)*. The blank determination was carried out simultaneously, and the number of electrons exchanged was calculated from the difference of the consumptions for the determination and the blank.

Spectrophotometric detection of the oxidation products. The filtrate after the separation of the manganese dioxide (see the previous paragraph) was transferred into a 1-l volumetric flask, diluted with distilled water to the mark, and the absorption spectrum of this solution was obtained in the region from 325 to 700 nm.

Verification of the Oxidation Reversibility

Thin-layer chromatography. To 10 ml of 0.01 *N* potassium permanganate, 0.5 ml of 0.5 *M* manganese (II) sulfate and 15 ml of 1 *N* hydrochloric acid were added. The reaction mixture was stirred, and 5 ml of a 0.005 *M* solution of an appropriate amine were added. The mixture was stirred with an electromagnetic stirrer and, after 2 min, 15 ml of a 0.01 *N* solution of ascorbic acid were added. The solution pH was then adjusted to 7 by adding solid sodium bicarbonate (using a universal pH paper), and the solution was extracted with three 10-ml portions of benzene. The joint extracts were evaporated to dryness at a decreased pressure, the residue was dissolved in 0.5 ml acetone, and about 0.5 μl amounts were placed at the start of the chromatogram. A standard solution, prepared by dissolving 10 mg of the original amine in 10 ml acetone, was simultaneously placed at the start. Ascending chromatography was performed in a ground-glass stoppered bottle, the atmosphere of which was saturated by the vapors of the solvent system using a strip of filter paper soaked in the solvents. The solvent system contained benzene and ethanol at a ratio of 4:1. The detection involved spraying with a 1% solution of *p*-dimethylaminobenzaldehyde.

IR spectroscopy. To 15 ml of 0.1 *N* potassium permanganate were added 7.5 ml of 0.5 *M* manganese (II) sulfate and 20 ml of 4 *N* hydrochloric acid. Then 100 ml of a 0.005 *M* solution of an appropriate amine were added. The mixture was stirred for 2 min with an electromagnetic stirrer and the quinonediimine that formed, together with the unreacted manganese dioxide, was reduced by adding 0.5 g of ascorbic acid. The solution pH was then adjusted to 7 by adding solid sodium bicarbonate (using a universal pH paper), and the solution was extracted with four 25-ml portions of benzene. The joint extracts were evaporated to dryness under vacuum, the residue was dissolved in chloroform, and its IR spectrum was obtained in the region from 600 to 3000 cm^{-1}. The spectrum of the initial diamine was obtained simultaneously.

Analytical Utilization of the Studied Reactions

An indirect determination based on the determination of the amount of oxidant consumed. The procedure for this determination is analogous to that employed during the measurement of the time dependence of the number of electrons exchanged. The sample was treated for 2 min with excess manganese dioxide.

An indirect determination based on determination of the amount of the oxidation product formed. To 10.00 ml of 0.01 *N* potassium permanganate were added 0.5 ml of 0.5 *M* manganese (II) sulfate, 15 ml of 1 *N* hydrochloric acid, and 5.00 ml of a 0.005 *M* solution of an appropriate amine. The mixture was stirred for 2 min with electromagnetic stirrer, the unreacted manganese dioxide was filtered off on an S4 frit, thoroughly

washed with seven 10 ml portions of distilled water, and the intensely colored quinonediimine was titrated visually in the filtrate with a 0.01 *N* solution of ascorbic acid to discoloration of the solution.

RESULTS AND DISCUSSION

The Study of the Stoichiometry

The time dependence of the number of electrons exchanged is given in Table 1; each value is the average of three measurements, the results of which did not differ by more than ±1%. It follows from the table that a two-electron exchange is virtually instantaneous and that deeper oxidation follows on prolonged treatment with excess manganese dioxide. This oxidation is most pronounced with *o,o'*-dianisidine, this can be explained by the predominating +M effect of the methoxy group, which renders the quinonediimine corresponding to *o,o'*-dianisidine more readily oxidizable, compared with benzidine or *o,o'*-tolidine.

The spectra of the oxidation products of the amines exhibit absorption maxima at 425, 437, and 441 nm for benzidine, *o,o'*-tolidine, and *o,o'*-dianisidine, respectively, which are identical with the spectra of the oxidation products of the studied substances after treatment with compounds of tervalent manganese and quadrivalent cerium *(3)*.

Thin-layer chromatography and IR spectroscopy have shown that the products of the oxidation of the studied substances with manganese dioxide can be re-reduced to the original amines by ascorbic acid. The location of the spots after chromatogram development is identical for the original amine and for the substance obtained by the reduction of its oxidation products with ascorbic acid. The IR spectra of the substances obtained by the reduction of the oxidation products with ascorbic acid are also identical with the spectra of the original amines.

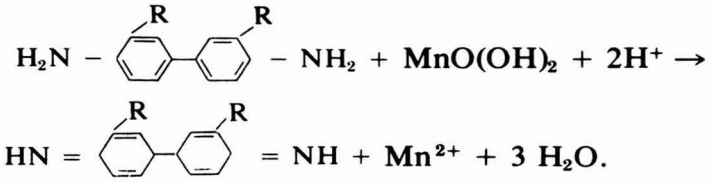
These facts and the finding that the amount of ascorbic acid consumed for quantitative reduction of the oxidation products is identical with the theoretical amount (see the paragraph on the determination of the studied

TABLE 1

THE TIME DEPENDENCE OF THE NUMER OF ELECTRONS EXCHANGED DURING THE OXIDATION OF BENZIDINE, *o,o'*-TOLIDINE, AND *o,o'*-DIANISIDINE BY MANGANESE DIOXIDE IN 0.5 *N* HYDROCHLORIC ACID AT LABORATORY TEMPERATURE

	The number of electrons exchanged		
t (min)	Benzidine	*o,o'*-Tolidine	*o,o'*-Dianisidine
2	2.01	2.00	2.02
5	2.02	2.00	2.08
10	2.07	2.01	2.16
30	2.14	2.03	2.32

substances by ascorbinometric titration of the oxidation products formed) indicate that the oxidation of the studied substances with manganese dioxide obeys the equation:



(R is H in benzidine, CH_3 in *o,o'*-tolidine and OCH_3 in *o,o'*-dianisidine).

Analytical Utilization of the Studied Reactions

The facts described above were used for the development of two indirect titration methods for the studied substances. The first method is based on the ferrometric determination of the unreacted excess manganese dioxide after its separation from the reaction mixture and conversion to the pyrophosphate complex of tervalent manganese. The other method involves the ascorbinometric determination of the quinonediimines formed by the oxidation of the studied substances by manganese dioxide.

The accuracy and reproducibility of the two new methods is summarized in Tables 2 and 3, from which it follows that both methods yield good results for benzidine and *o,o'*-tolidine, while an error, due to further oxidation of the quinonediimine formed, appears with *o,o'*-dianisidine. Further, it can be seen that the method based on ascorbinometric titration of the quinonediimines formed exhibits better accuracy and reproducibility.

It is important from the analytical point of view that only colored substances or substances that react with ascorbic acid interfere in the determination based on the ascorbinometric titration of the quinonediimines formed. This determination is thus more selective than that based on back-titration of the unconsumed manganese dioxide, where all substances oxidizable by this reagent interfere.

Compared with compounds of tervalent manganese or quadrivalent cerium, which have so far been used for the oxidimetric determination of

TABLE 2

THE ACCURACY AND REPRODUCIBILITY OF THE DETERMINATION OF BENZIDINE, *o,o'*/TOLIDINE AND *o,o'*-DIANISIDINE BASED ON THE CONSUMPTION OF MANGANESE DIOXIDE[a]

	Benzidine	*o,o'*-Tolidine	*o,o'*-Dianisidine
Taken, mg	4.606	5.308	6.108
Found, mg	4.555	5.266	5.942
Standard deviation, mg	0.024	0.021	0.053

TABLE 3

THE ACCURACY AND REPRODUCIBILITY OF THE DETERMINATION OF BENZIDINE, *o,o'*-TOLIDINE AND *o,o'*-DIANISIDINE BASED ON THE ASCORBINOMETRIC DETERMINATION OF THE QUINONEDIIMINE FORMED[a]

		Benzidine	
Taken, mg	6.909	4.606	2.303
Found, mg	6.872	4.581	2.270
SD, mg	0.026	0.016	0.012
		o,o'-Tolidine	
Taken, mg	7.961	5.308	2.654
Found, mg	7.919	5.289	2.611
SD, mg	0.026	0.011	0.015
		o,o'-Dianisidine	
Taken, mg	9.161	6.108	3.054
Found, mg	8.834	5.993	2.971
SD, mg	0.044	0.025	0.033

[a] The tabulated values are the averages of ten determinations, from which the standard deviations were also calculated.

the studied substances, an advantage of manganese dioxide is that an excess can be filtered off from the reaction mixture. Therefore, both the titrant consumption and the amount of the quinonediimine formed by the oxidation can be simultaneously determined on a single sample; this basically makes it possible to analyze a mixture of a conjugated aromatic diamine with another substance, which is oxidized by manganese dioxide with formation of products that do not interfere in the ascorbinometric determination of the quinonediimine formed.

SUMMARY

The oxidation of benzidine, *o,o'*-tolidine, and *o,o'*-dianisidine by manganese dioxide was studied in an acidic medium. It has been shown that the studied substances are quantitatively oxidized by the reagent to the corresponding quinonediimines and that this reaction can be utilized for the indirect titrimetric determination of benzidine and *o,o'*-tolidine, based either on ferrometric titration of unconsumed manganese dioxide or on ascorbinometric titration of the quinonediimine formed.

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Spectrophotometric Study of Both the Zimmermann Reaction and the Application of a Corrective Measure for Irrelevant Absorption[1]

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INTRODUCTION

Often, several methodologies are developed for the determination of a single constituent in a biological sample. Usually, these techniques are modifications of a single procedure in which treatment of the sample and the quantification reaction are basically similar in all of the variations. In addition, convenient commercial kit systems derived from the same procedure for that analyte offer the promise of speed, accuracy, and sensitivity, along with certain other claimed advantages. The determination of urinary 17-ketosteroids (17KS) by means of procedures which end up with a *m*-dinitrobenzene (MDB) reaction in alkaline medium to form a Zimmermann chromogen (ZC) presents the analyst with a gamut of possibilities for the clinical laboratory. In these methods, acid hydrolysis vies with enzymatic hydrolysis, extraction with batch chromatography, direct color reactions with extracted color reactions, and mathematical corrections for irrelevant absorption with uncorrected readings at peak maximum. In addition, the quantification step varies considerably in solvent makeup, wherein different organic solvent systems vie with a virtually all-aqueous reaction medium. Therefore, the present investigation was concerned with a consideration of several of these solvent systems, some of which offer evidence of greater sensitivity and selectivity than others offer, a comparison of the worth of extraction of the chromogen generated to nonextraction, and a study of the computations used for mathematical background correction when encountering known and commonly used therapeutic drugs or naturally occurring interferences of urine samples. In addition, the ability to achieve equal chromogenicity

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with the major 17KS such as dehydroisoandrosterone (DHI), etiocholanolone (E) and androsterone (A) is of prime importance, and the study of this phenomenon was included. The composition of the reaction mixture and its effect on reproducibility and molar absorptivity was another important consideration in the selection of a procedure.

MATERIALS AND METHODS

Reagents

The reagents used were identical to those previously described for the aqueous 17KS procedure *(15)*, the Vestergaard modification *(28)* of the Wendland and Lohmann procedure *(30)* and the pyridine–ethyl acetate procedure of Pontius *(23)*. Hyamine 1622 (H1622) solutions: Prepare 2.5 and 5% aqueous solutions. Potassium hydroxide solutions: prepare 2.5 *(23)* and 10.0 *N (15)* aqueous solutions. 17-Ketosteroid standards: Prepare methanolic standards containing 20–90 μg/ml of A, DHI, and E. Sodium hydroxide: Prepare a 0.1 *N* aqueous solution. Pyridine color reagent (Pontius): Dissolve 2.5 g of MDB in 2.5 ml of pyridine *(23)*. Pyridine color reagent (Vestergaard): Dissolve 208 mg of MDB in a mixture of 100 ml of purified pyridine *(27)* and 4.0 ml of 0.1 *N* NaOH *(28)*. Hyamine 1622 color reagent: Prepare a saturated solution of MDB in 5% H1622 *(12)*.

The pyridine used was of gas chromatographic quality, distilled in glass, and packed under nitrogen (Burdick and Jackson Laboratories, Inc., Muskegon, Michigan). All other organic solvents used were of spectral quality.

Procedures

The procedure for the aqueous H1622 reaction was carried out on the standards exactly as previously described *(12)*. Briefly, one-half ml of standard in methanol was treated with 0.2 ml of H1622-MDB reagent. This solution was mixed with 1.0 ml of 10 *N* KOH, which caused a precipitate to form instantly. The turbid mixture was allowed to stand for 15 min to ensure full color formation. It was diluted with 1.5 ml of 2.5% H1622 solution for solubilization, and this resultant clear solution was scanned against a reagent blank from 700–400 nm. When extraction was used, it was carried out by removal of the ZC into ether from the precipitated H1622 step instead of proceeding to the solubilization step.

The procedure for the pyridine reaction was carried out as described by Vestergaard *(28)* by incubating the standards with the alkaline pyridine color reagent for either 3 hr at 37°, or overnight at room temperature.

The mixed solvent procedure was followed as described by Pontius by dissolving standards and the color reagent in pyridine. The reaction was initiated by adding 2.5 *N* KOH, and then the mixture was incubated for 30 min at 45°. The chromogen formed was diluted with a mixture of ethyl acetate and pyridine as recommended *(23)*.

RESULTS AND DISCUSSION

It is now well established that the Zimmermann reaction for 17KS can take place in an aqueous medium, providing that the latter contains a substance such as a detergent to solubilize both the reactants and resultant product, because neither is ordinarily soluble in water *(9,11,12,15,26)*. Therefore, the concept of the use of an aqueous system seems in diametric opposition to a previously registered opinion on the effect of water *(7,10)*. Such ideas concerned with the presence of water led several investigators to develop techniques that excluded water from the reaction medium entirely. In doing so, they created problems that are not encountered in the proposed precipitating detergent system in which a small amount of methanol used to dissolve the extract residue is the only organic solvent required. In the latter circumstance, the need to prepare and maintain an alkali solution as carbonate-free as possible *(8)* was obviated because the carbonate in KOH does not cause turbidity in the aqueous systems *(12,13)*. The use of ethanol in the reaction was also avoided, as were positive interferences due to contaminants of that solvent *(3)* along with distorted and broadened 17KS spectra caused by the presence of this alcohol *(9)*. The reported negative influence of ethanol on the reaction by virtue of color fading *(7,13)* was eliminated. The strong alkali solution of KOH was stable when made up in water. It is not stable when prepared in ethanol *(4,7,13,31)*. The aqueous approach then, in contradistinction to previous opinions, results in favorable comparative reaction characteristics and these will be described.

The reaction mechanism of the precipitating detergent procedure appears to be somewhat different than one carried out under monophasic conditions. When KOH is added to an H1622-MDB mixture, an oily precipitate appears. At the same time, it can be observed that the ZC begins its comparatively rapid formation, comparative that is, to other reported modifications *(13,14,28)*. Several facts can be gleaned from a spectrophotometric study of this mixture. To begin with, there is a loss of 97% of the H1622 from solution to the precipitate as determined from ultraviolet spectrophotometric measurement of the residual H1622 remaining after precipitation with KOH in methanol. When the same process was repeated with MDB present in the sample tube, and the residuum measured in the ultraviolet region against a blank solution containing H1622, it was determined that approximately 60% of the MDB precipitated with the H1622. At anytime during the formation phase of ZC, if the solution were spun free of its precipitate, no purple color appeared in the aqueous fraction. From this, one might infer that the reaction took place on the precipitated H1622-MDB particulate matter, and that probably, the H1622 was in the hydroxide form acting now in the dual role of both required alkali and chromogen adsorbent. The ZC still can be formed in

the solution phase, for when 17KS is added to the lower phase, a solution now denuded by precipitation of most of its active ingredients, a reaction does occur even though it is considerably slower than when the precipitate is present to form a biphasic system. This, then, is a visual description of the aqueous reaction as it appears to take place. The reaction was selected over organic solvent systems for the reasons to be described subsequently.

One advantage of the H1622 aqueous system resides in the speed with which the reaction takes place at room temperature. When dilution resulting in solubilization of the precipitated detergent was tested at various time periods by measuring the absorbance immediately after the solubilization step was completed, the absorbance versus time graph of Fig. 1 showed a plateau of color formation at 10 min. This is considerably shorter than the 45–90 min described for other commonly accepted procedures *(7,13,14)*, and even much shorter when compared to the Vestergaard modification employing 3 hr at 37°, or overnight at room temperature *(28)*. Also, all of the points were obtained without resorting to a darkened container, so the aqueous reaction is not light sensitive, a feature not inherent in several other described methods *(7,14,20)*.

Another advantage of the aqueous system lies in the reproducibility of the color reaction. Reliability in the production of precise absorbance values can be exemplified by the following statistics. The absorbance values collected over a period of several years on 240 observations using a control showed an absorbance range of 0.39 to 0.45 *A*, a mean of 0.416A

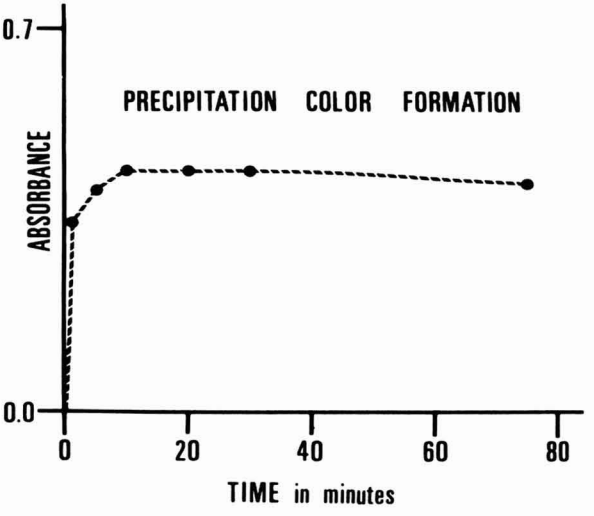

FIG. 1. Time study for the plateau obtained in chromogenic formation by the precipitation color formation procedure.

and a standard deviation of ±0.0103 *A*. Factors such as indeterminate changes in the reaction characteristics of the reagents that might cause poor day-to-day reproducibility of working standard appeared to be obviated.

The problem of the strength of the KOH solution and its effect on the reaction in terms of color generated is eliminated. The normality is kept high in order to precipitate the detergent. The latter adsorbs the chromogen, accelerates the rate of reaction, and increases the concentration of ZC obtained, which is a definite advantage in quantification. The precipitated detergent, once its function as a reaction accelerator is complete, is solubilized by the addition of a small volume of dilute detergent to decrease the alkalinity of the solution. By adhering to a plateau effect of alkalinity during the reaction phase, small changes in KOH normality have little or no effect on the reproducibility of the molar absorptivity attained.

These are several substantial reasons why the H1622 system was selected over other existing methodologies. However, two other procedures have appeared in which claims were made of superior reaction characteristics, higher molar absorptivities *(23,28)*, or more equivalent chromogenicity for the different 17KS *(28)*. In one of these, Pontius *(23)* described a sensitive chromogenic system for 17KS in which pyridine was the medium for the alkaline reaction with *m*-dinitrobenezene, and where the ZC formed was finally dissolved in a mixture of ethyl acetate and pyridine. He described two colors, blue and red, for an all pyridine or all ethyl acetate reaction, respectively. When this individual solvent effect was tested, it was found that when each solvent was used alone it resulted in red colors with a small bathochromic shift and a large hypochromic effect in going from all pyridine to all ethyl acetate. This phenomenon is shown in the bar graph of Fig. 2. Some instability of the final colors was noted for either solvent, so several mixtures bridging the one suggested by Pontius confirmed that his proportions of the two solvents (1:1) resulted in a more stable color reaction. When the stability was studied over an extended time period, the reproducibility was not always quite as good as the sensitivity, unlike the H1622 reaction, and this was related to the extreme purity required for solvents and *m*-dinitrobenzene *(22)*. In addition, pyridine is a rather obnoxious solvent to deal with in a routine situation. Therefore, in spite of its promise of heightened molar absorptivity, this mixed solvent system was abandoned in favor of the more easily reproducible though somewhat less sensitive H1622 color reaction. However, in spite of the described shortcoming, this procedure merits further study because of its sensitivity feature. Further insight into the mechanism of chromogenic sensitization obtained with organic bases may be gained from the investigation presently in progress.

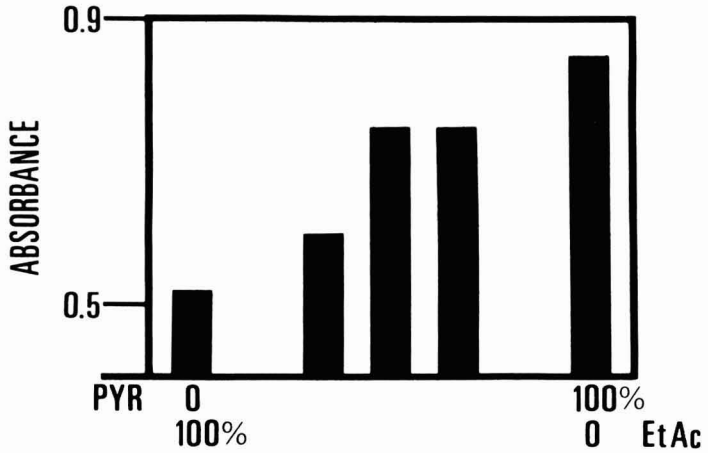

FIG. 2. Bar graph showing the hypochromic effect in going from all pyridine to all ethyl acetate plus solvent ratios of 2:1, 1:1, and 1:2 ethyl acetate: pyridine mixtures.

The Wendland and Lohmann technique *(30)* as described and modified by Vestergaard *(28)* was also studied. This is a procedure that employs just pyridine as the basic organic solvent in the reaction. Again, the involved purification technique required for preparing the highly purified solvent *(27)*, the obnoxious odor, the noxious properties of pyridine *(24)*, and the extremely slow reaction time made this an undesirable procedure for the routine circumstance. Therefore, it too was abandoned in favor of the H1622 reaction, which had none of the negative aspects cited.

The basic argument in favor of the modified Wendland–Lohmann reaction aside from sensitivity, was the claim of equal or nearly equal chromogenicity with either natural 17KS or those derived from 17-ketogenic steroids (17KGS) by oxidative action of bismuthate or periodate *(28)*. On the other side of the coin, descriptions in which different chromogenicities were obtained for several 17KS with modified Zimmermann reactions have been published. In two of these, both using an H1622 system, the reaction of DHI, A and E is shown to result in markedly dissimilar calibration slopes *(9,26)*. This is an important point to consider, in view of the fact that the molecular weights of the three compounds differ by approximately 0.7% at their extremes. If correct, these findings indicate that the marked dissimilarity in chromogenicity would cause measurements made on these mixtures to be empirical rather than absolute values. In one of the two procedures, the order of decreasing chromogenicity is shown as both DHI, E, and A as well as E, DHI, and A, an apparent contradiction *(9)*. In the other, the order of decreasing chromogenicity is E, A, and DHI *(26)*. Interestingly, since both procedures used versions of the H1622 system, one might expect each to show the same order of chromogenicity even though one *(9)* is an automated

continuous flow procedure with heating of the color reaction, while the other *(26)* is a manual procedure with the color reaction carried out at room temperature. Because neither procedure used the precipitating detergent system *(15)*, and because these findings seemingly contradict those described earlier for the H1622 detergent system *(11)*, equivalent amounts of the three 17KS were carried through the described procedure for generating ZC and spectral scans were made. The spectra graphed in Fig. 3 (lower) indicate clearly that the three steroids are equivalent in their chromogenicity for this reaction. Differences that have been encountered were determined to stem from impurities and wetness of the standards, primarily the latter. When equivalent concentrations of the acetate, sulfate, and free forms of DHI were treated in a similar manner, they also resulted in superimposable spectra. These are graphed in Fig. 3 (upper). One might conclude that purity of the steroids, or differences in the reaction characteristics, caused the two versions *(9,26)* of the H1622 technique to have varied chromogenicity and the original *(11)* to have equivalent chromogenicity with 17KS.

Several purification devices for the final chromogen obtained can be encountered in procedures for the determination of 17KS. Among these are included the use of batch chromatography using short XAD-2 columns

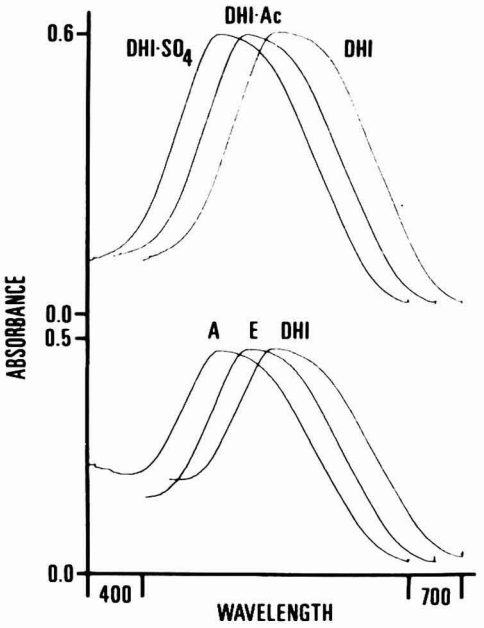

FIG. 3. The upper half shows the equivalent chromogenicities obtained for dehydroisoandrosterone (DHI), its acetate (DHI-Ac) and sulfate (DHI-SO4) and the lower half shows the same findings for dehydroisoandrosterone (DHI), androsterone (A) and etiocholanolone (E).

(*16*), extraction of the ZC after it is formed (*15,29*), and finally, the use of either the Allen correction (*2*) or the Medical Research Council correction (*20,25*), two mathematical systems for the elimination of irrelevant absorption of a special type. To test the validity or necessity of such actions, several experiments were carried out with the first used to test the efficacy of XAD-2 columns, where the column is a substitute for the extraction step. Two drugs, Cephalothin and Tegretol, previously tested because of reported interference in the Zimmermann reaction (*12*) were selected for this pilot study. They differ markedly from each other in aqueous solubility and chemical structure. The least soluble compound of the two, Tegretol, also has no methylene carbonyl group, which has been reported as necessary for a Zimmermann reaction (*19*). An amount of Tegretol approximating that expected to be encountered in urine samples was placed onto an XAD-2 column in acid medium as described (*5,16*). The solution represented an acid hydrolysate, in which 10 ml of sample mixed with 1 ml of concentrated HCl, is heated in a boiling water bath, cooled, and treated in a batch chromatographic manner as follows. After the liquid had percolated through the XAD-2 column, the latter was washed three times in order with 10 ml aliquots of water, 2 *N* sodium hydroxide, and 20% ethanol, respectively, and then the 17KS were eluted with 10 ml of 95% ethanol. The residue from 0.5 ml of the eluate was carried through the H1622 reaction system and the results are graphed in Fig. 4. It can be seen from the final results, that Tegretol was retained by the column, was resistant to the action of the three wash solutions, was eluted off with the 95% ethanol, and finally, was reacted to form a ZC. It must be remembered that in the described column procedure (*5*), 10 ml of eluate, not 0.5 ml, is normally used in the Zimmermann reaction, and the use of a 20-fold amount of Tegretol would have resulted in a reaction of swamping proportions. Obviously, this is a good example of an interference handled in unselective and nonspecific fashion in both the separation and chromogenic phases, so the use of the XAD-2 column for purification in a procedure of this type may be open to question. In this instance, the process concentrated the interference and ensured that it appeared in the reaction system. which is exactly the opposite of what such a purification step should accomplish. When the same procedure was repeated with Cephalothin, it was found that Cephalothin did not end up in the ethanolic eluate, as it was probably removed in column washings. It has previously been shown (*12*) that unmodified Cephalothin will not extract into ether from acidified urine, and therefore, the column adds nothing to the procedure with regard to this particular drug. However, Cephalothin, when added to urine and subsequently treated by periodate for a 17KGS determination, will form extractible products that will interfere in the ensuing colorimetric Zimmermann procedure. Since this modified compound is

extractible, unlike the parent compound, it may adsorb onto the column and imitate 17KS action as was shown for Tegretol. Tegretol is extractible into ether and will mimic the actions of a 17KS just as it does in the batch chromatography procedure. The difference in actions between the two drugs in the process described may be attributed in part to aqueous solubility. Cephalothin is very soluble in water whereas Tegretol is only slightly soluble. The former is apparently easily washed from the column, whereas the latter is as quantitative in its recovery and reaction as is a 17KS treated in the same manner. Some attention should be directed toward those compounds or drugs, like Tegretol whose actions and reactions parallel those of a 17KS. Otherwise, attempts to purify the analyte or even the resultant chromogen may result in wasted efforts. To recapitulate a bit, if Cephalothin or Tegretol are excreted unchanged, they will have roles in the 17KS procedure that are dependent on both their aqueous solubilities as well as their extractive and reactive capabilities. Neither drug was investigated as a function of potential metabolites that may react differently than the parent compounds from which they were derived.

The second kind of purification that may be used is extraction of the ZC *(28)*. Hopefully, this selectively leaves behind any other interfering colors. A favorable feature of the described aqueous system is the ease with which the precipitated ZC can be extracted by simple solvents such as ether *(12)*. The fact that there is very little of other organic solvents to

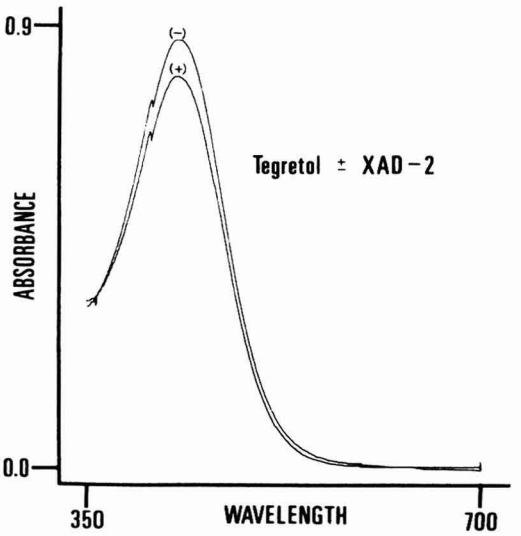

FIG. 4. Tegretol recoveries by H-1622 precipitation reaction, obtained by direct analysis without the use of a column (−XAD−2) and compared to adsorption onto a column (+XAD−2), followed by elution.

partition between the two phases also simplifies the extraction picture. If the organic solvent of reaction, ethanol in most cases, is salted into an extractant by the addition of a strong salt solution thereby diluting it, the potential signal size will be decreased and the medium in which the chromogen is isolated may yield a less stable color system than would be obtained if the extraction solvent were not diluted in this fashion. The kit system of Brinkman *(5)* based on the work of Levy and Schwartz *(16)* uses this technique. For example, several ml of extractant, methylene chloride, was used to remove the ZC from 20 ml of solution containing 11.4 ml of ethanol. But since 4 ml of the 20 ml was saturated NaCl, the extraction volume was increased several fold because the ethanol salted into it resulted in a smaller measured absorbance. In the case of the H1622 reaction, the absorbance of the extracted chromogen is virtually identical to that of the unextracted aqueous reaction mixture because little organic solvent was involved in the reaction phase. Therefore, in this procedure, the measurement in terms of the absorbance signal is almost toally unaffected if one resorts to extraction to attempt color purification. In addition, the ratio of the molar absorptivities obtained is 2:1 for the H1622 technique *(12,15)* over the Brinkman procedure *(5)*. However, there is some question as to the efficiency of purification by means of such a step. If an interfering color is generated and also extracted along with the 17KS chromogen, then the extraction step would seem to be an exercise in futility. That this extraction of an interfering chromogen obtained by a simultaneous reaction with MDB is possible has already been described for Tegretol *(12)*. It undergoes a Zimmermann reaction and its ZC extracts as easily into ether as do the 17KS reaction products. Another drug has been shown to extract partially after forming a ZC *(12)*, and therefore, the resort to extraction as a purification device may not always work as planned.

The last phase in purification involves mathematical corrections for irrelevant absorption. If a compound could mimic the actions and reactions of a 17KS, or if a nonspecific, usually yellow color appeared in the final reaction, it might still be possible under favorable spectral conditions to eliminate the error portion of the spectrum. The use of such mathematical corrections for interfering colors in the Zimmermann reaction is an important issue because they are frequently used *(2,20)*. However, an available mathematical test for validating whether or not the Zimmermann reaction can be corrected by the Allen computation has not been as commonly applied *(1)*. In effect, that method entails the use of an ever widening wavelength spread in the Allen correction using small wavelength increments to calculate whether a straight line relationship for the interference is present in a potentially contaminated spectrum *(1,21)*. The Allen correction equation is:

V. Ph. 12. July 1977

$$A_{Corr} = \frac{A_{520} - A_{580} + A_{460}}{2} ,$$

whereas the similar valdiating equation is:

$$A_{Corr} = A_{520} - \frac{[A_{520} + (n \times nm)] + [A_{520} - (n \times nm)]}{2}$$

Where n = number of 10-nm increments used to determine linearity *(1)*. In the described validation system, it was demonstrated by means of nine calculations on a standard plotted as the abscissa versus the same number of calculations on a sample plotted as the ordinate, that the plot could be used to determine whether or not a straight-line interference had been encountered *(1)*. This is a formidable effort to be made for each sample in conjunction with the numerous Allen calculations. And after all the absorbances were found and all the calculations made, if the resultant plot were not linear, indicating that the interference characteristics were nonlinear, there is no clear alternative approach for the analyst. There remains the dilemma of whether one should use peak readings to calculate, or whether one should still use the Allen correction even though the interference did not linearly bridge *(2)* the wavelength span. It is questionable that this validation technique is a common procedure for those who use the Allen correction formula. Yet, it has been pointed out that in order to use the Allen correction effectively, every ZC generated from a urinary sample that is to be corrected by the Allen calculation must be tested in this manner *(1,21)*.

A study that exemplifies the problems inherent in a limited correction for irrelevant absorption as represented by the Allen calculation or the Medical Research Council calculation *(20)* was carried out by contaminating DHI with the drugs, Cephalothin and Tegretol. Table 1 contains the recoveries of dehydroisoandrosterone made with these mixtures. The drugs were introduced directly into the reaction mixture containing the 17KS with the deliberate intent of contaminating the color produced by the latter. All results were calculated from peak absorbance readings, Allen corrected (AC) readings, or the somewhat similar appearing Medical Research Council (MRC) correction *(20,25)*. The Medical Research Council correction differs from the Allen correction in that it assumes that a correction is only necessary on the ultraviolet side of peak maximum, whereas the Allen correction also considers the infrared side. Looking at the Tegretol results, it is obvious that the Allen correction does not work because the calculated percent recovered continuously decreases as the Tegretol concentration increases resulting in a negative value in the worst instance. If those same results had been obtained from peak readings alone, then the values recovered would increase in a positive manner, but

would not have resulted in nearly as large an error even in the worst instance. However, if the readings were made a little to the infrared side of the peak maximum, the interference would decrease almost entirely, whereas the sensitivity would only decrease by a rather small percentage. This latter effect has been described previously *(12)*. When the same sort of experiment was carried out with Cephalothin, the results are somewhat better when the Allen correction was used, but nevertheless, they are far too inaccurate to be reliable in diagnosis. This again points out the inadequacy of the Allen correction.

Negative and positive interferences have been shown to occur *in vitro* in the MDB reaction for 17KS *(6)*. Although these aspects of the reaction are known, the fact that these cannot all be corrected by the Allen calculation are not fully described. The Allen correction can work only with an interference that is represented by a linear bridge of the 17KS spectrum as a positive absolute error. There are compounds that are known to result in relative errors in colorimetric reactions in which the interference does not itself react with the reagents. They take part in the reaction in a relative way by enhancement *(17)*, or inhibition *(18)*, and in these circumstances, cannot be corrected for by a device such as the Allen correction. They

TABLE 1

COMPARISON OF ALLEN CORRECTION, MEDICAL RESEARCH COUNCIL CORRECTION AND PEAK ABSORBANCE READINGS USING MIXTURES OF DEHYDROEPIANDROSTERONE AND EITHER TEGRETOL OR CEPHALOTHIN[a]

DIH (pres.)	Drug (pres.)[b]	Found (AC)	Found (direct)	Found (MRC)
90	25(T)	68.5	92.0	72.3
90	50(T)	50.0	93.3	54.7
90	75(T)	32.2	95.7	38.8
60	75(T)	1.0	67.6	—
40	75(T)	−20.2	45.6	—
20	75(T)	−39.2	26.8	—
20	50(C)	25.1	34.6	—
20	100(C)	31.3	48.8	—
20	200(C)	41.5	78.7	—
20	300(C)	54.6	107.9	—
40	100(C)	50.2	67.7	—
60	100(C)	70.3	88.8	—
90	100(C)	—	—	66.4
90	200(C)	—	—	44.6
90	300(C)	—	—	26.9
90	400(C)	—	—	3.4

[a] All values are mg/ml.

[b] T and C are Tegretol and Cephalothin.

would have to be handled by a separation scheme, or more easily, by the method of internal standardization.

Perhaps several disadvantages of the Allen correction can be summarized as follows based on the data reported elsewhere and in this manuscript. To begin with, it severely decreases the measured peak signal, and thereby, may magnify those errors due to small differences in absorbance. The severely decreased signal can be even smaller, if the reaction conditions are poorly chosen so that the spectrum is considerably widened. The wider the spectral curve, the higher the absorbance readings to be subtracted. Some curves are so wide that subtracting one-half the sum of the 460 and 580 nm readings from the 520 nm reading results in virtually no delta absorbance reading at all even for very high concentrations of 17KS *(9)*. It only works with a linear bridge of the 17KS spectrum and it breaks down if nonlinear interference appears at either side of the spectrum or beneath it. Validation of a linear interference is difficult, uncertain, and formidable *(1,21)*, therefore, few if any investigators resort to the multiple calculations required. Consideration is not given to a negative error of the absolute type, or negative or positive errors of the relative type, but only to absolute linear spectral errors of the positive type. That is an extremely narrow limitation of the correction. Finally, if one uses the Allen correction and in the process generates a value different from the one obtained by peak measurement, then the assumption is made that the peak calculation has been corrected in some advantageous manner. In fact, little hard evidence has ever been presented that a different answer is necessarily a more correct one.

SUMMARY

A spectrophotometric study of the Zimmermann reaction carried out in several solvent matrices has been described. The advantages of an aqueous system over those containing pyridine are detailed. Purification devices such as batch chromatography and color extraction used commonly in 17KS procedures have been examined, and the results obtained are shown as evidence that they are not always effective if the actions and reactions of the interference mimic those of 17KS. Finally, the study of mathematical corrections for irrelevant absorption resulted in values that indicated that the concept of such corrective action was not always easily applicable to the determination.

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On-Line Data Acquisition System for Perkin–Elmer 240 Carbon, Hydrogen, and Nitrogen Analyzer[1]

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Automation of carbon, hydrogen, and nitrogen analyses has greatly improved the efficiency of elemental analysis laboratories. The Perkin–Elmer 240 system, described by Condon *(4)*, has gained wide acceptance and demonstrated excellent reproducibility *(19)*. The instrument has proven satisfactory for a number of specialty applications, including analysis of petroleum compounds *(20)* and fluorine-containing organic materials *(15)*. Modification of the combustion train *(5)*, capsules for volatile samples, and attempts to improve ladle design *(1,22)* have continued to improve performance. The 240 has compared favorably with other commercially available CH and N analyzers, but slightly less reliable than classical methods *(3)*.

While other approaches to CH and N analysis have been suggested *(2,8,23)*, and improvements have been suggested for the combustion process *(10,12,24)*, the most significant advances and technological improvements have been made in automation and computerization of CH and N analysis. The combustion–gas chromatography system *(17)* has been modified to allow sample calculations with a dedicated computer *(21)*. A digital computer has been used to automate vacuum apparatus *(6)*, and a desk calculator can be used to assist determinations based on titration procedures *(16)*.

Electronic integration is used to assist in sample calculations with the F&M Model 185 *(7)*, and a data printing system has been employed to record output signals *(11)*. The Perkin–Elmer 240 system was updated to provide digital readout from a dedicated computer *(18)* and automated *(13)*. An in-lab minicomputer was used with three 240 instruments and two electronic microbalances *(14)*. This last system is elegant in design; however, it can be justified only for extremely high sample load situations. In

[1] A similar paper was presented at the International Symposium on Microchemical Techniques (1973) held at The Pennsylvania State University, University Park, Pennsylvania, August 19–24, 1973.

most cases, the computerization has failed to provide for permanent records or report formats suitable for direct transmission to the sample submitter.

Since 1971, the Perkin–Elmer 240 in this laboratory has been operated on-line with a remote computer. The system was integrated to allow the computer to monitor the output of the three thermal conductivity detectors, the Cahn electrobalance, and teletype used for communicating specific instructions to the computer. Recently, the computer system was updated by changing over to a PDP-15/76 computer equipped with an A to D converter and acquiring a Silent 700 (Texas Instrument) teletype. This teletype is always available for other uses *(9)*. Advantages to be realized from on-line computer acquisition of a routine analytical determination are: (A) cost savings, and (B) improved quality and reliability of results. The CH and N analyzer monitoring system consists of three programs: (A) data acquisition, (B) numerical analysis, and (C) input/out control. The teletype is used to enter sample identifications, to initiate specific tests such as standardization runs, and to receive output reports.

MATERIALS AND METHODS

The three detector pairs from the PE-240 are connected to the PDP-15/76 via shielded cable networks. Ten 1-sec readings of zero and sample signals are taken from each detector during an analysis. The readings for each output are statistically averaged.

All input signals required to calculate percent CH and N are directly transmitted to the computer. For automatic sample weight entry, and adjustable dc voltage proportional to the sample weight is fed to the computer. The Cahn G-2 Electrobalance (Ventron Instruments Corporation) is equipped with a dual mass potentiometer. The first potentiometer is the normal one for this balance; the second is connected to a 15-V dc power supply. The output of the power supply is set to read the same as the balance with course (10 k $\Omega \pm 3\%$) and fine (200 $\Omega \pm 3\%$) adjustment voltage dividers. The sample weight is entered into the system by pressing the interrupt button. The interrupt device is constructed with a momentary contact switch.

One shielded twisted pair of Belden electronic cable connects each detector with the analog/digital converted (ADC). These conductors pass through doubly shielded terminal boxes. In addition, each input signal is connected to an RC passive filtering network. This network is shown in Fig. 1.

Two pairs of unshielded cable are also used. One pair is wired to a relay inserted in the instrument circuit, which is triggered simultaneously with

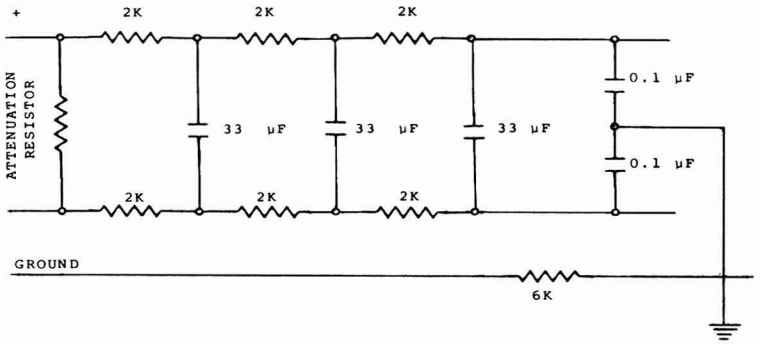

FIG. 1. Cell passive filter network.

the instrument DETECT lamp. The relay generates a contact closure that is sensed by the computer as a process interrupt, which initiates the data acquisition program. A monitor lamp is also lighted to confirm the interrupt (via the second pair of conductors). The lamp is turned off when the monitoring sequence is complete. The unshielded cables also pass through terminal boxes.

Upon completion of each run, the computer generates a complete report of the analysis. This report is sent directly to the sample submitter. Periodically, duplicate summary reports are generated, one for a central file, and the other for the analyst's notebook. The available computer storage space will retain 92 CH and N analyses.

Ten computer options are available to change any operating procedures the analyst deems necessary. To initiate any one of 10 options currently available, the operator types in the following:

*CHN/N/

where N represents any number from 1 to 10 corresponding to the options below.

Option 1 is used for blank runs. No additional information is required, and valid entries are confirmed with the message:

BLANK RUN—SYSTEM READY

The blank analysis is printed at the conclusion of the run. After the first blank run, all subsequent results are compared with the average of all previous results. If the three new blank values are within preset tolerances of the corresponding current average for that detector, a new average is calculated. The report contains the new values and the updated averages. If the tolerance limit is exceeded for any detector, an error is printed, and the report then shows the new values, but the previous averages. A blank run can be run any number of times and an example is shown in Fig. 2.

Option 2 is used for standard runs. After the initial request, the computer prints the message:

*CHN/1/

Blank Run—System Ready
Carbon–Hydrogen–Nitrogen Analysis
Date: 8-15-75 Time: 8:06 a.m.
Blank 2 is out of range.
Blank 3 is out of range.
New blank average of 1 run.

	Average	*This Run*
N	54.	64.
C	112.	41.
H	246.	113.

FIG. 2. Blank run.

STANDARD RUN—ENTER NUMBER/NAME/TOTAL WT/N PCT/C PCT/H PCT/

The operator then enters the requested information. Any number of letters, numbers, or special characters can be used for the sample name and number. The first 15 and 25 characters are printed on the final report for the number and name, respectively. A slash separates all entries.

The next four values must be numerical characters. Nothing else is allowed. In addition, each value must contain four characters, or less, with blanks being noted as zeros. The decimal point is assumed to be after the fourth digit. If an error is detected, the following message is printed:

ERROR IN INPUT DATA

It is then necessary to re-enter the entire line of information. It is not necessary to re-enter the initial operator request code.

If a valid entry is made, the operator is notified with the message:

CHN SYSTEM READY

A sample report for a standard run is shown in Fig. 3. Once again, the current results are printed, together with the cumulative average of sensitivities for each detector, if the new values fall within preset tolerance limits. Errors are printed, and a new average is not calculated if the tolerance limits are exceeded. For example,

STANDARD 1 IS OUT OF RANGE

A standard also can be run any number of times.

Option 3 is used for unknown samples. After the following message:

UNKNOWN RUN—ENTER NUMBER/NAME/SAMPLE WEIGHT/

the operator must enter the requested information according to the same restrictions described under Option 2. Valid input is acknowledged by printing:

*CHN/2/

Standard Run—Enter Number/Name/Total Wt/
N Pct/C Pct/H Pct/
3/AA/2487/1036/7109/671/
CHN System Ready
Carbon—Hydrogen—Nitrogen Analysis
Date: 8-15-75 Time: 8:48 a.m.

Standard Number 3
Standard Name AA
Average of 3 Runs

	Average	*This Run*
N	7.027	7.016
C	19.368	19.401
H	62.377	62.648

FIG. 3. Standard run.

CHN SYSTEM READY

A report, which is shown in Fig. 4, is printed at the conclusion of the test. The analysis is then stored permanently in a data file for future reference. When the sample weight is monitored directly by the computer, zero is inserted for this parameter.

All previously generated files are then searched for identical sample numbers and names. If any are found, the results are averaged and a second report is printed. Unknown analyses (as well as blanks and standards) are retained in a data file until they are erased.

Ninety-two unknown analyses can be stored at one time. If an attempt is made to exceed this number, the following message is printed:

NUMBER OF RECORDS IN DATA FILE EXCEED 92

The program is terminated, and the 93 run is lost.

Option 4 is used to erase the current cumulative average of blank values for the three detectors. Upon completion, a message confirms the operation and prints the current tolerance limits for blank runs.

BLANK FILE ERASED
BLANK CONSTANTS: N = XX.XXX C = XX.XXX H = XX.XXX

No provision has been made for the operator to alter these tolerances values. They can be changed only from the computer room.

Option 5 is identical to Option 4, but clears the current cumulative average of standard sensitivity factors. The confirming message is:

*CHN/3/

Unknown Run—Enter Number/Name/Sample Wt/
A-2127/7648-106-1 Wayner/2901/
CHN System Ready

Carbon—Hydrogen—Nitrogen Analysis
Date: 8-15-75 Time: 9:02 a.m.

Sample Number A-2127
Sample Name 7648-106-1 Wayner
Sample Weight 2901, mg

Element	*Sensitivity* (($\mu v/\mu g$)	*Weight* (μg)	*Percent*
N	7.027	120.245	4.144
C	19.368	1460.780	50.354
H	62.377	150.122	5.174

FIG. 4. Unknown run.

Carbon—Hydrogen—Nitrogen Analysis
Date: 8-15-75 Time: 16:18

Blank Values: N = 64.000 C = 60.000 H = 113.000

Sensitivity Values: N = 7.027 C = 19.368 H = 62.377

Total Wt.	N-Wt.	C-Wt.	H-Wt.	N-%	C-%	*H-%*
A-2127 7648-106-1 Wayner						
2901.	120.245	1460.780	150.122	4.144	50.354	5.174

FIG. 5. Summary.

STANDARD FILE ERASED
SENSITIVITY CONSTANTS: N = XX.XXX C = XX.XXX H = XX.XXX

Similarly, the sensitivity constants, or tolerances, cannot be altered by the operator.

Option 6 clears the unknown sample data file. A confirming message is also printed.

UNKNOWN FILE ERASED

Option 7 is included to allow for the sequential execution of Options 4, 5, and 6 with one request.

Option 8 calls for a summary to be printed of all information contained in all three data files (Fig. 5). This option is normally used at the end of each working day.

Option 9 permits the CHN operator to enter three blank values or three standard sensitivity factors directly into the appropriate data files. After his initial request, the following message is printed:

ENTER 1/ AND 3 BLANK VALUES - OR - 2/ AND 3 SENSITIVITY FACTORS

Input values must be separated by a slash, four numbers must be entered, and except for blanks and numeric characters, only plus signs, minus signs, and decimal points are accepted. This message results if an error is encountered:

ERROR IN INPUT DATA

The message:

NEW [BLANK VALUES / SENSITIVITY FACTORS] ACCEPTED

is printed if a valid entry is made.

Option 10 permits the analyst to enter two coefficients for a second-order equation relating millivolt values of the second mass potentiometer to micrograms of sample weight. The coefficients are stored in the appropriate file.

The procedure for determining the coefficients involves clearing past coefficients from the file and generating millivolt readings for corresponding microgram settings over the entire calibration range. When no coefficients are contained in the file, the vernier on the Cahn electrobalance is set for specific values. At each milligram value, the interrupt is activated and the teletype will printout the measured millivolt reading. The calibration range is from 0 to 3000 μg, and data points are generated at 200.0 μg increment over the range.

The pairs (microgram, millivolt) of data are fitted to the best second order equation through the origin with a standard statistical computer program. Coefficients from this equation are placed in the file in scientific notation by Option 10 by typing

*CHN/11/A/B/C/X/Y/Z

where A,X equal the numerical values of coefficients, first and second term, respectively; B,Y are the signs of exponent of coefficients, first and second term, respectively; C,Z are exponents of coefficients, first and

second term, respectively. For example, if the coefficients were 9.904893 and 0.00660917, the entry would be:

*CHN/11/0.9904893/+/1/0.660917/−/3/

If the coefficients are accepted, the computer will print out:

WT,MICROGRAMS = (0.9904893E+01)*X + (0.6609170E−03)*X**2
X = UNCORR WT, MICROGRAMS

Under experimental conditions, X is an analog signal from the power supply.

RESULTS

To demonstrate that the on-line data acquisition system is as good as, or better than, manual calculation procedures, a number of standard compounds were analyzed by both techniques. Data for three standard compounds were collected over a 4-week period. Ten samples of each compound were analyzed. Comparison of results from data acquisition system to results obtained from manual calculations for benzoic acid, acetanilide, and nicotinic acid are presented in Tables 1, 2, and 3, respectively.

TABLE 1
RESULTS FOR BENZOIC ACID

	Manual calculation	Data acquisitions system	Theoretical
Carbon (%)	68.69	68.84	68.846
Standard deviation[a]	0.27	0.18	—
Hydrogen (%)	5.10	5.03	4.952
Standard deviation	0.10	0.11	—

[a] Standard deviation, N = 10.

TABLE 2
RESULTS FOR ACETANILIDE

	Manual calculation	Data acquisitions system	Theoretical
Carbon (%)	71.46	71.19	71.09
Standard deviation[a]	0.30	0.17	—
Hydrogen (%)	6.86	6.82	6.71
Standard deviation	0.13	0.13	—
Nitrogen (%)	10.88	10.58	10.36
Standard deviation	0.16	0.19	—

[a] Standard deviation, N = 10.

TABLE 3
RESULTS FOR NICOTINIC ACID

	Calculation	Data acquisitions system	Theoretical
Carbon (%)	58.31	58.52	58.54
Standard deviation[a]	0.06	0.03	—
Hydrogen (%)	4.15	4.17	4.094
Standard deviation	0.13	0.02	—
Nitrogen (%)	11.72	11.70	11.38
Standard deviation	0.20	0.19	—

[a] Standard deviation, N = 10.

DISCUSSION

The on-line data acquisition system provided a significant cost savings compared to manual calculation methods. When manual calculations were employed, 18 samples could be analyzed daily for CH and N content. If the on-line system is used, 25 samples can be processed per day. This represents a 38% improvement in overall efficiency.

SUMMARY

The Perkin–Elmer 240 CH and N analyzer was adapted for on-line data acquisition with a remote computer. Sample weights from a Cahn electrobalance are directly entered into the computer via a power supply and interrupt system. The outputs from the three thermal conductivity detectors are monitored for 10 sec and signal averaged by the computer. The computer takes all data necessary for the calculation of percent CH and N and prints out a final report on a Silent 700 remote terminal. The report is suitable for direct transmission to the sample submitter. Duplicate reports are printed for permanent analytical records. The precision of results obtained with on-line data acquisition compare favorably to results calculated manually, and provides a 38% improvement in efficiency. The on-line systems employ a PDP-15/76 computer, which is available for other tasks. This provides an additional cost savings compared to the alternate approach of purchasing a small dedicated computer.

ACKNOWLEDGMENTS

We wish to thank Mrs. M. L. Decker, Mr. J. F. Gentile, and Dr. J. B. Worthington for their help and assistance in programming, systems engineering, and consultation in developing the initial on-line CH and N analysis system.

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MICROCHEMICAL JOURNAL 21, 70–74 (1976)

An Efficient Inexpensive Multisleeve Liquid Scintillation Counting System[1]

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The rapidly expanding use of the relatively inexpensive ^{3}H-labeled compounds has prompted a parallel search for simpler, less expensive, but highly efficient liquid scintillation counting methods. Most users employ the standard glass or plastic 20-ml counting vial, which can be expensive in terms of both the cost of vials and scintillation fluid. Two concepts investigated to date to minimize the cost of scintillation counting of aqueous samples have been the use of plastic bags (*3*) and minivials (*1,5*). Both of these systems place the counting vessel inside a standard glass vial and have the advantages of (1) reduced cost (smaller volumes), and (2) disposability of smaller volumes. The minivial system appears to have better reproducibility and efficiency than the plastic bag (*1,3,5*) when dealing with aqueous solutions.

The purpose of this study was to carry the minivial several steps further and investigate the applicability of a multisleeve counting system using ^{3}H-labeled compounds of aqueous solubility. This system was designed to see if readily available and less expensive glass vessels, which fit into the conventional 20-ml glass liquid scintillation vials, would maintain counting efficiency in small volumes of counting fluid; and further, to determine at what point counting efficiency would be markedly reduced relative to vessel size and volume.

METHODS AND MATERIALS

Reagent. BIO-SOLV (Beckman) counting solution was used in these studies. The solvent plus scintillator-solution mixture was as follows: 15 g of PPO, 2,5 diphenyloxazole and 0.9 g POPOP, *p*-bis (2-(5-phenyloxazoly) benzene per 3 l of toluene. The BIO-SOLV counting solution was prepared by adding 500 ml of BIO-SOLV (BBS-3) to 3 l of the prepared scintillation solution (*2*). Counting fluid was placed into the vessels by means of volumetric pipettes or by a 50-μl gas chromatography syringe.

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Radioactive Materials. The materials used were [^{3}H]adenosine (to give high counts) ^3H_2O (for low counts). The quantities added to the counting vessels were 5 μl of [^{3}H]adenosine or 10 μl of ^3H_2O. Either a 5 or a 10-μl Linderstrom, Lang, Levy pipette was used to add the radioactive material.

Counting Vessels. (A) Glass scintillation vials, volume 22 ml and internal diameter of 20 mm (Beckman Extra Vial, 22 mm, $65.00/500) to which 15 ml of counting solution was added. (B) Specimen vials with black molded plastic screw caps, volume 4 ml and internal diameter of 8 mm (Fisher Scientific, 45 × 15 mm, $6.84/144) to which 3 ml of counting solution was added. (C) Culture test tubes, volume 1 ml with internal diameter of 4 mm (Fisher Scientific, 50 × 6 mm, $13.11/1000) to which 0.75 ml of counting solution was added. (D) Blood collecting tubes cut to a length of 48 mm and fire polished at one end having a volume of 0.2 ml and a internal diameter of 2.7 mm (Arthur H. Thomas Co., 75 × 3.9 to 4.1 mm, $9.45/100) to which 0.15 ml of counting solution was added. (E) Capillary tubes, cut to a length of 50 mm and fire polished at one end to give a volume of 50 μl and an internal diameter of 1 mm (Arthur H. Thomas Co., 74.5 × 75.5 mm × 1.1 × 1.2 mm, $24.30/2000) to which 30 μl of counting solution was added.

It is known that the height of the counting solution is an important factor in efficiency *(4)*. Therefore, the volume of counting solution in all the various counting vessels was kept constant approximately at 3/4 full. It is also important when dealing with BIO-SOLV to keep the ratio of water and counting solution proportional in order to keep the solution clear *(2)*.

Multisleeve Counting Systems. In the experimental procedure, first, the radioactive material was added, then the counting fluid. Each vessel was shaken to distribute the radioactivity. The multisleeve counting systems (Fig. 1) used included: system A, standard glass 20-ml counting vial; system B, specimen vial inserted into the standard counting vial; system C, culture test tube fitted into sleeve system B; system D, blood collecting tube fitted into sleeve system C; system E, capillary tube fitted into sleeve system D.

Equipment. All counting was performed on a Packard liquid scintillation spectrometer (Model 3320).

RESULTS AND DISCUSSION

The mean counts (Table 1) and efficiencies (Fig. 2) were relatively the same for systems A, B, and C with an observed decrease in these parameters for systems D and E. This pattern was observed for both the [^{3}H]adenosine and the ^3H_2O. Thus, counting efficiency was maintained within the same range in volumes from 15 to 0.75 ml, while a decrease in efficiency did not occur until a volume of 0.15 ml was reached. We observed that the decrease in mean counts and effiency for systems D and E

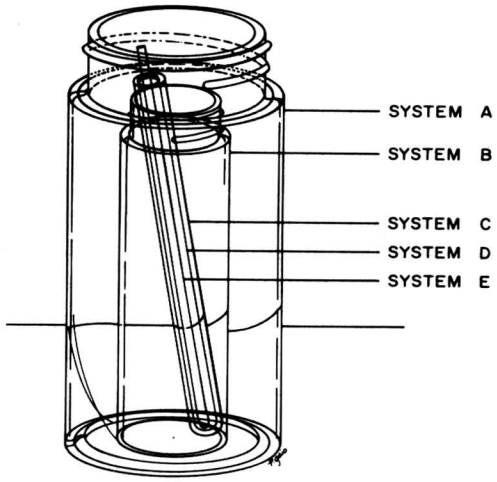

FIG. 1. Illustration of the multisleeve counting systems showing the relationship of the various counting vessels to one another. During experiments, systems C, D, and E were vertical and not slanted as illustrated.

TABLE 1
A COMPARISON OF THE VARIOUS MULTISLEEVE COUNTING SYSTEMS ON MEAN COUNTS

System	Volume of counting fluid	Mean counts 0.2 min [^{3}H]adenosine[a]	Mean counts/min ^3H_2O[a]
A	15 ml	642,000	7200
B	3 ml	630,000	7300
C	0.75 ml	570,000	6800
D	0.15 ml	410,000	3500
E	30 μl	440,000	3500

[a] Each sample was counted to give 1% variability of observed counts and the mean represents 9 different samples.

when compared to A, B, and C was related more to the decreasing volume of counting fluid than decreased transmittancy of light due to the thickness of glass layers involved in this system. This was tested by inserting just the blood collecting tube or the capillary tube into the standard glass vial and comparing it to systems D and E.

An interesting phenomenon was observed when testing system B. If, when inserting the capped specimen vial with an opening of 15 mm instead of 16 mm, which we used, the cap will catch and suspend the specimen vial 1.5 cm from the bottom of the counting vial. This suspended vial counted with almost identical efficiency to the nonsuspended vial (system B). A similar idea was reported previously *(5)*. This may indicate that there

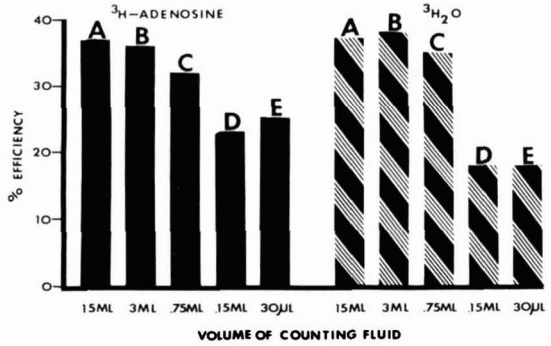

FIG. 2. Comparison of efficiencies of the various multisleeve counting systems. High counting efficiency is maintained by systems A (15 ml), B (3 ml), and C (0.75 ml) while a decline in counting efficiency is observed with systems (D (0.15 ml) and E (30 μl).

is a critical region observed by the scintillation counter, and below this region you lose efficiency while above it you neither gain nor lose efficiency.

The use of multisleeve counting systems B and C can offer several advantages over the use of just the standard glass counting vial. These systems maintain counting efficiency, for both high and low count radioactivity, within the same range obtained with the standard glass of vial but at volumes of 3 and 0.75 ml as compared to 15 ml. In a research laboratory, investigators would be able to interchange from one system to another depending on their needs. The use of systems B and C would reflect decreased expense in both counting vials and counting fluid cost. Finally, disposability of vials and their decreased volume of content would be much less of a problem.

SUMMARY

This paper reports a method that conserves greatly on the cost of counting radioactive samples for liquid scintillation counting. It accomplishes this by using several glass sleeves that are much more inexpensive than conventional counting and/or minivials, and because volumes of counting fluid are reduced the cost of the counting fluid is also markedly reduced. The usual vial system (A) uses 15 ml of counting fluid with 37% efficiency for tritum; the minivial uses .75 ml with 37% efficiency while 0.15 and 30 μl volumes gave approximately 21% efficiency. Another feature of this system is the capacity to reuse the outer sleeve(s) and dispose of the smaller counting vessel. This means that less counting fluid and glass will have to be disposed of in the environment.

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Microdetermination of Carbon and Hydrogen in Organic Compounds Using Flushed-Oxygen Combustion Tube

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INTRODUCTION

Radmacher and Haverath *(3)* established a quantitative method for organic carbon and hydrogen macroanalysis by applying Voigt's principle *(6)*, originally introduced in the determination of halogen and sulfur in organic compounds. It was later modified to microanalysis by Pfab *(2)*. In the latter method, a sample gasified by heat decomposition in a nitrogen stream was transported to a nozzle where oxygen gas was flushed for oxidation at high temperature. The carbonaceous residue produced in the nitrogen stream was then removed by passing oxygen as the finishing process. The combustion tube was stuffed with silica wool without any oxidation catalyst and was heated up to 1050°C. The flushing oxygen effectively shortened the time for the oxidation of the sample. The present author has taken account of an advantage of this combustion method and further modified the configuration of the flushing nozzle.

Comparing several types of the flushing nozzles, double installations of the bound-capillaries type was found to be most effective *(5)*. The proposed method has decreased the time for combustion, and has reduced the temperature of the combustion furnace from 1050°C with the Pfab method to 850–900°C, while preserving the equal oxidation efficiency. Removal of nitrogen oxides derived from some samples containing nitrogen has been currently attained by placing lead dioxide or manganese dioxide as the internal or external absorbent, respectively, It was noted, however, that these absorbents sometimes caused poor reproducibility, probably because of their characteristic behaviors for water and carbon dioxide. Therefore, the use of reduced copper was suggested to eliminate the above shortcomings, but the lifetime was expected to be considerably shortened when supplying excess oxygen during the combustion. The present author has found the minimum demand of oxygen for combustion with the proposed method and has suggested the necessary amount of reduced copper that would endure the appropriate numbers of analysis.

MATERIALS AND METHOD

Apparatus. The combustion tube was made of silica 12 mm in diameter and 30 cm in length, followed by the reduction tube and the absorption train as illustrated in Fig. 1(a). Either of the two nozzles fused into the middle portion of the combustion tube consisted of several capillaries tightly packed in a quartz tube through which oxygen gas flushed out. Some details of this portion are depicted in Fig. 1(b). The flow rates of the oxygen towards the entrance of the combustion tube and the two nozzles, I, II, were regulated by means of needle valves, V_1–V_3, respectively. The combustion tube was wound separately with nichrome wire, as were the sample heater and the stationary heater, the latter being covered with Ceramic Fiber (Isolite Insulating Product Co., Osaka) for heat-insulation, and kept at 850°C. The present author employed the same primitive style of combustion system as the preliminary test.

The entrance and the exit sides (5 cm. length) of the reduction tube (8.5 ± 0.2 mm i.d.) were filled with Sulfix (silver particles dispersed in cobaltic oxide granules) and were heated at 500°C, while a 16 cm length at the middle of the reduced copper was heated at 500−600°C. After the reduced copper was exhausted by absorption of oxygen and reduction of nitrogen oxide, it was reactivated at 200°C (± 10°C) in a hydrogen stream, the flow rate of which was regulated. A Mariotte bottle was used to check the flow rate through the combustion system and the absorption train. Two three-way stop cocks E_1 and E_2, were installed for replacing the oxygen flow with the nitrogen, and vice versa. The absorption tubes were designed by Sakamoto *(4)*.

Reagents. Oxygen, nitrogen, reduced copper wire (Merck), Sulfix (Kishida Chemicals, Osaka), Anhydrone, Ascarite.

Procedure. The nitrogen is first passed through the combustion tube and the absorption train, and the latter is disconnected and weighed. The absorption tubes are again connected to the combustion system. A sample of approximately 1 mg weighed in a platinum boat is put into the combustion tube. Oxygen gas is then supplied to the combustion tube via three valves V_1, V_2, and V_3, with the individual flow rates of 5, 30−40, and 10−5 ml/min, respectively. The sample is heated to 800°C for 2 min with the nichrome wire or a burner. The oxygen is quickly replaced with nitrogen by which all the combustion products are transported to the absorption train within 3 min. The absorption tubes are disconnected and reweighed.

RESULTS AND DISCUSSION

The quantity of oxygen demanded for the complete oxidation varied greatly from sample to sample. Some samples were determined with a very small volume of oxygen. For example, 2−3 mg of caffeine could be determined with oxygen amounts of 5−10 ml/min from the entrance, 3−5 ml/min from the nozzle I, and 2 mg of antipyrine could be determined with

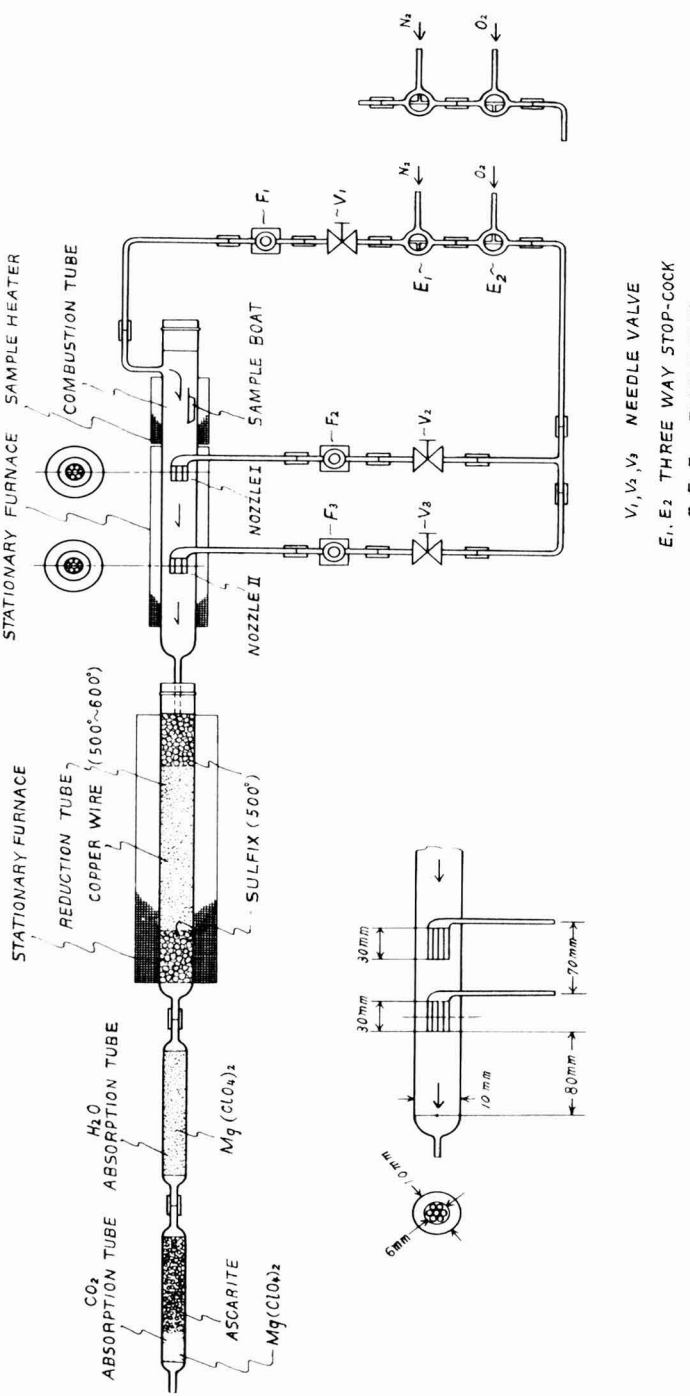

FIG. 1. (a) Schematic diagram of apparatus for determination of carbon and hydrogen in organic compounds by flushed-oxygen combustion tube method. (b) Nozzles of combustion tube.

5–10 ml/min of oxygen from the entrance and the nozzle I, while oxygen was not supplied from the nozzle II. In the case of cholesterol, however, 5 ml/min of oxygen supplied from the entrance, 35–40 ml/min from the nozzle I, and 10–5 ml/min from the nozzle II allowed only 1–1.5 mg of the sample to be determined. It has been revealed in the case of cholesterol, as illustrated in Fig. 2, that the complete oxidation of the sample was attained when more than nine times as much oxygen was supplied from the two nozzles combined than was supplied from the entrance of the combustion tube. The efficiency of employing two nozzles already has been mentioned *(4)*, but in the previous report, neither a flow meter nor a needle valve was applied to each nozzle, so that the quantity of the oxygen from each nozzle was not actually measured.

Taking into consideration the possible influence on the oxidation efficiency of the volume of oxygen flushing from each nozzle, the author examined the interrelation between the flow rate of oxygen from each nozzle and the results obtained. This type of nozzle effectively oxidizes the gasified sample with multiple jet streams from heated bound-capillaries. But it also must be noticed that the higher flow rate of flushing oxygen rapidly oxidizes the surface of the reduced copper, resulting in incomplete reduction of the nitrogen oxides. A compromise was made, therefore, so that the main stream of oxygen was supplied from the nozzle I, and the supplementary flow was from the nozzle II, to ensure the complete oxidation. Figure 3 shows the efficiency of the flow rate of oxygen from the nozzle II. When 1 mg of cholesterol was burned with the flow rate of oxygen at 5–8 ml/min from the entrance, and 30 ml/min from the nozzle I, more than 7 ml/min of oxygen from the nozzle II, was required to attain the complete oxidation.

Another effort was made to save the total volume of oxygen during the combustion. Most of the organic samples were decomposed in the sample heater within 1 min and it was found that 2 min of heating was sufficient to

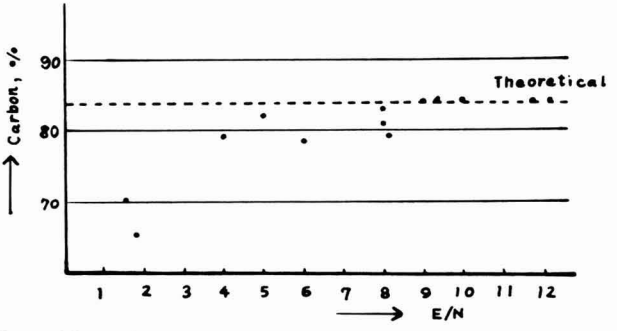

FIG. 2. Effect of combined flow rate of oxygen from two nozzles relative to flow rate from entrance. N/E: Flow ratio of two nozzles/entrance. Sample: cholesterol.

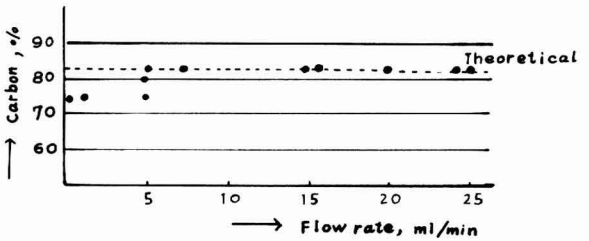

FIG. 3. Effect of flow rate of oxygen from nozzle II. Sample: cholesterol. Oxygen from entrance: 5 ml/min; nozzle I: 30 ml/min.

burn practically all of the organic samples of approximately 1 mg containing so-called refractory substances. The total volume of oxygen used in this method was 120–130 ml/analysis. On the other hand, an organic compound of 2 mg hydrocarbon demands not more than 2–5 ml of oxygen for its oxidative decomposition.

It may be readily construed that the rapid decomposition of the organic sample in the sample heater produces a reductive vapor zone that will be transported towards the nozzle I as a plug flow. The reductive vapor will be diluted by the flushing oxygen at the nozzle I, which necessarily results in excess oxygen throughout the time during which the reductive vapor merges with the flushing oxygen. Very high dilution with the flowed oxygen will ensure the oxidative condition throughout, but it will increase oxygen waste. Nozzle II then, plays as an important role in reducing the flow rate of nozzle I, and at the same time, acts as a safety guard for the occasional imperfection of the oxidative condition at the nozzle I. The allotment of the flow rates at the two nozzles described in the proposed procedure is one of the practical suggestions of this paper. The author has observed the average life of the reduced copper for 15–20 sequential analyses until it was reactivated by passing hydrogen.

When the sample size was raised to 2 mg, it sometimes caused imperfect combustion, yielding carbonaceous soot at the end of the combustion tube. To overcome the trouble, it was thought that the sample should be decomposed much more slowly, or that the flow rate of the flushing oxygen at nozzles I and II should be increased. The author suggests, however, to limit the sample size at around 1 mg, taking advantage of the rapid combustion and long life of the reduced copper. This sample size also calls for somewhat higher precision in weighing the samples than in the case of ordinary microanalysis. Therefore, it is advised to use a Mettler UM6, or an electrobalance, having equal precision for sampling, and a Mettler M5 for weighing the absorption tubes. However, standardization of the sensitivities of these balances with the same origin is essentially required in this case. Some analytical results with representative standard samples are listed in Table 1.

TABLE 1
ANALYTICAL RESULTS OF SOME STANDARD SAMPLES BY PROPOSED METHOD

Sample	Sample wt. (mg)	Theory C (%)	Theory H (%)	Found C (%)	Found H (%)	Deviation C (%)	Deviation H (%)
Hippuric acid	1.288	60.33	5.06	60.57	4.69	+0.24	−0.37
	1.194			60.45	4.87	+0.12	−0.19
p-Toluene-sulfonamide	1.287	49.11	5.30	49.36	5.08	+0.25	−0.22
	1.079			49.06	5.16	−0.05	−0.14
Caffeine	1.268	49.48	5.19	49.60	5.40	+0.12	+0.21
	1.035			49.30	4.94	−0.18	−0.25
Cholesterol	0.986	83.87	11.99	83.52	11.98	−0.35	−0.01
	1.145			83.94	11.86	+0.07	−0.13
2,4-Dinitrochlorobenzene	0.791	35.58	1.49	35.29	1.80	−0.29	+0.31
	0.941			35.44	1.32	−0.14	−0.17
Antipyrine	2.023	70.19	6.43	70.01	6.17	−0.18	−0.26
	1.966			70.45	6.42	+0.26	−0.01
p-Bromoacetanilide	1.297	44.89	3.77	45.10	4.03	+0.21	+0.26
	1.347			44.90	3.77	+0.01	0

The proposed method is classified basically as the empty tube method of Belcher and Ingram *(1)*. But they employed a vertical combustion chamber heated by a special furnace, and carried out the decomposition of the samples for 10 min. For the removal of nitrogen oxides, they also used manganese dioxide. Therefore, considerable modification has been made with the proposed method, in that the time of the decomposition of the sample is reduced to 2 min, and the nitrogen oxides are removed by reduced copper, which contributes to reproducible analytical results.

SUMMARY

A rapid method of microanalysis for carbon and hydrogen in organic compounds using an empty combustion tube fused with two nozzles for flushing oxygen and employing reduced copper for the reduction of nitrogen oxide was investigated. A sample was decomposed rapidly in a sample heater for 2 min, and the gasified vapor was transported to the stationary combustion zone heated at 850°C, where oxygen was flushing from two nozzles, the oxygen flow was then replaced with nitrogen for 3 min until all the combustion products were swept out towards the absorption train. The sample size was suggested to be around 1 mg for this rapid method, using a Mettler UM6, or an electrobalance having equal precision for the sampling.

ACKNOWLEDGMENT

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MICROCHEMICAL JOURNAL 21, 82–84 (1976)

Indirect Determination of Picogram Amounts of Germanium in Pure Water by Flameless Atomic Absorption (Mo) Spectroscopy

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INTRODUCTION

The determination of minute amounts of Ge is of importance in the analysis of ultra pure substances, semiconductor and laser materials.

It is possible to increase the sensitivity of determination of Ge by using an indirect (by Mo) method *(1)*. A method has been reported for the successive indirect (by Mo) determination of picogram amounts of P and As, employing specially made microextractors and flameless atomic absorption determination of Mo *(3)*.

This paper describes the indirect determination of picogram amounts of Ge, using the same analytical technique *(3)*. 2-Ethylhexanol already employed by Paul *(2)* was used as an extractant of germanomolybdic acid.

MATERIALS AND METHODS

Apparatus. The apparatus and conditions for AA determination have been described *(3)*. The microextractors made from polyethylene film *(3)*, being more practical, were preferred for extraction and reextraction.

Reagents. High purity reagents and deionized water, redistilled from a quartz still were used. The Mo standard solutions employed for calibration (as described *(3)*) and washing liquid were similar to these, previously used *(3)*. Ge (IV) stock solution was prepared by fusing 0.144 g of GeO_2 with 0.7 g of anhydrous Na_2CO_3 in a platinum crucible, dissolving the melt in 10 ml of water, adding 0.02 ml of 7 *N* H_2SO_4 to the solution, and diluting to 100 ml. The solution of 5.10^{-4} μg Ge/ml was prepared by dilution and used for preparing simulated samples, containing 25pg Ge in 0.4 ml ultrapure water. These samples were used to develop the following procedure.

Procedure. A water sample (0.4 ml), containing about 25pg Ge (IV) are introduced into a 2 ml Teflon beaker; 0.02 ml 18*N* H_2SO_4, and 0.02 ml 3% ammonium molybdate are added, and the mixture is allowed to stand for 5 min. A solution of 0.3 ml. 2-ethylhexanol is added, followed by the extraction of the molybdogermanic acid (in the large microextractor) by mixing for 1 min. The water phase is discarded. The organic phase is washed for

0.5 min with 0.3 ml washing liquid (by mixing in the same microextractor). This washing is repeated with a fresh portion of the washing liquid. The water phases are discarded and the molybdenum is reextracted from the organic phase by mixing it with 0.02 ml 4% NH_3 in the small microextractor. 20 μl of the ammonia extract are injected with a 20-μl micropipette into the prepared graphite furnace for the *AA* molybdenum determination. The Mo concentration is computed by the calibration equation (see Eq. (1) below) after substraction of the blank reading obtained using the procedure for Ge, but with the substitution of 0.04 ml ultrapure water for the sample. The Mo values obtained must be divided by 16 to obtain the amounts of Ge present in the original water sample.

RESULTS

The calibration equation used was *(3)*:

$$C = 4.0 \cdot H \tag{1}$$

where C is the amount of Mo present (ng/20 μl); and H is the peak height, in millivolts.

Table 1 presents statistical data resulting from the determination of samples containing 25 pg Ge. The mean of the molybdenum concentration conforming to 25 pg. Ge (on the basis of the stechiometric ratio Ge:Mo = 1:12) is $\bar{C}_{Mo}$ = (0,360 ± 0,067) ng. *(3)*.

The performed investigation showed that variations in pH within the region of 0.3−0.7 do not affect germanomolybdic acid recovery.

Possible interference resulting from the presence of heteropoly acids forming ions [P(V), As(V), Si(IV)] in the test solution and from the addition of ethanol to the solution before extraction of germanomolybdic acid into 2-ethylhexanol were studied: a 100-fold excess of P (V), a 50-fold excess of As (V), a 5-fold excess of Si (IV), and also, the addition of 0.07 ml of ethanol does not increase the error of the determination of 25 pg Ge.

On the other hand, a 100-fold excess of Ge(IV) does not interfere with the determination of 10 pg P(V), and 25 pg As(V) (3,4).

Obviously, this allows a successive determination of picogram amounts of P(V), As(V), and Ge(IV) in the presence of one another, namely: at

TABLE 1
DETERMINATION OF 25 pg. Ge. STATISTICAL DATA

Number of variates (peak heights)	Mean of variates $\bar{H}_i$, mV ± 5.10^{-3}	Mean of concen Mo $\bar{C}_{Mo}$ (ng/20 μl)	Variance V_{CMo}	Relative standard deviation S_r	Error $\epsilon_{\alpha,K}$ (α = 0,95; k = 5)	Percentage error (± $\epsilon/\bar{C}_{Mo} \cdot 100$)
6	$7,2 \cdot 10^{-2}$	$28,8 \cdot 10^{-2}$	$4,3 \cdot 10^{-3}$	$23 \cdot 10^{-2}$	$6,9 \cdot 10^{-2}$	24.0

first, P(V) and As(V) are determined (*3*); then, a small volume (0.02–0.03 ml) of H_2SO_4 is added to adjust the required pH, and Ge(IV) is determined by the procedure described above (starting from the 5-min interval before extraction).

In conclusion, it must be said that, as in the determinations of P and As (*3*), the success of the analysis for Ge requires minute attention to careful technique and to the prevention of contamination.

SUMMARY

The method involves the conversion of the Ge into yellow 12-germanomolybdic acid, extraction into 2-ethylhexanol, the decomposition of the heteropoly-compound with aqueous NH_3, and the back extraction of the liberated Mo into aqueous solution for flameless *AA* determination. 25 pg Ge in 0.4-ml samples of ultrapure water were determined with a percentage error of ±24.0%.

ACKNOWLEDGMENT

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MICROCHEMICAL JOURNAL 21, 85–91 (1976)

A Rapid Measure of Primary Antigen Binding Capacity of Antiserum

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INTRODUCTION

The observation that binding of a small ligand (cetylpyridium chloride) to bacteria could be quantitatively measured in a matter of minutes utilizing the concept of steady state transport suggested that a similar approach might be used in the measurement of primary antigen–antibody interaction. *(1)*.

The method allows the same measurements of primary antigen–antibody interaction as equilibrium dialysis, but again, in a much shorter period of time. The antigen–antibody mixture is placed in a minibeaker chamber through which hollow fiber membranes pass. The fiber membranes provide a large surface over which saline or buffer may be pumped at a constant rate. Under these conditions, unbound antigen passes from the chamber into the hollow fibers. The amount of antigen appearing in the effluent is directly proportional to the unbound antigen, allowing calculation of both the total free and the total bound antigen in the sample.

MATERIALS AND METHODS

Minibeaker. The Minibeaker (BioFiber 50 Minibeaker, Bio–Rad Laboratories) consists of an outer chamber through which hollow cellulose fibers pass. The nominal molecular weight cut-off of the fiber pores is 5,000.

Reagents. Radioactive ε-Dinitrophenyl-L-lysine (Phenyl-3,5-^{3}H) was purchased from New England Nuclear (sp act 1.1 Ci/mol, Lot No. 307-291). Cold ε-Dinitrophenyl-L-lysine was purchased from Sigma. Commercially prepared antibody to ε-Dinitrophenyl-L-lysine was purchased from Miles Yeda LTDC (1 mg Ab/ml, Lot No. R63-72).

Experimental Method. The method was previously described for the determination of the amount of CPC bound by bacteria *(1)*. Modifications in this study include the use of nonpyrogenic saline (0.9%) as the carrier effluent and the tritium labeled Ag. At the beginning of an experiment, the Minibeaker was washed for 5 min with saline. Samples (7 ml each), containing only Ag or Ab preincubated with Ag, were added to the outside

chamber with a syringe, and a 10 drop fraction was collected 2 min after the addition of the sample to the outside chamber. The buffer flow rate through the system was a constant 3 ml/min and all experiments were carried out at 25°C. Control experiments indicated that the steady state transport plateau was attained at $T = 2$ min, and that a 10 min incubation period was a sufficient period of time to permit equilibration of the Ag and Ab molecules. Control experiments to determine the effect of normal sera in this system were also conducted.

Calibration Curve. A calibration curve was obtained with different concentrations of Ag alone in the outer chamber. The steady state concentration of the dialysate (cpm) was plotted as a function of the total concentration of Ag (ng/ml) added to the outside chamber.

Ag–Ab Binding. In the Ag–Ab binding experiment, the concentration of free Ag $[Ag]_F$, was calculated (ng/ml) by comparing $T = 2$ min determinations obtained from Ag–Ab solutions with the calibration curve. The amount of Ag bound by Ab $[Ag]_B$, was calculated from the following equation:

$$[Ag]_B = ([Ag]_T - [Ag]_F) / C,$$

where $[Ag]_T$ is the total concentration of Ag (ng/ml), and C is the concentration of Ab (μg/ml).

Competition Experiments. Varying concentrations of cold Ag were incubated for 10 min with a constant concentration of Ab (1 μg/ml). Radioactive Ag (0.025 μCi/ml) was then added to the sample and incubated for an additional 10 min before injection of the sample into the Minibeaker. The ratio of $[Ag]_B/[Ag]_F$ was then plotted as a function of the cold concentration of Ag (pg/ml).

Regression Analyses. The regression analyses were performed with SAS (Statistical Analysis System, North Carolina State) on an IBM 370 computer.

RESULTS AND DISCUSSION

Figure 1 depicts the calibration curve obtained when varying concentrations of [^{3}H]DNP-lysine are placed in the outer chamber of the Minibeaker. There is a linear relationship between the concentration of free antigen in the chamber and the steady state concentration found at $T = 2$ in the effluent.

Confidence intervals for repetitive experiments fall on the line indicating that if any nonspecific binding of reagent to the Minibeaker occurs it is a constant factor.

Once the relationship between antigen concentration and steady state conditions was established, studies were conducted to determine if one could measure the presence of a specific Ab (anti-DNP) in this system. To obtain Fig. 2, concentrations of antibody of either 1 or 10 μg/ml were used

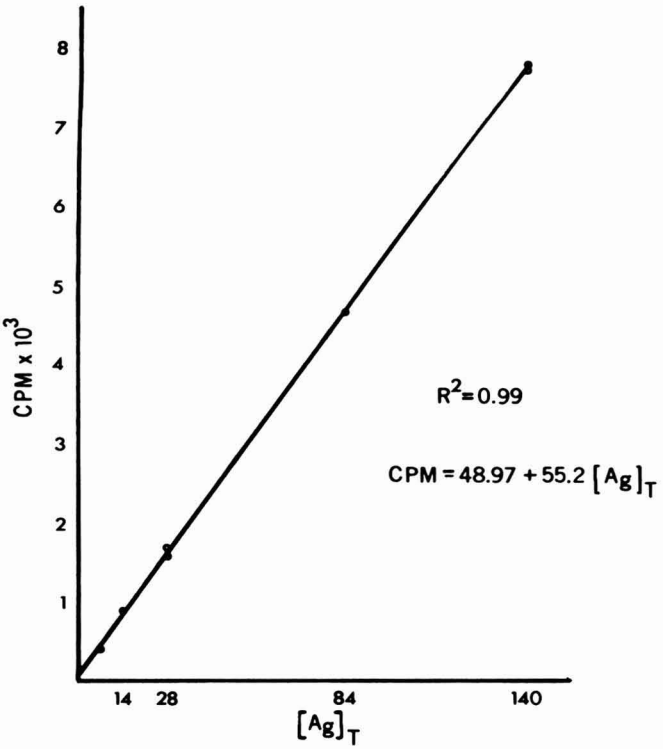

FIG. 1. Calibration curve for Σ-DNP-lysine. CPM values were obtained for the steady state concentrations of Σ-DNP-lysine in the dialysate. Concentrations on the abscissa are the total concentrations of Σ-DNP-lysine in the outer chamber $[Ag]_T$. The points represent experimental values (○, single observation; ●, two observations); the line was obtained from a least squares fit to CPM = $b + m\ [Ag]_T$.

in conjunction with antigen concentrations varying over a range of from 7 to 252 ng/ml (Table 1). The bimodal curve obtained when 1 μg of Ab is used indicates that the Ab population (unpurified) used in these studies consisted of at least two affinities, the detection of which was dependent upon ratios of Ag to Ab. One may speculate that a similar effect with concentrations of 10 μg/ml antibody is masked by the lower Ag/Ab ratio existing with the higher concentration of Ab. It appears that one may utilize this method to obtain the same information as provided by equilibrium dialysis, however, more quickly and more conveniently.

The data in Fig. 3 demonstrates a linear relationship between the amount of antigen bound and varying concentrations of Ab. Comparison of lines A, B, C indicate that the greater sensitivity for measurement of Ab occurs in what is probably antigen excess. Nonetheless, the kinetic relationship is such that antihapten antibody may be quantitated. The advantage offered by this method as compared to passive methods that are

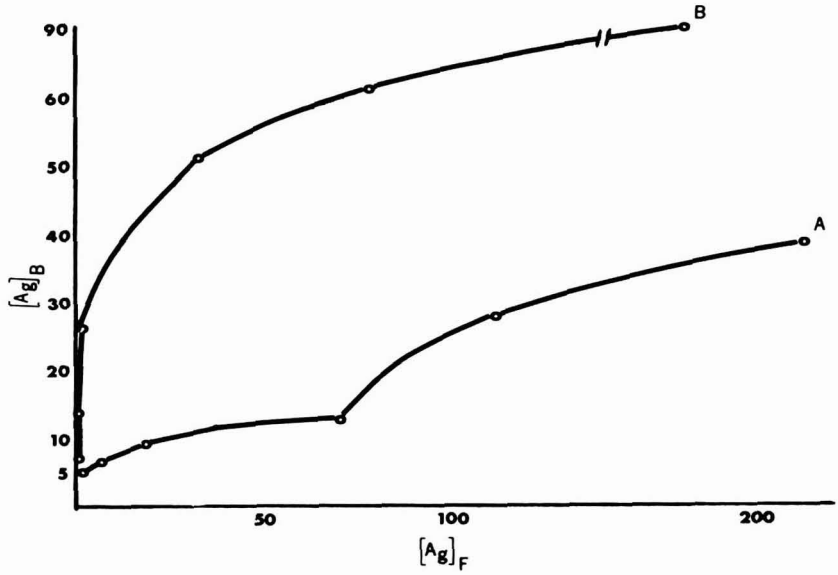

FIG. 2. Binding isotherm for the interaction of Σ-DNP-lysine with anti-DNP. The points represent experimental observations (line A, anti-DNP = 1 μg/ml; Line B, anti-DNP = 10 μg/ml). Concentrations on the ordinate are the total concentrations of ε-DNP-lysine bound in the system, $[Ag]_B$. Concentrations on the abscissa are the total concentrations of ε-DNP-lysine free in the system, $[Ag]_F$.

TABLE 1

EFFECTS OF CONCENTRATION ON THE BINDING OF ε-DNP LYSINE BY ANTI-DNP

$[Ag]_T$ (ng/ml)	$[Ag]_F$ (cpm)	$[Ag]_F$ (ng/ml)	[Ab] (μg/ml)	$[Ag]_B$ (ng/μg Ab)
7	165	2.10		4.9
14	475	7.72		6.3
28	1093	18.91		9.1
84	4003	71.63	1	12.3
140	6238	112.20		27.8
252	11837	213.50		38.5
7	44	0		0.7
14	68	0.34		1.37
28	161	2.03		2.60
84	1874	33.06	10	5.10
140	4409	78.99		6.10
252	8990	161.98		9.00

dependent upon secondary antigen–antibody interaction is the same as that offered by equilibrium dialysis *(4)*. However, the total time needed to obtain a quantitative measure of antibody is much shorter.

Radioimmune assay of a variety of biologically active substances has

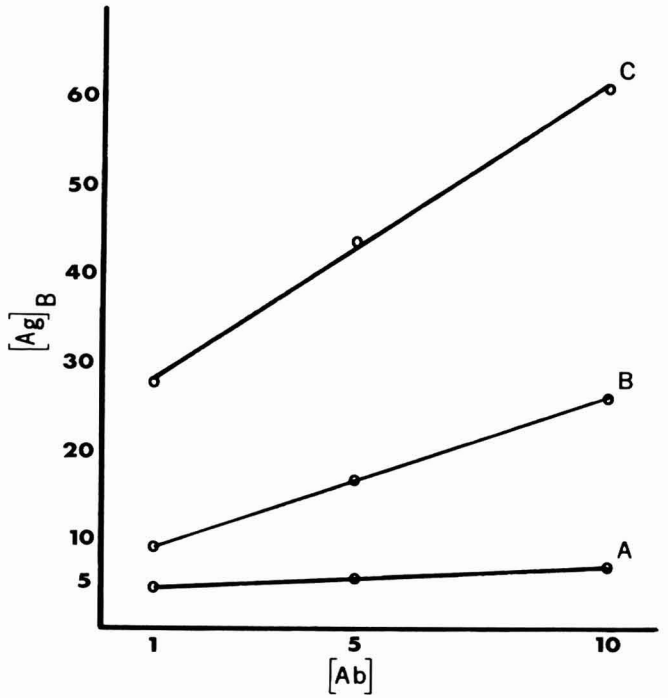

FIG. 3. Calibration curves for anti-DNP. $[Ag]_B$ (Ag_T − Ag_F) values were obtained for the steady state concentrations of ε-DNP-lysine in the dialysate with selected concentrations of anti-DNP. The relationship of 3, ε-DNP-lysine concentrations is also shown (line A, $[Ag]_T$ = 7 Ng/ml line B, $[Ag]_T$ = 28 Ng/ml line C, $[Ag]_T$ = 140 Ng/ml)

become an important technique in both clinical and experimental studies. Several authors *(2,3,5)* have pointed out several problems that exist, or procedural steps that are of critical importance: (1) damage to tracer compounds used during prolonged incubation periods, (2) nature of the separation systems used, (3) sensitivity and precision, and (4) time needed to obtain the results. As indicated in Fig. 4, it is possible to construct a standard curve for varying amounts of unlabeled antigen (Table 2). The *B/F* ratio is plotted against the level of cold DNP-lysine (a fairly common practice in RIA procedures) added to a system containing a constant amount of [^{3}H]DNP-lysine. The latter suggests the possibility of eliminating (1) second incubations (i.e., ppt. Ab), (2) prolonged separation techniques from saturation analysis, and (3) time needed to obtain results. The use of steady state transport requires only three procedural steps: (1) incubation of the antiserum with the cold (unknown) antigen, (2) a second incubation following the addition of labeled antigen, and (3) establishment of steady state transport with collection of the effluent sample.

The system utilized for this work required a total incubation time (steps 1 and 2) of 10 min each, and an additional 2 min to reach steady state. The

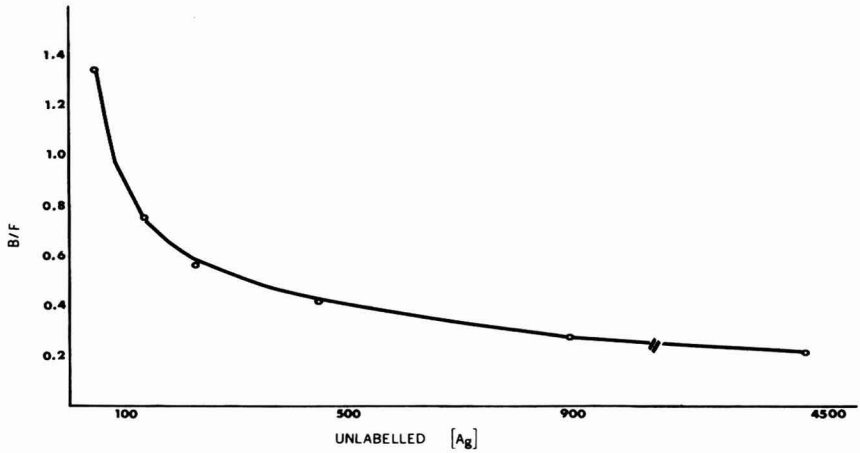

FIG. 4. Standard Curve for DNP assay. *B/F* ratio in standard solutions is plotted as a function of known ε-DNP-lysine concentration. From *B/F* ratios in assayed samples corresponding DNP concentrations are determined by reference to the standard curve. Unlabeled [Ag] is expressed in pg/ml.

TABLE 2
COMPETITION STUDY[a]

[Ab] (μg/ml)	[^{3}H]DNP μCi	DNP (μg/ml)	$[Ag]_F$ (cpm)	$[Ag]_F$ (pg/ml)	$[Ag]_B$ (pg/μg Ab)	$[Ag]_B/[Ag]_F$	Cold DNP (pg/ml)
1.0	0.025	7.0	210	26.14	36.33	1.34	43.58
1.0	0.025	7.0	270	35.79	26.67	0.75	133.8
1.0	0.025	7.0	297	40.06	22.41	0.56	223.2
1.0	0.025	7.0	320	43.89	18.36	0.42	445.15
1.0	0.025	7.0	350	48.65	13.82	0.28	893.4
1.0	0.025	7.0	367	51.39	11.08	0.22	4460.92

[a] Effect of varying concentrations of unlabeled ε-DNP on the binding of [^{3}H] ε-DNP-lysine by anti-DNP.

latter reduces the number of variables in the system, which should minimize the chances for technical error.

Though further evaluation of the technique is needed as it applies to other antigen–antibody systems, one may assume at this time that steady state conditions will provide a rapid, technically less demanding method for the determination of binding constants; various properties of antibody, antigen, and antibody concentrations; as well as a practical method for radioimmune assays. The most obvious limiting factors at this time is the molecular cut off of the fibers.

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MICROCHEMICAL JOURNAL 21, 92–97 (1976)

Semimicro Determination of Hydroxy and Amino Compounds Using Pyromellitic Dianhydride[1]

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INTRODUCTION

Siggia *et al. (8)* introduced pyromellitic dianhydride (PMDA) as a reagent for determining amines and hydroxyl groups. It has the advantage of a reaction rate comparable to that of acetic anhydride, and is unaffected by aldehydes and phenols. The reagent was originally prepared in tetrahydrofuran (THF), but bumping near the boiling point (65°C) occasionally caused a loss of sample. This problem was remedied *(7)* by substituting dimethyl sulfoxide (DMSO) for THF.

Siggia's method uses 50 ml of 0.5 *M* PMDA, samples containing 10 to 15 meq of hydroxyl or amine, and a blank titration on the order of 100 ml of 1 *N* base. In cases where only small samples are available, or where the sample contains only small amounts of hydroxyl or amine, the original method (with THF) was reduced by a factor of 5 *(6)*: 50 ml of 0.1 *M* PMDA was used, and the blank titration required 100 ml of 0.2 *N* base. Because of the more dilute reagent, a 50% increase in reaction time was recommended.

Mathur *(3)*, in his review on newer acylation methods, points out that the PMDA method has only been used on the macro scale. We investigated the feasibility of reducing this method to the microscale. Also, because a visual endpoint is difficult to ascertain in turbid solutions, a potentiometric titration monitored by a minicomputer was used when necessary.

[1] This work was performed under the auspices of the U. S. Energy Research and Development Administration, under contract No. W-7405-Eng-48. This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Energy Research and Development Administration, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights.

MATERIALS AND METHODS

Reagents

Reagent grade chemicals were used throughout. Pyromellitic dianhydride, 0.04 *M*. 8.72 g of the dried reagent (48 hr at 160°C) is dissolved in 550 ml of dry dimethyl sulfoxide, and the volume is made to 1 l with dry pyridine. The reagent is stored over molecular sieve (Linde, Type 4A).[2] Sodium hydroxide, 0.08 *N*, standardized against potassium acid phthalate.

Procedure

The reaction vessel, which also serves as the titration vessel, was designed for use with a Mettler DV11 digital buret drive and a DV105 50-ml buret. However, it also can be used for visual titrations using the phenolphthalein endpoint. The vessel is made from a 45/50 female joint and is approximately 4.5 in. high (excluding the joint). A sample containing 0.4 to 0.6 mEq of hydroxy or amino compound was weighed into the vessel, and 25 ml of 0.04 *M* PMDA reagent was pipetted in. Because this solution is fairly viscous, the same drainage times were allowed for samples and blank. The stopper was wetted with pyridine and loosely seated in the vessel. The flasks were placed on a hotplate at approximately 115°C for 30 to 40 min (the latter time is recommended for polyols). The flasks were removed from the hotplate, 10 ml of distilled water were added, and the solution then reheated for an additional 2 min. The stoppers and walls of the flasks were then rinsed with pyridine and cooled to room temperature. Colorless clear samples were then titrated visually to the phenolpthalein endpoint with 0.08 *N* base. Colored or turbid samples were titrated potentiometrically (Sargent–Welch miniature combination electrode, No. S-30070-10, or equivalent). At least one blank, in which only the sample was omitted, was run in the same manner as the samples.

Our potentiometric titration system was controlled by a conventional PDP-8/I minicomputer processor. A summary of its operation and a schematic of the equipment are found elsewhere *(1)*. Titration endpoints were calculated according to Savitsky and Golay *(4)*. A convolute was used for a third-order second derivative with 25 points. The zero-crossing was found by linear interpolation near the sign change.

A magnetic stirrer was used, and the motor was separated from the titration vessel by a water-cooling plate and an aluminum plate connected to ground.

CALCULATIONS

The common calculations for the acylation reaction were used:

[2] Reference to a company or product name does not imply approval or recommendation of the product by the University of California or the U. S. Energy Research and Development Administration to the exclusion of others that may be suitable.

$$\%OH = \frac{(\text{ml blank} - \text{ml titration})\,(\text{normality base})\,(1700.7)}{\text{mg sample}}, \quad (1)$$

$$\%\ NH_2 \text{ or } -NH \text{ (primary or secondary amine)} \quad (2)$$

$$= \frac{(\text{ml blank} - \text{ml titration})\,(N \text{ base})\,(1602.3)}{\text{mg sample}}.$$

The hydroxyl content is frequently defined by "hydroxyl number" that indicates the mg of KOH required to react with 1 g of sample. This is calculated from the OH%:

$$\text{hydroxyl number} = (\%OH)\,(32.99), \quad (3)$$

because each percentage of OH is equivalent to 10/17.007 mEq of KOH per gram of sample (or to 32.99 mg of KOH).

Conversely, for a given hydroxyl number, the amount required for analysis can be calculated from

$$\text{mg sample} = \frac{(\text{mEq desired})\,(561.1)\,(100)}{\text{hydroxyl No.}}, \quad (4)$$

where the factor 561.1 is 32.99×17.007.

RESULTS AND DISCUSSION

The reagent PMDA is convenient for the acylation of hydroxyl and primary or secondary amino groups. Unlike acetic anhydride, it is not volatile, and it reacts more rapidly than phthalic anhydride. Alcohols can be determined in the presence of phenols, and aldehydes do not interfere. The original method *(8)*, however, requires samples containing 10 to 15 mEq of alcohol or amine. A sample this size is not always available, particularly samples containing small amounts of hydroxyl or amine.

We previously *(5)* attempted to reduce the scale of Siggia's method. With limited experiments, we found the lower limit of the method to be near 1.5 mEq of alcohol when using 5 ml of 0.5 *M* PMDA. The method was thus scaled down by a factor of 10. However, a considerable increase in reaction time was required to compensate for the lower concentration of reagent.

This study was undertaken to investigate systematically the lower limit of the PMDA method. The total volume of the titration and the amount of PMDA reagent was reduced by 50%. The PMDA/OH ratio, however, was kept at the previous range of 3.3 to 5. The model compounds in our study were 1-octadecanol (Eastman Organic chemicals, *Anal* Calcd C 78.40%, H 14.10%; found C 78.32%, H 14.03%; OH Calcd 6.29%) and *l*-amphetamine (Aldrich Chemical Co., puriss., NH_2 Calcd 11.85%).

The reaction of PMDA with 1-octadecanol always produced a turbid solution, making recognition of the phenolphthalein endpoint difficult.

Such samples, therefore, were determined by the computer-controlled system. Visual titration was possible with clear solutions, such as *l*-amphetamine.

The reagent PMDA is very hygroscopic and should be dried at 160°C for at least 48 hr before use *(2)*. The absence of moisture becomes critical as the concentration is decreased below 0.05 *M*. Therefore, the solvents used in preparing the PMDA reagent, DMSO, and pyridine should be stored for several days over an activated molecular sieve. The hydroxyl determination is more susceptible to the presence of moisture than the amine determination, which requires less drastic conditions and can be determined with shorter reaction times.

The results of the experiments with 1-octadecanol are shown in Table 1. Under normal conditions, 115°C and 30 min, satisfactory recoveries were obtained when the method was scaled down by a factor of 25. When the method was scaled down by a factor of 30, the recovery was somewhat low, although still acceptable by microanalytical standard (±0.30%). However, if the reaction temperature was increased to 145°C, the recovery was theoretical. When the method was reduced by a factor of 35, even raising the reaction temperature to 140°C yielded a borderline acceptable −0.29%. The results shown in Table 2 indicate that those obtained from a polyol, Polymeg 1000, are more easily scaled down than the model compound 1-octadecanol. Good recoveries were obtained from Polymeg 1000 when the method was scaled down by a factor of 35. For hydroxyl compounds, we recommend scaling down by a factor of 25. If further scaling down is desired, then additional experiments are recommended to establish the reaction conditions.

The results with the model amine *l*-amphetamine are shown in Table 3. For amines, the method can be scaled down by a factor of 40 with the reaction conditions recommended. We found that more severe reaction conditions such as an increase in the reaction time and temperature gen-

TABLE 1
HYDROXYL DETERMINATION OF 1-OCTADECANOL[a]

Time (min)	Temp (°C)	N_{KOH}	M_{PMDA}	Scaling factor[b]	Found (% OH)	Standard deviation	Number of experiments	Error (%)
30	115	0.10	0.05	20	6.36	0.11	6	+0.07
30	115	0.08	0.04	25	6.37	0.05	6	+0.08
30	115	0.065	0.033	30	6.14	0.10	3	−0.15
30	145	0.065	0.033	30	6.29	0.02	3	0
30	140	0.058	0.029	35	6.09	0.10	3	−0.29

[a] Calcd. 6.29% OH.

[b] Factor by which method has been reduced from the original (1, 2).

TABLE 2
HYDROXYL DETERMINATION OF POLYMEG 1000[a]

Time (min)	Temp (°C)	N_{KOH}	M_{PMDA}	Scaling factor	Found (% OH)	Standard deviation	Number experiments	Error (%)
30	115	0.10	0.05	20	3.38	0.03	3	−0.02
30	130	0.058	0.029	35	3.32	0.01	2	−0.08
30	140	0.05	0.025	40	2.60		3	−0.80

[a] Quaker Oats Co., polytetramethylene ether glycol, molecular weight about 1000, (nominal 3.40% OH).

TABLE 3
AMINE DETERMINATION OF L-AMPHETAMINE[a]

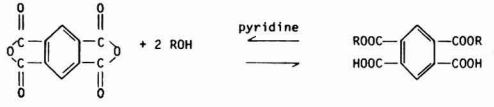
Time (min)	Temp (°C)	N_{KOH}	M_{PMDA}	Scaling factor	Found (% NH_2)	Standard deviation	Number of experiments	Error (%)
30	115	0.10	0.05	20	11.66	0.13	3	−0.19
25	115	0.08	0.04	25	11.69	0.10	3	−0.16
30	115	0.067	0.033	30	11.88	0.03	3	+0.03
30	115	0.05	0.025	40	11.76	0.06	3	−0.09
30	130	0.04	0.02	50	11.52	0.06	3	−0.33

[a] Calcd 11.85% NH_2

TABLE 4
HYDROXYL DETERMINATION OF SOME COMPOUNDS CONTAINING POLYGLYCOL ETHERS

Sample	OH (%)	Mean
MGM-14	6.54 6.49	6.52
MGM-18	7.23 7.31	7.27
AB-IV-5-1	3.19 3.19	3.19
AB-IV-5-2	5.67 5.57	5.62

erally do not facilitate scaling down the PMDA method. It is likely, however, that further scaling down is possible by increasing the PMDA/OH ratio, thus, shifting the equilibrium of the reaction:



Duplicated results for several polyglycol ether-containing materials are presented in Table 4. Note that for such compounds, perchloric acid catalysis cannot be applied because the materials are easily oxidized and yield highly erratic results *(6)*.

SUMMARY

The PMDA method for determining hydroxyl and primary and secondary amino groups was applied on the semimicro scale. A sample containing 0.4 to 0.6 mEq of amine or hydroxyl was reacted with 25 ml of 0.04 *M* PMDA in a routine analysis. The unconsumed PMDA was titrated with 0.08 *N* base. For colorless clear samples, the phenolphthalein endpoint was used; for colored or turbid samples, a potentiometric titration is recommended. Further scaling down of the method may be feasible by increasing the PMDA/OH ratio.

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MICROCHEMICAL JOURNAL 21, 98–105 (1976)

Spectrophotometric Determination of Antimony by Extraction of Reduced Molybdoantimonylphosphoric Acid with Butyl Acetate

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INTRODUCTION

Heteropoly acids, particularly the molybdates, have traditionally been utilized for the analysis of the central atom, P, As, Si, or Ge *(9)*. It is known, however, that mixed molybdophosphoric acids form with elements such as V, Sb, Bi, Ti, Th, Zr, Nb, Ce, and others *(2)*. In a few instances, the mixed heteropoly acid has been the basis for the analysis of the added element. Ti, Zr, and Th, for example, have been determined colorimetrically as the mixed molybdophosphoric acids *(6)*. More recently, Ce has been analyzed by atomic absorption and colorimetry as the molybdocerophosphoric acid *(5)*. These procedures are all, at some point, based upon the extraction of excess molybdophosphoric acid (MPA) from the mixed heteropoly.

A procedure for the trace analysis of phosphate which incorporates the use of antimony to increase the rate of reduction of the heteropoly acid, was reported by Murphy and Riley *(7)*. Molybdoantimonylphosphoric acid (MSbPA) initially formed is reduced by ascorbic acid. The molar ratios of the P, Sb, and Mo in the reduced mixed heteropoly acid was recently shown to be 1:2:10 *(2)*. In the reduced forms, both MPA and MSbPA are readily extracted by oxygenated solvents. It was recently shown that solution acidity as well as solvent identity is critical in the effect upon extractability *(1)*. It is possible, for example, by proper choice of acidity, to selectively extract MSbPA from MPA using butyl acetate.

In the classic Murphy and Riley procedure, antimony is used in large excess over phosphate. This study presents a unique variation on the Murphy and Riley technique in that phosphate is used in excess over antimony. The amount of reduced MSbPA formed is now determined by the amount of antimony present. Following reduction by ascorbic acid,

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the blue MSbPA can be selectively extracted from any reduced MPA. In fact, the reduction of MPA is quite slow and little is formed by the time that the MSbPA is extracted.

MATERIALS AND METHOD

Reagents

Antimony stock solution I. 0.1669 g of reagent grade potassium antimony tartrate is dissolved and diluted to 250 ml with distilled water. $[Sb^{+3}] = 2.00 \times 10^{-3}$ *M*.

Antimony stock solution II. Dissolve 0.1218 g of Sb metal in 50 ml of conc H_2SO_4, with heating. When fully dissolved, cool the solution and dilute to 500 ml with distilled water. $[Sb^{+3}] = 2.00 \times 10^{-3}$ *M*.

Stock acid solution. Add 83.3 ml of concentrated H_2SO_4 to 100 ml of distilled water, allow to cool and dilute to 250 ml with distilled water. $[H_2SO_4] \simeq 12$ *N*.

Stock phosphate solution. 0.1361 g of dried KH_2PO_4 are dissolved and diluted to 250 ml with distilled water. $[PO_4^{3-}] = 4.00 \times 10^{-2}$ *M*.

Stock molybdenum solution. 14.5 g of $Na_2MoO_4 \cdot 2H_2O$ are dissolved and diluted to 250 ml. $[MoO_4^{2-}] = 0.24$ *M*.

These stock solutions are stable for several weeks stored at room temperature.

Ascorbic acid. Prepare a 1% solution daily.

Mixed reagent. Add 10 ml of acid, 25 ml of phosphate, and 10 ml of molybdate solution to a 100-ml volumetric flask and dilute to volume with distilled water. This solution is prepared fresh daily.

Apparatus

Spectral measurements were made with a GCA-McPherson Model 727 spectrophotometer while photometric analyses measurements were made with a Beckman D.U. One centimeter silica cells were used.

Procedure

Using Table 1 as a guide, place the appropriate volume of sample into a separatory funnel, add the indicated volume of mixed reagent and 1 ml of ascorbic acid. Allow 20 min for formation, and extract with the indicated volume of solvent by shaking vigorously for 6 min. After the layers have separated, draw off the more dense aqueous phase and transfer the remaining organic phase to a 15-ml centrifuge tube and centrifuge briefly. Measure the absorbance of the organic phase at 651 nm in a 1-cm cell against a reagent blank carried through the entire procedure. Prepare a calibration curve using dilutions of either stock antimony solution I or II.

RESULTS AND DISCUSSION

Near Infrared Spectrum

The near ir spectrum of reduced MSbPA in butyl acetate has maxima at 651 and 728 nm. The molar absorptivities, based upon antimony, were

TABLE 1
PROCEDURE GUIDELINES FOR ANTIMONY ANALYSIS

Range (ppb)	Sample volume (ml)	Mixed reagent (ml)	Extractant (ml)
5–40	200	20	5[a]
10–100	200	20	10
40–400	100	10	10
100–1000	50	5	10

[a] Requires a restricted volume 1.00-cm cell.

9500 at both wavelengths. The spectrum has undergone a hypsochromatic shift relative to the aqueous spectrum as shown in Fig. 1. Corrections should be made for the blank absorbance which is normally about 0.012 for an aqueous/organic volume ratio of 10.

Formation and Extraction

The effect of acidity and molybdenum concentration upon the formation and extraction of reduced MSbPA was investigated. It has been previously shown that acidity and the molybdenum concentration are interrelated *(2)*. Optimum conditions for the formation of reduced MSbPA have been presented as being a $[H^+]/[MoO_4^{2-}]$ ratio of 70 ± 15.

In the initial studies, a phosphate concentration of 2×10^{-5} was chosen as an upper limit. A study of the amount of Mo required for complete formation and extraction was carried out. The results are shown in Fig. 2 and indicate that an Mo/P ratio of at least 75 is required. The $[H^+]/[MoO_4^{2-}]$ ratio was held constant at 70. The results also indicate that the formation and extraction of reduced MPA is negligible as expected.

To confirm that an $[H^+]/[MoO_4^{2-}]$ ratio of about 70 was optimum for both formation and extraction, further studies were conducted. The results shown in Fig. 3 indicated that at a constant molybdenum concentration of 0.0014 *M*, formation and extraction occurs between 0.06 and 0.15 *N* H_2SO_4, with a maximum occurring at 0.12 *N*. Subsequently, molybdenum concentration was varied at a constant acidity of 0.12 *N* H_2SO_4. The results shown in Fig. 4 indicate a plateau region of optimum formation and extraction. The plateau corresponds to an $[H^+]/[MoO_4^{2-}]$ range of 28 to 86. At the high molybdenum concentrations, it is probably that a molybdenum blue species is extracting. It was decided to use 0.12 *N* H_2SO_4 and 0.0024 *M* MoO_4^{2-} as the optimum formation conditions. This corresponds to an $[H^+]/[MoO_4^{2-}]$ ratio of 50 which is slightly lower than that reported by Going and Eisenreich *(2)*.

In order to develop a simple procedure, a mixed reagent, containing the necessary H_2SO_4, Na_2MoO_4 and KH_2PO_4 was developed. Initially prob-

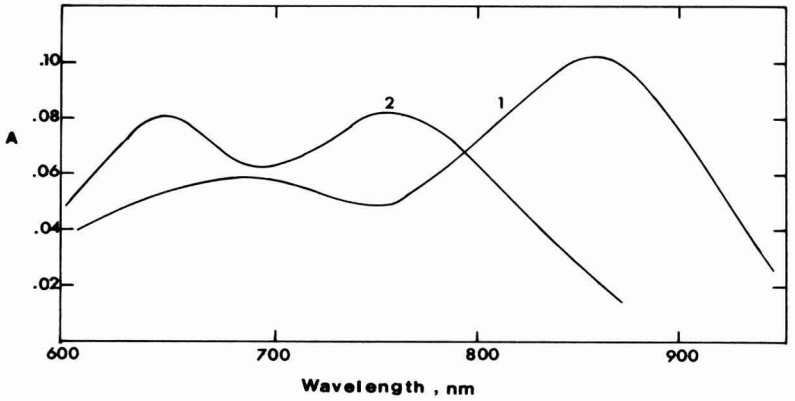

FIG. 1. Near infrared absorption spectra of reduced molybdoantimonylphosphoric acid. $[Sb^{+3}] = 8.22 \times 10^{-6}$ *M*. 1. Aqueous; 2. butyl acetate.

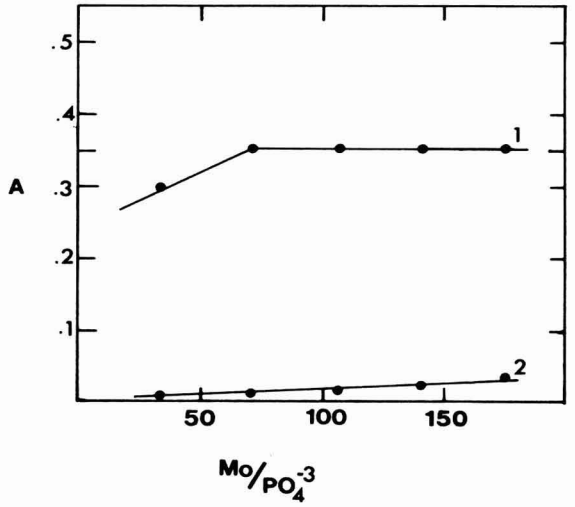

FIG. 2. Effect of the Mo/P ratio on formation and extraction of molybdoantimonylphosphoric acid. $[Sb^{+3}] = 4.00 \times 10^{-5}$ *M*; $[PO_4^{3-}] = 2.00 \times 10^{-5}$ *M*; $[H^+]/[MoO_4^{2-}] = 75$, $\lambda = 651$ nm. 1. With antimony; 2. without antimony.

lems were incurred in that yellow MPA acid eventually precipitated. Thus the three solutions were made up separately and appropriate volumes of the above solutions were added to form the mixed reagent. An optimal order of addition was found to be acid first, then phosphate, and finally the molybdenum. The mixed reagent was found to be stable for a period of 21 days. It was decided, however, to prepare the mixed reagent fresh daily so as to avoid the possibility of precipitation of oxidized MPA.

The solvent butyl acetate is less dense than water and consequently was the upper layer. Since it is more convenient for the extractant layer to be

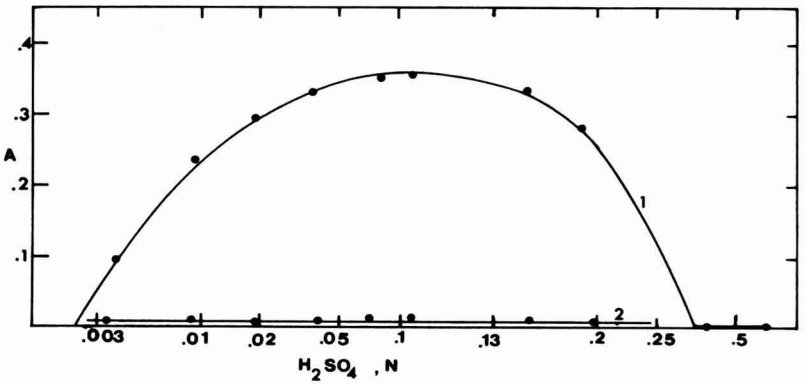

FIG. 3. Effect of acidity on formation and extraction of molybdoantimonylphosphoric acid. $[Sb^{+3}] = 4.00 \times 10^{-5}\,M$; $[PO_4^{3-}] = 2.00 \times 10^{-5}\,M$; $[MoO_4^{2-}] = 1.20 \times 10^{-3}\,M$, 651 nm. 1. With antimony; 2. without antimony.

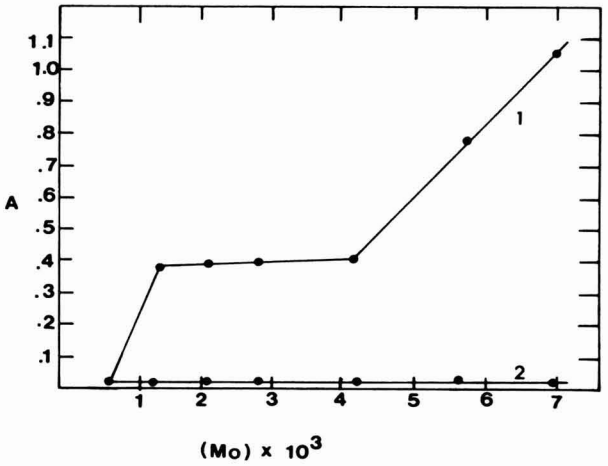

FIG. 4. Effect of molybdenum on formation and extraction of molybdoantimonylphosphoric acid. $[Sb^{+3}] = 4.00 \times 10^{-5}\,M$; $[PO_4^{3-}] = 2.00 \times 10^{-5}\,M$; $[H^+] = 0.12\,N$, $\lambda = 651$ nm. 1. With antimony; 2. without antimony.

more dense, an attempt to use a density enhancer was made. Chloroform has been used successfully for this purpose *(3)*. Initially a range of 5%–40% $CHCl_3$ was studied by adding the $CHCl_3$ to the butyl acetate before extracting. A solvent mixture of 30% $CHCl_3$ was required to be more dense than the aqueous phase. Unfortunately, the extraction of MSbPA began to diminish by 10% $CHCl_3$ and was largely incomplete at 30% $CHCl_3$. Addition of the $CHCl_3$ after extraction was also not effective.

Diverse Ion Studies

The potential interferences of a number of elements were studied and

are reported in Table 2. At 100 ppm tungsten, an intense blue complex, probably a tungstophosphoric acid, formed which was not extractable. The presence of zirconium yielded light blue precipitate. Thorium and bismuth yielded very blue complexes which were extractable. Mixed heteropoly acids of thorium *(6)* and bismuth *(4)* have been reported. Arsenic (V) will form a molybdoarsenic, MAsA, and molybdoantimonylarsenic acid, MSbAsA. The MSbAsA has very similar properties to MSbPA *(8)* and causes only minor interference.

Beer's Law

By variation of the aqueous/organic volume ratio, it was possible to determine antimony from 5 to 1000 ppb. The Beer's Law plots shown in Fig. 5 range from 1:1 to 40:1 in the aqueous/organic volume ratios. Molar absorptivity was consistently 9500 indicating negligible loss of butyl acetate at the high ratios.

Antimony Solutions

The Murphy and Riley procedure uses the tartrate complex of antimony as the source of the metal. In the analysis of antimony, it cannot be expected that the antimony would be present as the complex. Accordingly a standard solution of Sb^{+3} was prepared by dissolving the metal in sulfuric acid. This solution was used for the preparation of several calibration curves and it behaved in every respect like the potassium antimonyl tartrate solution.

TABLE 2
DIVERSE ION STUDY

Element	ppm	Error (%)
Ce^{+4}	1	− 8.41
Th^{+4}	10	21.02
	1	1.38
Bi^{+5}	1	61.92
W^{+6}	10	−71.03
	1	2.10
Zr^{+4}	10	20.79
	1	5.37
V^{+5}	100	20.60
	10	3.22
Fe^{+3}	100	− 6.65
	10	− 3.65
As^{+5}	100	9.66
	10	1.12
Si^{+4}	100	− 5.15
	10	− 1.29
Ti^{+4}	10	+ 0.90

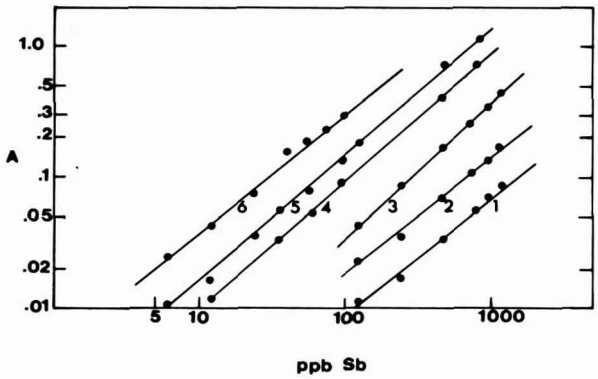

FIG. 5. Calibration curve for extraction of molybdoantimonylphosphoric acid at various V_{H_2O}/V_{org} ratios. (1) V_{H_2O}/V_{org} = 1:1; (2) V_{H_2O}/V_{org} = 5:1; (3) V_{H_2O}/V_{org} = 10:1; (4) V_{H_2O}/V_{org} = 20:1; (5) V_{H_2O}/V_{org} = 40:1.

TABLE 3
PRECISION STUDY[a]

Sb, ppb	S, ppb	S_{rel} (%)
200	1.85	0.925
20	2.10	10.52
10	0.840	8.40
5	0.534	10.68

[a] Vol_{aq}/Vol_{org} = 10.

Precision and Limit of Detection

A precision study was performed over a range of 5–200 ppb antimony to determine a limit of detection using an aqueous/organic volume ratio of 10. These results, shown in Table 3, indicate the precision that can be obtained. Subsequently, the limit of detection was determined to be 5 ppb antimony.

SUMMARY

Antimony can be determined colorimetrically by extraction of the reduced molybdoantimonylphosphoric acid with butyl acetate. The complex has absorption maxima at 651 and 728 nm and molar absorptivities of 9500. The optimum conditions were 0.12 *N* H_2SO_4 and 0.0024 *M* MoO_4^{2-}. Reduced molybdophosphoric acid does not form or extract to any significant extent. From 5–1000 ppb antimony can be analysed by varying the V_{H_2O}/V_{org} ratio from 40:1 to 5:1.

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MICROCHEMICAL JOURNAL 21, 106–117 (1976)

BOOK REVIEWS

Principles and Techniques of Scanning Electron Microscopy, Vol. 1. Biological Application. Edited by M. A. HAYAT. Van Nostrand Reinhold, New York, 1974. xi + 273 pp., $22.50.

This book and the others in the distinguished series are designed and intended to provide comprehensive, practical, and fully authoritative coverage of the applications of scanning electron microscopy (SEM) principles and techniques to the biological and biomedical fields. The series represents the most successful cooperation of numerous contributing scientists and experts who have varied backgrounds in biomedical and biophysical areas. It promises to become the standard reference in the field.

The book consists of 11 chapters. Each chapter has been written by experienced scientists or a group of authorities who have been using those particular techniques in the course of their professional work. Besides, each chapter is provided with an exhaustive list of references with complete titles. The full author and subject indexes are included at the end of the volume.

The opening chapter by J. T. Black describes the fundamental principles, theory, design, and construction of the SEM; the comparisons of the function of the SEM to those of the light (LM) and transmission electron microscopy (TEM); and the application of the SEM to the study of a wide variety of biological specimens. Some significant advantages of the SEM over the LM and the TEM are clearly shown here. For examples, the SEM provides a greater depth of field, the three/dimensional images, and a clear view of much larger specimens. These advantages have made the SEM an important tool for examining much larger biologic specimens, or specimens having irregular surfaces.
specimens having irregular surfaces.

The second chapter, by A. L. Cohen, deals with the critical point drying (CPD) method. The CPD technique is one striking example of the relatively recent improvements of major importance. Factors such as causes of distortion (volume and surface changes), properties of fluids, toxicity of intermediate and transitional fluids, safety precautions, preliminary treatment of specimens, specimen cleaning, fixation, storage, mounting, and coating, etc., which might influence the accuracy of the examination results are extensively and critically demonstrated.

Chapter 3, by T. Nei, is concerned with cryotechniques (freeze-drying). These techniques offer the opportunity for directly visualizing the internal three-dimensional structure of the native configuration of biological specimens with the SEM. Further development and application of this simple and useful technique are expected in the near future.

In Chapter 4, the frozen resin cracking method with its cytological application are reviewed by K. Tanaka. The method is an effective means for studying the intracellular structures with the SEM. The procedures include fixation and dehydration, embedding, cracking, drying, and metal-coating. The specimens, such as nucleus and nucleolus, endoplasmic reticulum, golgi complex, mitochrondria, secretory granules, cartilage, collagen fibers, and hydroxyl apatite clusters, have been examined by this method.

Chapter 5 by M. K. Nemanic considers the preparation of stereo slides from electron micrograph stereopairs. The advantages and disadvantages of stereo methods using orthogonally polarized light or red and green (or blue) light are completely and comparatively described. it is concluded that the polarized method is generally superior to the red–green method, although the latter provides a useful alternative.

Chapter 6 by H. F. Howden and L. E. C. Ling, discusses primarily the problems of

working at low magnifications using uncoated specimens. Also mentioned are methods of handling material and the types of microscope modification that may be needed in order to obtain reasonable results. It is suggested that further research in lessening the charging and distortion problems is necessary in this particular area.

In Chapter 7, the ideal use of the SEM in conjunction with the LM and the TEM to the study of spore morphology is presented in detail by A. W. Nickerson, L. A. Bulla, Jr., and C. P. Kurtzman. Following a thorough description of the procedures and precautions for specimens preparation, the exercises on the examination of spores of the actinomycetes, ascospores of aspergilli, yeast ascospores, fleshy fungi, bacteria, and myxomycetes, etc., are concisely illustrated.

Chapter 8 by P. J. Holloway and E. A. Baker considers the various techniques and problems associated with the SEM examination of the delicate surfaces of leaves, stems, and soft fruits. Also described are the detailed methods suitable for studying isolated cuticular membranes and cork layers of higher plants. Since a number of important physiological processes and other phenomena occur at these sites, the knowledge of the physical and chemical nature of plant surface structures easily obtainable with the SEM is importat in many fields of biological research.

Cahpter 9, by L. G. Briarty, emphasizes the practical application of the SEM to the investigation of plant cell walls and intracellular structures. It is recommended that a selective exposure of a particular intracellular component specifically for examining the structures is accomplished by means of mechanical dissection, ion beam etching, and chemical and enzymatic etching methods. Further, the structure–function relationships in plant cells can also be elucidated.

Chapter 10, by B. J. Panessa and J. F. Gennaro, Jr., is also concerned with the study of intracellular structures. It introduces the "tissue conductance technique" (TCT) for preparing stable and conductive specimens without metal coating. By using TCT, it has been possible to observe relatively smaller organelles and minute membranous channels as well as the cell internal structures.

In the last chapter, special techniques and detailed methods for preparing specimens from the structural material, "wood," for studying its structural properties are provided by K. Borgin. The value of using the SEM as a powerful tool for the investigation of micromorphology of wood, the structure and ultrastructure of the cell wall, and the single fibers is quite obvious here.

Overall, the book is well written and easy and pleasant to read. Paper, printing, and cloth binding are of good quality. Reading this book, leaves one with both an awareness of the wide vareity of techniques available for specimen preparations that come under the title *Principles and Techniques of Scanning Electron Microscopy* and the enormous range of biological materials and problems that the SEM can be applied to with success. The instructions provided for those excellent techniques and methods are straightforward and complete. The figures and micrographs for demonstration and illustration are reproduced in a particularly clear and lucid form. The methods have all been tested for their applicability and reliability. They are the best of those currently available. In addition, the book also points out alternative procedures and research areas and aspects of this progressing technology which are likely to be the forerunners of advances to come. Both the editor and the authors are to be congratulated sincerely on their successful work. This book is highly recommended for scientists, research workers, biochemists, biologists, and biophysicists as an invaluable reference book. The book is worth its price and should be on the bookshelves, not only of those already interested in the subject, but also of those who wish to know "what the scanning electron microscope is all about."

GEORGE W. C. HUNG, *The University of Tennessee, Center for the Health Sciences, Memphis, Tennessee 38163*

Principles and Techniques of Scanning Electron Microscopy, Vol. 2. Biological Applications. Edited by M. A. HAYAT. Van Nostrand Reinhold, New York, 1974. xviii + 172 pp. $19.95.

This is the second volume of a well-designed serial treatise on the principles and techniques employed for studying biological specimens with a scanning electron microscope (SEM). The purpose is to provide a comprehensive understanding of the usefulness, limitations, and potential of the preparatory procedures used for the SEM. The volume has been developed over the years through the joint effort of 13 distinguished scientists, representing five countries. As a part of the series, the volume promises to become the standard reference in the field.

The basic approach and format in this volume are similar to those in the first volume. The methods presented have been tested for their reliability, and they are the best of those currently available. The instructions for the preparation and use of various solutions, media, stains, coats, and apparatus are straightforward and complete. It enables the worker to prepare his specimens for the SEM examination without outside help. Further, some new viewpoints concerning the current problems and the potential usefulness of a new method are also critically described and suggested. The material and information thus presented in each chapter include a short introduction, theoretical backgrounds (whenever available), methods, possible applications, and several concluding sections. Each chapter is also provided with an exhaustive list of references with complete titles. Full author and subject indexes are included at the end of the book.

The book consists of 11 chapters. These cover the materials on methods and applications in cathodoluminescence of organic chemicals and herbicides, preparation and application of staining procedures using silver compounds, examination of specimens incubated in histochemical media, use of a single tissue specimens for both the SEM and transmission electron microscopy (TEM), soft tissues of marine teleosts, ciliated epithelia, embryonic and fetal tissues of vertebrates, methods in preparing for the SEM lung tissue, bone and other hard tissues, and fossil palynomorphs.

Specifically, the information and material cited in Chapter 1 (by M. De Mets) and Chapter 2 (by R. H. Falk) on the fundamental principles of cathodoluminescence phenomena observed with organic chemicals and herbicides are very interesting. These not only suggest a useful technique (as a detector) for examining biological specimens in a SEM-spectrometer combination but also indicate an effective means of conducting localization studies of herbicide dispersal on leaf surfaces. Further, Chapter 3 by H. D. Geissinger describes a specific silver-staining technique (using silver nitrate and silver halides) for investigation of specimens of blood platelets, intercellular "cement," and white blood cells with the SEM. This technique possesses a great potential in preparing microtome sections for the SEM.

Chapter 4 by T. Makita considers the exmination of Epon-embedded tissue sections (prepared for histochemical studies) with a *combination* of SEM operated in the transmission mode and electron prove x-ray microanalyzer. Also presented are methods for examining the treated and incubated unfrozen tissue sections in histochemical media. Chapter 5 by M. G. Wickham and D. M. Worthen is concerned with the manifold benefits offered by SEM–TEM *correlation* on the same specimens. These two chapters are of technologic importance and should be read with care.

In chapter 6–9 (by G. H. Dobbs, III, E. R. Dirksen, E. E. Waterman, and C. Kuhn, III, etc.), the design of fixing solutions and the appropriate fixative techniques for optimal specimen preparation from soft tissues are extensively and critically discussed. These are quite useful, since the successful examination of tissue structures with the SEM depends mostly on the effective preservation of the cell surface topography and/or the tissue surface morphology. The biological materials thus demonstrated cover marine teleosts, ciliated epithelia, embryonic and fetal tissues and lung tissues. The general process of specimen

preparation involves: (1) fixation with varied percentages of glutaraldehyde and buffers, (2) dehydration by various concentrations of ethanol or glycerol solutions and drying by freeze or Freon and liquid CO_2 critical point methods, (3) creation of conductive surface with silver paint or gold coat, and (4) observation of the specimens with reliable techniques. In addition, two special procedures, "rapid freezing and vascular perfusion," for fixation of lung specimens under physiologically defined conditions are also concisely described. By using these procedures the tissues can not only be preserved with a minimum of change (such as shrinkage or swelling) but also restored to a state which resembles the *in vivo* condition. It thus enables one to utilize more efficiently and effectively the capabilities of the SEM as a research tool and to obtain maximum information on structural–functional correlations from a single specimen.

Bone tissue is especially difficult to be studied with the SEM because it is a complex system of hard and soft tissues. However, A. Boyde and S. J. Jones have presented an excellent outline of the detailed procedures for preparation of bone tissue specimens. The outlined techniques are applicable to those highly mineralized tissues such as calcifiable cartilage, cementum, dentine, developing dental enamel, adult enamel, molluscs, echinoderms, and egg shells. A well-designed diagram showing principal routes for the preparation of bone and other hard tissues for the SEM and serving as the quickest and most complete guideline for performing examination work is provided. Therefore, for a comprehensive survey of structure, function, and surface morphology of bone and other hard tissues, Chapter 10 should be studied very carefully and thoroughly.

The last chapter by H. A. Leffingwell provides a stepwise procedural discussion on preparations of single grain or multigrain mounts from slurries of fossil palynomorphs for the SEM. The advantages of alternate using of LM, TEM, and SEM to examine this category of specimen more efficiently are comparatively reviewed and demonstrated.

In summary, this excellent book can be considered as a practical handbook of technology for preparation of biological specimens for the SEM. Throughout the volume, the materials are well organized, and the treatment of the subject matter is comprehensive, specific, and critical. There are no significant typographical errors. Paper, printing, and cloth binding are of good quality. The reproduced micrographs are particularly clear, lucid, and greatly worthwhile. Biologists, biochemists, biophysicists, research workers, scientists, and libraries, should own a copy of this book whether or not they already have the first volume.

GEORGE W. C. HUNG, *The University of Tennessee, Center for the Health Sciences, Memphis, Tennessee 38163*

The Mitochondria of Microorganisms. By DAVID LLOYD. Academic Press, New York and London, 1974. xii + 553 pp. $36.00 (£13.60).

This book offers the first complete coverage of the present knowledge of microbial mitochondria. It is divided into three major parts: structure and function, biogenesis, and evolution. Microtechniques are basic in microbiology; hence, this publication should be noted here. Some elegant X-ray and electron microscope techniques are described, but there is little else in the book that would interest anyone but a microbiologist.

DAVID B. SABINE, *185 Old Broadway, Hastings-on-Hudson, N. Y. 10706*

Current Topics in Micribology and Immunology. Vol. 68. Edited by W. Arber, R. Haas, W. Henle, P. H. Hofschneider, J. H. Humphrey, N. K. Jerne, P. Koldovsky, H. Koprowski, O. Maaloe, R. Rott, H. G. Schweiger, M. Sela, L. Syrucek, P. K. Vogt, E. Wecker. Springer–Verlag, Berlin New York, 1974, 174 pp., $30.40.

This is Volume 68 of a continuing series. The book contains five well-written reviews on various aspects of virology: (1) mouse type-C viruses (Sarma and Gazdar), (2) viral envelopes (Klenk), (3) satellite phage P4 (Goldstein *et al.*), (4) icosahedral cytoplasmic deoxyviruses (McAuslan and Armentrout), and (5) bacteriophage PM2 (Franklin). The volumes in this series continue to be overpriced.

Eugene D. Weinberg, *Department of Microbiology, Indiana University, Bloomington, Indiana 47401.*

Methods in Enzymology. By S. P. Colowick and N. O. Kaplan, Editors-in-Chief, Vol. XXIX, XXX. Edited by Lawrence Grossman and Kivie Moldave, Academic Press, New York, 1974. Vol. XXIX, xxi + 804 pp., $35.00; Vol. XXX, xxi + 808 pp., $35.00.

Volume XXIX and XXX represent parts E and F, respectively, of the nucleic acids and protein synthesis volumes of this monumental undertaking.

Volume XXIX contains the following sections:

I. Nucleic acid synthesizing systems: DNA directed DNA polymerases, RNA directed DNA polymerases, screening for mutants, proteins affecting the conformations of nucleic acid structure, cells with altered permeability properties.

II. Structural analysis of nucleic acids: primary sequencing methods; repeating primary sequence analysis.

III. *t*-RNA and Enzymes Acting on *t*-RNA.

Volume XXX contains the following sections:

I. Initiation factors in protein synthesis. II. Elongation factors in protein synthesis. III. Termination factors in protein synthesis. IV. Ribosome structure and function. V. Messenger RNA and protein synthesizing systems.

The series continues to be an indispensible source for those working in enzymology. The detailed experimental descriptions and excellent up-to-date referencing (e.g., 1973 references) continue to make these volumes one of the best starting points in a literature search on any topic in enzymology. The contributors are at the forefront of their fields so that the contributions not only give invaluable descriptions of experimental techniques, but also indicate the direction in which the particular research area is developing. This latter aspect can be especially helpful to the newcomer in a field.

Addition of these volumes to any research library can be highly recommended.

Frank Jordan, *Carl A. Olson Laboratory of Chemistry, Rutgers, The State University, Newark, New Jersey 07102*

Basic Principles in Nucleic Acid Chemistry. Edited by Paul O. P. Ts'o, Academic Press, New York, 1974. Vol. I, xii + 636 pp. $37.50; Vol. II, xi + 519 pp. $34.50.

These two volumes mark the beginnings of a series (projected to total four volumes) edited by Tsó. The first two volumes include 10 review articles of significant depth on topics of intense current interest to the nucleic acid researcher.

Volume I includes the following six chapters:

Chapter 1 (by Paul O. P. Ts'o) titled "In the Beginning" undertakes an analysis of the evolution of the nucleic acids both from a scientific and philosophical point of view and reviews the current beliefs on biological evolution. The ideas exposed in this chapter are of considerable interest to all, especially in view of the recent controversy surrounding "genetic engineering."

Chapter 2 (by Leon Goodman) is titled "Chemical Syntheses and Transformations in Nucleosides." It includes sections of synthetic methods yielding nucleosides: (condensation of sugar and base, construction of base after glycosylation, synthesis of C-nucleosides, sterochemistry of nucleoside synthesis); on chemical transformation of sugar moieties in nucleosides; chemical transformation of base moieties of nucleosides, and transformations involving both sugar and base (i.e., cyclonucleoside formation).

Chapter 3 (by James A. McCloskey) is concerned with "Mass Spectrometry" of bases alone, of nucleosides, of nucleotides and application of mass spectrometry to base sequence determination in oligonucleotides.

Chapter 4 (by M. Gueron, J. Eisinger and A. A. Lamola) is titled "Excited States of Nucleic Acids". It provides a theoretical framework for the discussion of excited state properties, then proceeds to present experimental results on such diverse topics as energy transfer in polymers, singlet and triplet states of nucleic acid polymers, and the photoreactions of pyrimidines (thymine dimerization and photohydration).

Chapter 5 (by Masamichi Tsuboi) is titled "Infrared and Raman Spectroscopy". After a brief introduction to the theory, the characteristic infrared and raman frequencies are described for base, ribose, and phosphate; and the applications of these methods to nuclei base interactions and nucleotide and conformational analysis are outlined. The future applications of Raman spectroscopy (already evident in the literature at the time of review) are forecast.

Chapter 6 (by Paul O. P. Ts'o) "Bases, Nucleosides and Nucleotides" discusses molecular structures (based on X-ray crystallography), tautomerism, ionization sites and especially conformation of sugar and around the glycosyl C–N bond (the *syn–anti* problem) in monomeric structures as well as intramolecular interactions. This is followed by solid state and solution evidence for base–base interactions (both hydrogen-bonding and stacking) in both aqueous and nonaqueous solvent systems. The evidence is presented for each problem (i.e., conformational or base–base interaction) according to the physical method employed and the conclusions drawn by the original contributions are critically evaluated. Since Professor Ts'o has made a very sizable contribution to the resolution of the questions raised, this chapter is of particularly great value.

Volume II includes the following five chapters:

Chapter 1 (by D. M. Brown) Chemical Reactions of Polynucleotides and Nucleic Acids." The first part concentrates on nucleophilic and electrophilic base modifications. Then follows a discussion of reactions affecting internucleotide linkage (phosphate diester hydrolysis reactions, phosphate elimination, and alkylation reactions), and finally, results on the influence of conformation on reactivity in the polynucleotides.

Chapter 2 (by C. Allen Bush) "UV, CD, ORD". First, the theoretical aspects of the title measurements are discussed, along with the interpretation of the phenomena observed especially as the phenomena apply to nucleic acid interactions. Next, the experimental techniques (UV absorption and those based on optical activity) are described including the well-known hypo- and hyperchronic effects, and the characteristics of the measurements used to elucidate helix–coil transitions in polymers and conformational and configurational aspects of the monomers and dimers.

Chapter 3 (by Henryk Eisenberg) "Hydrodynamic and Thermodynamic Studies". First, the theoretical aspects (thermodynamic, polyelectrolyte, transport phenomena) are reviewed (this also can be found, incidentally in several other recent books). Next, applica-

tions to the determination of molecular weight and flexibility of DNA are listed. Interaction of nucleic acids with small molecules (including drugs, ions, aromatic carcinogens) and polynucleotide conformation as reflected by the title techniques are then discussed.

Chapter 4 (by William Bauer and Jerome Vinograd) "Circular DNA". All aspects (tertiary structure, helix–coil transition, enzymic preparation, and occurrence, among others) are presented on this only recently exploited, topic.

Chapter 5 (by Paul O. P. Ts'o) rounds out the second volume. First, the experimental techniques commonly employed in studying the conformation and interactions of the substrates are presented. Next, the conformational studies and results are critically evaluated. The very recent X-ray crystallographic results by Rich and co-workers on G_pC and ApU are included. The author's own numerous contributions on the subject of conformations by nuclear magnetic resonance are detailed. Next, the interactions between dinucleotides and oligonucleotides, and between oligonucleotides and polynucleotides are discussed.

These two volumes were well conceived and written and can be highly recommended for any library, since the reviews presented are quite comprehensive in nature and provide excellent referencing to all but the last two years' literature.

FRANK JORDAN, *Carl A. Olson Chemistry Laboratories, Rutgers, The State University, Newark, New Jersey 07102*

Micromethods in Molecular Biology (Molecular Biology, Biochemistry and Biophysics, Vol. 14). Edited by V. Neuhoff. Springer–Verlag, New York Heidelberg Berlin, 1973. xiv + 428 pp., $40.20.

For those who study tissues or components available only in very small quantities, it has been necessary to develop laboratory techniques for "micro" quantities of material. Although these methods are often merely scaled-down standard techniques, application on the microscale usually turns out to involve special problems and methods for solving them. For example, how does one remove a 5 μl polyacrylamide gel from the tube? This volume contains chapters on Micro-Electrophoresis on Polyacrylamide Gels, Micro-Determination of Amino Acids and Related Compounds with Dansyl Chloride, Micro-Determination of Phospholipids, Micro-Diffusion Techniques, Capillary Centrifugation, Micro-Dialysis, Micro-Homogenisation, Wet Weight Determination in the Lower Milligram Range, Micro-Magnetic Stirrer, and Production of Capillary Pipettes all written by the editor, V. Neuhoff. In addition, the book contains chapters on Micro-Electrophoresis for RNA and DNA Base Analysis by J. -E. Edström and V. Neuhoff, Determination of the Dry Mass of Small Biological Objects by Quantitative Electron Microscopy by G. F. Bahr, The construction and use of Quartz Fiber Fish Pole Balances by G. F. Lehrer, Microphotometry by H.-G. Zimmer, Cytofluorometry by F. Ruch and U. Leemann, and Quantitative Autoradiography at the Cellular Level by P. Dörmer. Researchers with a need for these techniques, as well as those considering adopting them, should find this a very useful book. As Neuhoff points out, substantial savings of both time and money are an added benefit of micromethods. The chapters are well written with detailed step-by-step procedures for both building the specialized equipment needed and for performing experiments on the microscale. Many practical hints are included which can often make the difference between successful and unsuccessful applications of new procedures. Furthermore, in most cases, the authors have included reviews of the literature in which the micro techniques were used. All in all, this volume will be extremely helpful to scientists whose work requires micromethods. I would expect, however, that its $40 price tag would make most researchers think twice about purchase of the book for private use.

THOMAS BLUMENTHAL, *Department of Microbiology, Indiana University, Bloomington, Indiana 47401.*

Photoresist: Materials and Processes. By W. S. DeForest. McGraw-Hill, New York, 1975. x + 269 pp., $16.50.

"Photoresist: Materials and Processes," surveyed and compiled by an expert scientist and technologist (DeForest) presents the first practical "how-to-do-it" survey of all the basic photoresist processes, applications, and technology. It was written primarily to help solve day-to-day problems, and to assist in setting up and developing processes for more efficient operations.

The book consists of seven chapters covering the following main subjects: history of photoresists, types of photoresists, cleaning, negative resists, positive resists, dry-film photoresists, and microelectronic applications. To facilitate a full understanding, each chapter is written to stand alone with a minimum of cross referencing, so that all the essential material is concentrated in one place. Furthermore, the information is based on the author's actual experiences and know-how, which are focused on explaining in clear detail exactly what goes on during each process step. In addition, the book contains a great deal of reference material for those so inclined, and can be used as practical text in photoresist matters. An index is also provided.

The first chapter reviews early photoresists and how they were used in the printing industry. The key developments in circuit board technology, including simple and complex multilayer boards are well illustrated. The rapid transfer of such technology to the modern electronics industry is also discussed in detail.

The second chapter deals with the different types of resists function, covering resins, sensitizers, solvents and additives which enhance performance for both positive- and negative-acting resists. Resist constituents and how they work are discussed, based on the available information. A minimum of practical theory that is necessary to understand a process or an application is included. It is especially worthwhile for those interested in microelectronics.

In Chapter 3, a heavy emphasis is placed on metal cleaning and surface preparation, a critical but sometimes neglected step. Types of soils, basic cleaning theory and sequence, and a series of cleaning cycles for various metals, with emphasis on copper, are extensively demonstrated.

Chapter 4 is devoted entirely to processing information for negative-acting wet-film resists, such as the Kodak Photoresist. Topics such as coating methods, controls and light sources, comparison of exposure methods, and dyeing are critically mentioned. The following chapter deals with positive resists such as the Shipley AZ-119, with emphasis on roller coating. Processing sequence such as resist application, air drying, prebaking, exposure and development, rinsing, drying, examining and touch up, postbaking, etc. are thoroughly described.

Chapter 6 compares dry- and wet-film resists. It focuses on the three types of dry-film resists: solvent developing, aqueous developing, and peel-apart or thermally developing resins. Both compositional and processing information, ranging from cleaning and part preparation requirements unique to dry films, to lamination, such as oxygen effects, primers, exposure times and methods of determination and control, scum and its causes, multiple coatings, methods for evaluation strippers, and so forth, are comprehensively discussed.

Microelectronics, which has unique needs, is discussed in the final chapter. This chapter contains vital information for both positive and negative processing, including processing details for each type of resist, followed by resist application-whirling and roller coating, with emphasis on coating thickness and accuracy requirements. Other subjects such as prebaking and drying, exposure, light sources, collimation by distance and lens, developing solvents and solutions, rinses, postbaking, stripping, and pinhole testing for process evaluation, etc., are also covered.

Throughout the book, the materials are carefully organized with step-by-step explana-

tions, detailed presentations of practical examples, and frank discussions of practical uses and established practices. Tables and figures are well prepared. Prints and reproduced pictures are of high quality. There is no significant typographical error in the text. Therefore, with its wealth of easy-to-understand and easy-to-use information illuminating all major photoresist techniques and applications, this book is highly recommended as an invaluable reference to all whose daily work requires a working understanding of photoresists. Manufacturing personnel, production support groups, design engineerings, managers, salesmen, artists, engravers, photo-scientists, and others, are encouraged to have one on their shelves for daily use.

GEORGE W. C. HUNG, *The University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38163.*

Principles and Techniques of Scanning Electron Microscopy. Vol. 3. Biological Applications. Edited by M. A. HAYAT. Van Nostrand/Reinhold, New York, 1975. xi + 226 pp., $22.50

Edited by the famous electron microscopic biologist, Professor Hayat, and with the aid of nine contributing authorities who have varied backgrounds in biomedical and biophysical areas, this third volume is part of a comprehensive series designed to help secure the benefits that the scanning electron microscope (SEM) has over both the light microscope (LM) and the transmission electron microscope (TEM). The basic approach here is similar to that in the previous two volumes, in that the methods presented have been tested for their reliability, and are the best of those currently available. The book contains an extensive compilation of techniques developed and used by numerous scientists in their professional work.

The volume contains seven chapters. The materials presented include methods for isolating giant chromosomes, microdissection, preparing very small biological specimens, microorganisms, cultured and free-living cells, stereographic techniques, and application of a field emission source to SEM.

The first chapter deals with an attempt to freeze–dry isolated chromosome specimens at a very low temperature, to compare the results obtained by such method with those obtained after critical point drying (CPD), and to correlate the results with the known TEM micrographs of thin sections. It has been reported that carbon dixoide–CPD specimens show the same surface and thin-section structure as those that have been frozen with ethanol or liquid helium II.

Chapter 2 is devoted to the techniques of microdissection. The methodology, advantages, and disadvantages of this technique in SEM are discussed in detail. The possible combined use of the SEM's ability to image biological surfaces with the TEM's superiority in revealing intracellular detail is critically mentioned. Also, the potential application of coupling these techniques with the X-ray fluorescence analysis to pieces uncovered or removed from a sample by microdissection is recommended.

The application of SEM techniques to the studies of very small biological specimens such as blood and cultured cells in suspension, marine and aquatic microorganisms, lipoproteins and other large macromolecules, and unicellular organisms such as yeast, bacteria, and protozoans, are reviewed in Chapter 3. The specific techniques discussed include micropipetting, centrifugation, filtration, diffusion, CPD, freeze-drying (FD), mounting of specimens on stubs, and metal coating.

In Chapter 4, the practical application methods and detailed experimental procedure of utilizing the advantages of SEM to the investigation of the microstructure and surface structure of microorganisms are described. This chapter demonstrates the potential value of the SEM in the field of microbiology.

Chapter 5 is concerned with the studies of cell surface structure, cell–cell interactions, cell morphology, and distribution of surface antigenic determinants by SEM analysis. The chapter contains a brief discussion of principles, and a thorough consideration, in conjunction with step-by-step analysis, for several basic preparative pathways, such as FD, CPD, and freeze-substitution procedures. Some well-designed figures and plenty of excellent electromicrograms serve as the first-aid of illustration.

The principle and technique of stereomicrography are considered in Chapter 6. This technique has proven to be of inestimable value in both TEM and SEM applications. It provides an invaluable tool for assessing objects in three dimensions, both qualitatively and quantitatively. Readers are urged to study this chapter with great care.

The last chapter introduces the applications of the newly developed field emission source to SEM. The field emission system possesses several distinctive advantages over a thermionic emitter. These are improved depth of field with higher probe currents; improved resolution for low voltage operation; fast scan, superimposition recording; more gray scales and shorter recording durations and higher resolution. All these characteristics are comparatively and critically discussed.

In summary, as in the previous volumes, this book provides straightforward and complete instruction and information. It enables the user, without asking for outside help, to utilize its information on the preparation and use of various solutions, media, coats, and apparatus. Furthermore, the book points out alternative procedures and potential research areas, aspects of this rapidly progressing technology that are likely to be the forerunners of advances to come.

The series promises to become the standard reference in the field. It would be considered worthwhile, therefore, to keep a copy on the bookshelves for permanent use.

GEORGE W. C. HUNG, *The University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38163.*

Analytical Profiles of Drug Substances. Vol. 4. Edited by KLAUS FLOREY. Academic Press, New York, 1975. xi + 526 pp., $26.50.

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

Drug compounds mentioned in Volume 4 are: Cetazolin, Cephalexin, Chloramphenicol. Clorazepate Dipotassium, Cloxacillin Sodium, Diatrizoic Acid, Disulfiram, Estradiol Valerate, Hydroxyprogesterone Caproate, Isosorbide Dinitrate, Methaqualone, Norethindrone, Norgestrel, Phenformin Hydrochloride, Procainamide Hydrochloride, Reserpine, Spironolactone, Testosterone Enanthate, Theophylline, and Tybamate.

The drug substances mentioned in this book are defined as to identity, purity, strength, and quality in the official compendia (United States Pharmacopeia and National Formulary). However, the compendia normally does not provide other physical or chemical data, nor do they list methods of synthesis or pathways of physical or biological degradation and metabolism. To provide important supplemental information that contributes to the better understanding of drug substances, the Pharmaceutical Analysis and Control Section, Academy of Pharmaceutical Sciences, has compiled and published "Analytical Profiles of Drug Substances." The information presented in this volume is a collection of data scattered throughout the literature and files of pharmaceutical laboratories. The profiles provide information from important instrumentation including Infrared, Nuclear Magnetic Reso-

nances, Mass Spectroscopy, Differential Scanning Calorimetry, Thermogravimetric Analysis, and Gas–Liquid and High Pressure Liquid Chromatography. The profiles are sufficiently thorough and provide an authoritative source of information concerning the properties of drug substances as well as up-to-date references. The cumulative index started in Volume 3 and continued in Volume 4, provides a convenient method for quick reference, although the italic numerals used to refer to volume numbers are difficult to differentiate from the page numbers.

Volume four, as with previous volumes, is thoroughly done and provides a valuable and convenient reference. This profile should be welcomed by pharmaceutical chemists and research pharmacists.

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Principles and Techniques of Scanning Electron Microscopy. Vol. 4. Biological Applications. Edited by M. A. HAYAT. Van Nostrand/reinhold, New York, 1975. xii + 230 pp., $22.50.

This fourth volume is an up-dated volume of Professor Hayat's famous series of comprehensive treatises on the principles and techniques of scanning electron microscopy (SEM) employed in the biomedical areas. It is designed to provide the worker with an understanding of the usefulness, limitations, and potential of the preparatory procedures used for SEM without the necessity of outside help. It has been completed through the joint effort of eight distinguished author–scientists. Its purpose is to serve as an interntional authoritative source in the field, and to cover important new developments systematically.

The book consists of six chapters, and includes methods and technology for preparing and examining specimens at low temperature; thiocarbohydrazide-mediated osmium binding, a technique for protecting soft biological specimens in the SEM; replica techniques; SEM methods in studying spermatozoa; electron probe X-ray microanalysis; and scanning electron spectrometric microscopy. The format and basic approach in this volume are similar to those in the previous three volumes. That is, the methods presented have been tested for their reliability, and are the best of those currently available. The instructions for the preparation and use of various solutions, media, stains, coats, and apparatus are straightforward, complete, and easily applicable. In addition, areas of disagreement and potential research problems have been pointed out. Each chapter provides an exhaustive list of references with complete titles. Full author and subject indexes are available at the end of the book.

Specifically, Koch introduces the low-temperature techniques for preparation of soft biological specimens in the beginning chapter. This technique not only is unique in terms of its dynamics, but also, it differs from the more traditional fixation methods in terms of the equipment, skills, and methodology required for its application; samples are ideally suited to be examined by the SEM equipped with an X-ray spectrometer for microanalysis. A well-designed specimen preparation flow chart and some 27 fine figures and clearly reproduced electron micrograms help a great deal in demonstrations and provide a better understanding of entire procedures. A short chapter by Kelley, Dekker, and Bluemink recommend another more efficient and economical technique of ligand (thiocarbohydrazide) bound osmium for the preparation of soft tissue specimens for SEM. The technique possesses several apparent advantages, such as, (1) no special instruments are required; (2) specimen damage from heat and mechanical vibration is avoided; (3) it can be readily adapted to a variety of subsequent procedures; and (4) it permits maximum information retrieval from a single valuable specimen.

Chapter 3 by Pameijer reviews a special "replica" technique. A replica can be defined as

a copy of an original, obtained by means of a reproduction procedure. The result of the first impression is called a negative replica and the result of filling the negative imprint is called the positive replica. Both hard and soft replica techniques applicable *in vitro* and *in vivo* biological specimens study have been discussed in detail. One of the advantages of the replica technique is that it can bypass the tissue shrinkage problems, and will allow observation of the true morphology and surface topography of a specimen on which direct observations would have been impossible. Some 34 excellent electron micrograms are presented.

In Chapter 4, Baccetti presents a brief but clear discussion of preparing single cells for the SEM in spermatology. For a rapid processing of sperms, this author uses fixation with glutaraldehyde, dehydration with ethanol, and critical point drying on aluminum foils with Freons 113 and 13.

The longest chapter, Chapter 5 (by Marshall) is devoted entirely to the electron probe X-ray microanalysis method. X-ray microanalysis is a technique that permits a complete, nondestructive, chemical analysis of the distribution of elements to be made at the cellular and subcellular levels in biological specimens. A particular emphasis has been placed on the discussion of the fundamental principles and preparatory and operational techniques of the SEM with an energy dispersive spectrometer. Since this technique is of prime importance for the biologist, providing high detection efficiency and spatial resolution on the analysis of microstructures containing very low concentrations of elements, biological scientists are urged to study this chapter with great care. Some 132 references (to 1976) are provided.

The volume ends with a presentation of scanning electron spectrometric microscopy by Hart. This technique combines SEM with electron energy analysis, especially applied to those secondary electrons that have energies between approximately 50 and 150 eV. The instrumentation serves as a powerful analytical tool, which is able to chemically identify minute constituents of matter in a scanning electron micrograph, and with a spatial resolution comparable to that of the micrograph. The various aspects of this method, including its comparison to other analytical methods, its advantages, and its limitations, are critically discussed; its potential application to biological problems, both present and future, is encouraging and should prove to be most rewarding.

In summary, this volume is well written. The materials and information presented are up-to-date and most valuable. There is no significant typographical error in the text. The comprehensive treatment of the material on the new development of the SEM equipped with other analytical instrumentation is of particular interest and challenging, since the combined techniques for the chemical identification of specific elemental species in biologically significant molecular structures is vital to the understanding of the chemical complexities of living structures in health and disease.

Biologists, biochemists, biophysicists, chemists, and other health scientists who are interested in this particular field should keep a copy as a standard reference.

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Edited by MURRAY S. COOPER
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