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Microchemical Journal, Volume 22, Number 4, December 1977

Briefs

Bilirubin and Hemoglobin Interferences in Direct Colorimetric Cholesterol Reactions Using Enzyme Reagents. M. T. PERLSTEIN,[1,3] R. J. THIBERT,[2,3] AND B. ZAK,[2,3] [1] *Department of Laboratory Medicine, Sinai Hospital, Detroit, Michigan 48235,* [2] *Department of Pathology, Wayne State University, School of Medicine and Detroit General Hospital, Detroit, Michigan 48201, and* [3] *Department of Chemistry, University of Windsor, Windsor, Ontario, Canada N9B 3P4.*

The interferences of bilirubin and hemoglobin were tested in two cholesterol procedures in which enzymes were used as chemical reagents. Both compounds were shown to produce some interferences by reacting competitively with peroxide in both systems.

Microchem. J. **22**, 403–419 (1977).

A Simple Apparatus for Rapidly Initiating Spectrophotometrically Monitored Enzymatic Reactions. HARLOW H. DARON AND JOHN L. AULL, *Department of Animal and Dairy Sciences and Department of Chemistry, Auburn University, Auburn, Alabama 36830.*

Apparatus is described for rapidly initiating spectrophotometrically monitored reactions by adding a reagent to the cuvette without opening the sample chamber of the spectrophotometer. The apparatus allows the recording of initial reaction rate data within 2 sec after initiating the reaction.

Microchem. J. **22**, 420–425 (1977).

Indirect Polarographic Microdetermination of Alkoxyl Groups. Y. A. GAWARGIOUS, L. S. BOULOS, AND B. N. FALTAOOS, *National Research Centre, Dokki, Cairo, Egypt, A. R. E.*

The method is based on the conversion of the alkoxyl group to alkyl iodide which is subsequently oxidized with bromine to iodate. The latter is then estimated through polarographic recording of its cathodic reduction wave. Eleven alkoxyl compounds were analyzed satisfactorily with an average error of ± 0.34%.

Microchem. J. **22**, 426–433 (1977).

Spectrophotometric Investigation of Ti(IV) Complexes of *N*-Phenylacetylphenylhydroxylamine. SASWATI P. BAG AND ANUP K. KHASTAGIR, *Chemistry Department, Jadavpur University, Calcutta–700032, India.*

The yellow complexes are soluble in chloroform and exist with metal:ligand ratios of 1:2, 1:3, and 1:4 in the acidity ranges of 1–3, 4–7, and 7.5–9.0 *N* HCl, respectively. A method for the determination of trace amounts of titanium in the presence of diverse ions is described.

Microchem. J. **22**, 434–441 (1977).

Polarographic Microdetermination of Iron, Manganese, Lead, Copper, Bismuth, and Tin in Organic Compounds. Application to Analysis of Pharmaceuticals. S. W. BISHARA AND F. M. EL-SAMMAN, *Department of Chemistry, College of Science, Mosul University, Mosul, Iraq.*

Samples are combusted, suitable supporting electrolyte is added, and the cathodic reduction wave of the corresponding metal recorded. A determination requires less than 45 min.

Microchem. J. **22,** 442–450 (1977).

NMR Measurements of Stability Constants for Complexes of Carbon Tetrachloride and Some Aromatic Compounds. H. MODARESS AND M. EDRISSI, *Department of Chemistry, Tehran Polytechnic, Tehran, Iran.*

NMR spectroscopy was used to measure the stability of constants of complexes of carbon tetrachloride with toluene and with benzylcyanide.

Microchem. J. **22,** 451–456 (1977).

Methods for the Isolation and Characterization of Constituents of Natural Products. XXI. Use of a Celite–Potassium Methylate Column for Rapid Preparation of Methyl Esters From Microgram Amounts of Glycerides. DANIEL P. SCHWARTZ, *Eastern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, 600 East Mermaid Lane, Philadelphia, Pennsylvania 19118.*

A very small column of potassium methylate–Hyflo Super-Cel is used to convert microgram quantities of glycerides to methyl esters. Transesterification is complete in hydrocarbon but not in chlorinated solvents or in CS_2. The methyl esters can be recovered in 92–95% yield. Regardless of solvent used, the recovered methyl esters are representative of the original fatty acid composition of the glycerides.

Microchem. J. **22,** 457–462 (1977).

Luminol-Dependent Chemiluminescence Analysis of Cellular and Humoral Defects of Phagocytosis Using A Chem-Glo Photometer. KNOX VAN DYKE, MICHAEL TRUSH, MARK WILSON, PATRICIA STEALEY, AND PHILIP MILES,* *Department of Pharmacology and Physiology–Biophysics–ALOSH,* West Virginia University Medical Center, Morgantown, West Virginia 26506.*

Conditions are described under which luminol has been utilized to measure phagocytosis-associated metabolic events in activated human PMNs and rabbit and dog alveolar macrophages. Some possible applications are suggested.

Microchem. J. **22,** 463–474 (1977).

Microdetermination of Ascorbic Acid Using Bromine Monochloride in Water–Acetic Acid Medium. V. K. S. SHUKLA AND JØRGEN CLAUSEN, *Institute of Experimental Biosciences, Roskilde Universitetscenter, P.O. Box 260, 4000 Roskilde, Denmark.*

The sample is dissolved in acetic acid, an excess of bromine monochloride is added, after which the excess is back-titrated iodometrically.

Microchem. J. **22,** 475–478 (1977).

Spectrophotometric Determination of Cyanocobalamin (as Cobalt). G. S. VASILIKIOTIS, TH. A. KOUIMTZIS, AND A. VOULGAROPOULOS, *Laboratory of Analytical Chemistry, University of Thessaloniki, Thessaloniki, Greece.*

A method is described for the determination of cyanocobalamin in pharmaceutical preparations. After wet combustion, the cobalt is determined by reaction with 2,2′-dipyridyl-2-pyridylhydrazone.

Microchem. J. **22,** 479–483 (1977).

Oxidation of Organic Substances by Tervalent Manganese Compounds. VIII. Determination of Benzene Polyhydroxy and Aminohydroxy Derivatives with a Standard Solution of Hexaquomanganese(III) Ion in Perchloric Acid. J. BAREK, A. BERKA, AND J. KOREČKOVÁ, *Department of Analytical Chemistry, Charles University, Albertov 2030, 128 40 Prague 2, Czechoslovakia.*

The reaction of hexaquomanganese(III) ion with hydroquinone, *p*-aminophenol, metol, pyrochatechol, resorcinol, and phloroglucinol was studied. The first three compounds were oxidized quantitatively to the corresponding quinone and the reaction can be employed for the determination of the three. The same does not apply to the other three compounds.

Microchem. J. **22,** 484–488 (1977).

Determination of Nanogram Quantities of Cobalt in Complex Matrices by Flameless Atomic Absorption Spectrometry. CRAIG M. YOUNG AND JON M. BALDWIN, *Allied Chemical Corporation, Idaho Chemical Programs–Operations Office, 550 Second Street, Idaho Falls, Idaho 83401.*

Flameless atomic absorption spectrometry is used to determine as little as 10 pg of cobalt. The cobalt is extracted into ethyl acetate as the pyrrolidinedithiocarbamate complex. The system is effective in removing matrix effects due to high concentrations of various complexing agents, strong oxidants, and reductants that are present in the original samples.

Microchem. J. **22,** 489–497 (1977).

Kinetic Potentiometric Determination of Creatinine in Urine with a Picrate-Ion-Selective Electrode. E. P. DIAMANDIS, M. A. KOUPPARIS, AND T. P. HADJIIOANNOU, *Laboratory of Analytical Chemistry, University of Athens, 104 Solonos Street, Athens (144), Greece.*

The method is based on the creatinine–picrate reaction in alkaline medium. The reaction is monitored with a picrate-selective electrode, and the increase in electrode potential in 90 sec is measured and related directly to the creatinine concentration.

Microchem. J. **22**, 498–504 (1977).

Spectrophotometric Determination of Traces of Hydrogen Peroxide with the Ti(IV)–Xylenol Orange and the Ti(IV)–Chromazurol S Reagents. CHIYO MATSUBARA AND KIYOKO TAKAMURA, *Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan.*

The formation of mixed ligand complexes in Ti(IV)–xylenol orange (XO)–H_2O_2 and Ti(IV)–chromazurol S (CAS)–H_2O_2 systems was studied by spectrophotometry. In both cases, the absorbance at λ_{max} was stable for a long time and proportional to the concentration of hydrogen peroxide.

Microchem. J. **22**, 505–513 (1977).

The Automated Analysis of Catecholamines: An Improved Procedure for the Simultaneous Differential Analysis of Epinephrine and Norepinephrine in Tissues, Blood, and Gland Perfusates. ROBERT L. ROBINSON, *Department of Pharmacology, West Virginia University Medical Center, Morgantown, West Virginia 26506.*

A modified automated method is described for the determination of the two amines simultaneously in a sample during a single run through the AutoAnalyzer. The differentiation of the two is based on the use of two stabilizing agents and oxidation of the catecholamines at two quite different pHs.

Microchem. J. **22**, 514–527 (1977).

Assay of Diamine Oxidase by Amperometric Measurement of the Rate of Oxygen Depletion. LINDA B. MARSHALL AND GARY D. CHRISTIAN, *Department of Chemistry, University of Washington, Seattle, Washington 98195.*

A method for the rapid kinetic assay of diamine oxidase is described. Diamine oxidase catalyzes the direct oxidation of diamine compounds by oxygen. The rate of oxygen depletion is measured with a membrane oxygen electrode and the reaction rate is obtained directly by recording the derivative of the change in amperometric current.

Microchem. J. **22**, 528–534 (1977).

Amperometric and Constant-Current Potentiometric Determinations of the Landolt Effect. F. F. GAÁL, V. I. SÖRÖS, F. B. SZEBENYI, AND V. D. CANIĆ, *Institute of Chemistry, Faculty of Sciences, University of Novi Sad, Novi Sad, Yugoslavia.*

An amperometric method with one platinum indicator electrode as well as the constant-current potentiometric method with one and two indicator electrodes were successfully applied to the Landolt effect determination by means of the hydrogen peroxide–iodide–ascorbic acid and bromate–bromide–ascorbic acid indicator reactions.

Microchem. J. **22**, 535–540 (1977).

Catalytic Microdetermination of Iron. G. S. VASILIKIOTIS, C. PAPADOPOULOS, M. SOFONIOU, AND D. THEMELIS, *Laboratory of Analytical Chemistry, University of Thessaloniki, Thessaloniki, Greece.*

A reaction rate method is described for the microdetermination of iron. The method is based on the catalytic action of iron on the reaction of 2,4-diaminophenol with hydrogen peroxide. The effect of reagent concentration is studied and the maximum tolerable amounts of interfering ions are determined. The relative error is approximately 2% for the determination of 2.8×10^{-2} μg/ml.

Microchem. J. **22**, 541 – 547 (1977).

Design and Construction of a Novel Long-Path Spectrophotometer. G. E. O'BRIEN, J. V. HORNSTEIN, AND H. A. FLASCHKA, *School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332.*

The photometer described uses a light emitting diode (LED) as light source and a phototransistor as detector. The LED is pulsed by an ampere level dc current and the light is measured only during the pulse. In this way the LED can deliver up to 10 times the light energy as when operated with the usual current on the milliampere level, without showing the harmful effects of overheating and great deviation from linearity. The pulsing also aids in the exclusion of steady ambient light. Ambient light by incandescence and fluorescence lamps is excluded by intermittently measuring the ambient and LED light, storing the information in electronic units, and subtracting the two signals electronically. The housing is such that the LED and associated circuits are in one compartment and the detector with related circuits is in another. The two compartments, connected by extendable cables, can be separated and thus accomodate cells even of extreme length.

Microchem. J. **22**, 548 – 556 (1977).

Determination of Elemental Fluorine in Organic Compounds by Fluorine-19 Nuclear Magnetic Resonance Spectroscopy. DAVID B. STAIGER, RICHARD J. WARREN, AND JOHN E. ZAREMBO, *Smith Kline & French Laboratories, 1500 Spring Garden Street, Philadelphia, Pennsylvania 19101.*

Fluorine-19 NMR was used for the determination of covalently bonded fluorine in organic compounds of pharmaceutical interest. The accuracy and precision in addition to the rapidity of the analysis recommend the NMR method as the method of choice for fluorine analysis.

Microchem. J. **22**, 557 – 560 (1977).

Analytical Reactions of Substituted Cyanoferrates. 1. $Na_3[Fe(CN)_5(NH_3)]$. M. K. GADIA AND M. C. MEHRA, *Trace Contaminants Research Group, Chemistry Department, Université de Moncton, Moncton, New Brunswick, Canada E1A 3E9.*

Pentacyanoamminoferrate (PCAF) reacts in aqueous solution with some common cations and anions to produce colored species. A number of these are sensitive enough to permit their colorimetric determinations. These include Fe^{3+}, Fe^{2+}, Co^{2+}, VO^{2+}, Mo^{6+}, $S_2O_3^{2-}$, and NO_2^-.

Microchem. J. **22,** 561–566 (1977).

Performance of a New Automatic Carbon Dioxide Coulometer. EDWARD W. D. HUFFMAN, JR. *Huffman Laboratories, Inc., 3830 High Court, P.O. Box 777, Wheat Ridge, Colorado 80033.*

A new automatic carbon dioxide coulometer is shown to give accurate and reliable results. This article represents the first in a series of planned papers to describe the use of the instrument in a variety of analyses.

Microchem. J. **22,** 567–573 (1977).

Micromethod for the Gas Chromatographic Quantification of Blood Ethanol. RUTHANN P. STURTEVANT, *Departments of Anatomy and Surgery, Loyola University of Chicago, Stritch School of Medicine, 2160 South First Avenue, Maywood, Illinois 60153.*

A microadaptation for the head space analysis of ethanol in 20-μl blood samples is described. Ethanol concentration is determined by comparison of the derivatized ethanol peak area with that of a 1-propanol internal standard.

Microchem. J. **22,** 574–579 (1977)

A Simple Correction for Errors in Measuring Areas of Attenuated Chromatographic Peaks. HARLOW H. DARON, *Department of Animal and Dairy Sciences, Agricultural Experiment Station, Auburn University, Auburn, Alabama 36830.*

The method was used to determine the fatty acid composition of bacterial lipid extracts, where the relative amounts of the fatty acids varied greatly and low attenuation was needed for quantitation and identification of minor components, but when major components emerged the attenuation was increased in steps to allow their measurement.

Microchem. J. **22,** 580–584 (1977)

MICROCHEMICAL JOURNAL 22, 403–419 (1977)

Bilirubin and Hemoglobin Interferences in Direct Colorimetric Cholesterol Reactions Using Enzyme Reagents[1]

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Department of Laboratory Medicine, Sinai Hospital, Detroit, Michigan 48235[2], Department of Pathology, Wayne State University School of Medicine and Detroit General Hospital, Detroit, Michigan 48201[3], and Department of Chemistry, University of Windsor, Windsor, Ontario, Canada N9B 3P4[4]

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INTRODUCTION

Since enzymes can permit a certain measure of specificity, it would appear that the determination of cholesterol using enzymes as chemical reagents (*3, 12, 16*) might provide direct methods which are relatively interference-free when compared to acid systems (*7, 10, 22*) that involve similar direct serum handling. Two enzymatic cholesterol procedures using different colorimetric endpoint reactions were selected for study here because the latter are commonly employed in other peroxide-forming systems as well. Therefore, any concomitant errors which might result from the use of those reactions for the quantification of cholesterol (or other analytes) which ultimately depend on the generation of hydrogen peroxide in the final analytical step could also be studied. The determination of peroxide generated as a result of a specific enzymatic action on a particular serum constituent used as a substrate is a rather common practice (*4–6, 17, 21*). However, the ways in which the peroxide is treated for quantification may be varied for the different procedures (*5, 9, 13*).

Hemoglobin adds color to a sample and, at the same time, is capable of exhibiting pseudoperoxidase activity if peroxide is present (*8*). When acting like a peroxidase it may, like other peroxidases, undergo structural conversion leading to spectral changes (*15*) and, therefore, also seemed to be an interesting compound to study in these systems. In addition, hemoglobin's Soret band, which is a very strong absorbing chromophoric portion of the molecule, superimposes on the peak maximum of one of the two reactions to be described (*14*). Since the Soret band intensity can be shown to have some variability when peroxide is present, this will

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illustrate an obvious difficulty for any spectrophotometric blanking process. Even though moderately elevated concentrations of hemoglobin will be shown to have a small effect on the final cholesterol value, hemoglobin provides another means for examining ways in which a compound can take part in the chemistry of the reactions needed as the indicator portion of an analytical sequence.

Bilirubin has been aggravatingly troublesome in direct cholesterol methodologies involving oxidative acid systems (*23*). Because our preliminary findings (*11*) indicated that bilirubin reacted in a very interesting though similar manner in both of the enzyme procedures for cholesterol, it seemed important to conduct a thorough study of its reactivity. Samples submitted for cholesterol determinations sometimes contain abnormal concentrations of bilirubin, whose effect in other methodologies has been to elevate observed cholesterol values. However, the reports describing the effect of bilirubin on the coupled enzyme reactions using Trinder's reaction to quantify peroxide are somewhat confusing, and contradictory. In one study (*20*), a cholesterol-free serum was tested which was fortified with various amounts of bilirubin. From the data obtained these investigators concluded that bilirubin gave a slight positive interference. Subsequent experiments, to be described here, will demonstrate both why this should appear positive when cholesterol is absent as well as why it does not represent a natural circumstance when cholesterol is present. In another study it was shown that the effect of bilirubin on cholesterol in that enzymatic modification resulted in an apparent depression of the cholesterol value (*1*). Again our experiments will confirm that depression should be the case in the presence of bilirubin. In a third variation of a similar procedure proposed, there was a claim of no error at all when a very high concentration of bilirubin (300 mg/liter) was tested (*18*). In this case, our experiments will demonstrate that this cannot be what should be obtained under the conditions as described. Rather than zero error, the error should be negative. This dichotomy in findings from three groups of investigators for a bilirubin effect is interesting and obviously merits further investigation.

The purpose of the present report, then, is to show the spectrophotometric findings of the side reactions of bilirubin and hemoglobin in two cholesterol procedures which seemingly use virtually identical reaction conditions for cholesterol ester saponification and free cholesterol oxidation to cholestenone and peroxide. These steps are followed then by two different colorimetric approaches for the determination of the peroxide generated. In both of the endpoint reactions for the determination of peroxide, different enzymes, catalase for the Hantzsch technique (*14*) and peroxidase for the Trinder technique (*20*), are used for catalytic oxidation resulting in measurable colored compounds. The final analytical

steps are considerably different in the time of their reactions, the spectra generated, and the effect of hemoglobin on the measurement characteristics, but are similar in the final effect of bilirubin on the color reaction itself. They also are similar in the effect of subtraction of a serum blank, a procedural step suggested by several kit manufacturers for both methodologies.

MATERIALS AND METHODS

Reagents

Bilirubin. A lyophilized control solution of bilirubin in albumin (Dade, Division American Hospital Supply Corp., Miami, Fla.) was reconstituted. It contained 196 mg/liter of bilirubin.

Cholesterol standard solutions. An aqueous cholesterol standard solution (3000 g/liter) was used as prepared (Searle Diagnostic Inc., Columbus, Ohio) or diluted with diluent to make 2000 and 1000 mg/liter solutions.

Method A reagent. The enzyme reagent utilizing the Trinder reaction for measurement of peroxide can be prepared as described by Allain *et al.* (1). However, the enzyme reagent prepared lyophilized in vials by Abbott Laboratories, Diagnostic Division, North Chicago, Ill., was used in the studies described.

Method B reagent. The enzyme reagent (purchased from Boehringer Mannheim Corp.) using the Hantzsch reaction for quantification of peroxide can be prepared as described by Roeschlau *et al.* (*14*). However, the reagents prepared by the Boehringer Mannheim Corp. were used in the studies described.

Hemoglobin. Red blood cells were lysed and the ghosts removed by centrifugation. The hemolysate so prepared contained 30 g/liter hemoglobin and was demonstrated to be cholesterol-free. This stock solution was then diluted to give the final desired concentration and small aliquots were then added to the reagent.

Peroxide solution. A peroxide stock solution was prepared by diluting 10 ml of a 30% peroxide solution to 100 ml. The peroxide content was determined by titration with 0.1 *N* potassium permanganate and was found to be 2.52% (*19*). The stock solution was diluted 1/100 with distilled water and was used as a working solution (prepared fresh). This was equivalent to a cholesterol concentration of 7.40 m*M*/liter, (2860 mg/liter).

Albumin. Human serum albumin (250 g/liter) was obtained from the Red Cross, Windsor, Ontario, Canada.

Methods

Method A enzymatic assays were carried out as described by the procedural protocol. The reaction was started by the addition of a

cholesterol standard solution with a sample to reagent ratio of 10 μl sample/1.0 ml reagent. After a 10-min incubation period at 37°K, the spectrum of the completed reaction was recorded using the reagent solution as a reference blank.

Method B enzymatic assays were performed according to Roeschlau (*14*). In this procedure, the reaction was started by the addition of cholesterol oxidase, and was allowed to proceed for 60 min at 37°K in a precision water bath. The visible spectrum was then recorded using a sample blank as the reference solution. The sample blank consisted of the sample and all components of the reagent except the cholesterol oxidase. The sample to reagent volume ratio was 10 μl sample to 1.0 ml reagent.

Bilirubin and hemoglobin studies. The effect of bilirubin and hemoglobin was observed by reacting either by itself in either reagent without permitting peroxide generation (Method A, no cholesterol added; Method B, no cholesterol oxidase added). These spectra were then compared to the spectra obtained when the cholesterol oxidase reaction was progressing in the presence of bilirubin, and the spectra obtained with cholesterol standard solutions.

RESULTS AND DISCUSSION

The measure of cholesterol as an analyte in most of the presently used spectrophotometric procedures in which enzymes serve as reagents is based on the amount of peroxide generated by the action of cholesterol oxidase on its substrate. It may appear from the results generated by a single wavelength measurement that the absorbance values obtained at that wavelength may be entirely due to the peroxide action in a catalase-coupled, or peroxidase-coupled endpoint reaction, whereas the absorbance measured in reality may be due in part to the endpoint reaction and in part to the absorbance of a competing colored substrate in the peroxidase or catalase sequences of the procedures, which is only partly destroyed by peroxide reaction. An example of such a potential compensatory system would be one in which a colored reactant such as bilirubin, for peroxidase-activated peroxide, is present to compete with a second reaction present as a part of the reagents selected to determine cholesterol by a final coupled step. Apparently the effect of bilirubin in these reactions is twofold. It will be shown that it consumes generated peroxide and as a result undergoes a spectral change so that the reacted bilirubin no longer absorbs light at the measurement wavelength. The residual blank spectrum after reaction is due to unreacted bilirubin which partially compensates for the peroxide no longer available for the proposed endpoint reaction. If a serum blank is used to compensate for the background color, it would result in an overcorrection owing to the diminished spectrum of the bilirubin whose color vanished during the

coupled enzyme reactions which occurred in the presence of peroxide generated from cholesterol.

Upon casual examination, both enzyme systems for cholesterol measurement studied here appear to be similar; see reaction schemes. Each utilizes enzymes specific for hydrolysis of cholesterol esters (cholesterol esterase) and for the oxidation of cholesterol to the ketone and peroxide (cholesterol oxidase). Therefore, any resultant differences should reside in the mode of measuring the peroxide generated during the cholesterol oxidase reaction. In one case (Method A) using the Trinder reaction (*1*), peroxide is reduced by peroxidase and the reaction intermediates of this complex enzyme mechanism are used to oxidatively couple 4-aminoantipyrine to phenol to form a rose-colored chromogen that has an absorption maximum at 500 nm. The entire series of reactions from cholesterol esters to color formation is over in less than 10 min. In the other case (Method B), the measure of peroxide begins by reducing it with catalase in the presence of methanol to produce formaldehyde and water. The formaldehyde then reacts with ammonium ions and acetylacetone in a Hantzsch reaction (*14*) to produce the colored lutidine derivative, 3,5,-diacetyl-1,4-dihydrolutidine, which has an absorption maximum between 405 and 415 nm. This reaction is 95% complete after 30 min at 37°K, and the recommended reaction time is 60 min. Besides reaction times, another obvious difference between the two reactions is that Method A employs a reagent blank as a spectrophotometric reference, while the reaction itself is started with the introduction of the sample, while Method B utilizes a serum blank, and in this case, the reaction for cholesterol present commences on the separate addition of cholesterol oxidase. These differences in reaction conditions provide an interesting example of how an interference such as bilirubin can interact in these procedures as a reaction competitor, or how hemoglobin can interfere because its spectrum exhibits its Soret band in the same area as the chromogen formed in the Hantzsch reaction, a spectrum which can change because of its action as a pseudoperoxidase (*15*). The Soret band shows such a change when hemoglobin is a member of the mixture. The final color is therefore difficult to blank out because of this change in spectral fine structures (Figs. 4 and 5).

Figure 1 shows the spectra obtained when bilirubin in albumin is added to the enzymatic reagents for cholesterol determination. Since no reaction occurs, an unaltered spectrum of bilirubin results as a static blank. In comparing the absorbances at 500 and 410 nm, it can be seen that the 410-nm wavelength would have the greater absorbances to subtract if bilirubin were indeed a part of a static blank system.

In order to further demonstrate the effect of bilirubin as a static blank when it is added to the reagents of both enzymatic procedures tested

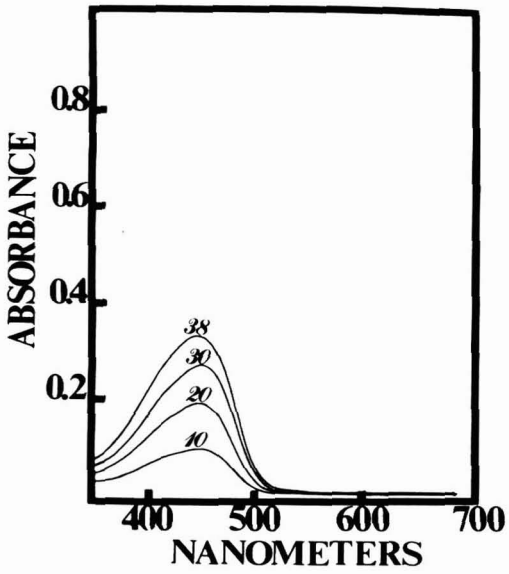


FIG. 1. Bilirubin spectra at several concentration levels in the reagent matrix of Method A.

here, different concentrations of bilirubin were added and measurements were made at the specified wavelengths of both procedures, Method B which uses cholesterol esterase (C.E.)–cholesterol oxidase (C.O.)–catalase plus methanol–acetylacetone at 410 nm, and Method A which uses C.E.–C.O.–peroxidase–4-aminoantipyrine plus phenol at 500 nm. In Fig. 2, curve B is the result for Method B and curve A is the result for Method A, where one is the static blank absorbance of the IR side of the bilirubin spectrum for Method A and the other, the UV side of that spectrum for Method B. Dynamic blanks representing oxidations of bilirubin by the respective reagents are shown for contrast for the Liebermann–Burchard (LB) and the iron reactions for cholesterol. An interesting aspect here is that the static blanks are not correct for either enzyme procedure simply because bilirubin does act as a competing reactant for peroxide and is therefore not inert. Use of the static mode of blanking would cause the results to obviously be even lower than they should be if it were ignored as a blank. This phenomenon, as stated earlier, will be further described in Figs. 3 and 7. The tendency therefore for both enzymatic procedures in the presence of bilirubin is to yield low values because the peroxide representing cholesterol is partially removed by oxidation of bilirubin.

The effect of bilirubin by itself in Method A reagent, approximates the one Witte and co-workers reported (*20*) using a cholesterol-free serum. Since peroxide is the substitute analyte, its interference cannot easily be corrected by preparing a serum blank because bilirubin reacts with

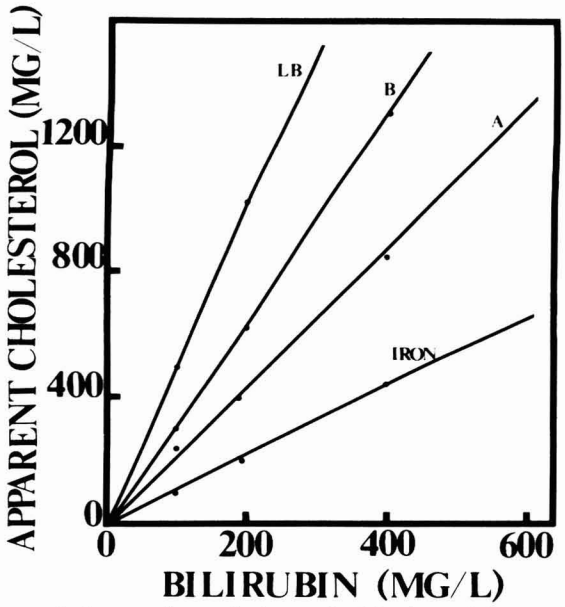


FIG. 2. Comparison of the static and dynamic blanks of four cholesterol reactions: Method A and Method B show calibration static blank curves A and B, respectively. Iron and Liebermann–Burchard reactions (LB) generate the dynamic blanks shown.

peroxide during the procedure in which cholesterol reacts to form that peroxide.

Figure 3 shows several results which fortify the previous findings. They were with bilirubin when it was included in the reaction medium under different conditions selected to show why bilirubin in albumin could appear to yield either a positive or negative interference depending on what circumstances for reaction were selected. Scan 5 shows the spectrum due to bilirubin when dissolved into the Method A reagent in an albumin matrix. Inspection of the IR side shows that there should be a positive interference from bilirubin with a cholesterol measurement if nothing happened to it while the endpoint reaction was taking place, that is, if it were truly a static blank interference. Scan 3 was obtained when bilirubin and cholesterol were reacted simultaneously. However, scan 1 was generated by the addition of bilirubin after the cholesterol reaction was complete and no peroxide remained as a potential reactor for bilirubin. Thus the bilirubin in this case exerted its total interfering effect (static) due to the IR side of its spectrum. The resultant absorbance at 500 nm, therefore, is the mathematical sum of the color given by cholesterol reaction (scan 2) and the absorbance contributed by the bilirubin spectrum of scan 5. The summation spectrum shows an apparent hypsochromic shift in peak maximum along with apparent hyperchromic

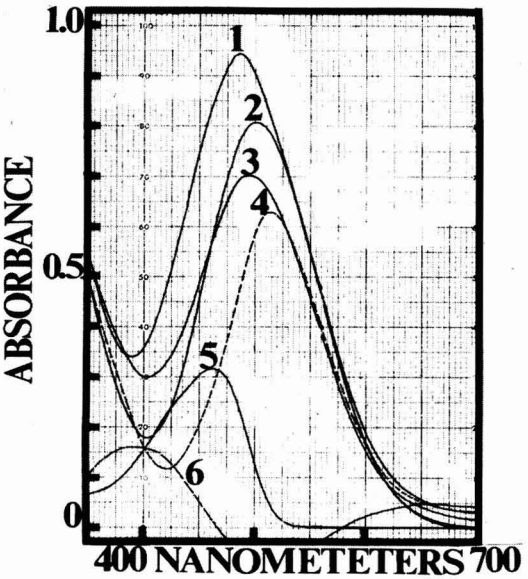


FIG. 3. Spectra 2 and 5 are the respective ones for cholesterol and bilirubin in Method A. Spectrum 3 was obtained by reacting cholesterol plus bilirubin simultaneously, whereas spectrum 1 was obtained by adding bilirubin after the cholesterol reaction was complete. Spectrum 4 is the difference spectrum between spectra 3 and 5 while spectrum 6 is the difference spectrum between spectra 3 and 2.

effect, and obviously shows a positive error at 500 nm, where the Trinder reaction is usually measured. Therefore scan 5 indicates what would result if the effect of bilirubin was only to add color to the system. Perhaps not surprisingly however, the spectrum obtained when bilirubin is present during the cholesterol reaction, shown as spectrum 3, does not represent the sum of the spectra of scan 2 and scan 5, and a competitive situation between the Trinder reactants and bilirubin has occurred. To emphasize this phenomenon, when the difference spectrum between the competing reactions of bilirubin and the peroxide obtained from cholesterol (scan 3) and the bilirubin "blank" (scan 5) were recorded using spectrum 5 solution as a reference, scan 4 was the residuum. This shows what one would actually obtain if one treated the data as if bilirubin did not compete in the reactions taking place during the step following peroxide generation involving color development. This spectrum of scan 4 demonstrates an overcorrection in blanking and lowers the measured cholesterol value. In this reaction, the use of a serum blank makes matters worse by correcting for a color that obviously is partly destroyed during the procedure. Similarly, the solution giving rise to spectrum 3 was scanned against the cholesterol standard as a reference in order to subtract the color due to the cholesterol reaction. This should have regenerated the spectrum of bilirubin, if bilirubin had not reacted. That

the resulting spectrum, scan 6, was not the same as bilirubin is clearly shown by comparing it to the spectrum of scan 5. The convincing evidence of this experiment is proof that bilirubin reacted and underwent a chemical conversion that caused its spectrum to be altered so that it no longer absorbed light at 500 nm. In the process of conversion, some peroxide was used up. Since peroxide formation and subsequent color reaction is the true measure of cholesterol, it becomes clear that cholesterol is not measured correctly when a bilirubin and cholesterol mixture is treated as described here. The resultant absorbance used to determine cholesterol must be summed up as due in part to peroxide and in part to any residual absorbance due to bilirubin.

Reinforcing evidence that bilirubin will indeed react with peroxide is easily demonstrated by substituting peroxide for the cholesterol it would have used to generate it. Figure 4, scan P, represents the peroxidase-coupled 4-aminoantipyrine–phenol reaction with peroxide. When peroxide plus bilirubin are put into the same reaction, scan (P + B) resulted. The spectrum for the same amount of bilirubin is shown as scan B. The difference spectra [(P+B) −B] and [(P+B) −P] result when the peroxide and bilirubin reaction are scanned with either the bilirubin blank or the peroxide reaction used as reference solutions. The difference spectrum [(P + B) −B] shows that in the presence of bilirubin less than the

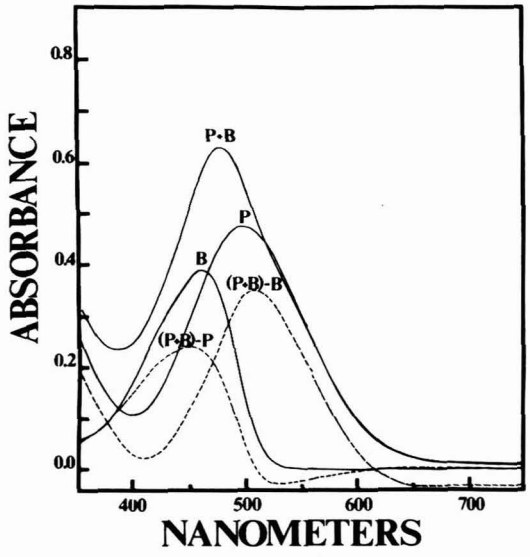
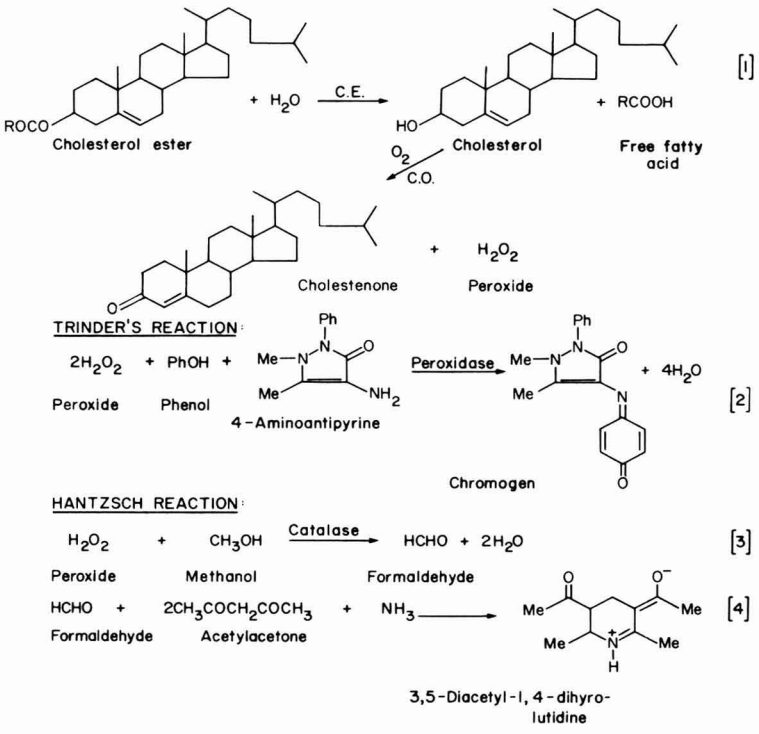


FIG. 4. Spectrum P results when peroxide is used to generate the color reaction instead of cholesterol. Spectrum B is the static blank obtained when bilirubin is present in the reagent. Spectrum (P + B) is the simultaneous reaction of bilirubin and peroxide with reagent. Spectra [(P + B) − B] and [(P + B) − P] are the difference spectra of the simultaneous reaction of bilirubin and peroxide with reagent when using either bilirubin alone in reagent as a blank or by using the peroxide-generated color as a blank, respectively.

anticipated color is formed, and one can infer that bilirubin does react during this endpoint step in the coupled reaction scheme. It is also evident that it is at this step that bilirubin undergoes a reaction since the difference spectrum [(P+B) −P] does not generate the true spectrum of bilirubin shown as scan B. These results are similar to those obtained when the cholesterol reaction was responsible for the formation of peroxide and subsequent color development. Interestingly, the effect of bilirubin in this peroxide system is not as pronounced as it is in the cholesterol system. The simultaneous reaction of peroxide and bilirubin results in more color than peroxide reacted by itself, but less than the mathematical sum of the separate bilirubin and peroxide spectra. There is a suggestion that the rapid addition of peroxide is not exactly the same as enzymatically generated peroxide during the cholesterol oxidase reaction. Also, the ratio of reactants favors bilirubin when peroxide is enzymatically generated whereas the addition of the peroxide in a single addition may upset this ratio by a considerable amount. Thus the above experiment suggests that the bilirubin interference is facilitated by the slower introduction of peroxide provided by the cholesterol oxidase generation of peroxide. Bilirubin could be competing for the substrate peroxide, and rapid addition of peroxide may somewhat overcome this effect if one



considered comparative reaction rates of bilirubin and peroxidase with peroxide.

An experiment similar to the one carried out with bilirubin was then performed with hemoglobin. Hemoglobin was pipetted into the Method A cholesterol reaction medium and the spectrum for hemoglobin (Hb) shown in Fig. 5 was generated for this matrix. If the effect of hemoglobin was simply to add color, the predicted absorbance for the combination of cholesterol and hemoglobin should have been the summation of the contributions of the two spectra at 500 nm. However, the observed absorbance for the simultaneous reaction shown as spectrum (C+Hb) was greater than the calculated sum. When this combination was scanned using the same concentration of hemoglobin in the reagent as a blank, the scan shown as [(C + Hb) − Hb] resulted. If no interaction had occurred, this should have approximated the cholesterol standard spectrum, but along with the small increase in peak reading, some spectral distortion is present on the IR side. Predictably then, when the mixture was scanned using the cholesterol standard reaction mixture as the reference solution, the hemoglobin spectrum was not regenerated. Oddly, the Soret band was hypsochromically shifted and at the same time underwent a hypochromic effect, which suggest that hemoglobin was altered as a result of these

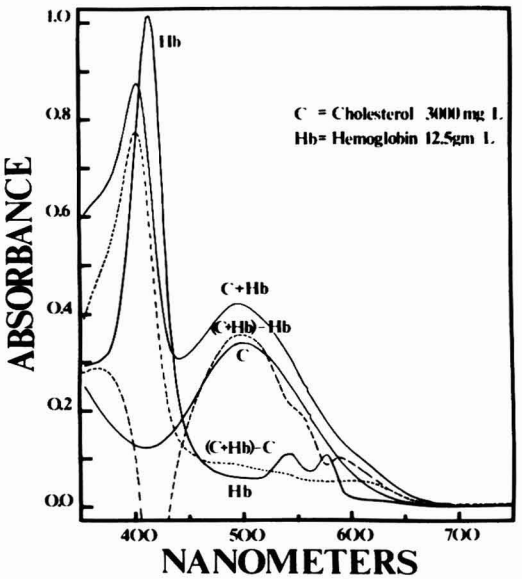


FIG. 5. Spectra C and Hb are those obtained individually with cholesterol and hemoglobin in the matrix of Method A, while spectrum (C + Hb) was obtained when both compounds were reacted simultaneously in the same medium. The difference curves of [(C + Hb) − Hb] and [(C + Hb) − C] were the respective attempts to regenerate the cholesterol and hemoglobin spectra.

coupled enzyme reactions. If the hemoglobin dissolved into the reagent was used as a blank as shown in spectrum [(C + Hb) − Hb] of Fig. 5, and its spectral contribution subtracted at 500 nm, the apparent cholesterol value would more closely approximate the true value than if it remained unblanked. Therefore, even if one uses such a blank it should only be partly correct for the effect of hemoglobin which may be present.

One might infer from the spectral characteristic exhibited by hemoglobin that it forms peroxide complexes similar to those formed between peroxidase and peroxide. The spectrum obtained after the cholesterol reactions are over, shown as scan [(C + Hb) − C], have similar characteristics to those reportedly observed after peroxidase had completely reduced peroxide, suggesting that hemoglobin is exhibiting a similar expected peroxidase activity. As further evidence in support of this finding, hemoglobin solution was added to a reagent mixture to which peroxide was added to generate color from the 4-aminoantipyrine–phenol coupled reaction which obviated the cholesterol–cholesterol oxidase reaction entirely. These results are shown in Fig. 6. In this case, the Soret band shift was not as marked as in Fig. 5, although it still showed a similar hypochromic effect, a fact which would complicate the subtraction of a serum blank in the Hantzsch reaction. As previously stated, during the cholesterol oxidase reaction with cholesterol, peroxide was slowly and continuously released over a period of time, unlike the sudden addition of

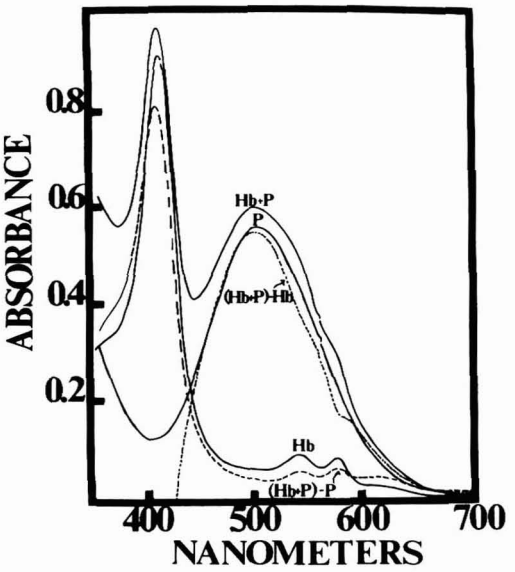


FIG. 6. Spectra P and Hb are those obtained with Method A reagents with hydrogen peroxide and hemoglobin, individually. The difference spectra [(Hb + P) − Hb] and [(Hb + P) − P] were the respective attempts to regenerate the peroxide and hemoglobin spectra.

peroxide shown here. Also, as when using a true peroxidase, the reaction of peroxide with the enzyme may be different, a factor which may be dependent on the mode of peroxide addition (*2, 21*). It has been reported that these types of changes in reaction conditions give rise to different mechanisms, and that the rapid interaction of peroxide with peroxidases produces different spectral changes in the visible region as compared to those changes where the reduction of peroxide is complete, as exhibited by the reactions shown in Fig. 6. Similar effects have also been reported with another heme protein, catalase. The blank reaction shown here as scan [(Hb + P) − P] is characteristic of peroxidase under similar reaction conditions (*15, 21*). In the case described here, however, the absorbance of the blank reaction at 500 nm is less than that shown for hemoglobin when it is mixed into the reagent (spectrum Hb). Therefore, the sum of the peroxide reaction and the contribution due to hemoglobin is greater than the observed absorbance when peroxide and hemoglobin are reacted simultaneously, scan (Hb + P). This evidence suggests that hemoglobin is behaving like a peroxidase, and its reaction with peroxide causes the spectral differences observed before and after the cholesterol reaction took place. Therefore, it can be concluded that hemoglobin is interacting in the peroxidase step of the reaction.

Figure 7 demonstrates how bilirubin can be shown to be an interference in the Method B procedure in a manner similar to that demonstrated in Fig. 3 for Method A. Scans 2 and 4 are the spectra obtained for cholesterol and bilirubin, respectively, using the reagent matrix of that system. Scan 4 represents a static reaction system, because without cholesterol to generate peroxide, nothing is present which will alter the bilirubin structure by oxidative action. Scan 1 is the spectrum obtained when the same concentrations of bilirubin and cholesterol are simultaneously present in the reaction. When the scan 4 bilirubin spectrum is used as a reference solution for the mixture of scan 1, scan 3 is the spectrum which is generated, which is considerably less than the theoretical spectrum shown as scan 2. The latter would be the resultant spectrum if bilirubin had no effect on the reaction. In similar fashion, if the solution which generated scan 2 is used as the reference for the mixture of scan 1, scan 5 is the spectrum which results. Since scan 4 is the expected spectrum if there was no interference from bilirubin, the difference between scans 4 and 5 again prove that bilirubin is a definite interference by competing for peroxide in the catalase reaction of Eq. [3]. A second experiment to show the effect of bilirubin on the cholesterol reaction was made, in which varying aliquots of bilirubin were added to a fixed concentration of cholesterol. In each case, the appropriate static blank was subtracted, each of which contained the same concentration of bilirubin as in the standard being measured. Those spectral results as

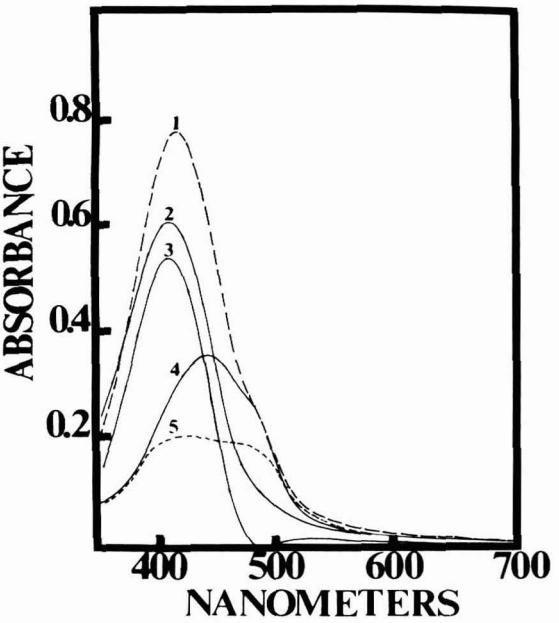


FIG. 7. Spectra 2 and 4 are those for cholesterol and bilirubin, respectively, using Method B. Spectrum 1 was obtained when the same concentrations of cholesterol and bilirubin were reacted simultaneously. Spectra 3 and 5 are the respective difference spectra obtained when curves 4 and 2 are each subtracted from curve 1 in an attempt to regenerate the spectra of cholesterol and bilirubin.

shown in Fig. 8. It can be seen that as the concentration of bilirubin increased, the apparent cholesterol peak absorbance diminished. If bilirubin had no effect in the overall reaction sequence, then the curves

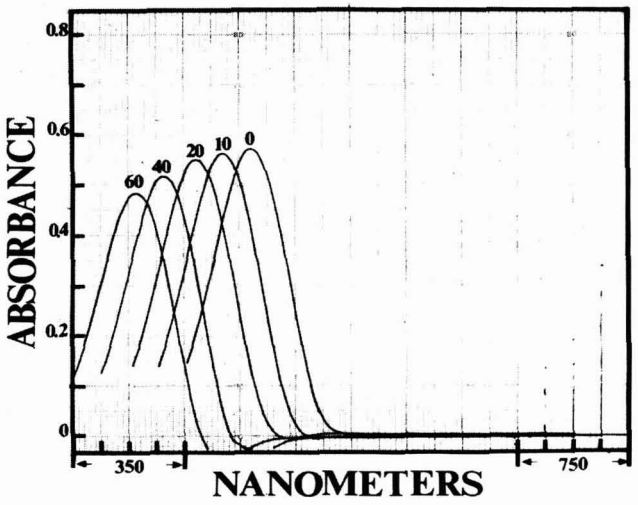


FIG. 8. Spectra obtained when increasing concentrations of bilirubin in albumin solution were added to fixed concentration of cholesterol and referenced against the appropriate static bilirubin blanks.

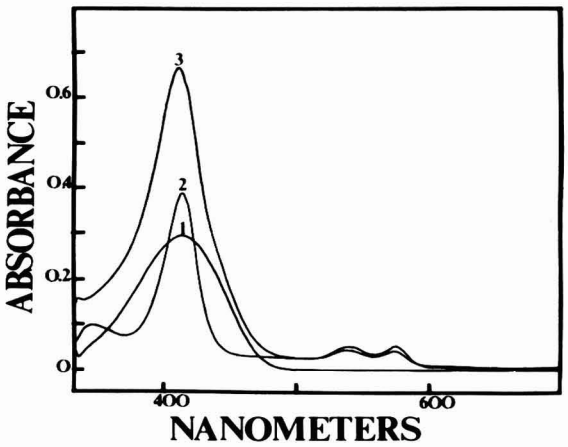


FIG. 9. The effect of hemoglobin in the cholesterol reaction of Method B.

should all have shown the same residual intensities. However, since some bilirubin interfered in each instance by reaction with peroxide in Eq. [3], then less formaldehyde was generated to react in Eq. [4]. If no sample blank were used here, the peak heights would have been more similar in intensity owing to the compensating error of the residual bilirubin solutions.

Figure 9 demonstrates the effect that hemoglobin could have on the cholesterol reactions of Method B. Scan 1 shows the spectrum of cholesterol, scan 2 shows the static blank spectrum of hemoglobin, while scan 3 shows the spectrum obtained when a mixture of the same two concentrations of each is present during the entire reaction process. It is quite apparent that the Soret band is a major interference to contend with in this particular endpoint reaction. Hemoglobin in this reaction also seems to exhibit the same nonadditive effect at peak absorbance as observed in Method A. This suggests the expected, that hemoglobin can also exert pseudoperoxidase activity in the Hantzsch reaction as well as in the Trinder reaction.

SUMMARY

The interferences of bilirubin and hemoglobin were tested in two cholesterol procedures in which enzymes were used as chemical reagents. Both procedures used similar approaches with cholesterol esterase to free cholesterol from its esters and cholesterol oxidase to generate hydrogen peroxide from the total free cholesterol resulting. From that common start, one procedure then used catalase to generate formaldehyde from methanol and the peroxide produced from cholesterol, and the formaldehyde was then reacted with acetylacetone to produce a yellow chromogen, while the other procedure used peroxidase to catalyze a reaction directly between peroxide and 4-aminoantipyrine plus phenol to generate a pink chromogen. Bilirubin and hemoglobin were shown to produce some interference by reacting competitively with peroxide in both systems and by contributing residual absorbance at the wavelengths of measurement of each of the chromogens. Since bilirubin showed a spectral change, static blanking with sample blanks caused overcorrections.

However, the elimination of a sample blank for either procedure could result in a favorable compensating error because the residual color of bilirubin could substitute in part at least for the lost reactivity of the peroxide used up in reaction with the bilirubin of the sample.

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MICROCHEMICAL JOURNAL 22, 420–425 (1977)

A Simple Apparatus for Rapidly Initiating Spectrophotometrically Monitored Enzymatic Reactions

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INTRODUCTION

In kinetic studies of enzyme-catalyzed reactions, it is generally advantageous to measure reaction rates as soon as possible after initiating the reaction, since the many factors which might influence the rate will not have changed appreciably from their initial conditions. This communication describes a relatively simple and inexpensive apparatus for rapidly initiating spectrophotometrically monitored reactions by adding enzyme (or substrate) to the cuvette without opening the sample chamber of the spectrophotometer. The apparatus has been used to study the steady-state kinetics of thymidylate synthetase, where it was necessary to measure initial rates at very low substrate concentrations. With this apparatus, reaction rates were conveniently recorded within 2 sec after initiating reactions.

MATERIALS AND METHODS

Materials were either available in the Machine Shop, Department of Physics, Auburn University, or were purchased from Small Parts Inc., Miami, Fla.

A photograph of the apparatus used with a Gilford Model 250 spectrophotometer is shown in Fig. 1. The plunger assembly holder is shown permanently mounted to the sample compartment cover of the spectrophotometer and positioned in such a way that when the plunger assembly is inserted, the plunger rod is directly over the center of the cuvette positioned in the light path. Details for the construction of the plunger assembly and its holder are given in Fig. 2. The plunger assembly holder (h) was constructed from an aluminum block, and an aluminum plug was placed in the hole when the instrument was being used for purposes not requiring the plunger assembly. The plunger rod housing (e) was constructed from aluminum. One end of the housing was threaded to accept a brass cap (d) which facilitated assembly and disassembly of the apparatus. The plunger rod (a) was made from either stainless steel or steel drill rod.

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FIG. 1. Gilford Model 250 spectrophotometer fitted with rapid add/mix apparatus.

The bearing surfaces and alignment of the movable plunger rod were provided by two holes drilled in the plunger rod housing and its cap, respectively. One end of the plunger rod was threaded to accept a sample holding and mixing device (j) designed to deliver up to 0.05 ml of sample to a cuvette and mix the cuvette contents, simultaneously. This device was modeled after a commercially available product (Add-A-Mixer, Precision Cells, Inc., Hicksville, N.Y.). The sample holder was made from Acrylite (polymethylmethylacrylate) and designed for use with semimicrocuvettes (12. 5 × 12.5 × 45 mm outside) with internal dimensions of 10 × 4 mm. Pushing down on the plunger rod compresses a spring (f) and inserts the sample holder in the cuvette. When the rod is released, the

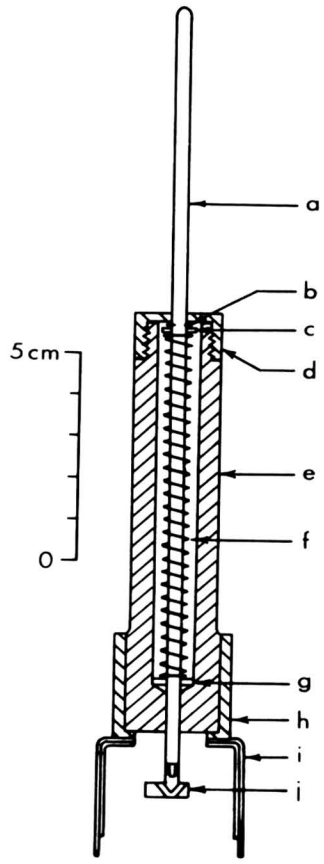

FIG. 2. Construction details of rapid add/mix apparatus. (a) plunger rod (stainless steel); (b) retaining ring; (c) upper washer (brass); (d) cap (brass); (e) plunger housing (aluminum); (f) compression spring; (g) lower washer (brass); (h) plunger assembly holder (aluminum); (i) spectrophotometer sample-chamber lid (Gilford); (j) sample holder/mixer.

compression spring returns the sample holder to a position over the cuvette. The bottom and top of the spring were supported with flat washers (c and g). A retaining ring (b) in a groove in the rod held the upper washer in place and restricted the upward distance that the plunger rod could travel.

The following general procedure was used to measure enzyme-catalyzed reaction rates. All components of the reaction mixture except the enzyme were added to a cuvette, mixed, and placed in the temperature-controlled spectrophotometer sample chamber. The sample chamber was covered with the sample-chamber lid. A small volume (0.05 ml or less) of enzyme solution was pipetted onto the sample holder (Fig. 3), and the plunger assembly was placed in the holder. Absorbance was

FIG. 3. Introduction of sample into sample holder/mixer.

recorded for several minutes prior to adding the enzyme to measure any background rate and to allow time for temperature equilibration of the reaction mixture and enzyme solution. The reaction was initiated by pushing the plunger rod down and rapidly moving the rod up and down several times to mix the reaction mixture components. The spring-loaded plunger rod automatically retracted the sample holder from the cuvette so that recording of rate data began shortly after releasing the plunger rod. In most instances rate data were recorded within 2 sec after initiating the reaction. When the assay was finished, the plunger assembly was removed from the holder, and the sample holder and lower portion of the

plunger rod were rinsed with distilled water in preparation for the next assay.

RESULTS AND DISCUSSION

To test for quantitative transfer, the apparatus was used to add 0.02 ml of a solution of bromphenol blue in 0.02 *M* piperazine-*N*,*N*′ -bis (2-ethanesulfonate) buffer, pH 6.8, to a cuvette containing 0.98 ml of distilled water. The mean (with standard deviation) of the absorbance at 592 nm for five trials was 1.787 ± 0.014 compared to 1.773 ± 0.011 when 0.02 ml of the same solution was pipetted directly into the cuvette (five trials). This result shows that the apparatus consistently transfers quantitatively,

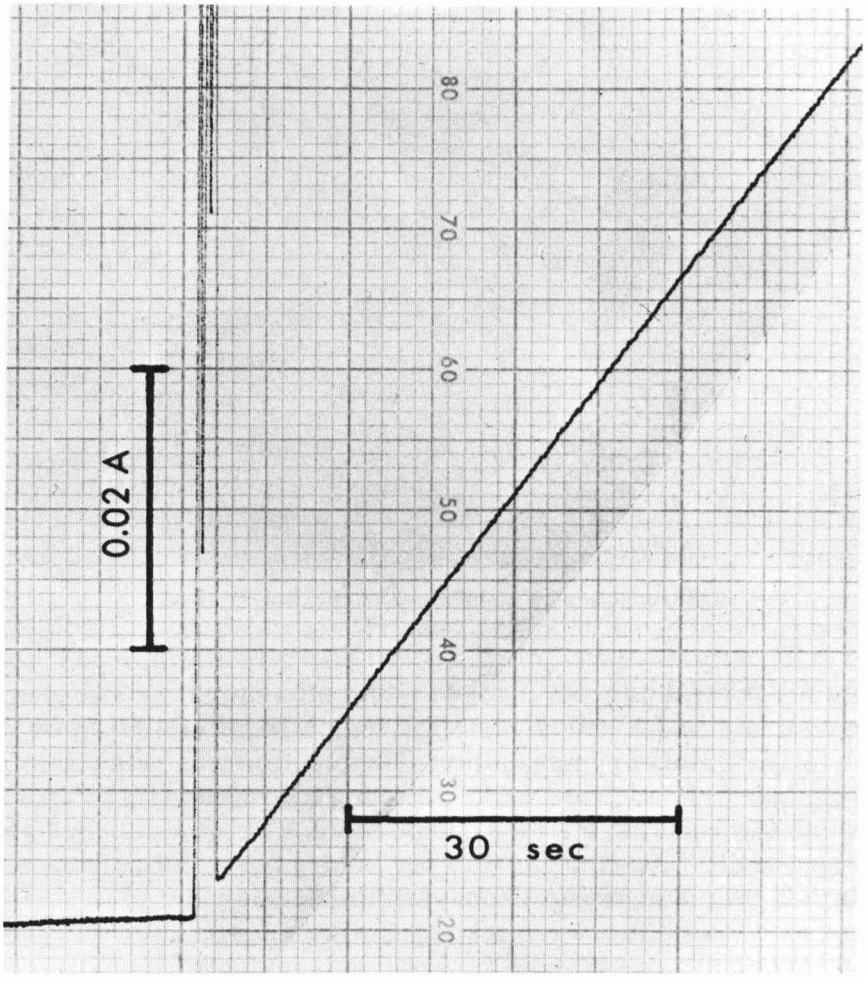

FIG. 4. Recording of a spectrophotometric assay of thymidylate synthetase initiated with the rapid add/mix apparatus.

since the standard deviation is less than 1% of the mean. The ability of the apparatus to mix the added reagent was tested by measuring the absorbancy after each of a series of plunger depressions. Values of 80.5, 97.1, 99.5, 99.7, and 100% of the final absorbance were obtained for the first five depressions, showing that mixing is essentially complete after three depressions.

The apparatus was used while conducting a kinetic analysis of the reaction catalyzed by thymidylate synthetase. The rate of the reaction is measured by recording the increase in absorbance at 340 nm due to the formation of dihydrofolate. A recording of an enzyme assay is shown in Fig. 4. The first part of the recording was made before enzyme was added and shows the nonenzymatic reaction rate. The sharp vertical deflection occurred when the sample holder entered the cuvette and marks the point at which the reaction was initiated. The plunger was rapidly depressed three times and, since the chart drive was uninterrupted throughout the entire recording, the elapsed time between initiation and data acquisition is seen to be less than 2 sec. After the addition of enzyme, the absorbancy increased at a constant rate.

The first apparatus had a plunger rod made of steel drill rod. When this was first used with the reaction mixture mentioned above, the rod was slightly discolored and the reaction mixture became slightly cloudy, which greatly interfered with obtaining accurate rate measurements. The problem was eliminated by coating the plunger rod with a thin film of silicone stopcock grease. A later modification had a stainless-steel plunger rod coated with Teflon from the retaining ring to the sample holder. The Teflon coating was applied by inserting the plunger rod through a length of heat-shrinkable Teflon tubing and heating it at approximately 170°C for 5 to 10 min.

SUMMARY

A relatively simple and inexpensive apparatus is described for rapidly initiating spectrophotometrically monitored reactions by adding a reagent to the cuvette without opening the sample chamber of the spectrophotometer. The apparatus allows the recording of initial reaction rate data within 2 sec after initiating the reaction.

ACKNOWLEDGMENT

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Indirect Polarographic Microdetermination of Alkoxyl Groups

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INTRODUCTION

Since the original method of Zeisel appeared, numerous modifications dealing with almost all aspects have been published. In essence, the method involves treatment of the organic compound with boiling hydriodic acid, in a special apparatus, to effect the splitting off of the alkoxyl group and its conversion into the respective alkyl iodide which is then determined gravimetrically (*7,18*), alkalimetrically (*19*), argentometrically (*17*), iodometrically (*2, 20, 27*) conductometrically (*4*), potentiometrically (*6*), spectrophotometrically (*1, 23*), or by gas chromatography (*22, 26*). Detailed reviews on the subject are already available in many text books (*5, 24, 28*).

However, the present work offers for the first time a new indirect polarographic method developed for the microdetermination of alkoxyl groups in various organic compounds. After converting the alkoxyl group to alkyl iodide and ultimate oxidation to iodic acid, following the modification described by Klimova and Zabrodina (*20*), the cathodic reduction wave of the iodate ion is measured polarographically. Also, a comparison is carried out between the iodometric and polarographic estimation of iodate as performed successively in the same analysis using one micro sample weight. Thus, a single determination provides finally two mutually confirmatory results or at least one correct answer.

EXPERIMENTAL

Apparatus. The design of the microscale glass apparatus, employed for the fission reaction of the alkoxyl compound, is the same as that described by Klimova and Zabrodina (*20*) which is a modified version of that of Večéra and Spévak (*25*).

Rotameter, used for adjusting the flow rate of the pure and dry nitrogen gas stream at 14–15 ml/min.

Polarograph, an Orion KTS 510 (Hungarian make) with accessories was used. The electrolytic vessel is an ordinary Kalousek cell with a cathode compartment that allowed sample solutions down to 4 ml to be recorded using a capillary (DME) of drop time 3–4 sec under an open head of 75 cm

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of mercury. The anode compartment (SCE) is filled with mercury and saturated potassium chloride solution.

Reagents. All reagents are of AR or MAR grade and doubly distilled water was used.

Absorption solution, prepared by dissolving 20 g of sodium acetate trihydrate in 200 ml of acetic acid (98%) followed by addition of 1 ml of bromine free from iodide.

Ascarite, 9 cm layer in a tube between two plugs of glass wool.

Formic acid (2%), methyl red (0.2% ethanolic solution), orthophosphoric acid (sp. gr. 1.70), phenol, potassium iodate (0.01 *M*), potassium iodide (solid), sodium acetate (20% aqueous solution), sodium hydroxide (5 *M*), sodium thiosulfate (0.01 *N*) standarized against potassium iodate solution of approximately the same normality, starch solution (0.5%), and sulfuric acid (10%).

Procedure. Introduce 3–6 mg of the alkoxyl compound into the reaction flask, add ca. 0.1 g of phenol and heat the mixture gently on a microflame till complete dissolution. Cool and add 1.2 g of potassium iodide and 2 ml of orthophosphoric acid. Moisten the lower ground joint of the condenser with H_3PO_4 and connect to the reaction flask, while the upper joint is moistened with water and connected to the ascarite tube. The latter is joined to the gas outlet tube which is then lowered, in turn, to the bottom of the receiver containing 6 ml of the absorption solution. Circulate the water in the condenser and connect the flask to the source of purified dry nitrogen which is allowed to flow through the reaction mixture at a rate of 14–15 ml/min. Simultaneously, heat the flask on an air bath at 195–200°C for 60 min; in case of colchicine and papaverine hydrochloride heating is conducted at 200–220°C for 90 min.

Pour the contents of the receiver into a 150-ml conical flask containing 10 ml of 20% sodium acetate. Rinse the receiver and gas outlet tube with ca. 10 ml of water, which is also poured into the conical flask. Add 1.8 ml of 2% formic acid to remove the excess of free bromine, close the flask with its ground stopper and mix the solution by stirring for 10 min over a magnetic stirrer. Add 0.2 ml of methyl red followed by dropwise addition of 5*M* sodium hydroxide solution till the orange (neutral) color appears and then add 1 ml in excess (pH ca. 10). Transfer the contents quantitatively into a 50-ml measuring flask and make up to the mark with water. Mix the solution thoroughly and introduce an aliquot into the cathode compartment of the Kalousek cell. Record the polarogram of the cathodic reduction wave of iodate using a starting potential of −0.9 V versus SCE as anode and a sensitivity of 20 μA full-scale deflection under otherwise appropriate polargraphic conditions. Run a blank experiment under exactly identical conditions excluding the alkoxyl sample.

Measure the height of the wave due to IO_3^- and calculate the alkoxyl content with the aid of calibration curves (Fig. 1) constructed previously between increasing amounts of vanillin (containing 0.618 to 1.428 mg OCH_3) or phenacetin (containing 0.838 to 2.013 mg OC_2H_5) as standard methoxyl or ethoxyl compounds, respectively, carried through the same procedure, and the corresponding height (in mm) of the cathodic reduction wave of the sodium iodate produced.

Calculate the percent alkoxyl group in the sample from the equation;

$$\% \text{ OR} = \frac{X}{Y} \times 100,$$

where, X = amount of methoxyl or ethoxyl functions in mg as deduced from the respective predrawn calibration curve; Y = weight of the alkoxyl compound analysed in mg; and R = CH_3 or C_2H_5.

Procedure for the successive polarographic and iodometric determinations. Proceed exactly as described above except for cancelling the addition of methyl red and sodium hydroxide solutions. Then, after dilution to 50 ml with water, divide the solution equally into two 25-ml portions for the polarography and iodometry.

(i) *For polarography.* To the first 25-ml portion, add 0.2 ml of methyl red and sodium hydroxide dropwise til pH 10, make up to a volume of 50 ml, and then polarograph an aliquot in the same manner described above except for using a sensitivity of 10 μA full-scale deflection.

(ii) *For iodometry.* To the second 25-ml portion, add 5 ml of 10% sulphuric acid and ca. 0.1 g of potassium iodide. Stopper the flask, shake well till complete dissolution of potassium iodide, and leave in a dark place for 5 min. Titrate the liberated iodine with 0.01 N sodium thiosulfate, as usual, by adding 1 ml of 0.5% starch solution prior to the end point. Run a blank similarly, but without alkoxyl sample, and test for complete removal of excess bromine by the persistence of the methyl red color. In this way, the blank value did not exceed ca. 0.03 ml of the thiosulfate solution.

Calculate the alkoxyl group content of the sample based on the following equivalence:

1 ml of 0.01 N $Na_2S_2O_3$ $\equiv$ 0.05172 mg of OMe, and
$\equiv$ 0.0751 mg of OEt.

RESULTS AND DISCUSSION

No polarographic methods, neither direct nor indirect exist for the determination of the alkoxyl function, primarily because it does not exhibit polarographic characteristics (21). Also although previous work has already established polarographic methods for the microdetermination of a

number of nonmetals (*8, 9, 14, 16*) metals (*3, 11*), and functions (*10, 12, 13, 15*), a method for alkoxyl determination is still lacking. The development of such a method has thus become a desideratum.

The sole approach, best to none, that seemed to lend itself for the purpose of indirect polarography, is simply offered by the iodometric determination of the alkoxyl group, which involves, as already known, final conversion to iodic acid according to the equations;

$$R'OR + HI \longrightarrow RI + R'OH \quad \ldots \ldots \quad (i)$$

$$RI + Br_2 \longrightarrow IBr + RBr \quad \ldots \ldots \quad (ii)$$

$$IBr + 2\,Br_2 + 3H_2O \longrightarrow HIO_3 + 5HBr \quad \ldots \ldots \quad (iii)$$

A comparison of the various iodometric methods (*2, 5, 20, 24*) available, as regards the simplicity of the apparatus and experimental conditions, revealed that the method of Klimova and Zabrodina (*20*) is most suitable for the purpose of the present study. The apparatus utilized in this method (*20*), which is a modified verison of that of Večéra and Spévak (*25*), is very compact and easy to manipulate. Also, the various difficulties associated with the use of hydriodic acid whether reagent grade or Analar, which necessitate special pretreatments (*2, 24*), no longer exist in the method (*20*), selected where potassium iodide and orthophoshoric acid are added to the reaction mixture in order to provide the required HI on heating.

As to the polarographic estimation of the iodate formed due to reactions (i)–(iii), the experimental conditions required for recording its cathodic reduction wave have, fortunately, been already established in previous work (*14*). According to this work, the cathodic iodate wave is recorded in a medium of pH ca. 10 and thus the iodate solution (of pH ca. 2.5) obtained in the present work was adjusted at this value by addition of sodium hydroxide solution (5 *M*) in presence of methyl red which serves also as a maxium suppressor (*21*).

Construction of a calibration curve between various [IO_3^-] and the corresponding wave heights gave rise to a linear relation passing by the origin and thus obeying the Ilkovic equation (cf. (*21*)) over the range 1.749 to 8.754 mg IO_3^-/50 ml. This range was selected as it covers the expected alkoxyl content of a microamount of any of the compounds analyzed. Next, the efficiency of the method was tested through constructing two parallel calibration curves (Fig. 1), one for vanillin (3.030 to 7.006 mg possessing 0.617 to 1.428 mg OCH_3) and the other for phenacetin (3. 335 to 8.014 mg containing 0.838 to 2.013 mg OC_2H_5) as representatives for methoxyl and ethoxyl compounds, respectively. As obvious, both concentration ranges gave rise to equivalent amounts of iodate which fall well within the range previously selected. Typical polarograms representing the cathodic wave of IO_3^- formed in case of vanillin and phenacetin are shown in Fig 2.

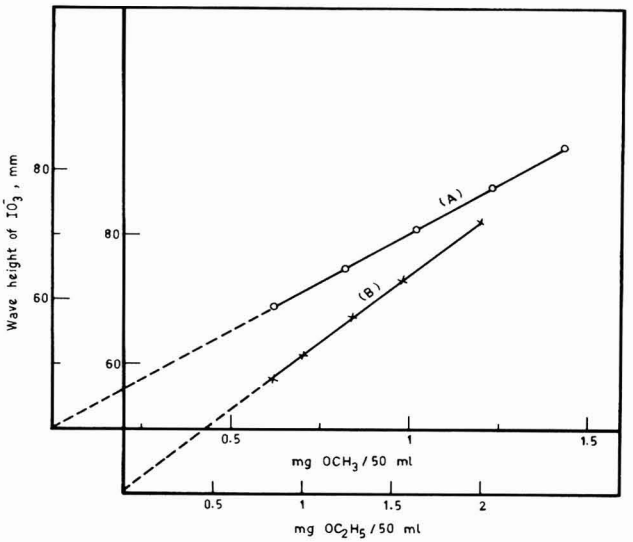

FIG. 1. Calibration curve for the indirect polarographic microdetermination of OR in organic compounds. (A) OCH_3 (B) OC_2H_5.

Application of the procedure finally developed, for the analysis of a wide series of 11 alkoxyl compounds, yielded satisfactory results (cf. Table 1) showing an average error of ± 0.34% and a maximum error not exceeding 0.41%. Whereas 10 methoxyl compounds containing up to four

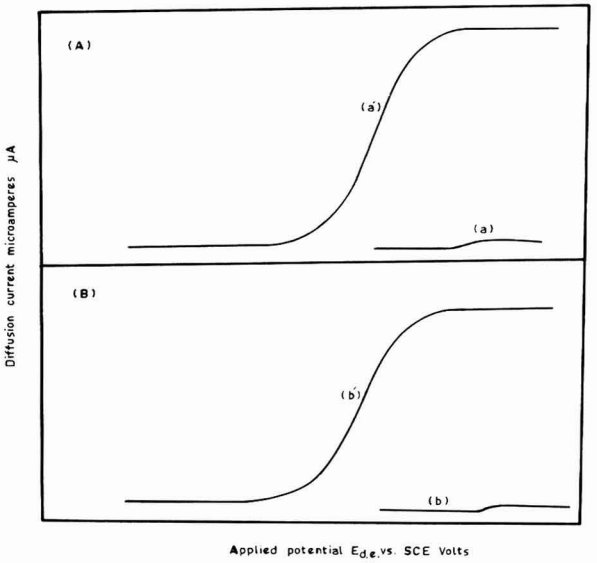

FIG. 2. (A) Cathodic wave of IO_3^-. (a) Blank (no sample). (a') Sample (6.035 mg of vanillin, 1.227 mg. OCH_3). (B) Cathodic wave of IO_3^-. (b) Blank (no sample). (b') Sample (6.235 mg of phenacetin, 1.566 mg OC_2H_5). Start, −0.9 V; sensitivity, 20 μA full scale deflection.

OCH_3 groups were analyzed, only one ethoxyl compound namely phenacetin was obtainable during the course of this study.

Although the method developed succeeded for the analysis of most of the compounds, yet colchicine and papaverine hydrochloride gave OCH_3 values low by ca. 4%. This is not surprising since the reaction conditions recommended by Klimova and Zabrodina (*20*) though proved efficient for the analysis of various alkoxyl compounds; yet the method seemed to cope only with compounds possessing not more than two OR groups in the same molecule. However, quantitative results (cf. Table 1) were obtained for colchicine and papaverine hydrochloride, each known to possess four OCH_3 groups, only after heating the reaction muxture at 220°C for 90 min.

The polarographic method developed is considered to be advantageous since the cathodic reduction of iodate at the DME is accompanied with

TABLE 1
POLAROGRAPHIC AND IODOMETRIC MICRODETERMINATION OF ALKOXYL GROUPS USING ONE SAMPLE

Compound	Sample wt. (mg)	OCH_3 (%) Calcd	OCH_3 (%) Found		Error (%)	
			Polar	Iodo	Polar	Iodo
Vanillin	3.010	20.38	19.97	20.10	−0.41	−0.28
	3.519		20.01	20.27	−0.37	−0.11
Xanthotoxin	4.814	14.33	14.67	14.55	+0.34	+0.22
	5.211		14.01	14.45	−0.32	+0.12
Visnaginone	3.600	15.13	15.54	15.51	+0.41	+0.38
	4.004		15.52	15.32	+0.39	+0.19
2-Amino-4-nitroanisole	4.020	18.89	18.52	18.62	−0.37	−0.27
	4.299		18.55	18.65	−0.34	−0.24
Khellin	3.015	23.83	23.53	23.95	−0.30	+0.12
	3.741		24.10	24.01	+0.27	+0.18
p-Azoxyanisole	3.501	24.02	24.41	24.18	+0.39	+0.16
	3.935		23.72	23.82	−0.30	−0.20
Isopimpinellin	3.810	25.09	25.39	25.29	+0.30	+0.20
	4.100		25.38	25.30	+0.29	+0.21
Khellinone	2.810	26.27	25.98	26.13	−0.29	−0.14
	3.650		25.91	26.34	−0.36	+0.07
Colchichine	3.310	31.06	30.71	30.96	−0.35	−0.10
	4.011		31.37	31.31	+0.31	+0.25
Papaverine hydrochloride	3.620	33.0	33.41	33.26	−0.41	+0.26
	3.851		32.80	33.10	−0.20	+0.10
Phenacetin[a]	3.701	25.12	25.45	25.01	+0.33	−0.11
	4.152		24.83	25.30	−0.29	+0.18
	5.150		25.50	25.22	+0.38	+0.10

[a] The results given represent the ethoxyl group content.

consumption of six electrons and owing to proportionality this contributes to a six-fold amplified wave height.

$$IO_3^- + 6H^+ + 6e \rightarrow I^- + 3H_2O \quad \ldots \ldots \quad \text{(iv)}$$

In this respect, the polarographic method resembles the iodometric procedure which involves also a 6-fold amplification.

$$IO_3^- + 5I^- + 6H^+ \rightarrow 3I_2 + 3H_2O \quad \ldots \ldots \quad \text{(v)}$$

Finally, a successive procedure combining both the polarographic and iodometric methods was developed. Application of this procedure for the analysis of alkoxyl compounds enabled two independent values for the OR group using one microamount of the sample. The results obtained by this successive polarographic-iodometric procedure are shown in Table 1. Although the accuracy of the iodometric method slightly exceeds that of the polarography, the results achieved by the latter are also reasonable being within the error limits acceptable for functional group analysis considering that it also offers an alternative possibility for alkoxyl group determination. Moreover, this successive procedure allows a double check of the alkoxyl content of one compound in the same analysis.

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Spectrophotometric Investigation of Ti(IV) Complexes of *N*-Phenylacetylphenylhydroxylamine

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INTRODUCTION

Review articles (*1,10,12*) and monographs (*2,11*) have well exemplified the distinctive role of several aryl hydroxamic acids and their *N*phenyl analogs as important analytical reagents for different metals. Till now, a very few pieces of works (*3,6,7,15,16*) have examined the formation of complexes of variable composition at various acidities. This paper presents an account of an extractive spectrophotometric investigation of Ti(IV) complexes of *N*-phenylacetylphenylhydroxylamine (NPacPHA) at different acidities. Applications of Job's and molar ratio methods to different sets of yellow colored complex solutions, extracted into chloroform, have established that the complexes having the compositions 1:2, 1:3, and 1:4 (metal:ligand) occur in the acidity ranges 1–3, 4–7, and 7.5–9.0 *N* HCl, respectively. Measurements were performed at 390 nm in the first two cases and at 400 nm in the last one. Yatsimirskii's (*18*), Leden's (*9*), and Harvey and Manning's (*8*) methods were employed to calculate the stepwise and overall formation constants of the complexes. A computer using FORTRAN programming was required for evaluation of formation constants of the 1:3 system following Yatsimirskii's method. The molar absorptivity, sensitivity, and optimum range were calculated for all the systems and also the effect of different foreign ions on absorbance measurements was examined. In the light of the above observations, the suitability of the reagent for analytical determination of titanium was envisaged.

EXPERIMENTAL METHODS

Reagents and Solutions

Using potassium titanyl oxalate (A.R.), a stock solution of titanium (2.18 mg/ml) was prepared and standardized (*17*). Metal solutions of various strengths for different measurements were made ready after proper dilution of the stock solution with twice distilled water.

[1] This work comprises a part of the Thesis submitted for the Ph.D. Degree of Jadavpur University in 1974.

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The reagent, NPacPHA (mP 90–91°C) was prepared following the method of Shome (*14*). Standard reagent solutions of desired strength in A.R. chloroform were used.

Other chemicals used in all spectrophotometric measurements were of A.R. or spectrograde quality.

Apparatus

Spectrophotometric measurements were carried out on a Hilger Uvispek spectrophotometer with matched 1.0 cm glass cells.

An IBM 1130 scientific and electronic computer, using FORTRAN programming, was employed for evaluation of the formation constants of the 1:3 system.

Extraction Procedure

An aliquot of titanium solution (8.352×10^{-5} *M*) was taken into a 100 ml separatory funnel and adjusted to the desired acidity. To 15 ml of the aqueous layer was added a desired amount of the reagent solution in chloroform. Equal volumes (15 ml) of nonaqueous and aqueous layer were used. The two layers were shaken thoroughly for 5 min to reach equilibrium. The yellow colored chloroform layer was drained out and dried over anhydrous sodium sulphate in a 50 ml beaker. The aqueous layer was further shaken with a 5 ml portion of chloroform. The combined extract, after being dried over sodium sulphate, was diluted to 25 ml in a volumetric flask. The absorbances of the solutions of the 1:2 and 1:3 complexes were measured at 390 nm and those of the 1:4 complex at 400 nm.

RESULTS AND REMARKS

Absorption Spectrum

The absorbance spectra of the solutions of the complexes, formed at 2, 6, and 8 *N* HCl, respectively, were measured in the wavelength region 350–520 nm. It is evident from the spectral curves that the complexes (Ti = 16 and 12 ppm, respectively), formed at 2 and 6 *N* HCl absorb the maximum at 390 and 370 nm, respectively, while the complex (Ti = 6 ppm) formed at 8 *N* HCl shows gradual decrease in value. Comparing the next absorbances of complex solutions with those of the reagent over the spectral range of wavelengths, other measurements were carried out at 390 nm for solutions extracted at 2 and 6 *N* HCl and at 400 nm for those at 8 *N* HCl. Figure 1 represents the spectral curves of Ti–NPacPHA complexes against the reagent blank and of the reagent (10 ml of 0.066 *M*) against the solvent blank.

Effect of Reagent Concentration

Using varied concentrations of the reagent, a fixed amount of the metal

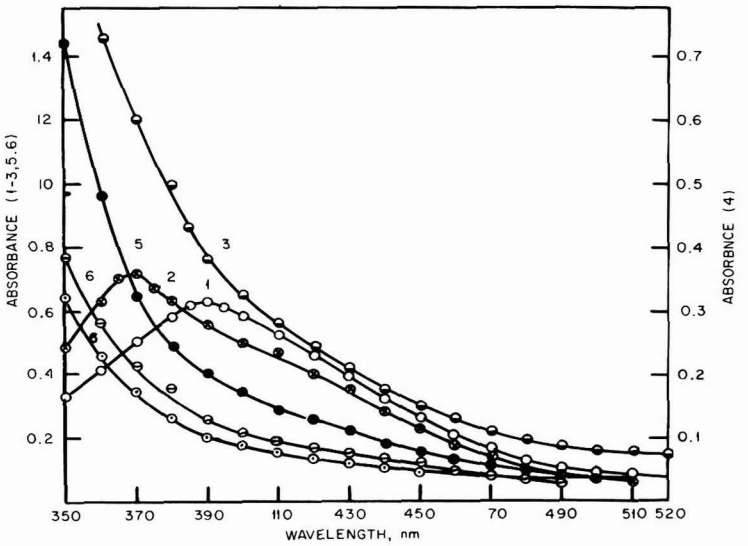

FIG. 1. Spectral curves of the Ti–NPacPHA complex against the reagent blank and those of NPacPHA against the solvent blank.

1. Absorbance of Ti–NPacPHA complex at 2 *N* HCl.
2. Absorbance of Ti–NPacPHA complex at 6 *N* HCl.
3. Absorbance of Ti–NPacPHA complex at 8 *N* HCl.
4. Absorbance of NPacPHA at 2 *N* Hcl.
5. Absorbance of NPacPHA at 6 *N* HCl.

R = 10 ml of 0.066 *M* in each case.

was extracted at 2, 6, and 8 *N* HCl, respectively. The results indicate 12 ml of 0.066 *M* reagent solution in chloroform were adequate for quantitative extraction by a single operation at 2 *N* HCl. For the same purpose at 6 and 8 *N* HCl, 6 and 4 ml of 0.044 *M* reagent solution were found necessary. The yellow colored complex solutions were stable for about 2 hr at all the acidities.

Effect of Acid on Compositions of the Complexes

Ti–NPacPHA complexes, yellow in color, can be extracted into chloroform from 1 to 10 *N* HCl. The complexes are extractable into isomyl alcohol and the color intensity is slightly lesser in it. Absorbance measurements (Job's method) at 390 nm of different sets' complementary solutions have shown the predominance of 1:2 and 1:3 (metal:ligand) complexes in the acidity ranges of 1–3 and 4–7 *N* HCl, respectively. The result was further corroborated from molar ratio plots. Similar measurements at 400 nm have confirmed that the 1:4 complex predominates in the acidity range 7.5–9 *N* HCl. Figures 2 and 3 represent the results of one of a few sets of the experiments.

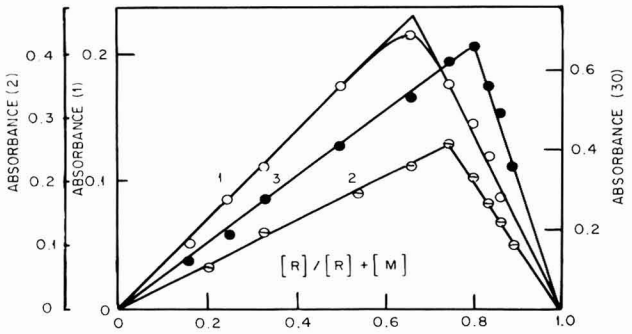

FIG. 2. Job's plots of Ti–NPacPHA complexes.
1. $M = R = 5.469 \times 10^{-2}$ *M*; acidity = 2 *N* HCl.
2. $M = R = 1.823 \times 10^{-2}$ *M*; acidity = 6 *N* HCl.
3. $M = R = 1.823 \times 10^{-2}$ *M*; acidity = 8 *N* HCl.

Calibration Curve, Optimum Range, and Photometric Error

Varied amounts of titanium were extracted at the respective acidities following the given procedure, and their absorbances were measured against the reagent blank at 390 and 400 nm, respectively. The concentration ranges in conformity to Beer's law were found to be 1–16, 1–14, and 1–13 ppm at 2, 6, and 8 *N* HCl, respectively.

The optimum ranges, evaluated from Ringbom's curves, were 6–16, 5–13, and 4–12 ppm at the respective acidities.

The relative analysis error per 1% absolute photometric error following Ayre's equation was found to be in the order of 2.72 in all the cases.

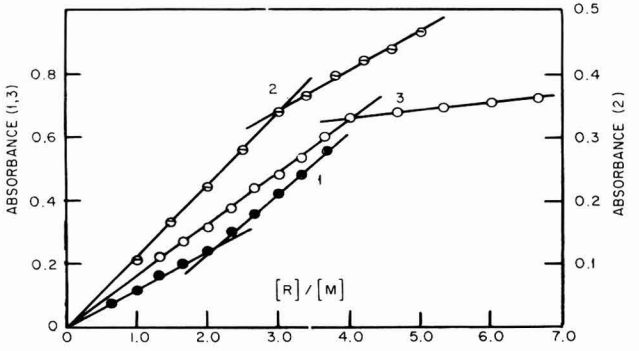

FIG. 3. Molar ratio plots of Ti–NPacPHA complexes:
1. $M = R = 6.563 \times 10^{-2} M$; acidity = 2 *N* HCl.
2. $M = R = 1.823 \times 10^{-2} M$; acidity = 6 *N* HCl.
3. $M = R = 1.094 \times 10^{-2} M$; acidity = 8 *N* HCl.
Metal taken = 1.0 ml in each case.

Molar Absorptivity and Sensitivity

The molar absorptivity of the complexes at 2 and 6 *N* HCl, calculated from Beer's law data at 390 nm, are $(1.84 \pm 0.01) \times 10^3$ and $(2.18 \pm 0.02) \times 10^3$ liters · $mol^{-1}cm^{-1}$ respectively, while the corresponding value of the complex, formed at 8 *N* HCl, at 400 nm is $(2.61 \pm 0.02) \times 10^3$ liters · $mol^{-1}cm^{-1}$. The molar absorptivity values of the 1:3 complex at 370 nm (λ_{max}) and that of the 1:4 complex at 350 nm appear to be 2.87×10^3 and 1.14×10^4 liters · $mol^{-1}cm^{-1}$, respectively. As the reagent absorbs strongly around the respective wavelengths mentioned earlier, measurements at 390 and 400 nm, respectively, are found to be advantageous.

The Sandell's sensitivities are 0.026, 0.023, and 0.016 μg per cm^2 at 2, 6, and 8 *N* HCl, respectively.

Effect of Diverse Ions

The influence of different ions on absorbances of NPacPHA complexes at different acid concentrations at the respective wavelengths were examined. The effect of commonly associated ions appears to be tolerable. Though the ions Fe(III), V(V), U(VI), MO(VI), and W(VI) influence the absorbance readings, the effects of the first two are most prominent at the higher acidity. Considering these effects and also the other features, the reagent can be recommended for determination of micro amounts of titanium at 8 *N* HCl as the 1:4 complex.

Formation Constants of the Complexes

Stepwise formation constants of 1:2 and 1:3 (metal:ligand) systems were calculated following both extended Yatsimirskii's and Leden's methods. The equilibrium ligand concentrations and the degree of complex formation were calculated from Beer's law data. Suitable functions were constructed and their values were evaluated following the procedure stated earlier (*4*).

Both the stepwise stability constants of the 1:2 system were evaluated with the help of each of the above methods.

For the 1:3 system, Leden's method yielded only two stepwise formation constant values as a straight line parallel to the abcissa resulted from the plot of Ψ_2 against $[L]$. Evaluation of the stepwise formation constants (K_1, K_2, and K_3), following Yatsimirskii's method were done by solving a fourth degree polynomial equation in K_2, obtained through the combination of the equations:

$$a_1 = \epsilon_1 K_1; \qquad b_1 = \epsilon_3;$$

$$a_2 = \epsilon_2 K_1 K_2 - \epsilon_1 K_1^2; \quad b_2 = \frac{\epsilon_2 - \epsilon_3}{K_3};$$

$$a_3 = \epsilon_3 K_1 K_2 K_3 - \epsilon_1 K_1^3; b_3 = \frac{\epsilon_1 - \epsilon_2}{K_2}.$$

TABLE 1

STEPWISE STABILITY CONSTANTS FOR TITANIUM COMPLEXES AT 27 ± 2°C

Method	For 1:2 (metal:ligand)			For 1:3				For 1:4				
	log K_1	log K_2	log β_2	log K_1	log K_2	log K_3	log β_3	log K_1	log K_2	log K_3	log K_4[a]	log β_4
Yatsimirskii's	1.73	1.95	3.68	2.08	2.59	2.25	6.92	—	—	—	—	—
Leden's	1.85	2.05	3.90	2.00	2.61	—	—	2.60	3.18	3.40	3.47	—
Harvey and Manning's	—	—	3.53	—	—	—	7.64	—	—	—	—	12.65

[a] K_4 = calculated from overall stability constants by Harvey and Manning's method.

The polynomial equation was solved by an IBM 1130 scientific and electronic computer using FORTRAN programming. Newton and Raphson's method was applied for the initial guess value of K_2.

For 1:4 systems, Leden's method yielded the first three stepwise constant values (K_1, K_2, and K_3) as the plot of Ψ_3 against $[L]$ led to a straight line parallel to the abcissa; K_4 was evaluated by subtracting β_3 from β_4 obtained by Harvey and Manning's method. The results for all the systems including the values of overall stability constants by Harvey and Manning's method are incorporated in Table I.

It is evident from the formula of the isolated complexes in the pH as well as the normality range of acidity that titanium exists in solution (*5,13,19*) in the form of TiO^{2+}. The formula of the 1:2 complex, on the basis of data from Table I and the nature of the isolated complex in the solid state, may be represented as $[TiOR_2]$ where H*R* stands for NPacPHA.

The formula of the 1:3 complex is, in all probability, $[TiOR_2]HR$, where the ligand molecules may be in resonance in such a way that two ligands always are attached to the titanium atom as a bidentate unit, the third one remaining associated by hydrogen bonding through the titanyl oxygen atom.

Similar considerations may be applied also to the 1:4 system. The probable formula of the complex is $H^+[TiO(R)_2R]$ H*R*, where two ligand molecules act as bidentate, one as monodentate, and the fourth one remaining associated through intermolecular hydrogen bonding.

SUMMARY

N-Phenylacetylphenylhydroxylamine complexes of titanium(IV), yellow in color and extractable into chloroform, with metal:ligand ratios 1:2, 1:3, and 1:4, exist in the acidity ranges of 1–3, 4–7, and 7.5–9.0 *N* HCl, respectively. The compositions of the complexes were found out by Job's and molar ratio methods. The stepwise and overall formation constants were evaluated following extensions of Yatsimirskii's, Leden's and Harvey and Manning's methods. A computer was used for evaluation of the formation constants of the 1:3 system following the first method. A spectrophotometric method based on solvent extraction technique, for determination of trace amounts of titanium in the presence of diverse ions, has also been described.

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Polarographic Microdetermination of Iron, Manganese, Lead, Copper, Bismuth, and Tin in Organic Compounds

Application to Analysis of Pharmaceuticals

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The increasing importance of organometallic compounds as fundamental products of many industries, e.g., pharmaceuticals, calls for development of alternative procedures suitable for their analysis. Albeit extensive methods are known for the estimation of metals, those dealing with the analysis of organometallics for their metal content are still very limited and not recognized in most standard textbooks (*4*). However, several reviews have been published (*7*).

In the analysis of such a class of compounds, two problems are involved (a) decomposition of the organic sample, and (b) the method of determination. Several techniques have been recommended for decomposition of organic compounds but the closed-flask method (*6*) is undoubtedly the most popular because of its simplicity and rapidity. It is known that combustion of some organometallics using the closed-flask method may lead to difficultly soluble metal oxides or to alloy formation through interaction with the platinum sample holder resulting in low metal recoveries. These difficulties had restricted application of the technique to decomposition of certain organometallics. In the present work, however, mixing the sample with a flux prior to combustion precluded the formation of insoluble oxides; sodium sulfite, sodium carbonate, and sodium nitrate served as combustion aids for decomposition of organic samples containing iron, manganese, and bismuth, respectively. Compounds containing iron, lead, and bismuth could be mineralized quantitatively using a Pyrex-glass sample holder. Organotin compounds were decomposed by open-flask digestion only.

Different methods of finish have been reported for estimating the metal content of organometallic compounds. Terenteva and Malolina (*11*) de-

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termined iron by differential polarography after complexation with pyrocatechol disulfonic acid. The same authors (*12*) determined manganese polarographically after decomposing the sample with H_2SO_4 and H_2O_2 followed by evaporation, dissolution in HCl, addition of ethanolamine then KOH to serve as a supporting electrolyte. Manganese and copper were analyzed iodometrically; the iodine consumption measured coulometrically (*2*). Reznitskaya and Burtseva (*10*) determined copper in an acidic medium of pH 4 through titration against complexon III. Organotin compounds were decomposed with H_2SO_4 and 30% H_2O_2; tin was determined photometrically with 3′pyridylfluorone (*1*) or reacted with oxine, the complex extracted in chloroform, and tin determined absorptiometrically (*9*).

From the various analytical tools, polarography seems to offer the most promise for the analysis of organometallic compounds. Cations of almost all metals in the periodic table exhibit well-defined polarographic waves suitable for analytical application. Since it is rather unusual that an organic compound comprises more than one metal, the danger of interference can be overlooked and the use of a widely applicable finish such as polarography is seen to its full advantage. Previous work (*3*) has described the polarographic microdetermination of cobalt, nickel, and antimony in organic compounds. The present work deals with the development of a simple and rapid procedure for the polarographic estimation of iron, manganese, lead, copper, bismuth, and tin in organic compounds and pharmaceuticals following closed-flask combustion except for organotin compounds which were decomposed by open-flask digestion.

EXPERIMENTAL

Apparatus

A Cambridge polarograph with accessories as usual was used. The polarograph was connected to an automatic Desktop recorder (Jasco RC-200) with a 250-mm chart. A universal U-shaped Kalousek cell was used as the electrolytic vessel; the anode was a saturated calomel electrode (S.C.E.). The cell had a negligible IR drop. The capillary used had a drop time of 3−5 sec under an open head of 60 cm of mercury. The dropping electrode, the polarographic cell, and the reagents were kept in an air thermostat at 20°C.

Microgram balance (Mettler M5) was used with accessories as usual.

Reagents and Materials

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

Ammonium chloride: a 5.5 *M* solution.

Gelatin: a 0.15% solution prepared every week.

Hydrochloric acid: concentrated, 1, and 5 *N* solutions.

Mannitol: a 10% solution.

Nitric acid: concentrated solution.

Nitrogen gas: O_2 free, obtained by passing stream of gas through purification train composed of five bubblers: the first three contained pyrogallol followed by one containing *N* hydrochloric acid and one empty.

Perchloric acid: a 72% solution.

Potassium hydroxide: a 5 *N* solution.

Sodium carbonate: 1 and 6 *N* solutions.

Standard solutions: ferric ammonium sulfate, manganese chloride, lead nitrate, copper chloride, bismuth nitrate, and tin were used for preparation of stock solutions; 1 ml of each solution contained exactly about 0.5 mg of the respective cation. Standard solution of tin was prepared by dissolving 511.31 mg of pure tin in a warm mixture of 50 ml of 12 *N* HCl and 25 ml of 15 *N* HNO_3; the solution diluted to 125 ml, boiled for a few minutes, treated with 30 ml of 12 *N* HCl, and diluted to 1 liter (*5*).

Sodium sulfite, sodium carbonate, and sodium nitrate: solid.

Bidistilled water was used throughout the present work.

Procedures

The organic sample (3–8 mg) was decomposed using a 500-ml Erlenmeyer flask fitted with a ground-glass stopper. A platinum gauze was used as the sample holder for compounds containing Mn or Cu and a Pyrex-glass spiral for samples containing Fe, Pb, or Bi. Organotin compounds were decomposed after wet digestion in a 100-ml Erlenmeyer flask fitted with a ground-glass stopper.

Iron. Mix the weighed amount of sample with 10–15 mg of solid sodium sulfite. Combust as usual (*6*); absorb the combustion products in 10 ml of 5 *N* HCl. Shake for 10 min. Rinse the stopper out and transfer quantitatively to a tall 100-ml beaker. Dip the spiral into the solution and add 4 drops of perchloric acid. Boil gently on a hot plate. Rinse out the holder and continue boiling until the reaction volume is 15–20 ml. Add 10 ml of a solution 5 *N* in KOH and 10% in mannitol. Adjust the pH of solution to fall along the range 9–10. Transfer quantitatively to a 50-ml volumetric flask. Add 3 ml of gelatin solution followed by water up to the mark. Mix well and transfer an aliquot to the cathode compartment of the polarographic cell. Bubble a stream of O_2-free nitrogen for 3 min. Record the cathodic reduction wave of iron starting from −0.9 V vs SCE. (Measure the wave corresponding to reduction of Fe^{2+} to Fe; $E_{1/2}$ = −1.3 V vs SCE.)

Manganese. Mix the weighed sample with 20–25 mg of solid sodium carbonate. After combustion, the products are absorbed in 5 ml of 5 *N* HCl. Shake for 10 min. Rinse the platinum gauze and transfer the absor-

bent solution quantitatively to a tall 100-ml beaker using 15–20 ml of water. Immerse the sample holder in solution and boil for 8–10 min on a hot plate. Rinse the holder out and allow the solution to cool. Add 2–3 drops of methyl red followed by 6 *N* Na_2CO_3 solution until color just turns orange. Add 1 *N* Na_2CO_3 solution dropwise till the first appearance of yellow color. Transfer quantitatively to a 50-ml volumetric flask. Introduce 3 ml of gelatin solution and complete to volume with water. Record the wave of manganese starting at −1.0 V vs SCE.

Lead or copper. The weighed sample is clamped using a Pyrex-glass spiral for organolead compounds and a platinum gauze for organocopper compounds. After combustion, shake for 10 min to absorb the gaseous products in 5 ml of 1 *N* HCl. Transfer quantitatively to a tall 100-ml beaker using 1 *N* HCl for washing. Immerse the sample holder in the absorbing medium and boil gently for 10 min. Rinse the holder out using 1 *N* HCl then allow to cool. Transfer to a 50-ml volumetric flask using 1 *N* HCl. Add 6 ml of gelatin solution and complete to the mark using 1 *N* HCl. Record the wave of lead starting at 0 V vs SCE. For copper, record the wave starting from +0.05 V vs SCE.

Bismuth. Mix the weighed sample with 10–15 mg of $NaNO_3$. Burn as usual using 5 *N* HCl (5 ml) as the absorbent. Shake for 10 min. Release the holder slightly and boil for 3–5 min. Rinse the spiral out using water and allow the solution to cool. Transfer quantitatively to a 50-ml volumetric flask using water. Add 2.5 ml of gelatin solution followed by water up to the mark. Record the wave of bismuth starting at +0.12 V vs SCE.

Tin. Place the weighed sample in a 100-ml conical flask. Add 1 ml of concentrated HNO_3. Heat on a hot plate till evolution of brown fumes ceases. Fit a reflux condenser and introduce through it 1 ml of perchloric acid followed by 5 ml of concentrated HCl. Reflux for 20 min or till a clear solution is obtained. Rinse and remove the condenser. Evaporate till the reaction volume is ca. 10 ml. Allow to cool then transfer quantitatively to a 50-ml volumetric flask using water. Add 25 ml of ammonium chloride solution and 1.5 ml of gelatin solution then water up to the mark. Record the wave of tin starting at −0.2 V vs SCE.

For the analysis of pharmaceuticals, weigh one tablet, grind to fine particles in an agate mortar, then weigh accurately 5–15 mg and proceed as for synthetic organic compounds. The variation in the metal percentage per tablet calculated for the same drug (Table 1) is due to slight differences in weight of individual tablets.

For every metal, construct a calibration curve. In four 50-ml volumetric flasks, introduce increasing volumes of the corresponding standard inorganic salt solution followed by the same other reagents employed in the sample run. For each of the six metals studied, the wave height changes linearly with the metal ion concentration (Fig. 1). The straight line calibra-

TABLE 1
ANALYSIS OF METAL-CONTAINING ORGANIC COMPOUNDS AND PHARMACEUTICALS

Compounds	Sample wt (mg)	Metal (%) Calcd	Found	Error	Std dev.
Ferrocene (dicyclopentadienyliron)	4.115	30.02	29.09	−0.93	±0.60
	5.741		29.37	−0.65	
	5.970		29.44	−0.58	
	7.626		29.88	−0.14	
	3.460		30.09	+0.07	
	3.138		29.64	−0.38	
Ferrosam[a]	7.135	14.72	14.85	+0.13	±0.21
	4.680	14.74	14.91	+0.17	
Ferro-Bifacton[b]	12.524	6.13	6.44	+0.31	±0.42
	7.335		5.85	−0.28	
Hepaferrovit[c]	13.660	3.77	3.65	−0.12	±0.80
	17.139	3.68	2.89	−0.79	
Manganese acetate	3.655	22.42	21.61	−0.81	±0.64
	5.961		22.48	+0.06	
	5.489		21.59	−0.83	
	5.134		22.79	+0.37	
	6.078		22.05	−0.37	
Manganese oxalate	3.100	30.69	29.80	−0.89	±0.93
	4.797		30.42	−0.27	
Manganeseglycerophosphate[d]	3.465	24.41	26.07	+1.66	±2.07
	4.245		22.19	−2.22	
	3.180		23.44	−0.97	
Lead acetate	4.133	54.62	54.68	+0.06	±0.51
	3.011		53.94	−0.68	
	5.405		55.19	+0.57	
	3.407		54.68	+0.06	
Lead oxalate	4.128	70.19	70.78	+0.59	±0.81
	3.994		69.63	−0.56	
Copper acetate	6.520	31.82	31.60	−0.22	±0.75
	4.612		32.52	+0.70	
	3.761		32.70	+0.88	
	3.699		31.20	−0.62	
Copper acetylacetonate	5.000	24.28	23.20	−1.08	±1.19
	3.785		23.78	−0.50	
Copper tetrapyridine dichloride	5.195	14.09	14.82	+0.73	±0.75
	5.111		14.28	+0.19	
Bismuth subgallate[d]	3.419	50.71	49.83	−0.88	±0.60
	4.200		50.35	−0.36	
	3.416		50.37	−0.34	

TABLE 1—*(Continued)*

Compounds	Sample wt (mg)	Metal (%) Calcd	Found	Error	Std dev.
	5.211		50.45	−0.26	
Tetraphenyltin	4.528	27.79	28.03	+0.24	±0.30
	5.383		27.97	+0.18	
Triphenyltin chloride	4.400		30.91	+0.12	
	5.200	30.79	30.73	−0.06	±0.11
	6.176		30.72	−0.07	
Tetracyclohexyltin	3.584		26.85	+0.55	
	4.228	26.30	26.23	−0.07	±0.66
	5.060		25.72	−0.58	
	6.390		25.49	−0.81	

[a] Provided from Samarra Drug Industries, Iraq. Each tablet contains 200 mg of ferrous fumarate.

[b] Product of Organon, Holland. Each tablet contains 100 mg of ferrous tartrate.

[c] Provided from Samarra Drug Industries, Iraq. Each tablet contains 250 mg of ferrous gluconate.

[d] Provided from Samarra Drug Industries, Iraq.

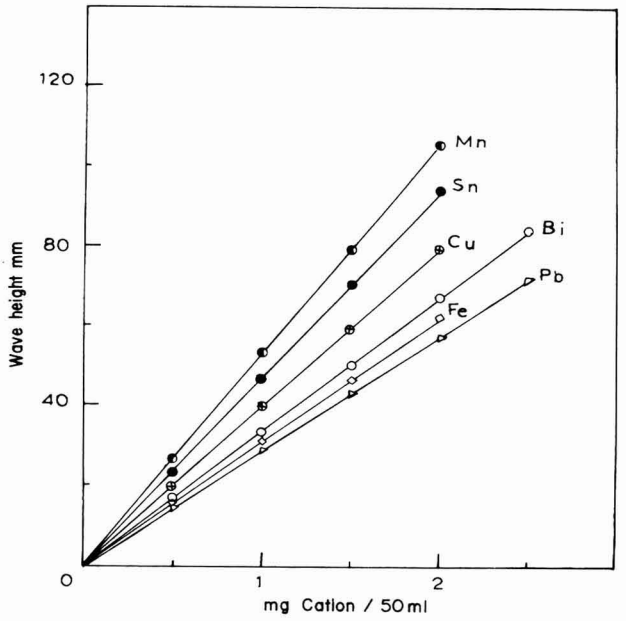

FIG. 1. Calibration curves.

tion curves pass through the origin and therefore satisfy the Ilkovic equation at least along the concentration range tested.

RESULTS AND DISCUSSION

To ensure quantitative transformation of iron into Fe(III), nitric acid was first used as oxidant. However, addition of 1 drop of the concentrated acid obliterated the upper plateau of the polarogram. Perchloric acid enabled complete oxidation without affecting the polarographic waves. It is known that ferric iron produces two successive waves: The first corresponds to reduction to ferrous iron and the second to reduction of ferrous to the metal. We preferred measuring the second wave which, as to be expected, was double the height of the first with one-electron transfer.

No trouble was encountered in the decomposition of organomanganese compounds using sodium carbonate as flux (*8*) and hydrochloric acid as absorbent.

The use of a Pyrex-glass spiral as sample holder during decomposition of organolead compounds prevented alloy formation reported previously between lead and the conventional platinum gauze. The successive turns of the spiral should be as close as possible to prevent the escape of uncompletely decomposed particles of the substance during combustion. On the other hand, glass spiral failed for mineralization of organically bound copper even after boiling of the absorbent with the spiral dipped in. Correct recoveries were obtained (Table 1) using a platinum gauze sample holder; elimination of the boiling treatment gives rise to low results for copper. Since lead and copper produce well-developed diffusion currents from 1 *N* hydrochloric acid, the same reagent served as absorbent and supporting electrolyte for either cations.

Bismuth, as oxide or as organically bound bismuth, is a fundamental constituent of some pharmaceuticals used as antiseptic, astringent, or antihemorrhoidal therapies. The importance of bismuth in the drug industry is evidenced from the large number of publications dealing with its determination in pharmaceuticals. Estimation of the metal content of organobismuth compound, e.g., bismuth subgallate, following closed-flask combustion has been tried using a Pyrex-glass spiral for holding the sample mixed with a flux; sodium sulfite, sodium metabisulfite, sodium carbonate, sodium sulfate, potassium nitrite, or potassium nitrate have been investigated. Potassium nitrate proved the best but the recoveries were still low (-0.5 to -1.5%). However, mixing with 10–15 mg of sodium nitrate gave correct results (Table 1).

Trials to mineralize tin using the O_2-filled flask method were unsuccessful. Mixing the sample with ca. 20 mg of potassium nitrate, potassium nitrite, potassium hydrogen sulfate, sodium sulfite, sodium carbonate, sodium hydrogen sulfate, sodium sulfate, sodium metabisulfite, or sodium nitrate could not allow accurate results. Mixed flux composed of 10 mg

each of sodium nitrate and sodium hydrogen sulfate or sodium nitrate and potassium nitrite did not improve the results. However, recoveries obtained using the latter mixture showed the least error but still with a negative bias. Increasing the amount of flux started to distort the wave of tin rendering its measurement uncertain. Different absorbing media, e.g., 10 ml of 5 *N* hydrochloric acid, 10 ml of concentrated hydrochloric acid plus 1 ml of concentrated nitric acid, or 5 ml of 5 *N* sodium hydroxide plus 5 ml of water, were tried but none proved suitable.

The compounds analyzed contained, besides carbon and hydrogen, nitrogen, chlorine, phosphorus, and oxygen; ferro-Bifacton contains copper as subcarbonate, hepaferrovit contains copper gluconate and manganese hypophosphite besides iron and other ingredients. None of these elements interfered with the determination. Even in the presence of an interfering element, proper selection of the supporting electrolyte would eliminate this effect. The proposed method is simple and expeditious; single determination of any of the six elements consumes not more than 45 min including the weighing step. The method, without modification, was successfully applied to analysis of some pharmaceutical preparations (Table 1).

Polarography, being a nondestructive tool of analysis, offers the advantage that after polarographic recording, the metal content may be redetermined in the same or in another aliquot by any other method. In this way, two confirmatory results are obtained for the same sample weight which contributes an economy both in time and in test material. Results given in Table 1 show reasonable accuracy. The average percent errors for 49 determinations for Fe, Mn, Pb, Cu, Bi, and Sn amount to ±0.38, ±0.85, ±0.42, ±0.62, ±0.46, and ±0.30, respectively.

SUMMARY

A micromethod for the polarographic estimation of Fe, Mn, Pb, Cu, Bi, and Sn in organometallic compounds is presented. The organic sample is decomposed using closed-flask combustion except for organotin compounds which require open-flask digestion. Pyrex-glass spiral is used for mineralizing organically bound Fe, Pb, and Bi. Mixing with a flux prior to combustion is necessary for compounds containing Fe, Mn, or Bi. After combustion, a suitable supporting electrolyte is added and the cathodic reduction wave of the corresponding metal recorded. The same procedure is applied for the analysis of pharmaceuticals. The method is simple and rapid; one determination consumes less than 45 min for any of the metals reported. Results obtained are satisfactory and the average percent errors for 49 determinations are ±0.38, ±0.85, ±0.42, ±0.62, ±0.46, and ±0.30 for Fe, Mn, Pb, Cu, Bi, and Sn, respectively.

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NMR Measurements of Stability Constants for Complexes of Carbon Tetrachloride and Some Aromatic Compounds

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INTRODUCTION

Carbon tetrachloride was known as an inert solvent, since it was believed that ideal solutions were formed on mixing of CCl_4 and aromatic compounds. Investigations on the solutions containing CCl_4 showed evidences of complex formation (*1*). The stability constants of these complexes have been measured by UV spectroscopy. In some cases, for a system different stability constant values have been reported (*5*).

In this work NMR spectroscopy is used to study the extent of complexation of CCl_4 and an equation is suggested for calculation of stability constants.

EXPERIMENTAL

All compounds were of Analar grade and were fractionally distilled twice through a 100 cm vigreux column in which the middle 50% was collected. The recovered materials were degassed by nitrogen free oxygen shortly before preparation of the solutions. All solutions were prepared by weighing out known amounts of compounds in stoppered flasks. NMR measurements were made with a Varian HA100 spectrometer operating at 100 MHz for ^1H. Sweep width calibrations were made with a signal generator and a Varian V4315 frequency counter. The chemical shifts were measured from internal and external references using the side band technique. The measured values were reproducible within 0.3 Hz. Temperature calibration was carried out by comparing the observed separation of two major peaks of ethylene glycol with the manufacturer's calibration graph.

RESULTS AND DISCUSSION

The measured chemical shifts are reported in Table 1. On the chemical shifts measured from an external reference the appropriate correction (*8*) for the bulk magnetic susceptibility differences was made. Using a least-squares based computer program, the stability constants (K^{AD}) were calculated via the Foster equation (*6*)

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TABLE 1

THE CHEMICAL SHIFTS (HZ) OF RING PROTONS (δ_R) AND SUBSTITUENT GROUPS (δ_S) MEASURED FROM DIFFERENT REFERENCES FOR (CCl_4,TOLUENE) AND (CCl_4,BENZYLCYANIDE) IN CYCLOHEXANE SOLVENT AT 25°C

Mole fractions		References					
		Cyclohexane (internal)		TMS (internal)		TMS (external)	
		δ_R	δ_S	δ_R	δ_S	δ_R	δ_S
Toluene	CCl_4						
0.029	0.000	561.955	83.042	705.865	226.948	722.252	243.343
	0.120	562.208	84.831	706.264	228.250	724.962	245.708
	0.226	562.520	85.520	706.550	229.053	728.438	251.236
	0.425	563.145	86.920	707.263	230.340	733.838	257.086
	0.630	563.989	87.716	708.114	231.278	739.576	263.103
	0.830	564.923	89.270	709.119	232.490	746.768	269.825
Benzylcyanide	CCl_4						
0.026	0.000	577.300	206.534	721.656	351.222	729.486	368.935
	0.117	578.307	208.973	722.856	353.344	743.383	373.608
	0.225	579.307	210.708	723.693	355.277	746.568	378.214
	0.435	581.462	214.652	725.537	358.422	753.693	386.802
	0.629	583.158	217.969	726.849	361.506	760.225	394.632
	0.844	585.034	221.631	728.279	364.737	767.990	403.714

$$\Delta/X_D^0 = -K^{AD}\,\Delta + K^{AD}\,\Delta_0 \quad . \tag{1}$$

This equation is applicable to the complexing reaction of type

$$A + D \rightleftharpoons AD \quad , \tag{2}$$

and for the situation of $X_D^0 \geqslant X_A^0 \geqslant X_{AD}$ where X designates a mole fraction at equilibrium and X^0 an initial value. The other symbols represent the following: $\Delta_0 = \delta_{AD} - \delta_A$, $\Delta = \delta - \delta_A$, where δ is the observed shift of a group in an A molecule in the mixture and δ_{AD} and δ_A are the chemical shifts of pure AD and A, respectively.

As Table 2 shows there are inconsistencies among the K^{AD}'s calculated from chemical shifts of aromatic and substituent groups in both systems. Also it can be seen that the K^{AD}'s are highly dependent on the nature of the references used.

The dependence of K^{AD}'s on the measured chemical shifts of different groups in a molecule has been reported by other workers (*4*). In most cases it has been ascribed to formation of higher complexes other than 1:1. However, considering the suggestion made by Homer and Ydava (*7*) the

TABLE 2

K^{AD} (MOLE FRACTION) CALCULATED VIA THE FOSTER EQUATION USING CHEMICAL SIFTS OF AROMATIC (δ_R) AND SUBSTITUENT GROUP (δ_S) FOR THE SYSTEMS STUDIED

	Reference					
	Cyclohexane (internal)		TMS (internal)		TMS (external)	
Systems	δ_R	δ_S	δ_R	δ_S	δ_R	δ_S
Benzylcyanide,CCl_4	−0.226	0.173	0.421	0.182	0.389	1.420
Toluene,CCl_4	−0.413	0.178	−0.346	0.640	−1.663	1.625

formation of higher complexes cannot be responsible for the disagreements in our results. Since, as Figs. 1 and 2 show, the plots of shifts of aromatic protons against those of substituent groups for both systems are good straight lines, the shifts in resonance position of each group are caused by the same CCl_4 molecule and only one equilibrium is involved. Thus we consider the effect of other factors on the chemical shifts.

The chemical shift which is the summation of several terms (*2,3*) can be represented as

$$\delta = \delta_g^0 + \delta_b + \delta_a + \delta_c + \ldots, \tag{3}$$

where δ_g^0 is the absolute shift in gaseous state and δ_c is due to complexation. δ_b and δ_a are, respectively, due to the shielding of bulk magnetic suscepti-

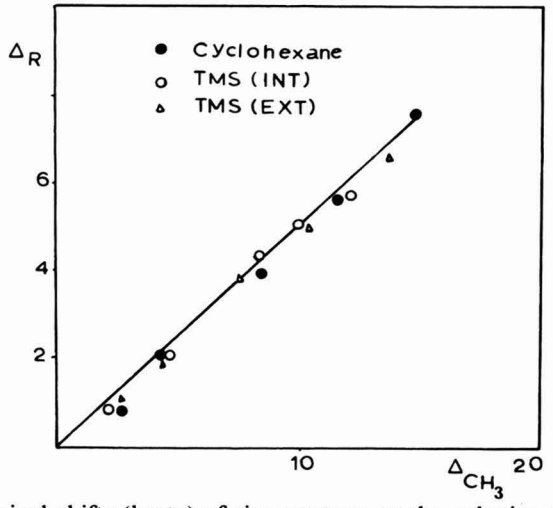

FIG. 1. The chemical shifts (hertz) of ring protons vs the substituent group for (toluene, CCl_4, cyclohexane) system at 25°C.

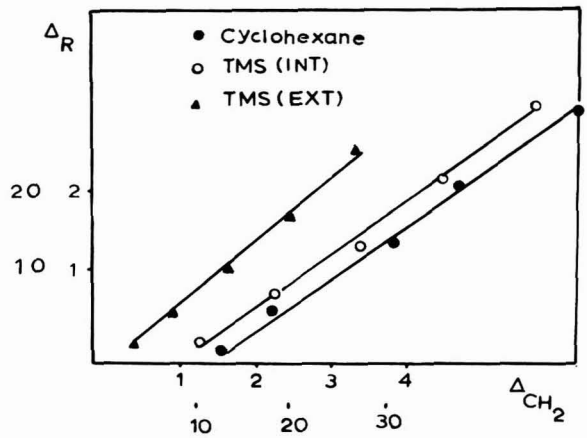

FIG. 2. The chemical shifts (hertz) of ring protons vs the substituent group for (benzylcyanide, CCl_4, cyclohexane) system at 25°C.

bility and magnetic anisotropy of media. The sum of these two terms, which is known as the shielding effect of media (δ_m), may have a more significant effect on variations of chemical shift and give rise to an incorrect value of K^{AD} (*9*).

The shielding effect of media can be eliminated if the chemical shifts are related to the gaseous state of reactants. For a very fast exchange of AD and its components A and D in gas phase, the chemical shift will be written as

$$\delta_g = P_A \, \delta_{g,A} + P_{AD} \, \delta_{g,AD} \quad , \tag{4}$$

where P_A and P_{AD} are fractions of uncomplexed and complexed A, respectively. Since $P_A + P_{AD} = 1$ and $\delta_g = \delta_g^0 + \delta_c$, then

$$\delta_g^{01} + \delta_c^1 = (X_{AD}/X_A)\,(\delta_{g,AD}^1 - \delta_{g,A}^1) + \delta_{g,A}^1 \quad , \tag{5}$$

where X_{AD}/X_A has been substituted for P_{AD} and the subscript 1 refers to the group 1 in the A molecule. Substitution for the left-hand side of the above equation from Eq. (3) gives

$$\delta^1 = (X_{AD}/X_A)\,(\delta_{g,AD}^1 - \delta_{g,A}^1) + \delta_{g,A}^1 + \delta_m^1 \quad . \tag{6}$$

Applying the above equation to another group in the same molecule and subtracting it from the above equation and considering that $\delta_m^1 = \delta_m^2$ will give

$$\delta^{1,2} = (X_{AD}/X_A)\,(\delta_{g,AD}^{1,2} - \delta_{g,A}^{1,2}) + \delta_{g,A}^{1,2} \quad , \tag{7}$$

TABLE 3
K^{AD} (MOLE FRACTION) CALCULATED VIA EQ. (7) FOR THE (CCl_4, TOLUENE) AND (CCl_4, BENZYLCYANIDE) SYSTEMS

Systems	References		
	Cyclohexane (internal)	TMS (internal)	TMS (external)
CCl_4,Toluene	4.732	4.572	4.754
CCl_4,Benzylcyanide	0.284	0.315	0.291

where $\delta^{1,2}$ is the differences in the chemical shifts of groups 1 and 2 in an A molecule in liquid state.

Equation (7) may be used to evaluate the stability constants of a complex in which one of its components has two measurable groups, such as systems used in our investigations. Using a computer program the X_{AD}/X_A values were calculated from the equilibrium expression for assumed values of K^{AD}. Then they were compared with $\delta^{1,2}$ in Eq. (7), seeking the line of the best fit.

The K^{AD}'s evaluated from Eq. (7) for (CCl_4, toluene) and (CCl_4, benzylcyanide) are reported in Table 3. The agreement of K^{AD}'s for different references indicates that the shielding effect of media has been eliminated.

SUMMARY

NMR spectroscopy was used to measure the stability constants of complexes of (carbon tetrachloride, toluene) and (carbon tetrachloride, benzylcyanide) in cyclohexane solvent. It was found that the results were dependent on the references used and this was attributed to the shielding effect of media. An equation was used for calculation of stability constants of these systems which eliminated the shielding effect of media.

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MICROCHEMICAL JOURNAL 22, 457–462 (1977)

Methods for the Isolation and Characterization of Constituents of Natural Products

XXI. Use of a Celite–Potassium Methylate Column for Rapid Preparation of Methyl Esters from Microgram Amounts of Glycerides

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INTRODUCTION

A number of publications from this laboratory have described column reactions that are useful in the analysis of microgram and sometimes submicrogram amounts of lipid constituents *(3–6)*. In continuing our efforts in this direction, a method is detailed below which utilizes a very small column of potassium methylate mixed with diatomaceous earth to transmethylate microgram amounts of glycerides. The procedure is rapid, simple, and convenient, and is executed without the use of methanol *per se*.

MATERIALS[2]

Hyflo Super-Cel (Fisher Scientific Co., King of Prussia, Pa.) was dried at 110°C for 48 hr; powdered potassium methylate (CH_3OK) was purchased from the Ventron Corp., Danvers, Mass.; melting-point capillaries open at both ends 1.6–1.8 × 100 mm and Critoseal (a vinyl plastic putty) were from A. H. Thomas Co., Philadelphia, Pa.; 100-μl volumetric capillaries sealed at one end and graduated in 10 μl increments were obtained from Friedrich and Dimmock, Inc., Millville, N.J.; solvents were ACS grade or better and used as received.

EXPERIMENTAL

Preparation of transmethylating powder. Hyflo Super-Cel (1.0 g) was ground thoroughly[3] in a mortar (8.2 cm o.d., 60 ml capacity) for 1 min with 0.3 g CH_3OK. The powder was transferred to a dry 5-ml screw cap vial and stoppered by pulling the sleeve portion of a rubber septum (sleeve 7 ×

[1] Agricultural Research Service, U.S. Department of Agriculture.

[2] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

[3] As much pressure should be applied to the pestle as possible, and the powder should be scraped from the sides about midway through the grinding period.

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11 mm) over the threads of the vial. The septum was punctured with a hypodermic needle and wire until a glass capillary could be inserted smoothly. The powder was kept at room temperature during the working day and at −18°C at other times.

Preparation of Microcolumn. A melting-point capillary was sealed at one end by pushing it into Critoseal. It was weighed,[4] the open end was inserted through the septum of the vial containing the transmethylating powder, and the capillary was dabbed into the powder several times. Approximately 8−10 mg was loaded into the capillary in this manner. If more powder was needed, the capillary was withdrawn, the powder pushed into the capillary with a tamper (paper clip), and the process repeated. The powder was finally pushed into the capillary so that 1−1.5 cm of space remained between the end of the capillary and the bed. The capillary was cut below the Critoseal plug, and, while the end portion of the bed was retained with a tamper, the powder was pressed into a compact column with another tamper. The capillary was then cut 4−5 cm above the bed.

Transmethylation of sample. The glyceride, fat, or oil was dissolved in petroleum ether, *n*-hexane, cyclohexane, or benzene at a concentration not exceeding 7 μg/μl. A volume[5] of the solution was applied to the column from a 10-μl syringe by placing the needle tip lightly on the bed and expelling the solution slowly into the column. The column was then eluted with any of the solvents mentioned above or with CS_2 or CH_2Cl_2 using N_2 pressure.[6] One of three procedures was used for collecting the effluent for subsequent analysis of the methyl esters: (a) if only a few micrograms of lipid had been applied to the column or when quantitative recovery of the methyl esters was not necessary, the column was eluted with 1 bed volume of solvent. The first 9−10 μl (about 9−10 mm) emerging was taken up as thoroughly as possible in a 10-μl syringe and analyzed; (b) when quantitative recovery of the methyl esters was desired and the expected esters were long chain ($\geq C_{14}$), the column was eluted with 3 bed volumes of solvent, the effluent was collected in a 2-ml vial, the solvent was removed at room temperature under a stream of N_2, and the residue was taken up in a definite volume of CS_2 for analysis; and (c) as in (b) except that the effluent was continuously removed from the capillary with a hypodermic syringe and transferred to a volumetric capillary. The

[4] In practice it may not always be necessary to obtain the weight of powder. Each millimeter of the compact bed is approximately equal to 1 mg.

[5] A volume of the solution was applied so that not more than 2 μg of lipid/mg of packing was present. Some unwetted portion of packing must remain after application. One microliter of solution will wet approximately 1 mg of packing.

[6] The capillary was inserted in a capillary holder such as those supplied with Microcaps or one made out of a septum fitted on a piece of glass tubing. A pressure of 3–4 lb/in.^{2} was used.

contents of the capillary were mixed by drawing up and expelling the solution with a hypodermic syringe prior to analysis. This procedure was used with lipids containing short chain acids such as milkfat.

Gas–liquid chromatography (GLC). GLC was performed on a Hewlett–Packard 5750 instrument with a flame ionization detector. The column was 8 ft × ⅛-in. silanized stainless steel packed with 7.5% stabilized ethylene glycol adipate plus 2% H_3PO_4 on 90–100 mesh Anakrom ABS. Helium was the carrier gas emerging from the column at 30 ml/min. Peak areas were determined with a Supergrator-2 (Columbia Scientific Industries, Wilmington, Del.), and quantitation was done by establishing standard curves prepared from pure methyl esters (NUCHEK Prep, Inc., Elysian, Minn.).

Thin-layer chromatography (TLC). TLC was carried out on precoated 250-μm layers of Silica Gel G (Analtech, Inc., Newark, Del.) with benzene as solvent. Spots were visualized by spraying with 8.5% aqueous H_3PO_4 containing 3% cupric acetate and heating at about 200°C.

RESULTS AND DISCUSSION

The method was applied to the following synthetic glycerides[7]: P-S-S, O-O-O, P-P-O, P-O-S, P-P-P, S-S-S, M-M-M, and L-L-L, and to milkfat, beef tallow, and corn, cottonseed, olive, safflower, and soybean oils.

The effect of the solvent used to apply the lipid to the column on the completeness of transmethylation is shown in Fig. 1. Transmethylation was complete in hydrocarbon solvents but incomplete in CCl_4, CH_2Cl_2, and CS_2. The reason for this is unclear. Regardless of the solvent used, however, the correct (within 1% of theory) ratio of fatty acids was always obtained when mixed synthetic glycerides were tested.

Recovery of methyl esters from synthetic triglycerides was between 92–95% of theory when the glycerides (26–39 μg) were applied to the column in hydrocarbon solvents and when procedure (b) or (c) was used to collect the effluent from the column for analysis. With the chlorinated solvents or CS_2, yields were only 42–70%.

Transmethylation apparently took place instantly upon contact of the glyceride with the powder. Longer contact times did not increase yields of methyl esters in those solvents in which incomplete transmethylation occurred. Consequently, it was possible to apply the glyceride in hydrocarbon solvents and elute with chlorinated solvents or CS_2 and obtain maximal yields of methyl esters.

Figure 2 shows a gas chromatogram of the methyl esters obtained from application of 2 μg of cottonseed oil to a 4 mg transmethylating column. The results (in area percent) agreed well with those obtained by the al-

[7] Abbreviations: L = linoleyl, M = myristoyl, O = oleoyl, P = palmitoyl, S = stearoyl.

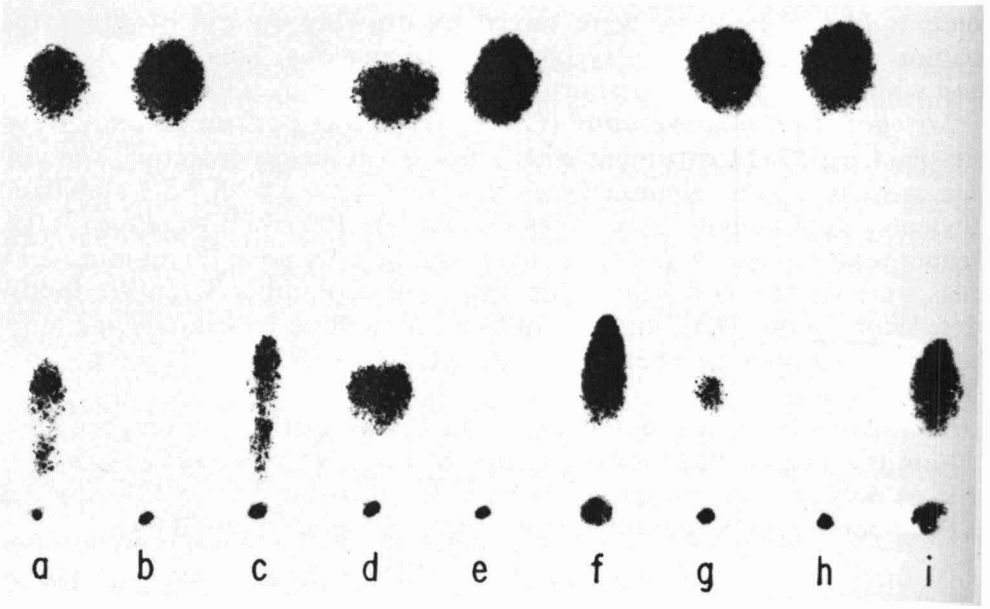

FIG. 1. Transmethylation of several fats and oils showing effect of solvent on completeness of reaction: (a) 31-μg milkfat in CS_2; (b) 31-μg milkfat in hexane; (c) milkfat control; (d) 32-μg corn oil in CH_2Cl_2; (e) 32-μg corn oil in benzene; (f) corn oil control; (g) 28-μg olive oil in CCl_4; (h) 28-μg olive oil in cyclohexane; (i) olive oil control.

kaline catalyzed procedures of Luddy *et al. (2)* and Christopherson and Glass *(1)*.

All other oils and fats also gave satisfactory analyses at the 1–2 μg level. A larger (5 μg) sample of milkfat was required, however, and the methyl butyrate and methyl caproate peaks were under the solvent peak. This situation could be circumvented by transmethylating a larger amount (10 μg) in CS_2; in this case about half of the glycerides were transmethylated and some intact glycerides were injected into the instrument, although no adverse effects were apparent.

The powder retained its initial transmethylating characteristics for about 3 weeks. During this period approximately 70 punctures of the septum were made. There was a slight but gradual increase of methanol in the powder due to admittance of small amounts of moisture each time the septum was penetrated. There was also a slight and gradual accumulation of an unidentified component with a retention time close to methanol. Both peaks emerged under the solvent peak when transmethylation was conducted in any solvent other than CS_2.

Attempts to prepare the transmethylating powder and methyl esters under strictly anhydrous conditions (i.e., all manipulations done in a dry box under N_2 and with chemically dried solvents) resulted in essentially

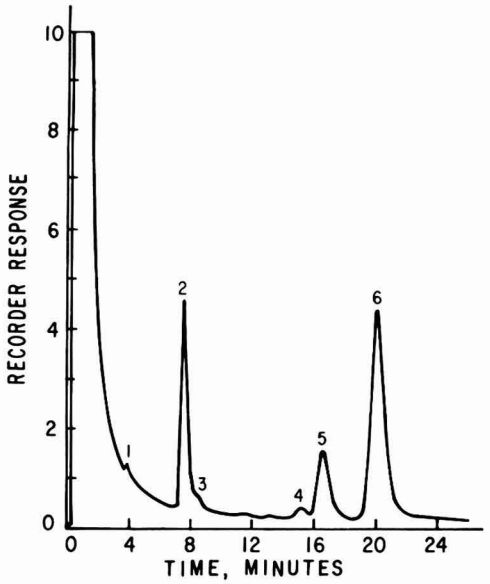

FIG. 2. Gas–liquid chromatogram of methyl esters obtained from application of 2 μg of cottonseed oil to 4 mg of transmethylating powder. Sample applied in 1-μl *n*-hexane and methyl esters were eluted with CS_2. Temperature, 170°C; range 10; attenuation × 4. Peak identification with area percent: (1) methyl myristate, 0.8; (2) methyl palmitate, 23.5; (3) methyl palmitoleate, 0.16; (4) methyl stearate, 2.2; (5) methyl oleate, 16.1; (6) methyl linoleate, 57.8. Values obtained by the method of Luddy *et al. (2)* were: (1) 0.8; (2) 23.9; (3) 0.14; (4) 2.1; (5) 16.0; (6) 57.2. Values obtained by the method of Christopherson and Glass *(1)* were: (1) 0.8; (2) 24.7; (3) 0.12; (4) 2.0; (5) 16.1; (6) 56.3.

no transmethylation. This is interpreted to mean that a certain minimum amount of moisture must be introduced into the powder initially to generate enough KOH to catalyze the reaction.

Hyflo Super-Cel was superior to some other types of diatomaceous earths for mixing with the CH_3OK. These included Celite 545, which never gave complete transmethylation with any of the lipids studied; analytical grade Celite, which gave variable results and some saponification; and Filter-Cel, which usually gave complete transesterification in all of the solvents studied but gave extensive (up to 40%) saponification. Powdered CH_3OK alone was incapable of causing any transmethylation.

Sodium methylate, when substituted for CH_3OK, was inferior to the latter, giving lower yields of methyl esters, but this could be due to the physical form of the powder and not necessarily to lower reactivity.

SUMMARY

In a convenient, rapid procedure, a very small column of potassium methylate–Hyflo Super-Cel is used to convert microgram amounts of glycerides to methyl esters. Transesterification is complete in hydrocarbon but not in chlorinated solvents or in CS_2. The methyl

esters can be recovered in 92–95% yield if desired. Regardless of the solvent used, the recovered methyl esters are representative of the original fatty acid composition of the glycerides.

ACKNOWLEDGMENT

Mr. Francis Luddy of this laboratory provided the glycerides and some transmethylated fats and oils prepared by the Luddy *et al.* procedure.

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MICROCHEMICAL JOURNAL 22, 463–474 (1977)

Luminol-Dependent Chemiluminescence Analysis of Cellular and Humoral Defects of Phagocytosis Using a Chem-Glo Photometer

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INTRODUCTION

Phagocytosis is the general process whereby host cells, such as polymorphonuclear neutrophils (PMNs) and macrophages (alveolar, peritoneal), engulf and destroy foreign material and constitutes an important first-line defense mechanism against invading and potentially pathogenic organisms (*8*). Figure 1 outlines the sequence of events which occur during the phagocytic process.

The importance of phagocytosis in host defense has led to the development of numerous methods designed to assess the phagocytic capabilities of individuals. These approaches include the reduction of nitroblue tetrazolium (NBT), as well as the determinations of oxygen consumption, particle uptake, and bactericidal activity. However, these methods are either equivocal, expensive, and/or time consuming. Recently, Allen *et al.* (*1*) described a chemiluminescence (CL) assay whereby the metabolic activities of PMNs may be assessed more rapidly. This light emission

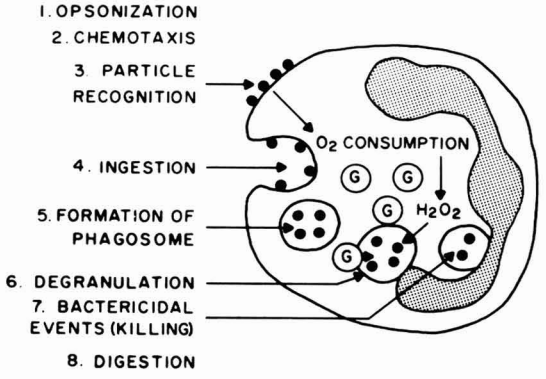

FIG. 1. Synopsis of phagocytosis. For further details, see (*8*).

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results from the generation by phagocytes of highly reactive and excited-state oxygen metabolites (superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen) during their interaction with various opsonized particles (such as bacteria and zymosan).

Subsequent reports have correlated the generation of CL with various defects in PMN activation and/or metabolism, including chronic granulomatous disease of childhood (CGD), myeloperoxidase deficiency, and diminished serum opsonic activity (*5, 6, 12*). Thus, it appears that measurement of CL may be a rapid and valuable parameter in assessing the metabolic and phagocytic capabilities of individuals.

The original CL system reported by Allen employed the use of a liquid scintillation counter set in the out-of-coincidence mode. The purpose of this communication is to modify this procedure in order to:

1. measure the emission of light (as an index of phagocytic activation and metabolism) using less costly equipment (Aminco Chem-Glo photometer);
2. make this modified procedure more generally applicable to the purpose of clinical laboratories by markedly reducing the volume of blood required (10 ml or less);
3. demonstrate that this procedure is useful in detecting defects in cellular metabolism and/or opsonic activity.

To accomplish the aforementioned goals it was necessary to add an agent that would increase the amount of measurable light. We chose luminol (3-aminophthalic hydrazide) to produce this desired effect, because it reacts with a high quantum efficiency with those species of oxygen generated during PMN activation. Previously, Allen and Loose published a procedure using the liquid scintillation counter and luminol (*2*) using normal cells (PMNs and alveolar macrophages). This work extends their observations to another instrument and demonstrates its applicability for detecting phagocytic defects of both cellular and humoral origin.

MATERIALS AND METHODS

Instrumentation

The instruments used in this study were the Chem-Glo photometer and integrator–timer available from the American Instrument Co., Silver Spring, Md. The Chem-Glo photometer was equipped with the large head which can hold plastic tubes with outside dimensions of 1.2 × 4.6 cm. The photomultiplier tubes used in this study will be designated as tube 1 (a selected 931 B, S-4 response) and tube 2 (a prototype 1P28, S-5 response).

In Fig. 2 three instruments can be seen from left to right: the Aminco integrator–timer, Aminco Chem-Glo photometer, and a Houston record-

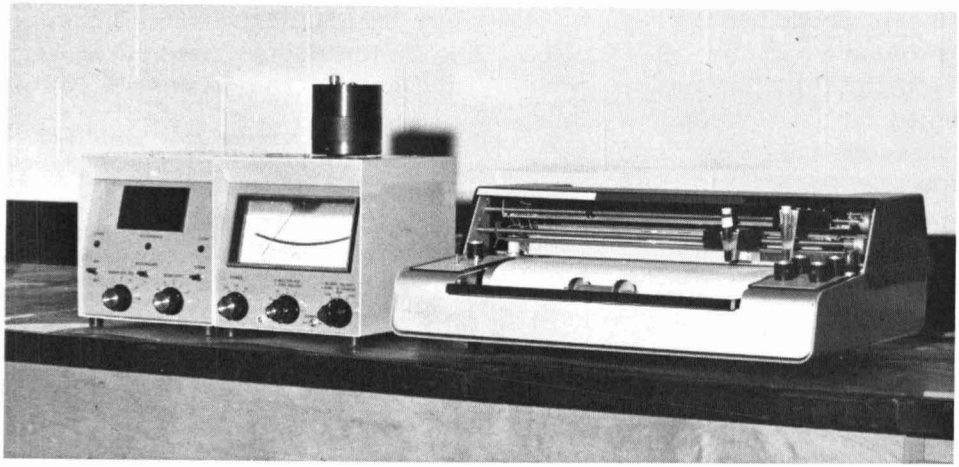


FIG. 2. Aminco integrator–timer, Aminco Chem-Glo photometer, and Houston recorder (from left to right). The black cylinder on the top of the photometer is where the sample is placed.

er, which is electronically attached to the system for continuous scanning. The recorder is not necessary and was not used except in the inhibitor study of the light response. The data can be recorded with the integrator–timer using the clear and accumulate button during a set interval of time. The integrated counts of light emission are the result of a multiplication from the meter multiplier times the number that appears on the integrator–timer per minute. In some studies a Packard liquid scintillation spectrometer Model 2002 was used in the out-of-coincidence mode (*10*).

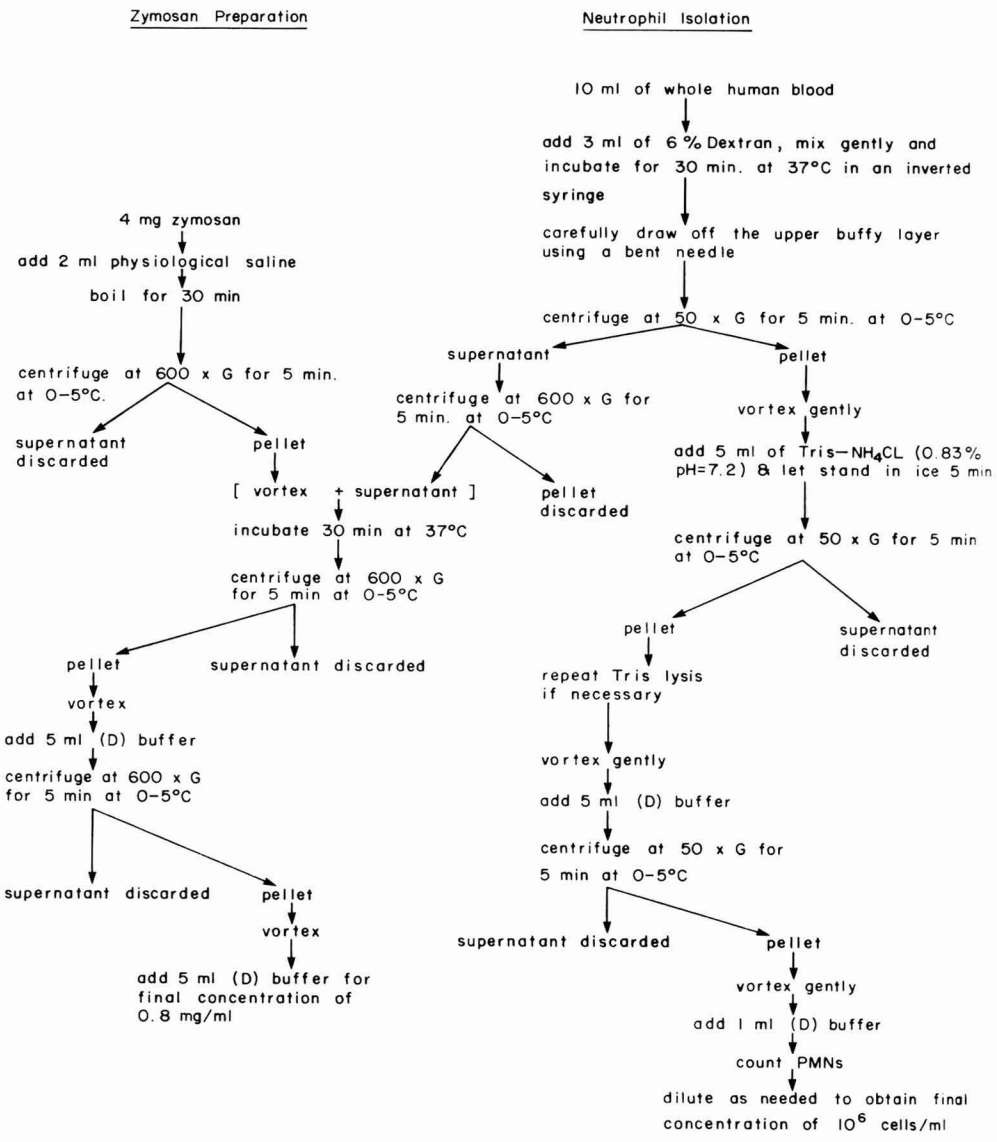
Cell Isolation Procedures

The methods for the preparation of granulocytes, macrophages, and the opsonization of zymosan are shown in the following flowcharts. (Cells were suspended in Dulbecco's (D) buffer and only plastic labware was used in this study.)

In this study we have chosen to use yeast-derived particles called zymosan because large amounts of the oxidative compounds are produced when they come in contact with the surface of the granulocyte or macrophage. With or without luminol, bacteria do not produce as much light as zymosan. The exact reason for this is unclear but may relate to the biochemical composition of the yeast cell wall.

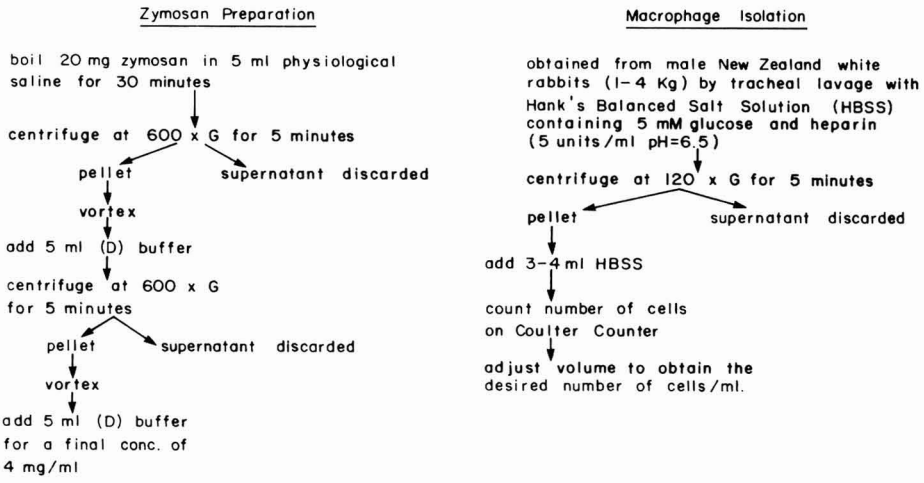
Measurement of Light

We found that it was necessary to check the photomultiplier tube in the Chem-Glo photometer for light response using a C^{14} light source supplied by Aminco. This would detect any change in the sensitivity of the tube



over a period of time. If the photomultiplier tube has not been exposed to large amounts of light, the response is quite consistent. We checked the instrument sensitivity to the C^{14} light source before and after each experiment.

Typical instrument settings for granulocyte and macrophage CL with the Chem-Glo photometer would be: power on high voltage; multiplier 1.0; blank, medium; and damping, on. For the integrator–timer, the usual settings would be: sensitivity 1.0 and counted for 60 sec.



When measuring granulocyte light, 1 ml of the cell preparation was mixed with 0.5 ml of the opsonized zymosan and 0.5 ml of (D) buffer then incubated for 5 min at 37°C. Immediately before counting 0.1 ml of luminol (1 mg/ml) was added to the mixture and the emitted light was measured for 10–15 min at 1-min intervals with the appropriate instrumentation. This same procedure was followed using superoxide dismutase or sodium benzoate to first obtain a blank. Then, instead of adding 0.5 ml (D) buffer, we substituted 0.5 ml of either S.O.D. or sodium benzoate. For the neonatal study two experiments were run. The first was a blank using adult granulocytes and zymosan opsonized with adult serum; the second used adult granulocytes with neonatal serum. Luminol solutions contained 0.1% DMSO.

RESULTS AND DISCUSSION

Figure 3 depicts the probable chemical reaction which occurs during

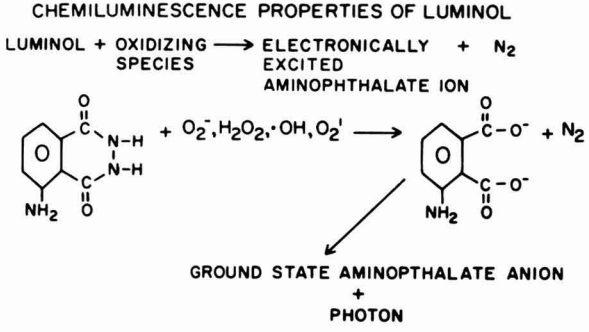


FIG. 3. Probable chemistry of the oxidation of luminol by various oxygen-containing species.

the interaction of luminol with various oxidizing species. This reaction leads to the formation of an electronically excited aminophthalate ion intermediate, which undergoes subsequent relaxation to its ground state with the concomitant release of a photon.

Figure 4 schematically represents the interaction between actively phagocytosing PMNs and luminol. Metabolic activation of these cells leads to the generation of reactive oxygen metabolites, which in turn react with luminol to produce photon emission as described in the Fig. 3. The association between the activation of luminol-dependent CL and the generation by phagocytosing PMNs of potent oxidizing species is shown in Fig. 5. This graph illustrates the effects of superoxide dismutase (S.O.D.) and sodium benzoate on luminol-dependent chemiluminescence. Both S.O.D., which catalyzes the dismutation of superoxide anion to hydrogen peroxide, and sodium benzoate, a hydroxyl radical scavenger, depressed the luminol responses of phagocytosing PMNs.

The results are supportive of our contention that luminol-dependent CL may be useful in monitoring oxidative metabolism in phagocytic cells. Several recent studies indicate that the production of phagocytosis-associated CL may relate to bactericidal activity of these cells (*3, 7*). This assay can also be applied to the assessment of serum opsonic deficiencies under appropriate conditions. Consequently, defective opsonization and/or PMN oxidative metabolism will lead to diminished CL responses using a liquid scintillation counter. The luminol-dependent CL assay can similarly be applied to the quantification of opsonophagocytic defects.

Figure 6 illustrates the luminol-dependent CL responses of PMNs from four patients, using 10^6 PMNs and zymosan (yeast cell wall) as the phagocytosable particle. The figure indicates that upon addition of luminol to actively phagocytosing PMNs, a rapid increse in photon emis-

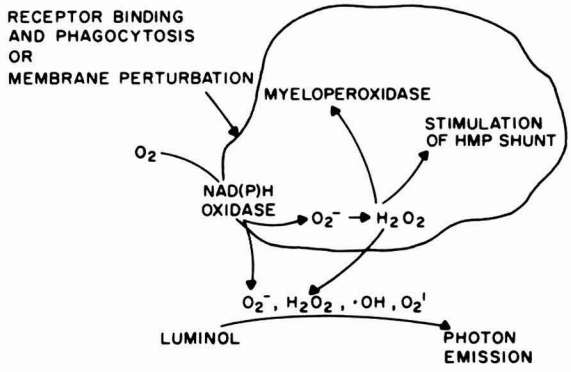


FIG. 4. Interaction between luminol and phagocytosing cells. This shows the relationship between activation of the cell with subsequent generation of reactive oxygen intermediates and their reaction with luminol to produce light.

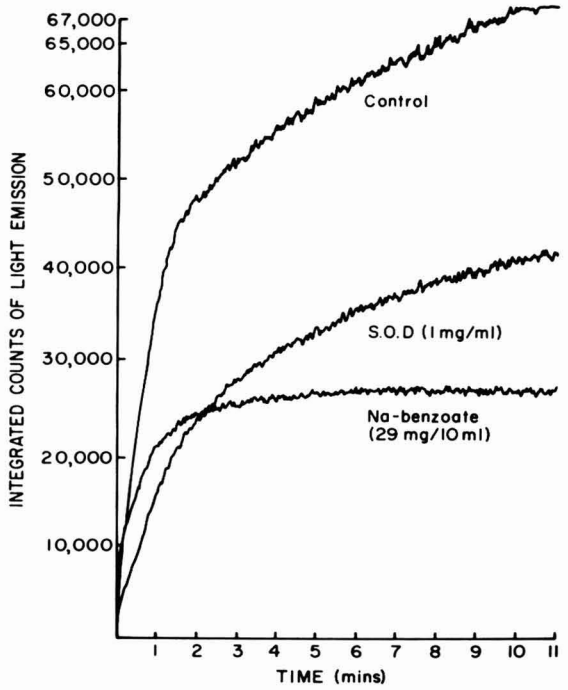


FIG. 5. Continuous recording of granulocyte chemiluminescence in the presence of zymosan particles and luminol. The control contains no inhibitors, the curve labeled S.O.D. was determined using superoxide dismutase, 0.5 ml, and the lowest curve was influenced by 0.5 ml of sodium benzoate.

sion is observed, reflective of the oxidative metabolic burst characteristic of these cells. The initial slopes of these responses appear to be quite similar in all four cases, although some variability is noted in the peak responses of these cells.

The oxidative metabolic activities of dog and rabbit alveolar macrophages were similarly studied using unopsonized zymosan as the phagocytosable particle. Figure 7 depicts the luminol-dependent CL responses of these cells. It may be seen that the CL responses of both rabbit and dog alveolar macrophages are considerably less than for PMNs. Further, dog alveolar macrophages appear to generate greater oxidative metabolic activity than their rabbit counterparts.

Figure 8 reflects the ability of the luminol-dependent CL assay system to discern both cellular metabolic defects and serum opsonic defects. Whether the defect lies in the oxidative metabolic pathways of the phagocyte or in the ability of serum factors to opsonize phagocytosable particles (which in turn leads to defective PMN activation), the luminol-dependent CL responses will be diminished. The ability of this system to detect cellular metabolic defects is exemplified in Fig. 9. This figure

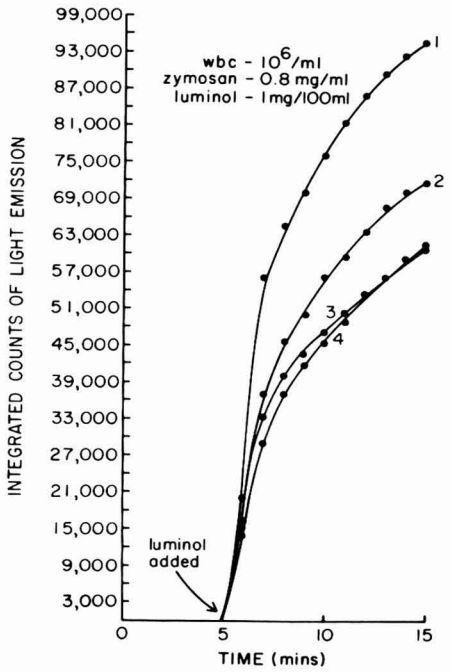


FIG. 6. Chemiluminescence (CL) response of normal human granulocytes in the presence of luminol. Cells were preincubated with opsonized zymosan particles for 5 min before the addition of luminol. Note the apparent lack of (CL) without the addition of luminol. The numbers 1–4 on each curve depict different individuals.

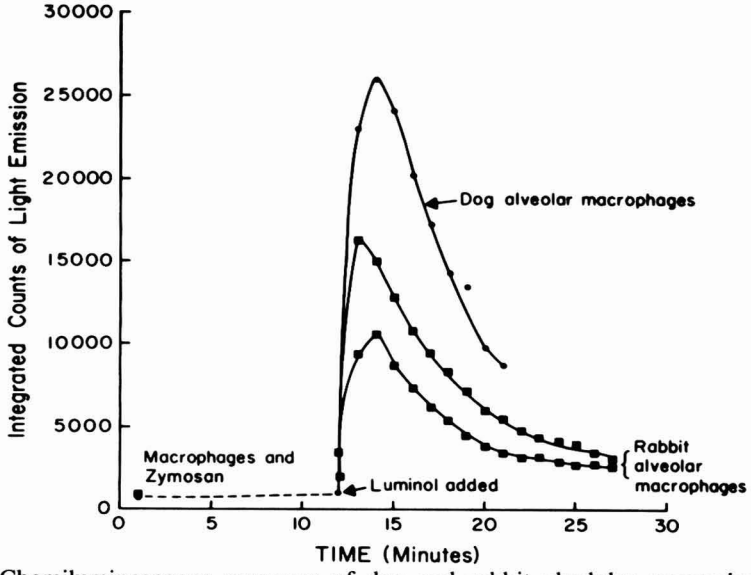


FIG. 7. Chemiluminescence response of dog and rabbit alvelolar macrophages in the presence of luminol. Note the absence of light response without the addition of luminol. PM tube 2 with an S-5 response was used while tube 1 gave no response.

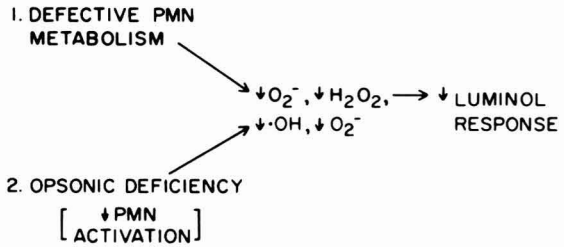


FIG. 8. Effect of opsonophagocytic defects on luminol response.

shows the luminol-dependent CL responses of normal and CGD PMNs. CGD PMNs are characterized by defects in oxidative metabolism and subsequent killing of certain species of organisms. As the graph indicates, these PMNs also showed greatly diminished (almost nonexistent) luminol responses. The luminol responses of normal and CGD responses in the Chem-Glo photometer are compared to the responses of these cells in a

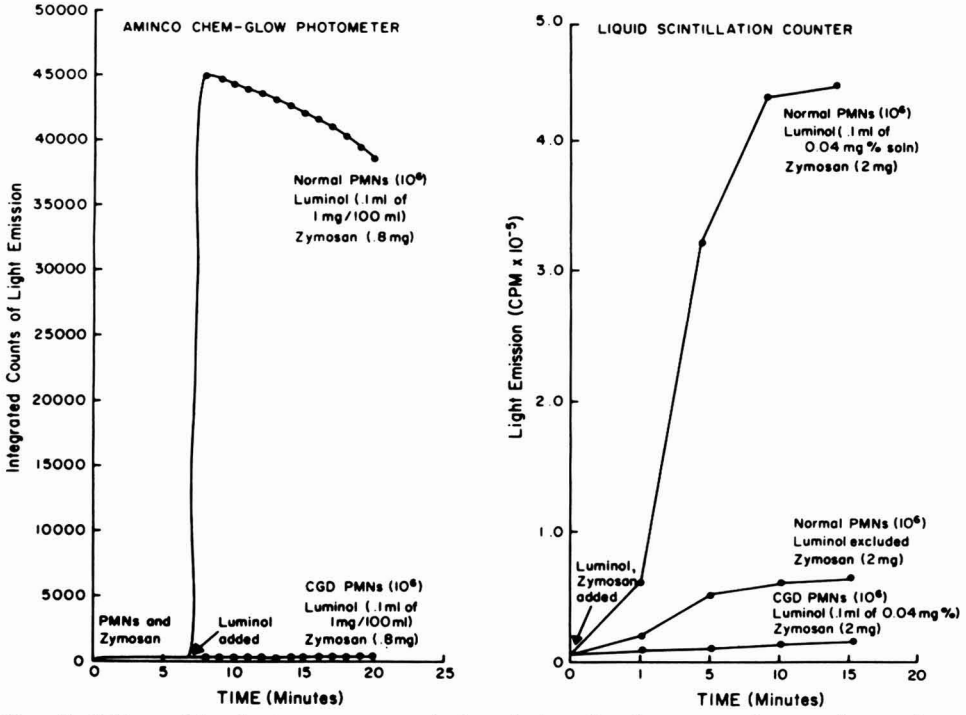


FIG. 9. Effect of luminol on zymosan-induced chemiluminescence from polymorphonuclear neutrophils from a chronic granulomatous disease (CGD) patient. On the left is the study using the Chem-Glo photometer. Note the absence of the response before luminol addition and the large response from normal cells with no detectable response from the CGD cells. On the right is the similar response with the liquid scintillation counter as the detection system. Note the similar response compared to the Chem-Glo photometer.

liquid scintillation counter. Comparable results are obtained with both assay systems.

Figure 10 demonstrates the use of the luminol-dependent CL assay in detecting opsonic deficiencies. In this example, zymosan was opsonized with fresh serum from either a normal adult or a premature human neonate. Neonatal serum has been characterized as having diminished opsonic capacity (*4*). The opsonized zymosan was then added to normal adult PMNs and the responses compared. The luminol responses in the Chem-Glo photometer were compared to the responses of these samples in a liquid scintillation counter in the absence of luminol. As in Fig. 9, the CL responses gave comparable results in both assay systems, with neonatal serum opsonins generating markedly less oxidative metabolic activity in normal PMNs.

These studies indicate that the luminol-dependent chemiluminescence assay, using the Chem-Glo photometer, may have clinical application in the detection of opsonophagocytic defects. The major advantages of this

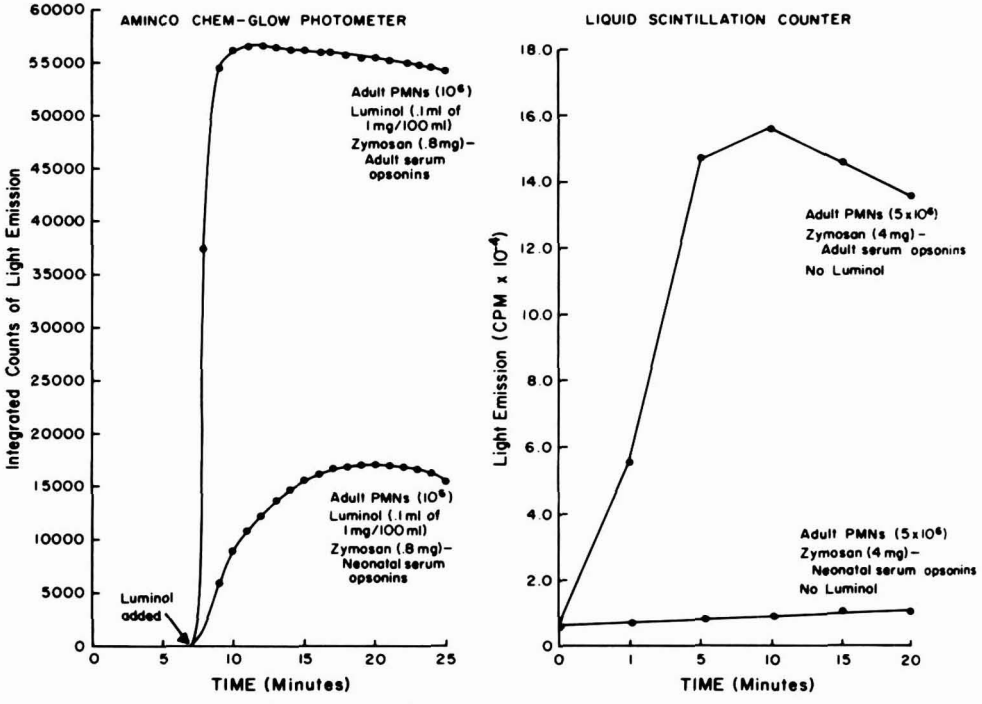


FIG. 10. Comparison of opsonic activity of normal and neonatal serum. On the left, adult granulocytes were preincubated with zymosan opsonized with either adult serum or neonatal serum. The Aminco Chem-Glo photometer with PM tube 1 was used for detection of light on the right is a similar experiment without luminol using a liquid scintillation counter.

system over the liquid scintillation system are in its requirement for smaller biological samples and in its markedly diminished expense.

SUMMARY

In summary, this report describes the conditions under which luminol has been utilized to measure phagocytosis-associated metabolic events in activated human PMNs and rabbit and dog alveolar macrophages. We feel that this system may have wide applicability to both clinical and experimental situations. Some possible applications are shown in the following table.

POTENTIAL APPLICATIONS OF LUMINOL-AMPLIFIED CHEMILUMINESCENCE

Cell type	Applications
Human polymorphonuclear leukocytes	(1) Detection of bactericidal defects, particularly chronic granulomatous disease (2) Detection of host opsonic defects (both immunoglobulin and complement −C3b opsonic defects). (3) Analysis of drug effects on host cellular and opsonic defenses (*9,11*). (4) Characterization of bacteria or other particulate matter in terms of ability to generate opsonic activity and/or be ingested by phagocytic cells (*3,7*).
Alveolar macrophages	(1) Detection of environmental pollutant effects on respiratory defense mechanisms (against both particulate and soluble matter). (2) Analysis of drug effects on respiratory defense mechanisms, particularly drugs administered in the treatment of respiratory diseases.

ACKNOWLEDGMENTS

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MICROCHEMICAL JOURNAL 22, 475–478 (1977)

Microdetermination of Ascorbic Acid Using Bromine Monochloride in Water–Acetic Acid Medium

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Received April 4, 1977

INTRODUCTION

A number of methods are available for the quantitative determination of ascorbic acid, depending upon various analytical principles. Bessey (2) reported a visual titrimetric method employing 2, 6-dichlorophenolindophenol. Deutsch and Weeks (3) described a microfluorometric method for the determination of ascorbic acid. Roe and co-workers (7) evolved a colorimetric method using 2, 4-dinitrophenylhydrazine. Various other polarographic (4), chromatographic (*8*), and turbidimetric (*6*) methods have also been recently investigated for assay of ascorbic acid. Bromine monochloride has been used as a brominating reagent for the determination of olefinic unsaturation in a number of compounds (1, 10). It has also been employed for the substitutive halogenation of aromatic compounds (*9*) in aqueous solutions, selective bromination of phenols (*5*), and volumetric determination of hydrazines and hydrazides (*12*), and has been recently used for the microdetermination of histamine and serotonin drugs (*11*). The aim of the present work is to report a rapid and convenient method for the microgram determination of ascorbic acid using bromine monochloride in a water–acetic acid medium.

MATERIALS AND METHODS

Reagents and Solutions

All chemicals used were of highest obtainable purity for analytical purposes and were purchased from British Drug Houses, Pool, Dorset, England. Unless otherwise stated solutions indicate aqueous solutions.

0.1 *N* Bromine monochloride: 1.3917 g of potassium bromate and 1.9835 g of potassium bromide were dissolved in 125 ml of water in a 500-ml volumetric flask. The solution was cooled in ice, then 100 ml of concentrated hydrochloric acid was added and the solution was diluted to the mark with distilled water.

Glacial acetic acid.

0.02 *N* Sodium thiosulphate solution.

Potassium iodide: 15% (w/v) solution of potassium iodide.

Starch solution: 1% (w/v).

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Sample solution: Stock solution of analytical grade ascorbic acid was prepared by dissolving an accurately weighed amount in glacial acetic acid in a 100-ml volumetric flask.

PROCEDURES

Determination of the stoichiometry of the reaction. The stoichiometry of the reaction was established as follows: 2–10 mg of ascorbic acid dissolved in glacial acetic acid was reacted with a known amount of bromine monochloride solution. The reaction was allowed to proceed for about 15 min in an ice bath, after which the remaining part of the reagent was assayed iodometrically. Results obtained are shown in Table 1.

Analysis of ascorbic acid. An aliquot containing 2–10 ml of the sample was placed in a 100-ml iodine flask. Five milliliters of glacial acetic acid followed by 5 ml of bromine monochloride solution were added and the flask was stoppered and shaken well. The flask was placed in an ice bath and the reaction mixture was allowed to cool for 15 min. After the reaction was over the stopper was washed with 5 ml of the potassium iodide solution and the liberated iodine was titrated with standard sodium thiosulphate solution, using starch as indicator.

A blank experiment was also run under the identical conditions except for the use of the sample. All the determinations were done in triplicate.

Calculation. Ascorbic acid (mg) = $88\, N_{Na_2S_2O_3}\, (V_B - V_S)$

where

$N_{Na_2S_2O_3}$ = normality of sodium thiosulphate solution;
V_B = volume of sodium thiosulphate required to titrate blank (ml);
V_S = volume of sodium thiosulphate required to titrate sample (ml).

Statistical evaluation. The statistical evaluation was made by means of regression analysis, as the material was found to have a normal (Gaussian) distribution.

TABLE 1
DETERMINATION OF THE STOICHIOMETRY OF THE REACTION

Weight of ascorbic acid (mg)	Moles of BrCl consumed per mole of ascorbic acid assayed titrimetrically
2.1100	1.0009
3.6925	1.0009
5.2750	0.9842
6.3300	1.0148
8.4400	1.0113
10.5500	1.0092

TABLE 2
MICRODETERMINATION OF ASCORBIC ACID

Sample no.	Sample weight (mg)		Deviation (%)
	Taken	Recovered	
1	2.1100	2.1120	+0.0947
2	3.6925	3.6960	+0.0947
3	5.2750	5.1920	−1.5734
4	6.3300	6.4240	+1.4849
5	8.4400	8.5360	+1.1374
6	10.5500	10.6480	+0.9289

RESULTS AND DISCUSSION

The above-mentioned procedure was applied for the microdetermination of ascorbic acid and the results of the determination are presented in Table 2. The maximum deviation is about 1.5% on the average. Figure 1 shows the relationship between the milligrams of ascorbic acid in the assay system and milliliters of BrCl consumed. The correlation coefficient was 99–98%, and the lower level of detection is below 0.5-mg ascorbic acid. Acetic acid (10–12) has previously been used as solvent in various reactions of bromine monochloride. Variation of acetic acid concentration has been studied and it has been found that the recoveries of the samples were unaffected. However, when the reaction was carried out in a large

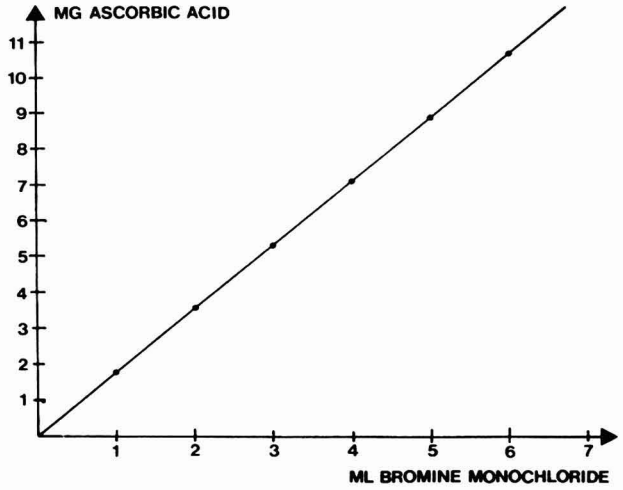

FIG. 1. The relationship between the amount of ascorbic acid (Y) and the volume of bromine monochloride (X) consumed. Regression analysis revealed that the best fit curve could be drawn from the equation $Y = 0.58\,X - 0.034$ ($r = 0.9998$).

excess of acetic acid (10–15 ml), the accurate detection of end point was difficult.

The reaction of bromine monochloride with ascorbic acid is not instantaneous, and bromine monochloride consumption was almost negligible when titrated back at once. After prolonging the reaction time, it was found that 15 min were required for the completion of the reaction. For determination, usually 0.1 *N* bromine monochloride had been used. The rate of the reaction was independent of the concentration of bromine monochloride.

SUMMARY

A micromethod for the determination of ascorbic acid has been developed. A 2–10 mg sample dissolved in glacial acetic acid is reacted with a known excess of bromine monochloride at ice-bath temperature, and the excess reagent is back-titrated iodometrically. The maximum deviation in the results is ± 1.5%.

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MICROCHEMICAL JOURNAL 22, 479–483 (1977)

Spectrophotometric Determination of Cyanocobalamin (as Cobalt)

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INTRODUCTION

The determination of cyanocobalamin in pharmaceutical preparations is usually done either directly by measuring the absorbance of its water solutions at 361 nm *(1,2)* or indirectly by a method which involves the determination of cobalt (on a weight percentage basis, cobalt being 4.35% of the total B_{12} molecule). The direct method is applied to pharmaceutical preparations that give clear water solutions. In the presence of other compounds that interfere with its determination, there have been suggested various methods for the separation of cyanocobalamin prior to its determination *(3,4)*.

For indirect determination various methods have been proposed. They are based on the decomposition of the samples and the subsequent determination of cobalt by spectrophotometric *(5–7)* and other methods such as isotopic dilution *(8)* and A.A.S. *(9,10)*. The last method can also be used without decomposition of the samples by direct aspiration of their water solution into the appropriate flame. In this case, the absorbance by cobalt into the flame could be affected by the presence of various organic compounds which enhance the absorption *(9)*.

The spectrophotometric determination of cobalt after the decomposition of the sample is usually done by use of a reagent such as nitroso-R salts *(5)*, 1-benzoyl-4-phenylthio-semicarbazidlosung *(6)*, or fast navy 2R *(7)*. With these reagents and the presence of other metal cations, interference in some extension was experienced in the determination of cobalt *(7,11)*.

In a recent paper *(12)* a new specific and very sensitive spectrophotometric method for cobalt determination was proposed. The method is based on the formation of a complex between cobalt and 2,2′-dipyridyl-2-pyridylhydrazone (DPPH). This reagent also forms colored complexes with a number of metal cations in solutions having pH>3. Addition of a strong acid (H_2SO_4, $HClO_4$) to solutions of these complexes results in the decomposition of all complexes except that of cobalt, which only changes its color from orange-yellow to pink. At 25% perchloric acid the molar

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absorptivity of the cobalt complex is 42,000 liter mol^{-1} at 500 nm. At this acidity, the presence of other metal cations does not interfere with the cobalt determination. The reagent itself does not absorb at 500 nm and this offers a considerable advantage over the group of nitrosonaphtholes and nitroso-R-salts.

In the work presented here, the application of the DPPH method for the indirect determination of cyanocobalamin in pharmaceutical preparation was studied.

EXPERIMENTAL

Reagents

2,2′-Dipyridyl-2-pyridylhydrazone (DPPH) was prepared as described previously *(12)*. Solutions of DPPH were prepared by dissolving the required weight in ethanol. The ethanolic solution of DPPH is stable and could be kept for several weeks in an amber glass bottle.

Standard solutions of cobalt(II) were prepared by dissolving the appropriate amount of cobaltous chloride hexahydrate in distilled water. The solutions obtained were standardized by EDTA titration.

Cyanocobalamin (Merck) was dried for 4 hr over silica gel at 50°C before its use. Various amounts of cyanocobalamin were accurately weighed and transferred to a 50-ml beaker for the wet ashing.

A standard solution of cyanocobalamin containing 4.35 ppm Co was prepared by dissolving 100 mg of cyanocobalamin in 1000 ml of distilled water. These solutions were tested either by the recommended procedure or by atomic absorption spectroscopy.

All other reagents used were of analytical grade.

Apparatus

Absorbance measurements were made in 10-mm quartz cells with a Zeiss M4QII spectrophotometer.

A Perkin–Elmer Model 403 atomic absorption spectrophotometer equipped with an air–acetylene burner head was also used in the experimental work.

Recommended Procedure

The appropriate amount of the pharmaceutical preparation (so as to contain 7 to 100 μg of cobalt) is weighed and transferred to a 50-ml beaker. Then 5 to 20 ml of concentrated sulfuric acid and about 0.2 g of potassium perchlorate are added. The solution is heated slowly with constant swirling of the beaker and is then boiled. If the hot digest is not colorless, a few crystals of potassium perchlorate are added, and the solution is boiled again. Finally the bulk of sulfuric acid is volatilized. At this stage all organic matter should have been completely decomposed. After cooling the digest, about 10 ml of distilled water are added followed by a concentrated solution of ammonium hydroxide until the pH exceeds 5. The solution is transferred to a 50-ml volumetric flask and 2 ml of 10^{-2} *M*

TABLE 1
COBALT DETERMINATION IN CYANOCOBALAMIN STANDARDS

B_{12} taken (mg)	Cobalt calculated (μg)	Cobalt found (μg)
0.200	8.7	7.9
0.400	14.4	15.8
0.800	34.8	32.4
1.384	60.2	60.8
1.809	78.7	76.5
2.191	95.3	94.4

ethanolic solution of DPPH are added. Then 10 ml of concentrated sulfuric acid are added and the flask is filled with distilled water. The absorbance of the resulting solution is measured at 500 nm and compared with the calibration curve which is obtained using standard solutions of cobalt.

In the case of injectable pharmaceutical preparations, an appropriate volume is transferred into the beaker, boiled nearly to dryness, and then treated as described above.

RESULTS AND DISCUSSION

The method developed has been applied to the determination of cobalt in cyanocobalamin standard samples. The results obtained are summarized in Table 1. For the first three samples listed in the table the appropriate volumes of the standard cyanocobalamin solution were taken and transferred to the beaker for the wet ashing. The other three samples were taken by weighing cyanocobalamin. The results show that the recovery of cobalt from the wet ashing is complete.

The method has also been applied to the determination of cyanocobalamin (as cobalt) in two pharmaceutical preparations coded as A tablets and B injections. The analysis of these two preparations has also been performed by A.A.S. The results obtained are summarized in Table 2.

TABLE 2
CYANOCOBALAMIN DETERMINATION (AS COBALT) IN PHARMACEUTICAL PREPARATIONS

Sample	Cobalt calculated (ppm)	Cobalt found (ppm)[a]	
		Present method	A.A.S.
A 125/μg B_{12}/tablet	17.6	16.2 ± 1.2	16.5 ± 1.4[b]
B 1 mg B_{12}/ml	43.5	42.4 ± 2.3	45.7 ± 1.8[c]

[a] Five determinations.
[b] After wet ashing.
[c] Direct aspiration.

These results confirm the recovery of cobalt and the precision of the method developed.

The procedure described is simple to use for routine testing in pharmaceutical preparations, mainly when these products are not dissolved in water or the solutions obtained produce cloudiness. It offers advantages over the other reagents used for this purpose because of the lack of interferences.

Since the present method determines the total cobalt present, the presence of cobalt compounds other than cyanocobalamin will give higher values. This is applied to all methods that determine cyanocobalamin as cobalt. In this case, ionic cobalt, if present, could be extracted into a $CHCl_3$ solution of 8-hydroxyquinoline prior to the wet ashing (*9*).

The method developed could also be applied to the determination of cobalt in various biological materials or food after the appropriate decomposition of the samples.

SUMMARY

A spectrophotometric method for the determination of cyanocobalamin (as cobalt) in pharmaceutical preparations has been developed. The sample is first decomposed with sulfuric acid and potassium perchlorate. The liberated cobalt is then determined using 2,2'-dipyridyl-2-pyridylhydrazone as reagent in solutions containing 20% sulfuric acid. The presence of other metal cations does not interfere with the determination of cobalt. The method has been applied to the determination of cobalt in pure cyanocobalamin and in two pharmaceutical preparations.

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Oxidation of Organic Substances by Tervalent Manganese Compounds

VIII. Determination of Benzene Polyhydroxy and Aminohydroxy Derivatives with a Standard Solution of Hexaquomanganese(III) Ion in Perchloric Acid[1]

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INTRODUCTION

Compounds of tervalent manganese belong among the newer oxidants whose stability and reactivity can be varied widely by means of the formation of variously stable complexes *(1,6)*. The most reactive form of tervalent manganese is the hexaquomanganese(III) ion in a noncomplexing medium of perchloric acid *(1)*. This reagent has so far been used for analytical purposes only in the indirect titrimetric determination of mandelic acid *(2)*.

In the framework of the systematic study of the oxidation of organic substances by tervalent manganese compounds, the present work was devoted to the investigation of the potential analytical use of this reagent for the determination of benzene polyhydroxy and aminohydroxy derivatives, using pyrocatechol, resorcinol, hydroquinone, phloroglucinol, *p*-aminophenol, and metol as model substances. Among tervalent manganese compounds, only the less reactive sulfate *(12,13)*, phosphate *(5,10)*, and diphosphate *(4,8)* complexes of tervalent manganese have been used for similar purposes.

EXPERIMENTAL

Reagents

A 0.005 *N* solution of hexaquomanganese(III) ion in 6 *M* perchloric acid and 0.4 *M* manganous perchlorate was prepared by the procedure described earlier *(3)* and its titer was determined using ferrous sulfate *(9)*.

Pyrocatechol, resorcinol, hydroquinone, phloroglucinol, *p*-aminophenol, and metol were used as 0.01, 0.005, and 0.001 *M* solutions in 6 *M* perchloric acid. The titers of hydroquinone, *p*-aminophenol, and metol

[1] Part VII: Oxidation of Malachite Green by the Diphosphate Complex of Tervalent Manganese. *Collect. Czech. Chem. Commun.*, in press.

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were determined cerimetrically *(11)*, those of pyrocatechol, resorcinol, and phloroglucinol by a bromination method *(7)*.

The titers of 0.1, 0.01, and 0.005 *N* solutions of ferrous sulfate in 0.5 *N* sulfuric acid were determined daily by potentiometric titration of potassium dichromate standard solutions of the same normalities.

All chemicals used were of p.a. purity.

Apparatus

The potentiometric titrations were carried out on a Multoscop V vacuum-tube millivoltmeter (Laboratorní přístroje, Prague), with a bright platinum indicator and a saturated calomel reference electrode.

PROCEDURES

Direct Potentiometric Titration

To 5.00 ml of a 0.01 to 0.001 *M* solution of the test substance in 6 *M* perchloric acid, 20 ml of 6 *M* perchloric acid were added and the solution was titrated with a 0.005 *N* solution of hexaquomanganese(III) ion in 6 *M* perchloric acid and 0.4 *M* manganous perchlorate.

Indirect Determination

To 20.00 ml of a 0.005 *N* solution of hexaquomanganese(III) ion in 6 *M* perchloric acid and 0.4 *M* manganous perchlorate, 5.00 ml of 0.005 *M* hydroquinone, *p*-aminophenol, or metol or 2.00 ml of 0.001 *M* pyrocatechol, resorcinol, or phloroglucinol in 6 *M* perchloric acid were added. The solution was allowed to stand at laboratory temperature for a time interval *t* and then the excess oxidant was potentiometrically back-titrated with 0.01 *N* ferrous sulfate. The blank experiment was carried out simultaneously and the oxidant consumption in equivalents per mole of the substance oxidized was calculated from the difference.

RESULTS

Direct Potentiometric Titration

Whereas in the titration of hydroquinone, *p*-aminophenol, and metol in 6 *M* perchloric acid the potentials stabilized sufficiently rapidly and the end point break amounted to about 150 mV per 0.02 ml of a 0.005 *N* solution of hexaquomanganese(III) ion, the titration curves of pyrocatechol, resorcinol, and phloroglucinol were poor, and the method could not be used for the determination.

The accuracy and reproducibility of the direct potentiometric determination of hydroquinone, *p*-aminophenol, and metol with the hexaquomanganese(III) ion standard solution are summarized in Table 1, the values given being the averages of seven measurements, from which the standard deviations were also calculated. One milliliter of 0.005 *N* hexaquo-

TABLE 1

ACCURACY AND REPRODUCIBILITY OF THE DIRECT DETERMINATION OF HYDROQUINONE, *p*-AMINOPHENOL, AND METOL WITH A STANDARD SOLUTION OF HEXAQUOMANGANESE (III) ION IN NONCOMPLEXING MEDIUM OF PERCHLORIC ACID[a]

	Hydroquinone		
Taken	5.505	2.753	0.550
Found	5.507	2.751	0.556
SD	0.007	0.005	0.008
	p-Aminophenol		
Taken	7.279	3.639	0.728
Found	7.283	3.635	0.720
SD	0.009	0.006	0.011
	Metol		
Taken	8.658	4.329	0.866
Found	8.656	4.333	0.859
SD	0.006	0.010	0.008

[a] All values are milligrams.

manganese(III) ion corresponds to 0.2752 mg of hydroquinone, 0.3628 mg of *p*-aminophenol, and 0.4329 mg of metol.

Indirect Determination

As pyrocatechol, resorcinol, and phloroglucinol could not be directly titrated with the solution of hexaquomanganese(III) ion, the possibility of the indirect determination based on back-titration of the unreacted excess of the oxidant was investigated. For the sake of comparison, the time dependence of the oxidant consumption was also measured for hydroquinone, *p*-aminophenol, and metol. These dependences, in equivalents of the reagent per mole of the test substance, are given in Table 2, each being the average of three measurements which did not differ by more than ±0.5%.

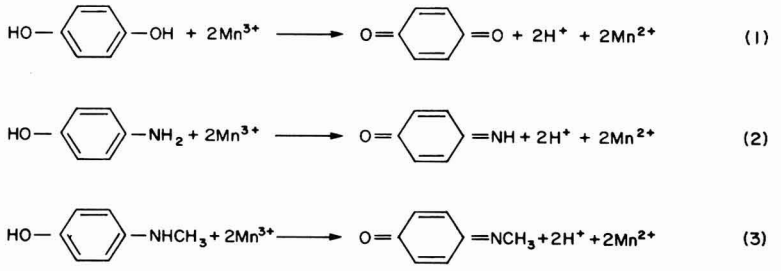
TABLE 2

TIME DEPENDENCE OF THE CONSUMPTION OF HEXAQUOMANGANESE (III) ION, IN EQUIVALENTS PER MOLE OF THE SUBSTANCE OXIDIZED

Substance oxidized	2 min	5 min	10 min	30 min	60 min	120 min	240 min
Hydroquinone	2.00	2.00	2.01	2.02	2.02	2.05	2.08
p-Aminophenol	2.00	2.00	2.01	2.02	2.03	2.06	2.10
Metol	2.00	2.00	2.00	2.01	2.01	2.02	2.02
Pyrocatechol	3.2	4.7	5.9	6.8	7.2	7.5	7.6
Resorcinol	4.1	8.4	9.5	10.6	11.5	11.8	12.7
Phloroglucinol	3.7	5.5	7.7	9.4	11.0	12.3	12.8

DISCUSSION

It follows from the above results that hydroquinone, *p*-aminophenol, and metol are oxidized with the exchange of two electrons to the corresponding quinone compounds, according to Eqs. (1) to (3); the products are resistant to further oxidation.



On the other hand, with resorcinol and phloroglucinol, which cannot form quinone compounds during the oxidation, the aromatic system is destroyed more deeply and the reaction stoichiometry is undefined. Pyrocatechol can form a quinone substance on oxidation, but the *o*-benzoquinone formed undergoes further nonstoichiometric oxidation, which prevents analytical use of the reaction.

On the basis of the above findings it can be expected that the reactions of hexaquomanganese(III) ion with other *p*-dihydroxy and *p*-aminohydroxy derivatives of benzene will also be analytically useful.

SUMMARY

The reaction of hexaquomanganese(III) ion with hydroquinone, *p*-aminophenol, metol, pyrocatechol, resorcinol, and phloroglucinol was studied. It has been found that the first three substances are oxidized quantitatively with the exchange of two electrons to the corresponding quinone compounds and that this reaction can be employed for direct or indirect titrimetric determination of these substances. On the other hand, pyrocatechol, resorcinol, and phloroglucinol are oxidized nonstoichiometrically with a deeper destruction of the aromatic system, which prevents analytical use of these reactions.

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Determination of Nanogram Quantities of Cobalt in Complex Matrices by Flameless Atomic Absorption Spectrometry

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INTRODUCTION

Components of nuclear reactor systems at ERDA's Idaho National Engineering Laboratory are descaled with a series of solutions containing high concentrations of various strong oxidants, reductants, and organic complexing agents. As part of the evaluation of the descaling operation, cobalt must be determined in these solutions at about the 200 ng/ml level. Conventional atomic absorption analysis with a flame as the absorption cell provides a detection limit of 200 ng/ml thus limiting its utility. In addition, severe and complicated matrix effects are encountered necessitating matrix matching, standard additions, or chemical separation. Finally, the radioisotope content of the solutions makes it desirable to minimize the volume of sample handled and the complexity of sample treatment.

Direct determination of Co has been reported by Maessen *et al. (3)*. Untreated blood plasma was introduced directly into a mini-Massmann-type atomizer and in the absence of a means for background correction it was necessary to use the ramp heating mode to separate peaks due to molecular decomposition products from the Co atomic absorption peak. Sensitivity was reduced by the slow heating rate. Their detection limit appears to have been about 5 ppm with a 0.5–μl sample, or 250 ng absolute. Muzzarelli and Rocchetti *(5)*, using a Massmann furnace with a deuterium lamp background corrector, have determined Co in dry-ashed blood serum. A standard additions technique was used to correct for matrix interferences. Simmons *(7)* has determined as little as 10 ng of Co in plant material by wet ashing, extraction of the 2-nitroso-1-naphthol complex, and atomization in a Massmann furnace. McElhaney *(4)* has determined as little as 0.7 ng of Co in aluminum metal with a Massmann-type atomizer.

In this paper, we describe work leading to the development of a method

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for the determination of cobalt in the following four matrices: (1) alkaline permanganate (AP), 0.076 *M* $KMn0_4$ and 0.15 *M* NaOH; (2) hydrazine/Versene (HV), 0.5 *M* hydrazine, 0.5 *M* NH_4OH, and 0.25 *M* ethylenediaminetetraacetic acid; (3) citrate/Versene (CV), 0.033 *M* monoammonium citrate and 0.033 *M* EDTA; (4) a composite of matrices (1) through (3), plus 3 g/liter of potassium pyrosulfate. The samples contain as well the elements Fe, Ni, and Cr in 50- to 100-fold excess of the Co. The method to be described provides a more than adequate detection limit (10 pg of Co), can be performed on 5.0 1μl of sample and elminates the need for matrix matching or standard additions techniques.

MATERIALS AND METHODS

A. Atomic Absorption Measurement

This work was conducted with a Varian AA-5 atomic absorption spectrophotometer equipped with a Varian Model 63 carbon rod atomizer mounted in place of the burner. The source of atomic resonance radiation was a commercial cobalt hollow cathode with neon filler gas, operated at 10 mA. The 240.7-nm cobalt resonance line was observed with a spectral bandpass of 0.17 nm (50-μm slit width). Background correction was accomplished with a Varian BC-6 simultaneous background corrector and a hydrogen continuum lamp. Transient absorption signals were recorded on a Varian Model A-25 strip chart recorder with 0.5-sec full-scale pen response, or on a Houston Model 2000 X–Y recorder.

Dry, ash, and atomization times and temperatures varied according to sample matrix. Variations in N_2 sheath gas flow rate in the range 1.5 to 6.0 liters/min produced no significant change in atomic absorption peak shape or amplitude. A flow of 4 liters of N_2/min was chosen as a compromise between N_2 consumption and oxidative damage to graphite atomizer components. The effect of varying H_2 flow, at a constant flow of 4 liters/min N_2 is shown in Fig. 1. There is little or no effect of H_2 flow between 0 and 2 liters/min, followed by a gradual decrease in peak absorbance that we presume is due to more efficient cooling of the atomizer by the higher thermal conductivity of the sheath gas. However, a detailed mechanistic study of the cobalt atomization *(9)* has shown the presence of H_2 in the sheath gas mixture to be of profound benefit in the atomization of the cobalt–ammonium pyrrolidinedithiocarbamate complex. For this reason, a H_2 flow of 1 liter/min was used throughout these studies.

B. Temperature Measurement

Maximum temperatures below 800°C were measured with an iron–constantan thermocouple; those above 800°C were measured with a Pyro micro-optical pyrometer. The temperatures actually measured were, in all cases, that of the atomizer tube outer surface, and in no case correspond

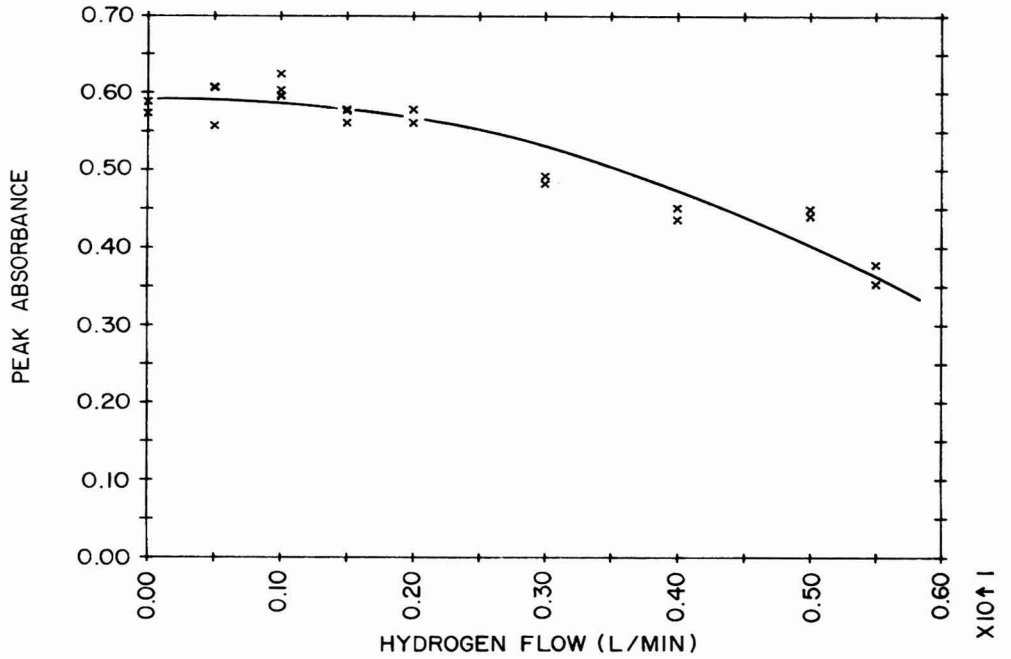

FIG. 1. Effect of H_2 flow rate on Co peak absorbance. N_2 flow rate is 4 liters/min.

to the temperature of the surface on which the sample was deposited. The sample surface temperatures are about 20% higher than the termperatures measured in this work (J. M. Baldwin and D. A. Pavlica, unpublished observations).

C. Reagents

All chemicals were analytical reagent grade materials with the exception of ammonium pyrrolidinedithiocarbamate, which was of unspecified purity. All were used without further purification. Cobalt stock solution was prepared by dissolution of the spectrographically pure metal in reagent grade nitric acid. Working solutions were prepared by dilution of the stock solution with the appropriate matrix. All aqueous solutions were prepared with water from a Millipore Milli-Q water system.

RESULTS AND DISCUSSION

A. Matrix Effects in Direct Atomization

Attempts to atomize Co directly from the various matrices produced evidence of complicated chemical behavior in the atomizer. The atomization conditions are summarized in Table 1, and recorder traces of the transient absorbance signals for the entire heating sequence with each matrix are shown in Fig. 2. All five traces are for samples containing 0.15

TABLE 1
CONDITIONS FOR DIRECT ATOMIZATION OF COBALT FROM COMPLEX MATRICES[a]

	Max. temperature (°C)	Time (sec)
Dry step	120	30
Ash step		
Water	600	15
AP	1050	15
HV	850	15
CV	600	15
Composite	1050	15
Atomize Step	2300	4

[a] Atmosphere: 4 liters/min N_2, 1 liter/min H_2.

μg of Co/ml. The water solution produces only one peak, due to the atomic absorption of cobalt, while the other matrices all exhibit molecular absorption and/or scattering of varying complexity during the ash step. In addition, the apparent recovery of Co as measured by peak height varies considerably with the matrix. Apparent recoveries are summarized in Table 2. All are significantly lower than 100%, and are lowest for those matrices that required the highest ash temperature. Loss by volatilization

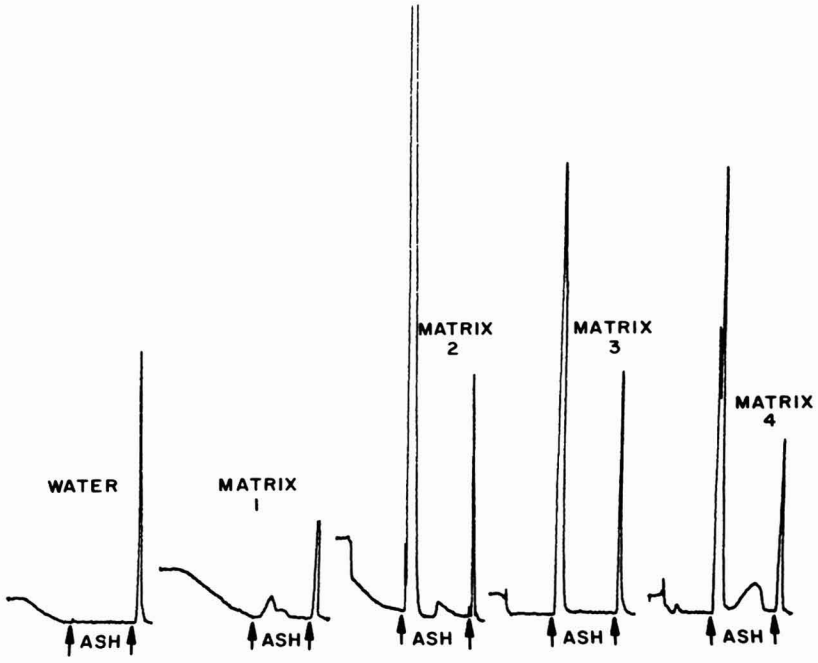

FIG. 2. Transient absorbance signals for direct atomization. Water, (1) AP matrix, (2) HV matrix, (3) CV matrix, (4) composite matrix. No background correction.

TABLE 2
APPARENT RECOVERY OF COBALT BY DIRECT ATOMIZATION FROM VARIOUS MATRICES

Matrix	Peak height (arbitrary units ± σ_{mean})	Recovery (% of water matrix ± σ_{mean})
Water	442.5 ± 6.1	—
AP	76.0 ± 7.0	17.2 ± 1.6
HV	379.5 ± 13.4	85.8 ± 3.2
CV	370.0 ± 11.4	83.6 ± 2.8
Composite	306.3 ± 17.1	69.2 ± 4.0

of cobalt during the ash step is suspected as at least a partial cause for the low recoveries. Low recoveries were in fact observed over the entire analytical range, as typified by the calibration curves from cobalt standards in water and in the composite matrix (Fig. 3).

B. Solvent Extraction of the Co–APDC Complex

The data of the previous section clearly indicated the desirability of a chemical separation of cobalt to eliminate the need for matrix matching. Kinrade and Van Loon *(2)* have reported the use of a number of systems, among them ammonium pyrrolidinedithiocarbamate (APDC)/methyl

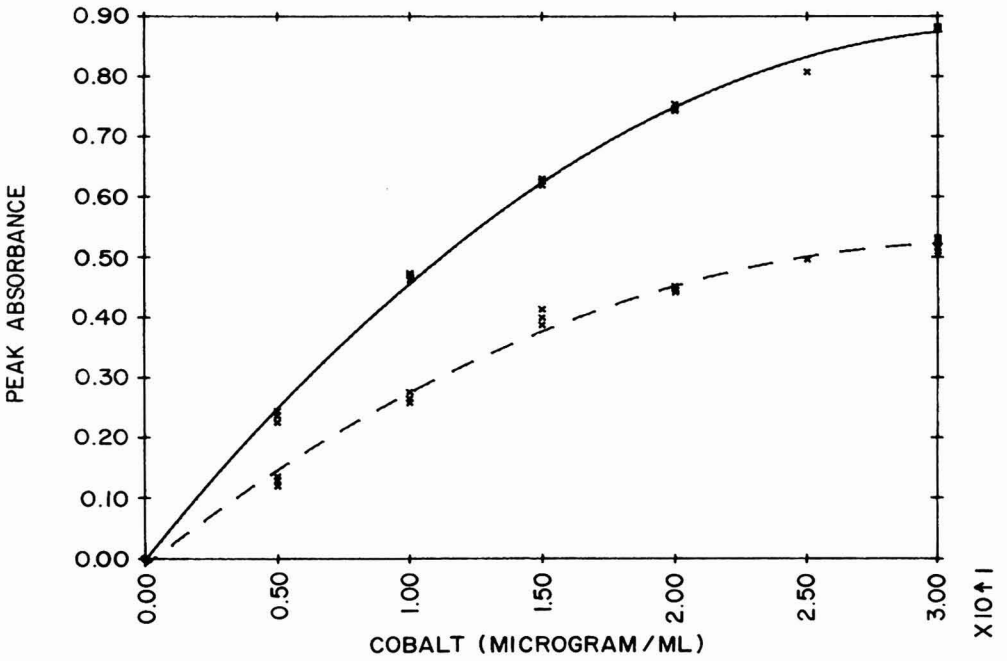

FIG. 3. Calibration curves for direct atomization of cobalt from the composite matrix (dashed line) and from water (solid line).

isobutyl ketone (MIBK) and APDC/ethyl acetate for the extraction of cobalt from natural waters for flame atomic absorption measurement. Popova *et al. (6)* have applied the APDC/MIBK system to the measurement of Co in wet- or dry-ashed animal feeds by flame atomic absorption. Delves *et al. (1)* have used this same system for determination of cobalt in blood.

In the present case, extraction under acidic conditions is indicated due to the presence of EDTA in some of the matrices *(8)*. The effect of pH on the recovery of Co from the composite matrix is shown in Fig. 4 for extraction of the Co–APDC complex into ethyl acetate. The phthalate buffer system was used to control pH in the range 1–6, and the phosphate system above pH 6. No cobalt was recovered above pH 6. Precipitation occurred below pH 2, so a pH of 3 was chosen for all further work. The phthalate and acetate buffer systems performed equally well in this system at pH 3. The phthalate system was used in all further work because of the greater available buffer capacity at pH 3.

Various organic solvents have been used for the extraction of the Co–APDC complex from aqueous solutions. The behavior of several solvents was examined in this instance. Benzene, chloroform, carbon tetrachloride, and methyl isobutyl ketone all have surface tension too low to

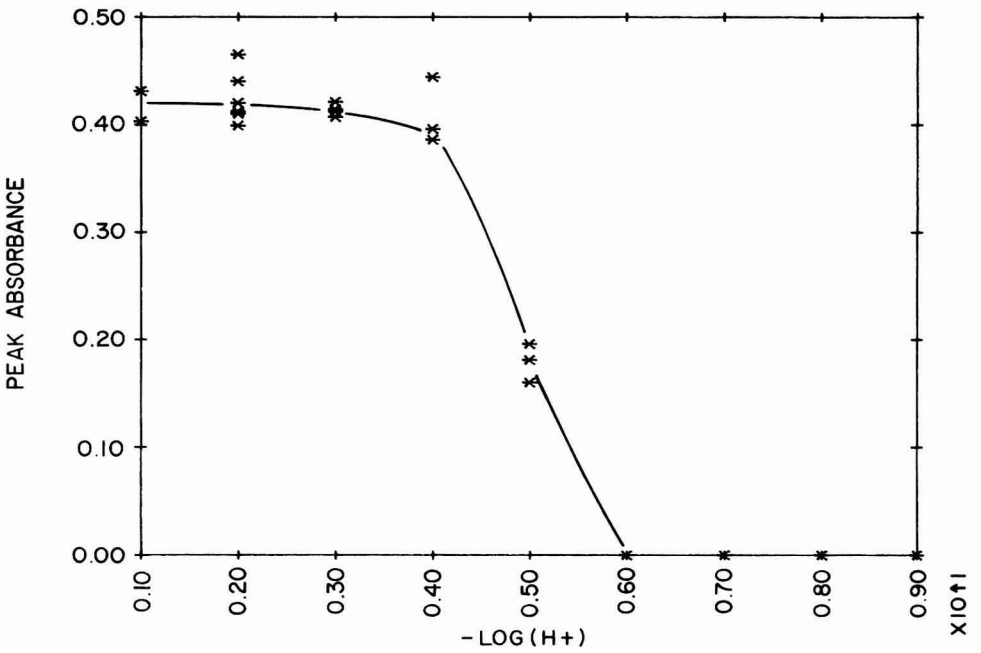

FIG. 4. Effect of pH on recovery of cobalt from the composite matrix. Extraction of the Co–APDC complex into ethyl acetate.

give good deposition characteristics on the atomizer. Ethyl acetate performed suitably and was used in all further work.

C. Analysis Procedure

In the analysis procedure as finally developed, the sample aliquot is mixed with 10 ml of 0.25 *M* potassium hydrogen phthalate and the pH adjusted to 3.00 ± 0.05. Five milliliters of a 1% (w/v) APDC solution in water are added and mixed. The aqueous phase is extracted for 2 min with 10 ml of ethyl acetate. A 5-μl aliquot of the organic phase is pipetted into the carbon rod atomizer. The sample is dried for 15 sec at a maximum temperature of 70°C, ashed for 15 sec at a maximum temperature of 250°C, and atomized for 4 sec at a maximum temperature of 2300°C.

With this procedure, a detection limit of 10 pg of cobalt was obtained when background correction was used. Typical calibration curves are shown in Fig. 5 for extractions from water solution and from the composite matrix. There is no significant difference in the working curves obtained with standards extracted from water and from any of the complex matrices.

A point of interest in comparing the calibration plot for the water matrix in direct atomization, Fig. 3, with that for the extracted standards, Fig. 5,

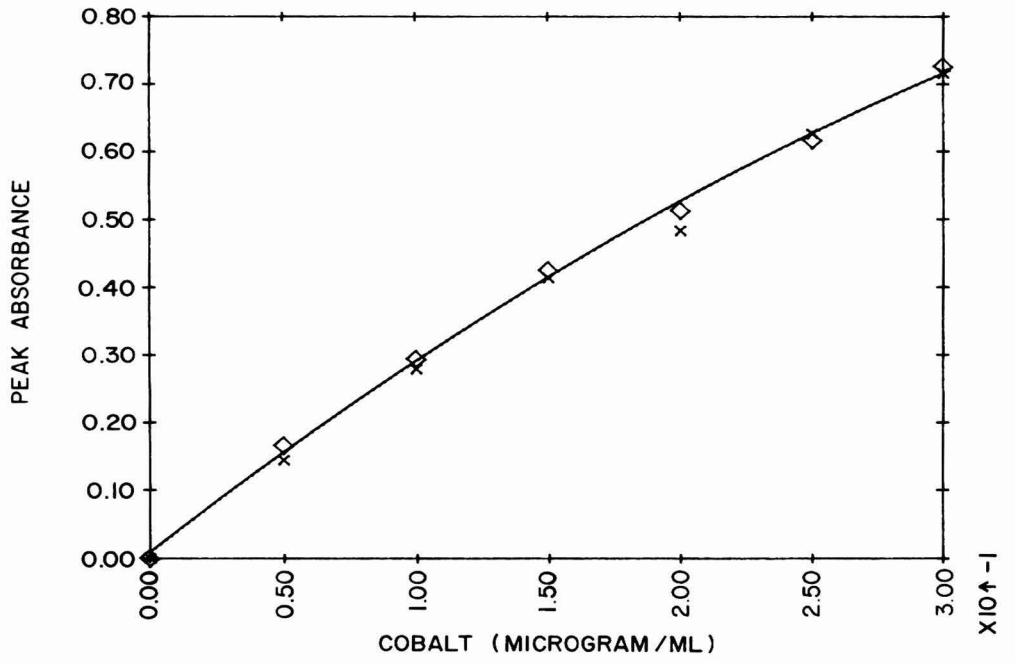

FIG. 5. Calibration data for samples extracted from the composite matrix (×) and from water (◇).

TABLE 3
APPARENT RECOVERY OF COBALT FROM EXTRACTS OF VARIOUS MATRICES

Matrix	Peak height (arbitrary units ± σ_{mean})	Recovery (% of water matrix ± σ_{mean})
Water	407.2 ± 11.7	—
AP	379.7 ± 14.1	93.2 ± 4.4
HV	374.6 ± 13.3	92.0 ± 4.2
CV	401.7 ± 6.6	98.6 ± 3.3
Composite	403.2 ± 15.2	99.0 ± 4.7
Composite + Fe, Ni, Cr	395.0 ± 10.0	97.0 ± 3.7

is the lesser curvature in the latter case. Oscilloscopic observation of the atomic absorption transient in each case revealed that under identical heating programs, the risetime for the transient from the directly atomized matrix was approximately 370 msec, while that from the extract was about 700 msec *(9)*. This would be manifested as decreased curvature of the calibration plot due to the 0.5-sec time constant of the normal readout electronics. The reasons for these differences in transient response will be discussed in detail in a future publication.

Additional evidence is given for lack of significant matrix effects in the extraction procedure in Table 3, where recoveries are shown for 0.15 μg of Co/ml in each of the matrices. Also shown in Table 3 is evidence for lack of significant matrix effect by a 100-fold excess over Co of each of the elements Fe, Ni, and Cr, all of which were present in real samples.

Finally, Table 4 shows a comparison of analyses on actual samples by the extraction procedure, by matrix matching, and by a colorimetric procedure based on formation and extraction of the cobalt–2-nitroso-1-naphthol complex. The agreement is in all cases acceptable. The extraction procedure is, however, more sensitive than the colorimetric procedure, gives somewhat better precision than flameless atomization with matrix matching, and is faster than either of the others.

TABLE 4
COMPARISON OF RESULTS BY DIFFERENT ANALYTICAL METHODS[a]

Sample no.	Extraction procedure (μg/ml)	Matrix matching (μg/ml)	Colorimetry (μg/ml)
654	0.205 ± 0.004	0.197 ± 0.013	0.21
659	0.092 ± 0.004	0.106 ± 0.014	0.08
667	0.024 ± 0.001	0.024 ± 0.001	—[b]
671	0.104 ± 0.001	0.107 ± 0.012	0.10

[a] Precision stated as ± 1σ.
[b] Below detection limit.

SUMMARY

The application of flameless atomic absorption spectrometry to determination of a little as 10 pg of Co is described. Cobalt is extracted into ethyl acetate as the pyrrolidinedithiocarbamate complex. This system is effective in removing matrix effects due to high concentrations of various complexing agents, strong oxidants, and reductants that are present in the original samples.

ACKNOWLEDGMENT

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Kinetic Potentiometric Determination of Creatinine in Urine with a Picrate-Ion-Selective Electrode

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INTRODUCTION

Numerous methods have been described for determining creatinine in biological fluids. Many of the currently used procedures are based on the color reaction which takes place between creatinine and sodium picrate in an alkaline medium (Jaffé reaction). These methods give erroneous results in the presence of proteins, ketone substances, and glucose *(6)*. Thus, methods employing preliminary separation, reaction kinetic procedures, polarographic techniques, and specific enzymes in conjunction with ammonia or enzyme electrodes have been developed to minimize the effects of other Jaffé-positive materials which are found in serum and urine *(1,3,5,6,8,)*.

Recently we reported a new picrate-selective membrane electrode and its applications to the potentiometric titration of silver and thiourea *(2,4)*. The rapid response of the picrate-selective electrode to changes in picrate concentration makes it a valuable sensor for following the rate of picrate reactions.

In this paper, a kinetic potentiometric method is described for the determination of creatinine in urine, based on the Jaffé reaction, which is monitored with a picrate-selective electrode. Under controlled conditions, a linear relation exists between the increase in electrode potential within a fixed period of time (90 sec) and the amount of creatinine present. The method is simple, accurate, and sensitive, and no sample pretreatment is required. Recovery and comparison experiments gave satisfactory results.

MATERIALS AND METHODS

Reagents

All solutions were prepared with bidistilled deionized water from reagent grade materials.

Picric acid solution, 0.020 M. Dissolve 4.58 g of picric acid (Fluka purum) in 1 liter of water. No standardization is required.

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Standard creatinine solutions. (a) Stock solution, 4.000 g/liter. Dissolve pure creatinine (Merck) dried at 100°C in water. (b) Working standards, 0.5, 1.5, 2.5, and 3.5 g/liter. Prepare from the stock solution by dilution. All creatinine standards must be stored in a refrigerator.

Ionic strength adjustment composite buffer, pH 12.6. Dissolve 257.8 g of $Na_2SO_4 \cdot 10H_20$ (Merck), 214.5 g of $Na_2HPO_4 \cdot 7H_2O$ (Merck), and 36.0 g of NaOH (Merck) in about 800 ml of water and heat to boiling. Cool at 37°C and dilute to 1 liter with water.

Apparatus

The picrate-selective electrode was constructed, used, and stored as previously described *(4)*. The outer chamber of the reference electrode was filled weekly with a 10% NaCl solution.

The reaction cell was a 50-ml double-wall glass cell thermostated at 37.0 ± 0.2°C. The temperature was checked with a thermometer in the reaction cell. The distance between the electrodes and their position in the reaction cell were rigidly maintained during all measurements. The reaction cell was emptied by suction. All measurements were taken under constant stirring with a magnetic stirring bar, at such a rate that no bubbles or vortex were formed. The electrode potential was measured and printed with an Orion Ionanalyzer (Model 801 digital pH/pIon meter) coupled to an Orion digital printer (Model 751) which was adjusted to print the potential every 30 sec. The Heath–Schlumberger recording system *(4)* was used to monitor the electrode signal and record the reaction curve. Appropriate coupling of the two units allows synchronous printing and recording of the potential. For pH measurements a Metrohm Model E-350 B pH meter with a combination glass electrode (pH range 0–14) was used. Spectrophotometric measurements were carried out with a Bausch and Lomb Spectronic 20 spectrophotometer.

Procedure

Into the thermostated reaction cell, kept at 37.0°C, pipet 25.00 ml of the composite buffer and 1.00 ml of picric acid solution. Start the stirrer, and after the potential has stabilized and the solution temperature reached 37.0°C (1–2 min), inject 1.00 ml of the standard or sample solution into the cell and *immediately* press the "start" switch of the printer. After 120 sec (five potential measurements) stop the printer, empty the cell by suction, rinse the electrodes and the cell with water, and dry by suction. Repeat the procedure for each standard or sample analysis.

Calculations

Working curves are prepared to read grams per liter of creatinine by plotting ΔE values ($\Delta E = E_{120} - E_{30}$) against the creatinine concentrations of the standards.

RESULTS AND DISCUSSION

The effect of pH on the Jaffé reaction rate was studied. Recorded curves for the reaction at various pH levels are shown in Fig. 1. It can be seen that the reaction is very slow below pH 11, whereas above pH 11 the reaction rate increases significantly with increasing pH. A pH of 12.6 was chosen as optimum to ensure small measurement times and satisfactory

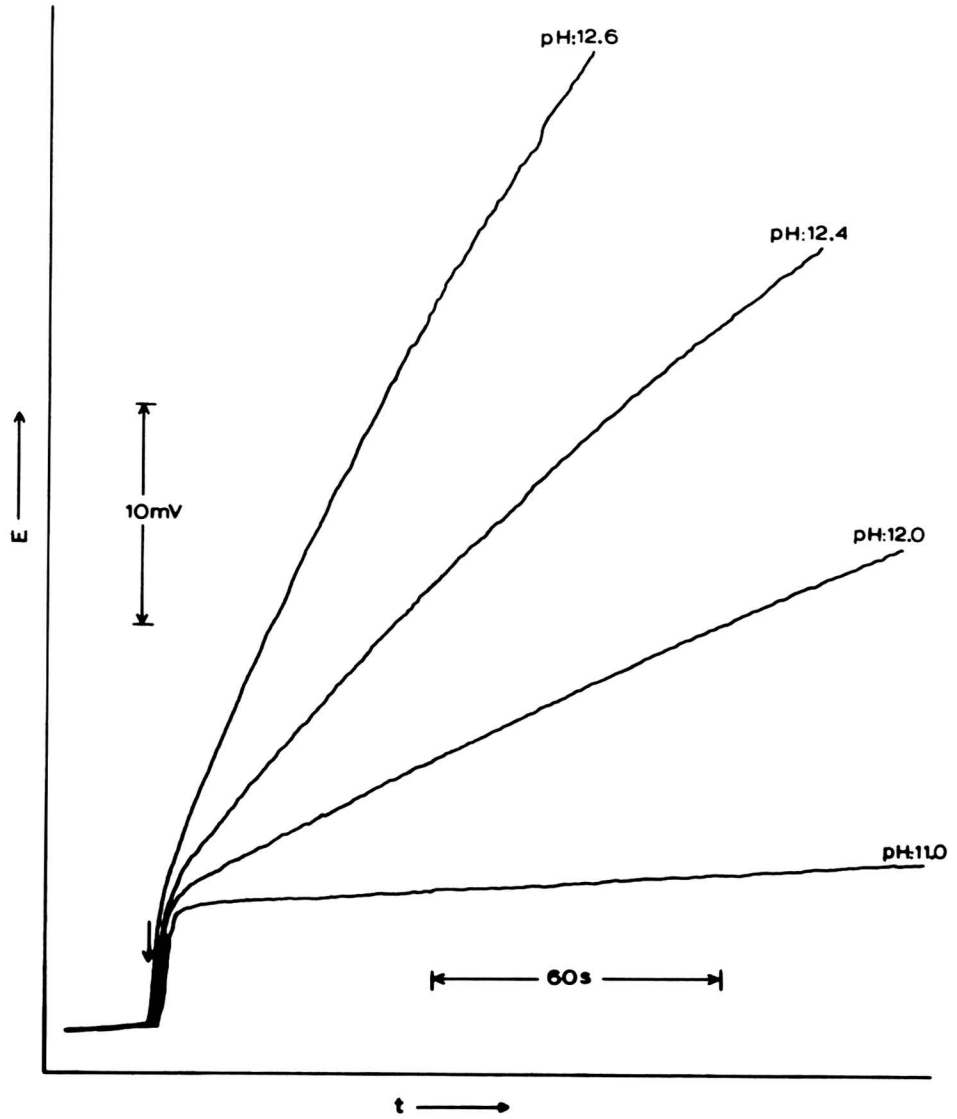

FIG. 1. Recorded curves of cell voltage vs time for the creatinine–picrate reaction at various pH levels. Creatinine concentration, 2.50 g/liter. Other conditions as under Procedure.

electrode operation in the alkaline pH range. Since the Jaffé reaction is critically pH-dependent, a buffer with sufficient buffer capacity must be used for the pH matching of standards and samples. So a final phosphate concentration of 0.74 M was chosen.

The rate of the creatinine–picrate reaction increases with increasing ionic strength.

The concentration of picrate chosen, 7.4×10^{-4} M, is a compromise to ensure a linear potential change of at least 30 mV before approaching the lower limit of the linear response of the picrate-selective electorde.

The rate of the creatinine–picrate reaction increases with increasing temperature. A temperature of 37°C was chosen as a compromise to avoid sedimentation at lower temperatures.

A premeasurement time of 30 sec was chosen to ensure that a "kinetic separation" of interfering substances present and reacting at the same time with different rates is achieved.

Figure 2 shows typical recorded curves and the corresponding working

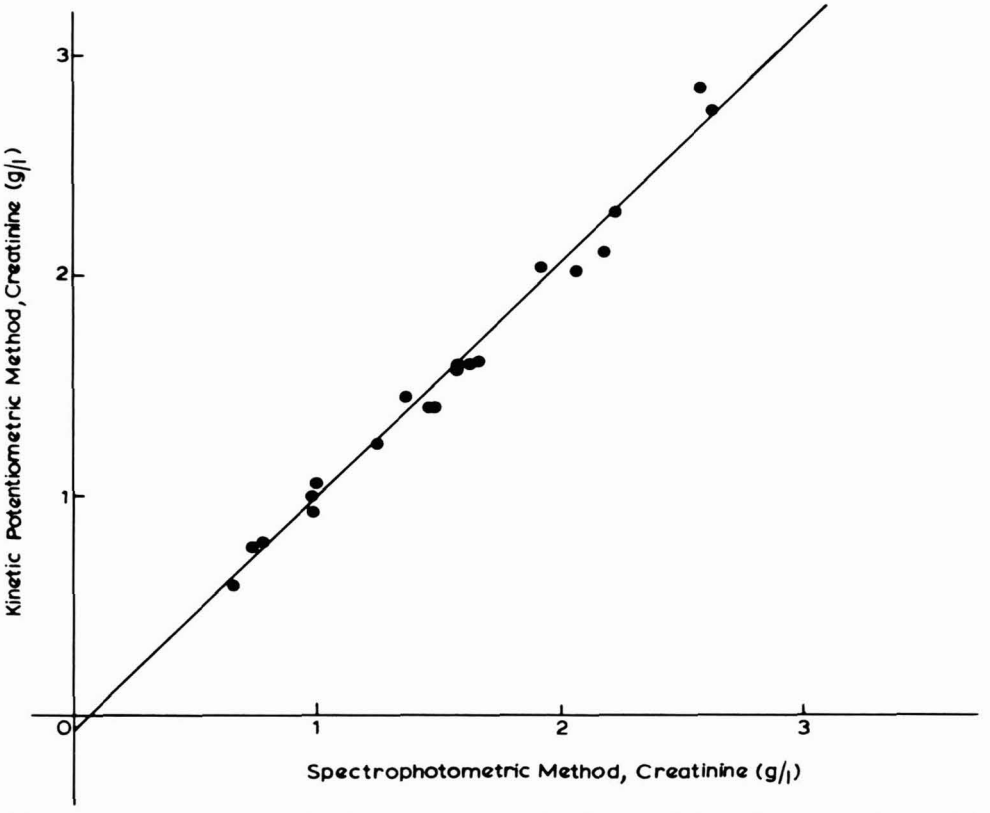

FIG. 2. Recorded curves of cell voltage vs time for the creatinine–picrate reaction and the corresponding working curve. Other conditions as under Procedure.

TABLE 1
ANALYTICAL RECOVERY OF CREATININE ADDED TO EIGHT URINE SAMPLES AND ONE URINE CONTROL SAMPLE

Creatinine (g/liter)				
Initially present[a]	Added	Total	Found[a]	Recover (%)
1.00[b]	0.75	1.75	1.75	100
	1.50	2.50	2.46	97
1.58	1.04	2.62	2.54	92
	1.90	3.48	3.29	90
1.43	1.05	2.48	2.34	87
	1.93	3.36	3.13	88
0.68	1.12	1.80	1.77	97
	2.05	2.73	2.66	97
1.17	1.08	2.25	2.21	96
	1.97	3.14	3.03	94
0.51	1.14	1.65	1.66	101
	2.08	2.59	2.59	100
0.66	1.12	1.78	1.80	102
	2.06	2.72	2.72	100
1.44	1.05	2.49	2.47	98
	1.93	3.37	3.20	91
1.27	1.07	2.34	2.29	95
	1.95	3.22	3.11	94
			Av	95.5

[a] Average of two values.
[b] Urine control sample.

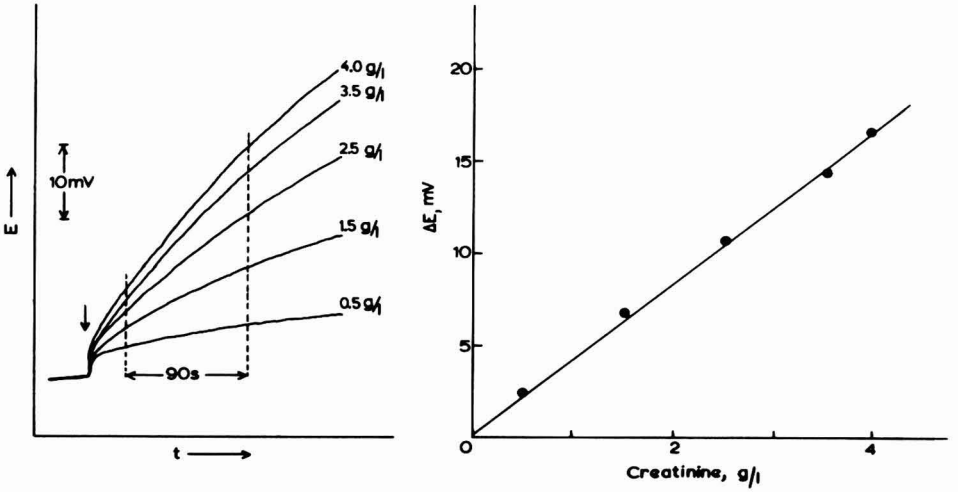

FIG. 3. Correlation between kinetic potentiometric method and spectrophotometric method for the determination of creatinine.

curve for the creatinine–picrate reaction. There is good linearity between reaction rate and creatinine concentration in the range 0.5–4 g/liter.

The response characteristics of the picrate-selective electrode were discussed elsewhere *(4)*. However, because of the extreme conditions required for the creatinine determination (high ionic strength, pH 12.6), the electrode behavior under such conditions was reexamined. With Orion perchlorate membranes (92-81-04) consisting of polycellulose acetate the slope of the working curve was 51 mV at 37°C, the lower linear response limit was 2.4×10^{-4} *M*, and the operative life of the electrode was about 15 days. When Teflon membranes (Millipore 10-μm Teflon LCWPO 1300) were used, the response characteristics of the electrode were improved. Thus, a near-Nernstian slope of 58 mV was obtained, the lower linear response limit was decreased to 1.6×10^{-5} *M*, and the operative life of the electrode was increased to about 1 month. Therefore, Teflon membranes were chosen for the construction of the picrate-selective electrode.

The accuracy of the method was checked with recovery experiments, in which creatinine was added to urine samples (normal human urine or urine control samples). The recovery of creatinine (amount added, 0.75–2.08 g/liter) ranged from 86 to 101%, with an average value of 95.5%. Typical results of analytical recovery studies are shown in Table 1.

The accuracy of the potentiometric kinetic method was further tested by comparing values of urine with those obtained with a spectrophotometric method *(9)*. Results from this comparison are shown in Fig. 3. The correlation coefficient r was 0.992, and the regression line was found to be of the form $y = 1.0676\,x - 0.0819$.

The relative standard deviation for a urine control sample containing 0.97 g/liter was 2.9% ($n=5$).

In conclusion, the proposed kinetic method is faster than the end point potentiometric method. The method permits analysis of about 20 samples per hr.

SUMMARY

A new kinetic method for the potentiometric determination of creatinine in urine based on the creatinine–picrate reaction in alkaline medium (Jaffé reaction) is described. The reaction is monitored with a picrate-selective electrode, and the increase in electrode potential within a fixed period of time (90 sec) is measured and related directly to the creatinine concentration. Analytical recovery of creatinine added to urine was 86–101% (average, 95.5%). Comparison with a spectrophotometric method gave a correlation coefficient of 0.992 over a concentration range of 0.6–2.6 g/liter.

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Spectrophotometric Determination of Traces of Hydrogen Peroxide with the Ti(IV)–Xylenol Orange and the Ti(IV)–Chromazurol S Reagents

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INTRODUCTION

Rapid and simple methods for analysis of trace amounts of hydrogen peroxide are important to fill practical needs. Colorimetric analysis due to the formation of yellow complexes with titanium (IV) and hydrogen peroxide is a well-known example of such methods *(10)*, but this method is inadequate to apply to a small quantity of hydrogen peroxide below 10^{-4} *M* in concentration. Even in other methods, such as iodometry *(12)* and chemiluminescence spectrophotometry *(1)*, some difficulties in terms of precision and selectivity still arise due to the influence of some oxidative substances.

The formation of mixed ligand complexes in ternary systems such as M^{m+}–*Y*–H_2O_2 (M^{m+} and *Y* denote metal ion and chelating reagent, respectively) was first studied by Sweeter and Bricker *(11)*. Cheng and Lott found the M^{m+}–*Y*–H_2O_2 complexes as reaction products between hydrogen peroxide and some metal chelates (M^{m+}: titanium (IV), iron (III); *Y*: ethylenediaminetetraacetic acid (EDTA), xylenol orange (XO)) *(3)*. While previous investigations on the formation of the mixed ligand complexes have been applied to the trace analysis of metal ions *(2,6,7,9,13)*, only a few papers have dealt with their application to the spectrophotometric determination of hydrogen peroxide *(4,8)*.

During the course of our investigation of the mixed ligand complexes formed in some M^{m+}–*Y*–H_2O_2 systems *(5)*, it became favorable to use the mixtures of Ti(IV)–XO and Ti(IV)–chromazurol S (CAS) as reagents for the colorimetric determination of hydrogen peroxide. The determination of hydrogen peroxide by means of the Ti(IV)–XO–H_2O_2 complex formation was attempted by Gupta *(4)*, but his method does not seem to offer a high sensitivity because of the low molar absorptivity of the absorption peak used. As to the complexes formed in the Ti(IV)–CAS–H_2O_2 system, little attention has so far been paid to them.

This paper is concerned with the formation of the mixed ligand complexes in the Ti(IV)–XO–H_2O_2 and Ti(IV)–CAS–H_2O_2 systems and the

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usefulness of the Ti(IV)–XO and Ti(IV)–CAS reagents for the colorimetric determination of traces of hydrogen peroxide.

EXPERIMENTAL

Reagents and Apparatus

Reagents. All the chemicals were reagent grade and were used without further purification.

Stock titanium(IV) solution (1.00 m*M*): 0.55 ml of titanium(IV) chloride was dissolved in 500 ml of 4 *M* hydrochloric acid; the solution was standardized by titration with EDTA. The solution was further diluted with 4 *N* hydrochloric acid to 1.00 m*M* titanium(IV) solution, which was used as a stock solution of titanium(IV).

Stock XO and CAS solutions (1.00 m*M*): Stock solutions of XO and CAS were prepared by dissolving sodium salts of XO (0.0675 g) and CAS (0.0606 g) in 100 ml of water.

Stock hydrogen peroxide solution (100 m*M*): 2.6 ml of 30% hydrogen peroxide was diluted with water to 500 ml; the solution was standardized by titration with potassium permanganate.

Ti(IV)–XO reagent: The Ti(IV)–XO reagent was prepared by mixing the stock solutions of titanium (IV) and XO in a volume ratio of 1:1.

Ti(IV)–CAS reagent: The Ti(IV)–CAS reagent was prepared by mixing the stock solutions of titanium(IV) and CAS in a volume ratio of 1:4.

Apparatus. Visible absorption spectra were recorded on a Hitachi double-beam spectrophotometer, Model 323. Absorbance at a fixed wavelength was measured on a Hitachi spectrophotometer, Model 139, in a 10-mm quartz cell. The pH of the test solution was obtained by measurement with a Toshiba–Beckmann pH meter, Model SS-2.

Procedure for Determination of Hydrogen Peroxide

To make a test solution, a known amount of hydrogen peroxide (up to 2.5×10^{-3} μg) was diluted with water to 25 ml together with 2.5 ml of the Ti(IV)–XO or 5.0 ml of the Ti(IV)–CAS reagent. The pH of the test solution was adjusted to 4.0 (in the case of XO) or to 4.9 (CAS) using acetate buffer, and the ionic strength was made 0.01 by the addition of sodium chloride. Then hydrogen peroxide in the Ti(IV)–XO–H_2O_2 and the Ti(IV)–CAS–H_2O_2 systems was determined by measuring the absorbance at 562 and 557 nm, respectively. Corrections for reagent blank were made on all measurements.

RESULTS AND DISCUSSION

Light Absorption of Ti(IV)–XO–H_2O_2 and Ti(IV)–CAS–H_2O_2 Systems

Spectrophotometric studies of the complex formation in both the Ti(IV)–XO–H_2O_2 and the Ti(IV)–CAS–H_2O_2 systems were done for the purpose of deciding the optimum conditions for the determination of hy-

drogen peroxide by using the Ti(IV)–XO and the Ti(IV)–CAS reagents.

Absorption spectra. Figure 1 gives the absorption spectra of complexes formed in the solutions involving titanium(IV), hydrogen peroxide, and XO or CAS. In the case of the Ti(IV)–XO–H_2O_2 system, the absorption spectrum exhibits an absorption maximum at 562 nm (curve a). The absorption maximum was virtually invariant irrespective of pH over the range of pH 2 to 5 and its molar absorptivity was 1.5×10^4. At pH values above 5, the absorbance at 562 nm decreased with increasing pH and a new absorption peak appeared at 520 nm at the same time. The latter peak was applied to the determination of hydrogen peroxide by the method of Gupta *(4)*, but the molar absorptivity was only half as compared with the former peak. Further, those peaks were no longer distinguishable when the ratio of $[H_2O_2]/[Ti(IV)]$ was less than 0.05. Then the use of the absorption peak at 562 nm obtained at pH 2–6 is highly recommended for the determination of hydrogen peroxide with higher sensitivity.

In the case of the Ti(IV)–CAS–H_2O_2 system also, the absorption spectrum is characterized by a sharp peak having a maximum at 557 nm at pH values above 4.5 (curve b, corrected for reagent blank), suggesting the formation of only one species as a complex involving hydrogen peroxide in this system. Contrary to this, a broad absorption band at around 590 nm was observed for the Ti(IV)–CAS reagent. This implies the presence of some Ti(IV)–CAS complexes of different compositions in the reagent itself *(7)*.

Effect of pH. As a first step in deciding the appropriate conditions to utilize the reagents for the colorimetric determination of hydrogen peroxide, the effect of pH on the absorbance was examined.

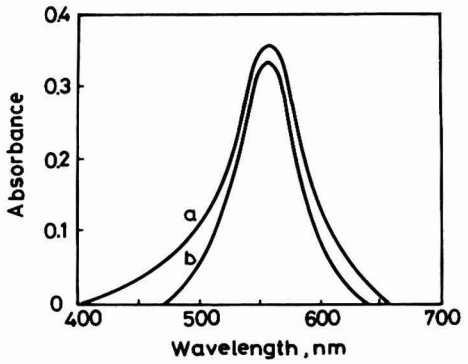

FIG. 1. Absorption spectra of Ti(IV)–XO–H_2O_2 (a) and Ti(IV)–CAS–H_2O_2 (b) complexes vs reagent blanks. Conditions are as listed below.

	[Ti(IV)] (m*M*)	[XO] or [CAS] (m*M*)	[H_2O_2] (m*M*)	pH
a	0.050	0.050	0.022	4.0
b	0.040	0.160	0.022	4.9

As shown in Fig. 2, constant absorbance was obtained in the pH ranges 2 to 5 and 4.5 to 5.2 for the Ti(IV)–XO–H_2O_2 and the Ti(IV)–CAS–H_2O_2 systems, respectively. In the latter system, the absorption peak gradually shifted to the shorter wavelengths with the lowering of absorbance at pH values below 4.5. Then pH values of 4.0 and 4.9 are employed as optimum conditions for the determination of hydrogen peroxide using the Ti(IV)–XO and the Ti(IV)–CAS reagents, respectively (cf. Table 1).

Effect of composition of the reagents. The absorption maxima of Ti(IV)–XO–H_2O_2 and Ti(IV)–CAS–H_2O_2 complexes were found to the affected by the concentration ratio of XO or CAS to titanium(IV). Then the effect of XO or CAS concentration in the reagents on the absorbance of the complexes was examined keeping the concentrations of both titanium (IV) (0.05 or 0.025 m*M*) and hydrogen peroxide (0.02 m*M*) constant.

The compositions of the complexes have already been proved by Otomo *(9)*: Only a 1:1 complex is formed in the solution containing titanium (IV) and XO at pH 3–4.5, and only a 1:1:1 complex in the solution containing titanium (IV), XO, and hydrogen peroxide at ca. pH 2. Actually in the present experiment, the absorbance at 562 nm obtained for the Ti(IV)–XO–H_2O_2 system was found to remain constant irrespective of the XO concentration when [XO]/[Ti(IV)] $\geq$ 1 (Fig. 3, curve a), so that the presence of excess XO seems to be favorable for the determination of hydrogen peroxide. However, the addition of XO in a large excess will resulting in a lowering of the precision of the absorption data because of the higher value of the reagent blank. Then the [XO]/[Ti(IV)] ratio is fixed to be unity in the Ti(IV)–XO reagent.

The maximum value of the absorbance of the Ti(IV)–CAS–H_2O_2 complex was obtained at 557 nm using the Ti(IV)–CAS reagent in the range of

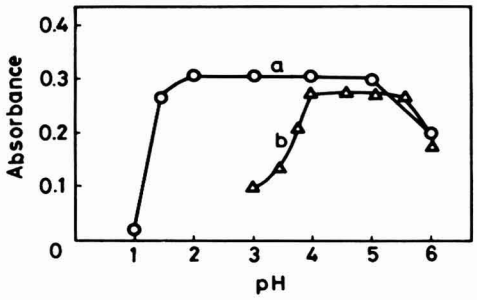

FIG. 2. Effect of pH on absorbance of Ti(IV)–XO–H_2O_2 (a) and Ti(IV)–CAS–H_2O_2 (b) complexes. Conditions are as listed below.

	[Ti(IV)] (m*M*)	[XO] or [CAS] (m*M*)	[H_2O_2] (m*M*)	λ (nm)
a	0.050	0.050	0.02	562
b	0.040	0.160	0.02	557

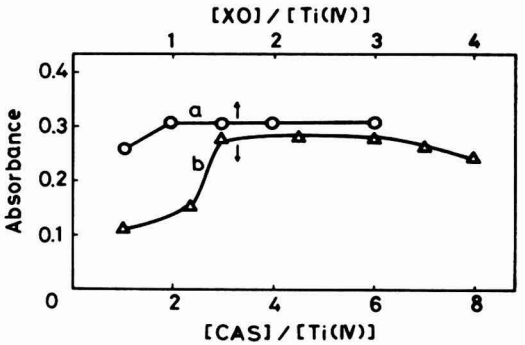

FIG. 3. Effect of concentration of XO or CAS on absorbance of Ti(IV)–XO–H_2O_2 (a) and Ti(IV)–CAS–H_2O_2 (b) complexes. Conditions are as listed below.

	[Ti(IV)] (m*M*)	[H_2O_2] (m*M*)	pH	λ (nm)
a	0.050	0.020	4.0	562
b	0.025	0.020	4.9	557

[CAS]/[Ti(IV)] ratios of 3 to 6 (Fig. 3, curve b). Nishida proposed the composition of the complex which is responsible for the absorption at 557 nm as Ti(IV):CAS:H_2O_2 = 1:2:1 *(7)*. The changes in the absorption peak were observed under the conditions in which the [CAS]/[Ti(IV)] ratio was greater than 6 or less than 3, probably because of the formation of other complexes. From such facts it will be noted that the [CAS] [Ti(IV)] ratio in the Ti(IV)–CAS reagent must be kept within the range of 3–6.

Time course of the absorbance. The constant values of absorbance for both Ti(IV)–XO–H_2O_2 and Ti(IV)–CAS–H_2O_2 complexes were obtained within a few minutes after adding the reagents, and the absorbance remained unchanged over 24 hr. No appreciable changes in the absorbance were observed for both the reagents on standing at room temperature for a month, so no special precaution is needed upon storage of the reagents for a long time.

Determination of Hydrogen Peroxide

Based on the experimental results, the conditions for the colorimetric determination of hydrogen peroxide with both the reagents were decided upon and are listed in Table 1.

Plots of the absorbance against the hydrogen peroxide concentration give straight lines as shown in Fig. 4, and those reagents are found to be applicable to the determination of hydrogen peroxide in the following concentration ranges: the Ti(IV)–XO reagent, 3×10^{-6}–1×10^{-4} *M* H_2O_2; the Ti(IV)–CAS reagent, 2×10^{-6}–5×10^{-5} *M* H_2O_2. The data are reliable with a coefficient of variation below 3%. In order to determine hydrogen peroxide, a colorimetric method based the formation of Ti(IV)–H_2O_2

TABLE 1
OPTIMUM CONDITIONS FOR THE DETERMINATION OF H_2O_2 USING Ti(IV)–XO AND Ti(IV)–CAS REAGENTS

Reagent	Optimum conditions			$[H_2O_2]$ (M)
	Concentration (mM)	pH	λ (nm)	
Ti(IV) – XO	[Ti(IV)] = 0.01 – 0.1 [XO]/[Ti(IV)] = 1	4.0	562	$3 \times 10^{-6} - 1 \times 10^{-4}$
Ti(IV) – CAS	[Ti(IV)] = 0.05 – 0.1 [CAS]/[Ti(IV)] = 4	4.9	557	$2 \times 10^{-6} - 5 \times 10^{-5}$

complexes has been widely used. However, the advantage of Ti(IV)–XO–H_2O_2 or Ti(IV)–CAS–H_2O_2 complexes over that of Ti(IV)–H_2O_2 complexes is their greater molar absorptivities: the molar absorptivities of Ti(IV)–XO–H_2O_2 and Ti(IV)–CAS–H_2O_2 complexes are 1.5×10^4 and 1.48×10^4 M^{-1} cm^{-1}, respectively; both are much greater than the absorptivities of Ti(IV)–H_2O_2 complexes.

From all the results stated above, the utility of the Ti(IV)–XO and the Ti(IV)–CAS reagents in the determination of trace amounts of hydrogen peroxide, for example, in the practical analysis of hydrogen peroxide in foods or biological samples such as animal serums and plasmas, can be expected. Thus further experiments were performed to examine the effect of some foreign substances which are liable to cause interference in the practical analysis on the determination of hydrogen peroxide with those reagents. The results are given in Tables 2 and 3.

As seen in Table 2, some ions such as Na^+, K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Cl^-, NO_3^-, and SO_4^{2-} scarcely affected the absorption measurements even

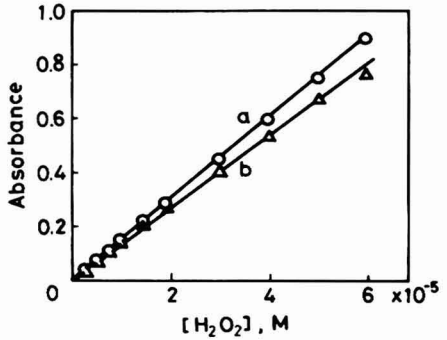

FIG. 4. Relation between concentration of H_2O_2 and absorbance of mixed ligand complexes formed through the reactions with Ti(IV)–XO (a) and Ti(IV)–CAS (b) reagents. Wavelengths: 562 (a) and 557 (b) nm.

TABLE 2
EFFECT OF INORGANIC IONS ON THE DETERMINATION OF H_2O_2[a]

Inorganic ion	Concentration (M)	$[H_2O_2]_{found} \times 10^{-5}$ M (error, %)	
		Ti(IV) – XO	Ti(IV) – CAS
Na^+	1.0×10^{-3}	1.00 (0)	1.00 (0)
K^+	1.0×10^{-3}	1.00 (0)	1.00 (0)
Mg^{2+}	1.0×10^{-5}	1.00 (0)	1.00 (0)
	1.0×10^{-3}	1.03 (+ 3)	1.02 (2)
Ca^{2+}	1.0×10^{-5}	1.00 (0)	1.00 (0)
	1.0×10^{-3}	1.03 (+ 3)	1.00 (0)
Mn^{2+}	1.0×10^{-5}	0.98 (– 2)	1.00 (0)
	1.0×10^{-3}	0.98 (– 2)	0.99 (– 1)
Ni^{2+}	1.0×10^{-3}	0.70 (– 30)	0.66 (– 34)
Zn^{2+}	1.0×10^{-5}	1.10 (+ 10)	0.80 (– 20)
	1.0×10^{-3}	1.22 (+ 22)	1.50 (+ 50)
Al^{3+}	1.0×10^{-5}	2.50 (+150)	2.10 (+110)
	1.0×10^{-3}		4.00 (+300)
Fe^{3+}	1.0×10^{-5}	2.14 (+114)	2.15 (+115)
Cl^-	1.0×10^{-3}	1.00 (0)	1.00 (0)
NO_3^-	1.0×10^{-3}	1.00 (0)	1.00 (0)
SO_4^{2-}	1.0×10^{-3}	0.98 (– 2)	1.00 (0)
PO_4^{3-}	1.0×10^{-5}	0.96 (– 4)	0.95 (– 5)
	1.0×10^{-3}	0.63 (– 37)	0.20 (– 80)

[a] $[H_2O_2]_{added} = 1.00 \times 10^{-5}$ M.

TABLE 3
EFFECT OF ORGANIC COMPOUNDS ON THE DETERMINATION OF H_2O_2[a]

Organic compound	Concentration (M)	$[H_2O_2]_{found} \times 10^{-5}$ M (error, %)	
		Ti(IV) – XO	Ti(IV) – CAS
Acetic acid	1.0×10^{-3}	1.00 (0)	1.00 (0)
Ascorbic acid	1.0×10^{-3}	1.00 (0)	0.98 (– 2)
Citric acid	1.0×10^{-5}	0.95 (– 5)	0.32 (–68)
Cysteine	1.0×10^{-5}	0.88 (–12)	0.85 (–15)
Glycine	1.0×10^{-4}	0.98 (– 2)	1.00 (0)
Glycolic acid	1.0×10^{-4}	1.03 (+ 3)	1.00 (0)
Glyoxylic acid	1.0×10^{-4}	1.02 (+ 2)	1.02 (+ 2)
Isonicotinic acid	1.0×10^{-5}	1.00 (0)	1.00 (0)
Nicotinamide	1.0×10^{-5}	1.00 (0)	1.03 (+ 3)
Pyruvic acid	1.0×10^{-3}	1.00 (0)	1.00 (0)
Succinic acid	1.0×10^{-5}	1.05 (+ 5)	1.05 (+ 5)
Tartaric acid	1.0×10^{-5}	0.09 (–10)	0.42 (–58)
Thiourea	1.0×10^{-3}	1.05 (+ 5)	1.00 (0)
Urea	1.0×10^{-4}	1.00 (0)	1.00 (0)

[a] $[H_2O_2]_{added} = 1.00 \times 10^{-5}$ M.

when present in large amounts compared with hydrogen peroxide. On the other hand, the presence of Al^{3+} and Fe^{3+} led to serious errors due to complex formation between those metal ions and the reagents, i.e., due to overlapping of the absorption bands of titanium(IV)-and those metal complexes.

Among the organic compounds listed in Table 3, citric acid, tartaric acid, and cysteine gave rise to fairly large negative errors, even when present in small quantities. Increasing the amount of those compounds resulted in the disappearance of the original colors of the complexes between the reagents and hydrogen peroxide, suggesting ligand substitution by such compounds.

The usefulness of the Ti(IV)–XO and the Ti(IV)–CAS reagents for the colorimetric determination of traces of hydrogen peroxide is thus proved. This method is straightforward and simple to use, and gives good results unless some particular substances are contained in sample solutions.

SUMMARY

The formation of mixed ligand complexes in Ti(IV)–xylenol orange (XO)–H_2O_2 and Ti(IV)-chromazurol S (CAS)–H_2O_2 systems was studied by spectrophotometry. The former system gave constant absorbance (λ_{max} = 562 nm) under the condition of [XO]/[Ti(IV)] = 1 in the pH 2–4 region. In the latter system, a distinct maximum at 557 nm was observed when [CAS]/[Ti(IV)] = 4 in the pH range of 4.5–5.2. In both cases, the absorbance at λ_{max} was stable for a long time and proportional to the concentration of hydrogen peroxide. From those facts, the usefulness of the mixtures of Ti(IV)–XO and Ti(IV)–CAS as the colorimetric reagents for the determination of hydrogen peroxide can be expected. The conditions for the use of the Ti(IV)–XO and the Ti(IV)–CAS reagents were examined in detail, and both reagents were found to be available for trace analysis of hydrogen peroxide with high sensitivity.

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The Automated Analysis of Catecholamines: An Improved Procedure for the Simultaneous Differential Analysis of Epinephrine and Norepinephrine in Tissues, Blood and Gland Perfusates[1]

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INTRODUCTION

The fluorometric methods in common use today for the differential determination of epinephrine and norepinephrine are modifications of the trihydroxyindole procedure introduced by Ehrlén *(5)* and first successfully applied to biological samples by Lund *(8, 9)*. The catecholamines in slightly acid medium were oxidized by potassium ferricyanide or iodine to the corresponding chromogens and then tautomerized to the fluorescent lutines by strong alkali. In order to stabilize the fluorescent products, an antioxidant such as ascorbic acid was added to the alkali. Improvements in the ability to determine both catecholamines in the same sample were introduced by von Euler and Floding *(19)* and Cohen and Goldenberg *(4)*. The differential determination of epinephrine and norepinephrine in mixtures was achieved by taking advantage of differences in the rate of oxidation of the two amines when oxidation was carried out at two pHs or in their excitation–emission characteristics when two sets of filters were used.

Many modifications of these earlier procedures have been forthcoming, primarily because of certain inherent difficulties with the trihydroxyindole (THI) procedure, such as instability of the fluorescence products and of the alkaline–stabilizer mixture. The THI procedure, therefore, requires that certain steps be timed quite precisely *(6, 11)*. An important advance in the analysis of catecholamines was the development of the automated method and the introduction of the selective stabilization of norepinephrine fluorescence with thioglycolic acid by Merrills *(13, 14)*. Following the adaptation of the automated THI method for the differential analysis of catecholamines from tissues and plasma as well as urine by Robinson

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and Watts *(17)*, several modifications have appeared *(2, 10, 12, 15, 22)*. Major changes made in the automated procedure include the elimination of dialysis to increase sensitivity and in-line pH adjustment.

It has been the practice in this laboratory to test each new modification that appeared and to utilize those features that improve the method. The original method of Robinson and Watts *(17)* was modified only slightly until the excellent study of Andersson *et al. (2)*. Using this method as a starting point, a manifold has been developed in which the in-line separation of a sample into two equal parts makes possible the simultaneous analysis of both amines using two fluorometers and recorders. Furthermore, in order to achieve a more reliable differentiation of the catecholamines, the method has been modified so that the fluorescence of each amine can be developed with a minimal contamination from the other. This was accomplished by the use of two filter systems and oxidation at quite different pHs.

It is the purpose of this communication to describe these modifications in detail and their use in analyzing catecholamines derived from tissues, plasma, and adrenal perfusates.

MATERIALS AND METHODS

Various steps in the procedure were tested on biological material primarily adrenal venous blood.

Apparatus

Technicon AutoAnalyzer equipment was used. A fluorometer I was modified to increase its sensitivity by replacing the standard mercury lamp with a Turner blue lamp (No. 110-853) and substituting the more sensitive 931 B for the 931 A photomultiplier tube. The fluorometer I with the Turner No. 47-B narrow pass filter, which peaks at 436 nm, as the primary filter and the Turner No. 2A-12 sharp cut filter, which passes all wavelengths longer than 510 nm, as the secondary filter was used to measure epinephrine fluorescence. A fluorometer II was used to measure norepinephrine fluorescence. The primary filter was a Baird Atomic, Inc., Type B3A interference filter which peaks at 400 nm. The secondary filter was a Wratten No. 8 sharp cut filter, which passes all wavelengths above 485 nm. Additional equipment included a proportioning pump II, a sampler II, and two recorders. The sampler was operated at 30 samples per hour using a 30/hr, 1:1 timing cam.

Reagents

Glass-distilled water was used throughout. All chemicals used were of the highest grade available and required no further purification.

I. For chromatography.

1. Aluminum oxide (British Drug House, Brockman activity II for

chromatographic adsorption analysis). The aluminum oxide was used without acid washing.

2. Sodium metabisulfite, 10 mg/ml.
3. Phosphate buffer, 0.1 *M*, pH 6.5.
4. Ethylenediaminetetraacetic acid disodium salt (EDTA).
5. Acetic acid, 0.25 *N*.

II. For chemical analysis.

1. Phosphate buffer containing citrate, pH 7.4 (0.5 *M* KH_2PO_4 + 10 m*M* citric acid and 0.5 *M* K_2PO_4 + 10 m*M* citric acid). The buffer was adjusted to pH 7.4 by mixing these solutions, using a pH meter. Citric acid was added to prevent the precipitation of aluminum hydroxide in the manifold. Brij 35 (0.75 ml/liter) and toluene (0.25 ml/liter) were added to the buffer. Brij 35 was added as a wetting agent and toluene was added to retard the growth of bacteria in the buffer.

2. Acetate buffer containing citrate and zinc sulfate, pH 3.5 (0.05 *M* acetic acid + 10 m*M* citric acid and 0.05 *M* sodium acetate + 10 m*M* citric acid). The buffer was adjusted to pH 3.5 by mixing these solutions. To each liter of the buffer 15 mg of zinc sulfate ($ZnSO_4 \cdot 7H_2O$) was added. Zinc sulfate markedly increased the fluorescence due to epinephrine without increasing that due to norepinephrine.

3. Acetic acid, 0.25 *N*.

4. Potassium ferricyanide (0.1%, w/v) and (0.5%, w/v). To each solution Brij 35 (0.75 ml/liter) was added.

5. Sodium hydroxide, 4 *N*.

6. Sodium hydroxide, 2.5 *N*, containing 0.05 *M* formaldehyde.

7. Ascorbic acid (0.1%, w/v), from Eastman. The solution was prepared fresh each week and stored in a refrigerator.

8. Thioglycolic acid (mercaptoacetic acid, 4%) from Sigma. The solution was prepared fresh each week and stored in a refrigerator.

9. Standard solutions: Epinephrine stock solution, 100 μg of free base/ml, was prepared by dissolving 18.2 mg of *l*-epinephrine bitartrate (Winthrop) in 100 ml 0.01 *N* HCl. Norepinephrine stock solution, 100 μg of free base/ml, was prepared by dissolving 19.9 mg of *l*-norepinephrine bitartrate monohydrate (Winthrop) in 100 ml of 0.01 *N* HCl. The stock solutions were stable for 1 year if stored in a refrigerator. Working standard solutions (50 ng/ml) were prepared by diluting 10 ml of the stock solutions to 100 ml with 0.01 *N* HCl. The final standard solutions were made by taking 1 ml of the above solutions and diluting them to 200 ml with 0.25 *N* acetic acid. The working standard solutions were stable for at least 6 months if stored in a refrigerator.

Preparation of Samples

Tissues. The excised tissue was freed of fat and connective tissue,

blotted dry on filter paper and weighed. The tissue was homogenized in 10 ml of cold 0.4 *N* perchloric acid in a glass Ten Broeck tissue grinder submerged in an ice bath. The homogenate was transferred to a 50-ml polyethylene centrifuge tube containing 10 mg of metabisulfite and centrifuged at 10,000*g* at 4°C for 10 min. The homogenate was frozen until adsorption on aluminum oxide was carried out. After thawing, the clear supernatant was transferred to a 50-ml beaker containing 200 mg of EDTA, 1 ml of phosphate buffer, pH 6.5, and 500 mg of aluminum oxide which had been suspended in 20 ml of glass-distilled water three or four times, decanting each wash to remove fine particles. The total volume of fluid in the beaker should not exceed 30 ml. The mixture was adjusted to a pH between 8.3 and 8.5 with 1 *N* NaOH using a pH meter. The pH adjustment was carried out with vigorous stirring with a motor-driven glass stirring rod; a magnetic stirring bar should not be used since this results in grinding the aluminum oxide to a fine powder. The pH was maintained at 8.3 to 8.5 for 5 min after which the entire contents of the beaker were transferred to a 50-ml polyethylene tube and centrifuged in a clinical centrifuge at low speed for 4 min. During this period, the pH adjustment of the next sample was begun. A number of samples could be processed almost simultaneously. Following centrifugation the supernatant was removed using a water aspirator. The precipitated aluminum oxide was transferred to a 15-ml glass tube with glass-distilled water. The tube was capped and mixed on a rotary mixer for 2 min. The uncapped tube was then centrifuged for 2 min in a clinical centrifuge at low speed to hasten settling of the aluminum oxide, and the supernatant was aspirated. Washing of the aluminum oxide was repeated a minimum of four times. Following the fourth wash, the catecholamines were eluted by rotation of the capped tube for 15 min with 5 ml of 0.25 *N* acetic acid on a rotary mixer. The aluminum oxide was allowed to settle and the supernatant was transferred to a heavy-walled glass centrifuge tube. A second elution was carried out with an additional 5 ml of 0.25 *N* acetic acid, and the supernatant was added to the first eluate. Although double elution with a total of 10 ml of acetic acid was chosen as the standard procedure for tissue preparation, a single elution with 5 ml of acid gave good results.

The eluate was centrifuged at 30,000*g* at 4°C for 10 min to remove a faint haze due to fine particles of aluminum oxide *(3)*. This step was essential in order to eliminate a high blank reading. The sample was frozen until the analysis was performed.

Blood. Heparinized blood was collected in a centrifuge tube immersed in an ice bath. The sample was centrifuged as soon as possible in a clinical centrifuge at 2500*g* at 4°C for 20 min. The plasma was removed with a Pasteur pipet and 1 ml of sodium metabisulfite (10 mg) was added. The plasma was frozen until adsorption on aluminum oxide was carried out.

The thawed plasma was diluted to 20 ml with glass-distilled water and adsorption on aluminum oxide was carried out as described for tissues. The catecholamines were eluted with 5 ml of 0.25 *N* acetic acid. The eluate was frozen and stored until analysis could be carried out.

Simultaneous Differential Automated Analysis of Epinephrine and Norepinephrine with the AutoAnalyzer

The flow diagram of the AutoAnalyzer system used for the simultaneous fluorometric determination of epinephrine and norepinephrine is shown in Fig. 1. The epinephrine and norepinephrine manifolds described by Andersson *et al. (2)* have been combined so that the sample in 0.25 *N* acetic acid was split into two equal parts. One-half of the sample was pumped through the epinephrine manifold with ascorbic acid stabilization. Oxidation was carried out at a pH of 3.5, using an acetate buffer containing zinc sulfate to catalyze the oxidation of epinephrine. Under these conditions only the fluorescence due to adrenolutine developed. The other half of the sample was pumped through the norepinephrine manifold with thioglycolic acid stabilization. Oxidation was carried out at pH 7.4 using phosphate buffer. Under these conditions the formation of noradrenolutine fluorescence was accompanied by a small fluorescence due to adrenolutine formation amounting to no more than 4% of that due to noradrenolutine.

Solutions of 0.25 *N* acetic acid containing 50 ng/ml of epinephrine or norepinephrine were chosen as standards for adjusting the sensitivity of the fluorometers. No more than 10 samples were analyzed without rerunning both standards. A slow decrease in sensitivity amounting to 1 to 2% per hour occurred generally. If the catecholamine content of a sample was greater than the sensitivity of the system, a 1-ml aliquot of the sample was diluted with 0.25 *N* acetic acid and the sample was rerun.

Blanks

A faded (oxidation) epinephrine blank was obtained by replacing ascorbic acid with water and rerunning all samples after a washout period of 20 min to remove ascorbic acid from the system. The norepinephrine blank was obtained by multiplying the epinephrine blank by a correlation factor. The correlation factor was constant as long as the filters, apertures, and amplification settings on the fluorometer were unchanged *(2)*. The correlation factor under the conditions described was 0.5.

Calculations

The epinephrine concentration in the eluate was determined directly from the fluorescence reading obtained with the epinephrine manifold using the equation

$$(\text{Epi}) = (A/E_a) \text{ ng/ml eluate},$$

and norepinephrine was determined from the difference between the

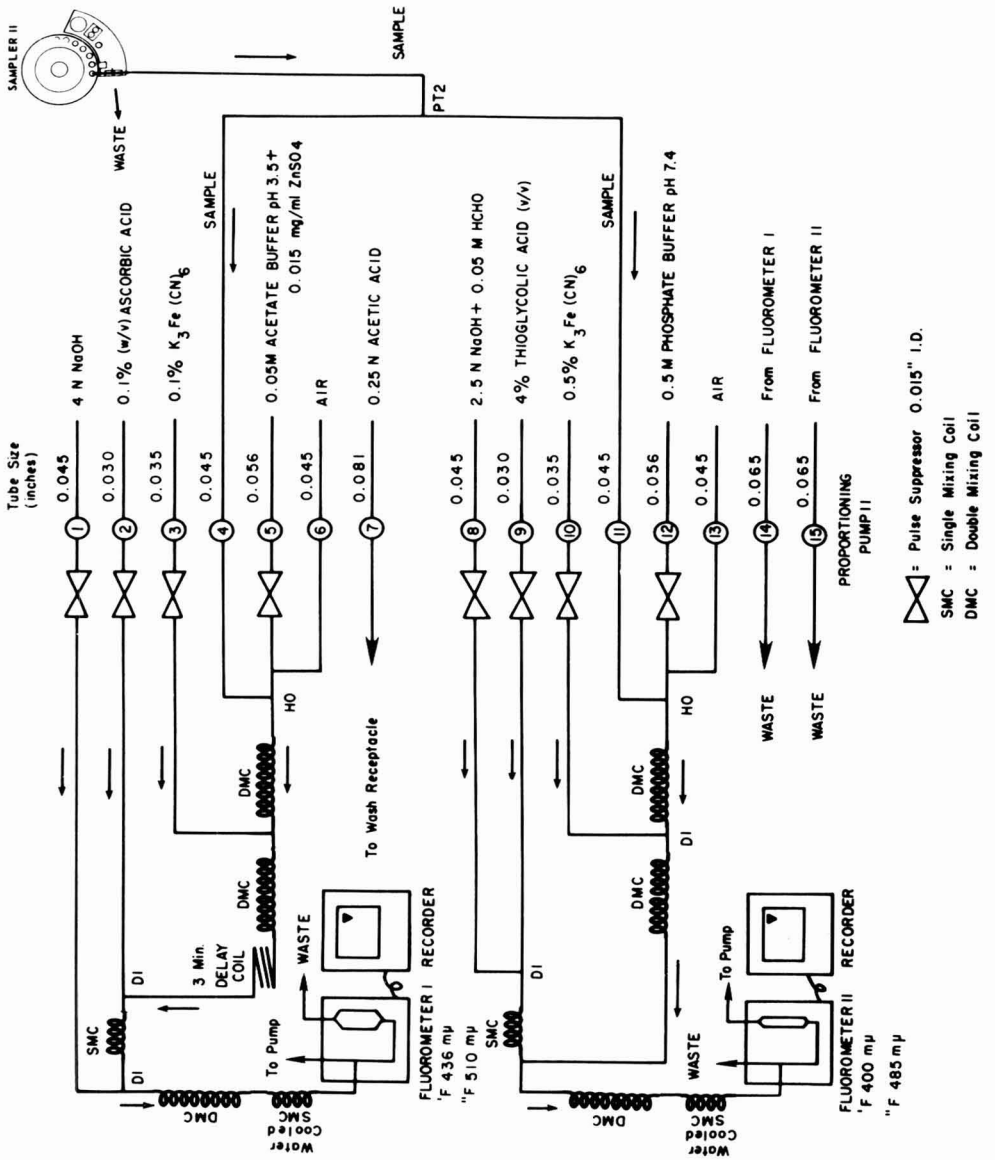

FIG. 1. Flow diagram of automated system for the simultaneous differential analysis of epinephrine and norepinephrine, showing tube sizes (inches) and reagent concentrations.

fluorescent reading obtained with the norepinephrine manifold and the epinephrine concentration using the equation

$$(\text{Nor}) = (B - (\text{Epi})E_t)/N_t) \text{ ng/ml eluate},$$

where A was the fluorescent reading of the eluate obtained with the epinephrine manifold (ascorbic acid stabilization at pH 3.5), corrected for the baseline and blank; B was the fluorescent reading of the eluate ob-

tained with the norepinephrine manifold (thioglycolic acid stabilization at pH 7.4), corrected for the baseline and blank; (Epi) was the unknown concentration of epinephrine in the eluate; (Nor) was the unknown concentration of norepinephrine in the eluate; E_a was the fluorescent reading of the epinephrine standard when the stabilizing agent was ascorbic acid, corrected for the baseline, divided by the concentration of the standard (nanograms per milliliter); E_t was the fluorescent reading of the epinephrine standard when the stabilizing agent was thioglycolic acid, corrected for the baseline, divided by the concentration of the standard (nanograms per milliliter); N_t was the fluorescent reading of the norepinephrine standard when the stabilizing agent was thioglycolic acid, corrected for the baseline, divided by the concentration of the standard (nanograms per milliliter).

Since there was a minimum epinephrine fluorescence with thioglycolic acid stabilization, it was feasible to determine the norepinephrine concentration directly from the fluorescent reading obtained with the norepinephrine manifold when analyzing tissues which contain little or no epinephrine, such as the heart or vas deferens. The equation used was

$$(\text{Nor}) = B/N_t \text{ ng/ml eluate.}$$

The total epinephrine and norepinephrine content of the sample was determined by multiplying the values calculated above by the volume of the eluate and any dilution made.

Adaptation of the AutoAnalyzer for the In-Line Analysis of Catecholamines in the Effluent from the Perfused Adrenal

The isolated perfused adrenal is frequently used to study the secretory process in the adrenal medulla *(16)*. The method described can be adapted for the in-line differential analysis of catecholamines in the gland effluent. Dialysis of the effluent was required in order to eliminate macromolecules released from the gland. Dialysis was added to the system without altering the basic manifold. The flow diagram of the perfusion and dialysis module is shown in Fig. 2. One set of dialysis plates with a type-C membrane was used. The effluent from the gland, 0.25 *N* acetic acid containing Brij 35, and air were pumped separately, mixed, and delivered to the upper surface of the dialysis membrane. Acetic acid (0.25 *N*) containing Brij 35 and air were pumped to the lower membrane. The catecholamines which passed through the membranes into the recipient stream were directed into the manifold after air and excess fluid were removed by a debubbler and vent system. When a differential analysis of catecholamines in the effluent from perfused adrenals was carried out, the sensitivity of the fluorometers was adjusted using epinephrine and norepinephrine standards in the range of 200 to 500 ng/ml.

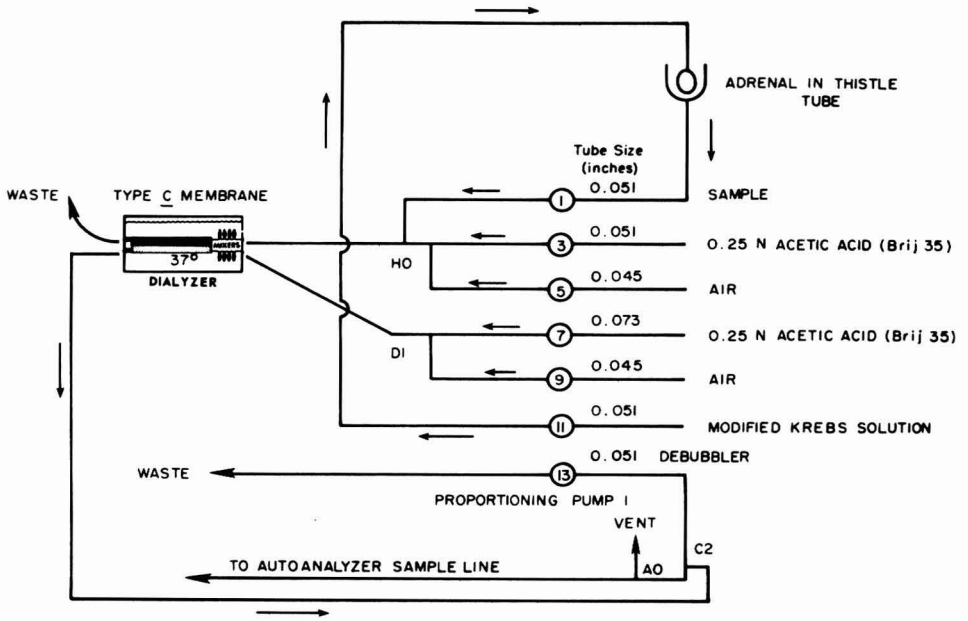

FIG. 2. Flow diagram of isolated adrenal gland perfusion module, showing tube sizes (inches).

RESULTS AND DISCUSSION

The Manifolds

The formation of adrenochrome in the epinephrine manifold at pH 3.5 in the presence of zinc sulfate was found to proceed at a slower rate than when oxidation was carried out at a pH near neutrality (Fig. 3). In order to obtain optimal oxidation of epinephrine, a 3-min-delay coil was inserted in the oxidation stream of the epinephrine manifold. Since the run-through time of the norepinephrine manifold was 3 min shorter than that of the epinephrine manifold, the use of a two-pen recorder was not feasible.

Water cooling of the last mixing coil before entry of the reaction stream into the fluorometers significantly increased the fluorescent reading of both epinephrine and norepinephrine *(2,12)*. The temperature of the water bath was maintained at 10–12°C with water pumped from an ice bath. Care was required to prevent greater fluctuations in the temperature. If the temperature was lowered below 10°C, condensation of water on the cuvettes occurred.

Chromatography

The low-speed centrifugation of the aluminum oxide (British Drug House) during the washing steps following adsorption of the catecholamines improved their recoverability. The high-speed centrifuga-

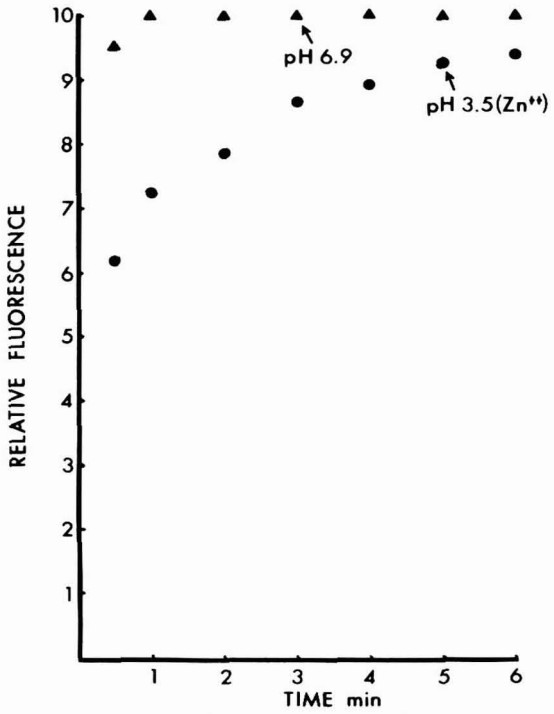

FIG. 3. Effect of oxidation time on fluorescence of epinephrine at pH 6.9 and at pH 3.5 in the presence of zinc sulfate.

tion of the eluate virtually eliminated blank readings. British Drug House aluminum oxide has given consistent results without the inconvenience of acid washing.

Blanks

Andersson *et al. (2)* recommended that faded (oxidation) blanks should be obtained before unknown samples were analyzed by running glass-distilled water in place of the stabilizing agents, ascorbic acid and thioglycolic acid. This was often impractical, however, since the unknown samples may contain quite different amounts of catecholamines, some of which may exceed the sensitivity of the system and require dilution. It was also impractical to obtain norepinephrine blanks after analyzing unknown samples because of the long washout time required to remove traces of thioglycolic acid from the norepinephrine manifold. Because of the high correlation between the epinephrine and norepinephrine blanks and the rapid washout of ascorbic acid, this problem was circumvented by measuring the faded blank in the epinephrine manifold and multipying this blank by the appropriate correlation factor to obtain the norepinephrine blank.

A nonoxidation blank may be obtained by replacing the potassium ferricyanide reagents with water containing ascorbic acid and thioglycolic acid in the appropriate manifolds. The stabilizing agents were necessary because some oxidation of catecholamines occurred within the Auto-Analyzer system even in the absence of potassium ferricyanide.

Linearity

The linearity of the standard curves for epinephrine and norepinephrine stabilized with ascorbic acid and thioglycolic acid is shown in Fig. 4. When samples were diluted because of high catecholamine content, the linear relationship still existed.

Recovery and Discrimination between Epinephrine and Norepinephrine

The recovery of epinephrine and norepinephrine was tested by adsorption of known mixtures of the two catecholamines on aluminum oxide. The percentage recoveries of amines from acid, tissue homogenates, and

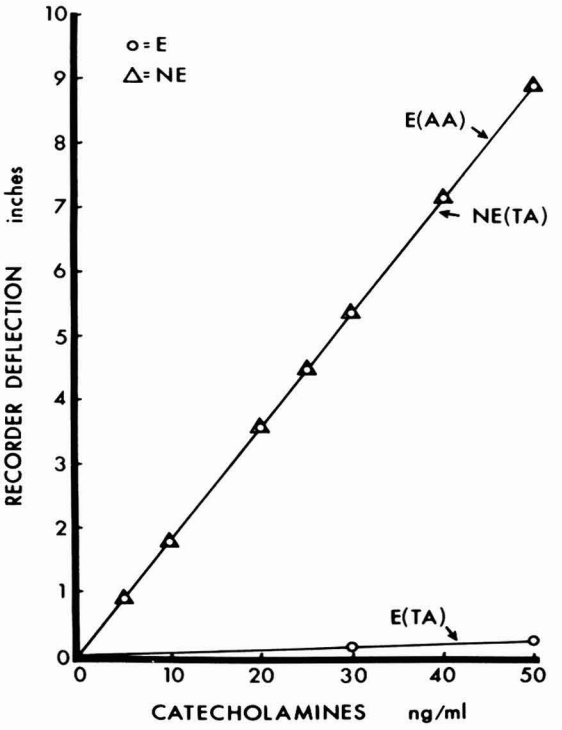

FIG. 4. Standard curves of epinephrine (E) and norepinephrine (NE), with ascorbic acid (AA) stabilization at pH 3.5 and thioglycolic acid (TA) stabilization at pH 7.4. The norepinephrine with ascorbic acid stabilized is not shown since it does not rise above the baseline.

TABLE 1
RECOVERY OF EPINEPHRINE (E) AND NOREPINEPHRINE (NE) FROM ACID, TISSUE, AND PERIPHERAL PLASMA FOLLOWING ADSORPTION ON ALUMINUM OXIDE AND FROM ACID WITHOUT ADSORPTION ON ALUMINUM OXIDE

	Catecholamines added (ng)			Recovery (%)[b]		
	E	NE	n[a]	E	NE	Percentage of E[b]
Acid	250	250	5	74.39 ± 0.10	72.04 ± 1.99	51.5 ± 0.5
	500	500	7	77.00 ± 0.90	78.31 ± 1.26	49.6 ± 0.4
	1000	1000	4	87.78 ± 1.49	86.30 ± 1.97	50.4 ± 0.8
Tissue	500	500	5	74.85 ± 0.36	73.86 ± 1.09	50.34 ± 0.29
	1000	1000	5	76.42 ± 1.04	73.36 ± 0.85	51.02 ± 0.48
Plasma	250	250	5	76.70 ± 0.83	69.56 ± 1.53	52.4 ± 0.58
	500	500	7	72.23 ± 1.34	74.20 ± 0.61	49.3 ± 0.32
	1000	1000	16	74.57 ± 1.09	72.77 ± 0.94	50.6 ± 0.28

[a] n = Number of samples.
[b] Mean ± SE.

plasma were of the same order of magnitude (Table 1). The recovery of catecholamines from plasma after precipatation of proteins with perchloric acid was reduced by 10 to 15%. Since the percentage recovery of epinephrine and norepinephrine from aluminum oxide was approximately the same for both standards and samples, no correction for recovery was made.

The ability of the present method to discriminate between epinephrine and norepinephrine is shown in Table 2. As little as 1 ng of either amine could be distinguished in the presence of 50 ng of the other.

TABLE 2
DISCRIMINATION BETWEEN EPINEPHRINE (E) AND NOREPINEPHRINE (NE) ADDED TO ACID IN VARYING MIXTURES

	Amount added (ng/ml)		Amount found (ng/ml)[b]		
n[a]	E	NE	E	NE	Percentage of E[b]
6	50.00	1.00	52.53 ± 1.93	1.50 ± 0.13	97.53 ± 0.53
6	50.00	4.00	51.85 ± 0.26	4.22 ± 0.25	92.78 ± 0.35
6	50.00	5.00	50.67 ± 0.46	5.20 ± 0.07	90.50 ± 0.17
5	1.00	50.00	0.84 ± 0.19	49.20 ± 0.28	2.00 ± 0.07
4	5.00	50.00	5.30 ± 0.20	53.25 ± 0.23	9.04 ± 0.27
4	10.00	50.00	11.35 ± 0.29	50.58 ± 0.46	18.10 ± 0.04
3	40.00	50.00	41.20 ± 0.03	51.20 ± 0.12	44.67 ± 0.03

[a] n = Number of samples.
[b] Mean ± SE.

TABLE 3
EPINEPHRINE (E) AND NOREPINEPHRINE (NE) CONTENT OF TISSUES

	Number of determinations	E[a] (μg/g)	NE[a] (μg/g)	Percentage of E[a]
Rat heart	4	0.08 ± 0.03	1.05 ± 0.04	3.94 ± 1.58
Rat vas deferens	9		10.93 ± 0.78	
Guinea pig vas deferens	7		14.68 ± 0.91	
Rat adrenal	7	772.63 ± 94.66	164.45 ± 21.12	81.10 ± 1.63
Cat adrenal	33	877.42 ± 51.54	588.22 ± 45.27	60.36 ± 1.38

[a] Mean ± SE.

Analysis of Tissues and Adrenal Perfusates

The results of the analysis of the catecholamine contents of several tissues are shown in Table 3. The results obtained were in good agreement with those reported in the literature *(3, 18, 20, 21)*.

Figure 5 is an AutoAnalyzer record showing the secretory response of the isolated perfused dog adrenal gland to 17.5 m*M* KCl. The record is a composite drawing from the two recorders showing the time relationship of the release of epinephrine and norepinephrine. Ackerly *et al. (1)* have recently reported a modification of the automated procedure of Robinson

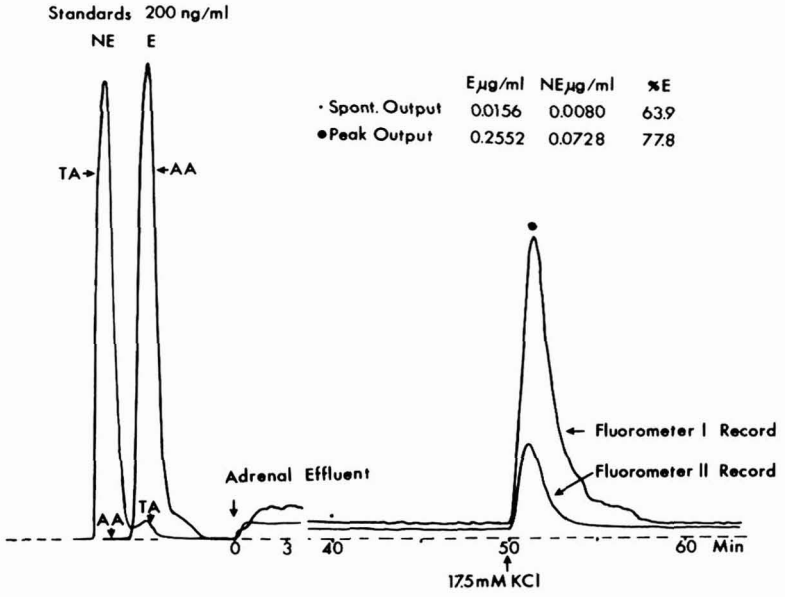

FIG. 5. AutoAnalyzer record of secretory response of isolated perfused dog adrenal gland stimulated for 10 sec with 17.5 m*M* KCl. Epinephrine (E) and norepinephrine (NE) 50 ng/ml standards run with ascorbic acid (AA) and thioglycolic acid (TA) stabilization are shown.

and Watts *(17)* which permitted the continuous in-line analysis of catecholamines from perfused adrenal glands.

The automated procedure described made possible the simultaneous analysis of epinephrine and norepinephrine during a single passage of a sample through the AutoAnalyzer. Blank readings have been found to be negligible for aluminum oxide adsorbed samples from tissues and adrenal vein plasma.

A prime goal in the development of the present method was to improve the ability of the method to distinguish betwen epinephrine and norepinephrine. Optimal conditions for the differential quantitation of the two amines were obtained with a mimimum of cross contamination by the use of two combined manifolds. The oxidation of epinephrine at a pH of 3.5 in an acetate buffer *(3, 10, 15)* containing a trace of zinc sulfate *(19)* resulted in the formation of adrenolutine but not noradrenolutine. An increase in the oxidation time at pH 3.5 was an important consideration because of the slower rate of oxidation at that pH.

The epinephrine content of the sample could be determined directly from the fluorescence peak obtained with the epinephrine manifold. Norepinephrine oxidation was carried out at pH 7.4 as recommended by Andersson *et al. (2)*. The use of the 400-nm interference filter as suggested by Hathaway *et al. (7)* coupled with thioglycolic acid stabilization nearly abolished the fluorescence due to epinephrine.

SUMMARY

The automated method of Andersson *et al. (2)* for the determination of epinephrine and norepinephrine in urine has been modified to allow the simultaneous quantitation of the two amines in a sample during a single run through the AutoAnalyzer. The differentiation of epinephrine and norepinephrine was based on the use of two stabilizing agents and oxidation of the catecholamines at two quite different pHs. As a result epinephrine could be measured directly from the recorded fluorescence peak. Norepinephrine fluorescence was accompanied by a minimal contamination by epinephrine fluorescence, amounting to less than 4% of the total fluorescence.

The method has been applied to the determination of the amines in tissues, adrenal vein blood, and perfused adrenal effluents.

ACKNOWLEDGMENTS

I thank Mr. Warren Cooper and Miss Rebecca Martin for their excellent technical assistance.

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Assay of Diamine Oxidase by Amperometric Measurement of the Rate of Oxygen Depletion

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INTRODUCTION

Diamine oxidase (EC 1.4.3.6) is an enzyme which catalyzes the oxidation of diamines and histamine according to the reaction

$$R-CH_2-NH_2+O_2+H_2O \xrightarrow{DAO} R-CHO+NH_3+H_2O_2 .$$

It has been purified from hog kidney and plasma and human placenta. Diamine oxidase levels in human serum have been shown to increase during pregnancy *(8)* and its assay is of prognostic value in threatened abortion *(1)*. Increased activities also can occur in cases of cancer of the uterus and ovaries *(3)*, bronchial carcinoma *(4)*, and anaphylactic shock *(10)*.

A number of methods for diamine oxidase assay have been described, including measurement of ammonia formed *(5,11,12)*, manometric measurement of oxygen consumption *(9)*, radiochemical measurement of [^{14}C]putrescine consumption *(13)*, and a number of colorimetric and spectrophotometric procedures *(2,7)*. A fluorometric method has also been described *(6)*.

MATERIALS AND METHODS

Apparatus

The rate of oxygen consumption was measured with a Clark oxygen electrode in a Beckman glucose analyzer (Beckman Instruments, Inc., Fullerton, Calif. 92634) in conjunction with a strip-chart recorder (Linear Instruments Corp., 17282 Eastman Ave., Irvine, Calif. 92714).

Reagents

Phosphate buffer, 1 M. 17.42 g of K_2HPO_4 in 100 ml of deionized water. Adjust the pH to 7.5 by adding 1 *M* KH_2PO_4 solution (containing 13.61 g of KH_2PO_4 per 100 ml of deionized water). Store in refrigerator at 4°C to prevent bacterial growth.

Diamine oxidase (EC 1.4.3.6). Dissolve 100 mg of hog kidney diamine oxidase (Sigma Chemical Co.) in 1.0 ml of deionized water to make a

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solution with diamine oxidase activity of 100 IU/liter. Dilute this solution further to make standard solutions with activities ranging from 0 to 100 IU/liter.

Substrates. The following 1 *M* substrate solutions were prepared with deionized water for studying the relative response of diamine oxidase toward each: 161.1 mg/ml of putrescine dihydrochloride, 184.1 mg/ml of histamine dihydrochloride, 175.2 mg/ml of cadaverine dihydrochloride, and 10.9 ml of benzylamine diluted to 100 ml with deionized water.

Procedure

Preparation of calibration curve. Add 0.500 ml of phosphate buffer and 0.500 ml of diamine oxidase standard (containing 100 IU/liter) to the cell of the analyzer. Let the oxygen in the solution come to equilibrium with atmospheric oxygen (indicated by a stable reading on the analyzer's digital display and by a stable baseline on the recorder chart). The recorder is operated at 1 V full scale. Inject 10 μl of 1 *M* putrescine into the cell and record the signal maximum, i.e., the distance from the baseline to the derivative peak height. Repeat for samples in the concentration range from 0 to 50 IU/liter, and plot the relative peak heights as a function of diamine oxidase activity.

Measurement of unknown sample. Follow the procedure above, using 0.500 ml of sample, and find the corresponding diamine oxidase activity from the calibration curve.

RESULTS AND DISCUSSION

Electrode Response

The electrode signal is maximized by using the high-sensitivity "U-mode" of the analyzer and by setting the "Air Adjust" control to give the maximum response level. The calibration curve (Fig. 1) varied very little from day to day, but for best results a new curve should be prepared on the day of the analysis.

Effect of pH

Optimum pH values ranging from 7 to 9 have been reported for diamine oxidase activity, depending on the substrate. We found the pH range for optimum activity, using putrescine as the substrate, to be 7.4–7.6 (Fig. 2).

Effect of Buffer Concentration

The rate of oxygen consumption varies significantly with the phosphate buffer concentration (pH 7.5), increasing with increasing concentration and leveling off above 0.50 *M* (Fig. 3).

Effect of Substrate

Diamine oxidase activity was compared for 1 *M* solutions of histamine, cadaverine, and putrescine. The response toward putrescine was slightly

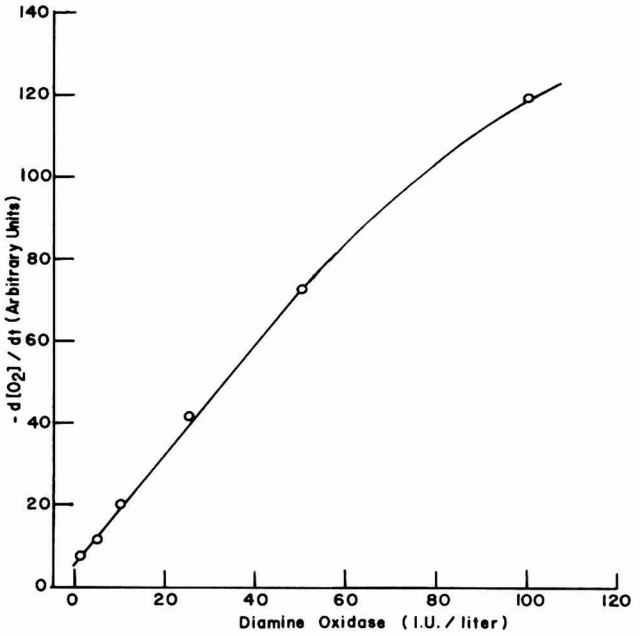

FIG. 1. Calibration curve for aqueous diamine oxidase standards (0.500-ml sample).

greater than toward the other substrates. The effect of putrescine concentration was studied (Fig. 4). Using 0.010 ml of putrescine added to the sample cell containing 0.005 IU of diamine oxidase in 0.3 *M* phosphate

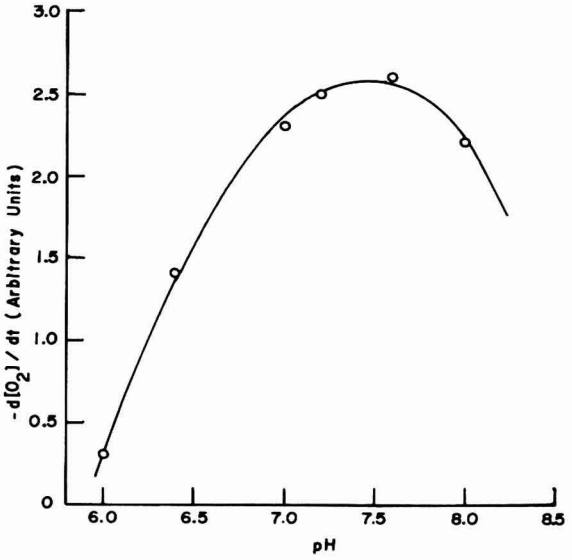

FIG. 2. Effect of pH on rate of oxygen consumption using putrescine substrate.

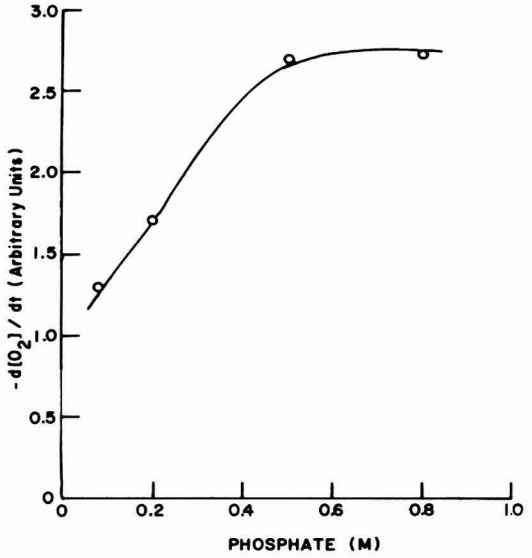

FIG. 3. Effect of phosphate buffer concentration on rate of oxygen consumption.

buffer (pH 7.4), the rate of oxygen consumption increased with increasing putrescine concentration, leveling off above 1.0 *M*.

Linearity

Figure 5 shows recorded signals ($-d[0_2]/dt$ vs time) for aqueous diamine oxidase standards. The peak heights represent the maximum rate of oxy-

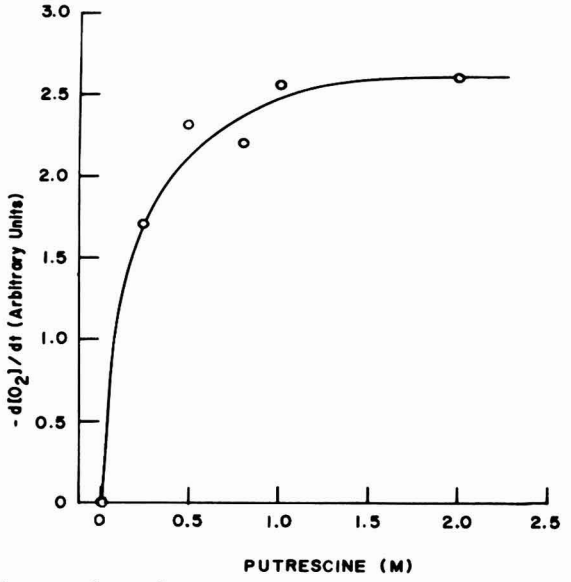

FIG. 4. Effect of putrescine substrate concentration on rate of oxygen consumption.

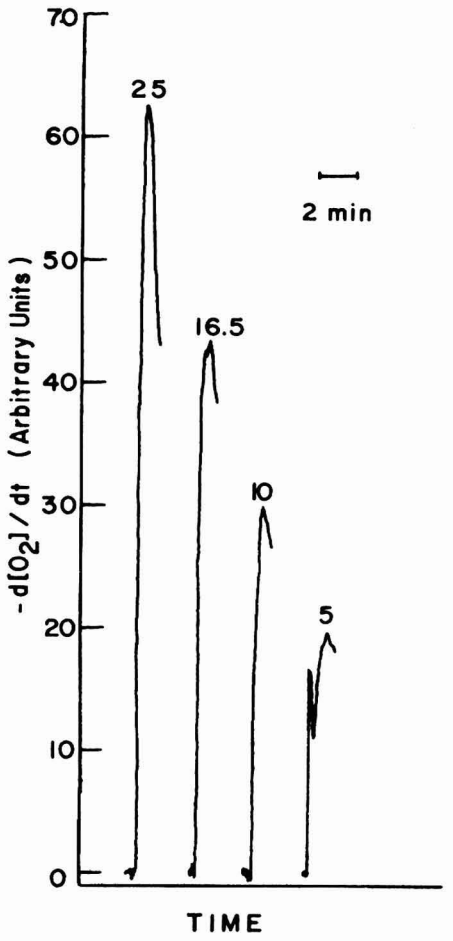

FIG. 5. Signals recorded for aqueous 0.500-ml diamine oxidase standards. Numbers on peaks represent activities of diamine oxidase samples, international units per liter.

gen consumption. As shown in the calibration curve, a linear relationship between peak height (maximum rate) and diamine oxidase activity was observed from 0 to 50 IU/liter, using 0.500-ml samples (0–0.025 IU in the analyzer cell). At activities greater than 50 IU/liter, the response begins to deviate from linearity. A small blank signal occurs in the absence of diamine oxidase activity and begins to affect the reproducibility of measurements below 0.0025 IU in the sample cell.

Precision

A coefficient of variation of 6% was calculated for 30 determinations of a 10 IU/liter diamine oxidase standard solution. This compares favorably with other methods (Table 1).

TABLE 1
COMPARISON OF DIAMINE OXIDASE METHODS

Method	Number of steps required	Precision	Reference
Amperometric			
Oxygen depletion, putrescine substrate	1	6%	—
Spectrophotometric			
p-Dimethylaminomethyl-benzylamine substrate	1	7.2%	*2*
Spectrophotometric			
o-Aminobenzaldehyde substrate	1	8%	*7*
[^{14}C]Putrescine method	>3	3.9%	*13*

Recovery from Serum

Table 2 shows recoveries of hog kidney diamine oxidase standards added to pooled human serum and analyzed by our procedure. Excellent recovery was obtained for diamine oxidase activities ranging from 5 to 25 IU/liter, with an average relative error of −0.8%.

CONCLUSIONS

Table 1 shows a comparison of the present method with other currently used diamine oxidase methods. Diamine oxidase assay by amperometric measurement of the rate of oxygen consumption has the advantages of being inexpensive and simple to perform and not requiring incubation or sample pretreatment, and it offers precision comparable to that of other methods. This method requires only a few minutes for an analysis whereas most other methods require lengthy incubation periods.

TABLE 2
RECOVERY OF DIAMINE OXIDASE FROM 0.5 ml OF SERUM

Added (IU)	Recovered (IU)		Relative error (%)
0.0125	0.0121		−3.2
0.0083	0.0083		0.0
0.0050	0.0048		−4.0
0.0025	0.0026		4.0
		Av	−0.8

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Amperometric and Constant-Current Potentiometric Determinations of the Landolt Effect

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INTRODUCTION

Landolt-type reactions, according to the investigations of many authors, have proved to be suitable indicator reactions in kinetic analysis. For many reactions applied up to now, the Landolt effect was determined visually *(1)*. But it was not possible to apply this method to the case of slow evolution of halogens at the moment of the Landolt effect intervention, due either to the nature of the indicator reaction or to low concentrations of the indicator components. Hence the instrumental methods have proved to be more convenient for the Landolt effect determination. In our previous articles, an application of thermometric *(2–5)* and biamperometric *(2)* methods to the Landolt effect determination has been described. The present paper deals with new possibilities of the Landolt effect determination applying the amperometric method and the potentiometric method at a small constant current. As indicator reactions, the hydrogen peroxide–iodide–ascorbic acid and bromate–bromide–ascorbic acid reactions were investigated. The possibilities of application of these methods are illustrated by determination of small amounts of iron(III), vanadium(V), and molybdenum(VI).

MATERIALS AND METHODS

Reagents

All chemicals used were of p.a. purity.

Reducing solution I: 4.350 g of potassium iodide and 0.775 g of ascorbic acid were dissolved and diluted to 250 ml with acetate buffer of pH 4.5 (for the determination of iron(III) with acetate buffer of pH 3.6).

Reducing solution II: 25.194 g of potassium bromide and 0.924 g of ascorbic acid were dissolved and diluted to 250 ml with citrate buffer of pH 2.1.

Reducing solution III: 12.500 g of potassium bromide and 0.550 g of ascorbic acid were dissolved and diluted to 250 ml with buffer of pH 1.6 (potassium chloride–hydrochloric acid buffer).

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Oxidizing solution I: 21.0 ml of 30% hydrogen peroxide and 50.0 ml of 1 *M* sulfuric acid were mixed and diluted to 100 ml with water.

Oxidizing solution II: 7.500 g of potassium bromate were dissolved in 100 ml of water.

Stock solutions of metal ions, i.e., of catalysts, were prepared with the following salts: $FeCl_3 \cdot 6H_2O$, NH_4VO_3, and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. From these stock solutions the appropriate diluted solutions were prepared.

All solutions were made with double-distilled water.

Apparatus

The usual apparatuses were used in the amperometric and the potentiometric determinations of the reaction rate. In the amperometric determinations the indicator electrode was the platinum needleshaped electrode, coupled with S.C.E. In potentiometric determinations at a small constant current, the indicator electrode was the platinum square electrode, also coupled with S.C.E., or the indicating electrode system consisting of two identical platinum square electrodes.

In potentiometric determinations at a small constant current, a current of 2 μA was selected.

Indicating current changes were registered by a Servogor Type RE 511 recorder and the potential changes by a Servogor S Type RE 541 recorder of the Goerz-Electro production. The reaction mixture was thermostated by the Veb. Prüfgerätwerk U3 thermostat.

Procedure

The reaction mixture was prepared by mixing 5.0 ml of the corresponding catalyst salt solution with 5.0 ml of reducing solution I or reducing solution II in amperometric determinations, or reducing solution III in potentiometric determinations, and 10.5 ml of water. The reaction mixture was thermostated to 26.0 ± 0.1°C, and after some time 0.5 ml of the appropriate oxidizing solution was added to initiate the reaction.

RESULTS AND DISCUSSION

Amperometric Determinations

In the hydrogen peroxide–iodide–ascorbic acid reaction, the change of concentration of ascorbic acid and iodide was monitored according to their anodic waves at the potential of +600 mV vs S.C.E. Figure 1a shows the amperometric curves obtained at this potential. The limiting current of ascorbic acid decreases during the incubation period. The break at the curve marking the Landolt effect intervention appears at the moment of complete consumption of ascorbic acid. The further current decrease corresponds to anodic oxidation of iodide.

The change of iodine concentration was followed by its cathodic wave at an indicator electrode potential of −200 mV vs S.C.E. (Fig. 1b). At the moment of Landolt effect intervention when rapid iodine evolution is started, the sharp break observed in the curve is due to an increase in the limiting current of iodine reduction.

On the basis of data marking the moment of the Landolt effect intervention, the calibration curves are constructed by plotting the reciprocal of time against the concentration of the metal ion determined. For the concentration range investigated, a linear graph was obtained. Some results achieved in determinations of metal ions by application of the hydrogen peroxide–iodide–ascorbic acid reaction are given in Table 1.

In the bromate–bromide–ascorbic acid reaction at an indicator electrode potential of +700 mV vs S.C.E., the ascorbic acid concentration change was followed by its anodic wave. During the reaction ascorbic acid is consumed on the reduction of bromine resulting in a decrease of its limiting current. At the moment of Landolt effect intervention a sharp break in the curve is observed, affected by a rapid increase of limiting current of the reduction of bromine evolved.

The cathodic wave of bromine was used to follow its concentration change at the potential of +200 mV vs S.C.E. At the start of the Landolt effect, the intensive bromine evolution caused a sharp break in the curve due to an increase in the limiting reducing current of bromine.

On the basis of data obtained in amperometric determinations of the indicator reaction rate in the presence of metal ions, the corresponding calibration curves are plotted. Results obtained are given in Table 2.

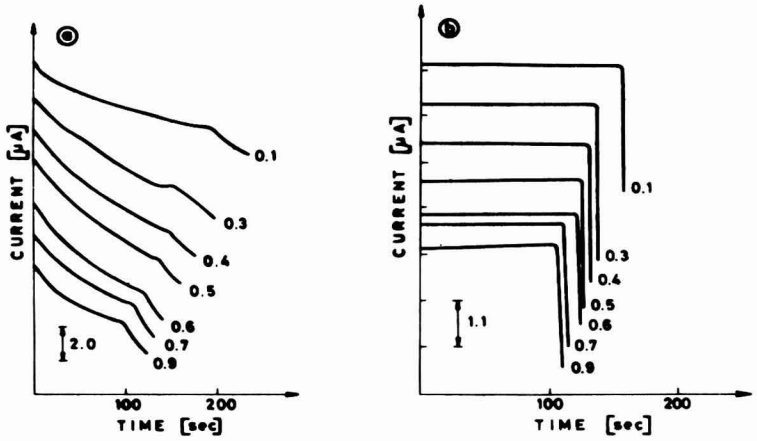

FIG. 1. Amperometric kinetic curves obtained in determinations of molybdenum by application of the hydrogen peroxide–iodide–ascorbic acid indicator reaction at indicator electrode potentials of: (a) +600 mV vs S.C.E.; (b) −200 mV vs S.C.E. Numbers on the curves show the amounts of molybdenum in micrograms per milliliter.

TABLE 1

RESULTS OF AMPEROMETRIC DETERMINATIONS OF METAL IONS BY MEANS OF THE HYDROGEN PEROXIDE – IODIDE – ASCORBIC ACID REACTION

Ions	Useful range (μg/ml)	Taken (μg/ml)	Indicator electrode potential +600 mV vs S.C.E. Found[a] (μg/ml)	Error (%)	Average deviation (%)	Indicator electrode potential −200 mV vs S.C.E. Found[a] (μg/ml)	Error (%)	Average deviation (%)
Fe(III)	0.1 – 1.0	0.400	0.395	−1.2	8.8	0.422	+5.5	0.7
		0.600	0.620	+3.3	3.7	0.605	+0.8	1.3
V(V)	1.0 – 10.0	4.00	4.10	+2.5	0.1	4.08	+2.0	1.1
		6.00	6.15	+2.5	1.2	5.85	−2.5	2.5
Mo(VI)	0.1 – 1.0	0.400	0.392	−2.0	1.8	0.390	−2.5	0.2
		0.600	0.606	+1.0	0.2	0.600	0.0	0.2

[a] Average of four determinations.

Potentiometric Determination at a Small Constant Current

In Fig. 2 are illustrated the constant-current potentiometric curves of vanadium determination applying the bromate–bromide–ascorbic acid indicator reaction. The curves are obtained by means of the platinum indicator electrode polarized anodically (a) and cathodically (b). At the moment of the Landolt-effect intervention, a rapid increase or decrease of potential, correspondingly, can be observed on these curves.

The curves of vanadium determination applying the same indicator reaction obtained with two identical indicator electrodes, presented in Fig. 3, show the appearance of sharp breaks at the moment of Landolt effect intervention. Using the calibration curve method, metal ions can also be

TABLE 2

RESULTS OF AMPEROMETRIC DETERMINATIONS OF METAL IONS BY MEANS OF THE BROMATE – BROMIDE – ASCORBIC ACID REACTION

Ions	Useful range (μg/ml)	Taken (μg/ml)	Indicator electrode potential +700 mV vs S.C.E. Found[a] (μg/ml)	Error (%)	Average deviation (%)	Indicator electrode potential +200 mV vs S.C.E. Found[a] (μg/ml)	Error (%)	Average deviation (%)
Fe(III)	1.0 – 10	4.00	3.95	−1.2	0.5	4.13	+3.2	0.6
		6.00	5.85	−2.5	1.6	5.85	−2.5	0.8
V(V)	0.1 – 1.0	0.400	0.388	−3.0	0.9	0.397	−0.8	1.0
		0.600	0.605	+0.8	0.7	0.588	−2.0	0.4
Mo(VI)	100 – 900	400	415	+3.8	0.2	426	+6.5	1.5
		600	592	−1.3	2.7	625	+4.2	1.5

[a] Average of four determinations.

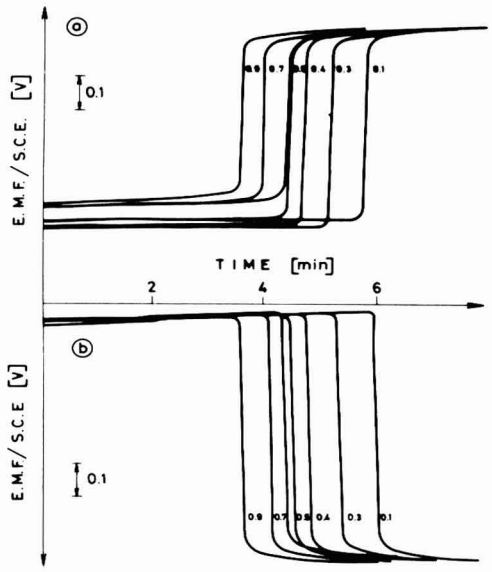

FIG. 2. Constant-current (2 μA) potentiometric kinetic curves obtained in determinations of vanadium by application of the bromate–bromide–ascorbic acid indicator reaction with the indicator electrode polarized: (a) anodically; (b) cathodically. Numbers on the curves show the amounts of vanadium in micrograms per milliliter.

estimated in this case. Results of the determination of vanadium by means of these methods are given in Table 3.

It can be concluded that the amperometric method can successfully be applied to the Landolt effect determination by means of these reactions. The method proved to be more suitable for following the indicator reaction rate at the potential of the indicator electrode at which the cathodic current or halogens can be registered. Thus, the intervention of the

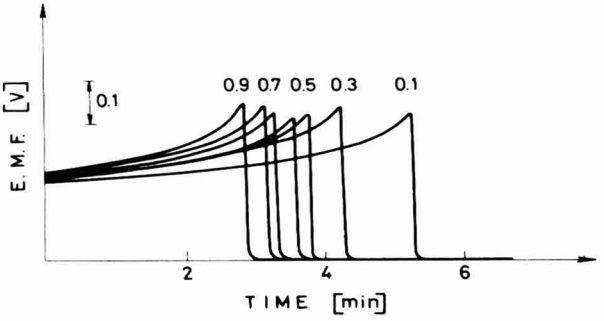

FIG. 3. Constant-current (2 μA) potentiometric kinetic curves of vanadium determinations by application of the bromate–bromide–ascorbic acid indicator reaction obtained with two identical indicator electrodes. Numbers on the curves show the amounts of vanadium in micrograms per milliliter.

TABLE 3

RESULTS OF POTENTIOMETRIC DETERMINATIONS OF VANADIUM(V) AT A SMALL CONSTANT CURRENT (2 μA) BY MEANS OF THE BROMATE – BROMIDE – ASCORBIC ACID REACTION

Indicating system	Useful range (μg/ml)	Taken (μg/ml)	Found[a] (μg/ml)	Error (%)	Average deviation (%)
Pt +/S.C.E. –	0.1 – 0.9	0.400	0.400	0.0	0.7
		0.600	0.585	−2.5	0.9
Pt –/S.C.E. +	0.1 – 0.9	0.400	0.385	−3.8	0.5
		0.600	0.600	0.0	0.4
Pt +/ Pt –	0.1 – 0.9	0.400	0.380	−5.0	0.4
		0.600	0.585	−2.5	0.2

[a] Average of four determinations.

Landolt effect can easily be determined according to sharp breaks in the curves. Besides, the curves based on the cathodic current of halogens are more reproducible due to the lesser dependence of shape on the surface conditions of the indicator electrodes. On the basis of the results obtained, it can also be concluded that the potentiometric method at a small constant current proved to be convenient for the Landolt effect determination by means of these indicator reactions.

SUMMARY

An amperometric method with one platinum indicator electrode as well as the constant-current potentiometric method with one and two indicator electrodes were successfully applied to the Landolt effect determination by means of the hydrogen peroxide-iodide-ascorbic acid and bromate–bromide–ascorbic acid indicator reactions. The possibilities of application of these methods are illustrated by the determination of small amounts of iron(III), vanadium(V), and molybdenum(VI) with satisfactory accuracy and precision.

ACKNOWLEDGMENT

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MICROCHEMICAL JOURNAL 22, 541 – 547 (1977)

Catalytic Microdetermination of Iron

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INTRODUCTION

Several kinetic methods are known for the microdetermination of iron *(2,3)*. The most sensitive of them are based on the accelerated oxidation of arylamines or phenols by an oxidant in the presence of iron ions. The oxidation of these compounds leads to the formation of colored products, so the kinetics of these reactions are usually followed spectrophotometrically. In a previous work *(1)*, the oxidation reaction of 2,4-diaminophenol (DAP) by hydrogen peroxide was used for the determination of copper. We have found that iron(III) ions catalyze the same reaction too.

In the present work we report the optimal conditions for the above reaction in the presence of iron(III) ions, in order to propose a kinetic method for the microdetermination of iron.

EXPERIMENTAL

Instruments

A PYE–UNICAM SP-700 recording double beam spectrophotometer, connected with a Sargent thermonitor thermocirculating bath, was used for absorbance measurements. For pH measurements a Beckmann 1019 research pH meter was used with absolute accuracy limits of pH measurement defined by NBS buffers.

Reagents

The reagents used were of analytical grade and doubly distilled boiled out water was always used.

The stock iron solution, 0.001 M, was prepared from ferric chloride (Merck) and was determined gravimetrically. Hydrolysis was prevented by adjusting the pH to about 1 by HCl.

Hydrogen peroxide solution was prepared by a proper dilution of Merck p.a. reagent and was standardized against potassium permanganate solution.

The 2,4-diaminophenol solution, 1.25×10^{-2} M, was prepared by dissolving doubly recrystallized 2,4-diaminophenol dihydrochloride (Merck, zür Synthese) in 0.2 N hydrochloric acid.

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Constant ionic strength buffer solutions were obtained by diluting glycine, NaCl and HCl, each 1 *M* *(4)*.

Pure hydrochloric acid, 1 *M*, was prepared by dilution Titrisol Merck solution.

All solutions were prepared immediately before use and were brought to 25±0.1°C by immersion in a water bath.

Procedure

The following procedure was adopted for the study of the kinetics of the selected reaction.

The spectrophotometer was switched on for at least 2 hr before commencing measurements, to ensure good stability of the light source.

A mixing flask, with three side arms beneath, was used to mix the reactants *(5)*. The DAP solution was placed in the first side arm, H_2O_2 solution in the second, and iron ion solution in the third. All solutions were placed very carefully to avoid any contamination. Proper volumes of buffer solution were added to the arms which contained H_2O_2 and iron ion solutions in order to make the final volume 50 ml. This flask with the reactants was thermostated at 25± 0.1°C for 20 min. At the end of this period, the contents of the three arms were vigorously mixed in the stoppered flask and the time was recorded. The mixture produced was transferred to a 10-mm path length cell which was placed in the thermostated cell holder of the spectrophotometer. One minute after mixing, the change in absorbance at 500 nm *(1)* was recorded automatically, as well as the time intervals.

RESULTS AND DISCUSSION

It was observed that the rate reaction depends on the concentrations of DAP, H_2O_2, iron ions, and hydrogen ions as well on the temperature. The effect of each variable was studied by using the arrangements as described under procedure.

The influence of DAP concentration is presented in Fig. 1. It shows that, as the DAP concentration increases, the reaction rate increases up to a certain value and then becomes DAP-concentration independent.

Figure 2 presents reciprocal DAP concentration vs reciprocal reaction rate. This curve shows that a 1:1 complex is formed between the metal ions and the ligand molecules according to the equation of Yatsimirskii *(5)*.

A similar curve was obtained (Fig. 3) showing the effect of hydrogen peroxide concentration on the observed reaction rate constant (designated as K^*).

Figure 4 presents the effect of hydrogen ion concentration on the observed reaction rate constant. The optimum conditions as obtained from

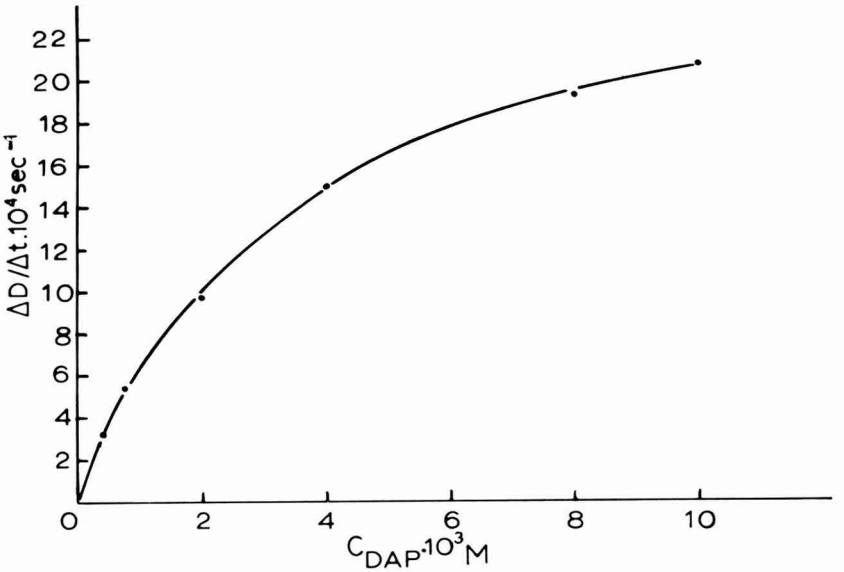

FIG. 1. Influence of DAP concentration on the reaction rate (Fe = 5×10^{-5} *M*, 5 ml; H_2O_2 = 3.39 *N*, 1 ml; pH = 2.88; buffer, glycine + HCl, ionic strength *I* = 0.1, 40 ml; temperature, 25±0.1°C).

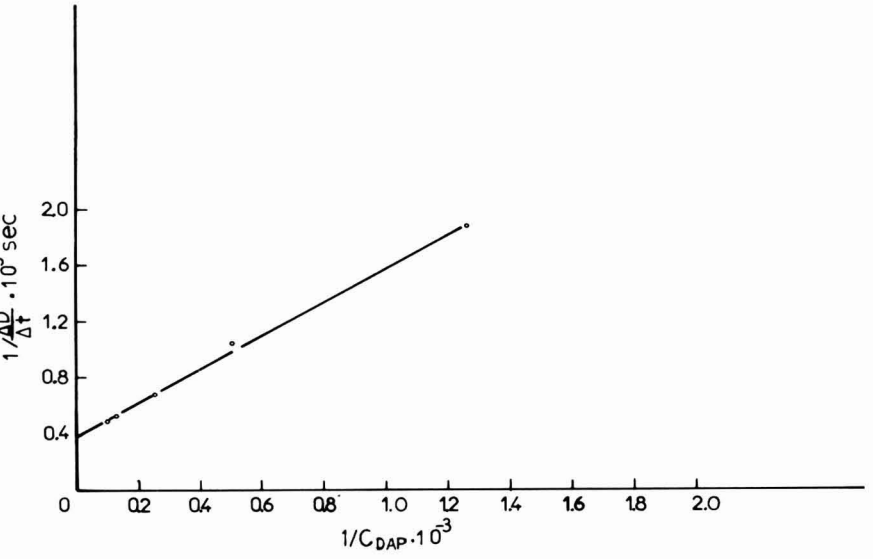

FIG. 2. Plot of the reciprocal of reaction rate against the reciprocal DAP concentration (Fe = 5×10^{-5} *M*, 5 ml; H_2O_2 = 3.39 *N*, 1 ml; pH = 2.88; buffer, glycine + HCl, ionic strength *I* = 0.1, 40 ml; temperature, 25±0.1°C).

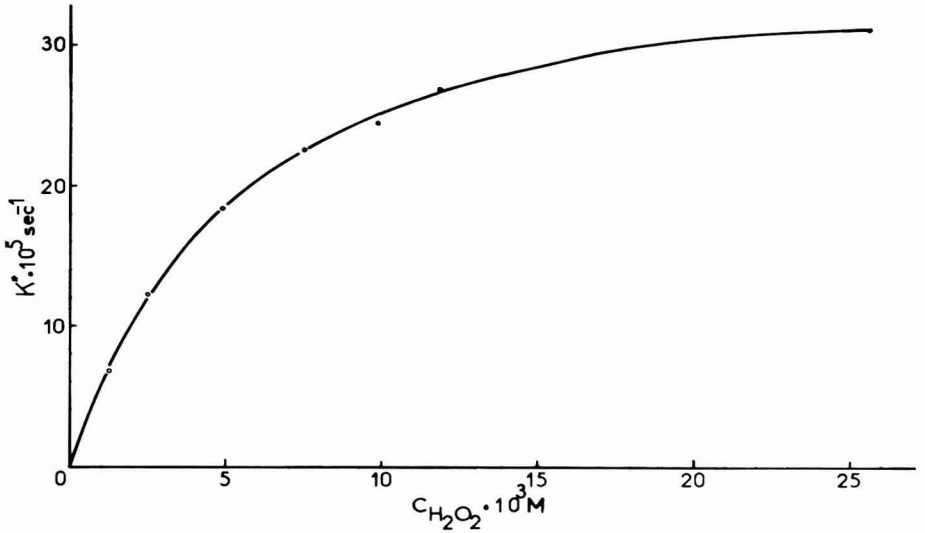

FIG. 3. Influence of hydrogen peroxide concentration on the observed reaction rate constant (DAP = 2.5×10^{-2} *M*, 4 ml; Fe = 1×10^{-5} *M*, 5 ml; pH = 2.88; buffer, glycine + HCl, ionic strength $I = 0.1$, 40 ml; temperature, 25±0.1°C).

Figs. 1-4 were: $C_{DAP} = 2 \times 10^{-3}$ *M*, $C_{H_2O_2} = 3 \times 10^{-2}$ *M*, pH = 2.88, temperature 25±0.1°C. These conditions were used to determine the Fe^{3+} ion concentration.

The results are summarized in Fig. 5. It was found that for the selected

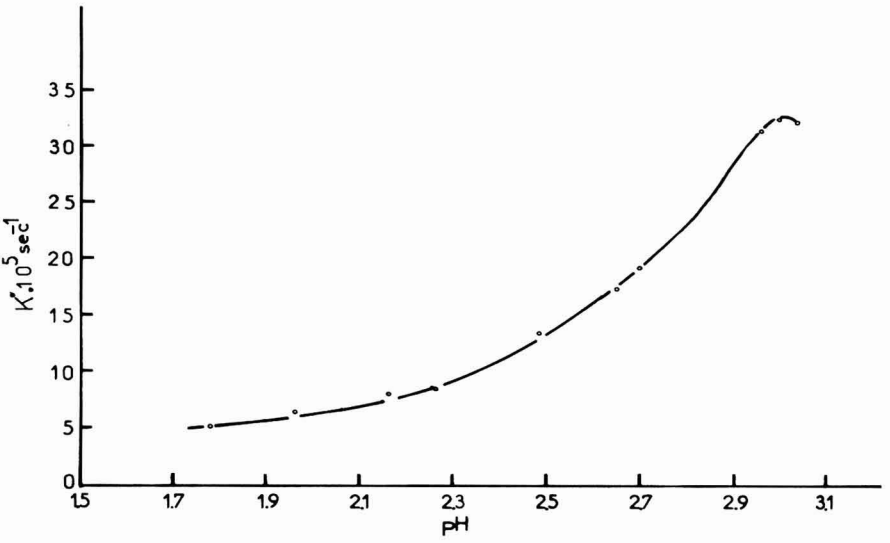

FIG. 4. Influence of pH on the observed reaction rate constant (DAP = 2.5×10^{-2} *M*, 4 ml; Fe = 5×10^{-5} *M*, 5 ml; $H_2O_2 = 2.57 \times 10^{-1}$ *N*, 1 ml; temperature, 25±0.1°C).

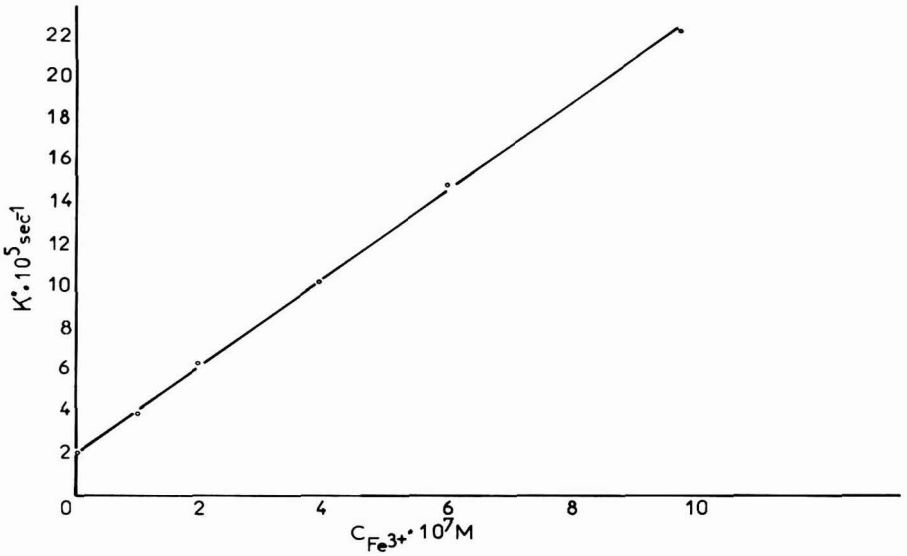

FIG. 5. Working curve for iron determination (DAP = 2.5×10^{-2} *M*, 4 ml; H_2O_2 = 3×10^{-2} *M*, 1 ml; pH = 2.88; buffer, glycine + HCl, ionic strength $I = 0.1$, 40 ml; temperature, 25±0.1°C).

set of conditions, the reaction is first order with respect to iron concentration from 0.977×10^{-7} to 9.77×10^{-7} *M*.

Using the curve in Fig. 5 as reference, the results of iron determination in aqueous solutions are given in Table 1. In Table 2 are listed the determined percentages of iron in artificial iron samples (Smith and Underwood Co.).

In both cases, the precision and accuracy were found to be within 1–3%. The sensitivity of the method is found to be 2.8×10^{-3} μg/ml.

Interferences

The effect of various metal ions that interfere in the determination of iron was also investigated. Sn^{2+}, MoO_4^{2-}, CrO_4^{2-}, Co^{2+}, Mn^{2+}, which cata-

TABLE 1
RESULTS FOR AQUEOUS IRON SOLUTIONS[a]

Iron (μg)		
Taken	Found[b]	Relative error (%)
2.00	1.98	−1.00
4.00	4.06	+1.50
8.50	8.36	−1.74
15.50	15.70	+1.30

[a] Conditions as under Procedure.
[b] From straight-line working curve.

TABLE 2
DETERMINED PERCENTAGE OF IRON IN LIMESTONE AND CEMENT SAMPLES[a]

	Iron (%)		
	Taken	Found	Relative error (%)
Cement No. 13	0.180	0.185	+2.77
Limestone 416	0.105	0.102	−2.85
Limestone 406	0.126	0.123	−2.38

[a] Conditions as under Procedure.

TABLE 3
INFLUENCE OF DIVERSE IONS: MAXIMUM CONCENTRATIONS OF IONS WHICH CAUSE NO INTERFERENCE[a]

Ion	Concentration (*M*)
Oxalate	2×10^{-7}
Mn^{2+}	3.6×10^{-5}
Co^{2+}	2.5×10^{-7}
CrO_4^{2-}	1×10^{-5}
Sn^{2+}	5×10^{-6}
MoO_4^{2-}	2×10^{-5}
Citrate	1×10^{-6}

[a] Solutions: DAP = 10^{-3} *M*, Fe^{3+} = 4×10^{-7} *M*, H_2O_2 = 0.766 *N*, pH = 2.72 at 25 ± 0.1°C.

lyze the reaction, interfere seriously. The anions citrate and oxalate show an appreciable interfering action. Table 3 gives the minimum amount of ions which causes a deviation greater than the accuracy of the method.

SUMMARY

A reaction rate method is described for the microdetermination of iron. The method is based on the catalytic action of iron on the reaction of 2,4-diaminophenol with hydrogen peroxide. The effect of reagent concentration is studied and the maximum tolerable amounts of interfering ions are determined. Procedures for the determination of 2.8×10^{-3} to 2.8×10^{-2} μg/ml are given.

Quantities of 2.8×10^{-3} to 2.8×10^{-2} μg/ml could be determinated with a relative error of about 2%.

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Design and Construction of a Novel Long-Path Spectrophotometer

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INTRODUCTION

Long-path photometry *(5–7,12)* has proven to be a valuable tool to lower the limits of detection and to increase the sensitivity in photometric determinations and titrations *(9)*. The use of solid-state optoelectronic devices has in many cases simplified these methods. Simple instruments can be built when light emitting diodes (LED) are used as radiation sources and photodiodes or phototransistors *(8)* are used as detectors. Such instruments have low power requirements and the necessary circuits involve a minimum of components. This combination of simplicity, portability, and inherent ruggedness makes these instruments well suited for field analysis.

The length of the cell is theoretically unlimited. In practice, however, the situation is different. Unless perfectly collimated light is used (e.g., a laser beam), losses of radiant energy are unavoidable. With certain instrumental settings, as the cell length is increased a point is reached at which the energy received by the detector is insufficient to set 100% transmittance with the blank in the cell. The simple and seemingly most expedient approach of increasing the driving current to the LED fails. Although the intensity of the light emitted by a LED is directly proportional to the forward current, the conversion efficiency has a temperature coefficient of -0.3 to -2% per degree. This means that at higher ambient temperature a lower output is obtained. Thermal effects will occur even when the ambient temperature is kept constant because self-heating becomes a factor, especially when approaching driving currents close to the rated dc maximum. The internal conversion efficiency will greatly decrease before the LED reaches the blowout point. Cooling the LED to the lowest possible operating temperature of $-55°C$ could, therefore, be considered as a means of increasing the output. The approach is impractical because the gain in the output is not commensurable with the additional equipment required and the operating inconvenience incurred. Another possibility is to increase the amplification of the output current of the detector. To do so, without adversely affecting the benefits in the improvement of the analytical signal derived from the use of long-path-length cells, would be difficult within the constraints of the preselected components.

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There is a method for obtaining more radiation from a LED while avoiding overheating. A gain of an order of magnitude in intensity can be realized by pulsing the LED with a low-duty-cycle ampere-level current.

Pulsing has been reported *(1)* for the purpose of excluding the influence of ambient light through the use of tuned detector circuits. The pulsing circuitry required to achieve an increase in light output is somewhat different from that required for solely the exclusion of the influence of ambient light. Of course, exclusion of ambient light would be an added benefit in the former case.

PRINCIPLES OF OPERATION[1]

The principal idea is to apply a high current pulse which yields a large light output but is of such a short duration that the heating effect is held below the level where inefficiency becomes noticeable or LED burnout possible. The high current pulse increases the peak radiative power of the LED, but the average radiative power is decreased. Saturation effects are more pronounced, but this problem is minor when compared to the thermal heating effects. During the dark cycle that follows, the pulse heat is dissipated. A large increase in light energy recepted is possible when photometric measurements are made using a pulse-synchronized detector. The pulse exciter circuit and the tuned amplifier are derived from the simple circuits employed in previous instruments. The miniature components with multiple functions (integrated circuit chips), now readily and relatively inexpensively available, make the project possible, and the benefits derived more than compensate for the additional costs and efforts encountered in building the instrument *(2)*.

Operating a LED in a pulsed manner as desired generates a more intense light output during the period of the pulse than could be achieved by operating the LED at its maximum dc rating. In addition, the spectral shift due to temperature changes, as well as effects that reduce the output intensity, are minimized. However, deviation from the linear current–light intensity relationship at high current levels may occur as a result of saturation effects.

The optical transmittance measurements are made using a pulse-synchronized detector circuit; that is, the actual measurement is made only during the brief interval when the LED is on. Sampling techniques can be used in the detector circuit to measure only the desired signal and reject ambient light signals. The pulse frequency for the LED is determined by

[1] Here only the basic principles and components as well as their functions are discussed. Detailed schematics of circuitry, instrumental layout, and building and operating instructions can be obtained by sending a stamped, self-addressed envelope to the senior author (G.E.O.).

considering the duty cycle and the linear response time of the detector circuit. The LED duty cycle, that is, the ratio of LED on-time to off-time, should be approximately 0.1% and the pulse width should be sufficiently long to allow the detector (phototransistor) and its associated preamplifier to respond linearly to the signal. When these factors were taken into consideration, a pulse width of 30 μsec and frequency of 30 Hz were chosen.

When one attempts to exclude the influence of ambient light, it is necessary to discriminate the three major types present in most laboratories: steady ambient light, ambient light from incandescent lamps, and ambient light from fluorescent lamps. Most of the effect of any ambient light is excluded because of the angular response characteristics of phototransistors or photodiodes *(4,11)*. Light must be incident upon the receptor surface of the detector within certain narrow angular limits to be recepted. If these angular response characteristics did not exist, saturation effects would preclude the general use of phototransistors as detectors. Steady ambient light, e.g., daylight, is automatically excluded by the tuned detector. Light from the now less frequently used incandescent lamps has a 60-Hz low-amplitude output, while the most commonly employed fluorescent lamps have a 120-Hz high-amplitude output. The 30-Hz pulsing signal could be generated by an oscillator circuit incorporated for this purpose. Then, however, a beat frequency could result from differences between this 30 Hz and the 60 and 120 Hz of the ambient light if either of the two, oscillator or ambient light, deviates slightly from the assumed nominal value. Such beat frequencies would have to be filtered out. This problem can possibly be encountered in the instrument developed by Anfält and co-workers *(1)*, in case their 120-Hz oscillator is not in complete synchronization with the ambient light frequency. When, however, the 30-Hz pulsing is derived from the same source as the ambient light, namely, the main power source, the two are always synchronized and no beat frequency can develop. The added benefit of such circuitry is greater simplicity.

The use of the main power source to derive the 30-Hz pulsing frequency also lends field applicability and portability to the instrument. It is possible to use the photometer in the field by operating it from whatever main power source is available. The most convenient method of field operation is realized by using a 12-V car battery and a dc-to-ac inverter. The only caveat is that exceptionally strong sunlight should be avoided in order to prevent saturation effects.

The transmittance measurements are made only during the 30-μsec pulse. The phototransistor will not respond within this time unless it is operated at a current level higher than the photocurrent produced by the light pulse. Therefore, the phototransistor base lead and control loop are employed to regulate the phototransistor emitter current at approximately

0.5 mA. This arrangement is necessary to ensure sufficiently rapid response. The photocurrent representing the *%T* signal is added to this quiescent current. Photocurrents generated by ambient light sources also contribute to the emitter current, requiring less base current from the control loop than would be necessary when operating in an environment devoid of ambient light. Thus, the phototransistor operating point is stabilized and some rejection of ambient light is achieved by regulating the phototransistor emitter current in this manner. Two solid-state electronic sample and hold units (S&H) and a differential amplifier are used to provide additional rejection of ambient light and also of the control loop error. The S&H designated as unit 2 is used to store the sum of the dark signal and the photocurrent signal voltage. Unit 1 operates on those cycles of the 60-Hz line frequency when the LED is off. Unit 2 operates during the cycles when the LED is on. The differential amplifier subtracts the signal stored in unit 1 from the signal stored in unit 2 and amplifies the difference signal. The rejection of ambient light is sufficiently high that the correctness of the measurement and the sensitivity of the instrument are limited only by the random noise inherent in the phototransistor.

All of the system timing and control pulses are generated by appropriate logic and derive from the 60-Hz line frequency. A 120-Hz half-wave input is produced by rectifying the secondary voltage of the power transformer. The signal is shaped using appropriate logic to produce square-wave outputs of 30 and 60 Hz. The 60-Hz square wave is used to time all the components except the S&H's and the LED. These components are timed from the 30-Hz signal.

The LED driver circuit is designed to produce a constant current pulse to power the LED. Circuitry is provided to allow the operator to preset the pulse current amplitude. A forward-biased Zener diode is included to serve as a level shifter, which is required to provide a wide dynamic range (20 V), which is necessary because of the high forward voltage drop present in some LEDs when they are operated at ampere-level currents. A circuit is included to warn the operator when the driving circuit has reached its voltage limit, because beyond this voltage the LED current will deviate from the preset value. This warning circuit is necessary because the driving circuit cannot drive some LEDs at their maximum-rated pulsed current.

The detector preamplifier and bias control circuit is designed with two goals in mind: to provide the flexibility necessary to allow the use of phototransistors of widely varying characteristics and to aid in the rejection of ambient light. A bias control trimpot is used to compensate for beta variation between different FPT-120 transistors and to maintain a constant loop gain. Ambient light that reaches the phototransistor generates a photocurrent which is amplified by the current gain of the photo-

transistor. This results in an increase in the emitter current which is compensated for by the bias control, maintaining the emitter current at 0.5 mA. The frequency response of this control loop is sufficiently high to null the 120-Hz light signal arising from the fluorescent lights. Most of the system noise results from the phototransistor and is independent of the control loop gain. A circuit is provided to allow the operator to select one of three feedback resistors to adjust low, medium, and high gain to provide the best possible signal-to-noise ratio.

The signal processing circuit is designed to interact with the S&H circuits and to have the *%T* signal as the output. The same circuit allows the operator to set 0 and 100*%T* by adjusting the zero and the gain of the differential amplifier. The output is fed to a bipolar 1.999-V full-scale digital voltmeter for direct readout of the *%T*. The voltmeter can also be used to obtain a direct readout of various operating parameters.

INSTRUMENT LAYOUT AND OPERATION

A front view drawing of the spectrometer is given in Fig. 1. This instrument consists of two compartments that are separable. One contains the LED mounting post and the electronic control circuitry. The other contains the phototransistor mounting post and magnetic stirrer. When placed together, the total length is 38.5 cm. Any cell up to this length or a titration vessel can be placed on top of the instrument between the centrally mounted LED and detector. For longer cells, the compartments are separated to the needed distance. The connecting cable stored in the stirrer–phototransistor compartment unfolds and thus great flexibility is secured. The mounting posts will accept nearly any LED or phototransistor commercially available.

The power switch, A, controls the power flow to all components except the LED. The LED has its own control switch, B, and adjustment knob, C. The LED switch allows the LED to be turned off for 0*%T* adjustments. The LED current knob allows the LED pulsing current to be adjusted from 0 to 3 A and can also serve as a coarse control for setting 100*%T*. Two limit lights are provided to warn the operator of undesirable operating conditions. The PT limit light, D, will come on when the ambient light correction circuitry is not working. The LED limit light, E, will come on when the forward voltage drop across the LED has reached 20 V, the limit of the drive circuits. It will also come on when the LED is burned out; this warning occurs at very low current levels (<0.1 A). The two "SET" control knobs are used to adjust the readout on the digital display when transmittance values are being presented. The set 0*%T* knob, F, is used to adjust the display to zero for either the 100 or 20*%T* range. The set 100*%T* knob, G, is used as a fine adjustment control. The display selector knob, H, selects the function of the digital display. In the PT bias position, the operating bias of the phototransistor control circuit is shown.

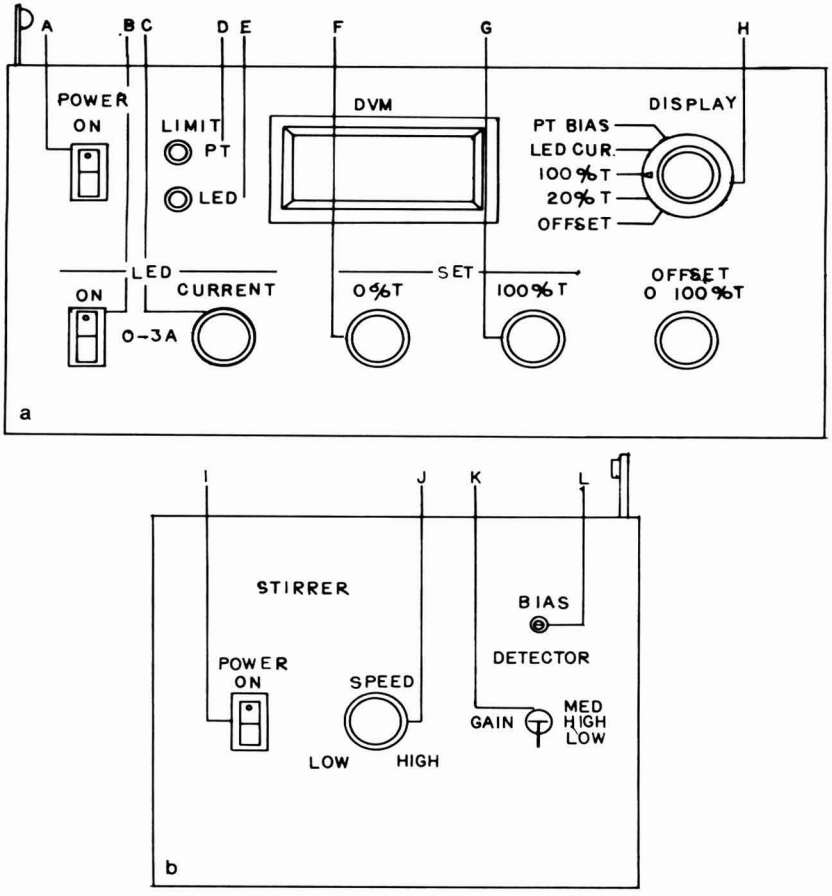

Fig. 1. (a) LED-circuitry compartment; (b) stirrer–photodetector compartment.

The LED current position allows the display of the pulsed current level. The 100 and 20%*T* positions provide for a readout of the percentage transmittance values. The offset position is used to display a direct reading in %*T* of the suppression set by the offset control. The stirrer controls are used to turn the stirrer motor on or off, I, and to control its speed, J. The gain switch, K, is used to allow the selection of one of the three feedback resistors included in the amplifier circuit. The lowest gain which allows a 100%*T* setting within the current rating of the LED should be used. The bias control, L, is provided to allow the operator to adjust the bias voltage in the detector circuitry. A screw adjustment is used to prevent any accidental movement of this control.

TESTING

The pulsed photometer was tested with regard to the exclusion of ambient light, stability, and linearity of response. No attempt was made to evaluate the effects of changing ambient temperature.

The ambient light rejection capability of the photometer was tested by operating the instrument under different ambient light conditions. The photometer was operated with no cell in the optical path for these tests. Readings were taken for operation in a darkened room, a room lighted with a combination of incandescent and fluorescent lamps, and with a high-intensity lamp, which was shown upon the detector within the constraints of the angular response characteristics of the phototransistor used. The LED current was held constant for each gain setting used. No readings were taken for the "HIGH" gain setting. The *%T* could not be adjusted at this setting under the conditions of the test. The results are summarized in Table 1.

The stability of the photometer was evaluated by connecting it through appropriate circuitry to a strip chart recorder set at its highest sensitivity. The photometer was again operated under the conditions of illumination outlined above. The resulting recording was compared to a recording produced by the strip chart recorder alone. No discernible differences were detected over the test period of 8 hr.

The photometer in a field test was powered from a 12-V dc car battery using a Micronta dc-to-ac inverter. When the phototransistor detector was exposed directly to very bright sunlight, saturation effects became noticeable. Under all other conditions, normal operation was possible. The photometer was tested for stability in the same manner as previously described and the output was found to be stable over the test period of 2 hr.

Linearity of response in the laboratory and the field was evaluated from calibration curves using solutions of copper nitrate. The LED used, a Hewlett–Packard 5082-4658 *(10)*, had a peak emission at 635 nm. The detector used was a Fairchild FPT-120 *(4)*. The measurements had to be made on a sloping portion of the absorbance curve because the copper(II)–aquo complex has its absorbance maximum at 739 nm. Solutions of various known concentrations were prepared and absorbances read against doubly deionized water. For a 40-cm cell, solutions in the concentration range 1.0×10^{-6} to 5.0×10^{-5} F were used. The absorbance measurements

TABLE 1
EFFECT OF ILLUMINATION ON THE PULSED PHOTOMETER

Illumination conditions	*%T*	
	"LOW" gain	"MEDIUM" gain
Darkened room	50.0	50.0
Normal lighting	50.7	50.7
Normal lighting plus high-intensity lamp	50.8	50.8

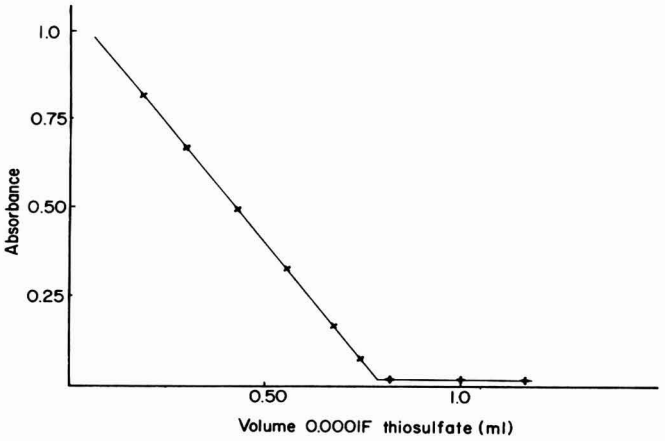

Fig. 2. Curve for the titration of iodine with thiosulfate.

were made using the "HIGH' gain detector setting and a LED current of 1.0 A.

The data were plotted and a best-fitting straight line could be placed through the points without difficulty. The line went through the origin, and the point with the greatest deviation was off from the line only by 0.015 absorbance units and by 1×10^{-7} F. The data were also analyzed by a computer program developed by Dobbs *(3)*. The intercept value reported was +0.039 and the degree of fit 0.74, thus reaffirming the very favorable impression gained from the plot.

The photometer was also used in a photometric titration of iodine with thiosulfate using starch as an indicator. The thiosulfate solution was 0.0001 F and was delivered using a No. 4834 Kimax buret (5 ml, 0.01-ml divisions) fitted with a platinum capillary tip. The titration vessel used was a 25-ml Erlenmeyer flask. Ten milliliters of a 1 μg/ml iodine solution was titrated. The expected volume of thiosulfate at the endpoint was 0.788 ml; the actual volume of the titrant used was 0.785 ml. The titration curve is shown in Fig. 2.

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This work was performed under a National Science Foundation Grant (GP-37376). Mr. Coulter was involved in the initial planning and building of the instrument and provided valuable advice. Mr. Tom Courtney of Fairchild Semiconductor provided the FPT-120 phototransistors free of charge. All this aid is gratefully acknowledged.

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MICROCHEMICAL JOURNAL 22, 557–560 (1977)

Determination of Elemental Fluorine in Organic Compounds by Fluorine-19 Nuclear Magnetic Resonance Spectroscopy

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INTRODUCTION

The versatility of high resolution nuclear magnetic resonance (NMR) spectroscopy in quantitative analysis is well established. Many of the applications and techniques for proton-containing substances have been discussed by Kassler (4). These principles when applied to fluorine-19 NMR spectroscopy have proven very useful in the quantitative analysis of fluorine-containing compounds. For example, Brance and Yeager determined the weight percentage of each component in a mixture of fluorine-containing polymers using the internal standard 2,5-dichlorobenzotrifluoride (*2*). In this paper, we describe the merits of fluorine-19 NMR as a quantitative tool for the determination of covalently bonded fluorine in organic compounds of pharmaceutical interest.

Generally, fluorine in organic materials is quantitated spectrophotometrically (*1,3,6,7*) or titrimentrically using a specific-ion electrode as an end point indicator (*5*). These methods are tedious, time consuming, and beset by many difficulties. For instance, the combustion of the sample to form fluoride ions is sometimes incomplete and a step where losses may occur (*7*). In the preparation of the standard curve, considerable time is involved and optical density readings must be taken promptly since color fading is possible. Interfering ions which are inherent in the sample or created during sample preparation must be extracted in some cases. The proposed fluorine-19 NMR method is rapid, accurate, and highly specific and does not destroy the sample. Interferences are possible from other covalently bonded fluorine in the sample; however, this is unlikely because of the wide range of chemical shifts for fluorine resonances.

EXPERIMENTAL

The fluorine-containing compounds used in this study were prepared in these laboratories and have been shown by other methods to be of highest purity (*8*). Trifluoroacetanilide (TFFA), a convenient internal standard,

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was obtained from BDH Microanalytical Reagents. The NMR solvent dimethylsulfoxide-d^6 was obtained from Merck.

The NMR spectra were run under continuous-wave conditions on a Perkin–Elmer R32 equipped with a fluorine-19 accessory. Fluorine resonances fall at 84.6 MHz at 21.1 kG, the field strength of this instrument. All spectra were obtained on a 20-ppm sweep width with the sample signal approximately 18 ppm upfield from trifluoroacetanilide. A 180-sec sweep rate was used for the integrations. This was sufficient to prevent saturation.

The NMR solutions were prepared by dissolving 10–25 mg of trifluoroacetanilide and 10–40 mg of sample in 0.8 ml of dimethylsulfoxide-d^6. This solution was transferred to a standard 5-mm-o.d. NMR tube and the fluorine-19 spectrum taken. Fluorine resonances of the sample and standard were integrated five times and the average was used for calculating the fluorine content by the following general formula[1,2]:

$$\text{Percentage of fluorine} = \text{ratio} \times \frac{\text{atomic weight of fluorine}}{\text{equivalent weight of TFAA}} \times \frac{\text{weight of TFAA (mg)}}{\text{weight of sample}} \times 100.$$

RESULTS AND DISCUSSION

Figure 1 shows a typical ^{19}F spectrum of a compound of pharmaceutical interest. The fluorine signal of the sample is separated from the reference material (trifluoroacetanilide) by an amount sufficient to allow good integration. The data obtained by the NMR method for 10- to 40-mg samples are comparable to spectrophotometric and titrimetric methods. In addition, there are the added advantages of nondestructive analyses, specificity (the spectrum may also be used for qualitative analysis), and absence of any interfering ions. Table 1 summarizes the results obtained by the NMR method for three compounds of pharmaceutical interest. The accuracy and precision in addition to the rapidity of the analysis recommend the NMR method as the method of choice for fluorine analysis.

It would be possible to operate with much smaller sample sizes if one has access to a pulsed Fourier transform NMR system equipped with fluorine accessories, but for routine fluorine analysis a continuous-wave instrument is adequate.

[1] Ratio = Integration value of fluorine signal in sample/integration value of fluorine signal in TFFA.

[2] Equivalent weight of TFAA = 189.1/3.

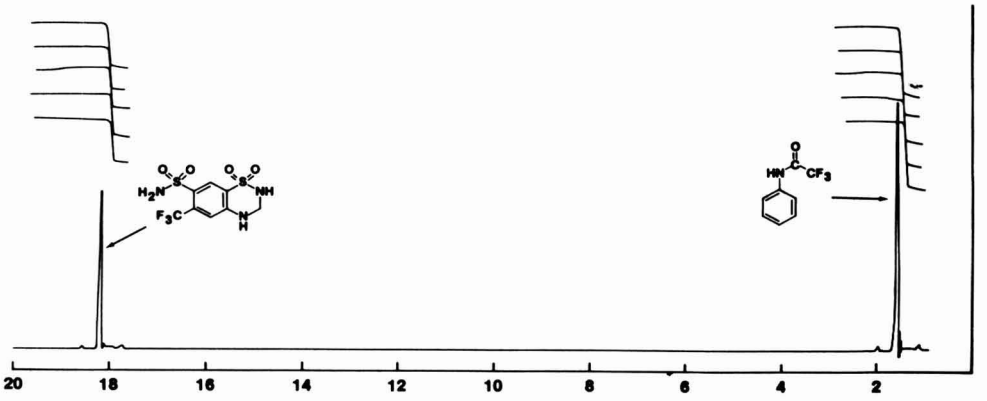
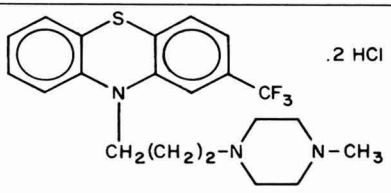
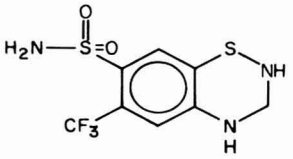
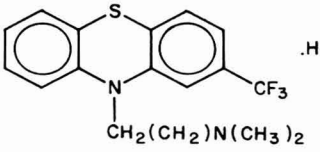


FIG. 1. ^{19}F spectrum of compound of interest and that of reference compound, trifluoroacetanilide.

TABLE 1

	Percentage of F			
	Theory	^{19}F NMR method	C.V. (%)[a]	Accuracy (%)
 .2 HCl	11.87	11.89	3.6	100.2
	17.20	17.30	4.7	100.6
 .HCl	14.66	14.89	5.4	101.6

[a] C.V. is coefficient of variation for five trial integrations.

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Analytical Reactions of Substituted Cyanoferrates

I. $Na_3[Fe(CN)_5(NH_3)]$

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INTRODUCTION

The qualitative and quantitative analytical reactions of cyanoferrates (II) and (III) have been known for quite some time. These reagents produce, in general, insoluble precipitates with heavy-metal cations, but at some extreme, dilution-colored colloidal suspensions are also produced with some transition-metal cations. The latter have been used in the colorimetric analyses of these cations *(8, 13, 18)*. The metal cyanoferrates have also found their use in analytical separations as ion exchangers for lighter elements *(7, 9, 19)*, as reagents for organic compounds *(3)*, and even as collectors for radionuclides *(5, 11, 15)*. In an analogous manner, one can expect the substituted cyanoferrates to behave in somewhat similar fashion. Surprisingly, very scanty information is available in the literature on the analytical interactions of the substituted cyanoferrates with cations or anions, although a series of such compounds has been synthesized in recent years *(12)*.

In this communication we report the analytical reactions of the reagent pentacyanoamminoferrate (II), termed as PCAF, with some cations and anions. The only reported information on its use as a qualitative reagent for cations stems from the work of Harrop and Herington *(10)*, who have characterized some of its colored reactions in conjunction with reubeanic acid. However, this reagent has been employed in the quantitative determination of some compounds such as cyanamide *(4)*, hydroxyurea *(1)*, arylhydroxylamine *(2)*, and methionine *(14)*.

MATERIALS AND METHODS

The reagent pentacyanoamminoferrate was procured from Fisher Scientific Co. A stock solution, 0.01 *M*, was prepared fresh for each set of analyses by accurately weighing 0.7433 g in 250 ml of double-distilled water. In general, it was protected from direct sunlight.

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The cation solutions of appropriate concentration were prepared by dilution from the standard atomic absorption stocks (1000 ppm) also supplied by Fisher Scientific Co. The solutions of Mo^{6+} and VO^{2+} were prepared by weighing pure analytical grade ammonium molybdate and vanadyl sulfate compounds of Fisher Scientific Co.

The buffer employed in this study was prepared by dissolving 62.5 g of CH_3COONH_4 in 175 ml of CH_3COOH and made up to 250 ml volume.

The absorption data were recorded on a Bausch and Lomb Spectronic-70 spectrophotometer using optically matched 1-cm rectangular quartz cells.

PROCEDURE

Place 5 ml of the reagent PCAF in a 50-ml flask and add 2 ml of the buffer solution. Dilute to 40 ml and add 1–5 ml of the diluted cation solution. Bring to final volume with double-distilled deionized water, mix the solution thoroughly, and allow the system to equilibrate for 30 min. Measure the absorbance of each cation–PCAF system at the specified wavelength (cf. Table 1). A similar procedure was followed for anion analyses.

The experimental procedure was slightly modified in the case of Mo^{6+}, since erratic data were obtained for it in acetate medium. In the modified procedure for molybdenum, HNO_3 (pH 6) was employed to control the acidity of the system.

RESULTS AND DISCUSSION

The analytical reactions of PCAF with Fe^{3+}, Fe^{2+}, Co^{2+}, Cu^{2+}, VO^{2+}, UO_2^{2+}, and Mo^{6+} produced colored species in solution in the $<$ 15 ppm

TABLE 1

COMPARATIVE DATA ON ANALYTICAL REACTIONS OF CYANOFERRATES[a,b]

Metal Ion	Color of Complex	$[Fe(CN)_5NH_3]$ ϵ	λ_{max} (nm)	$[Fe(CN)_6]^{4-}$ ϵ	λ_{max} (nm)	$[Fe(CN)_6]^{3-}$ ϵ	λ_{max} (nm)	Medium
Fe^{3+}	Blue	4620	760	1456	760	NR	760	Acetate buffer
Co^{2+}	Pale green	2270	420	1615	420	1917	420	Acetate buffer
VO^{2+}	Bright yellow	3550	420	2280	420	NR	420	Acetate buffer
Mo^{6+}	Brown	6000	500	2110	420	NR	420	HNO_3
Reagent	Light yellow	300	420	NR	420	NR	420	Acetate buffer

[a] ϵ = Molar extinction coefficient.

[b] NR = No reaction observed.

range. However, solutions of Cu^{2+} and UO_2^{2+} are very unstable, such that their colored species start fading minutes after the reagents are mixed. The colored species of iron, vanadium, cobalt, and molybdenum, on the other hand, remain stable for at least 0.5 hr. The reactions of the reagent PCAF, when compared with the unsubstituted cyanoferrates, do show a strong similarity. However, a careful look at the comparative data in Table 1 reveals that the molar extinction coefficients of the PCAF complexes with cations are higher than those observed with cyanoferrates(II) and (III) under similar experimental conditions. This is important since use of the reagent PCAF in an analytical determination would naturally offer an improved sensitivity.

The quantitative data do support this concept since linear spectrophotometric responses have been obtained for Fe^{2+} (0.2–5 ppm), Fe^{3+} (0.1–3.2 ppm), VO^{2+} (0.25–11.0 ppm), Mo^{6+} (1–12 ppm), and Co^{2+} (0.2–6.4 ppm) at the respective peaks of absorption of these ions (cf. Table 1). It is also interesting to note that the coefficient of variation for 0.1-ppm Fe^{3+} was only 6.5%, while it was of the order of 2–4% for other ions at their lowest concentration analyzed. At higher concentrations of these ions, one generally observes colored precipitates which can be employed for gravimetric analyses of these metals. The spectra of the two oxidation states of iron are similar, since both absorb at 760 nm. It is thus evident that iron(III) is reduced to iron(II) in the presence of PCAF. This has been further confirmed by following the iron(III)–ferrozine reaction, which in the presence of PCAF forms the known iron(II)–ferrozine complex, while no reaction is seen with Fe^{3+} in the absence of PCAF *(17)*. This observation has been utilized in the quantization of the ionic forms of iron in some natural aqueous samples *(6)*. The data cited above certainly suggest the possibility of the rapid quantitative determinations of Fe^{3+}, Co^{2+}, Mo^{6+}, and VO^{2+} ions employing their colored colloidal suspensions produced with the reagent PCAF. In our study it has been found to be true provided the other experimental parameters such as pH, period of contact, and reagent concentration are properly regulated.

The system is influenced by the acidity and the period of contact. In general the pH range 3–6 was found adequately suited, and an acetate buffer (pH 4.8) was selected for this purpose. As mentioned earlier in the case of Mo^{6+}, erratic data were obtained in acetate medium; consequently, a change to pH 6 in HNO_3 medium was made to avoid any experimental dispersions. The actual data for Mo^{6+} and VO^{2+} are shown in Table 2. It is seen that at proper pH stabilized readings are recorded up to 30 min. or more after the reagents are mixed. The data for iron and cobalt parallel the vanadium behavior.

The reagent concentration though not critical should remain below 0.001 *M* in the final volume. Any increase either adds to the background

TABLE 2
ACIDITY AND TIME PERIOD EFFECT ON COLOR INTENSITY

Element	pH	Absorbance						
		5 min	10 min	20 min	30 min	40 min	50 min	60 min
Mo^{6+} (500 nm, HNO_3 medium, 8 ppm)	3	0.335	0.320	0.300	0.280	0.270	0.260	0.250
	6	0.333	0.337	0.340	0.343	0.341	0.343	0.330
VO^{2+} (420 nm, acetate medium, 9 ppm)	4.8	—	0.410	0.414	0.413	0.414	0.399	0.388

or accelerates the decomposition of the colored species. The rapid fading is seen as well at lower concentrations of the reagent. The 0.001 *M* concentration thus appears optimal for such determinations.

The absorption peaks of iron and vanadium differ by at least 340 nm. This suggested the possibility of simultaneous colorimetric analysis of these two elements in the same mixture. To demonstrate such a possibility synthetic mixtures of these two ions were analyzed through the system of simultaneous equations *(16)*, since each absorbs somewhat at the absorption peak of the other as seen in Fig. 1. The experimental data in Table 3 do show that one can simultaneously analyze Fe^{3+} and VO^{2+} in

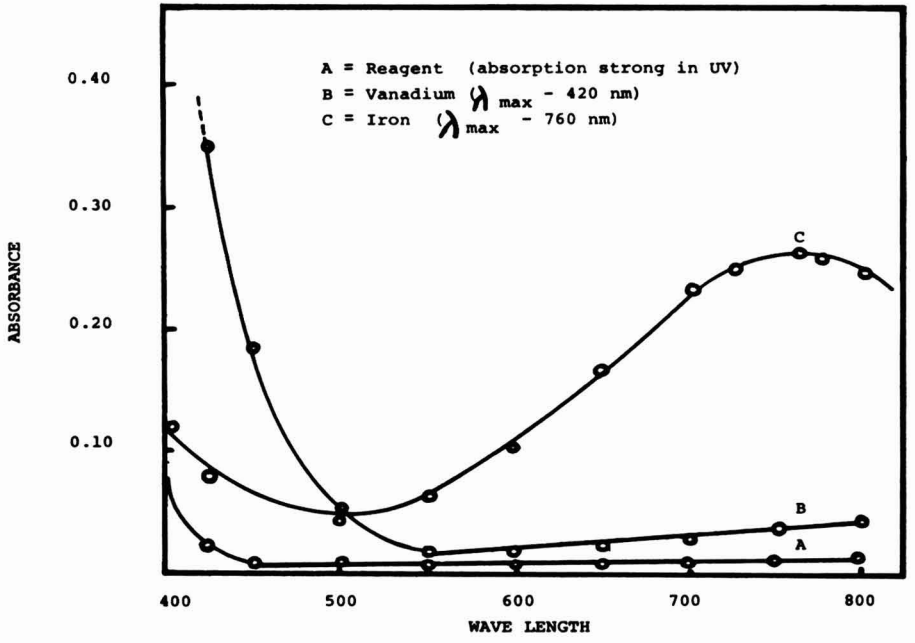

FIG. 1. Absorption characteristics of Fe^{3+} and VO^{2+} in aqueous medium (0.001 *M* PCAF).

TABLE 3
SIMULTANEOUS ANALYSIS OF Fe^{3+} AND VO^{2+}[a]

Amount added (ppm)		Amount found (ppm)	
Fe^{3+}	VO^{2+}	Fe^{3+}	VO^{2+}
2.0	4.0	2.0	4.0
1.0	5.0	1.1	5.0
0.7	8.0	0.7	8.0
0.5	10.0	0.5	10.1
1.7	6.0	1.7	6.1

[a] Molar extinction coefficients: Fe^{3+} = 4620 (760 nm) and 505 (420 nm); VO^{2+} = 640 (760 nm) and 3550 (420 nm).

the same mixture. One may also visualize simultaneous analyses of Fe–Co or Fe–Mo systems in an analogous manner.

In addition, the reagent PCAF has been found reactive toward some anions. It produces a rose-red complex with NO_2^- and a green complex with $S_2O_3^{2-}$ ion. The former absorbs at 540 nm, and in all probability $[Fe(CN)_5(NO)]^{2-}$ ion is produced by replacement of ammonia by NO_2^-. The nitroprusside ion itself absorbs at the same wavelength. The continuous variation plot in the case of $S_2O_3^{2-}$ shows a 1:1 reaction, thus suggesting that the new complex formed has stoichiometry $[Fe(CN)_4(S_2O_3)]^{4-}$. These reactions are not very sensitive but do respond linearly in the milligram range. It appears from these observations that such substitution reactions are possible because of the nonsymmetric character of the reagent molecule $[Fe(CN)_5NH_3]^{3-}$, where ammonia is displaced by a stronger ligand. Many other characteristic reactions can be envisaged if ammonia is replaced by another ligand in the coordination sphere of the reagent. The use of such potential analytical reagents is currently under investigation in our laboratory.

SUMMARY

The reagent pentacyanoamminoferrate (PCAF) in its aqueous reactions with some common cations and anions produces colored species in solution. It has been observed that the reactions of Fe^{3+}, Fe^{2+}, Co^{2+}, VO^{2+}, Mo^{6+}, $S_2O_3^{2-}$, and NO_2^- are sensitive enough to permit their colorimetric determinations. In addition, the reagent has been found useful in the simultaneous trace analysis of Fe^{3+} and VO^{2+} present in the same solution.

A comparative study has been made of the reactions of PCAF and the other unsubstituted cyanoferrates(II) and (III).

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Performance of a New Automatic Carbon Dioxide Coulometer

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INTRODUCTION

The analytical chemist has used a wide variety of methods for the determination of carbon dioxide. The classical gravimetric and volumetric methods, although somewhat tedious, have the distinct advantage of being based on theoretical considerations rather than empirical calibration with known amounts of CO_2. Methods such as thermal conductivity, infrared absorption, electrical conductivity, conversion to methane, and detection with a flame ionization detector all require empirical calibration with known amounts of carbon dioxide and, at least under optimum conditions, are generally less accurate than the classical gravimetric approach. This paper describes a coulometric CO_2 titrator that has the theoretical advantages of classical gravimetric and volumetric methods, but, since the unit is fully automated with digital readout and is computer compatible, the analysis is much easier to perform and more reliable.

Direct titration methods for CO_2 include absorption in nonaqueous or partially aqueous media, usually containing monoethanolamine for conversion of CO_2 to the stronger acid hydroxyethylcarbamic acid. The absorbed CO_2 is titrated with bases such as sodium methanolate *(1,2,11)* or tetrabutyl ammonium hydroxide *(7)*. A problem with the titrimetric methods is that the titrant must be standardized regularly. The author has found that the titrant proposed by Merz *(7)* must be standardized at least daily. In performing large numbers of analyses, large volumes of absorbent and titrant must be dealt with.

If 100% coulometric efficiency can be obtained, coulometry can be used as a standard reference method without the necessity of empirical sample standardization. Several coulometric procedures which are basically extensions of some of the titrimetric procedures have been proposed. Nakamura *et al. (9)* used a coulometric generation of base in an aqueous barium perchlorate medium to maintain a pH of 10 as CO_2 was absorbed. Boniface and Jenkins *(3)* developed a procedure for the determination of carbon in steel using a coulometric titration of CO_2 in a dimethylformamide medium. Ottaway *et al. (10)* mention the use of pyridine-based

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solution for the absorption medium and coulometric titration of CO_2. The dimethylformamide and pyridine solvents have been found by us and others *(8)* to have a significant instability and high solution blank. Isopropanol has been proposed as an alternative solvent *(4,8)*. We have found isopropanol to be a stable solvent, but it requires replenishment after limited usage because of insolubility of the reaction products.

Coulometrics, Inc.[1] has recently placed on the market a CO_2 coulometer and an associated proprietary coulometer solution. The coulometer is designed for high accuracy CO_2 titration and the solution is stable and has a high capacity for the coulometric titration of CO_2. The purpose of this report is to evaluate the performance of the Coulometrics, Inc. CO_2 titrator.

INSTRUMENTATION

Figure 1 shows the subfunctions of the coulometer in block form. A colorimetric end point indicator (thymolphthalein) is used to detect the end point of the titration. The solution is clear with excess CO_2 and turns deep blue as the end point is reached. The titration end point is detected by a photometer set at 612 nm. The output of the photometer drives a 0 to 100% *T* meter on the front panel. The photometer output is compared to preset voltages to determine how far the solution is from the end point. The comparator outputs are decoded by a logic block that drives the current source at an appropriate rate. The current passing through the cell is converted to a frequency by a current to frequency converter. The pulse output is scaled and fed to a counter whose output is a digital display which reads directly in micrograms of carbon. Figure 2 shows the CO_2 coulometer.

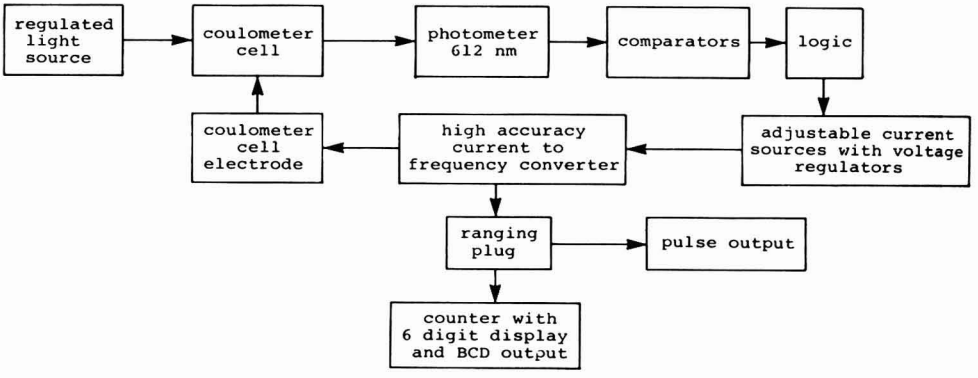

FIG. 1. Block diagram of coulometer system.

[1] Coulometrics, Inc., P. O. Box 544, Wheat Ridge, Colo. 80033.

FIG. 2. Carbon dioxide coulometer.

Calibration of the current to frequency converter is performed electrically by passing an accurately known current through the current to frequency converter and accurately determining the frequency output. The calibration factor is calculated from these data using Faraday's law. As will be shown later, the calibration is stable over long periods and the coulometric efficiency is 100%. Empirical calibration of the coulometer is not required. Since the coulometer requires no sample calibration, it is often useful to use it to check out the rest of the analytical system. Low carbon results generally suggest a leak, whereas high results suggest contamination.

The coulometer was operated as specified in the Coulometrics, Inc. instruction manual.

MATERIALS AND METHODS

The carbon dioxide generator apparatus shown in Fig. 3 was used to generate known amounts of CO_2 for evaluation of the coulometer's capability. Samples of dried primary standard grade calcium carbonate (Mallinckrodt No. 4071) were weighed into porcelain boats. The reaction apparatus was placed in the position shown in Fig. 3 and the sample boat

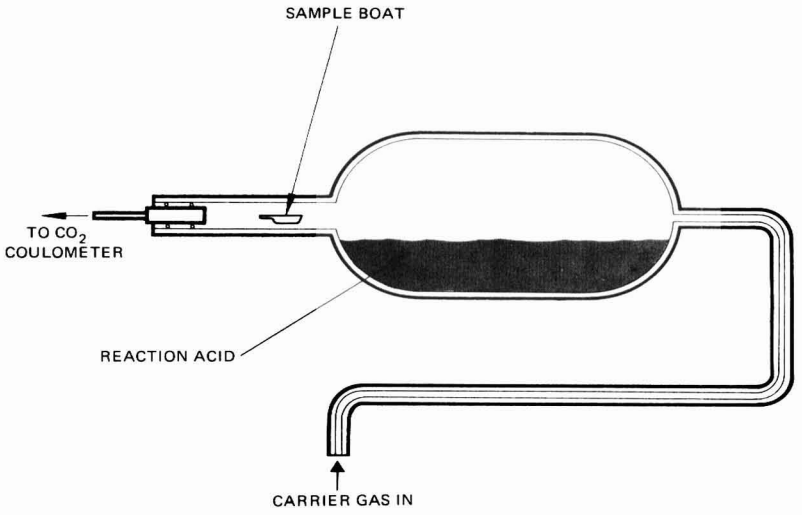

FIG. 3. Carbon dioxide generator.

was placed in the apparatus. The exit plug was replaced and any introduced CO_2 was allowed to sweep out of the apparatus. The apparatus was then inverted so that the sample boat dropped into the reaction acid. The acid was swirled to contact any sample spilled on the wall of the apparatus. A few of the experiments were performed with a Coulometrics, Inc. mineral carbon apparatus which is similar in function to the CO_2 generator described above. The reaction acid used was 1 *N* $HClO_4$ or 2 *N* H_2SO_4.

Carrier gases used include nitrogen, oxygen, helium, and purified (CO_2-free) air. The flow rates were calibrated using a soap-bubble-type flowmeter.

Potentially interfering gases include H_2S, SO_2, NO_2, HCl (or other halogen), Cl_2 and water vapor. Hydrogen sulfide was generated by dropping iron sulfide into the reaction acid. SO_2 was generated by dropping sodium bisulfite into the reaction acid. Nitrogen dioxide was generated by replacing the reaction acid with concentrated nitric acid. Chlorine gas and HCl were introduced to the coulometer by placing gas sampling bottles that had previously been filled with the appropriate gas in the carrier gas line to the coulometer.

RESULTS

The general accuracy and precision of the coulometer are shown in Table 1. Twenty-one runs on standard $CaCO_3$ were randomly selected from data obtained during the running of unknowns. First runs of the day are not included. The data are from 11 different days and 3 different

TABLE 1
RESULTS OBTAINED ON $CaCO_3$ (12.00% C) BY THREE OPERATORS ON DIFFERENT DAYS USING THE COULOMETRICS MINERAL CARBON APPARATUS

Number of runs	Average (% C)	Total range (% C)	Standard deviation (% C)
22	12.01	11.95 – 12.04	0.02

operators. A standard deviation of 0.02 is demonstrated. These results show the high precision of the coulometer and that the coulometer has 100% coulometric efficiency.

Table 2 shows carbon results obtained for calcium carbonate as a function of sample size at a constant carrier gas flow of 200 cm^3/min. In this system, introduction of CO_2 into the coulometer is rapid. However, the coulometer titrates at a maximum rate of about 775 μg/min. Increasing sample size shows the capacity of the solution to hold CO_2 for subsequent titration. Table 2 shows that greater than 99.6% absorption was obtained for up to 8 mg of C. At the 16 mg C level, absorption dropped to 99.1%. As will be shown subsequently, the absorption figures can be increased by decreasing the carrier flow rate or decreased by increasing flow rate. The somewhat larger deviations at low levels are probably due to relatively larger error in the sample weights and lack of correction for the small system blank. The total capacity of the cell before the solution must be changed is over 100 mg C.

TABLE 2
RESULTS OBTAINED ON $CaCO_3$ (12.00% C) AS A FUNCTION OF SAMPLE SIZE AT A CARRIER GAS (AIR) FLOW RATE OF 200 cm^3/MIN

$CaCO_3$ (mg)	C (mg)	C found (mg)	C (%)
1.122	0.135	0.1355	12.08
1.492	0.179	0.1798	12.05
2.077	0.249	0.2495	12.01
4.415	0.530	0.5301	12.01
8.107	0.973	0.9733	12.01
10.844	1.301	1.3041	12.03
15.967	1.916	1.4165	12.00
20.558	2.467	2.4719	12.02
29.195	3.503	3.5104	12.02
29.893	3.587	3.5872	12.00
39.671	4.761	4.7734	12.03
40.366	4.844	4.8346	11.98
53.160	6.379	6.3669	11.98
70.616	8.474	8.4489	11.96
139.733	16.768	16.6149	11.90

Table 3 shows the effect of flow rate on absorption of CO_2 by the coulometer. The results show that absorption efficiency is inversely proportional to sample size and flow rate. Absorption is very efficient at flow rates of less than 200 cm^3/min which are normally used in analytical systems for carbon analysis. For small amounts of carbon, as may be found in trace analysis, it is obvious that higher flow rates can be used.

Interferences were found for H_2S, SO_2, NO_2, HCl, and Cl_2. Moderate amounts of water vapor do not interfere with normal operation of the coulometer. In some cases the coulometer appeared to titrate the interfering gases but these substances also appear to affect the colorimetric end point detection of the system. Most of these interfering gases can be removed with either an aqueous or dry silver scrubber. Nitrogen oxides are best removed by oxidation to NO_2 by a reagent such as sulfuric acid–dichromate on pummice *(6)* and subsequent absorption of NO_2 on specially prepared manganese dioxide *(5)*.

The electrical calibration of the coulometer appears quite stable. Our units have maintained their calibration, within 0.1%, for over 1 year.

SUMMARY

The Coulometrics Inc. CO_2 coulometer has been shown to be an accurate and reliable CO_2 measuring device. The coulometric efficiency is essentially 100%. This means that the method can be considered as a standard reference method for CO_2. As with a CO_2 absorption tube, certain potential interferences must be considered, however, the removal of these interferences is well documented in the literature.

The CO_2 coulometer has found a variety of applications in the author's

TABLE 3
EFFECT OF FLOW RATE ON ABSORPTION OF CO_2 BY COULOMETER

$CaCO_3$ (mg)	C (mg)	Flow rate (cm^3/min)	C found (mg)	C (%)
27.989	3.359	100	3.3669	12.03
28.604	3.432	200	3.4343	12.00
29.259	3.511	300	3.5149	12.01
33.808	4.057	400	4.0381	11.94
5.629	0.675	500	0.6760	12.01
10.311	1.237	500	1.2337	11.96
15.647	1.878	500	1.8706	11.95
35.214	4.226	500	4.1982	11.92
40.733	4.888	500	4.8212	11.84
59.678	7.161	500	7.0263	11.77
30.386	3.646	780	3.5941	11.83
29.781	3.574	780	3.5361	11.87
28.113	3.374	1150	3.2534	11.57

laboratory. The applications, in addition to the determination of carbonate as described here, include total carbon analysis from parts per million to 100% levels, a carbon readout for organic elemental microanalysis, a readout for a modified Unterzaucher oxygen analyzer, and a readout for two different types of trace carbon in water (TOC) analyzers. These applications will be described in detail in future publications.

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Micromethod for the Gas Chromatographic Quantification of Blood Ethanol

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INTRODUCTION

A growing number of reports over the last several years has established the gas chromatograph as the instrument of choice for the quantitative determination of ethanol in biological samples. However, certain considerations made each of the gas chromatographic methods described in the literature inappropriate for use in our laboratory. Frequent blood samples taken from a single laboratory rat over periods of 12 or more hours necessitated limitation of sample size to 20 μl in order to avoid severe changes in the animal's total blood volume. This quantity of blood is less than half that described for most micromethods *(4, 5, 8)*. The need to monitor the constantly changing levels of blood ethanol precluded time-consuming procedures such as distillation, protein precipitation, or extraction of the plasma component of the sample. In addition, the latter procedure ignores variations in hematocrit values, which are known to fluctuate in a circadian fashion. We chose to avoid on-column injection of whole blood since this procedure has been shown to effect changes in column composition and to shorten column life. Some authors *(1, 3, 6)* have circumvented this objection by the use of a variety of filters or other preinjection accessories. However, such devices were unsuitable due to instrument design, lack of availability, or budgetary limitations. The latter consideration precluded the immediate acquisition of an automatic "Head Space Analysis" accessory, although the advantages of injecting only the gaseous components of a biological mixture are obvious. Thus we sought to develop a highly sensitive and reproducible analytical procedure that would allow the rapid quantification of ethanol in very small samples of blood. This has been achieved by modification of the technique of Gessner *(2)*, in which the senstivity of the analysis was enhanced by the conversion of the ethanol and propanol components, presumably to nitrite derivatives. The further addition of an internal standard served as a reference in computing the ethanol concentration of the sample.

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MATERIALS AND METHODS

Reagents

Oxalic acid, sodium nitrite, urea, and 1-propanol, all analytical grade, were purchased from Mallinckrodt Chemical Division of Scientific Products. Absolute alcohol (ethanol), 2-ml ampoules, was obtained from Abbott Laboratories (North Chicago, Ill.).

Apparatus

A Perkin–Elmer Model 910 gas chromatograph with dual hydrogen flame detectors was used. Helium, delivered at 30 ml/min, was the carrier gas. Stainless-steel columns, 6 ft. × ⅛-in. o.d., were packed with Poropak Q/R (Waters Associates, Inc.), 80–100 mesh, and conditioned overnight at 250°C. The operating conditions were column temperature 170°C, injector temperature 190°C, and detector temperature 210°C. A signal fed from the flame detector to a calculating integrator (Spectra Physics, Model 23000-050) and then to a recorder provided both peak areas and heights, as well as elution times. Many workers have determined ethanol concentration in an unknown sample by a comparison of the peak height to that of an internal standard. However, the shape of the peaks may change with variations in operating conditions, injection techniques, or other variables *(7)*. A comparison of the ethanol and internal standard peak areas, as reported by the integrator, was found to give a more reliable estimation of blood-ethanol concentrations than a comparison of their peak heights. However, with the combined use of an integrator and a recorder, the data for either method are available, one serving as a check on the other.

Standard Solutions

An ethanol standard solution, 10 μg/μl, was made by weighing 1.0 g of absolute ethanol from a newly opened 2-ml vial into a 25-ml vial containing about 10 ml of distilled water. This solution was then transferred quantitatively to a 100-ml volumetric flask and made up to volume with distilled water. A 1-propanol internal standard solution was prepared by diluting 1.0 ml to 100 ml with distilled water in a volumetric flask. The above solutions were refrigerated in closely stoppered containers.

Each day the following procedures were followed. Small aliquots of both the above solutions, as well as blood-bank blood, were allowed to come to room temperature. One milliliter of internal standard was diluted to 50 ml with distilled water in a volumetric flask. Five milliliters of this solution were then added to a vial containing 3.5 g of urea. A working reference solution, 400 μg of ethanol/ml of blood was prepared by adding 2.5 ml of propanol–urea solution, 0.5 ml of blood-bank blood, and 20 μl of ethanol standard solution to a small vial.

Assay Procedure

Using a disposable pipet (Dade Diagnostics, Inc.), 100 μl of the propanol–urea internal standard were introduced into a 16 × 100-mm Vacutainer tube (Becton, Dickinson and Co.). With a 20-μl disposable pipet, blood was withdrawn from the amputated tip of a rat's tail and quickly mixed with the propanol by repeated flushing of the pipet. Fifty microliters of a saturated sodium nitrite solution were immediately added with a disposable pipet, the tube was stoppered, wrapped with aluminum foil up to the level of the rubber stopper, and the contents were mixed for 0.5 min on a Vortex mixer. Using a tuberculin syringe, 0.1 ml of saturated oxalic acid was added by piercing the rubber stopper. The tube was again placed on the Vortex mixer for 1.5 min. To complete vaporization of the propanol, the foil-wrapped tube was warmed in a 60°C stationary water bath. At the end of a 10-min period, a 2.0-ml gas syringe (Model A-2, Pressure Lok series, Precision Sampling Corp., Baton Rouge, La.), was inserted through the stopper, and the plunger was withdrawn and depressed twice to mix the gaseous contents of the tube. With the third withdrawal of the plunger to the 2-ml mark, the syringe valve was held in the open position for 15 sec, the valve was closed, and the plunger was depressed to about the 0.4-ml mark. This compression of the gas in the syringe barrel allowed the rapid introduction of a bolus of gas onto the column with resultant sharper peaks. Between successive injections, the syringe was held in a syringe cleaner (Hamilton Co.) that was attached to a vacuum line. This served to remove any condensation that formed in the syringe barrel.

From time to time throughout the day, 100 μl of the working reference solution were transferred to a Vacutainer, mixed with 50 μl of sodium nitrite and 0.1 ml of oxalic acid and heated, following the procedure described for the unknown solution.

RESULTS AND DISCUSSION

The retention time of ethanol was 130–135 sec, and the retention time of propanol was 275–283 sec. The ratios of the area of the ethanol to the area of the propanol in the unknown sample (E/P_u) and in the working reference solution (E/P_r) were used to calculate the concentration of ethanol in the unknown sample (C_u) as follows:

$$C_u \text{ (mg/ml)} = (E/P_u)/(E/P_r) \times 0.4 .$$

The finding of Gessner *(2)* and Komers and Šiř *(3)* that the addition of sodium nitrite and oxalic acid greatly increased peak height was confirmed in our experience. The vapor phase over a heated solution of nitrous acid

with ethanol produced a peak with the same retention time as ethanol. Similarly, the vapor phase over propanol plus nitrous acid produced a peak with the same retention time as propanol alone. A linear response in the E/P ratios was produced by a series of ethanol dilutions in the 25–1600 μl/ml range (Fig. 1). Table 1 shows the reproducibility of multiple determinations made on each of two different concentrations of ethanol.

A 200 or 300% increase in the quantities of sodium nitrite and oxalic acid solutions produced no significant change in the E/P ratios. However, when these same volumes were increased by 400%, the gas pressure that evolved explosively ejected the Vacutainer stopper during the heating process.

The 16 × 100-mm (10-ml draw) Vacutainer proved to be the most suitable size for the range of blood-ethanol concentrations measured in our studies. The volume of smaller Vacutainers could not contain the gas

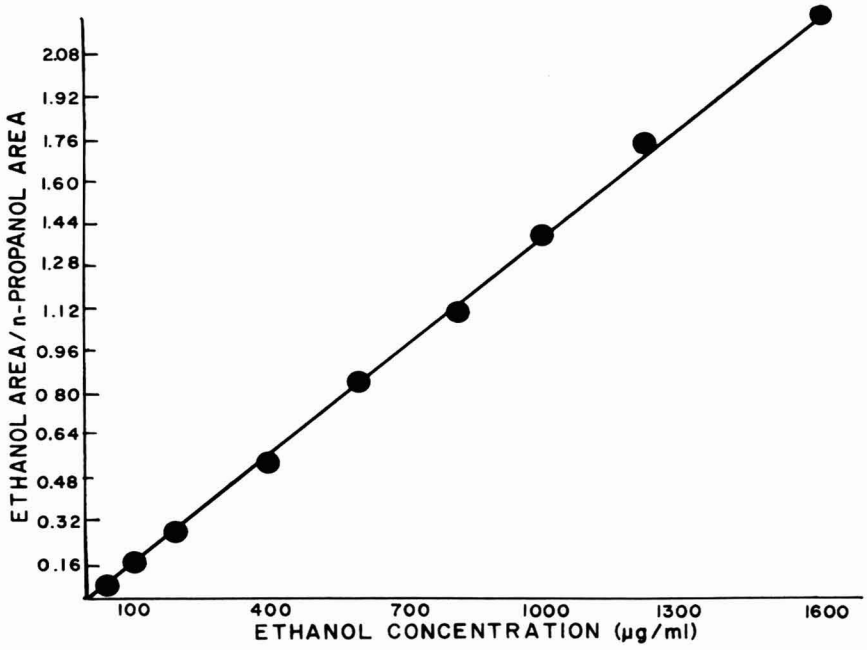

FIG. 1. Values of ratio of ethanol peak area: 1-propanol peak area obtained following chromatographic analysis of 2-ml samples of vapor phase in contact with samples warmed at 60°C for 10 min. Each sample contained 100 μl of a mixture of 20 μl of ethanol in stated concentrations, 0.5 ml of blood-bank blood, and 2.5 ml of 1-propanol (0.2 mg/ml) containing 1.75 g of urea, to which 50 μl of sodium nitrite and 100 μl of oxalic acid had been added. Conditions: 6-ft stainless-steel column, ⅛-in. diameter, packed with Poropak Q/R, 80–100 mesh; column temperature 170°C; injection temperature 190°C; flame ionization detector 210°C. Helium flow was 30 ml/min.

TABLE 1
PRECISION OF ANALYSIS ON TWO BLOOD SAMPLES

Blood contained 0.4 mg/ml of ethanol and 0.2 mg/ml of 1–Propanol ($n=15$)	
Mean (ethanol area/1–propanol area)	0.5300
Standard deviation	0.020
Standard deviation (%)	3.7
Blood contained 0.1 mg/ml ethanol and 0.2 mg/ml 1–propanol ($n=12$)	
Mean (ethanol area/1–propanol area)	0.1245
Standard deviation	0.002
Standard deviation (%)	2.2

evolved. The additional length of 16 × 127-mm tubes presented difficulty in flushing the blood from the 20-μl pipet.

Ten minutes was found to be the optimal time for complete vaporization of the propanol in the mixture, as the E/P ratio was inconsistent in samples removed from tubes heated for a shorter period. However, multiple samples from a single tube heated for periods of 1–2 hr resulted in E/P ratios reproducible to within 4%. In establishing the linearity of the standard curve dilutions, four or five runs were made on each dilution.

Since Gessner *(2)* reported a stabilizing effect of urea on the reaction, it was incorporated into the present adaptation. Similarly, we followed Gessner's technique of wrapping the reaction container in aluminum foil so as to decrease the light, presumed to have a catalytic effect upon the reaction. In addition, the foil served to insulate the tube and thus to contain the heat of reaction. Gessner was troubled with the appearance of large negative peaks, which he avoided by purging the reaction vessel with nitrogen. As we did not encounter this problem, possibly due to differences in the carrier gas employed or other chromatographic conditions or to the smaller sample size, this step was eliminated.

It was found that samples of blood mixed with internal standard could be stored at 4°C for up to 12 hr, if necessary, without significant discrepancy between the E/P ratios of these samples and of duplicate samples analyzed immediately. Later, the refrigerated samples were allowed to warm to room temperature for 5 min, sodium nitrite was added, and the sequence of mixing, heating, and injecting into the chromatograph proceeded, as outlined above. If the sodium nitrite, alone or in combination with oxalic acid, was added prior to refrigeration, E/P values were significantly different from similar samples analyzed immediately.

SUMMARY

A microadaptation for the head space analysis of ethanol in 20-μl blood samples is described. Ethanol concentration is determined by comparison of the derivatized ethanol peak

area with that of a 1-propanol internal standard. Reaction of the alcohols with nitrous acid followed by a warming of the mixture greatly enhances peak sizes. The method is rapid, sensitive, and highly reproducible for the concentrations encountered during the linear phase of the ethanol elimination curve in laboratory rats.

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A Simple Correction for Errors in Measuring Areas of Attenuated Chromatographic Peaks

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One of the simplest and most convenient methods of manually measuring the areas under chromatographic peaks is by assuming that the normal distribution curve is approximated by a triangle (*1*). The area is then calculated as the product of the peak's height and its width at one-half its height. The area determined in this manner is 6% less than that obtained by integrating the normal distribution equation,[1] but this is immaterial when percentage compositions are being calculated, since the areas of all peaks are underestimated by the same factor.

Peaks with vastly different areas can be measured in the same chromatographic run if the larger peaks are attenuated during their recording. This, however, involves some prior knowledge of the relative amounts of the various components so that the appropriate attenuation can be selected before the emergence of the peak. A simpler way to do this is initially to set the attenuation to the value appropriate for the lowest peaks (the least attenuation), and when higher peaks emerge, increase the attenuation as the recorder goes off scale. The attenuation is decreased in steps to its original value after the peak maximum has passed, producing a recorder tracing like that shown in Fig. 1.

When the baseline coincides with the recorder zero, the peak height is simply the height (measured from the baseline), h_b, times the attenuation (this is 4 in Fig. 1), and the width at one-half height is most easily measured as shown in Fig. 1. Thus, the area is given by Eq. (1),

$$A = 2^n h_b w_m, \tag{1}$$

where 2^n is the attenuation (n is zero or an integer) and w_m is the width measured as described. However, when the baseline does not coincide with the recorder zero, these measurements underestimate the peak height by $b_0(2^n - 1)$, where b_0 is the displacement of the baseline from zero. This

[1] The area obtained by integration of the normal distribution curve is $A = hw\,(\pi/(4 \ln 2))^{1/2} = 1.064\,hw$, where h and w are the peak's height and width at one-half height, respectively.

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is partially compensated for in the calculated area because the width is measured below one-half of the peak height and is, therefore, overestimated. Although it would be possible to obtain correct measurements for the peak height and width, this would involve measuring the width at a height that would have to be computed. It is far easier to make measurements at the easily discernible places shown in Fig. 1 and then correct for their inadequacies when calculating the area.

The correct equation for the area of an attenuated peak is Eq. (2) (see Appendix for the derivation),

$$A = 2^n h_b w_m \left[\left\{ 1 + r\left(\frac{2^n - 1}{2^n} \right) \right\} \left(\frac{\ln 2}{\ln\left(2 \left\{ \frac{2^n + r(2^n - 1)}{2^n + r(2^n - 2)} \right\} \right)} \right)^{1/2} \right], \qquad (2)$$

where r is b_o divided by h_b. Equation (2) reduces to Eq. (1) when r is zero; i.e., when the baseline coincides with the recorder zero. It is also apparent that the correct area can be obtained by multiplying the area calculated from Eq. (1) by the factor in brackets. This factor does not depend on the absolute values of the peak height, the width at one-half height, or the

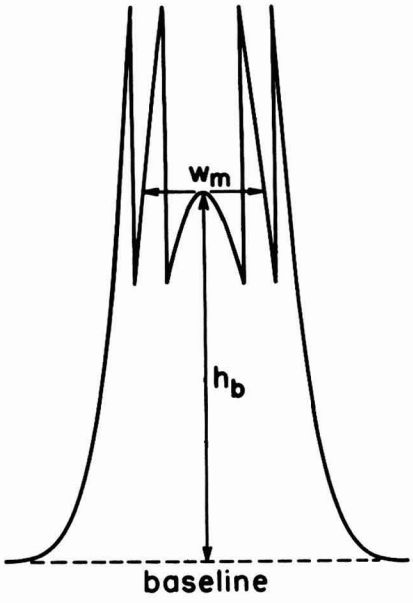

FIG. 1. Diagram of a recorder tracing of an attenuated chromatographic peak.

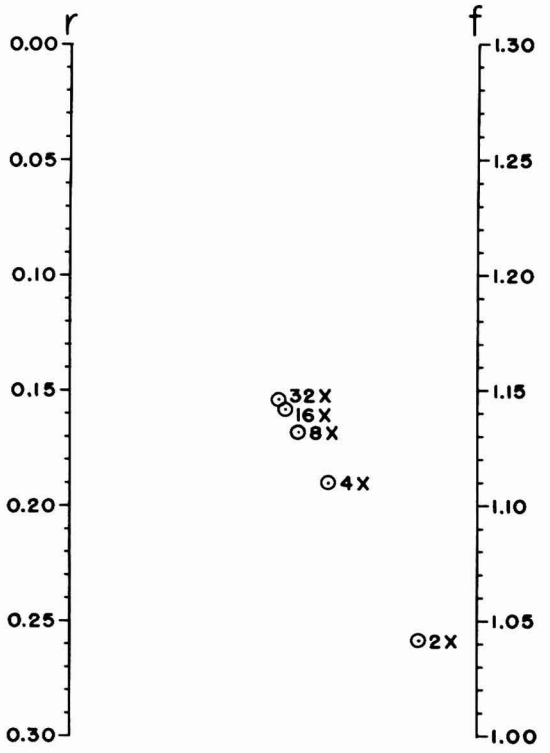

FIG. 2. Nomograph for correcting areas of attenuated chromatographic peaks. Calculate r from the values of b_0 and h_b (see text). Draw a straight line from this value of r (on the left scale) through the point corresponding to the attenuation at which the peak was recorded to the f scale (on the right). Read the value from the f scale and multiply the uncorrected area by this factor.

displacement of the baseline from zero. It depends only on the baseline displacement relative to the attenuated peak height and the attenuation. A nomograph for obtaining this factor is provided in Fig. 2. Figure 3 shows the error for various relative displacements of the baseline at several attenuations which results when Eq. (1) is used to calculate the area.

This method was used to determine the fatty acid composition of bacterial lipid extracts, where the relative amounts of the fatty acids varied greatly and low attenuation was needed for quantitation and identification of minor components, but when major components emerged the attenuation was increased in steps to allow their measurement. Since the baseline did not always correspond to the recorder zero, the areas of the attenuated peaks were multiplied by an appropriate factor to compensate for underestimation.

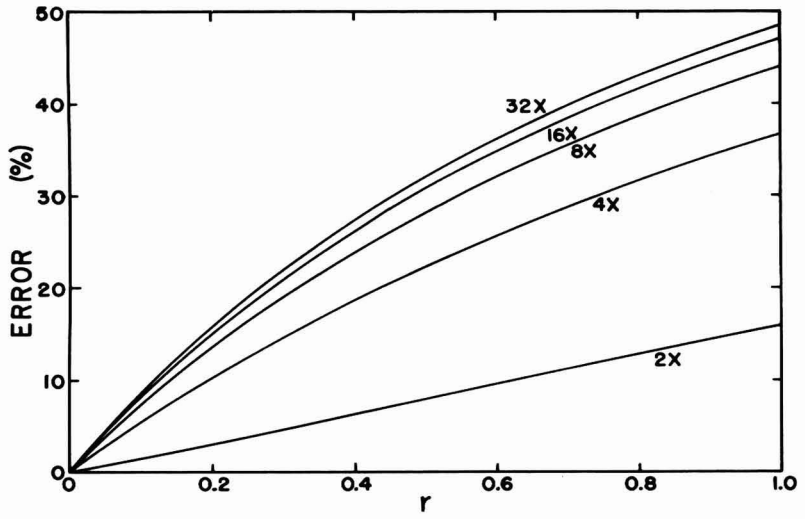

FIG. 3. Error involved when the area is calculated from Eq. (1) (see text) at various attenuations and values of r. The error is related to the correction factor, f, by the equation, error $=100\ (1 - (1/f))$.

APPENDIX

The distances from the recorder zero to the tops of the peaks that would be traced at attenuations of $2^0, 2^1, 2^2, \ldots, 2^i, \ldots, 2^n$ are designated $d_0, d_1, d_2, \ldots, d_i, \ldots, d_n$. They are related to each other by Eq. (A1).

$$d_i = 2^{j-i} d_j, \tag{A1}$$

A corresponding equation, relates the distances from zero to the baselines at the various attenuations (Eq. (A2)),

$$b_i = 2^{j-i} b_j. \tag{A2}$$

The height of the peak at any attenuation is the difference between d_i and b_i (Eq. (A3)),

$$h_i = d_i - b_i. \tag{A3}$$

If $j=n$ in Eq. (A1), then d_i is expressed in terms of d_n, which is the sum of the two measured quantities h_b and b_o (see Fig. 1), and setting $j=0$ in Eq. (A2) allows b_i to be expressed in terms of b_o. If these values for d_i and b_i are employed in Eq. (A3), Eq. (A4) is obtained:

$$h_i = 2^{n-i} h_b + b_o \left(\frac{2^n - 1}{2^i} \right). \tag{A4}$$

The height of the unattenuated peak (Eq. (A5)) is obtained by letting b_o/h_b be represented by r and $i=0$ in Eq. (A4),

$$h_o = 2^n h_b \left\{1 + r\left(\frac{2^n - 1}{2^n}\right)\right\} . \quad \text{(A5)}$$

The width at one-half height is calculated from the measured width by the following consideration. The equation for the normal distribution curve is given in Eq. (A6),

$$y = y_o e^{-(4x^2 \ln 2)/\omega^2} \quad \text{(A6)}$$

where y_o is the maximum height and w is the width at one-half height. Equation (A7) is obtained by solving Eq. (A6) for w:

$$w = 2x\left(\frac{\ln 2}{\ln y_o/y}\right)^{1/2} . \quad \text{(A7)}$$

When the width is measured as shown in Fig. 1, y_o is obtained from Eq. (A4) by letting $i=n-1$ (Eq. (A8)),

$$y_o = h_{n-1} = 2h_b + 2b_o\left(\frac{2^n - 1}{2^n}\right) , \quad \text{(A8)}$$

and the measured width, w_m, equals $2x$ when y equals $d_n - b_{n-1}$. Since d_n is the sum of h_b and b_o, and b_{n-1} can be expressed in terms of b_o (Eq. (A2)), an expression for y is obtained (Eq. (A9)):

$$y = h_b + b_o\left(\frac{2^n - 2}{2^n}\right) . \quad \text{(A9)}$$

The equation for the width (Eq. (A10)),

$$w = w_m \left(\frac{\ln 2}{\ln\left(2\left(\frac{2^n + r(2^n - 1)}{2^n + r(2^n - 2)}\right)\right)}\right)^{1/2} , \quad \text{(A10)}$$

is obtained by replacing b_o by rh_b in Eqs. (A8) and (A9) and making the appropriate substitutions for y, y_o, and $2x$ in Eq. (A7). The area (by triangulation) is the product of Eq. (A5) and (A10) (Eq. (A11)):

$$A = 2^n h_b w_m \left[\left\{1 + r\left(\frac{2^n - 1}{2^n}\right)\right\}\left(\frac{\ln 2}{\ln\left(2\left\{\frac{2^n + r(2^n - 1)}{2^n + r(2^n - 2)}\right\}\right)}\right)^{1/2}\right] . \quad \text{(A11)}$$

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MICROCHEMICAL JOURNAL 22, 585–591 (1977)

BOOK REVIEWS

Handbook of Intermediary Metabolism of Aromatic Compounds. By B. L. GOODWIN. Wiley (Halsted), New York, 1976. ix + 142 + 648 pp. $75.00.

This is really two books within one cover. The smaller unit gives a concise and well-organized review of the enzymes and reactions involved in the metabolic processes of aromatic compounds. Each enzyme or reaction is treated separately with an appropriate bibliography. For example, tyrosine hydroxylase is given a full page of text and a full page of references, whereas the reduction of *N*-hydroxyamides receives two lines and one citation. There is a separate index for this section.

The remaining portion of the book (over 80%) is arranged in dictionary fashion. A compound name is given followed by an indication of its source and/or its fate, sometimes the organism involved is cited and finally a list of references.

About 7000 compounds have been listed. The author was faced with two major problems: first, which compounds to include. An arbitrary decision was made to exclude all aromatic compounds not having at least one six-membered aromatic carbon ring. Thus nicotinic acid was omitted.

The second problem was what name to use for each entry. No structures or empirical formulas are used. The author considered that the users would not be conversant with the details of systematic nomenclature and chose to favor trivial names, those most likely to be known and used by biochemists and pharmacologists rather than to try to please the pure chemists. He has suggested that the user might consult such a reference as the "Merck Index" for alternate names.

This handbook was not meant to be a critical review and some entries are not confirmed. However, this is a convenient starting point since there are ample references.

In view of the rather hefty price, this reviewer would like to see the first and smaller section published as a separate entity. The pagination indeed suggests that this may have been contemplated.

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Principles and Techniques of Electron Microscopy, Volume 6. Biological Applications. Edited by M. A. HAYAT. Van Nostrand Reinhold, New York, 1976. xix + 355 pp. $27.50.

Volume 6 of the Hayat's continuing series discusses the principles and application of eight major techniques employed in the study of the structure, composition, and location of cellular components. The areas covered are high voltage electron microscopy (EM), the principles of high resolution EM, contrast and image formation of biological specimens, the analysis of biological structure with X-ray diffraction techniques, tilting experiments in the EM, electron autoradiography of free specimens, cryoultramicrotomy, and electron interference microscopy.

The basic approach and format in this volume are similar to those in the previous five volumes. It consists of eight chapters presenting the materials of eight major techniques

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which have been tested for their reliability, and are the best of those currently available. It has been developed over the years through the joint effort of nine distinguished author–scientists and is considered as one of the most comprehensive compilations of methods developed and used by a large number of competent scientists in the fields specified. Besides, the instructions given in the text for the preparation and use of various solutions, media, stains, and apparatus are straightforward and complete, and will enable the worker to prepare his specimens for EM study without outside help. In addition, whenever possible, it also suggests some new concepts and points out the areas of disagreement and current potential research problems. Each chapter is provided with an exhaustive list of references with complete titles. Full author and subject indexes are included at the end of the book.

The beginning chapter by C. Humphreys introduces some of the important aspects of high voltage EM in biological applications. General principles, instrumentation, specimen preparation, and practical applications such as environmental wet cells and living specimens are concisely demonstrated with the aid of some 17 fine electron micrographs.

In Chapter 2, J. M. Cowley discusses critically the principles of high resolution EM. High resolution refers to the imaging of the distribution of molecules or atoms from the point of view of the specimens. The chapter therefore places a particular emphasis on this aspect. Both ideal imaging such as Abbe theory, phase contrast, optimum defocus–bright field, dark field images, chromatic aberration effects, and strong phase objects and practical considerations on radiation damage, minimum-exposure microscopy, scanning transmission EM (STEM), and inelastically scattered electrons are extensively described and illustrated. This thus provides the reader with a background of ideas and a sketch of some theoretical concepts for the appreciation of the potential future developments as well as the present accomplishments. Future developments are certainly going to be very great in a number of fields.

Chapter 3 by R. E. Burge is concerned with imaging by phase contrast of biological specimens. It includes some aspects of imaging by both elastically and inelastically scattered electrons. Three principal problems with possible solutions in image analysis and interpretation are comprehensively considered here first, the determination of image wavefunction as two-dimensional projected (through the specimen thickness) distributions of wave amplitude and phase relative to the incident electron beams; second, the relationship between the image wavefunction and the object wavefunction; third, the relationship between the object wavefunction and the object structure. The argument is, however, primarily directed towards TEM, although many of the points are also relevant to STEM.

In the longest chapter, Chapter 4, A. McPherson, Jr. reviews and describes in detail the use of X-ray diffraction techniques in elucidating biological fine structure. The materials of the chapter are well organized and the subject matter is comprehensively treated. The important subjects include principles of X-ray diffraction analysis, preliminary analysis, data collection, methods for structural determination, the image mapping, and interpretation. The complete discussion of the three proven techniques for obtaining the structure of a given molecule in its crystal diffraction intensities by (1) combination of the Patterson techniques and the heavy atom method, (2) direct mathematical methods, and (3) isomorphous replacement is very useful and valuable. Some 62 intelligently designed figures and micrographic patterns are provided and 200 references are cited. This chapter deserves to be studied carefully by those scientists who are interested in structure analysis.

The succeeding chapter by R. H. Lange deals with tilting experiments in EM. It clearly demonstrates that specimen tilting is an efficient means for studying all kinds of anisotropic structures which are important constituents, (e.g., membranes, vesicles, filaments, and crystals) of cells and tissues.

Chapter 6 by N. M. Maraldi considers the ultrastructural analysis of free specimens by

electron autoradiography. It reports the application of autoradiography to isolated macromolecules using shadow-casting, positive staining, and negative staining techniques. Some advantages of these techniques are also critically evaluated and verified.

In Chapter 7, R. Simard reviews all the necessary steps involved in cryoultramicrotomy and describes a few applications in cytochemistry and autoradiography. With a clear understanding of its possibilities and limitations, cryoultramicrotome technique can be utilized in many fields of research concerned with the elucidation of structure–function correlation problems.

The last chapter, by T. Hibi and K. Yada, recommends a new development of electron interference microscope techniques. Instrumentation, its advantages and limitations, and applications are extensively illustrated. It is anticipated that this technique will be actively utilized in local inner potential determination, thickness estimation of samples, and electron holography in both biological and nonbiological fields in the future. Some 33 illustrative figures and micrographs are presented.

In brief, as presented in the previous volumes, this book provides straightforward and complete instruction and information concerning the efficient use of EM techniques to the study of biological systems. It should promise to become a standard reference in the field. Biologists, biomedical scientists, biochemists, biophysicists, structural chemists, and other health scientists and research workers should keep an up-to-date volume for permanent use.

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Wilson and Wilson's Comprehensive Analytical Chemistry, Volume 7. Edited by G. SUEHLA. Elsevier, Amsterdam New York, 1976. ix + 322 pp. $49.95.

The aim of Wilson and Wilson's series on comprehensive analytical chemistry, of which this is the seventh volume, is to present monographs on analytical techniques which are essentially complete reference works for the analytical scientist. In this volume, two highly useful analytical techniques are presented by internationally known experts in these areas.

The book consists of two parts: Part I, "Thermal Methods in Analytical Chemistry" by C. Duval of the Laboratoire de Recherches Microanalytiques, Paris, France; and Part II, "Substoichiometric Analytical Methods" by J. Stary of the Fakulta Jaderna a Fyzikalne Inzenyrska, Kafedra Jaderna Chemie, Praha, Czechoslovakia and J. Ruzicka, Technical University of Denmark. Part I includes six sections on thermogravimetry, applications of the thermobalance, precautions to be observed in using the thermobalance and differential and simultaneous thermal analysis. Part II also includes six sections on the theory of substoichiometric analytical methods, instrumentation used, methodology, and practical applications. The book includes a number of useful tables, figures, and illustrations plus extensive lists of references.

This book is a welcome addition to the available reference works on analytical chemistry techniques and in keeping with the previous excellent volumes in the series on comprehensive analytical chemistry.

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A Dictionary of Chromatography. By R. C. DENNEY. John Wiley & Sons, New York, 1976. xi + 191 pp. $14.50.

In every field of specialization there inevitably is created a vocabulary and usage of words that have a unique meaning for those actively working in the area. This "jargon" of the field can act as a formidable barrier to the student entering the field or for the nonspecialist wishing to gain further information about the field. The author has prepared the dictionary to meet the needs of these individuals for easily accessible information on chromatographic terms and concepts.

The book consists of 176 pages of short discussions of chromatographic terms and concepts. The terms range from "absolute detector sensitivity" to "zones." In order to further aid our understanding of the concepts presented, a total of 321 references to books and articles on chromatography are included. The book also includes a two-page list of symbols pertinent to chromatography. The book is packed with illustrations which greatly aid the reader's understanding of the terms and concepts presented. Each term or concept is clearly and concisely discussed and cross-referenced to related terms or concepts.

Although some may quarrel as to the value of some of the terms or concepts included or excluded, the book includes most of those which the student or nonspecialist might encounter and would definitely be helpful.

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Principles of Mössbauer Spectroscopy. By T. C. GIBB. Wiley (Halsted Press), New York, 1976. 254 pp. $27.50.

Atomic resonant fluorescence, in which a photon emitted during a change in the electronic state of an atom is absorbed by another atom, was discovered early in this century. Predictions of similar phenomena involving nuclear excited states were made some fifty years ago, but observation of these nuclear resonances was difficult prior to Rudolf Mössbauer's experiments in 1957. The problem is that nuclear energy levels have extremely narrow line widths, and the recoil of the nuclei upon emission or absorption of photons is sufficient to destroy the resonance conditions. In addition, Doppler broadening, due to the thermal motion of the nuclei, spreads the lines and decreases the overlap of the emission and absorption energy distributions. Mössbauer discovered that these problems could be obviated by having the nuclei bound in a crystal at low temperatures. Under these conditions, the recoil momentum for a substantial portion of the photons is taken up by the crystal as a whole, with the result that very little energy is transferred to the lattice vibrations, and the resonance is greatly enhanced. It is possible to measure such small frequency shifts as those induced by variations in the chemical environment of the nuclei or those produced by the action of gravity upon the photons. The technique is to restore resonance by producing a Doppler shift by imparting a relative velocity of the order of centimeters per second to the source and absorber.

This book, by T. C. Gibb of the University of Leeds, demonstrates the great versatility and tremendous power of Mössbauer spectroscopy. The emphasis is on applications to chemistry, but uses in physics, biology, and metallurgy are also treated in considerable detail.

The first chapter presents the background and the basic theory. Very little actual derivation

is given, but the essential equations are accompanied by lucid descriptions of the physical principles involved. For example, about two pages are devoted to a discussion of lattice dynamics, culminating in the formula for the recoil-free fraction that results from the Debye model; more details are given in a subsequent chapter, but this is adequate coverage of the essential points at this stage.

The origin and theory of hyperfine interactions are discussed in Chapter 2. Since the Mössbauer technique is able to measure such tiny frequency shifts (1 part in 10^{12}), it is possible to observe the effect of the chemical surroundings on the energy levels of the nucleus. The so-called chemical isomer shift is produced by the interaction of the nucleus with *s* electrons, which have a finite probability of being inside the nucleus. The total electron configuration influences the extent of this shift because *p*, *d*, and *f* electrons shield the nucleus from the *s* electrons. Thus, the shift is sensitive to variations in the chemical environment. Magnetic hyperfine splitting of a Mössbauer spectrum results from the interaction of the magnetic moment of the nucleus with a magnetic field. If the spin quantum number of the nucleus is I, the magnetic field splits each energy level into $2I + 1$ sublevels. The magnetic field may be applied externally, or it may be natural to the crystal, as, for example, in a substance containing paramagnetic ions. The third hyperfine interaction discussed in this chapter is the interaction between the nuclear quadrupole moment and the electric field gradient at the nucleus. This effect also produces line splitting, and the magnitude of the interaction depends upon the chemical environment of the nucleus.

The remaining nine chapters of the book describe the chemical applications of Mössbauer spectroscopy. Chapter 3, on "Molecular Structure," discusses structural investigations containing ^{57}Fe (the most important Mössbauer nuclide), ^{119}Sn, and ^{129}I. Several pages are devoted to the iron carbonyls, but other examples are reported too, including the Mössbauer proof of the identity of Prussian blue and Turnbull's blue. Chapter 4 discusses diamagnetic compounds, where the chemical isomer shift and quadrupole splitting provide valuable information about oxidation states and chemical bonding. The paramagnetic compounds (mainly of iron) treated in Chapter 5 have the additional complications of a hyperfine splitting produced by the internal magnetic field and a quadrupole interaction that is temperature dependent.

Chapter 6, entitled "Dynamic Effects," describes the information that Mössbauer spectroscopy can give on lattice vibrations, on diffusion, and on paramagnetic relaxation. A shift in energy, called the second-order Doppler shift, is produced by the atomic vibrations; the energy decrease is proportional to the mean-square velocity of the atoms, and the mean-square displacements of the atoms are related to the recoil-free fraction of emissions. Although the problems of lattice dynamics are far too formidable for any simple technique, Mössbauer spectroscopy can provide some useful data; an example given in the book is the determination of the anisotropy of vibrations of cobalt impurity atoms in a crystal of zinc. Atomic diffusion is manifested in Mössbauer studies by line broadening. Paramagnetic relaxation processes that are sufficiently slow can result in hyperfine interactions between the nuclear and electronic spins; examples are the temperature-dependent spin–lattice relaxation of Fe^{3+} ions and the concentration-dependent spin–spin relaxation involving neighboring ions.

The next two chapters are on specific chemical systems. Chapter 7 treats "Oxides and Related Systems," in which the dominating structural feature is the close packing of anions. Extensive consideration is given to the stoichiometric spinels with formula AB_2O_4, the nonstoichiometric spinels, and the rare-earth iron garnets. Chapter 8 is on "Alloys and Intermetallic Compounds," which is one of the principal areas of application of Mössbauer spectroscopy. The Mössbauer technique probes the interactions between nearby atoms, and it is thus sensitive to the details of structure and ordering.

Chapter 9 describes some "Analytical Applications." A unique analytical advantage of Mössbauer spectroscopy is that it can distinguish nondestructively between oxidation states of cations, and several examples of such cases are given. Also included are applications to silicate analysis and to the study of surfaces. Next is a chapter on "Impurity and Decay

After-effect Studies," which discusses two main topics: (1) methods of monitoring the properties of a material by incorporating a small amount of a Mössbauer nuclide into the host lattice, and (2) the effects of the decay of the Mössbauer precursor upon the chemical environment. The final chapter is on applications to the very important but difficult biological systems.

This book will serve both as an excellent introduction to the chemical uses of Mössbauer spectroscopy and as a reference book for the active researcher. It is concise without being superficial. It does not purport to offer a comprehensive review, but it does provide 232 literature references with citations up to 1973. Another valuable feature is a bibliography of 14 books and 17 review articles. This is an excellent book; it may be recommended as a place to start learning about this powerful chemical technique, as a means of refreshing one's knowledge, or as a summary of what is new and exciting in the field.

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Life's Basis: Biomolecules. By GARY E. PARKER AND THOMAS R. MERTENS. John Wiley & Sons, New York, 1973. viii + 158 pp. (paperback). $2.95.

This booklet is not a text but can be so used by the teacher; neither is it just a workbook but can be used as such by the student. There is no running text. The five divisions (carbohydrates, proteins, nucleic acids, lipids, and energy) consist of self-explanatory diagrams and informative statements, each statement being followed by a question (and answer). A self-test follows each division and, at the end of the book, there is a final self-test (both with answers). There are two prefaces—one an explanation of the program and the other a guide for its use, plus a bibliography and an adequate index.

The book is elementary—a high school background is sufficient—but the approach is ingenious and should be very successful.

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Current Topics in Microbiology and Immunology. Volume 72. Edited by W. ARBER, 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, AND P. K. VOGT. Springer–Verlag, Berlin, 1975. 194 pp. $33.70.

This is Volume 72 of a continuing series. The five articles are: Cell Division and DNA Replication in Synchronous Tetrahymena Cultures (H. A. Andersen, L. Rasmussen, and E. Zeuthen); Humoral Factors Abrogating Cell-Mediated Immunity in the Tumor-Bearing Host (R. W. Baldwin and R. A. Robins), Inhibitory *T* Cells (G. L. Asherson and M. Zembala); The Slow Infection Caused by Visna Virus (A. T. Haase); and Transfer of Antitumor Immunity by "Immune" RNA (Y. H. Pilch, D. Fritze, S. R. Waldman, and D. H. Kern). The high standards of writing and editing, as well as the timeliness of the topics typical of previous volumes have been maintained in this one.

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Handbook of Analysis of Organic Solvents. By VÁCLAV ŠEDIVEC AND JAN FLEK. Halsted Press, New York, 1976. 456 pp. $42.50.

The first eighty-three pages of this handbook are devoted to presenting general principles of sampling and treatment of samples, determination of basic physical properties, procedures for the analysis of an unknown or of an unknown two- or three-component system, and a brief review of chromatography.

At the end of the volume, there are a series of tables of physical properties, a list of trade names and composition of solvents, a list of 1012 references, and an index.

The great bulk of the book is divided into sixteen chapters, each of which deals with an individual solvent class. Each class, alkanes, alcohols, etc., is described in general terms; methods of detection, characteristic derivatives, and methods of determination are presented, and finally, important solvents in the class are described with special reactions of individuals. Tables of properties are included.

Since this is the type of book likely to be used by inexperienced chemists, the reviewer feels that safety was passed over rather lightly.

One other complaint is that several pages in the index were blank.

The authors seem to have achieved a compact one volume source book on the analysis of organic solvents.

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ANNOUNCEMENT

INTER/MICRO Returns to Chicago

INTER/MICRO-78, the annual international conference on microscopy, returns to the United States from the 1977 conference in Cambridge, England. It will be held at the McCormick Inn at 23rd Street and the Lake, Chicago, Illinois 60616.

The meeting, to be held 23–27 July 1978, is expected to be attended by about 250 microscopists and other scientists who will hear and see approximately 60 technical papers describing the most recent advances in light, electron, and X-ray microscopy applied in biology, metallography, mineralogy, chemistry, and medicine.

An exhibition including all major manufacturers of microscopes and accessories will feature the newest microscopes and accessories.
Contact:

INTER/MICRO-78
McCrone Research Institute
2508 South Michigan Avenue
Chicago, Illinois 60616

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