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Microchemical Journal

devoted to the application of microtechniques in all branches of science

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Briefs

Inverse Polarographic Determination of Creatinine with Alkaline Picrate and 3,5-Dinitrosalicylic Acid. K. G. BLASS AND R. J. THIBERT, *Department of Chemistry, University of Windsor, Windsor, Ontario N9B 3P4, Canada.*

The determination of creatinine involves the measurement of disappearance of diffusion current for the picric acid wave when creatinine is added. Levels of 0–5 μg/ml can be measured accurately. 3,5-Dinitrosalicylic acid was found to be more reactive and more useful than 3,5-dinitrobenzoic acid for the inverse polarographic determination of creatinine at levels of 0–30 μg/ml.

Microchem. J. **19,** 1 (1974).

Cholesterol Study: Reaction Plateau and Kinetic Determinations. A. MANASTERSKI AND B. ZAK, *Departments of Biochemistry and Pathology, Wayne State University School of Medicine and the Department of Pathology, Detroit General Hospital, Detroit, Michigan 48201.*

Reaction plateaus and kinetic modes for measuring cholesterol have been compared. A similar investigation of bilirubin reactions under the same procedural conditions was also carried out, because this compound is a major interference which is frequently encountered in abnormal serums.

Microchem. J. **19,** 8 (1974).

The Microdetermination of Molecular Weight by Vapor Pressure Equilibrium. H. SWIFT, *Atomic Weapons Research Establishment, Aldermaston, Berkshire, Great Britain.*

An improved apparatus for the determination of molecular weight is described. Three solutions of the same solvent and different solutes equilibrate by distillation to equal vapor pressure in a common container.

Microchem. J. **19,** 18 (1974).

Effect of the Nonionic Surfactant on the Fluorometric Determination of Gallium Using Lumogallion. KENYU KINA AND NOBUHIKO ISHIBASHI, *Department of Applied Analytical Chemistry, Faculty of Engineering, Kyushu University, Higashi-ku, Hakozaki, Fukuoka, Japan.*

The sensitive fluorometric determination of gallium using lumogallion and the nonionic surfactant, polyethylene glycol monolauryl ether. The effect of a large number of interfering ions was studied.

Microchem. J. **19,** 26 (1974).

A New Type of Platinum Basket for Combustion of a Sample in a Closed Flask. JOSEPH F. ALICINÓ, *The Squibb Institute for Medical Research, Princeton, New Jersey 08540.*

A new type of basket is described prepared from perforated platinum sheet. It offers several advantages over the conventional type prepared from platinum wire.

Microchem. J. **19,** 32 (1974).

Nano-scale Column Partition Chromatography. R. H. WALTER, *Department of Food Science and Technology, New York State Agricultural Experiment Station, Cornell University, Geneva, New York 14456.*

Column chromatography was adapted to natural product isolates in which the components were present in concentrations of the order of 10^{-9} g. Use for this technique has been found in purification of nanogram quantities of gas chromatographic effluents and in resolution and concentration build-up of compounds which may otherwise be difficult to identify by gas chromatography–mass spectrometry.

Microchem. J. **19,** 34 (1974).

Determination of Calcium in Organometallic Compounds by Atomic Absorption Spectroscopy. V. KOVAČ, M. TONKOVIĆ, AND Z. ŠTEFANAC, *Institute "Rudjer Bošković," Bijenička c. 54; and Institute for Medical Research, Yugoslav Academy of Sciences and Arts, Moše Pijade 158, 41000 Zagreb, Yugoslavia.*

Oxygen flask is used for the decomposition of the organic sample and atomic absorption spectroscopy of the metal in aqueous medium.

Microchem. J. **19,** 37 (1974).

Delayed Luminescence Analysis (DLA) of Purine and Pyrimidine Ribose and Deoxyribose Nucleotide Triphosphates in Picomole Quantities. MADHU S. P. MANANDHAR AND KNOX VAN DYKE, *Department of Pharmacology, West Virginia University Medical Center, Morgantown, West Virginia 26506.*

A new system that enables the determination of separated nucleotide triphosphates in picomole quantities is described. This system of delayed luminescence analysis (DLA) is sensitive to both purine and pyrimidine ribose and deoxyribose nucleotide triphosphates. A crude luciferin–luciferase (substrate–enzyme) preparation from firefly lanterns, in the presence of nucleotide triphosphate, is utilized to generate light that is detected by a liquid scintillation counter using a single photomultiplier tube. Light is produced in a delayed fashion, the maximum emission being dependent on the type of nucleotide.

Microchem. J. **19,** 42 (1974).

Small-Scale Cell for External Generation of Coulometric Titrants. G. F. ATKINSON, *Department of Chemistry, Faculty of Science, University of Waterloo, Waterloo, N2L 3G1, Ontario, Canada.*

Electrodes are inserted into the stopper by piercing the stopper with a hypodermic needle containing a stylet, removing the stylet, inserting the electrode through the lumen of the needle, and then withdrawing the needle while holding the electrode in place.

Microchem. J. **19,** 52 (1974).

Fluorometric Assay of Ultramicro Quantities of Glucose with Somogyi Filtrate and Hexokinase. ARTHUR J. TOMISEK AND SAMUEL NATELSON, *Department of Biochemistry, Michael Reese Hospital and Medical Center, Chicago, Illinois 60616.*

A procedure is described for the determination of glucose in 3 μl of serum or plasma. It is based on the phosphorylation of glucose with ATP mediated by hexokinase. The TPNH generated with glucose-6-phosphate dehydrogenase is measured fluorometrically.

Microchem. J. **19,** 54 (1974).

A Study of Oxidation of Benzidine, *o,o'*-Tolidine, and *o,o'*-Dianisidine. LUDEK DOHNAL AND JAROSLAV ZÝKA, *Department of Analytical Chemistry, Charles University, Prague, Czechoslovakia.*

This paper is a continuation of the series of studies of oxidation reactions of organic compounds with salts of trivalent cobalt. The study was done in glacial acetic acid. The effect of ultraviolet light on the compounds in solution was studied spectrophotometrically and chromatographically and compared with the effect of Co(III) acetate.

Microchem. J. **19,** 63 (1974).

Kinetic Differential Determination of 2-Ketohexoses by Their Reaction with Cysteine–H_2SO_4. DONALD L. BISSETT, THOMAS E. HANSON, AND RICHARD L. ANDERSON, *Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823.*

2-Ketohexoses can be distinguished from each other by their reaction with cysteine–H_2SO_4. Sorbose gives a distinctive absorption spectrum, whereas fructose, psicose, and tagatose can be distinguished from each other by their differential rates of absorbance increase at 412 nm.

Microchem. J. **19,** 71 (1974).

Evaluation of Analytical Methods Using Signal–Noise Ratio as a Statistical Criterion. T. FUJIMORI, T. MIYAZU, AND K. ISHIKAWA, *Department of Reaction Chemistry, Faculty of Engineering, University of Tokyo, Bunkyo-ku, Tokyo, 113, Japan; and Technical Research Center, Nippon Kokan K. K., Kawasaki, 210, Japan.*

Signal–noise ratio is useful in the case of physical tests. It has little advantages for

the evaluation or selection of usual chemical analysis methods, because signal-noise ratio is varied in proportion to the differences of precision of each method.

Microchem. J. **19,** 74 (1974).

The Oxidation of Aminophenazone and Phenazone by Ferricyanide. HANA TOMÁNKOVÁ AND JAROSLAV ZÝKA, *The State Institute for Control of Drugs, Prague and the Department of Analytical Chemistry, Charles University, Prague, Czechoslovakia.*

By monitoring the prolonged oxidation of an aqueous solution of aminophenazone with potassium ferricyanide, it was found that the degree of oxidation depends on the pH and the temperature of the medium and on the amount of oxidant present. The oxidation of phenazone differs from that of the above.

Microchem. J. **19,** 86 (1974).

MICROCHEMICAL JOURNAL 19, 1–7 (1974)

Inverse Polarographic Determination of Creatinine with Alkaline Picrate and 3,5-Dinitrosalicylic Acid

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Received June 2, 1973

INTRODUCTION

The Jaffé (7) reaction for creatinine with alkaline picrate is not specific and numerous interferences which produce similar chromogens exist (*17, 20*). Picric acid is a very reactive molecule with various types of reactions that it can undergo (*5, 11, 20*). Reactivity at the meta position of picric acid by acetone (*10–13*) and probably other molecules produces chromogens which interfere in the spectrophotometric determination of creatinine. Meisenheimer (*1*) complex formation also produces interfering chromogens.

Since the specificity of the colorimetric determination is decreased by many types of interfering chromogens, we propose a new indirect polarographic determination. The polarographic reduction of picric acid has been investigated by a number of researchers (*19, 21, 22*). Three well-defined polarographic waves can be seen in an alkaline medium. After reacting the alkaline picrate with an aqueous solution of creatinine, a shift in half-wave potential and a lowering of the picric acid diffusion current are observed. This decrease in diffusion current is directly proportional to the concentration of creatinine. Meisenheimer complex formation and the effect of the interferences that attack at the meta position are minimized in this new indirect polarographic determination of creatinine. In 1936, three different reports appeared on the reaction of alkaline 3,5-dinitrobenzoic acid with creatinine (*2, 3, 16*). The investigators described 3,5-dinitrobenzoic acid as a more specific reagent than picric acid for the determination of creatinine. However, limitations of low reactivity and instability of the purple color formed by the reaction were major disadvantages noted in the literature. Bollinger (*3*) attributed greater color stability to the alcoholic 3,5-dinitrobenzoic acid reaction of Benedict and Behre (*2*).

Since color stability is not a necessary requirement for polarographic investigation of the effect of creatinine upon the reduction waves of the nitro groups, an examination was undertaken to find a

reagent which is more specific than picric acid for the polarographic determination of creatinine. An investigation of 3,5-dinitrosalicylic acid (DNSA) was also undertaken to improve reactivity and selectivity of the new inverse polarographic determination of creatinine.

MATERIALS AND METHODS

Reagent grade creatinine was purchased from Fisher Scientific Company, Don Mills, Ontario, Canada. The NaOH was "Baker Analyzed" Reagent grade from J. T. Baker Chemical Co., Phillipsburg, N. J. Reagent picric acid crystals (under water) were purchased from Allied Chemical of Morristown, N. J. Laboratory Reagent 3,5-dinitrosalicylic acid was from the British Drug Houses Ltd., Toronto, Ontario, Canada. The ACS NaCl was obtained from Fisher Scientific Company, Don Mills, Ontario, Canada. Nitrogen 99.996% pure was from Liquid Carbonic of Canada Ltd. Triple distilled mercury was obtained from Engelhard Industries of Canada Ltd.

A Sargent (Sargent-Welch Scientific Co.) Model XVI Polarograph was employed for this investigation. The concentration study was carried out in a 5 ml Heyrovsky cell. The characteristics of the capillary used were: $m = 1.767$ mg s^{-1}; $t = 4.68$ s; $m^{2/3}t^{1/6} = 1.890$ $mg^{2/3}$ $s^{-1/2}$. The height of the mercury column was 71.5 cm. The cell was placed in a water bath maintained at 25 ± 0.1°C with a Haake Model ED "UNITHERM" constant temperature circulator.

A flask of stock saturated picric acid solution was prepared by adding distilled water to an excess amount of wet picric acid. The flask was immersed in the 25°C water bath until the temperature had equilibrated. Eight milliliters of this solution were added to a liter flask and brought up to volume with distilled water to form a picric acid working standard solution. To prepare the first blank solution, three milliliters of picric acid working standard and 4 ml 0.5 *N* NaOH solution were added to a 10-ml volumetric flask which was filled to the mark with distilled water. This solution (5 ml) was pipetted into the Heyrovsky cell for polarographic analysis. Creatinine standard was prepared by adding 0.025 g of creatinine to a 250-ml volumetric flask and made up to volume with distilled water. Solutions of various concentrations of creatinine (1–5 μg/ml) were prepared by adding appropriate alliquots of the standard creatinine to a 10-ml volumetric flask containing the picric acid working standard and the sodium hydroxide supporting electrolyte as in the blank. All solutions were deaerated for a period of 15 min with nitrogen prior to polarography.

A stock 3,5-dinitrosalicylic acid solution was prepared just prior to use by adding 0.050 g of DNSA to a 500-ml volumetric flask and bringing to volume with distilled water. The NaCl supporting electrolyte was prepared by adding 2.922 g of NaCl to a 100-ml volumetric flask and filling the flask to volume with distilled water. Similarly, 0.050 g of creatinine was weighed and made up to volume just prior to use in a 100-ml volumetric flask with distilled water. In the concentration study, 1 ml of NaCl solution, 5 ml of DNSA solution, and from 0.0 to 0.5 ml in 0.1 ml increments of creatinine solution (equivalent to 0.50 μg/ml) were added to a 10-ml volumetric flask and made up to volume with distilled water. The solution was mixed, and a 5-ml aliquot was pipetted into the Heyrovsky cell for polarographic analysis. A 15 min deaeration using nitrogen gas was accomplished by passing the gas through a water tower at room temperature, and then through the Heyrovsky cell which was kept at 25°C in a water bath. Duplicate analyses were performed for each concentration studied, with three polarograms obtained for each run.

RESULTS

A linear inverse relationship was observed between the diffusion current of the picric acid nitro group reduction wave at $E_{1/2} = -0.501$ V vs Hg pool with increased creatinine concentration. The results of this concentration study are listed in Table 1. A slight change to a less negative $E_{1/2}$ was observed upon reacting the alkaline picric acid with creatinine. The $E_{1/2}$ for the reduction of the first nitro group of the alkaline picric acid is -0.501 V, whereas the creatinine bound product has an $E_{1/2} = -0.496$ V.

TABLE 1
EFFECT OF CREATININE CONCENTRATION ON THE DIFFUSION CURRENT OF THE FIRST REDUCTION WAVE FOR PICRIC ACID

Creatinine concn (μg/ml)	Diffusion current (μA)[a] of the picric acid wave $E_{1/2} = -0.501$ V vs Hg pool
0	2.381
1	2.30
2	2.221
3	2.118
4	2.031
5	1.93

[a] The values reported represent an average of two samples at each concentration studied, with three polarograms run for each sample.

TABLE 2
EFFECT OF CREATININE CONCENTRATION ON THE DIFFUSION CURRENT OF THE DNSA WAVE AT $E_{1/2} = -0.674$ V

Creatinine concn (μg/ml)	Diffusion current[a] (μA) of the DNSA wave at $E_{1/2} = -0.674$ V vs Hg pool
0	2.785
10	2.265
20	1.884
30	1.435
40	1.263
50	1.070

[a] The values reported represent an average of two samples at each concentration studied, with three polarograms run for each sample.

The effect of creatinine concentration on the diffusion current of the DNSA wave at $E_{1/2} = -0.674$ V vs Hg pool is shown in Table 2. This study shows that the diffusion current is inversely proportional to concentration in the range of 0–30 μg/ml of creatinine. The half-wave potentials of the two nitro group reduction waves for DNSA were found to be -0.674 V and -1.75 V vs Hg pool. A shift of $E_{1/2}$ was observed upon reacting the DNSA with creatinine. The shift in $E_{1/2}$ values for the first nitro group reduction wave of the DNSA-creatinine study was from -0.674 V to -0.700 V vs Hg pool.

DISCUSSION

The polarographic reduction of the nitro groups of picric acid to hydroxylamine groups has been shown to involve the transfer of four electrons per nitro group (*14*). Inhibition of this reduction by creatinine causes a decrease in diffusion current of four electrons per molecule of creatinine. Because of this sharp decrease in the diffusion current, along with the fact that only the first nitro wave is affected by creatinine at low concentrations (1:4 C/P) accounts for the sensitivity of this new indirect polarographic determination.

Polarographic investigation is able to clarify whether the reaction between creatinine and picric acid forms a 1:1 or a 2:1 product. The difficulty in determining whether it is a 1:1 or a 2:1 product lies in the fact that creatinine can form both depending on the relative amounts of each of the chemicals present. At a low creatinine to picric acid ratio, a red 1:1 product is formed. At ratios of 1:1, a mixture consisting mainly of the 1:1 product is present, but approximately 10% is in the 2:1 form. This is easily observed by

polarography of picric acid-creatinine solutions and comparison of the reduction waves of picric acid. Spectrophotometric determination did not show clearly that the reaction product was a 1:1 or 2:1 adduct because the red product is unstable and changes to a yellow product upon dilution. Similarly, it was observed that the orange 2:1 product, which was first isolated by Greenwald (*6*), is also converted to a yellow color when it is dissolved in water and other solvents. Indeed, a solvent could not be found in which the red and orange products were soluble and at the same time retained their color in dilute solutions.

In 1936, three independent investigators (*2, 3, 16*) found that 3,5-dinitrobenzoic acid will produce a purple color with creatinine in alkaline solution. This method was shown to be more specific toward creatinine, but it was less sensitive. Polarographic examination of this reaction revealed, that both reduction waves corresponding to each of the nitro groups, would decrease at the same time when creatinine was added. This suggested that the reaction of 3,5-dinitrobenzoic acid was similar to that of picric acid. Since creatinine addition to 3,5-dinitrobenzoic acid affected both reduction waves at the same time, and also because of its lowered reactivity, the 3,5-dinitrobenzoic acid proved unsuitable compared to picric acid as a reagent for the indirect polarographic determination of creatinine.

To examine a similar molecule with greater reactivity 3,5-dinitrosalicylic acid was tried. This reagent was much more reactive, in that it gave a greater decrease in the diffusion current with creatinine than did 3,5-dinitrobenzoic acid. DNSA did not produce the purple color which occurs with 3,5-dinitrobenzoic acid and creatinine in the presence of base. It was found advantageous to eliminate the addition of base and to substitute NaCl as the supporting electrolyte for the polarographic determination of creatinine with DNSA.

An experimentally insignificant change in $E_{1/2}$ is observed for the first nitro group reduction wave of picric acid when creatinine is added under the conditions described in this paper. However, in the DNSA study, a shift of 0.26 V (to a more negative potential) was observed between the $E_{1/2}$ of the first nitro group reduction wave and the corresponding wave in the presence of creatinine. Although correlation between shifts in $E_{1/2}$ and the extent of conjugation have been well documented in the literature (*4, 9, 23*), such a conclusion cannot be drawn from the data presented in this paper. The shift in $E_{1/2}$ in the DNSA-creatinine study is believed to be due to a change in pH. An increase in pH would decrease the availability of protons for the reduction of the nitro group to the hydroxylamine (*14*), and therefore a change to a more negative potential would be expected and was ob-

served. The effect of changes in pH versus $E_{1/2}$ for nitro group reductions have been tabulated (*8, 15, 18*). Further investigation of the red Jaffé reaction product, the $E_{1/2}$ of the Jaffé reaction product, as well as, the mechanism of the Jaffé reaction are presently being examined in this laboratory.

SUMMARY

A selective and highly sensitive inverse polarographic method for the determination of creatinine has been developed, involving the measurement of disappearance of diffusion current for the picric acid wave ($E_{1/2} = -0.501$ V) when creatinine is added. Creatinine levels of 0–5 μg/ml can be measured accurately.

Investigation of 3,5-dinitrobenzoic acid and 3,5-dinitrosalicylic acid revealed the latter to be more reactive and useful for the inverse polarographic determination of creatinine at levels of 0–30 μg/ml.

ACKNOWLEDGMENT

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Cholesterol Study: Reaction Plateau and Kinetic Determinations[1]

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Time and temperature studies of the Liebermann-Burchard (LB) reaction for both cholesterol and bilirubin were described in a previously published spectrophotometric report (*14*). From these findings, one could easily deduce that the reactions for both compounds in the described reagent systems had excellent kinetic properties which might be useful in lessening the effect of interference of bilirubin in the determination of cholesterol. In addition, the iron reaction for cholesterol in which full color formation occurs somewhat slowly also appears workable in a kinetic mode since it is basically several times more sensitive than the LB reaction however the latter is performed (*13*). Not much has yet occurred with the kinetic approach for the determination of cholesterol, although at least two brief reports have been described (*9, 11*). Other serum constituents aside from enzymes have been determined by kinetic mode in recent years and this trend has caused investigators to lean toward that methodological direction in an attempt to lessen interference whenever such a possibility presented itself (*3, 6, 15*). The use of kinetic analysis could have certain analytical advantages over an endpoint technique in properly designed circumstances. For example, if the rate of reaction of the interfering substance has a lag phase where the reaction for the desired constituent is totally linear then the kinetic measurement could be made during that time period. If the interfering compound reacted much slower or could be made to react slower than the desired constituent even though both reactions were linear, its effect as an interference could be minimized and kinetic measurements would be more favorable than an end reaction determination if they were made while each was reacting at those different speeds. If the interfering substance reached its peak more rapidly than the desired

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[2] In partial fullfillment of the PhD in Biochemistry.

constituent, then the kinetic measurements could be made after a short time delay to obviate the interference entirely. Still another possibility to consider would be that case in which either the interference or the desired constituent yields an unstable color reaction while the other reaction was stable. It would then be possible to measure the rate of fading of color with time after both compounds had achieved peak maxima or if the compound of choice yielded a stable color, a reasonable wait for the unstable interfering compound to fade could be attempted and a correction then made for it. This idea is possibly applicable to cholesterol and bilirubin by LB reaction since biliverdin is quite stable once formed from bilirubin while the cholesterol reaction is relatively unstable. It is also possible that its fading rate could be accelerated by an increase in temperature (*12, 14*). On the other hand if the rate of change of absorbance with time for both interference and desired constituent stayed relatively the same, the kinetic measurement would be no more valuable than the endpoint reaction. Still another factor to consider is wavelength of measurement. It would be a fortunate circumstance if the interference and desired constituent could form peak maxima at wavelengths which are quite different one from the other. Ideally, this could either eliminate the interference entirely, or at least make a mathematical correction simpler. This report will therefore describe a study carried out using two systems, one in which both the interference, bilirubin, and the desired constituent, cholesterol, show broad absorbance maxima at virtually the same wavelength (*4, 7*) and one in which they peak at somewhat different wavelengths (*12*). Interestingly, in the latter case, and partly because of the smaller peak maxima overlap, it will be demonstrated that bilirubin is really a minor interference in the majority of jaundiced serums. This is important because several reports have indicated contrary opinions (*1, 2, 8*) and at least one (*8*) has suggested a correction far in excess of what should be the theoretical one even if all of the bilirubin were available for the reaction. Molar absorptivity comparisons will be derived which demonstrate that this correction (2.8 mg of cholesterol/mg of bilirubin) is excessive even if all of the bilirubin transferred to the filtrate.

MATERIALS AND METHODS

Reagents

Cholesterol standards. Two hundred milligrams of cholesterol were dissolved in glacial acetic acid or isopropyl alcohol and diluted to a final volume of 1 dl with the corresponding solvent. In the case of the former, standards were reacted without evaporation, and in the case

of the latter, standards were evaporated to dryness before reacting them.

Bilirubin standard. Ten milligrams of bilirubin were carefully dissolved in refrigerated chloroform to a final volume of 1 dl. This solution was kept refrigerated when not used.

Cholesterol color reagent Liebermann-Burchard (LB). The reagent was prepared by pouring 350 ml of glacial acetic acid into a flask and slowly adding 550 ml of acetic anhydride. The mixture was refrigerated at 5°C overnight, and then 100 ml of similarly cooled H_2SO_4 was carefully added while the solution was slowly mixed.

Ferric chloride color reagent. This was prepared exactly as previously described (*12*).

Procedure

Kinetic and endpoint LB reactions. Aliquots representing the concentrations of cholesterol and bilirubin shown in the figures were pipetted into test tubes and refrigerated LB reagent was added with or without water present. The tubes were mixed and the changing absorbance at 620 nm was recorded continuously vs. a reagent blank in an automatic recording spectrophotometer after a 1 min wait in reaction time. The procedure was the same for the endpoint determination except that the absorbance was read after a fixed reaction time as indicated in the corresponding figures.

Kinetic and endpoint iron reactions. Appropriate aliquots representing cholesterol and bilirubin were pipetted into 3 ml of $FeCl_3$ color reagent. The tubes were mixed, 2 ml of H_2SO_4 were added, and the tubes again mixed for 1 min. Two minutes after the reaction began the changing absorbance was measured continuously versus a reagent blank at 560 nm in an automatic recording spectrophotometer. The procedure was the same for the endpoint determination except that the absorbance was read after a fixed period of time as shown in the corresponding figures.

RESULTS AND DISCUSSION

A comparison of the effects of time and water content on the rates of reaction was determined for the LB procedure (*4*) for both cholesterol and bilirubin when each was measured continuously in an automatic recording spectrophotometer. Two kinds of LB systems were studied which differed one from the other by the presence or absence of H_2O. This was deemed necessary because the LB reaction is temperature dependent and the presence of H_2O in the standard can increase the temperature on reaction with the color reagent. In addition, it can be shown that the presence of H_2O will decrease the con-

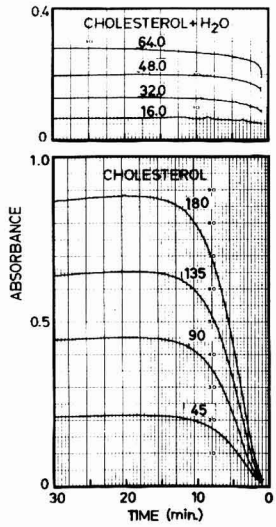

FIG. 1. The lower curves show the changing absorbances of the LB reaction for cholesterol without H_2O present for 45, 90, 135 and 180 μg/ml of cholesterol as measured by a continuous scan at 620 nm. The curves at the top show the changing absorbances of the LB color reaction at 620 nm for 16, 32, 48 and 64 μg/ml of cholesterol with water present in the reaction.

centration of color obtained. Attention is paid to the effect of H_2O in some direct procedures (*5*) but not in others (*4, 10*). Figure 1 illustrates the rate of color formation at 620 nm for several cholesterol concentrations both with no water present shown in the lower graph as well as with H_2O diluted samples shown in the upper graph. The recordings in each case were begun 1 min after mixing the standard with the cold LB reagent. Both color reactions appeared reasonably stable although some evidence of fading occurred after 15–20 min. The formation of the peak color was more rapid when water was present owing to the generation of heat, but the reaction sensitivity was decreased by some 13%. However, a kinetic approach, if desired, would be superior in the slower reacting system where the delta absorbance per unit time yielded steep and linear curves.

Figure 2 shows a similar graphic example of the LB reaction used with several concentrations of bilirubin when each was reacted as described for cholesterol with and without the presence of water. The rate of reaction was faster when water was present again owing to a heat buildup (*14*), but as the previous figure demonstrated, some reasonable linear measure of the change in absorbance per unit time is more apparent for the water-free system.

The calibration curves for delta absorbance of both bilirubin and

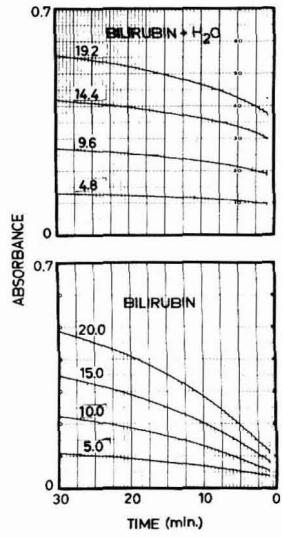

FIG. 2. The lower curves show the changing absorbances of the LB reaction for bilirubin with H_2O present for 5, 10, 15 and 20 μg/ml of bilirubin by a continuous scan at 620 nm. The top curves show the formation of color at 620 nm for 4.8, 9.6, 14.4, 19.2 μg/ml of bilirubin when water is present in the LB reaction.

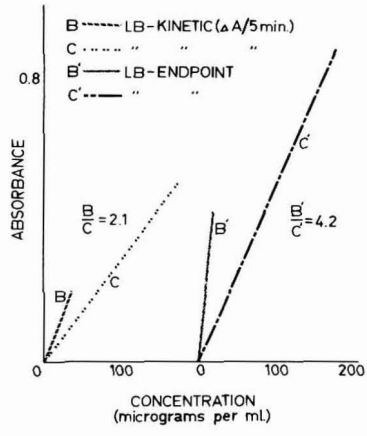

FIG. 3. Plot of absorbance versus cholesterol and bilirubin concentrations using the LB reaction. Lines B and B′ are the standard curves for bilirubin with a kinetic (ΔA/5 min) and an endpoint determination, respectively. Lines C and C′ are the standard curves for cholesterol in a kinetic (ΔA/5 min) and endpoint determination, respectively.

cholesterol in the H_2O-free system are graphically displayed in Fig. 3 (left). The slope of the line for cholesterol is lower than for bilirubin and both have an intercept of zero. The ratio of absorbance signals as a comparative measure of these two slopes is 2.1. In the case of the endpoint reaction for the same system using absorbance measurements made after 20 min, the calibration curves for cholesterol and bilirubin are shown in Fig. 3 (right). The slope of the line for cholesterol is still less than the slope of the line for bilirubin but in this case the ratio of absorbance signals for bilirubin to cholesterol is increased to 4.2. This means that the interference of bilirubin here using the data derived from the same time studies as in the same figure on the left is twice that obtained by the kinetic mode indicating that the rate method may be superior in terms of interference problems even though it appears less sensitive with respect to comparative slope characteristics. Obviously, the kinetic approach would lead to a smaller error from the interfering compound, bilirubin, than would the endpoint technique measuring at the plateau of formation of color for cholesterol. The presence of H_2O, the generation of heat, the concomitant increase of reaction velocity associated with mixing the sample into the H_2O reacting LB medium, and the loss of some reaction sensitivity owing to the presence of the H_2O seem somewhat self defeating in this comparison with the kinetic example.

Figure 4 illustrates the calibration characteristics for both cholesterol and bilirubin when a system containing the amount of H_2O which would be present in a common automated procedure by LB reaction (*7*) was followed by measuring absorbance versus time con-

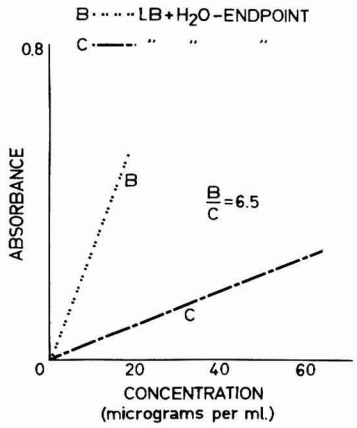

FIG. 4. Plot of absorbance versus bilirubin and cholesterol concentration with an endpoint LB reaction. Lines B and C are the standard curves for bilirubin and cholesterol respectively with water present in the LB reaction.

tinuously over a period of minutes in an automatic recording spectrophotometer. The formation of color was so rapid that it would be difficult to attempt to present this data as a delta plot of absorbance vs time. However, endpoint calibration plots of final absorbance attained for both bilirubin and cholesterol indicate that the ratio of the slopes for the reactions of the two compounds was 6.5 as shown. The slope of the calibration line for bilirubin here was much greater than cholesterol as previously described. However, in each instance when using this LB reaction with and without the presence of H_2O, bilirubin is a more sensitive reactant than is cholesterol no matter how the data is handled. The point to consider here is that bilirubin and cholesterol are measured at their wavelength maxima since the reaction products for both are spectrophotometrically similar throughout the visible range.

Although the ferric chloride reaction for cholesterol is more sensitive than the LB reaction when considered from a reaction plateau point of view, the fact that it is a more rapid reaction as described than is the LB reaction makes it appear less sensitive when considered from a kinetic point of view. This can be inferred from the graphings of Fig. 5. The viscosity of the medium and the temporary presence of air bubbles made it necessary to wait 2 min before the continuous scans at 560 nm were begun. The reactions were now near plateau levels, they slowed up and the plot of delta absorbance

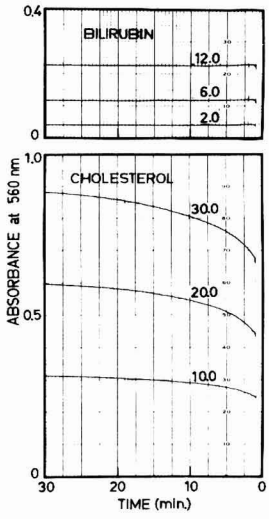

FIG. 5. Continuous scan of the iron reaction with cholesterol and bilirubin. Lower curves show the formation of color at 560 nm for 10, 20, and 30 μg/ml of cholesterol. Upper curves show the formation of color at 560 nm for 2, 6, and 12 μg/ml of bilirubin.

versus time in the steeper areas of the curves appeared to be less than were those of the less sensitive but slower reacting LB system previously described in Fig. 1. By this time (2 min), bilirubin shown in the upper part of the graph was completely reacted and had plateaued and stabilized without any evidence of fading. However, since bilirubin is measured at an absorption minimum while cholesterol is measured at an absorption maximum, bilirubin interference is minimal unless bilirubin values become excessive, i.e., much greater than 20 mg/dl.

The $FeCl_3$ reaction like the H_2O-free LB reaction can also be plotted in either a reaction plateau or a kinetic mode as shown in Fig. 6. The initial part of the reaction is very fast as these continuously recorded graphings indicate but the delta absorbance vs concentration plots for the different levels is still linear. The sensitivity of the iron kinetic method is virtually identical to the more ideal LB reaction when the actual concentrations of cholesterol are considered. But, this would certainly be a far better system if the initial part of the iron reaction with cholesterol could be slowed somewhat, especially since the bilirubin is totally oxidized to biliverdin very rapidly. When examining the endpoint calibrations versus the kinetic calibrations, it is apparent that the error from bilirubin by iron reaction as described is much smaller than it would be by LB reaction since in the former case the ratio of the calibration slopes of bilirubin to cholesterol is less than one and in the latter case it is always greater than one.

The measure of the comparative sensitivity of the iron reaction

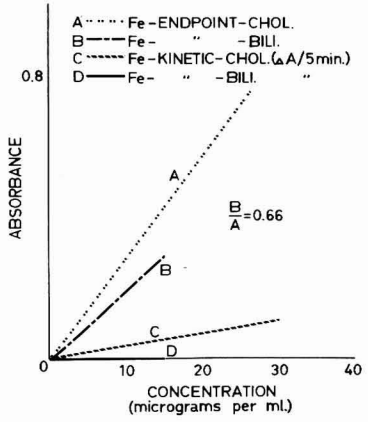

FIG. 6. Plot of kinetic and endpoint determination of cholesterol and bilirubin with iron reagent. Lines A and C are the standard curves for cholesterol in an endpoint or kinetic ($\Delta A/5$ min) determination, respectively. Lines B and D are the standard curves for bilirubin in an endpoint and kinetic determination, respectively.

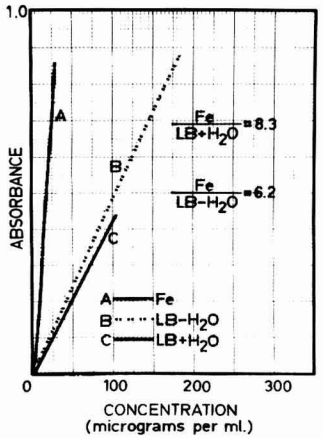

FIG. 7. Plot of the iron and LB reactions for cholesterol. Line A is the calibration curve for cholesterol with iron reagent. Lines C and B are the calibration curves for cholesterol using the LB reagent with and without water present, respectively.

versus the LB reactions plus and minus water is further described in Fig. 7. The ratios of the slopes obtained is quite favorable to the iron reaction over the other two reactions, with the H_2O-free LB system showing the best sensitivity of the two LB determinations and the least interference.

SUMMARY

A study has been described of a comparison between reaction plateaus and kinetic modes for measuring cholesterol. A similar investigation of bilirubin reactions under the same procedural conditions was also carried out because this compound is a major interference which is frequently encountered in abnormal serums. The findings indicate that bilirubin is a more sensitive reactant than cholesterol by LB reaction but that its effect as an interference can be lessened by a kinetic approach providing that the effect of H_2O on reaction velocity and molar absorptivity can be minimized. An iron reaction even though more sensitive proved less workable in the kinetic mode owing to reaction velocity and the presence of air bubbles. However, its reaction plateau characteristics were superior to the LB reaction because of a favorable ratio of colors generated for the two reacting constituents. Based on this study, it is predictable that a kinetic approach is quite workable for the LB reaction and could be made more workable for the iron reaction if, in the latter case, conditions perhaps such as temperature and reaction media, could be altered to slow the rate of color formation.

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MICROCHEMICAL JOURNAL 19, 18–25 (1974)

The Microdetermination of Molecular Weight by Vapor Pressure Equilibration

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INTRODUCTION

When two or more solutions containing known weights of solute are placed separately in a closed evacuated container and maintained at a constant temperature, the vapor pressures of the solutions eventually come to equilibrium by a distillation process. If the molecular weight of one of the solutes is known and provision is made for the measurement of the volume or weight of solvent at the end of the experiment, then the molecular weight of each unknown solute may be calculated.

Methods for both the volumetric (*2*, *6*) and gravimetric (*1*, *3*–*5*, 7) determination are available. The volumetric approach is based on the work of Signer, later modified by Clark. The gravimetric method was initially used by Robinson and Sinclair (*5*, *7*) for work on activity coefficients and vapor pressures of solutions. Morton *et al.* (*4*) adapted Sinclair's technique for molecular weight determinations and from this produced sound results. Later Boraman (*1*) claimed more rapid attainment of equilibration and equal accuracy from an apparatus similar to Morton's. This form of apparatus has been further modified in this laboratory to give steady control of temperature, more ease of manipulation and no loss of solvent in weighing.

METHOD

Apparatus

The boiling vessel A (Fig. 1) consists of a litre round-bottomed wide neck flask. Inside the flask and sealed to its neck are two concentric wells B and C. The flange D of the lower well C and the lid E resting on it are both of flat sight glass and provide an efficient seal when lubricated with silicone grease. At a point on the upper exterior wall of the neck of the flask provision is made for a socket joint F which is used to accommodate a short efficient condenser G. The lid of the container has a Rotaflow Teflon tap H sealed to its centre in such a manner that when the lid is in position the greater part of the

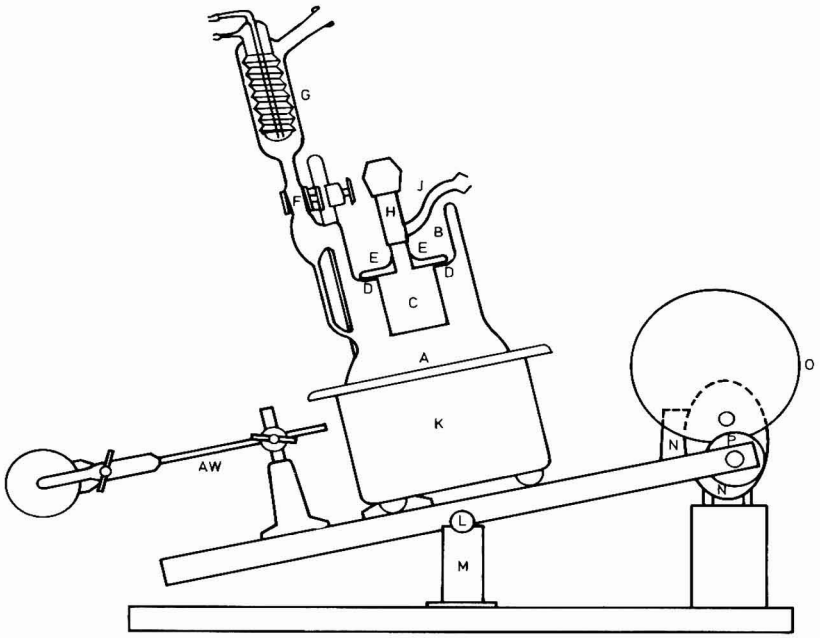

FIG. 1. Scale 1:8. (A) Boiling vessel; (B) Upper well; (C) Lower well; (D) Flange; (E) Flat circular lid; (F) B19 joint; (G) Condenser; (H) Rotaflow Teflon tap; (J) Exhaust arm; (K) Heating mantle; (L) Steel rod; (M) Grooved pillar; (N) Electric motor; (O) Large wheel; (P) Free wheel; (AW) Weight adjustable 1 kg.

exhaust arm J is well down inside the upper well of the boiling vessel.

The bottle holder (Fig. 2) is a brass cylinder in which equally disposed wells Q have been drilled to accommodate three glass bottles. The depth of each well is equal to the height of a bottle without the stopper. The base of each well is $\frac{1}{8}$ in. or 3 mm deep. The central section Y of the holder has been removed leaving only a small pillar at its centre R. By removing this unwanted metal the cooling time is decreased and it is also possible to see the bottle and its contents at all times. On the upper surface of the holder are three ridges S which connect the three wells and upon which the stoppers of the bottles rest. The handling rod T has a thick shoulder at its base and this provides a rest for the moveable stopper holder U. The three holes in the latter are of sufficient diameter to accommodate the stoppers at their widest part while the overall diameter of the stopper holder's body, slightly smaller than the bottle holder, enables it to be turned freely in the glass container C (Fig. 1) without sticking. A metal rod carrying a soft iron head passes through a hole in the stopper holder into a locating hole Z in the bottle holder. This secures the stoppers

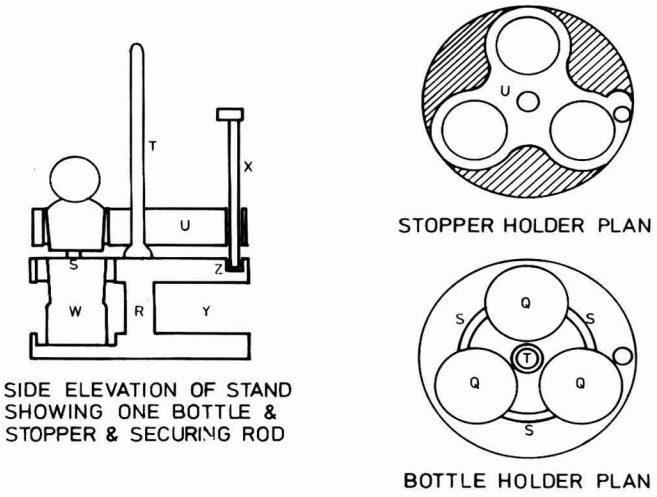

FIG. 2. Scale 1:2. (Q) Well; (R) Pillar; (S) Ridge; (T) Handling rod; (U) Movable stopper holder; (W) Glass bottle; (X) Securing rod; (Y) Disposed Waste; (Z) Locating hole—bottle holder.

during the experiment. In order to allow free movement of vapor the stopper holder is cut to the shape shown in Fig. 2 (unshaded area).

The glass bottles W (Fig. 2) are made from B_{14} soda joints.[1] Soda glass joints were chosen in preference to borosilicate because the static charges which the latter is liable to collect makes it difficult to obtain constant weight. The sockets are shortened to 10 mm, as are the cones; for ease of removal the handle of the stopper is flat in the vertical plane. The total height of the bottle and stopper is 35 mm. Each bottle and stopper is clearly numbered.

The heating mantle K (Fig. 1) for the boiling vessel is fixed to the centre of a rectangular board. The latter has a steel rod L screwed to its underside across its centre and each side of the rod pivots on a grooved pillar M. One end of the board is cut away to accommodate an electric motor N geared to 16 rpm. To the spindle of the motor, a 6 in. diameter wheel O is eccentrically attached. This in turn contacts a smaller 2 in. diameter, freely revolving wheel P fixed to the pivotted board. A small ridge on each side of its periphery prevents the eccentric wheel from slipping off the free wheel. The boiling vessel is secured by an adjustable 1 kg weight at AW. Air is removed from the container C by evacuating a large desiccator and attaching it to the container via a rotameter which indicates when the pressures are equalized. Controlled evacuation is preferred in order to minimize bubbling which sometimes occurs.

[1] Soda glass joints may be purchased from Messrs Gallenkamp, England.

Materials

Boiling fluids. Dichloromethane AR. bp 40.1°C for most low boiling point solvents, chloroform AR bp 61.3°C for higher boiling solvents.

Solvents. Acetone AR. bp 56.5°C dried, chloroform AR bp 61.3°C.

Procedure

Weigh 5–10 mg of a known pure material in a tared stoppered bottle. Introduce a similar amount of each of two unknown materials into the other bottles. Set each bottle into the bottle holder with its respective stopper resting on an adjacent ridge and secure the stopper holder by passing the rod carrying the soft iron head X (Fig. 2) through the securing hole into the locating hole of the bottle stand. Introduce 0.4 ml of solvent (0.5 ml in the case of highly volatile solvents such as ethyl ether) into each bottle and place the stand inside the lower well of the boiling flask. With the aid of a syringe filled with high vacuum silicone grease, pipe a narrow band of grease around the bottom facing edge of the glass lid E, (Fig. 1). Pipette 0.3 ml of solvent onto the walls of the well C (Fig. 1), 0.5 ml in the case of highly volatile solvents, and place the lid firmly in position. Rock the apparatus for a few minutes to dissolve the samples. Connect the exhaust arm J (Fig. 1) to the vacuum desiccator which has been previously evacuated to 150 mm pressure (300 mm when highly volatile solvent, e.g., ether, is used) and carefully evacuate the well until the pressures are equalized between it and the desiccator. Screw in the Rotaflow tap, and cover the lid with a thick wad of cotton wool.

With the apparatus secure, set the motor in motion and switch on the heater until the boiling fluid is seen to condense and run back into the flask. Set the control to maintain a steady boiling.

After 48 hr stop the motor, remove the cotton wool, and with the aid of a magnet lift the securing rod from its position in the bottle holder socket and rotate the stopper holder so that the stoppers drop into their respective bottles. Admit air and with a twisting motion remove the glass lid. Withdraw the complete bottle holder, and, after an initial cooling, allow the three bottles to stand, preferably in a balance room, on a brass cooling block for 5 min before weighing. Weigh each bottle to the nearest 0.01 g.

Calculation

Use the formula $M_u = M_k W_u S_u / W_u S_k$ to calculate the molecular weight, where

M_u and M_k	molecular weight of the unknown and known substances,
$W_u W_k$	weight in mg of unknown and known substances,
$S_u S_k$	weight in mg of solvent in unknown and known bottles.

TABLE 1
TABLE OF RESULTS[a]

	Compound	Wt (mg)	Solvent	Temp (°C)	Wt solvent (mg)	Time (hr)	Molecular weight Found	Molecular weight Theoretical
1.	Phenacetin	6.800	Acetone	40.1	61.08	7		179
	azobenzene	6.958			64.73		173	182
	benzil	7.006			57.83		195	210
	Phenacetin				153.70	24		
	azobenzene				157.29		179	182
	benzil				139.54		203	210
	Phenacetin				296.14	48		
	azobenzene				303.31		179	182
	benzil				264.08		207	210
2.	Phenacetin	5.358	Acetone	40	72.45	48		179
	benzoic acid	4.987			101.96		119	122
	salicylic acid	6.600			119.64		134	138
3.	Benzoic acid	6.206	Acetone	40	239.30	48		122
	salicylic acid	6.501			228.95		134	138
	cinnamic acid	6.377			203.73		147	148
4.	Benzil	4.541	Chloroform	40	165.23	48		210
	triphenylphosphine	5.766			171.21		258	262
	dibenzyl disulfide	5.985			187.94		244	246

4a.	Benzil	4.779	Chloroform	40	131.57	48		
	Triphenyl phosphine	7.852			172.17		264	262
	Dibenzyl disulfide	7.850			186.75		243	246
4b.	Benzil	5.791	Chloroform	40	210.33	48		
	Dibenzyl disulfide	7.486			232.99		245	246
	Triphenyl phosphine	7.737			225.70		262	262
5.	Triphenyl phosphine	7.061	Acetone	40	207.16	48		262
	Dibenzyl disulfide	6.452			203.36		244	246
	Atropine	7.625			200.51		293	289
6.	Triphenyl phosphine	7.039	Chloroform	40	256.95	48		262
	Dibenzyl disulfide	5.291			208.77		243	246
	Atropine	9.247			275.86		321[b]	289
7.	Azobenzene	5.352	Ether	ca. 20	99.098			182
	Dibenzyl disulfide	7.509		i.e. R.T.	102.671		247	246
	Triphenyl phosphine	9.149			114.982		268	262

[a] The first compound in each triplet was used as the reference substance of known molecular weight.

[b] See Discussion.

DISCUSSION

Of various materials examined to seal the lid of the containing vessel C, silicone grease was found to be satisfactory for acetone, chloroform and methanol solvents. Silicone grease was attacked by benzene and if this solvent must be used then a silicone rubber O ring is the most satisfactory sealant. Ethyl ether would not normally be selected as a solvent but to cover the event of necessity trials were carried out with the equilibrating apparatus at ambient temperatures and satisfactory results were obtained.

Sometimes chloroform gave high results and cannot be considered reliable. An example of this is atropine (see Table 1). This behavior has been reported elsewhere (*8*).

Samples under test are usually equilibrated in 48 hr although acceptable results are often obtained after 24 hr. So far, no substances encountered have required longer than 48 hr. Should it be found necessary to allow a longer period of equilibration, a further 0.3 ml of solvent may be added to each bottle and a further 0.3 ml of solvent added to the container. The solutions may then be equilibrated as described in the method.

SUMMARY

An improved apparatus for the determination of molecular weight is described. It uses the isopiestic principle in which three solutions of the same solvent and different solutes equilibrate by distillation to equal vapor pressure in a common container. The main advantages claimed for this apparatus are ease of manipulation and more precise weighing of the solutions.

ACKNOWLEDGMENTS

The author thanks Mr. J. M. Donaldson for his helpful suggestions and critical appraisal of this paper, Messrs. A. Dobson and P. Begbie for practical assistance, and the Director of this Establishment for permission to publish this paper.

Note added in proof. Since this paper was submitted for publication, it has been found that a 2 : 1 mixture of graphite and glycerol provides a suitable seal when benzene is used as solvent. In addition, it is preferable when using this sealant to use dichloromethane as the boiling fluid and to extend the time for equilibrium to 3–4 days.

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MICROCHEMICAL JOURNAL 19, 26–31 (1974)

Effect of the Nonionic Surfactant on the Fluorometric Determination of Gallium Using Lumogallion

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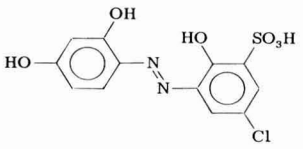
INTRODUCTION

The *o,o'*-dihydroxy azo compound, 4-chloro-6-(2,4-dihydroxyphenylazo)-1-hydroxybenzene-2-sulfonic acid, commonly known as the lumogallion which is designated by the following structure,



has been used for the fluorometric determination of niobium, aluminum and gallium (*1*, *5*, *6*) or masking reagent of titanium and tantalum in the spectrophotometric determination of niobium (*3*). Previously we reported on the fluorometric determination of the micro amount of aluminum which was based on the sensitivity enhancement effect of the nonionic surfactant. The present paper describes the highly sensitive fluorometric determination of gallium by the use of lumogallion and surfactant.

MATERIALS AND METHODS

Reagents

The standard solution of gallium, 1.0×10^{-2} *M*, was prepared by dissolving gallium oxide (Mitsuwa Pure Chemicals) in dilute hydrochloric acid. It was diluted to the desired concentration in use. The lumogallion and the nonionic surfactant, polyethylene glycol monolauryl ether (PGME), were obtained from Dojindo Co. Ltd., and Tokyo Kasei Co. respectively.

All water used was deionized and then distilled by using an all glass distilling apparatus.

[1] Correspondence should be addressed to N.I.

Equipment

Fluorescence intensity measurement was made using a Hitachi 204 spectrofluorophotometer fitted with a 150-W xenon arc lamp. Fused quartz cells ($10 \times 10 \times 40$ mm) were used throughout. A fluorescence intensity was compared with that of the setting solution of fluorescein (0.4 μg/ml).

The pH measurements were performed by using an HM-5A pH meter (TOA Electronics Ltd.) equipped with a combined glass electrode.

Procedure

An aliquot of the gallium solution was transferred into a 50 ml beaker, and 1 ml of the 1×10^{-4} *M* lumogallion solution and 4 ml of the 5% PGME was added. The pH of the solution was adjusted to 3.5 with acetate buffer. The solution was heated for 60 min at 80°C in a water bath. The solution was transferred to a 50 ml volumetric flask after cooling and was diluted to the mark with water. The fluorescence intensity was measured against the fluorescein setting solution.

Spectral Characteristics

Figure 1 shows excitation and emission spectra of the gallium–lumogallion complex in cases of the presence and absence of the surfactant. The addition of the nonionic surfactant to the solution of gallium–lumogallion chelate complex causes the increase of fluorescence intensity and the marked wavelength shift (17 nm) in the emission maximum of the complex to shorter wavelength, but no detectable shift was observed in the fluorescence excitation spectra. The

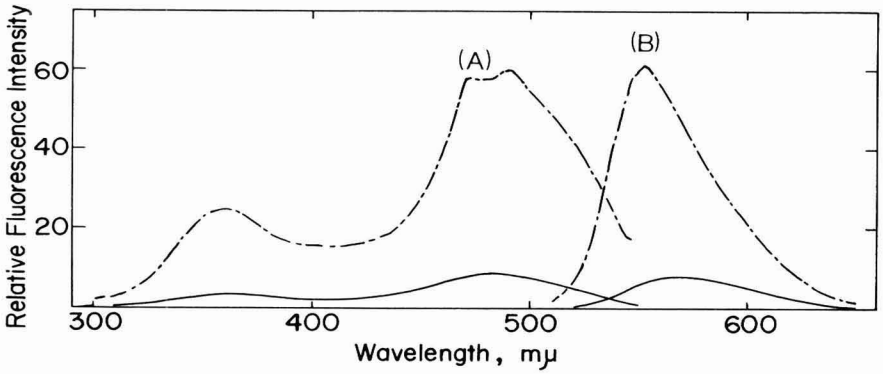


FIG. 1. Excitation (A) and emission (B) Spectra of the Ga-lumogallion chelate. (—) in the absence of surfactant; (— -) in the presence of nonionic surfactant (PGME).

shift of the emission maximum indicates that the interaction of the surfactant with the complex gave rise to the energy change of the excited state of the complex. The interaction between surfactant and ground state of the complex seems to be so small that it may be undetected spectrophotometrically (*7*). The enhancement effect of the nonionic surfactant can be used for the high-sensitive determination of gallium.

Effect of Surfactant

An addition of cationic surfactant to the solution of the complex also produced a large increase in the fluorescence intensity, whereas the anionic surfactant (dodecylbenzenesulfonate) showed a slight decrease of fluorescence. The effect of the cationic surfactant (tetradecyldimethylbenzylammonium chloride), however, was temporary; its enhancement effect on the fluorescence was large immediately after the surfactant was added, but it gradually decreased with time-lapse. Such unstable influence of the cationic surfactant is due to the dissociation of the complex (*2*). Increased fluorescence by the nonionic surfactant was stable at least 2 days. The nonionic surfactant, therefore, was proved to be useful from an analytical point of view. The fluorescence intensities increased with the increasing concentration of PGME, and reached to a constant and maximum intensity by the addition of more than 4 ml of 5% PGME for the 50 ml sample solution.

Effect of pH on Fluorescence Intensity

Fluorescence intensities of the sample solutions of various pH were measured at 553 nm with excitation at 490 nm. As shown in Fig. 2, the fluorescence intensity of the complex was constant in the pH range 3.1–4.3. The pH was adjusted to the value of 3.5 with acetate buffer solution for other studies.

Determination of Gallium

The effect of the lumogallion concentration was examined using 3 ml of 10^{-5} *M* gallium solution, 4 ml of 5% PGME, and various amounts of 10^{-4} *M* lumogallion solution at pH 3.5. Twofold excess of the lumogallion to the concentration of gallium was proved to be necessary in order to obtain a constant and maximum fluorescence. For the determination of 10^{-7} *M*–10^{-6} *M* of gallium in the 50 ml sample solution, 1 ml of 10^{-4} *M* lumogallion solution should be used. Figure 3 shows the working curves for gallium determination in both cases of the presence (A) and absence (B) of surfactant (PGME).

It is almost impossible to determine the micro quantities of gallium

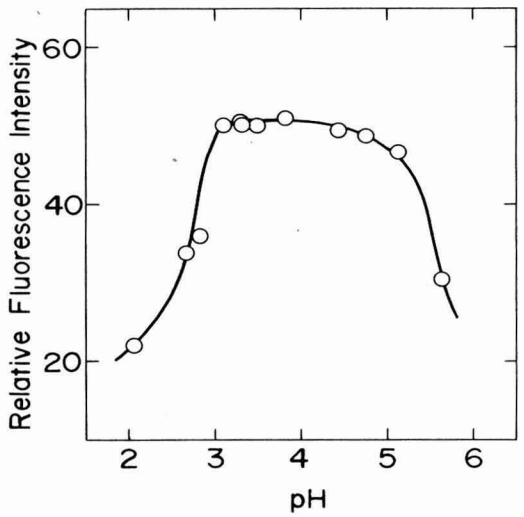


FIG. 2. Effect of pH.

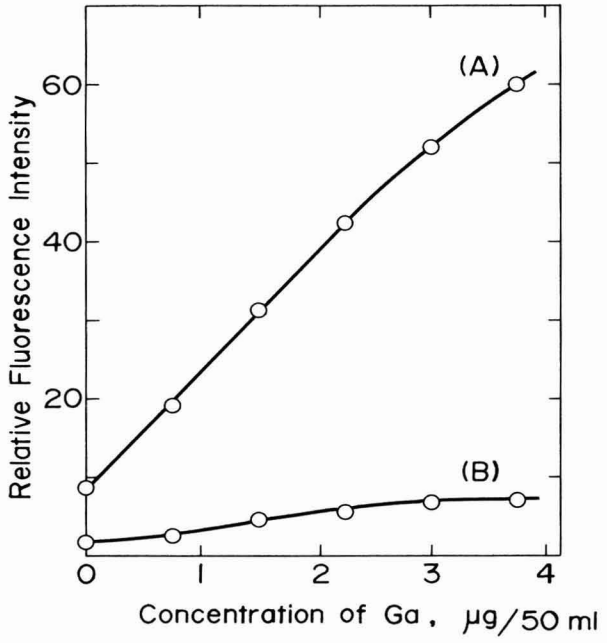


FIG. 3. Working curves for gallium determination. (A) in the presence of the nonionic surfactant: (B) in the absence of the surfactant. $\lambda_{Ex} = 490$ nm, $\lambda_{Em} = 553$ nm.

TABLE 1
EFFECT OF DIVERSE IONS[a]

Ions	Added as	Ga found (μg/50 ml)
Ni^{2+}	$Ni(NO_3)_2$	3.0
Co^{2+}	$CoSO_4$	5.2
Mn^{2+}	$MnCl_2$	1.8
Pb^{2+}	$Pb(NO_3)_2$	1.3
Cu^{2+}	$CuSO_4$	0
Al^{3+}	$KAl(SO_4)_2$	—
Fe^{3+}	$FeCl_3$	0
Sn^{2+}	$SnCl_2$	0.8
Cr^{3+}	$CrCl_3$	1.2
		1.5[b]
Zn^{2+}	$ZnSO_4$	2.3
Sr^{2+}	$SrCl_2$	2.8
		2.3[b]
Mg^{2+}	$MgSO_4$	2.6
		2.3[b]
Ba^{2+}	$Ba(NO_3)_2$	2.3
Ca^{2+}	$CaCl_2$	2.3
Cd^{2+}	$Cd(NO_3)_2$	2.3

[a] Each sample contained 2.3 μg of gallium in 50 ml of solution. The diverse ion of each sample was 100 μg in 50 ml of solution.

[b] 50 μg of diverse ions were added in 50 ml of the solution.

by the curve (B). The determination of 0.008 to 0.06 μg of gallium, however, can be carried out by using the curve (A).

Effect of Diverse Ions

All experiments on the effect of diverse ions were carried out with 2.3 μg/50 ml gallium. The results are summarized in Table 1. The ions, Zn^{2+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, did not interfere. The presence of Mg^{2+} or Sr^{2+} in 50 μg or less did not interfere. The ions, Al^{3+}, Cu^{2+}, Fe^{3+}, Sn^{2+}, Cr^{3+}, Pb^{2+}, Mn^{2+} interfered. Especially, Fe^{3+}, Cu^{2+}, and Sn^{2+} completely quench the fluorescence of the gallium complex. The Al^{3+} ion forms a very strong fluorescent complex with lumogallion (*1*, *2*). These interfering ions, therefore, must be masked or removed before the analysis. Shigematzu *et al.* have attempted the masking of iron with *o*-phenanthlorine (*6*). Separation of gallium from aluminum and the interfering ions is possible by using the ion-exchange resins or the solvent-extraction method (*4*).

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A New Type of Platinum Basket for Combustion of a Sample in a Closed Flask

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Combustion of a sample in oxygen in a closed flask, generally referred to as the Schöniger method (*2*), is universally accepted as an excellent means of combusting organic compounds. The literature on the application of this method is too extensive to detail here. In this laboratory alone the method is used in the determination of such diverse elements as sulfur, chlorine, bromine, iodine, fluorine, phosphorus, selenium, boron, and several metals.

A disadvantage is the cost of maintenance. In daily use, the platinum baskets must be replaced after several months at a considerable cost. Therefore, experiments were conducted with some new types of commercially available materials in an effort to reduce this high cost. Such a material (*1*) was found and proved to be superior in use to the wire-form platinum basket. This material, known as perforated platinum sheet, is sold in bulk by the square inch and can be cut easily with scissors to any desired size. Eight different designs are available (A–J, inclusive). The design found to be most appropriate for the purpose is No. F, the specifications for which are 0.062 in. diam holes, 144 holes/in.^{2}, 0.005 in. sheet thickness, and 1.0 g/l $\times$ 1 in. sheet.

Through the center of a section of sheet approximately 0.5 $\times$ 1 in. is threaded the platinum wire, which is then sealed to the glass stopper. After being folded in the center in sandwich fashion, the sheet is ready for use.

Over a period of months, more than a thousand combustions were performed, without any signs of deterioration of the platinum sheet. The size of the sheet may be varied for different sized flasks. Successful ignitions have been carried out in flasks ranging in size from 100 to 1000 ml, utilizing platinum sheets ranging in size from 1 to 2 in.^{2}.

The sample size varied from 1 to nearly 100 mg. Even with large samples, the presence of any charred material due to incomplete com-

bustion has not been observed, although it is observed sometimes when the standard wire-form platinum basket is used. The perforated platinum sheet provides a better container for the sample (wrapped in filter paper) than does the conventional basket type, due to the sheet's much greater surface. This greater surface might lead to a greater catalytic effect and the success of this rapid uncontrolled combustion possibly depends on the magnitude of the catalytic effect.

In comparison with the wire-type platinum basket, the perforated sheet has the following advantages: (a) larger surface area per dimension; (b) lower cost; (c) greater durability; (d) more intimate contact of sample, paper, and platinum; (e) no falling char that might escape ignition; and (f) option to increase or decrease size.

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Nano-scale Column Partition Chromatography

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INTRODUCTION

The chemical elucidation of flavor and aroma sometimes requires that micro-quantities of a compound be isolated from kilogram amounts of a starting material prior to analysis. It is not unusual for such conversions to result in accumulation of interfering contaminants. This problem is frequently encountered in spectroscopic methods of analysis which utilize gas chromatography for the purposes of isolation and purification. Incomplete resolution of peaks may also frustrate attempts at identification solely by interpretation of a mass spectrum obtained by coupled gas chromatography–mass spectrometry.

The smallest scale application of column partition chromatography has been to isolate volatiles from a 30–100 μl sample on $9 \times \frac{3}{16}$ in. i.d. Teflon tubing (*1*). In the absence of simple complements to gas chromatographic separation and purification, flavor complexes have seldom been fully elucidated. This communication reports the application of column partition chromatography to the improved resolution of nanograms of isolate.

EXPERIMENTAL

Two and one-half microliters of a natural product, gas chromatographic isolate was eluted through a 360×2 mm, i.d., glass capillary, packed with silica gel (0.05–0.2 mm) to a height of 240 mm. The remainder of the capillary was filled with an eluent which was selected to give the subfractions a small elution volume. Aliquots of 0.25 ml each of eluate were collected in three Microflex tubes (Kontes Glass Co., Vineland, NJ), evaporated under nitrogen to 2–3 μl, and rechromatographed by capillary-column gas chromatography under the following conditions: 10^3 ft $\times$ 0.03 in. o.d., stainless steel column of Carbowax 20 M; flow rate (He), 10 ml/min; program rate, 75–170°C at 2°C/min.

RESULTS AND DISCUSSION

Figure 1a represents two segments from the 40–60 min section of a gas chromatogram of the natural product isolate. Calculations by computerized area normalization indicated concentrations of the order of 10^{-9} g for each peak. Subfractions b and c in Fig. 1 are the identical segments after elution and concentration of the second and third 0.25 ml volumes of eluate from silica gel. Elution time was 2.5 hr. The improved separation of b and c was attributed to capillarity through the silica gel, and the resulting absence of diffusion. It was then possible to obtain relatively purer mass spectra by coupled gas chromatography–mass spectrometry. In one case (left side of Fig. 1), resolution was between terpene isomers, while in the other, the compounds were structurally unrelated. In Fig. 2, comparison is made between the 50 min segment of the gas chromatogram before (upper chromatogram) and after subfractionation of the first 0.25 ml on the silica gel. It indicates the ability of column partition chromatography

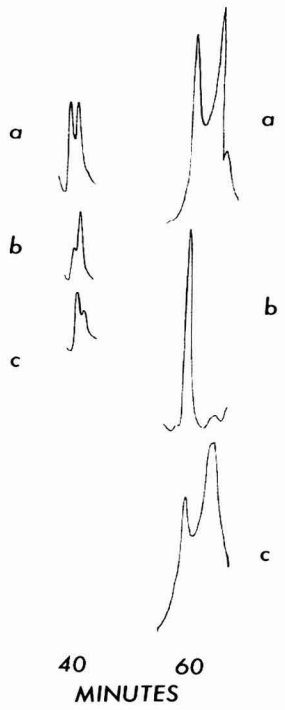

FIG. 1a. Segments (40 and 60 min) of a natural product, gas chromatographic isolate; gas chromatograms: (b) 2nd subfraction of 0.25 ml and (c) 3rd subfraction of 0.25 ml represent (a), after subfractionation of 2.5 μl by silica gel in a 2 mm i.d. glass capillary.

FIG. 2. Relative concentrations of peaks in the 50 min section of a gas chromatogram, before (upper chromatogram) and after subfractionation by silica gel in a 2 mm i.d. glass capillary.

in fine capillaries to effect large shifts in nano-scale concentrations of sample.

Thus, by adapting column partition chromatography to nano-scale quantities, it is possible to reduce the incidence of loss by contamination of samples of this magnitude, to obtain a high degree of resolution as a preparatory step to instrumentation, and to lower the threshhold of mass spectrometric identification for a given sample.

This technique is simple, compact, and has the advantage of a direct retrieval of solute.

SUMMARY

Column chromatography was adapted to natural product isolates in which the components were present in concentrations of the order of 10^{-9} g. Use for this technique has been found in purification of nanogram quantities of gas chromatographic effluents, and in resolution and concentration build-up of compounds which may otherwise be difficult to identify by gas chromatography–mass spectrometry.

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Determination of Calcium in Organometallic Compounds by Atomic Absorption Spectroscopy

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INTRODUCTION

Calcium determination by atomic absorption spectroscopy in organic compounds and industrial materials is not burdened with lasting preparations of sample for analysis. Presumably, simple dissolution in a suitable solvent should be sufficient. However, enhancement or depressive effects have been reported for organometallics in organic and aqueous solutions and this is the reason why the problem of an adequate standard arises (*1–4*). Moreover, the availability of a substance with acceptable properties and sufficiently soluble in a definite solvent sometimes represents a serious hindrance (*1*, *5*).

To avoid these difficulties a preliminary decomposition of the sample and a final determination in aqueous medium might be applied. In the present paper the usual wet digestion of the organic sample as well as the unhandy oxidation mixture are replaced by a fast flask combustion procedure (6, 7). Numerous determinations performed with dissolved and pyrolyzed samples of the same series of calcium containing compounds enable a mutual comparison and evaluation. Critically compared are also the results obtained by oxygen stream combustion with the addition of sulfuric acid and weighing of calcium sulfate (*8*) as well as those obtained by the spectrophotometric determination after dissolution of the residue with diluted acid (*9*).

MATERIALS AND METHODS

Reagents

Hydrochloric acid, 0.1 *M*.

Methanol (E. Merck A.G.).

$CaCl_2$ standard aqueous solution, 4.1 mg Ca/ml. This solution was standardized by complexometric titration with 0.1 *M* EDTA. The standard solutions of Ca in methanol were prepared by dilution of standard aqueous solution with methanol.

Dimethyl sulfoxide (DMSO) (E. Merck A.G.).

Methyl isobutyl ketone (MIBK) (E. Merck A.G.).

Standard solutions of Ca in MIBK; 571.80 mg $CaCl_2$ were dissolved in 100 ml DMSO. The aliquots were prepared by dilution of standard Ca solution in DMSO with MIBK.

All reagents used were of analytical reagent grade.

Apparatus

A Jarrell-Ash 82-500 atomic absorption spectrophotometer equipped with a Jarrell-Ash laminar flow (Tri-Flame) burner for air–acetylene flame and a Beckman potentiometric recorder were used throughout. A calcium hollow cathode lamp (422.7 nm) was used as a light source.

Other instrumental settings agree with recommendations of the instruction manual for this spectrophotometer.

Procedure

Calcium was determined by atomic absorption after dissolving samples in suitable solvents (0.1 *M* HCl, methanol, methyl isobutyl ketone) or after oxygen flask combustion. One to 2 mg of the substance was wrapped in filter paper and decomposed by ignition in an oxygen filled flask. After about 10 min the absorption solution (10 ml 0.1 *M* HCl) was transferred into a calibrated flask and filled up to a volume of 25 ml.

DISCUSSION OF RESULTS

The results of a series of calcium determinations by AAS in simply dissolved organometallic compounds are shown in Table 1. In some

TABLE 1
RESULTS OF CALCIUM DETERMINATIONS IN DISSOLVED SAMPLES

Compound	No. of determinations	Ca (%) Calcd	Found (mean value)	SD	SE
Ca-metacycline[a]	10	7.20	8.00	0.15	0.05
Ca-metacycline[b]	10	7.20	7.57	0.16	0.05
Ca-lactate × H_2O[a]	10	16.96	16.87	0.48	0.15
Ca-gluconate × H_2O[a]	10	9.27	7.85	0.19	0.06
$CaNa_2EDTA \times 2.5\ H_2O$[a]	10	9.56	8.36	0.22	0.07
Ca-sulfonate[c]	7	~13	15.25	0.29	0.11

[a] Dissolved in acid.
[b] Dissolved in methanol.
[c] Dissolved in MIBK.

compounds the results show an appreciable difference between the theoretical and found values. This is evident in the case of Ca-gluconate, $CaNa_2EDTA$ and Ca-metacycline dissolved in 0.1 *M* hydrochloric acid. More accurate results are obtained for Ca-metacycline dissolved in methanol. The present results suggest that the nature of the substance or the solvent used can cause systematic errors in direct calcium analysis by AAS in agreement with experiences of other investigators (*4*). Even though the reasons for this effect are not known so far, it is without doubt that in using atomic absorption technique the analyst should be aware that an organometallic in aqueous medium and in an organic solvent may behave differently than expected. To obtain accurate results it would be necessary to search for an adequate solvent and standard (e.g., Ca-metacycline in methanol). Another possibility is to use oxygen flask combustion as the initial step.

The results of calcium determinations after oxygen flask combustion of the same compounds by identical final procedure are shown in Table 2. The values found for calcium are in good agreement with the theoretical values with the exception of Ca-gluconate. However, even in this case the difference between the theoretical and found values is smaller than when the substance is dissolved in hydrochloric acid. As regards the accuracy, standard deviations and standard errors, the values for calcium listed in Table 2 are comparable to those obtained by the spectrophotometric determination after the compound is burnt in a stream of oxygen and the residue is dissolved in diluted acid (*9*). As the combustion products cause difficulties in the spectrophotometric determination, the flask combustion procedure was replaced by oxygen stream pyrolysis. These problems were not realized when AAS was applied as final procedure following flask combustion. Moreover, in AAS the problem of the phosphate simultaneously present in calcium compounds can be simply circumvented by the addition of lanthanum.

TABLE 2
RESULTS OF CALCIUM DETERMINATIONS IN PYROLYZED SAMPLES

Compound	No. of determinations	Ca (%) Calcd	Found (mean value)	SD	SE
Ca-metacycline	22	7.20	7.43	0.28	0.06
Ca-lactate × H_2O	20	16.96	16.86	0.46	0.10
Ca-gluconate × H_2O	20	9.27	8.64	0.20	0.05
$CaNa_2EDTA$ × 2.5 H_2O	20	9.56	9.11	0.33	0.07
Ca-sulfonate	8	~13	11.78	0.28	0.10

TABLE 3
RESULTS OF CALCIUM DETERMINATIONS IN A LUBRICANT OBTAINED BY DIFFERENT METHODS

Method	No. of determinations	Ca (%) found (mean value)	SD	SE
AAS				
Sample dissolved in MIBK	7	15.25	0.29	0.11
Sample pyrolyzed in oxygen filled flask	8	11.78	0.28	0.10
Sample pyrolyzed in oxygen stream	2	11.01		
Sample burnt in an oxygen stream				
With H_2SO_4; $CaSO_4$ wt detn	6	9.51		
Residue dissolved; spectrophotometric detn	3	9.23		

The results obtained with all methods mentioned in the introduction using an identical lubricant are shown in Table 3. Different combustion procedures combined with different final determinations—gravimetric, spectrophotometric, atomic absorption—performed in three different laboratories gave results nearly within ±1% of the expected value.

In the AAS method by direct dissolution of the sample the aberrations are more serious (>5%) despite the fact that in the preparation of standards the matrix characteristics were respected, but evidently not enough.

It can be concluded that when adjusted solvent and standard are to be found for individual samples and sporadic analyses, the combustion of the sample in an oxygen filled flask and AAS determination of calcium in aqueous medium represent the fastest and easiest procedure.

SUMMARY

A simple and relatively rapid procedure for the estimation of calcium in organometallic compounds is described. The oxygen filled flask for decomposition of the organic sample and AAS determination of the metal in aqueous medium without any effect of the organic portion of the molecule or solvent is recommended. The results of calcium determination in a lubricant obtained by different combustion procedures and different final determinations are also compared.

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Delayed Luminescence Analysis (DLA) of Purine and Pyrimidine Ribose and Deoxyribose Nucleotide Triphosphates in Picomole Quantities

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The light emission system using firefly lanterns has been used extensively for the measurement of adenosine triphosphate (ATP) (*12, 3, 2*), adenosine tetraphosphate (AP_4) (*6, 7*), and guanosine triphosphate (GTP) (*6, 8*). Both AP_4 and GTP are analyzed by delayed luminescence analysis (DLA) in which the maximal intensity of the light occurs after a period of minutes following the mixing of nucleotide and the firefly extract (*7, 8*). This report demonstrates that this (DLA) system can be extended to include all the major nucleotide triphosphates both purines and pyrimidines of either ribose or deoxyribose composition.

There have been previous attempts at indirectly measuring nucleotide triphosphate pools but these techniques are less useful and more cumbersome to perform (*3, 5, 10, 11*).

MATERIALS

Nucleotides (triphosphates) were purchased from Sigma Chemical Company (St. Louis, MO). Freeze-dried firefly lanterns were prepared from locally caught fireflies or purchased from Sigma Chemical Co. Precoated MN-polygram CEL 300 PEI/UV 254 thin-layer chromatograms were obtained from Brinkmann Instruments, Inc. (Westbury, NY) and developed in a Kodak Sandwich Apparatus. All other reagents were of analytical grade.

METHODS

Commercial nucleotide triphosphates GTP, dGTP, dATP, ITP, UTP, dUTP, CTP, dCTP, and TTP must be purified before use on PEI plates (10 × 20 cm). Usually 0.5 mg/ml solution of the nucleotide was prepared (*7*) and 10 μl spotted on PEI-plates previously washed in H_2O. The nucleotides were developed first in water and again in 1.4 *M* LiCl in the same dimension. Total development time was about 50 mins.

Nucleotides were viewed under short wave ultraviolet (260 nm) light and cut out, eluted with 0.5 ml of 0.08 N K_2CO_3 and appropriately diluted with phosphate buffer (KH_2PO_4 10 mM, $MgSO_4$ 4 mM, pH 7.4) and stored frozen at −20°C.

Assay. Firefly luciferin–luciferase was prepared as described previously (*7*). The reaction mixture contained 0.4 ml nucleotide triphosphate of varying concentrations (see text) and 7.4 ml of phosphate buffer in a scintillation vial. The enzyme preparation (0.2 ml) was

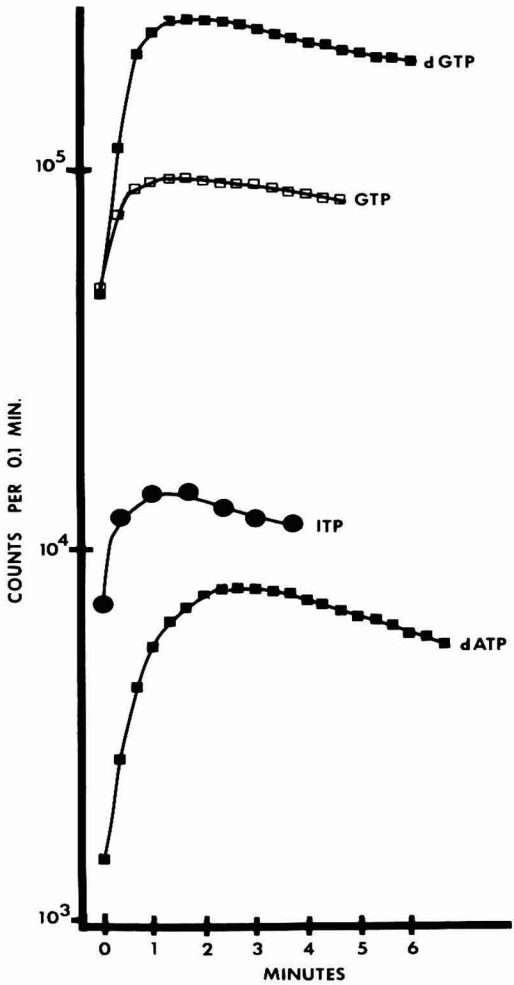

FIG. 1. Semilog plot of delayed light emission reaction kinetics of dGTP, ITP, and dATP. Concentrations of nucleotides in moles/ml are dGTP, 4.7×10^{-11}; ITP, 3.5×10^{-11}; and dATP, 1.7×10^{-11}. The plots were generated by repeated counting of sample for 0.1 min and 3 times every minute. Reaction was started by addition of firefly extract to the substrate in phosphate buffer (10 mM KH_2PO_4 and 4 mM $MgSO_4$, pH 7.4) and assayed at 6°C maintained in the spectrometer.

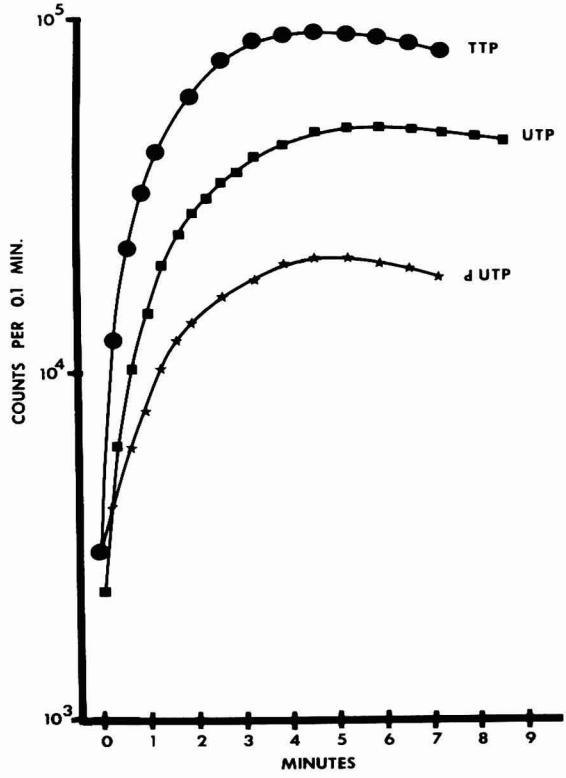

FIG. 2. Semilog plot of delayed light emission reaction kinetics of TTP, UTP, and dUTP. Concentrations of nucleotides in moles/ml are TTP, 3.3×10^{-11}; UTP, 1.5×10^{-11}; and dUTP 1.75×10^{-11}. The plots were generated by repeated counting of sample for 0.1 min and 3 times every minute. Reaction was started by addition of firefly extract to the substrate in phosphate buffer (10 m*M* KH_2PO_4 and 4 m*M* $MgSO_4$, pH 7.4) and assayed at 6°C maintained in the spectrometer.

added to start the reaction and counted in a Packard Liquid Scintillation Counter Model 3320 equipped with RCA 4501-V3 bialkali photomultipliers. The counter was set with photomultiplier coincidence (switch in the back of the instrument) turned off, window of the first channel A-B adjusted to 60–65, and a 100% gain. Time (approx 20 sec) required from the addition of the enzyme to actual counting was held constant. This precise timing was achieved by counting an empty vial and as soon as the adder printed, enzyme was added to the next vial in the series mixed, etc.

RESULTS

Our data indicate that light is produced in the presence of a large variety of nucleoside triphosphates whether they be purines or pyrim-

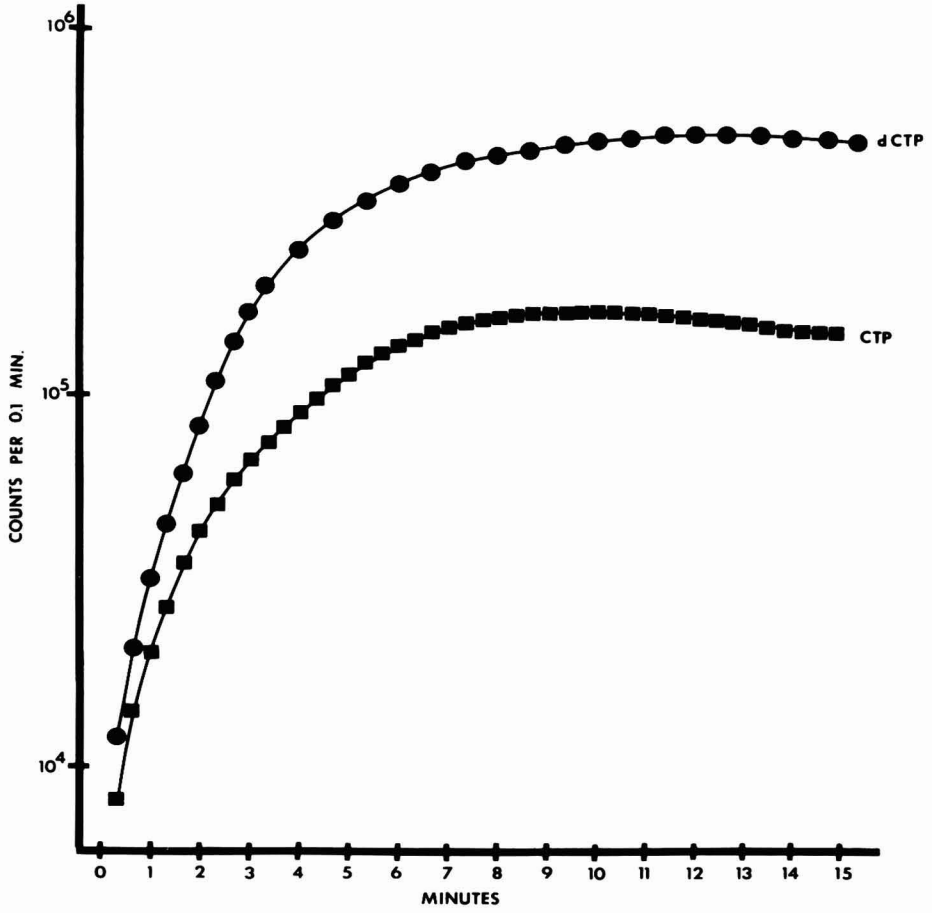

FIG. 3. Semilog plot of delayed light emission reaction kinetics of dCTP (1.75×10^{-11} moles/ml) and CTP (1.75×10^{-11} moles/ml). The plots were generated by repeated counting of sample for 0.1 min and 3 times every minute. Reaction was started by addition of firefly extract to the substrate in phosphate buffer (10 m*M* KH_2PO_4 and 4 m*M* $MgSO_4$, pH 7.4) and assayed at 6°C maintained in the spectrometer.

idines or whether they be deoxyribose or ribose compounds. However, in all cases with the exception of adenosine triphosphate (*7*), the peak of the light that is produced occurs at a point in time after the initial mixing of enzymes, substrates, and cofactors (Figs. 1–3). This time required for maximal light emission is constant for a given nucleotide and is characteristic of the compound. Earlier we reported this delayed maximal light emission property with GTP (*6*, *8*) and AP_4 (*6*, *7*).

A composite picture of these peak-emission times for each nucleo-

TABLE 1
TIME OF MAXIMAL LIGHT INTENSITY (DELAYED RESPONSE) BY NUCLEOTIDES

	RNA nucleotides	Time (min)	DNA nucleotides	Time (min)
Purines	ATP	0 (*7*)	dATP	2
	GTP	2 (*8*)	dGTP	2
	ITP	2	–	–
Pyrimidines	UTP	5	dUTP	5
	CTP	10	dCTP	12
	–	–	TTP	5

tide tested is given in Table 1. Purine nucleotides both DNA and RNA types (with the exception of ATP) emit maximum light in approximately 2 min after the initial mixing of the reaction components. The pyrimidine nucleotides UTP, dUTP, and TTP all required 5 min for their maximal light emission whereas CTP and dCTP required 10 and 12 min, respectively. Therefore the delayed luminescence of these nucleotides generally falls at 2 min, 5 min, and 10–12 min time categories.

In Table 2, we note the "amplification" of light associated with the maximal response (time of maximal light emission). Amplification refers to the ratio between the amount of light emitted at peak time and the light emitted at the initial or zero time. At the present time amplification appears to be slightly variable and we believe that this is due to the fact that between runs, the concentration of the enzyme is not held constant. Also, more work needs to be done to understand the stability properties of enzyme(s) involved. Despite this variability, it is to be noted that if amplification is not observed similar to the

TABLE 2
AMPLIFICATION OF LIGHT ASSOCIATED WITH MAXIMAL RESPONSE[a]

	RNA nucleotides	Amplification	DNA nucleotides	Amplification
Purines	ATP	– (*7*)	dATP	3
	GTP	2–4 (*8*)	dGTP	8
	ITP	2–4	–	–
Pyrimidines	UTP	17	dUTP	8
	CTP	30	dCTP	110
	–	–	TTP	30

[a] Amplification denotes ratio between the amount of light emitted at peak time and the amount of light at zero time.

quantities shown in Table 2, other factors in the analysis need to be checked (see Discussion). In our experience we have found that contamination from other nucleotides will alter the amplification factor.

Standard curves developed for each of the nucleotides tested are given in Figs. 4–7. They all give a straight line relationship when plotted on a log × log scale therefore making it possible to determine concentrations of the nucleotides based on the amount of light

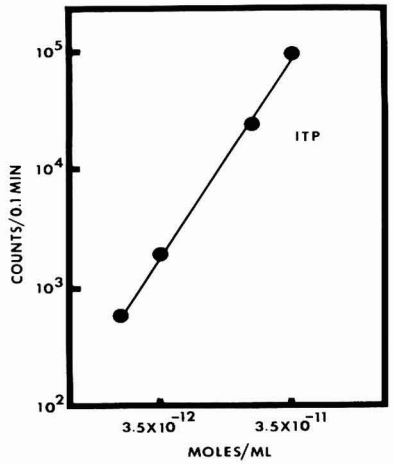

FIG. 4. Log × log relationship between concentration of ITP and number counts. Reaction was started by addition of firefly extracts to ITP in phosphate buffer and assayed at 6°C and 2 min after the addition of enzyme.

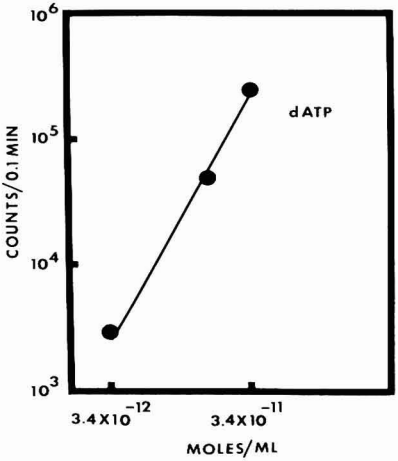

FIG. 5. Log × log relationship between concentration of dATP and counts. Reaction was started by addition of firefly extract to dATP in phosphate buffer and assayed at 6°C, 2 min after the addition of enzyme.

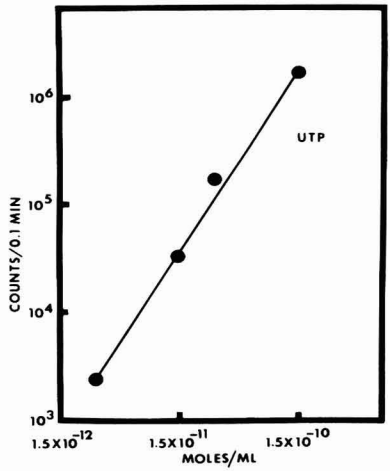

FIG. 6. Log × log relationship between concentration of UTP and counts. Reaction was started by addition of firefly extract to UTP in phosphate buffer and assayed at 6°C, 5 min after the addition of enzyme.

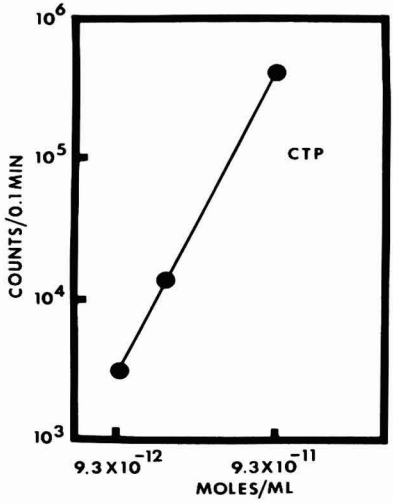

FIG. 7. Log × log relationship between concentration of CTP and counts. Reaction was started by addition of firefly extract to CTP in phosphate buffer and assayed at 6°C, 10 min after the addition of enzyme.

emitted or the number of counts. It is to be noted that separate standard curves need to be developed for each assay as discussed previously (*7*).

When testing for purified nucleotide triphosphates in unknown materials, once the identity of the nucleotide is clear, it is advisable that purified nucleotide be added to the preparation under study to de-

tect the possibility of inhibiting substances that could interfere with the DLA light emission. To simply use light emission as a criterion of quantitation of nucleotide triphosphate is to miss the maximal quantitative use of the system. In a previous paper (*8*) concerning analysis of GTP we detailed a procedure for using added GTP as an internal standard.

DISCUSSION

Firefly luciferin–luciferase light reaction system has been extensively used to analyze ATP primarily because it requires a relatively short experimental time yet a high degree of sensitivity is achieved. It was reported to exhibit a high degree of specificity for ATP (*4*), reacting with iso-ATP at a fraction of efficiency of ATP and nonreactive with all other purine tri- and tetraphosphates tested (*4*, *9*).

Recently, we showed a crude system is not specific for ATP, instead it emits light in the presence of GTP as well as AP_4. This enabled us for the first time to extend the application of the luciferin–luciferase system to analyze these nucleotides with the same degree of sensitivity and efficiency as ATP.

Our continued investigation to develop sensitive methods to measure purine and pyrimidine triphosphates have unfolded that the method could be used to analyze all naturally occurring nucleotide triphosphates, both purines and pyrimidines of either ribose or deoxyribose composition.

Amplification is an inherent characteristic and fundamental to the basis of DLA. Purines give less amplification than pyrimidines but low amplification is not an indication of lower sensitivity. The lower amplification with purines is probably due to their initial high activity.

Application of this phenomenon of DLA is the single most important reason that permitted analysis of a variety of nucleotides tested in picomole concentrations. Methods available to quantitate these compounds were not nearly as sensitive yet highly time consuming and expensive to run (*3*, *5*, *11*).

Each of the nucleotides exhibited a definite time after the addition of the enzyme when a maximum amount of light was emitted. This specificity and the associated sensitivity of DLA is therefore in sharp contrast to the relatively insensitive and nonspecific ultraviolet absorption analysis of these compounds more frequently encountered (*1*).

Factors necessary for DLA of nucleotides were found to be the same as for standard luciferin–luciferase reaction, i.e., it required: (a) nucleotide (tri- or tetraphosphate), (b) firefly lantern extract (containing luciferin), (c) Mg^{2+}, and (d) O_2.

Investigations to explain the mode of action of luciferin–luciferase with the nucleotides are presently underway.

DLA has made it possible to determine the pool size of these nucleotides efficiently and with a high degree of sensitivity. Nucleotide triphosphates, particularly the deoxyribose compounds, which occur in smaller concentrations than their ribose counterparts can be quantitated without the use of labeled compounds.

DLA of all nucleotides can be automated like the one similarly developed for ATP analysis (*15*) and can be used essentially as a readout system. Such a readout system will be several orders of magnitude higher in sensitivity than the conventional UV absorption analysis. Therefore a readout of this type could be attached to high pressure liquid chromatography (*1*) or to sequence polynucleotides (RNA, DNA) by converting the free base to triphosphate level by established enzymatic methods (*14*).

The system may be adaptable to monitoring growth of living organisms because all organisms require the various nucleotide triphosphates for their vital life processes. As such this system could be important in problems of environmental contamination. It can be important in discovering the importance of the nucleotide tri- and tetraphosphates in the life processes. Particularly, we have used these systems in our research in pharmacology to try to understand the role of nucleotides in nerves and how drugs and physiological parameters affect nucleotide content. Also, these nucleotides that the DLA system reacts with may be important in storage of blood cells to maintain viability or in storage of cell lines and organ preservation.

This system fills an analytical void previously unattainable and the possible uses of this system include: (a) measurement of cellular nucleotide triphosphate pool-size, (b) sequence analysis of DNA and RNA, (c) measurements of cyclic nucleotides, e.g., cyclic 3′5′-AMP and cyclic 3′5′-GMP via coupled analysis, and (d) as a detector for high pressure liquid chromatography using DLA thereby increasing analytical sensitivity in the order of 10^3–10^4.

SUMMARY

A discovery is reported of a new system that enables one to quantitate the amounts of separated nucleotide triphosphates in picomole quantities. This system of delayed luminescence analysis (DLA) is sensitive to both purine and pyrimidine ribose and deoxyribose nucleotide triphosphates. A crude luciferin–luciferase (substrate–enzyme) preparation from firefly lanterns, in the presence of nucleotide triphosphate, is utilized to generate light that is detected by a liquid scintillation counter with the coincidence of the photomultiplier tubes turned off. Light is produced in a delayed fashion, the maximum emission being dependent on the type of nucleotide. Purine nucleotides (GTP, ITP, dATP, dGTP) give maximal light emission at approximately 2 mins; with the

pyrimidine nucleotides the time required for maximal light emission was 5 min for UTP, dUTP, and TTP, 10 min for CTP, and 12 min for dCTP. A linear relationship on a log–log plot of light emission vs. concentration of nucleotide is demonstrated with ITP, dATP, UTP, and CTP.

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Small-Scale Cell for External Generation of Coulometric Titrants

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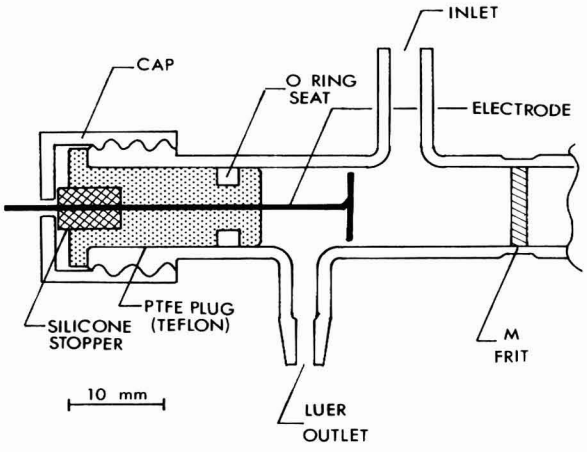
The device shown in Fig. 1 was made by sealing a fritted glass disc between the tops of two screw-capped centrifuge tubes and then adding the inlet and outlet arms on each side. The Teflon plugs were machined to fit and bored to accept Kontes Glass Company silicone rubber stoppers. Electrodes were inserted by piercing the stopper with an 18 gauge needle containing a stylet, removing the stylet, inserting the electrode through the lumen of the needle, and then withdrawing the needle while holding the electrode in position.

A separate inlet for each half-cell permits the addition of feedstock solution containing the titrant precursor through a variety of fittings ranging from a simple Y-tube and metering pump if electrode gassing is not expected to more elaborate devices to permit the escape of gases when needed.

The electrode most often used with this cell is a flat spiral of platinum wire but a screen or perforated disc of platinum could be substituted to vary the available electrode surface.

The electrolyzed solution is delivered to the titration vessel through a Teflon tube with Luer taper syringe needle hub.



This cell has proved useful for generating acid or base to maintain the appropriate pH during preparative electrolyses in a larger cell and for generation of iodine titrant from iodide precursor. In the latter case, two tubes may be used in the peristaltic feed pump, and non-iodide-bearing sodium bicarbonate solution may thus be fed to the cathode chamber so iodide is not wasted.

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Fluorometric Assay of Ultramicro Quantities of Glucose with Somogyi Filtrate and Hexokinase[1]

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In a previous paper a generalized system was set up for microanalysis in the Pediatric Laboratory (*3*). It was pointed out that the frequency with which turbidity, high bilirubin content, and hemolysis occur in serum or plasma from the neonate all contributed to the fact that procedures were needed to circumvent interference from these substances. In addition a high degree of sensitivity was necessary because of the small sample available from a premature infant.

It was apparent that in order to achieve this purpose interfering substances had to be removed. This was readily accomplished by precipitation with zinc hydroxide. Hemoglobin, protein, bilirubin, lipids, uric acid, and a host of other substances are removed leaving a clear supernatant.

When the toluidine (*1*, *6*), glucose oxidase (*2*), and hexokinase (*5*, *7*) methods were applied for glucose analysis, using the spectrophotometer, sensitivity was not adequate at the level where the test was performed. The NADPH formed in the hexokinase reaction may also be measured by fluorescence (*4*). Fluorescence yields a factor of sensitivity of at least 20 under practical conditions over the procedure using absorbance in measuring NADPH.

MATERIALS AND METHODS

Reagents

Barium hydroxide (*0.5 N*). Dissolve 7.89 g of $Ba(OH)_2 \cdot 8H_2O$ in CO_2-free water and make up to 1 l.

Zinc sulfate (*0.5 N*). Dissolve 7.19 g of $ZnSO_4 \cdot 7H_2O$ in water and make up to 1 l. When this solution is mixed in equal quantities with the barium hydroxide solution, the pH should be 7.0–7.2.

[1] Supported in part by the NIH Training Grant No. GM 2124-03 and the Morris Natelson Research Fund.

Tris buffer (pH 8.0, 0.25 M). Dissolve 3.03 g tris(hydroxymethyl)aminomethane (Sigma's TRIZMA) in about 70 ml water. Add 1 *N* HCl until the pH is 8.0 (Approx. 13 ml). Make up to 100 ml with water. Store in refrigerator.

Magnesium chloride (0.2 M). Dissolve 1.0 g $MgCl_2 \cdot 6H_2O$ in 25 ml water.

Albumin solution. Dissolve 10 mg of crystalline bovine serum albumin (Sigma) in 100 ml of lukewarm water. Store frozen.

Adenosine triphosphate (0.01 M). Dissolve 60 mg ATP disodium dihydrogen trihydrate (Sigma, Grade I) in 10 ml water. Store frozen.

NADP (10^{-3} M). Dissolve 13 mg NADP in 15 ml of water. (Sigma, Nicotinamideadenine dinucleotide phosphate monosodium dihydrogen, β-form, 98–100% purity). Store frozen.

Hexokinase (20 U/ml). Dissolve the contents of one 500-unit vial (Sigma, Type F, 300 U/mg) with 25 ml of the albumin solution. Stable frozen for 3 months.

G-6-P-DH (1 U/ml). A 100-unit vial's contents are washed out and diluted to 100 ml with water. (Glucose-6-phosphate dehydrogenase 200–400 U/mg, Type XV, Sigma). Transfer to small vials and store frozen. Stable frozen for 1 month.

Substrate. To 4 ml Tris buffer add 1 ml of each: ATP, NADP, hexokinase, albumin, and G-6-P-DH solutions and mix. Add 1 ml of the magnesium solution and mix again. This mixture has a half-life of four days in the refrigerator.

Commercial Substrates

(Note: these may be used in place of the *substrate* prepared above.)

Sigma. To one single assay vial (Cat. No. 15-1) add 6 ml of deionized water. Mix well. If the 10-assay vial (Cat. No. 15-10) is used, wash into a glass stoppered bottle with 60 ml of water. The half-life of this reagent in the refrigerator is 7 days.

Worthington. To one 5-test vial (U.V Glucose Reagent, Cat. No. 7490) add 15 ml of the buffer supplied with the kit. The half-life of this reagent is 7 days in the refrigerator.

Calbiochem. To a 5-test vial labeled A (Glucose Stat-Pack, Cat. No. 869204) add 10 ml of deionized water. Add 10 ml of water also to the B-Vial. Mix the contents of both vials. The half-life of this reagent is 10 days.

General diagnostics. To one 10-test vial of "Glucostrate" add 15 ml of deionized water. Mix well by rotating on a rotator. The half-life of this reagent is more than 30 days in the refrigerator. Note that the color reagent supplied with this kit is not used in this procedure.

PROCEDURE

With Protein Precipitation

By means of a sampler–diluter fitted with a double dispenser (Rohe Sci. Co., Santa Ana, CA) sample 10 μl and dispense 300 μl of water from the sampler diluter and 0.7 ml each of barium hydroxide and zinc sulfate from the dispenser. Mix and let stand for 10 min and centrifuge. To 0.5 ml of the supernatant add 1 ml of substrate into a disposable 10 × 75 mm Kimble glass test tube. Incubate at 37°C for 15 min.

For the blank and standards treat 10 μl of water and the standards in the same manner as for the unknown. Prepare a standard curve with 50, 100, 150, 200, and 250 mg/100 ml glucose standards. With the Farrand fluorometer (Mark I) maximum excitation was at 353 nm and emission at 455 nm.

Set the instrument to zero with water and to maximum scale deflection with the 250 mg/100 ml standard. Read the blank and the various unknowns. Subtract the blank reading from all of the other readings to obtain the net reading. This value is converted to mg/100 ml by comparison with the standard curve which is a straight line.

Direct Assay

From the sampler–diluter sample 3 μl and dispense 500 μl of water into the disposable test tube. Add 1 ml of the reagent and proceed as above. Treat 3 μl of water for the blank, and 3 μl of the standards as for the unknown to obtain the standard curve.

RESULTS

As can be seen from Table 1 the fluorometric hexokinase procedure correlates well with the procedure using the *ortho*toluidine after protein precipitation. When the t test was applied to the results on the thirty serums no significant difference was found between the means at the 5% probability level. Similarly the F test showed no significant difference between the reproducibility of the procedures. The correlation coefficient, comparing results by the two procedures for the same specimen was +0.97.

Bilirubin levels as high as 40 mg/100 ml do not interfere in the procedure even when protein is not precipitated. This can be readily seen in Table 2. Hemolysis, however, will result in a significant overestimation of the glucose level when the hemoglobin level rises above 200 mg/100 ml (Table 3). This calculates to a hemolysis of approximately 1.5% of the cells initially present. After protein precipitation the supernatant is colorless and this source of interference is eliminated.

TABLE 1
COMPARISON OF GLUCOSE VALUES WITH THE HEXOKINASE AND TOLUIDINE PROCEDURES, BOTH AFTER PROTEIN PRECIPITATION[a]

Specimen no.	Hexokinase	Toluidine	Specimen no.	Hexokinase	Toluidine
1	51.5	46.0	16	120	112
2	71.0	61.5	17	114	117
3	73.3	75.5	18	102	126
4	88.3	85.5	19	135	131
5	86.8	87.5	20	147	134
6	88.5	88.0	21	149	135
7	93.0	89.0	22	133	139
8	96.0	89.0	23	133	143
9	111.0	94.0	24	155	154
10	95.0	96.0	25	151	156
11	86.0	97.0	26	175	180
12	88.5	103	27	189	195
13	105	105	28	218	201
14	122	105	29	223	217
15	112	111	30	250	242
			Mean	125.7	123.8
			Coeff. of variation	±2.26%	±2.70%

[a] Results are the mean of duplicates expressed in mg/100 ml.

TABLE 2
EFFECT OF BILIRUBIN ON THE GLUCOSE FLUOROMETRIC HEXOKINASE PROCEDURE WITH AND WITHOUT PROTEIN PRECIPITATION[a]

Bilirubin (mg/100 ml)	Glucose (mg/100 ml)	
	Pptd.	Nonpptd.
2.7	77	77
5.4	63	59
7.8	73	71
11.2	118	113
18.1	85	85
21.1	90	85
40.0	382	377

[a] Results are means of duplicate determinations. Serums are from the routine laboratory. Bilirubin levels are assayed values.

The standard curves obtained with this procedure are straight lines going through the origin to levels in excess of 250 mg/100 ml. This can be seen in Table 4, where recovery of added glucose is quantitive within the limits of experimental error of the procedure, when 3 μl are being analyzed.

TABLE 3
EFFECT OF HEMOLYSIS ON THE RESULTS OBTAINED WITH THE HEXOKINASE SYSTEM WITHOUT PROTEIN PRECIPITATION[a]

	Hb (mg/100 ml)					
	0	100	200	300	1000	2000
Glucose (mg/100 ml)	69.0	71.9	75.3	79.3	87.9	95.7

[a] Analysis of the same specimen with varying amounts of added hemolysate.

TABLE 4
RECOVERY OF ADDED GLUCOSE IN THE HEXOKINASE SYSTEM[a]

Serum concn (mg/100 ml)	Glucose added (mg/100 ml)	Found (mg/100 ml)	Recovered (mg/100 ml)	Recovery (%)	S. D. (%)
82.3	160	247.7	165.3	103.3	±3.50

[a] Results are the mean of ten replicates.

TABLE 5
COMPARISON OF THE VARIOUS HEXOKINASE ENZYME MIXTURES FOR SPECIFICITY IN GLUCOSE ANALYSIS[a]

Preparation	Sucrose, 200 mg/100 ml	Mannose, 100 mg/100 ml	Fructose, 100 mg/100 ml
This paper	0	0.2	18.9
Sigma	0	0.1	4.7
Worthington	0	0	6.2
Calbiochem	0.5	0.8	15.5
Gen. Diagnostics	1.1	2.4	85.5

[a] Values are glucose equivalents in mg/100 ml.

The specificity of the hexokinase procedure was explored using various sugars with the substrate mixture described in this paper and the various commercially available enzyme preparations, diluted as described in the experimental part of the paper (Table 5). Incubation time was 60 min. The table lists those where some interference was observed. Note that fructose will interfere to some extent in all of the preparations. However, in view of the low fructose level in plasma only the preparation of General Diagnostics could introduce significant differences.

Table 6 illustrates the fact that blood can be drawn with fluoride as preservative since no interference is detected.

TABLE 6
STUDY OF FLUORIDE INTERFERENCE IN THE HEXOKINASE REACTION[a]

Method	No. samples	Mean (mg/100 ml)	Coeff. of variation (%)
Hexokinase	85	133.9	2.97
Ferricyanide (Autoanalyzer)	85	134.2	3.45

[a] Blood contained 10 mg NaF per ml. The coefficient of correlation between the two procedures was +0.964.

Figure 1 demonstrates the rate of NADPH formation with time for the various substrate mixtures at 25°C. It should be noted that any of these preparations could be accelerated by increasing the concentration of the reagents. The dilution chosen in each case is the greatest which will still complete the reaction in 5–8 min. This is done to reduce cost to a minimum.

The "half-life" figures for the stability of the various substrate solutions is that time which results in a 100% increase in the time required to complete the reaction in the procedure described.

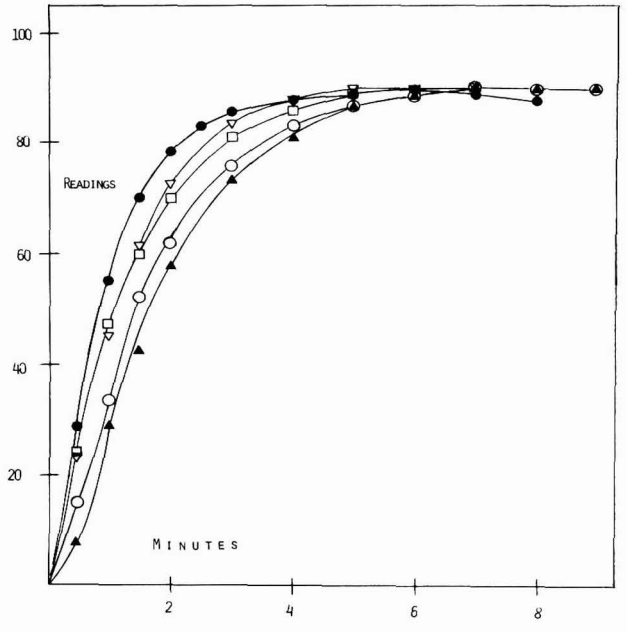

FIG. 1. Plot of the relative increase in fluorescence with time for the various substrate mixtures used. The reagents used were ○—○, General Diagnostics; □—□, Sigma; ▲ — ▲, Worthington; ▽ — ▽, Calbiochem; ● — ●, our preparation.

DISCUSSION

The use of fluorometry for the determination of the NADPH formed in the hexokinase system for the detection of glucose permitted the assay of 3 μl of serum in a final volume of 1.5 ml of solution. For a normal glucose level of 100 mg/100 ml this corresponds to 3 μg of glucose. With the instrumentation used, the fluorometer was set at low sensitivity. If the reaction is carried out in a final volume of 100 μl, which can be used in the Farrand fluorometer, it is practicable to assay 5 ng of glucose readily. This brings glucose estimation into the range where it can be used for analyzing nanoliter volume specimens such as those obtained from glomerular filtrates in animals with the micromanipulator.

It is of interest to note from Table 2 that bilirubin values as high as 40 mg/100 ml do not interfere even if protein is not precipitated initially. However, severe hemolysis will result in elevated values probably due to light scattering and release of other fluorescing materials from the red cells. Slight to moderate hemolysis will not interfere (Table 3). All of this is readily removed when the protein is precipitated before analysis.

The results (Table 1) correlated well with those obtained by the toluidine procedure using 200 μl of serum and precipitating the proteins. This latter procedure was chosen for comparison because it is now widely recommended as a standard procedure. The coefficient of variation of the duplicates was $\pm 2.26\%$. This compares with a value of $\pm 2.70\%$ found with the toluidine procedure, using 200 μl of serum. The toluidine procedure is not sensitive enough to assay the small volumes used with the hexokinase procedure. The toluidine procedure also requires heating followed by cooling to room temperature which more than doubles the time required for the analysis.

The hexokinase procedure is linear to glucose levels as high as 250 mg/100 ml. In the recovery experiment (Table 4) the initial glucose level of 82.3 mg/100 ml is raised to 247.7 mg/100 ml or three times its value and recovery of added glucose is within $\pm 3.5\%$ using a linear curve in the calculations. A contributing cause for this linearity is the fact that with the minute amounts of substrate being acted on in the fluorometric procedures, substrate concentration remains fairly constant and the products of the reaction do not accumulate in high enough concentration to significantly inhibit the enzyme system.

Table 5 demonstrates that the specificity of the system depends upon the purity of the enzymes employed. For example, using a 100 mg/100 ml solution, detection of fructose varied from 4.7, with Sigma's prepared reagent, to 85.5 mg/100 ml with the General Diagnostics reagents. Mannose and sucrose did not react appreciably in any of the preparations. In human serum, except in rare condi-

tions (e.g., fructosuria) urine and blood do not contain significant amounts of fructose as compared to glucose. This might not be the case when applied to commercial problems. For application to a specific problem this points up the need for testing the specificity of the enzyme preparation being used.

Interference from impurities in other reagents was also noted. The tris used as the buffer needs to be free of fluorescing material. Sigma's TRIZMA is satisfactory. Tris from some other sources interfered significantly in the test, producing high background fluorescence.

It is interesting to note from Table 6 that fluoride does not interfere in the test. Magnesium is a necessary cofactor in the system and fluoride acts as an inhibitor. However, if blood in which 10 mg/ml of NaF is used only 1.5 μequiv of fluoride is added to each tube, as compared to 40 μequiv of Mg. When the $Zn(OH)_2$ is used as a protein precipitant, fluoride also forms the poorly dissociated zinc fluoride which further removes this interference. This permits the collection of blood with fluoride for glucose preservation as is done with other procedures.

The procedure described permits the rapid assay of minute samples of serum, with a specific procedure which removes interfering substances such as fats, hemoglobin, and other components which may fluoresce or produce turbity. The blood may be drawn with fluoride and analyzed at leisure. Lesser amounts of reagent are used for the fluorometric procedure and elevated temperatures are not required. The reagents are stable for substantial periods of time and are readily available from several sources.

SUMMARY

A procedure is described for the determination of glucose in 3 μl of serum or plasma. It is based on the phosphorylation of glucose with ATP mediated by hexokinase. The TPNH generated with glucose-6-phosphate dehydrogenase is measured fluorometrically.

Sensitivity is greatly improved over the corresponding procedure using UV absorption for the determination of NADPH. Bilirubin does not interfere. Interference from hemolysis is eliminated by precipitation with $Zn(OH)_2$ Blood taken with fluoride can be utilized. Fluoride interference is removed with the $Zn(OH)_2$ and with excess Mg in the substrate. Positive interference by fructose is negligible with some enzyme preparations but is appreciable with others.

The results correlate well with those obtained with an ortho toluidine method, after protein precipitation with trichloroacetic acid. The method is especially useful in the Pediatric Laboratory.

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MICROCHEMICAL JOURNAL 19, 63–70 (1974)

A Study of Oxidation of Benzidine, *o,o'*-Tolidine, and *o,o'*-Dianisidine

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INTRODUCTION

The present paper is a continuation of the series of studies of oxidation reactions of organic compounds with salts of trivalent cobalt, published in this journal (*2, 3, 7, 8*), and deals with an investigation of the possibility and the course of the oxidation of benzidine, *o,o'*-tolidine, and *o,o'*-dianisidine with Co(III) acetate, chiefly in glacial acetic acid, considering the formation of semiquinones and quinonediimines. The effect of uv light on solutions of these compounds in various solvents was also investigated and comparison of the results with the action of other oxidants (*6*) attempted. For determination of the number of electrons exchanged in the oxidation of one molecule of the amines studied, a cobalt(III) acetate standard solution was used, or an excess of the same which was back-titrated potentiometrically with a ferrous salt. Spectrophotometry in the visible region and thin-layer chromatography (TLC) were employed for monitoring the decomposition products of these compounds with uv light.

EXPERIMENTAL

Reagents

The Co(III) acetate solution was prepared electrolytically according to Budĕšínský *et al.* (*2*).

0.001*M* solutions of benzidine, tolidine, and dianisidine were prepared by dissolving the exactly weighed amine of p.a. purity in 1 liter of glacial acetic acid.

The *p*-dimethyl aminobenzaldehyde spray reagent for TLC was prepared according to Ref. (*5*).

The glacial acetic acid and all other chemicals were of p.a. purity.

Apparatus

The potentiometric titrations were carried out using platinum indicator and saturated calomel reference electrodes, and the Multoscop V pH meter (Metra, Czechoslovakia).

The spectrophotometric measurements were performed on Unicam SP-800 instrument using 1 cm cuvettes.

For study of the uv light effect on the solutions of the amines investigated, a long-wave uv lamp was employed.

Silufol uv 254 plates (Kavalier, Votice, Czechoslovakia) were used for TLC. A 7:3 benzene–acetone mixture was used as an eluent.

RESULTS AND DISCUSSION

Attempts to potentiometrically titrate benzidine, tolidine, and dianisidine directly with Co(III) in a glacial acetic acid (HAc) medium, 80% HAc, 50% HAc, 50% HAc plus 0.1 to 5.0 *M* $HClO_4$, and 50% HAc plus 0.1 to 5.0 *M* HCl were unsuccessful. Catalysis of the reaction with silver nitrate proved to be ineffective. The potentiometric curves did not exhibit any potential break corresponding to some oxidation state.

During these titrations, blue coloration was temporarily formed with benzidine and *o,o'*-tolidine after the first titrant addition, probably corresponding to formation of the semiquinone. On standing, the blue coloration turned slowly to yellow, most probably corresponding to the formation of quinonediimine (*6*). With *o,o'*-dianisidine, red coloration appeared after the first reagent addition, which also turned to yellow on standing. Any other possible color changes during the titration could not have been observed because of the coloration of the excess cobalt(III) salt.

The amines studied were also oxidized by excess Co(III) acetate, using the following procedure: 100 ml of the 0.001 *M* amine and 30 ml of about 0.1 *N* Co(III) acetate were pipetted into the reaction vessel. At various time intervals, an aliquot part of the reaction mixture was taken and the unreacted Co(III) was determined according to Buděšínský *et al.* (*2*), by reduction with excess $FeSO_4$ solution and titration of the unreacted Fe(II) with a potassium bichromate standard solution.

Since Co(III) acetate has only limited stability, a blank was measured in all the experiments.

The number of equivalents of Co(III) per mole of the respective amine (i.e., the number of the electrons exchanged, *n*) is given in Fig. 1 in dependence on the reaction time.

It is evident from Fig. 1 that oxidation with excess Co(III) acetate proceeds very slowly in a glacial acetic acid medium at laboratory temperature, *o,o'*-dianisidine being oxidized most rapidly and benzidine most slowly. Benzidine is oxidized with exchange of 8 electrons, tolidine with exchange of 12 electrons, and dianisidine with exchange of 14 electrons. This is far more than originally expected

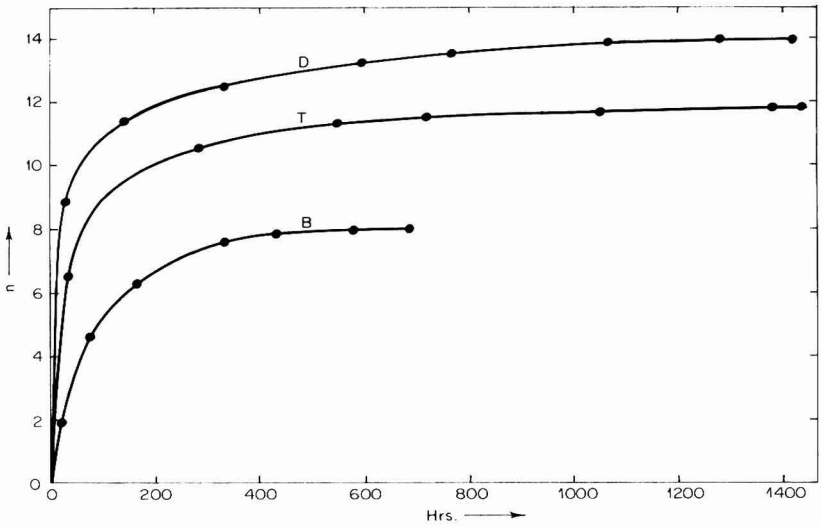

FIG. 1. The dependence of the number of electrons exchanged, n, in oxidation with excess Co(III) acetate, on the reaction time in glacial acetic acid medium at laboratory temperature. (B) benzidine, (T) *o,o'*-tolidine, (D) *o,o'*dianisidine.

two-electron exchange, obtained during action of other oxidants (*5*, *6*). Thus, it is evident that although the formation of semiquinones and quinonediimines as intermediates is very probable, much deeper oxidizing action takes place during reaction with excess Co(III) acetate.

The great number of exchanged electrons can be explained by some of the following hypotheses:

It is possible that during the action of Co(III) acetate, oxidation of the primary amino group takes place with formation of azo-, azoxy-, nitroso-, and finally nitro-compounds. This oxidation of the primary amino group on the aromatic ring, with formation of these products, is known, and it was also observed in the case of benzidine, *o,o'*-tolidine, and *o,o'*-dianisidine, during action of strong oxidants, (e.g., organic peroxo-acids or hydrogen peroxide (*1*).

It is also known that C-hydroxylation on the aromatic ring can take place during oxidation of compounds similar to the amines studied (*5*); however, this reaction course is rather improbable in the oxidation with Co(III) acetate, since in glacial acetic acid a sufficient amount of water, taking part in the C-hydroxylation, is not available.

During oxidation of some aromatic compounds in acetic acid media, methylation and acetylation of the aromatic ring was also observed; first the methylation of the ring and then the acetylation of the methyl group occurs. The reaction proceeds most probably through a

radical mechanism, when the methyl and acetyl radicals are formed by oxidation of acetic acid, and it was described for the oxidation of toluene, anisol, 2-methyl naphthalene, and *p*-methoxytoluene with trivalent manganese in the glacial acetic acid medium (*4*). The formation of the methyl and acetyl radicals in the reaction between Co(III) and acetic acid was also observed (*10*) and thus it is probable that similar reactions also take place in the oxidation of benzidine, *o,o'*-tolidine, and *o,o'*-dianisidine.

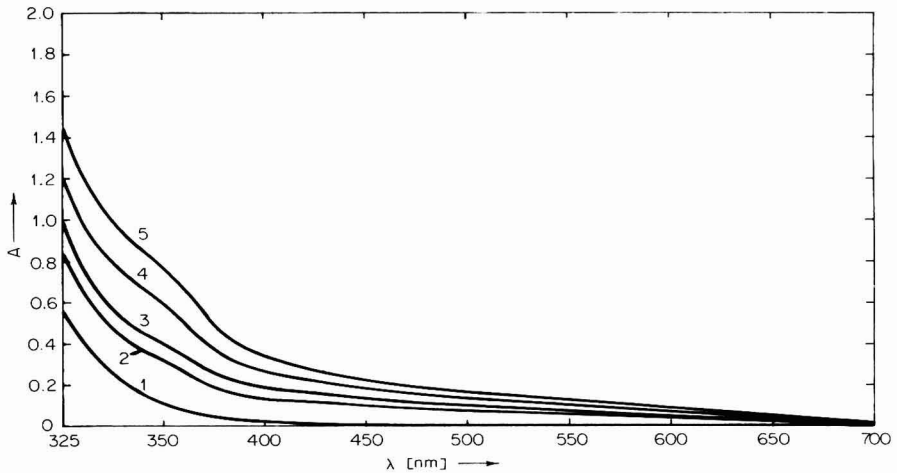

FIG. 2. The spectrum of 0.001 *M* benzidine in glacial acetic acid (against glacial acetic acid). The solution was irradiated with uv light. (1) fresh solution, (2) after 2 days, (3) after 6 days, (4) after 9 days, (5) after 12 days.

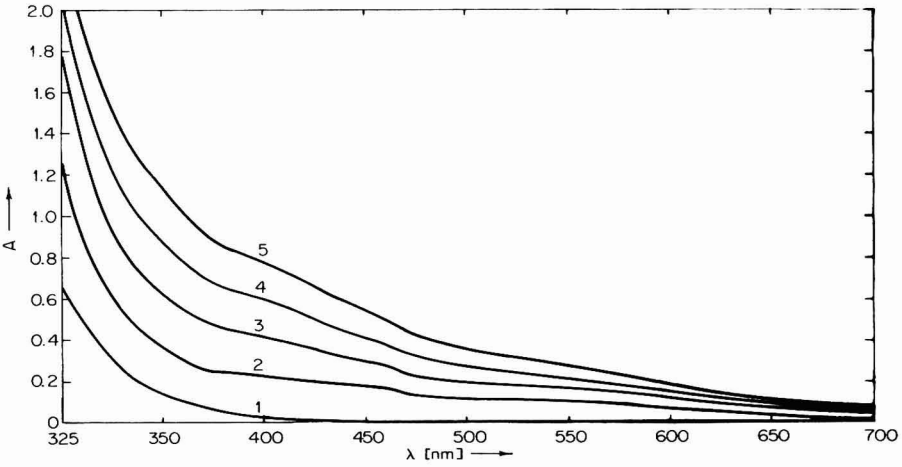

FIG. 3. The spectrum of 0.001 *M* *o,o'*-tolidine in glacial acetic acid (against glacial acetic acid). The solution was irradiated with uv light. (1) fresh solution, (2) after 2 days, (3) after 6 days, (4) after 9 days, (5) after 12 days.

In order to find out to what degree the studied substances change with time in the nonaqueous solvent alone, we paid our attention to the photochemical decomposition of the studied amines. Fresh 0.001 *M* solutions of benzidine, tolidine, and dianisidine were prepared in glacial acetic acid and in benzene. The solutions in closed glass flasks were irradiated with uv light. The flasks were placed 10 cm from the lamp. The absorption spectra of these solutions were measured at various time intervals. The results are shown in Figs. 2–7.

It was found that on irradiation of the solutions of the amines studied in glacial acetic acid and in benzene, colored substances are formed in all cases, probably products of photochemical oxidation. Spectrophotometric investigation of these solutions was supplemented by TLC.

To 2 ml of the solution of the amine in glacial acetic acid, which

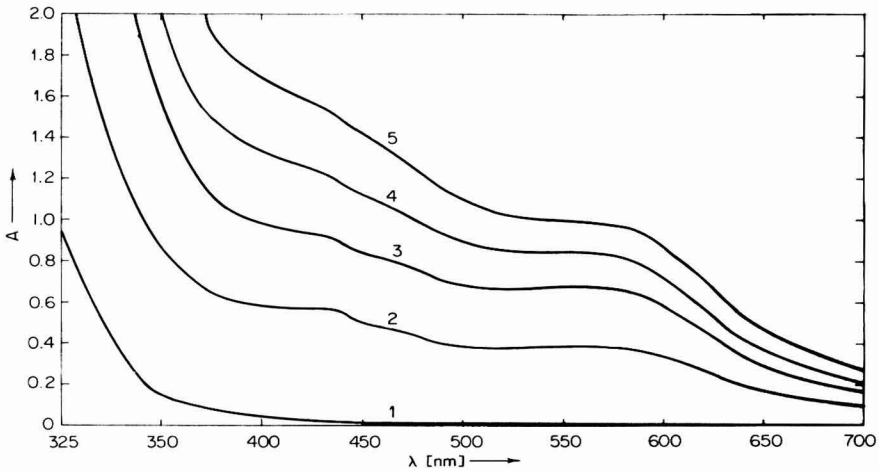

FIG. 4. The spectrum of 0.001 *M* *o,o'*-dianisidine in glacial acetic acid (against glacial acetic acid). The solution was irradiated with uv light. (1) fresh solution, (2) after 2 days, (3) after 6 days, (4) after 9 days, (5) after 12 days.

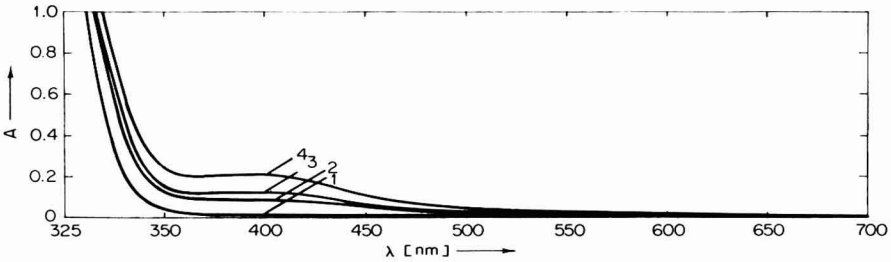

FIG. 5. The spectrum of 0.001 *M* benzidine in benzene (against benzene). The solution was irradiated with uv light. (1) fresh solution, (2) after 4 days, (3) after 9 days, (4) after 16 days.

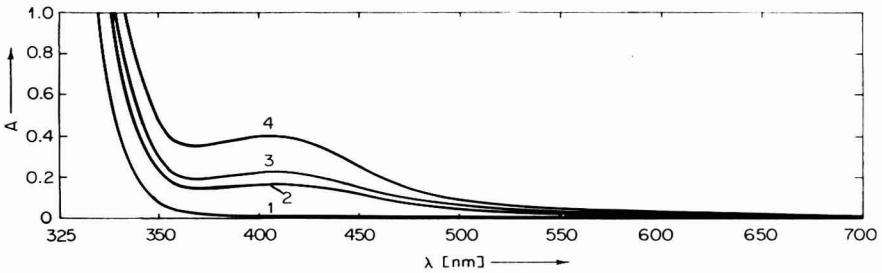

FIG. 6. The spectrum of 0.001 *M* *o,o'*-tolidine in benzene (against benzene). The solution was irradiated with uv light. (1) fresh solution, (2) after 4 days, (3) after 9 days, (4) after 16 days.

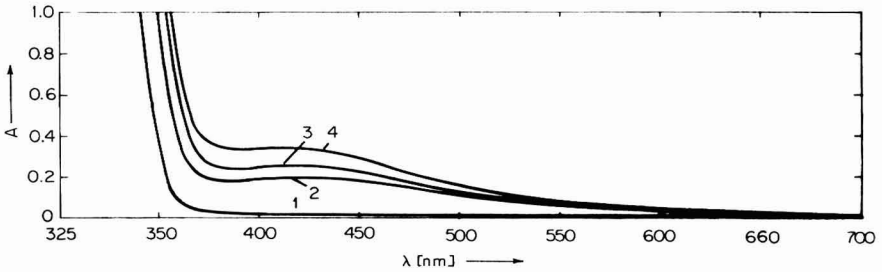

FIG. 7. The spectrum of 0.001 *M* *o,o'*-dianisidine in benzene (against benzene). The solution was irradiated with uv light. (1) fresh solution, (2) after 4 days, (3) after 9 days, (4) after 16 days.

was irradiated for 12 days by uv light, were added 10 ml of 15% sodium hydroxide. The alkaline solution was extracted with 10 ml of benzene for 10 min. The benzene phase was evaporated to about 0.5 ml at 40°C and under decreased pressure. About 10 μl were then spotted on the TLC plate. After development, the colored components remained at the origin. Visualization was carried out by spraying the chromatogram with a *p*-dimethyl aminobenzaldehyde solution. The results are given in Table 1.

Further, about 5 μl of the benzene solutions of the amines studied,

TABLE 1
THE CHROMATOGRAPHY OF THE PRODUCTS OF THE STUDIED AMINES DECOMPOSED BY UV LIGHT IN GLACIAL ACETIC ACID[a]

Amine studied	R_f	
Benzidine	0.17	*0.26*
o,o'-Tolidine	0.18	*0.33*
o,o'-Dianisidine	0.28	*0.40*

[a] The values in italics correspond to the spots of the original amines.

which were irradiated with uv light for 16 days, were placed at the start. The colored components remained at the start after the chromatography. The detection was carried out by spraying with *p*-dimethyl aminobenzaldehyde reagent. The results are summarized in Table 2.

In the case of benzidine and *o,o'*-tolidine in glacial acetic acid, the spectra do not exhibit more pronounced maxima. The colored product of the *o,o'*-dianisidine decomposition has a maximum around 570 nm. These changes are accompanied, as follows from Table 1, by formation of colorless compounds with a primary amino group on the aromatic ring, which has a lower R_f than the original amine. It is evident from Table 2 that colorless products are formed among other things. These colorless products with a primary amino group on the aromatic ring have a larger R_f than the original amine, in contrast to the previous case in glacial acetic acid medium.

In both cases, the effect of uv light probably leads to reversed benzidine rearrangement with formation of a compound of the hydrazobenzene type, or to some kind of semidine or diphenyline rearrangement, with formation of a compound of the aminodiphenylamine or diaminodiphenyl type. Formation of azo-compounds, polymeric compounds, or substances of the phenazine type cannot be excluded either.

The spectra of the colored products, formed by the action of uv light on the benzene solutions of benzidine, *o,o'*-tolidine, and *o,o'*-dianisidine, resemble each other very much and exhibit identical absorption maximum around 400 nm. In this case, quinonediimines are most probably formed. The measured absorption maxima are in good agreement with the literature data (*6*).

Thus it may be concluded that solutions of benzidine, *o,o'*-tolidine, and *o,o'*-dianisidine are rather unstable. Changes take place in them, which are dependent, among other things, on the solvent used and especially on the presence of light of shorter wavelengths. Both colorless amino-compounds, and colored substances, which seem to be products of photochemical oxidation of the studied compounds, are formed in the solution.

TABLE 2
CHROMATOGRAPHY OF THE PRODUCTS OF THE STUDIED AMINES DECOMPOSED BY UV LIGHT IN BENZENE[a]

Amine studied	R_f	
Benzidine	*0.26*	0.48
o,o'-Tolidine	*0.33*	0.54
o,o'-Dianisidine	*0.40*	0.59

[a] Values in italics correspond to the spots of the original amines.

SUMMARY

The oxidation of benzidine, *o,o'*-tolidine, and *o,o'*-dianisidine by the acetate complex of trivalent cobalt was studied chiefly in glacial acetic acid medium, considering the possible formation of semiquinone and quinonediimine compounds. The effect of uv light on the solutions of these compounds in glacial acetic acid and in benzene was followed spectrophotometrically and chromatographically and compared with the effect of Co(III) acetate. Certain possibilities explaining the course of the studied reactions are discussed.

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Kinetic Differential Determination of 2-Ketohexoses by Their Reaction with Cysteine–H_2SO_4

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INTRODUCTION

There are several microcolorimetric or spectrophotometric procedures described for the determination of 2-ketohexoses in the presence of aldohexoses (*2, 3, 6, 7*). Sensitive methods for differentiating among the 2-ketohexoses, however, have not been published. Reports of identification by gas chromatography (*9, 10*) have dealt with only two or three of the 2-ketohexoses; and although paper chromatography can distinguish among all four 2-ketohexoses (*4*), its sensitivity is less than 1% that of certain colorimetric procedures. Dische and Devi (*3*) reported that the cysteine–H_2SO_4 method could distinguish sorbose from other ketohexoses, but did not attempt further differentiation. The present communication describes the use of the cysteine–H_2SO_4 method for the differentiation of fructose, tagatose, psicose, and sorbose.

MATERIALS AND METHODS

D-Tagatose was a gift from Henry A. Lardy. D-Psicose was prepared from 6-amino-9-D-psicofuranosylpurine (*8*), which was a gift from the Upjohn Co. Other materials were from commercial sources.

The reaction mixture consisted of 0.18 ml of a 4.17% solution of cysteine·HCl (freshly prepared), 2 ml of 75% by volume of H_2SO_4 (prepared by adding 225 ml of H_2SO_4 to 95 ml of H_2O), and 0.01 ml of 1–5 m*M* ketohexose. The first two components were mixed thoroughly and cooled to 25°C, and then the ketohexose solution was added. The reactants were immediately mixed and poured into a 4-ml silica cuvette (1.0-cm light path), and the increase in absorbance at 412 nm was monitored with a Gilford absorbance-recording spectrophotometer thermostated at 25°C. Time zero was taken as the time the final solution was mixed.

RESULTS AND DISCUSSION

Dische and Devi (*3*) showed that sorbose can be distinguished from fructose and tagatose on the basis of its relatively high ratio of absorbance at 605 nm to 412 nm in the reaction with cysteine–H_2SO_4. We have confirmed this and have found that psicose behaves similarly to fructose and tagatose in this reaction. Thus, the procedure described by Dische and Devi (*3*) distinguishes fructose, tagatose, and psicose from sorbose, but not from each other. The former three 2-ketohexoses can be distinguished by this procedure, however, by monitoring the rate of absorbance increase at 412 nm (Fig. 1). The distinguishing parameter in the identification of a 2-ketohexose by this procedure is the time required to reach half-maximal color development ($T_{1/2}$). This value will be independent of the concentration of the particular 2-ketohexose being analyzed. In Fig. 1, the $T_{1/2}$ values in minutes are 6.7 for tagatose, 14.5 for psicose, and 45.7 for fructose.

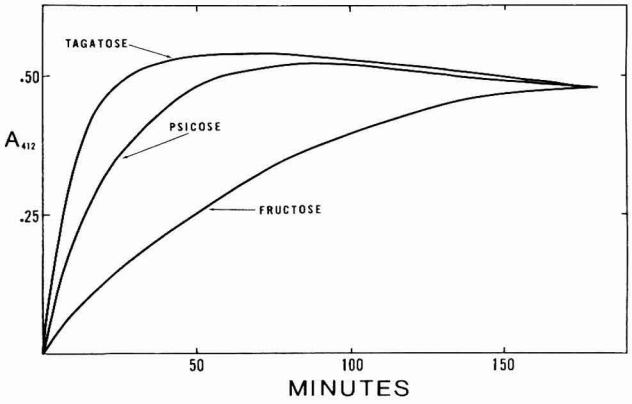

FIG. 1. Time course of color development in the reaction of cysteine–H_2SO_4 with 37 nmoles each of fructose, psicose, and tagatose.

Although these values vary with variations in the H_2SO_4 concentration (for example, increasing the H_2SO_4 concentration from 75 to 81% decreases the $T_{1/2}$ values to 3.4, 7.2, and 23.1 min), the relative $T_{1/2}$ values are constant at 1.0:2.1:6.8, and, thus, only one of the 2-ketohexoses need be used as a standard. The consistency of these values is sufficient to allow the application of simultaneous equations to the resolution of unknown mixtures of these 2-ketohexoses. This refinement was not pursued, however, because mixtures have not been encountered in our investigations (*1*, *4*, *5*).

The sensitivity of the method can easily be increased 10-fold by scaling down the reaction and using microcuvettes, and another 10-fold by switching to the 0.1 absorbance unit full-scale setting on

the recording spectrophotometer. Another advantage of this method derives from the fact that the reaction is carried out at room temperature, thereby allowing continuous recording of its progress. Furthermore, the reaction is specific, not being subject to interference by aldoses, ketopentoses, or ketoheptoses (*3*). The method has already found application in our laboratory in the elucidation of new routes for the metabolism of D-fructose (*4*), L-sorbose (*5*) and D-galactose (*1*).

SUMMARY

2-Ketohexoses can be distinguished from each other by their reaction with cysteine–H_2SO_4. Sorbose gives a distinctive absorption spectrum, whereas fructose, psicose, and tagatose can be distinguished from each other by their differential rates of absorbance increase at 412 nm.

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Evaluation of Analytical Methods Using Signal–Noise Ratio as a Statistical Criterion[1]

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INTRODUCTION

It is the purpose of this paper to point out the advantages and disadvantages of signal–noise (SN) ratio as a criterion in the evaluation or selection of various testing methods. SN ratio (η) suggested by Taguchi (*2, 3*) is expressed as the following formula[4]:

$$\eta = \sigma_M^2/\sigma_E^2,$$

where, σ_M^2 is a variance between different samples, and σ_E^2 is a variance of analytical error.

These variances are calculated by ordinary analysis of variance. SN ratio can, therefore, be regarded as a kind of discriminating capability of testing methods. We had pointed out the following facts (*1*) that SN ratio would be an especially effective criterion in evaluating fuel-testing methods because the determined values are frequently empirically defined, as with proximate analysis or coke strength tests. In such cases the most important factor is the detecting capability for the differences between samples; and not accuracy because no true value originally exists.

On the other hand, when a true value exists, as with ultimate analysis, the SN ratio cannot be the deciding factor because of the primary importance of accuracy.

It should be noted that the comparative evaluation of testing methods on the basis of SN ratio may sometimes be dangerous when V_E (variance of analytical error) varies apparently with the sensitivity of the testing method or the rounding-off method for the results since

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[4] η is usually expressed as decibel unit, because it is convenient to compare the SN ratio with each other.

V_E calculated by "analysis of variance" is a component of the SN ratio. Our theoretical considerations are made with regard to these problems.

EXPERIMENTAL METHODS

Example 1

The case of weighing using different types of balance. Four balances were used to weigh 13 boats five times to compare the detecting capabilities of each balance. Seven platinum boats of approximately equal weight were weighed by even, chemical, semimicro, and microbalances, respectively. The order of each weighing was made at random. Six boats of different weights were weighed five times using these four balances. The order of repeats in weighing was made at random. Factors and levels of this experiment are summarized in Table 1.

Example 2

The case of the determination of ferrous oxides in blast furnace slags using different methods. In order to compare the four analytical methods, seven samples of blast furnace slag were analyzed in duplicate. Factors and levels of the experiment are summarized in Table 2.

TABLE 1

Factors		Levels
Controllable factor	A: Balance	A_1: Even; A_2: chemical A_3: semimicro; A_4: micro
Signal factor	M: Boats	M_1–M_7: Approximately equal wt M_8–M_{13}: Different wt

TABLE 2

Factors		Levels
Controllable factor	A: Methods	A_1: Spectrophotometric determination using NaSCN A_2: Volumetric method using 0.02 N $K_2Cr_2O_7$ A_3: Volumetric method using 0.02 N $KMnO_4$ solution A_4: Volumetric method using 0.1 N $KMnO_4$ solution
Signal factor	M: Samples	M_1–M_7: Blast furnace slags obtained from routine operations

Example 3

The case of evaluation and selection of a routine strength tests for coke. The experiment was carried out to select the best method among the following three coke strength tests.

Coke, sample retained on a specified sieve, is subjected to treatment under standardized conditions in a rotating drum. The results of size analysis of the treated coke are used as indices of its resistance to breakage on impact and by abrasion. Standardized conditions for the three different tests are summarized in Table 3.

In this experiment, tests were carried out in triplicate on each sample and method respectively. Factors and levels of the experiment are summarized in Table 4.

Example 4

The case of draftation of a new coke-strength test. Four procedures were proposed to detect the resistance of metallurgical coke at a high temperature for process control of blast furnace.

The experiment was carried out on eight samples, using each method twice.

Table 5 is the summary of four proposed procedures.

TABLE 3
OUTLINES OF COKE-STRENGTH TEST METHODS

	Method 1	Method 2	Method 3
Standard	JIS K2151	Revised JIS K2151	ASTM D294
Sample size	+50 mm, 10 kg	+50 mm, 10 kg	2–3 in., 22 lb
Drum size	1.5 m diam × 1.5 m	1.5 m diam × 1.5 m	36 in. diam × 18 in.
Rotating speed (rpm)	15	15	24
Total revolutions	30	150	1400
Sieve	15 mm	15 mm	$\frac{1}{4}$ in.
Symbol of index	DI_{15}^{30}	DI_{15}^{150}	T_6

TABLE 4

Factors		Levels
Controllable factor	A: Methods	A_1: Method 1; A_2: Method 2, A_3: Method 3 (shown in Table 3)
Signal factor	M: Samples	M_1–M_{30}, different kinds of coke

TABLE 5
PROCEDURES OF TESTING METHODS OF COKE-STRENGTH AT A HIGH TEMPERATURE

Apparatus and procedures	Testing methods to be compared			
	A_1	A_2	A_3	A_4
Sample size (mm)	5.66–10 mm			
Sample wt (g)	150 g			
Rotating drum				
Diameter (mm)	150			
Length (mm)	150			
Inside atmosphere	Nitrogen gas			
Rotating speed (rpm)	150			
Rotating time (min)	40	40	20	20
No. of steel balls	35	35	25	25
Aperture of sieve used after testing (mm)	0.5	2.83	0.5	2.83

RESULTS AND DISCUSSION

Example 1

"True values" exist in the case of usual chemical analysis, though unknown. Accuracy is, therefore, considered the most important criterion for reference methods or methods employed for trade. Precision may be more important for methods employed for process control, but even in that case, SN ratio is not as effective for evaluation or selection of various analytical methods. This may be proved by the following example, which is the most simplified forms of chemical analyses. A part of the actual data obtained is shown in Tables 6 and 7.

The results of "analysis of variance" for data obtained by the even balance are summarized in Table 8.

Sum of squares are omitted in Table 8, because only variance is needed for the calculation of SN ratio in the formulae.

The results for the other balances are summarized in Tables 9, 10, and 11, respectively, in the same manner.

Error variances of semimicro or microbalances are smaller than that of even or chemical balances in Table 11. Therefore, F values observed are higher than for those of even or chemical balances, as a matter of course.

Standard deviations of weighing errors and boat weights are shown in Fig. 1.

TABLE 6
A PART OF RESULTS OF EXPERIMENTS (WEIGHTS OF BOATS M_1–M_7 ARE APPROXIMATELY EQUAL)

Balances	Even (A_1)	Chemical (A_2)	Semimicro (A_3)	Micro (A_4)
Wt of boats M_1–M_7 (g)				
	1.00	1.0041	1.00400	1.003849
	1.00	1.0040	1.00400	1.003853
M_1	1.00	1.0041	1.00398	1.003858
	1.00	1.0041	1.00400	1.003860
	1.00	1.0039	1.00397	1.003863
M_2–M_6	—	—	—	—
	1.00	1.0072	1.00732	1.007146
	1.00	1.0071	1.00723	1.007149
M_7	1.00	1.0072	1.00720	1.007150
	1.00	1.0073	1.00731	1.007152
	0.99	1.0073	1.00720	1.007150

TABLE 7
A PART OF RESULTS OF EXPERIMENTS (WEIGHTS OF BOATS M_8–M_{13} ARE DIFFERENT)

Balances	Even (A_1)	Chemical (A_2)	Semimicro (A_3)	Micro (A_4)
Wt of boats M_8–M_{13} (g)				
	0.99	1.0001	1.00013	1.000006
	1.00	1.0001	1.00008	1.000012
M_8	0.99	1.0001	1.00008	1.000011
	0.99	1.0001	1.00010	1.000012
	0.99	1.0001	1.00008	1.000013
M_9	—	—	—	—
M_{10}	—	—	—	—
	4.89	4.9052	4.90541	4.905231
	4.88	4.9052	4.90539	4.905237
M_{11}	4.89	4.9053	4.90542	4.905239
	4.90	4.9053	4.90544	4.905248
	4.89	4.9052	4.90546	4.905235
M_{12}	—	—	—	—
	8.50	8.4978	8.49768	8.497609
	8.47	8.4976	8.49764	8.497600
M_{13}	8.47	8.4977	8.49774	8.497593
	8.50	8.4977	8.49762	8.497601
	8.48	8.4979	8.49766	8.497598

TABLE 8
ANALYSIS OF VARIANCE FOR EVEN BALANCE (A_1)[a]

Sources of variation	Weighing of boats M_1–M_7 Degrees of freedom	Mean squares	F_0	Weighing of boats M_8–M_{13} Degrees of freedom	Mean squares	F_0
Wt of boat (M)	6	228571×10^{-10}	1.333	5	348800×10^{-4}	5979×10^2
Error (E)	28	171428×10^{-10}		24	583333×10^{-10}	

[a] $\eta(A_1) = \dfrac{(V_M - V_E)/5}{V_E} = \dfrac{(228571 - 171428)/5}{171428}$

$= 0.066663$

$= -11.8$ (dB).

$\eta(A_1) = 119589$

$= 50.8$ (dB).

TABLE 9
ANALYSIS OF VARIANCE FOR CHEMICAL BALANCE (A_2)[a]

Sources of variation	Weighing of boats M_1–M_7 Degrees of freedom	Mean squares	F_0	Weighing of boats M_8–M_{13} Degrees of freedom	Mean squares	F_0
Wt of boat (M)	6	285740×10^{-10}	5129	5	349685×10^{-4}	5995×10^6
Error (E)	28	557142×10^{-14}		24	583333×10^{-14}	

[a] $\eta(A_2) = 1025.5349$

$= 30.1$ (dB).

$\eta(A_2) = 11989 \times 10^5$

$= 90.8$ (dB).

TABLE 10
ANALYSIS OF VARIANCE FOR SEMIMICRO BALANCE (A_3)[a]

Sources of variation	Weighing of boats M_1–M_7 Degrees of freedom	Mean squares	F_0	Weighing of boats M_8–M_{13} Degrees of freedom	Mean squares	F_0
Wt of boat (M)	6	288284×10^{-10}	10728	5	349676×10^{-4}	2280×10^7
Error (E)	28	268714×10^{-14}		24	153333×10^{-14}	

[a] $\eta(A_3) = 2145.45$

$= 33.3$ (dB).

$\eta(A_3) = 456099 \times 10^4$

$= 96.6$ (dB).

TABLE 11
ANALYSIS OF VARIANCE FOR MICROBALANCE (A_4)[a]

Sources of variation	Weighing of boats M_1–M_7 Degrees of freedom	Mean squares	F_0	Weighing of boats M_8–M_{13} Degrees of freedom	Mean squares	F_0
Wt of boat (M)	6	289379×10^{-10}	2966×10^3	5	349681×10^{-4}	1082×10^9
Error (E)	28	975714×10^{-17}		24	323166×10^{-16}	

[a] $\eta(A_4) = 593163.9 = 57.7$ (dB). $\eta(A_4) = 216413 \times 10^6 = 113.3$ (dB).

Weighing errors, i.e., repeatability of weighings, are different according to the types of balance; but standard deviations for weights of different boats are almost the same with only one exception for the even balance. This exception can be explained by the insufficiency of sensitivity of the even balance when it was used for the weighing of approximately equal weights. The significance of Fig. 1 is that SN ratio should be influenced by only σ_E, and not by σ_M, because SN ratio is nothing but a ratio of σ_M^2 and σ_E^2. These relations may be explained more clearly in Fig. 2.

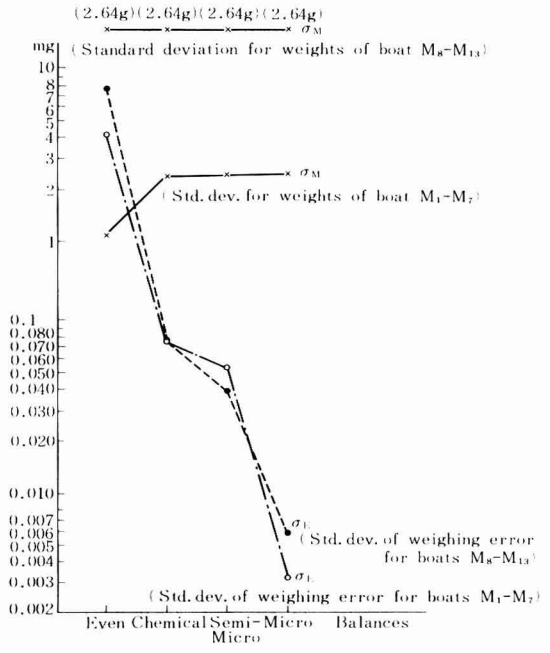

FIG. 1. Standard deviations of the boat weights and the weighing errors in example 1.

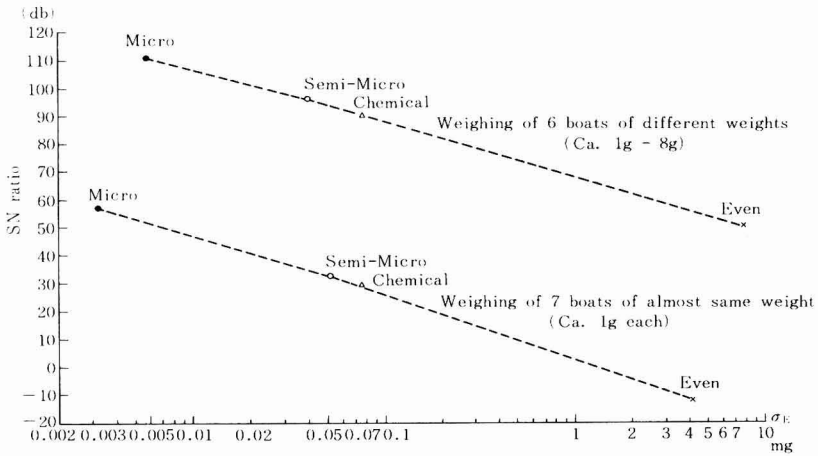

FIG. 2. Relations between weighing error σ_E and SN ratio η (dB) in example 1.

It is clearly seen from Fig. 2 that SN ratios increase in proportion to the decreases in weighing error, σ_E.

These experimental facts indicate that SN ratio has little meaning when true values exist and can be estimated. In such cases it is sufficient to compare the precision of methods for their evaluation or selection. This can be explained more theoretically by Table 12.

Table 12 explains that SN ratio has little meaning in the ordinary chemical analysis for the following reasons:

A. Standard deviation of true values of each sample, σ_μ, should be constant as a matter of course.

B. In the actual analysis, observed standard deviations $\sigma_{\bar{x}_1}$ and $\sigma_{\bar{x}_2}$ obtained by analytical methods A_1 and A_2 are not always the same because of the difference of errors in each method;

C. However, σ_M obtained by deducting σ_E from σ_x should be substantially constant, because the expected value of σ_M^2 is nothing but σ_μ^2;

D. Therefore, SN ratio is not influenced by σ_M^2 but by σ_M^2, in this case.

Example 2

Standard deviations of analytical error and those between different samples are shown in Fig. 3.

The standard deviations of ferrous oxide contents, σ_M between different samples, calculated from "analysis of variance" on 7 samples are not so varied, and SN ratios, therefore, are mainly changed in proportion to the changes of analytical error σ_E. These results also support the above conclusions.

SN ratio is considered as the most effective criterion when ob-

TABLE 12

THE REASON WHY σ_M IS CONSIDERED CONSTANT IN THE CASE OF CHEMICAL ANALYSIS FOR THE DETERMINATION OF SOME ELEMENT[a]

Samples	M_1,	$M_2, \ldots, M_i, \ldots, M_n$	Expected value
True value	μ_1,	$\mu_2, \ldots, \mu_i, \ldots, \mu_n$	$\sigma_\mu^2 = \text{const.}$
Mean values obtained by analytical method A_1	$\bar{x}_{11.}$,	$\bar{x}_{12.}, \ldots, \bar{x}_{1i.}, \ldots, \bar{x}_{1n.}$	$\sigma_{M_1}^2 = \sigma^2 - \frac{\sigma_{E_1}^2}{r}$
Structure of $\bar{x}_{1i}$	$\mu_1 + a_1 + \bar{e}_{11.}$	$\mu_2 + a_1 + \bar{e}_{12.}, \ldots, \mu_i + a_1 + \bar{e}_{1i.}, \ldots, \mu_n + a_1 + \bar{e}_{1n.}$	$E(\sigma_{M_1}^2) = \sigma_\mu^2$
Mean value obtained by analytical method A_2	$\bar{x}_{21.}$	$\bar{x}_{22.}, \ldots, \bar{x}_{2i.}, \ldots, \bar{x}_{2n.}$	$\sigma_{M_2}^2 = \sigma^2 - \frac{\sigma_{E_2}^2}{r}$
Structure of $\bar{x}_{2i.}$	$\mu_1 + a_2 + \bar{e}_{21.}$	$\mu_2 + a_2 + \bar{e}_{22.}, \ldots, \mu_i + a_2 + \bar{e}_{2i.}, \ldots, \mu_n + a_2 + \bar{e}_{2n.}$	$E(\sigma_{M_2}^2) = \sigma_\mu^2$

[a] where, r: No. of replications of analysis; a_1, e_1: bias and dispersion of method A_1, respectively; a_2, e_2: bias and dispersion of method A_2, respectively; $E(\sigma_{M_1}^2)$, $E(\sigma_{M_2}^2)$: expected values of σ_M^2 (variance between samples) obtained by methods A_1 and A_2, respectively.

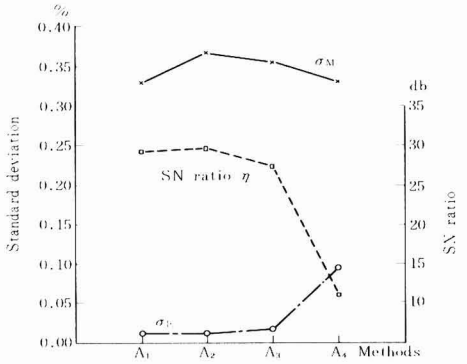

FIG. 3. Results of experiment on the analysis of blast furnace slags.

served values are a kind of definition, i.e., in the case when no true values substantially exist. This may be proved by the following examples:

Example 3

The experimental results calculated as in (*1*) are shown in Fig. 4.

From Fig. 4, it is clear that σ_M and σ_E are both varied according to the methods employed. SN ratios are, therefore, influenced by both σ_M and σ_E. This means that SN ratio is superior to precision, as a criterion for the evaluation or selection of testing methods.

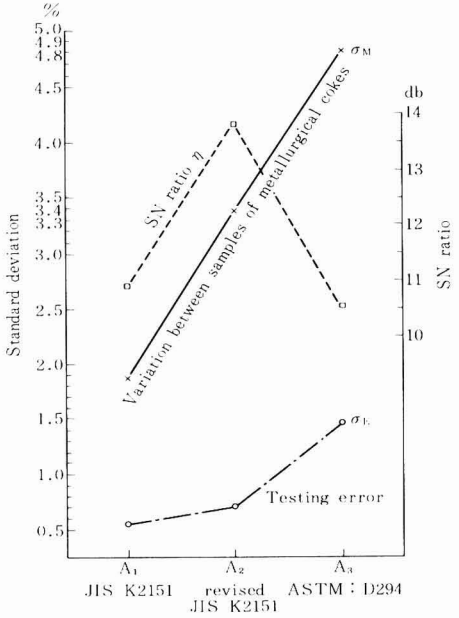

FIG. 4. Results of experiment on the strength test for metallurgical coke.

TABLE 13
RESULTS OF EXPERIMENT IN EXAMPLE 4[a]

Coke sample	Methods to be compared			
	A_1	A_2	A_3	A_4
M_1	48.1	49.4	27.4	69.8
	49.7	47.6	28.4	68.6
M_2	48.4	49.6	24.2	70.9
	49.4	48.4	25.7	71.4
M_3	49.1	49.0	28.1	69.0
	44.6	52.3	22.5	74.8
M_4	46.8	51.4	23.0	73.9
	45.6	51.4	27.1	70.3
M_5	62.1	34.8	36.9	56.2
	60.1	36.7	39.3	53.8
M_6	44.4	52.3	28.7	68.8
	45.3	52.6	24.5	73.2
M_7	56.8	41.7	32.8	64.2
	54.8	43.3	35.9	60.8
M_8	64.8	31.8	38.4	54.8
	64.2	32.2	37.4	56.0

[a] Unit: wt %.

Method A_1 is the best judging from a traditional criterion: precision. Method A_2 is, nevertheless, considered the best one for discriminating the difference between coke samples, judging from the other viewpoint, namely, SN ratio.

Example 4

Weight percentages of coke retained on the specified sieves after rotating through the four procedures to be compared, are shown in Table 13.

TABLE 14
RESULTS OF STATISTICAL ANALYSIS FOR EXAMPLE 4[a]

Sources of variation	Degrees of freedom	Mean squares				Expected values of mean squares
		A_1	A_2	A_3	A_4	
Samples (M)	7	109.6725	119.0899	67.1442	108.3956	$\sigma_E^2 + 2\sigma_M^2$
Error (E)	8	2.1512	1.3743	5.3393	5.4006	σ_E^2

[a] $\eta(A_1) = 24.99 = 14.0$ (dB). $\eta(A_2) = 42.83 = 16.3$ (dB). $\eta(A_3) = 5.788 = 7.63$ (dB). $\eta(A_4) = 9.536 = 9.80$ (dB).

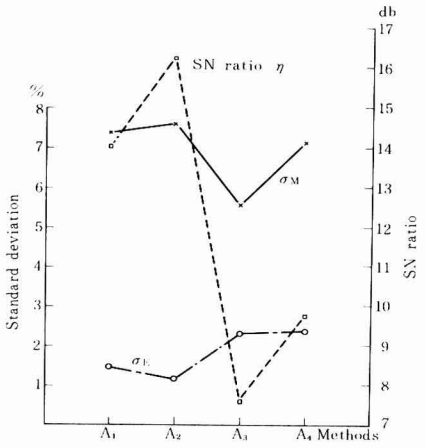

FIG. 5. Results of experiment in example 4.

Notice that these are not definite values but variable ones. In other words, no true values substantially exist. Which procedure is the best to discriminate the difference between samples in such a case? A part of the "analysis of variance" is shown in Table 14.

Relations between SN ratio and σ_M of σ_E are shown in Fig. 5.

From Fig. 5, it is clear that σ_M and σ_E both vary according to the methods employed. Method A_2 has the best precision, by chance, in this example.

SUMMARY

SN ratio is undoubtedly an interesting criterion and is especially useful in the case of physical tests in which values obtained are a kind of definition. It has little advantage for the evaluation or selection of the usual chemical analysis methods, because SN ratio varies in proportion to the differences of precision of each method.

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The Oxidation of Aminophenazone and Phenazone by Ferricyanide

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INTRODUCTION

The reactions of 4-aminophenazone derivatives with various oxidants have been followed by a number of authors (*1*, *2*, *5*, *8*, *12*). Those 4-aminophenazone derivatives, which have analgetic effects, have aroused the greatest interest. The reaction mechanism of the oxidation of 4-aminophenazone derivatives is very complex and has not so far been unambiguously clarified. Only a few of the above cited papers mention a probable reaction mechanism for the oxidation of 4-aminophenazone derivatives. From them it follows that, in addition to complete degradation of aminophenazone (APH) to phenol, formaldehyde, nitrogen, dimethylamine, and acetic and oxalic acids, a number of intermediates is formed. The structure of some intermediates has been demonstrated {*N*-methylamino antipyrine, bis [*N*-methyl (antipyrinyl) amino] methane} (*5*), but a number of the oxidation products still remains unidentified. In addition to oxidative demethylation on N-4, oxidative demethylation on N-2 or coupling of two molecules with formation of imino-bis-phenazone sometimes occurs (*8*).

Some authors used oxidants even for determination of 4-aminophenazone derivatives (*3*, *4*, *9*, *10*, *11*, *12*, *14*, *16*). Pál (*11*) describes the formation of dioxypyramidone by four-electron oxidation of APH with ceric sulfate. Schulek (*13*) assumes formation of dioxypyramidone by APH oxidation with potassium permanganate.

Awe (*1*) attempted to explain the formation of the temporary blue-purple coloration of APH solutions on action of a number of oxidants. An intermediate, which in the author's opinion causes the blue-purple coloration, is converted into colorless bis (pyrazolonyl-3,3′) ethane by excess oxidant.

In the present work, the time course of the long-term oxidation of aqueous solutions of APH and phenazone (PH) with potassium ferricyanide was qualitatively followed as a function of pH, temperature,

and the amount of oxidant. The APH and PH solutions were always oxidized with excess oxidant at laboratory and elevated temperatures, and the unconsumed oxidant was back-titrated after various time intervals. In similar way, the oxidation of some substances assumed to be decomposition products (e.g., phenol) was also followed. Other oxidation products of the reactions were followed using thin-layer chromatography (TLC) and UV spectra.

EXPERIMENTAL PROCEDURE

Reagents and Solutions

Aminophenazone from the firm VCHZ Synthesia, Pardubice-Semtín, purity: 99.89%

Phenazone (VEB, GDR), purity: 99.92%

Potassium iodide, p.a., Lachema, Czechoslovakia

Phenol, p.a., Lachema, Czechoslovakia

N-Phenyl anthranilic acid, VÚFB Prague

0.1 *N* solution of potassium ferricyanide (the titer was checked once a week)

0.1 *N* Solution of sodium thiosulfate

0.1 *N* Solution of ceric sulfate

0.4% Solution of starch

50% Sulfuric acid, Merck

15% Aqueous solution of ferric chloride

1% Aqueous solution of potassium ferricyanide

Methanol, chloroform, benzene, acetone, p.a., Lachema

Systems: (a) Benzene:methanol (95:5); (b) chloroform:methanol (90:10); (c) methanol:acetone:chloroform (3:30:70) (6)

Plates: Silufol UV 254, (Kavalier, Votice, Czechoslovakia) (15 × 15 cm); DC-Fertigplatten Kieselgel Merck (without fluorescence indicator and with the UV 254 indicator; 20 × 20 cm, 5 × 20 cm, layer thickness, 0.25 mm).

APH Oxidation in Alkaline Medium

An APH aqueous solution (2×10^{-4} mol) was oxidized by sufficient excess of a 0.1 *N* solution of potassium ferricyanide (see Results), in a medium of 0.05, 0.1, and 1 *N* sodium hydroxide. The degree of oxidation at laboratory temperature was monitored in time intervals of 20, 24, 48, and 72 hr, and at 95°C (95–98°C) in 1-hr intervals for 1–6 hr. The unconsumed amount of potassium ferricyanide was determined iodometrically, by acidifying the solution with 50% sulfuric acid to a final concentration of about 2 *N*, cooling to laboratory temperature, adding potassium iodide, and titrating with a 0.1 *N*

sodium thiosulfate solution, using a starch solution as the indicator. The oxidation at an elevated temperature was carried out in ground-glass stoppered flasks. Blank determinations were always performed.

APH Oxidation in Acidic Medium

An APH aqueous solution (5×10^{-4} mol) was oxidized with a sufficient excess of a 0.1 *N* potassium ferricyanide solution (see Results) in media of 0.1, 1, 2, and 3 *N* sulfuric acid. The unconsumed potassium ferricyanide was determinated iodometrically in the same manner as in the APH oxidation in alkaline medium. The degree of APH oxidation was monitored at laboratory temperature in time intervals of 3, 6, 24, and 48 hr, and after 60 and 90 min at an elevated temperature (65–68°C). Blanks were performed in each case.

Phenol Oxidation in Alkaline Medium

An aqueous phenol solution (2.1×10^{-4} mol, i.e., the amount theoretically formed by the oxidation of 2×10^{-4} mol APH according to ref. *2*) was oxidized with ferricyanide in parallel with an APH aqueous solution for 5 hr at 95°C, in a medium of a 0.05 *N* sodium hydroxide solution. (The same excess of oxidant as in the APH oxidation was employed.)

Phenol Oxidation in Acidic Medium

An aqueous phenol solution [4.8×10^{-4} mol, i.e., the amount which should theoretically result by the oxidation of 5×10^{-4} mol APH (*2*) was oxidized with a 0.1 *N* potassium ferricyanide solution in a medium of 1 *N* sulfuric acid under the same conditions as the APH solution, at 65°C for 60 min, using an identical excess of the oxidant.

Extraction of the APH and Phenol Oxidation Products

The oxidized solutions of APH and phenol (after 5-hr oxidation at 95°C in a medium of a 0.05 *N* solution of sodium hydroxide) were cooled to laboratory temperature, the contents of the flasks transferred into separatory funnels, and extracted with 2×15 ml of chloroform. The combined chloroform extracts were evaporated on a water bath. The remaining aqueous layers were acidified with 1 *N* sulfuric acid and again extracted with 2×15 ml of chloroform. The combined chloroform extracts were evaporated on a water bath.

The extraction of the oxidation products of APH and phenol, formed during the oxidation of APH and phenol in a medium of 1 *N* sulfuric acid at 65°C for 60 min, was carried out identically.

Thin-Layer Chromatography of the Oxidation Products

The individual residues after extraction of the oxidized solutions of APH and phenol were dissolved in 1 ml of chloroform and 0.1 ml of this solution was applied to the TLC plate.

The plates were always washed with the system used prior to sample application. The developing chamber was saturated for 2 hr and the chromatogram was developed in the upward direction to a distance of 12–15 cm. Visualization performed either in UV light (UV 254, UV 366, Fluotest Universal of the firm Hanau) or using a spray reagent consisting of a 1 : 1 mixture of 15% ferric chloride solution with a 1% solution of potassium ferricyanide (6). (This detecting agent is stable for 5 min.)

In system (a) (see Reagents), phenol (10 μg, in chloroform solution) was chromatographed with the oxidation products of APH and phenol. In systems (b) and (c), APH (5 and 10 μg, respectively, in chloroform solution) was chromatographed with the APH oxidation products.

Extraction of the Oxidation Products from the Chromatogram and Their UV Spectra

Two substances were extracted from the chromatograms, with $R_f = 0.63$ and $R_f = 0.76$. The detection of the substances was carried out in the UV region; the spots of these substances (2 × 2 cm) were gradually extracted with methanol, up to a total volume of 10 ml. A blank was performed by the same extraction procedure. For obtaining the UV spectra of the substances with $R_f = 0.63$ and $R_f = 0.76$, the chromatography was always carried out using Kieselgel Merck plates (0.2 ml of the sample was applied). The UV spectra of the extracted substances were recorded with a Unicam SP-700 instrument, against a blank. The UV spectrum of the substance with $R_f = 0.63$ was recorded within the region from 450 to 320 nm, that of the substance with $R_f = 0.76$ in the region, 420–350 nm.

RESULTS AND DISCUSSION

The course of the oxidation of an APH aqueous solution (2×10^{-4} mol APH) with a 0.1 *N* ferricyanide solution at laboratory temperature is given in Table 1. It can be seen in the table that the oxidation of the APH solution proceeds much faster during the first hours than during later time intervals.

The effect of potassium ferricyanide as an oxidant on APH depends strongly on the pH of the medium. While in a medium of 1 *N*

TABLE 1
THE OXIDATION OF AN AQUEOUS APH SOLUTION (2×10^{-4} mol) WITH A 0.1 *N* POTASSIUM FERRICYANIDE SOLUTION IN AN ALKALINE MEDIUM AT LABORATORY TEMPERATURE, IN DEPENDENCE ON TIME

	20 hr	24 hr	48 hr	72 hr
Medium	No. of exchanged electrons			
0.05 *N* NaOH	11.25[a]	11.32	11.55	11.85
0.1 *N* NaOH	12.3	12.7	12.9	13.2
1 *N* NaOH	16.7	17.9	18.8	19.25

[a] After 17 hr.

sodium hydroxide at laboratory temperature 18 electrons are exchanged after 24 hr oxidation of the APH solution, this falls to 13 electrons in a medium of 0.1 *N* sodium hydroxide, 11 electrons in 0.05 *N* sodium hydroxide, and only 6 electrons in a medium of 1 *N* sulfuric acid (see Fig. 1, and Table 2).

Similar to alkaline media, most of the APH is oxidized during the first several hours in acidic media; then the reaction proceeds only very slowly. Media more acidic than 1 *N* sulfuric acid were not suitable for the APH oxidation, since the potassium ferricyanide solution decomposed quickly. For this reason we studied only the 1 *N* sulfuric acid medium.

APH oxidation in an alkaline medium at an elevated temperature is shown in Fig. 2 and Table 3. The effect of temperature on the oxida-

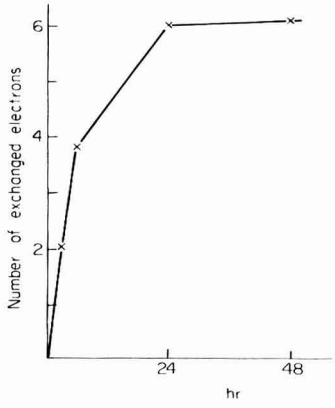

FIG. 1. The oxidation of an aqueous APH solution (5×10^{-4} mol) with a 0.1 *N* potassium ferricyanide solution in 1 *N* sulfuric acid medium at laboratory temperature, in dependence on time.

TABLE 2
THE OXIDATION OF AN AQUEOUS APH SOLUTION (5×10^{-4} mol) WITH A 0.1 *N* POTASSIUM FERRICYANIDE SOLUTION IN AN ACIDIC MEDIUM AT LABORATORY TEMPERATURE, IN DEPENDENCE ON TIME

	3 hr	6 hr	24 hr	48 hr
Medium	No. of exchanged electrons			
1 *N* H_2SO_4	3.6	4.8	6.0	6.2

tion of the APH solution follows from comparison of the results in Tables 1 and 3. While 12 electrons are exchanged during APH oxidation in 0.05 *N* sodium hydroxide at laboratory temperature only after 72 hr, the same number are exchanged already after 1 hr oxidation at 95°C. The character of the APH oxidation at an elevated temperature remains the same as that in oxidation at laboratory temperature. In both cases the solution is oxidized rapidly during the first hours and then only slowly. Pronounced slowing down of the APH oxidation occurs in the 0.05 *N* sodium hydroxide medium at 95°C after 5 hr oxidation when 16 electrons are exchanged. Therefore we concentrated our attention on studying the products or the reaction course in this medium and in the 1 *N* sulfuric acid medium, where 6 electrons are exchanged. We also followed the oxidation of an aqueous solution of PH at an elevated temperature, in addition to the oxidation of the aqueous APH solution; the PH solution was not oxidized in the 0.05

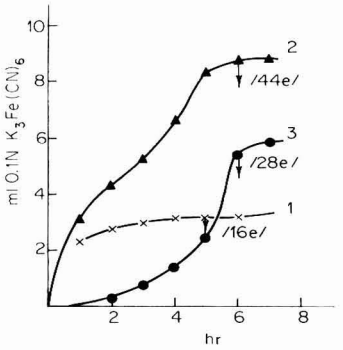

FIG. 2. The oxidation of an aqueous APH solution (2×10^{-4} mol) and of an aqueous PH solution (2×10^{-4} mol) with a 0.1 *N* potassium ferricyanide solution in an alkaline medium at 95°C, in dependence on time. (1) the oxidation of the APH aqueous solution in 0.05 *N* NaOH medium; (2) the oxidation of the APH aqueous solution in 1 *N* NaOH medium; (3) the oxidation of the PH aqueous solution in 1 *N* NaOH medium; and ( ), number of exchanged electrons.

TABLE 3
THE OXIDATION OF AQUEOUS APH (2×10^{-4} mol) AND PH (2×10^{-4} mol) SOLUTIONS WITH A 0.1 *N* POTASSIUM FERRICYANIDE SOLUTION IN AN ALKALINE MEDIUM AT 95°C, IN DEPENDENCE ON TIME

		1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	7 hr
		No. of exchanged electrons						
APH	0.05 *N* NaOH	12.0	13.5	14.7	15.8	16.1	16.4	17.0
	1 *N* NaOH	16.3	22.0	26.6	33.6	42.3	44.0	44.9
PH	1 *N* NaOH	–	1.8	4.4	7.4	12.5	28.3	29.5

N sodium hydroxide medium at 95°C. Its oxidation took place only in more alkaline media. The course of the oxidation of the PH solution in the 1 *N* sodium hydroxide medium at 95°C is given in Fig. 2 and Table 3. The different behavior of the PH and APH solutions toward the oxidant may be seen from Fig. 2. The difference is due first to a slower oxidation rate of the PH solution and secondly to a different oxidation character. While 16 electrons are exchanged during 1 hr oxidation of the APH solution in the 1 *N* NaOH medium at 95°C, no oxidation of PH takes place under identical conditions. This fact can be utilized for oxidative determination of APH in the presence of PH. The PH solution is also oxidized very slowly in acidic media at an elevated temperature (Table 4). The potassium ferricyanide solution rapidly decomposed in acidic media at higher temperatures; for this reason the oxidation of the APH and PH solutions could not be monitored for a longer time interval. The easier "oxidizability" of the APH molecule is, in our opinion, due to the substitution on C-4.

On the basis of the experimental data it has been found that the degree of oxidation of APH depends not only on the pH of the

TABLE 4
THE OXIDATION OF AQUEOUS APH AND PH SOLUTION (BOTH 5×10^{-4} mol) WITH A 0.1 *N* POTASSIUM FERRICYANIDE SOLUTION IN 1 *N* SULFURIC ACID MEDIUM AT 65°C, IN DEPENDENCE ON TIME

	60 min	90 min
	No. of exchanged electrons	
APH	5.68	6.28
PH	–	0.95

medium and its temperature, but also on the amount of oxidant added: With increasing oxidant amounts, the APH oxidation proceeds more rapidly. The experiments have shown that increasing the reagent excess to more than five times (molar ratio) with respect to APH already has no effect on the first, faster phase of the oxidation (i.e., up to the exchange of 16 electrons in alkaline or 6 electrons in acidic media), and has practically no effect on the final, not exactly characterized, complete APH oxidation.

During the APH oxidation with potassium ferricyanide in alkaline media, temporary blue-green coloration of the solution appears immediately after addition of the oxidant. This coloration was not observed in the APH oxidation in acidic media. The coloration also did not appear during the PH oxidation in alkaline and acidic media. The formation of temporary coloration in APH solutions due to action of oxidants is explained by Awe (*1*) by formation of a radical compound, in which the electrons of the dimethylamino group in the C-4 position participate. On the basis of this assumption of a radical compound responsible for the APH solution coloration it is possible to explain why the PH molecule does not yield any coloration on action of oxidants. During the APH oxidation in alkaline media, the original yellow coloration of the oxidized solution deepened to a yellow-brown color. The PH solution remained unchanged in color during the whole oxidation time.

In all oxidations, the unconsumed potassium ferricyanide was determined iodometrically, since the iodometric determination was less time consuming than, e.g., the titanometric (which could also be used). In order to avoid the possibility of APH oxidation with the liberated iodine, the solution to be titrated was already acidified before the addition of potassium iodide. In addition to the back-titration of excess potassium ferricyanide, we also monitored the degree of APH oxidation by determining the amount of ferrocyanide formed. The determination of the ferrocyanide present was carried out by titration with ceric sulfate, both potentiometrically (a Pt–calomel electrode pair) and visually using *N*-phenyl anthranilic acid as the indicator (*12*). The visual titration was not suitable since the indicator color transition at the equivalence point was very drawn out in the yellow colored oxidized APH solution. We stopped following the APH oxidation by determining the ferrocyanide formed for the following reason: according to our own experience and on the basis of the literature data (*11*), APH can easily be oxidized with ceric sulfate in acidic media. Thus APH was also oxidized with ceric sulfate, together with the ferrocyanide formed, and the picture of the APH oxidation with potassium ferricyanide was distorted.

THE CHROMATOGRAPHIC SEPARATION OF THE APH OXIDATION PRODUCTS

The APH oxidation products were followed chromatographically in the slowest stage of the oxidation. In the 0.05 *N* sodium hydroxide medium, the APH oxidation proceeds most slowly after 5 hr of reagent action at 95°C, when 16 electrons were exchanged. In 1 *N* sulfuric acid at 65°C, the APH oxidation proceeded most slowly after 1 hr, when 6 electrons were exchanged. When systems (b) and (c) were used, it was found that the APH solution, oxidized under the above conditions, did not contain APH, and by the use of system (a) it was found that it did not contain even phenol in this phase of the oxidation. (R_f APH in system (b) = 0.52, R_f APH in system (c) = 0.27, R_f phenol in system (a) = 0.23. (The given values were obtained on Silufol UV 254 plates.)

By TLC it was found that a number of oxidation products are formed by the oxidation of an APH aqueous solution (R_f of the oxidation products formed during the oxidation in the 0.05 *N* NaOH medium at 95°C after 5 hr are 0.044, 0.22, 0.27, and 0.63; in the 1 *N* sulfuric acid medium at 65°C after 60 min they are 0.13, 0.21, 0.51, 0.76, and their study will be the topic of further investigation.

Phenol is considered by many authors to be one of the final products of the APH oxidation, but, since it is itself easily oxidizable (*7*), we assumed that it will not be detectable in the oxidized solution, but that its oxidation products will be detectable. This assumption was confirmed by an experiment, in which a phenol aqueous solution was oxidized with potassium ferricyanide under identical conditions to the APH solution, namely, (a) in the 0.05 *N* sodium hydroxide medium at 95°C for 5 hr. In the oxidized phenol solution, no phenol was detected, but three of its oxidation products were found. The R_f values of the phenol oxidation products are given in Table 5. In the APH ox-

TABLE 5
THE TLC SEPARATION OF THE PHENOL OXIDATION PRODUCTS[a]

	Phenol oxidation products	
	1 *N* H_2SO_4	0.05 *N* NaOH
R_f	0.13 × 3	0.05
	0.21	0.11
	0.76	0.63

[a] Phenol (2.1×10^{-4} mol) oxidized with a 0.1 *N* potassium ferricyanide solution in 0.05 *N* sodium hydroxide medium at 95°C for 5 hr. Phenol (4.8×10^{-4} mol) oxidized with a 0.1 *N* potassium ferricyanide solution in 1 *N* sulfuric acid medium at 65°C for 1 hr. System: benzene + methanol (95:5); DC-Fertigplatten Kiesegel Merck (with the UV 254 fluorescence indicator, layer thickness, 0.25 mm).

TABLE 6
THE UV SPECTRA OF THE SUBSTANCES WITH $R_f = 0.63$ AND $R_f = 0.76$[a]

R_f	Max (nm)	Min (nm)
63	229	250
	258	266
	282	
76	234	247
	250	252
	255	259
	261	

[a] Unicam SP-700, medium: methanol, $d = 2.000$ cm.

idized solution, a substance was chromatographically detected, whose R_f value, 0.76, was identical with the R_f of one of the phenol oxidation products. (b) in 1 N H_2SO_4, for 1 hr, at 65°C. No presence of phenol was found, but its 3 oxidation products (for R_f see Table 5) were detected. One compound with $R_f = 0.76$, the R_f value of which was identical with that of the oxidation products of phenol was found in the oxidized APH.

After extraction of the substance with $R_f = 0.63$ from the oxidized APH solution and from the phenol oxidized solution, its UV spectrum was recorded; it exhibited 3 maxima and 2 minima, as shown in Table 6. From Fig. 3 it is evident that the substances with $R_f = 0.63$, isolated from the APH and the oxidized phenol solutions, yield entirely identical spectra.

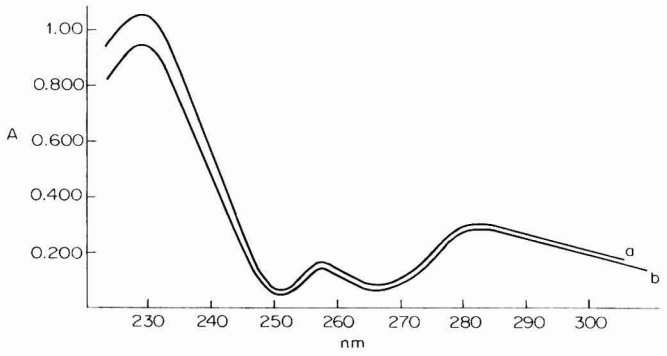

FIG. 3. The UV spectrum of the substance with $R_f = 0.63$ (Unicam SP 700, medium: methanol, $d = 2.000$ cm). (a) the UV spectrum of the substance with $R_f = 0.63$, isolated from the APH oxidized solution (2×10^{-4} mol); (b) the UV spectrum of the substance with $R_f = 0.63$, isolated from the phenol oxidized solution (2.1×10^{-4} mol). (The solutions were oxidized with a 0.1 N potassium ferricyanide solution in 0.05 N NaOH medium at 95°C for 5 hr.)

After extraction of the substance with $R_f = 0.76$ from the APH and oxidized phenol solutions, its UV spectrum was obtained, which is more complex than that of the substance with $R_f = 0.63$ (Fig. 4). From Fig. 4 it is evident that the substance with $R_f = 0.76$, isolated from the APH and oxidized phenol solutions, give identical spectra. The spectrum of the substance with $R_f = 0.76$ exhibited 4 maxima and 3 minima (see Table 6).

Both the substance with $R_f = 0.63$ and that with $R_f = 0.76$ were detectable in the UV region at 254 nm (they quenched the fluorescence) and at 366 nm (they fluoresced intensely with a blue color). These substances gave blue spots on the yellow background with a detection reagent consisting of a mixture of $FeCl_3$ with $K_3Fe(CN)_6$ (see above).

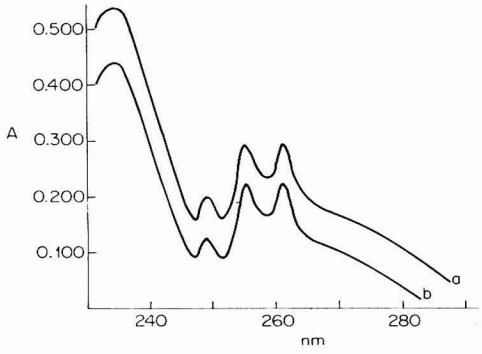

FIG. 4. The UV spectrum of the substance with $R_f = 0.76$ (Unicam SP 700, medium: methanol, $d = 2.000$ cm). (a) the UV spectrum of the substance with $R_f = 0.76$, isolated from the APH oxidized solution (5×10^{-4} mol); (b) the UV spectrum of the substance with $R_f = 0.76$, isolated from the phenol oxidized solution (4.8×10^{-4} mol). (The solutions were oxidized with a 0.1 *N* potassium ferricyanide solution in 1 *N* H_2SO_4 medium at 65°C for 1 hr.)

CONCLUSIONS

By monitoring the prolonged oxidation of an aqueous solution of aminophenazone (APH) with potassium ferricyanide it was found that the degree of APH oxidation depends on the pH and the temperature of the medium and on the amount of oxidant present. These dependences are shown in Tables 1–4 and Fig. 2.

The character of the oxidation of the APH aqueous solution differs from the course of the phenazone (PH) oxidation (Fig. 2). The APH molecule undergoes faster oxidation compared to PH. The easier oxidizability of the APH molecule is due to the substitution with the dimethyl amino group on C-4.

The APH oxidation products were followed chromatographically during the slowest phase of the oxidation, i.e., after 5 hr oxidation in the 0.05 *N* sodium hydroxide medium at 95°C, when 16 electrons were exchanged, and after 1 hr oxidation in the 1 *N* sulfuric acid medium at 65°C, when 6 electrons were exchanged. It has been proved that, in this phase of the APH oxidation with potassium ferricyanide, the oxidized APH solution does not contain phenol but its oxidation products. After the oxidation of the APH aqueous solution under the given experimental conditions, (a) a substance with $R_f = 0.63$ was detected in the alkaline medium, and (b) a substance with $R_f = 0.76$ in the acidic medium. It has been found that both these substances are oxidation products of phenol. Their UV spectra are shown in Table 6 and Figs. 3 and 4.

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Book Reviews

The Use of the Scanning Electron Microscope. By J. W. S. HEARLE, J. T. SPARROW, AND P. M. CROSS. Pergamon, New York, 1973. xi + 278 pp. $26.50.

This book is superb. It matches its title in advising the reader to uses of the scanning electron microscope. The coverage of the material is excellent and the illustrations, of which there are a large number of sharp, well produced photographs as well as line drawings, help elucidate the material. The quality of the printing is high; the publisher also took pride in the effort and deserves congratulations. Most of the chapters were written by the authors; several chapters have been written by experts specializing in particular areas. Appropriate literature references are supplied throughout.

The first three chapters by J. W. S. Hearle, D. C. Northrup and J. E. Castle, respectively, provide background information on the basic principles, theory, design and construction of the scanning electron microscope, with comparisons to the optical and direct (conventional) electron microscope. Chapter 4 by Mrs. P. M. Cross treats specimen preparation with emphasis on coating procedures. In Chapter 5 Mrs. Cross covers procedures on specialized uses of the scanning electron microscope such as surface topography, chemical composition of surface layers and more specialized application such as studies on the electrostatic and magnetic fields associated with surfaces, current carrying patterns, and computer coupling of the microscope output signal which, unfortunately, is only briefly treated. Chapter 6 by J. E. Castle pertains to metallurgical applications such as eutectic phase studies, fracture studies (stress, corrosion, fatigue, cleavage, embrittlement), powder metallurgy processes, corrosion studies, oxide layers, etc. Chapter 7 written by J. T. Sparrow has applications to fibers and polymers. The advantages of the scanning electron microscope are well shown here, with the great sharpness and depth of focus compared to the other microscopic techniques. Applications here include twist breaks of fibers, surface fatigue studies, cross section and internal structures, as well as studies of bulk polymers. In addition considerable information is given on sample preparation techniques. Chapter 8 by D. C. Northrup covers applications to solid state electronics treating homogeneous, near homogeneous materials and semiconductor devices.

Chapter 9 by P. Echlin considers the specialized area of the biological materials with particular emphasis on methods of sample preparation and ways of circumventing the difficulties of treating cellular material. Chapter 10, a trouble shooting chapter, written by Mrs. Cross, gives information on how to recognize electronic problems, means of possibly correcting vacuum leakage, causes of distorted images or low quality images, beam damage, and vacuum damage of the specimen, mechanical faults such as vibration, etc. Photographs are included showing the resultant deteriorated image when the system is at fault. Chapter 11, by G. S. Lane, covers dimensional measurements for which the scanning electron microscope is well suited because of its large depth focus. Stereoscopic methods for three-dimensional viewing are described as well as quantitative lateral and height measurements. Stereographic equations are derived and the use of the equations is discussed. A. J. Sherrin, wrote Chapter 12 which pertains to the business aspects of office organization, management, use scheduling, and general operating procedures that have been found useful through past experience. This information will be very valuable to prospective purchasers of this equipment. The final

chapter of the book by J. W. S. Hearle and D. C. Northrup has a discussion on the future of scanning electron microscopy, with an insight to both future and current instrumentation, developments, techniques, element identification by X-ray attachments, electron channeling, stroboscopic techniques, signal processing with again only brief mention of computer techniques. A list of manufacturers and supplies with addresses (both U. S. and European) is included in the book.

The reviewer was highly impressed by the book. Obviously, it will not make an electron microscopist out of the reader; it will give the reader a clear insight as to how scanning electron microscopy can be employed and what is involved. To anyone in the field or entering the field, the book is highly recommended.

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The Radiation Chemistry of Macromolecules. Vol. 2. Edited by MALCOLM DOLE. Academic Press, New York, 1973. xvii + 406 pp. $25.00.

This second volume, the better-half of "The Radiation Chemistry of Macromolecules," completes the fulfillment of an extensive, up-to-date survey of the chemical and physical effects of high-energy radiations (including high speed electrons, gamma rays, and X-rays) on substances of macromolecules. It is devoted chiefly to the description and elucidation of the radiation chemistry of specific types of macromolecular systems such as substituted vinyl polymers, polyamides, polyesters, polysiloxanes, and biological macromolecules and the effects of radiations on oxidation rates, mechanical properties, and on single crystals. It consists of 17 chapters divided into four parts. In addition, many useful references to the literature for the specific topics are provided at the end of each chapter. Author index, subject index, and contents of Volume 1 are also available for easy use of the text and the whole set.

Part I deals with radiation chemistry of substituted vinyl polymers (Chaps. 1–6, 117 pp., 281 refs.). Polypropylene (PP) which is intermediate in structure between polyethylene (PE), which is predominantly cross-links, and polyisobutylene (PIB), which only degrades when subjected to ionizing radiation is first reviewed by D. O. Geymer. The general consequences of ionizing radiation such as the evolution of gas (hydrogen and methane), the introduction of unsaturation, the production of free radicals and their reactions other than cross-linking and decay of radicals, reactions with small molecules and so forth are discussed from the practical aspects. The cross-linking and chain scission, thermoluminiscence and electron trapping, which are characteristics of the effects of ionizing radiation on PP are also critically demonstrated. Following the same patterns and format, the reviews on polypropylene oxide (PPOx) by M. Dole; on polyvinyl chloride by R. Salovey; and on polyvinyl acetate by W. W. Graessley are subsequently presented. However, a particular emphasis has been placed on the discussion of the characteristic physicochemical properties, experimental conditions and measurements, chemical mechanisms and products of radiation reactions for each particular system. In Chapter 5, one of the most radiation-resistant polymers, polystyrene, is reviewed by W. W. Parkinson and R. M. Keyser. Polystyrene, because of its resistance, its chemical simplicity with aromatic stabilization, its abundant use in many applications was studied early in the investigation of the effects of radiation on materials. The process of energy transfer and dissipation in polystyrene system give rise to the chemical processes and are utilized in the operation of scintillation radiation detectors. Thus, the fundamental chemical processes, electrical phenomena, and the resulting changes in physical and mechanical properties are reviewed in some details. (However, the theoretical treatment has been presented in Chapter 11, Vol. 1.) Chapter

6 by M. Dole considers polymers such as polymethylmethacrylate (PMMA) and PIB that are primarily degraded on irradiation. Factors that promote degradation and that inhibit cross-linking [for examples, lacks of α-hydrogen atom and due to steric hindrance for the type $-(CH_2\overset{\overset{CH_3}{|}}{\underset{\underset{R}{|}}{C}}-)_n$] are briefly mentioned. The experimental determination of G values for main chain fracture, $G(S)$, from M_n, M_w, and intrinsic viscosity measurements; from ultracentrifuge and spectroscopic studies are concisely discussed. Chemical yields, the results of uv and ir studies, and free radicals and mechanisms studies are summarized for better understanding of the effects of ionizing radiations on these polymeric systems.

Part II is concerned with the radiation chemistry of some miscellaneous polymers (Chaps. 7–12, 142 pp., 342 refs.). In Chapter 7, the physical results of irradiation of polyamides including some degradation and cross-linking, different gaseous products and yields, mechanisms of free radicals formation, the principles governing typical grafting reactions (e.g., by soaking), etc., are reviewed by J. Zimmerman. Chapter 8 by D. T. Turner treats the studies of electrical conductivity on charged species, the ESR spectroscopic studies on free radicals and concludes with product analysis for the irradiated polyethylene terephthalate (PET) with ^{60}Co γ-rays. Chapter 9 by M. Dole discusses the crystallinity, degradation, free radical formation, reaction mechanisms, cross-linking of the irradiated polytetrafluoroethylene (PTFE), which is characterized by its chemical resistance to attack by solvents and high thermal stability. Chapter 10 by A. A. Miller reviews briefly the different mechanisms and results of radiolysis of polydimethylsiloxane (PDMS) in the liquid and solid states and that of phenylmethylsiloxane. In Chapter 11, M. Dole reports the results of solubility, ir, GLC, NMR measurements and the kinetics of free radical decay on the irradiated polyoxymethylene (POM). The last chapter of the part by G. G. A. Böhm describes in detail the radiation chemistry of elastomers. It covers the materials on transient intermediates, gas evolution, changes in microstructure, cross-linking, and physical properties of polyisoprene (natural rubber), butadiene rubbers and copolymers, and polyisobutylene and copolymers.

Part III considers the effect of radiation on oxidation, mechanical properties, and physical state (Chaps. 13–16, 60 pp., 129 refs.). In Chapter 13, questions concerning oxidation G values, oxidation in the crystalline or amorphous phases of the polymers, oxidation kinetics and mechanisms, and the effect of irradiation oxidation on physical properties for PE are reviewed by M. Dole. While in Chapter 14, B. J. Lyons and F. E. Weir report the changes in mechanical properties both above and below crystalline melting points caused by ionizing radiation in materials which cross-link. These changes have been discussed in terms of elastic properties (above its melting point), long-term properties (screep, etc.), intermediate-time properties (quasi-static), and the dynamic mechanical properties on the basis of a model for spherulitic, semicrystalline materials. Chapter 15 by R. Salovey describes the structural studies on PE single crystals which were exposed to ionizing radiation by utilizing electron microscopy and electron and X-ray diffraction. Finally, in Chapter 16, M. Dole reports some recent results of ESR measurements on the irradiated polyamide fibers and other polymer fibers.

The last part, Chapter 17 (54 pp., 179 refs.) by L. S. Myers, Jr., is concerned primarily with determining the mechanisms of typical gross radiation effects including breakage of main chains, formation of cross-links, degradation of side chains, cleavage of side chains, and breakage of hydrogen bonds on biological macromolecules (such as nucleic acids, proteins, and polysaccharides). However, the main emphasis is on the solid state with discussion of aqueous solutions limited to recent advances in the forma-

tion, structure, and properties of intermediate radical species. This chapter is therefore very useful to biological chemists who are interested in the area of biological effects of ionizing radiation.

In summary, this book represents a successful work reviewing and reporting the practical aspects of the most current advances in radiation chemistry of typical polymeric materials by a number of experts in the fields concerned. Throughout the book, the treatment of subject matter is comprehensive, specific but comparative, and critical. There is no significant typographical error. Therefore, it should be considered as an invaluable reference source for radiological chemists, polymer chemists, scientists, research workers, and radiological biochemists who are interested in this particular area. Moreover, it is suggested that this volume should be read very carefully together with Volume I (which treats the fundamental theories) in order to get a complete understanding of the effects of high-energy radiations on substances of macromolecules.

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Chemistry (Imagination and Implication). By A. TRUMAN SCHWARTZ. Academic Press, New York, 1973. iv + 571 pp. $10.95.

Why review a freshman chemistry text, particularly for the *Microchemical Journal? Well, this is not an ordinary text. Dr. Schwartz has authored a* highly stimulating monograph which is an elegant exposition of some of the important developments in chemistry from the time of the phlogiston theory to the present. The reader will see at once that this book is remarkably different from others in its class by the manner in which Dr. Schwartz foreswears the inclusion of a paragraph on "the" scientific method, explaining that, "First, the workings of science and scientists are too diverse and too individualistic to permit a simplistic summary. And, Second, this entire book is an attempt to describe the ways (not way) in which science is done." He also flings down the gauntlet in challenging most chemistry laboratories as "chemical calisthenics which emphasize obedience rather than independence, which just as many texts teach memorization rather than understanding." In this reviewer's opinion, Dr. Schwartz' text is not only beautifully written but would serve as an excellent text, not only for the nonscience majors, for whom it is written, but also as a supplementary volume that chemistry students and professional chemists alike would find a great benefit.

Dr. Schwartz interweaves historical and logical approaches to the science of chemistry and should, if nothing else, develop a sense of the tradition of science in chemistry which, with the usual haste to cram as much "solid information" into our courses as possible, is usually absent.

While the book is delightfully readable, it is demanding. This is a book that will tax the vocabulary and general cultural level of students and might possibly lead to the introduction of a literacy test in place of (or in addition to) the usual arithmetic test sometimes given in chemistry placement. The exercises for "thought and action" given throughout the book are indeed thought provoking and interesting. The traditionalists among chemistry teachers (and here I must place myself) will be disappointed with the relatively small amount of descriptive inorganic chemistry. There is an occasional introduction of terms before they are defined. The book is attractively produced and relatively free of typographical errors (page 88, $CaCO_4$ where $CaSO_4$ was intended).

In summary, then, this is a book that merits favorable review in the *New York Times* book review section as well as in the *Microchemical Journal.*

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Principles of Organic Mass Spectrometry. By DUDLEY H. WILLIAMS AND IAN HOWE. McGraw-Hill, New York, 1973. 245 pp. $15.00.

The main objective of this relatively small book (245 pages) is to explain the basic concepts of formation and "reaction" of the positive ions in a mass spectrometer. The explanations are aimed primarily at the organic chemist who would use mass spectrometry for structure elucidation. This basic material (preceded by a succinct chapter on instrumentation) is covered under the following chapter headings: ionization and energy transfer, metastable ions, the quasi-equilibrium theory, and energetics of decompositions.

A subsidiary objective is to provide the organic chemist with a summary of the "relationship between fragmentation pattern and structure" in a chapter so titled. Additional information is provided in short chapters: isotopic labeling, collision processes, field ionization, ion structures, gas chromatography–mass spectrometry, and use of the computer in mass spectrometry.

Within the strictures of space set for themselves, the authors have done a magnificent job. The writing is uncluttered, and the figures are clear and abundant. Pitfalls and limitations are delineated, and the beginner is thoroughly disabused of the notion that a molecule is neatly dissected into bits characteristic of all members of its class, ready for facile reassembly. On the other hand, the extraordinary power of the tool, properly applied, is suggested in numerous examples.

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Determination of pH. Theory and Practice. By R. G. BATES. Wiley, New York, 1973. vii + 479 pp. $19.95. 2nd edition.

In the more frivolous jargon of Hollywood circles, Roger Bates undoubtedly would sail under the name "Mr. pH." He has devoted several decades of intensive work to the theoretical and practical aspects and problems of all facets of acidity. Among numerous publications resulting, a book that appeared in 1954 may be singled out: "Electrometric pH Determinations." It soon became THE reference work for anyone seeking information on background and know-how related to pH. Ten years later much material was added and an even more indispensable volume appeared: Determination of pH. Another enlargement and updating has been necessary and has led to the second edition at hand.

It is rare that one person is able to master both theory and practice to such an astounding degree. This mastery paired with a good hand for writing makes a very readable style and brings even difficult topics to a quite ready grasp. Especially elucidating is the treatment concerning philosophy, necessity and establishment of the operational definition and scale of pH. Acidity and pH are concepts that are of importance to more and more fields of science and it becomes increasingly difficult to make all the realms equally broad and exhaustive. While sections have been enlarged and added (e.g., those on glass electrodes, acidity concepts in nonaqueous media, or precision measurement on biochemical systems), others had to be modified and reduced as that on the description of commercial instruments. However, where a representative of a special discipline may consider his particular questions somewhat short-answered he will find the copious literature citations of great help.

The new volume will again be the foremost reference work like its forerunners and even more so. Proofreading was seemingly done with great care and the make of the book fits well with the overall excellence.

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Advances in Organometallic Chemistry. Vol. 11. Edited by F. G. A. STONE AND ROBERT WEST. Academic Press, New York, 1973. xi + 510 pp. $27.00

This continuing series in this volume presents five chapters dealing with transition metal complexes and one each on boranes, thallium and radiochemistry.

"Boranes in Organic Chemistry" by H. C. Brown is based on his 1971 Roger Adams Award Address. It is primarily a review of his group's work in this area and makes no claim for a complete treatment. That can be found in the recently published book by Brown bearing the same title as this chapter.

E. C. Taylor and A. McKillop have surveyed the major trends in organothallium chemistry during the past 20 years with particular emphasis on the utility of thallation and oxythallation in organic synthesis.

"The Radiochemistry of Organometallic Compounds" is an attempt by D. R. Wiles to acquaint the organometals chemist with the radiochemist. The expressed hope was that each could benefit from the exchange. The review covers the physical aspects of the nuclear reaction and subsequent atomic processes which occur in times too short to constitute conventional chemistry. In addition there is a short summary of work of historical or practical interest.

The recent literature on transition metal–isocyanide complexes is covered by Paul Treichel. Reactions of a general nature and specific to the complexes of individual metals are reported.

An equally thorough and complete coverage of the literature dealing with the insertion of carbon monoxide into transition metal–carbon bonds by A. Wojcicki focuses particularly on the kinetic and stereochemical aspects.

Cundy, Kingston and Lappert have reviewed the literature of transition metal complexes involving silicon ligands. The chemistry of both metal–silicon and metal–carbon–silicon bonds is surveyed as well as the transition metal catalyzed homogeneous hydrosilylation reaction.

The main aim of the review, "Organocobalt (III) Complexes," by Pratt and Craig is to survey the reactions by which the cobalt–carbon bond is made, broken or modified and which may be used for preparative purposes or in catalytic processes. This is not a review of vitamin B_{12} chemistry but interested chemists will find much that is helpful.

The review of the sources of literature for organo-transition metal chemistry for 1950–1970 by Bruce has been extended in this volume to cover 1971.

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Modern Methods in the Study of Microbial Ecology. Edited by THOMAS ROSSWALL. Swedish Natural Science Research Council, Stockholm, 1973. 511 pp. SKr 35.

This paperback volume consists of 80 brief papers presented at a symposium in 1972. They are grouped in six sets: techniques for observing microorganisms in soil and water, isolation and characterization of microorganisms, techniques for determination of microbial activity in relation to ecological investigations, estimation of microbial growth rates under natural conditions, model systems, mathematical models and systems analysis in microbial ecology. Aspects of soil microbiology are considered in 60% of the papers; aquatic microbiology in 12%; air, gases, and outer space in 8%; and laboratory studies on pure cultures in the remaining 20%. Although ecological aspects of microorganism–host interactions are not included, many of the techniques described could readily be applied to this somewhat neglected area of microbial ecology.

The methods generally are clearly described and most of the authors have included references that amplify and provide background for their techniques. A great diversity of methods are included, but there are no papers on detection of microbial poisons (toxins, antibiotics) that might be synthesized in natural environments.

The editing, printing, charts, and pictures are excellent, and the publishers should be commended for making the volume available within a year of the symposium presentation. Every student and professional worker in microbial ecology should have access to this volume.

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Encyclopedia of Industrial Chemical Analysis. Vol. 17. Edited by F. D. SNELL AND L. S. ETTRE. Wiley (Interscience), New York, 1973, xii + 644 pp. Each volume $45.00 ($35.00 by subscription).

Topics treated in Volume 17 include phenol, phosphorus (and inorganic phosphorus compounds), phthalic acids and derivatives, pigments, platinum group metals (and their compounds), polyamides (and fibers), polycarbonates, potassium and potassium compounds, pyridine and pyridine derivatives, radium, rare earths (and compounds), rhenium and technetium, rubidium and rubidium compounds, scandium, selenium and tellurium, and sensory testing methods.

The 37-page article on sensory testing methods presents a well-balanced introduction to the use of human subjects in the evaluation of the appearance, color, taste, flavor, and texture of food materials or products. Many analysts may not view sensory testing as a facet of industrial *chemical* analysis, the central topic of this encyclopedia, yet, the chemical basis of the senses seems established.

Although the contributors to this volume include staff members from 8 U.S. firms, and one western German firm, and one U.S. university, a substantial portion of the text (55%) is the work of a single author.

To recapitulate the review of earlier volumes, this encyclopedia is recommended to libraries having collections and clientele oriented to chemistry and chemical technology. With 17 volumes issued in the period 1965–1973 (3 on general methods and 14 on products), the analyst should consider this work a first place to look for many ready-to-use procedures.

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

Practical Fluorescence: Theory, Methods, and Techniques. By GEORGE G. GUILBAULT. Dekker, New York, 1973. xi + 664 pp. $29.50.

As stated in the preface the author's goal in writing this book is to introduce the reader to the entire field of luminescence spectroscopy. As such the title of the book probably should have been "Practical Luminescence: Theory, Methods, and Techniques." Also, in the preface the sentence, "This volume starts with an introduction to luminescence and then proceeds to a discussion of luminescent instrumentation, structural and environmental effects of luminescence, and phosphorescence," is confusing because of the phrase "and phosphorescence." Luminescence is a general term that includes both fluorescence and phosphorescence. In many parts of the book the words "fluorescence" and "luminescence" are used interchangeably.

There are 16 chapters in the book, 3 of which are written by other authors. Chapter 1

deals with a general discussion of luminescence theory and analysis at an elementary level beginning with a brief three paragraph historical introduction taken verbatim from the preface and ending with the advantages and limitations of fluorescence.

Chapter 2 entitled "Instrumentation" covers light sources, monochromators, cell compartment, cell configuration, slits, detectors, filter instruments, grating instruments, instruments for measuring decay times, and modification of cell compartment. Ten pages are devoted to filters, and a general survey of fluorometers and spectrofluorometers as to characteristics and cost is given. The comment on p. 32, "Thus any good spectrophotometer can be adapted to fluorescence work at a small additional cost," is misleading because the adaptation depends on the degree of sophistication needed.

Chapter 3 written by E. L. Wehry emphasizes the effects of molecular structure and molecular environment on fluorescence and briefly considers phosphorescence. This is a well written chapter, but the author occasionally uses "internal conversion" and "intersystem crossing" interchangeably, which is confusing. The chapter ends with an interesting and critical discussion of fluorescence and phosphorescence in rigid media.

Chapter 4 on the practical aspects of measurement is a short chapter that contains much useful information such as emission from cuvettes, light scattering, and correction of spectra.

Chapter 5 covers historical development, theoretical considerations, instrumentation, and analytical applications of phosphorescence. The section on structural effects is superfluous because the same examples were discussed in Chapter 4. The section on "Choice of Experimental Conditions; Signal-to-Noise Ratio Theory" is taken almost verbatim from an earlier book edited by the author, "Fluorescence: Theory, Instrumentation, and Practice," Dekker, New York, 1967. The numbers listed in Table 37 are ambiguous because it is not mentioned that they are percentage relative standard deviation values. However, Chapter 5 is well written and gives many important contributions to the book especially the section on applications.

Chapter 6 is a survey of fluorometric determinations of inorganic ions by direct analysis, fluorescent chelates, and quenching reactions through 1971. The section on fluorescent chelates and quenching reactions takes up the major part of the chapter covering 58 inorganic ions. Table 45 gives an extensive listing of fluorescence methods, sensitivities, and references. In the chapter 284 references are given.

A review of the assay of organic compounds through 1971 appears in Chapter 7. Three hundred fifteen references are given and several tables listing the fluorescent properties of the organic compounds or their derivatives are presented. Equal emphasis is placed on organic compounds and biochemical compounds.

Chapter 8 is concerned with fluorescence in enzymology and contains a general survey of the determination of enzymes, substrates, activators, cofactors, and inhibitors. In the first two pages of the chapter there is a good discussion of the advantages and disadvantages of the analytical uses of enzymes. The remainder of the chapter is concerned mainly with the determination of biochemical compounds.

Atomic fluorescence flame spectrometry is covered in Chapter 10 emphasizing atomic fluorescence theory, experimental systems, use of signal-to-noise ratio, and analytical results. Large sections such as II and III were taken verbatim from the previously mentioned book edited by the author; consequently these sections are not updated. The conclusion in section VII, "So far AF has been useful only for a limited number of elements. . ." obviously does not do justice to the many recent advancements made in atomic fluorescence flame spectrometry. Despite the above objections, after reading the chapter one should have a good understanding of the fundamentals of atomic fluorescence flame spectrometry.

Chapter 11 on electrogenerated luminescence is a short but interesting chapter covering experimental set up, formation of luminescent species, and ending with some suggestions for analytical applications of electrochemluminescence. Most of the chapter was taken from the previously mentioned book edited by the author.

Chapter 12 is a comprehensive chapter written by R. F. Chen on extrinsic and intrinsic fluorescence of proteins giving 447 references and emphasizing the great utility of fluorescence in studying the structure of proteins. Several references are made to his research work.

Govindjee, G. Papageorgiou, and E. Rabinowitch wrote Chapter 13, which is concerned with chlorophyll fluorescence and photosynthesis. They show by several examples from the literature how fluorescence can be used to study photosynthetic pigments *in vivo* in a variety of plants and algae as well as energy transfer phenomena in photosynthesis. Several references are made to their research work.

Chapter 14 is a short chapter dealing with analysis on solid surfaces such as TLC plates, paper chromatograms, and potassium bromide disks. The author presents extensive treatment of analysis on silicone rubber pads, a unique approach developed by the author and co-workers.

Chapter 15 gives a classical treatment on fluorescent indicators and discusses some newer applications.

The final chapter on forensic and environmental analysis consists of seven pages and the latest reference is from 1965.

In the preface the author states, "Throughout the book, emphasis is placed upon fundamental principles and actual analytical applications." Certainly he has accomplished this goal. By reading the entire book one will appreciate the applicability of luminescence and acquire fundamental knowledge of many aspects of luminescence.

Since the book is a survey of the entire field of luminescence spectroscopy, few topics are covered in depth, but the book contains sufficient information, numerous tables, figures and references to satisfy several workers with differing interests in luminescence. In general the field of luminescence analysis is covered through 1971 with an occasional reference to 1972.

The book does have some drawbacks. The price is very high and as a result it will probably be purchased primarily by libraries and those definitely interested in luminescence analysis. More serious is the number of typographical errors; over 65 were detected. For example, several references to figures in the text were incorrect and the first sentence on p. 64 is not a complete sentence. The author has taken sections almost verbatim from the previously mentioned book he edited and not given adequate credit to the authors that wrote these sections. Such a statement as "Taken from . . ." might have appeared in the appropriate sections in Chapters 5, 10, and 11.

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Phytochemistry. Vol. 2. Edited by LAWRENCE P. MILLER. Van Nostrand Reinhold, New York, 1973. xiv + 445 pp. $24.50.

This is the second excellent volume of a three-volume series in phytochemistry. As in the first volume, which was devoted to photosynthesis and carbohydrates, a distinguished group of authors have been assembled and they have made lavish use of structural formulae, pathways, and tabular material.

The second volume is subtitled "Organic Metabolites," and it discusses amino acids; proteins of flowering plants; purines, pyrimidines, and their derivatives; alkaloids; steroids; lipids; terpenes; rubber, gutta percha, and chicle; flavonoids; and miscella-

neous volatile substances of plants. The occurrence, structure and properties, role, metabolism, biosynthetic pathways, and in many cases, the taxonomic significance of each important group of metabolites is discussed and supported by abundant references.

The editor has provided valuable indexes according to subject, author, and, happily, plants and animals.

This volume and its companion Volume 1 should be valuable aids to the plant chemist, pharmacologist, and other botanical researchers, and particularly apropos as supporting texts for courses in phytochemistry.

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Phytochemistry. Vol. 3. Edited by LAWRENCE P. MILLER. Van Nostrand Reinhold, New York, 1973. xiv + 448 pp. $24.50.

This volume completes the excellent three-volume series in phytochemistry. The editor has again assembled a distinguished group of authors who have covered their subjects expertly and have made excellent use of structural formulae and appropriate tabular material.

The final volume is subtitled "Inorganic Elements and Special Groups of Chemicals," and includes chapters on the role of minerals in phytochemistry, sulfur compounds, nonvolatile organic acids, naturally occurring acetylene, lignin, cutins and suberins, surface waxes, vitamins, mitochondria and plant respiration, endogenous plant growth regulators, the gibberellins, molecular taxonomy, importance of secondary plant constituents as drugs, importance of plant chemicals in human affairs, and a retrospective and prospective look at phytochemistry.

Although each chapter is well-written and presented a good review of the subject, this reviewer was particularly pleased by the chapters on mitochondria and plant respiration, sulfur compounds, the gibberellins, the importance of secondary plant constituents as drugs, the importance of plant chemicals in human affairs, and the short closing chapter, entitled "Phytochemistry: Retrospect and Prospect."

This three-volume series should be a welcome addition to the libraries of plant chemists and pharmacologists, and to the teachers of phytochemistry.

In the final chapter, Professor Fowden mentions that in summarizing an account of alkaloid synthesis, a researcher quoted Robert Frost:

> The woods are lovely, dark and deep,
> But I have promises to keep,
> And miles to go before I sleep,
> And miles to go before I sleep.

Perhaps this quotation also summarizes the status and need for research in many areas of this fascinating field.

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Determination of Organic Structures by Physical Methods. Vol. 5. Edited by F. C. NACHOD AND J. J. ZUCKERMAN. Academic Press, New York, 1973. 365 pp. $26.00.

Each volume of this series presents contributed reviews that describe new and recent physical methods for the establishment of chemical structures and delineate applica-

tions, limitations, and prospects. The series is directed primarily to inorganic and organic chemists and biochemists involved in structural studies.

To the analytical chemist, more oriented to compositional and gross structural analysis, Volume 5 of this work is of bewildering complexity. The series obviously belongs in libraries having substantial holdings in physical and theoretical chemistry, but probably will be of limited utility to most analytical chemists.

Electron diffraction is considered by J. Karle of the U. S. Naval Research Laboratory in 74 pages, with some 346 references cited; this technique can provide useful information as to the configuration and conformation of organic compounds. Spin saturation labeling (or transfer) is treated in a 23-page article by J. W. Faller of Yale University (43 references). The technique has power for the elucidation of reaction mechanisms and has been exploited for this purpose only in recent years.

Chemical and electromagnetically induced dynamic nuclear polarization is covered by R. G. Lawler and H. R. Ward of Brown University in a 52-page article (over 115 references). Interest in DNP underwent striking change in 1967 with the finding that with conventional NMR instruments enhanced NMR absorption and emission can be observed for samples that are undergoing rapid free radical reactions. This chemically induced technique, given the acronym CIDNP, is still in its infancy, but applications appear promising.

Ion cyclotron resonance spectroscopy is the subject of a 17-page chapter (38 references) by J. I. Brauman and L. K. Blair of Stanford University. ICR is finding increasing use in the study of ion–molecule reactions.

M. G. Voronkov and V. P. Feshin of the Latvian Academy of Sciences and the Siberian Division of the Academy of Sciences of the USSR, respectively, provide a major contribution to this volume. In a 65-page chapter provided with an 18-page updating appendix, they review nuclear quadrupole resonance in organic and metalloorganic chemistry and cite over 360 papers. NQR is one of the latest physical methods for the study of the structural features of crystalline substances. To the chemist, possibly the greatest interest is in the shifts in NQR frequencies associated with bond changes to a quadrupolar nucleus, such as a halogen.

Mössbauer spectra is considered by N. W. G. Debye and J. J. Zuckerman of the State University of New York at Albany in a 49-page article (196 references). After a short introduction to Mössbauer spectroscopy, this review focuses on selected applications to problems in the chemistry of organometallic compounds.

S. I. Sasaki of Myasi University (Sendai, Japan) in a 37-page article sketches work toward automated chemical structure systems involving computer-based high-level analysis acting as an "artificial intelligence." Major inputs proposed include mass, NMR, and IR spectra. The outputs sought are the detailed structure of organic molecules, including compounds of unknown structure. Computer-based assessment of spectral data coupled with compositional and physical property data may be a part of the "tomorrow" for the organic microchemist.

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Annual Reports in Organic Synthesis—1972. Edited by J. MCMURRY AND R. B. MILLER. Academic Press, New York, 1973. xii + 273 pp. $7.50 paper.

This is the second year of publication of this series. The organic synthetic literature is surveyed through 50 worldwide publications. References into 1973 are in evidence.

The reactions are grouped in the following manner: carbon–carbon bond forming reactions, oxidations, reductions, heterocyclics, protecting groups, useful synthetic

preparations (functional group preparation and multistep transformations constitute this chapter), and very brief sections on miscellaneous reactions and miscellaneous reviews.

Each chapter is further broken down into well accepted categories. By far, most references are concerned with carbon–carbon bond formation processes.

Each reference is accompanied by a representative equation and very often also by percentage yields on more common substrates. Helpful sentences often indicate the limitations of the cited reactions.

Thanks to the relatively low price and up-to-date referencing apparent in this volume, this book should be accessible in all chemical libraries and on the shelves of many a synthetic organic chemist.

With the often lamented literature explosion such reference collections are a welcome help in literature search.

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Comprehensive Biochemistry: Enzyme Nomenclature. Edited by MARCEL FLORKIN AND ELMER H. STOTZ. Elsevier, New York, 1973. xii + 219 pp. $17.75. 3rd edition.

This is the third edition of Volume 13 of the series, Comprehensive Biochemistry. Starting out as the Report of the International Commission on Enzymes set up by the International Union of Biochemistry in 1956 to standardize, define and classify the nomenclature of enzymes, the first report appeared in 1961. Extensive editing and modifications as well as expansion resulted in a second edition in 1965. The growth of enzymology has now required that a third edition be prepared.

The first part of this report is devoted to definitions, classification schemes and reaction kinetic symbols.

Following are a group of four appendices listing the documents consulted, recommended symbols for reaction kinetics, a list of cytochromes, and a key to numbering and classifying enzymes.

The bulk of the book, 130 pages, is devoted to the 875 enzymes which are classified as oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Each of these groups is subdivided into specific areas of application, e.g., in the transferases is a subgroup involved with transferring a phosphorus containing group. Within each subgroup are listed the individual enzymes.

The entry for each enzyme lists the systematic code number and name, a recommended trivial name, other nonrecommended names, the chemical reaction involved and finally notes on the specificity and other comments.

There is also included a comprehensive alphabetical index of all of the names listed in the tables with the corresponding code number and a general subject index.

Some researchers may miss the listing of specific literature references for each enzyme, however, for anyone checking the literature or writing in this field this book should be indispensible.

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Rare Earth Intermetallics. By W. E. WALLACE. Academic Press, New York, 1973. 266 pp. $22.50.

This book probably provides the most complete coverage to date on this subject. Primary emphasis is placed upon the magnetic properties of rare earth intermetallic compounds, and the book covers these in three parts. Part II surveys the compounds

formed by rare earth metals and the nonmagnetic metals, such as RAl_2, RIn_3, and RCu_5, etc., and gives extensive details upon their magnetic properties. Part III covers the transition metal compounds, including the technologically important RCo_5, R_2Co_{17}, and RNi_5 series. The $SmCo_5$ and Sm_2Co_{17} compounds are now being employed as high strength magnet materials, having an energy product some five times greater than the best Alnico material.

Part I is perhaps the best concise description that I have encountered, and summarizes the nature of magnetic interactions. The explanation of the calculation of the crystal field interaction is excellent, including the results in the Appendix by E. Segal for: energies, eigenfunctions, and magnetic moments of rare earth ions in a hexagonal field. Early in the book, the author refers to the RKKY mechanism (sometimes called superexchange) and uses the mechanism to explain the various types of changes in magnetic coupling of electrons in the numerous compounds covered.

A great deal of experimental data is included as graphs and the tables on magnetic data are extensive. The bibliographies at the end of each chapter are said to be complete to August, 1971.

This is an excellent book for those involved with, or interested in, magnetic properties, and should be a welcome addition to any scientific bookshelf.

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Nuclear Magnetic Resonance Shift Reagents. Edited by ROBERT E. SIEVERS. Academic Press, New York, 1973. xii + 410 pp. $9.50.

This work is one of the finest compilations of recent advances in lanthanide shift reagents (LSR). For a relatively low price, one receives a complete library of the recent findings of research groups working on NMR shift reagents. There are a total of 58 contributors describing their work in 16 papers. These papers describe applications of lanthanide shift reagents to such problems as structural and conformational studies in solutions, and run the gamut from "Chiral Shift Reagents" through "The Structure of a Cholesterol-Shift Reagent Complex in Solution" to "Chemically Induced Dynamic Nuclear Polarization in the Presence of Paramagnetic Shift Reagents."

The extensive bibliography is uniquely presented, such that the reader may look under various aspects of LSR directly. The comprehensive bibliography is divided into the following subdivisions: Fundamental Aspects of Shift Reagent Chemistry (110 references), Chemical and Physical Properties of Shift Reagents (14 references), X-Ray Crystallographic Structural Determinations (11 references), General Applications of Shift Reagents (159 references), Applications of Shift Reagents to Studies of Biologically Significant Molecules (23 references), Determination of Enantiomeric and Diastereomeric Compositions and Related Phenomena (25 references), Kinetic Studies Involving Shift Reagents (5 references), Isotope Effects in Shift Reagent Chemistry (6 references), Applications of Shift Reagents to the Study of Stereochemistry (67 references). There is also a three page Glossary of terms and symbols used in the text.

This book will prove indispensable in the library of anyone interested in lanthanide shift reagents.

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High Pressure Liquid Chromatography, Biochemical and Biomedical Applications. By PHYLLIS R. BROWN. Academic Press, New York, 1973. xi + 202 pp. $11.50.

High pressure liquid chromatography is the most recently developed method for the separation of complex mixtures and is, by all accounts, the most powerful technique yet

discovered. In this fairly short book on the method, Mrs. Brown has introduced the subject, defined the appropriate terms, given a brief theoretical background and described some of the technical aspects. She has discussed qualitative and quantitative applications and described a number of separations, many of them taken from her own experimental work.

As the subtitle indicates, the book is strongly slanted toward biochemical and biomedical research. However, the discussions of technique and equipment are more general and appear to be exceedingly well done. There is no mention of preparative separations, perhaps because such problems seldom arise in this type of work. This, however, is a serious lack for those of us interested in synthetic chemistry.

The overall quality of the writing and production of the book is high. It would be most useful to anyone working in any aspect of biochemistry, medical research or forensic work.

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Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 3. Edited by T. S. WORK AND E. WORK. Amer. Elsevier, New York, 1972, 610 pp. $24.

This book is divided into two parts, each independent of each other. *It is therefore two separate books under one cover.*

Part I is written by G. G. Brownlee and is entitled "Determination of Sequences in RNA." This part consists of 275 pages. Following a brief introduction, which includes a general approach to sequence determination procedure, the author goes directly into the techniques of sequencing.

The techniques used with nonradioactive RNA and radioactive RNA are compared. The methodology is detailed for using enzymes so as to hydrolyze at specific linkages to produce the oligonucleotides and finally the individual nucleotides. The techniques using ion exchange and gel columns to separate and isolate the various digests are reviewed. Finally, the use of the spectrophotometer in locating the peaks and identifying them by their ultraviolet spectrum is covered in detail.

High voltage electrophoresis is then described. Patterns obtained by two-dimensional electrophoresis with cellulose acetate and then DEAE-cellulose are shown. The location of low molecular weight nucleotides by these techniques such as the di-, tri-, tetra-, and penta-nucleotides are given.

A typical problem, that of the elucidation of the structure of 5S RNA of *E. coli* is selected, and the reader is taken through the various steps used in sequencing this 120 unit polynucleotide. Subsequent studies present problems associated with the sequencing of the high molecular weight polynucleotides. Terminology, reagents, and specific techniques are given in the appendix for carrying out the various studies.

The text written by Brownlee is in fact an exhaustive laboratory manual on modern RNA sequencing technology. For those engaged in or entering this field it is of major importance. Since the techniques described are useful in all phases of analytical and biochemical research it is a valuable general source book in methodology. It should also be of value in designing experiments for the university undergraduate and graduate teaching laboratory.

The second text under the cover is entitled "Techniques of Lipidology" and is authored by Morris Kates. The experimental procedures used in the study of lipid metabolism are presented in 341 pages.

At first, classification of the various lipids is presented in conventional form, illus-

trating the types with structural formulas. This follows classical lines starting with hydrocarbons, alcohols, aldehydes, acids, etc. The neutral esters and ethers of glycerol and other alcohols are listed followed by the complex lipids containing carbohydrates, phosphorus, sulfur, and nitrogen.

The basic equipment is then described including specifications for glassware, pipets, and flasks. The various techniques for evaporation of lipid extracts is described in detail. This is followed by a section on the analytical instrumentation employed. This includes among others, spectrophotometers, mass spectrometers, NMR equipment, GLC equipment and the spectropolarimeter. In each case a commercial company making the equipment is listed.

Techniques are then described for extraction of the lipids from various biological sources. This is followed by a detailed description of the various analytical procedures for assaying for the various components of the lipids including total nitrogen, sulfur, phosphorus, carbohydrate, vicinal glycols, glycerol, cholesterol, etc.

The methodology used for the separation of lipid mixtures is then described. This includes the use of thin layer, column, and gas chromatography. In addition the classical chemical techniques such as solvent fractionation are also detailed.

The procedure for obtaining and preparing radioisotopically labeled lipids is then considered. This includes the methodology for counting the labeled lipids. It also includes radioactive scanning of the chromatograms and autoradiography.

In the final chapter, the techniques described in the text are applied to the identification of individual lipids. Tables are included giving the R_f values of various lipid types, the staining behavior of the lipids, and their special characteristics in the infrared. Infrared and NMR tracings of the various lipid classes are also shown. Specific techniques for identifying chain length, branching, double bond position and configuration, identification of functional groups such as aldehydes and ketones, etc., so as to delineate structure, are described in specific detail based upon the experiences of the author.

This book on lipidology is a practical laboratory manual for use in any biochemical laboratory. Published in paperback, it would make a valuable addition to the library of the college senior or graduate student majoring in organic or biochemistry. It is also an excellent reference book for the organic analyst faced with the problem of identifying the structure of an organic compound.

It would have been preferable if each of these excellent texts were published under a separate cover.

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