แผนกห้องสมุด กรมวิทยาศาสตร์
กระทรวงอุตสาหกรรม

64 V

VOLUME 5, 1963

ANALYTICAL BIOCHEMISTRY

An International Journal

EDITOR: *Alvin Nason*

ADVISORY BOARD:

Samuel P. Bessman
Harry P. Broquist
Melvin Calvin
Osamu Hayaishi
Evan C. Horning
Rollin D. Hotchkiss
Walter L. Hughes
Vernon M. Ingram
Andrew A. Kandutsch
Olov Lindberg
Hugh J. McDonald
Edward F. MacNichol, Jr.
Manfred M. Mayer
Howard K. Schachman
Donald D. Van Slyke
Sidney Udenfriend
Theodor Wieland
Lemuel D. Wright

ACADEMIC PRESS
New York and London

Analytical Biochemistry

An International Journal

EDITOR: *Alvin Nason*

ADVISORY BOARD

Samuel P. Bessman
Harry P. Broquist
Melvin Calvin
Osamu Hayaishi
Evan C. Horning
Rollin D. Hotchkiss
Walter L. Hughes
Vernon M. Ingram
Andrew A. Kandutsch
Olov Lindberg
Hugh J. McDonald
Edward F. MacNichol, Jr.
Manfred M. Mayer
Howard K. Schachman
Donald D. Van Slyke
Sidney Udenfriend
Theodor Wieland
Lemuel D. Wright

VOLUME 5
1963

ACADEMIC PRESS
New York and London

Copyright©, 1963, by Academic Press Inc.

ALL RIGHTS RESERVED

No part of this volume may be reproduced in any form, by photostat, microfilm, or any other means, without written permission from the publishers

Made in the United States of America

CONTENTS OF VOLUME 5

NUMBER 1, JANUARY 1963

E. D. SCHILLING, P. I. BURCHILL, AND R. A. CLAYTON. Anomalous Reactions of Ninhydrin 1

SAM KATZ. A Device for Generating Zero, Linear, or Exponential Gradients or Their Combinations 7

V. MOSES AND K. K. LONBERG-HOLM. A Semiautomatic Device for Measuring Radioactivity on Two-Dimensional Paper Chromatograms 11

DAVID M. UPDEGRAFF AND WILLIAM B. HUCKABAY. A Rapid Micro Gas Analysis System for Carbon Dioxide, Oxygen, Hydrocarbon Gases, and Hydrogen 28

ABRAMO C. OTTOLENGHI. Phospholipase C Determination by Egg Yolk Turbidimetry 37

ALAN R. WILLIAMSON AND STEPHEN ZAMENHOF. Detection and Rapid Differentiation of Glucosamine, Galactosamine, Glucosamine Uronic Acid, and Galactosamine Uronic Acid . . . 47

W. B. QUAY. Differential Extractions for the Spectrophotofluorometric Measurement of Diverse 5-Hydroxy- and 5-Methoxyindoles 51

MARTIN FLAVIN. Purification of Labeled *N*-Ethyl Maleimide . . 60

JOSEPH R. DAVIS AND ROBERT N. MORRIS. Rapid Colorimetric Determination of Adenine Compounds 64

HELEN W. WARREN, MILLARD J. HORN, AND AMOS E. BLUM. Microbiological Determination of Alanine in Proteins and Foods . 70

JOSEPH J. KOLB, MARY A. WEIDNER, AND GERRIT TOENNIES. Microdetermination of Lipid Phosphorus as a Measure of Bacterial Membrane Substance 78

SHORT COMMUNICATIONS

HAROLD A. RAMSEY. Thin-Layer Starch Gel Electrophoresis on Glass Slides 83

ARTHUR L. BABSON AND WILLIAM CAMPBELL. A Convenient Cuvette Rinsing Device 86

IRVIN L. SHAPIRO AND DAVID KRITCHEVSKY. Radioassay of Cholesterol-C^{14} Digitonide 88

NUMBER 2, FEBRUARY 1963

SUSANNE VON SCHUCHING AND CARL W. KARICKHOFF. Low-Level Carbon-14 Determination by Improved Schöniger Combustion and Ionization Chamber 93

David A. Turner, G. E. Seegar Jones, I. J. Sarlos, Allan C. Barnes, and Richard Cohen. Determination of Urinary Pregnanediol by Gas Chromatography 99
Bruce B. Stowe. Synthesis of High Specific Activity C^{14}-Carboxyl Indoleacetic Acid and of C^{14}-Nitrile Indoleacetonitrile . . . 107
Gary J. Nelson. An Apparatus for Production of Concave Gradients and Its Application to Chromatography of Lipids . . . 116
W. R. Slaunwhite, Jr., and Lavalle Neely. Analysis of Estrogens with Bromine-82 133
Hugh L. Spitzer and John A. Balint. A Rapid Quantitative Separation of Phosphatidyl Ethanolamine and Phosphatidyl Serine by Column Chromatography 143
L. Lepoutre, J. Stockx, and L. Vandendriessche. Influence of Coprecipitation on Quantitative Estimations of Ribonuclease Activities of Precipitation Methods 149
E. H. Mosbach, J. Blum, E. Arroyo, and S. Milch. A New Method for the Determination of Dihydrocholesterol in Tissues 158
John W. Mehl. A Simple Design for a Fraction Collector . . 170

Short Communication

L. Stewart and P. Nordin. Determination of the Degree of Polymerization of Oligosaccharides 175
Erratum 178

Number 3, March 1963

G. Rindi and V. Perri. Separation and Determination of Thiamine and Pyrithiamine in Biological Materials by Chromatography on Polyethylene Powder 179
B. Filipowicz, Maria Gross, and Bogna Skoczylas. Micromethod for Simultaneous Determination of Nitrogen and Phosphorus in Single Samples of Nucleoproteins and Their Derivatives . 187
Bernard A. Koechlin and Lucius D'Arconte. Determination of Chlordiazepoxide (Librium) and of a Metabolite of Lactam Character in Plasma of Humans, Dogs, and Rats by a Specific Spectrofluorometric Micro Method 195
George G. Guilbault, David N. Kramer, and Paul L. Cannon, Jr. A New, General Electrochemical Method of Determining Enzyme Kinetics. Kinetics of the Enzymic Hydrolysis of Thiocholine Iodide Esters 208
Hildegard Wilson and Mortimer B. Lipsett. Use of Periodate Oxidation in the Clinical Analysis of Urine Corticoids . . 217
P. A. Charlwood. Applications of Radioactively Labeled Marker Proteins in Density Gradient Ultracentrifugation 226

EDWARD S. REYNOLDS AND RICHARD E. LINDE. Colorimetric Determination with Murexide of Microgram Amounts of Calcium in Tissues 246

SHORT COMMUNICATIONS

R. L. LARSON AND H. P. BROQUIST. A Color Reaction between Reduced Pyridine Nucleotides and *o*-Aminobenzaldehyde of Potential Analytical Use 255

SIDNEY UDENFRIEND, PEROLA ZALTZMAN-NIRENBERG, AND G. L. CANTONI. Fluorometric Assay of 2-Dimethylamino-6-hydroxypurine (Dimethylaminoguanine) in the Presence of Guanine 258

DEREK WATSON. A New Method for Microanalysis of Blood Galactose 260

SOLON A. ELLISON AND HERBERT S. ROSENKRANZ. An Apparatus for Sampling after Cesium Chloride Density Gradient Centrifugation in the Preparative Ultracentrifuge . 263

SOMA KUMAR AND REMEDIOS M. AVENA. Paper Chromatographic Separation of Acetyl and β-Hydroxybutyryl Hydroxamates 265

RICHARD S. RIVLIN AND HILDEGARD WILSON. A Simple Method for Separating Polar Steroids from the Liquid Scintillation Phosphor 267

NUMBER 4, APRIL 1963

MYRON K. BRAKKE. Photometric Scanning of Centrifuged Density Gradient Columns 271

YUTAKA KOBAYASHI. Determination of Histidine Decarboxylase Activity by Liquid Scintillation Counting of $C^{14}O_2$ 284

ROBERT LEE SCHEIG, RITA ANNUNZIATA, AND LEROY A. PESCH. Studies on the Separation of Pyridine Nucleotides by Thin-Layer Chromatography 291

J. H. MCCLENDON AND J. H. KREISHER. Chromatography on Cellulose Phosphate of Polysaccharide Hydrolases from Fungi . 295

T. H. SIMPSON AND R. S. WRIGHT. The Characterization of Estrogens 313

H. C. MCALLISTER, JR., Y. C. WAN, AND J. LOGAN IRVIN. Electrophoresis of Histones and Histone Fractions on Polyacrylamide Gels 321

IRWIN H. SEGEL AND MARVIN J. JOHNSON. Synthesis and Characterization of Sodium Cysteine-*S*-sulfate Monohydrate . . 330

SIMON FREED AND MARY H. VISE. On Phosphorimetry as Quantitative Microanalysis with Application to Some Substances of Biochemical Interest 338

SHIZUO WATANABE, WILLIAM FRANTZ, AND DONNA TROTTIER. Fluorescence of Magnesium-, Calcium-, and Zinc-8-quinolinol Complexes 345

SHORT COMMUNICATIONS

R. J. PLANTA AND M. GRUBER. A Simple Estimation of Cathepsin C Using a New Chromogenic Substance . . 360

CLYDE M. WILLIAMS AND REID H. LEONARD. Microanalytical Determination of Dihydroxy Aromatic Acids by Gas Chromatography 362

JAKE BELLO. Convenient Apparatus for Protein Hydrolysis and Greaseless Freeze-Drying 366

NUMBER 5, MAY 1963

YUNG S. SHIN. Silicic Acid Column Chromatography for the Micro determination of Cholesterol, Cholesterol Ester, and Phospholipid from Human Cerebrospinal Fluid 369

JOHN A. JOHNSON, JOEL D. NASH, AND RAMON M. FUSARO. An Enzymic Method for the Quantitative Determination of Glycogen 379

DAVID KRITCHEVSKY, DOLORES S. MARTAK, AND GEORGE H. ROTHBLAT. Detection of Bile Acids in Thin-Layer Chromatography 388

CHARLES H. BURNS, A. D. NEVILL, AND G. HOYT WHIPPLE. The Photometric Estimation of Luciferase in Luminous Bacterial Cells 393

ENRICO FORCHIELLI, GUISEPPE SORCINI, MARY STYLIANOU NIGHTINGALE, NORMA BRUST, RALPH I. DORFMAN, WILLIAM H. PERLOFF, AND GERT JACOBSON. Testosterone in Human Plasma . . . 416

SHOGO ICHII, ENRICO FORCHIELLI, WILLIAM H. PERLOFF, AND RALPH I. DORFMAN. Determination of Plasma Estrone and Estradiol-17β 422

D. T. MASUOKA, W. DRELL, H. F. SCHOTT, A. F. ALCARAZ, AND E. C. JAMES. Separation of Catecholamines and Their Metabolites 426

M. A. JERMYN. Anthrone as a Reagent for the Determination of *S*-Carboxymethylcysteine in Proteins 433

PAMELA M. BELL. A Critical Study of Methods for the Determination of Nonprotein Nitrogen 443

FRANCES B. NESBETT AND ADELBERT AMES, III. Increase in Sodium Contamination from Glass As a Function of Time after Cleaning 452

SHORT COMMUNICATIONS

KAROLY G. PINTER, JAMES G. HAMILTON, AND O. NEAL MILLER. Liquid Scintillation Counting with Glass Fiber Paper 458

DIANNA CUPPY AND LAMAR CREVASSE. An Assembly for $C^{14}O_2$ Collection in Metabolic Studies for Liquid Scintillation Counting 462

NUMBER 6, JUNE 1963

FRANCISCO R. VEGAS. Chromatographic Behavior on Ion-Exchange Paper of Bilirubin Conjugated with Sulfate and Glucuronide . 465
SYDNEY M. FRIEDMAN AND F. K. BOWERS. A Computer Solution for Cation Analysis with Glass Electrodes 471
ROBERT A. HARRIS AND DAVID GAMBAL. Fluorometric Determination of Total Phospholipids in Rat Tissues 479
B. SCALES. Liquid Scintillation Counting: The Determination of Background Counts of Samples Containing Quenching Substances 489
TOSISUKE HIRAOKA AND DAVID GLICK. Studies in Histochemistry. LXXI. Measurement of Protein in Millimicrogram Amounts by Quenching of Dye Fluorescence 497
KENNETH H. IBSEN, RUSSELL L. SAUNDERS, AND MARSHALL R. URIST. Fluorometric Determination of Oxytetracycline in Biological Material 505
N. BURR FURLONG. A Rapid Assay for Nucleotide Kinases Using C^{14}- or H^3-Labeled Nucleotides 515
M. MAKITA AND W. W. WELLS. Quantitative Analysis of Fecal Bile Acids by Gas-Liquid Chromatography 523
R. A. DILLEY AND F. L. CRANE. A Specific Assay for Tocopherols in Plant Tissue 531
T. R. SATO, J. F. THOMSON, AND W. F. DANFORTH. Electrochromatographic Separation of Inorganic Phosphate, Adenosine Monophosphate, Adenosine Diphosphate, and Adenosine Triphosphate 542
JOHN R. SHERMAN. Rapid Enzyme Assay Technique Utilizing Radioactive Substrate, Ion-Exchange Paper, and Liquid Scintillation Counting 548

ERRATUM 554

AUTHOR INDEX 555

SUBJECT INDEX 557

ANALYTICAL BIOCHEMISTRY 5, 1–6 (1963)

Anomalous Reactions of Ninhydrin[1]

E. D. SCHILLING, P. I. BURCHILL, AND R. A. CLAYTON

From the James Ford Bell Research Center, General Mills, Inc., Minneapolis, Minnesota

Received March 22, 1962

INTRODUCTION

Largely as a result of the work of Spackman, Stein, and Moore (1), the automatic analysis of complicated protein hydrolyzates has become a routine analytical procedure in many laboratories. It is somewhat surprising, therefore, that little research effort has been directed toward the characterization of the ninhydrin-positive anomalies which frequently appear in these ion-exchange chromatograms. Schram *et al.* reported the presence of an unidentified ninhydrin-"positive" peak in the amino acid chromatograms of food hydrolyzates as early as 1953 (2). Since this compound was eluted early in the chromatogram, i.e., before aspartic acid, it did not confuse the standard amino acid elution pattern and little attention was given the observation. These authors suggested that levulinic acid, present as an artifact in protein hydrolyzates, was responsible for the strange "red peak." The observation that authentic levulinic acid exhibited chromatographic behavior identical to the putative anomaly gave credibility to their assumption.

Recently, Schilling *et al.* (3) reported the results of preliminary studies on the elucidation of the *structure* of the ninhydrin-levulinic acid reaction product. More recently, Zacharius and Talley (4) unequivocally demonstrated that levulinic acid is the compound responsible for the wine-red colored peak. In this communication, we wish to present further evidence for the structure of this reaction product. We wish also to report the results of a study which would suggest that caution should be exercised in the evaluation of the significance of the well-known ninhydrin reaction. In this study we have observed the ninhydrin-"positive" reaction of more than 20 nitrogen-free aldehydes, ketones, keto acids, and monosaccharides.

EXPERIMENTAL

Ninhydrin-Levulinic Acid Reaction

A 5.0-gm sample of levulinic acid adjusted to pH 5.0 with 1 *N* sodium

[1] Paper No. 290, Journal Series, Central Research Laboratories, General Mills, Inc.

hydroxide was mixed with an aqueous solution of 2.56 gm of ninhydrin (3:1 molar ratio). The volume of the reaction mixture was 35 ml. After the solution had been heated in a boiling water bath for 20 min, a deep wine-red color was produced. The reaction mixture was then subjected to a 16-hr diethyl ether liquid-liquid extraction; flash evaporation of the ether extract yielded 3.5 gm of a dark red syrup. The syrup was dissolved in several milliliters of acetone, absorbed on 2.0 gm of Celite 545[2] and the solvent evaporated. A 20 mm diameter column was prepared with 20.0 gm of Celite 545, slurried in Skellysolve B[3] and packed under 3–4 lb air pressure. Stepwise gradient elution was carried out from Skellysolve B to chloroform. The elution sequence was as follows: Skellysolve B (125 ml); Skellysolve B-chloroform 75:25 (375 ml); Skellysolve B-chloroform 50:50 (100 ml); chloroform (250 ml). Reduced pressure concentration of the pooled fractions yielded yellow to red colored oils. Cooling of the oily materials from the Skellysolve B-chloroform (75:25) fraction resulted in the crystallization of 87 mg of yellow prisms, m.p. 145–149° dec. Colorless prisms were obtained by recrystallization from *n*-butyl alcohol, m.p. 148–149° dec. Prior to analysis, the substance was dried over P_2O_5 for 6 hr at 100° and 0.5 mm Hg.

Anal. Calcd. for $C_{14}H_{10}O_5$: C, 65.11; H, 3.90. Found: C, 65.34; H, 3.96.

Absorption Spectra

Visible and ultraviolet absorption spectra were determined in 95% ethyl alcohol using a Cary Model 11 M spectrophotometer.

The nuclear magnetic resonance (NMR) spectrum was examined in a deuterochloroform solution with tetramethylsilane added as an internal reference. The spectrum was obtained on a Varian Associates HR 60 spectrometer.

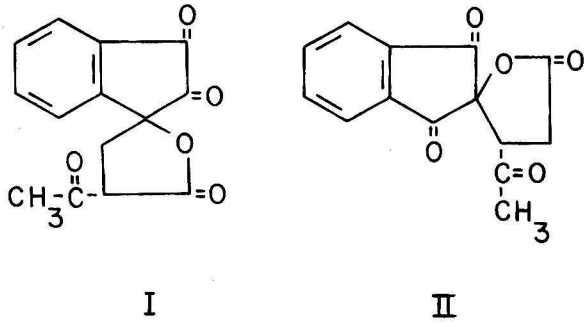
RESULTS AND DISCUSSION

The colorless crystalline compound isolated from the ninhydrin-levulinic acid reaction gave analytical values agreeing with the formula $C_{14}H_{10}O_5$. The elemental analysis is consistent with either of the two structures shown in Fig. 1.

Attempts to acetylate the colorless compound were unsuccessful. This observation was in agreement with the absence of a hydroxyl band in the infrared spectrum. A sharp band at 5.5 μ suggested the presence of a lactone carbonyl, whereas mass spectral as well as infrared data indicated the presence of the ninhydrin ring in the compound. Angelica lactone (γ-lactones of 4-hydroxy-2-pentenoic and -3-pentenoic acids) are

[2] Johns-Manville Co.

[3] Petroleum ether, b.p. 60–72°, Skelly Oil Co.



I II

FIG. 1. Proposed structures for the colorless ninhydrin-levulinic acid reaction product.

the most reactive derivatives of levulinic acid. Therefore, consideration was directed to the structures which contained α or β angelica lactones attached to the ninhydrin ring.

Analysis of the substance by NMR in deuterochloroform proved most helpful. Signal positions are reported relative to the tetramethylsilane internal standard. A sharp signal was observed at 129 cps, characteristic of a methyl ketone group. Signals between 170–225 cps were typical of a CH—CH_2 group. A peak at 479 cps indicated the presence of four aromatic protons. The total number of protons in the molecule was ten as determined by electronic integration of the signals; this was in agreement with the results of ultimate analysis.

The infrared, NMR, and mass spectral data all point to, but do not differentiate between, the proposed structures (Fig. 1). Normally, α-diketones are colored substances with absorption maxima above 350 mμ. The putative compound was colorless and exhibited an ultraviolet maximum at 233 mμ (similar to ninhydrin, 228 mμ) with no absorption above 350 mμ. Therefore, we have ascribed structure II (Fig. 1) to the colorless ninhydrin-levulinic acid reaction product.

Recently, we reported (3) the separation of a number of colored products from the ninhydrin-levulinic acid reaction. A flocculent reddish material precipitated upon acidification of the wine-red reaction mixture. Silicic acid chromatography of this precipitate by gradient elution from chloroform to 10% *n*-butyl alcohol in chloroform resulted in the development and elution of a host of multicolored bands. These compounds, or mixtures of compounds, were nondialyzable and because of lack of volatility, could not be analyzed by mass spectroscopy. It would appear, therefore, that the end product(s) of the ninhydrin-levulinic acid reaction are polymeric in nature. It is not entirely untenable to assume that

the characterized colorless compound might well be the repeating unit in such a macromolecule.

Additional Ninhydrin Reactions

In view of the reactivity of levulinic acid with ninhydrin, it was of interest to determine whether other nonnitrogenous compounds would react "positively" with ninhydrin. This problem was of special interest to us since our chromatograms of cereal protein hydrolyzates, as determined with an automatic amino acid analyzer,[4] have repeatedly indicated several anomalous peaks. Of particular interest was the observation that these anomalies were detected at 440 mμ, a wavelength generally reserved for proline and hydroxyproline. These peaks were eluted prior to aspartic acid and did not correspond to any known amino acid. In preliminary work, milligram quantities of the test compound were dissolved in 1.5 ml of the ninhydrin reagent (1) and heated in a boiling water bath for 20 min. Air was bubbled through the test solution for 5 min to eliminate the possibility of simple ninhydrin reduction with concomitant false color production. If a positive reaction was noted, a separate sample of the test compound was dissolved in 0.2 *N* sodium citrate buffer (pH 2.2) and analyzed with the automatic amino acid analyzer. Elution times and peak areas (Table 1) refer to development of the 150-cm Dowex 50-X8 column with 0.2 *N* citrate buffer (pH 3.25).

It can readily be seen from the variety of nonnitrogenous compounds listed in Table 1 that the "positive" ninhydrin reaction is certainly less specific than originally presumed. Thus, α- and γ-keto acids, α-ketodicarboxylic acids, aldehydes, ketones, hydroxy acids, aldonic and uronic acid derivatives, and hydroxy ketones as well as monosaccharides are among the classes of compounds which react positively to ninhydrin and are recorded as discrete peaks by the amino acid analyzer. Because of a lack of solubility in the citric acid buffer, several compounds which gave positive reactions in the preliminary test tube experiments could not be run on the amino acid analyzer. Typical of such compounds were α-keto acids above α-ketovalerate, diacetyl, 2,4-pentanedione, diacetone alcohol, and dimethyl malonate. Of the water-soluble compounds studied, it would appear that four classes of compounds are capable of yielding a positive ninhydrin reaction in the amino acid analyzer. These classes include hydroxy or keto acids and hydroxy aldehydes and hydroxy ketones. It should be emphasized, however, that not all such compounds give a positive ninhydrin reaction. Thus, glucose, glycolic acid, and 5-hydroxymethylfurfural are examples of "class" compounds which do not give a positive reaction.

[4] Phoenix Precision Instrument Co., Model K5000.

TABLE 1
BEHAVIOR OF NITROGEN-FREE COMPOUNDS ON AN AUTOMATIC AMINO ACID ANALYZER

Compound	Sample size (μmoles)	Elution time (min)	(H × W)[a]
Proline (reference)	1	366	3.9
Levulinic acid	50	155	10.8
Methyl levulinate	50	171	2.0
Pyruvic acid	125	97	1.3
α-Ketobutyric acid	202	98	0.18
α-Ketovaleric acid	508	132	0.02
α-Ketoglutaric acid	50	100	14.5
Oxalacetic acid	100	102	5.1
Phenylpyruvic acid	610	115	5.7
p-Hydroxyphenylpyruvic acid	609	108	6.1
Ethyl acetoacetate	205	169	8.2
γ-Ketopimelic acid	100	160	98.7
Acetoin	335	148	17.4
Dihydroxyacetone	98	169	15.2
γ-Valerolactone	267	151	1.13
Glucuronolactone	50	142	3.9
D,L-Glyceraldehyde	50	132	5.8
Fructose	222	124	2.2
Ascorbic acid	113	130	28.5
5-Ketogluconic acid	125	112	29.0

[a] (H × W) determined according to the method of Spackman *et al.* (1).

It will be seen from the data in Table 1 that the nonnitrogenous test compounds exhibited relatively low color yields with ninhydrin. Thus, the (H × W) value for proline is ca. 20 times greater than that for levulinic acid. Because of their elution positions and low extinction coefficients, it is not likely that these compounds would interfere with the quantitative interpretation of the chromatograms of hydrolyzates of pure proteins. It *is* important, however, to recognize the danger in assuming that a chromatographic peak must be associated with a nitrogen-containing compound. This is especially true since the analyzer is now being used as an analytical tool in research areas other than protein work. Thus, in our laboratories we have used the instrument in connection with the characterization of fermentation acids, the quantitation of keto acids in food products, and structure elucidation studies of complex lipid fractions.

SUMMARY

The structure of a colorless ninhydrin-levulinic acid reaction product has been established.

The ninhydrin-"positive" reaction of more than 20 nitrogen-free alde-

hydes, ketones, keto acids, and monosaccharides has been discussed. The chromatographic behavior of these compounds was determined with an automatic amino acid analyzer.

The results of this study suggest that caution should be exercised in the interpretation of "positive" ninhydrin reactions.

ACKNOWLEDGMENTS

The authors are indebted to Varian Associates for the NMR spectrum, to Mrs. Rachel Puntriano for technical assistance, and to Dr. L. E. Peterson and Mr. Robert Fisher for interpretation of the mass and infrared spectra.

REFERENCES

1. SPACKMAN, D. H., STEIN, W. H., AND MOORE, S., *Anal. Chem.* **30**, 1190 (1958).
2. SCHRAM, E., DUSTIN, J. P., MOORE, S., AND BIGWOOD, E. J., *Anal. Chim. Acta* **9**, 149 (1953).
3. SCHILLING, E. D., BURCHILL, P. I., COFFMAN, J. R., AND CLAYTON, R. A., *Biochem. Biophys. Research Communs.* **5**, 217 (1961).
4. ZACHARIUS, R. M., AND TALLEY, E. A., *J. Chromatog.* **7**, 51 (1962).

ANALYTICAL BIOCHEMISTRY 5, 7–10 (1963)

A Device for Generating Zero, Linear, or Exponential Gradients or Their Combinations[1]

SAM KATZ

From the Naval Medical Research Institute,[2] National Naval Medical Center, Bethesda, Maryland, and The Departments of Surgery and Biochemistry,[3] Albert Einstein College of Medicine, Yeshiva University, New York, New York

Received April 10, 1962

The considerable advantage accruing from the use of linear and/or exponential liquid gradients or combinations of same for column chromatography and zonal density gradient electrophoresis is well established (1, 2). However, the apparatus required for generating these gradients either is less than flexible in operation or else it is expensive and complicated. The following simple modification of the stand-pipe principle has been used successfully to produce zero, linear, or exponential gradients, or any of these combinations. The apparatus requires no attention after being placed in operation except when a change of gradient is desired.

Prior to describing this unit, a restatement of some of the fundamental relationships involved in creating these gradients is in order. The equation for the rate of change of concentration of solution, dc/dt, in a reservoir can be derived from the following conservation equation:

$$\frac{d(cV_1)}{dt} = \frac{r_2c_2 - r_1c_1}{V_0 + (r_2 - r_1)t}$$

where r_1 = rate that solution M leaves reservoir B1, r_2 = rate that solution N leaves reservoir B2, V_0 = initial volume of liquid in B1, V_2 = initial volume of liquid in B2, $V_1 = V_0 + (r_2 - r_1)t$ = volume of liquid in B1 at time t, c = concentration of solute in B1 at time t, and c_2 = concentration of solute in B2.

[1] The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

[2] Present mailing address.

[3] This problem was initiated while at the Albert Einstein College of Medicine, New York. During this period the investigation was supported in part from grants by the U. S. Public Health Service CY 3599 and G. D. Searles and Co.

Condition I: Exponential gradient. $r_1 = r_2$

$$c = c_2\left[1 - 10^{-\frac{0.434tr_1}{V_0}}\right]$$

Condition II: Linear gradient. $r_1 = 2r_2$

$$c = c_2\left[1 - \frac{V_1}{V_0}\right]$$

Condition III: Variable gradients. $r_1 \neq r_2$

$$c = c_2\left[1 - V_1/V_0^{\frac{r_2}{r_1 - r_2}}\right]$$

The solution of this equation has been adequately described elsewhere (3); thus this report will be confined to the description and method of operating the apparatus to achieve the desired sequence of events. In the subsequent discussion it is recognized that these gradients[4] can be attained only if there is adequate mixing in reservoir B1 (Fig. 1).

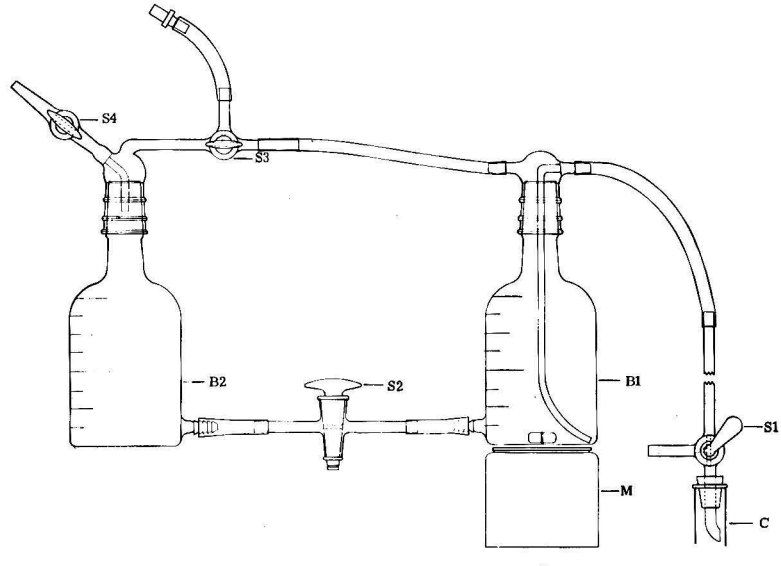


FIG. 1. Device for generating gradients.

[4] The gradients referred to are those produced in the chromatographic column; the correct term is the time rate of change of concentration observed in the mixing reservoir.

Conditions for linear gradient: In order to achieve a linear gradient it is essential that the geometry of both reservoirs be identical and that the densities of the solutions used do not differ by more than 5%.[5] Each reservoir is filled to the same height, resulting in equal hydrostatic pressures in both reservoirs. A given solution is added to Bl by initially introducing it into B2 via S4. Then S4 is closed and S1 and S2 are opened. Either nitrogen or air pressure is then applied through the S3 so that the solution is transferred to B1. The procedure for the addition of the second solution to B2 is self-evident. To establish the desired gradient, stopcocks S1 and S2 are opened, S4 is shut, and S3 is adjusted so that B1 and B2 are exposed to the same pressure, be it atmospheric or elevated.

A convenient check on the linearity of the gradient is provided by the fact that the liquid levels in both reservoirs should decrease identically during the course of the production of the gradient.

Condition for exponential gradient: The solvents are added to their respective reservoirs as indicated previously. Stopcock 4 is closed; S1 and S2 are opened while S3 is adjusted so that only B2 is exposed to atmospheric or elevated pressure as desired. Thus the flow rates for both reservoirs should be equal, resulting in an exponential gradient. During the course of this experiment, the volume of solution in reservoir B1 will remain constant until the solution has completely drained from reservoir B2.

This unit is so constructed that solutions may be introduced, transferred, or drained from the different reservoirs and still maintain the column under a positive gas pressure. Thus it is a simple matter to establish any of several combinations sequentially of zero, exponential, or linear gradients as desired during the course of a single experiment with a minimum of manipulation. It should be noted that variable gradients, Condition *III*, may be produced either by introducing glass inserts into the reservoirs or by the use of chambers of different geometry.

ACKNOWLEDGMENTS

I wish to acknowledge my indebtedness to Mr. M. B. Bergey for introducing me to the concepts of gradient elution, and to Captain R. H. Lee, MSC, USN, for his

[5] Approximate linear gradients can be produced with solutions whose densities differ by as much as 15%. In those circumstances the amounts of solution introduced should be such that the initial hydrostatic heads in each reservoir are equal. The deviation from linearity during the first portion will be in the order of 10%; however, during the progress of the experiment the deviation from linearity will become progressively smaller since the densities will tend to approach each other and thus exert a self-correcting effect.

assistance with the mathematics. The apparatus was constructed according to specifications by Labtician Products Co., Hollis 23, New York.

REFERENCES

1. PETERSON, E. A., AND SOBER, H. A., *Anal. Chem.* **31,** 857 (1959).
2. SVENSSON, H., *in* "Analytical Methods of Protein Chemistry" (Alexander, P., and Block, R. J., eds.), Vol. I, p. 211. Pergamon Press, New York, 1960.
3. LAKSHMANAN, T. K., AND LIEBERMAN, S., *Arch. Biochem. Biophys.* **45,** 235 (1953).

ANALYTICAL BIOCHEMISTRY 5, 11–27 (1963)

A Semiautomatic Device for Measuring Radioactivity on Two-Dimensional Paper Chromatograms

V. MOSES AND K. K. LONBERG-HOLM

From the Bio-Organic Chemistry Group, Lawrence Radiation Laboratory, University of California, Berkeley, California

Received April 27, 1962

INTRODUCTION

The automatic measurement of radioactivity in spots of labeled compounds on two-dimensional paper chromatograms is technically much more difficult than performing the same task with one-dimensional separations. In the latter instance resolution is required in one dimension only (i.e., along the direction of solvent travel) and with a suitable slit width of the masking shield, speed of movement of the chromatogram past the detector, and sensitivity of the detecting and recording equipment, a resolution can be achieved similar to that obtained with x-ray film radioautography, while simultaneously obtaining a continuous quantitative estimate of the radioactivity on the paper. The record may be obtained either as a continuous count-rate tracing with a pen recorder, or as a printed tape if the pulses received for predetermined intervals of time are integrated and automatically printed. Such machines have been in use for many years and are available commercially.

Two-dimension chromatograms present an entirely different problem. Unless spots on a chromatogram are widely separated, resolution adequate to distinguish between them is extremely difficult to obtain by any type of scanning technique. The smaller the detector the higher is the resolution, but the lower the number of events detected per unit time. To achieve sufficient resolution to distinguish between adjacent compounds on chromatograms of the type routinely obtained in this laboratory (Plate 1), in which many spots lie within about 2 mm of each other and frequently have irregular margins which may even dovetail together, the detector would need to be no larger than about 1 mm in diameter. The number of emitted particles entering such a minute detector per unit time would be relatively small, demanding greatly prolonged counting times at each position in order to achieve any reasonable statistical accuracy; alternatively, such accuracy must be sacrificed. As one cannot predict the most suitable directions in which to scan the chromatogram

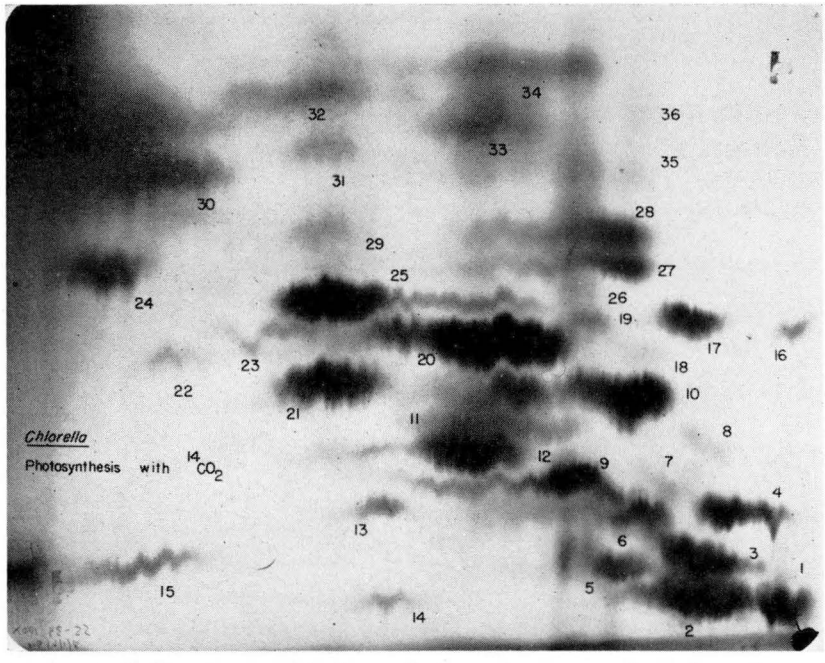


PLATE 1. Radioautogram of the radiochromatogram used to investigate the reproducibility of manual and automatic counting. The radioautogram (14 × 17 in.) was obtained with Kodak Single-Coated Blue-Sensitive Medical X-Ray Film, exposed for 8 days.

unless the distribution of the spots is known (in which case scanning becomes superfluous), it is inevitable that no matter in which pair of dimensions at right angles to each other the scanning is performed, resolution will be incomplete unless spots are sufficiently widely separated and there is no dovetailing at the margins.

The difficulties of attempting simultaneously to scan and evaluate automatically a two-dimensional radiochromatogram are well illustrated by the apparatus designed by Chain *et al.* (1). They used a pair of opposed mica end-window Geiger tubes with a window size of 1 cm^2. Pocchiari and Rossi (1) do not mention the distance between the two windows, but in a commercial model based essentially on this design (Bi-Dimensional Scanner, Model 460, Packard Instrument Company, Inc., La Grange, Ill.), in which the window size may be set at 8.4 × 10.2 mm or 4.2 × 10.2 mm, the distance between the two windows is 5.6 mm; this in itself is quite sufficient to ensure that the resolution would be far poorer than 2 mm. We have demonstrated conclusively that a radiochromatogram similar to the one shown in Plate 1 cannot be scanned

and evaluated quantitatively with the Bi-Dimensional Scanner; all the compounds numbered 1–8, for instance, were recorded as only two spots.

Most workers faced with this problem have located the exact positions, sizes, and shapes of the spots on radiochromatograms by radioautography, and it continues to be our experience that this is the most satisfactory method for obtaining the location of labeled compounds with sufficient accuracy and resolution. Equipment has been designed to locate radioactive spots by making a facsimile of the chromatogram (2–4). In the designs by Aronoff (3) and Perkins and Tyrrell (4), a Geiger tube is moved across the chromatogram, which is fastened to a drum, while simultaneously an electrically energized stylus is moved over electrosensitive paper wrapped around the opposite end of the drum. Each pulse received by the Geiger tube from a certain point on the chromatogram is recorded as a black dot on a corresponding position on the electrosensitive paper. In this way a facsimile of the radiochromatogram resembling a radioautogram is produced on the electrosensitive paper, though with a somewhat reduced degree of resolution. The main advantage of such a technique is the reduction in time required to produce a facsimile of the radiochromatogram compared with the exposure required for an x-ray film. Perkins and Tyrrell (4) illustrate one facsimile requiring 62 hr for preparation compared with the corresponding radioautogram exposed for 192 hr. The facsimile scanner is thus mainly of value when the location of the radioactive compounds on a small number of chromatograms is urgently required, since 10, 50, or 100 radioautograms could have been exposed in the same 192 hr, yet scanning would have occupied 620, 3100, or 6200 hr, respectively, if only one machine were available. Such comparisons are admittedly only approximate, since a radioautogram might require any period from minutes to months for adequate exposure.

Having located the positions of the radioactive compounds on the chromatogram, two main types of technique are used to determine the radioactivity in each compound. In the first the compound may be eluted, and after suitable manipulation, assayed on a planchette, in a scintillation counter, or by conversion to some volatile compound and counted in a gas counter; or it may be counted by one of these methods without previous elution from the paper. Each of these requires time, limits the number of samples which can be handled to a low figure, and frequently results in a loss of the sample for further manipulation, though many of the counting devices have high efficiencies and a count is obtained of correspondingly high accuracy.

In the second technique, reviewed by Moses and Edwards (5), a large diameter (2.25 in.) end-window Geiger-Müller tube (6), fitted

with a thin replaceable Mylar polyester window (density about 1 mg/cm^2) and flushed with an appropriate counting gas, is placed on the chromatogram at each position known to be the location of a radioactive substance. The number of counts from each location is recorded for a given period, often 1 min. Many spots on the chromatogram are frequently too large to be counted with the detector tube in a single counting operation, and part of the spot must then be shielded in a suitable manner (index cards are used for C^{14}) while another part is counted. The position of the shield is then moved so that the area previously shielded may be counted and the area previously counted is shielded. Because of the sizes of many spots, it is frequently necessary to count them in several, rather than in two, operations. Spots on the chromatogram lying close to the one being assayed must also be shielded lest they interfere with the one being counted. Finally, a spot will usually give a different count on the two sides of the paper, probably due to differences in the rates of evaporation from the two surfaces of the solvent used for development of the chromatogram. It is therefore necessary, in all investigations requiring a reasonably quantitative treatment, for each spot to be counted on both sides of the paper.

A typical chromatogram obtained in an investigation of the pathways of intermediary metabolism may have as many as forty or fifty spots on it (Plate 1). Perhaps one-half of these spots will be too large to be counted in one positioning of the Geiger tube, and will need to be counted in sections. Thus, on both sides of the paper, there may be a total of more than 120 different positions to be counted, each, say for 1 min, and in practice 30 sec must be added to each counting period for aligning the shields and recording the results, making some 3 hr in all for each chromatogram. It is difficult to keep more than one counter in operation for 1-min counts as the operator rapidly becomes confused and over-all efficiency and accuracy of placing the masking shields deteriorates considerably. Equally important is the general tedium of the whole operation, and, although it may be theoretically possible to count three such chromatograms in one working day, this places an undue mental strain on the operator, again with a fall in efficiency and an increase of carelessness. In addition, a 1-min counting period is insufficient to achieve a high degree of statistical accuracy, while increasing the counting period to, say, 5 or 10 min would slow the whole operation down to a quite unacceptable degree if this were done manually.

Some alleviation of these difficulties can be obtained by cutting out the spots from the chromatogram and placing them one at a time, if necessary in sections, between two Geiger tubes mounted with their windows opposed, and connected in parallel to one scaling unit. This eliminates

the need for careful masking of parts of the spot not being counted, and of adjacent spots, while at the same time enabling both sides of the paper to be counted simultaneously (Moses, unpublished). However, once the spots have been cut out it then becomes possible to count them sequentially in an entirely automatic apparatus, the description of which forms the purpose of this communication.

PRINCIPLE

Two pieces of equipment are required: a "loader" and a "counter."

Loader. The principle of the loader is shown in Fig. 1; a photograph is shown in Plate 2. A strip of Mylar (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.), 5 in. wide and 0.00025 in. thick, is unwound

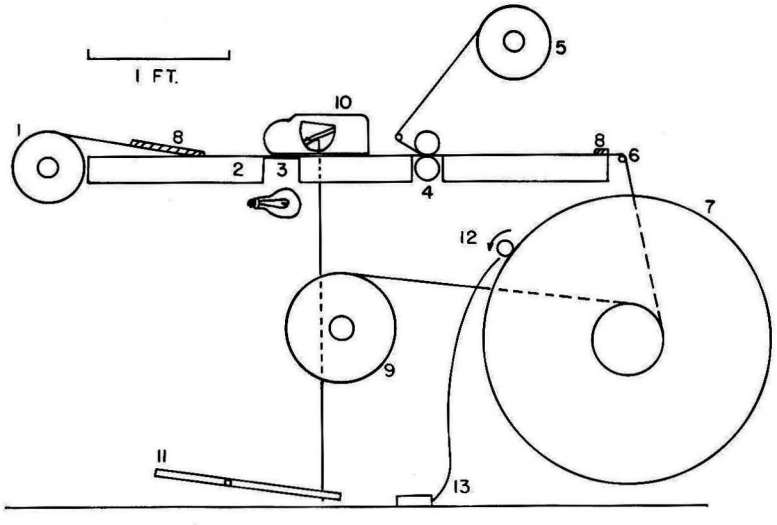


FIG. 1. Diagrammatic representation of loading machine: (1) supply spool of Mylar for bottom layer; (2) wooden table top faced with plastic; (3) illuminated window; (4) opposed rubber-covered rollers; (5) supply spool of Mylar for upper layer; (6) guide roller; (7) takeup spool; (8) felt pads; (9) supply spool of kraft paper; (10) machine for dispensing marker tape; (11) foot pedal for same; (12) motor for driving takeup spool; (13) foot switch for same.

from a spool (1) along a table (2). The Mylar passes over a window (3-in. diameter) in the table (3), illuminated from below by an electric light bulb, and then runs between two opposed rubber-covered rollers (4). At this point another similar strip of Mylar from a second spool (5) is also fed between the rollers, and comes to lie exactly on top of the first Mylar strip. The double band of Mylar passes over another roller (6) and is wound onto a large takeup spool (7). Two felt pads (8) remove

แผนกห้องสมุด กรมวิทยาศาสตร์
กระทรวงกลาโหม

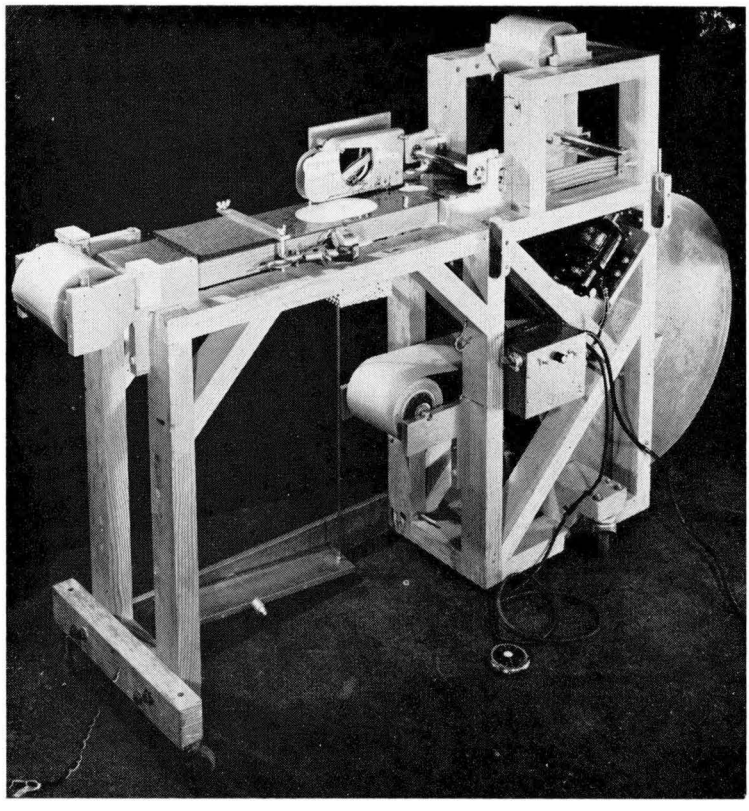


PLATE 2. Apparatus for mounting chromatogram spots between two continuous layers of Mylar film.

wrinkles from the Mylar. A strip of kraft paper, also 5 in. wide, is simultaneously unwound from a spool (9), and wound onto the takeup spool (7) together with the now double Mylar strip. At a particular position in relation to the illuminated window (3) a tape-dispensing machine is mounted (10), operated by a foot petal (11) which dispenses small rectangles of opaque adhesive tape onto the bottom strip of Mylar before it is covered with the upper strip. The chromatogram spot, cut out of the whole chromatogram paper, is placed on the stationary lower strip of Mylar over the illuminated window (3), with a small drop of fast-drying glue applied to the leading edge of the spot. The glue serves to attach the paper spot securely to the Mylar and also helps to keep the upper and lower layers of Mylar in a constant relation to one another. A piece of opaque tape is dispensed from the tape dispenser (10) and the Mylar strip is then advanced by means of the motor (12), controlled by a foot

pedal (13), operating on the rim of the takeup spool. The motor (12) can be operated continuously or only when the foot pedal (13) is depressed.

As the Mylar is so thin, it has very little resistance to bending and folding. It is therefore important that the axles of all the spools and rollers are accurately aligned or the combined strip will not wind uniformly onto the takeup spool. The purpose of the kraft paper is to provide a continuously smooth surface on which to roll up the combined Mylar strip onto the take-up spool. As chromatogram paper (Whatman No. 4) is about 15 times as thick as the double layer of Mylar, and the chromatogram spots are confined to the center of the strip, a bulge develops in the accumulated strip on the takeup spool unless the backing of kraft paper is provided.

With the size of the takeup spool currently in use (24-in. over-all diameter, 6-in. core diameter), the total capacity is about 3000 ft of combined tape plus kraft paper, or about 7200 spots spaced at 5-in. intervals.

Counter. In the counter (Fig. 2, Plates 3 and 4) the strip is unwound from the loaded takeup spool (1), which has been transferred from the

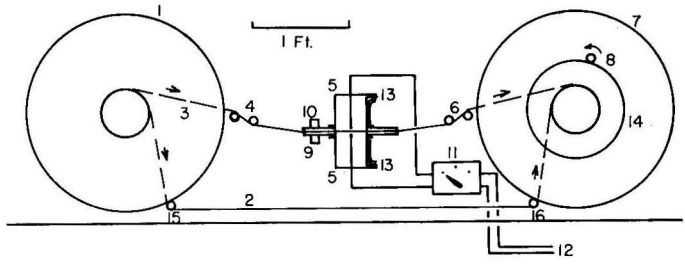


FIG. 2. Diagrammatic representation of counter assembly: (1) supply spool bearing kraft paper, and chromatogram spots between two layers of Mylar; (2) kraft paper; (3) double layer of Mylar bearing chromatogram spots; (4) guide rollers; (5) opposed detector tubes; (6) guide rollers; (7) takeup spool; (8) motor drive for takeup spool; (9) light for photocell; (10) photocell; (11) switch for selecting either or both detector tubes; (12) cables for connections to scaler and high voltage supply; (13) gas inlet and outlet tubes; (14) knurled drive wheel for takeup spool; (15, 16) guide rollers for kraft paper.

loader, the kraft paper (2) is separated from the double layer of Mylar (3), and the latter, after alignment by passage over two rollers (4), is passed between two end-window gas-flow counter tubes (5). The Mylar is passed over two more rollers (6), brought into contact again with the kraft paper and the two taken up together as in the loader onto another

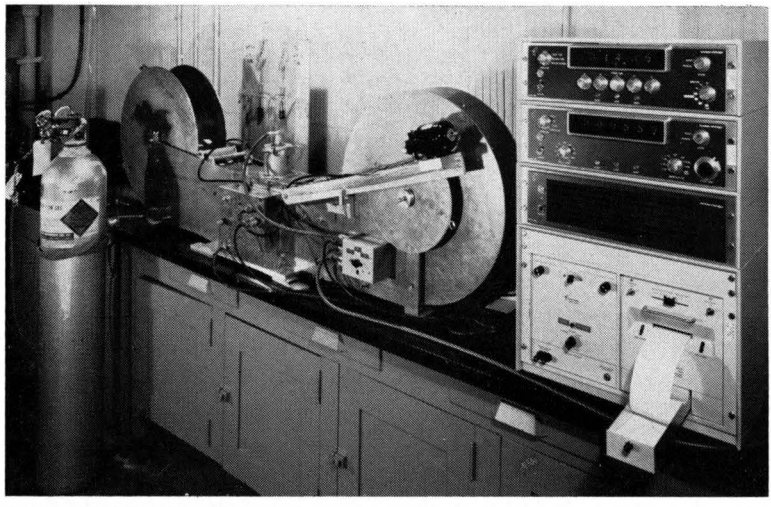


PLATE 3. Equipment for counting chromatogram spots mounted between two continuous layers of Mylar film: general view.

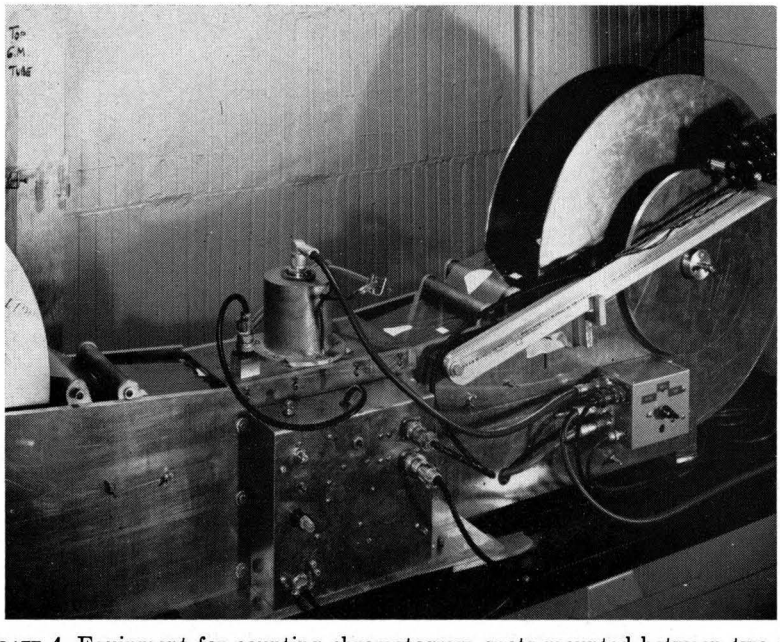


PLATE 4. Equipment for counting chromatogram spots mounted between two continuous layers of Mylar film: closeup showing details of detector tubes, rollers for alignment, and mounting of drive motor.

takeup spool (7) driven by a motor (8). A photoelectric cell (9) and a light (10) are so mounted that they bear the same relation to the center of the counter tubes that the opaque tape-dispensing machine (10, Fig. 1) bears to the illuminated window (3, Fig. 1) on the loader. Thus, when a piece of opaque tape blocks light coming into the photoelectric cell, and a chromatogram spot lies exactly between the two counter tubes, the drive motor (8) is automatically switched off and counting commences. The output from the counter tubes in parallel is fed into a scaling, timing, and automatic printout circuit. The chromatogram spots are counted according to a predetermined program, and the elapsed time and recorded counts automatically printed out onto tape.

In the longest loading operation to date, 1250 counting positions were loaded onto tape. The counting of this tape took 8 days to complete and required no attention on the part of the operator except to renew a cylinder of counting gas.

CONSTRUCTIONAL DETAILS

Counter

(*a*) *Mechanical.* Sectional and plan diagrams showing the details of the design and mounting of the counter tubes are shown in Figs. 3 and 4.

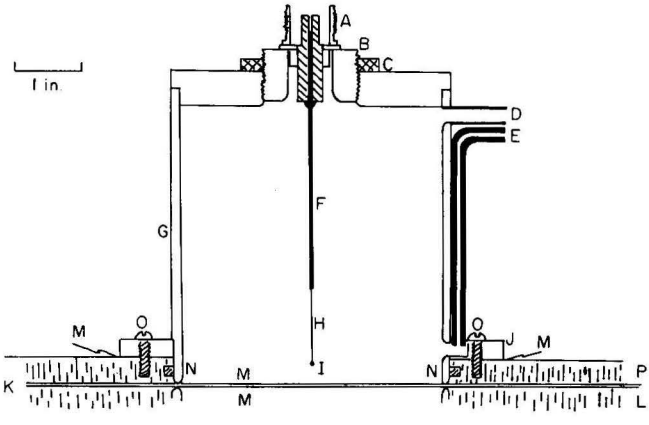


FIG. 3. Section through detector tube showing details of mounting: (A) amphenol plug (UG-560/U) with Teflon insulator; (B) threaded insert holding anode wire; (C) knurled locking ring for threaded insert; (D) gas inlet tube; (E) gas outlet tube; (F) pillar supporting anode; (G) body of tube; (H) anode wire (0.005-in. diameter tungsten); (I) glass bead; (J) flange soldered to tube body; (K) distance between base plates is 0.063 in.; (L) lower base plate; (M) gold-sputtered Mylar windows; (N) rubber O-ring (4.25 × 0.125 in.); (O) six bolts holding flange to base plate; (P) upper base plate.

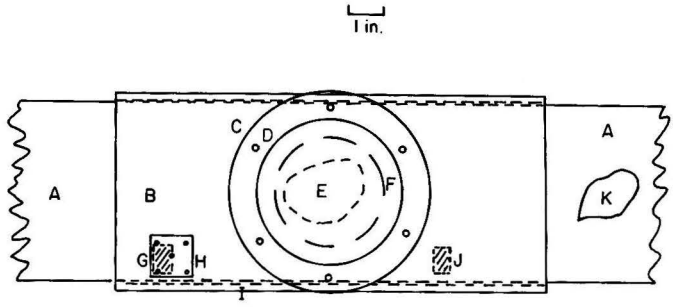


FIG. 4. Plan of detector tube mounting: (A) Mylar band; (B) base plate; (C) outer diameter of flange on tube; (D) outer diameter of tube; (E) chromatogram spot No. 1 in counting position; (F) maximum permitted diameter of counting position; (G) marker tape for chromatogram spot No. 1; (H) photoelectric cell assembly; (I) spacer strip (0.063 in. thick) separating base plates; (J) marker tape for chromatogram spot No. 2; (K) chromatogram spot No. 2 after counting. Mylar band moves from left to right.

The counter tubes are made of brass tubing, 3.75 in. internal diameter and 4 in. external diameter. The brass plates holding the counter tubes are mounted in the frame 0.063 in. apart. The tubes are flushed with a gas mixture containing 0.95% (v/v) isobutane in helium (The Matheson Co. Inc., East Rutherford, N. J.). A manometer is placed on the outlet side of each counter tube to measure the gas pressure within the tubes. The counting gas is exhausted to the atmosphere via tubes containing glycerol about 0.75 in. deep. These bubbler tubes prevent air diffusing into the counter tubes and also enable a measure of the gas-flow rate to be made. The pressure of the counting gas inside the tubes is maintained at about 10 cm of ethanol above atmospheric, this being controlled from manometers on the outlet side of each tube. This pressure is sufficient to bow out the flexible Mylar windows of the counter tubes toward each other until between them they compress the Mylar tape bearing the chromatogram spots, thereby ensuring that the sensitive volumes of the counter tubes are brought into the closest possible contact with the Mylar tape, and simultaneously smoothing out any wrinkles which arise in the tape as it is wound from one spool to the other. A gas-flow rate of 1 bubble/sec/tube is sufficient to maintain the proper gas composition inside the counter tubes.

The Mylar used for the windows is 0.00025 in. thick, and is sputtered on each side with metallic gold (Atomic Development & Machine Corp., College Point, N. Y.). The over-all density of the metallized Mylar is 1.0 mg/cm^2. The metallized Mylar windows of the counter tubes have withstood the movement past them of at least 2500 ft of Mylar tape with no damage save for slight abrasion marks.

The central anode wire (0.005-in. diameter tungsten) of each counter tube terminates in a glass bead. As the anode assembly is mounted in the roof of the counter tube on a screw-threaded insert, the distance from the glass bead to the window may readily be adjusted; 0.25 in. has been found to be the most satisfactory distance.

The large spools for winding up the Mylar tape containing the chromatogram spots together with the kraft paper have wooden cores 5.125 in. wide and 6 in. in diameter, and flanges of aluminum 24 in. in diameter and 0.1875 in. thick. The axles of these spools are held in Oilite bearings which are mounted on the main framework of the counter assembly.

A knurled disk (14, Fig. 2) is mounted on the axle of the takeup spool and serves as a friction drive from a 9.6 rpm motor (7-lb torque). The drive shaft of the motor is fitted with a 1-in. rubber tire which rests on the knurled disk. The motor is mounted at the end of a long metal arm fixed to the frame with a pivot as shown in Plates 3 and 4.

(*b*) *Electrical.* In the operation of the sample changer control, a command pulse is sent from the scaler on completion of counting (preset time, preset count, or time/count) to the printer and to the control box on the counter assembly; the latter starts the drive motor and turns on the light for the photocell circuit. After being switched on, the motor drives for at least 2.2 sec, regardless of the light beam, to permit the opaque tape to clear the photocell. The motor runs until the light beam is interrupted by a piece of opaque tape; this stops the motor drive by removing the power and applying a DC dynamic brake, switches off the light, and starts the timer and scaler.

The output signals are taken independently from each counter tube, and a selector switch (11, Fig. 2) selects either or both tubes.

The timing, scaling, and printing units used are as follows: timer, Nuclear-Chicago Corp. (Des Plaines, Ill.) Model 8600; scaler, Nuclear-Chicago Model 8250; analog output, Nuclear-Chicago Model 8400; digital recorder, Hewlett-Packard (Palo Alto, Calif.) Model 560A; high voltage supply, John Fluke Manufacturing Co. Inc. (Seattle, Wash.) High Voltage D.C. Power Supply Model 408 A/U. The timer and scaler are cooled by a Nuclear-Chicago Fan Unit Model 8905.

Loader

The takeup spool of the loader is driven on its outer rim by a motor (48 rpm, 7.1-lb torque) fitted with a 1.25-in. tire. The motor is mounted on a hinged plate and the drive wheel is pressed against the rim of the takeup spool by the weight of the motor.

The opaque tape is dispensed by a Trig-a-Tape Model T-1 (Dymo Industries, Inc., Berkeley, Calif.). This is mounted on an aluminum bracket and is operated by a rod connected to a foot pedal. This machine

dispenses pieces of tape 0.5 × 0.75 in., the long direction of the piece of tape being at right angles to the direction of travel of the Mylar tape.

The two layers of Mylar together with the chromatogram spots are pressed together by being passed between two rubber-covered rollers, each 1.875 in. in diameter. The ends of the two roller axles are mounted 3.75 in. apart in an Oilite bearing block. The positions of these bearing blocks are adjustable to permit alignment of the Mylar strips. The two supply spools of Mylar are similarly mounted on adjustable bearings.

PERFORMANCE

Characteristics of Counter Tubes

Due to the large size of the counter tubes we have carefully investigated their characteristics.

Using a scaler input sensitivity of 20 mv, the plateau characteristics of both tubes in parallel was determined for a sample of material containing C^{14}. The tubes showed a plateau between 1450 and 1775 volts. At the operating voltage (1600 volts) the slope of the plateau was 0.64%/100 volts. If the scaler input sensitivity was set at 250 mv, the normal value for Geiger tube operation, the plateau was shorter (1600–1750 volts), the operating voltage higher (1675 volts), and the slope greater (5.22%/100 volts). Examination of the pulses with an oscilloscope showed that when the scaler input sensitivity was adjusted to 20 mv many proportional, as well as Geiger-Müller pulses, were being recorded.

Figure 5 shows the counting efficiency when a spot of [C^{14}] glucose, 0.15 in. in diameter, was counted at intervals along a diameter of the tubes. Both tubes together were uniformly sensitive over an area of 3.5-in. diameter. However, in order to allow for a possible incorrect alignment of chromatogram spots in the counting position, the maximum permissible area for mounting the spots on the loading machine is a circle of 3-in. diameter, thus allowing for a margin of error.

No shielding against background radiation is provided for the counter tubes. The background count rate with each tube is usually 140–150 cpm. The reproducibility of the background count rate agrees with statistical expectation. The background count of both tubes in parallel was 205 ±3.8 cpm for 10-min counts.

With the tubes removed from their mounts and separated from each other, the background of both tubes together is equal to the sum of the background counts with each tube separately. However, when the tubes are mounted on the assembly, one tube being directly above the other,

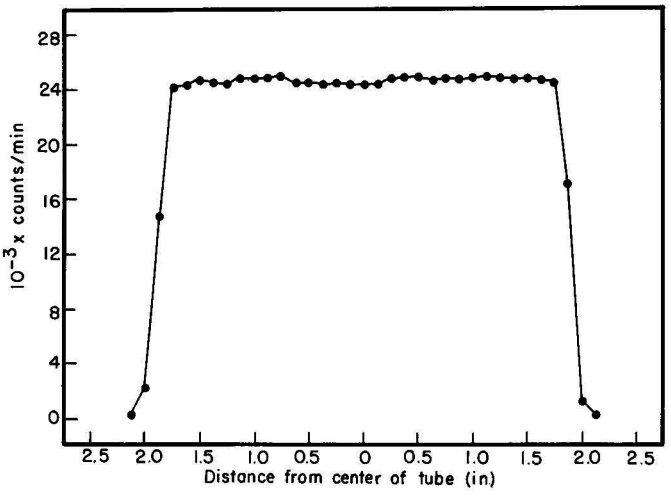


FIG. 5. Sensitivity of both tubes in parallel across face of gold-sputtered windows.

the background count rate of both tubes together is only about 70% of the sum of both tubes separately. The explanation for this effect is probably that many background counts originating from cosmic sources give rise to pulses produced simultaneously in both tubes. Since the scaling circuit has a definite resolving time (1 μsec), only one of these pairs of pulses is recorded; presumably about 60% of the background counts are due to cosmic rays arriving from a vertical direction, and half of these are not recorded. With the tubes separated in space this anticoincidence effect does not operate, and hence the background count with both tubes operating together equals the sum of those with the individual tubes. An anticoincidence circuit, if provided, could be expected to reduce the background with both tubes to about 120 cpm. Further reduction in the background would require extensive shielding and the use of special radioactivity-free metal for the construction of the counter tubes.

At a scaler input sensitivity of 20 mv, the resolving time of the complete system was 53 μsec. This unusually low value was due to a combination of two effects: the detectors were operating partly as proportional tubes, and one tube remained sensitive while the other was recovering.

With a C^{14}-labeled compound spotted onto Whatman No. 4 filter paper lying between the two layers of the Mylar strip, and counted simultaneously by two tubes fitted with gold-sputtered Mylar windows,

10.69% of all disintegrations are recorded after correction for coincidence loss and deduction of the background count. The sensitivities of the two tubes did not differ by more than 1.5%.

Reproducibility of Counting

Background Counting. The rate of background counting is stable enough so that determination of a background count for 10 min every 1 or 2 hr during an automatic counting sequence is sufficient to provide a reliable measure of this value (Fig. 6).

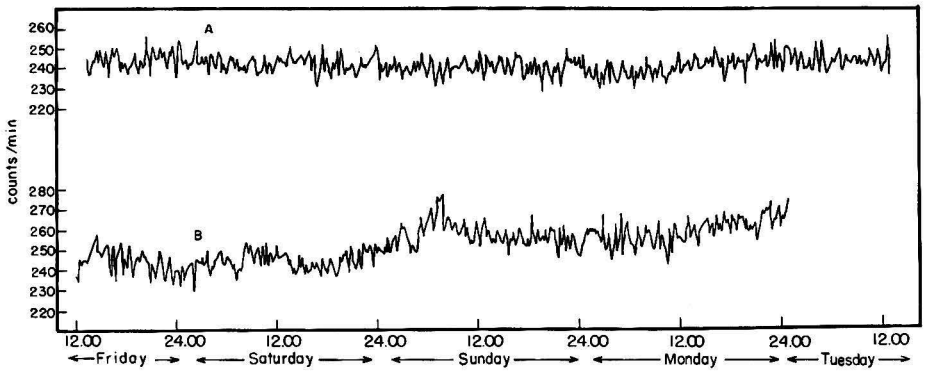


FIG. 6. Stability of background count rate. Each point represents the background count rate for a period of 0.01 day. (A) Mylar band stationary throughout; (B) Mylar band moved 5 in. between counts.

Counting of Chromatogram Spots. A comparison was made between manual and automatic counting of 36 spots on the radiochromatogram shown in Plate 1. Manual counting for 1-min intervals was performed without cutting the spots out of the paper. One side of the paper was counted at a time with a single Geiger-Müller tube of 2.25-in. internal diameter. Individual index card masks were prepared for each of the 36 spots; as well as saving time, since each spot was to be counted several times, the preparation of individual masks probably increased accuracy compared with arranging loose cards to mask the unwanted areas. However, cutting out each mask required 1–2 min. With this procedure, counting all 36 spots on one side of the chromatogram took 1.75 hr; the chromatogram was actually counted 8 times on each side. The background was deducted from each count, but the counts were not corrected for coincidence losses, since it was the reproducibility which was required, not the absolute value. With the particular counter tube used, 7.79% of all disintegrations were recorded; a summary of the findings is reported in Table 1.

TABLE 1

COMPARISON OF COUNTING REPRODUCIBILITY USING A MANUAL SYSTEM, AND AN AUTOMATIC SYSTEM, AT THREE DIFFERENT PROGRAM SETTINGS

Thirty-six spots of compounds labeled with C^{14} on a two-dimensional paper chromatogram were counted manually for 1-min periods, 8 times on each side of the paper. The spots were excised from the chromatogram and were automatically counted 8 times on each of three program settings, both sides of the paper being counted simultaneously. The automatic results have been corrected for coincidence losses and background has been deducted; in the manual results coincidence correction has not been applied, but background has been deducted. The results for each technique are presented as the average standard deviation for the spots in question expressed as a percentage of the respective mean, together with the range of standard deviations found, again as percentages of the mean. The ranges of actual rates of counting are also presented, as well as a measure of the total C^{14} in the sum of all 36 spots.

Technique	Manual	Automatic		
Program	60 sec (av. of both sides)	60.000 sec	600.00 sec or 10000 counts	1200.0 sec or 20000 counts
All 36 compds.				
Av. S.D. (as % of means)	2.23	1.78	1.02	0.74
Range of S.D. (as % of means)	0.90–9.09	0.60–6.81	0.31–2.27	0.34–1.74
15 most active compds. (together containing about 90% of all the C^{14})				
Range of activities (cpm)	11840–138600		16900–279000	
Av. S.D. (as % of means)	1.59	0.91	0.86	0.63
Range of S.D. (as % of means)	1.20–2.04	0.60–1.43	0.39–1.40	0.34–1.00
21 least active compds. (together containing about 10% of all the C^{14})				
Range of activities (cpm)	417–11377		517–16293	
Av. S.D. (as % of means)	2.69	2.40	1.14	0.81
Range of S.D. (as % of means)	0.90–9.09	0.98–6.81	0.31–2.27	0.43–1.74
Total C^{14} in all 36 compds. (cpm)	586131 ± 7992 (8) 570833–595919	944321 ± 7269 (8) 932909–957317	936226 ± 3985 (8) 928105–940372	940399 ± 2323 (8) 937056–944484

The spots were next excised from the chromatograms (30–60 sec/spot), and were loaded onto the Mylar tape. Owing to the sizes of some of the spots these had to be subdivided; in all a total of 72 counting positions was required, including 10 background positions. Once the spots were ready to be applied to the Mylar tape, the actual loading operations took less than 19 min. Three series of counts were made, each replicated 8 times to correspond with hand counting: (1) each counting position was counted for 60.000 sec; (2) each position was counted for 600.00 sec, or 10,000 counts, whichever was less; (3) each position was counted for 1,200.0 sec, or 20,000 counts, whichever was less. These results were corrected for coincidence losses, and background was deducted. The results are reported in Table 1.

Even with a similar counting period of 1 min, automatic counting is considerably more reliable than manual counting. This can probably be explained as being partly due to a more accurate location of the chromatogram spot in the counting position in the automatic system, and much higher reproducibility of the timing system in the automatic equipment. For routine use a program of 600.00 sec or 10,000 counts, whichever is reached first, has been found to be satisfactory. Assuming a background rate of 210 cpm, the standard deviation on even the lowest count (a background) would not exceed 2% of the mean value.

Further details concerning the construction, operation, and performance of this equipment may be obtained on application to the authors.

SUMMARY

A technique has been developed for the automatic measurement of radioactivity on areas of paper from paper chromatograms. The positions of radioactive compounds on the chromatograms are determined by radioautography and the appropriate areas are cut out from the chromatogram. The excised chromatogram spots are sandwiched between two continuous bands of thin plastic polyester film in an apparatus designed for this purpose.

In another apparatus, the accumulated double band of polyester film bearing the chromatogram spots is automatically passed in a discontinuous manner between two closely opposed detector tubes. Each spot remains stationary between the detector tubes for a predetermined counting period. For each chromatogram spot the number of counts recorded, together with the elapsed time, is automatically printed onto paper tape. The double band of polyester film is then automatically advanced until the next spot lies between the detector tubes, and the cycle is repeated.

ACKNOWLEDGMENTS

The work reported in this paper was sponsored by the United States Atomic Energy Commission. The authors would like to express their thanks to Mr. Frank Upham, Mr. Loren Cadra, and Mr. Ralph Norman for their invaluable efforts regarding the electronic, metal-working, and wood-working aspects of the apparatus.

REFERENCES

1. Pocchiari, F., and Rossi, C., *J. Chromatog.* **5**, 377 (1961).
2. Wingo, W. J., *Anal. Chem.* **26**, 1527 (1954).
3. Aronoff, S., *Nucleonics* **14**, 92 (1956).
4. Perkins, H. J., and Tyrell, C., *Canad. J. Biochem. and Physiol.* **39**, 1183 (1961).
5. Moses, V., and Edwards, R. W. H., *in* "Chromatographic and Electrophoretic Techniques" (I. Smith, ed.), Vol. 1, p. 484. Heinemann, London, 1960.
6. Fuller, R. C., *Science* **124**, 1253 (1956).

ANALYTICAL BIOCHEMISTRY 5, 28–36 (1963)

A Rapid Micro Gas Analysis System for Carbon Dioxide, Oxygen, Hydrocarbon Gases, and Hydrogen

DAVID M. UPDEGRAFF[1] AND WILLIAM B. HUCKABAY[2]

From the Magnolia Petroleum Company, Field Research Laboratory, Dallas, Texas

Received April 30, 1962

INTRODUCTION

Of the many excellent gas analysis apparatuses available, none fulfilled our particular requirements for use in a study of the oxidation of gaseous hydrocarbons or hydrogen by bacteria. This study required a simple, inexpensive, micro or semimicro apparatus capable of determining gaseous paraffin hydrocarbons and hydrogen as well as carbon dioxide and oxygen. Rapidity of analysis was of prime importance in order to analyze large numbers of bacterial culture gases within a single day's work. We were willing to sacrifice some accuracy in order to obtain rapidity.

Instrumental methods of gas analysis such as mass spectrometry, infrared spectrometry, and gas chromatography were not considered for our purposes because of the time and labor involved in their operation. The Orsat gas analysis apparatus and related variations were rejected because a large gas volume was required, and the combustion methods employed for hydrogen and hydrocarbons were slow and laborious.

The Blacet and Leighton (2) method, and the Spence (8) method are capable of giving excellent results, but require combustion methods for determining hydrogen and methane. Berg (1), Scholander (7), Lewis (4), and Pitts, DeFord, and Recktenwald (6) developed simple, rapid micro or semimicro apparatuses for carbon dioxide and oxygen which do not provide for the determination of gaseous hydrocarbons and hydrogen.

Of all the systems described, that of Berg (1) seemed to be the best point of departure. The first modification was the employment of an aqueous retaining medium and aqueous absorption reagents instead of mercury and solid reagents. We then increased the size of the buret and the absorption chamber, and added two stopcocks and a sliding scale upon which percentage composition of the components in a gas mixture

[1] Central Research Department, Minnesota Mining and Manufacturing Company, St. Paul, Minnesota.

[2] Scientific Service Laboratories, Inc., P. O. Box 175, Dallas 21, Texas.

were read directly. These changes resulted in a major gain in simplicity and rapidity of operation at a small sacrifice in precision and accuracy. Dworkin and Foster (3) have referred to the use of the apparatus described here in a paper on the bacterial oxidation of methane.

METHODS

Apparatus

The gas analysis apparatus is constructed entirely of Pyrex glass, and is fastened to a frame of Plexiglas which is attached to a base of Micarta plastic. The gas buret is made from an 0.1-ml pipet graduated at 0.01-ml intervals. Measurements are made on a graduated sliding scale constructed in such a manner that the line of the buret subtends equal distances between each of the 100 scale divisions for each setting of the scale. By means of this scale, direct percentage readings are obtained, thus eliminating calculations. A photograph of the apparatus, approximately one-half actual size, is shown in Fig. 1.

Procedure

Analysis is conducted as follows: (*1*) The buret is flushed with 0.1 *N* sulfuric acid containing sufficient methyl red to give a perceptible red color in the absorption chamber D, by opening stopcock A, and all the gas bubbles are removed from absorption chamber D with the hypodermic syringe carrying a bent needle (F). (*2*) The gas sample to be analyzed is collected under 0.1 *N* sulfuric acid in the hypodermic syringe and the sample is introduced through a 1-mm opening in the bottom of chamber D. (*3*) The gas sample is slowly drawn into the gas buret, E, by turning screw C, and the measuring scale is adjusted so the menisci rest upon zero at one end of the scale and 100 at the other end. (*4*) The bubble is returned to the absorption chamber, and 5% KOH is admitted through cock B. The reagent washes the bubble and drops out of the opening in the bottom of chamber D into a Petri dish used as waste receptacle. There is always sufficient capillarity between the liquid and the 1-mm opening to prevent drainage of liquid out of the chamber. (*5*) The bubble is returned to the buret until one meniscus falls at 100. The other meniscus reading gives per cent carbon dioxide in the sample. (*6*) The bubble is returned to the absorption chamber, and a mixture of 0.5 ml of saturated aqueous pyrogallol with 9.5 ml of 5% potassium hydroxide is added through cock B. (*7*) The bubble is returned to the 100 mark on the buret as before, and the reading on the meniscus minus per cent carbon dioxide gives per cent oxygen in the sample. (*8*) The gas bubble is returned to the absorption chamber and washed with 2 to

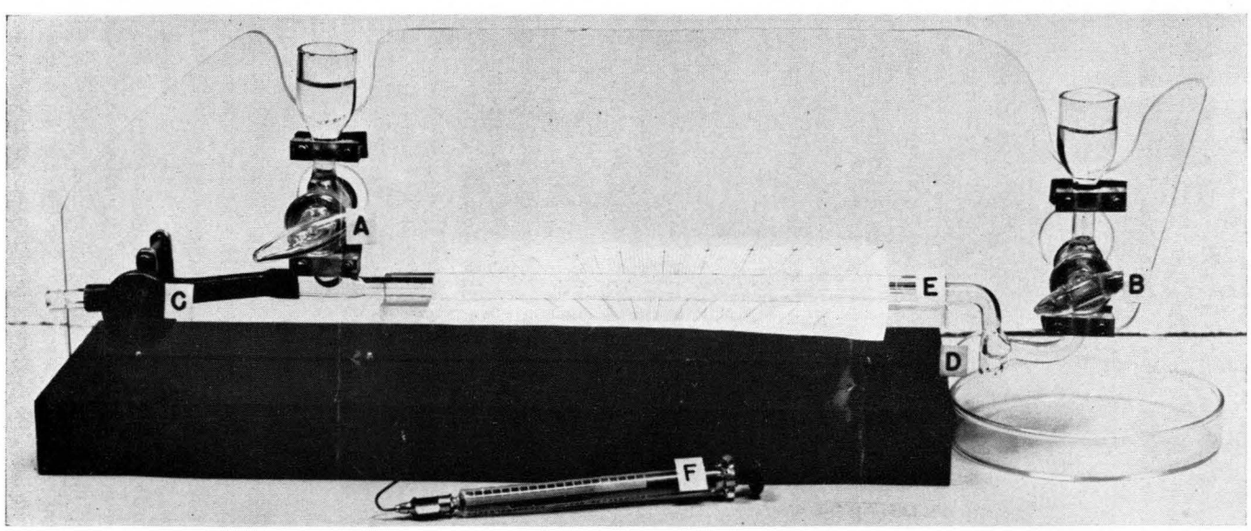


FIG. 1. Micro gas analysis apparatus (approximately ½ actual size): (A) Stopcock to reservoir for dilute H_2SO_4. (B) Stopcock to reservoir for absorption reagents. (C). Screw clamp on rubber tube. (D) Gas absorption chamber. (E) Gas buret. (F) One-ml syringe with bent needle for introduction of the gas sample, and for washing gas bubble with water-immiscible solvents.

20 ml of fuel oil (boiling range 416 to 608°F) contained in a 1-ml syringe until successive 1-ml washes produce no change in gas volume. (*9*) The gas bubble is returned to the buret as before, and the reading minus the sum of oxygen and carbon dioxide gives per cent of gaseous hydrocarbons. (*10*) The gas bubble is returned to the absorption chamber, and washed over a period of 5 to 7 min with 2 to 10 ml of 1% palladous chloride solution by introducing small volumes repeatedly around the bubble through cock B. (*11*) The gas bubble is returned to the buret. The reading minus the sum of carbon dioxide, oxygen, and hydrocarbons, gives per cent of hydrogen. The final reading gives the residual inert gas in per cent.

The fuel oil proved equal or superior to any of 15 solvents tested for extraction of the gaseous hydrocarbons. Volatile solvents are unsatisfactory, but decane and dodecane are good. After a large number of analyses, small droplets of oil may accumulate in the gas buret, thus introducing a small error in measurement. The buret may be easily cleaned by injecting 1 to 2 ml of a detergent solution into it through chamber D while stopcock A is open, followed by flushing with the 0.1 *N* sulfuric acid solution.

Because of the high solubility of carbon dioxide in the dilute sulfuric acid special precautions are necessary where appreciable concentrations are present. The sample should be at the same temperature as the water in the buret, should be transferred in the 1-ml syringe under mercury, and should be measured as quickly as possible after transfer to the absorption chamber. Samples containing high concentrations of carbon dioxide can be recognized, even before treatment with the 5% potassium hydroxide, by the fact that they rapidly decrease in volume upon washing with 0.1 *N* sulfuric acid in the absorption chamber.

RESULTS

Table 1 presents data from a number of analyses. All gases used for these analyses except air were analytically pure research grade materials. The hydrocarbon gases used were analyzed on a mass spectrometer and found to be 99.7% pure or better. The analyses in Table 1 were performed with both the 0.1 *N* sulfuric acid and the fuel oil saturated with air. Errors are within ±0.5% of the total sample for carbon dioxide and oxygen. Those for gaseous hydrocarbons and hydrogen are much greater Dashes in the table indicate omission of those parts of the analytical procedure omitted for specific samples.

Examination of the literature for solubility relationships of the gases in water and organic solvents reveals that all the gases mentioned have an appreciable solubility in water, and a higher solubility in organic

TABLE 1

ANALYSES OF KNOWN GASES WITH SOLVENTS SATURATED WITH AIR
(PERCENTAGE COMPOSITION)

Gas	Found						Anticipated					
	CO_2	O_2	HC[a]	H_2	Residual	Total	CO_2	O_2	HC[a]	H_2	Residual	Total
Air	0	21.5	0	—	78.5	100.0	0.03	21.0	0	—	79.0	100.03
Air	0	20.8	0	—	79.2	100.0	0.03	21.0	0	—	79.0	100.03
Air	0	21.2	0	—	78.8	100.0	0.03	21.0	0	—	79.0	100.03
Air	0	21.0	0	—	79.0	100.0	0.03	21.0	0	—	79.0	100.03
Oxygen	0	99.7	—	—	0.3	100.0	0	100	0	—	0	100
Carbon dioxide	99.8	0	—	—	0.2	100.0	100	0	0	—	0	100
Methane	—	—	79	—	21[b]	100	0	0	100	—	0	100
Ethane	—	—	97.0	—	3.0	100.0	0	0	100	—	0	100
Ethane	—	—	96.7	—	3.3	100.0	0	0	100	—	0	100
Ethane	—	—	96.4	—	3.6	100.0	0	0	100	—	0	100
Ethane	—	—	96.4	—	3.6	100.0	0	0	100	—	0	100
Propane	—	—	97.0	—	3.0	100.0	0	0	100	—	0	100
Propane	—	—	97.2	—	2.8	100.0	0	0	100	—	0	100
Hydrogen	—	—	—	95.2	4.8	100.0	0	0	—	100	0	100

[a] Hydrocarbon.

[b] Washed with five times the usual volume of fuel oil.

TABLE 2

ANALYSES OF KNOWN GASES WITH DEGASSED SOLVENTS

(PERCENTAGE COMPOSITION)

Gas	Found						Anticipated					
	CO_2	O_2	HC	H_2	Residual	Total	CO_2	O_2	HC	H_2	Residual	Total
Methane	—	—	96.0	—	4.0	100.0	0	0	100	—	0	100
Methane	—	—	96.9	—	3.1	100.0	0	0	100	—	0	100
Ethane	—	—	99.9	—	0.1	100.0	0	0	100	—	0	100
Ethane	—	—	99.7	—	0.3	100.0	0	0	100	—	0	100
Ethane	—	—	99.7	—	0.3	100.0	0	0	100	—	0	100
Hydrogen	—	—	8	92	0	100	—	—	0	100	0	100
Hydrogen	—	—	—	100	0	100	—	—	0	100	0	100
Air	—	—	10	—	90	100	—	—	0	—	100	100
Air	—	—	9	—	91	100	—	—	0	—	100	100
Air	—	—	11	—	89	100	—	—	0	—	100	100

solvents, including higher hydrocarbons. Methane differs markedly from ethane and propane in solubility, having a slightly greater solubility in water, but a much lower solubility in hydrocarbon solvents. The fact that the methane is so much less soluble in the fuel oil than the other gaseous hydrocarbons made it necessary to wash with a much larger volume of fuel oil before the volume ceased to decrease. It is believed that gas exchange between this larger volume of fuel oil and the methane resulted in the contamination of the methane with sufficient air to explain the low value obtained for gaseous hydrocarbons in the methane. The same reasoning applies to the low values obtained for hydrogen.

In order to test this hypothesis, the fuel oil and the palladium chloride solution were degassed for 5 to 10 min *in vacuo* (1 to 10 mm Hg), and analyses were made as before on ethane. Results were better, but still 1 to 2% low. The 1 *N* sulfuric acid solution was therefore degassed in the same manner, and analyses of methane, ethane, air, and hydrogen were conducted as before. Results are presented in Table 2. Dashes in the table indicate omission of parts of the analytical procedure.

By this method pure methane gives low results to the extent of 3.1 to 4.0%, ethane only 0.1 to 0.3% low, hydrogen 8% high, and air 9 to 11% high, in the hydrocarbon analysis. The large difference between the two analyses for hydrogen was caused by the fact that the fuel oil extraction step was performed on the first analysis only. The degassed fuel oil dissolved some of the hydrogen, giving a false value of 8% hydrocarbons and a value 8% low for hydrogen.

DISCUSSION

In order to give more accurate results it is necessary for the 0.1 *N* sulfuric acid to be near equilibrium saturation with the gas mixture to be analyzed, and for the fuel oil to be near equilibrium saturation with all the gases in the gas mixture at the time of extraction except for the hydrocarbon gases.

A suggested procedure would be to determine the approximate composition of the mixture using solvents saturated with air. Then a more accurate analysis could be obtained by degassing the solvents *in vacuo,* and allowing the 0.1 *N* sulfuric acid to come to equilibrium with a gas mixture of the determined composition under 1 atm pressure. The fuel oil should be equilibrated with a gas mixture containing all the components except the hydrocarbons at the same partial pressures, and the palladium chloride with a gas mixture containing all the components except the hydrocarbons and hydrogen at the same partial pressures. However, the system using air-saturated liquids should yield results

within 1 or 2% of the total sample for gas mixtures containing less than 20% of gaseous hydrocarbons plus carbon dioxide provided that methane is absent, or present in a concentration of less than 5%. Many types of gas mixtures may be analyzed by these simple procedures using air-saturated liquids and requiring only 2 to 15 min per determination, depending on the number of gases present in the mixture. For the routine determination of 2 or 3 components in a series of gas mixtures all containing only the same 3 or 4 gases, the reagents may be equilibrated with the appropriate gas mixtures as indicated by the average composition of the samples under analysis. Fairly accurate results may then be obtained without sacrificing rapidity. Small vacuum desiccators are satisfactory for the equilibration and storage of reagents.

The gas buret is not water jacketed. However, the buret and all reagents were kept together in an air-conditioned room. Under less stable temperature conditions, a water jacket might be necessary around the buret.

The palladous chloride solution used for the removal of hydrogen is a slow reagent (5). However, under these efficient absorption conditions, with a very small gas bubble and a large excess of reagent, it satisfactorily removes the hydrogen in 5 to 10 min, where it might be quite impractical in a macro system.

SUMMARY

The apparatus described is applicable to multicomponent systems. The percentage of carbon dioxide, oxygen, hydrocarbon gases, hydrogen, and inert gases may be determined in a sample of 50 to 100 μl with a precision of $\pm 0.5\%$ of the total sample for carbon dioxide, somewhat better than this for oxygen, and from 1 to 10% of the total sample for gaseous hydrocarbons or hydrogen, depending on the concentrations present. Analysis requires approximately 15 min if all five components of the mixture are determined, but is ordinarily much more rapid if one or more of the components are known to be absent. For example, respiratory gases containing only carbon dioxide, oxygen, and inert gases or bacterial fermentation gases containing only carbon dioxide, hydrogen, and methane, can be analyzed in 2 to 3 min. Calculations are eliminated by the use of a sliding scale on the gas buret, which reads percentage composition directly.

ACKNOWLEDGMENT

The authors are particularly indebted to Dr. Paul P. Reichertz, Manager, Field Research Laboratory, Magnolia Petroleum Company, for encouraging the publication of this work.

REFERENCES

1. BERG, W. E., *Science* **104,** 575 (1946).
2. BLACET, F. E., AND LEIGHTON, P. A., *Ind. Eng. Chem., Anal. Ed.* **3,** 266 (1931).
3. DWORKIN, M., AND FOSTER, J. W., *J. Bacteriol.* **72,** 646 (1956).
4. LEWIS, V. M., *Anal. Chem.* **21,** 635 (1949).
5. MULLEN, P. W., "Modern Gas Analysis." Interscience, New York, 1955.
6. PITTS, J. N., DEFORD, D. D., AND RECKTENWALD, G. W., *Anal. Chem.* **24,** 1566 (1952).
7. SCHOLANDER, P. F., *J. Biol. Chem.* **167,** 235 (1947).
8. SPENCE, R., *J. Chem. Soc.,* **1940,** 1300.

ANALYTICAL BIOCHEMISTRY 5, 37–46 (1963)

Phospholipase C Determination by Egg Yolk Turbidimetry

ABRAMO C. OTTOLENGHI

From the Sidney Hillman Medical Center, Philadelphia, Pennsylvania

Received April 30, 1962

During work on a phospholipase from *Bacillus cereus* the need for a rapid and accurate enzyme assay method became critical. The method which had been previously used by us (1) assayed the enzyme in terms of its inhibitory action on the blood-clotting mechanism. The main disadvantages of this method were the complex reaction system and the uncontrollable variability of the substrate (tissue thromboplastin). The determination of the decrease in acid-insoluble phosphorus caused by enzyme action on phospholipid or phospholipoprotein suspensions although accurate, is too slow for routine determinations.

The ability of certain strains of *Bacillus* to produce turbidity in egg suspensions was reported in 1948 by McGaughey and Chu (2) and later by Chu (3). This phenomenon had previously been observed for anaerobic bacilli by Macfarlane *et al.* (4, 5) and a method for the semiquantitative determination of phospholipase action described by van Heyningen (6). All these authors ascribed the turbidity produced in egg yolk suspensions by these bacterial strains to the production of an exocellular phospholipase C. The method was used by Heimpel (7) for the quantitative comparison of phospholipase production by different strains of insect pathogens and was evaluated by Kushner (8) for both clostridial and bacillus phospholipases. More recently Orlowska *et al.* (9) examined further the egg yolk system in terms of its quantitative application. This same general method has been used recently by Ellner (10) and Stephen (11) to assay for phospholipase (α-toxin) activity of clostridial preparation.

In attempting the turbidimetric assay of the enzyme from *B. cereus* using a commercial egg yolk preparation we found that the turbidity produced in a given period of time was not proportional to the enzyme concentration. A detailed examination of the system was undertaken in order to devise a suitable assay procedure and to identify at least in part the reason for the nonlinear behavior.

MATERIALS AND METHODS

Egg Yolk Suspension. The egg yolk suspension used in these experiments is marketed by Difco Laboratories under the name "Colbeck EY Broth" and is prepared according to the procedure of Colbeck (12). It is a modification of the medium of McGaughey and Chu (2). The prepared medium comes in sterile form, internally buffered (phosphates) at pH 7.0 and has proved stable (when unopened) on our shelves at room temperature for up to two months.

Phospholipase. Preparations used were the supernatant culture fluids from growing cultures of *B. cereus,* or solutions of lyophilized powders prepared from supernatants.

Turbidity Test. The incubation system consisted of 2.5 ml of the appropriate dilution of Colbeck EY broth and 0.2 ml of enzyme solution. The reference cell (of a Beckman DB spectrophotometer) contained 2.5 ml of diluted substrate and 0.2 ml of water or enzyme-free culture fluid. Readings were made at 560 mμ.

The sample cuvette was allowed to equilibrate at the temperature of the spectrophotometer compartment (28–29°C) prior to the addition of enzyme. It was found that there was no appreciable increase in temperature of the sample even after an additional 0.5 hr in the compartment.

The recorder (Beckman Instruments) and the spectrophotometer were started, the enzyme added, and the changes in optical density (OD) recorded continuously.

Modification for Nonrecording Instruments. Although the method as described has been applied to a continuously registering instrument, an effective modification using nonrecording equipment can be devised simply by determining the time required for turbidity to increase from a given OD to another arbitrarily set level. Care must be taken to choose levels which are on the linear portion of the curve. In our experience levels below 70% transmittance (T) could be used.

RESULTS

A. Kinetics of Turbidity Production

Figure 1 shows a series of curves obtained with varying enzyme dilutions. It must be noted that this figure represents raw data and that these data when replotted on a linear scale (Fig. 2) show the same lag in turbidity production which is apparent in Fig. 1. By using Fig. 1 and extrapolating the linear portion of the curves to zero turbidity it may be observed that the lag time is roughly inversely proportional to the

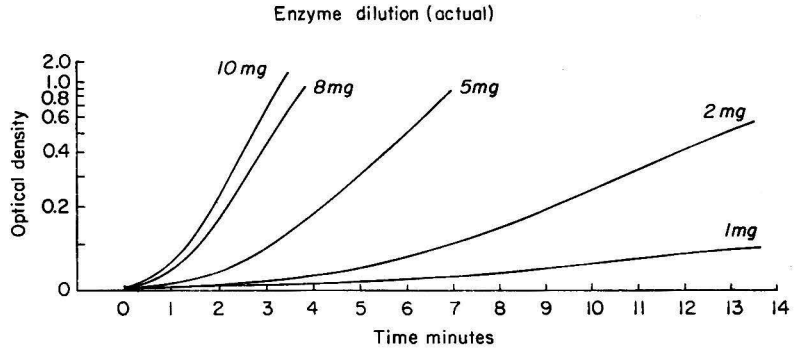


FIG. 1. Turbidity production curves as observed (raw data) with different amounts of enzyme. System in text.

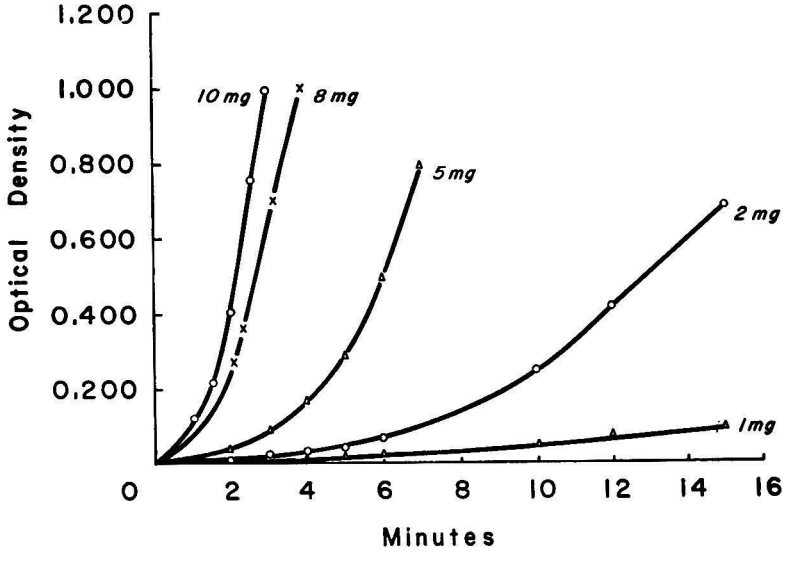


FIG. 2. Data derived from Fig. 1 and replotted on a linear density scale.

enzyme concentration. When the maximum slopes of the curves shown in Fig. 1 (raw data) were determined by direct measurement and then plotted against enzyme concentration the linear relationship shown in Fig. 3 was obtained. In our discussion the term "slope" will be used as an expression of rate denoting the relationship $\Delta\%T$ per unit time measured in cm on the graph paper.

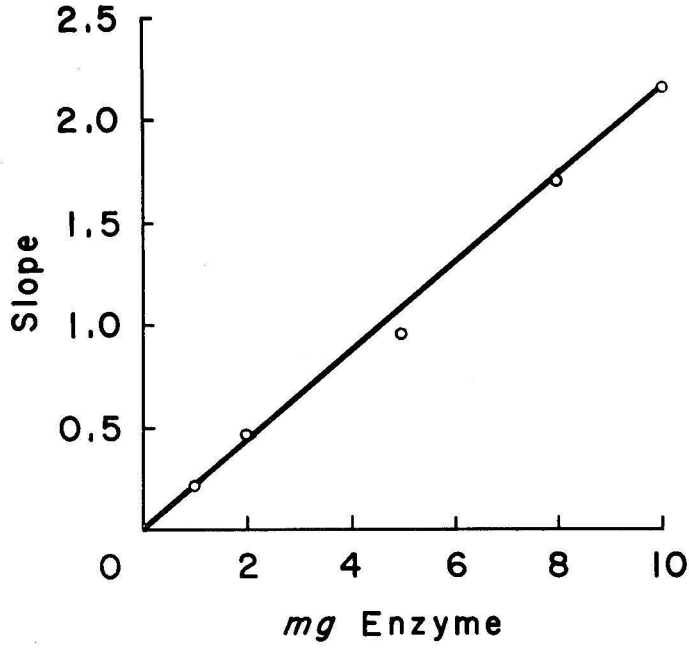


FIG. 3. Plot of the maximal slopes ($\Delta\%T$/with time as as cm/cm) obtained as function of enzyme concentration.

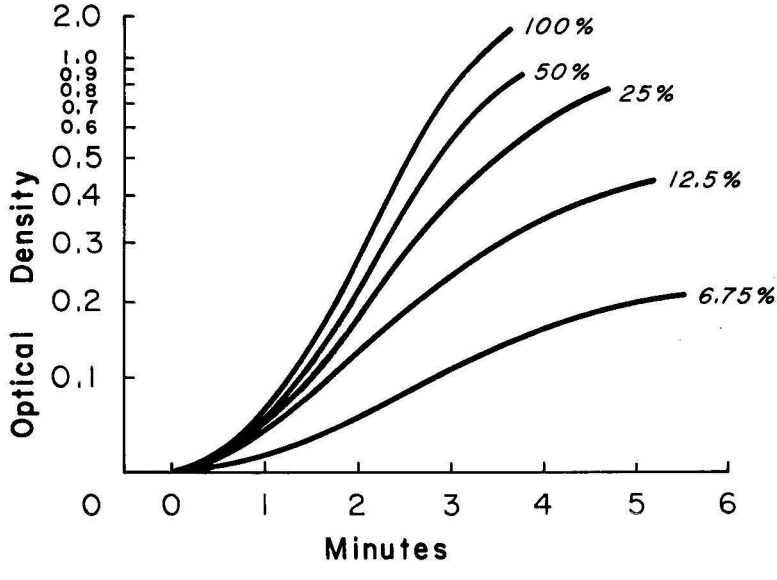


FIG. 4. Curves of raw data obtained with different substrate concentrations (% Colbeck EY broth).

B. Effect of Egg Yolk Concentration

Figure 4 shows the effects of varying concentrations of egg yolk. When a semilogarithmic plot was made of the egg yolk concentration (%) vs. slope at maximal velocity the straight line shown in Fig. 5 was obtained.

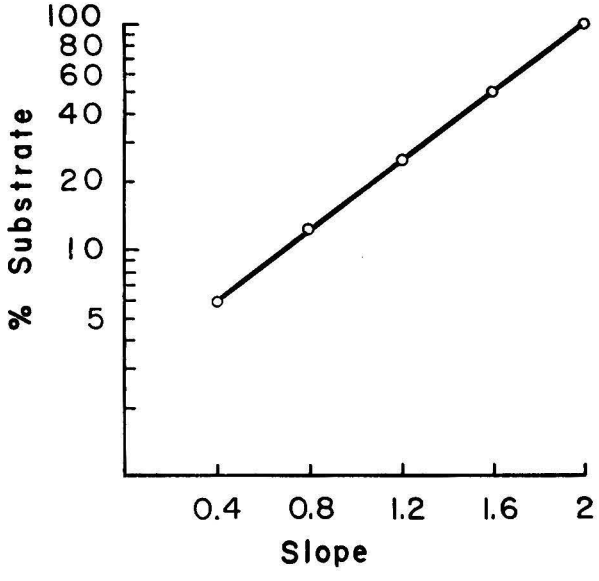


FIG. 5. Semilogarithmic plot of % substrate vs. maximal slope (reaction rates) as derived from curves on Fig. 4.

C. Effect of Egg Yolk on Reaction Lag

To determine whether the lag was a function of the presence of material introduced with the substrate or the enzyme, the effect of fresh substrate (added together with enough new enzyme to make up for system dilution) on a reaction already in progress was investigated. Results are shown in Fig. 6.

It can be seen that addition of egg yolk (and enzyme) resulted in an initial dilution effect followed by a new lag period. The addition of new enzyme in water did not produce a new lag and the reaction proceeded to attain maximal velocity. Confirmation of the findings and their interpretation is offered by the experiment shown in Fig. 7. In this experiment the instrument was used as a differential analyzer of simultaneous reaction rates in the two cuvettes.

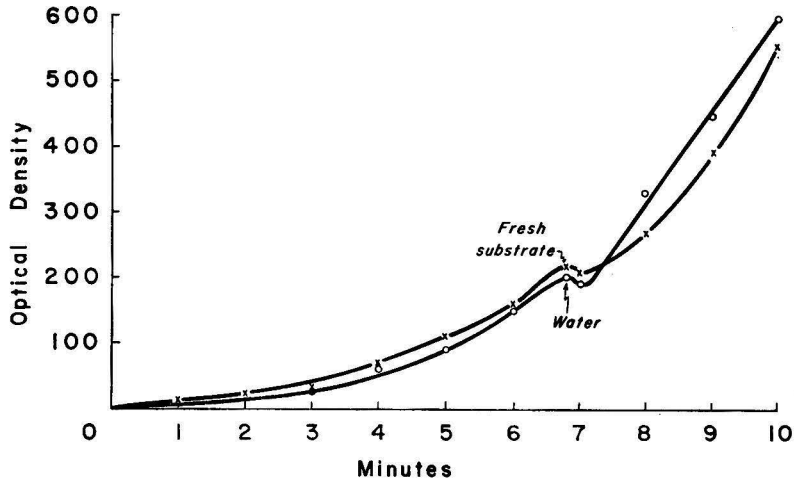


FIG. 6. Effect of substrate addition after termination of lag. System as described in text except that in one experiment (○—○) 0.5 ml water and enzyme to compensate for dilution was added after 6.5 min and in the other (×—×) 0.5 ml fresh 100% Colbeck EY broth and enzyme to compensate for dilution was added after the same period of time.

D. Specificity of the Reaction

The specificity of the turbidimetric reaction as described herein was tested in relation to both the tissue thromboplastin destruction test (blood-clotting inhibition) and the decrease in acid-insoluble phosphorus from phospholipid suspensions. Results showed good agreement (Table 1).

TABLE 1

Phospholipase prepn.	Slope (max.) (%)	Decrease in acid-insol. phosphorus (μg/10 min)	Thromboplastin destroyed (%/10 min)
A	0.28	7.6	52
B	0.20	5.0	40
C	0.42	10.2	60

Contrary to the results reported by Kushner (8) for his enzyme preparations, with our enzyme preparations there was no residual turbidity generating activity after autoclaving at 121°C for 15 min.

E. Behavior of Different Manufacturer Lots of Colbeck EY Broth

Different lots of a preparation as complex as that used in these experi-

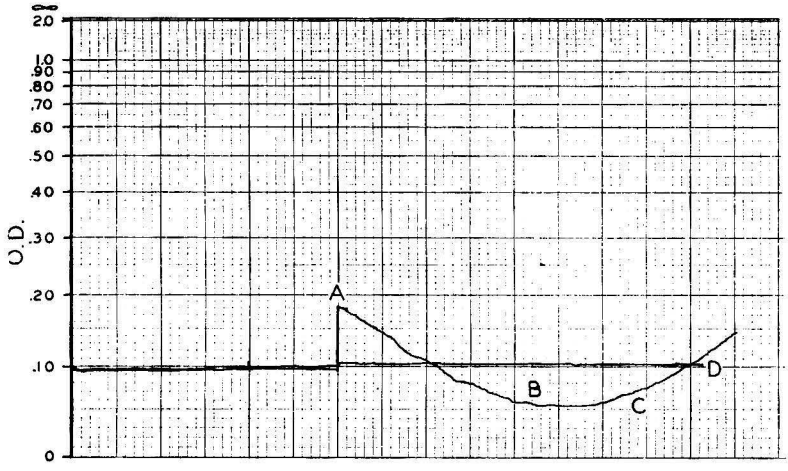


FIG. 7. Demonstration of reaction slowing by addition of new substrate. Differential analysis of turbidity produced in two different systems simultaneously. *Systems:* As described in text in both the reference (R) and sample (S) cuvettes of the Model DB Beckman spectrophotometer. Time 0 to A, equal reaction in both cuvettes. Time A, addition of 1 ml H_2O + sufficient enzyme to compensate for dilution to cuvette R and 1 ml 100% Colbeck EY broth + sufficient enzyme to compensate for dilution to cuvette S. The increase in turbidity observed at A is due to the different turbidities of water and Colbeck EY broth. The decrease in turbidity shown by the A–B segment of the curve indicated that turbidity production is more rapid in the R (H_2O) cuvette than in the S (Colbeck EY broth) cuvette. The rates of turbidity production become identical after B, and the increase in turbidity seen after C reflects the greater amount of substrate available in cuvette S. Curve D is a control where 1 ml 100% Colbeck EY broth was added to both cuvettes at time A.

ments might differ as to their substrate contents. To test this possibility and to determine its significance for the assay we carried out the following experiments:

(*1*) *Determination of total "apparent available substrate" concentration:* An appropriate dilution of the sample was incubated with a given amount of enzyme for 2 hr at room temperature to give maximal turbidity. The observed OD multiplied by the dilution factor gave an arbitrary "apparent available substrate factor" (ASF).

(*2*) *Determination of rate of reaction as function of "apparent available substrate" concentration:* It was found that incubation of different lots of undiluted Colbeck EY broth with a given enzyme concentration did not give the same slope.

Appropriate dilution so that all samples used had the same "apparent available substrate" concentration resulted in identical slopes for all lots tested. It was also found that the slope obtained with the undiluted

preparations was related (as previously described for substrate concentration, Fig. 5), to the ASF of each individual lot. These results are summarized in Table 2.

TABLE 2
DETERMINATION OF "AVAILABLE SUBSTRATE"

Colbeck EY broth lot	OD	Dilution factor	ASF	Max. slope observed (undild. substrate)
A	0.75	20	15	2
B	1.3	1	1.3	0.6
C	1.0	1	1	0.45
D	0.8	1	0.8	0.28

DISCUSSION

Our procedure for the assay of phospholipase C from *B. cereus* has shown that this system is subject to variables which can greatly influence the results obtained. Briefly these variables are: (*1*) the duration and nature of the lag period; (*2*) the "apparent available substrate" of each preparation of egg yolk suspension.

Until now, quantitative measurements by means of the egg yolk test have been derived by measuring the attained turbidity in a given period of time (7–9), by determining the minimal amount of enzyme which would still give turbidity after neutralization with a given amount of antiserum (13–14), or by comparing the OD reading obtained after a given time interval with that obtained with a "standard" enzyme preparation (10–11).

In our system, and presumably in at least some of the similar assays, this approach will introduce systematic errors that will increase as the differences in enzyme concentrations which are compared become greater. Also, the shorter the period of elapsed time used before readings are taken, the greater the error introduced.

It is possible that the lag period reflects the time necessary for the minimum release and aggregation of split product before turbidity is registered (15). An autocatalytic reaction caused by the release of an activator or accelerator would also produce an apparent lag. Finally a complex substrate material containing two substrates with different enzyme affinities, only one of them turbidogenic, could also produce a lag.

The results of the experiments shown in Figs. 6 and 7 support the last explanation. Addition of new substrate results in the slowing of the reaction rate, a new lag period, and then resumption of the maximal

velocity. The new lag must be explained on the basis of something added with the substrate and then destroyed or neutralized, since the equivalent dilution of the control system fails to produce a lag.

The suggestion of Orlowska *et al.* (9) that the lag is due to temperature equilibration would not account for these results.

The meaning of the variations in effective substrate concentration among batches of commercial substrate is not clear. It could be due to actual differences in substrate concentrations or presence of a required and as yet unknown cofactor in the substrate preparation, or it could be explained on the hypothesis that the reaction occurs at the surfaces of suspended particles (micelle) and the variation in "apparent available substrate" could reflect a variation in particle size or surface charge.

Considering the kinetics observed, the egg yolk assay should be performed by comparing maximal slopes of the turbidity curve for different enzyme preparations under conditions in which the lag is taken into proper consideration, and in which a given substrate batch has sufficient "apparent available substrate" to saturate the enzyme.

The assay can also conveniently be performed with continuous recording instruments by measuring the maximal slopes of the transmittance curve, provided that the maximal slope can always be read at roughly the same transmittance, since:

$$\mathrm{OD} = -\log T$$

or:

$$d\mathrm{OD} = -\frac{dT}{T}$$

so that for a constant T:

$$\frac{dT}{d\mathrm{OD}} = \text{constant}$$

SUMMARY

The egg yolk assay for phospholipase from *B. cereus* has been examined. The existence of an initial lag in turbidity increase was shown. The theoretical implications and practical consequences of this phenomenon are discussed. The utilization of maximal reaction velocity for enzyme assay rather than single point determination is suggested as a modification for greater accuracy of the method.

ACKNOWLEDGMENTS

Thanks are due Dr. D. Ezekiel, Albert Einstein Medical Center, Philadelphia, and Dr. D. J. Kushner, National Research Council, Ottawa, for their suggestions and critique of this work. This work was supported in part by NIH Grant E 3701.

REFERENCES

1. OTTOLENGHI, A., GOLLUB, S., AND ULIN, A., *Federation Proc.* **19,** 64 (1960).
2. MCGAUGHEY, C. A., AND CHU, H. P., *J. Gen. Microbiol.* **2,** 334 (1948).
3. CHU, H. P., *J. Gen. Microbiol.* **3,** 255 (1949).
4. MACFARLANE, M. G., AND KNIGHT, B. C. J. G., *Biochem. J.* **35,** 884 (1941).
5. MACFARLANE, M. G., OAKLEY, C. L., AND ANDERSON, C. G., *J. Pathol. Bacteriol.,* **52,** 99 (1941).
6. VAN HEYNINGEN, W. E., *Biochem. J.* **35,** 1246 (1941).
7. HEIMPEL, A. M., *Can. J. Zool.* **33,** 311 (1955).
8. KUSHNER, D. J., *J. Bacteriol.* **73,** 297 (1957).
9. ORLOWSKA, B., MEDUSKI, J., AND USPIENSKAYA, W. D., *Bull. acad. polon. sci.* **6,** 463 (1958).
10. ELLNER, P. D., *J. Bacteriol.* **82,** 275 (1961).
11. STEPHEN, J., *Biochem. J.* **80,** 578 (1961).
12. COLBECK, J. C., *Can. Serv. Med. J.* **12,** 563 (1956).
13. MEISEL, H., ALBRYCHT, H., AND RYMKIEWICZ, D., *Bull. acad. polon. sci.* **7,** 395 (1959).
14. MEISEL, H., ALBRYCHT, H., MIERZEJEWSKA, H., AND RYMKIEWICZ, D., *Bull. acad. polon. sci.* **8,** 227 (1960).
15. SEVAG, M. G., "Immuno-catalysis," p. 342. C. C Thomas, Springfield, Ill., 1951.

ANALYTICAL BIOCHEMISTRY 5, 47–50 (1963)

Detection and Rapid Differentiation of Glucosamine, Galactosamine, Glucosamine Uronic Acid, and Galactosamine Uronic Acid[1]

ALAN R. WILLIAMSON AND STEPHEN ZAMENHOF

From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York

Received May 1, 1962

INTRODUCTION

The widespread occurrence of glucosamine and galactosamine as monosaccharide components of polysaccharides and mucopolysaccharides has evoked many methods of distinguishing between these hexosamines. Both paper chromatographic (1–3) and electrophoretic (4, 5) separations have been described for the free hexosamines and various *N*-substituted derivatives, but one of the simplest and best methods of differentiation, on a micro scale, is that based on ninhydrin degradation and chromatographic separation of the resultant pentoses (6).

Recently galactosamine uronic acid (7) and glucosamine uronic acid (8) have been found in naturally occurring polysaccharides. While these hexosamine uronic acids are readily distinguished from other acidic amino sugars, such as muramic acid or neuraminic acid derivatives, a simple micro technique was sought for their differentiation from each other. Previously paper chromatographic separation has been described (7), but only small differences in their R_f values were obtained.

The method presented here permits the rapid differentiation, not only of the two hexosamine uronic acids, but also of glucosamine and galactosamine present in the same mixture. It consists of the separation, by paper electrophoresis in borate buffer (9), of their deamination products.

Also presented, is a new specific spray reagent for the detection of 2-amino-2-deoxy sugars. The method is based on the color produced by the action of indole on their deamination products (10).

MATERIALS

Glucosamine was obtained from Nutritional Biochemicals Corporation. Galactosamine was kindly supplied by Dr. K. Meyer, of this department.

[1] This work was supported by a research grant No. G-15637 from the National Science Foundation.

Glucosamine uronic acid and galactosamine uronic acid were synthesized by K. Heyns *et al.* (11, 12); we are very grateful to Prof. Heyns, University of Hamburg, for generous gifts of these substances.

Reagents

A, 5% (w/v) sodium nitrate solution.

B, 33% (v/v) acetic acid.

C, 12% (w/v) ammonium sulfamate.

D, 1% (w/v) indole in 95% ethanol containing 5% (v/v) concentrated hydrochloric acid. .

E, 1:1 mixture of *A* and *B*, prepared as required.

Reagent *D* is stable for at least 6 weeks at room temperature.

METHODS

Differentiation of the Hexosamines and Hexosamine Uronic Acids

An aliquot (20–30 μl) of a solution containing the amino sugar (ca. 25 μg of each) was treated with an equal volume of reagent *E* for 10 min at room temperature. The mixture was then applied to Whatman No. 1 paper and subjected to electrophoresis in borate buffer, pH > 10 (7.44 gm boric acid/liter 0.1 *N* NaOH) (9) at 20 volts/cm for 2–3 hr.

After drying, the paper was sprayed with reagent *D* and heated at 100°C for 1 min; the anhydro sugars show as orange-yellow spots.

Specific Spray for 2-Amino-2-Deoxy Sugars

1. Spray thoroughly with *E*, leaving paper wet, at room temperature, for 1–2 min before drying at 100°C.
2. Spray with *C* to wet the paper completely, and dry at 100°C.
3. Spray with *D*, and heat at 100°C for 1 min or 80°C for 2 min.

2-Amino-2-deoxy sugars give bright orange-yellow spots on a pale pink ground.

RESULTS

A typical separation of the deamination products of the four amino sugars is shown in Table 1. The relative mobilities of the four compounds were found to be reproducible and various mixtures of them were easily separable. Compact spots were obtained.

The lower limit of the quantities of amino sugars which can be separated is dependent on the sensitivity of the indole spray reagent which will detect 15 μg in a spot having an area of 1.5 cm^2.

DISCUSSION

Deamination of 2-amino-2-deoxyhexoses results in 2,5-anhydrohexoses,

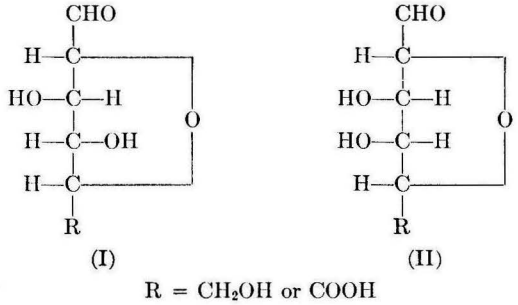
TABLE 1

SEPARATION OF DEAMINATION PRODUCTS OF 2-AMINO-2-DEOXY SUGARS

Amino sugar	Migration[a] (cm)
Glucosamine	0.0
Galactosamine	12.5
Glucosamine uronic acid	15.7
Galactosamine uronic acid	21.4

[a] Electrophoresis on Whatman No. 1 paper in borate buffer, pH > 10, for 3 hr at 20 volts/cm.

with Walden inversion at C-2 (13, 14). The products from glucosamine (structure I, R = CH_2OH) and from glucosamine uronic acid (I, R = COOH) have *trans*-3- and 4-hydroxyl groups which do not complex with borate, while the 3- and 4-hydroxyl groups of the products from galactosamine and galactosamine uronic acid (II, R = CH_2OH and COOH, respectively) are *cis* related, and thus give an anionic complex with borate (4).



R = CH_2OH or COOH

Since the separation of the four products depends mainly on the configuration of the 3- and 4-hydroxyl groups, the method is certainly not a rigorous structural proof for unknown amino sugars. However, since only two hexosamine uronic acids are known and the hexosamines occurring naturally have predominantly gluco and galacto configurations, the method affords a rapid initial screening test for the presence of these four amino sugars.

SUMMARY

A method is presented for the rapid differentiation, on a micro scale, of glucosamine, galactosamine, glucosamine uronic acid, and galactosamine uronic acid by paper electrophoresis of their deamination products in borate buffer. A new spray reagent for the specific detection of the 2-amino-2-deoxy sugars is also described; this is based on the color given by their deamination products with indole.

REFERENCES

1. AMINOFF, D., AND MORGAN, W. T. J., *Nature* **162**, 579 (1948).
2. KAYE, M. A. G., AND STACEY, M., *Biochem. J.* **48**, 249 (1951).
3. FISHER, F. G., AND NEBEL, H. J., *Z. physiol. Chem.* (*Hoppe-Seyler's*) **302**, 10 (1955).
4. FOSTER, A. B., AND STACEY, M., *J. Appl. Chem.* (*London*) **3**, 19 (1953).
5. SCOTT, J. E., *Biochem. J.* **82**, 43p (1962).
6. STOFFYN, P., AND JEANLOZ, R. W., *Arch. Biochem. Biophys.* **52**, 373 (1954).
7. HEYNS, K., KIESSLING, G., LINDENBERG, W., PAULSEN, H., AND WEBSTER, M. E., *Chem. Ber.* **92**, 2435 (1959).
8. WILLIAMSON, A. R., AND ZAMENHOF, S., in preparation.
9. FOSTER, A. B., *Chem. & Ind.* (*London*) **1952**, 828, 1050.
10. DISCHE, Z., AND BORENFREUND, E., *J. Biol. Chem.* **184**, 517 (1950).
11. HEYNS, K., AND PAULSEN, H., *Chem. Ber.* **88**, 188 (1955).
12. HEYNS, K., AND BECK, M., *Chem. Ber.* **90**, 2443 (1957).
13. LEVENE, P. A., AND LA FORGE, F. B., *J. Biol. Chem.* **21**, 345, 351 (1915).
14. LEVENE, P. A., *J. Biol. Chem.* **36**, 89 (1918).

ANALYTICAL BIOCHEMISTRY 5, 51–59 (1963)

Differential Extractions for the Spectrophotofluorometric Measurement of Diverse 5-Hydroxy- and 5-Methoxyindoles[1]

W. B. QUAY

From the Department of Zoology, University of California, Berkeley, California

Received May 4, 1962

INTRODUCTION

A highly sensitive and selective chemical method for the measurement of the 5-hydroxyindoleamine, serotonin, was described by Bogdanski, Pletscher, Brodie, and Udenfriend (1) based upon the discovery by Udenfriend, Weissbach, and Bogdanski (2) that this compound fluoresces at 550 mμ when in 3 N HCl and activated at 295 mμ. It was discovered subsequently in this and other laboratories that this fluorescence in 3 N HCl at 540–550 mμ with activation maximum at 295 mμ is a distinctive characteristics of all available 5-hydroxy- and 5-methoxyindoles. Compounds of this group, in addition to serotonin, have been found recently to occur naturally in appreciable amounts in mammalian tissues, especially in the pineal gland (3). This report describes methods for the selective extraction and measurement of six of the currently available 5-hydroxy- and 5-methoxyindoles which occur in mammals. It should be noted at the outset that these methods are indebted to recent improvements, especially those of Halevy (4), in the extraction and measurement of serotonin.

MATERIALS AND METHODS

Materials

Reagents found previously to be the most satisfactory in the extraction and measurement of serotonin (4) were used in the procedures reported here and include: HCl (36.5–38.0%, Du Pont), n-butanol (Eastman or Fisher), n-heptane (pure grade, 99 mol. % min., Phillips Petroleum Co.), EDTA (= ethylenedinitrilotetraacetic acid disodium salt, Eastman),

[1] This work has been supported by a grant from the National Institutes of Health, U. S. Public Health Service, RG-5219.

and L-ascorbic acid (Eastman). Additional reagent-grade solvents whose origins were found to be critical in regard to purity include: diethyl ether (Merck), *tert*-amyl alcohol (= 2-methyl-2-butanol, b.p. 100–102°C, Matheson Coleman & Bell) and *p*-cymene (terpene-free, Eastman). Other chemicals as well were of reagent grade and all aqueous solutions were made with deionized, distilled water.

Indole standards used in these studies include: 5-hydroxyindole, 5-methoxyindole, and 5-hydroxyindole-3-acetic acid from Nutritional Biochemicals Corporation, Cleveland, Ohio; serotonin creatinine-H_2SO_4 complex, DL-hydroxytryptophan·H_2O, and bufotenine monooxalate hydrate from California Corporation for Biochemical Research, Los Angeles, Calif.; melatonin and 5-methoxytryptamine from Regis Chemical Co., Chicago, Ill.; and *N*-acetylserotonin and 5-methoxyindole-3-acetic acid from Dr. A. Hofmann of Sandoz AG. Approximately twenty other indoles and indole derivatives were obtained for spectrophotofluorometric comparisons. Concentrated standard solutions were prepared from indoles weighed with a quartz helix balance, dissolved in a small amount of ethanol when necessary, and diluted (250 μg/ml) with either 0.1 *N* HCl or 0.1 *N* HCl, 0.5% ascorbic acid. Working standards were prepared by diluting 200 λ of the concentrated standards to 10 ml (= 5 μg/ml) with the same acid solutions. All standards and samples were refrigerated in the dark when not in use.

The primary buffer used, pH 10, was made from boric acid (59.6 gm), NaOH (41.7 gm), water (2 liters). Other buffers used experimentally were not found to be necessary in the procedures outlined below.

Experimental Methods

The differential extraction of indoles, including the ten listed above, was tested with different solvents and at different pH values according to several systems and using 500-λ aliquots (2.5 μg) of standards in separate tubes. The volume of the aqueous, acid phase in the final tube after the extraction was measured and a 250-λ aliquot was transferred to a quartz cuvette. Concentrated HCl (75 λ) was added to this and the fluorescence at the 540–550 mμ maximum was measured with an Aminco Bowman Spectrophotofluorometer and activation at 295 mμ. The fluorescence of the extracted standards was compared with that of known dilutions of the parent unextracted standards. The fluorescence of pineal and brain tissues (to be reported elsewhere) alone or with added indole standards was examined using tissue samples in 0.1 *N* HCl or 0.1 *N* HCl, 0.5% ascorbic acid. Tissue fragments were crushed on the insides of the extraction tubes with glass pipets after quantitative transferral from the collection tubes. Metal instruments were never brought in contact with

the standard or sample solutions. Tissues or body fluids with very low indole content may require additional preliminary purification steps to remove interfering substances. The investigator is referred to other publications for such methods.

Recommended Methods

General. In each series of tissue samples two or three sets of standards of two or three concentrations should be extracted and treated in the same manner as the samples. The concentrations of the standards should be close to and span the range expected of the unknown samples. The mean fluorescences (units of deflection on the photometer) of the extracted standards are plotted against their concentration, and the concentrations of the unknown samples are read from this graph. When such internal standards are not used in tissue solutions comparable to those of the unknown samples it is desirable to test the recoverability of the indoles from the kind of tissue solution being studied before proceeding with the determinations.

In determining the per cent recovery of indole standards from different extraction procedures it is necessary in some cases to correct for dilution of the final aqueous phase. When 500 λ equals the volume of the standard solution extracted, the corrected per cent recovery equals 500 × % recovery/vol in λ of final aqueous phase.

Variations in fluorescence readings can be minimized by using a single quartz cuvette for an entire series of samples and standards, marking the lip of the cuvette so that it may be placed in the light path in exactly the same way each time, and ensuring that there is no play between cuvette and adapter and between adapter and fluorometer housing.

I. Serotonin. As in the original procedure of Bogdanski *et al.* (1), it was found that highest recoveries of serotonin were obtained with extraction by *n*-butanol of an alkaline, NaCl-saturated solution. However by this and related methods, 40–80% of the *N*-acetylserotonin, 5-methoxytryptamine, 5-hydroxytryptophan, and bufotenine present were extracted also. *N*-acetylserotonin can be removed with diethyl ether before extraction and 5-methoxytryptamine, bufotenine, and most of the 5-hydroxytryptophan can be removed with an additional wash with ether after increasing the pH as follows:

(*1*) In a 5-ml glass-stoppered centrifuge tube place, in order, 100 λ freshly dissolved ascorbic acid (3%) in 1% EDTA, 500 λ standard or sample in 0.1 *N* HCl, 0.5% ascorbic acid, 3 ml solvent (diethyl ether), and 300 mg NaCl.

(*2*) In a 15-ml glass-stoppered centrifuge tube place 8 ml heptane and 500 λ 0.1 *N* HCl, 0.5% ascorbic acid.

(*3*) Each tube from step (*1*) is treated continuously: (a) shake 1 min, centrifuge 1 min, and discard ether phase; (b) add another 3 ml ether and repeat step (a); (c) add another 3 ml ether and 500 λ pH 10 buffer (saturated with NaCl and diethyl ether) and immediately repeat step (a); (d) repeat step (b); (e) immediately add 3 ml *n*-butanol, shake 1 min, centrifuge 1 min, and transfer butanol phase to 15-ml centrifuge tube [from step (*2*)].

(*4*) Shake this tube 2 min and centrifuge.

(*5*) Measure the volume of the aqueous phase in this tube and transfer 250 λ to a quartz cuvette; add 75 λ concentrated HCl and read fluorescence at 540–550 mμ max. with activation at 295 mμ.

II. N-Acetylserotonin. *N*-Acetyl serotonin is extracted best by diethyl ether. The melatonin which accompanies it is removed with cymene, and 5-hydroxyindole-3-acetic acid and bufotenine are excluded by using a double extraction with ether; at a higher pH 5-hydroxyindole-3-acetic acid is excluded and at an acid pH bufotenine is excluded.

(*1*) As above but *p*-cymene is used as solvent.

(*2*) As above.

(*3*) Each tube from step (*1*) is treated continuously: (a) shake 1 min, centrifuge 1 min, discard cymene phase; (b) add another 3 ml cymene and repeat (a); (c) add 3 ml diethyl ether, shake 1 min, centrifuge 1 min, and transfer ether phase to 15-ml centrifuge tube from step (*2*).

(*4*) Shake this tube 2 min; centrifuge; keep cool and dark until all tubes reach this stage.

(*5*) (a) The aqueous phase from each tube from step (*4*) is transferred to a 5-ml glass-stoppered centrifuge tube containing 300 mg NaCl and 3 ml diethyl ether; (b) add 250 λ pH 10 buffer (saturated with NaCl and diethyl ether) and immediately shake 1 min, centrifuge 1 min; and transfer ether phase to a 15-ml glass-stoppered centrifuge tube containing 8 ml heptane and 500 λ 0.1 *N* HCl, 0.5% ascorbic acid.

(*6*) Shake this tube 2 min; centrifuge.

(*7*) Measure volume of aqueous phase and read fluorescence as above.

III. 5-Hydroxytryptophan. At an acidic pH *tert*-amyl alcohol extracts serotonin, *N*-acetylserotonin, 5-methoxytryptamine, and bufotenine, as well as 5-hydroxytryptophan. An alkaline wash with *tert*-amyl alcohol takes up these other indoles and leaves behind all but about 5% of the 5-hydroxytryptophan.

(*1*) As above but *tert*-amyl alcohol is used as solvent.

(*2*) As above.

(*3*) Each tube from step (*1*) is treated continuously: (a) add 250 λ pH 10 buffer (saturated with NaCl and *tert*-amyl alcohol); (b) immediately shake 1 min, centrifuge 1 min, and discard alcohol phase; (c)

add another 3 ml *tert*-amyl alcohol and repeat step (b); (d) add 200 λ 1.0 *N* HCl, mix, add 3 ml *tert*-amyl alcohol; (e) shake 1 min, centrifuge 1 min, and transfer alcohol phase to 15-ml centrifuge tube from step (*2*).

(*4*) Shake this tube 2 min and centrifuge.

(*5*) Measure volume of aqueous phase and read fluorescence as above.

IV. 5-Hydroxyindole-3-acetic Acid. An acidic extraction with diethyl ether takes up 5-hydroxyindole-3-acetic acid, melatonin, and *N*-acetyl serotonin. The latter two compounds are removed by previous alkaline washing with diethyl ether.

(*1*) As above but diethyl ether is used as solvent.

(*2*) As above.

(*3*) Each tube from step (*1*) is treated continuously: (a) add 250 λ pH 10 buffer (saturated with NaCl and diethyl ether); (b) immediately shake 1 min, centrifuge 1 min, and discard ether phase; (c) add another 3 ml ether and repeat step (b); (d) add 150 λ 1.0 *N* HCl, mix, add 3 ml diethyl ether; (e) shake 1 min, centrifuge 1 min, and transfer ether phase to 15-ml centrifuge tube from step (*2*).

(*4*) Shake this tube 2 min and centrifuge.

(*5*) Measure volume of aqueous phase and read fluorescence as above.

V. 5-Methoxyindole-3-acetic Acid. Acidic extraction with *p*-cymene recovers the largest per cent of 5-methoxyindole-3-acetic acid. The accompanying melatonin is removed with an alkaline diethyl ether wash.

(*1*) As above with diethyl ether as solvent.

(*2*) As above.

(*3*) Each tube from step (*1*) is treated continuously: (a) add 250 λ pH 10 buffer (saturated with NaCl and diethyl ether); (b) immediately shake 1 min, centrifuge 1 min, and discard ether phase; (c) add another 3 ml ether and repeat step (b); (d) add 200 λ 1.0 *N* HCl, mix, add 3 ml *p*-cymene; (e) shake 1 min, centrifuge 1 min, and transfer cymene phase to 15-ml centrifuge tube from step (*2*).

(*4*) Shake this tube 2 min and centrifuge.

(*5*) Measure volume of aqueous phase and read fluorescence as above.

VI. Melatonin. Selective extraction of melatonin occurs with *p*-cymene and an alkaline solution.

(*1*) As above but with *p*-cymene as solvent.

(*2*) As above.

(*3*) Each tube from step (*1*) is treated continuously: (a) add 250 λ pH 10 buffer (saturated with NaCl and *p*-cymene); (b) immediately shake 1 min, centrifuge 1 min, and transfer cymene phase to 15-ml centrifuge tube from step (*2*).

(*4*) Shake this tube 2 min and centrifuge.

(*5*) Measure volume of aqueous phase and read fluorescence as above.

RESULTS

The per cents of indole standards recovered by the methods recommended above are listed in Table 1. The ranges in per cent recovery are

TABLE 1

MEAN PER CENT RECOVERIES BY SELECTIVE EXTRACTION METHODS OF STANDARDS IN 0.1 *N* HCl, 0.5% ASCORBIC ACID

Compounds	Methods					
	I	*II*	*III*	*IV*	*V*	*VI*
Serotonin	*92*	3	3	0–1	0–1	0–1
N-Acetylserotonin	0–1	*69*	2	0–1	1	2
5-Hydroxytryptophan	30	3	*59*	0–1	0–1	0–1
5-Hydroxyindole-3-acetic acid	0–1	2	12	*84*	2	0–1
5-Methoxyindole-3-acetic acid	0–1	2	0–1	13	*27*	0–1
Melatonin	0–1	6	3	0–1	2	*73*
5-Methoxytryptamine	3	2	3	0–1	0–1	5
Bufotenine	0–1	3	3	0–1	0–1	0–1

less than ±5% for the larger mean per cent recoveries and about ±1% for the smaller means. Neither 5-hydroxy- nor 5-methoxyindole is listed, because only traces of these compounds were recovered from fresh standard solutions by the extractions. It should be noted, however, that these are the most labile of the indoles studied and that older standard solutions of them yielded compounds that were extracted by some of the methods and fluoresced at 530–550 mμ.

Results of extractions of single indoles from mixtures of the eight listed in Table 1 and from similar mixtures containing, in addition, fresh pineal tissue, are shown in Table 2. The fluorescences of the extracts of mixtures are expressed as per cents of the fluorescences of the extracts of the individual indoles. The somewhat greater fluorescences of extracts of mixtures by methods *I*, *III*, and *IV* reflect low recovery of compounds in addition to those being more selectively extracted. The level of this additional fluorescence conforms to what would be expected according to the data of Table 1. The fluorescences of extracts of indoles containing two ♀ (10–12 week old) rat pineals exceeds the fluorescences of extracts of indole mixtures lacking pineals, to a significant degree in regard to at least several of the procedures. The significance, if any, of the lower fluorescences of extracted mixture + pineals by method *V* is not apparent at this time. The pineal indole contents listed in Table 2 are derived from diverse extraction series, including the ones averaged here in column three of Table 2. Experimental analysis of the factors

TABLE 2

COMPARISONS OF FLUORESCENCE OF EXTRACTED SINGLE INDOLES (500 NG/TUBE), EXTRACTED MIXED INDOLES (500 NG OF EACH/TUBE), AND EXTRACTED MIXED INDOLES PLUS TWO ADULT RAT PINEALS

(With estimates of pineal contents and sensitivities of methods)

Method	Indole	Single indole	Mixed indoles	Mixed indoles + pineals	Pineal contents		Sensitivity (ng)
					ng/pineal	ng/mg	
I	Serotonin	100	130	295	80→300	100→350	10
II	*N*-Acetylserotonin	100	99	104	<15	<21	30
III	5-Hydroxytryptophan	100	114	114	<10	<14	20
IV	5-Hydroxyindole-3-acetic acid	100	109	115	5→15	7→21	10
V	5-Methoxyindole-3-acetic acid	100	95	91	<5	<7	10
VI	Melatonin	100	103	113	<5→20	<7→28	15

that modify the pineal's content of these indoles will be presented later.

The sensitivities listed in Table 2 for the extraction and fluorometry of these indoles are approximate minimum detectable amounts. Differences between the sensitivities of the methods result primarily from differences in the fluorescence intensities of the compounds and the per cents recovered by the selective extraction procedures. In studies of quantitative differences, or experimentally produced changes in tissue indoles, levels at least ten times those listed here under sensitivity would be desirable.

In general, bufotenine and 5-methoxytryptamine demonstrated similar solubility characteristics according to the pH values and solvents that I used. Similar recoveries of the two were obtained by the solvents, arranged from about 90 to 30% yield: *tert*-amyl alcohol, *n*-butanol, cyclohexanol, amyl acetate, diethyl ether, methyl benzoate, *p*-xylene. For the last 5 or 6 of these, significant recoveries were dependent upon pH's greater than 7. It seems possible that some minor differences in the solubilities of these compounds could be utilized in their quantitative differential measurement. The fluorescences obtained from two solvent systems (A and B) could be expressed as simultaneous equations in which X and Y equal the amounts of the two indoles present in the sample solution and P_{xa}, P_{xb}, P_{ya}, P_{yb} the per cents recovered of standards of X and Y by these solvents: $A = XP_{xa} + YP_{ya}$. $B = XP_{xb} + YP_{xb}$. Substitution of one equation into the other, and the solution of the equations for the unknowns, X and Y, would follow. Measurement of

serotonin in the presence of 5-hydroxytryptophan with extraction method *I* could be aided similarly, by the use of equations, in this instance employing figures from extraction methods *I* and *III* (see Table 1).

DISCUSSION

As noted previously in the extraction of serotonin by Halevy (4), EDTA and ascorbic acid in the samples or standards to be extracted increased the reliability and per cent of the indole recovery. Solubilization of serotonin in organic solvents in the presence of plasma is influenced by divalent cations and disodium EDTA (5). Inclusion of ascorbic acid in the final aqueous phase in the methods described here increases the stability of the more labile indoles, especially during activation. And the fluorescence peaks of the 5-methoxy- and 5-hydroxyindoles at 540–550 mμ are not distorted by the ascorbic acid nor by scatter at near maximum sensitivity.

Butanol extraction is optimal only in the cases of method *I*, extraction of serotonin. Nevertheless, it is possible to fractionate by different methods aliquots of an *n*-butanol extract and obtain some of the other indoles, but at recoveries lower than those provided by the methods described here. It is also possible to combine some of the extraction methods described here, in order to measure several of the indoles from the same sample. For example: a sample solution could be extracted first with *p*-cymene for melatonin (with an alkaline ether wash) and *N*-acetylserotonin (with additional acid and alkaline ether extractions), then with acid ether for 5-hydroxyindoleacetic acid, and finally with alkaline *n*-butanol for serotonin. The washing procedures and multiple extraction steps are not necessary or desirable in extractions of samples that are known to lack significant amounts of the compounds for which these steps were designed. They may be by-passed too by the use of countercurrent distribution techniques.

Some of the reagent-grade solvents used for extractions varied significantly in quality from one commercial source to another. Repeated cleaning of the solvents with 1 *N* NaOH, 1 *N* HCl and deionized water frequently improved them, but they were still inferior to unpurified samples from other sources. For example, extractions with *p*-cymene from one source yielded 11% of the melatonin present (method *VI*); and multiple purifications could not increase the yield beyond 49%. However, unpurified Eastman *p*-cymene yielded 73%. Variations in purity of commercial reagent-grade solvents appeared to affect the per cent recovery of the primary indole solutes much more than the specificity of the extractions.

Specificity of fluorescence at 540–550 mμ with activation at 295 mμ

to 5-hydroxy- and 5-methoxyindoles in 3 *N* HCl appears to be good since the following closely related compounds did not show this characteristic: tryptophan, tryptamine, 5-methylindole, 3-methylindole, indole-3-acetic acid, 5-methyltryptophan, 4-hydroxy-*N*-dimethyltryptamine, *o*-phosphoryl 4-hydroxy-*N*-dimethyltryptamine, 4-hydroxytryptamine, 6-hydroxytryptamine, 4-hydroxytryptophan, and 6-methoxyindole.

SUMMARY

Serotonin, *N*-acetylserotonin, 5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid, 5-methoxyindole-3-acetic acid, and melatonin can be each selectively extracted from tissue or standard solutions in 0.1 *N* HCl or 0.1 *N* HCl, 0.5% ascorbic acid, and measured by their fluorescence at 540–550 mμ in 3 *N* HCl with activation at 295 mμ. Possibilities for measurement of 5-methoxytryptamine and bufotenine and the specificities of the methods are discussed.

ACKNOWLEDGMENTS

I am grateful to Peter Baker for skillful technical assistance, to Dr. A. Hofmann for providing certain of the indole standards, and to Dr. A. Halevy for helpful comments on the manuscript.

REFERENCES

1. Bogdanski, D. F., Pletscher, A., Brodie, B. B., and Udenfriend, S., *J. Pharmacol. Exptl. Therap.* **117**, 82 (1956).
2. Udenfriend, S., Weissbach, H., and Bogdanski, D. F., *Science* **122**, 972 (1955).
3. Quay, W. B., and Halevy, A., *Physiol. Zoöl.* **35**, 1 (1962).
4. Halevy, A., *Research Repts. of VA Palo Alto* No. 18 (Veterans Administration Hospital, Palo Alto, Calif.), 1961.
5. Kerby, G. P., and Taylor, S. M., *Proc. Soc. Exptl. Biol. Med.* **107**, 936 (1961).

ANALYTICAL BIOCHEMISTRY 5, 60–63 (1963)

Purification of Labeled *N*-Ethyl Maleimide

MARTIN FLAVIN

From the Enzyme Section, National Heart Institute, Bethesda, Maryland

Received May 18, 1962

INTRODUCTION

Isotopically labeled *N*-ethyl maleimide (NEM) has been used to trap and quantitatively determine mercaptans, particularly cysteine (1–3). Because of its instability with respect to imide hydrolysis, and to other reactions (4), we thought that a method for its purification might prove useful to other workers, particularly when it is to be used for quantitative determinations.

In the solid state NEM is readily purifiable by sublimation. However, the only labeled material now commercially available (Schwarz) is provided as a dilute solution in 50% aqueous ethanol. Because of its high solubility in many solvents, and its volatility, it is difficult to isolate NEM from the above solvent mixture. Thus, although crystals will separate from the residual water after ethanol has been removed in an air stream, the yield is very low. We have found, however, that it can be obtained in crystalline form in 50% yield, by sublimation after extraction into a very low boiling solvent.

METHODS

The procedure was carried out in a —14° room, and glassware and reagents were placed in the cold room the day before use to avoid condensation of water. Nine-tenths ml of the 50% ethanol solution of NEM-1-C^{14} (4.3 mg, 26 μc) was placed in a glass-stoppered centrifuge tube containing 26.3 mg of resublimed carrier NEM (the procedure should be equally effective without addition of carrier, but the sublimation method used was less suited to the manipulation of such small amounts of material). The solution was then extracted twice with 2-ml portions of ethyl chloride (USP, available from pharmacies as a local anesthetic). After clarifying emulsions by brief centrifugation, the ethyl chloride was pipetted into a cork-stoppered glass PR-2 International high speed head centrifuge tube, containing about 2 ml of 12-mesh anhydrous calcium chloride. The latter removes at least the major part of the ethanol, which is extracted together with NEM into ethyl chloride. This suspen-

sion was stirred occasionally over a period of several hours at —14°, and then centrifuged at —17° for 10 min at 10,000 rpm. The precipitate was stirred with 3 to 4 ml of fresh ethyl chloride, and centrifuged again. The extract and wash were pipetted into a small glass evaporating dish, without a lip, which was then placed in a hood at room temperature. The dish remains quite cold while the solvent is evaporating (b.p. 12–13°). As soon as evaporation was complete, the dish was covered with a slightly larger watch glass, and a small chunk of dry ice was placed in the center of the latter. NEM was allowed to sublime, over a period of 1 to 2 hr, by placing the dish in a sand bath at about 45°. Care is needed to avoid losses in collecting the large and very light crystals.

RESULTS

The yield was 16.1 mg (53%), and the product was chemically pure as judged by absorbancy at 302 mμ (2) and absence of titratable acidity up to pH 6. In two preparations, from different batches of starting material, the yield of isotope was, respectively, 11 and 12% less than the chemical yield. It should be emphasized that the proportions of impurities could probably be much larger after longer periods of storage.

Labeled NEM has been used to ascertain the proportions of cysteine and homocysteine liberated enzymically from cystathionine (5).[1] The experiment described in Table 1 shows that the apparent recovery of cysteine is lower using unpurified NEM-1-C^{14}.

One μmole of cysteine was added to 1 ml of phosphate buffer, pH 7.3, containing $5 \times 10^{-3} M$ purified NEM-1-C^{14} of known specific radioactivity (Expts. 1 and 2, Table 1), or the same amount of resublimed carrier plus unpurified NEM-1-C^{14} (Expt. 3), the specific radioactivity of the latter being calculated on the assumption that all the C^{14} present was in NEM (amount determined by absorbancy at 302 mμ). Experiment 2 also contained enzyme and the other cystathionine cleavage re-

[1] It may be of interest that we have not found it possible to use iodoacetate-1-C^{14} in place of labeled NEM, in experiments of this type. Thus, when comparable reaction mixtures containing iodoacetate-1-C^{14} and homocysteine (which is more resistant to air oxidation than cysteine) were chromatographed, recovery of labeled *S*-carboxymethylhomocysteine was only 13% of theoretical, and ninhydrin-positive, strongly radioactive spots appeared near the origin of the chromatogram. Similar unknown labeled components were present after incubation of cysteine-S^{35} with unlabeled iodoacetate. These side products appeared to be formed during the application of the solution containing excess iodoacetate to the paper. After removal of excess iodoacetate by incubation with alkaline piperdine prior to chromatography (5), the only radioactive spots appearing corresponded to *S*-carboxymethylhomocysteine and *N*-carboxymethylpiperidine. However, recovery of homocysteine was still only 36%.

TABLE 1
CYSTEINE DETERMINATION WITH LABELED *N*-ETHYL MALEIMIDE

Expt.[a]	Specific radioactivity of NEM-C^{14} (counts/μm × min)	Total radioactivity in NEM-cysteine-C^{14} (cpm)	Cysteine recovered (%)
1a	85,000	65,300	77
1b	85,000	53,300	63
1c	85,000	51,100	60
2b	85,000	57,100	67
2c	85,000	45,100	53
3a	232,000[b]	149,000	64
3b	232,000	129,000	56
3c	232,000	129,000	56

[a] The results of Expts. a were obtained after incubation of cysteine with NEM-C^{14} for 10 min at 30°, of Expts. b after incubation for 100 min, and of Expts. c for 100 min followed by a procedure for deproteinization. See text for other experimental conditions.
[b] Calculated value.

action components (5). In the experiments, incubation was for 10 min at 30° (a), for 100 min (b), and (c) for 100 min followed by a procedure for deproteinization: addition of perchloric acid, followed by enough $KHCO_3$ to give a final pH of 4 (to precipitate excess perchlorate). Aliquots from each mixture were chromatographed for 40 hr descending on Whatman No. 1 in: 3° butanol (70), 90% formic acid (15), water (15). The labeled NEM-cysteine adduct was located by radioautography, eluted with water, and assayed in a scintillation counter. The total C^{14} recovered indicates the amount of cysteine.

Recovery of cysteine is less than quantitative under all conditions, perhaps in part due to oxidation in the interval between neutralizing it and adding it to the NEM-C^{14} (the experiments were aerobic). However, the experiment demonstrates that the average apparent recovery was 12% less with unpurified NEM-C^{14} (Expt. 3) than with purified (Expt. 1). Also, at least five nonvolatile radioimpurities were seen in the radiogram from Expt. 3a, which were absent in 1a. The three of these which migrated farther than the NEM-cysteine adduct also appeared after longer incubation (at pH 7.3) of purified NEM-C^{14} (Expt. 1b), and may have been imide hydrolysis products.

SUMMARY

Commercially available preparations of radioactive *N*-ethyl maleimide can be purified after extraction at low temperature with ethyl chloride and removal of ethanol with calcium chloride. The residue from evapo-

ration of this low boiling solvent can be readily obtained free of impurities by sublimation.

REFERENCES

1. GUTCHO, M., AND LAUFER, L., *in* "Glutathione" (S. Colowick *et al.*, eds.), p. 79. Academic Press, New York, 1954.
2. GREGORY, J. D., *J. Am. Chem. Soc.* **77,** 3922 (1955).
3. ROBERTS, E., AND ROUSER, G., *Anal. Chem.* **30,** 1291 (1958).
4. SMYTH, D. G., NAGAMATSU, A., AND FRUTON, J. S., *J. Am. Chem. Soc.* **82,** 4600 (1960).
5. FLAVIN, M., *J. Biol. Chem.* **237,** 768 (1962).

ANALYTICAL BIOCHEMISTRY 5, 64–69 (1963)

Rapid Colorimetric Determination of Adenine Compounds[1]

JOSEPH R. DAVIS AND ROBERT N. MORRIS[2]

From the Department of Pharmacology and Therapeutics, Stritch School of Medicine, and Graduate School, Loyola University, Chicago, Illinois

Received May 28, 1962

Numerous spectrophotometric methods employing the ultraviolet range have been described for the determination of adenine. Since the absorption maximum of all the nucleic acid bases varies between 250 and 290 mμ, one disadvantage to this method is the lack of specificity for adenine in a mixture of nucleic acid bases. In addition, many other biological materials can absorb at similar wavelengths and thus interfere with the determination of adenine. Differential spectrophotometry has been reported by Kalckar (1) to differentiate adequately a number of purine compounds by changes in the ultraviolet absorption of these compounds resulting from actions of specific enzymes. A specific colorimetric analysis for adenine has been reported by Woodhouse (2) employing reduction with zinc dust and coupling with *N*-(1-naphthyl)ethylenediamine. Although these methods have been found to give satisfactory determinations of adenine, they are both complicated and time consuming. For these reasons, studies have been carried out in this laboratory on a rapid and specific colorimetric analysis of adenine which would serve not only as a quantitative assay but also as a convenient spot test for this substance.

In the course of determining citrate content in tissue slices by the pentabromacetone ether extraction method of Natelson *et al.* (3), it was observed that a clear yellow color appeared after the addition of hydrogen peroxide when ATP had been added to the incubation medium. The possibility that this color reaction was due to some color complex involving adenine was confirmed by using the same procedure with adenine alone. The present experiments were designed to ascertain the

[1] These studies were supported in part by Institutional Grants from the American Cancer Society (IN-52) and the General Research Support Grant of the Public Health Service.

[2] Predoctoral Trainee, United States Public Health Service, Training Grant 2G-77.

specificity of the color reaction for adenine and the sensitivity of the analysis, as well as to investigate the optimal conditions of the reaction.

METHODS

Apparatus. A Beckman DU spectrophotometer was used for absorbance measurements.

Purines and Pyrimidines. Adenine, guanine, xanthine, hypoxanthine, cytosine, 5-methylcytosine, uracil, and thymine were obtained from the California Corporation for Biochemical Research (Los Angeles, Calif.). Purine, uric acid, caffeine, theobromine, theophylline, 6-chloropurine, adenosine, adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate were obtained from Mann Research Laboratories (New York, N. Y.). 6-Mercaptopurine was obtained from Nutritional Biochemicals Corporation (Cleveland, Ohio). In all instances, 0.1 *N* H_2SO_4 was employed as the solvent.

Reagents. Stock solutions were employed for 18 *N* H_2SO_4, 0.2 *M* KBr, 1 *N* $KMnO_4$, and 6% H_2O_2. The 1 *N* solution of potassium permanganate was obtained from the Fisher Scientific Co. (Chicago, Ill.) and contained 31.61 mg $KMnO_4$/ml by sodium oxalate reference standard.

Procedure. A 1.5-ml aliquot of the sample to be analyzed was transferred to a centrifuge tube with a conical bottom and 0.2 ml of 18 *N* H_2SO_4 was added. The contents were mixed and 0.1 ml of 0.2 *M* KBr was added. Following shaking, 0.3 ml of 1 *N* $KMnO_4$ was added and after waiting 5 min 6% H_2O_2 was introduced dropwise to decolorize the excess permanganate. The final volume was then adjusted to 3.0 ml with distilled water and the optical density was measured within 15 minutes at 330 mμ against a reagent blank.

RESULTS AND DISCUSSION

A yellow color appeared almost immediately following the decolorization of the excess permanganate with hydrogen peroxide in the reaction mixture containing adenine. The absorption of light by adenine before and after the outlined procedure is shown in Fig. 1. A solution of untreated adenine in 0.1 *N* H_2SO_4 had a single absorption maximum of 262 mμ. Following color development, the adenine solution had maximal absorption at wavelengths of 254, 330, and 420 mμ. The absorption peak at 330 mμ was chosen as the optimal wavelength for sample readings, although the yellow color resulting from the absorption at 420 mμ was sufficient to carry out a rapid qualitative test for the presence of adenine. In order to determine the reproducibility of the method, ten samples of adenine (two different commercial lots) were treated with the above reagents and the optical density at 330 mμ determined. The average

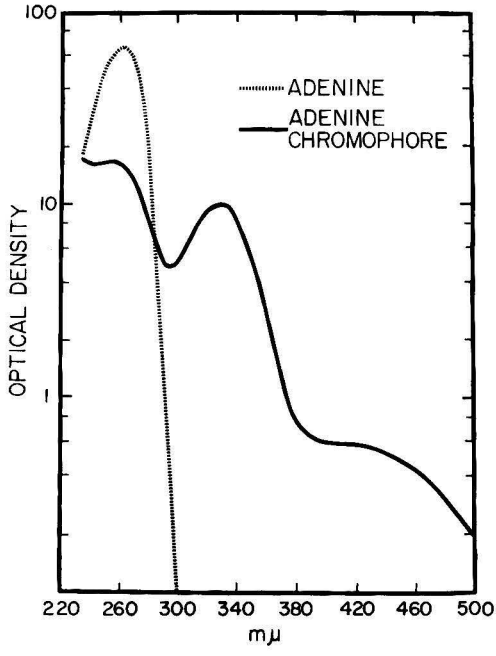


FIG. 1. Absorption of light by adenine before and after treatment according to the outlined procedure. In the regions of high absorption, measurements were made on appropriate dilutions of a solution which was originally $5 \times 10^{-3} M$ adenine.

molar extinction coefficient was found to be 2170 (standard error of ± 20) at a concentration of $5 \times 10^{-4} M$ adenine. Because of the nonstoichiometric nature of the reaction, the molar extinction coefficient varied somewhat with adenine concentration.

Figure 2 is a standard curve of optical density versus adenine concentration. A straight line was obtained from 10 to 50 μg of adenine/ml of reaction mixture.

The stability of the adenine chromophore was investigated with regard to its absorption at 330 mμ. Essentially no change in optical density occurred up to 60 min following the final addition of hydrogen peroxide.

Figure 3 indicates the effect of varying amounts of potassium bromide on the yield of the adenine chromophore. An inverse relationship occurred between the absorption at 262 mμ as compared to the absorption at 330 mμ, which was dependent upon the concentration of KBr in the reaction mixture. Similar relationships were also found to be dependent upon the concentration of H_2SO_4 and $KMnO_4$ in the reaction mixture. These data indicate that the absorption peak at 330 mμ is due to the

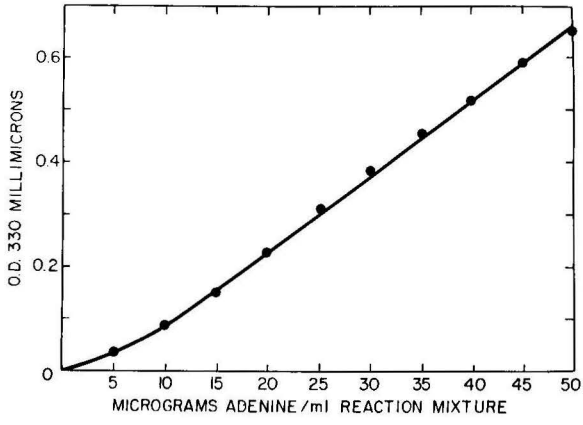


FIG. 2. Standard curve for adenine chromophore plotted in μg adenine/ml reaction mixture against the optical density measured at 330 mμ.

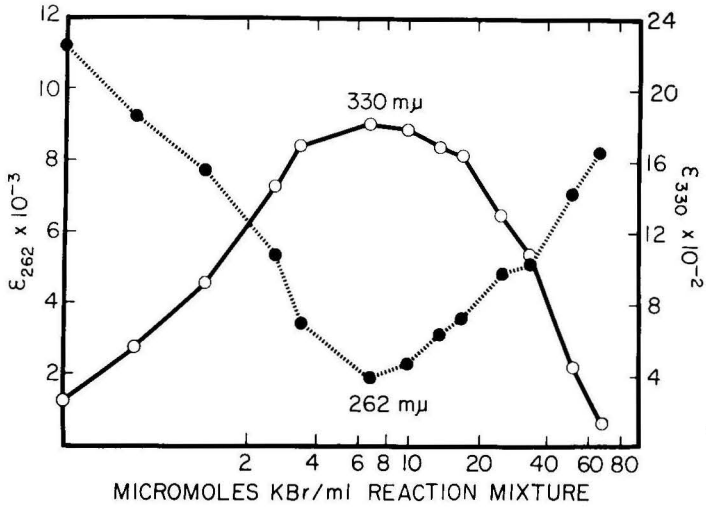


FIG. 3. Effect of varying amounts of potassium bromide on yield of chromophore from $5 \times 10^{-4} M$ adenine.

formation of the adenine chromophore. It would appear, however, that the absorption at 262 mμ may represent not only unreacted adenine but a portion of the adenine chromophore as well.

The specificity of the reaction for adenine was investigated by testing a number of purines and pyrimidines (Table 1). All of the compounds

studied were found to give a negative test with respect to both the visible yellow color and an ultraviolet absorption peak at 330 mμ.

TABLE 1
NONINTERFERING COMPOUNDS[a]

Guanine	6-Chloropurine
Hypoxanthine	6-Mercaptopurine
Xanthine	Uracil
Purine	Thymine
Uric acid	Cytosine
Caffeine	5-Methylcytosine
Theobromine	
Theophylline	

[a] Each of the compounds listed was mixed at $5 \times 10^{-4} M$ with adenine and the absorbancy after color development was compared with that for an adenine control.

In contrast to the results obtained with purines and pyrimidines other than adenine, it was found that such adenine-containing compounds as adenosine, adenylic acid, adenosine diphosphate, and adenosine triphosphate all produced a visible yellow color and the characteristic ultraviolet absorption peak at 330 mμ when treated with the outlined procedure. The molar extinction coefficients at 330 mμ for these compounds were 102, 97, 91, and 94%, respectively, of the corresponding molar extinction coefficient obtained for adenine itself. The fact that these adenine nucleosides and nucleotides reacted positively without prior hydrolysis, such as refluxing for 1 hr with $1 N$ H_2SO_4, eliminates this time-consuming step when a rapid quantitative or qualitative analysis is desired for these substances.

In order to test the applicability of the present method to biological fluids, recoveries were made of known quantities of adenine added to protein-free filtrates of rat serum, kidney, and spleen. Tungstic acid was employed as the precipitating agent. The recovery of added adenine ranged between 98 and 102% in these tissues. The total tungstic acid-soluble adenine of the kidney was found to be 171 μg/gm wet weight tissue with a standard error of ±12, while the total tungstic acid-soluble adenine of the spleen was found to be 179 μg/gm wet weight tissue with a standard error of ±14. No demonstrable adenine was found in samples of rat serum employing a 1:10 dilution of the original material.

SUMMARY

A procedure for both a rapid qualitative and a quantitative determination for adenine in a mixture of various purines and pyrimidines is described. In addition, the method can also be applied for the rapid

detection and quantitative estimation of a number of adenine nucleosides and nucleotides without prior hydrolysis of these compounds.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. Norton C. Melchoir and Dr. Frederick W. Pairent of the Department of Biochemistry of Stritch School of Medicine, Loyola University, for their many helpful suggestions.

REFERENCES

1. KALCKAR, H. M., *J. Biol. Chem.* **167,** 445 (1947).
2. WOODHOUSE, D. L., *Arch. Biochem.* **25,** 347 (1950).
3. NATELSON, S., LUGOVOY, J. K., AND PINCUS, J. B., *J. Biol. Chem.* **170,** 597 (1947).

ANALYTICAL BIOCHEMISTRY 5, 70–77 (1963)

Microbiological Determination of Alanine in Proteins and Foods

HELEN W. WARREN, MILLARD J. HORN, AND AMOS E. BLUM

From the Human Nutrition Research Division, United States Department of Agriculture, Washington, D. C.

Received June 5, 1962

INTRODUCTION

The microbiological determination of alanine in foods has been considered by many as one of the most difficult (1). Some of the early workers used *Streptococcus faecalis* as the test organism (2). Sauberlich and Baumann (3) used *Leuconostoc citrovorum* 8081[1] and found that it required a purified liver preparation (reticulogen) in addition to a synthetic medium containing 17 amino acids. We found in attempting to use several of the published methods that consistent results on different kinds of foods could not be obtained.

In our previous work on synthetic mixtures of amino acids that simulated protein hydrolyzates of foods (4), more consistent results and better curves were obtained when L-amino acids were used in the medium, than when DL-amino acids were used. L-Amino acids furnished by an acid hydrolyzate of decystinized casein also gave more consistent results in determinations of cystine by Horn and Blum (5). The present report gives the results of using all L-forms of the amino acids and *L. citrovorum* 8081 as the test organism for determination of alanine. The strain of *L. citrovorum* 8081 used here did not require folinic acid. When crystalline folic acid was used (20 μg/liter of medum) the addition of folinic acid gave no increased response.

PROCEDURE

The hydrolyzates of foods were prepared as described by Horn *et al.* (6). The preparation of samples after hydrolysis was identical with those described in previous papers (7, 8). The humin was filtered off at pH 4. The composition of the basal medium shown in Table 1 includes all substances, with the exception of alanine, essential for the growth of *L. citrovorum* 8081. Since exact conditions of autoclaving the basal

[1] *Leuconostoc citrovorum* 8081 has been identified as *Pediococcus cerevisiae* 8081.

TABLE 1
BASAL MEDIUM

One liter of double strength basal medium contains:			
Compound	Gm	Compound	Mg
Dextrose	20.00	Adenine sulfate	100.0
Sodium acetate	12.00	Guanine hydrochloride	100.0
K_2HPO_4	1.00	Uracil	100.0
KH_2PO_4	1.00	$MgSO_4 \cdot 7H_2O$	400.0
	Mg	NaCl	20.0
L-Arginine HCl	72.5	$MnSO_4 \cdot 4H_2O$	20.0
L-Aspartic acid	60.0	$FeSO_4 \cdot 7H_2O$	20.0
L-Cystine	30.2	Ammonium chloride	370.0
L-Glutamic acid	100.0	*p*-Aminobenzoic acid	0.400
Glycine	60.0	Biotin	0.010
L-Histidine $HCl \cdot H_2O$	81.1	Calcium pantothenate	0.400
L-Isoleucine	69.8	Folic acid	0.020
L-Leucine	100.0	Nicotinic acid	0.800
L-Lysine HCl	75.6	Pyridoxine HCl	0.400
L-Methionine	17.6	Pyridoxal HCl	0.040
L-Phenylalanine	82.6	Riboflavin	0.400
L-Proline	60.0	Thiamine HCl	2.000
L-Serine	60.0	Adjusted to pH 6.6, made up to 1 liter with distilled water, and autoclaved at 15 lb for 15 min.	
L-Threonine	44.4		
L-Tryptophan	16.0		
L-Tyrosine	60.4		
L-Valine	69.8		

medium seems to be critical for both alanine and cystine a detailed description of this procedure is given here. After the initial autoclaving, which was adjusted to pH 6.6, the medium stood overnight at room temperature in a cotton-stoppered flask. The following day the medium was readjusted to pH 6.6 and 5 ml of medium was placed in each tube. Varying amounts of standard or hydrolyzate were added and the volume in each tube was made up to 10 ml. The tubes were autoclaved at 15 lb for 15 min and inoculated the following day. The organism was grown on stabs of yeast dextrose agar and transferred to liver infusion containing liver, yeast, and dextrose; it was made 24 hr before it was to be used and incubated in a water bath at 36.5°C. The organism was washed once with normal saline. The centrifuged organism was resuspended in 5 ml of normal saline. One drop of this suspension was used as the inoculum. The tubes were incubated for 72 hr at 36.5°.

The curve shown in Fig. 1 was developed for use in determining the response of the organism to the hydrolyzate of the foods, and was reproducible. The titration value for the zero point was not altered by

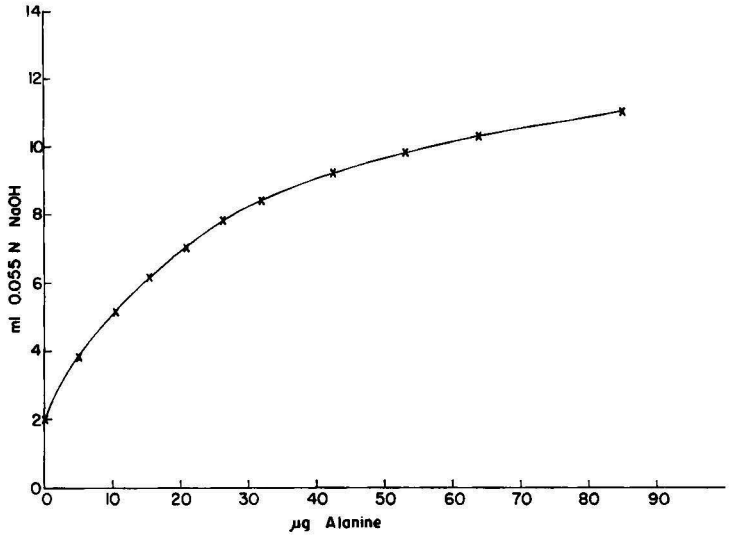


FIG. 1. Standard curve for alanine with *Leuconostoc citrovorum* 8081.

addition of 400 µg of any of the other 17 amino acids. Titration values at other levels were not altered upon doubling the essential or non-essential amino acids or both in the basal medium. D-Alanine gave a similar curve to L-alanine when added in graded amounts to this medium. However, this does not create a problem when analyzing proteins and foods as only the L-form is in the hydrolyzate. Titration values for the L-alanine curve are similar with or without pyridoxine in the medium. However, when an acid hydrolyzate of casein was tested with the three media (no B_6, pyridoxine, and pyridoxine plus pyridoxal) the values for alanine content obtained with pyridoxine and pyridoxine plus pyridoxal checked each other but were higher than the results obtained on the B_6-free medium.

RESULTS AND DISCUSSION

Table 2 gives values for alanine at different assay levels of the food for various foods containing different constituents and varying nitrogen levels. There was no drift in values at increasing levels of the food and little variation among levels all along the curve from 4 to 40 µg.

Alanine added before hydrolysis to casein and red kidney beans, as examples of two widely different materials, gave recoveries of 101% for casein and 95% for red kidney beans (Table 3).

Data on the reproducibility of values found for a number of materials

TABLE 2

ALANINE CONTENT OF SOME PROTEINS AND FOODS DETERMINED AT DIFFERENT ASSAY LEVELS OF THE FOOD

Coconut globulin		Cottonseed globulin		Glycinin		Zein		Barley		Red kidney beans		Lima beans		Peanuts	
μg	%	μg	%	μg	%	μg	%	μg	%	μg	%	μg	%	μg	%
—	—	—	—	—	—	9.7	9.70	—	—	—	—	—	—	—	—
—	—	—	—	—	—	20.2	10.10	—	—	—	—	—	—	—	—
10.0	4.00	10.0	4.00	9.1	3.64	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	30.7	10.20	—	—	—	—	—	—	—	—
—	—	—	—	—	—	40.2	10.05	—	—	—	—	—	—	—	—
20.5	4.10	20.0	4.00	18.4	3.68	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	7.5	1.25
31.2	4.16	30.2	4.02	28.6	3.81	—	—	—	—	7.7	1.03	7.5	1.00	—	—
41.5	4.15	40.2	4.02	39.0	3.90	—	—	4.5	0.45	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	13.0	1.08
—	—	—	—	—	—	—	—	—	—	15.5	1.03	14.0	0.93	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	20.7	1.15
—	—	—	—	—	—	—	—	9.0	0.45	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	23.5	1.04	20.7	0.92	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	28.2	1.18
—	—	—	—	—	—	—	—	13.5	0.45	31.0	1.03	27.5	0.92	—	—
—	—	—	—	—	—	—	—	18.2	0.45	—	—	—	—	—	—
e	4.10		4.01		3.76		10.01		0.45		1.03		0.94		1.16

TABLE 3
RECOVERY OF ALANINE ADDED TO CASEIN AND RED KIDNEY BEANS BEFORE HYDROLYSIS

Food hydrolyzate	Alanine				
	In hydrolyzate (μg/tube)	Alanine added (μg/tube)	Calculated (μg/tube)	Analyzed (μg/tube)	Recovery (%)
Casein	1.43	4.04	5.47	5.30	97
	2.86	8.08	10.94	10.70	98
	4.29	12.12	16.41	17.30	105
	5.72	16.16	21.88	23.30	106
	7.15	20.20	27.35	27.70	101
					Av. 101
Red kidney beans	3.87	9.80	13.67	12.70	93
	7.74	19.60	27.34	26.00	95
	11.61	29.40	41.01	39.00	95
	15.48	39.20	54.68	51.30	94
	19.35	49.00	68.35	66.80	98
					Av. 95

when determined on different hydrolyzates are given in Table 4. Each hydrolyzate was tested at 4 levels with 3 tubes each. The figure reported is the average of these 36 values. Included in this table are the standard

TABLE 4
REPRODUCIBILITY AND STANDARD DEVIATION OF ALANINE CONTENT BY SEPARATE ASSAY[a]

Material	Assay 1 (%)	Assay 2 (%)	Assay 3 (%)	Average (%)	S.D.	C.V. (%)
Beans, red kidney	1.034	1.043	1.034	1.037	±0.0052	0.5
Casein	3.030	3.001	2.900	2.977	±0.068	2.3
Corn, whole	0.879	0.818	0.877	0.858	±0.035	4.1
Cottonseed meal	1.340	1.489	1.503	1.444	±0.093	6.4
Flour, wheat	0.411	0.370	0.382	0.388	±0.021	5.4
β-Lactoglobulin	6.600	6.575	6.325	6.500	±0.143	2.2
Milk	1.075	1.129	1.053	1.086	±0.039	3.6
Spanish peanuts	1.244	1.165	1.155	1.188	±0.049	4.1
Soybean	1.695	1.639	1.554	1.619	±0.072	4.4
Zein	10.40	10.64	10.01	10.35	±0.32	3.2
						Av. 3.6

[a] Values given are per cent of alanine in 100 gm of food.

deviation (S.D.) and coefficient of variation (C.V.) for some representative materials. The average coefficient of variation from the mean is 3.6%.

Table 5 gives the values for alanine found in some proteins and the values found in the literature for similar materials. In many cases no

TABLE 5
ALANINE CONTENT OF PROTEINS

Protein	Nitrogen (%)	Alanine content (gm)		
		Per 100 gm protein	Per gm N	Lit. (calcd. to 1 gm N)
Arachin	18.30	4.23	0.231	0.250 (13)
Casein	16.07	2.98	0.185	0.231 (3)
				0.203 (12)
Coconut globulin	17.42	4.10	0.235	
Conarachin	18.20	2.88	0.158	
Cottonseed globulin	18.00	4.01	0.223	
Edestin	18.55	4.32	0.233	0.231 (12)
				0.250 (3)
Gelatin (Bacto)	18.32	8.98	0.490	0.500 (3)
				0.515 (12)
				0.450 (14)
Glycinin	17.30	3.76	0.218	
Lactalbumin	15.39	5.14	0.334	0.412 (3)
β-Lactoglobulin	14.22	6.50	0.457	0.426 (12)
				0.456 (10)
				0.397 (11)
Ovalbumin	15.98	6.57	0.411	0.475 (3)
				0.426 (12)
Ox muscle protein	16.00	6.03	0.377	
Peanut total globulins	18.01	3.89	0.216	
Phaseolin	16.07	3.26	0.203	
Wheat bran globulin	17.76	4.28	0.241	
Zein	16.00	10.35	0.647	0.770 (3)
				0.612 (15)

alanine values are reported and those reported varied widely. For β-lactoglobulin, Keston *et al.* (10) using the isotope method obtained 0.456 compared to our value of 0.457 per gram of nitrogen, and Brand *et al.* (11) reported 0.397 for the same material using *Streptococcus faecalis*. The values obtained by the partition chromatography method for alanine of β-lactoglobulin, egg albumin, edestin, gelatin, and casein agree closely with those obtained by the method reported here (12). Table 6 gives the alanine values found in some foods. The values summarized by Orr and Watt give the range of values for beef as 0.205 to 0.400 and for fresh pork as 0.185 to 0.400 per gram total nitrogen. The values obtained in this study were 0.351 for beef and 0.362 for pork per gram total nitrogen.

TABLE 6
ALANINE CONTENT OF FOODS

Protein source	Nitrogen (%)	Alanine content (gm)	
		Per 100 gm food	Per gm N
Cereal			
Barley (*Hordeum sativum*)	1.68	0.45	0.267
Corn germ	3.93	1.42	0.361
Corn, whole (*Zea mays*)	1.78	0.86	0.483
Oat, whole (*Avena sativa*)	2.42	0.67	0.277
Rice, brown (*Oryza sativa*)	1.27	0.33	0.260
Rice, white	1.19	0.30	0.252
Rye, whole (*Secale cereale*)	2.03	0.54	0.266
Wheat, flour (70% extraction, *Triticum vulgare*)	2.22	0.39	0.176
Wheat germ (commercial sample)	5.15	1.62	0.314
Wheat, whole	1.83	0.42	0.230
Egg, whole dried	6.03	1.67	0.277
Legume			
Bean			
Lima (*Phaseolus lunatus*)	3.46	0.94	0.271
Navy (*Phaseolus vulgaris*)	3.57	1.01	0.283
Red kidney	3.96	1.04	0.262
Soy (*Glycine hispida*)	6.21	1.62	0.260
Lentil	4.48	0.98	0.219
Pea (*Vigra sinensis*)			
Blackeye (Cow pea) (*Pisum saliorem*)	3.41	0.92	0.269
Green split	3.78	0.98	0.259
Meat			
Chicken, without skin			
Dark	3.07	0.99	0.322
Light	3.60	1.25	0.347
Hamburger	3.25	1.14	0.351
Lamb, leg	3.22	1.18	0.366[a]
Lamb, loin	2.89	0.99	0.343
Pork, loin	3.70	1.34	0.362
Pork, ham	3.48	1.30	0.373[a]
Turkey, without skin			
Dark	3.38	1.29	0.381
Light	3.87	1.53	0.395
Milk, nonfat dried	5.51	1.09	0.198
Nut, oil seed			
Brazil nut meal (defatted) (*Bertholletia excelsa*)	7.49	1.53	0.204
Cottonseed meal (defatted) (*Gossipum*)	6.66	1.44	0.216
Peanut (*Arachis hypogaea*)	4.59	1.19	0.259
Yeast (dried brewers')	6.82	2.96	0.434

[a] Alexander *et al.* (16) report 0.387 for lamb leg and 0.381 for pork ham calculated to 1 gm of nitrogen.

SUMMARY

A microbiological method is described for the determination of alanine in proteins and foods using *Leuconostoc citrovorum* 8081 as the test organism and using all L-amino acids in the medium. Alanine added to casein and red kidney beans before hydrolysis was recovered 101 and 95%, respectively. Reproducibility of values for a number of materials is reported and the average coefficient of variation from the mean is 3.6%. Consistent results were obtained over a wide range of values. Data are given for the alanine content of 48 proteins and foods.

REFERENCES

1. BARTON-WRIGHT, E. C., "Microbiological Assay of Vitamin B-Complex and Amino Acids," p. 179. Pitman, New York, 1952.
2. SNELL, E. E., "Microbiological Methods in Amino Acid Analysis," *Ann. N. Y. Acad. Sci.* **47,** Art. 2, 161 (1946).
3. SAUBERLICH, H. E., AND BAUMANN, C. A., *J. Biol. Chem.* **177,** 545 (1949).
4. HORN, M. J., AND WARREN, H. W., *J. Nutrition* **74,** 226 (1961).
5. HORN, M. J., AND BLUM, A. E., *Cereal Chem.* **33,** 18 (1956).
6. HORN, M. J., BLUM, A. E., GERSDORFF, C. E. F., AND WARREN, H. W., *J. Biol. Chem.* **203,** 907 (1953).
7. HORN, M. J., JONES, D. B., AND BLUM, A. E., *J. Biol. Chem.* **166,** 321 (1946).
8. HORN, M. J., JONES, D. B., AND BLUM, A. E., *J. Biol. Chem.* **169,** 71 (1947).
9. ORR, M. L., AND WATT, B. K., "Amino Acid Content of Foods," *Home Econ. Research Rept. U. S. Dept. Agr.,* No. 4 (1957).
10. KESTON, A. S., UNDENFRIEND, S., AND CANNON, R. K., *J. Am. Chem. Soc.* **71,** 249 (1949).
11. BRAND, E., SAIDEL, L. J., GOLDWATER, W. H., KASSALL, B., AND RYAN, F. J., *J. Am. Chem. Soc.* **67,** 1524 (1945).
12. BRAND, E., "Amino Acid Composition of Simple Proteins," *Ann. N. Y. Acad. Sci.* **47,** Art. 2, 187 (1946).
13. BLOCK, R. J., AND BOLLING, D., "The Amino Acid Composition of Proteins and Foods," 2nd Ed. C. C Thomas, Springfield, Ill., 1951.
14. CHIBNALL, A. C., *J. Intern. Soc. Leather Trades' Chemists* **30,** 1 (1946).
15. OSBORNE, T. B., AND LIDDLE, L. M., *Am. J. Physiol.* **26,** 295 (1910).
16. ALEXANDER, J. C., BECKNER, C., AND ELVEHJEM, C. A., *J. Nutrition* **51,** 319 (1953).

ANALYTICAL BIOCHEMISTRY 5, 78–82 (1963)

Microdetermination of Lipid Phosphorus as a Measure of Bacterial Membrane Substance[1]

JOSEPH J. KOLB, MARY A. WEIDNER, AND GERRIT TOENNIES

From the Institute for Cancer Research, Philadelphia, Pennsylvania

Received June 11, 1962

In studies of the effect of environment on bacterial anatomy and physiology (1) a method for the quantitative determination of bacterial membrane substance was desired. Studies on *Micrococcus lysodeikticus* indicate that bacterial membrane is rich in non-nitrogenous phospholipid (phosphatidic acid), which accounts for most of its phosphorus content (2), and that the membrane accounts for most of the lipid content of the whole cells (3).

Having found (4) that the membrane of *Streptococcus faecalis* resembles that of *M. lysodeikticus* in regard to phosphatidic acid content and that it is difficult to determine membrane substance by quantitative isolation, we considered the possibility that the membrane may be the sole seat of bacterial phospholipid. If this were the case, then determination of lipid phosphorus in whole bacteria or bacterial fractions might provide a quantitative index of the amount of membrane substance present.

Utilizing products obtained by a mechanical fractionation procedure (5), the experiments to be described showed that the wall is essentially free of lipid and that cytoplasm does not contain more than 4% of the cellular lipid phosphorus. Therefore, lipid phosphorus may serve as a true index of membrane substance.

The analytical procedure used in these studies was developed on the basis of existing methods, adapted to the task of reproducibly determining 2–20 μg lipid phosphorus in the presence of a twentyfold excess of nonlipid phosphorus and in samples corresponding to 10–20 mg bacterial substance. Lyophilized cells or membrane of *S. faecalis* are refluxed with 95% methanol in order to split protein-lipid complexes (6) and the resulting extract is evaporated to dryness. In order to eliminate all nonlipid P, the evaporation residue is taken up in petroleum ether and the

[1] Aided by a research grant of the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service (E-1935), and a research contract (Nonr-244) with the Office of Naval Research. Presented at the Fourth Delaware Valley Regional Meeting of the American Chemical Society, January, 1962.

solution is equilibrated with water (7). Then a portion of the clear petroleum ether solution is evaporated to dryness, a Kjeldahl digest made (8), and the phosphoric acid determined colorimetrically with molybdate hydrazine reagent (9).

METHOD

For reasons of ready extractability and avoidance of denaturation, lyophilized cellular material should be used. The samples are dried to constant weights. A pair of plastic closures from 2-dram size Kimble No. 60975 L vials, with their top surfaces removed, make convenient closed weighing bottles (1.9 gm) for hydroscopic products. Samples are transferred to 40- or 50-ml conical centrifuge tubes. The inside surface of these tubes is sand blasted to a height of 6–8 mm to ensure fast and smooth boiling (10). The sample is wetted with a few drops of 95% methanol and stirred with a rod. This is followed by washing rod and walls with 4 ml 95% methanol and refluxing for 2 hr. A set of four of the pointed tubes, with reflux condensers, is conveniently heated in a Petri dish containing a 1-cm layer of aluminum pellets which is placed on an electric hot plate set at 475–500°F. After 1 hr of boiling, solids deposited above the surface of the boiling liquid should be washed down by gentle shaking, or a little more solvent, just enough to cover the ring, should be added through the top of the condenser. At the end of 2 hr the tubes are raised and allowed to cool. Deposited material is scraped down into the liquid and the rod or microspatula used is rinsed off with a little 95% methanol. The capped tubes are centrifuged at 3000 rpm (about $2000 \times g$) for 10 min and the supernatants are decanted into 50-ml centrifuge tubes. As a washing procedure the packed material is stirred in the remaining liquid, resuspended by squirting in 4 ml 95% methanol, and refluxed for 15 min, followed by centrifuging and decanting.

The combined supernatant and wash of each sample is evaporated to near dryness in a water bath of 80–85°C under a stream of nitrogen. The nitrogen enters through disposable capillary glass pipets (145 mm long) inserted to about 1 cm below the surface and maintained at that depth to prevent deposition of substance too high up on the pipet. When 0.05–0.10 ml liquid remains, the tubes, with the pipets still inside, are placed in an inclined position into a desiccator with silica gel for drying under vacuum overnight.

The following day the pipet tips are cut off 3.5–4 cm long and the tips returned to their tubes. The tubes are stoppered and weighed to the nearest centigram. Ten-ml petroleum ether (b.p. 30–60°C) is pipetted into each tube. The tubes are stoppered and weighed, and then set up in the pellet bath for 30-min refluxing. The cooled and stoppered tubes

are weighed again and any weight loss is made up by addition of petroleum ether. Immediately thereafter 30–40 ml ice cold water is added, and the tubes are stoppered, thoroughly shaken, and allowed to stand for 3–4 hr. This equilibration period for the removal of water-soluble contaminants is essential in the case of lipid nitrogen determinations, and advisable also in the case of lipid phosphorus analyses.

The petroleum ether layers are transferred with a syringe to cool 15-ml graduated conical centrifuge tubes and the capped tubes are spun in a refrigerated centrifuge for 10 min at 3000 rpm. Seven ml of the clear upper layers is then transferred to 10-ml Kjeldahl flasks and evaporated to dryness under the hood, by immersing the flasks to ¾ of their length in a hot water bath. Two-tenths ml 1:1 sulfuric acid is used for Kjeldahl digestion (8). After addition of 2 ml water to the cooled digest it is boiled for a minute or more to hydrolyze pyrophosphates (11). The digest is then brought to 10 ml in a volumetric flask, including four 1.5-ml washings of the Kjeldahl flask.

Phosphate standards containing 0.15, 0.3, 0.6, 0.9, 1.2, and 2.4 μg phosphorus are diluted to 2 ml with water and 1 ml of molybdate hydrazine reagent is added. The tubes are capped and, together with 0.5, 1, 1.5, and 2 ml of the digests treated the same way, heated for 10 min in a boiling water bath.

The reagent is made by mixing equal volumes of 1.1 gm ammonium molybdate in 100 ml 6 N sulfuric acid and 36 mg hydrazine sulfate in 100 ml water. The separate solutions are stable for months. The reaction mixtures are cooled in a water bath to room temperature and read against reagent blanks at 830 mμ in a Beckman DU spectrophotometer. The colors are stable for several hours. The average deviation of individual values from the mean is about $\pm 2\%$. The advantages of the Boltz and Mellon method are a high degree of stability of the color and the fact that the intensity of the color read at 830 mμ is three times that measured at 720 mμ in other procedures.

RESULTS

1. *Lipid Phosphorus of Whole Cells.* In order to evaluate the practical usefulness of the method an attempt was made to determine the lipid P content of rapidly growing cells of *S. faecalis* 9790 at different stages of the exponential phase. In our buffered synthetic medium (12) this organism grows with constant duplication time of about 32 min beyond an adjusted optical density (AOD) of 2000, which corresponds to 0.8 mg dry weight/ml medium. Samples are removed at different stages, rapidly chilled to stop growth, and centrifuged in the cold. After decanting the supernatant medium the bacterial pellet was lyophilized. Since the

medium used contains no lipid P, the cells were not washed. Their weight was estimated from the volume of culture used and its net AOD, i.e., the AOD of the culture at the moment of chilling minus the AOD of the supernatant, and the value of 0.39 μg dry weight/ml/AOD (13).

TABLE 1
LIPID PHOSPHORUS OF EXPONENTIALLY GROWING CELLS

Sample No.	Turbidity (AOD)	Sample size (ml)	Dry wt., est. (mg)	Lipid P found	
				μg	%
1	119	350	13.48	18.37	0.136
2	119	350	13.78	19.38	0.141
3	420	120	17.08	22.74	0.133
4	420	120	16.96	23.65	0.139
5	1701	30	19.62	23.9	0.122
6	1701	30	19.22	25.6	0.133

The analytical results are shown in Table 1. They indicate that throughout the exponential growth phase the lipid phosphorus is essentially constant. On the basis of the nucleic acid content (1), the estimated total P value is about 3.2%. Therefore, the lipid P represents only 4% of the total phosphorus, and in the above series could be determined with a precision of about $\pm 4\%$, i.e., $\pm 0.16\%$ of the total phosphorus.

2. *Lipid Phosphorus of Cell Membrane.* Membrane material made in this laboratory (4) gave lipid P values of $0.85 \pm 0.01\%$, $1.00 \pm 0.05\%$, and $1.17 \pm 0.02\%$ for the membranes of exponential, threonine-depletion, and valine-depletion cells, respectively.

3. *Lipid Phosphorus of Cell Wall.* Four samples, weighing 11.3–16.6 mg, of different lyophilized cell wall preparations (1) gave lipid P values of 0.00, 0.00, 0.00, and 0.03%. Since wall substance comprises 25–44% of the total cell substance, we may conclude that the pure wall substance is free of lipid P.

4. *Lipid Phosphorus of Soluble Cell Fraction.* A pool, weighing 363 mg, of different lyophilized preparations of the soluble cytoplasmic fraction (1), dissolved in 25 ml water was centrifuged for 1 hr at about 150,000 $\times g$. A washed lyophilized pellet corresponding to 15.6% of the substance used was obtained. The supernatant and the pellet were separately subjected to lipid P determination, with the following results:

307 mg supernatant substance gave 24 μg lipid P
56 mg insoluble pellet gave 48 μg lipid P

Since the total "soluble fraction" represents 56–75% of the total cell

substance (1), the sample used corresponds to at least 480 mg cell substance and, on the basis of the value of 0.13% lipid P (Table 1), would contain about 625 μg lipid P. We may assume that the high speed pellet represents a mixture of ribosomes and of finely fragmented walls and membranes. On the basis of our lipid P data on membrane substance the lipid P values of supernatant and pellet would indicate that they contain about 0.9 and 9%, respectively, of membrane substance.

If the lipid P of the supernatant did not represent membrane substance, the fact that it accounts for no more than 4% of the total cellular lipid P does not invalidate our notion that lipid P may serve as a quantitative index of membrane substance. Different bacterial cell types are now being investigated.

SUMMARY

A microprocedure for the precise determination of bacterial lipid phosphorus under conditions where it represents less than 5% of the total phosphorus, is presented, together with analytical results which indicate that bacterial lipid phosphorus may serve as a quantitative index of bacterial membrane substance.

REFERENCES

1. TOENNIES, G., BAKAY, B., AND SHOCKMAN, G. D., *J. Biol. Chem.* **234**, 3269 (1959).
2. GILBY, A. R., FEW, A. V., AND MCQUILLEN, K., *Biochim. et Biophys. Acta* **29**, 21 (1958).
3. MACFARLANE, M. G., *Biochem. J.* **79**, 4P (1961).
4. SHOCKMAN, G. D., KOLB, J. J., BAKAY, B., CONOVER, M. J., AND TOENNIES, G., *J. Bacteriol.*, in press.
5. SHOCKMAN, G. D., KOLB, J. J., AND TOENNIES, G., *Biochim. et Biophys. Acta* **24**, 203 (1957).
6. REICHERT, R., *Helv. Chim. Acta* **27**, 961 (1941).
7. FOLCH, J., ASCOLI, I., LEES, M., MEATH, J. A., AND LEBARON, F. N., *J. Biol. Chem.* **191**, 833 (1951).
8. MILLER, G. L., AND MILLER, E. E., *Anal. Chem.* **20**, 481 (1948).
9. BOLTZ, D. F., AND MELLON, M. G., *Anal. Chem.* **19**, 873 (1947).
10. KOLB, J. J., AND TOENNIES, G., *Chemist-Analyst* **50**, 82 (1961).
11. UMBREIT, W. W., "Manometric Techniques and Related Methods for the Study of Tissue Metabolism," p. 165. Burgess, Minneapolis, 1945.
12. SHOCKMAN, G. D., KOLB, J. J., AND TOENNIES, G., *J. Biol. Chem.* **230**, 961 (1958).
13. SHOCKMAN, G. D., *J. Biol. Chem* **234**, 2340 (1959).

ANALYTICAL BIOCHEMISTRY 5, 83–91 (1963)

SHORT COMMUNICATIONS

Thin-Layer Starch Gel Electrophoresis on Glass Slides[1]

Starch gel electrophoresis, which was introduced by Smithies (1, 2), has been used widely to separate different proteins. In the modification described below, the flexibility of this technique as an analytical tool has been extended by pouring the gel in a thin layer on glass slides.

Hydrolyzed starch[2] is suspended in buffer at the rate of 10 gm/100 ml. This mixture is placed in a water bath and heated to boiling while being stirred continuously. The hot solution of starch is kept in the water bath and deaerated under reduced pressure. A large pipet is filled with starch solution, and 5 to 6 ml is poured in a uniformly thin layer, approximately 1.5 mm, on each of several glass slides, $25 \times 150 \times 1$ mm. Prior to pouring the gel, the slides are placed on a glass tray supported over a layer of water in a plastic box. The lid of the box is closed subsequently to prevent evaporation from the surface of the gel. The slides of gel are stored in the box at room temperature until they are used. Concentrations of starch higher than 10 gm/100 ml yield solutions that are too viscous to flow well from the pipet and over the surface of the glass slides.

The surface of the gel is covered as shown in Fig. 1 with two pieces of Saran Wrap,[3] the edges of which are folded beneath the slide. The sample slot (Fig. 1) is made by removing a narrow strip of gel from the slide. A special cutter is required, which is fashioned from two razor blades that have a cutting edge of 15 mm. The blades are placed parallel to each other on either side of a plastic spacer, 0.5 mm thick, and bound together with tape. The cutter is pressed vertically into the gel until the cutting edges touch the glass slide, thus forming two parallel slits. After withdrawing the cutter, a very slender spatula having a square tip is

[1] This investigation was supported in part by a research grant, A-2230, from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

[2] Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada.

[3] Dow Chemical Company, Midland, Michigan.

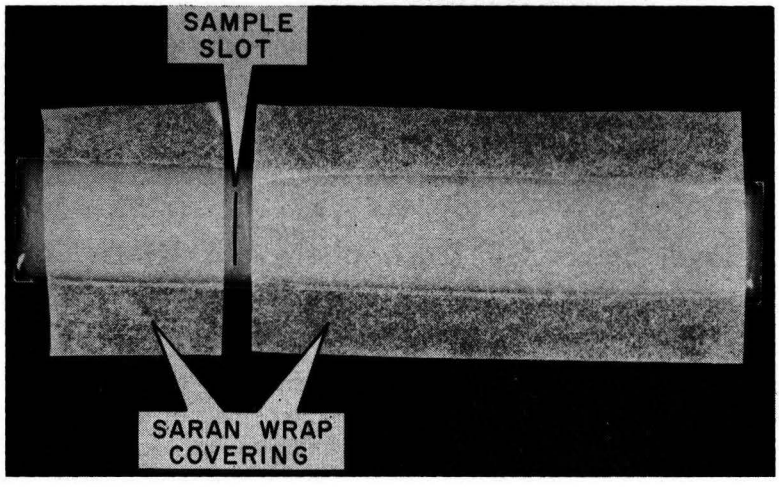


FIG. 1. Slide of gel showing the position of the sample slot and the Saran Wrap covering. For clarity of illustration, waxed paper has been substituted for Saran Wrap.

pressed vertically into the gel at either end of the parallel slits, thus creating the ends of the sample slot. With care, one end of the narrow gel strip is compressed slightly, the spatula is slipped beneath it, and the strip is lifted from the sample slot.

Approximately 0.015 ml of sample (tissue extract, blood serum, etc.) is placed in the slot. Both the slot and the surrounding area of exposed gel are sealed immediately with melted petrolatum dispensed from a pipet. The petrolatum should be just warm enough to flow freely from the pipet. The slides thus prepared are placed horizontally in an electrophoresis tray, and the exposed ends of gel are connected to the buffer chambers with flannel bridges (Fig. 2). After electrophoresis, the Saran Wrap covering and the petrolatum seal are lifted carefully from the surface of the gel. For enzyme location, the slide is turned glass-side up and immersed in an appropriate incubation medium. The gel loosens in a few minutes, and the glass is removed. If the gel adheres to the glass, the slide probably was not cleaned adequately prior to use. For protein location, the gel is loosened in distilled water, and surface of the gel is flooded with a staining solution.

Electrophoresis in a thin layer seems to offer several advantages over the use of a thicker gel. First, it lessens the effect of electrodecantation during the entry of migrating proteins into the gel, although this effect is eliminated completely in the vertical procedure of Smithies (2). Higher voltage gradients can be applied across a thin layer because heat dissipa-

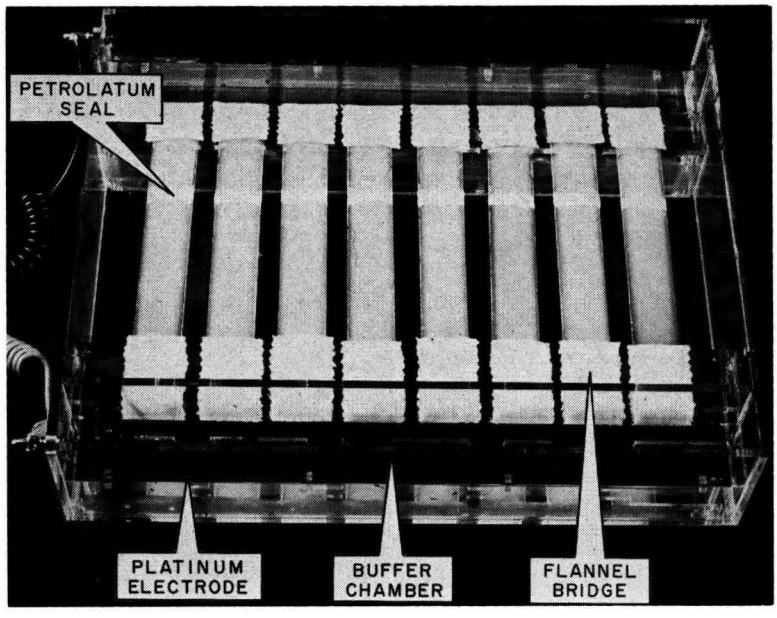


FIG. 2. Electrophoresis tray with the slides of gel and the flannel bridges in place.

tion is more rapid per unit volume of gel. For example, a gradient of 15 volts/cm has been used satisfactorily in gel prepared with 0.025 *M* borate buffer, pH 8.55. With both the reduced concentration of starch in the gel and the higher voltage gradient, the rate of protein migration is

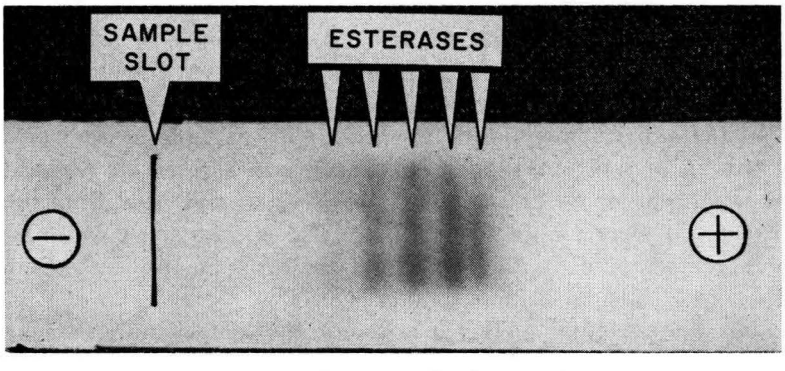


FIG. 3. Electrophoretic pattern of esterases in the duodenal contents of the young calf. Gel buffer, 0.025 *M* borate, pH 8.55. Voltage gradient, 12 volts/cm. Electrophoresis time, 90 min. Substrate, 1-naphthyl butyrate. Coupling agent, tetrazotized *o*-dianisidine.

increased markedly. Thus, the time required for electrophoresis is reduced to approximately 2 hr.

This technique of thin-layer starch gel electrophoresis has been used extensively by the author to study esterases in various biological materials. An example of the separation of five esterases in the duodenal contents of the young calf is illustrated in Fig. 3. In general, the quality of resolution has been equal to that obtained with a lower voltage gradient, 5 volts/cm, in gels containing 12 to 15% starch.

REFERENCES

1. SMITHIES, O., *Biochem. J.* **61**, 629 (1955).
2. SMITHIES, O., *Biochem. J.* **71**, 585 (1959).

HAROLD A. RAMSEY

Department of Animal Science
North Carolina State College
Raleigh, North Carolina
Received July 19, 1962

A Convenient Cuvette Rinsing Device

The present communication describes a simple device which could be constructed by any competent glass blower to efficiently rinse the inside of 10-mm square cuvettes with a minimum of handling. The cuvette rinser is illustrated in Fig. 1. It consists of an outer bowl 70 mm in diameter which is connected through an outlet tube to a waste sink. Fused to the bowl is a central tube with a 15-mm ball float valve. The valve is activated by the weight of the cuvette depressing a long extension of the ball which projects above the thin part of the central tube. The bottom of the central tube is connected to a reservoir of water or other wash solution by means of a rubber stopper and a length of tubing. The reservoir should be a foot or more higher than the cuvette rinser. Four notches are cut in the top of the central tube to direct the wash solution to all inside surfaces of the cuvette.

In use, the cuvette is merely inverted on the central tube. The cuvette contents and wash solution are caught in the outer bowl and carried to a drain or waste receptacle. The inside of the cuvette is continuously flushed until the cuvette is removed. At no time do the outside surfaces of the cuvette get wet. To dry the cuvettes rapidly another rinser can be

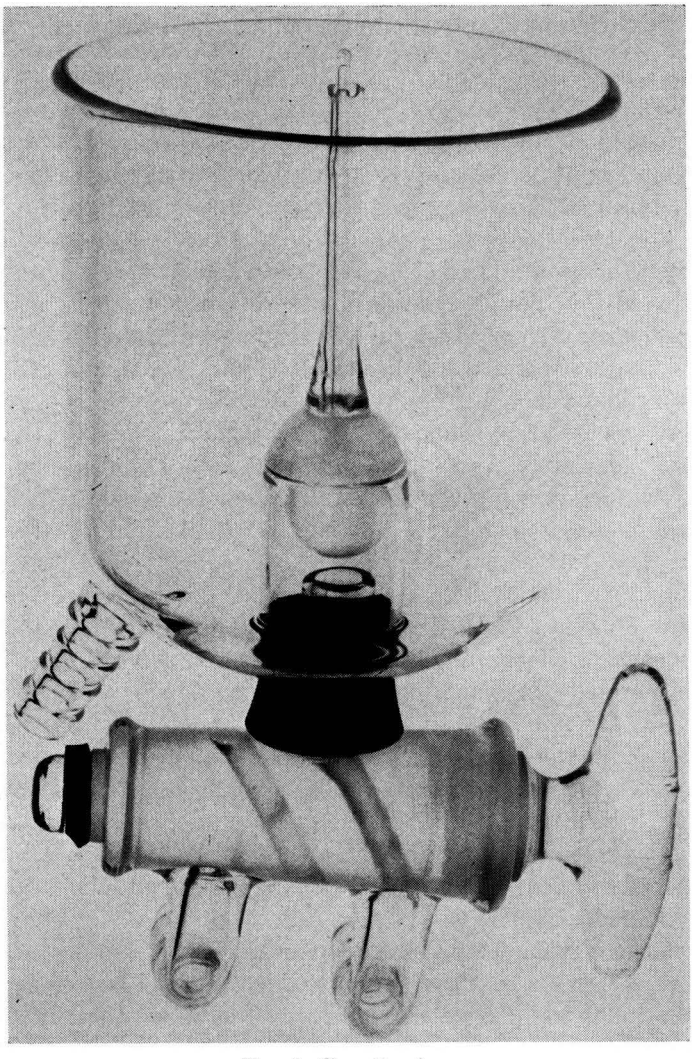


FIG. 1. Cuvette rinser.

set up with acetone as the wash solution. Alternatively, a three-way stopcock can be incorporated in the solution line to allow the use of two different solutions with a single cuvette rinser.

ARTHUR L. BABSON
WILLIAM CAMPBELL

The Warner-Lambert Research Institute
Morris Plains, New Jersey
Received August 6, 1962

Radioassay of Cholesterol-C^{14} Digitonide[1]

In the course of an investigation involving the isolation and assay of a large number of samples of cholesterol-C^{14} as the digitonide, it was deemed advisable to investigate all methods available for solubilization of the cholesterol-digitonin complex in order to ascertain which was most suited to rapid and efficient radioassay. Cholesterol digitonide has been assayed by liquid scintillation spectrometry after solution in H_3PO_4-*p*-dioxane (1) or methanol (2) or by sealing the solid in plastic which is immersed in the solution of liquid scintillator (3). Since cholesterol digitonide can be split by heating with pyridine (4), xylene (5), benzene (6), or pyridine:acetic anhydride 1:1 (7)—all materials which are completely miscible in common liquid scintillation solvent systems—we compared them for efficiency and ease in radioassay of cholesterol-C^{14} digitonide. The digitonide was prepared by the method of Sperry and Webb (8). Assays were carried out in a Packard Tri-Carb Liquid Scintillation Spectrometer using a solution of 0.6% PPO and 0.02% dimethyl POPOP in toluene.

The quenching effects of a number of potential solvents were studied. Pyridine lowered the counting efficiency too much to be of value; Hyamine and acetic anhydride could be of potential utility if small (less than 1 ml) volumes were used. In the volume range under consideration (1–2 ml) glacial acetic acid, methanol, and ethanol gave comparatively little quenching. The findings with pyridine, methanol, and ethanol are in accord with those of other authors (9, 10). Cholesterol, cholesterol dibromide, cholesterol digitonide, and digitonin did not give appreciable quenching.

To test the efficiency of the solvent systems under study, triplicate samples of a standard amount of digitonide (0.8 mg) were treated with one of the following: 0.5 ml Hyamine, 2 ml each of ethanol, methanol, boiling pyridine, boiling acetic acid, boiling acetic anhydride, and boiling acetic anhydride-pyridine (1:1) or 4 ml each of boiling xylene, benzene, or toluene. Complete solution was effected only with Hyamine, methanol, acetic acid, pyridine and acetic anhydride-pyridine (1:1). After 2 min the solution was transferred to the scintillation counting vial and the residue washed with ether, which was added to the solution to be assayed. While all the solvents used will split the digitonide on prolonged heating, only those listed above gave complete solution under our

[1] This work was supported (in part) by grants H-3299 and H-5209 from the National Institutes of Health, U. S. Public Health Service.

conditions of treatment. In every case in which a residue remained, it was dissolved in methanol and the resulting solution accounted for the counts not recovered in initial treatment. Samples were also prepared using phosphoric acid-*p*-dioxane (1:99). Since Funt and Hetherington (11) have shown that some insoluble materials can be dispersed for counting with the aid of gel formation, samples of cholesterol digitonide were suspended in the scintillation solution containing either 2.4% Cab-O-Sil or 2.5% Thixcin R. Recovery of radioactivity is summarized in Table 1. Corrections have been made to account for quenching effects

TABLE 1
RECOVERY OF CHOLESTEROL-C^{14} FROM CHOLESTEROL DIGITONIDE

Solvent[a]	Dpm recovered[b]	Recovery (%)
Original solution	19,514	—
Acetic anhydride-pyridine (1:1)	19,302	99
Phosphoric acid-*p*-dioxane (1:99)	19,152	98
Hyamine	18,882	97
Methanol	18,721	96
Ethanol	18,609	95
Acetic anhydride	18,416	94
Thixcin R	14,531	74
Toluene	13,203	68
Acetic acid	11,489	59
Pyridine	10,972	56
Cab-O-Sil	10,200	52
Xylene	6,314	32
Benzene	3,518	18

[a] Treatment for 2 min with 2–4 ml of solvent (except for Hyamine, 0.5 ml used).
[b] Average of triplicate determinations. Corrected by internal standardization.

of the various solvents. The extent of solubilization of digitonide by the various treatments is reflected in the recovered radioactivity. It is evident that with ethanol and methanol one can achieve the most rapid quantitative assay of cholesterol from labeled cholesterol digitonide.

In a study of the solubility of cholesterol and its digitonide, Schoenheimer and Dam (4) found that 1 ml of methanol could dissolve 6 mg of cholesterol and 5 mg of the digitonide, while 1 ml of ethanol could dissolve 19 mg of cholesterol but only 0.7 mg of the digitonide. In our study, 0.8 mg of digitonide was used. One milliliter of Hyamine will dissolve as much as 100 mg of cholesterol digitonide. From these considerations methanol or Hyamine would appear to be best suited for direct assay of cholesterol digitonide.

The radioassay of cholesterol-C^{14} digitonide by gas flow technique

was also investigated using methanol as the solvent for solution and plating. A Tracerlab CompuMatic II gas flow counter was used. Construction of self-absorption curves for cholesterol-C^{14} and cholesterol-C^{14} digitonide was carried out, with results shown in Fig. 1. Infinite

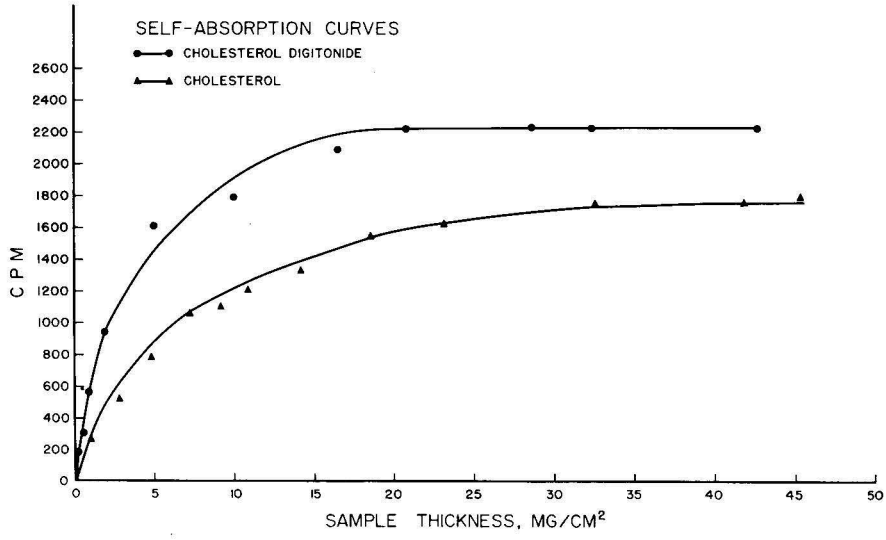


FIG. 1. Self-absorption curves for cholesterol-C^{14} and cholesterol-C^{14} digitonide.

thickness for cholesterol was reached at 30 mg/cm² and for the digitonide at 20 mg/cm². Plating of duplicate aliquots of cholesterol-C^{14} digitonide from methanol gave an average recovery of 3443 cpm as compared with 12,684 cpm for aliquots assayed by liquid scintillation counting. Thus it would appear that the plating and flow counting method is practical for the assay of cholesterol-C^{14} digitonide when no liquid scintillation spectrometer is available or when it is desired to recover the sample for subsequent chemical manipulations.

ACKNOWLEDGMENT

We are indebted to Hoffmann-La Roche Co., Nutley, New Jersey, and to Merck and Co., Rahway, New Jersey, for generous gifts of the digitonin used in this study.

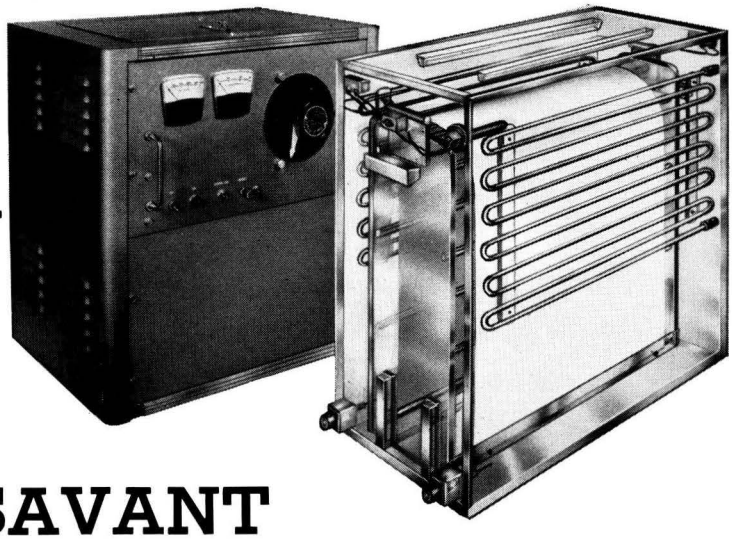
REFERENCES

1. KABARA, J. J., *J. Lab. Clin. Med.* **50,** 146 (1957).
2. WILSON, J. D., AND SIPERSTEIN, M., *Am. J. Physiol.* **196,** 596 (1959).
3. GRIMES, E. T., *Anal. Chem.* **34,** 1175 (1962).
4. SCHOENHEIMER, R., AND DAM, H., *Z. physiol. Chem.* **215,** 59 (1933).
5. WINDAUS, A., *Z. physiol. Chem.* **65,** 110 (1910).

6. KELSEY, F. E., *J. Biol. Chem.* **127,** 15 (1939).
7. WINDAUS, A., *Z. physiol. Chem.* **101,** 276 (1918).
8. SPERRY, W. M., AND WEBB, M., *J. Biol. Chem.* **187,** 97 (1950).
9. KERR, V. N., HAYES, F. N., AND OTT, D. G., *Intern. J. Appl. Radiation Isotopes* **1,** 2846 (1957).
10. STITCH, S. R., *Biochem. J.* **73,** 287 (1959).
11. FUNT, B. L., AND HETHERINGTON, A., *Science* **131,** 1608 (1960).

IRWIN L. SHAPIRO
DAVID KRITCHEVSKY

The Wistar Institute of Anatomy and Biology
Philadelphia, Pennsylvania
Received September 11, 1962



SAVANT

(formerly SERVONUCLEAR CORP.)

HIGH VOLTAGE PAPER

ELECTROPHORESIS

SYSTEMS—*up to 10,000 VOLTS*

For

- *Identifying and characterizing Protein Material*
- *Amino Acid Analysis*
- *Peptide (Fingerprinting)*
- *Nucleotides*
- *Nucleic Acids*

Investigate today, write for Bulletin AB-1004 for complete information.

Five complete systems for every type of laboratory. Tanks are constructed of Lucite or Fibreglass as the main structural material. They are inert to most chemicals used in Electrophoresis application and the Lucite tank has the distinct advantage of being transparent. The investigator sees at all times what is taking place.
Tank racks will accept paper from 8″ to 60″ in length, up to 20″ in width. The paper is cooled by a high flash point liquid, aliphatic-hydrocarbon, Savant EC-123. It is now possible to achieve the maximum resolution through the application of higher voltages, up to 10,000 Volts. Today, laboratories around the world are using Savant's Lucite and Fibreglass tanks with confidence.

SAVANT INSTRUMENTS, INC.

221 PARK AVENUE • HICKSVILLE, NEW YORK

Automatic Protein
and/or
Peptide Analyses

(Either Individual Samples or Column Effluent)

Quantitate...

Total Nitrogen by Kjeldahl

Total Protein by Biuret

Total Protein by Folin–Ciocalteau (Lowry modification)

Amino Groups by Ninhydrin

Tyrosine by Folin–Ciocalteau

Histidine by Pauly Diazo Reaction

Arginine by Sakaguchi

Glutamic Acid by Decarboxylase

Lysine by Decarboxylase

Albumin by HABA Dye [2-(4′ hydroxyazobenzene) benzoic acid]

Analyze...

Biological Fluids directly

Effluent from DEAE or Resin Chromatographic Column

Effluent from Poraeth Electrophoretic Column

Electrophoretic Starch Block Fractions

Electrophoretic Paper Chromatogram Segments

Check point stages in Protein Fractionation

Counter Current Distributions

with the TECHNICON®

AutoAnalyzer®

Any or all of these determinations may be run on the same AutoAnalyzer: Takes only two minutes to change from one type of analysis to another. Any combination may be run simultaneously from the same sample by adding additional standard AutoAnalyzer modules. The response time is such that most of the analyses may be run at 40 samples per hour.

For information, select area of interest and write to

TECHNICON CHROMATOGRAPHY CORP.
22 RESEARCH PARK • CHAUNCEY, NEW YORK

BIOCHEMICAL APPLICATIONS OF GAS CHROMATOGRAPHY

By **H. P. BURCHFIELD,** *Southwest Research Institute, San Antonio, Texas and* **ELEANOR E. STORRS,** *Boyce Thompson Institute, Yonkers, New York*

1962, 680 pp., $22.00

This book fulfills a need for a text that supplies both theoretical background and details of the applications of gas chromatography to biochemical problems. Specific procedures are supplied together with enough background information to enable the experimentalist to improvise adaptations without an extensive study of the literature. Although the work is organized along biochemical lines, information is included that will be of value to workers in other fields, such as air polution and petroleum chemistry.

Contents

General Techniques, Conventions, and Instrumentation. Permanent Gases and Condensable Organic Vapors. Volatile Components of Tissues and Biological Fluids. Cyclic Compounds. Essential Oils. Resin Acids. Lipids. Nonvolatile Components of Tissues. Review of Miscellaneous Applications Related to Biochemistry. Appendixes: Manufacturers of Gas Chromatographs and Auxiliary Equipment; Comparison Table of United States, British, and German Sieve Sizes with Tyler Equivalents; Hazards Associated with the Use of Diazomethane. Author Index–Subject Index–Methods Index. Index of Stationary Liquids Used in Methods.

"The book is well laid out, of a convenient size to handle despite its 680 pages and the print both clear and comfortable to read. Altogether I find this an admirable book and I hope that this will be made apparent to the authors by the number of copies sold; it should be in every library used by chemists."

S. R. Elsden in *Chemistry and Industry*

ACADEMIC PRESS, New York and London

111 Fifth Avenue, New York 3, New York

Berkeley Square House, London, W. 1, England

AP 1053

Biological Transmission of Disease Agents

Symposium Held under the Auspices of the Entomological Society of America, Atlantic City, 1960

Edited by KARL MARAMOROSCH
Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York

March 1962, 192 pp., $7.00

The biological transmission of disease agents is of considerable interest and importance to workers in branches of biology, particularly to microbiologists, zoologists, virologists, plant pathologists, entomologists, veterinarians, and physicians. The chapters in this work, contributed by biologists of widely different interests and experience, cross the traditional and professional boundaries between workers engaged in plant and animal disease study.

CONTENTS

Agents of Plant Disease

Contributions by: L. M. BLACK, WILLIAM B. HEWITT, D. J. RASKI, K. SAKIMURA, JOHN T. SLYKHUIS, and EDWARD S. SYLVESTER

Agents of Human and Animal Diseases

Contributions by: DARRELL W. ANTHONY, HERBERT C. BARNETT, D. BLASKOVIC, A. W. LINDQUIST, CORNELIUS B. PHILIP, WILLIAM C. REEVES, J. REHACEK, R. TRAUB, and K. C. WILLETT A. W. LINDQUIST, CORNELIUS B. PHILIP, WILLIAM C. REEVES, J. REHÁCEK, R. TRAUB, and K. C. WILLETT.

ACADEMIC PRESS, New York and London

111 Fifth Avenue, New York 3, New York
Berkeley Square House, London, W.1, England

AP-874

ANALYTICAL BIOCHEMISTRY 5, 93–98 (1963)

Low-Level Carbon-14 Determination by Improved Schöniger Combustion and Ionization Chamber

SUSANNE VON SCHUCHING AND CARL W. KARICKHOFF

From the Radioisotope Service, Veterans Administration Center, Martinsburg, West Virginia

Received February 27, 1962

A procedure for measuring low levels of C^{14} radioactivity, diluted with large amounts of inactive carbon in heterogeneous samples, has been developed. It was used during a metabolic balance study of L-ascorbic-1-C^{14} acid in monkeys (9) and humans. The techniques employed are described below, in particular the modifications developed in the use of a Schöniger (1, 2) type of oxidation flask for the conversion of liquid and solid samples to barium carbonate.

APPARATUS

The design of the combustion flask is shown in Figs. 1 and 2. The thickwalled round-bottom flask withstands pressures better than an Erlenmeyer flask. The ignition head was changed from its original design (3) by the addition of a platinum mesh basket and the construction of four prongs to hold glass slides. The apparatus for conversion of barium carbonate to carbon dioxide and the ionization chamber for measuring its activity are shown in Fig. 3.

PREPARATION OF SPECIMEN

In addition to the preparation of specimens on glass wool, on filter paper (4), or in a dialysis bag (5), we have used a microscope slide for drying liquid specimens when it is not desirable to add extra carbon. It is very important that the dried sample on the glass slide touches the ignition coil at one point.

COMBUSTION PROCEDURE

Before insertion of the specimen, the flask containing 30 ml of 0.5 *N* sodium hydroxide is flushed with oxygen for 3–4 min at a flow rate of 5 liters/min. Then the ignition head with the sample in place is inserted into the flask and a large wire spring is placed over the top of the rubber stopper (Fig. 2). The electrical connections are made, the flask on the

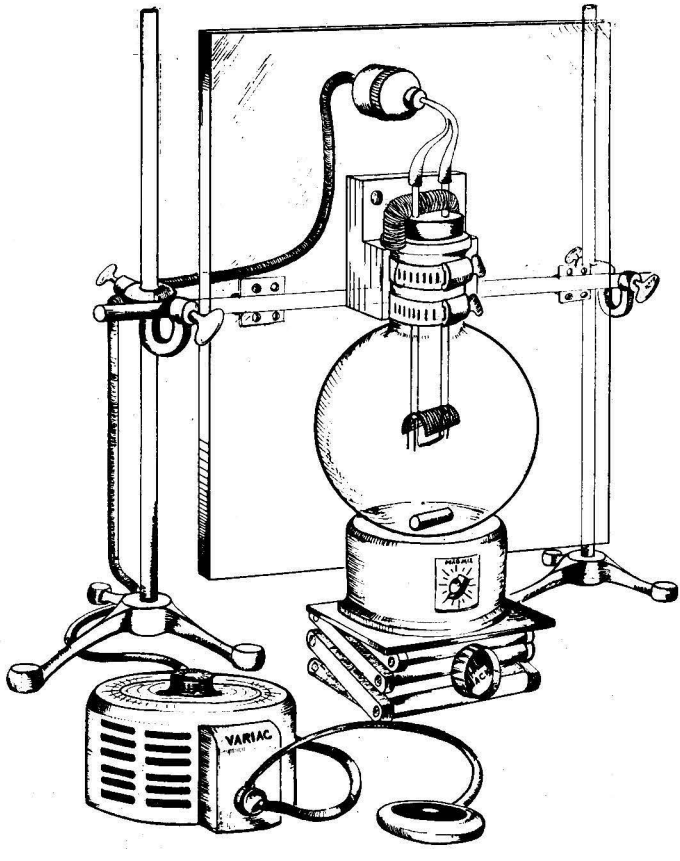


FIG. 1. Complete assembly of combustion apparatus.

safety shield is carefully inverted through 180°, and a current of 2 amp at 9–10 volts is applied. The wire becomes bright red and the progress of the combustion can be followed visually. The flask is rotated (6) to its upright position and stirred with a magnetic stirrer for 10 min, while still attached to the safety shield, immediately following the combustion, in order to complete the absorption of carbonate in the alkali.

The life of the ignition coil depends on the heat generated by the combustion of the sample. The average life of the coil is between 10–20 combustions, but it was found that compounds like 2,4-dinitrophenyl hydrazones necessitated replacement of the coil after 1–2 combustions. If glass wool is used to hold the sample, the wool must be spread very thin to allow an adequate amount of oxygen to reach the sample. It was found that a flux of glucose is not required (5).

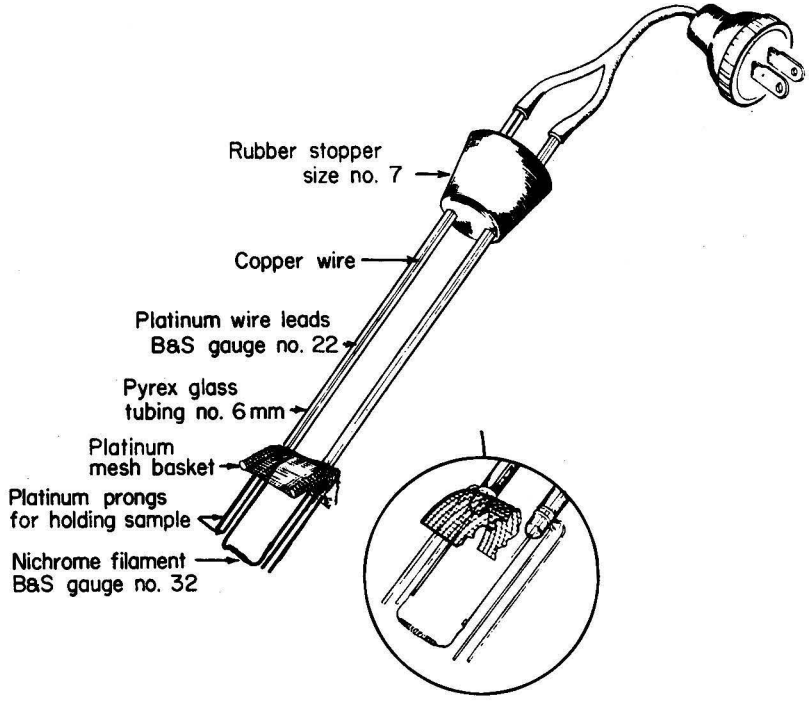


FIG. 2. Details of ignition head.

LIBERATION OF CARBON DIOXIDE INTO THE IONIZATION CHAMBER (7)

Twenty-five milliliters of the contents of the combustion flask are pipetted into a 100-ml round-bottom flask which contains a magnetic stirring bar. The contents of the flask are frozen by surrounding it with dry ice to allow evacuation of the system without loss from the sample, and the flask is attached to the ionization chamber, as shown in Fig. 3. A drying tube, filled with anhydrous calcium sulfate, between the flask and the ionization chamber protects the latter from moisture. A side arm funnel allows the addition of lactic acid into the flask. The system (flask and ionization chamber) is evacuated by means of a mechanical pump. Following a period of evacuation stopcock 3 (Fig. 3) between the pump and the system is closed. Ten milliliters of lactic acid is added slowly to the flask through the funnel. A seal of lactic acid is left above stopcock 4 so that no air is introduced into the system. The mixture is allowed to thaw and carbon dioxide is gradually liberated and expands into the ionization chamber. The reaction is allowed to continue with

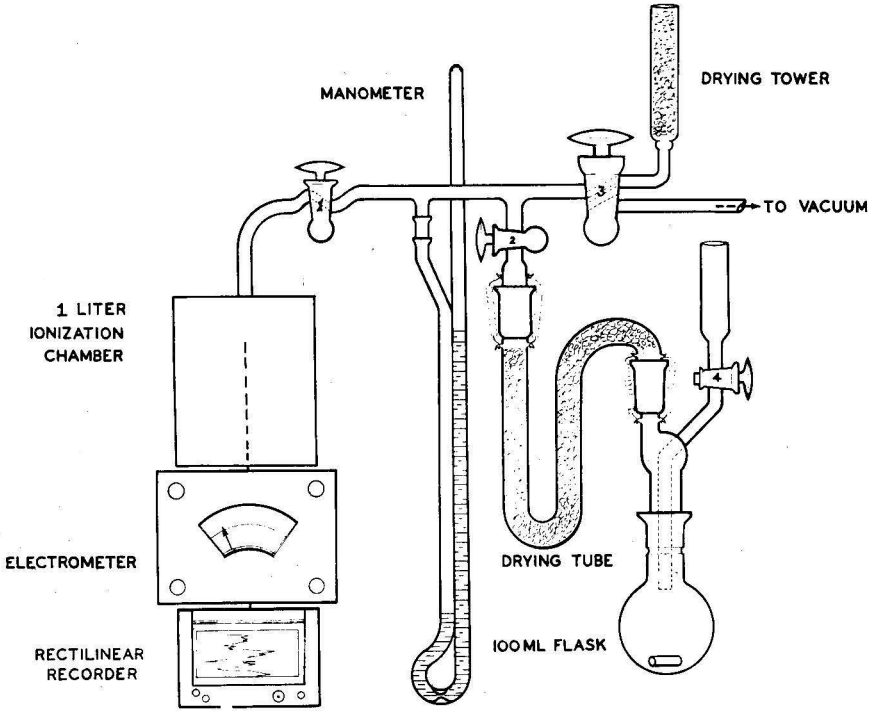


FIG. 3. Ionization chamber filling apparatus for $C^{14}O_2$.

stirring until there is no further rise in pressure as shown by the manometer, indicating the complete liberation of $C^{14}O_2$.

When no more $C^{14}O_2$ is developed, a slow stream of air is allowed to enter through stopcock 4 (Fig. 3) until the ionization chamber is at atmospheric pressure. Next, stopcock 1, between the ionization chamber and the flask, is closed, the ion collector is disconnected, and the ion chamber discharge is recorded. Radioactivity is recorded in divisions per minute, where 1 μc of C^{14} gives 2.839×10^3 net division per minute.

RESULTS AND DISCUSSION

The general procedure described above is particularly useful for all types of heterogenous samples encountered in biological experiments. The following examples illustrate this point.

The wide range of samples and recovery of specific activity can be noted in Table 1. If the sample weight is below 25 mg, extra carbon is added by combusting it with a piece of filter paper, weighing 25–75 mg.

Samples such as $C^{14}O_2$ from exhaled air absorbed in 10% NaOH

TABLE 1

SPECIFIC RADIOACTIVITY OF HETEROGENEOUS SAMPLES OF VARYING WEIGHTS

Sample	Weight (mg)	Radioactivity (div/min)	Specific radioactivity		Standard deviation (μc/mg)
			div/min/mg	μc/mg	
C^{14} plastic standard attached to Pt	26.5	83.8	3.15	1.109×10^{-3}	
prongs (New England	59.4	192.0	3.22	1.134×10^{-3}	
Nuclear Corp.) Manu-	109.0	350.0	3.21	1.131×10^{-3}	
facturers information:	170.7	565.0	3.33	1.173×10^{-3}	
1.182×10^{-3} μc/mg	201.5	636.0	3.16	1.113×10^{-3}	
0.3916 mg carbon/mg	301.1	968.0	3.22	1.134×10^{-3}	$1.132 \times 10^{-3} \pm 0.0146$
Ascorbic-1-C^{14} acid (20 λ = 0.8 mg)	52.0 (Filter paper)	1145	1.4312×10^{3}	5.08×10^{-1}	
placed on filter paper	92.0	1093	1.3662×10^{3}	4.82×10^{-1}	
	87.0	1135	1.4188×10^{3}	5.01×10^{-1}	$4.97 \times 10^{-1} \pm 0.135$
Ascorbic-1-C^{14} acid placed	25.0	972	3.88×10^{1}	1.32×10^{-2}	
on glass wool	15.0	578	3.85×10^{1}	1.35×10^{-2}	
Bis-2,4-dinitrophenylhydrazone of ascorbic acid					
On glass wool	50.0	148	2.96	1.04×10^{-3}	
	123.0	386	3.14	1.10×10^{-3}	
On filter paper	8.0	24	3.01	1.06×10^{-3}	$1.07 \times 10^{-3} \pm 0.0264$
C^{14} in muscle as residue dried at	405 wet wt.	238	5.88×10^{-1}	2.07×10^{-4}	
100°C placed on glass wool	250 wet wt.	145	5.8×10^{-1}	2.05×10^{-4}	
C^{14} in bone	231.0	59.3	2.57×10^{-1}	9.07×10^{-5}	
C^{14} in org. material only placed on glass wool	190.0	46.6	2.45×10^{-1}	8.64×10^{-5}	
C^{14} urine in unknown products					
Dried in plastic bag	1.0 ml	2690	2.69×10^{3}/ml	9.47×10^{-1}/ml	
Dried on microscope slide	1.0 ml	2670	2.67×10^{3}/ml	9.47×10^{-1}/ml	

or $CaC^{14}O_3$ from bone which do not require combustion, are acidified with lactic acid and the liberated $C^{14}O_2$ is measured in the ionization chamber. $C^{14}O_2$ from radioactive calcium oxalate is liberated with permanganate (8).

Cross contamination in this type of apparatus is very low. If the ionization chamber is flushed repeatedly with air the same background is obtained even if it has been previously filled with a sample of high activity. We have used the added precaution of using a separate 2-liter flask for the combustion of high activity samples and always wash the glassware with Radiac (Atomlab Products Company, Center Moriches, N. Y.) solution.

ACKNOWLEDGMENT

The authors wish to acknowledge the encouragement of Dr. Arthur F. Abt during the course of this study.

REFERENCES

1. HEMPEL, W., *Z. angew. Chem.* **5**, 393 (1892).
2. SCHÖNIGER, W., *Mikrochim. Acta* **1955**, 123; **1956**, 896.
3. JUVET, R. S., AND CHIN, J., *Anal. Chem.* **32**, 130 (1960).
4. MARTIN, A. J., AND DEVERAUX, H., *Anal. Chem.* **31**, 1932 (1959).
5. KELLY, R. G., PEETS, E. A., GORDON, S., AND BUSYKE, D. A., *Anal. Biochem.* **2**, 267 (1961).
6. A. H. Thomas Apparatus Co., Catalogue, 61st Ed., p. 637.
7. CHINARD, F. P., TAYLOR, W. R., NOLAN, M. F., ENNS, T., *Am. J. Physiol.* **196**, 535 (1959).
8. WILLARD, H. W., AND FURMAN, N. H., "Elementary Quantitative Analysis," p. 98. Van Nostrand, New York, 1935.
9. ABT, A. F., VON SCHUCHING, S., AND ENNS, T., *Nature* **193**, 1178 (1962).

ANALYTICAL BIOCHEMISTRY 5, 99–106 (1963)

Determination of Urinary Pregnanediol by Gas Chromatography[1]

DAVID A. TURNER, G. E. SEEGAR JONES, I. J. SARLOS, ALLAN C. BARNES, AND RICHARD COHEN

From the Biochemistry Research Division, Sinai Hospital of Baltimore, Inc., and the Department of Gynaecology and Obstetrics, Johns Hopkins Hospital, Baltimore, Maryland

Received March 12, 1962

Since Horning *et al.* (1) first published on the use of gas chromatography in the identification of urinary steroid hormone metabolites a number of papers have appeared on the subject, outstanding work being by Lipsky (2). However, little work has appeared using these techniques in biological material. Horning has published the chromatogram of the urinary 17-ketosteroid from a normal female (3) and Wotiz has reported that analysis of urinary estrogens is under way (4).

Pregnanediol, the major urinary metabolite of progesterone, is excreted in relatively large amounts, 2 to 5 mg/24 hr, during the normal menstrual cycle following ovulation, and as much as 50 mg is usually found per 24 hr in late pregnancy. Because of the quantities present and also because the compound is fully saturated and stable, it seemed an ideal steroid to attempt to identify and quantitate from biological sources by gas chromatography. For this purpose, a gas chromatography column has been prepared which will satisfactorily separate the major groups of urinary steroid metabolites (5). Presumably because of the inert nature of the particular liquid phase used, no apparent degradation of the steroids occurs (1).

The retention times of fourteen urinary steroid metabolites has been determined and related to the retention times of pregnanediol. Standard solutions of pregnanediol have been quantitated. Twenty-four hour urine specimens from patients with different clinical conditions have been analyzed to determine whether the technique is applicable to biological material, or if there are interfering substances, and a quantitative recovery of both pregnanediol and conjugated pregnanediol sodium glucuronide added to urine and aqueous solutions has been attempted.

[1] This work was supported in part by Public Health Service Grant HTS-5399 and by a grant from the Maryland Division of the American Cancer Society.

METHODS

The steroid metabolites in urine or aqueous extracts were hydrolyzed either with 15% hydrochloric acid for 10 min under toluene, or with β-glucuronidase,[2] 1000 units/ml at pH 4.6, 37°C, for 24 hr. Following hydrolysis, the urine was extracted three times with 10 ml of toluene per 100 ml of urine, the toluene extract evaporated, and the crude residue taken up in 2 ml of ethanol. Depending on the concentration of the sample, 2- to 10-μl aliquots were then placed directly on the gas chromatography column. For the purpose of quantitative analysis, a pregnanediol standard of known concentration was chromatographed, prior to the analysis of each biological sample, and the peak area calculated by triangulation and expressed as μg/ml. Areas of pregnanediol peaks of unknown samples were measured and compared with the standards. The differences in the retention times of pregnanediol which appear in the figures are a reflection of experimental changes in flow rate or column temperature. The other conditions of analysis were identical in each case. *Column conditions:* 6 ft × 4 mm i.d. glass column; 2% silicone gum rubber containing 50 mole % cyanoethylpolysiloxane[3] on 100–120 mesh Gas Chrom P; argon ionization detector; column temperature 205°C; inlet pressure 12 psi; outlet flow 45 ml/min; flash heater 275°C; voltage 1000.

EXPERIMENTAL

Comparison of Retention Times for Urinary Steroid Metabolism

The retention times for the following steroids, which are common urinary metabolites, have been determined: androsterone, etiocholanolone, dehydroepiandrosterone, tetrahydrocortisone, estradiol, estrone, estriol, Δ^5-androstenediol, Δ^4-androstenedione, 11β-hydroxyandrosterone, 11β-hydroxyetiocholanolone, 11-ketoetiocholanolone, allopregnanediol, and pregnanolone. Only allopregnanediol and pregnanolone, both of which are also metabolic products of progesterone, offer any difficulty in separation from pregnanediol under the conditions suitable for pregnanediol identification (Table 1). An effort was made, without success, to effect a separation of these compounds using a 5% column (Fig. 1).

Relation of Peak Area to Concentration

Pregnanediol standards containing 1, 0.5, and 0.25 mg/ml were prepared and quantitatively measured by gas chromatography. Good agree-

Warner Chilcott & Co., Ketodase.

Synthesized by Dr. A. C. Martellock of Silicone Products Division, General Electric Company, Waterford, New York. Material Code #287-108-949.

TABLE 1

COMPARISON OF RETENTION TIMES FOR SOME URINARY STEROID HORMONE METABOLITES IN RELATION TO PREGNANEDIOL

Standard	T_r (relative)
Δ^5-Androstene-3β-17α-diol	0.650
5β-Androstan-3α-ol-17-one (androsterone)	0.712
5β-Androstan-3α-ol-17-one (etiocholanolone)	0.712
Dehydroepioandrosterone	0.766
5β-Pregnane-3α,20α-diol	1.000
5β-Pregnan-3α-ol-20-one	1.010
5α-Pregnane-3α,20α-diol (allopregnanediol)	1.021
Tetrahydrocortisone	1.521
11-keto-5β-androstan-3α-ol-17-one (11-ketoetiocholan-ol-17-one)	1.524
17β-Estradiol	1.553
Δ^4-Androstene-3,17-dione	1.570
Estrone	1.691
11β-Hydroxy 5β-androstan-3α-ol-17-one (11β-hydroxyetiocholanolone)	2.030
11β-Hydroxy 5α-androstan-3α-ol-17-one (11β-hydroxyandrosterone)	2.290
Estriol	5.532

Column conditions: 6 ft × 4 mm i.d. glass column; 2% silicone gum rubber containing 50 mole % cyanoethylmethylpolysiloxane[3] on 100–120 mesh Gas Chrom P; argon ionization detector; column temperature 205°C; inlet pressure 12 psi; outlet flow 45 ml/min; flash heater 275°C; voltage 1000.

ment could be obtained on repeated determinations in five separate experiments. A representative experiment is shown in Table 2.

Identification and Quantitation in Biological Material

As biological material often presents problems different from experimental standards, pregnanediol identification was made in urine

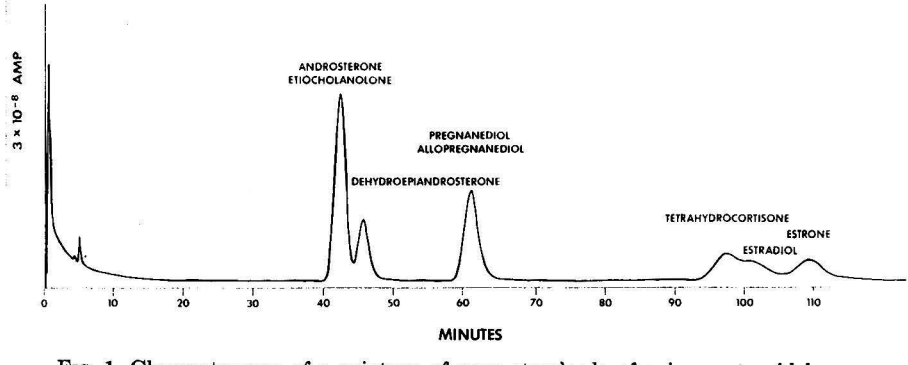


FIG. 1. Chromatogram of a mixture of pure standards of urinary steroid hormone metabolites. Column temperature 200°C, cell temperature 250°C, flash heater 280°C, inlet pressure 15.5 psi, flow rate 75 ml/min.

TABLE 2
QUANTITATION OF PREGNANEDIOL STANDARDS BY TRIANGULATION OF PEAKS

Concn. standard (μg/ml)	Amt. injected (λ)	Area (mm^2)
250	10	94
500	10	214
1000	5	212

collections from pregnant women, menopausal women, amenorrheal women, normal cyclic women, and men. A peak with identical T_r to pregnanediol or related metabolites appeared in all instances in which progesterone metabolites were expected (Fig. 2). When a pure pregnane-

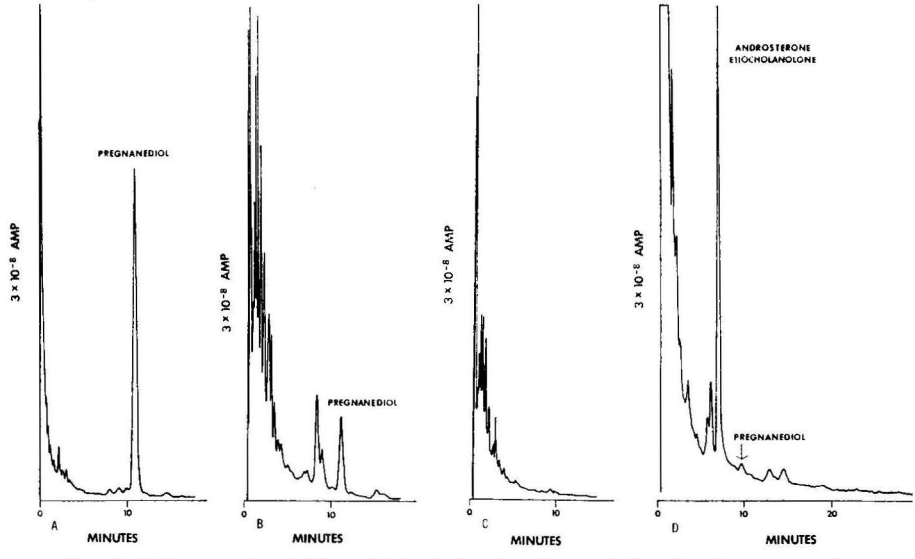


FIG. 2. Toluene-extractable acid hydrolyzates from: (A) urine in a normal pregnancy; (B) urine during the post-ovulatory phase of the menstrual cycle; (C) urine of an amenorrheal woman; (D) male urine. The peaks marked pregnanediol are also known to contain allopregnanolone.

diol standard was added to urine containing negligible quantities of pregnanediol, 0.05 mg/24 hr, under the conditions and sensitivity used the peaks again appeared as expected (Fig. 3).

To test the quantitative recovery, known amounts of pregnanediol and sodium pregnanediol glucuronide were added to urine samples found to contain negligible amounts of pregnanediol, 0.05 mg/24 hr. This urine was from an amenorrheal patient with gonadal agenesis who was excreting

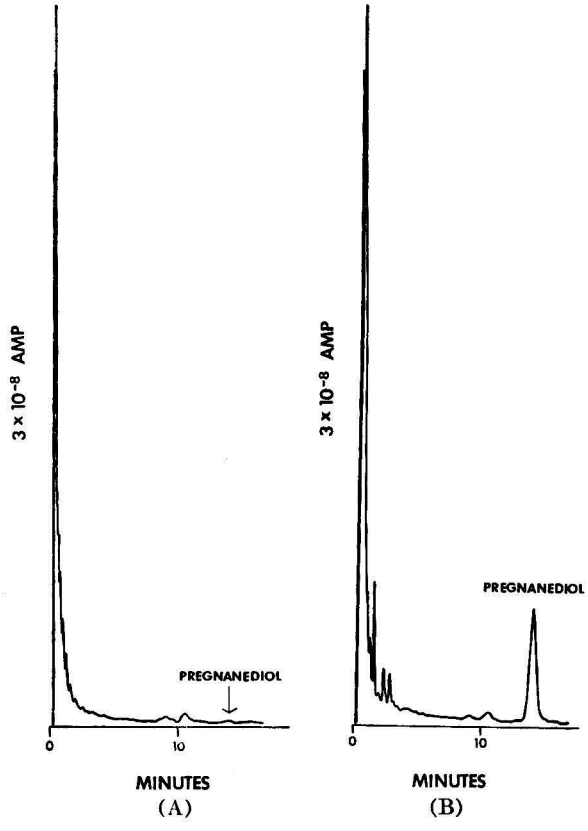


FIG. 3. (A) Toluene-extractable acid hydrolyzate of urine of postmenopausal woman. (B) Same sample with pregnanediol added.

about 2 mg of total 17-ketosteroids per 24 hr. To obtain any peak of pregnanediol in urine from this patient it was necessary to concentrate the alcoholic extract of 100 ml of urine to 0.5 ml and inject 10 μg of this on the column. The steroid metabolites of urine were hydrolyzed either by acid, as described in the Astwood-Jones method (6), or by β-glucuronidase, extracted with toluene which was then evaporated under vacuum, and the crude residue redissolved in ethanol. The alcoholic solution was analyzed directly by gas chromatography. One of the best experiments is reproduced in Table 3. A comparable recovery experiment was attempted from distilled water. With acid hydrolysis recoveries from urine ranged from 69 to 95%. Recovery from distilled water was 80% for sodium pregnanediol glucuronide, and 85% for unconjugated pregnanediol. Using enzymic hydrolysis on one occasion only,

TABLE 3
RECOVERY OF SODIUM PREGNANEDIOL GLUCURONIDE ADDED TO URINE[a]

Expt.	Volume of urine hydrolyzed and extracted (ml)	Pregnanediol expected[b] (μg)	Pregnanediol obtained (μg)	Per cent recovery
1A	100	290	250	86.4
1B	100	290	280	96.6
1C	100	290	250	86.4
2	700	2090	2000	95.7
Total	1000	2960	2780	93.3
Control	100	0	0	0

[a] 5 mg NaPG/liter added to urine containing 0.05 mg pregnanediol per 24 hr.
[b] Calculated from sodium pregnanediol glucuronide with a factor of 0.597.

recovery from urine was 84%. Quantitative recovery was possible when as little as 0.25 mg of pregnanediol was added to a 24-hr urine specimen. Under these circumstances the 24-hr volume of 1720 ml was divided into two 800-ml volumes after the removal of 100-ml control fraction and the steroids in these volumes were hydrolyzed and extracted. An average recovery of 0.115 mg was obtained from the expected 0.125 mg.

Reproducibility

The reproducibility of measurements as well as recovery can be seen in Table 4. Urine samples from two pregnant patients were run in

TABLE 4
REPRODUCIBILITY: RECOVERY OF PREGNANEDIOL IN DUPLICATE ALIQUOTS HYDROLYZED AND EXTRACTED SEPARATELY FROM URINE OF PREGNANT PATIENTS A AND B

Sample	Peak area (mm^2)		Pregnanediol (mg/100 ml)	
A. 100 ml[a]	124.8	127.3	0.56	0.56
100 ml	166.1	167.6	0.74	0.74
200 ml	149.7	142.7	0.66	0.60
200 ml	120.3	119.7	0.54	0.54
B. 100 ml[b]	67.2	71.0	1.7	1.8
	67.8	71.0	1.7	1.8
100 ml	71.0	64.0	1.6	1.8

[a] Final volumes of urine sample extracts were adjusted so that peak areas and volumes injected would be of the same order of magnitude.
[b] Final residue dissolved in 5 ml absolute alcohol and two 2-ml aliquots removed for individual quantitation.

duplicate; in one instance two 200-ml aliquots and two 100-ml aliquots from the same specimen, and in the other case two 100-ml aliquots, were compared. In each experiment the two measurements for the same sample showed close agreement.

DISCUSSION

In 1941 (6), when determinations were made on the recovery of pregnanediol after acid hydrolysis, it was found that an average recovery of 68% could be expected. When recovery was made from distilled water the loss was 20%. This loss could not be accounted for theoretically as the toluene extraction was found to be complete and the layer of toluene during acid hydrolysis should protect against destruction during hydrolysis. With the simplified procedure reported here, the recovery rates after acid hydrolysis varied between 69 and 95% and the best experiments indicated that a recovery of close to 90% might be possible. Analysis of the observed variability showed that it is probably a result of loss or destruction during hydrolysis and extraction, rather than due to errors in quantitation following gas chromatographic analysis (Table 4).

Gas chromatographic identification and quantitation was performed

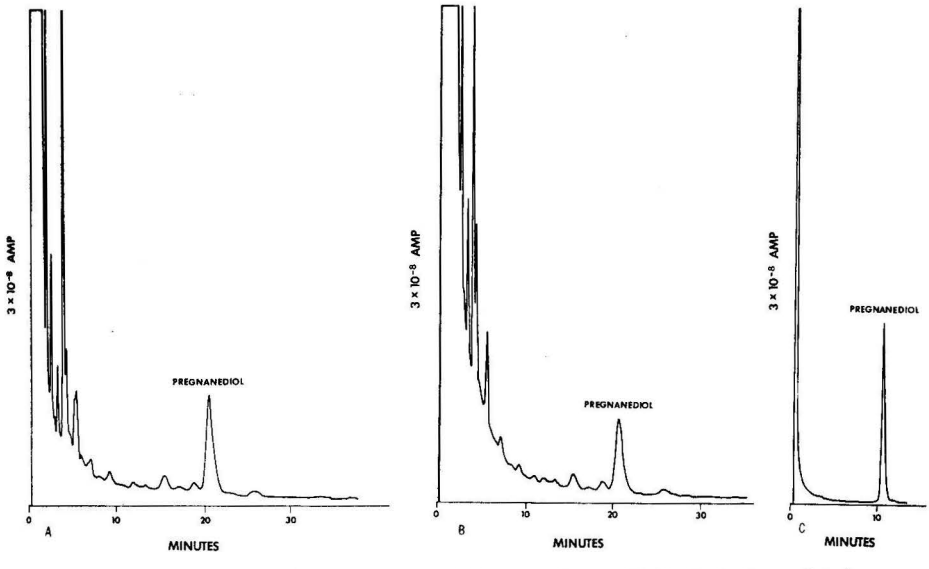


FIG. 4. Comparison of purification techniques after acid hydrolysis and toluene extraction: (A) acid hydrolysis and toluene extraction only; (B) followed by sodium methanol wash; (C) aqueous alcohol precipitation by the Astwood gravimetric method.

on samples after various states of purification in the Astwood-Jones method, e.g. (*1*) following removal of the phenolic fraction with sodium methanol, and (*2*) after two aqueous alcohol precipitations. Some differences in purity was obvious in the tracings (Fig. 4); however, there was no enhancement of ability to calculate the pregnanediol by area triangulation.

CONCLUSIONS

A gas liquid chromatographic method for the identification and quantitation of urinary pregnanediol has been described. Recovery of the metabolite seems to be consistent and sensitivity is satisfactory, as little as 0.25 mg in a 24-hr sample being quantitatively recovered. The method is not specific for pregnanediol as closely related steroid metabolites, allopregnanediol, and pregnanolone have retention times on the column used which are too similar to allow for separation, although later work indicates that a 2% SE-30 (General Electric Co.) column will separate the three major metabolites of progesterone satisfactorily. Vanden Heuvel *et al.* (7) were unable to do this with a 0.75% SE-30 column but could do so using a 1% Q. F. 1 phase (Dow-Corning Corp.).

REFERENCES

1. VANDEN HEUVEL, W. J. A., SWEELEY, C. C., AND HORNING, E. C., *Biochem. Biophys. Research Communs.* **3**, 33 (1960).
2. LIPSKY, S. R., AND LANDOWNE, R. A., *Anal. Chem.* **33**, 818 (1961).
3. HAAHTI, E. O. A., VANDEN HEUVEL, W. J. A., AND HORNING, E. C., *Anal. Biochem.* **2**, 182 (1961).
4. WOTIZ, H. H., AND MARTIN, H. F., *J. Biol. Chem.* **236**, 1312 (1961).
5. SJOVALL, J., MELONI, C. R., AND TURNER, D. A., *J. Lipid Research* **2**, 317 (1961).
6. ASTWOOD, E. G., AND SEEGAR JONES, G. E., *J. Biol. Chem.* **137**, 397 (1941).
7. VANDEN HEUVEL, W. J. A., HAAHTI, E. O. A., AND HORNING, E. C., *J. Am. Chem. Soc.* **83**, 1513 (1961).

ANALYTICAL BIOCHEMISTRY 5, 107–115 (1963)

Synthesis of High Specific Activity C^{14}-Carboxyl Indoleacetic Acid and of C^{14}-Nitrile Indoleacetonitrile[1]

BRUCE B. STOWE

From the Department of Botany, J. W. Gibbs Laboratory, Yale University, New Haven, Connecticut

Received May 22, 1962

INTRODUCTION

Studies of transport, metabolism, and mode of action of the plant growth hormone indoleacetic acid (IAA) and one of its natural precursors, indoleacetonitrile (IAN), would be greatly facilitated if high specific activity C^{14}-labeled material were readily available. Several radiosynthetic methods have been published, but each has certain limitations. Furthermore, commercially available C^{14}-labeled indoleacetic acid has been found wanting both in specific activity levels and in purity. This paper describes a convenient one-day synthesis of pure indoleacetic acid with sufficient specific activity for use at physiological concentrations. Alternatively, the indoleacetonitrile produced as an intermediate in the synthesis may be recovered and used for physiological investigations.

Syntheses for radioactive IAA labeled in the methylene carbon have been published by Heidelberger (1), Gordon (2), Stutz *et al.* (3), and Weygand and Linden (4), in the 2-C of the pyrrole ring by Pichat *et al.* (5), and in the benzene ring by Robinson (6). The last method also has been used by Andreae *et al.* (7) for carboxyl-labeled IAA. Similar reaction sequences have been used for the synthesis of animal indole hormones (8). A specific activity of 1 mc/mmole has been attained in several studies; the maximum specific activity has been reported by Stutz *et al.* (3), who obtained one lot of methylene-labeled IAA at 6.5 mc/mmole with a 69% radiochemical yield.

Labeling the carboxyl position is preferable for many purposes. It is well established that the carboxyl group of IAA is lost as carbon dioxoide when the hormone is inactivated by the common enzyme indoleacetic

[1] This work was aided by institutional grants to Harvard University and to Yale University from the American Cancer Society, and by N. S. F. Grant G9084 to Harvard University.

acid oxidase. For this reason, the amount of radioactive CO_2 or conventional decarboxylation products which appeared after treatment of plant tissue with carboxyl-labeled IAA would provide a measure of the *in vivo* activity of this enzyme. Any other radioactivity would represent either free intact growth hormone or still potentially biologically active conjugates. Andreae *et al.* (7) have carried out comparisons of this nature and cite papers of other workers. The use of carboxyl-labeled IAA in other physiological investigations is detailed and reviewed by Goldsmith and Thimann (9), and Gillespie and Thimann (10).

RESULTS

Of the synthetic methods for IAA, those proceeding from gramine by the replacement of its dimethylamine group with cyanide are the most attractive. C^{14}-Cyanide can be obtained at high specific activities; the methods give high yields and are relatively simple. The following procedures have been investigated.

Gramine. The method of Kühn and Stein (11) gave nearly quantitative yields from indole without major modifications. It was found possible to recover additional product by making the first filtrate alkaline, then cooling. The reacting materials should be kept out of strong light and the gramine washed thoroughly. Gramine holds an unusual amount of water; therefore it takes a long time to dry and relatively large amounts of a basic drying agent should be added to the desiccator. The product is sufficiently pure to be used in the next step without recrystallization.

Existing IAA Syntheses. The synthetic method used by Heidelberger (1) for methylene-labeled IAA could be used to label IAA in the carboxyl position. However, the method was reported to be unsatisfactory by Gordon *et al.* (2), even after a number of modifications. The method of Andreae *et al.* (7) requires a labeled organic acid as starting material. Therefore other routes were investigated.

The direct method of Snyder and Pilgrim (12) at first seemed suitable because it is carried out in one step. Repetition of their procedure confirmed that high yields were attained only when the five times excess of cyanide recommended by the authors was used. In the present work, yields of no more than 20% were obtained when equimolar amounts of cyanide and gramine were used, and the workup was complicated by the unreacted gramine. Evidently much of the cyanide decomposed during the long period of boiling, which would result in an expensive loss of radioactivity if $KC^{14}N$ were used. Carrying out the synthesis at various alkaline pH values did not lead to any improvement. Gordon *et al.* (2) also reported difficulty with this procedure. Accordingly, the single-step method was abandoned.

Quaternary Gramine Derivatives. An alternative synthetic route is to carry out the amine replacement with cyanide via a quaternary gramine salt. Two salts have been used, the methiodide and the methosulfate. When gramine methiodide was synthesized by the method of Potts and Robinson (13), recrystallization of the product was erratic and a large loss always occurred in the next reaction. These difficulties are believed due to the bi-indolyl salt, which predominates in the product, as pointed out by Geissman and Armen (14) and discussed further by Snyder and Cook (15).

The methosulfate thus seemed the more promising intermediate and the procedure of Schöpf and Thesing (16) was finally adopted. The first attempt at this synthesis resulted in a light red oil which resisted all efforts at recrystallization. However, when scrupulous care was taken to keep the reaction anhydrous, and the tetrahydrofuran and dimethyl sulfate were freshly redistilled, the reaction proceeded ideally. White crystals resulted after the mixture was left overnight at −10°C. The yields of gramine methosulfate were 87–98%. After washing with ether and filtration, these crystals must be kept completely dry and in weak light, or they rapidly become red. They are best used as is; recrystallization from various solvents always resulted in an increase in color.

IAN from Gramine Methosulfate. Two methods were investigated. That of Hill (17) carries out the reaction in dimethylformamide solution and, although good results were obtained, the workup was judged inconvenient with highly radioactive material. Thesing and Schülde (18) used an aqueous solution. The latter seemed preferable for radioactive work, as the subsequent hydrolysis to indoleacetic acid could be carried out directly in the same flask. Thesing and Schülde's conditions specify that three moles of cyanide should be used per mole of the methosulfate. A test confirmed this need, as 14, 54, 72, and 92% yields of IAN from gramine methosulfate were obtained with 1.0, 1.5, 2.0, and 3.0 mole equivalents of cyanide, respectively. Since the final pH of the reaction mixture became acid (2) the effect of various salts as buffers was tested. When KH_2PO_4 or concentrations of other buffering salts insufficient to keep the pH greater than 7 were employed, the globules of IAN formed were very dark and at best 20–40% yield from cyanide was obtained as IAA after hydrolysis; more often the yields were negligible. But if K_2HPO_4, $Na_4P_2O_7$, or $NaBO_3$ was used (concentrations near 0.2 *M* were suitable and not critical), yields ranged from 50 to 80% in many trials. K_3PO_4 proved to be too basic and the yield again dropped below 50%. Basic media also increase the radiation stability of IAA (19). Accordingly, 0.2 *M* K_2HPO_4 was adopted as the medium, and other variables investigated.

Keeping the reaction mixture under nitrogen had little effect on yield, but the appearance of the product was improved. Efforts to drive the reaction to completion, by removing the volatile trimethylamine produced (via self-distillation into a sulfuric acid solution placed in the same chamber), did not significantly increase the yields even though a number of procedural modifications were tried. Increasing the time of the reaction was unproductive and higher temperatures led to a more colored product. It is likely that decomposition of cyanide during the course of the reaction is largely responsible for the incomplete reaction. This cannot be the entire explanation, however, since a slight odor of cyanide is noted when the mixture is acidified subsequent to the alkaline hydrolysis.

Purification of IAN. IAN is usually obtained by vacuum distillation, but this would have been difficult with the small quantities used here. Instead, modifications of the method of Henbest *et al.* (20) were investigated. The picrate derivative they used, probably because of its acidity, readily degrades the IAN and consequently needs careful handling. More convenient was the trinitrobenzene adduct, which formed in 80% yield upon cooling a solution of 100 mg of IAN and 200 mg of 1,3,5-trinitrobenzene in 5 ml of ethanol. The adduct recrystallized beautifully, upon alternate crystallization from water and from CCl_4, in long orange needles, m.p. 139–141°C. The adduct is a convenient storage form for IAN, but must be decomposed for further use. Henbest *et al.* (20) used a gypsum column for this purpose which was not reinvestigated. Fractional elution from silica gel as outlined in the procedure below also was found to be suitable. Subsequently, it was found that the use of the adduct as an intermediate is unnecessary, since IAN can be directly purified via the column. The IAN fraction, detected by testing drops with the Salkowski reagent (21), can be dissolved in ether-hexane and upon storage at —10° it crystallizes. This crystallization step was omitted from the radioactive synthesis as it did not perceptibly increase the purity of the product and would have allowed more time for autoradiodecomposition.

IAN to IAA. This conversion is easily carried out by refluxing IAN in strongly alkaline aqueous solution, and, if the liquid volume is kept small, yields of greater than 90% are readily obtained. The intermediate product indoleacetamide, obtained by some authors (13, 15), has never been observed in our manipulations. Also, paper chromatograms prepared as described by Stowe and Thimann (21) did not reveal it.

Modifications Necessary for Use with Radioactive Materials. Because rubber tubing was found to pick up a high count of C^{14}-cyanide, an all-glass apparatus is preferable. Once radioactive IAA has been obtained

the crystalline material rapidly colors and eventually resinifies [R. E. Stutz, S. A. Gordon—personal communications; cf. (5)]. On the assumption that this is due to autoradiosensitivity, storage in dilute alcoholic solution has been used, but decomposition still continues [J. P. Nitsch, P. M. Ray—personal communications; cf. (22)]. Moreover, residual traces of alcohol interfere with plant hormone bioassays (23). Accordingly, a search was made for a free-radical quenching solvent with suitable properties. A study of the free radicals produced by irradiating a variety of organic solvents was carried out by Prévost-Bérnas *et al.* (24), who found propionitrile to be one of the least affected. Acetonitrile, assumed to have similar properties, was selected for our purpose, as it is miscible in water, easily evaporated, and relatively nontoxic to plants (25). This solvent is a highly effective stabilizer for labeled IAA at a radioconcentration of 10 μc/ml, and 0.96 mc/mmole IAA has now been stored in it under N_2 at $-10°$ for two years with no perceptible change in biological, paper chromatographic, or ultraviolet absorbing properties.

In over thirty nonradioactive trials of the procedure recommended below (or minor modifications), the over-all yields were within the range of 50–80%, calculated on the basis of cyanide. The radioactive synthesis was similarly satisfactory up to 0.1 mc/mmole, where 53% yield was obtained. But at ca. 1 mc/mmole only 34 and 61% resulted, and from the three syntheses at highest specific activity (16.9 mc/mmole), using 3.9 mc of $KC^{14}N$ per 2 ml of reaction medium, only 18, 25, and 20% of IAA were recovered by the identical procedure. An independent run of our procedure has confirmed the drop in yield when the high specific activity cyanide is substituted for unlabeled cyanide (P. Albersheim—personal communication). This consistently reduced yield makes it very likely that, at the higher radioactivity levels, extensive decomposition of the indole system is taking place in the aqueous reaction medium. In fact Allsopp and Wilson (26) found indole relatively radiosensitive, several hundred molecules being decomposed for each ion pair formed in the medium. They concluded that free radicals formed from the water initiated the chain mechanism. IAA apparently has a higher order of stability (19), but in our case the major dose of radiation must be received during the IAN synthesis. At this time IAN globules are forming and autodecomposition is probably at a maximum. It seems then that optimal yields at high specific activities will be obtained only if the synthesis is carried out diluted in a nonaqueous solvent under free-radical quenching conditions. Probably the dimethyl formamide reaction medium employed by Hill (17) could be modified to this purpose.

Despite the losses experienced at the highest radioactivity levels, white microcrystalline IAA of excellent quality was obtained by the procedure

given below. Melting points were in the range 165–170° (Hoffman-LaRoche IAA measured 169–172°). Tests of purity (kindly performed by Dr. Mary Helen M. Goldsmith) showed that in all but one case paper chromatograms (21) had 97–98% of the radioactivity in one spot, the residual activity being distributed uniformly along the strips. Since the Salkowski indole reaction (21) similarly detected only one spot, unlabeled indolic impurities also should be less than 1%. The single impurity discovered by chromatography of material obtained in one synthesis is described by Goldsmith and Thimann (9), who devised a method for its removal. The *Avena* curvature test gave satisfactory quantitative bioassays with the material. The ultraviolet spectrum of the IAA solution was identical with that of Hoffmann-LaRoche IAA, with one exception. One batch (that of 18% yield above) whose crystals were slightly pinkish upon precipitation, showed a peak at 320 mμ whose optical density was 13% of that of the 280 mμ IAA peak. All commercially available radioactive IAAs which we have examined have one or more major impurities readily detected by the above tests.

METHODS

Indoleacetic Acid. A 5-ml $\overline{\$}$ 10 round-bottom flask equipped with a $\overline{\$}$ 10 distillation head is fitted with a glass capillary drawn from a $\overline{\$}$ 10 inner joint placed in the upper joint so that its point is at the base of the flask. A $\overline{\$}$ 10 outer joint is then drawn to another capillary and fitted to the side arm joint in such a manner that the capillary fits into a 5-ml test tube containing 3 ml of 1 *N* H_2SO_4 to provide a pressure relief outlet and bubble counter. All steps are carried out in a hood equipped for radioisotope work, and wearing face mask and gloves. Gramine methosulfate (70 mg) and 15 mg of $KC^{14}N$ (New England Nuclear NEC-79H) are then added to the 5-ml flask and 2 ml of 0.2 *M* K_2HPO_4 (previously equilibrated with N_2) added by syringe, washing down the joint and sides of the flask. The mixture is agitated with a slow stream of N_2 bubbles until all solids are dissolved, and then the stream reduced to the minimum possible rate. The apparatus is then placed in a water bath and brought to a temperature of 65°. After 4 to 6 hr, the originally cloudy solution clears, and a single slightly colored droplet of IAN forms at the bottom of the flask. No increase in yield is obtained after the cloudiness disappears. If IAN is the desired product the flask is removed at this point and treated by the alternative procedure below.

For IAA, still head and side arm are removed, one pellet of KOH and a silicon carbide boiling chip are added, and a reflux condenser is attached. The flask is then placed in a 50-ml beaker with just enough mineral oil to coat the base of the flask and brought to reflux temperature on the hot plate. Exceptional care must be taken with this small

volume to ensure that connections are tight, that the condenser is operating efficiently, and that overheating does not occur. After 1 hr the flask is removed, and the contents filtered through a 2-ml 10-mm coarse sintered-glass funnel into a 10-ml beaker. Any loss is removed as resin on the filter at this stage. Flask and filter are syringed down with two 0.5-ml aliquots of water. The combined filtrates, chilled to ice temperature, are acidified with cold 85% H_3PO_4 (5–6 drops) to pH 1.5 (by Hydrion narrow range pH paper). The thick white IAA precipitate which forms is especially sensitive to light under these conditions and should be kept in dim light during 30–60 min of further chilling. The precipitate is then collected on another 2-ml 10-mm coarse sintered-glass funnel, the beaker is washed with the first filtrate, and the washing is refiltered. The crystals are then rinsed once with 0.5 ml cold 0.1 *N* H_3PO_4 and twice with 0.5 ml cold water, syringing all parts of the funnel thoroughly. The precipitate is sucked dry for a few seconds; then the funnel is placed in a desiccator evacuated by mechanical pump and left standing over BaO for 1 hr. The funnel is removed and weighed to 0.1 mg, and a small crystal of IAA is removed for microscopic melting point determination. The funnel is then placed in a stopper and set in a 1-liter suction flask over a graduated 50-ml test tube. Several aliquots of CH_3CN are then added to the funnel and sucked down into the tube. Any residual phosphate salts are left behind on the funnel and the weight of IAA is now obtained by redrying the funnel and obtaining the difference in weight. The test tube may now be filled with CH_3CN to an appropriate calibration mark and known volumes of the solution transferred by syringe to serum bottles or vaccine vials for storage under nitrogen at a concentration of 10 μc/ml acetonitrile.

Indoleacetonitrile. The IAN synthesized according to the first paragraph of the IAA procedure above is taken into solution by adding 3 ml of benzene to the reaction flask and stirring with a stream of N_2. The benzene supernatant is removed with a fine capillary pipet and transferred to a 25-ml Erlenmeyer flask. The extraction is repeated twice more, dissolving all but a trace of dark gum. A small quantity of powdered CaH_2 is added to the combined extracts in the Erlenmeyer flask and let dry until gas evolution ceases (about 3 hr). In the meantime, a $\frac{3}{8}'' \times 6''$ Fisher-Porter chromatograph column is packed to a depth of 3″ with 100/200 mesh Davison silica gel suspended in anhydrous benzene. Then, in 5-ml aliquots, the following are added to the column: 25 ml benzene, 10 ml 1% ether-benzene, 10 ml 2% ether-benzene, 25 ml 4% ether-benzene, 25 ml 8% ether-benzene, and 20 ml 16% ether-benzene. The drops forming on the drip tip at ca. 1 ml/min are monitored continuously by a thin window probe placed 5 mm away. The radioactive fraction which comes off in the 8% ether-benzene elution is

collected in a tared flask and evaporated over a warm water bath in a N_2 stream and then taken to dryness *in vacuo*. The residual radioactivity remains largely on the column and negligible amounts are found in the other eluted fractions. The IAN is then dissolved in acetonitrile and stored at 10 μc/ml under N_2 in a manner similar to that employed for IAA. This synthesis was performed once with radioactive cyanide (1.1 mc/mmole) and a yield of 61% from cyanide obtained.

DISCUSSION

IAA at 16.9 mc/mmole specific activity, as synthesized here, should permit a statistically valid study of concentrations of IAA well below physiological concentrations or even in as few as 2 oat coleoptile sections. For instance, the studies of Goldsmith and Thimann (9), which were carried out with out 0.96 mc/mmole IAA, could thus be modified to ten- to twenty-fold lower concentration (i.e., 5×10^{-8} to 10^{-7} *M* IAA) or to correspondingly less plant material. To give perceptible curvature of 12 coleoptiles in the *Avena* curvature bioassay, ca. 2×10^{-3} μg IAA is required. Using the procedures detailed by Goldsmith and Thimann (9), radioactivity equivalent to only 10^{-4} μg IAA with a specific activity of 16.9 mc/mmole can be detected. This IAA increases the threshold of sensitivity twenty-fold over the most sensitive biological assay, but care will be needed to ensure that it is unaltered IAA that is being detected. The extension to other studies, and to animal material (8), should be similarly benefited.

SUMMARY

Existing methods which can be modified for the synthesis of C^{14} carboxyl-labeled indoleacetic acid have been surveyed and the conditions necessary to obtain the maximum radiochemical yield from C^{14}-cyanide were determined. A major problem is the autoradiosensitivity of the product, which can be minimized by storage in dilute acetonitrile solution. Detailed procedures are given for a convenient one-day synthetic method for high purity indoleacetic acid and indoleacetonitrile. The former has been prepared with specific activities up to 16.9 mc/mmole. At the highest specific activity levels, autoradiodecomposition during the course of synthesis apparently limits the yield obtained.

ACKNOWLEDGMENTS

The encouragement of Professor K. V. Thimann, in whose laboratory this work was initiated, is greatly appreciated. I am grateful to Dr. E. W. Samuel for helpful advice and to Dr. Mary Helen M. Goldsmith for cooperation in testing purity of samples and criticizing this manuscript. Professor G. R. Wyatt is thanked for the use of radioisotope facilities.

REFERENCES

1. HEIDELBERGER, C., *J. Biol. Chem.* **179,** 139 (1949).
2. GORDON, S. A., STUTZ, R., AND WEBER, R., *U. S. Atomic Energy Comm. Rept.* ANL-4401, 147 (1949–50).
3. STUTZ, R. E., ATKINSON, D. E., AND GORDON, S. A., *U. S. Atomic Energy Comm. Rept.* ANL-4710 (1951).
4. WEYGAND, F., AND LINDEN, H., *Z. Naturforsch.* **9b,** 682 (1954).
5. PICHAT, L., AUDINOT, M., AND MONNET, J., *Bull. soc. chim. France* **1954,** 85.
6. ROBINSON, J. R., *Can. J. Chem.* **35,** 1570 (1957).
7. ANDREAE, W. A., ROBINSON, J. R., AND VAN YSSELSTEIN, M. W. H., *Plant. Physiol.* **36,** 783 (1961).
8. KVEDER, S., AND MCISAAC, W. M., *J. Biol. Chem.* **236,** 3214 (1961).
9. GOLDSMITH, M. H. M., AND THIMANN, K. V., *Plant Physiol.* **37,** 492 (1962).
10. GILLESPIE, B., AND THIMANN, K. V., *Experientia* **17,** 126 (1961).
11. KÜHN, H., AND STEIN, O., *Ber. deut. chem. Ges.* **70,** 567 (1937).
12. SNYDER, H. R., AND PILGRIM, F. J., *J. Am. Chem. Soc.* **70,** 3770 (1948).
13. POTTS, K. T., AND ROBINSON, R., *J. Chem. Soc.* **1955,** 2675.
14. GEISSMAN, T. A., AND ARMEN, A., *J. Am. Chem. Soc.* **74,** 3916 (1952).
15. SNYDER, H. R., AND COOK, P. L., *J. Am. Chem. Soc.* **78,** 969 (1956).
16. SCHÖPF, C., AND THESING, J., *Angew. Chem.* **63,** 377 (1951).
17. HILL, R. K., Ph.D. Thesis, Harvard Univ., Cambridge, Mass., 1953.
18. THESING, J., AND SCHÜLDE, F., *Chem. Ber.* **85,** 324 (1952).
19. GORDON, S. A., AND WEBER, R. P., *Plant Physiol.* **30,** 200 (1955).
20. HENBEST, H. B., JONES, E. R. H., AND SMITH, G. F., *J. Chem. Soc.* **1953,** 3796.
21. STOWE, B. B., AND THIMANN, K. V., *Arch. Biochem. Biophys.* **51,** 499 (1954).
22. HENDERSON, J. H. M., *Nature* **192,** 1076 (1961).
23. BACH, M. K., AND FELLIG, J., WITH STREET, H. E., GRIFFITHS, D. J., THRESHER, C. L., AND OWENS, M., *Nature* **182,** 1359 (1958).
24. PRÉVOST-BÉRNAS, A., CHAPIRO, A., COUSIN, C., LANDLER, Y., AND MAGAT, M., *Discussions Faraday Soc.* **12,** 98 (1952).
25. STOWE, B. B., *Plant Physiol.* **35,** 262 (1960).
26. ALLSOPP, C. B., AND WILSON, J., *Discussions Faraday Soc.* **12,** 299 (1952).

ANALYTICAL BIOCHEMISTRY 5, 116–132 (1963)

An Apparatus for Production of Concave Gradients and Its Application to Chromatography of Lipids[1]

GARY J. NELSON[2]

From the Donner Laboratory of Biophysics and Medical Physics, University of California, Berkeley, California

Received May 24, 1962

Gradient elution, first introduced by Alm *et al.* (1, 2) in 1948, has only recently been applied to column chromatography of lipids (3–5). Early studies with gradient elution systems considered only gradients in which the concentration of the polar or eluting solvent was increased very rapidly at the start of a run and then slowly approached 100% concentration asymptotically in the latter part of the run; this is often termed a convex gradient. This type of gradient is relatively simple to produce in the laboratory, but is necessarily a much poorer system than the corresponding linear or concave gradient in which the eluting solvent is introduced gradually at first and then more rapidly so that a continually increasing rate of change of concentration of the eluting solvent is presented to the column.

In the study of serum lipids being carried out in this laboratory (6, 7), stepwise silicic acid column chromatography of phospholipids has been used. However, the nonlinearity of the absorption characteristics of lipoid substances on silicic acid columns coupled with the disturbances caused by solvent discontinuities in stepwise procedures impose limitations on the resolution obtainable by such method. These problems, along with that of automating stepwise systems, prompted the development of a concave gradient elution silicic acid column procedure for the separation of complex lipid mixtures. This paper will present the details of this system, certain aspects of column performance will be discussed, and the results of chromatographic runs on standard mixtures and natural samples will be shown.

THEORETICAL CONSIDERATION

Martin and Synge (8) developed a theory for partition chromatogra-

[1] This work was supported in part by the United States Atomic Energy Commission and the U. S. Public Health Service, Grant Number H-2029 (C-6).

[2] Postdoctoral Fellow of the National Heart Institute, United States Public Health Service.

phy which, while not rigorous for adsorption, can be adapted to adsorption processes. Lakshmanan and Lieberman (9) presented a discussion of gradient elution chromatography utilizing partition chromatography theory and concluded that concave gradient elution will provide the best possible elution characteristics if the substance has an adsorptivity described by a nonlinear isotherm of the type proposed by Freundlich (10) for batch processes and mathematically represented by the equation:

$$C_s = K(C_m)^n \tag{1}$$

where C_s is the amount of solute adsorbed by a given amount of absorbent, C_m is concentration of the solute, and k and n are constants and n is less than 1.

A more rigorous treatment applying to absorption chromatography *per se* has been proposed by deVault (11) and provides additional theoretical support for the belief that concave gradient elution will give optimum resolution in absorption column chromatography. If diffusion effects are neglected, a single solute with a Freundlich adsorption isotherm will elute off an adsorption column with a sharp front and a gradually tapering trailing edge. In practice diffusion effects will tend to spread the boundary further. deVault has shown that a substance with a linear adsorption isotherm will elute off a column with the shape identical to the initial adsorption band on the column, which will be rectangular in a well-packed column, if diffusion effects are neglected.

Concave gradient elution can be considered a way to increase the linearity of a nonlinear isotherm. For a single substance a gradient can be constructed to keep the adsorptivity of that substance almost always in the linear region of the absorption isotherm. With complex mixtures this is not possible, but by varying the shape of the gradient in critical regions much improved separations can be obtained over stepwise elution schemes.

EXPERIMENTAL APPARATUS

Lakshmanan and Lieberman (9) proposed a modification of the double reservoir system in the apparatus described by Alm *et al.* (1) in which the rate into, R_1, and out of, R_2, the mixing were independently variable. In this manner they obtained concave, convex, or linear gradients depending on whether R_1 was greater, equal to, or less than R_2, and devised rigorous mathematical solutions for each case. More recently several other authors have described apparatus for the production and maintenance of complex gradients (12–14). Perhaps the ultimate in flexibility for the production of gradients was achieved in the Varigrad designed by Peterson and Sober (15).

Wren (3) proposed a system for the specific production of a concave gradient adapted from a proposal of Boman (16). While Wren's system gave a wide, uniformly varying gradient, it was not easily adaptable to close regulation of flow rates or an exact mathematical description of the resulting gradient.

When the siphons and pressure differentials in Wren's system are replaced by controlled volume pumps, the shape of the gradient produced in this system is then given by the differential equation in terms of C_t, the concentration of eluting solvent leaving the mixing chamber:

$$C_t = \frac{\int C_0 R_1 dt - \int C_t R_2 dt}{V_0 + \int R_1 dt - \int R_2 dt} \tag{2}$$

where R_1 and R_2 are as given previously, C_0 is the concentration of eluting solvent added to the mixing chamber, and V_0 is the volume of carrier solvent in the mixing chamber at time zero. This equation is similar to that given by Lakshmanan and Lieberman (9) except that R_1 is a variable of the form:

$$R_1 = at \tag{3}$$

where a is an arbitrary constant determined by a pump setting and has units of ml/min^2.

Substituting Eq. (3) into Eq. (2) and differentiating yields:

$$\frac{dc}{(C_0 - C_t)} = \frac{atdt}{\left(V_0 + \frac{at^2}{2} - R_2 t\right)} \tag{4}$$

which can be solved immediately by integration. The forms of the solutions are:

If $2aV_0 = R_2^2$

$$C_t = C_0 \left[1 - \frac{R_2^2 e^{2\left(1+\frac{R_2}{at-R_2}\right)}}{(at - R_2)^2}\right] \tag{5}$$

If $2aV_0 < R_2^2$

$$C_t = C_0 \left[1 - \frac{V_0 \left(\frac{R_2 + \sqrt{R_2^2 - 2aV_0}}{R_2 - \sqrt{R_2^2 - 2aV_0}}\right)^{\left(\frac{R}{\sqrt{R_2^2 - 2aV_0}}\right)}}{\left(V_0 + \frac{at^2}{2} - R_2 t\right)\left(\frac{at - R_2 - \sqrt{R_2^2 - 2aV_0}}{at - R_2 + \sqrt{R_2^2 - 2aV_0}}\right)^{\left(\frac{R_2}{\sqrt{R_2^2 - 2aV_0}}\right)}}\right] \tag{6}$$

If $2aV_0 > R_2^2$

$$C_t = C_0\left[1 - \frac{V_0 e^{\frac{2R_2}{\sqrt{2aV_0 - R_2^2}}\tan^{-1}\left(\frac{-R_2}{\sqrt{2aV_0 - R_2^2}}\right)}}{\left(V_0 + \frac{a}{2}t^2 - R_2 t\right)e^{\frac{2R}{\sqrt{2aV_0 - R_2^2}}\tan^{-1}\left(\frac{at - R_2}{\sqrt{2aV_0 - R_2^2}}\right)}}\right] \tag{7}$$

The three solutions are necessary because the radical $[\pm (R_2^2 - 2aV_0)]^{1/2}$ appears and is indeterminate where $\pm (R_2^2 - 2\ aV_0)$ assumes a negative value or zero. Figure 1 gives examples of the curves obtained

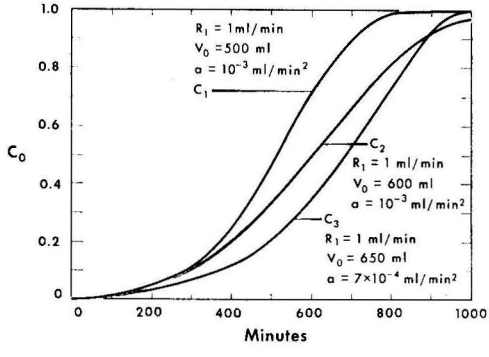


FIG. 1. Graphic presentation of gradients obtained under conditions indicated on the figure for the three forms of the solution to Eq. (2) in the text. Curves C_1, C_2, and C_3 are solutions of Eq. (5), (6), and (7), respectively. The values of the parameters a, V_0, and R_2 are listed for each solution.

from each solution with various initial conditions. The curves are sigmoidal over the full range.

Design of Apparatus

Figure 2 is a diagrammatic sketch of the apparatus capable of producing the gradients described above mathematically. The critical parts of the assembly are the gradient and monitor pumps and the control for the gradient pump. Both pumps are "chromatographic miniPumps" No. CH-MM-29B designed by Milton Roy Company, Philadelphia, Pa. These pumps have no organic soluble substances in the liquid head and are capable of pumping quite accurately liquids of low viscosity. The pumping action is accomplished by a reciprocating piston whose stroke can be varied from zero to 100% of capacity by a micrometer adjustment.

The motor on the gradient pump is a $\frac{1}{15}$ hp-DC motor made by Bodine

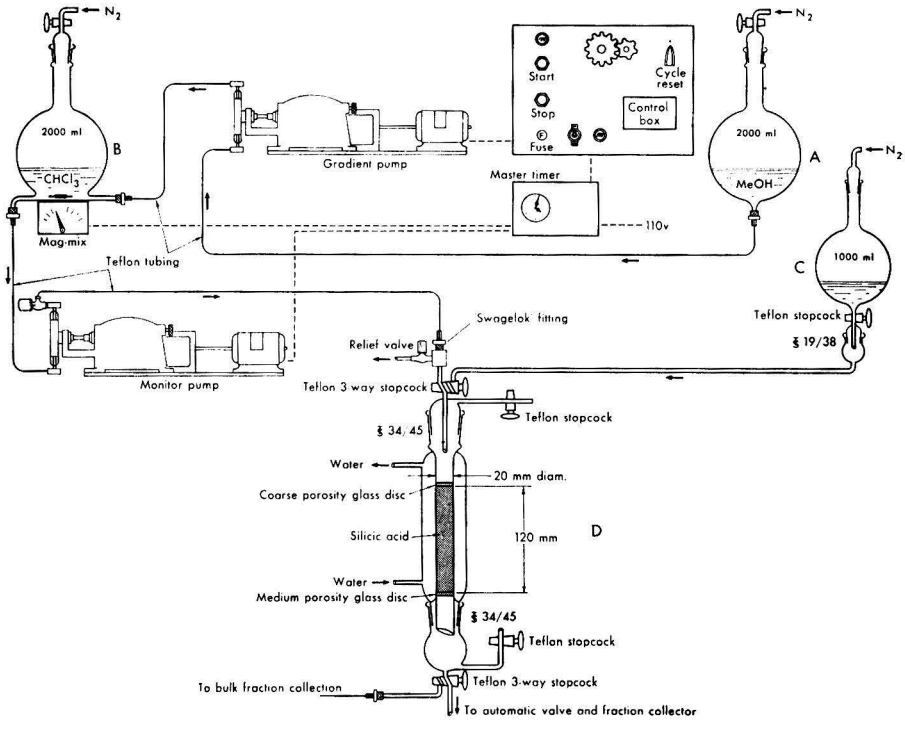


FIG. 2. Diagrammatic sketch of the gradient elution column chromatographic system described in the text. A is the eluting solvent reservoir from which the solvent is transferred to the mixing chamber (B) by the gradient pump. A specific volume of carrier solvent is placed in the mixing chamber before the start of the run. The exact amount is determined by shape of the gradient and length of the run and calculated by Eqs. (5), (6), or (7) given in the text. The outflow of the mixing chamber is pumped to the column (D) by the monitor pump. C is an additional solvent reservoir for nongradient elution.

Electrical Company, Chicago, Ill., and is connected to an automatic control which increases the rpm of the motor linearly with time, starting at very low rpm. Figure 3 is the schematic circuit diagram of this control.

A linear increase in rpm of the motor produces a linear increase in pumping rate. The total volume, V, delivered by the pump at time, t, is:

$$V = (1/2)at^2 \tag{8}$$

a can be calculated from this relationship by collecting the volume delivered by the pump during a specific interval. The time for the pump motor to reach its maximum rpm is taken as t_{max}; t_{max} is varied by a

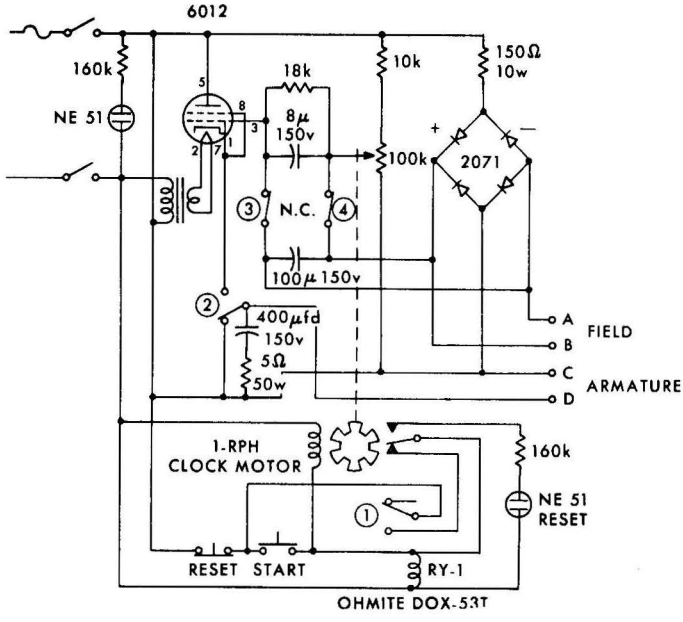


FIG. 3. Schematic circuit diagram for the gradient pump motor control.

gear change on the motor control. a can be varied independently of t_{max} by changing the length of the piston stroke.

The motor is started, however, at an rpm somewhat greater than zero, approximately $\frac{1}{20}$ of its maximum rpm. This produces a small deviation in the value of a predicted by Eq. (8), and consequently in the gradient for time close to zero. For runs with t_{max} equal to or greater than 1000 min the effect of this small deviation from the theoretical gradient should be negligible. However, on short runs corrections must be applied.

The second or monitor pump, also a "chromatographic miniPump" identical to the gradient pump except that a constant speed AC motor is used in place of variable speed motor on the gradient pump, is used to transfer the eluting solvent from the mixing chamber to the column at a constant rate, R_2. Experimentally, this pump delivered 1 ± 0.08 ml/min when pumping a gradient which varied from 100% chloroform to 100% methanol during 1000 min.

Column Housing

A glass column housing which was water jacketed and similar to the design of Hirsch and Ahrens (17) was used. A medium-porosity fritted glass disk held in place by a carefully machined Telflon gasket which in

turn rested on a slight constriction at the bottom of the column supported the packing in the column. A coarse porosity disk was used to protect the top of the column from disturbance after packing. Teflon stopcocks[3] were used throughout the apparatus to prevent leakage and eliminate the need for stopcock grease.

Preparation of Column Packing

As the monitor pump is capable of maintaining a constant flow rate against high back-pressures, no filter aid was added to the silicic acid packing. Columns were packed with pure silicic acid obtained from Mallinckrodt Chemical Co., 100 mesh, which was resieved to give a fraction passing 100 mesh but retained by a 325-mesh screen. The sieved silicic acid was then washed with successive portions of distilled water, methanol,[4] and acetone, dried for 24 hr at 120°C, and then stored in a sealed jar.

To pack a column, 20 gm of silicic acid was weighed and heated between 110 to 130°C for 48 hr in a vacuum oven immediately before use, then cooled and slurried with a solvent, and poured as the slurry into the column housing. A minimum amount of additional solvent was used to wash the packing down the column and the coarse-porosity glass disk placed on top of the packing. The disk was pressed down until the column appeared closely packed under visual inspection. The extra solvent above the level of the disk was removed, and the charge added to the column.

Chromatographic Procedure

The lipid extract was added to the column in the same solvent used to prepare the column packing. If neutral lipids were being analyzed, normal hexane was used; if only the phospholipids were of interest, chloroform was used. The charge was added to the column in 2 ml of solvent and washed onto the column with an additional 2 ml. When neutral lipids are being fractionated, gradient elution with a gradient of ethyl ether in normal hexane is begun immediately after the charge is placed on the column. When the gradient reaches pure ethyl ether, a methanol in chloroform gradient can be started to fractionate the phospholipids.

If, as was the case in most studies carried out here, neutral lipids were not separated in the same run, the column was prepared with chloroform.

[3] Kimax brand Teflon stopcocks obtained from Kimble Glass Co. through various laboratory supply houses.

[4] All solvents used in all phases of this work were reagent-grade commercial solvents. The chloroform contained 0.75% ethanol.

Before the gradient separation of the phospholipids was begun, 1000 ml of chloroform was run through the column to remove all neutral lipids in a single fraction (8). The flow rate through a 20-gm column was 1 ml/min. The charge added to the column yielded good resolution for most mixtures when it was between 3 and 4 mg of phospholipid per gram of silicic acid. No single component should exceed 3 mg/gm silicic acid in this system.

In most runs in which multiple fractionation was carried out 10-ml aliquots were collected. As the flow rate through the column was constant at 1 ml/min, time indexing was used to collect fractions.[5]

Lipid Samples

Red blood cells to be extracted were first resuspended and washed twice with 0.9% saline solution. Packed cells (20 ml) were then transferred to 180 ml of 50:50 methanol-chloroform solution and homogenized for 2 min in a Waring Blendor. The subsequent extraction steps and the extraction of normal human serum were carried out as described (7, 18).

Standard samples of L-α-dipalmitoyl phosphatidyl ethanolamine and L-α-diplamitoyl phosphatidyl choline were obtained from California Corporation for Biochemical Research, Los Angeles, Calif. Sphingomyelin standards were prepared by the method of Rapport and Lerner (19) and rechromatographed on silicic acid. Lysolecithin was prepared by the method of Long and Penny (20). Monophosphoinositol was prepared from mouse liver (18). No pure phosphatidyl serine or cardiolipin standards were obtained.

Neutral lipid standards were obtained from the following sources: palmitic acid and tripalmitin, Hormel Foundation, Austin, Minn.; free cholesterol, Armour Laboratories, Chicago, Ill. Cholesteryl laurate was synthesized from the free cholesterol and lauric acid. Saturated mono- and diglycerides were prepared from hydrogenated cottonseed oil by partial hydrolysis in 1 *N* NaOH for 15 min at 37°C and isolated by silicic acid chromatography.

Analytical Methods

Neutral lipids were characterized by infrared spectrophotometric techniques (21, 22) and thin layer chromatographic methods (23). Phospholipids were also analyzed by these methods and fractions were monitored by chemical phosphorus determinations performed as described previously (24). The solvent system used for separation of the phospholipid

[5] The automatic fraction collector with time indexing unit was obtained from Research Specialties Co., Richmond, California.

mixture on the thin layer chromatograms was chloroform-methanol-water (60:40:4 v/v). Spots were developed by spraying the dried plates with concentrated H_2SO_4 and charring (18).

In addition, specific chemical determination were performed on aliquots taken from various chromatographic fractions by the methods listed below: glycerol was determined by the method of Van Handel and Zilversmit (25) as modified by Van Handel (26), ethanolamine and serine by the method of Dittmer *et al.* (26), and choline by the procedure of Wheeldon and Collins (28); sugars were analyzed by the anthrone reaction of Radin *et al.* (29). Sphingosine was determined by its infrared spectra after extraction from the neutralized aqueous phase of the trans-methylation procedure (7) with chloroform.

RESULTS

Figure 4 shows the elution pattern of standard mixture of neutral lipids from a 20-gm silicic acid column. Table 1 gives the recoveries of the various component on a weight basis. The cholesteryl esters and triglycerides were fully resolved, while the free fatty acids, cholesterol, and diglycerides peaks overlapped slightly, which is reflected in the lower

TABLE 1

RECOVERIES OF NEUTRAL LIPID FROM CHROMATOGRAPHY OF STANDARD MIXTURE SHOWN IN FIG. 4

(Gravimetric Determination)

Lipid	Collection tube Nos.	Wt. added to column (mg)	Wt. recovered (mg)	Per cent recovery
Cholesteryl laurate	2–10	58.3	58.1	99.6
Tripalmitin	11–20	59.1	60.7	102.8
Palmitic acid	21–30	21.2	19.8	93.4
Free cholesterol	31–46	37.9	40.3	105.9
Diglyceride Monoglyceride	47–110	12.3	11.1	90.2
Total		188.8	190.0	100.7

recovery of free fatty acids and diglycerides while the cholesterol is greater than 100%.

Figure 5 is the elution pattern of a known mixture of phospholipids obtained from the sources listed above. All components except lecithin and spingomyelin are well resolved. And even these hard to separate compounds are fairly well resolved. Infrared spectrophotometric measurements indicated that collection tubes 80 through 95, the sphingomyelin peak, contained 5% of the lecithin added to the column while tubes 69 through 86, the lecithin peak, contained approximately 3% of the

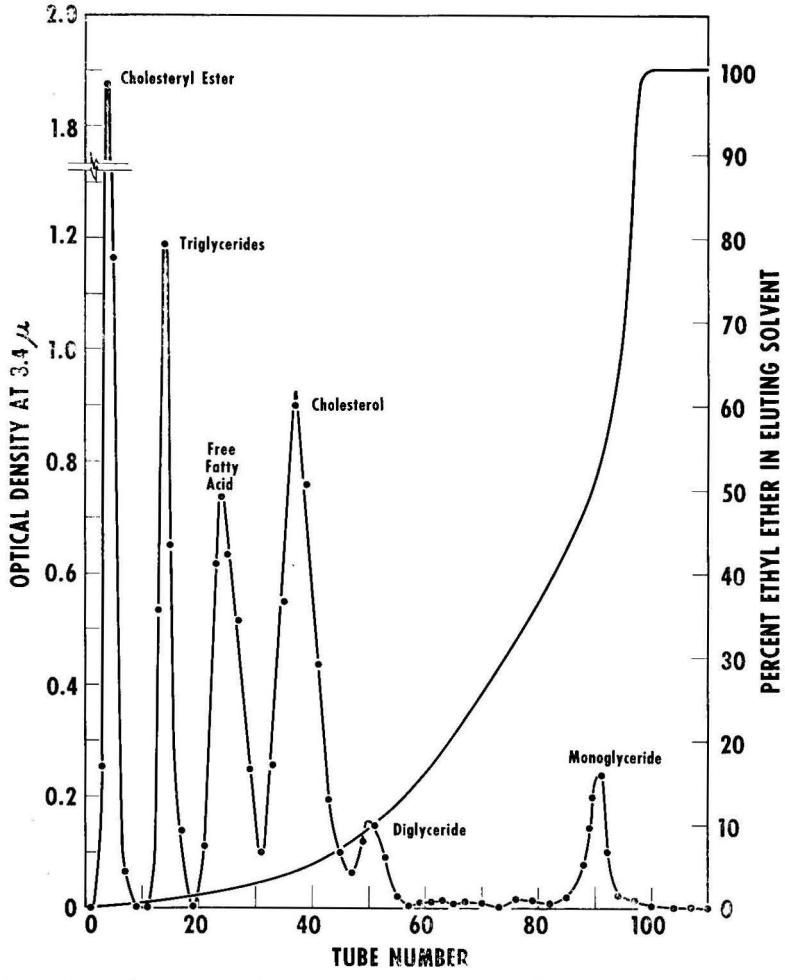


FIG. 4. Experimentally determined elution pattern obtained by gradient elution chromatography of a known mixture of neutral lipids from a 20-gm silicic acid column. The gradient was diethyl ether in normal hexane. The shape of the gradient is shown on the figure. The left ordinate is the optical density of a 0.15-ml solution of the material, recovered from 1.0 ml of eluant solvent, in CCl_4 in a 3.0-mm path length cell. The various peak heights are dependent on the specific absorption coefficient of each substance and not directly comparable to each other in the figure. The right ordinate is per cent of diethyl ether in the gradient. See text for details of method and recovery data.

sphingomyelin. Most of the contamination is in tubes 77 through 85. Table 2 gives the recoveries of the various phospholipids in terms of mole % phosphorus added to the column.

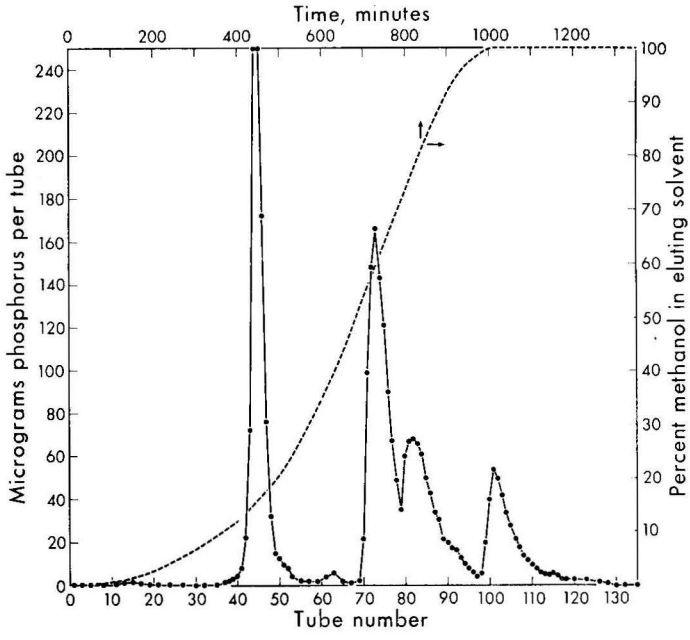


FIG. 5. Experimentally determined elution pattern obtained by gradient elution chromatography (20-gm silicic acid column) of a known mixture of phospholipids obtained from sources listed in the text. The shape of gradient is indicated by the broken line. The gradient was methanol in chloroform, and determined theoretically by Eq. (6) of the text. The values for the various parameters were: $a = 0.0007$ ml/min^2; $R_2 = 1$ ml/min; $V_0 = 650$ ml; and $t_{max} = 1000$ min. After 1000 min, elution was continued with pure methanol for an additional 400 min. Collection tubes 40 to 50, 69 to 85, 75 to 97, and 98 to 120 contained phosphatidyl ethanolamine, lecithin, sphingomyelin, and lysolecithin, respectively. See text for detail of method and recovery data.

TABLE 2

RECOVERIES OF PHOSPHOLIPIDS FROM CHROMATOGRAPHY OF STANDARD MIXTURE SHOWN IN FIG. 5

(Mole % Phosphorus Added to Column)

Phospholipid	Collection tube Nos.	P added to column (μg)	P recovered (μg)	Per cent recovery
L-α-(Dipalmitoyl) phosphatidyl ethanolamine	35–55	880	885	100.7
L-α-(Dipalmitoyl) phosphatidyl choline	69–85[a]	1976	1940	98.1
Sphingomyelin	75–97[a]	735	738	100.4
Lysolecithin	98–120	370	394	106.5
Total		3961	3957	99.9

[a] Overlapping tubes analyzed by infrared spectrophotometry.

In Figs. 6 and 7 the elution patterns for the phospholipids obtained from normal human red cells and serum lipids, respectively, are shown.

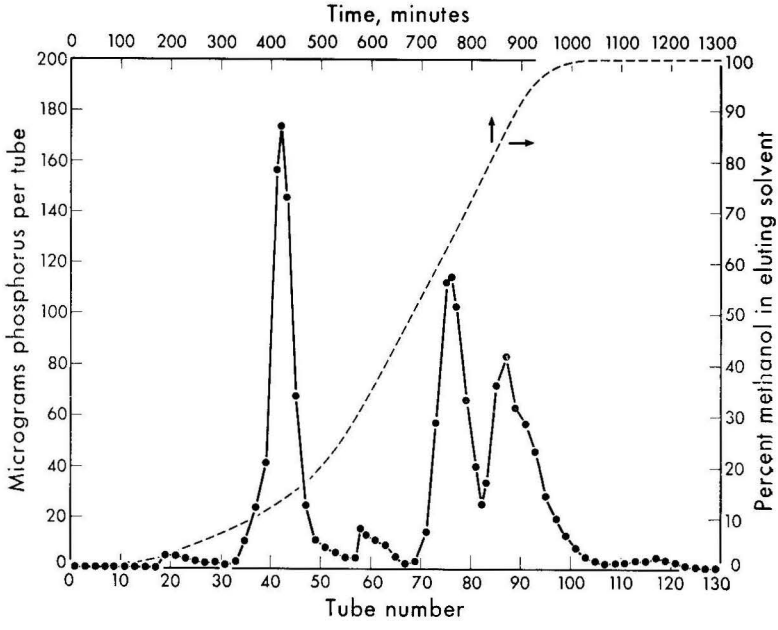


FIG. 6. Experimentally determined elution pattern obtained by gradient elution chromatography of phospholipids extracted from normal human erythrocytes. Chromatographic conditions are described in the captions to Fig. 5. Weight added to column, 2.73 mg P; recovery, 2.77 mg P. Collection tubes 18 to 30 and 56 to 66 contained polyglyceryl phosphatides and monophosphoinositol, respectively. Main components can be located by reference to Fig. 5.

From comparisons of these runs and others (not shown) on lipids extracted from liver (18) and serum lipoprotein fractions it was determined that the system is relatively unaffected by the source of the sample. In all chromatographic runs with identically activated packing the major lipid components were eluted in the same position, plus or minus two collection tubes (20 ml).

Table 3 compares the recovery of phospholipids from a duplicate analysis of normal human serum sample. Serum (50 ml) was extracted and 384.1 mg of lipid recovered, of which 119.5 mg was phospholipid as determined by chemical phosphorus measurements (21). The extract was divided into two equal parts and each half chromatographed in an identical manner. The B run in Table 3 was performed two weeks after the A run. Although the sample was stored under N_2 at 0°, some deteri-

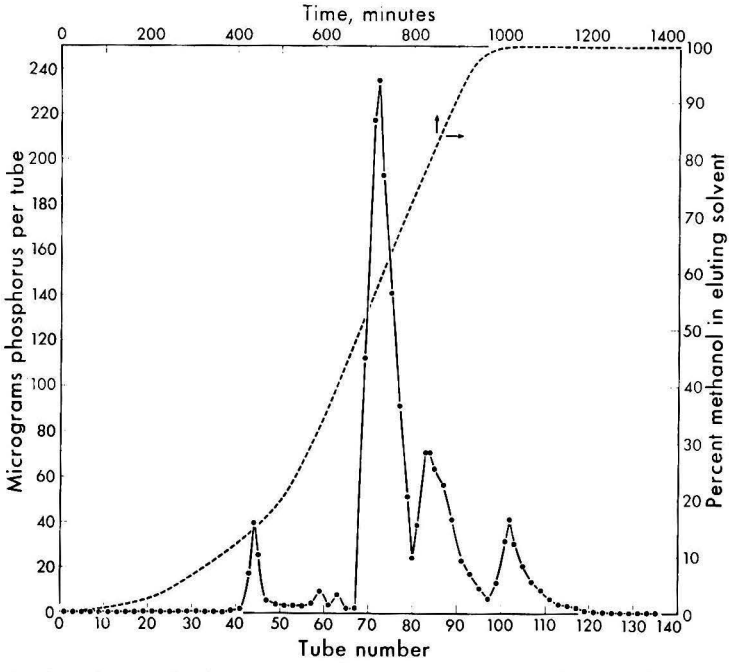


FIG. 7. Experimentally determined elution pattern obtained by gradient elution of phospholipids extracted from normal human serum. Chromatographic conditions are described in the caption to Fig. 5. Weight added to column, 2.39 mg P; recovery, 2.40 mg. Components can be located by reference to Figs. 5 and 6.

TABLE 3

DUPLICATE ANALYSIS ON SAME SAMPLE OF PHOSPHOLIPIDS EXTRACTED FROM NORMAL HUMAN SERUM

Phospholipid	Column A		Column B	
	μg P	%	μg P	%
Phosphatidyl ethanolamine[a]	106	4.4	94	4.0
Monophosphoinositol	12	0.5	7	0.3
Phosphatidyl choline	1637	68.3	1553	66.5
Sphingomyelin	470	19.6	485	20.8
Lysolecithin	172	7.2	192	8.2
Total	2397	100	2331	100

Wt. added to columns, 2389 μg. Recovery: column A, 100.3%; column B, 97.8%.

[a] Contain phosphatidyl serine and plasmalogens of ethanolamine and serine.

oration may have occurred, accounting for the slightly lower recovery in that run. Even so, the results still exhibited good agreement.

The resolution of the column was determined by using thin-layer

silicic acid chromatography and the analytical procedures listed above on aliquots from the column fractions. Table 4 presents the results of such analysis on the run shown in Fig. 6 of red blood cell phospholipids. Except for the phosphorus determination the tests were not performed quantitatively.

TABLE 4

CHEMICAL ANALYSES OF COLUMN FRACTIONS FROM GRADIENT ELUTION CHROMATOGRAPHY OF NORMAL HUMAN RED BLOOD CELL PHOSPHOLIPIDS SHOWN IN FIG. 6

	Column fractions					
	1	2	3	4	5	6
	Tubes					
Chemical analysis[a]	18 to 30	31 to 54	55 to 67	68 to 81	82 to 104	105 to 130
Phosphorus (μg)	15	882	132	797	624	57
Glycerol	+	+	+	+	Trace	+
Ethanolamine	0	+	Trace	0	0	0
Serine	0	+	Trace	0	0	0
Choline	0	0	Trace	+	+	+
Sphingosine	Trace	Trace	+	Trace	+	Trace

Wt. added to column, 2581 μg P. Recovery, 97.0%.

[a] + indicates strong position reaction; and 0 indicates no observable reaction.

In the samples investigated here no material eluted off the column after lysolecithin, although the triphosphoinositol reported recently by Tomlinson and Ballou (30) and Dittmer and Dawson (31) would be expected to elute after lysolecithin if it can be eluted from silicic columns at all.

In chromatography of the red blood cell phospholipids shown in Fig. 6 cerebroside-like material as indicated by its infrared spectrum was found in tubes 15 through 20 and 45 through 60, and amounted to approximately 10% of the total lipids extracted from the erythrocytes by methanol-chloroform. While no detailed studies of the elution characteristics of the cerebrosides were attempted, it is feasible that these sphingolipids can also be resolved from phospholipids by this procedure.

DISCUSSION

The results presented in this study indicate that concave gradient elution provides a technique capable of separating lipid mixtures with good resolution and without the necessity of multiple solvent changes during the run. Not only are so-called "false peaks" avoided but the method lends itself to complete automation. Once a run is started no attention need be paid to it until it is finished, thus allowing a run to

continue overnight and for extended periods not feasible in manual operations.

The activation of the column packing to the same state is absolutely necessary to obtain reproducible results from run to run, and a packing is good for only one run in this system. No solvent rejuvenation was able to return a packed column to its initial degree of activation. Furthermore, a column packing that was not fully activated showed poorer resolving power and shorter elution times than fully activated columns. The most prominent change observed with deactivated columns was a shortened retention time for the various lipids, either neutral lipids or phospholipids.

Several reports of the silicic acid column fractionation of neutral lipids by stepwise (32, 34) or convex gradients (17) have appeared which yield separation equal to that obtained in this study. Generally the cholesterol esters and triglycerides are well resolved while the fatty acids, cholesterol, and diglycerides overlap slightly. In the present work only the single gradient shown in Fig. 4 was investigated. Changes in shape of the gradient may yield better separation of the slower eluting component in the neutral lipid series.

From the data given in Figs. 4, 5, and 6 and Tables 2, 3, and 4 this system appears capable of separating mixtures of phosphatidyl ethanolamine, monophosphoinositol, phosphatidyl choline, and lysolecithin completely and also a fast eluting component that may be either polyglycerol phosphatide or phosphatidic acid. Lecithin and sphingomyelin overlap slightly. Phosphatidyl ethanolamine and phosphatidyl serine and the plasmalogen analogs of the various phospholipids are not separated by this method. Stepwise elution procedures described by Hanahan *et al.* (35), Phillips (36), and Newman *et al.* (37) yield somewhat similar separations as those shown here, but these methods are difficult to automate and the columns cannot operate untended for long periods.

Variations in the ratios of the phospholipids present in the sample will effect the resolution, but as long as the total load is less than 3 mg/gm packing, the effect is negligible.

Other authors (36–38) have added water to their solvents in stepwise elution of phospholipid from silicic acid columns. In the recent scheme of Newman, Liu, and Zilversmit (37), the authors indicated that addition of 3% H_2O to their methanol greatly improved the resolution of the system. Addition of 3% H_2O to the methanol (the commercial reagent-grade methanol used here had 0.05% H_2O) produced no noticeable improvement in resolution with the gradient elution system reported here.

The initial choice of a gradient is arbitrary. When some prior knowl-

edge of the elution characteristics of the system is available, a gradient picked to approximate a stepwise scheme can be chosen. If two components are not well resolved, a reduction in the slope of the gradient will often provide a better separation. Sometimes it is possible to separate compounds having identical elution times under one set of conditions by appropriate changes in the shape of the gradient.

In operation the system was found to be very reliable. A reliable automatic fraction collector and indexing device is a necessity for monitoring runs during developmental stages of an investigation. Batch collection can be used after the reproducibility of the system for a given mixture has been determined.

The use of this system for producing concave gradients is not restricted to silicic acid column chromatography of lipids. Any column chromatographic system would be applicable, as would many ion-exchange separations. Piez and Morris (39) have already indicated that gradient elution of amino acid hydrolyzates on an ion-exchange column using buffered solutions of different ionic strength greatly enhances the operation.

SUMMARY

An automatic method for the column chromatographic separation of lipid mixtures, particularly phospholipids, utilizing concave gradient elution is described in detail. The gradient in this system is produced by an automatic pumping system using accurate controlled volume pumps and variable pumping rates. A discussion of the theoretical aspects of gradient elution in relation to nonlinear absorption isotherms is included, and several chromatographic runs using the system are presented. The system is capable of excellent resolution of naturally occurring phospholipid mixtures and is quite reproducible. Considerable flexibility is available in shape of the gradient produced by the pumping system allowing modification for particular tissue extracts containing phospholipids in varying ratios. The system is completely automatic and, once a run is started, operates untended until completion.

REFERENCES

1. ALM, R. S., WILLIAMS, R. J. P., AND TISELIUS, A., *Acta Chem. Scand.* **6**, 826 (1952).
2. TISELIUS, A. W. K., *Endeavour* **11**, 5 (1952).
3. WREN, J. J., *Nature* **184**, 816 (1959).
4. SCHWARZ, H. P., DREISBACH, L., BARRIONUEVO, M., KLESCHICK, A., AND KOSTYK, I., *J. Lipid Research* **2**, 208 (1961).
5. KARNOVSKY, J. L., AND WALLACH, D. F. H., *J. Biol. Chem.* **236**, 1895 (1961).
6. NELSON, G. J., AND FREEMAN, N. K., *J. Biol. Chem.* **235**, 578 (1960).
7. NELSON, G. J., *J. Lipid Research* **31**, 71 (1962).

8. MARTIN, A. J. P., AND SYNGE, R. L. M., *Biochem. J.* **35,** 1358 (1941).
9. LAKSHMANAN, T. K., AND LIEBERMAN, S., *Arch. Biochem. Biophys.* **53,** 758 (1954).
10. FREUNDLICH, H., "Colloid and Capillary Chemistry," 3rd Ed. Metheun, London, 1926.
11. DEVAULT, D., *J. Am. Chem. Soc.* **65,** 532 (1943).
12. VESTERGAARD, P., *J. Chromat.* **3,** 560 (1960).
13. PIEZ, K. A., *Anal. Chem.* **28,** 1451 (1956).
14. BOCK, R. M., AND LING, N. S., *Anal. Chem.* **26,** 1543 (1954).
15. PETERSON, E. A., AND SOBER, H. A., *Anal. Chem.* **31,** 857 (1959).
16. BOMAN, H. G., *Biochim. et Biophys. Acta* **16,** 245 (1955)
17. HIRSCH, J., AND AHRENS, E. H., JR., *J. Biol. Chem.* **233,** 311 (1958).
18. NELSON, G. J., *J. Lipid Research* **3,** 256 (1962).
19. RAPPORT, M. M., AND LERNER, B., *J. Biol. Chem.* **232,** 63 (1958).
20. LONG, C., AND PENNY, I. F., *Biochem. J.* **65,** 382 (1957).
21. NELSON, G. J., AND FREEMAN, N. K., *J. Biol. Chem.* **234,** 1375 (1959).
22. FREEMAN, N. K., *N. Y. Acad. Sci.* **65,** 131 (1957).
23. MALINS, D. C., AND MANGOLD, H. K., *J. Am. Oil Chemists' Soc.* **37,** 576 (1960).
24. GRISWOLD, B. L., HUMOLLER, F. L., AND MCINTYRE, A. R., *Anal. Chem.* **23,** 192 (1951).
25. VAN HANDEL, E., AND ZILVERSMIT, D. B., *J. Lab. and Clin. Med.* **50,** 152 (1957).
26. VAN HANDEL, E., *Clin. Chem.* **7,** 249 (1961).
27. DITTMER, J. C., FEMINELLA, J. L., AND HANAHAN, D. J., *J. Biol. Chem.* **233,** 862 (1958).
28. WHEELDON, L. W., AND COLLINS, F. D., *Biochem. J.* **70,** 43 (1958).
29. RADIN, N. S., LAVIN, F. B., AND BROWN, J. R., *J. Biol. Chem.* **217,** 789 (1955).
30. TOMLINSON, R. V., AND BALLOU, C. E., *J. Biol. Chem.* **236,** 1907 (1961).
31. DITTMER, J. C., AND DAWSON, R. M. C., *Biochem. J.* **81,** 535 (1961).
32. FILLERUP, D., AND MEAD, J., *Proc. Soc. Exptl. Biol. Med.* **83,** 574 (1953).
33. BARRON, E. J., AND HANAHAN, D. J., *J. Biol. Chem.* **231,** 493 (1958).
34. HORNING, M. G., WILLIAMS, E. A., AND HORNING, E. C., *J. Lipid Research* **1,** 482 (1960).
35. HANAHAN, D. J., DITTMER, J. C., AND WARASHINA, E., *J. Biol. Chem.* **228,** 685 (1957).
36. PHILLIPS, G. B., *Proc. Natl. Acad. Sci. U. S.* **43,** 566 (1957).
37. NEWMAN, H. A. T., LIU, C. T., AND ZILVERSMIT, D. B., *J. Lipid Research* **7,** 403 (1961).
38. RHODES, D. N., AND LEA, C. H., *Biochem. J.* **65,** 526 (1957).
39. PIEZ, K. A., AND MORRIS, L., *Anal. Biochem.* **1,** 187 (1960).

ANALYTICAL BIOCHEMISTRY 5, 133–142 (1963)

Analysis of Estrogens with Bromine-82[1,2]

W. R. SLAUNWHITE, JR., AND LAVALLE NEELY

From the Department of Medicine C, Roswell Park Memorial Institute, Buffalo, New York

Received June 6, 1962

INTRODUCTION

Present methods (1–3) of analysis of estrogens (*1*) are largely restricted to the three components—estrone, estradiol-17β, and estriol, (*2*) destroy the compounds in the process of fluorometric or colorimetric analysis; and (*3*) often fail to take advantage of high resolution techniques due to the limitations imposed by the minute quantity of material available and the complexity of high resolution techniques. Two exceptions to the last-named characteristic are the recent use of gradient elution partition chromatography (4, 5) and vapor-phase chromatography (6). Both fluorometric and colorimetric assays contain a certain degree of specificity (7, 8) which, although useful in certain situations, limits their applicability to some of the phenolic steroids recently identified in human urine.

It is our intent to elaborate a method which is completely general for phenolic steroids, yet is highly specific when combined with a chromatographic technique. Due to the minute amounts of estrogens present in blood and frequently in urine, the use of a radioactive label is mandatory. For ease of counting and conservation of material, a gamma-emitting isotope would be preferred. To minimize the formation of artifacts, such as the isomerization of 16α-hydroxyestrone to 16-keto-estradiol-17β, hydrolysis, extraction, and labeling procedures should, if possible, avoid strongly acidic or basic conditions. For obvious reasons the label must be applied in the laboratory after collection of specimens has been completed.

Halogenation was chosen as the reaction most closely meeting these specifications. Because of its long half-life, iodine-131 was our first choice, but preliminary investigation with unlabeled iodine revealed that

[1] Aided by grants from the American Cancer Society (P-235) and the National Institutes of Health, U. S. Public Health Service (CY-3559).

[2] Presented in part at the annual meeting of The Endocrine Society, June, 1961, New York, New York.

iodoestrogens were so unstable as to preclude chemical characterization. This was not true of bromoestrogens, and hence bromine-82 was selected for halogenation. Estrone and estradiol-17β brominate in the 2 and 4 positions (9). Unpublished work from our laboratory has shown that estriol behaves similarly.

EXPERIMENTAL

Reagents

Radioactive Potassium Bromide. Approximately 4 mg of accurately weighed potassium bromide, ACS reagent-grade, is placed in 6 mm i.d. quartz tubing, which is sealed and placed in the neutron beam (flux ca. 10^{13} neutrons/cm/sec). The sample is usually irradiated for 7.5 hr on two consecutive days, then removed from the reactor. This leads to a specific activity of ca. 0.2 mc/mg of KBr-82. A limiting specific activity of 1.2 mc/mg could be attained by continuous irradiation for 100 hr before removal, but this has not been found necessary.

Manganese Dioxide. This was prepared as directed (10), using 1/10 the quantities specified. Its activity was assayed periodically by oxidizing 1.0 gm of nonradioactive potassium bromide (8.4 moles) in 1 ml of water with 400 mg of manganese dioxide (4.6 moles) in 1 ml of concentrated sulfuric acid according to the procedure described below. That portion of the bromide which is not oxidized to bromine is swept over as hydrogen bromide and trapped with the bromine in glacial acetic acid. After completion of the reaction the bromide content of the acetic acid is determined gravimetrically as silver bromide. Approximately 20% of the bromide escapes oxidation with a satisfactory preparation of manganese dioxide. Many commercial preparations are unsatisfactory since 50–90% of the bromide escapes oxidation.

Alumina. Acid-washed Woelm alumina was deactivated by the addition of 8 gm of water to 100 gm of alumina.

Bicarbonate-bisulfate Solution. To 100 ml of saturated sodium bicarbonate solution 20 gm of sodium bisulfate was added.

Other Reagents. Solvents were ACS reagent-grade except for ethanol, which was USP. All solvents were used without purification.

Standards. Estrone, estradiol-17β, and estriol meeting USP standards were used after two crystallizations. The 2,4-dibromo derivatives of these compounds were synthesized (9). A solution of 1.5 mg of each in 10 ml of 5% ethanol in benzene was prepared fresh daily.

Procedure

Dissolve the extract to be brominated portionwise in a total of 2 ml of

glacial acetic acid and transfer to the reaction vessel. To the bromine generator (Fig. 1) add ca. 1 mg of manganese dioxide, 1 ml of concen-

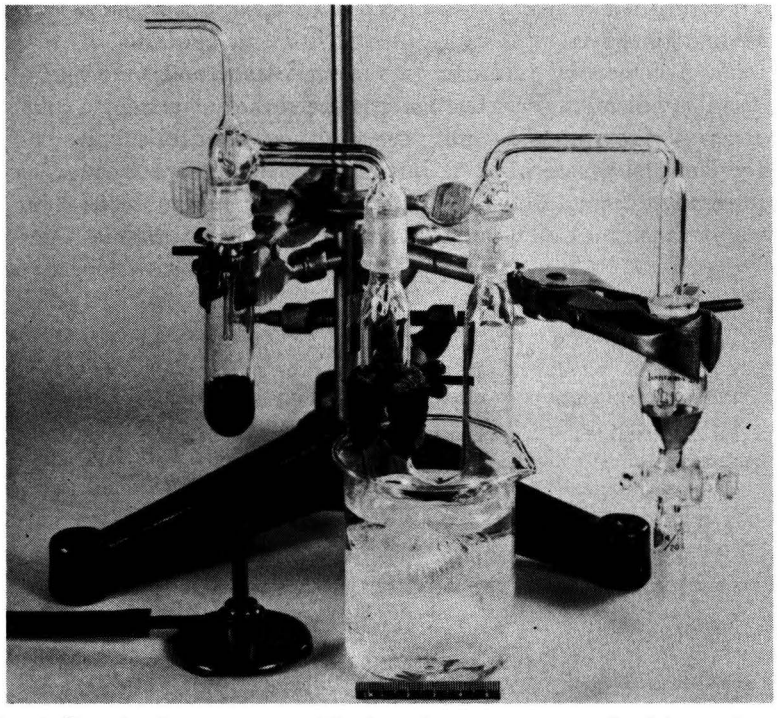


FIG. 1. Bromination apparatus. The bromine generator at the left consists of a 2 cm o.d. × 7 cm tube with a 1 mm i.d. inlet tube for the air sweep. The trap immersed in an ice-water mixture in a 600-ml beaker is a U-tube of 2 cm o.d. × 10 cm high. The ice bath is cold enough to condense most of the water vapor and acid fumes swept over from the generator, but not cold enough to condense bromine vapor. A 5-ml pipet serves as a trap in case the glacial acetic acid solution contained in the 10-ml separatory funnel is accidentally sucked up. (A small Kjeldahl bulb might have been a better trap than the pipet.) All joints are 14/20. After completion of the bromination, the separatory funnel is used in a conventional manner for extraction of the brominated steroids.

trated sulfuric acid, and 1 ml of potassium bromide-82 solution[3] in the order given. Start the air sweep and boil gently for 10 min. Dilute the acetic acid in the reaction vessel cautiously with 2 ml of the bicarbonate-bisulfate solution and extract the brominated estrogens three times with

[3] From a solution of radioactive potassium bromide in 1.0 ml of water is taken 0.1 ml, which is diluted to 10.0 ml. This working solution has ca. 40 μg/ml (0.3 μmole/ml) and contains ca. 8 μc/ml.

2-ml portions of chloroform. Wash the combined chloroform extract twice with 2 ml of 3% (v/v) acetic acid. Evaporate the chloroform extract to dryness, keeping the temperature below 50°C. The procedure to the point of evaporation is done in a hood behind ¼″ lead shielding.

Dissolve the residue in 1 ml of benzene and add 1 ml of the stock solution of carrier dibromoestrogens. Transfer to a 1 × 7.5 cm column of Woelm alumina (ca. 7.5 gm). Complete the transfer by rinsing once with two drops of ethanol *and* 1 ml of benzene, and once with 1 ml of benzene. After the transfer is complete, elute with the gradient defined by the following conditions: mixing chamber, 200 ml benzene; reservoir, 50%

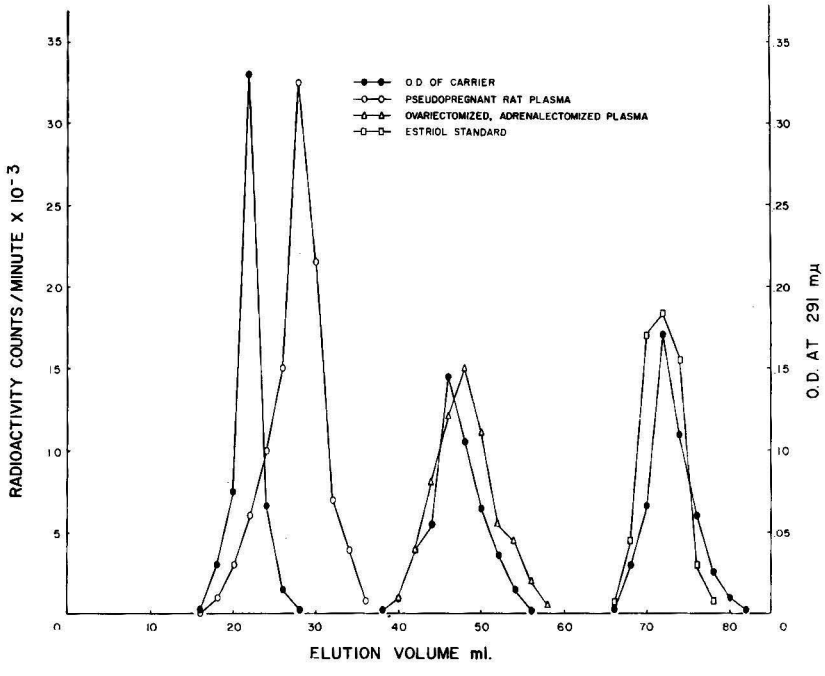


FIG. 2. Composite of three chromatograms. (—●—) Optical density of carrier measured at 291 mμ; the dibromoestrone peak is located at 22 ml, dibromoestradiol at 46 ml, and dibromoestriol at 72 ml. Radioactive products (—○—). The principal component from an extract of plasma from pseudopregnant rats (courtesy of Dr. Keith Vance); (—△—) only component from an extract of plasma from oophorectomized, adrenalectomized women which was shown not to be dibromoestradiol by dilution of the contents of the three peak tubes with more dibromoestradiol and recrystallization; (—□—) authentic estriol from a recovery experiment. Notice that "estrone-like" and "estradiol-like" substances were easily recognized and excluded.

ethanol in benzene; inflow rate: outflow rate of mixing chamber = 1:7; column flow rate, 2 ml/min. Collect sixty 2-ml fractions.

Upon completion of the chromatography assay the radioactivity in a suitable pulse-height analyzer (Nuclear-Chicago Model 132b, base = 500, window off). Evaporate the solvent, dissolve the residue in 3.0 ml of 95% ethanol, and measure the absorption of the carriers at 291 mμ.

When a peak of radioactivity coincides with an absorption peak of carrier (Fig. 2), add 50 mg of the appropriate carrier and crystallize several times from benzene-petroleum ether to constant specific activity. If desired, other derivatives, such as esters and carbonyl derivatives, may also be recrystallized to constant specific activity.

Calculate the quantity of each estrogen present on the basis of the radioactivity yielded by 100 mμg of authentic estrone, correcting for the decay of radioactivity when necessary.

RESULTS AND DISCUSSION

The reliability of any technique is a composite of its accuracy, precision, sensitivity, and specificity (11). Simplicity and speed, although desirable, are of secondary importance.

Accuracy, the closeness of measurements to the *true* value has been assessed by recovery experiments under a variety of conditions and with varying amounts of estrogens. Table 1 shows the recovery of a mixture

TABLE 1

PER CENT RECOVERY OF A MIXTURE OF AUTHENTIC ESTROGENS CONTAINING 1–100 MμG OF EACH ESTROGEN

Each number is the average of three determinations. A value of 100 has been arbitrarily assigned to 100 mμg of estradiol.

Steroid	Amt. (mμg)	Operator	
		1	2
Estrone	100	96	98
	10	101	97
	1	96	84
Estradiol	100	100	100
	10	100	100
	1	72	72
Estriol	10	100	98
	1	97	90

of authentic estrogens. Recovery is uniformly very satisfactory throughout the range from 1–100 mμg (1 mμg = 10^{-9} gm) for the three estro-

gens tested although there is a tendency for poorer recoveries at the 1 mμg level. The recoveries are relative to that for 100 mμg of estradiol, which is arbitrarily accorded the value of 100. (Both operators obtained the same value.) Values less than 96% may be regarded as primarily due to manipulative losses. Such values occurred only at the 1-mμg level, where the small quantity of material was conducive to loss. Recovery of carrier—and hence of radioactivity—from the column was essentially quantitative when fresh solutions of the carriers were employed. The bromination reaction also proceeds in good yield, being consistently 85-90% for quantities from 50 μg to 500 mg. It is of especial importance that all three estrogens react equally so that separate standardization is not necessary.

TABLE 2

PER CENT RECOVERY OF AUTHENTIC ESTROGENS ADDED TO EXTRACT OF URINE OBTAINED FROM AN OOPHORECTOMIZED, ADRENALECTOMIZED PATIENT

An equivalent of 100 ml of urine was used in each determination. Each number is the average of three determinations and is corrected for "endogenous steroid." The recoveries are based on the value obtained for 100 mμg of pure estrone.

Steroid	Amt. (mμg)	Operator 1	Operator 2
Estrone	100	108	100
	10	88	92
Estradiol	100	82	79
	10	90	(63)
Estriol	100	90	94
	10	109	82
	av.	96	90

Recovery of pure estrogens added to a urinary extract (Table 2) is more variable than for pure estrogens (Table 1); but on the average they are satisfactory, especially when the ratio of extraneous material to estrogen is appreciated. This ratio is estimated to be between 10^5 and 10^7. The area under each peak was determined by the baseline method. Accurate determinations at the 1-mμg level was impossible because the "background" of radioactivity from the column was of the order of that given by 10 mμg of estrogen.

Recovery of added estrogens from a plasma extract was more satisfactory (Table 3) than from the urinary extract, perhaps due to the fact that the former contained less interfering material or that less volume (6 ml) was used. There was some radioactivity (equivalent to 80 mμg)

TABLE 3

PER CENT RECOVERY OF AUTHENTIC ESTROGENS ADDED TO EXTRACT OF POOLED PLASMA OBTAINED FROM OOPHORECTOMIZED, ADRENALECTOMIZED PATIENTS

An equivalent of 6 ml of plasma was used in each determination. Due to the limited quantity of this type of plasma, only operator 2 performed recovery experiments in duplicate; both values are shown. The recoveries are based on the value obtained for 100 mμg of pure estrone.

Amt. (mμg)	Steroid		
	Estrone	Estradiol	Estriol
100	96, 97	96, 96	96, 96
10	99, 99	99, 98	98, 97

associated with the estradiol peak, but it was obviously not estradiol (Fig. 2). Otherwise, there was very little radioactivity and determinations at the 1-mμg level would have been attempted except for the lack of plasma extract.

Precision, the variation within replicate analyses, has been calculated in one instance. Using the data from which the values in Table 2 were derived, the percentage deviation from the average was calculated and summed for each estrogen over the three amounts of added estrogen (1, 10, and 100 mμg). The standard deviations for operator 1 were 3.1, 3.6, and 3.4% and for operator 2 were 2.8, 4.0, and 2.3% for estrone, estradiol, and estriol, respectively. The precision varies a little with the amount of estrogen being determined. The values cited are typical for 10 mμg. The precision is somewhat better at the 100-mμg level and somewhat poorer at the 1-mμg level. As frequently happens, the precision at low levels is adequate while the accuracy sometimes is not.

Sensitivity, the smallest detectable amount which differs significantly from zero, cannot be categorically stated unless several parameters are first defined. The sensitivity will vary with the specific activity of the bromine used in labeling, the sharpness of the peak eluted from the column, the sensitivity of the detection system, and the amounts of interfering substances eluted with or in close proximity to the compound being analyzed. Using potassium bromide having a specific activity of 0.2 mc/mg, 100 mμg of estrogen yields approximately 10^5 counts/min. Since background is about 10^2 counts/min, theoretically 0.1 mμg should be detectable in our counting system, assuming no interfering substances and elution in a single tube. In practice, the long counting times combined with the short half-life of the isotope and a customary peak width of 2–5 tubes makes such a determination unrealistic. The availability of bromide with a limiting specific activity of 1.2 mc/mg would make the

assay of 0.1 mμg practical. This assay is, of course, two or three orders of magnitude more sensitive than present methods (1–3).

Specificity, the assurance of identity, is accomplished in several discrete steps. The first is gradient elution chromatography on alumina of the labeled extract admixed with suitable carriers. Identity of radioactive and ultraviolet peaks permits a tentative identification. Then, confirmation of the assignment may be made by further addition of carrier to the peak tube(s) and crystallization to constant specific activity. Finally, further confirmation may be obtained by making any number of derivatives and repeating the process. An important feature of this technique is that no material is consumed in assays, a vital requirement when the amount of material is limited.

The urinary extract used was obtained by an enzymic (beef β-glucuronidase) hydrolysis followed by solvolysis at pH 1 with ether. The ether extract was washed only with sodium bicarbonate. Toluene-alkali partition was purposely omitted as we wish to add, from time to time, other estrogens, which would be altered by the alkali, to the determination. In addition, a large amount of any 2-methoxyestrone present would be lost by an alkali partition (12). The quantity of urine extract used (100 ml equivalent) was a compromise. Since urines from males and postmenopausal females contain 1–6 μg of estrogen per day, of which 90–95% is estrone and 5–10% estradiol (13), use of 100 ml of urine would result in the availability of 60–100 mμg of estrone and 3–10 mμg of estradiol for analysis. The latter is near the limit of detection of the method. Increasing the amount of urine would not help much since contaminants are also increased.

Plasma extracts were prepared by precipitating the protein with alcohol, removing the alcohol, and processing the remaining aqueous residue in exactly the same manner as urine.

Details of the methodology of hydrolysis and extraction are not included here for two reasons. First, it is felt that the bromine-82 technique is compatible with any method of extract preparation. Even though we believe that the future lies with mild hydrolytic methods (or no hydrolysis at all), we do not wish to imply that acid hydrolysis is incorrect. Second, the whole problem of hydrolysis is currently under investigation in our laboratory and will be published in a separate communication.

This report is limited to the "classical" estrogens. Investigation of the bromination of the 2-methoxy- and 16-oxyestrogens is currently in progress. Some compounds may fail to separate adequately upon column chromatography, but it is felt that most of the newer metabolites of estradiol or estrone can be accommodated by the method.

Although of lesser importance, it is gratifying to report that the methodology is both simple and rapid to anyone already acquainted with the techniques. Bromination and chromatography require 1.5 hr. Assay of radioactivity and carrier requires an additional 2–3 hr. For satisfactory results, however, thorough standardization and scrupulous attention to detail are required.

Although this technique is more reliable and simpler than previous methods, it remains at present too involved to be used as a routine clinical procedure. If the chromatography and subsequent steps could be omitted, the procedure would be simple enough for routine clinical application. Such omissions would be valid if estrogens and estrogen metabolites are the principal phenolic compounds present in the extract. Preliminary results indicate that such is probably true in the case of plasma extracts. Many more extracts, representing a wide variety of clinical conditions, must be examined before a definitive statement can be made.

SUMMARY

A new method for analysis of phenolic steroids is reported. It consists of dibromination with bromine-82, a gamma emitter with a half-life of 36 hr. This method is very sensitive (1 mμg), reasonably accurate (>80% recovery from urine, >95% recovery from plasma at the 10-mμg level), quite precise (±3%), and highly specific. Specificity is assured by gradient elution chromatography, followed by dilution with carrier and recrystallization to constant specific activity.

ACKNOWLEDGMENTS

The encouragement, support, and advice of our colleague, Dr. Avery A. Sandberg, is gratefully acknowledged. We should also like to thank the personnel of the Western New York Nuclear Research Center at the University of Buffalo for their cooperation in supplying radioactive potassium bromide. Mr. Robert Ehrke and Mr. James Sheehe rendered invaluable technical assistance.

REFERENCES

1. Engel, L. L., Slaunwhite, W. R., Jr., Carter, P., and Nathanson, I. T., *J. Biol. Chem.* **185**, 255 (1950).
2. Brown, J. B., *Biochem. J.* **60**, 185 (1955).
3. Bauld, W. S., *Biochem. J.* **63**, 488 (1956).
4. Preedy, J. R. K., and Aitken, E. H., *J. Biol. Chem.* **236**, 1300 (1961).
5. Engel, L. L., Cameron, C. B., Stoffyn, A., Alexander, J. A., Klein, O., and Trofimow, N. D., *Anal. Biochem.* **2**, 144 (1961).
6. Wotiz, H. H., and Martin, H. F., *J. Biol. Chem.* **236**, 1312 (1961).
7. Slaunwhite, W. R., Jr., Engel, L. L., Olmstead, P. C., and Carter, P., *J. Biol. Chem.* **191**, 627 (1951).

8. BAULD, W. S., GIVNER, M. L., ENGEL, L. L., AND GOLDZIEHER, J. W., *Can. J. Biochem. and Physiol.* **38,** 213 (1960).
9. SLAUNWHITE, W. R., JR., AND NEELY, L., *J. Org. Chem.* **27,** 1749 (1962).
10. ATTENBURROW, J., CAMERON, A. F. B., CHAPMAN, J. H., EVANS, R. M., HEMS, B. A., JANSEN, A. B. A., AND WALKER, T., *J. Chem. Soc.* (*London*) **1952,** 1094.
11. DICZFALUSY, E., *Acta Endocrinol., Suppl.* **31** (1957).
12. ENGEL, L. L., BAGGETT, B., AND CARTER, P., *Endocrinol.* **61,** 112 (1950).
13. SLAUNWHITE, W. R., JR., AND NEELY, L., unpublished observations.

ANALYTICAL BIOCHEMISTRY 5, 143–148 (1963)

A Rapid Quantitative Separation of Phosphatidyl Ethanolamine and Phosphatidyl Serine by Column Chromatography[1]

HUGH L. SPITZER AND JOHN A. BALINT

From the Department of Medicine, University of Alabama Medical Center, Birmingham, Alabama

Received June 11, 1962

INTRODUCTION

During the course of our investigation of phospholipid metabolism in various pathological tissues the need arose for a rapid quantitative separation of the cephalin fraction into its component parts. The only complete quantitative separation of phosphatidyl ethanolamine and phosphatidyl serine by column chromatography, described in the literature to date, has been that of Rouser *et al.* (1). The procedure described here is a modification of that method and its application to 100-μg quantities of sample.

PROCEDURE

Rat liver was used as the source of material. Normal male rats weighing 250–350 gm were sacrificed, and their livers immediately removed, and perfused with normal saline. The tissue was then freeze-dried, homogenized in normal saline, and then extracted with 20 vol of chloroform:methanol (2:1) overnight; the extract was washed with normal saline, and the chloroform layer then dried over anhydrous sodium sulfate. The chloroform phase was then reduced to 50 ml under vacuum and stored at 2°C until used. This procedure of rapid extraction of fresh tissue and storage of the final chloroform extract in the cold effectively limited decomposition of the lipids.

Human erythrocyte lipids were extracted as described by Balint *et al.* (2) and stored in chloroform in the cold until used.

The crude lipid was first fractionated on a Unisil[2] column, using 1 gm

[1] This work was supported by Grant A-5323 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland.

[2] Activated Unisil, 100–200 mesh obtained from Clarkson Chemical Company, Williamsport, Pennsylvania.

silicic acid for 10–15 mg lipid. Neutral lipids were eluted with chloroform, and the phospholipids were separated by increasing concentrations of methanol in chloroform as described by Balint *et al.* (2). The chloroform eluate was checked for phosphorus with negative results.

Chemical analyses were performed for phosphorus by the Bartlett procedure (3) for amino nitrogens by the method of Lea and Rhodes (4) and for esters by a combination of the Synder and Stephens (5) and Rapport and Alonzo method (6) [reagents from (5) and procedure from (6)].

The fraction eluted in 25% methanol in chloroform was found to be cephalin. In rat liver the phosphorus to ester ratio of this fraction was found to be 1:1.96 to 1:2.05 and in erythrocytes 1:1.70 to 1:1.90. The amino nitrogen to phosphorus ratio in the cephalin fraction from both tissues was in the range of 1:0.87 to 1:1.15. It was felt that since the amino nitrogen to phosphorus ratio approximated 1:1 there was no significant contamination by phosphatidylinositol or cardiolipin. In view of this consideration no further efforts were made to check for phosphatidylinositol or cardiolipin.

The cephalin fraction was further checked for purity by paper chromatography. A 25-μg sample when placed on silicic acid impregnated Whatman No. 1 filter paper in a diisobutyl ketone:acetic acid:water (40:25:5) or chloroform:methanol (3:1) plus 2% water ascending system (8) gave a single spot when stained with rhodamine G. This was ninhydrin positive and choline negative when stained with phosphomolybdic acid followed by stannous chloride. The cephalin gave an R_f value similar to an appropriate cephalin[3] standard. It has been our experience that the phosphatidyl ethanolamine and serine are not separable in the solvent systems described.

Two sizes of ammonium silicate columns were used in separating the cephalin fraction into its component parts: a large one for 50-mg samples and a small one for less than a 5-mg load. The large column was prepared by slurrying 15 gm (height 180 mm, diameter 22 mm) of Unisil in chloroform:methanol (4:1) on to a Hirsch-Ahrens type of column, (7). The column was then washed with exactly 70 ml of a chloroform:methanol:ammonia mixture that was prepared by adding 10 ml of concentrated aqueous ammonia to 1 liter of chloroform:methanol (4:1). The small column of 5 gm (height 125 mm, diameter 10 mm) of Unisil was prepared in a similar manner but washed with exactly 20 ml of the chloroform:methanol:ammonia mixture.

[3] Synthetic dipalmitoyl ethanolamine from Mann Research Laboratories, Inc., New York 6, New York, and Californina Biochemical Research Corporation, Los Angeles 63, California. Phosphatidyl serine was purified from human brain by the method of Rouser *et al.* (1).

The cephalin fraction was dissolved in a small amount of chloroform: methanol (4:1) and applied to the column. A mixture of chloroform: methanol (4:1) was used to elute the phosphatidyl ethanolamine; the solvent volume varied with amount of phosphatidyl ethanolamine present. Phosphatidyl serine was eluted with 200 ml of 100% methanol after phosphorus values in the eluate in 4:1 chloroform:methanol had reached baseline (Fig. 1).

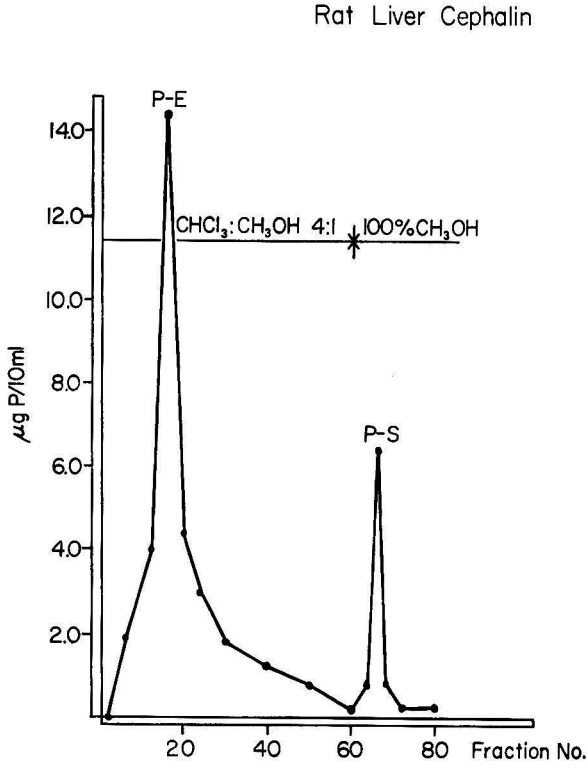


FIG. 1. Five-gram ammonium silicate column: 4.6 mg cephalin applied; (P-E) phosphatidyl ethanolamine recovered 3.6 mg; (P-S) phosphatidyl serine recovered 1.0 mg. Column monitored by phosphorus on 1-ml aliquot from every other 10-ml fraction.

RESULTS AND DISCUSSION

Figure 1 shows a typical separation of phosphatidyl ethanolamine and phosphatidyl serine on a 5-gm ammonium silicate column. By monitoring elution with the Bartlett procedure for phosphorus (3) the time required to separate the cephalin fraction into its component parts takes

about 1.5 hr from the slurrying of the column to the completion of elution. The 15-gm column for larger samples gives similar results and takes only 2 to 2.5 hr. Neither the large nor small column needed external pressure to maintain an adequate flow rate. In both, a flow rate of 5 ml/min was used with complete success. Recovery from both columns ranged from 94.4 to 106.0% in 6 determinations.

Because the ratio of phosphatidyl ethanolamine to phosphatidyl serine is disproportionately one sided in rat liver the ratio was artificially altered to check its effect on elution. Purified phosphatidyl ethanolamine and phosphatidyl serine when added in varying ratios had no effect on separation. In all instances recovery and separation were complete (see Table 1).

TABLE 1

SEPARATION AND RECOVERY OF PHOSPHATIDYL ETHANOLAMINE AND PHOSPHATIDYL SERINE ON AN AMMONIUM SILICATE COLUMN

Source	Expt. No.	Column size (gm)	Column load (mg)	Phosphatidyl ethanolamine (mg)	Phosphatidyl serine (mg)
Rat liver cephalin	1	15	45.8	36.2	9.2
	2	5	4.6	3.6	1.0
	3	5	15.3	13.3	2.8
Human erythrocyte cephalin	4	5	3.6	2.1	1.1
	5	5	3.7	2.1	1.6

Artificial mixture from rat liver applied to 5-gm column

	Applied (mg)	Recovered (mg)
Phosphatidyl ethanolamine	2.4	2.6
Phosphatidyl serine	1.8	1.9

It should be noted that the amount of chloroform:methanol:ammonia mixture used to prepare the ammonium silicate column must be precise. Small deviations from the amounts prescribed, ± 1.0 ml for the large column and ± 0.5 ml for the small column, will result in either poor separation if too little is used, or an inability to remove the sample from the column if an excess is used. In the latter case it was found that the phosphatidyl serine was tenaciously held by the column and required acidified methanol for its removal.

Paper chromatography on silicic acid impregnated Whatman No. 1 filter paper run in a descending system of chloroform:methanol (4:1), containing 10 ml of concentrated aqueous ammonia and 1 gm of ammonium acetate per liter, was used to check the completeness of separation on the ammonium silicate column. In all instances no traces of

phosphatidyl ethanolamine could be detected in the phosphatidyl serine, nor phosphatidyl serine in the phosphatidyl ethanolamine. As described by Rouser *et al.* (1) the phosphatidyl serine remains at the origin and the phosphatidyl ethanolamine gives an R_f value comparable to an appropriate standard.[3] Running time for the paper chromatograms was 2–2.5 hr. The best results were obtained on freshly impregnated silicic acid paper, not over two weeks old.

By using Unisil, a commercially prepared activated silicic acid for making an ammonium silicate column, the necessity of solvent washing and heat activation described by Rouser *et al.* (1) has been eliminated. We have found no variation in the Unisil from batch to batch. To minimize any possible uptake of water the Unisil is stored under vacuum over silica gel.

The simplicity of the method described here has reduced the quantitative separation of cephalin into its component parts by column chromatography to a rapid routine procedure. In practice it is possible to separate the cephalin fraction from other phospholipids and transfer it to an ammonium silicate column, split it into phosphatidyl ethanolamine and phosphatidyl serine, and subject aliquots of each component to paper chromatography all in one day. The rapidity of the method minimizes oxidative decomposition to an undetectable level.

Comparison of the data shown here on rat liver cephalin is in agreement with that of Artom (9). This method also has been used to separate erythrocyte cephalin and the figures for normal controls have been in agreement with those obtained by Reed *et al.* (10). The differences in experiments 4 and 5 in Table 1 indicate a wide variation in the composition of erythrocyte cephalins. It should be noted that the apparent discrepancy is probably due to incomplete recovery in experiment 4.

SUMMARY

A rapid quantitative separation on an ammonium silicate column of phosphatidyl ethanolamine from phosphatidyl serine has been described. It is more convenient and less time consuming than earlier methods, and the separations obtained have been as complete as those described by Rouser *et al.* (1). It is a procedure readily adaptable in routine phospholipid analysis. The separation has been found to be complete when checked by paper chromatography. The method gives results which are in agreement with the findings of other authors.

REFERENCES

1. Rouser, G., O'Brien, J., and Heller, D., *J. Am. Oil Chemists' Soc.* **38**, 14 (1961).

2. BALINT, J. A., NYHAN, W. L., LIETMAN, P., AND TURNER, D. A., *J. Lab. and Clin. Med.* **58,** 548 (1961).
3. BARTLETT, G. R., *J. Biol. Chem.* **234,** 466 (1959).
4. LEA, C. H., AND RHODES, D. N., *Biochim. et Biophys. Acta* **17,** 416 (1955).
5. SNYDER, F., AND STEPHENS, N., *Biochim. et Biophys. Acta* **34,** 244 (1959).
6. RAPPORT, M. M., AND ALONZO, N., *J. Biol. Chem.* **217,** 193 (1955).
7. HIRSCH, J., AND AHRENS, E. H., JR., *J. Biol. Chem.* **233,** 311 (1958).
8. MARINETTI, G. V., AND STATZ, E., *Biochim. et Biophys. Acta* **21,** 168 (1956).
9. ARTOM, C., *J. Biol. Chem.* **157,** 595 (1945).
10. REED, C. R., SWISHER, S. N., MARINETTI, G. V., AND EDEN, E. G., *J. Lab. and Clin. Med.* **56,** 281 (1960).

ANALYTICAL BIOCHEMISTRY 5, 149–157 (1963)

Influence of Coprecipitation on Quantitative Estimations of Ribonuclease Activities by Precipitation Methods

L. LEPOUTRE, J. STOCKX, AND L. VANDENDRIESSCHE

From Laboratorium voor Fysiologische Scheikunde, Rijksuniversiteit, Gent, Belgium

Received June 18, 1962

Many precipitation methods are in use for the quantitative estimation of RNase[1] activities (1–12). In these methods the undegraded RNA and the less degraded RNA fragments are precipitated and the breakdown is followed either by measurement of the optical density of the supernatant, which contains the nonprecipitable degradation products, or by determinations of the inorganic phosphate after mineralization of an aliquot of the supernatant. Attention has been drawn to an interfering influence on the results obtained through such methods by various compounds, e.g., cations or anions (11–13) or polyanionic substances like heparin and sulfated pectins (13). As a consequence of these findings no activity determinations by precipitation methods can be trusted unless the same concentration of the products whose effect is under investigation is added to the blank determinations before precipitation. When these interfering substances are precipitable themselves, their influence on the activity determinations will originate most frequently from their capacity of withdrawing from the supernatant a certain amount of otherwise soluble degradation products, e.g., through the formation of less soluble salts or by their increased adsorption on the precipitate; when the interfering substances are not precipitated by the precipitating reagent used, they can render the less soluble fragments more soluble, e.g., through complex formation.

It is necessary to correct for both effects in order to rule out apparent activations or inhibitions, uniquely due to such alterations of the solubilities of the various mono-, oligo-, and polynucleotides present at any stage of the breakdown.

A question which arises in this connection is the following: how can results be influenced by various additions of RNA or still precipitable

[1] Abbreviations: RNA, yeast ribonucleic acid; RNase, ribonuclease; P.RNase, pancreatic ribonuclease; Ph.a.RNase, ribonuclease of *Phaseolus aureus* Roxb.

degradation products? Such a situation arises, e.g., in kinetic experiments, where the influence of substrate concentration on the activity of RNases is investigated. It seems to be a rather common practice in such cases to use a constant RNase concentration with various RNA concentrations and to precipitate the incubation mixtures as such (10, 14). Frisch-Niggemeyer and Reddi (14) and Dickman and Ring (10) find substrate inhibition, respectively, for pancreatic RNase and for the RNase of tobacco leaves, by a precipitation method; they do not mention whether the various RNA concentrations were adjusted to a same value before precipitation. Holden and Pirie (6) also used a precipitation method in their study of the RNase of pea leaves. They showed that the so-called "apparent hydrolysis" of RNA became greater for more dilute substrate concentrations. Accordingly they precipitated the solutions directly when the incubation mixtures contained no more than 20 mg P/liter; on the other hand they diluted the solutions once when they contained more than 400 mg P/liter. Pierpoint (15) used this method in an investigation on the influence of RNA concentrations on the activity of pea leaf RNase; he diluted or added RNA to the incubation mixtures until they had approximately the same RNA concentration as before precipitation.

RESULTS AND DISCUSSION

Precipitation of Mixtures Containing a Constant Amount of Degradation Products and Varying Amounts of RNA

We started an investigation on the influence of substrate concentration on the activity of P.RNase and Ph.a.RNase. In this study we used two precipitation reagents containing $Ba(ClO_4)_2$ in organic solvents (9, 12). Our interest went first to the influence of RNA concentration on the results of the precipitation method itself. The net effect of different (precipitable) RNA concentrations on a same amount of (nonprecipitable) degradation products can be evaluated by direct precipitation of mixtures containing a constant amount of degradation products (obtained from exhaustive digestion of RNA by P.RNase or Ph.a.RNase, dialysis, and lyophilization of the dialyzate) and different amounts of added RNA. These mixtures simulate, within a certain approximation, those that are obtained when an RNase has liberated the same amount of essentially nonprecipitable degradation products in the presence of various RNA concentrations; they exclude the kinetic influence of the substrate concentration on RNase activity itself. In each case definite and well-known proportions of nonprecipitable to precipitable material are present. The first precipitation method tested was the $Ba(ClO_4)_2$-Cellosolve-water method (12). We used as nonprecipitable material the

degradation products of RNA by Ph.a.RNase. Mixtures of nonprecipitable products and RNA in varying proportions were made as shown in Table 1. Two stock solutions in 0.1 *M* cacodylate (pH 5.8) were used: one was a 0.075% solution of degradation products and the other a 6.25% RNA solution (sodium nucleate Merck). All dilutions were made with the original 0.1 *M* buffer, so that differences in ionic strength (except those contributed by RNA itself) could not influence the results. The range of supernatant extinctions tested was that which is currently used in the determination of RNase activities.

TABLE 1

COMPOSITION OF MIXTURES OF DEGRADATION PRODUCTS AND RNA PRECIPITATED BY $Ba(ClO_4)_2$-CELLOSOLVE-WATER REAGNET

Stock soln. degradation products (μl)	Stock soln. RNA (μl)	0.1 *M* cacodylate, pH 5.8 (μl)	Final concn. degradation products (%)	Final concn. RNA (%)
500	—	2.000	0.015	—
500	2.000	—	0.015	5.00
—	2.000	500	—	5.00
500	1.000	1.000	0.015	2.50
—	1.000	1.500	—	2.50
500	400	1.600	0.015	1.00
—	400	2.100	—	1.00
500	300	1.700	0.015	0.75
—	300	2.200	—	0.75
500	200	1.800	0.015	0.50
—	200	2.300	—	0.50
500	100	1.900	0.015	0.25
—	100	2.400	—	0.25
500	40	1.960	0.015	0.10
—	40	2.460	—	0.10

The results are shown in Fig. 1. The upper curve shows the results obtained for a constant amount of degradation products precipitated in the presence of various RNA concentrations; it is apparent that with increasing RNA concentration of the solutions more and more soluble degradation products are removed from the supernatant. The lowest curve shows that RNA contains some nonprecipitable material and that the blank values increase with RNA concentration, although not linearly. The intermediate (broken) curve was obtained by subtracting the blank values from the experimental values of the upper curve. These values are those used in fact for the evaluation of the amount of degradation in enzymic experiments. The decreasing E_{260} values of the supernatant shown with increasing RNA concentrations make one suspicious about

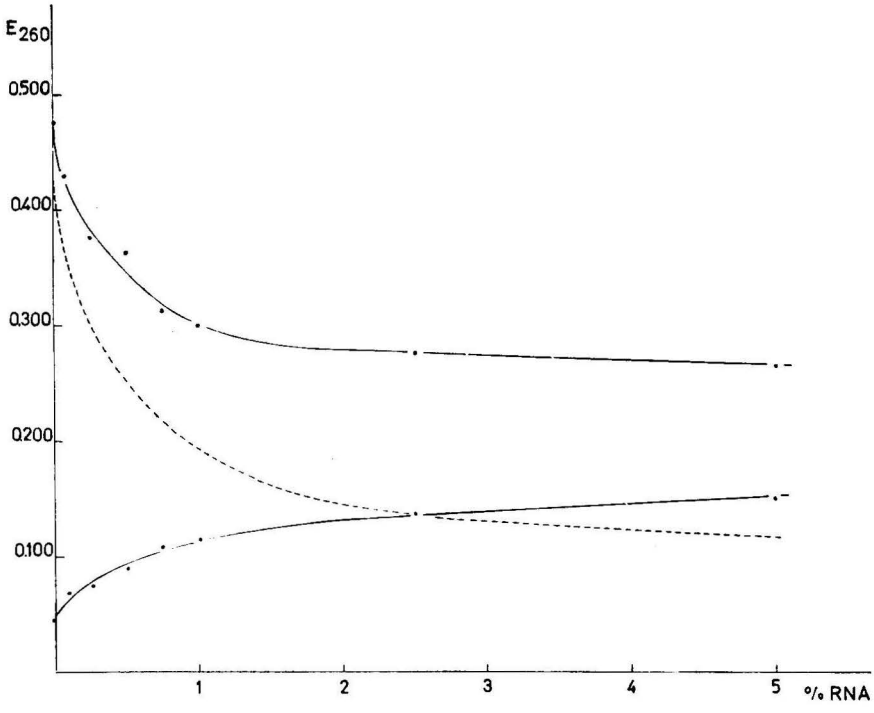


FIG. 1. Influence of RNA concentration on determination of degradation products obtained from hydrolysis of RNA by Ph.a.RNase. *Precipitation reagent:* 1 vol 80% $Ba(ClO_4)_2$ in H_2O + 9 vol Cellosolve (ethyl glycol); 400 μl precipitated with 2 ml of the reagent. *Upper curve:* precipitation of 0.015% degradation products + varying amounts of RNA (see Table 1); extinction values of the supernatant at 260 mμ, measured against water as a blank. *Lowest curve:* precipitation of varying amounts of RNA (see Table 1); extinction values of the supernatant at 260 mμ, measured against water as a blank. These are the blank values for the assays on mixtures containing RNA as well as degradation products. *Middle curve:* values obtained by subtracting the RNA blank values (lowest curve) from those of the mixtures containing degradation products and RNA (upper curve). These are the values actually used in the estimation of RNase activities.

the meaning of substrate inhibitions found in certain experiments in which the incubation mixtures are precipitated without having been brought to the same substrate concentration. It is clear that an apparent substrate inhibition can thus be found which is exclusively due to coprecipitation, and this because the amount of the coprecipitated substances becomes greater when more precipitable material is present in the solution. It was easy to show that measurements performed after a preliminary dilution to the same RNA concentration give, within the experi-

mental limits, the same value for the supernatant when the results are multiplied with the dilution factor that was applied.

In the same way the precipitation method with $Ba(ClO_4)_2$-isopropanol-ethanol (9) was tested. Here we used as nonprecipitable degradation products those obtained after dialysis of an RNA solution which had been exhaustively digested by P.RNase; this time a stock solution of 0.25% was made up (this precipitation method gives in general E_{260} values of the supernatant that are about 2.3 times lower than those found with the previous precipitating agent). The buffer used was 0.1 *M* acetate of pH 5.0, instead of 0.1 *M* cacodylate of pH 5.8. The results are shown in Fig. 2. They are essentially the same as those obtained with the

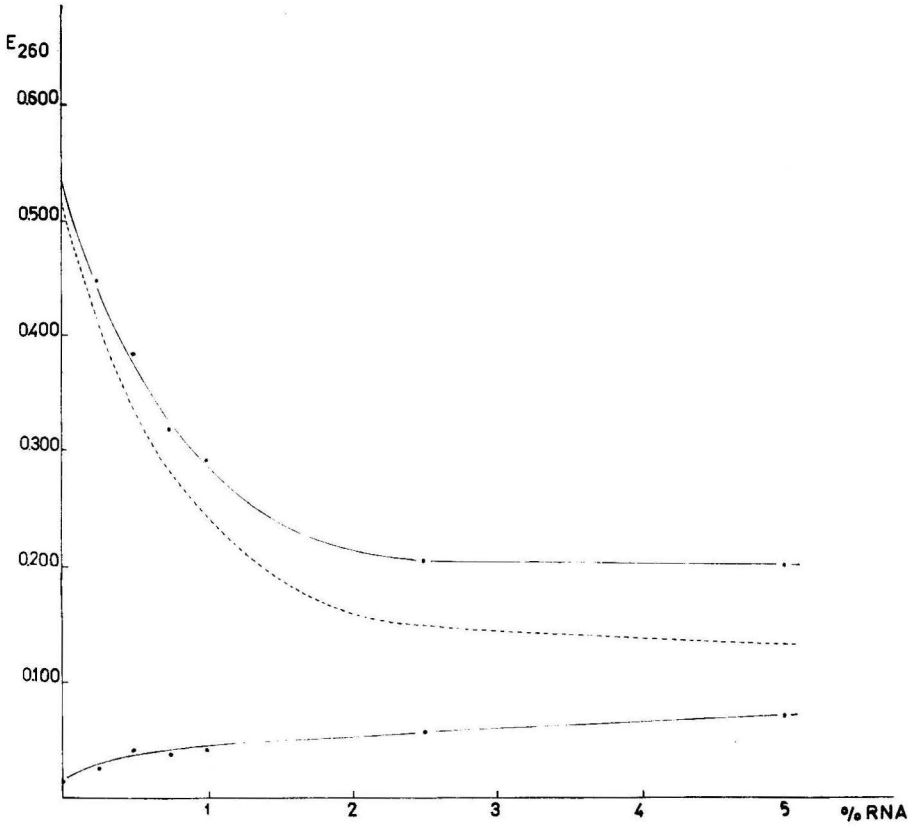


FIG. 2. Influence of RNA concentration on determination of degradation products obtained from hydrolysis of RNA by P.RNase. *Precipitation reagent:* 5 vol 83.5% $Ba(ClO_4)_2$ in H_2O + 10 vol isopropanol, diluted to 100 vol with ethanol; 1 ml precipitated with 5 ml of the reagent. The order and the significance of the curves is the same as in Fig. 1. The stock solution of degradation products was 0.25%.

previous method, although quantitative differences are apparent. The blank values, especially, are appreciably lower. The conclusions reached with the former precipitation method are valid qualitatively here too: greater amounts of precipitable material withdraw greater amounts of nonprecipitable substances from the supernatant.

A third precipitation method using glacial acetic acid-*tert*-butanol (1:2) as the precipitating reagent (10) was finally investigated according to the same principles (Fig. 3). The buffer used was 0.1 *M* acetate,

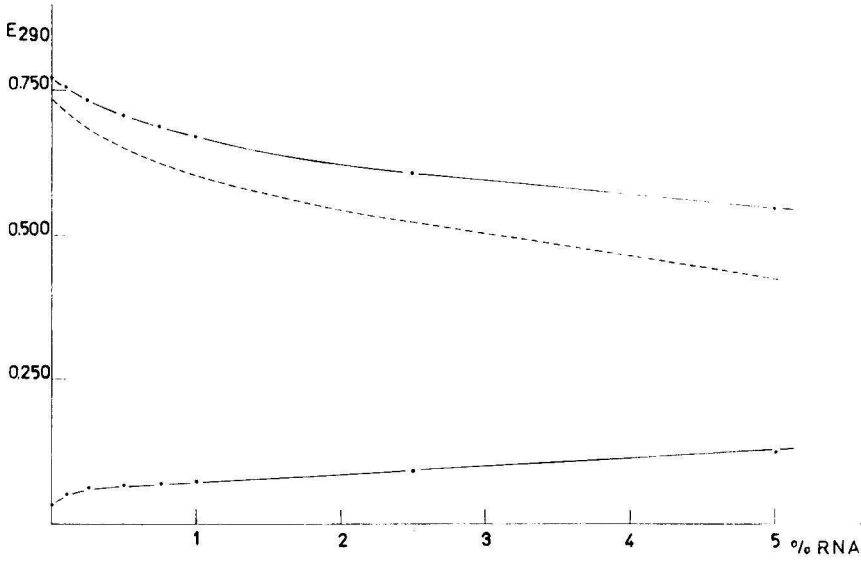


FIG. 3. Influence of RNA concentration on determination of degradation products obtained from hydrolysis of RNA by P.RNase. *Precipitation reagent:* 1 vol acetic acid + 2 vol *tert*-butanol; 1 ml was precipitated with 3 ml of the reagent. The order and the significance of the curves is the same as in Fig. 1. Determinations were done at 290 instead of 260 mμ; the stock solution of degradation products was 0.20%.

pH 5.0. Here too the results were qualitatively the same as those found for the two preceding methods.

All three precipitation methods tested are thus endowed with the disadvantage of showing appreciable coprecipitation effects. The amount of coprecipitation depends on the method used and it will interfere with any determination in which the RNA concentration is not kept constant.

The nature of this coprecipitation is such that it produces results that may be interpreted as proof of substrate inhibition where this phenomenon does not at all occur; in some cases this inhibition will be erron-

eously suspected at lower substrate concentrations than where it really occurs. Meaningful kinetic experiments in relation to the influence of substrate concentration can only be performed provided the incubation mixtures are all brought as closely as possible to the same substrate concentration before precipitation.

Precipitation of Mixtures Containing Various Amounts of Degradation Products and a Constant Amount of RNA

In the preceding section we studied the coprecipitation of a fixed amount of nonprecipitable degradation products as a function of the concentration of precipitable, undegraded RNA. We also studied quantitatively the coprecipitation as a function of the concentration of degradation products, the amount of precipitable material (RNA) being kept constant.

For this investigation we used the precipitation method of Dickman

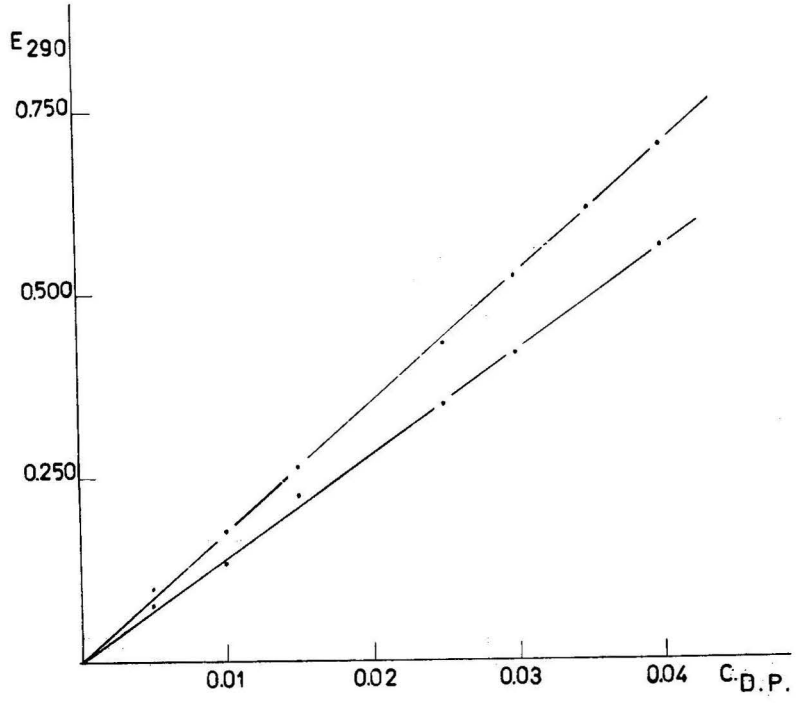


FIG. 4. Precipitation of various concentrations of degradation products (obtained from hydrolysis of RNA by P.RNase) as such (*upper curve*) and in the presence of 1% RNA (*lower curve*). The concentration of degradation products (as %) is shown along the abscissa.

and Ring (10). Various amounts of degradation products (the dialyzed degradation products obtained from RNA digestion by pancreatic RNase) were added to 1% RNA in a final acetate concentration of 0.1 *M* (pH 5).

One-ml quantities were precipitated with 3 ml of glacial acetic acid-*tert*-butanol (1:2), centrifuged, and the optical density of the supernatant measured directly at 290 mμ. The results are shown in Fig. 4.

The amount of degraded material coprecipitated along with a constant amount of precipitable material is obviously a linear function of the amount of nonprecipitable material present, at least in the concentration range investigated. This concentration range is the one which is used practically in the precipitation method tested. Coprecipitation there obeys the Langmuir equation:

$$\frac{X}{m} = \frac{k_1 C}{1 + k_2 C}$$

The rectilinear relationship of the amount of adsorbed degradation products to their concentration in the solution is merely due to the fact that $k_2 C << 1$ with the result that:

$$X/m \cong k_1 C$$

where X = amount of adsorbed essentially nonprecipitable degradation products, C = concentration of these products, m = amount of precipitated products, and k_1 and k_2 are constants. It is probable that the same relationship holds for other precipitation methods, at least in the most useful region of their applicability where $k_2 C$ is smallest.

Whenever precipitation methods are used in conditions under which the proportion of nonprecipitable to precipitable material varies considerably, the interfering effect of coprecipitation may not be kept out of view. Its influence may be considerable, as will be shown in a forthcoming paper on the alleged substrate inhibition of P.RNase.

SUMMARY

We investigated coprecipitation effects occurring in three precipitation methods used for the determination of RNase activities. In this study we examined exclusively the coprecipitation of the nonprecipitable degradation products themselves along with the precipitable substrate. This was done in two ways: (*1*) by precipitating RNA in various concentrations in the presence of the same amount of nonprecipitable degradation products; (*2*) by precipitating the same amount of RNA in the presence of various concentrations of degradation products.

It was shown that the results yielded by precipitation in media con-

taining different amounts of substrate cannot be trusted. In kinetic studies on the influence of substrate concentration of RNase activities they will occasionally lead to erroneous conclusions and reveal apparent substrate inhibitions that do not exist or exist only at higher substrate concentrations. From our data it follows that such studies can be performed using those techniques, provided that the proportion of non-precipitable to precipitable substances is kept as constant as possible (e.g., by adequate dilution of the more concentrated solutions). It was also shown in one case that the coprecipitation is a rectilinear function of the concentration of degradation products in the range of the practical application of the method. In this way it fits the Langmuir adsorption isotherm.

REFERENCES

1. KUNITZ, M., *J. Gen. Physiol.* **24,** 15 (1940).
2. PIROTTE, M., AND DESREUX, V., *Bull. soc. chim. Belg.* **61,** 167 (1952).
3. SCHNEIDER, W. C., AND HOGEBOOM, L. E., *J. Biol. Chem.* **210,** 551 (1952).
4. ANFINSEN, C. B., REDFIELD, R. R., CHOATE, W. L., PAGE, J., AND CARROLL, W. R., *J. Biol. Chem.* **207,** 201 (1954).
5. SCHUCHER, R., AND HOKIN, L. E., *J. Biol. Chem.* **210,** 551 (1954).
6. HOLDEN, M., AND PIRIE, N. W., *Biochem. J.* **60,** 39, 53 (1955).
7. DICKMAN, S. R., AROSKAR, J. P., AND KROPF, R. B., *Biochim. et Biophys. Acta* **21,** 539 (1956).
8. BRODY, S., *Biochim. et Biophys. Acta* **24,** 502 (1957).
9. FIERS, W., AND STOCKX, J., *Naturwissenschaften* **44,** 115 (1957).
10. DICKMAN, S. R., AND RING, B., *J. Biol. Chem.* **231,** 741 (1958).
11. FIERS, W., AND MØLLER, K M., *Compt. rend. trav. Lab. Carlsberg, Sér. chim.* **31,** 507 (1960).
12. FIERS, W., *Anal. Biochem.* **2,** 126 (1961).
13. STOCKX, J., AND VANDENDRIESSCHE, L., *Arch. intern. physiol. et biochem.* **69,** 493, 521, 545 (1961).
14. FRISCH-NIGGEMEYER, W., AND REDDI, K. K., *Biochim. et Biophys. Acta* **26,** 40 (1957).
15. PIERPOINT, W. S., *Biochim. et Biophys. Acta* **21,** 136 (1956).

ANALYTICAL BIOCHEMISTRY 5, 158–169 (1963)

A New Method for the Determination of Dihydrocholesterol in Tissues

E. H. MOSBACH, J. BLUM, E. ARROYO, AND S. MILCH

From the Division of Laboratory Diagnosis, Public Health Research Institute of the City of New York, Inc., and the Bureau of Laboratories, New York City Health Department, New York, New York

Received June 25, 1962

INTRODUCTION

Dihydrocholesterol (cholestan-3β-ol) has been found in the nonsaponifiable fraction of all mammalian tissues studied so far (1). The biological origin and functions of this sterol are not known but it has been suggested that dihydrocholesterol is involved in the pathogenesis of atherosclerosis and cholelithiasis. This conclusion is based upon reports claiming that relatively large concentrations of dihydrocholesterol were present in atherosclerotic lesions and cholesterol gallstones. For example, Schoenheimer (2) found 1.2–1.9% dihydrocholesterol in the sterol fraction isolated from human gallstones and 5.1–5.3% dihydrocholesterol in the sterols of human atherosclerotic aortas. McArthur (3) found 11.7–12.9% dihydrocholesterol in the nonsaponifiable matter obtained from human atherosclerotic aortas. The results of animal experiments support the assumption that dihydrocholesterol may play a role in the etiology of cholelithiasis and atherosclerosis. It is known, for example, that the feeding of dihydrocholesterol to rabbits and mice will produce cholecystitis and cholelithiasis (4), and this sterol has further been shown to be atherogenic in rabbits and chicks (5, 6).

Studies of the metabolism of dihydrocholesterol in mammalian tissues required a method for the quantitative determination of this sterol in the presence of a large excess of cholesterol, since these two sterols always seem to occur together. The bromination method of Schoenheimer (2) was found to be unsatisfactory when the dihydrocholesterol content of the sterol fraction was less than 3% (which is usually the case in most tissues). The present report describes a new method for the determination of dihydrocholesterol. This method is based upon the finding that oxidation of cholesterol-dihydrocholesterol mixtures with performic acid (7) converts cholesterol to cholestane-3β,5α,6β-triol leaving the saturated

sterol, dihydrocholesterol, unchanged. The oxidation mixture is resolved into its components by chromatography on silicic acid columns (8). Dihydrocholesterol in the column effluent is determined as the digitonide, either gravimetrically or colorimetrically by the anthrone reaction (9). By means of this procedure as little as 0.2 mg of dihydrocholesterol can be determined with good accuracy even if a 100-fold excess of cholesterol is present.

MATERIALS

Formic acid, Fisher Scientific Company, #A-118. Dilute with water to make an 85% solution (v/v).

n-Hexane, spectroanalyzed, Fisher Scientific Company. #H-334.

Hydrogen peroxide, 30% solution, Becco Chemical Division, Food Machinery and Chemical Corporation, Buffalo 7, N. Y.

Ethyl ether, anhydrous, absolute.

Silicic acid, Bio-Rad, California Corporation for Biochemical Research, Los Angeles 63, Calif.

Digitonin, Hoffman LaRoche, Nutley, N. J.

Cholesterol, free of dihydrocholesterol, prepared from cholesterol, USP, by the bromination procedure of Schoenheimer (2).

Dihydrocholesterol, free of cholesterol, prepared by the procedure described in "Organic Syntheses," Coll. Vol. 2, p. 191 (1943).

Cholesterol-4-C^{14}, Nuclear-Chicago. Purified by chromatography on silicic acid (8).

METHOD

1. Extraction of Sterol Fraction from Tissues

(*a*) *Serum.* The serum was heated with 5 vol of 5% (w/v) KOH in 95% ethanol at 60 ± 1°C for 1 hr. An equal volume of water was added and the solution was extracted 3 times with ½ its volume of *n*-hexane. An aliquot of this solution was taken for determination of total sterol concentration by digitonin precipitation. After evaporation of the solvent (60°C water bath, under a stream of nitrogen) this nonsaponifiable fraction was used directly for the oxidation step (step *2*).

(*b*) *Other Tissues.* The tissues were hydrolyzed with 5 or 10% KOH (w/v) in 95% ethanol using at least 5 ml of KOH solution per gram of tissues. In the case of cholesterol gallstones 25 ml of alcoholic KOH per gram of stone was required to achieve complete solution. In all cases the tissues were refluxed for 3 hr, and after cooling to room temperature an equal volume of water was added. The nonsaponifiable fraction was obtained by extracting the solution 3 times with ½ its volume of *n*-

hexane. An aliquot of the hexane extract was used for the determination of total sterol by digitonin precipitation. In most cases the nonsaponifiable fraction of tissues was used directly for the oxidation step. However, if the nonsaponifiable material was highly colored or contained oily material it was first purified, either via the digitonide or by silicic acid chromatography.

2. Oxidation

The dry sterol mixture, or nonsaponifiable fraction, was placed in an Erlenmeyer flask (a 50-ml flask for samples containing up to 25 mg sterol, and a 125-ml flask for samples up to 100 mg of sterol), containing a Teflon-covered stirring bar. A solution of cholesterol-4-C^{14}, previously purified by silicic acid chromatography (8), (0.05 μc; 1 μg/ml of benzene, 30,000 cpm/ml under our conditions of radioactivity assay) was added to serve as an indicator for completeness of oxidation. The benzene was evaporated at 60°C under a stream of nitrogen. This radioactive cholesterol must be purified by silicic acid chromatography; otherwise trace impurities will appear in the dihydrocholesterol fraction. Radioactive contamination of this fraction will make it impossible to obtain an accurate measure of the amount of unoxidized cholesterol.

Following evaporation of the benzene, formic acid (85%, v/v) was added in the proportion of 1 ml of acid per milligram of sterol. The minimum volume of formic acid used was 10 ml. The flask was placed on a thermostatically controlled hot plate-magnetic stirrer combination. The surface temperature of the hot plate had previously been adjusted to 170°C (thermometer). The reaction mixture was stirred until all of the sample had dissolved, and stirring was continued 1 min longer, for a total period of 6–10 min, depending upon sample size. These conditions were chosen so as to avoid excessively prolonged heating periods yet assure complete dissolution of the sterols and allow the formylation reaction to proceed at 90–95°C for 2–3 min. This is the most critical step in the procedure since overheating will lead to lowering of the oxidation efficiency.

The sample flask was then transferred to an unheated magnetic stirrer, a thermometer was inserted into the solution, and when the temperature reached 75 ± 5°C a 30% solution of hydrogen peroxide (1 ml of peroxide per 20 ml of formic acid solution) was added dropwise while stirring. After addition of the peroxide solution was completed (about 1 min) stirring was continued for 30 min. (The temperature at which the peroxide solution is added is not as critical as the formylation temperature. It is necessary, however, to keep within the 10° range to avoid

either destruction of the dihydrocholesterol which takes place above 80°C, or poor oxidation efficiency observed at low temperatures due to incomplete solution of the sterols.)

After the sample had come to room temperature (usually at the end of the 30-min oxidation period) a volume of water equal to that of the formic acid solution was added, plus an extra 25 ml. This aqueous solution was transferred to a separatory funnel and extracted twice with ½ its volume of *n*-hexane. (Some of the *n*-hexane is used to rinse the thermometer.) The sample was then further extracted with 3 portions of 20% *peroxide-free* ethyl ether in *n*-hexane (v/v), again using ½ the volume of the formic acid solution. All five hexane and hexane-ether extracts were combined and washed with 10 ml of water, twice with 10% (w/v) $NaHCO_3$ solution, and 3 times with 10 ml of water.

The organic solvents were evaporated on a 60°C water bath under nitrogen, and the sample was then dried *in vacuo* at room temperature for at least 1 hr. The mixture of sterol formates obtained is unstable in solution and should be used for the next step (silicic acid chromatography) as soon as possible. Storage for 24 hr at room temperature was found to be permissible both for the dry samples or for hexane solutions.

3. Silicic Acid Chromatography

The mixture of dihydrocholesterol formate, cholestane-3β,5α,6β-triol 3,6-diformate and any cholesterol formate which remained unoxidized was chromatographed on silicic acid, using columns 20 cm in length and 15 mm in diameter, at 25°C. The columns were prepared and conditioned as described by Hirsch and Ahrens (8). The sample was put on the column in 2–3 ml of *n*-hexane, and the flask containing the sample was rinsed 3 times with 2-ml portions of *n*-hexane; these washings were used for rinsing the column. Elution was carried out with 1% anhydrous ethyl ether in *n*-hexane. Dihydrocholesterol formate (plus traces of cholesterol formate, if present) was eluted first, requiring approximately 50 ml of eluent. Triol formate was eluted with 4 or 8% ethyl ether in *n*-hexane or, more rapidly, with methanol. In order to obtain reproducible results each lot of silicic acid must be tested with known compounds since the retention volumes vary from batch to batch. The effluent was collected in 5-ml fractions and the solvent was evaporated under nitrogen at 60°C.

The fractions containing a visible residue of dihydrocholesterol formate (2–3 tubes) were selected and rinsed with absolute ethanol. The rinses were combined and made up to a volume of 5.0 ml. The tubes containing cholestanetriol diformate were combined in a similar manner, and the

weight of the diformate (divided by 1.233) was used as a measure of the initial weight of cholesterol. Recoveries of cholesterol should range from 94–99%.

4. Determination of Dihydrocholesterol as the Digitonide

(*a*) *Gravimetric Method.* If the dihydrocholesterol content of the sample was 0.5 mg or greater, the sterol was determined gravimetrically as the digitonide. The solution containing dihydrocholesterol formate usually will have a volume of 5.0 ml; for samples of high dihydrocholesterol content a 5-ml aliquot should be taken so that the sterol content of the sample will not exceed 1 mg/ml. The dihydrocholesterol formate first must be hydrolyzed as follows: 0.3 ml of 33% KOH (w/v) solution in water is added to 5 ml of the dihydrocholesterol formate solution, and this alkaline solution is then heated in a 60°C water bath for 1 hr. The solution is cooled to room temperature and 1 drop of a 0.5% solution of phenolphthalein in 95% ethanol is added. Glacial acetic acid is then added dropwise to neutrality, followed by an additional drop of glacial acetic acid. An equal volume of a 1% solution of digitonin in 80% ethanol (v/v) is then added, and quantitative precipitation of dihydrocholesterol digitonide is found to be complete after the sample has stood for 30 min at room temperature. The digitonide is suction-filtered on a Whatman No. 42 tared filter paper, 2.4 cm in diameter, using a Millipore No. XX 1002500 filtration apparatus. The filter paper must be equilibrated in room air for 30 min before weighing. In the present experiment a Mettler No. H-16 balance having a precision of ± 0.01 mg was used. The digitonide was washed first with 3–5 ml portions of boiling water until the wash water no longer foamed (this is essential for removing all traces of potassium acetate and excess digitonin which otherwise will produce erroneously high weights of the digitonide). Washing was completed with 5-ml portions of 80% alcohol, acetone, and ethyl ether. The digitonin precipitate on the filter paper was then dried at 60°C for 1 hr *in vacuo*. The digitonide was allowed to equilibrate at room temperature for 0.5 hr before weighing. The weight of the digitonide was multiplied by the factor of 0.239 to obtain the weight of dihydrocholesterol in the sample.

The digitonide was next counted in a Nuclear-Chicago D-47 flow counter to determine the amount of unoxidized cholesterol. If the radioactivity of the sample, corrected for background and self-absorption, was 3 cpm or less the oxidation of the sample was assumed to be complete. If the radioactivity was greater than 3 cpm this was assumed to be due to unoxidized cholesterol and the weight of dihydrocholesterol was corrected accordingly.

(*b*) *Anthrone Method.* When the total dihydrocholesterol content of the sample was less than 0.5 mg the sterol was determined colorimetrically by the anthrone color reaction. As before (Section *4a*) the dihydrocholesterol formate fraction was made up to a volume of 5.0 ml, but only 4.0 ml was used for digitonin precipitation, using a 0.5% digitonin solution in 50% aqueous alcohol. The remaining 1.0 ml was used for radioactivity determination by liquid scintillation counting (Nuclear-Chicago Model 702).

The determination of the sterol digitonide by the anthrone method was carried out exactly as described by Vahouny *et al* (9).

RESULTS

The validity of this method was established by (*1*) analysis of known binary mixtures of cholesterol and dihydrocholesterol, (*2*) replication experiments, (*3*) recovery experiments, and (*4*) infrared spectrophotometry of dihydrocholesterol samples isolated from tissues.

Table 1 provides a summary of 67 analyses of known binary mixtures of cholesterol and dihydrocholesterol, ranging in dihydrocholesterol content from 0–100%. When care was taken during the formylation step oxidation efficiencies of 99.9 to 100% could be obtained with samples containing 0.5 to 2.0% dihydrocholesterol, thus making it possible to achieve good accuracy. As will be seen below, samples with this low dihydrocholesterol content are typical of most biological sterol mixtures with the exception of certain rabbit tissues. Table 1 further indicates that the cholestanetriol weights served as a fair measure of the original cholesterol content of the sterol mixture, although, as pointed out above, this information is usually available from the original determination of total sterol content.

When the weight of dihydrocholesterol in the sample to be analyzed is low, ranging from 0.2–0.4 mg, the anthrone method has proved useful for the determination of this sterol. The data listed in Table 2 demonstrate that good agreement with the theoretical values could be obtained even without the use of labeled cholesterol as an indicator of oxidation efficiency. This is feasible since very careful attention to the formylation procedure results in negligibly small corrections for unoxidized cholesterol. This possibility of achieving consistent and high oxidation efficiencies is also demonstrated in the reproducibility study shown in Table 3. It can be seen that the procedure gives good agreement among duplicate or triplicate determinations even when the amount of dihydrocholesterol is quite small.

Table 4 lists the results of 8 recovery experiments in which known amounts of dihydrocholesterol were added to the nonsaponifiable extracts

TABLE 1

ANALYSIS OF KNOWN CHOLESTEROL-DIHYDROCHOLESTEROL MIXTURES
(GRAVIMETRIC METHOD)

DHC[a] (%)	No. of samples	DHC (mg)	Total sterol (mg)	DHC recovered (mg)	σ^b	DHC recovered (%)	Cholestanetriol recovered (%)	σ^b	Oxidation (%)	σ^b
0.00	2	0.00	100.00	0.00	—	—	—	—	100.00	0.00
0.5	4	0.50	100.00	0.488	0.020	97.6	96.9	0.98	99.97	0.02
1.0	12	1.00	100.00	0.978	0.040	97.8	95.8	3.03	99.85	0.39
2.0	10	2.00	100.00	1.914	0.095	95.7	94.0	9.01	99.89	0.09
5.0	7	2.50	50.00	2.41	0.06	96.2	97.1	1.14	99.74	0.23
10.0	9	5.00	50.00	4.67	0.12	93.6	97.5	1.67	99.89	0.14
20.0	5	10.00	50.00	9.81	0.27	98.1	96.6	3.03	99.33	0.39
50.0	8	25.00	50.00	24.78	0.80	99.1	96.3	3.52	98.62	2.07
80.0	4	40.00	10.00	38.62	1.42	96.6	99.1	—	99.04	0.65
100.0	6	2.00	2.00	1.965	0.052	98.3	—	—	99.80	0.11

[a] DHC = dihydrocholesterol.
[b] σ = standard deviation.

TABLE 2
ANALYSIS OF KNOWN DIHYDROCHOLESTEROL-CHOLESTEROL MIXTURES (ANTHRONE METHOD)

DHC[a] (%)	DHC (mg)	Total sterol (mg)	DHC recovered (mg)	DHC recovered (%)	Av. DHC recovery (%)
0.5	0.20	40.0	0.194	97.0	
	0.20	40.0	0.182	91.0	
	0.30	60.0	0.294	98.0	
	0.40	80.0	0.389	97.3	95.8
1.0	0.20	20.0	0.186	93.0	
	0.20	20.0	0.198	99.0	
	0.30	30.0	0.287	95.6	
	0.40	40.0	0.387	96.7	96.1
2.0	0.20	10.0	0.175	87.5	
	0.20	10.0	0.199	99.5	
	0.30	15.0	0.285	95.0	
	0.40	20.0	0.384	96.0	94.5
100.0	0.20	0.20	0.189	94.5	
	0.20	0.20	0.177	88.5	
	0.30	0.30	0.284	94.7	
	0.40	0.40	0.377	94.2	93.0

[a] DHC = dihydrocholesterol.

TABLE 3
REPRODUCIBILITY STUDY

Sample	Total sterol (mg)	DHC[a] (mg)	DHC (%)	Oxidation (%)
Human gallstone	100.42	0.454	0.452	99.98
	100.47	0.460	0.458	99.98
	100.90	0.471	0.467	99.97
Intima of human thoracic aorta	112.25	0.225	0.200	99.96
	112.25	0.234	0.208	99.95
	109.00	0.237	0.218	99.97
Human serum pool	62.7	0.220	0.350	99.98
	62.5	0.220	0.352	99.98

[a] DHC = dihydrocholesterol.

of the tissue studied. It is apparent that, regardless of the biological origin of the sample, 96–104% recovery could be obtained.

Table 5 summarizes the results obtained when the method was applied to various mammalian tissues and to human cholesterol gallstones.

TABLE 4
RECOVERY EXPERIMENTS

Tissue	Total sterol (mg)	DHC[a] (mg)	DHC (%)	DHC added (mg)	DHC recovered (mg)	DHC recovered (%)
Rabbit liver	116.3	4.04	3.47	3.00	3.04	101.3
	91.2	3.82	4.19	3.00	3.12	103.9
	103.9	3.60	3.47	2.00	2.07	103.3
Gallstone	100.5	0.468	0.465	2.00	2.45	96.8
Human aorta	175.1	0.683	0.390	0.994	1.012	101.8
	99.99	0.645	0.648	0.994	0.985	99.1
Human serum	54.95	0.481	0.876	1.490	1.550	103.8
	53.40	0.467	0.876	1.00	1.04	104.0

[a] DHC = dihydrocholesterol.

TABLE 5
DIHYDROCHOLESTEROL CONTENT OF TISSUES

Tissue	No. of samples	Total sterol (mg/gm)	DHC[a] (%)	Range (%)
Rabbit				
Liver	8	2.00	4.25	1.39–4.65
Small intestine	7	1.65	5.28	1.54–7.53
Adrenal	2	51.50	8.24	5.76–10.72
Serum	2	0.289	2.08	1.68–2.48
Kidney	1	3.11	1.97	—
Human gallstones	5	998	0.475	0.327–0.599
Intima, human aorta	6	33.8	0.341	0.158–0.646
Media, human aorta	2	4.79	0.530	0.422–0.639
Human serum				
Hypercholesterolemic	1	8.57	1.030	—
Pool A	1	2.34	0.88	—
Pool B	1	2.51	0.35	—
Human red cells, pool	1	1.60	0.32	—
Dog serum, pool	1	1.75	0.563	—

[a] DHC = dihydrocholesterol.

Dihydrocholesterol samples isolated from rabbit liver, rabbit small intestine, and rabbit adrenals by the method described were subjected to infrared spectroscopy. The spectra obtained were identical with the spectrum of pure dihydrocholesterol, indicating that the isolated material was at least 95% pure and that the removal of cholesterol was practically complete.

Table 6 illustrates experiments comparing the results of the present

TABLE 6
COMPARISON OF SCHOENHEIMER AND OXIDATION METHODS

Sample	Method used[a]	Sample wt. (mg)	DHC[b] wt. (mg)	Per cent contamination with cholesterol[c]
Cholesterol gallstone	B	300.0	13.6	63.9
	O	300.0	0.98	0.02
Sterol fraction of human intima				
A	B	106.5	1.43	64.7
	O	99.4	0.38	0.00
B	B	113.1	4.40	77.0
	O	94.3	0.23	0.00

[a] B, bromination; O, oxidation.
[b] DHC = dihydrocholesterol.
[c] Determined with labeled cholesterol.

method with those obtained by the Schoenheimer procedure. It is apparent that the dibromination method gives high results due to the fact that a considerable quantity of the cholesterol and perhaps of other sterols escape dibromination and appear in the dihydrocholesterol fraction. Inspection of this table suggests that some of the high dihydrocholesterol concentrations reported in the past may be due, at least in part, to this factor.

DISCUSSION

The method described in this report is based upon a method for the preparation of cholestane-3β,5α,6β-triol from cholesterol published by Fieser and Rajagopalan (7). In order to adapt the performic acid oxidation employed by these workers to the determination of dihydrocholesterol on a milligram scale the oxidation had to be carried out at elevated temperatures since the solubility of dihydrocholesterol-cholesterol mixtures in formic acid solutions is low at room temperature, resulting in relatively poor oxidation efficiencies. It was surprising, therefore, that the critical step in this procedure is not the peroxidation itself but rather the formylation step which precedes the oxidation. Apparently the formylation reaction goes to completion only at temperatures close to the temperature at which these sterols undergo decomposition in 85% formic acid.

The present procedure requires at least 0.2 mg of dihydrocholesterol for accurate measurement. Therefore, for samples containing approximately 1% dihydrocholesterol the weight of total sterol in the sample

must be at least 20 mg. This constitutes a serious limitation of the method since, for example, an entire rat liver (containing 2 mg of sterol per gram wet weight) or 10 ml of human serum (having a cholesterol concentration of 200 mg per 100 ml) are required for a single analysis. It should be possible, however, to adapt the procedure to much smaller samples by employing gas chromatography to analyze the components of the oxidized mixture (10). The biological data presented in Table 5 are of interest since they serve to correct a widely held opinion that gallstones and atheromata usually are of high dihydrocholesterol content. The results of the present study indicate that the sterol fractions obtained from these materials have a surprisingly low concentration of dihydrocholesterol. Other human tissues also may prove to have a low dihydrocholesterol content since the results of the serum analyses showed a range of dihydrocholesterol in the total sterol fraction ranging from 0.35–1.03%. The latter value was obtained with a sample of hypercholesterolemic serum having a total cholesterol concentration of 857 mg per 100 ml.

The relatively high proportion of dihydrocholesterol in rabbit tissues was likewise unexpected. This finding suggests that the rabbit will be a useful species for further studies dealing with the metabolism of dihydrocholesterol.

SUMMARY

1. A new method has been developed for the analysis of dihydrocholesterol in the sterol fraction of mammalian tissues and in gallstones.

2. The method is based on the finding that cholesterol-dihydrocholesterol mixtures can be oxidized with performic acid, which converts the cholesterol quantitatively to cholestane-3β,5α,6β-triol, leaving the dihydrocholesterol unchanged. The oxidation products can then be separated by chromatography on silicic acid, and the amount of dihydrocholesterol can be measured by digitonin precipitation either gravimetrically or by the anthrone method.

3. The feasibility of this method has been demonstrated by analysis of known cholesterol-dihydrocholesterol mixtures, by recovery experiments, and by infrared spectrophotometry of the isolated dihydrocholesterol.

4. It has been shown that previous concepts of the high dihydrocholesterol content of gallstones and atheromata may be in error since these materials frequently contain less than 0.5% dihydrocholesterol.

5. It has further been shown that rabbit tissues, particularly the intestinal wall and adrenals, may contain as much as 5–10% of dihydrocholesterol in the total sterol mixture obtained from these tissues.

REFERENCES

1. BLADON, P., in "Cholesterol" (R. P. Cook, ed.), pp. 21–22.. Academic Press, New York, 1958.
2. SCHOENHEIMER, R., VON BEHRING, H., AND HUMMEL, R., *Z. physiol. Chem.* **192,** 93 (1930).
3. MCARTHUR, C. S., *Biochem. J.* **36,** 559 (1942).
4. MOSBACH, E. H., AND BEVANS, M., *Am. J. Path.* **37,** 631 (1960).
5. NICHOLS, C. W., JR., SIPERSTEIN, M. D., AND CHAIKOFF, I. L., *Proc. Soc. Exptl. Biol. and Med.* **83,** 756 (1953).
6. NICHOLS, C. W., JR., LINDSAY, S., AND CHAIKOFF, I. L., *Proc. Soc. Exptl. Biol. and Med.* **89,** 609 (1955).
7. FIESER, L. F., AND RAJAGOPALAN, S., *J. Am. Chem. Soc.* **71,** 3938 (1949).
8. HIRSCH, J., AND AHRENS, E. H., JR., *J. Biol. Chem.* **233,** 311 (1958).
9. VAHOUNY, G. V., MAYER, R. M., ROE, J. H., AND TREADWELL, C. R., *Arch. Biochem. Biophys.* **86,** 210 (1960).
10. BLANKENHORN, D. H., personal communication.

ANALYTICAL BIOCHEMISTRY 5, 170–174 (1963)

A Simple Design for a Fraction Collector

JOHN W. MEHL

From the Department of Biochemistry, University of Southern California, Los Angeles, California

Received July 10, 1962

Although there are some fraction collectors available for large volumes and for operation under refrigeration, the instrument described here has the advantage of being simple and readily fabricated without any demanding machine work. It is also sufficiently compact that the space required in a lateral direction is little more than that occupied by the bottles in which the collection is made, and will fit readily in a domestic-type refrigerator.

APPARATUS

A diagram of the fraction collector is shown in Fig. 1. The heart of the instrument is a Sigma Cyclonome stepping motor (Sigma Instruments, Inc., 9AB03-220) shown at *A*. The shaft of this motor rotates by 18° at each reversal of polarity of the applied voltage. Consequently, there are 20 positions at which the motor will stop during one revolution. These positions are matched by two sets of 20 collecting holes, one on a radius of 2¼″ and the other on a radius of 3⅛″ in the Plexiglas plate *B*. The plate is mounted on brass bushings *C*, which are 1½″ long, and through which three machine screws run from holes (not shown) in the Plexiglas plate and into the threaded mounting holes on the motor. The Plexiglas plate is ½″ thick, and the collecting holes are drilled 5/16″ in diameter through the upper half and 3/16″ in diameter through the lower half. Lengths of Tygon tubing (⅛″ i.d., 3/16″ o.d.) are cemented into the bottom halves of the holes with cyclohexanone. These tubes, only two of which are shown, and one of which is marked *D*, serve to carry the column effluent which is dripped into the collecting holes to bottles of an appropriate size.

The shaft of the Cyclonome is extended with a 2¼″ length of brass rod *E* which is drilled on one end to give a tight fit on the motor shaft and fixed with a set screw. The upper end of the brass rod carries a piece of flat brass stock, 1/16″ × ¼″ × 3½″. This is attached by drilling a hole in its center, turning down the end of the rod to fit through this

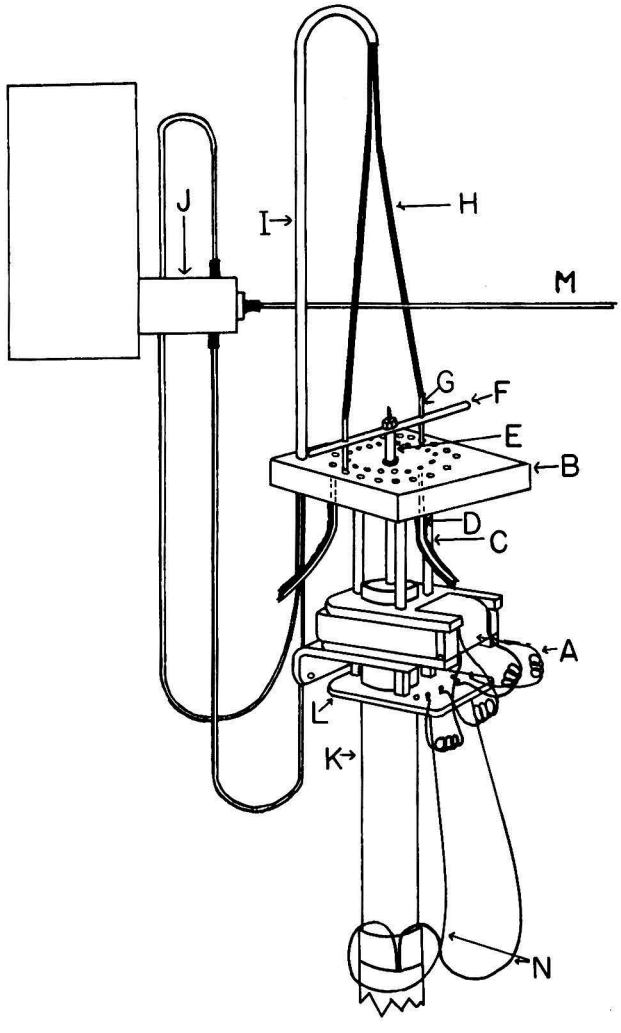


FIG. 1. Drawing of fraction collector. See text for description of parts and dimensions.

hole, and threading the ends of the rod to take a nut. This allows the rotating bar to be aligned with the collecting holes, after which it can be fixed in position with a drop of solder. The bar carries two ¾″ lengths of stainless-steel hypodermic tubing *G* which make a press fit into holes in the bar, one over the outer row of holes and one over the inner. If the fit is sloppy, the needles can, of course, be soldered in position. The purpose of the needles is to direct the polyethylene catheter tubing *H*,

which comes from the column, to the proper position over each collecting hole. This tubing should turn freely in the stainless tubing, and we have used polyethylene catheter tubing (Clay Adams Intramedic, PE 160) and 5/64" o.d. hypodermic tubing. A short sleeve of slightly larger polyethylene is fitted tightly over the delivery tubing to keep the end from projecting too far below the lower end of the steel tubing. In this way, the effluent from the column can be delivered immediately above the collecting holes.

The polyethylene delivery tubes are brought to a position about 12" above the extended end of the motor shaft through a piece of brass tubing *I*, which is cemented into a hole in the Plexiglas plate. The delivery tubes are then led to the two outlets of a solenoid valve *J* (Microvalve Instruments, Inc., Tulsa, Oklahoma, Model 198B) which directs the flow from the column, coming through *M*, to the inner or outer row of collecting holes. As the collector is being operated on time-flow, the switching of the solenoid from one row to the other can be made after one complete revolution with a suitable timer.

The height of the assembly can be made readily variable by mounting the motor on a pair of telescoping aluminum tubes. Tubing of 7/8" o.d. will fit snugly through the hole in a standard terminal board which is mounted on the lower end of the motor, and the tubing can conveniently be cemented to the terminal board with epoxy cement. Leads from the motor coils are shown at *N*, taped to the lower section of the telescoping tubing, and leading on out to the motor power supply.

The motor is powered with a simple circuit suggested by the manufacturer (Fig. 2). The components are indicated in the drawing, except for

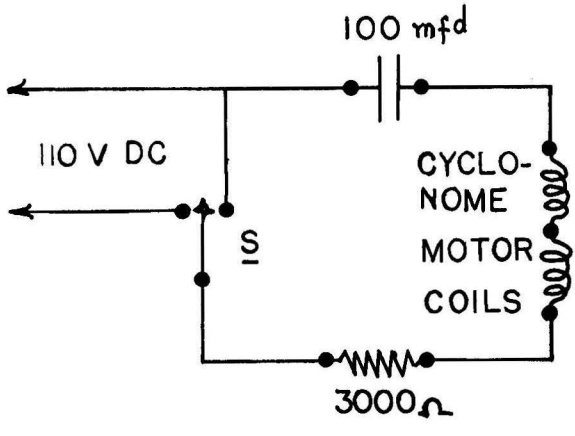


FIG. 2. Schematic diagram of power supply for stepping motor.

S, which is a Honeywell Micro Switch. Any one of several single-pole, double-throw switches with a roller arm may be used. The roller arm is actuated by a cam and motor (not shown), and we have used cams with actuating sectors over one half or over alternate quarters of the cam circumference. Driving the cam with a ½ rph motor (Synchron, 5 watt, 110 volt, 60 cycle), the switch will be on one pole for an hour and the other for the following hour in the case of the first cam. The motor rotates by one position at each change of switch poles, and, except for the leakage through the electrolytic condensors, power is drawn from the DC source only during the charging period of the condensor at alternate steps. The low current drain makes it possible to operate with a set of radio "B" batteries or a relatively simple power supply. Since the Synchron motors are quite inexpensive and available in a wide range of speeds, it is probably simpler to change motors than to build any very complicated device for providing a wide range of collection periods.

The set of forty collecting tubes is directed into appropriate bottles grouped at the base of the collector. The identification of tubes with holes can be more readily done if the tubes are held out somewhat by a ring of wire which takes a turn around each tube, goes to the adjacent tube, and turns around it, and thus around the complete circle. In order to get free flow down the collecting tubes, the ends should be placed high enough in the collecting bottles that they are not covered as the bottle fills.

DISCUSSION

This fraction collector was built primarily to fill a need for collections of 100- to 500-ml samples. It has worked well at flow rates of 450 ml/hr, and would probably work at still higher rates. Samples of 225 or 450 ml have been collected, and failure of the collecting tubes to drain completely does not introduce a significant error for these large fractions. It might be pointed out that it could provide an equally suitable system for small samples if the collecting tubes were replaced by test tubes held in holes in the Plexiglas plate, and the column mounted directly over the collector. The use of still smaller catheter tubing to deliver the effluent would minimize mixing in the delivery system.

The limitation to 40 fractions has not been serious in the present application, since a new set of bottles may conveniently be put on after 18 hr. However, it would be relatively simple to increase the number of samples by having a single delivery tubing which moved in a helix rather than in a series of circles. If only one piece of hypodermic tubing were used to position the catheter tubing, it could be mounted on the rotating bar so that it could be driven inward a fixed distance with each change in radial position. This could be readily accomplished by attach-

ing a movable needle assembly to a piece of flexible wire which would be shortened as the bar rotated by wrapping the wire around a fixed cylinder at the center of the Plexiglas plate.

ACKNOWLEDGMENT

The design and construction of this instrument was made possible by a Research Grant from the United States Public Health Service, A 2609.

ANALYTICAL BIOCHEMISTRY 5, 175–178 (1963)

SHORT COMMUNICATION

Determination of the Degree of Polymerization of Oligosaccharides

The alkaline copper phosphate reagent for glucose determination, described by Somogyi (1), has been used widely. It has also been applied to many other reducing sugars (2). In this paper an extension of this method to linear starch oligosaccharides is described. The reagent, like dinitrosalicylic acid (3), attacks only the reducing group of the oligosaccharide. This technique, coupled with the anthrone method, described by Dimler *et al.* (4), provides a method of determining the degree of polymerization of these oligosaccharides.

The oligosaccharide mixtures were prepared from α-amylase digests of Lintner soluble starch. The oligosaccahrides were separated by chromatography on Whatman No. 17 paper, eluted, and freeze-dried (5).

To determine the reducing values, Somogyi's (1) alkaline copper phosphate reagent was prepared and 5 ml of 1 N KIO_3 was added to each liter of reagent. An appropriate amount of oligosaccharide sample (between 10 and 500 μg glucose equivalent) was mixed with 5 ml of the reagent in a 25 $\times$ 200 mm Pyrex tube. A marble was placed in the mouth of the tube and the tube heated by immersion in a boiling water bath for 32 min. The tube was then withdrawn and allowed to cool. Two milliliters of 2 N sulfuric acid and exactly 0.5 ml of 2.5% potassium iodide were added in succession to the tube. The solution was then titrated to a starch end point with 0.0005 N sodium thiosulfate. The thiosulfate titer was subtracted from a blank and compared with a glucose standard to obtain the glucose quivalent.

The anthrone method (4), was used to determine total carbohydrate. Careful consideration should be given to temperature control in this method. This can be done by placing the test tube in an ice bath and layering the water and then the sample over the reagent. When all the pipetting is complete the test tubes should be mixed and placed simultaneously in a boiling water bath for exactly 10 min, then cooled rapidly to room temperature.

TABLE 1
DEGREE OF POLYMERIZATION OF OLIGOSACCHARIDES

Sample	Total carbohydrate[a] (μg glucose/ml)	Glucose[b] (equiv. μg/ml)	Degree of polymerization
Glucose	43.6	44.6	0.98
Maltose	926	459	2.02
Maltotriose	477	158	3.02
Maltotetraose	525	130	4.04
Maltopentaose	670	133	5.03
Maltohexaose	2460	398	6.18

[a] By Anthrone method.
[b] By Somogyi method.

The data in Table 1 show the results obtained by combining the two methods with oligosaccharides obtained from a sorghum α-amylase digest (6). The data were calculated on the assumption that the reducing end of the sugars are exclusively responsible for the reduction of the copper reagent:

$$\text{D.P.} = \frac{\text{Total glucose by anthrone method}}{\text{Glucose equivalent by Somogyi method}}$$

where D.P. is the degree of polymerization. In some experiments the oligosaccharides were weighed. The results checked very well with the anthrone method. In many instances, however, it is impossible to determine total carbohydrate by weighing and recourse to the anthrone method is a convenient way to determine it.

The anthrone reagent hydrolyzes the oligosaccharides to glucose, and reacts with the glucose to give the characteristic color. The Somogyi reagent on the other hand was expected to react stoichiometrically with the reducing glucose moiety. This assumption is verified by the good agreement between calculated D.P. and the known D.P. The slight departure from theoretical of maltohexaose can be attributed to poor resolution on the chromatogram.

The heating time in the Somogyi method is important. The effect of heating time was determined for maltotriose, maltotetraose, and an oligosaccharide mixture obtained by partial acid hydrolysis of amylose. Equal amounts of oligosaccharide were placed in 25 × 200 mm tubes containing 5 ml of the Somogyi reagent. These tubes were heated together in a boiling water bath. Tubes were withdrawn at 4-min intervals and titrated according to the Somogyi procedure (1). The reaction was considered to have approached completion when the net titrant volume no longer increased with heating time. The data appearing in Fig. 1 indicate that the reaction went to completion with maltotriose after 16 min and with malto-

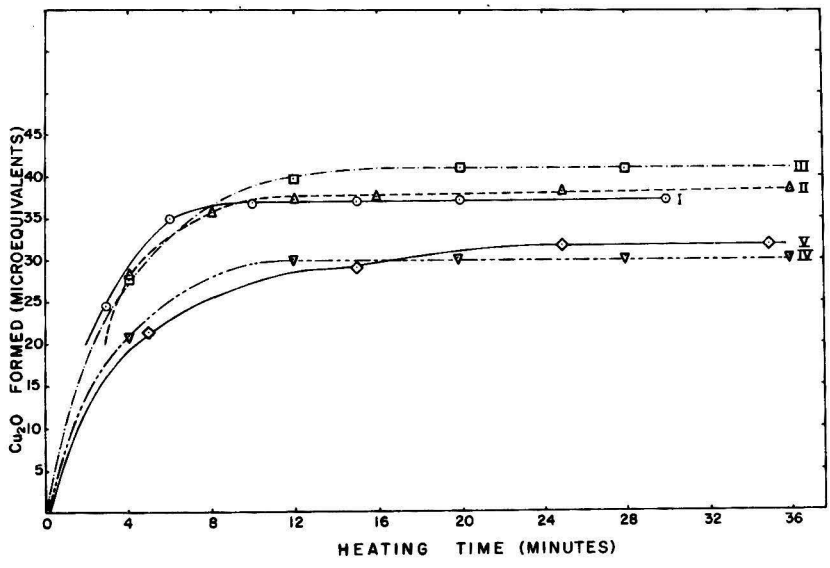


FIG. 1. Heating curves: (I) glucose; (II) maltose; (III) maltotriose, 1.39 mg; (IV) maltotetraose, 1.35 mg; (V) oligosaccharide mixture, 40 mg. Curves I and II are taken from Somogyi (2), concentration unknown.

tetraose after 20 min. The oligosaccharide mixture, which contained significant quantities of the glucose homologs of the maltose series from D.P. 1 to at least D.P. 15, showed no significant increase in cuprous oxide formation after 28 min. This indicated that, by further heating, no appreciable error was introduced into the reducing value determination. In practice, therefore, a heating time of 30 to 32 min was considered to be optimal.

The combination of these two techniques should have some useful applications. The D.P. of unknown reducing oligosaccharide could be determined. An α-amylase digestion could be followed with the Somogyi titration with some assurance that the true rate was being measured since, for every bond hydrolyzed, a new reducing group is created.

Extension of the method to dextrins of higher D.P. could be made. A problem inherent here is the diminished number of reducing sites. However, this is at least partly compensated for by the high reproducibility of the Somogyi method. It has been noted that duplicate samples of maltotetraose and maltotriose check fully as well as duplicate samples of glucose or maltose.

REFERENCES

1. SOMOGYI, M., *J. Biol. Chem.* **160**, 61 (1945).

2. MILLER, G. L., AND BURTON, A. L., *Anal. Chem.* **31**, 1790 (1959).
3. MILLER, G. L., SLATER, R., BIRZGALIS, R., AND BLUM, R., *Anal. Biochem.* **2**, 531 (1961).
4. DIMLER, R. J., SCHAEFER, W. E., WISE, C. S., AND RIST, C. E., *Anal. Chem.* **24**, 1411 (1952).
5. STEWART, L. M., AND NORDIN, P., unpublished data.
6. DUBE, S. K., AND P. NORDIN, *Arch. Biochem. Biophys.* **94**, 121 (1961).

L. STEWART
P. NORDIN

Department of Biochemistry
Kansas State University
Manhattan, Kansas
Received April 27, 1962

Erratum

Richard E. Maxwell and Vera Lewandowski, "Modified Fibrinolytic Technique for Analysis of the Plasmin System." *Anal. Biochem.* **4**, 407–412 (1962).

Page *408:* line 17 from the top, "action of plasma on fibrin" should read "action of plasmin on fibrin."

BIOCHEMICAL APPLICATIONS OF GAS CHROMATOGRAPHY

By **H. P. BURCHFIELD,** *Southwest Research Institute, San Antonio, Texas and* **ELEANOR E. STORRS,** *Boyce Thompson Institute, Yonkers, New York*

1962, 680 pp., $22.00

This book fulfills a need for a text that supplies both theoretical background and details of the applications of gas chromatography to biochemical problems. Specific procedures are supplied together with enough background information to enable the experimentalist to improvise adaptations without an extensive study of the literature. Although the work is organized along biochemical lines, information is included that will be of value to workers in other fields, such as air polution and petroleum chemistry.

Contents

General Techniques, Conventions, and Instrumentation. Permanent Gases and Condensable Organic Vapors. Volatile Components of Tissues and Biological Fluids. Cyclic Compounds. Essential Oils. Resin Acids. Lipids. Nonvolatile Components of Tissues. Review of Miscellaneous Applications Related to Biochemistry. Appendixes: Manufacturers of Gas Chromatographs and Auxiliary Equipment; Comparison Table of United States, British, and German Sieve Sizes with Tyler Equivalents; Hazards Associated with the Use of Diazomethane. Author Index–Subject Index–Methods Index. Index of Stationary Liquids Used in Methods.

"The book is well laid out, of a convenient size to handle despite its 680 pages and the print both clear and comfortable to read. Altogether I find this an admirable book and I hope that this will be made apparent to the authors by the number of copies sold; it should be in every library used by chemists."

S. R. Elsden in *Chemistry and Industry*

ACADEMIC PRESS, New York and London

111 Fifth Avenue, New York 3, New York

Berkeley Square House, London, W. 1, England

AP 1053

ANALYTICAL BIOCHEMISTRY 5, 179–186 (1963)

Separation and Determination of Thiamine and Pyrithiamine in Biological Materials by Chromatography on Polyethylene Powder

G. RINDI AND V. PERRI

From the Institute of Human Physiology, University of Pavia, Pavia, Italy

Received April 6, 1962

During research on the *in vivo* action of pyrithiamine, it became important to have a reliable method for determining thiamine and pyrithiamine in the same sample. In a previous paper (1), the problem was only partially resolved by utilizing the different behavior of the two compounds toward alkaline incubation. While thiamine was completely destroyed in 1 hr at 37°C, pyrithiamine was quite resistant and, successively, it could be oxidized by alkaline ferricyanide to a fluorescent pigment and fluorometrically determined. Obviously, this method allows only the determination of pyrithiamine, but not of thiamine.

The chromatographic separation of pyrithiamine and thiamine is not easy owing to the very similar physicochemical properties of the two compounds. Thus on ion-exchange resin (Amberlite IRC 50) they are adsorbed together and cannot be separated (2). With paper chromatography, their separation could be achieved only using alkaline solvents (2), which, however, destroy thiamine (2). We were able to obtain a sharp chromatographic separation of the oxidation products of thiamine (thiochrome) and of pyrithiamine [2,9-dimethyl-8-hydroxyethylpyrichromine (DHP)] (3, 4) by chromatography using polyethylene powder, which appeared very suitable also for their quantitative determination. Polyethylene powder has been successfully employed for the chromatography of other lipophilic substances (5–8).

In this paper we will describe the method we used for the determination of thiamine and pyrithiamine in pure solutions as well as some examples of its application to biological materials (liver and urine of rat).

MATERIALS

Polyethylene powder: Hostalen W, a type of low pressure polyethylene powder, produced by Farbwerke Hoechst A. G., Frankfurt a. M., Hoechst

(Germany), was washed three times in methanol, and filtered off in a Büchner funnel. After drying in the air, the powder was stored at room temperature in an amber bottle.

Thiamine: Thiamine chloride hydrochloride, Hoffmann-La Roche (Basel).

Pyrithiamine: Pyrithiamine bromide hydrobromide, California Biochemical Corporation, Los Angeles 63, California.

Asbestos: Woolly asbestos was thoroughly washed with methanol, filtered, and dried in the air.

Columns: The chromatographic columns (30 × 1.2 cm i.d.) were made by Pyrex glass tubes fitted with sintered-glass disk (No. 1 porosity) and fused to a ground-glass stopcock. Suitable separatory funnels could be attached to the tubes by means of ground-glass joints.

Adsorbent charcoal: This was prepared using an activated charcoal (3 SL, produced by Italian C. E. C. A., Milan), which was treated with cholesteryl stearate as described by Rindi and de Giuseppe (9).

Amberlite IRC 50 resin: The resin (British Drug Houses, Ltd.) was used in a carboxylic form, after treatment with 2 *N* HCl, followed by washing with water until disappearance of the Cl^- reaction.

EXPERIMENTAL

(*1*) *Separation and Determination in Pure Solution*

Principle: Thiamine and pyrithiamine were oxidized by alkaline ferricyanide to fluorescent pigments, which were separated by chromatography on a polyethylene powder column, using methanolic solutions in 0.1 *N* NaOH at different concentration as eluents. The fluorescence was measured in the collected fractions.

(*a*) *Preparation of chromatographic columns:* The polyethylene powder was slurried in methanol and poured in a chromatographic column. The powder, allowed to sediment completely by opening the column stopcock without eliminating all the methanol, was packed with a suitable glass rod to have a compact column of length 19–20 cm, on top of which a thin layer of washed asbestos was placed. The column was thoroughly washed with 100 ml of water, applying a gentle N_2 pressure.

(*b*) *Chromatography and determination:* 2–20 μg of pyrithiamine and 3–5 μg of thiamine, dissolved, together or separately, in 3 ml of water, were oxidized with 0.5 ml of 0.2% (w/v) $[K_3Fe(CN)_6]$ in 10 *N* NaOH, for 4 min, in a small beaker. The oxidation was stopped by adding 0.1 ml H_2O_2 solution containing 0.1 ml of Merck Perhydrol in 10 ml of H_2O (10).

The solution was then placed in a separatory funnel, the small beaker

was thoroughly washed with 0.1 *N* NaOH, and the solution was allowed to pass through the polyethylene column without pressure (5–7 drops/min). The column was successively washed with 35 ml of 0.1 *N* NaOH at the same flow rate.

The two pigments were now visible as sharp fluorescent bands (blue, thiochrome; greenish yellow, DHP) on the top of the column, when irradiated with Wood's light (365 mμ). The displacement of the bands during the elution could be easily followed under Wood's light.

Separation of the pigments was accomplished with 50 ml of 5% (v/v) methanol on 0.1 *N* NaOH, followed by 60 ml of 8% (v/v) methanol in

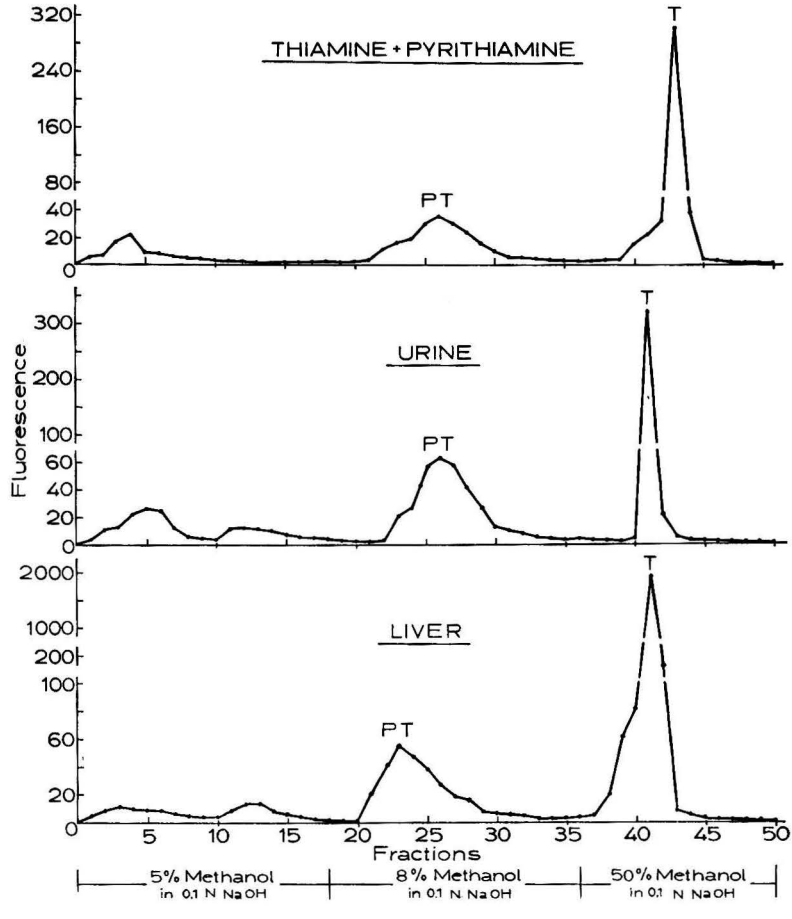


FIG. 1. Separation of thiamine (T) and pyrithiamine (PT) in pure solution (T, 5 μg; PT, 20 μg) and in biological materials by chromatography on polyethylene powder column: 3-ml fractions were collected with an automatic fraction collector.

the same solvent, which completely eluted DHP without displacing thiochrome. This was eluted by 30–50 ml of 50% (v/v) methanol in 0.1 *N* NaOH. The 8% methanol eluates and the 50% methanol eluates were concentrated to small volume in a rotating evaporator and then made up to volume in a 25-ml volumetric flask with 50% (v/v) methanol on 0.1 *N* NaOH. The fluorometric measurements of the solutions were performed in a Beckman DU model spectrophotometer, equipped with fluorometric accessories, setting the instrument with a quinine sulfate solution (0.3 μg/ml) in 0.1 *N* H_2SO_4.

A typical separation of the two compounds in pure solutions, obtained with the above technique, collecting 3-ml fractions by an automatic fraction collector, is reported in Fig. 1.

(*c*) *Recovery experiments:* Recovery from the column was evaluated by comparison of the eluate fluorescence, dissolved with 25 ml of 50% methanol in 0.1 *N* NaOH, with that of the same compounds separately oxidized and dissolved in the same volume of solvent, but not chromatographed. In a few experiments thiochrome and DHP prepared following Barger *et al.* (11) were used. The results of all the recovery experiments are reported in Table 1.

TABLE 1

RECOVERY OF THIAMINE AND PYRITHIAMINE THROUGH THE ENTIRE ANALYTICAL PROCEDURE

The compounds were in the same solution. For details see text.

Thiamine (μg)			Pyrithiamine (μg)		
Chromatographed	Recovered	Recovery (%)	Chromatographed	Recovered	Recovery (%)
2.00	2.01	100.5	5.00	4.59	91.8
2.00	1.80	90.0	5.00	4.47	89.6
2.00	1.94	97.0	5.00	4.60	92.0
2.00	2.09	104.5	5.00	4.51	90.2
6.10[a]	7.12	116.7	22.40[b]	22.40	100.0
24.40[a]	25.49	104.4	5.51[b]	5.17	93.8
		Av. 102.1			Av. 92.9

[a] Thiochrome.

[b] 2,9-Dimethyl-8-hydroxyethylpyrichromine (DHP).

(*2*) *Separation and Determination in Biological Materials*

(*a*) *Liver and other tissue:* The trichloroacetic extract of the tissue was purified by chromatography on special charcoal and on Amberlite IRC 50 resin, and then it was oxidized and chromatographed as indicated for the pure solutions.

Male albino rats (90–110 gm), reared on a balanced diet, were killed by decapitation 24 hr after an intraperitoneal injection of 1 mg of pyrithiamine in 0.2 ml of saline solution. The liver (5–6 gm), rapidly excised and weighed, was homogenized with an M.S.E. blade homogenizer[1] in 2.5 vol of 5% (w/v) cold trichloroacetic acid. The residue after centrifugation was again homogenized in 1 vol of 5% trichloroacetic acid and centrifuged. The combined supernatants, brought to pH 4.5 with 2.5 *M* sodium acetate, were incubated at 37° for 12 hr after the addition of 1 ml of a freshly prepared Takadiastase (Parke and Davis) solution (100 mg/ml) in water. Then they were neutralized (pH 6.8) with 40% (w/v) NaOH and adsorbed on a cholesteryl stearate treated charcoal, washed with 30 ml of water, and eluted with 25 ml of 10% (v/v) *n*-propanol (9).

The eluate was passed through a column (3 × 0.9 cm) of Amberlite IRC 50 resin (H^+), washed with 30 ml of water and eluted with 25 ml of 0.1 *N* HCl (1). The final eluate, concentrated under vacuum in a rotating evaporator to 3 ml, was oxidized and chromatographed on polyethylene powder as indicated for the pure solutions. The fluorescence measurements was performed as above described.

The content was evaluated by comparison with standard solutions containing pyrithiamine (20 μg) and thiamine (5 μg), which were separately oxidized and brought to 25 ml in a volumetric flask with 50% methanol in 0.1 *N* NaOH. A typical chromatographic separation of the two compounds extracted from rat livers is represented in Fig. 1.

For the quantitative assay of pyrithiamine it seemed necessary to evaluate the extraneous fluorescent materials present in the purified extract that could interfere in the fluorometric measurement. Thus a fluorescence value was obtained through all the above procedure with livers of rats which were not treated with pyrithiamine. This value, expressed as pyrithiamine, corresponded to 2.46 ± 0.66 μg/gm wet tissue (average of 6 rats) and it was substracted from the values obtained for tissues of treated rats. The corrected results of the determinations on pyrithiamine-injected rat livers are shown in Table 2.

(*b*) *Urine:* All the determinations were made on rat urine collected during 24 hr after the 1-mg intraperitoneal injection of pyrithiamine.

The preliminary purification on charcoal and ion-exchange resin was not necessary. The urine was diluted to 25 ml with water in a volumetric flask, and 1 ml of the solution, brought to 3 ml with water, was oxidized and chromatographed on the polyethylene column as described for pure solutions (Fig. 1). The fluorometric measurements of the eluted

[1] Made by Measuring and Scientific Equipment Ltd., London, England.

TABLE 2

THIAMINE AND PYRITHIAMINE CONTENTS OF LIVER AND URINE OF RATS 24 HR AFTER INTRAPERITONEAL INJECTION OF 1 MG PYRITHIAMINE, AND OF UNTREATED CONTROLS

Pyrithiamine content was corrected for nonspecific fluorescence (see text). Each value is the average of 6 rats ± S.E.

	Content of			
	Liver (μg/gm of wet tissue)		Urine (μg/24 hr)	
Compound	Treated	Untreated	Treated	Untreated
Thiamine	2.16 ± 0.14	5.41 ± 0.24	41.39 ± 5.73	1.99 ± 0.14
Pyrithiamine	8.74 ± 0.31	—	623.90 ± 55.98	—

fraction for the quantitative assay was performed as indicated above.

For the urine, too, a correction for the nonspecific fluorescence was introduced. The correction value was determined by carrying a urine sample of untreated rats through the complete procedure. The value of the fluorescence, expressed as pyrithiamine, was found to correspond to 104.25 ± 20.77 μg (average of 6 different samples).

In Table 2 the contents of pyrithiamine and thiamine in urine 24 hr after intraperitoneal injection of 1 mg of pyrithiamine in rats, corrected as indicated, are reported.

DISCUSSION

Thiochrome and DHP, the pigments produced by alkaline oxidation of thiamine and pyrithiamine, are lipophilic substances extractable in isobutanol (1, 12). But their liposolubilities differ greatly according to the different structures, so that it is possible to have a good separation through chromatography. Polyethylene powder was chosen as chromatographic support because it appeared particularly suitable for chromatography in aqueous solvents (3–6). Of the several types of polyethylene powder tried, Hostalen W was the most suitable as to reproducibility of results and recovery; however, it could not be used for more than one chromatography, since a long contact with alkaline medium seems to modify its properties. The separation of DHP from thiochrome is very sharp both in pure solutions and in tissue extract and urine, as is shown in Fig. 1, where the chromatographic patterns in all three conditions are reported. However, we found it essential to use alkaline eluents for a good separation, since alkalinity deeply influenced the sharpness of the separation as well as the rate of elution; moreover, the compounds are poorly fluorescent in neutral or acid solution.

As is shown in Table 1, the sensitivity of the proposed method is quite satisfactory, allowing the separation and determination of few μg of

pyrithiamine and thiamine, with good recovery. The higher sensitivity toward thiamine depends on the higher fluorescence of thiochrome, which is 5.7 times superior, on a molecular basis, to that of DHP (12).

The chromatography of pyrithiamine and thiamine from biological materials was very similar to that of the pure compounds, provided preliminary purification was made, a step which must be considered necessary (Fig. 1).

In order to increase the specificity of the method toward pyrithiamine, particularly when the quantitative determination on biological materials must be carried out, it was important to evaluate the interference of nonspecific fluorescent substances displaying the same chromatographic behavior of the DHP. This was possible by chromatographing liver extracts and urine from untreated animals. The results indicate that the percentage of the interference, expressed as pyrithiamine, was of the same order in urine as in liver, where the value, corresponding to 2.46 μg pyrithiamine/gm, was similar to that we obtained in previous research with a different method (1).

The thiamine and pyrithiamine contents in liver and urine of rats 24 hr after intraperitoneal injection of 1 mg of pyrithiamine are in good accordance with those we reported in previous papers, where we determined only one of the two compounds [for thiamine, see Refs. (13) and (14); for pyrithiamine, see Ref. (1)].

The present results again emphasize the great efficiency of pyrithiamine in displacing thiamine from tissues and thus increasing its urinary excretion.

A point emerging from the results obtained by the present method is that most (62%) of the injected pyrithiamine is excreted after 24 hr in the urine. Therefore, the amount taken up by the tissues was 374 μg (38% of the injected dose), and displaced 41.4 μg of thiamine, which was found in urine. Hence the molecular ratio thiamine/pyrithiamine, calculated as bases, was 1:7, very similar to 1:5 reported by Cerecedo *et al.* as an optimum ratio for the competitive action of pyrithiamine in experiments on oral administration to mice (15).

Note: While this manuscript was with the editor, Airth and Foerster (16) published a spectrofluorometric method for the simultaneous determination of thiamine and pyrithiamine.

SUMMARY

A method for the chromatographic separation and quantitative determination of pyrithiamine and thiamine in the same sample (pure solutions, liver and urine) is described. The compounds, after alkaline oxidation, are separated by chromatography on polyethylene powder, eluted

with different methanolic solutions in 0.1 N NaOH, and fluorometrically determined in the collected fractions. Twenty-four hours after intraperitoneal injection of 1 mg of pyrithiamine in rat, 62% of the antivitamin was found in urine together with 41.39 ± 5.73 μg of thiamine, 1 mole of thiamine being displaced by 7 moles of pyrithiamine. The liver contained, under the same conditions, 2.16 ± 0.14 μg/gm of thiamine and 8.74 ± 0.31 μg/gm of pyrithiamine.

ACKNOWLEDGMENT

We are greatly indebted to Farbwerke Hoechst A. G. for the generous gift of Hostalen W used in this work.

REFERENCES

1. RINDI, G., AND PERRI, V., *Biochem. J.* **80**, 214 (1961).
2. RINDI, G., AND PERRI, V., unpublished.
3. FUJITA, A., NOSE, Y., UEDA, K., AND HASEGAWA, E., *J. Biol. Chem.* **196**, 297 (1952).
4. PATTERSON, A. M., CAPELL, L. T., AND WALKER, D. F., "The Ring Index," p. 431. American Chemical Society, Washington, D. C., 1960.
5. FREEN, G., HORWITT, F. O., AND PRESTON, R., *Chem. & Ind.* **1955**, 591.
6. RINDI, G., *Intern. Z. Vitaminforsch.* **28**, 191 (1957).
7. WISS, O., AND GLOOR, U., *Z. physiol. Chem.* (*Hoppe-Seyler's*) **310**, 260 (1958).
8. WAGNER, H., WYLER, F., RINDI, G., AND BERNHARD, K., *Helv. Physiol. et Pharmacol. Acta* **18**, 438 (1960).
9. RINDI, G., AND DE GIUSEPPE, L., *Biochem. J.* **78**, 602 (1961).
10. BURCH, H. B., BESSEY, O. A., LOVE, R. H., AND LOWRY, O. H., *J. Biol. Chem.* **198**, 477 (1952).
11. BARGER, G., BERGEL, F., AND TODD, A. R., *Ber.* **86**, 2257 (1935).
12. SEALOCK, R. R., AND WHITE, H. S., *J. Biol. Chem.* **181**, 303 (1949).
13. DE CARO, L., RINDI, G., PERRI, V., AND FERRARI, G., *Intern. Z. Vitaminforsch.* **26**, 343 (1956).
14. DE CARO, L., RINDI, G., PERRI, V., AND FERRARI, G., *Intern. Z. Vitaminforsch.* **28**, 252 (1958).
15. CERECEDO, L. R., SOODAK, M., AND EUSEBI, A. J., *J. Biol. Chem.* **189**, 293 (1951).
16. AIRTH, R. L., AND FOERSTER, G. E., *Anal. Biochem.* **3**, 383 (1962).

ANALYTICAL BIOCHEMISTRY 5, 187–194 (1963)

Micromethod for Simultaneous Determination of Nitrogen and Phosphorus in Single Samples of Nucleoproteins and Their Derivatives[1]

B. FILIPOWICZ, MARIA GROSS, AND BOGNA SKOCZYLAS

From the Department of Physiological Chemistry, Academy of Medicine, Lódz, Poland

Received June 14, 1962

The N/P ratio is an essential proof of the chemical composition of nucleoproteins, nucleic acids, nucleotides, and phosphoproteins. It also expresses, among others, the degree of purification of NP[2] from other tissue proteins, and the degree of purity of NA and nucleotide preparations. It is useful in comparing the composition of different NP and NA samples, and so on. There are but few reports dealing with simultaneous nitrogen and phosphorus determinations in mineralized samples (1–4). In most laboratories, samples in which nitrogen and phosphorus are to be determined are mineralized separately and then each is also estimated separately.

Such a procedure presents many disadvantages: (*a*) Preparations of macromolecular compounds—especially the NP gels, being inhomogeneous, cannot be pipetted or weighed quantitatively. (*b*) The N/P ratio is charged with additional errors of measuring vessels and weighing when samples are prepared and analyzed separately. These errors are eliminated by simultaneous mineralization. Even nonquantitative transferring of material from Kjeldahl flasks to volumetric flasks exerts no influence on the N/P value in this case.

These considerations brought us to working out a method for simultaneous nitrogen and phosphorus determinations in a mineralized sample. The nitrogen determination had an essential influence upon the choice of a digestion mixture. As is known from numerous publications (5–11), many organic nitrogen compounds are very resistant to digestion by the Kjeldahl method. Especially resistant are purine and pyrimidine rings and some amino acids (lysine, tryptophan), the com-

[1] This work was supported by the Biochemical Committee of the Polish Academy of Sciences.

[2] The following abbreviations are used: NP, nucleoproteins; DNP, deoxyribonucleoproteins; NA, nucleic acids; DNA, deoxyribonucleic acids.

pounds which are attractive to us. Some biocatalysts that are useful in one case fail in another. Methods recommending mineralization in sealed ampoules (2, 3) give 100% nitrogen recovery, but are inconvenient to use. Similarly inconvenient are methods in which the components of the digestion mixture are weighed (7–14).

Vendrely's selenomercuric reagent (9) seemed to us to be the most suitable for this purpose. It permits a correct purine nitrogen determination, including only one pipetting. We have accepted for colorimetric phosphorus determination the method of Horecker, Ma, and Haas (12). It is characterized by a high molar absorption coefficient, colorfastness, and simplicity.

EXPERIMENTAL

I. Mineralization

Glassware and equipment: 25–200 ml Kjeldahl flasks, Pyrex or Jena, with a platinum wire placed in the bottom for protecting against overheating. The platinum wire, being a good conductor of heat, causes local vigorous bubbling, ensuring gentle boiling of the contents.

Digestion oven as described by Friedrich (15) and Kabat (16).

Flasks with side outlets connected, through the wash bottle filled with concentrated NaOH solution, to a water pump.

Pipets, Exax and Jena, retested, for pipetting standard solutions, error below 0.3%; 25-ml volumetric flasks, Jena, retested.

Reagents:

R-1, *selenium reagent according to Vendrely* (9): 10 gm of a carefully ground mixture comprising 1 gm Se (pure, Toscat, England), 5 gm $HgSO_4$ (pure, F. O. Ch., Poland), and 30 gm K_2SO_4 (analytical-grade, Riedel, Germany), boiled for 15 min with 100 ml concentrated H_2SO_4 prepared from fuming, analytical-grade H_2SO_4 (Riedel, Germany), and transferred to a hot, glass-stoppered bottle. A clear solution from above the salt excess was used.

R-2, *selenium reagent according to Roth* (14): 1 gm Se (see above) was ground carefully with 7 gm K_2SO_4 (see above).

R-3, 2 ml saturated $CuSO_4$ solution (analytical-grade, Merck, Germany) was added to 100 ml concentrated H_2SO_4 (see above). After 48 hr a clear solution from above the $CuSO_4$ precipitate was decanted.

R-4, 2% glucose solution.

R-5, *mercury catalyst according to Miller and Houghton* (7): 50 gm K_2SO_4 (see above) was ground with 5 gm HgO (pure, F. O. Ch., Poland).

R-6, 30% NaOH solution containing 5% $Na_2S_2O_3$ (analytical-grade, F. O. Ch.).

R-7, 30% H_2O_2 solution.

Methods:

Samples containing 200–1000 μg nitrogen (1–7 mg of substance) were used for mineralization. The appropriate Kjeldahl flasks, according to their volumes, were employed, and—depending on the method—the needed amounts of the corresponding reagents were used.

A: 0.5 ml H_2SO_4 saturated with $CuSO_4$ (R-3) and about 0.25 gm K_2SO_4 were taken for about 1 mg of the substance. Optimal digestion time—2 hr after white fumes appear.

B: 0.5 ml selenium reagent (R-1) was used for about 1 mg of substance. Optimal digestion time—2 hr after white fumes appear.

C: As under *B*, but with 0.2 ml glucose added (R-4). Optimal mineralization time—30 min after the contents of the flasks cleared.

D: 0.75 ml concentrated H_2SO_4 and 0.28 gm mercury catalyst (R-5) were taken for about 1 mg of substance. Mineralization time—2 hr.

E: As under *D*, but during the distillation NaOH with addition of 5% $Na_2S_2O_3$ (R-6) was used in accordance with Miller and Houghton (7).

F: 0.5 ml concentrated H_2SO_4 was used for about 1 mg of substance. After the appearance of white fumes, the sample was cooled, 1 drop of H_2O_2 (R-7) was added, and the mixture was heated again. This procedure was repeated several times until clearing of the sample, according to Miller and Miller (8), and heated for further 15 min.

G: As under *A*, but with addition of 0.2 ml glucose (R-4). Mineralization time—30 min after the sample cleared.

H: As under *B*, but with addition of 0.2 ml glucose (R-4). Digestion time—30 min after the sample cleared.

Each mineralized sample was transferred quantitatively to a 25-ml volumetric flask and adjusted to the mark with distilled water. Samples of suitable volumes were taken from the flask for nitrogen (usually 5 or 10 ml for one distillation) and phosphorus determinations (usually 0.1–1 ml for one determination).

II. Nitrogen Determination

This was carried out by the Kabat method (16), with certain modifications.

Glassware and equipment: Electrically heated Kjeldahl distillation apparatus evacuated by means of a water pump, 1 ml Exax buret (retested), pipets, and 50-ml Erlenmeyer flasks (receivers).

Reagents: A solution for filling the receivers—saturated H_3BO_3 solution with methyl red and methylene blue added (13) in the amount of 2 ml for one sample, 0.02 *N* HCl solution, 30% NaOH solution or 30% NaOH solution containing 5% $Na_2S_2O_3$.

Determination: A mineralized sample, containing 50–250 μg nitrogen, was transferred quantitatively into the apparatus and then 5 ml 30% NaOH—5% $Na_2S_2O_3$ solution was added. The whole was distilled and the distillate collected to constant volume in the receiver, filled with H_3BO_3 solution (1.5–2 min). The distillate was titrated with 0.02 *N* HCl solution until a blue-violet color appeared, the same as in the titrated blank. The normality of the acid was established by titration of the distillates of $(NH_4)_2SO_4$ standard solutions, or by titration of known amounts of NaOH solutions of a known concentration.

III. Phosphorus Determination

According to the method of Horecker *et al.* (12).

Glassware and equipment: Unicam SP 500 spectrophotometer, centrifuge for 3000 rpm, 10-ml calibrated test tubes, water bath for the test tubes, retested pipets.

Reagents: H_2SO_4 (analytical-grade); 5% $(NH_4)_2MoO_4$ (pure, F. O. Ch., Poland) solution; 0.2% "Eikonogen" (1-amino-2-naphthol-6-sulfonic acid—recrystallized from $NaHSO_3$) (17) in 12% $NaHSO_3$ solution with 1.2 gm Na_2SO_3 (pure, F. O. Ch.) added.

Determination: The mineralized solution, comprising 0.03–0.2 μmole of phosphorus, was introduced into the 10-ml test tube and the concentrated H_2SO_4 was added in such amounts that the final normality of the solution was 1.3–1.7n. The color intensity is independent of pH in this range of concentration. The following reagents were then added: water to about 8 ml of total volume, 0.5 ml molybdate, and 0.5 ml "Eikonogen." The whole was then adjusted to the mark with water, and mixed and heated for 20 min on a boiling water bath. The spectrophotometric determination was carried out at $E_{1cm}^{820\ m\mu}$. It sometimes happened, when more than 0.2 ml of the mineralized solution was used for the determination, that a faint red precipitate (Se compunds) fell down after boiling. Such samples were centrifuged (10 min, 3000 rpm) or left for decantation.

RESULTS

I. Nitrogen Determination

The following were used for the experiments:

1. Lysine monohydrochloride (analytical-grade, K & K Lab, Inc., USA).

2. Mixture of cytosine and thymine (pure, "Fluka").
3. Peptone ("Mikrokolor").
4. RNA isolated from yeast.
5. Adenylic acid (pure, "Fluka").
6. Cytydylic acid (pure, "Fluka").
7. DNA isolated from thymus.

In all these substances the nitrogen was determined according to Dumas and then—after mineralization by different methods—nitrogen was determined by the Kjeldahl method. The results presented in Table 1 are expressed in percentages of N in the substance and of N recovery

TABLE 1
NITROGEN IN SOME PROTEIN AND NUCLEIC COMPOUNDS MINERALIZED BY DIFFERENT METHODS

Substance	Mineralization	N (%)	Recovery (%)
Lysine HCl	According to Dumas	15.16	100.00
	A	14.71	97.04
	B	14.61	96.39
	C	14.82	97.79
	D	14.04	92.50
	E	14.22	93.80
	F	14.08	82.86
	G	14.56	96.04
	H	7.70	50.80
Pyrimidines (mixture)	According to Dumas	17.34	100.00
	A	16.59	93.95
	B	16.54	95.42
	C	17.22	99.30
	D	16.99	98.01
	E	17.04	98.27
	F	16.67	96.17
	G	15.42	90.17
RNA	According to Dumas	11.00	100.00
	C	11.01	100.90
Adenylic acid	According to Dumas	19.16	100.00
	C	19.44	101.45
Cytydylic acid	According to Dumas	12.43	100.00
	C	12.24	98.48
Peptone	According to Dumas	16.00	100.00
	C	15.95	99.69

in comparison with results obtained by the Dumas method (taken as 100). Afterward, the two most convenient methods, *A* and *C*, were employed for nitrogen determination in DNA preparations. The reproducibility of the results was confirmed by larger amounts of the

mineralized samples of the same preparations. The results are summarized in Table 2. The mean deviation:

$$\sigma = \sqrt{\frac{\Sigma(x - \bar{x})^2}{r}}$$

has been accepted as a measure of the reproducibility of the results.

TABLE 2
REPRODUCIBILITY OF RESULTS OF NITROGEN DETERMINATIONS IN DNA PREPARATIONS MINERALIZED BY TWO METHODS

Soln. No.	Method of mineralization	No. of detns.	Mean N content in 1 ml 0.08% soln. (μg)	σ
1	*A*	8	100.50	1.603
2	*A*	21	95.60	1.090
3	*C*	8	132.30	0.900
4	*C*	8	129.90	0.980

II. Phosphorus Determination

The simultaneous mineralization of several substances by methods chosen for nitrogen determination was carried out (*B* and *C*). The typical method accepted for phosphorus determination used perchloric acid. We showed that the results of phosphorus determinations in samples mineralized by means of these methods agree. This fact was taken as sufficient proof that phosphorus may also be correctly determined according to Horecker *et al.* (12) in the material mineralized by methods *B* and *C*. The results are listed in Table 3.

TABLE 3
COMPARISON OF RESULTS: PHOSPHATE STANDARD SOLUTIONS MINERALIZED WITH NITROGENOUS SUBSTANCES BY A METHOD USING CATALYST OR PERCHLORIC ACID

Soln. No.[a]	Method of mineralization	Mean P content in 1 ml (μg) of soln.	No. of detns.	σ
1	$HClO_4$	6.98	8	0.282
1	*B*	6.97	12	0.100
1	*C*	6.96	8	0.100
2	Without mineralization	18.30	10	0.510
2	*C*	18.13	8	0.100

[a] 1, DNA solution. 2, Protein solution with some phosphorus added.

DISCUSSION

It may be seen in Table 1 that the best nitrogen recovery was obtained when selenomercuric Vendrely reagent (R-1) with addition of glucose was employed. The positive influence of glucose is also to be observed. Contrary to the data given by Miller and Miller (8), only 93% of lysine N, instead of 99 and 96% of pyrimidine N, was recovered when carrying out the mineralization of lysine by means of many doses of H_2O_2. Other methods, which—according to these authors—yielded 100% lysine N recovery (7), have also been found to be deceptive.

The selenomercuric method gave satisfactory results in peptone, RNA, adenylic acid, and cytidylic acid examinations. The results obtained by this method were highly reproducible (Table 2). The data given in Table 3 suggest that this method is also suitable for phosphorus determinations in the preparations. The results obtained are in accordance with those found after mineralization with sulfuric and perchloric acids. The procedure described is very convenient and time saving.

SUMMARY

A method for mineralization of samples of nucleoproteins and their derivatives, aiming at the simultaneous determination of N and P, has been described. The influence of different metallic catalysts and H_2O_2 upon mineralization and correct determination of P has been examined. Good results for pyrimidines, purines, and proteins, using Se as catalyst, have been obtained. The recovery of lysine was 97.79%.

ACKNOWLEDGMENT

The authors would like to extend their appreciation for the able technical assistance of Mrs. Maria Ziemichód.

REFERENCES

1. DAMLE, S. P., AND KRISHNAN, P. S., *Anal. Chim. Acta* **11**, 225 (1954).
2. WHITE, L. M., AND LONG, M. C., *Anal. Chem.* **23**, 363 (1951).
3. GRUNBAUM, B. M., SCHAFFER, F. L., AND KIRK, P. L., *Anal. Chem.* **24**, 1487 (1952).
4. HOUBEN, J., "Methoden der organischen Chemie." Reprinted by Edwards Brothers, 1960.
5. VAN SLYKE, D. D., MACFADYEN, D. A., AND HAMILTON, P. B., *J. Biol. Chem.* **141**, 671 (1942).
6. JONNARD, R., *Ind. Eng. Chem., Anal.. Ed.* **17**, 246 (1945).
7. MILLER, L., AND HOUGHTON, J. A., *J. Biol. Chem.* **159**, 373 (1945).
8. MILLER, G. L., AND MILLER, E., *Anal. Chem.* **20**, 481 (1942).
9. VENDRELY, R., *Biochim. et Biophys. Acta* **1**, 95 (1947).
10. VAN SLYKE, D. D., HILLER, A., AND DILLON, R. T., *J. Biol. Chem.* **146**, 137 (1942).

11. DUNN, MAX S., AND ROCKLAND, LOUIS B., *in* "Advances in Protein Chemistry" (M. L. Anson and John T. Edsall, eds.), Vol. III, p. 347. Academic Press, New York, 1947.
12. HORECKER, B. L., MA, T. S., AND HAAS, E. J., *J. Biol. Chem.* **136,** 775 (1940).
13. EISNER, A., AND WAGNER, E. C., *Ind. Eng. Chem., Anal. Ed.* **6,** 473 (1934).
14. ROTH, H., *Angew. Chem.* **51,** 120 (1938).
15. FRIEDRICH, A., "Die Praxis der qantitativen organischen Micro-Analyse." Deuticke, Leipzig und Wien, 1933.
16. KABAT, E. A., AND MEYER, M. M., "Experimental Immunochemistry." C. C Thomas, Springfield, Ill., 1948.
17. PREDTECZENSKIJ, E. W., BOROWSKAJA, W. M., AND MARGOLINA, L. T., "Metody badań laboratoryjnych." P.Z.W.L., Warszawa, 1953.

ANALYTICAL BIOCHEMISTRY 5, 195–207 (1963)

Determination of Chlordiazepoxide (Librium) and of a Metabolite of Lactam Character in Plasma of Humans, Dogs, and Rats by a Specific Spectrofluorometric Micro Method

BERNARD A. KOECHLIN AND LUCIUS D'ARCONTE

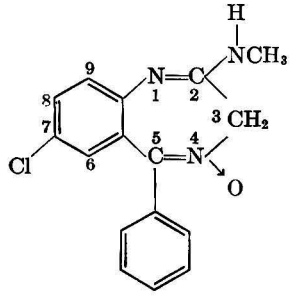
From the Department of Pharmacology, Hoffmann-La Roche Inc., Nutley, New Jersey

Received June 21, 1962

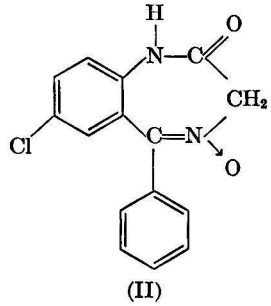
INTRODUCTION

Chlordiazepoxide (Librium) is the first and most widely studied representative of a new class of chemical compounds showing powerful antianxiety effects in humans (1–3) and unique taming action in animals combined with muscle relaxant and certain anticonvulsant properties (4, 5). With its clinical evaluation and wide use the need arose for a sufficiently sensitive and specific method for its determination in blood and other body fluids.

Synthesized by Sternbach and co-workers (6, 7), chlordiazepoxide is 7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepine-4-oxide, the structural formula of which is shown in (I). For the development of a



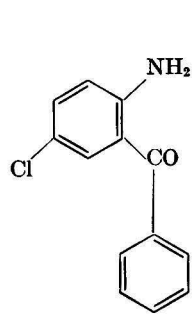
(I)



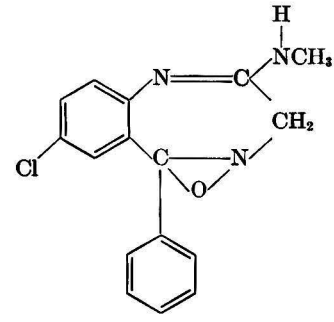
(II)

quantitative assay of chlordiazepoxide the following of its chemical characteristics were important:

(*1*) Due to the methylamino group at C-2 the compound is a weak base which forms water-soluble salts. The free base is selectively extracted into lipid solvents at pH's above 7 and is re-extracted from such solvents into dilute aqueous acid.



(III)



(IV)

(*2*) On mild acidic hydrolysis this basic grouping is split off with formation of a lactam (II). This lactam, 7-chloro-1,3 dihydro-5-phenyl-2*H*-1,4-benzodiazepine-2-one 4-oxide, referred to hereafter as lactam, has been described by Sternbach *et al.* (8). It exhibits weak acidic properties, and it can be extracted from ether into aqueous alkali at a pH above 10. Lactam (II), like chlordiazepoxide, is preferentially lipid-soluble at neutral physiological pH's.

(*3*) The chlordiazepoxide molecule exhibits a strong UV absorption which shows a characteristic dependence on pH and the solvent. In isopropanol, chlordiazepoxide exhibits a maximum molar extinction at 265

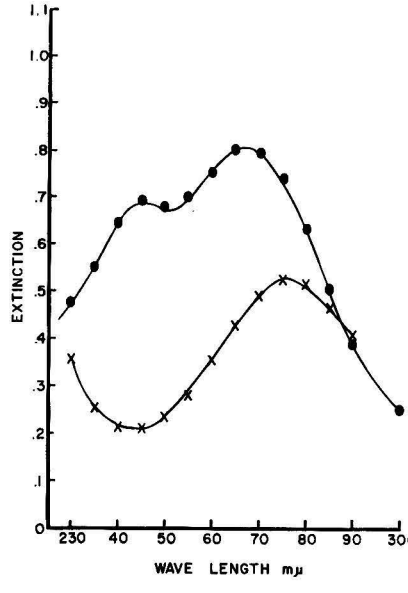


FIG. 1. UV absorption spectra in isopropanol of chlordiazepoxide base (●) and of its 4,5-epoxide isomer formed by light exposure (×). Concentrations = 10 γ/ml.

$m\mu$ of 2.5×10^4 (Fig. 1). A strong absorption in the UV is shared by most of the chlordiazepoxide derivatives of pharmacological interest (9).

(*4*) By drastic acidic hydrolysis the diazepine ring is cleaved at C-2 and at C-5 to produce the aromatic amine, 2-amino-5-chloro-benzophenone (III).

(*5*) The *N*-oxide grouping at position 4 can undergo rearrangement under the influence of light which results in the formation of an isomer (IV) in which a 4,5-epoxide group is present. The loss of the double bond in 4,5 position in chlordiazepoxide causes a change of the UV spectrum (Fig. 1). This reaction has been elucidated conclusively (10). Indications of similar rearrangements of other related *N*-oxides including lactam (II) have been obtained as will be shown below.

Randall (11) has reported a quantitative colorimetric assay for chlordiazepoxide, applicable to biological materials. It is based on the hydrolytic cleavage of the compound following its selective extraction to the aromatic amine (III). This amine is diazotized and coupled with *N*-(1 naphthyl)ethylenediamine to form an azo dye which is measured colorimetrically. A similar procedure was described more recently by Bäumler and Rippstein (12).

An alternate, more sensitive and selective, procedure is described in the present report. It is based on the quantitative conversion of chlordiazepoxide to the lactam (II) under controlled hydrolytic conditions followed by rearrangement of the *N*-oxide grouping in alkaline solution under the influence of light to a fluorescent derivative which is measured spectrofluorometrically. Preceding this treatment chlordiazepoxide is extracted selectively from the biological medium.

The procedure, although very specific for chlordiazepoxide, can be modified to permit an equally selective determination of the lactam (II). This derivative is a chemical degradation product and, also a metabolite of chlordiazepoxide which is present in blood and in the urine of chlordiazepoxide-treated subjects. Compared to the assay of Randall (11) the present assay is more specific and more sensitive. It permits measuring the concentrations of chlordiazepoxide encountered in the plasma of humans over a 48-hr period following oral administration of therapeutic doses ranging from 10–30 mg. Plasma levels of chlordiazepoxide and of lactam measured in human subjects, dogs, and rats by this method under different dosage conditions are reported.

ANALYTICAL PROCEDURES

Spectrofluorometry

The Aminco-Keirs Spectrophosphorimeter (American Instrument

Company) set up as a fluorometer was used in most of the experiments described. A xenon lamp served as a light source and an IP 21 type tube as a photomultiplier. Intensities were measured with slit openings of 5 mm throughout and a sensitivity setting of 50. Changes of the over-all sensitivity of the instrument were checked and corrected by measuring fluorescent standards, activated and emitting in the same wavelength range along with each set of readings.

Solutions and Reagents

Phosphate buffer pH 7.2, 0.2 *M*.

Glycine/hydrochloride buffer, pH 4.8 0.1 *N*: 7.505 gm of glycine (analytical-grade recrystallized from ethanol) and 5.85 gm NaCl dissolved in water, titrated to pH 4.8 with 0.1 *N* HCl, and diluted to 1 liter with water.

Ether, peroxide-free, distilled over sodium wire and stored in the dark.

Sodium hydroxide, 0.1 *N*, freshly prepared and checked prior to each experiment for fluorescence at 380 mμ activating and 480 mμ fluorescence wavelength.

Procedure for Chlordiazepoxide

(*1*) One milliliter of plasma separated immediately after drawing of the blood (heparinized or oxalated) is mixed with 3 ml of pH 7.2 buffer in a 10–15 ml glass-stoppered centrifuge tube. This mixture is extracted with 5 ml of ether by shaking mechanically for 10 min (stoppers are sealed with a drop of water).

(*2*) After short centrifugation a 4-ml aliquot of the clear ether phase is transferred into a second identical tube.

(*3*) The solvent is evaporated by immersing the tubes into a 50°C water bath. The dry residue is taken up in 2 ml of pH 4.8 glycine/HCl buffer and the solution is heated for 4 hr in a boiling water bath. (The stoppers are inserted and tightened after temperature equilibration.)

(*4*) After cooling, 5 ml of ether is added and the lactam formed is extracted by shaking for 10 min as described (step *1*).

(*5*) After centrifugation a 4-ml aliquot of the clear ether phase is transferred to a third identical tube and is extracted with 4 ml of 0.1 *N* NaOH. After centrifugation the ether layer is removed completely by aspiration.

(*6*) The remaining alkaline aqueous extract is exposed, in the same tube, to bright daylight for 1 hr.

(*7*) The extract is then transferred into a 1-cm quartz cell and the fluorescence intensity is measured in the spectrofluorometer at 380 mμ activating and 480 mμ fluorescence wavelength as described above.

(*8*) A solution of lactam in 0.1 *N* NaOH containing 5 γ/ml is prepared fresh for each series of determinations by diluting a stock solution of 500 γ/ml in absolute ethanol with 0.1 *N* NaOH. It is exposed to light and measured together with the other solutions as a check on the instrument and the adequacy of the light exposure. The former is checked also by reading the fluorescence of a solution of 0.1 γ of quinine sulfate in 0.1 *N* H_2SO_4 at 450 mμ activating at 350 mμ.

If no lactam standard is at hand, a chlordiazepoxide standard should be run through steps *3* to *7* along with the plasma extracts.

All the unknown plasmas are assayed in duplicate together with duplicate control plasma and the same control plasma to which an internal standard of 5 γ chlordiazepoxide hydrochloride has been added. The calculations of the unknowns are based on this internal standard.

The dilution of the final solution over the original plasma is 4/25 = 0.16; the ratio of the molecular weight of chlordiazepoxide HCl and of lactam is 336.2/286.8 = 0.853. Thus, if we assume quantitative extraction, an original plasma concentration of 1 γ/ml will result in a concentration 0.16 × 0.853 = 0.136 γ of lactam per ml of the final 0.1 *N* NaOH solution.

Duplicate 1-ml samples of freshly separated human plasma to which were added 0, 0.25, 0.5, 1.0, 2.0, and 5.0 γ of chlordiazepoxide hydrochloride were analyzed by the above procedure. The intensities obtained plotted against concentration fell on a practically straight line. From these readings the average recovery on extraction from plasma was calculated to be 73%.

Procedure for Lactam (II)

The lactam procedure differs from the chlordiazepoxide procedure by the omission of the steps *2*, *3*, and *4*.

The dilution factor of the final solution over the original plasma is ⅕ = 0.2 here and a concentration of 1 γ of lactam per 1 ml of plasma results in a concentration of 0.2 γ/ml in the final 0.1 *N* NaOH solution.

By analyzing increasing concentrations of lactam added to 1 ml of control plasma, a standard curve was obtained which revealed a nearly linear relationship of concentration to fluorescence intensity within a concentration range of 0.25–5 γ/ml.

DISCUSSION OF THE PROCEDURE

The sensitivity of the chlordiazepoxide assay in plasma is 0.25 γ/ml when taking a reading of two times blank as the minimum measurable quantity. The quality of the plasma which determines the blank reading is the limiting factor here. For 1 ml of fresh plasma, separated

from nonhemolyzed blood, blanks are usually about 1.5–2 times the reading obtained for reagent blanks. The latter is essentially that of the 0.1 *N* NaOH. This solution should be checked daily before use for the presence of fluorescent contaminants.

Chlordiazepoxide is extracted to over 95% from aqueous solutions at pH's above 7 by equal volumes of ether and is re-extracted quantitatively from this solvent into 0.1 *N* HCl. Quantitative re-extraction directly into buffer of pH 4.8 is not possible. Lactam is extracted quantitatively into ether from neutral and acidic aqueous media and can be re-extracted quantitatively from dilute ethereal solutions into dilute aqueous NaOH under formation of a sodium salt which is reversible. Furthermore, chlordiazepoxide and lactam can be separated by extraction at neutral pH with 2 vol of heptane containing 1.5% isoamyl alcohol whereby 85% of the former but only 4% of the latter are extracted. These different solubility properties suggest several selective extraction procedures leading to assays specific for either one of the two related compounds. In our procedure, the generally more selective heptane-isoamyl alcohol solvent mixture did not offer advantages over ether; it did not reduce the level of the fluorescent contaminants which were found occasionally to occur in plasma.

Attempts to achieve quantitative conversion of chlordiazepoxide into lactam by mild controlled treatment with HCl invariably lead to hydrolysis beyond the lactam stage, while heating with formic acid did not attack either compound. Heating aqueous solutions of chlordiazepoxide hydrochloride in absence of excess HCl lead quantitatively to the desired conversion product. However, the formation of the hydrochloride from γ quantities of the base which is obtained when the compound is extracted from biological material and freeing it from excess HCl in a reproducible manner proved to be difficult. The required conversion was achieved most readily by hydrolysis in aqueous solution in the presence of glycine hydrochloride buffer. As is shown in Fig. 2 a pH of 4.8 and heating to 100° for 4 hr was necessary for quantitative reaction. At lower pH's the yields did not exceed 50%, probably as a result of a nonuniform reaction.

The lactam gives rise to fluorescence only following its exposure to light and only in strongly alkaline aqueous solutions. As is shown in Table 1 a solution prepared and kept in the dark exhibited blank fluorescence. On continued irradiation by any source of visible light a maximal stable and reproducible fluorescence intensity was obtained which is seen (Table 1) to be proportional to the lactam concentration within the concentration range of 0.1–10 γ/ml. Using normal diffuse daylight as a light source the conversion was complete within 30–60 min.

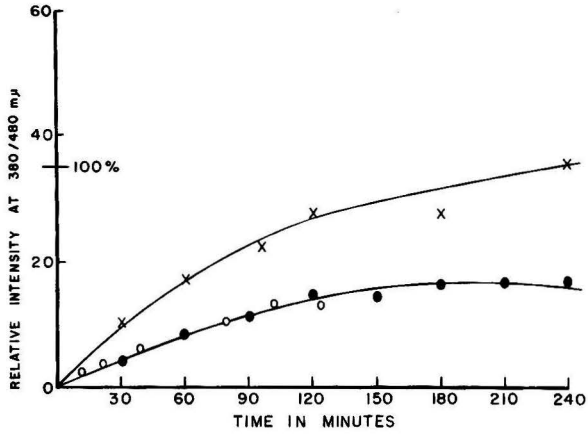


FIG. 2. Conversion of chlordiazepoxide to lactam in glycine/HCl buffers at 100°C as a function of time at pH 1.45 (●), at pH 1.95 (○), and at pH 4.8 (×).

The fluorescence of such an irradiated solution disappears instantaneously on acidification to reappear immediately on restoring alkaline conditions. The underlying reaction appears to correspond to the rearrangement of the *N*-oxide function to a 4,5-epoxide which has been shown (10) to occur with chlordiazepoxide under the influence of light. While this light-induced rearrangement per se is not pH dependent the fluorescence becomes manifest only in an alkaline medium. None of the many other members of this class of compounds and of their degradation products which we have investigated give rise to fluorescence of similar

TABLE 1
FLUORESCENCE INTENSITY OF LACTAM (II) AS A FUNCTION OF LIGHT EXPOSURE, CONCENTRATION, AND pH

Lactam (γ/ml) concn.	Medium	Time of light exposure	Rel. fluor. intensity (380/480 mμ)
10	0.1 *N* NaOH	0	1.32
1	0.1 *N* NaOH	0	0.32
1	0.1 *N* NaOH	1 hr	13.20
1	0.1 *N* NaOH	2 hr	12.90
	0.1 *N* NaOH	4 days	13.20
0.1	0.1 *N* NaOH	1 hr	1.59
0.01	0.1 *N* NaOH	1 hr	0.41
0	0.1 *N* NaOH	1 hr	0.35
1	0.1 *N* NaOH	1 hr	11.40
	Same soln. acidified	—	1.72
	Same soln. made alk.	—	11.20

intensity under analogous conditions. The assay based on this conversion is, therefore, highly specific for chlordiazepoxide or the lactam, depending on the preceding extraction procedure, and on the inclusion or omission of the hydrolysis step.

In the course of routinely analyzing larger numbers of plasma samples collected from different human subjects it was observed that the fluorescence intensities for the same internal standards, after correcting for the instrumental variations, varied significantly. In order to establish the cause of this variation the following experiment was run, the design and results of which are presented in Table 2. These results

TABLE 2

FLUORESCENCE INTENSITY READINGS (AT 380/480 mμ) OBTAINED FOR INTERNAL STANDARD OF CHLORDIAZEPOXIDE IN HUMAN PLASMA (5 γ/ML)

[as a function of: (1) procedural reproducibility and (2) individual plasma specimen]

	Plasma donor							
			MS		BK		GK	
	I[a]	II[a]	I[a]	II[a]	I[a]	II[a]	I[a]	II[a]
Lactam standard 5 γ/ml, 0.1 *N* NaOH	34.0	34.0						
Same corr. for diln. (× 0.138)[b]	4.6	4.6						
Plasma blank			0.28	0.20	0.28	0.25	0.38	0.22
Plasma + 5γ internal standard			3.40	3.40	4.15	3.95	4.35	3.90
Plasma + internal standard − plasma blank			3.12	3.20	3.88	3.70	3.97	3.68
% recovery[b]	100	100	68.0	69.5	84.0	80.0	86.0	80.0

[a] All the test samples in duplicate were taken through the total procedure as one series and read together with a lactam standard which had been light exposed simultaneously (I). The same set of samples was rerun as before, 2 days later, keeping the plasma frozen between experiments (II).

[b] Recoveries were based on the lactam standard (5 γ/ml of 0.1 *N* NaOH) times a dilution and conversion factor of 0.138 (see text).

demonstrate that the corrected readings for the same internal standards in the same plasma could be closely reproduced from one experimental series and from one day to the other, but that, consistently, a lower reading was obtained with one plasma than with the two others. This might be due to quenching or chemical interaction factors present in individual plasmas to a varying degree. To compensate for these factors it is suggested, if possible, to base the evaluation of test sample readings

on internal standard readings obtained with control plasma collected from the corresponding individuals.

Determination of Chlordiazepoxide and Lactam in Urine

While in the dog and in man only a fraction of 1% of an ingested chlordiazepoxide dose is recovered intact in urine, lactam is an important urinary excretion product, as will be reported in a later paper. The accuracy of the spectrofluorometric determination of this metabolite in urine suffers, however, from the high and variable blank fluorescence of this medium. Although conditions were found for selective adsorption of lactam, quantitative recovery of γ amounts on re-elution has so far not been possible. A reproducible routine fluorometric procedure which could match the colorimetric method of Randall (11) is at present not available.

DETERMINATION OF PLASMA LEVELS OF LIBRIUM AND OF LACTAM

(*1*) *In Humans*

A first group of 3 human subjects[1] received a single oral dose of 30 mg of chlordiazepoxide hydrochloride. Blood specimens were drawn before and 2, 4, 8, 24, and 48 hr after dosing. Chlordiazepoxide was then determined in the plasma. The logarithms of the concentrations were plotted against time (Fig. 3). The data for these 3 patients were in close agreement. They indicated maximum plasma levels of 1.3–2.0 γ of chlor-

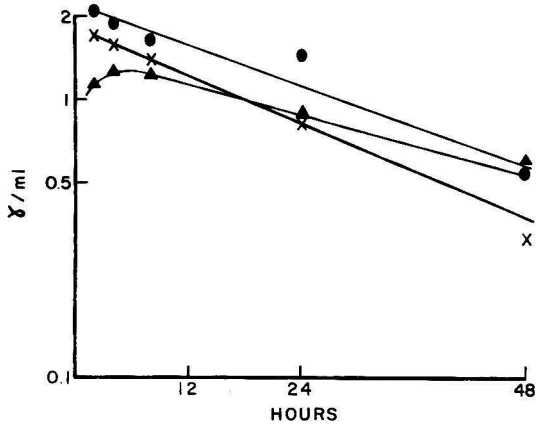


FIG. 3. Chlordiazepoxide plasma concentrations in 3 human subjects following a single oral dose of 30 mg chlordiazepoxide hydrochloride.

[1] These experiments were carried out in collaboration with Dr. W. B. Abrams at Martland Medical Center, Newark, N. J.

diazepoxide/ml plasma, occurring 2–4 hr after administration of this dose, and half-lives of 20–24 hr for the unchanged drug in plasma. These values could be closely reproduced in a larger number of similarly treated subjects.

The same plasma samples of two of the three above subjects were assayed for lactam. No measurable concentrations of this metabolite were present. In a single instance, following an accidental overdose of 500 mg of chlordiazepoxide, a chlordiazepoxide concentration of 25.3 γ/ml and a lactam concentration of 2.05 γ/ml were measured. This ratio extrapolated to a normal therapeutic dose suggests that here the concentration of the metabolite would fall below the threshold of our assay (< 0.2 γ/ml). To a second group of patients 30 mg of chlordiazepoxide was administered in divided doses: 10 mg at 0, 3, and 6 hr. Plasma was again collected before and 2, 4, 8, 24, and 48 hr after the first dose and analyzed. The results indicated a greater variation between individual subjects. Also, the concentrations in plasma which again reached maxima of 1–2 γ/ml appeared to be maintained somewhat longer than after a single high dose.

(2) *In Dogs*

Oral doses of 4 and 10 mg of chlordiazepoxide (base) were administered to dogs (beagles) weighing 8–12 kg. Chlordiazepoxide plasma concentrations were analyzed at intervals as indicated. The resulting semilogarithmic time curves are presented in Fig. 4.

Maxima of 6–12 γ/ml were reached 2–4 hr after dosing. The half-lives

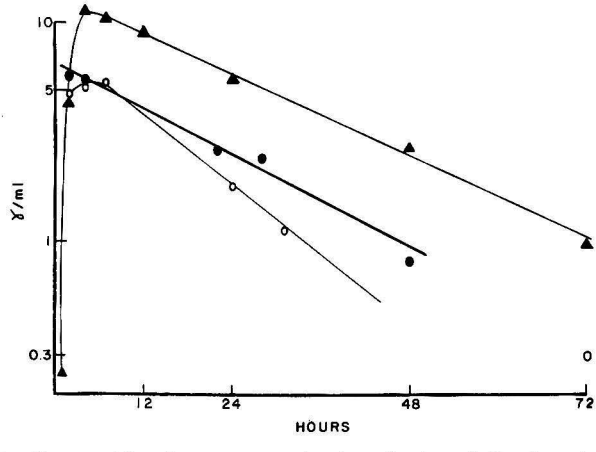


FIG. 4. Chlordiazepoxide plasma concentrations in dogs following single oral doses of 10 mg/kg (●, ▲) and 4 mg/kg (○).

ranged from 12–20 hr in these experiments. In one instance both chlordiazepoxide and lactam were determined 4 hr after a 10 mg/kg dose of chlordiazepoxide base. With a chlordiazepoxide plasma concentration of 8.8 γ/ml that of the metabolite was 0.6 γ/ml.

A single oral dose of 10 mg/kg of lactam was administered to 1 dog. Plasma levels of lactam were measured in plasma at regular intervals up to 48 hr after dosing. The semilogarithmic plot of the obtained values (Fig. 5) indicates that maximum plasma concentrations of this metabo-

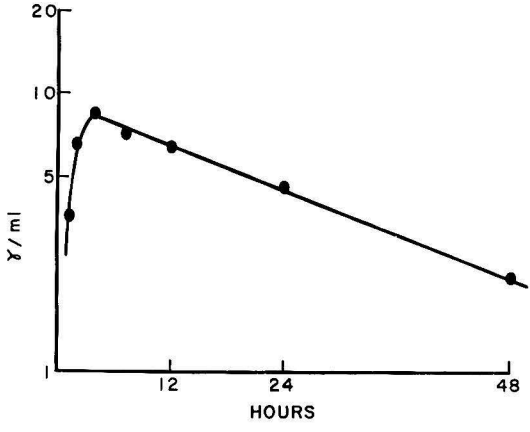


FIG. 5. Plasma concentrations of lactam in a dog following a single oral dose of 10 mg lactam/kg.

lite and its half-life are very similar to those obtained with chlordiazepoxide after equivalent doses.

(3) *In Rats*

A group of 10 rats (starved 24 hr) received single oral doses of 15 mg chlordiazepoxide (HCl) per kg. Groups of 2 animals were sacrificed 1, 2, 4, 6, and 12 hr after dosing. The plasmas were analyzed and the resulting chlordiazepoxide concentrations plotted semilogarithmically against time (Fig. 6). From this curve a half-life for chlordiazepoxide of 4–6 hr could be estimated.

SUMMARY

A sensitive and selective spectrofluorometric method has been developed for the determination of chlordiazepoxide in blood plasma. It is based on a selective extraction of the compound followed by controlled hydrolysis to a lactam derivative which is converted, under the influence

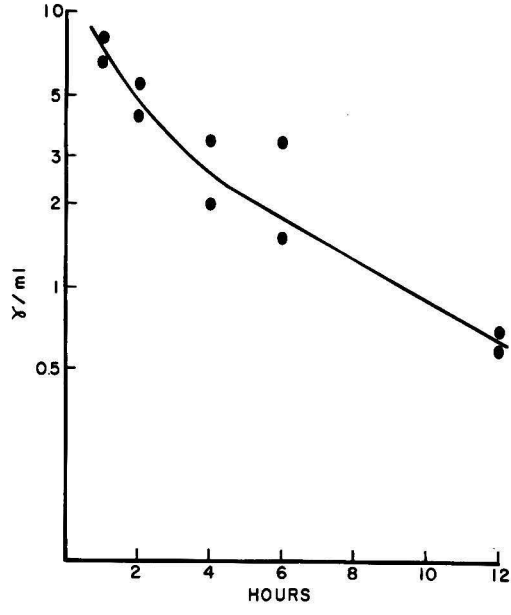


FIG. 6. Chlordiazepoxide plasma concentrations in rats following single oral doses of 15 mg chlordiazepoxide/kg.

of light, to a compound exhibiting a characteristic fluorescence in alkaline solution.

This lactam, which is also a major metabolite of chlordiazepoxide in man and in the dogs can be measured separately by a modification of this procedure.

With a sensitivity limit of 0.25 γ/ml of plasma for either compound, the method was found to be adequate for measuring plasma levels of chlordiazepoxide as encountered after normal therapeutic doses in man (10–30 mg).

Plasma levels of chlordiazepoxide and the lactam after various oral doses were determined in human subjects, in dogs, and in rats. The half-lives of chlordiazepoxide were 20–24 hr in humans, 14–20 hr in dogs, and 4–6 hr in rats. The lactam when administered orally to dogs showed a half-life similar to that of chlordiazepoxide.

REFERENCES

1. HARRIS, T., *J. Am. Med. Assoc.* **172**, 1162 (1960).
2. TOBIN, J. M., BIRD, I. F., AND BOYLE, D. E., *Diseases Nervous System* **21**, (suppl. 3), 11 (1960).
3. TOBIN, J. M., AND LEWIS, N. D. C., *J. Am. Med. Assoc.* **174**, 96 (1960).

4. RANDALL, L. O., SCHALLEK, W., HEISE, G. A., KEITH, E. F., AND BAGDON, R. E., *J. Pharmacol. Exptl. Therap.* **129**, 163 (1960).
5. RANDALL, L. O., *Diseases Nervous System.* **21**, (suppl. 3), 7 (1960).
6. STERNBACH, L. H., KAISER, S., AND REEDER, E., *J. Am. Chem. Soc.* **82**, 475 (1960).
7. STERNBACH, L. H., AND REEDER, E., *J. Org. Chem.* **26**, 1111 (1961).
8. STERNBACH, L. H., AND REEDER, E., *J. Org. Chem.* **26**, 4936 (1961).
9. RANDALL, L. O., *in* "Psychosomatic Medicine, the First Hahnemann Symposium" (J. H. Nodine and J. H. Moyer, eds.), pp. 476–479. Lea & Ferbiger, Philadelphia, Pennsylvania, 1962.
10. STERNBACH, L. H., KOECHLIN, B. A., AND REEDER, E., *J. Org. Chem.*, in press.
11. RANDALL, L. O., *Diseases Nervous System* **22**, (suppl. 7), 1 (1961).
12. BÄUMLER, J., AND RIPPENSTEIN, S., *Helv. Chim. Acta* **44**, 2208 (1961).
13. DUGGAN, D. E., BOWMAN, R. L., BRODIE, B. B., AND UDENFRIEND, S., *Arch. Biochem. Biophys.* **68**, 1 (1957).

ANALYTICAL BIOCHEMISTRY 5, 208–216 (1963)

A New, General Electrochemical Method of Determining Enzyme Kinetics. Kinetics of the Enzymic Hydrolysis of Thiocholine Iodide Esters[1]

GEORGE G. GUILBAULT, DAVID N. KRAMER, AND PAUL L. CANNON, JR.

From the Protective Development Division, Chemical Research and Development Laboratories, Army Chemical Center, Maryland

Received July 16, 1962

INTRODUCTION

Because of the importance of enzymes in catalyzing many reactions in the animal body, studies on the kinetics and mechanism of enzymic hydrolysis reactions have received increasing attention in the past decade. Techniques used to follow the rates of these reactions include measurement of the acid production of pH change (1) or manometrically through carbon dioxide liberation (2), and colorimetric methods (3, 4).

Polarographic methods have been used to study the kinetics of fast, nonenzymic reactions by Delahay (5), Berg (6), and Brdicka (7). Kern has discussed the use and limitations of heterogeneous rate constants in polarographic kinetics (8).

In the present study, the technique of constant-current voltammetry is used to follow the kinetics of enzymic hydrolysis reactions. Application to various enzyme-substrate systems is shown.

The use of the new galvanostatic technique affords a neat and simple instrumentable method for the determination of enzyme kinetics. Since the current density used is low and constant, the method probes directly and undisturbingly the dynamic changes in substrate and products, without introduction of significant concentrations of extraneous reagents capable of affecting the enzymic activity. Moreover, it is to be noted that the method is of general applicability in that a variety of enzyme reactions may be determined by proper choice of substrate and electrochemical mediator.

[1] Presented at the Biological Chemistry Division, 142nd Meeting, American Chemical Society, Atlantic City, New Jersey, September 11, 1962.

EXPERIMENTAL

Substrates. Acetylthiocholine iodide and butyrylthiocholine iodide solutions were prepared by dissolving the proper amount of salt (Dajac Laboratories) in 0.1 *M* Tris buffer, pH 7.40. Solutions of propionylthiocholine iodide (K & K Laboratories) were prepared in the same manner.

Cholinesterase. Solutions were prepared by dissolving horse serum cholinesterase (Armour and Co.) in 0.1 *M* Tris buffer, pH 7.40. The material was obtained by the Strelitz procedure (9), and had an activity of 1.9 μmoles of acetylcholine hydrolyzed per milligram of enzyme per minute.

Acetylcholinesterase. Purified enzyme from Bovine Erythrocytes (Winthrop Laboratories, activity 1.95 μmoles mg^{-1} min^{-1}) was dissolved in Tris buffer as described above.

Kinetic Measurements. The rates of hydrolysis were determined by measuring the rate of depolarization of a platinum anode by the thiocholine iodide produced upon enzymic hydrolysis of the thiocholine iodide ester by cholinesterase and acetylcholinesterase at pH 7.40 in 0.1 *M* Tris buffer. A sample of substrate, containing approx. 10^{-3} *M*, is dissolved in 25 ml of 0.1 *M* Tris buffer, and the resulting solution is magnetically stirred. The two platinum electrodes and a standard calomel electrode (S.C.S.) are immersed into the solution, and a constant current of 25 μa is applied across the platinum electrodes (Beckman Instrument Co., No. 39271) by a 135-volt battery across 270-kohm and 2-megohm resistors in a circuit as described previously (10). The voltage observed at the anode is measured versus the S.C.E. with a vacuum tube voltmeter and automatically recorded.

At zero time, 1.0 ml of enzyme is added to effect the catalytic hydrolysis of substrate to thiocholine iodide and acid, as illustrated:

$$(CH_3)_3N(I)CH_2CH_2SCOR \xrightarrow[\text{pH } 7.40]{\text{ChE}} (CH_3)_2N(I)CH_2CH_2SH + RCOOH$$

This thiocholine produced causes a depolarization of the electrodes, and a reduction in the potential of the system. From the slopes of these curves, $\Delta E/\Delta t$, the rates of hydrolysis may be calculated (10).

To check the accuracy of the electrochemical method, duplicate determinations of the reaction rates were made spectrophotometrically, following the rate of production of the thiocholine iodide, using the yellow color of the thiol-5,5′-dithiobis(2-nitrobenzoate) compound at 412 mμ (4). The reaction rates were recorded with a Beckman DB spectrophotometer, equipped with a Beckman adaptor and recorder. Thus the rate of spontaneous hydrolysis was automatically substracted from all readings.

Since all runs were made at pH 7.40 in Tris buffer, there was very little spontaneous hydrolysis occurring. The rates obtained electrochemically, $\Delta E/\Delta t$, agree well with those obtained spectrophotometrically, thus showing that the electrode reaction is not the rate-determining reaction, but is following the progress of reaction.

All solutions were thermostated at $25.0 \pm 0.1°C$ both before and during hydrolysis, using a constant-temperature bath. After each run, the electrodes were cleaned by rinsing with distilled water.

RESULTS

The new, proposed electrochemical method of following enzymic hydrolysis rates was used to elucidate the kinetics of the hydrolysis of acetyl-, propionyl-, and butyryl-thiocholine iodide by the enzymes acetylcholinesterase and cholinesterase.

Graphic plots of the initial rate of hydrolysis versus the enzyme concentrations, expressed in mg/ml of total solution, yielded straight lines for all three enzyme-substrate reactions, as predicted from the kinetic expression:

$$v = k_3[\mathrm{E}]_0[\mathrm{S}]_0/(K_m + [S]_0) \qquad (1)$$

Since the maximum velocity of these hydrolysis reactions, V_{max}, equals $k_3[\mathrm{E}]_0$, the constants k_3 for the rate of disproportionation of the substrate-enzyme complex, [ES], may be calculated. These values are reported in Table 1.

Figure 1 shows the relation of the reaction velocity to substrate concentration. As predicted from Eq. (1), the rate is proportional to $[\mathrm{S}]_0$ at low concentrations, then increases to a maximum, V_{max}. At high concen-

TABLE 1

RATE CONSTANT FOR HYDROLYSIS OF SUBSTRATES ACETYLTHIOCHOLINE AND BUTYRYLTHIOCHOLINE IODIDE BY CHOLINESTERASE AND ACETYLCHOLINESTERASE AT pH 7.40 IN 0.1 M TRIS

Substrate	V_{max} (M min^{-1} × 10^5)		K_m (M × 10^4)		k_3 (min × 10^{-5})	
	ChE[a]	AChE[a]	ChE	AChE	ChE	AChE
Acetylthiocholine	6.0	4.6	10.0	1.4	5.5	9.2
Butyrylthiocholine	6.1	—	3.1	—	6.5	—

[a] V_{max} determined using a 1.08×10^{-10} M ChE and a 0.5×10^{-10} M AChE solution. The molarity was calculated using the formula:

$$M = \text{activity(concentration)}(10^{-3})/\text{TN}$$

where the activity is expressed in μmoles/mg/min, concentration is in mg/ml, and M is in moles/liter. The turnover number (TN) used is 3.0×10^5 min^{-1} for ChE and 7.4×10^5 min^{-1} for AChE (11), based on the corresponding choline derivatives.

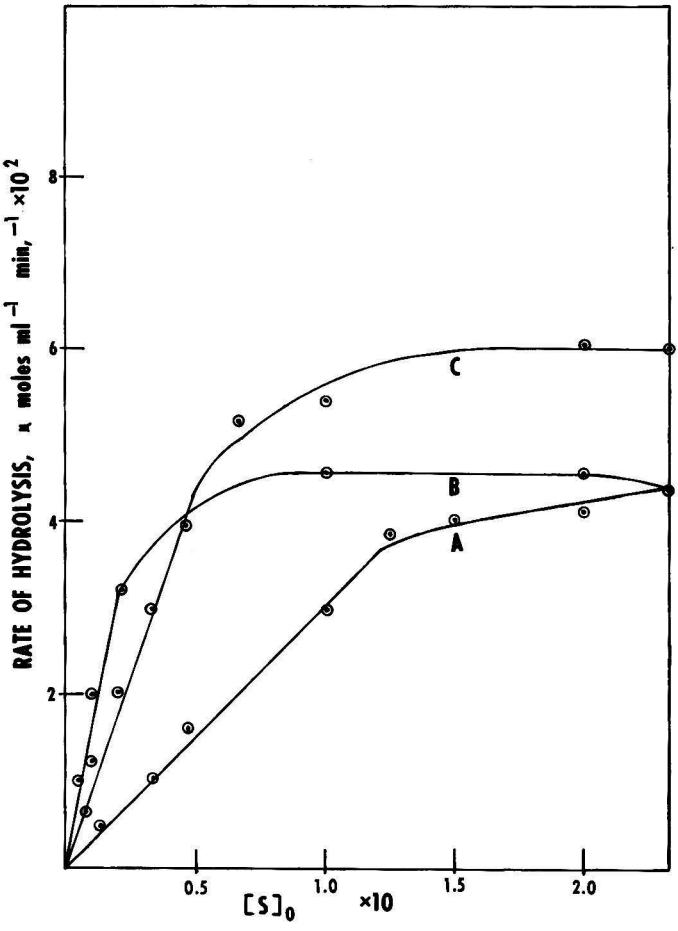


FIG. 1. Variation of rate of hydrolysis of thiocholine iodide esters with substrate concentration at 25.0°C. pH = 7.40. Concentration of enzyme = 0.02 mg/ml of total solution. Line A = acetylthiocholine iodide and cholinesterase. Line B = acetylthiocholine iodide and acetylcholinesterase. Line C = butyrylthiocholine iodide and cholinesterase.

trations of substrate, the enzyme becomes competitively inhibited by excess substrate, and the rate decreases. When the reaction velocity, v, is plotted versus $v/[S]_0$ according to the Michaelis-Menten equation, expressed as:

$$v = vK_m/[S]_0 + V_{max}$$

the Michaelis constant, K_m, may be calculated. These values are reported in Table 1.

The effect of temperature upon the rates of enzymic hydrolysis is shown in Fig. 2. A constant, initial substrate concentration of $2 \times$

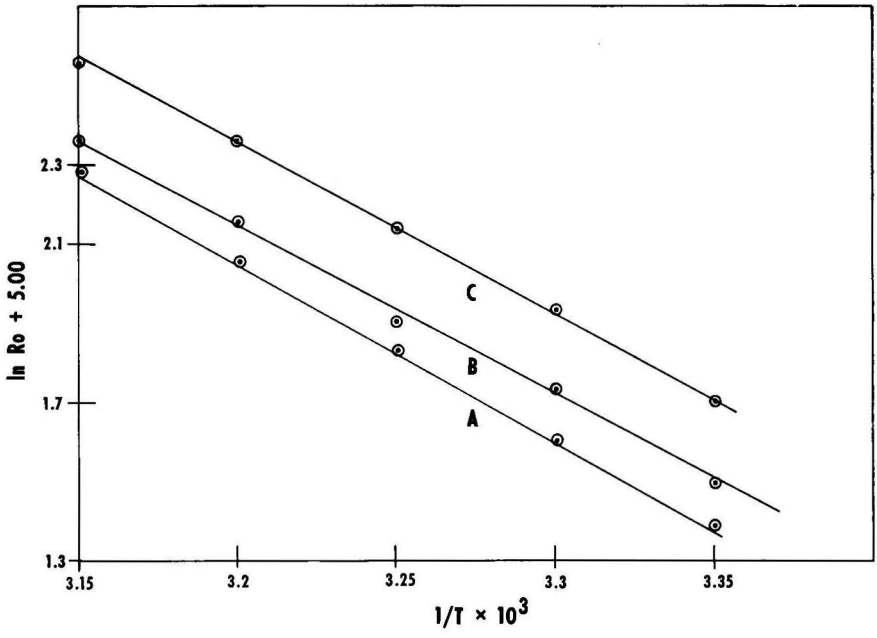


FIG. 2. Variation of ln R_0 with $1/T$. pH = 7.40. Substrate concentration = $2 \times 10^{-3} M$. Enzyme concentration = 0.0178 mg/ml. Line A = acetylthiocholine iodide and cholinesterase. Line B = butyrylthiocholine iodide and cholinesterase. Line C = acetylthiocholine iodide and acetylcholinesterase.

$10^{-3} M$, and an enzyme concentration of 0.0178 mg per each milliliter of total solution was maintained. Calculation of the activation energies from the slopes of Arrhenius plots of ln R_0 versus $1/T$ $(= -E/R)$ yielded values of 8.50 and 9.00 ± 0.20 kcal/mole for the hydrolysis of acetylthiocholine iodide by the enzymes acetylcholinesterase and cholinesterase, respectively, and 8.55 ± 0.20 kcal/mole for the hydrolysis of butyrylthiocholine iodide by cholinesterase.

The effect of acyl chain length on the rate of hydrolysis of thiocholine iodide esters by the enzymes cholinesterase (B) and acetylcholinesterase (A) is shown in Fig. 3. These same observations have been noted in the hydrolysis of the corresponding choline esters (12). It is evident that the horse serum cholinesterase is a more specific enzyme for the butyrylthiocholine iodide than for acetylthiocholine iodide, the order of the rate

of hydrolysis being butyryl > propionyl > acetyl. But with acetylcholinesterase, the exact opposite is observed. Although cholinesterase

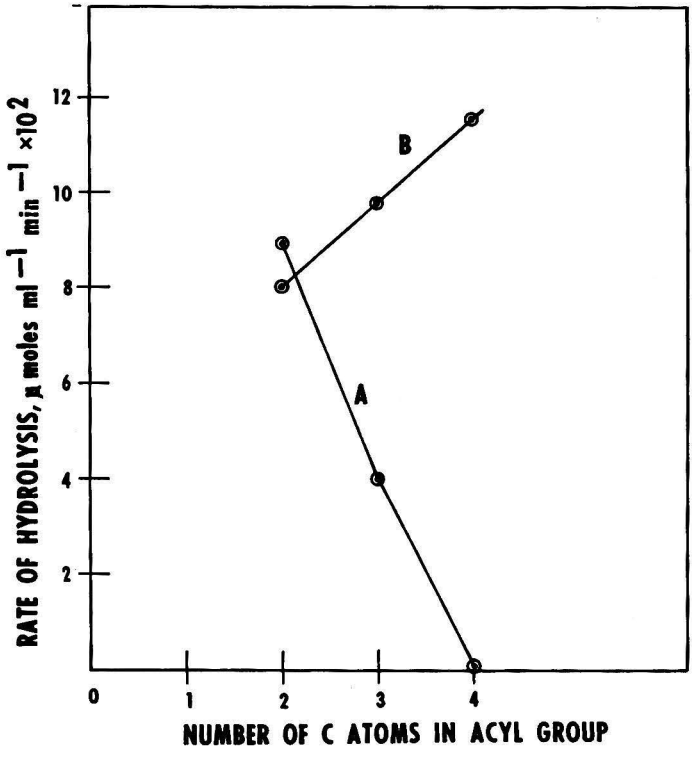


FIG. 3. Effect of acyl chain length on rate of hydrolysis of thiocholine iodide esters by enzyme. Line A = acetylcholinesterase. Line B = cholinesterase.

will hydrolyze acetylthiocholine iodide to an appreciable extent, the action of acetylcholinesterase upon butyrylthiocholine is negligible.

DISCUSSION

Kinetic Constants. In previous work, Heilbronn (13), Ellman (14), and Gal and Roth (15) used titrimetric, manometric, and colorimetric procedures to elucidate the kinetics of the hydrolysis of acetylthiocholine by acetylcholinesterase. It was of interest to ascertain whether the new, simpler electrochemical technique yielded results in agreement with previous workers. The values of K_m and V_{max} found using the proposed method agree well with those given by the above workers for

the ASCh-AChE reaction. In addition, kinetic constants were found for the hydrolysis of other thiocholine esters by both cholinesterase and acetylcholinesterase.

The values of k_3 and K_m given in Table 1 indicate that the enzyme-substrate complex disproportionates into products and free enzyme in the order:

$$[\text{ASCh—AChE}] > [\text{BuSCH—ChE}] > [\text{ASCh—ChE}]$$

and dissociates into substrate and enzyme in the reverse order. Thus the relative rates of hydrolysis follow those of the kinetic constants, k_3.

Temperature. The temperature coefficients, Q_{10}, calculated for the hydrolysis of acetylthiocholine iodide by acetylcholinesterase and cholinesterase were 1.53 and 1.57, respectively, and a value of 1.54 was obtained for the hydrolysis of butyrylthiocholine iodide by cholinesterase. These values, and the activation energies obtained, agree well with those found in other enzymic hydrolysis reactions (17).

Current. As a current of 25 μa is passed across two platinum electrodes, immersed in the substrate solution, a potential, E_1, of about 0.45 volt is recorded, due to oxidation of the iodide ion, which acts as a mediator, and establishes the initial potential. As enzyme is added, the potential drops to a steady value, E_2, of about 0.20 volt, due to formation of a more easily oxidizable product, the thiocholine iodide (16).

The slope of the depolarization curve, $\Delta E/\Delta t$, which is a measure of the reaction rate, is greatly dependent on the applied current, decreasing from a value of 12.0 mv/sec at 3.4 μa to a value approaching zero at very high currents (10). This observation may be explained by the following. The applied current causes oxidation of iodide to iodine, which can then react with the thiocholine produced upon hydrolysis. At low currents, little iodine is formed, so the change in potential with time is large. At high currents, appreciable amounts of iodine are formed, which immediately react with the electroactive thiocholine, removing it from solution, and causing a small $\Delta E/\Delta t$.

For constant, reproducible results, some iodide ion must be present in solution. If the original sample is present as the chloride or bromide, 1 ml of a 10^{-3} M solution of potassium iodide must be added. Chloride ions and bromide ions do not affect the results, but moderate amounts of cyanide adversely affect the reproducibility.

The excellent agreement with previous literature results indicates the new, proposed electrochemical method to be a good one for following the kinetics of enzymic reactions. Advantages include simplicity of apparatus, speed, accuracy, and noninterference in the enzymic processes. The accuracy obtainable by the method is, in most cases, better than

1%. This technique, furthermore, is not limited to this particular system, but should prove to be a useful, general tool for following any reactions of the type:

$$A \xrightarrow{\text{Mediator}} B + C$$

provided B and/or C and either A or a mediator are electroactive materials. In the hydrolysis of acetylthiocholine by cholinesterase, the substrate was not electroactive, so a mediator—iodide ion—was used. Likewise, in the application to the glucose-glucose oxidase system, diphenylaminesulfonic acid acts as a mediator in the reaction, glucose being relatively electroinactive.

In other systems studied, as the peroxide-peroxidase, the substrate is electroactive, and no mediator is used. The analytical results on these systems will be published in the near future.

SUMMARY

A new, electrochemical method is described for determining the rates of fast, enzymic hydrolysis reactions. A constant current of 25 μa is applied across two platinum thimble electrodes, and the change in potential upon hydrolysis is recorded versus time. From the slopes of the resulting depolarization curves, $\Delta E/\Delta t$, the relative rates of hydrolysis may be calculated. This method has been applied to the hydrolysis of several thiocholine iodide esters by the enzymes cholinesterase and acetylcholinesterase at pH 7.40 in 0.1 M Tris buffer. The rates determined electrochemically agree well with those obtained by other methods, showing that the electrode system is following the rate of enzymic hydrolysis. The method is a general one, and application to other enzyme-substrate systems is shown.

ACKNOWLEDGMENT

The authors wish to thank Dr. W. H. McCurdy, Jr., for his help on some phases of this work.

REFERENCES

1. Stedman, E., Stedman, E., and Easson, L., *Biochem. J.* **26**, 2056 (1932). Willstätter, R., *et al.*, *Z. physiol. Chem.* **167**, 303 (1927).
2. Mendel, R., and Rudney, H., *Biochem. J.* **37**, 59 (1943).
3. Kramer, D. N., and Gamson, R. M., *Anal. Chem.* **30**, 251 (1958).
4. Heilbronn, E., *Acta Chem. Scand.* **10**, 337 (1956).
5. Delahay, P., *J. Am. Chem. Soc.* **74**, 3506 (1952).
6. von Berg, H., and Kapulla, H., *Z. Elektrochem.* **64**, 44 (1960).
7. von Brdicka, R., *Z. Elektrochem.* **64**, 16 (1960).
8. Kern, D. H., *J. Am. Chem. Soc.* **75**, 2473 (1953).
9. Strelitz, F., *Biochem. J.* **38**, 86 (1944).

10. KRAMER, D. N., CANNON, P. L., AND GUILBAULT, G. G., *Anal. Chem.* **34**, 842 (1962).
11. WILSON, I., AND HARRISON, M., *J. Biol. Chem.* **236**, 2292 (1961).
12. ADAMS, D., *Biochim. et Biophys. Acta* **3**, 1 (1949). ADAMS, D., AND WHITTAKER, V., *Biochim. et Biophys. Acta* **3**, 358 (1949). WRIGHT, C., AND SABINE, J., *J. Pharmacol.* **93**, 230 (1948).
13. HEILBRONN, E., *Acta Chem. Scand.* **13**, 1547 (1959).
14. ELLMAN, G., COURTNEY, D., ANDRES, V., AND FEATHERSTONE, R., *Biochem. Pharmacol.* **7**, 88 (1961).
15. GAL, E., AND ROTH, E., *Clin. Chim. Acta* **2**, 316 (1957).
16. FISEROVA-BERGEROVA, V., *Collection Czech. Chem. Commun.* **27**, 693 (1962).
17. DIXON, M., AND WEBB, E. C., "Enzymes," p. 614. Academic Press, New York, 1958.

ANALYTICAL BIOCHEMISTRY 5, 217–225 (1963)

Use of Periodate Oxidation in the Clinical Analysis of Urine Corticoids

HILDEGARD WILSON AND MORTIMER B. LIPSETT

From The Endocrinology Branch, National Cancer Institute, Bethesda, Maryland

Received July 23, 1962

The most widely accepted method for the determination of urinary 17-hydroxycorticosteroids is the modification, by Appleby *et al.* (1), of the method devised by Norymberski *et al.* (2). There are several advantages of this method over procedures employing enzyme hydrolysis and the Porter-Silber reaction (3), chief among which are: (1) inclusion of corticoid metabolites having the 17,20,21-triol side chain in the determination, (2) oxidative removal of the two-carbon side chain before extraction of the urine, thus permitting hydrolysis of conjugates by hot acid.

Recently Few (4) studied the oxidation of periodate of steroids with the 17,20-diol and 17,21-dihydroxy-20-ketone side chains and pointed out that, following reduction with sodium borohydride, sodium metaperiodate would oxidize the same urinary C_{21}-steroids to 17-ketones as did sodium bismuthate. Since the sequence of reactions necessary in the periodate method avoids hot acid hydrolysis and results in greater specificity than methods employing bismuthate oxidation, we have adapted this periodate oxidation method to routine clinical use. The above advantages as well as the technical simplicity of this clinical method warrant its presentation.

MATERIALS AND METHODS

Reagents

(1) Sodium hydroxide solutions: 0.1, 2.5, and 6.0 *N*.

(2) Potassium borohydride, 10% w/v in 0.1 *N* NaOH. This solution is freshly prepared for each determination.

(3) Acetic acid, 25% and 4% aqueous v/v. We have used acetic acid distilled over CrO_3, but this may not be necessary with certain brands of glacial acetic acid.

(4) pH test papers, for pH 6.0–8.0, and "wide range" for pH 1.0–12.0.

(5) Sodium metaperiodate, 10% w/v, aqueous.

(6) Ethyl ether, peroxide free: The commercial ether is washed once with ⅕ volume of nearly saturated ferrous sulfate solution and then washed 4 times with ⅕ volume of distilled water. It is stored in metal cans without being freed of dissolved water. This preparation remains free of peroxide for at least 3 or 4 days. *Test for peroxides:* Five grams of potassium iodide is dissolved in 10 ml water. To 10 ml ether 0.5 ml KI solution is added, and the mixture shaken for 30 sec. A brown or yellow color indicates the presence of peroxides.

(7) Ethyl alcohol: U. S. Industrial Chemical Co. "U.S.I. Absolute pure ethyl alcohol," USP-NF reagent quality.

(8) Dichloromethane: Purified by passing through a large silica gel column. Three and one half pounds of silica gel (Fisher Chemical Co. 28/200 mesh) will suffice for 24 to 32 kg of dichloromethane.

(9) Sodium sulfate, anhydrous.

(10) Dinitrobenzene: "Specially prepared for the Zimmermann reaction in steroid determinations, Grade V," Sigma Chemical Co., St. Louis 18, Mo. No purification of this product is necessary. A 1% solution in absolute ethanol is used.

(11) Alcoholic potassium hydroxide solution, 2.5 *N*, prepared according to Wilson and Carter (5) or Hamburger (6).

(12) Dehydroepiandrosterone: The stock solution contains 50 mg in 50 ml absolute ethanol. A 1:10 dilution, containing 10 μg per 0.1 ml is used as a working standard.

General Procedure

The urine is first treated with potassium borohydride, which reduces the ketone groups to hydroxyl groups. After decomposing the excess KBH_4 with acetic acid, oxidation with periodate is carried out. This converts C_{21} 17,20,21-triols and 17,20-diols to the corresponding 17-ketones, but does not affect the C_{19} 17β-OH steroids, which were formed by reduction of the preformed 17-ketosteroids. In addition, the steroid glucosiduronates are converted either to free alcohols or to the corresponding formates. The remaining formates are saponified by a short incubation with alkali. The free steroids are then extracted with ether and the ether is washed with alkali and dried with anhydrous sodium sulfate. All of these operations are carried out in the same tube.

The neutral ether extract is decanted into another tube, evaporated to dryness, and assayed by a modification of the Zimmermann reaction as adapted by Peterson and Pierce (7).

Procedure in Detail

Measurement and reduction of urine: Duplicate 5.0-ml aliquots of

each sample are pipetted into 100-ml graduated tubes, (28.5 mm o.d., with $\overline{\$22}$ glass stoppers; Kontes Glass Co., Vineland, N. J.). The pH of each urine is tested with pH 6.0–8.0 paper. If less than 7.0, it is adjusted to pH 7.0 by the addition of about 0.4 ml of 0.1 *N* NaOH. To each tube 0.5 ml of KBH_4 solution is added with mixing. The stoppered tubes are then allowed to stand 1 hr at room temperature.

Decomposition of potassium borohydride: Following reduction, 0.4 ml of 25% acetic acid is added. When tested with wide-range pH paper, the paper should remain orange and not turn yellow, assuring an acid pH. After mixing well, the solutions are kept at room temperature for 15 min. It is important that all the KBH_4 be decomposed, as it will reduce the periodate and prevent oxidation.

Oxidation with sodium metaperiodate: To each tube 2.0 ml of freshly prepared 10% $NaIO_4$ solution is added. After mixing, the pH is tested with pH 6.0–8.0 paper and adjusted to pH 6.6 with about 0.5 ml of 2.5 *N* NaOH. The stoppered tubes are then placed in an incubator at 37°C for 75 min.

Saponification of formates: Immediately on removal from the incubator, 0.2 ml of 6.0 *N* NaOH is added to each tube with mixing. They are then incubated at 37° for an additional 20 min.

Extraction with ether: After cooling, 40 ml of peroxide-free ether is added to each tube. The tubes are stoppered, vigorously shaken for 30 sec, then allowed to stand 5 min for separation of the 2 phases.

Aspiration of urine layer: A fine tipped 1.0-ml graduated pipet is attached to gentle suction through a large suction flask trap. With a strong pinch clamp closing the rubber connection, the pipet is inserted into the urine layer, which is then carefully aspirated. No ether should be removed during this or succeeding steps.

Washing the ether extract: The ether is successively washed with 4.0 ml of 2.5 *N* NaOH, 2.0 ml of 4% acetic acid, and 4.0 ml of distilled water. Each wash is well mixed, then aspirated as above. The final wash should be at pH 6.5–7.0.

To the washed ether 3 or 4 gm anhydrous sodium sulfate is added through a powder funnel. After stoppering, the tubes are swirled until the ether appears clear. All the ether extract is then carefully decanted into 50-ml conical centrifuge tubes, with rinsing of the lip of the extraction tube. The remaining Na_2SO_4 is washed with 4–5 more milliliters of ether, which is added to that already decanted. The ether is evaporated from each tube under a stream of air in a warm water bath. The sides of each tube are rinsed down with 2–3 ml methylene chloride, which is also evaporated off. Finally the tubes are placed in a vacuum oven at 50°C for 0.5 hr to assure complete dryness.

Zimmerman reaction[1]*:* For reference standards and reagent blanks, color development is carried out directly in calibrated 19 × 160 mm cuvettes (Coleman Instruments Inc., Maywood, Ill.). With urine extracts the reaction takes place in the 50-ml centrifuge tubes containing the dried extract and the final colored solution is transferred to the 19 × 160 mm cuvettes. Readings are made on a Coleman Universal Spectrophotometer, Model 4, set at 520 mμ.

Graded amounts of dehydroepiandrosterone are prepared in duplicate by pipetting 0.2, 0.4, and 0.6 ml of the working standard (20, 40, and 60 μg) into 6 of the 19 × 160 mm cuvettes. These 9 tubes are dried down in a warm water bath under a stream of air, and placed in the vacuum oven together with the urine extracts. Reagent blanks are prepared in duplicate by adding 0.4 ml of 1% *m*-dinitrobenzene to 2 cuvettes and an ethanol blank is prepared by adding 0.4 ml absolute ethanol to a third tube.

To each of the tubes with the dried DHA or urine extract, 0.4 ml of the 1% *m*-dinitrobenzene is added. The time is noted and 0.2 ml of the 2.5 *N* alcoholic KOH is added to each tubes, with mixing. Color development is allowed to proceed in the dark at room temperature for 1 hr. Then 1.0 ml of distilled water and 4.5 ml of dichloromethane are added to each tube, followed by thorough shaking.[2] The tubes are allowed to stand for 3 or 4 min for separation of phases. Aqueous layers, varying in depth of brown coloration, will rise to the top while pale purple or almost colorless solvent layers are seen below. The upper aqueous layers are carefully aspirated off and 1.5 ml absolute ethanol is added to the remaining solvents. On shaking, purple colors of excellent spectral purity develop immediately. The solution should be perfectly clear. If not, a few crystals of anhydrous Na_2SO_4 or a few drops of ethanol may be added.

Colorimetry: The contents of the 50-ml centrifuge tubes are now transferred to 19 × 160 ml cuvettes for reading at 520 mμ. Reagent blanks are first checked against the ethanol blank set at zero optical density. They should not read over 0.050. A reagent blank tube is then set to zero and all other tubes read against it.

Calculations

Chromogenic values for DHA, or "*K* values," are calculated for each amount used and averaged:

$$K_{\text{DHA}} = \frac{\text{OD at } 520 \times 1000}{\mu\text{g DHA}}$$

[1] The procedure for extraction of the final Zimmermann chromogens and the development of the color was devised by Mr. Silas Jackson of this laboratory.

[2] A "Vortex Jr." mixer (Scientific Industries, Inc., Queens Village, N. Y.) is useful for all mixing steps in this procedure.

Since the chief 17-ketosteroid reacting is 11β-hydroxyetiocholanolone, a standard solution of this steroid was included in a series of assays, together with the DHA standards. The average chromogenic capacity of DHA was 1.35 times as great as that of 11β-hydroxyetiocholanolone. Having established this factor, DHA was used as a standard. This factor may differ with other techniques of carrying out the Zimmermann reaction.

To convert milligrams of 11β-hydroxyetiocholanolone equivalents to approximate milligrams of C_{21} cortisol metabolites, a further factor of 1.2 was used because of the difference in molecular weights.

Thus the calculations of mg C_{21} corticosteroids/24 hr may be summarized as follows:

$$\text{mg 17-OHCS/24 hr} = \frac{\text{OD of sample} \times 1.62 \times \text{24-hr urine vol.}}{K_{\text{DHA}} \times 5 \text{ (ml urine aliquot)}}$$

where OD = optical density at 520 mμ read against the reagent blank, and 1.62 = a factor combining 1.35 to correct for the lower chromogenicity of 11β-hydroxyetiocholanolone compared to DHA, and 1.2 to convert mg C_{19} to C_{21} steroids.

Comments on the Procedure

One hour's reduction with KBH_4 was as adequate as 2 hr when judged by quantitative paper chromatography of the final products. Oxidation of corticoids was complete since no reducing areas were found on paper chromatograms. No 17-ketosteroid formates were detected either with or without the saponification step; however, the yield of Zimmermann chromogens was 5–10% higher if this step was included.

Ethyl ether extracts were less pigmented than dichloromethane extracts, and recoveries of added steroids were as good. Two extractions did not increase the yield.

The procedure for removal of the nonspecific Zimmermann chromogens is superior to others we have tried. With few exceptions, the final Zimmermann color derived from the urine extracts was free of nonspecific brownish chromogens. Both stability of color and intensity per unit amount of reactant are good.

Colorimeter readings were sometimes below the optimum range in subjects with low 17-OHCS excretion and occasionally in subjects with normal levels and large urine volumes. If greater precision is desired in such cases, 10 ml of urine rather than 5 ml may be processed. Appropriate adjustments in the volume of reagents must be made.

Few (4) points out that high concentrations of glucose may interfere with the determination but that dilution of the urine may obviate this

problem. Presumably initial yeast fermentation of the glucose would also be satisfactory.

RESULTS

Reliability of the Method

Specificity: Paper chromatography of the final neutral extract revealed only 11β-hydroxyetiocholanolone in the region of 11-oxygenated 17-ketosteroids. This is the expected oxidation product of cortol and β-cortol, which would be the sole reduction products of tetrahydrocortisol, tetrahydrocortisone, cortolone, and β-cortolone. With the aliquots used for these chromatograms, a weak Zimmermann-reacting spot was seen corresponding in position to etiocholanolone. This is the expected metabolite of such steroids as 17α-hydroxypregnanolone, pregnanetriol, and tetrahydro substance S, which are present in small amounts in urine from normal subjects. Δ^5-Pregnenetriol would not be freed from its sulfate or extracted (4).

TABLE 1
ESTIMATES OF PRECISION

Range (mg/24 hr)	No. of duplicate determinations	S.D.
0–5.0	54	.76
5.1–10	87	.89
10.1–15	79	1.3
15.1–20	34	1.0
20.1–30	37	1.6
30.1–59.4	20	3.1

Precision: In Table 1, the results of 311 pairs of duplicate determinations over a wide range have been analyzed. The standard deviation (S.D.) has been calculated by Snedecor's formula (8), S.D. = $d^2/2n$, where d is the difference between duplicate determinations and n is the number of duplicate determinations. Except in the lowest range, the S.D. averaged about 10% at such levels. This is a satisfactory precision for clinical urinary steroid determinations (9).

Accuracy: The over-all accuracy of the procedure could not be evaluated since the glucosiduronates of tetrahydrocortisol and tetrahydrocortisone were not available to us. Few (4) presented evidence that oxidation of the glucosiduronates was more than 92% complete. When tetrahydrocortisone was added to urine in our study, the recovery was 94.2 $\pm$ 11% (average of 12 determinations).

Sensitivity: Since the S.D. is 0.76 mg in the range 0–5 mg/24 hr, the

95% level of confidence would be 1.5 mg/24 hr. Thus, in practice, values below 2.0 mg/24 hr should be considered indistinguishable from zero. In one adrenalectomized subject maintained in small doses of Dexamethasone, the corticoid excretion averaged 1.1 mg/24 hr.

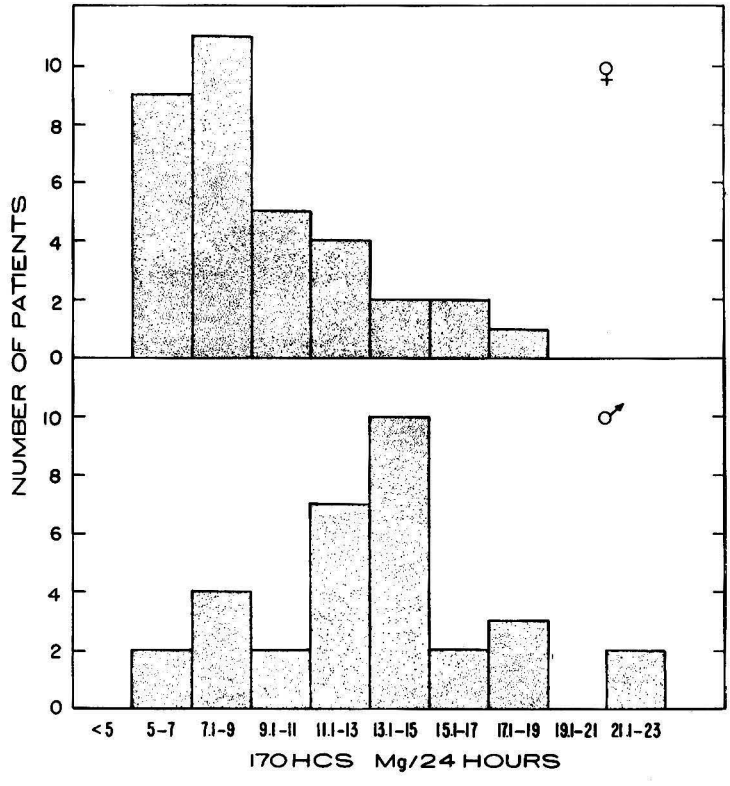


FIG. 1. 17-Hydroxycorticoid excretion by normal adults.

In Fig. 1 the 17-hydroxycorticoid excretion of 66 normal adults between the ages of 20 and 60 are depicted. For the 32 men, the mean excretion of 17-hydroxycorticoids was 13.0 mg/24 hr and the median was 13.2. The mean excretion for the 34 women was 9.4 mg/24 hr, the median being 8.4. The 2 male subjects with the highest excretion of 17-hydroxycorticoids excreted 21.1 and 21.2 mg, respectively. These values are comparable to, or a little lower than, those reported by Few for 11-oxy- plus 11-deoxysteroids (4).

One patient with Cushing's syndrome due to an adenoma excreted 35 and 42 mg of 17-OHCS. The response of 6 normal subjects to an 8-hr

infusion of 25 I.U. of Armour's ACTH gave values of 2–4 times the control levels. Suppression of 17-OHCS in the normal subject with 0.5 mg of dexamethasone 4 times a day yielded 17-OHCS titers below 3.0 mg/24 hr.

DISCUSSION

The presentation of a new method when an accepted technique already exists demands that the proposed method offer advantages as well as meet criteria of reliability. The method proposed here adequately meets criteria of reliability for a clinical procedure. The precision, accuracy, and sensitivity are approximately those that have been found in an evaluation of the Appleby-Norymberski technique (10).

The advantages of this method over those using bismuthate oxidation are greater specificity and a simpler and more reliable oxidation technique. Periodate has been shown not to oxidize the 11β-hydroxy groups except in acid solution (11) and at higher concentrations (4) and our failure to find any 11-ketoetiocholanolone confirms these findings. The elimination of acid hydrolysis prevents the variable dehydration at C-9,11 which results in compounds of differing chromogenicity. This increase in specificity means that the final color can be related directly to that obtained with 11β-hydroxyetiocholanolone in the Zimmermann reaction.

With respect to technique, the following advantages seem significant: The oxidation is carried out in solution with a reagent of standard grade, avoiding the variations in oxidizing capacity found in various lots of bismuthate. The removal of the excess of oxidizing agent is easy and does not require centrifugation. The results reported in this paper were obtained by one technician with little experience in this field. Twenty to 30 samples can be analyzed in duplicate in 1½ working days.

Roughly, 10–20% of the Zimmermann chromogen obtained by this method for normal urine is etiocholanolone (4). This seems to be a fairly constant fraction in normal urine and should not account for any large variation from normal. It is apparent, of course, that in clinical situations such as congenital adrenal hyperplasia or adrenal carcinoma periodate oxidation may result in a high titer of 17-ketosteroids because of the excess of such compounds as pregnanetriol and tetrahydro substance S, rather than metabolites of cortisol.

SUMMARY

A method for the routine clinical determination of urinary 17-hydroxycorticoids is described and its reliability analyzed. The method consists of reduction with potassium borohydride followed by oxidation with

sodium metaperiodate as described by Few, extraction with ether, and performance of the Zimmermann reaction. The advantages of this method are greater specificity of the oxidant, the avoidance of hot acid hydrolysis, the technical simplicity, and the removal of interfering chromogens from the final Zimmermann color.

ACKNOWLEDGMENTS

We wish to thank Mr. Herman Carter for his able assistance in the early phases of this work and to acknowledge the fine technical performance of Mr. James Long in performing these assays.

REFERENCES

1. APPLEBY, J. I., GIBSON, G., NORYMBERSKI, J. K., AND STUBBS, R. D., *Biochem. J.* **60**, 453 (1955).
2. NORYMBERSKI, J. K., STUBBS, R. D., AND WEST, H. F., *Lancet* **1**, 1276 (1953).
3. PORTER, C. C., AND SILBER, R. H., *J. Biol. Chem.* **185**, 201 (1950).
4. FEW, J. D., *J. Endocrinol.* **22**, 31 (1961).
5. WILSON, H., AND CARTER, P., *Endocrinology* **41**, 417 (1947).
6. HAMBURGER, C., *Acta Endocrinol.* **9**, 129 (1952).
7. PETERSON, R. E., AND PIERCE, C. E., *in* "Lipids and Steroid Hormones in Clinical Medicine" (F. W. Sunderman and F. W. Sunderman, Jr., eds.), p. 158. Lippincott, Philadelphia, 1960.
8. SNEDECOR, G. W., *Biometrics* **8**, 85 (1952).
9. MARRIAN, G. F., *Proc. Intern. Congr. Biochem., 3rd, Brussels, 1955* **9**, 511 (1956).
10. BIRKE, M. D., DICZFALUSY, E., AND PLANTIN, L.-O., *J. Clin. Endocrinol. Metab.* **18**, 736 (1958).
11. HARKNESS, R. A., AND FOTHERBY, K., *Experientia* **17**, 253 (1961).

ANALYTICAL BIOCHEMISTRY 5, 226–245 (1963)

Applications of Radioactively Labeled Marker Proteins in Density Gradient Ultracentrifugation

P. A. CHARLWOOD

From the National Institute for Medical Research, The Ridgeway, Mill Hill, London, England

Received July 26, 1962

INTRODUCTION

Use of a density gradient to stabilize the sedimentation of solutes present in very low concentration was described by Pickels (25), but the application of this principle in ultracentrifugation did not become widespread until the last decade. Brakke (4) pointed out its advantages in preparative work, and Meselson *et al.* (22) developed the equilibrium version into an elegant means of distinguishing macromolecules of slightly different densities. A very useful review of the whole subject, but with special reference to cell particles, has been compiled by de Duve *et al.* (12).

Density gradient ultracentrifugation in preparative tubes may have one (or more) of three main objectives. The first of these is the isolation of a component of distinctive size, the second is the establishment of an approximate value for the sedimentation coefficient of a biologically active material present in too low a concentration to be investigated by conventional optical analytical methods, and the third is the possible differentiation (on a size basis) of components possessing distinguishable kinds of biological properties.

Although Kunkel (16) identified the macroglobulin zone of serum, after an experiment of this nature, by its light scattering, this method of detection is of limited scope, because many proteins do not scatter sufficient light, particularly when present in low concentration. A more general approach by Stanworth *et al.* (27), which involves the use of dye-labeled reference proteins in a gradient tube prepared and treated in the same manner as the experimental tubes, is dependent on accurate reproducibility from tube to tube. It is also not easy to define the zones with very great precision because of the low intensity of color on the fringes. Martin and Ames (21), by careful control of variables and use of the swinging bucket type of rotor, have successfully used density

gradient ultracentrifugation as an absolute method for measuring sedimentation coefficients, and obtained results for three enzymes in very good agreement with accepted figures. They also mentioned the simplification which can be achieved if well-characterized enzymes are mixed with the sample to be examined and relative movements measured. Use of internal standards, then, allows close control to be exercised, and the sensitivity of radioactivity measurements would enable this to be done with the introduction of a minimum of foreign material which would sometimes in preparative work be nearly entirely confined to rejected fractions anyway. While there are alternative techniques, such as partition cells, for the estimation of sedimentation coefficients using biological assays, a comparison of the sedimentation rates of unknown and marker proteins, as described below, has certain advantages (see *Discussion*). It seemed desirable, therefore, to examine some readily available proteins covering as wide a range of size as possible for suitability as markers, to confirm that the introduction of the radioactive label gives products which are valid comparison substances, and to assess the degree of resolution attainable. This paper describes such work, together with examples of some specific cases in which the radioactive markers have been of value. Iodine isotopes were used, as they can be introduced with a minimum of alteration in the properties of many proteins (19).

MATERIALS AND METHODS

Proteins. The lysozyme, ovalbumin, bovine plasma albumin (fraction V), bovine γ-globulin, and bovine fibrinogen used were all Armour products (batches DN 2451, 24289, EB 1370, CH 2470, and EG 1771, respectively). Mercaptalbumin (10× cryst.), edestin (2× cryst.) and beef liver catalase were from Nutritional Biochemicals (batches 9499, 2163, and 1514, respectively). Human transferrin was either that supplied by Behring (1227/34) or a less pure sample obtained by gradient elution from a DEAE cellulose column loaded with serum. Macroglobulin was isolated from normal rabbit or human serum by density gradient ultracentrifugation, controlled by the use of labeled bovine γ-globulin as described later in this paper. The commercial catalase contained slower sedimenting impurities, which were easily eliminated by a preliminary density gradient experiment. Arachin was prepared by the method of Johnson (15) from ground-nut meal freed from oil by cold extraction with petroleum ether. The β-lactoglobulin, from L. Light and Co. (3× cryst.) had no identifying number.

Iodination. This was done with carrier-free I^{131} or I^{125} mixed with buffered iodine monochloride, either using the procedure of McFarlane (20) or in borate buffer (14) at pH 8.3. Low levels of carrier iodine

(often no more than one atom per molecule of protein) were nearly always used, and no special efforts were made to obtain high efficiencies of labeling. Only when products of low specific activity were being prepared for analytical ultracentrifugation was an attempt made to estimate the degree of incorporation. The iodinated proteins were either dialyzed or put through an anion-exchange column to remove free iodide (19).

Analytical ultracentrifuge experiments were done in a Model E Spinco machine according to the usual technique (8) to check the sedimentation coefficients both of untreated proteins and of many iodinated ones. A few measurements were made at lower concentrations by the use of ultraviolet optics.

Density gradients were established in preparative tubes for fixed angle rotors (usually No. 40, but occasionally No. 30) of Model L Spinco ultracentrifuges. In earlier experiments the gradients were formed by a device equivalent to that of Britten and Roberts (6) but uniform distribution into six or twelve tubes presented difficulty. When it became necessary to prepare simultaneously gradients of different sucrose concentrations, sometimes with different buffer salts in them, recourse was made to the simpler procedure of allowing three superimposed layers to diffuse for about 24 hr. Tests on the density distribution in such a tube were made by running out fractions and comparing the positions of sample drops from these with the positions of drops of standard sucrose solutions in an oil gradient. The departure from linearity of the sucrose gradient was not sufficient to affect seriously any of the subsequent discussion where it is based on the assumption of a linear gradient. The gradients ranged from 100 gm/liter sucrose at the top of the tube to 200–500 gm/liter at the bottom. Instead of sucrose, sodium potassium tartrate was used in some experiments (18), at a concentration of 0.5–1.0 or 0.5–2.0 M. Samples, which were if necessary diluted to contain no more than 30 mg/ml of protein (16), were layered on the top by means of a capillary with tip bent to give a horizontal outflow. A low hydrostatic head controlled the rate of sample introduction. The ultracentrifuges were run overnight ($\sim$16 hr) at speeds of 23,000 to 40,000 rpm according to the conditions of the particular experiment. No special precautions were taken during acceleration, but the brake was not used at the end of the centrifugation. On one machine it was possible to adjust the refrigeration so that the rotor remained at approximately the temperature of the room (18°C), but with minimum refrigeration on the other machine the rotor was quite cold to touch when the chamber was opened. This drop in temperature did not result in any obvious distortion of the zones, although they moved more slowly because of the higher

viscosity of the solvent. At the conclusion of centrifuging the tubes were sampled by a modified version of the Syzbalski (28) piercing device. The set screw was removed from the tube cap, and a seal formed by an O-ring between the cap and the top of the sampler. The needle was forced upward by a concentric screw through a molded elastomer pad which was superior to rubber bungs. Rate of outflow was controlled by manual operation of a screw-actuated syringe attached by polythene tubing to the top of the sampler, and by a clip on thin polythene tubing attached to the needle. Between twelve and twenty fractions (generally of equal size) were taken from each tube with the aid of an electronic drop counter, which was also used in experiments which demonstrated that drop size variations were negligible [cf. ref. (21)].

Fraction Analysis. The radioactivity of fractions was measured in scintillation counters, a pulse-height analyzer being required when I^{131} and I^{125} were present together. Details of this type of measurement were as given by Cohen and Freeman (9). When protein estimations were also required, fractions were diluted with 3.0 ml of appropriate buffer solution and the optical densities determined at 280 mμ wavelength in a Beckman DU spectrophotometer. If the protein concentration was too low for this, optical densities were measured at 215 and 225 mμ in a Unicam instrument. The difference between values at these wavelengths is proportional to protein concentration (31), provided absorption due to solvent is negligible. As tartrate interfered, it was dialyzed away, any alteration in sample volume being corrected for by means of a second radioactivity determination.

Chemicals. These were of "Analar" quality. The buffer used most frequently was 0.1 ionic strength sodium phosphate, pH 7.0, 7.5, or 8.0, but 0.4 M sodium chloride was added when seed globulins were used, and 0.025 M trisodium citrate when fibrinogen was involved. A few experiments were done in 0.1 ionic strength sodium acetate buffer pH 5.2, containing also 0.1 M sodium chloride. When the gradient was made of sodium potassium tartrate, no buffer salts were added, although there is no objection to including them if it is important to maintain a particular pH.

THEORETICAL CONSIDERATIONS

Several simplifying assumptions are made in the following sections. Zones are treated as if they were sedimenting parallel with the walls of the tube as they do (approximately) in the swinging bucket type of rotor, and the initial zone is taken as being infinitely sharp. Sedimentation rates are assumed to be constant (see *Discussion*), S_1 and S_2 referring to the faster and slower components, respectively. The peak of the faster

component is assumed to have travelled a fixed distance, h, measured radially.

(*a*) *Single Isotope*

Pedersen (23) worked out the relations which apply when virtually complete resolution is attainable in the analytical ultracentrifuge. Although he was concerned with the variation of concentration gradient across a boundary, whereas here the measurements give the concentration distribution across a zone, the two are mathematically equivalent. However, owing to the various disturbing factors, such good resolution is not obtained in the preparative tubes as is possible in the analytical cell. In both instances the most important factor is the relative difference in sedimentation velocity, but boundary spreading is also implicated. The distance between peaks, which is equal to $h(S_1 - S_2)/S_1$ at the end of centrifuging, is proportional to the relative difference of sedimentation velocity, $(S_1 - S_2)/S_1$, henceforth written as V. As a first approximation V was calculated for all the systems used to see how well it correlated with the observed success or failure to resolve a second peak.

(*b*) *Two Isotopes*

As the main interest here lies in peaks which are fairly close together, the diffusion coefficients of the two components will not differ greatly. If, as seems likely, other boundary spreading influences affect both components to a similar extent, the apparent diffusion coefficients will be even closer together. For these reasons it is assumed that the curves representing the distribution of the two components are of identical shape. Further, this shape is taken to be Gaussian and the linear unit of measurement the standard deviation of the curves. Since measurements are made on zones of finite width, results should strictly be plotted as histograms. However, we can equally correctly derive the ratio of the two components in any given zone by making use of the appropriate probability integrals, and refer this ratio R, to the center of the zone involved. The parameters which influence R are the peak separation, x, zone width, y, and the arbitrary level at which the first zone starts, z (Fig. 1). Elementary considerations show that, for given values of x and y, R is a smooth function of the position of a zone (i.e., of the location of its center). Therefore, if y is not too large, the variation of R from one edge of the peak to the other is nearly independent of z. In fact y can be as large as one unit without making this statement very much in error. It is, therefore, only the dependence of R on x and y that has been considered (Table 1).

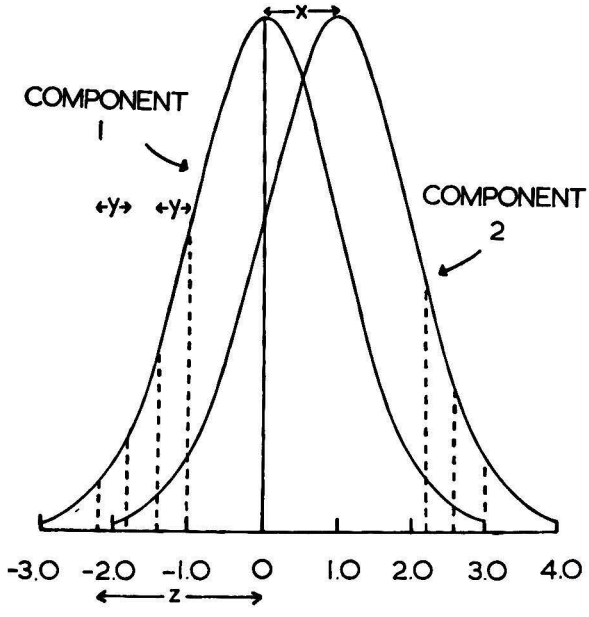


FIG. 1. Factors involved in calculating theoretical ratios of concentrations of two components with different sedimentation rates. Peak separation = x, fraction size = y, level at which first fraction begins = z.

TABLE 1

RELATIVE CONCENTRATIONS, R, OF COMPONENTS IN ZONES

x, peak separation[a]	y, zone width[a]	Zone center position[a]						
		−1.5	−1.0	−0.5	0.0	0.5	1.0	1.5
1.0	0	0.135	0.223	0.368	0.607	1.000	1.649	2.718
1.0	0.1	0.136	0.224	0.368	0.607	1.000	1.648	2.716
1.0	1.0	0.157	0.251	0.398	0.631	1.000	1.584	2.512
0.5	1.0	0.446	0.562	0.708	0.891	1.122	1.412	1.779
0.2	1.0	0.744	0.817	0.896	0.982	1.076	1.180	1.294
0.1	0	0.856	0.901	0.946	0.995	1.046	1.100	1.156
0.1	1.0	0.867	0.908	0.951	0.995	1.042	1.091	1.143

[a] Distances are expressed in terms of the standard deviation of the concentration-height curve (see Fig. 1).

RESULTS

A summary of the results of the main experiments is given in Table 2. When figures quoted in the literature for the sedimentation coefficients of the untreated proteins showed much scatter, or it was thought that the

TABLE 2
ASSESSMENT OF PROTEINS FOR SUITABILITY AS MARKERS

Protein	$S^\circ_{20,w}$ (Svedberg units)	Ref. quoting $S^\circ_{20,w}$	Sucrose gradient		Tartrate gradient
			pH 7–8	pH 5	
Lysozyme	1.9	(26)	[a]	[a]	[a]
Ovalbumin	3.4	(10)	[a]	[b]	[a]
Albumin (bovine)	4.4	(24)	[a]	[a]	[a]
Transferrin (human)	5.3[d]	(24)	[a]		
γ-Globulin (bovine)	6.8[d]	(24)	[a]	[a]	[a]
Fibrinogen (bovine)	7.8	(24)	[a]	[c]	
Catalase (beef liver)	11.3[e]	(7)	[a]	[a]	[a]
Arachin	13.5[e]	(5)	[a]	[c]	[b]
Edestin	14.0[e]	(5)	[a]	[c]	
Macroglobulin (normal, human)	19.6	(24)	[a]	[a]	[b]

[a] Satisfactory, i.e., specific activity variations negligible.
[b] Slight difference between iodinated and untreated protein.
[c] Unsatisfactory because of very low solubility or aggregation.
[d] Sedimentation coefficient, at a low concentration (ultraviolet absorption optics), of batch used.
[e] Sedimentation coefficient of batch used, estimated from measurements at several concentrations.

quality of preparations might not be strictly reproducible, determinations were done on the particular batch used for the density gradient work.

All the proteins studied could be iodinated satisfactorily, although in a few cases it was necessary to adhere more strictly to certain details. Edestin and arachin gave very low efficiencies if treated with less than one atom of iodine per molecule, but a more satisfactory incorporation if, say, five atoms were used. A low efficiency of iodination (often due to oxidation of sulfhydryl groups) results in a relatively high proportion of free iodide in the protein effluent from the anion-exchange column, and unless this iodide is dialyzed out it affects the distribution of activity in the density gradient fractions. Ovalbumin is known to be similar to the seed globulins in iodinating rather inefficiently (11, 32), and also to lose gradually some of the iodine it initially accepts (11). Comparison of ovalbumin which was used immediately after passage through a resin column, and some which had been dialyzed for several days, may be made by reference to Figs. 2 and 6a.

The shapes and positions of peaks confirmed that the gradients exerted a satisfactory stabilizing effect even when minute amounts of proteins were concerned. When trace amounts of a labeled protein were mixed with 2–8 mg of the same but untreated protein, a comparison of the distribution of activity and ultraviolet absorption among the fractions

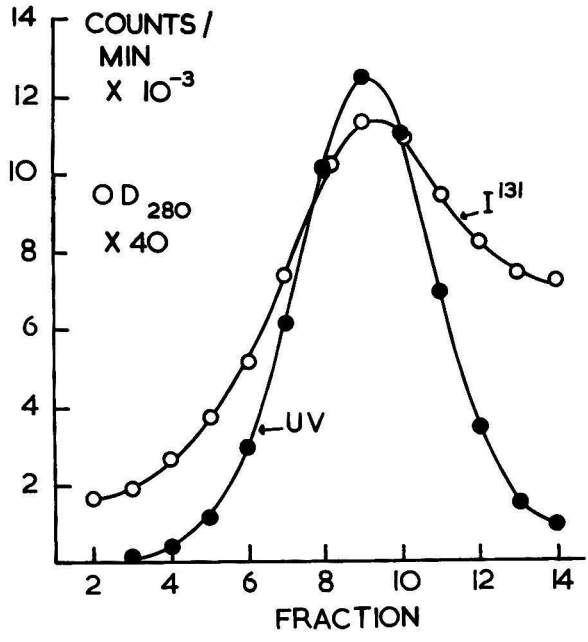


FIG. 2. Effect of free iodide on apparent distribution of iodinated ovalbumin as compared with the untreated protein. Centrifuged at 40,000 rpm for 16 hr in 100–200 gm/liter sucrose. Temperature ~18°. Phosphate buffer, pH 7.0, $I = 0.1$.

generally confirmed that iodination had no detectable effect on the sedimentation of the protein (e.g., Fig. 3 and Table 2). The absence of significant variations of specific activity in about four of the most concentrated fractions was the evidence sought (see *Discussion*). Even in the case of ovalbumin containing free iodide, the maximum activity and ultraviolet absorption occurred in the same tube (Fig. 2). It also transpired that the preliminary purification of the catalase could have been omitted, since the fastest peak of a portion iodinated as received coincided with the equivalent peak of the fractionated portion. Only mercaptalbumin and β-lactoglobulin failed to show close correspondence between radioactivity and ultraviolet absorption in phosphate buffered gradients of sucrose.

The mercaptalbumin showed two peaks according to light absorption, but activity was confined to the slower (Fig. 4a). When a trace of labeled mercaptalbumin was mixed with bovine plasma albumin (Fraction V), sedimentation rates were indistinguishable (Fig. 4b). As an analytical ultracentrifuge experiment confirmed the presence of an unusually high proportion of (presumed) dimer in the original mercaptal-

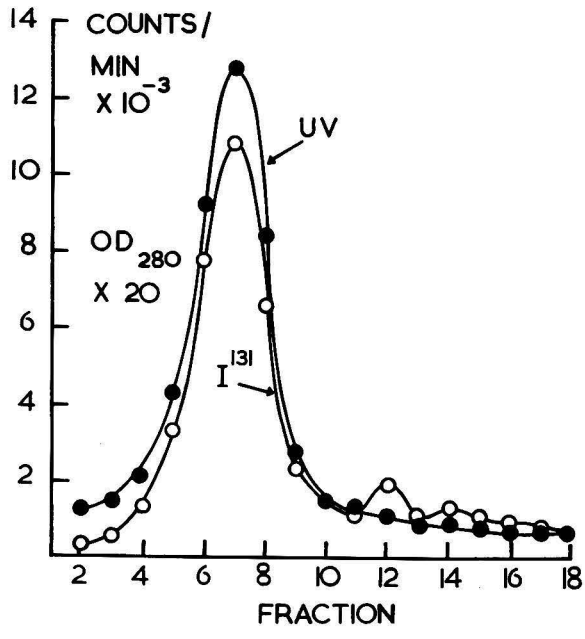


FIG. 3. Coincidence of sedimentation rates of fibrinogen and iodinated fibrinogen. Centrifuged at 40,000 rpm for 15 hr in 100–400 gm/liter sucrose. Temperature ~18°. Phosphate buffer ($I = 0.1$) + 0.025 M trisodium citrate, pH 7.0.

bumin, it was concluded that either dimer did not iodinate, or, much more probably, dimer was converted to monomer during the iodination procedure. Analytical runs subsequently showed that the faster component was nearly entirely lacking after iodination and that mere exposure to higher pH in absence of iodine had a similar effect. Thus the labeled product from mercaptalbumin is a satisfactory marker, corresponding to the normal monomer, in spite of the complication.

When β-lactoglobulin was treated with a small proportion of iodine, much of the product sedimented faster than usual, presumably due to formation of dimers or other aggregates (Fig. 5a). Higher iodine levels eliminated this effect, but the iodinated form now moved slightly more slowly than usual (Fig. 5b), a finding confirmed in the analytical ultracentrifuge.[1] Owing to this, and to its generally complex behavior, especially in marked pH and temperature dependence of sedimentation

[1] The lower sedimentation coefficient is conceivably a consequence of the incorporation of iodine in an imidazole residue, since Tanford *et al.* (29) have postulated that this type of residue is involved in the configurational change attendant on an increase of pH from 7 to 8, which is also accompanied by a decrease in sedimentation coefficient.

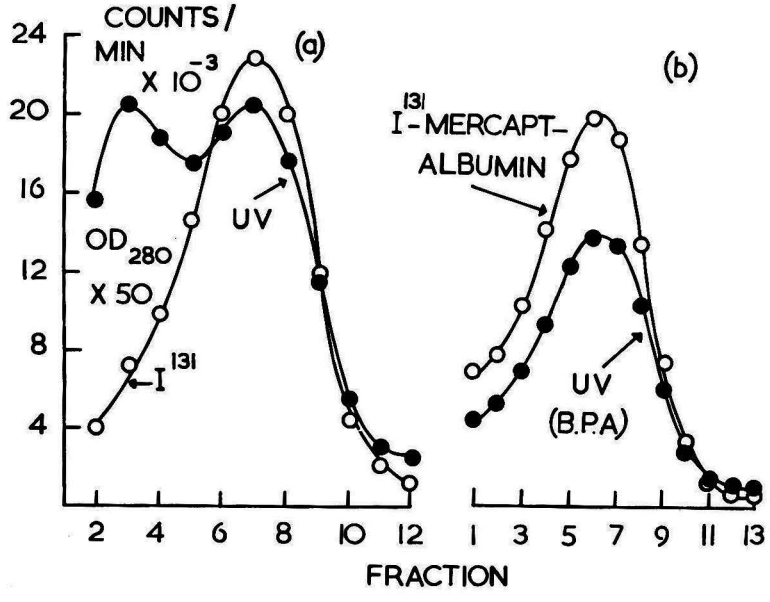


FIG. 4. Behavior of iodinated bovine mercaptalbumin compared with (a) the untreated protein (b) bovine plasma albumin (B.P.A.) (Fraction V). Both experiments at 40,000 rpm for 16 hr in 100–200 gm/liter sucrose. Temperature ~18°. Phosphate buffer, pH 7.0, $I = 0.1$.

characteristics (30), β-lactoglobulin is not to be recommended as a marker.

Every protein mentioned in Table 2 was studied in binary mixtures with the ones immediately below and above. Provided different isotopes were used in labeling, the peaks of activity were always displaced relatively by at least one tube in the sense expected, although the absolute rates of movement were low for lysozyme and ovalbumin (Fig. 6a) and V is only about 13% for γ-globulin and fibrinogen (Fig. 6b). A single exception to this general finding was that edestin and arachin, which have sedimentation coefficients differing by only a few per cent (Table 2), consistently sedimented at practically indistinguishable rates, although the relative concentrations altered across the peak in the manner anticipated from Tables 1 and 2. When proteins were labeled with the same isotope or, what was theoretically equivalent, curves pertaining to different isotopes were sometimes summed, separate peaks were less easily detected than when two isotopes were used. The criterion of resolution chosen here was the existence of two maxima with a definite minimum between them. Table 3 summarizes the results of experiments of this

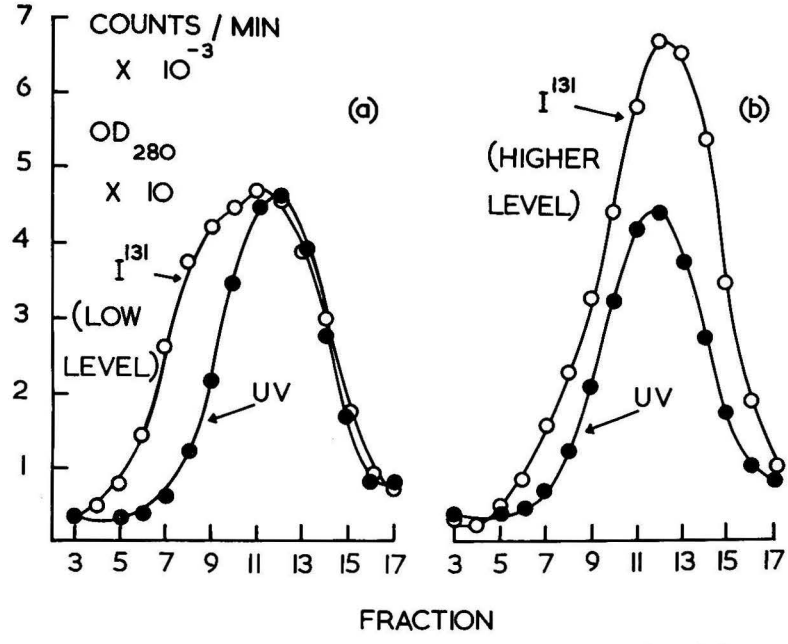


FIG. 5. Effect of different proportions of iodine on the properties of the product obtained with β-lactoglobulin. (a) and (b) represent, respectively, 0.1 and 1.0 atom of iodine added per molecule of protein (M.W. assumed 36,000). Iodinated in borate buffer, pH 8.3. Centrifuged at 40,000 rpm for 15 hr in 100–200 gm/liter sucrose. Temperature ~18°. Phosphate buffer, pH 7.0, $I = 0.1$.

TABLE 3

POSSIBILITY OF RESOLVING TWO PROTEINS LABELED IDENTICALLY

Components		$(S_1 - S_2)/S_1$	Resolution
Edestin	Arachin	0.035	No
Fibrinogen	γ-Globulin	0.128	No
Arachin	Catalase	0.163	No
Transferrin	Albumin	0.170	No
Edestin	Catalase	0.193	No
Albumin	Ovalbumin	0.223	No
Macroglobulin	Edestin	0.285	No
Catalase	Fibrinogen	0.310	No
Fibrinogen	Transferrin	0.321	Yes
γ-Globulin	Albumin	0.354	Yes
Transferrin	Ovalbumin	0.358	Yes
Catalase	γ-Globulin	0.398	Yes
Arachin	Fibrinogen	0.422	Yes
Ovalbumin	Lysozyme	0.441	No[a]
Edestin	Fibrinogen	0.443	Yes
Albumin	Lysozyme	0.569	Yes
Macroglobulin	γ-Globulin	0.653	Yes

[a] This result was obtained under conditions which did not fulfil the stipulation originally made (see *Discussion*).

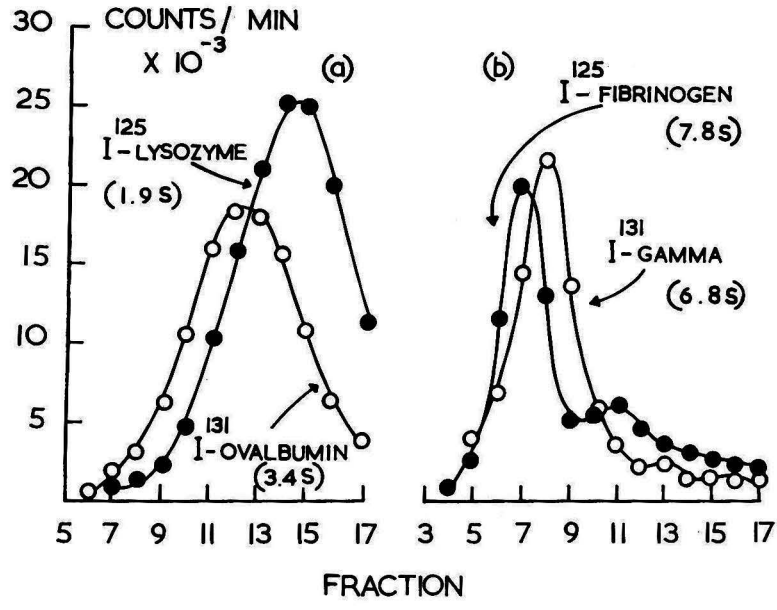


FIG. 6. (a) Differentiation of proteins with small sedimentation coefficients (1.9 and 3.4 S. Gradient 0.05–1.0 *M* sodium potassium tartrate. (b) Differentiation of proteins having rather similar sedimentation coefficients (6.8 and 7.8 S). Gradient 100–400 gm/liter sucrose. Phosphate buffer ($I = 0.25$) + 0.025 *M* trisodium citrate, pH 7.0. Both experiments for 15.5 hr at 40,000 rpm and ~18°.

type, involving mixtures containing approximately the same amounts of activity associated with each protein. It shows that at V values below 0.31 there was never any resolution but at V values above 0..31 resolution occurred in all cases but one, which will be referred to in the *Discussion*. In many cases experiments were made with ternary mixtures, the central component being labeled with one isotope, the flanking ones with the other isotope. The central peak was, of course, readily located, but whether or not the outer peaks separated depended on the corresponding V value.

In control of separations of both normal and abnormal macroglobulins from serum, trace-labeled ordinary (7 S) γ-globulin was used to decide which fractions could be pooled to give a maximum yield for any specified level of contamination, since separation is never perfect owing to diffusion, tailing, and other interfering phenomena. Figure 7 shows the quality of two preparations obtained in single-stage processes. In Fig. 7b the large amount of macroglobulins in the pathological serum made it possible to pool fractions containing 1.4% of the ordinary γ-globulin to give a final product containing a negligible proportion. In Fig. 7d the

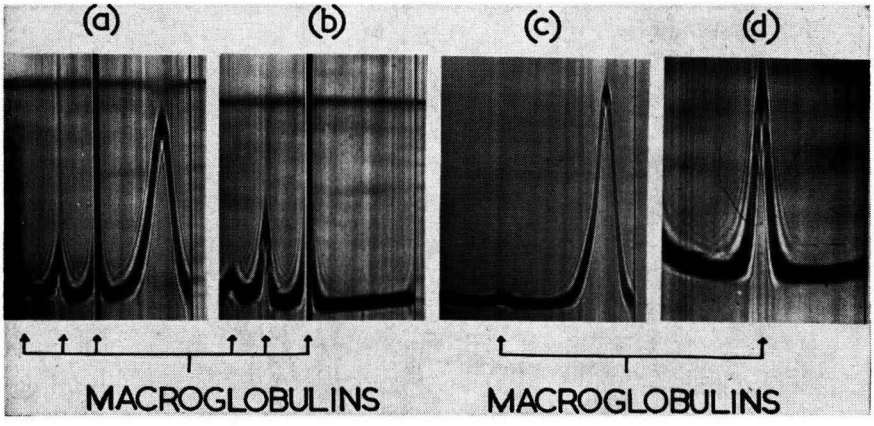


FIG. 7. Control of macroglobulin preparations. Ultracentrifuge patterns obtained with (a) diluted pathological human serum, (b) the isolated pathological macroglobulins, (c) diluted normal rabbit serum, (d) isolated rabbit macroglobulin. Sedimentation (right to left) at 52,640 rpm in phosphate buffer (pH 7.5, $I = 0.1$) at approximately 20°. Exposures taken at (a) 34, (b) 31, (c) 38, and (d) 33 min after attaining speed.

macroglobulin was separated from serum which contained 5–10 times as much 7 S γ-globulin, so that a more stringent condition had to be imposed ($<0.3\%$ of the activity was included).

Size classification of antibodies is very straightforward by the present technique. Figure 8 shows that anti-insulin antibodies can be obtained in both the macroglobulin and 7 S varieties, and Fig. 9a that certain antinuclear antibodies (2) belong to the 7 S class. The corresponding nuclear antigen (3) moved more slowly than ovalbumin (Fig. 9b), indicating a sedimentation coefficient of only about 2 S. A viral antigen associated with mouse encephalomyocarditis virus (13) was found to sediment at almost the same rate as arachin (Fig. 10), indicating a sedimentation coefficient of about 14 S and a minimum molecular weight of about 260,000.

DISCUSSION

The method used here, being essentially comparative, makes it unnecessary to attempt to obtain accurate temperature control of the preparative ultracentrifuge. It would also automatically reveal any unexpected disturbances which might occur. Although fixed angle rotors must detract from zone sharpness, owing to the changes in orientation and swirling which take place at various stages of the operations, other workers have also used them with success in density gradient work, and they do have the following advantages over the swinging-bucket type.

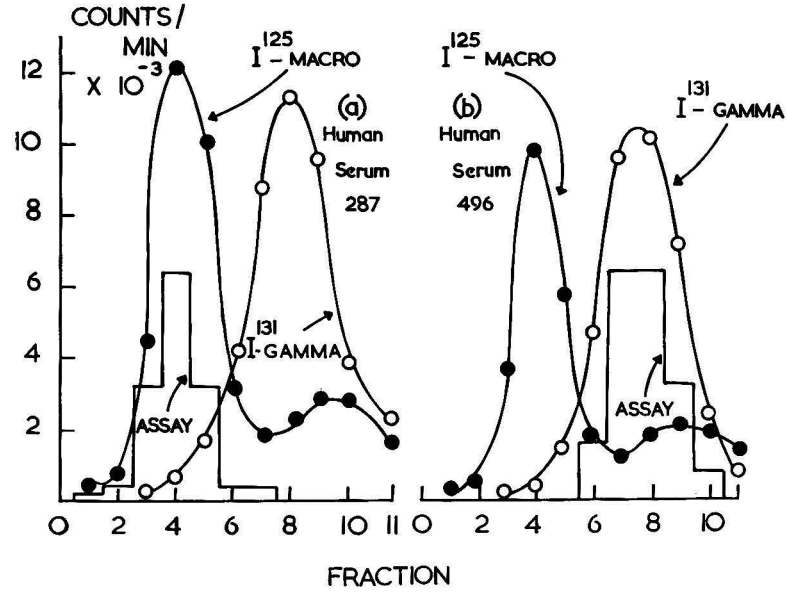


FIG. 8. Comparison of sedimentation rates of anti-insulin antibodies, 7 S γ-globulin, and macroglobulin. Centrifuged at 23,000 rpm (rotor 30) for 15 hr in 100–300 gm/liter sucrose containing phosphate buffer (pH 7.0, $I = 0.1$). Temperature initially ~18°, finally ~10°. Sera were diluted with an equal volume of the buffer before 3.0-ml samples were superimposed on the gradients. Two tubes were used for each serum, corresponding fractions being pooled before counting. Counted fractions were dialyzed free of sucrose and assayed by both passive cutaneous anaphylaxis and (the more sensitive) hemagglutination tests. Assays based on twofold serial dilutions by the latter method are recorded here as histograms.

For preparative work the capacity, especially of rotor 30, is very much higher and for screening experiments, for instance on sera, a dozen different samples can be dealt with simultaneously. This is not intended to imply that there is any reason why markers, as advocated here, should not be used in swinging-bucket tubes, which might indeed be essential if very large molecules (liable to pellet) were being studied in this way.

Although the proteins sediment through layers of steadily increasing viscosity, the higher gravitational field at greater radial distances is a compensating factor in helping to keep rates of movement approximately constant. Both de Duve *et al.* (12) and Martin and Ames (21) have recommended conditions which are nearly optimal in this respect. Computations showed that the lower sucrose concentrations used in this work justify the assumption of approximately constant sedimentation rates (see *Theoretical*). Although the viscosity factor tends to predominate

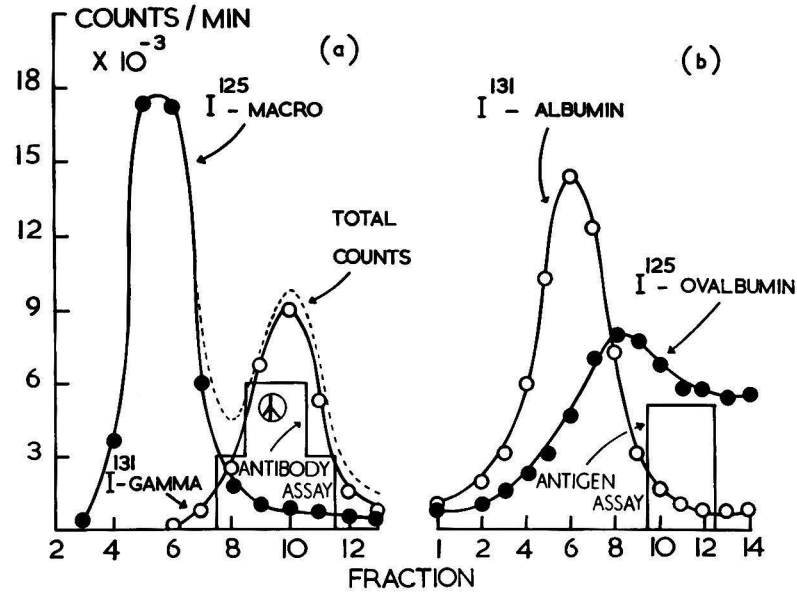


FIG. 9. Comparison of sedimentation rates of: (a) Antinuclear antibodies, 7 S γ-globulin, and macroglobulin; (b) Corresponding antigen, bovine plasma albumin, and ovalbumin; centrifuged (rotor 40) for 15.5 hr at ~18°. (a) 25,000 rpm in 100–300 gm/liter sucrose, phosphate buffer (pH 7.0, $I = 0.1$). (b) 40,000 rpm in 100–200 gm/liter sucrose, phosphate buffer (pH 7.0, $I = 0.1$). Fractions dialyzed free of sucrose were assayed semiquantitatively by the methods of Beck (2, 3).

at higher sucrose concentrations, it is not essential to use these. They were merely a technical convenience with certain combinations of samples. Generally, with larger molecules the sucrose gradient (100–200 g/liter) was preferred, the ultracentrifuge running for a shorter time or at reduced speed. It has been pointed out by Martin and Ames (21) that partial specific volume does not often affect the results appreciably, because of the relatively small range of densities of the sucrose solutions and the small variations among the partial specific volumes of most ordinary proteins. Should the material possess an unusually high or low partial specific volume, measurements in stronger sucrose solutions could reveal the fact.

The protein markers need be present in such low amounts that the sedimentation coefficient (where there is significant concentration dependence) can be taken as that extrapolated to zero concentration in the buffer used, but this refinement will not often be justified. The assumption has been made that the gradient-producing component has no effect on sedimentation coefficients (apart from the inevitable viscosity and

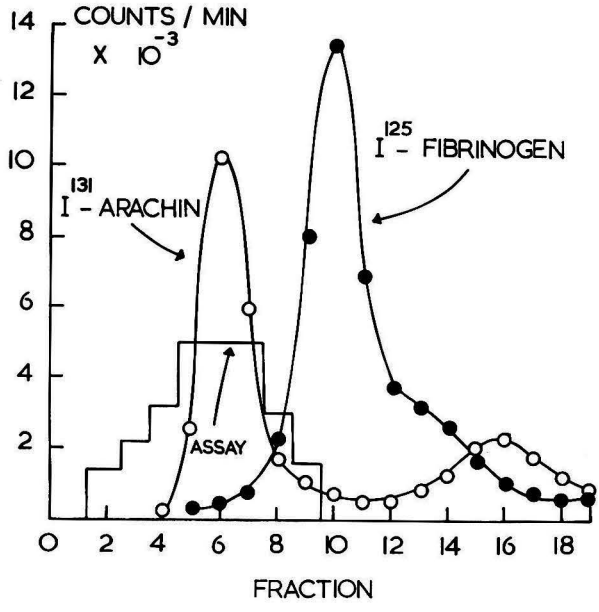


FIG. 10. Viral antigen compared with fibrinogen and arachin markers. Centrifuged at 40,000 rpm for 15 hr in 100–500 gm/liter sucrose at ~18°. Solvent 0.4 *M* sodium chloride, 0.025 *M* trisodium citrate, phosphate buffer (pH 7.0, $I = 0.1$). Sample was distributed among six tubes, corresponding fractions from which were pooled prior to counting. After removal of sucrose, fractions were assayed by an inhibition method, but figures have not been related to a standard curve obtained with dilutions of a single solution. The histogram plotted shows a broader distribution than would be given if the corrected figures were available.

buoyancy factors). Although this is probably justified for sucrose, it might not be true always in solutions of very high salt concentration.

If the substance being investigated has a very high activity, the estimations may be done at such a high dilution that it is unnecessary to attempt to remove the sucrose from the fractions. Otherwise, it can generally be dialyzed out without trouble. Should it be necessary to eliminate all sucrose, such as in an investigation of a glycoprotein, either tartrate or a salt gradient can be used, e.g., sodium chloride by Kunkel *et al.* (17). It is obviously essential to confirm that marker substances used do not interfere with the biological activity. If they do, it may be possible by means of higher specific radioactivities to employ lower, permissible concentrations. Otherwise, of course, alternative markers must be sought. The list of those examined is not intended to be exhaustive, merely illustrative. Where iodination involves complications, chemically modified proteins could no doubt be used if required. Suitable

synthetic polypeptides, if they could be produced in a range of well-controlled molecular sizes, might be very convenient.

The orthodox statistical approach to the problem of the resolution of two curves such as depicted in Fig. 1 is to express their separation in terms of their standard deviations. For various reasons this did not seem to be the best approach with the concentration curves obtained in density gradients. For instance, since points on the tails of the curves are less reliable, it is wise to confine attention to the rather small number of central fractions. Statistical curve fitting to a small number of points like this is not advisable. The ratio method seemed less open to criticism.

Table 1 shows that a reduction below one unit in the zone width, y, of each fraction has relatively little effect on the range of R, provided we ignore zones distant from the origin. This is fortunate, because, if y becomes very small, the numbers of fractions to be assayed or counted rises correspondingly, whereas the proportion of the original sample in any particular fraction decreases to a level at which it is likely to be more difficult to determine with the same degree of accuracy.

Although the variation of specific activity across a peak can be used as a sensitive indication of change in a protein on iodination (e.g., for metabolic studies), results must be interpreted with caution. Thus, there might be extraneous material in the solution affecting the ultraviolet absorption measurements or, as with ovalbumin, loss of activity over a period of time might result in misleading tailing. Deviations of specific activity in the center of a peak are, therefore, those of greatest significance.

The V values in Table 3 correlate well with observed separations. The exceptional case, lysozyme with ovalbumin, should strictly have been excluded because insufficient time was allowed for sedimentation, with the result that the ovalbumin had not moved the standard distance, h. Moreover, both these small proteins have high diffusion coefficients, which will diminish the resolution—a secondary factor ignored in calculating V. This is one reason why V is merely a guide to likely attainable resolution. Another is that it is proportional to a difference of sedimentation coefficients, and will therefore be subjected to relatively larger errors than an individual coefficient. There is also always the possibility that, since resolution is never complete or unequivocal, the apparent peak of concentration of one or both components may not coincide with the true position. In practice it has been found that the fractions in which the maximum concentrations occurred were the same, so that any displacement is fairly small.

Table 3 and Fig. 9a show that in locating antibodies by use of labeled "7 S" and "19 S" globulins, it is permissible to use the same isotope,

thus simplifying the procedure in this case. In a preliminary investigation of an unknown, if one wishes to use three markers, the value of V will indicate whether it is likely that the fastest and slowest ones can be used satisfactorily bearing the same label. Alternatively, if one has enzymic activity, this property can be taken advantage of (21) although enzymes are susceptible to traces of inhibitors and often require much stricter control of conditions than do isotope measurements. If the unknown is plentifully available it may be simpler to compare its rate of sedimentation with the markers individually in different tubes, although interpolation is not then possible.

When material of very high specific activity is prepared for tracer experiments, it is often mixed with a large amount of carrier protein immediately after iodination in order to minimize radiation damage. This makes it more difficult to check the product, but the present method is suggested as one such test. Evidence of a contaminant differing in molecular size from, and iodinating much more readily than, the major protein component would be revealed far more easily by a density gradient experiment than by analytical ultracentrifugation. If any substantial amount of dimer (or higher polymer) were formed during iodination its presence could be detected. For spherical molecules sedimentation coefficients of dimer and monomer are in the ratio $2^{2/3}$. If $S_1 = 2^{2/3}S_2$, the value of V is 0.371. Thus a dimer should certainly resolve even though, of course, labeled with the same isotope as monomer, provided it is present in a concentration comparable with that of the monomer. This statement is on the pessimistic side because a shoulder will often be discernible under conditions in which resolution is incomplete because of a smaller content of dimer, or if the faster peak has not been allowed time to move the minimum distance h, e.g., as with β-lactoglobulin (Fig. 5a).

Sometimes a biologically active material is believed to have been isolated in pure form, and its physical characteristics are carefully measured. It is not always easy to confirm that the material so examined is the entity sought, which may be only a minor constituent not normally resolved by the analytical technique used. The present method is useful to confirm that the active material sediments at the same rate as the isolated component.

The method described here is applicable to relatively impure materials having a characteristic activity which can be estimated at least semiquantitatively. In this respect it resembles the partition cell method (33) but the result is much less dependent on the accuracy of assay of the substance in question, although the more accurately the estimation of (relative) concentration can be made, the more nearly correct will be the

sedimentation coefficient. A linear interpolation between the positions of two known markers should be quite reliable, for reasons given in a previous paragraph.

Even with a crude extract of active material, the present method (yielding a sedimentation coefficient), in conjunction with a method such as that of Allison and Humphrey (1) to measure diffusion, can give an approximate molecular weight which is probably as accurate as can at present be obtained under such limitations.

SUMMARY

The general advantages of using internal reference substances to study rate of sedimentation in density gradients in the preparative ultracentrifuge have been pointed out, and the particular suitability of radioactively labeled ones referred to.

Some easily obtainable proteins, ranging in size from lysozyme to serum macroglobulin, have been iodinated and shown to be valid markers under various conditions. When two different isotopes were used, and in certain cases with only one, two markers could be used in the same tube.

Applications have been made of this principle in controlling the preparation of normal and pathological macroglobulins, and in studying the size of anti-insulin antibodies, antinuclear antibodies and their antigen, and a viral antigen.

ACKNOWLEDGMENTS

The author is grateful to Miss L. Martin for technical assistance in all aspects of this work. He also thanks Mr. C. Hay for designing and constructing the sampler, Drs. A. S. McFarlane and V. Bocci for sera, and Dr. T. Freeman for the transferrin. Ground-nut meal was kindly supplied by British Oil and Cake Mills, Ltd. The results of assays of anti-insulin antibodies, antinuclear activity, and viral antigen are incorporated by the courtesy of Mrs. V. Jones, Dr. J. S. Beck, and Mr. I. Kerr, respectively.

REFERENCES

1. Allison, A. C., and Humphrey, J. H., *Immunology* **3**, 95 (1960).
2. Beck, J. S., *Lancet* **i**, 1203 (1961).
3. Beck, J. S., *Lancet* **i**, 241 (1962).
4. Brakke, M. K., *J. Am. Chem. Soc.* **73**, 1847 (1951).
5. Brand, B. P., Goring, D. A. I., and Johnson, P., *Trans. Faraday Soc.* **51**, 872 (1955).
6. Britten, R. J., and Roberts, R. B., *Science* **131**, 32 (1960).
7. Cecil, R., and Ogston, A. G., *Biochem. J.* **43**, 205 (1948).
8. Charlwood, P. A., *Can. J. Chem.* **33**, 1043 (1955).
9. Cohen, S., and Freeman, T., *Biochem. J.* **76**, 475 (1960).
10. Creeth, J. M., and Winzor, D. J., *Biochem. J.* **83**, 566 (1962).
11. Cunningham, L. W., and Nuenke, B. J., *J. Biol. Chem.* **235**, 1711 (1960).

12. DE DUVE, C., BERTHET, J., AND BEAUFAY, H., *Progr. Biophys. Biophys. Chem.* **9,** 325 (1959).
13. FAULKNER, P., MARTIN, E. M., SVED, S., VALENTINE, R. C., AND WORK, T. S., *Biochem. J.* **80,** 597 (1961).
14. HELMKAMP, R. W., GOODLAND, R. L., BALE, W. F., SPAR, I. L., AND MUTSCHLER, L. E., *Cancer Research* **20,** 1495 (1960).
15. JOHNSON, P., *Trans. Faraday Soc.* **42,** 28 (1946).
16. KUNKEL, H. G., *in* "The Plasma Proteins" (F. W. Putnam, ed.), Vol. 1, p. 279. Academic Press, New York-London, 1960.
17. KUNKEL, H. G., FRANKLIN, E. C., AND MULLER-EBERHARD, H. J., *J. Clin. Invest.* **38,** 424 (1959).
18. MCCREA, J. F., EPSTEIN, R. S., AND BARRY, W. H., *Nature* **189,** 220 (1961).
19. MCFARLANE, A. S., *Ann. N. Y. Acad. Sci.* **70,** 19 (1957).
20. MCFARLANE, A. S., *Nature* **182,** 53 (1958).
21. MARTIN, R. G., AND AMES, B. N., *J. Biol. Chem.* **236,** 1372 (1961).
22. MESELSON, M., STAHL, F. W., AND VINOGRAD, J., *Proc. Natl. Acad. Sci. U. S.* **43,** 581 (1957).
23. PEDERSEN, K. O., *in* "The Ultracentrifuge" (T. Svedberg and K. O. Pedersen, eds.), p. 16. Clarendon Press, Oxford, 1940.
24. PHELPS, R. A., AND PUTNAM, F. W., *in* "The Plasma Proteins" (F. W. Putnam, ed.), Vol. 1, p. 143. Academic Press, New York-London, 1960.
25. PICKELS, E. G., *J. Gen. Physiol.* **26,** 341 (1943).
26. SOPHIANOPOULOS, A. J., RHODES, C. K., HOLCOMB, D. N., AND VAN HOLDE, K. E., *J. Biol. Chem.* **237,** 1107 (1962).
27. STANWORTH, D. R., JAMES, K., AND SQUIRE, J. R., *Anal. Biochem.* **2,** 324 (1961).
28. SYZBALSKI, W., *Experientia* **16,** 164 (1960).
29. TANFORD, C., BUNVILLE, L. G., AND NOZAKI, Y., *J. Am. Chem. Soc.* **81,** 4032 (1959).
30. TILLEY, J. M. A., *Dairy Sci. Abstr.* **22,** 111 (1960).
31. WADDELL, W. J., *J. Lab. Clin. Med.* **48,** 311 (1956).
32. WINZOR, D. J., AND CREETH, J. M., *Biochem. J.* **83,** 559 (1962).
33. YPHANTIS, D. A., AND WAUGH, D. F., *J. Phys. Chem.* **60,** 630 (1956).

ANALYTICAL BIOCHEMISTRY 5, 246–254 (1963)

Colorimetric Determination with Murexide of Microgram Amounts of Calcium in Tissues[1]

EDWARD S. REYNOLDS[2] AND RICHARD E. LINDE[3]

From the Armed Forces Institute of Pathology, Washington, D. C.

Received July 30, 1962

Murexide (acid ammonium purpurate), proposed initially as an indicator in complexiometric titrations for quantitative analysis of calcium (1), can also be employed as a direct colorimetric reagent for this alkaline earth (2, 3). As such, it is extremely sensitive, 0.004 μg calcium/cm^2 corresponding to log I_0/I of 0.001 at 495 mμ, pH 12.0. Adaptation of this method to measurement of calcium content of tissues allows use of minute sample sizes, including single small organisms and organs.

Quantitative determination of calcium in tissue ashes is complicated by the presence of relatively large amounts of magnesium and inorganic phosphate (4). Calcium isolation by precipitation is impractical for the separation of such low concentrations from interfering ions. Flame spectrophotometry, at least as sensitive for calcium as murexide, requires removal of inorganic phosphate from ashed samples (5, 6) or acid extraction of calcium from unashed tissues (7, 8) for full recovery of calcium and, in addition, correction for heterochromatic background radiation due to the presence of sodium and potassium in the sample (5, 7).

The colorimetric method for calcium using murexide described in this paper is tolerant to the presence of magnesium (9). Phosphate, which interferes with this determination of calcium, is removed by precipitation as stannic phosphate (6). Interfering concentrations of other cations present in tissues that form colored complexes with murexide are avoided by small sample size. The limit of detection of this method, which requires ashing of the sample, is 0.05 μg calcium in 25 mg liver or muscle.

METHODS

"Metal-free" water, used throughout the entire procedure, was prepared by passage of distilled water through a Dowex-50 column (Dow

[1] Research supported in part by Grant E-201, American Cancer Society.

[2] Captain, MC, USAR. Present address: Department of Anatomy, Harvard Medical School, Boston, Massachusetts.

[3] Present address: Medical College of Virginia, Richmond, Virginia.

Chemical Co.). Glassware and polyethylene were cleaned as previously described (6). Prior to initial use, all glassware was soaked for one month in warm concentrated nitric acid (70°C). This procedure effectively reduced reagent blanks to small, reproducible values. Polyethylene containers were used for storage whenever possible.

Perfused liver homogenates (6) were obtained from 200-gm male Sprague-Dawley derived rats (Walter Reed Army Institute of Research). Ten per cent liver homogenate was prepared by diluting 10.0 gm to 100 ml with water. Skeletal muscle was obtained by excision of the medial head of the vastus lateralis muscle from hindlimbs of anesthetized white mice.

Reagents

All chemicals used were of ACS reagent grade unless otherwise specified.

Standard Calcium Solutions. Stock solutions of approximately 10,000 μg calcium/ml were prepared by dissolving a known weight of $CaCO_3$ (previously dried 24 hr at 104°C) in an equivalent amount of concentrated hydrochloric acid. Working standard solutions of 10 μg calcium/ml were prepared by dilution with water.

Murexide (Acid Ammonium Purpurate). 5×10^{-4} *M* stock solutions in 70% ethanol were prepared by dissolving 40 mg acid ammonium purpurate in 75 ml water and diluting with 175 ml absolute ethanol. Solutions were stable for approximately two months at 0–4°C.

Stannic Chloride. A solution of 2% stannic chloride in ethanol was prepared by dissolving 0.4 gm $SnCl_4 \cdot 5H_2O$ in 15 ml absolute ethanol. Fresh solutions were made immediately prior to use.

Piperidine Buffer, pH 12.0. 0.5 *M* piperidine buffer, pH 12.0, was prepared by diluting 12.5 ml piperidine (purified, Fisher Scientific Co.) and 0.3 ml concentrated hydrochloric acid to 250 ml with water. Twenty-fold dilution of this stock buffer with 0.01 *N* NaOH resulted in a pH of 12.0 ± 0.1 at 23°C.

Solutions of sodium hydroxide were prepared by dissolving sodium hydroxide (electrolytic pellets) in water. Dilute concentrations of hydrochloric acid were prepared by diluting concentrated hydrochloric acid in water. Potassium cyanide was dissolved in 1 *N* NaOH to a final concentration of 2.5% (w/v).

Analyzed samples of argillaceous limestone (Sample 1A; 29.5% Ca, 1.31% Mg) and burned magnesite (Sample 104; 2.4% Ca, 51.4% Mg) were obtained from the National Bureau of Standards, Washington, D. C. Working solutions of each, containing approximately 3 μg calcium/ml, were prepared by dissolving known weights of samples (previously

dried 24 hr at 104°C) in equivalent amounts of concentrated hydrochloric acid and dilution with water.

Procedure

Tissue samples containing not more than 6 μg calcium were placed in 5-ml micro-Fernbach flasks (Kimble Glass Co., Catalog No. 26502), weighed, and heated gently at 90°C for 30 min following the addition of 0.5 ml 2:1 concentrated nitric acid:high-purity perchloric acid (G. F. Smith Chemical Co.). The temperature was gradually increased to 200°C over a 2-hr period and the samples allowed to go to dryness upon continued heating (usually overnight). The ash was dissolved in 1.0 ml 0.02 N hydrochloric acid, transferred with two subsequent 0.5-ml water washes to 3-ml centrifuge tubes scored at 2.0 ml, and the volume adjusted to 2.0 ml with water; 0.5 ml 2% stannic chloride in ethanol was added, the tube was stoppered with a polyethylene cap, and the contents were mixed by inversion. Stannic phosphate is precipitated (6). Excess stannic chloride was precipitated by heating the stoppered tube in a 70°C water bath for 30 min. Following cooling, precipitates were removed by centrifugation.

Portions (2.0 ml) of the supernatants were transferred to 5.0-ml volumetric flasks into which were pipetted 2.0 ml 25% ethanol in water (v/v) and sufficient 1 N NaOH containing 2.5% KCN to attain a pH of 12.0 ± 0.2; 0.25 ml 0.5 M piperidine buffer, pH 12.0, was then added to each flask. Immediately prior to reading, 0.5 ml 5×10^{-4} M murexide was added rapidly while the contents of the flask were mixed in a stream of bubbling nitrogen. The contents were then adjusted to 5.0 ml with water and mixed by inversion, and their optical density was determined at 495 and 510 mμ. All optical density measurements were carried out within 5 min after addition of the dye.

For each set of analyses a reagent blank was carried through the entire procedure. Calcium standards were carried through from the beginning of the stannic chloride precipitation step. Calcium content of each sample was directly determined from the standard calibration curve and corrected for the calcium content of the reagent blank.

All pH measurements were performed with a Beckman Model G pH Meter equipped with type E-2 glass electrodes (Beckman No. 40308).

Colorimetry. Colorimetric determinations were performed in a constant-temperature room in a Beckman DU spectrophotometer, using acid-cleaned fused silica cuvettes of 1-cm path length; 5×10^{-5} M murexide in 25% ethanol:water at pH 12.0 exhibits an absorption spectrum with a maximum at 545 mμ. The formation of calcium

murexide results in an absorption peak at 515 mμ at which the absorption of murexide alone is approximately three-quarters that of the peak value. To obtain the maximum difference in absorption between calcium murexide and murexide, measurements were performed at 495 mμ (Fig. 1).

Standard curves, determined each time with known amounts of calcium standard, are linear over the range 0 to 8 μg calcium/sample, 8 μg resulting in an increased optical density of approximately 0.325 at 495 mμ. Analysis of calcium in increasing amounts of liver pulp and standard calcium solutions give identical linear calibration curves, demonstrating that both systems satisfy the Beer-Lambert Laws. Duplicate determinations, carried out in all instances, agreed within 2% as a rule.

RESULTS

Ethanol markedly reduces the fading rates of murexide and calcium murexide at pH 12.0. At this pH, aqueous solutions of dye become colorless within an hour. Although their fading rates are reduced to 0.057 and 0.093 optical density units per hour, respectively, in 6.5% ethanol (the final concentration of ethanol added with stock dye solution), the addition of stock dye to solutions not containing ethanol results in rapid, erratic fading prior to complete mixing. The presence of 25% ethanol in the calcium-containing solutions prevents erratic fading during mixing and further decreases fading rates to 0.023 and 0.031 optical density units per hour for murexide and calcium murexide. Increasing ethanol concentration beyond 25% results in precipitation of calcium murexide.

The difference spectrum of murexide with calcium obtained from liver is shown in Fig. 1 with that of a corresponding amount of murexide with calcium obtained from a standard calcium solution. These spectra are identical in the range from 440 to 660 mμ. Magnesium, the major interfering cation present in liver, does not complex with murexide under the conditions of the assay. Interference due to magnesium murexide appears upon decreasing pH, with the formation of an absorption peak at 480 mμ and decreased absorption for calcium murexide at 515 mμ. Determination of calcium at a pH at which magnesium does not interfere permits use of wavelengths where differences in absorption between calcium murexide and murexide are maximal (495 mμ, Fig. 1). Determination of calcium content at wavelengths other than 495 mμ are within 2% of that value obtained at pH 12.0. With decreasing pH, increasing discrepancies between the values obtained at the different wavelengths appear.

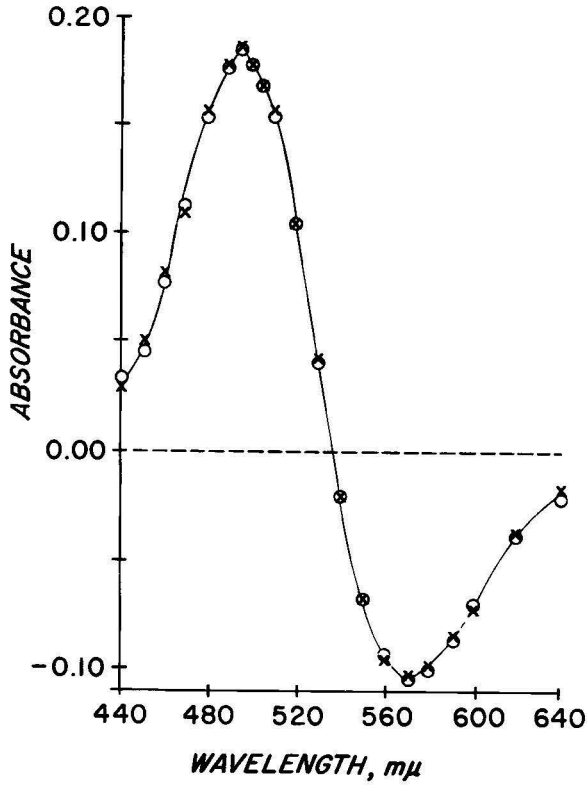


FIG. 1. Difference spectra of calcium murexide in 25% ethanol: water at pH 12.0, obtained with a Beckman DU spectrophotometer, using 1-cm path length cells. Reference cells contain the same concentration of murexide as the solutions containing the calcium. (×) Obtained from 120 mg liver homogenate. (○) Obtained from 4.36 μg calcium from calcium standard.

Interferences

Table 1 lists cations and anions with their valence states that were tested for possible interference with analysis of tissue calcium. Solutions of cations as chlorides and anions as sodium salts were added to 1.0 ml 10% liver homogenate (100 mg liver homogenate/ml) containing 2.72 ± 0.14 μg calcium. The maximum amount that could be tolerated is given in micrograms. Interference was defined as a deviation of more than two standard deviations (±0.28 μg calcium) from the mean calcium values of the control samples.

Analysis of calcium in liver and muscle requires removal of inorganic phosphate from tissue ash. Full recovery of calcium from argillaceous limestone to which increasing amounts of inorganic phosphate have been added is directly proportional to the amount of stannic chloride added

TABLE 1
SPECIFICITY OF METHOD FOR CALCIUM IN PRESENCE OF OTHER CATIONS AND ANIONS

Maximum amount of each cation or anion that may be added to 100 mg of liver pulp containing 2.7 μg calcium is shown. At these levels interference is not observed.

Cation	Micrograms	Anion	Micrograms
Sr^{++}	1	PO_4^{---}	300
Cd^{++}	6	SO_4^{--}	600
Zn^{++}	10		
Pb^{++}	10		
Ba^{++}	20		
Mg^{++}	30		
Mn^{++}	30		
Hg^{++}	30		
Cu^{++}	60		
Ni^{++}	100		
Fe^{++}, Fe^{+++}	300		
Ag^{+}	300		
Na^{+}	500		
K^{+}	500		
NH^{+}	500		
Co^{4++}	1000		

(8 mg $SnCl_4$/mg PO_4, Fig. 2). Slightly lower recoveries occur with increasing stannic chloride additions. Although 5 mg of stannic chloride permits full recovery of calcium from 100 mg liver, twice this amount was employed routinely.

Precision

Eleven replicate determinations of calcium in 150-mg liver samples ranged from 3.80 to 4.06 μg calcium with a mean of 4.01 μg and a standard deviation of ±0.08 μg. Calcium content of 12 replicates of burned magnesite containing 3.86 μg calcium ranged from 3.65 to 3.96 μg with a mean of 3.79 μg calcium and a standard deviation of ±0.11 μg, a recovery of 98.3% calcium in the presence of a twentyfold excess of magnesium (84 μg).

Over a period of several weeks the calcium content of a homogenate of normal liver was determined a total of 24 times on 6 different occasions. Values ranged from 2.35 to 3.02 μg calcium, with a mean of 2.72 μg and a standard deviation of ±0.14 μg.

Accuracy and Recoveries

Recovery of calcium added to liver homogenates was determined in 9 consecutive analyses. In each instance 4.0 μg calcium from a calcium standard was added to 100 mg liver homogenate containing 2.72 μg

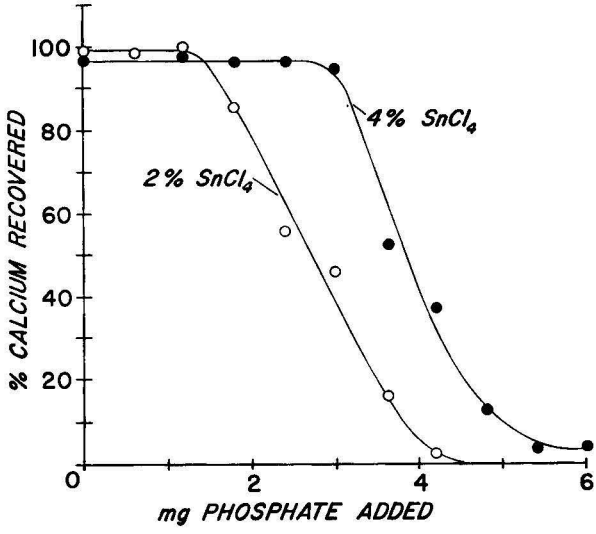


FIG. 2. Recovery of calcium following removal of phosphate with stannic chloride. A solution containing a known amount of calcium was prepared by dissolving a weighed sample of argillaceous limestone (National Bureau of Standards Analyzed Sample 1A) in 12 *N* HCl. Following dilution, 1.00 ml contained 4.52 μg calcium. Prior to ashing, phosphate was added to the calcium-containing solution in the quantities indicated from a solution of NaH_2PO_4 containing 5.0 mg PO_4/ml. A Beckman DU spectrophotometer with 1-cm path length cells, at 495 mμ, was used for calcium determination. (○) 0.5 ml 2% stannic chloride added to precipitate phosphate. (●) 0.5 ml 4% stannic chloride added to precipitate phosphate.

calcium. Recoveries ranged from 94 to 104%, the mean and standard deviation being 97.2 ± 3.1%.

Calcium contents of analyzed samples of argillaceous limestone (Fig. 2) and burned magnesite obtained from the National Bureau of Standards determined by this method were 29.1 and 2.36%, respectively. These values are within 0.4% of the given values for these samples, 29.5 and 2.40%.

Calcium Content of Tissues

Calcium contents of livers of normal rats and skeletal muscle of normal mice were determined. The calcium contents of livers from 10 normal rats ranged from 15.5 to 25.2 μg calcium/gm liver, with a mean of 21.8 μg and a standard deviation of ±3.3 μg. The calcium content of skeletal muscle from 9 normal mice ranged from 80.3 to 112.3 μg calcium, with a mean of 99.4 μg and a standard deviation of ±10.4 μg. Sample size for the determination of liver calcium content ranged between 50 and 150 mg and, for muscle, 20 and 40 mg.

DISCUSSION

Colorimetric determination of calcium with murexide at high pH avoids interference due to magnesium (9) and can be performed at wavelengths at which differences between molar extinction coefficients of murexide and calcium murexide are maximal. At lower pH, calcium analysis in the presence of magnesium requires the use of isosbestic wavelengths for murexide and magnesium murexide (2, 3). In practice, determination of calcium in the presence of magnesium below pH 12.0 is unreliable, as bathochromic shifts in absorption of murexide, calcium murexide, and magnesium murexide occur (1). This shift, caused by the tertiary ionization of murexide and the metal murexide complexes (pK_3's $\cong 11$) (1), renders their molar extinction coefficients at these wavelengths variable with slight pH differences in this pH range.

Magnesium interference at pH 12.0 is due to its precipitation as a colloidal suspension of magnesium hydroxide (K_{sp} 1.2×10^{-11}) (10), which can be removed by centrifugation, if necessary. In tissue analysis, however, the slight turbidity observed does not interfere with the determination of calcium.

Interfering concentrations of added elements of biologic importance (Table 1) are from 2 to 200 times in excess of those normally present in the amounts of liver and muscle analyzed (11, 12). Because of this, this method is regarded as highly specific for calcium under the conditions delineated. Potassium and magnesium show the lowest ratios between interference and normal metal content. Potassium interference, as well as that due to ammonium, sodium, and sulfate, is of the magnitude of contamination of these salts with calcium.

In addition to calcium and magnesium, strontium, cadmium, zinc, barium, manganese, copper, nickel, cobalt, tin, and iron form colored complexes with murexide (1). Strontium, which interferes in least amount (Table 1), is not usually found in soft tissues in such high concentrations (12). Of these metals, zinc and iron form insoluble hydroxides at this pH (10), and, in addition, iron appears to be deposited as an insoluble oxide during the ashing procedure. Cyanide, which renders calcium recovery from tissues more complete and reproducible, complexes any copper, cadmium, and cobalt that may be present (13) and may also complex any tin remaining in solution. Manganese, in amounts many times greater than those found in liver (11), interferes directly by its conversion to permanganate during ashing (Table 1). Interfering amounts of cobalt are so large that cobalt may be lost during ashing.

The calcium contents of livers of normal rats determined by this method compare favorably and are less variable (mean and standard deviation 21.8 ± 3.3 μg calcium/gm liver) than those values obtained

by emission spectroscopy (11), flame spectrophotometry (6), or complexiometric titrations employing murexide as indicator (14) (17.4 ± 11.4, 18.0, and 30.4 ± 9.6 μg calcium/gm liver, respectively). Although it is possible that the calcium contents of the livers of animals used were less variable, the fact that livers belonging to this group were accumulated from normal rats sacrificed over a 21-month period indicates that rat liver calcium contents may be more constant than previously found (6, 11, 14).

Mean calcium content of skeletal muscle, 99.4 μg calcium/gm muscle, is one-third lower than that obtained by gravimetric techniques (14, 15) and by complexiometric titrations (15). The histochemical demonstration of calcium in the region of the sarcoplasmic reticulum, a cytoplasmic component of the muscle cell, may account, in part, for the fourfold greater calcium content of skeletal muscle (16) than liver.

SUMMARY

A simple, direct colorimetric method employing murexide has been developed for determination of calcium in tissues. This method, which requires ashing of the samples and precipitation of phosphate, is insensitive to magnesium and is capable of detecting 0.05 μg calcium. Other metals, in concentrations normally found in tissue samples, do not interfere. The means and their standard deviations for calcium content of fresh rat liver and mouse skeletal with this method are 21.8 ± 3.3 and 99.4 ± 10.4 μg calcium per gram of tissue, respectively.

REFERENCES

1. SCHWARZENBACH, G., AND GYSLING, H., *Helv. Chim. Acta* **32**, 1314 (1949).
2. TAMMELIN, L. E., AND MORGENSEN, S., *Acta Chem. Scand.* **6**, 988 (1952).
3. WILLIAMS, M. B., AND MOSER, J. H., *Anal. Chem.* **25**, 1414 (1953).
4. SANDELL, E. R., "Colorimetric Metal Analysis," 3rd Ed., p. 366. Interscience, New York, 1959.
5. MARGOSHES, M., AND VALLEE, B. L., *Anal. Chem.* **28**, 180 (1956).
6. THIERS, R., REYNOLDS, E. S., AND VALLEE, B. L., *J. Biol. Chem.* **235**, 2130 (1960).
7. GEYER, R. P., AND BOWIE, E. J., *Anal. Biochem.* **2**, 360 (1961).
8. MORI, K., *Arch. Biochem. Biophys.* **83**, 552 (1956).
9. CHILCOTE, M. E., AND WASSON, R. D., *Clin. Chem.* **4**, 200 (1958).
10. "Handbook of Chemistry and Physics," 43rd Ed. Chemical Rubber Publishing Co., Cleveland, 1961.
11. THIERS, R., AND VALLEE, B. L., *J. Biol. Chem.* **226**, 911 (1957).
12. SPECTOR, W. S., "Handbook of Biological Data." Saunders, Philadelphia, 1956.
13. GORSUCH, T. T., AND POSNER, A. M., *Nature* **176**, 268 (1955).
14. KRATZING, C. C., DUNSTONE, J. R., MADSEN, N. P., MACDONALD, P., AND BELL, H., *Biochem. Pharmacol.* **3**, 272 (1960).
15. HINES, H. M., AND KNOWLTON, G. C., *Am. J. Physiol.* **104**, 379 (1933).
16. REYNOLDS, E. S., AND BAUER, W., unpublished observations.

ANALYTICAL BIOCHEMISTRY 5, 255–269 (1963)

SHORT COMMUNICATIONS

A Color Reaction between Reduced Pyridine Nucleotides and o-Aminobenzaldehyde of Potential Analytical Use

The reaction of *o*-aminobenzaldehyde with certain pyrroline compounds to give characteristic orange-colored dihydroquinazolinium derivatives has been extensively studied by Schopf and collaborators (1). *o*-Aminobenzaldehyde has been used to trap and identify Δ^1-pyrroline 5-carboxylate (2, 3) and Δ^1-piperideine 6-carboxylate (4, 5) as intermediates in the biosynthesis of proline and lysine, respectively. We have recently described a cell-free yeast enzyme system that converts α-aminoadipic acid to Δ^1-piperideine 6-carboxylate in the presence of ATP, TPNH (DPNH), glutathione, and a divalent cation (Mn^{++}) (5). One criterion used as evidence for this reaction was the formation of color when *o*-aminobenzaldehyde was present. During one study of this reaction, a solution of the above cofactors plus *o*-aminobenzaldehyde in the absence of yeast enzyme was acidified and allowed to stand. Unexpectedly, this mixture also turned orange. Further investigation disclosed that the color resulted from a reaction of TPNH or DPNH with *o*-aminobenzaldehyde in acid solution.

Figure 1 compares the visible spectra of the reaction product of DPNH plus *o*-aminobenzaldehyde with that of the dihydroquinazolinium derivative of Δ^1-piperideine-2-carboxylic acid (the cyclized form of α-keto-ϵ-aminocaproic acid). Color was obtained only with the reduced form of the pyridine nucleotides and was proportional to nucleotide concentration (Fig. 2A). This reaction was therefore applied to the assay of a pyridinenucleotide-dependent dehydrogenase enzyme system. A conventional spectrophotometric assay of glucose 6-phosphate dehydrogenase is compared with a simultaneous *o*-aminobenzaldehyde assay in Fig. 2B.

If adequate controls are exercised to rule out other substances that may react with *o*-aminobenzaldehyde, the ability of DPNH and TPNH to form colored compounds with *o*-aminobenzaldehyde may have certain

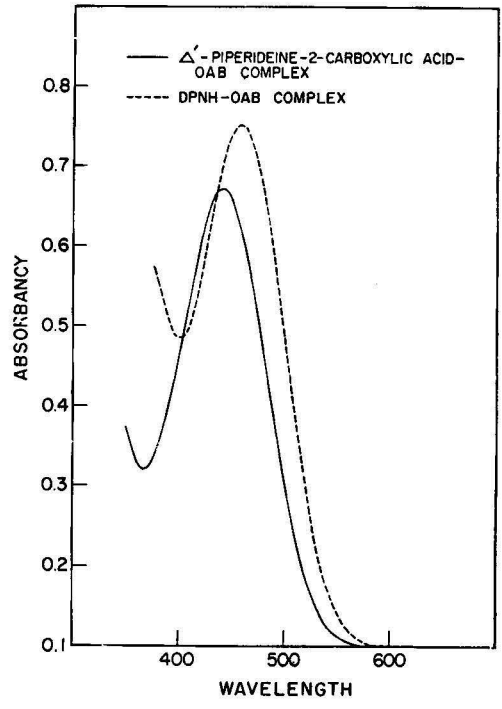


FIG. 1. Absorption spectra of *o*-aminobenzaldehyde (OAB) adducts of Δ^1-piperideine-2-carboxylic acid and DPNH. Spectral determinations were made with the Carey recording spectrophotometer (0.05 *N* HCl blank) of the following: a 0.05 *N* HCl solution, 0.5 mg/ml, of purified dihydroquinazolinium derivative of Δ^1-piperideine 2-carboxylate and OAB prepared as described elsewhere (5), and a solution (diluted 1:2) in which 2.5 μM DPNH had been standing with 3.3 μM OAB in 2 ml 0.05 *N* HCl for 1 hr. *o*-Aminobenzaldehyde was purchased from K and K Laboratories, Inc.

analytical advantage over present methods. The *o*-aminobenzaldehyde assay is only about a third as sensitive as the direct spectrophotometric assay of reduced pyridine nucleotides and requires a 30–60 min interval for maximum development of color. On the other hand only a colorimeter is required and an alternative wavelength for the measurement of TPNH and DPNH is provided. These observations may be of particular use in those instances (e.g., teaching) in which it may be desired literally to visualize reactions in which these co-enzymes participate.

ACKNOWLEDGMENT

This work was supported in part by a Grant, A-3156, from the National Institutes of Arthritis and Metabolic Diseases of the National Institutes of Health, United States Public Health Service.

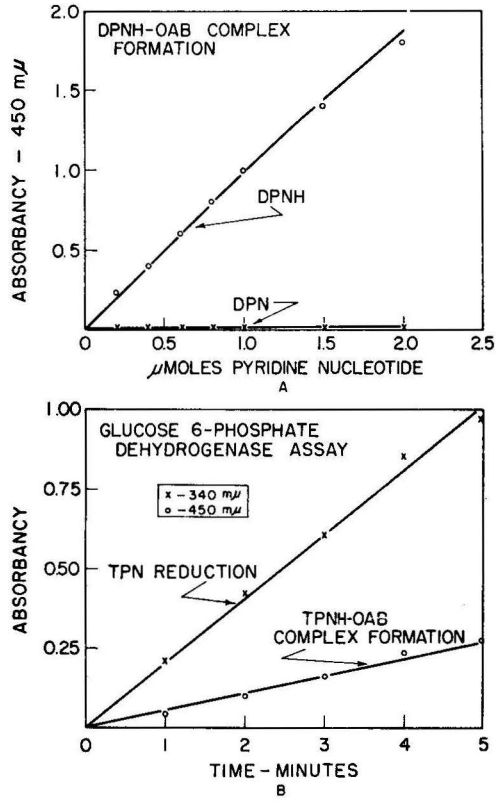


FIG. 2(A). Formation of DPNH-*o*-aminobenzaldehyde (OAB) adduct as a function of DPNH concentration. To a series of tubes containing 3.3 μM OAB in 0.5 ml 0.2 N HCl, increments of DPNH or DPN were added as indicated, the tubes brought to 2 ml with water, mixed, and let stand for 1 hr. Color formation was measured with a Spectronic 20 spectrophotometer at 450 mμ. (B) Glucose 6-phosphate dehydrogenase assay as followed by TPN reduction or formation of TPNH-OAB adduct. To 0.4 ml water in a 2-ml quartz cell having a 1-cm light path was added 0.5 ml TPN ($1.5 \times 10^{-3} M$), 0.3 ml 0.04 M glycylglycine buffer (pH 7.5), 0.2 ml $MgCl_2$ (0.1 M), 0.5 ml glucose 6-phosphate (0.02 M) and 0.1 ml glucose 6-phosphate dehydrogenase (0.1 K unit, Schwartz). Optical density changes were recorded at 1-min intervals in a Beckman Model DU spectrophotometer at 340 mμ with results as indicated in the upper curve of B. To measure TPNH formed as the OAB complex, the components of the system just described were added in 5-fold greater quantities to a test tube, but, following addition of the enzyme and mixing, 1.5-ml aliquots were removed at 1-min intervals by pipetting directly into tubes containing 3.3 μM OAB in 0.5 ml 0.2 N HCl. After 1 hr, color formed in these tubes was measured spectrophotometrically at 450 mμ with results as shown in the lower curve of B.

REFERENCES

1. SCHOPF, C., KOMZAK, A., BRAUN, F., AND JACOBI, E., *Ann. Chem.* **559**, 25 (1948).
2. VOGEL, H. J., AND DAVIS, B. D., *J. Am. Chem. Soc.* **74**, 109 (1952).
3. STRECKER, H. J., *J. Biol. Chem.* **225**, 825 (1957).
4. YURA, T., AND VOGEL, H. J., *J. Biol. Chem.* **234**, 339 (1959).
5. LARSON, R. L., SANDINE, W. D., AND BROQUIST, H. P., *J. Biol. Chem.* (in press).

R. L. LARSON
H. P. BROQUIST

Laboratory of Biochemistry
Department of Dairy Science
University of Illinois, Urbana
Received November 23, 1962

Fluorometric Assay of 2-Dimethylamino-6-hydroxypurine (Dimethylaminoguanine) in the Presence of Guanine

In a recent report (1) it was pointed out that 2-dimethylamino-6-hydroxypurine (dimethylaminoguanine, DiMG) is a highly fluorescent compound, the quantum efficiency at pH 11 being several times greater than that of guanine. Furthermore, fluorescent characteristics of DiMG are different from those of guanine, the excitation and fluorescence maxima of DiMG being 290 and 368 mμ as compared to 275 and 350 mμ for guanine (corrected). Recent findings of DiMG as one of the minor components in sRNA (2, 3) make desirable a specific fluorometric method for its assay. We have found that it is possible to measure small amounts of DiMG in the presence of large amounts of guanine by treating a mixture of the two purines with nitrous acid. By this procedure DiMG is unchanged whereas guanine is converted to xanthine, which is not fluorescent under the conditions employed.

S-RNA samples were hydrolyzed in 1 *N* HCl at 100°C for 30 min. Aliquots of the hydrolyzate containing 10–15 μg of sRNA in 0.1 to 0.3 ml were transferred to glass-stoppered centrifuge tubes and the volume brought to 0.3 ml with 1 *N* HCl, when necessary. The tubes were then placed in a boiling water bath and 0.1 ml of a solution of sodium nitrite (1 mg/ml) was added. Additional (0.1 ml) aliquots of the sodium nitrite were added at 3-min intervals for a total of four additions. Three minutes

after the final addition the solutions were cooled, neutralized with 1 *N* NaOH, and made up to 2 ml with 2 *N* ammonia. Standards and reagent blanks were carried through the entire procedure. Fluorescence of DiMG was measured in an Aminco-Bowman spectrophotofluorometer using optimal excitation and fluorescence settings (see above) of the instrument. Measurements were also made with excitation set at 280 mμ and fluorescence at 340 mμ, where DiMG has little fluorescence, to correct for traces of residual guanine. This was usually about 2 to 3% of the initial guanine. However, since the fluorescence efficiency of DiMG at 290 and 365 mμ is 25 times greater than that of guanine at the same wavelength settings this represents an error of less than 3% in estimating 2–3 parts of DiMG in the presence of 100 parts of guanine.

DiMG has been identified in alkaline hydrolyzates by chromatography on Dowex-1 formate (as well as by paper chromatography). In confirmation of this identification the peaks identified as 2′- and 3′-ribotides of 2-dimethylamino-6-hydroxypurine were shown to contain, after hydrolysis, only a single fluorescent moiety, stable to nitrous acid and with the same fluorescent properties of DiMG (Fig. 1). Direct fluoro-

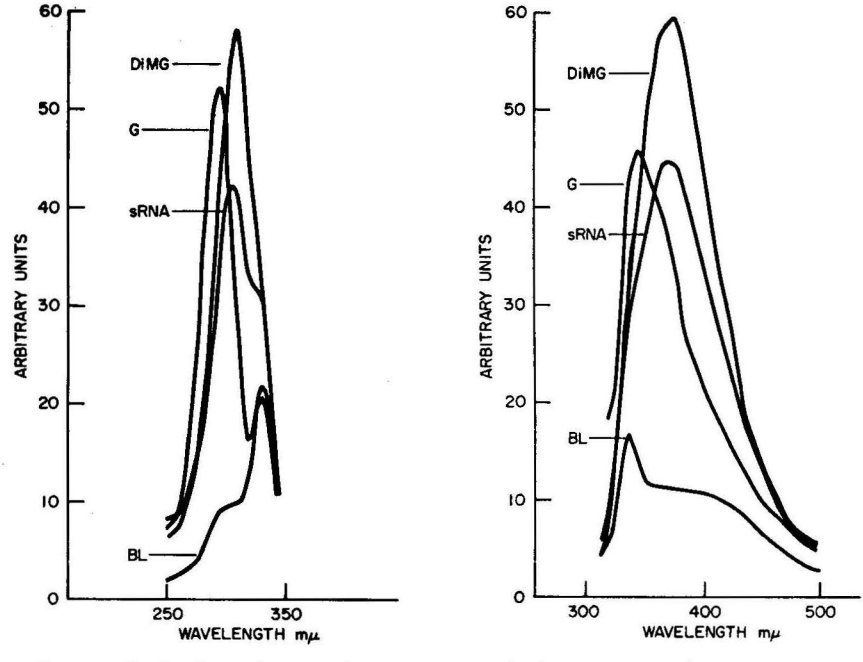


FIG. 1. Excitation (left) and fluorescence (right) spectra of dimethylguanine (DiMG), guanine (G), and S-RNA hydrolyzate carried through the nitrous acid procedure (S-RNA), and a reagent blank (BL).

metric assay of DiMG in a hydrolyzate of rabbit liver sRNA (without chromatography) gave a value of 2.3 to 2.5 moles per 100 moles of guanine. This value compares reasonably well with estimated values of 4.0 obtained by chromatographic procedures (3). In direct fluorometry, DiMG can be readily distinguished from guanine but interference would occur if monomethylaminoguanine were present, as has been suggested (2). The monomethyl compound is only partly destroyed by nitrous acid and is also fluorescent. Other methylated purines, such as 1-methylguanine and 6-methylaminopurine, are not fluorescent under these conditions.

The use of spectrofluorometry with or without preliminary chromatography should be of considerable help in evaluating the biochemical significance and distribution of dimethylaminoguanine.

REFERENCES

1. UDENFRIEND, S., AND ZALTZMAN, P., *Anal. Biochem.* **3**, 49 (1962).
2. SMITH, J. D., AND DUNN, D. B., *Biochem. J.* **72**, 294 (1959).
3. CANTONI, G. L., GELBOIN, H. V, LUBORSKY, S. W., RICHARDS, H. H., AND SINGER, M. F., *Biochim. et Biophys. Acta* **61**, 354 (1962).

SIDNEY UDENFRIEND
PEROLA ZALTZMAN-NIRENBERG
G. L. CANTONI

National Institutes of Health
Bethesda, Maryland
Received October 12, 1962

A New Method for Microanalysis of Blood Galactose

The results of a recent study (1) of modern methods for blood "sugar" indicate that Hultman's condensation reaction with *o*-aminotoluene (2) can be used in a simple, sensitive, and reasonably rapid colorimetric method for the determination of galactose in blood or other body fluid. On a molecular basis the color equivalents of glucose and lactose are 45 and 48%, respectively, that of galactose in this reaction.

Preparation of Glucose- and Protein-Free Blood Supernatant Fluid: 0.20 ml heparinized blood is incubated at 56°C with 1.7 ml Fermcozyme[1]

[1] Analytical grade 653A, obtainable from Hughes and Hughes (Enzymes) Ltd., London.

and 5 ml *M* acetate buffer pH 5.6. After 20 min, 0.3 ml 0.34 *M* zinc sulfate and 0.2 ml 0.3 *N* NaOH are added. The mixture is thoroughly shaken and then centrifuged for 5 min at 500 × *g* to obtain a clear supernatant.

Color Reaction: 1.0 ml supernatant fluid is mixed with 7.0 ml of Hyvarinen and Nikkila's color reagent (3). The latter contains 6% (v/v) *o*-aminotoluene and 0.15% (w/v) thiourea in pure acetic acid. After heating for 8 min in a boiling water bath, the mixture is cooled and its optical density measured at 630 mμ against a reagent blank. A solution of galactose (500 μg/ml) and one containing galactose (500 μg/ml) and glucose (1 mg/ml) are prepared in deionized serum (4). When treated exactly as for blood, they provide a suitable standard and control for the analysis.

Figure 1 shows that increase in extinction (2-cm cell, 630 mμ) is

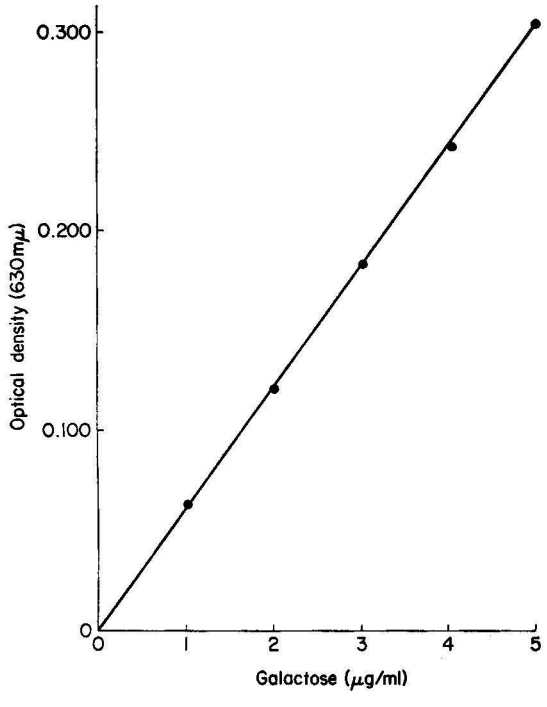


FIG. 1. Relationship between optical density and concentration of galactose in the reaction mixture.

linear with respect to galactose up to a concentration of 5 μg/ml (i.e., corresponding to blood galactose level of 500 μg/ml). Recovery of added galactose (100 μg/ml blood) was 95%, S.D. 3%, and *n* 20. No appreci-

able color develops in the presence of 1 mg/ml maltose, fructose, glutathione, or glucuronic acid, nor do those concentrations of sulfonamides, salicylates, or chloramphenicol likely to be found in blood affect the recovery of added galactose. Galactose estimation in the blood of 40 infants aged 1–5 days varied between 20 and 100 μg/ml.

Up to the present, the only safe biochemical tests to confirm the diagnosis of congenital galactosemia in the newborn infant involve the detection of red-cell abnormalities. In the erythrocytes of the galactosemic infant, the hexose 1-phosphate uridylyl-transferase activity may be abnormal (6). However, this metabolic process is not readily investigated in the routine hospital laboratory. Furthermore, a serious disadvantage of the red-cell qualitative tests is the delay necessarily involved if a galactosemic infant has been transfused with normal blood, since the erythrocytes of these infants may give false negative results for as long as 3 months after the transfusion (7). The quantitative method described here may overcome these difficulties by reason of its simplicity and its capacity to measure smaller changes in whole blood galactose than was previously possible.

REFERENCES

1. WATSON, D., AND STEVENSON, M. E., *Proc. Australian Assoc. Clin. Biochem.* **1,** 8 (1963).
2. HULTMAN, E., *Nature* **183,** 108 (1959).
3. HYVARINEN, A., AND NIKKILA, E. A., *Clin. Chim. Acta* **7,** 140 (1962).
4. LEVY, A. L., *Clin. Chem.* **8,** 174 (1962).
5. ANDERSON, E. P., KALCKAR, H. M., KURAHASHI, K., AND ISSELBACHER, K. J., *J. Lab. Clin. Med.* **50,** 469 (1957)
6. SCHWARZ, V., HOLZEL, A., AND KOMROWER, G. M., *Lancet* **1958-I,** 24.
7. ANDERSON, E. P., KALCKAR, H. M., AND ISSELBACHER, K. J., *Science* **125,** 113 (1957).

DEREK WATSON

Department of Pathology
The Royal Women's Hospital
Carlton, Victoria, Australia
Received September 29, 1962

An Apparatus for Sampling after Cesium Chloride Density Gradient Centrifugation in the Preparative Ultracentrifuge

Since the initial report of Meselson *et al.* (1), dealing with the banding of deoxyribonucleic acid (DNA) in a gradient of cesium chloride (CsCl), this method has been used with increasing frequency on the preparative scale to isolate and characterize DNA, hybrids of RNA and DNA, and bacterial and animal viruses. For this technique to attain its utmost usefulness the sampling procedure must give reproducible results, and preferably it should be simple. Several procedures have been described for piercing the bottom of the centrifuge tube and collecting the emerging drops (2–4). One of these methods, described by Szybalski (4), permits regulation of the flow rate of the drops by maintaining a hydrostatic pressure on top of the centrifuge tube.

The present report deals with inexpensive modifications of Szybalski's apparatus. One of these makes use of a metering pump to maintain a constant air pressure on top of the centrifuge tube and leaves the operator's hands free for the manipulation of the collecting tubes.

Apparatus: (*1*) Metering pump (Sigmamotor Pump, Model T-8 with speed changer and vernier; Sigmamotor, Inc., 3 North Main Street, Middleport, N. Y.) *or* 10-cc syringe (Becton, Dickinson and Co.) with automatic syringe attachment (Scientific Industries, Inc., Springfield 3, Mass., No. S-210-2). (*2*) Tygon tubing; Grade R-3602, $\frac{1}{32}$ in. i.d. × $\frac{1}{32}$ in. wall. (*3*) Becton, Dickinson and Co. (*a*) Adaptor, L/609, (*b*) 3-way stopcock, B-D MSO8, and (*c*) 18-gage needle, disposable.

Procedure 1. The apparatus is assembled as shown in Fig. 1. The end of the Tygon tubing is inserted between the steel fingers of the metering pump. After a tube is removed from the centrifuge rotor (Spinco swinging rotor SW 39 L), it is placed in a holder of polyurethan foam. The rubber stopper (Fig. 1) through which the needle assembly—already inserted into the pump—has been placed is inserted tightly into the tube. The 3-way valve is turned so that the tube is open to the atmosphere, thus equalizing the pressure, and is then closed. Next, the bottom of the tube is pierced with a hot 23-gage needle. Since the internal pressure has been equalized, the usual rapid initial flow rate is completely eliminated. The pump is then started at a suitable rate and the drops are collected in sequence. The rate of drop collection is determined by the air pressure applied on top of the centrifuge tube via the metering pump. The collec-

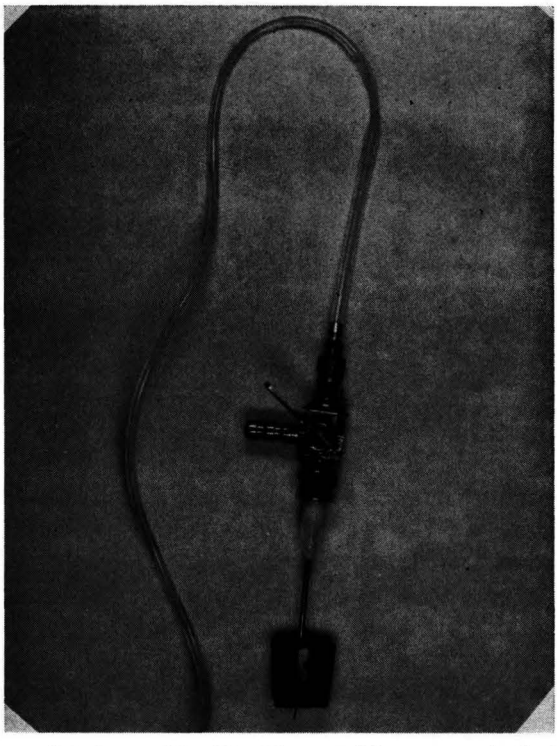


FIG. 1. The completed assembly. The Tygon tubing, one end of which attaches to the pump (or an automatic syringe), is coupled to the 3-way stopcock through a short length of polyethylene cannula tubing joined to the adapter. The needle goes through a soft rubber stopper of appropriate size.

tion may be interrupted at any time by stopping the pump, without loss of a partially emergent drop.

Procedure 2. In this procedure, the end of the Tygon tubing (shown in Fig. 1) is inserted by means of an 18-gage needle into a 10-cc syringe fitted with an automatic attachment, the adjusting screw of which has been turned counterclockwise until only 3 mm of it is within the assembly shell. The rubber stopper is inserted as above and the pressure is equalized. Prior to the piercing of the tube a negative pressure is applied by one counterclockwise turn of the adjusting screw. Following piercing, any flow rate can be obtained simply by turning the adjusting screw in a clockwise direction.

REFERENCES

1. MESELSON, M., STAHL, F. W., AND VINOGRAD, J., *Proc. Natl. Acad. Sci. U. S.* **43**, 581 (1957).

2. WEIGLE, J., MESELSON, M., AND PAIGEN, K., *J. Mol. Biol.* **1**, 379 (1959).
3. IFFT, J. B., VOET, D. H., AND VINOGRAD, J., *J. Phys. Chem.* **65**, 1138 (1961).
4. SZYBALSKI, W., *Experientia* **16**, 164 (1960).

SOLON A. ELLISON
HERBERT S. ROSENKRANZ

Department of Microbiology
College of Physicians and Surgeons
Columbia University
New York, New York
Received June 14, 1962

Paper Chromatographic Separation of Acetyl and β-Hydroxybutyryl Hydroxamates[1]

Many of the coenzyme A esters which are intermediates in metabolism of lipids have been identified as their reaction products with hydroxylamine. Thompson (1) has described various solvent systems for the paper chromatographic separation of the hydroxamates of fatty acids of different chain lengths. None of these was found to give a satisfactory resolution of acetyl and β-hydroxybutyryl hydroxamic acids. The solvent mixtures, butanol:acetic acid:water (4:1:5, v/v) and octanol:formic acid:water (3:1:3, v/v) used by Hele, Popják, and Lauryssens (2) for the separation of acetyl, β-hydroxybutyryl, butyryl, and other hydroxamates, were found in this laboratory to be adequate for the resolution of the hydroxamates of all the short-chain acids other than those of acetic and β-hydroxybutyric acids. The hydroxamate of these two acids were found to have almost the same R_f values under varying experimental conditions.

A systematic investigation with different solvents and combinations of solvents led to the solvent mixture, methylene chloride:*n*-butanol:acetic acid:water (80:20:15:30, v/v) which satisfactorily resolved these hydroxamates. Figure 1 shows the resolution. Aliquots of an aqueous solution containing 0.2 to 0.5 μmole each of the hydroxamates were spotted on Whatman No. 1 paper strips, 5 × 40 cm. The spots were dried and the

[1] Supported by the U. S. Atomic Energy Commission Contract, AT (30-1) 2412 and the National Institutes of Health, U. S. Public Health Service Research Grant, A-3504.

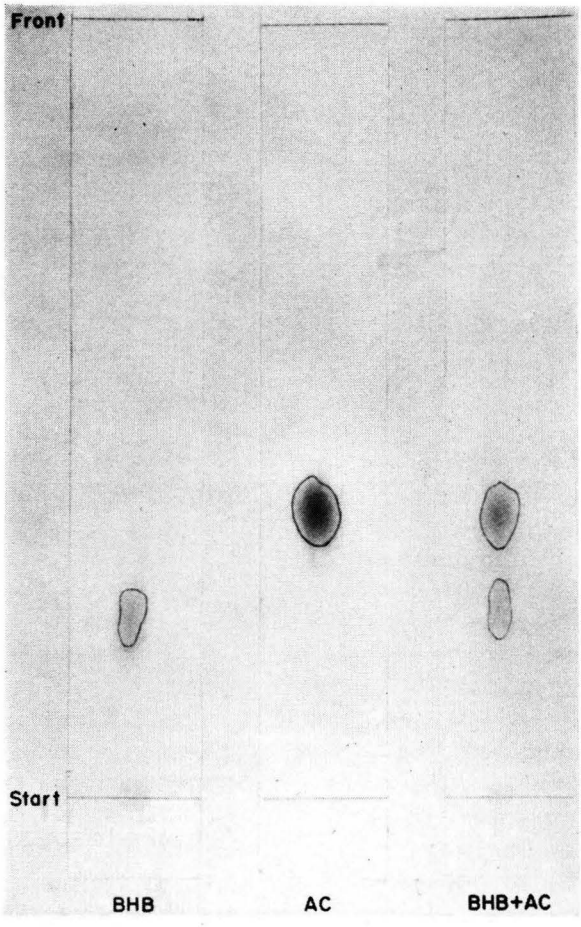


FIG. 1. Chromatograms showing the resolution of acetyl (AC) and β-hydroxybutyryl (BHB) hydroxamates using methylene chloride: *n*-butanol: acetic acid: water (80:20:15:30, v/v).

paper strips were humidified for 2 hr with the aqueous phase of the solvent system. The chromatograms were then developed by ascending chromatography using the organic phase of the solvent mixture, the solvent flow being perpendicular to the machine direction of the paper. The development of the chromatogram took 5 to 6 hr at about 25°C. The strips were then dried and sprayed with 1% methanolic solution of $FeCl_3$ which was 0.1 *N* with respect to the HCl. Blue-Violet spots indicated the location of the hydroxamates.

The chromatographic behavior of the hydroxamates of some of the

intermediates of fatty acid metabolism were checked and the R_f values obtained with the solvent mixture described are given in Table 1. The

TABLE 1
R_f VALUES OF ACYL HYDROXAMATES AND METHYL ISOXAZOLONE IN METHYLENE CHLORIDE:BUTANOL:ACETIC ACID:WATER

Compound	R_f
Hydroxamates:	
Malonyl mono-	0.0
β-Hydroxybutyryl-	0.23 ± 0.02
Acetyl-	0.35 ± 0.02
β-Hydroxy-β-methylglutaryl mono-	0.68 ± 0.02
Butyryl-	0.75 ± 0.02
Hexanoyl-	0.90 ± 0.02
Methyl isoxazolone	0.50 ± 0.03

table includes the R_f of methyl isoxazolone, the reaction product of acetoacetyl CoA and hydroxylamine (3).

REFERENCES

1. THOMPSON, A. R., *Australian J. Sci. Res.* **3A**, 128 (1950).
2. HELE, P., POPJÁK, G., AND LAURYSSENS, M., *Biochem. J.* **65**, 348 (1957).
3. AVENA, R. M., AND KUMAR, S., *Anal. Biochem.* **4**, 514 (1962).

SOMA KUMAR
REMEDIOS M. AVENA[2]

Chemistry Department
Georgetown University
Washington, D. C.
Received December 5, 1962

[2] This work was included in a thesis submitted to Georgetown University in partial fulfillment of the degree of Doctor of Philosophy.

A Simple Method for Separating Polar Steroids from the Liquid Scintillation Phosphor

In studies involving small amounts of radioactive steroids, it may be useful to recover the products which have been used for liquid scintillation counting. The method described below was designed to separate

phosphor in the 5 to 10 ml toluene from testosterone and more polar steroids by means of thin-layer chromatography (1–3). Relatively large amounts of phosphor (20 mg PPO in 5 ml solution) can be separated from much smaller amounts of corticoid by this method.

Glass plates, 20 × 20 cm, are coated with Silica Gel "G" (Brinkmann Instrument Co.) to 0.3-mm thickness. One plate suffices for each counting vial of 5 ml phosphor, although better separations are obtained when the sample is divided into 2 portions of 2.5 ml and each chromatographed separately. The toluene is removed by evaporation under a stream of air. The residual solids in the counting vial are dissolved in 0.3 ml ethyl acetate, and applied to the plate in a thin band. Three additional washes of 0.3 ml ethyl acetate are employed to ensure complete transfer of the material. Quantitative transfer to the plate is complete, as determined by the subsequent addition of a fresh sample of the toluene-phosphor mixture to each vial and by testing for residual radioactivity. The plate is developed in 100% ethyl acetate for 50 min, removed, allowed to dry in air, and then reinserted in the chamber for a second 50-min period.

Since the phosphor gives a strong visible blue fluorescence under ultraviolet light, its location on the thin-layer plate can be determined readily. With the technique described here, it occupies a band about 2 cm wide at the farthest side of the plate. The virtual absence of fluorescence in any region except the main phosphor band suggests that no significant trailing has occurred. Steroids having slower mobilities than the phosphor can be located by the usual methods, such as spraying with alkaline triphenyltetrazolium chloride, blue tetrazolium, or the Zimmermann reagents. If the radioactive steroids are present in extremely small amounts, they can be located more readily by the addition of 500 to 750 μg of nonradioactive steroid to the counting vial before evaporation of the toluene.

Elution of the areas of the plate containing steroid is achieved by shaking with warm (40–45°C) 95% absolute ethanol 3 times, centrifuging, and decanting each time. The ethanol fractions are then concentrated and dried down in a counting vial, when further measurements of radioactivity are required.

The separation of phosphor from the corticoids is effective, and the individual bands are clearly defined. The average mobilities of common steroids relative to the phosphor band are approximately: testosterone—0.85; cortisol—0.60; and tetrahydrocortisone—0.55. It is important not to exceed the 50-min period of development, as this tends to decrease the distance on the plate between steroids of different mobilities, and to render their separation more difficult. The mobility of

the steroids does not appear to be appreciably retarded by the large amounts of phosphor present.

This method employing thin-layer chromatography has enabled radioactive materials to be reutilized and purified further, once the initial measurements have been obtained. The method has found particular utility in situations in which the radioactivity of the original sample is so low that the entire amount, rather than an aliquot, must be employed for liquid scintillation counting. The technique probably could be adapted to the separation of phosphor from nonsteroid compounds as well.

REFERENCES

1. Stahl, E., Schröter, G., Kraft, G., and Renz, R., *Pharmazie* **11**, 633 (1956).
2. Demole, E., *J. Chromatog.* **1**, 24 (1958).
3. Adamec, O., Matis, J., and Galvánek, M., *Lancet* **I**, 81 (1961).

Richard S. Rivlin
Hildegard Wilson

Endocrinology Branch
National Cancer Institute
National Institutes of Health
Bethesda, Maryland
Received December 4, 1962

ANALYTICAL BIOCHEMISTRY 5, 271–283 (1963)

Photometric Scanning of Centrifuged Density Gradient Columns[1]

MYRON K. BRAKKE[2]

From the Crops Research Division, Agricultural Research Service, United States Department of Agriculture, and the Nebraska Agricultural Experiment Station, Lincoln, Nebraska

Received May 28, 1962

INTRODUCTION

Density gradient centrifugation as applied to plant viruses has been used mainly as a preparative technique and not for quantitative analysis. Brakke (4) presented scanning patterns showing absorbance at 400 mμ vs. depth in gradient columns. These results indicated a potential usefulness for quantitative analysis, but the light-measuring technique was not sufficiently accurate.

This paper presents results obtained with an apparatus designed to record the absorbance at 253.6 mμ vs. depth in a gradient column. Results are also presented on the relative amount of mixing when samples were removed from a gradient column with this apparatus as compared with samples removed through a hypodermic needle pierced through the side of the tube. Numerous methods have been used to remove samples from centrifuged gradient columns [(2), (8); for more references see (5)], but few or no data have been presented on the amount of mixing during sampling.

Automatic scanning of centrifuged gradient columns has not been reported but the Spinco Division of Beckman Instruments[3] has marketed quartz tubes which can be used for density gradient centrifugation in

[1] Published with the approval of the Director as Paper No. 1194, Journal Series, Nebraska Agricultural Experiment Station. Based on cooperative investigations between the Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, and the Nebraska Agricultural Experiment Station. Supported in part by a grant from the National Science Foundation.

[2] Chemist, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture; stationed at Plant Pathology Department, University of Nebraska, Lincoln, Nebraska.

[3] Mention of specific equipment, trade products, or a commercial company does not constitute its endorsement by the United States Government over similar products or companies not named.

their SW 39 rotor. The quartz tubes can then be placed in a special adapter and the optical density at all depths determined in a spectrophotometer. These tubes were not available until work reported here was essentially complete.

MATERIALS AND METHODS

Viruses

Tobacco mosaic virus (TMV) (AC No. 2) (1) was purified from tobacco plants by the method of Boedtker and Simmons (3). Southern bean mosaic virus (SBMV) (AC No. 17) (1) was purified by differential centrifugation from frozen extracts of diseased beans, *Phaseolus vulgaris* L., "Bountiful" (10). Brome mosaic virus (BMV) (AC No. 66) (1) was purified by differential centrifugation of a frozen extract of diseased barley, *Hordeum vulgare* L., "Moore."

Great Northern No. 59 beans were used for infectivity assay of SBMV. At least 6 randomly selected leaves were inoculated with each of several 10-fold dilutions in 0.01 *M* phosphate buffer with 1% Celite[3] of each preparation to be assayed. Relative virus titers were calculated from transformed numbers of lesions [$\log \frac{1}{2}\ (y + 5 + \sqrt{y^2 + 10y})$ where y is number of lesions; (9)] by the method given by Finney (7) for parallel line assays. Data from leaves inoculated with a given dilution were discarded if more than one leaf had no lesions or had merged lesions. Concentrations of purified virus were also determined by measuring the absorbance at 260 mμ in the Beckman DU spectrophotometer. The measured absorbances were converted to dry-weight concentrations with the following values for absorbance of 1 mg/ml solutions: TMV, 3.2; SBMV, 5.8; BMV, 4.8. The absorbances of 1 mg/ml solution at 253.6 mμ were 3.0 for TMV, 4.5 for BMV, and 5.5 for SBMV.

Density Gradient Centrifugation

Gradient columns were prepared by floating layers of 4, 7, 7, and 7 ml of solutions of 100, 200, 300, and 400 mg sucrose/ml in 1 × 3 in. plastic centrifuge tubes. The sucrose was dissolved in 0.01 *M* neutral phosphate buffer or 0.01 *M* Tris-HCl (trishydroxymethylaminomethane-hydrochloride), pH 7.0, with 0.001 *M* $MgCl_2$. Reagent-grade sucrose was used in columns for optical scanning. The layered sucrose solutions were allowed to stand overnight. One or two milliliters of virus solutions was floated on them and they were centrifuged at 22,000 to 23,000 rpm at 5°C for an appropriate time in the SW 25.1 rotor of the Spinco Model L ultracentrifuge.

ISCO Gradient Column Fractionator and Flow Densitometer[4]

A sketch of the ISCO gradient column fractionator and flow densitometer is shown in Fig. 1. The syringe is driven by a reversible synchronous motor through a gear train. The particular instrument used in experiments reported here is equipped with gears that can be shifted to give 8 different speeds ranging from 0.75 to 12 ml/min with a 50-ml syringe. A flow rate of 3 ml/min was used in experiments reported here. The needle from the syringe passes through sponge rubber compressed and held to the centrifuge tube by black plastic electrician's tape, and then through the side of the tube. The compressed sponge rubber prevents leaks. The bottom of the Lucite adapter is flanged to give a tight fit with the top of the centrifuge tube. An adapter is available for $\frac{1}{2} \times 2$ in. tubes. A mirror (not shown) is beneath the flexible support of the centrifuge tube. Some zones are thus made visible by scattered light. Light from a low-pressure mercury vapor lamp passes through the absorption cell, through filters, and to a photoelectric cell. Practically all the energy of the lamp transmitted by the filters has a wavelength of 253.6 mμ. The energy of the light of other wavelengths striking the photocell is less than 0.1% of the energy of the light of 253.6 mμ (R. Allington, personal communication). The response of the phototube is transformed by an electrical circuit and recorded as absorbance. The recorder is not shown in Fig. 1.

The channel from the centrifuge tube to the absorption cell has a circular cross section of 1 cm diameter. The absorption cell has a rectangular cross section of 5 by 10 mm, the light path being 10 mm. Tapered surfaces between the cylindrical channel below the absorption cell and the rectangular channel of the absorption cell reduce mixing to a minimum. The height of the absorption cell is 7 mm, and its volume is 0.35 ml. Thus the volume of the sample in the absorption cell corresponds to a disk of liquid 0.7 mm thick in the 1-in. diameter centrifuge tubes or 2.8 mm thick in 0.5-in. diameter centrifuge tubes. However, the light beam is easily reduced to a height of 1 mm with masks so that the volume of liquid in the light path is reduced to 0.05 ml, which would occupy a disk 0.4-mm thick in 0.5-in. diameter tubes.

A second Lucite adapter without an absorption cell may be used for collecting samples. The needle from the syringe passes through this

[4] This apparatus was designed by Robert Allington and constructed on special order by the Instrumentation Specialties Company (ISCO), Lincoln, Nebraska. It will be referred to as the ISCO density gradient column fractionator and flow densitometer, or more briefly as the ISCO scanner.

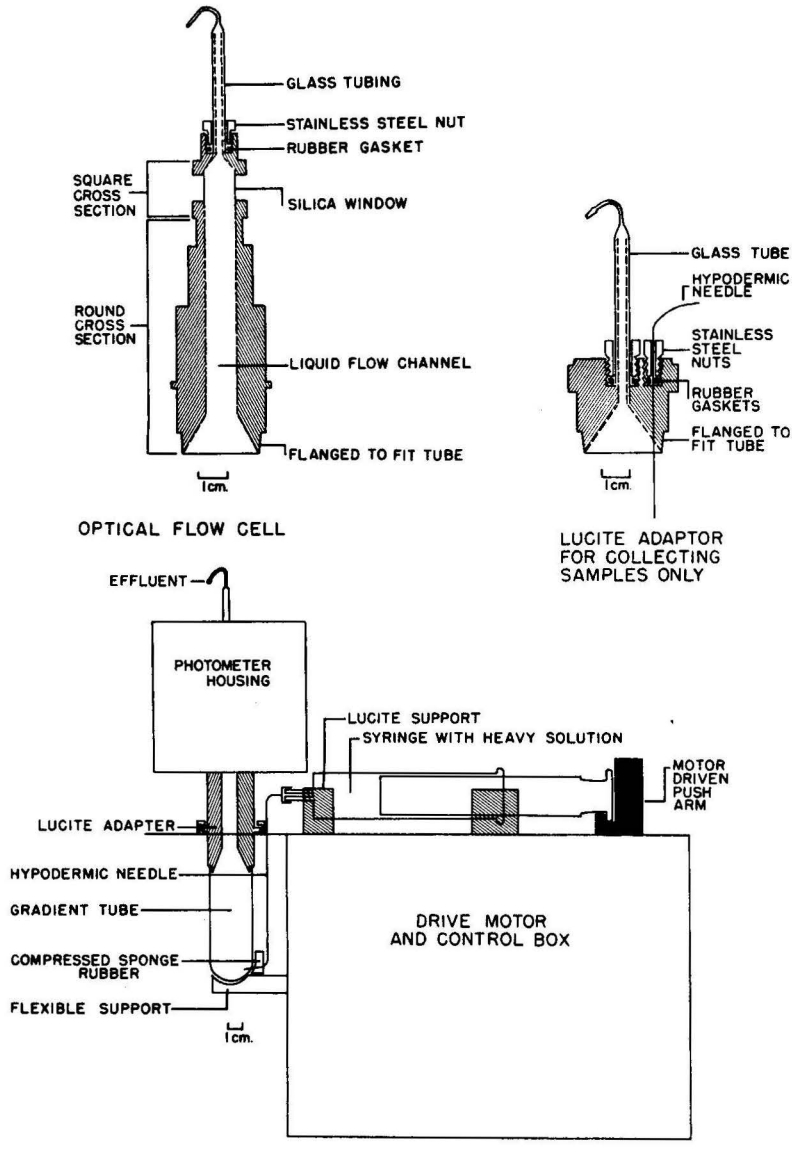


FIG. 1. Schematic representation of ISCO gradient column fractionator and flow densitometer.

adapter instead of through the side of the tube. The adapter is shaped like an inverted funnel with a stem of about 3 mm i.d. A 4-mm o.d. glass tubing fits into the top of the adapter. The top of the glass tubing is

pulled into a capillary and bent into a hook to deliver solution into a tube. The hypodermic syringe and the 4-mm glass tubing are held leak-tight in the Lucite adapter by rubber washers compressed by a stainless-steel nut. Samples may also be collected from a similar tube on top of the absorption cell.

The syringe may be run by the motor or manually. If the primary purpose is to record the absorbance, the syringe is driven at a constant and continuous speed by the motor. In some experiments in which the primary purpose is to collect samples, the syringe plunger is moved intermittently, manually or by motor, and stopped between samples. The apparatus may also be used with an automatic fraction collector. The syringe drive-motor stops and the recorder needle marks "zero" when the turntable indexes.

RESULTS

Convection in Removal of Samples

It is sometimes desirable to remove consecutive samples from a gradient tube for assay of one or more properties or activities. A profile of activity vs. depth may thus be obtained. If two activities have matching profiles, they must be associated with particles sedimenting at similar rates. Any procedure for separating all the contents of a gradient column into consecutive samples may produce some mixing. The amount of mixing with the ISCO fractionator was determined on samples removed from gradient columns containing zones of virus, sodium ribonucleate, or methylene blue. The Lucite adapter without the absorption cell was used.

Approximately 2 mg of SBMV was centrifuged for 3 hr at 23,000 rpm through a gradient column to give a zone 2.1–3.0 cm deep. Two-milliliter samples were removed from this zone and from a 4.0-cm depth through a 25-gage needle pierced through the side of the tube. Samples removed slowly through a needle pierced through the side of the tube should have less contamination than samples removed in most other ways because the needle does not pass through solution above or below that removed, nor does the sample flow through a channel that previously had a higher concentration of virus.

Samples were also removed with the ISCO fractionator and samples corresponding to depths of 2.1–3.0 and 3.6–4.0 cm were saved. The 3.6- to 4.0-cm sample was collected with the ISCO fractionator after the virus zone had been removed. There should have been more contamination from the virus zone in this sample than in samples preceding the virus zone. The samples from below the zone always had less than 1% as much

virus as the samples from the zone (Table 1). The sample removed with the ISCO fractionator always had slightly more virus than the sample removed with a needle, though the difference was not consistently statistically significant when individual assays were compared. Calcula-

TABLE 1

CONCENTRATION OF SOUTHERN BEAN MOSAIC VIRUS IN SAMPLES 0.6 TO 1.0 CM BELOW BOTTOM OF VISIBLE VIRUS ZONE IN CENTRIFUGED DENSITY GRADIENT COLUMNS

Method for removal of sample[a]	Logarithm of ratio of concn. of virus in sample from below visible zone to that in sample from visible zone		
	Expt. 1	Expt. 2	Expt. 3
Needle through side of tube	-2.58 ± 0.44[b]	-3.18 ± 0.22	-2.91 ± 0.21
ISCO fractionator			
1	-2.35 ± 0.44	-2.90 ± 0.25	-2.68 ± 0.21
2	-2.04 ± 0.44	-3.12 ± 0.25	-2.77 ± 0.21

[a] The visible virus zone was 2.1 to 3.0 cm below the meniscus. The sample comprising the liquid from 3.6 to 4.0 cm depth was removed from one tube through a hypodermic needle pierced through the tube wall at 4.0 cm depth and from 2 tubes by the ISCO fractionator.

[b] 95% confidence interval.

tion of a weighted average showed the difference to be real and significant. For this calculation, logarithms of the ratios of virus concentrations in the samples removed with the ISCO fractionator to the virus concentration in the samples removed with a needle, the latter being used as the standard, were calculated (7). The weighted average of the logarithms of the ratios was —0.19 with a 95% confidence interval of 0.11. That is, the samples removed with the ISCO fractionator from 0.6 to 1.0 cm below the visible virus zone had 1.55 (95% confidence interval 1.2–2.0) times as much virus as the samples removed from a similar depth with a needle pierced through the wall.

The virus in the sample that was removed from below the visible zone with the needle probably represents aggregated virus that sedimented to that depth during centrifugation. The additional virus in the sample removed with the ISCO fractionator is a measure of the amount of mixing.

In a second series of experiments, zones were created by floating 1 ml of solution of methylene blue or sodium ribonucleate at a density shelf created by removing solution from the middle of a gradient column in a $1 \times 3''$ centrifuge tube. These columns were not centrifuged but were immediately placed on the ISCO fractionator and consecutive 1-ml samples were removed. These were diluted and the dye or ribonucleate

concentration determined from absorbance values measured in the Beckman Model DU spectrophotometer. The amount of material in samples from below the zones was 1.3, 1.3, and 2.0% of the total in 3 experiments with methylene blue and 3.9, 1.1, 3.2, and 1.3% of the total in 4 experiments with sodium ribonucleate. The concentration of dye or ribonucleate decreased rapidly in successive samples below the zone, being less than 1% the concentration in the zone in the second and all succeeding samples after removal of the zone. Much of the mixing with the methylene blue was obviously due to adsorption and elution from the plastic walls of the tubes. The amount of such mixing is markedly influenced by flow rate, being less at slower rates.

Optical Scanning of Centrifuged Gradient Columns

To obtain more homogeneous virus for testing, purified SBMV, TMV, or BMV was centrifuged through one gradient column, and solution from the virus zone was removed, diluted, and floated on a second gradient column. This second gradient column was scanned after centrifugation. Some typical scanning patterns are shown in Fig. 2.

A single peak was usually obtained from the recycled viruses. The depths of the peaks and their size were nearly independent of other constituents. Trace A is of an extract from young wheat leaves clarified by centrifuging for 20 min at 10,000 rpm before density gradient centrifugation. This particular extract gave several peaks. The patterns obtained from extracts of healthy plants have varied with the physiological state of the tissue and the smaller peaks seen in trace A have not always been observed. Addition of 0.12 mg of TMV to the healthy wheat extract gave trace B, which is very nearly the sum of traces A and C. Trace C was obtained from 0.12 mg of TMV by itself.

Pattern D in Fig. 2 was obtained when BMV, SBMV, and TMV were in the same tube. The SBMV and TMV were not pure and absorption outside the area of the main peaks was due to the breakdown products of these two viruses. Experiments with mixtures have shown that the area under a peak is not markedly affected by a second virus if the peaks do not overlap. If the peaks overlap, the baseline is difficult to draw and areas cannot be determined as accurately. When 100 μg of the SBMV used for pattern D (Fig. 2) was centrifuged by itself, the virus peak corresponded to 37 μg. In the presence of 0.4 mg of BMV and 0.37 mg of TMV, the area under the SBMV peak corresponded to 42 μg. With 0.5 or 0.25 these amounts of BMV and TMV, the area under the SBMV peak corresponded to 37 μg.

The area under the peaks was proportional to the concentration of virus. Several concentrations of SBMV were centrifuged, three replicate

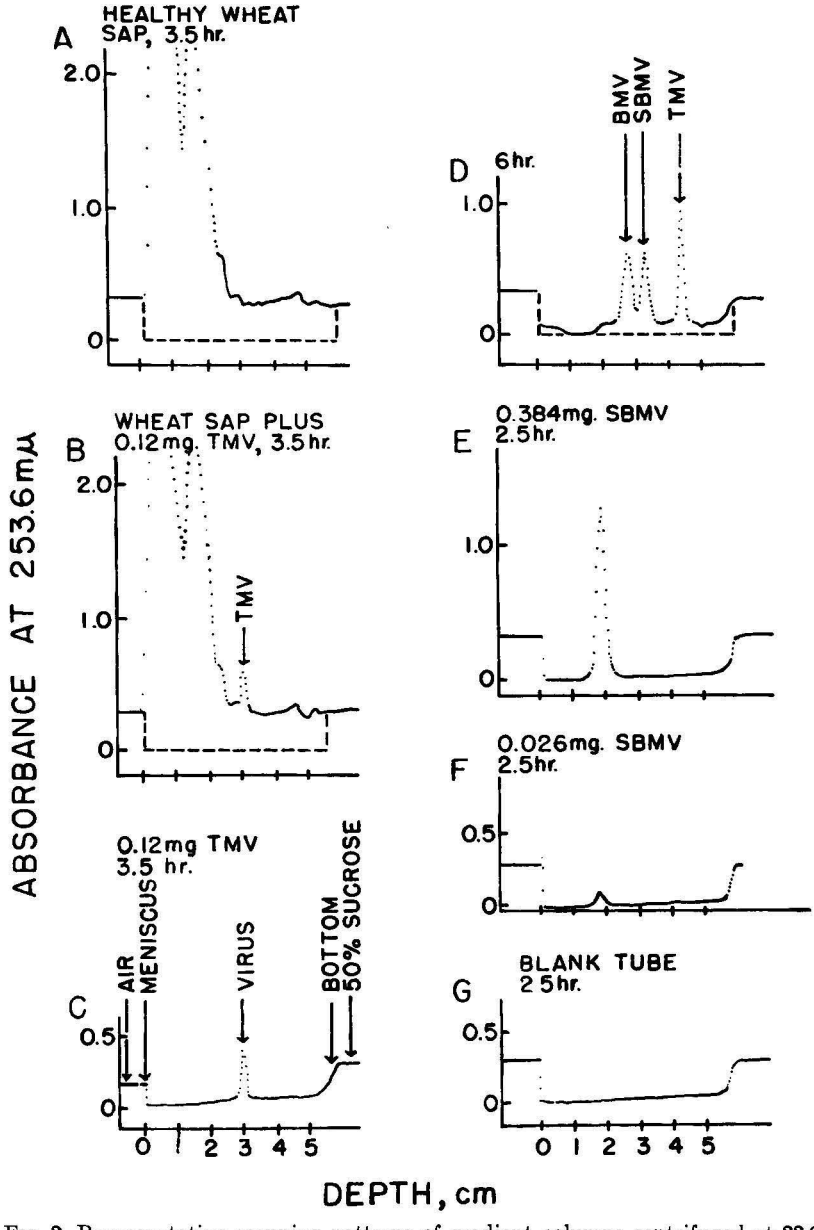


FIG. 2. Representative scanning patterns of gradient columns centrifuged at 22,000 rpm for indicated times. *Patterns A and B:* Young wheat leaves were macerated with twice their weight of 0.01 *M* phosphate buffer. The juice was expressed through cheesecloth and centrifuged for 20 min at 10,000 rpm in a Servall SS-4 centrifuge. Samples of the supernatant fluid were mixed with 1/9 volume TMV (pattern B)

tubes being used for each concentration. Scanning patterns of two of the tubes are shown in E and F of Fig. 2. Trace G shows a scanning pattern of a control tube centrifuged after water had been floated on it. The readings on the recorder were standardized by filling the absorption cell with the original virus solution and then with water. To accomplish this, a small beaker of the solution or of water was placed under the effluent tube and the motor reversed. The solution was drawn into the absorption cell on top of 50% sucrose solution and, therefore, with little mixing. The absorbance of the virus solution was determined in the Beckman Model DU spectrophotometer. The areas under the peaks were converted to milligrams of virus and plotted against the milligrams of virus added to each tube (Fig. 3). The data fit a straight line, calculated by least squares, of $y = -0.004 + 0.89x$, where y is milligrams of virus corresponding to the area under the peak and x is milligrams of virus added to the tube. Thus, 89% of the added virus was recovered in the virus zone. In this experiment, the standard deviation of the area under a single peak was 4.4 μg of virus, the average difference between duplicates was 7.3 μg, and the average percentage difference between duplicates was 5.04%. The average percentage difference between duplicates having more than 0.1 mg of virus was 2.3%. The percentage error between duplicates was obviously larger for smaller amounts of virus, and for the calculation of the equation for the straight line and the standard deviation it was assumed that the error expressed as micrograms virus was independent of the amount of virus. This absolute error may have been larger for larger amounts, but this conclusion was not obvious from a casual inspection of the data nor were the data extensive enough for a statistical test of this hypothesis.

The recovery of virus in this experiment was 89%. The other 11% may have been impurities, it may represent an inherent inaccuracy in the method, or it may have resulted from incorrect procedure. Since the

---

or 1/9 volume buffer (pattern A) and 2 ml was floated on a gradient column. *Pattern C* was given by a tube with an equivalent amount of TMV only. *Pattern D* represents a mixture of 0.20 mg of BMV, 0.38 mg of SBMV, and 0.64 mg of TMV. The SBMV had been dialyzed 24 hr vs. 0.01 *M* Tris-HCl, pH 7.0, with 0.001 *M* $MgCl_2$ and contained considerable breakdown products as shown by scanning patterns of gradient columns containing this preparation of SBMV only. This preparation of TMV also gave evidence of heterogeneity when centrifuged alone. The areas under the SBMV and TMV peaks in pattern D correspond to only 40% of the amounts added. The area under the BMV peak corresponds to 75% of the amount added. *Patterns E and F* are of 2 gradient columns with different amounts of SBMV from the experiment reported in Fig. 3. *Pattern G* is of a centrifuged gradient column on which water had been floated.

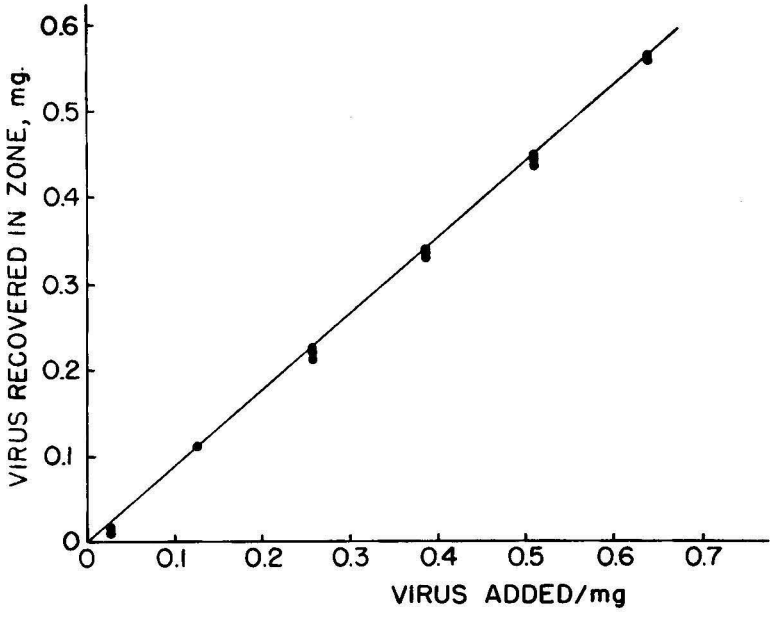


FIG. 3. The areas under the virus peaks of the scanning patterns converted to mg SBMV and plotted against the mg SBMV floated on the columns. Three replicate columns were centrifuged 2.5 hr at 22,000 rpm for each concentration.

ISCO scanner was standardized with the virus solution which had been floated on the gradient columns, an error in the spectrophotometer reading would not have affected the calculated percentage recovery.

An attempt was made to increase the percentage recovery by further recycling SBMV. SBMV recycled twice gave 92% recovery, recycled three times 88%, and recycled four times 62%. SBMV dialyzed vs. the buffer used in the gradient column, 0.01 *M* Tris-HCl, pH 7.0, with 0.001 *M* $MgCl_2$, gave 40% recovery and two distinct peaks of breakdown products. These results suggest that at least some of the 11% loss was the result of impurities or virus breakdown due to increased instability of the virus with further purification in this buffer.

The area under the peak of 0.44 mg SBMV after a 1.5-hr centrifugation indicated 88% recovery, whereas after a 4.5-hr centrifugation the recovery was 82%. In a second experiment, the recovery was 87% after both 1.5- and 6-hr centrifugation. Similar results were obtained with TMV. In one experiment the recovery was 86% after either 1.5- or 4.5-hr centrifugation, and in another experiment the recovery was 10% less after 4.5 hr than it was after a 1.5-hr centrifugation. The reason for the

differences between experiments is not known, but it may have resulted from partial breakdown of the virus. Minor variations in technique, previously thought unimportant, often led to scanning patterns indicating partial breakdown or aggregation of the viruses.

A detectable peak was obtained with as little as 10 μg of TMV. The minimum amount of a material detectable would, of course, depend on how strongly it absorbs light at 253.6 mμ. Ten micrograms of TMV dissolved in 1 ml should have an absorbance of 0.03 at 253.6 mμ. That amount of any substance having an absorbance of 0.03 at 253.6 mμ when dissolved in 1 ml should be detectable if it is located in a narrow zone.

DISCUSSION

The continuous recording of absorbance as the solution of a density gradient column flows through an absorption cell has proved to be an accurate and sensitive method for analysis of the results of density gradient centrifugation. Results obtained with the flow cell were considerably more accurate than those obtained by determining the absorbance of diluted consecutive samples in a conventional spectrophotometer. Use of the flow cell with density gradient centrifugation promises to be a useful technique for centrifugal analysis of components large enough to be sedimented in available swinging-bucket rotors.

The highest recovery of southern bean mosaic virus in the virus zone was 89%. This low recovery may be due to an instrumental or calibration error, or it may be an indication that 11% of the added material did not sediment with the bulk of the virus. Inspection of absorbance recordings indicated that there could have been a low concentration of material below the zone and near the bottom of the tube. Furthermore, material sedimenting to the pellet may have been incompletely resuspended and therefore not measured. Earlier experiments (6) showed that the amount of infectious southern bean mosaic virus sedimenting to positions below the virus zone was less than 1.07% even after a 7-hr centrifugation in gradient columns of the same composition as the ones used in the present experiments. The missing 11% of the material in present experiments was probably not infectious virus but may have been denatured, aggregated virus. This conclusion is consistent with the fact that the recovery of virus in the virus zone decreased very little with additional centrifugation. Extrapolation of percentage recovery vs. time of centrifugation did not give 100% recovery at zero time as would be expected if the low recovery were due to a phenomenon such as convection caused by wall effects.

The fact that the percentage recovery of southern bean mosaic virus in the virus zone decreased with successive cycles of density gradient

centrifugation suggests that something stabilizing the virus was being lost. The same conclusion is supported by the fact that half the virus was degraded after dialysis overnight against the buffer used for dissolving the sucrose for the gradient columns. Further critical experiments on the recovery of added virus in the virus zone during density gradient centrifugation will depend on finding a buffer in which southern bean mosaic virus or some other virus is sufficiently stable. Tobacco mosaic virus has not been completely satisfactory because of some aggregation in buffers which have been tried.

The percentage recovery in the virus zone is important if the method is to be used as a criterion for purity of virus preparations and proteins. Usually, the absence of components giving a distinguishable peak after centrifugal or electrophoretic analysis is used as the criterion of purity. A small amount of heterogeneous material might not give a discernible peak; and, therefore, the determination that all the material added is in a single peak is a more critical test of purity than the determination that only one discernible peak exists. For many other purposes, 89% recovery is entirely satisfactory.

The present optical scanner will detect only components absorbing light of 253.6-mμ wavelength. Fortunately, most large particles of biological origin absorb or scatter light of this wavelength. Modifications in the equipment would permit use of other wavelengths.

SUMMARY

Results obtained with an apparatus for recording the ultraviolet absorbance at all depths in a centrifuged density gradient column and for collecting consecutive samples are presented. A heavy sucrose solution is forced from a motor-driven syringe into the bottom of a gradient column. The gradient column floats on the heavy sucrose solution and is slowly forced up through a plastic adapter shaped like an inverted funnel and through a cell 1 cm thick with silica windows. As the solution passes through the cell, the light absorption at 253.6 mμ is measured and automatically recorded on a chart as absorbance. Samples of effluent from the optical cell may be collected manually or with an automatic fraction collector. The areas under the virus peaks on the charts recording absorbance were reproducible with a standard error corresponding to 4.4 μg of virus and were directly proportional to the concentration of southern bean mosaic virus up to 0.6 mg. As little as 0.01 mg of tobacco mosaic virus could be detected.

ACKNOWLEDGMENTS

The technical assistance of Mrs. Audrey Wacha and Mr. Stanley Jensen is gratefully acknowledged.

REFERENCES

1. "Catalog and Registry of the Plant Viruses," 2nd Ed. American Type Culture Collection, 2112 M St., N. W., Washington 7, D. C., 1958.
2. BANCROFT, J. B., *Virology* **14**, 296–7 (1961).
3. BOEDTKER, HELGA, AND SIMMONS, N. S., *J. Am. Chem. Soc.* **80**, 2550–6 (1958).
4. BRAKKE, M. K., *Arch. Biochem. Biophys.* **45**, 275–90 (1953).
5. BRAKKE, M. K., *Advan. Virus Res.* **7**, 193–224 (1960).
6. BRAKKE, M. K., *Arch. Biochem. Biophys.* **93**, 214–9 (1961).
7. FINNEY, D. J., "Statistical Method in Biological Assay." Hafner, New York, 1952.
8. HECKLY, R. J., *Anal. Biochem.* **1**, 97–102 (1960).
9. KLECZKOWSKI, A., *J. Gen. Microbiol.* **13**, 91–8 (1955).
10. PRICE, W. C., *Am. J. Botany* **33**, 45–54 (1946).

ANALYTICAL BIOCHEMISTRY 5, 284–290 (1963)

Determination of Histidine Decarboxylase Activity by Liquid Scintillation Counting of $C^{14}O_2$ [1]

YUTAKA KOBAYASHI

From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts

Received June 15, 1962

INTRODUCTION

The enzyme, L-histidine decarboxylase, can be assayed manometrically by the production of carbon dioxide, or by the formation of histamine (1, 2). The conventional methods available are not sufficiently sensitive to detect the low enzyme activity in tissues such as rat lung, skin, or intestines. A radioactive method has been used by Schayer *et al.* (3–5) which depends on the production of labeled histamine from labeled L-histidine. The radioactive method is the most sensitive available, but the procedure is relatively tedious and time consuming. This paper describes another radioactive method which appears to be as sensitive as that used by Schayer *et al.* and has the advantage of speed and simplicity.

MATERIALS AND METHODS

Uniformly labeled L-histidine (Volk) was diluted tenfold with cold carrier and purified by paper chromatography. The histidine peak was eluted with water and concentrated to give approximately 120,000 dpm/μg/0.1 ml.

Bacterial histidine decarboxylase, 12 units/mg. (Worthington Biochemical Corp.) was used at pH 4.5 at 37°C in air. Four mg of enzyme was used per flask.

The preparation of histidine decarboxylase from rat tissues was as follows: the tissue was excised, washed quickly with physiological saline, and minced with 3 vol (1 gm tissue/3 ml buffer) of 0.1 *M* phosphate buffer, pH 7.4. The tissue was homogenized in an Omnimixer for 1 min at maximum speed, then frozen and thawed three times. The entire brei was centrifuged at approximately 20,000 $\times$ g for 30 minutes at 4° and

[1] Supported, in part, by a grant E-1135 from the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service, National Institutes of Health, and Contract AT(30-1) 2085 with the U. S. Atomic Energy Commission.

the cell-free solution decanted. The pH of the cell-free preparation was adjusted to 6.9 with 0.01 *N* HCl.

Hyamine hydroxide, 10×, 1 *M* in methanol (Packard Instrument Co.) used for the absorption of carbon dioxide was concentrated to approximately half volume with nitrogen and reconstituted to its original volume with water. This prevented the gelling of Hyamine carbonate in methanol when used in the center well of a Warburg vessel.

All radioisotope counting was done in a Packard Tricarb liquid scintillation spectrometer, Model 314X using special low-potassium vials (Wheaton Glass Co.). The temperature of the spectrometer was maintained between —5° and 0° to prevent the counting mixture from solidifying.

A Warburg apparatus was used to provide a closed system. In a typical assay, the flasks were prepared as follows: 2 ml of rat enzyme, 30 μg of pyridoxal phosphate, and in one side arm 1 μg of C^{14} L-histidine and 0.3 ml of 1 *M* citric acid in the other. Aqueous Hyamine hydroxide used to absorb carbon dioxide was placed in the center well in one of two ways: (a) 0.2 ml in the center well or (b) 0.01 to 0.02 ml placed on a strip of Whatman No. 1 filter paper, 7.5 by 25 mm. The filter paper was edged with a thin strip of paraffin wax to prevent the Hyamine from touching the walls of the vessel. The final volume of each flask was usually 2.60 ml. The reaction time was 2 hr at 37°, after which the reaction was stopped with citric acid. The flasks were shaken for an additional hour to ensure quantitative absorption of the carbon dioxide by Hyamine hydroxide. Longer absorption times did not result in increased recovery of carbon dioxide as measured by the amount of radioactivity found.

When the bacterial enzyme was used, the incubation and gas absorption period was 2 hr. Additional time was not required since the pH maximum of this enzyme is 4.5 and carbon dioxide is not soluble at this pH.

When Hyamine hydroxide was placed directly into the center well, the Hyamine solution was washed out quantitatively into a counting vial with water such that Hyamine plus the wash water weighed 1.5 gm on a triple beam balance; 15 ml of counting solution was then added. The counting solution consisted of 6 parts dioxane, 1 part anisole, and 1 part dimethoxyethane; 500 ml of this solvent mixture contained 3.5 gm of diphenyloxazole (Pilot Chemical Co.) and 25 mg of 1,4 bis-2(5-phenyloxazolyl)benzene.

When Hyamine hydroxide on paper was used, 0.5 ml of water was added to each counting vial followed by 15 ml of the scintillation solvent described above.

RESULTS

The accuracy and precision of the radioactive carbon dioxide measurements was tested by decarboxylating uniformly labeled L-histidine using bacterial histidine decarboxylase and calculating enzyme activity by two methods: (a) the amount of radioactive carbon dioxide trapped by Hyamine hydroxide and (b) by the amount of radioactive histamine formed measured as the dibenzenesulfonyl derivative (4). The dibenzenesulfonylhistamine was recrystallized twice to achieve constant specific radioactivity. The results are given in Table 1. It can be seen that both

TABLE 1
COMPARISON OF CARBON DIOXIDE METHOD WITH "BSH" METHOD FOR DETERMINATION OF HISTIDINE DECARBOXYLASE ACTIVITY

Sample	CO_2 (dpm)	% metab. based on CO_2 (22,000 dpm)[a]	Histamine as "BSH" (dpm)	% metab. based on histamine (110,000 dpm)[a]	Ratio: histamine/ CO_2
1	9,925	45.1	45,494	41.4	4.58
2	8,886	44.9	49,012	44.6	4.95
3	9,731	44.2	49,375	44.9	5.07
4	9,356	42.5	49,364	44.9	5.27
5	10,511	47.7	51,425	46.8	4.89
				Av.	4.95

[a] Randomly labeled C^{14}-L-histidine = 132,000 dpm.

methods gave comparable results and that the average ratio of the histamine dpm to the carbon dioxide dpm was very close to the theoretical value of 5.0.

The reproducibility of the carbon dioxide determination was checked by running two experiments in which twelve replicates were prepared. The results (Table 2) show that the reproducibility of the determination is adequate for the purpose intended.

The method using 0.2 ml of Hyamine hydroxide in the center well was found to be dependent on quantitative washing of the center well with a constant amount of water. In order to minimize sources of error, and to simplify the determination, 0.01 to 0.02 ml of aqueous Hyamine hydroxide was adsorbed on paper, as described under *Methods,* and placed in the center well. After the experiment, the paper was removed from the center well and placed directly into a counting vial for assay. The counting efficiency was determined using C^{14} toluene as the internal standard (6). The counting efficiency was approximately 50%. A typical experiment showing the results obtained by the two methods on identical

TABLE 2

TWO SERIES OF TWELVE REPLICATES OF AN ESTIMATION OF BACTERIAL HISTIDINE DECARBOXYLASE ACTIVITY BY ABSORPTION OF RADIOACTIVE CARBON DIOXIDE WITH HYAMINE HYDROXIDE

	Counts/min.	
	Series 1	Series 2
	4977	5214
	4912	5114
	4600	5131
	4766	4959
	4986	5164
	4821	5146
	4826	5171
	4784	5089
	4957	5215
	4877	5216
	4978	5164
	4661	5014
Av.	4845	5133
S.D.	±127 (2.6%)	±80 (1.6%)

samples are presented in Table 3. It can be seen that the paper method gave slightly lower results (approximately 3%) than the aqueous wash method.

The application of the Hyamine hydroxide-filter paper technique for histidine decarboxylase estimation of some rat tissues is shown in Table 4. These data indicate the sensitivity of this method; 72 cpm represents the decarboxylation of about 0.001 μg L-histidine in 2 hr by the rat stomach preparation. No decarboxylase activity was found for the intestinal extract at pH 6.9, but some activity was found at pH 6.0.

TABLE 3

ASSAY OF BACTERIAL HISTIDINE DECARBOXYLASE ACTIVITY

Hyamine hydroxide in center well				Hyamine hydroxide on filter paper			
Sample	Net cpm	% eff.	Net dpm	Sample	Net cpm	% eff.	Net dpm
1	4643	42.80	10,849	1	5120	49.64	10,314
2	4777	41.48	11,516	2	5498	49.30	11,152
3	4643	38.34	12,110	3	5362	46.36	11,566
4	4524	40.21	11,251	4	5376	46.21	11,634
5	4985	42.43	11,749				
Background	55				36		
		Av.	11,495			Av.	11,166

TABLE 4
HISTIDINE DECARBOXYLASE ACTIVITY OF SOME RAT TISSUES

Tissue	Net cpm recovered as CO_2	Av. cpm
Stomach	29, 42, 46, 49, 60, 63, 76, 88, 109, 112, 131	72
Lung	10, 10, 16, 17, 21, 28, 42	20
Intestine	0, 0, 0, 0	0
Background		50

Conditions: as described under methods. Hyamine-paper technique used to trap carbon dioxide.

DISCUSSION

The method for radioactive carbon dioxide described here should be applicable to any determination of decarboxylase activity and is limited only by the availability of carboxyl-labeled substrates. The measurement of carbon dioxide formed by a decarboxylase must be the most accurate estimation of decarboxylase activity by definition. Estimation of decarboxylase activity by the estimation of the amine produced is always subject to the possibility of amine destruction either chemically or by the presence of amine oxidase and may thus lead to low values. Histamine can also be destroyed *in vitro* by methylation (7, 8) of the imidazole ring.

The carbon dioxide method for the determination of histidine decarboxylase is based on the assumption that the radioactive carbon dioxide collected originates from the carboxyl carbon of histidine. This assumption is supported by the fact that the known major and minor routes of *in vitro* mammalian histidine metabolism do not lead to the production of carbon dioxide with the exception of histidine decarboxylase. Although mammalian enzymes which oxidize the imidazole ring to produce carbon dioxide have been reported by Brown *et al.* (9) in rats *in vivo*, their existence *in vitro* have not been described. It would be expected that such an oxidation would require oxygen. Therefore, testing for the presence or absence of histidine decarboxylase using randomly labeled L-histidine should first be done in a nitrogen atmosphere to minimize the possible involvement of undescribed oxidases. When carbon dioxide evolution is observed, a simultaneous analysis for histamine would confirm the presence of histidine decarboxylase activity, provided the histamine is not further metabolized. The use of carboxyl-labeled L-histidine would obviate the need for these precautions.

The data presented show that the radioactive carbon dioxide method is as sensitive and reliable as the dibenzenesulfonylhistamine method originally used by Schayer *et al.* (4) for histidine decarboxylase assay. It would be expected that when the dibenzenesulfonylhistamine method

indicates histidine decarboxylase activity, the carbon dioxide method run under the same conditions must necessarily give the same result, although the converse may not always be true. The sensitivity of any radioactive method is determined by the specific radioactivity of the labeled reagent or substrate. In the case of the histidine used in these experiments, the sensitivity was approximately 0.001 μg of L-histidine decarboxylated. This figure is based on the assumption that 120 dpm above a background of 120 dpm can be detected with confidence. Since the histidine was diluted tenfold with carrier, the sensitivity can be increased by a factor of ten if the labeled histidine were used without dilution.

The paper technique for carbon dioxide has been used routinely for the past year in this laboratory. The convenience of this method over the original method of adding Hyamine hydroxide directly into the center well took precedence over the slightly lower recovery of carbon dioxide experienced by using paper.

The use of Hyamine hydroxide on filter paper for carbon dioxide determination is not new. Its use in the field of liquid scintillation counting has been reviewed by Rapkin (10). Other carbon dioxide absorbents such as phenylethylamine will probably substitute for Hyamine hydroxide, but these possibilities were not investigated. The primary task of this laboratory was to develop a rapid and accurate assay for histidine decarboxylase. The method using filter paper described in this report fulfilled this requirement and its application to biological samples has been demonstrated with rat tissues (Table 4).

SUMMARY

L-Histidine decarboxylase activity can be determined using randomly or carboxyl-labeled L-histidine in a closed system. The radioactive carbon dioxide produced by the enzyme is trapped by aqueous Hyamine hydroxide spread on filter paper edged with paraffin wax. The radioactive Hyamine carbonate is assayed in a liquid scintillation counter. This method can detect the decarboxylation of 0.001 μg L-histidine.

ACKNOWLEDGMENTS

The author gratefully acknowledges the technical assistance of James F. Lingley, LeSetta M. Shaw, and Heidrun Paschke.

REFERENCES

1. BLASCHKO, H., *Advan. Enzymol.* **5**, 67 (1945).
2. GALE, E. F., *Advan. Enzymol.* **6**, 1 (1946).
3. SCHAYER, R. W., DAVIS, K. J., AND SMILEY, R. L., *Am. J. Physiol.* **182**, 54 (1955).
4. SCHAYER, R. W., *Am. J. Physiol.* **186**, 199 (1956).

5. SCHAYER, R. W., ROTHSCHILD, Z., AND BIZONY, P., *Am. J. Physiol.* **196**, 295 (1959).
6. KOBAYASHI, Y., "Liquid Scintillation Counting—Some Practical Aspects," Tracerlab, Inc., Waltham, Mass., 1961.
7. KOBAYASHI, Y., AND SCHAYER, R. W., *Arch. Biochem. Biophys.* **63**, 480 (1956).
8. KOBAYASHI, Y., *Arch. Biochem. Biophys.* **77**, 275 (1958).
9. BROWN, D. D., SILVA, O. L., MCDONALD, P. B., SNYDER, S. H., AND KIES, M. W., *J. Biol. Chem.* **235**, 154 (1960).
10. RAPKIN, E., Technical Bulletin, January 1962, Packard Instrument Company, Inc., La Grange, Ill.

ANALYTICAL BIOCHEMISTRY 5, 291–294 (1963)

Studies on the Separation of Pyridine Nucleotides by Thin-Layer Chromatography[1]

ROBERT LEE SCHEIG, RITA ANNUNZIATA, AND LEROY A. PESCH

From the Department of Internal Medicine, School of Medicine, Yale University, New Haven, Connecticut

Received June 20, 1962

Thin-layer chromatography has evolved as an analytical method having several advantages over separations using paper chromatography. The main advantages are speed of analysis and ease of recovery of the sample from the supporting media. The present report describes a thin-layer chromatographic method for the separation of oxidized and reduced diphosphopyridine and triphosphopyridine nucleotides together with data concerning the stability of these compounds under the conditions of tissue extraction procedures.

METHODS

Ascending one-dimensional chromatography on 20 × 20 cm glass plates was used. The plates were uniformly coated with Silica Gel G to a thickness of 150 μ.[2] The coated plates were activated for 1 hr at 120°C, after which they were stored over silica gel in a desiccator before use. An aqueous solution containing 0.01–0.04 μmole of pyridine nucleotide was applied at the origin with a 10-μl syringe,[3] and the plate was then placed in a $10\frac{3}{8} \times 2\frac{3}{4} \times 10\frac{1}{4}$″ chromatography jar[4] containing 150 ml of solvent. Localization of the compounds on the plate was achieved by spraying lightly with 50% sulfuric acid and heating in an oven at 200°C for about 10 min until charring occurred. Staining of the nucleotides was also readily accomplished by putting the plates in a covered chromatography jar containing resublimed iodine crystals. While samples appear as yellow spots within 2–3 min by this method, the iodine sublimes rapidly and staining is no longer visible.

Quantitative recoveries of the individual compounds from the ad-

[1] Supported by research grants A-714 and A-5526 from the National Institutes of Arthritis and Metabolic Diseases, United States Public Health Service.

[2] Brinkman Instruments, Inc., Great Neck, New York.

[3] Hamilton Company, Whittier, California.

[4] Arthur H. Thomas Company, Philadelphia, Pennsylvania.

sorbant were carried out as follows. Plates were air-dried, placed briefly in the iodine jar to outline the compounds, and the appropriate delineated areas scraped into a test tube. DPN was eluted from the Silica Gel G with 0.1 *M* Tris buffer, pH 10.1, while 0.1 *M* phosphate buffer, pH 7.5, was used for DPNH, TPN, and TPNH. DPN, TPN, and TPNH were assayed enzymically by the method of Ciotti and Kaplan (1) and DPNH by the method of Kornberg (2).

Sodium isocitrate, isocitric dehydrogenase, oxidized glutathione, glutathione reductase, sodium pyruvate, DPN, DPNH, TPN, TPNH, and muscle lactic dehydrogenase were obtained from Sigma Chemical Company. Crystalline yeast alcohol dehydrogenase was obtained from C. F. Boehringer.

RESULTS

The solvent systems and resultant separations are shown in Figs. 1 and 2. Within a running time of 2 hr, diphosphorylated nucleotides are easily separated from the triphosphorylated nucleotides; however, 5 hr

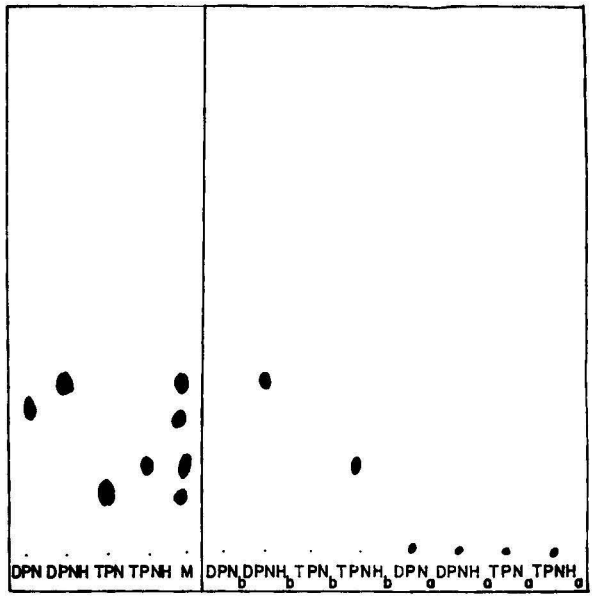


FIG. 1. Tracing of chromatoplate showing separation of pyridine nucleotides using isobutyric acid:concentrated ammonium hydroxide:water (66:1:33) (3) as the solvent system; 0.03-μmole sample applied at origin. M contains 0.03 μmole of each nucleotide, mixed before applying. Subscript b refers to the nucleotides treated with sodium carbonate and subscript a refers to the nucleotides treated with trichloroacetic acid as described in text. Running time 5 hr. Developed in iodine fumes.

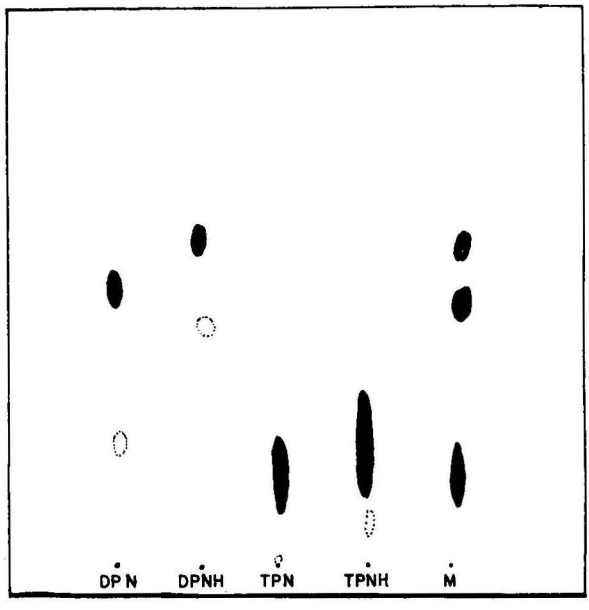


FIG. 2. Tracing of chromatoplate showing separation of pyridine nucleotides using ethanol:1.0 *M* ammonium acetate (pH 7.5) (50:50) as solvent system; 0.03-μmole sample applied at origin. M contains 0.03 μmole of each nucleotide, mixed before applying. Open circles mark location of compounds after migration in solvent system of ethanol:ammonium acetate (pH 7.5) (70:30). Running time 5 hr. Developed in iodine fumes.

was required to effect separation of oxidized and reduced diphospho- and triphosphopyridine nucleotides. Slight variation in migration was noted from plate to plate with this solvent system, thus making it necessary to chromatograph known compounds simultaneously with unknowns. For this reason R_f values are not reported. Increasing the polarity of the solvents improves separation, increases distance of migration, but also increases migration time, while varying the pH of the solvent systems had little effect on any of these parameters.

After separation with isobutyric acid:ammonium hydroxide:water, 100% of the DPN and 75% of the TPN that had been applied could be recovered by elution. The reduced nucleotides could not be recovered, nor had they been converted to their oxidized counterpart. These results parallel the experience of Burton and San Pietro using paper chromatography (4).

Mixtures of pure pyridine nucleotides were treated under conditions similar to those employed in the usual tissue extraction methods (1). To 1 μmole of each pyridine nucleotide in water was added 5 vol of

0.1 *M* sodium carbonate. This solution was placed in a boiling water bath for 30 sec, immediately chilled in ice, neutralized, and the equivalent of 0.03 μmole of nucleotide applied to the plate. The resultant chromatograph is shown on the right in Fig. 1 labeled with subscript b. It demonstrates that only the reduced nucleotides were stable after this treatment. Alternatively to 1 μmole of each pyridine nucleotide in water was added 5 vol of 5% trichloroacetic acid. This solution was then neutralized and an aliquot containing 0.03 μmole of nucleotide was applied. As can be seen from the far right of Fig. 1 labeled subscript a, no pyridine nucleotide separation could be seen, though staining was frequently noted at the origin or extending from the origin to the solvent front.

It is concluded that thin-layer chromatography offers a rapid and convenient way by which the oxidized and reduced pyridine nucleotides can be separated. After treatment of purified pyridine nucleotides by techniques employed in tissue extraction, only the reduced nucleotides remain chromatographically stable. This suggests that reasonable caution should be exercised in the interpretation of data pertaining to quantitative measurement of pyridine nucleotides in such tissue extracts.

REFERENCES

1. Ciotti, M. M., and Kaplan, N. O., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. III, p. 890. Academic Press, New York, 1957.
2. Kornberg, A., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. I, p. 441. Academic Press, New York, 1957.
3. Pabst Laboratories Circular O.R-18, April, 1961.
4. Burton, R. M., and San Pietro, A., *Arch. Biochem.* **48**, 184 (1954).

ANALYTICAL BIOCHEMISTRY 5, 295–312 (1963)

Chromatography on Cellulose Phosphate of Polysaccharide Hydrolases from Fungi[1]

J. H. McCLENDON AND J. H. KREISHER[2]

From the Department of Agricultural Biochemistry and Food Technology, University of Delaware, Newark, Delaware

Received July 6, 1962

The purpose of this paper is the demonstration of a method for the chromatography of proteins which has some unique features. In demonstrating this method, study is made of a crude commercial fungal preparation containing hydrolases acting on a select group of polysaccharides.

We have done sufficient work, in addition to that described here, to be confident of the applicability of the method to a variety of enzyme preparations. Indeed, provided the salt concentration can be properly adjusted, crude preparations can be separated directly on the column without prior dialysis. However, in such a case the ultraviolet absorption record is less meaningful.

The chromatography of proteins has been reviewed (1, 2), and the preparation of ion-exchange celluloses has been described (3). Cellulose phosphate has found but occasional or incidental use in protein adsorptions (4–8). It has been used as an exchanger for inorganic cations (9–15), and it is commercially available. We turned to the use of cellulose phosphate for two reasons. First, we wanted a cation exchanger so as to automatically eliminate adsorption of contaminating acidic polysaccharides or nucleic acids. Second, the isoelectric points of some of the enzymes of interest to us were too low to permit complete adsorption on carboxymethylcellulose. The cellulose phosphates are relatively resistant to cellulase (16).

We have used a gradient elution scheme for column operation. Such systems have been described before for various purposes, but a new feature, so far as we are aware, is the use of a pH gradient covering the range 2.2 to 7. We have considered pH as more fundamental than salt concentration and have not tried salt gradients. The gradient is accomplished with the simplest of equipment and so should be widely useful.

[1] Published as Miscellaneous Paper No. 424 with permission of the Director of the Delaware Agricultural Experiment Station.

[2] Present address: Tufts University School of Medicine, Boston, Massachusetts.

Although the enzymes came off the column in overlapping zones, the pH of the effluent associated with peaks of enzyme activity appeared to be characteristic of the enzymes concerned and was modified only to a minor extent by other components of the sample. The elapsed time for the actual chromatography was only about 8 hr.

The enzymes studied were chosen mainly from our interest in the analysis of plant cell wall polysaccharides. The pectolytic enzymes have been frequently reviewed (17, 18). Amylases and proteolytic enzymes are well known, and cellulases and xylanases have been reviewed (19, 20); but little is known about enzymes acting on galactan, araban, and galactomannan.

METHODS AND MATERIALS

Analyses

Polygalacturonase was determined on chromatographic fractions with a cup-plate assay (21, 22) using 0.05 *M* citrate buffers. Reducing group assay was done by the method of Willstätter and Schudel (23). Cellulase was determined by viscometry using carboxymethylcellulose solutions. The hydrolysis of galactomannan (guar gum) was determined by viscometry (0.2% solutions), and by the elimination of the ability to gel with borate. Xylan, araban, and galactan hydrolyses were detected by paper chromatography of the hydrolysis products of 2% commercial xylan or a pectate rich in galactan and araban (lupine seed). Amylase was determined by the loss of the iodine test (0.2% amylopectin) or sometimes by viscometry (2% amylopectin). Gelatin hydrolysis was determined on a 2% solution by viscometry or by inducing gelation of the unchanged gelatin at reduced temperature.

The nitrogen content of dried preparations was determined by digestion and direct Nesslerization. Protein in chromatographic fractions was determined by the phenol-biuret test (24).

Paper chromatography of the simple sugars was done by standard methods. The separation of galacturonic acid and the oligouronides from the partial hydrolysis of pectate was done using a solvent system of isopropanol, acetic acid, and water (2:2:1, parts by volume). The papers were dipped in color developer (2.5% aniline trichloroacetate, dissolved in a mixture of 1 part acetic acid and 9 parts acetone), and heated for 3–4 min at 100°C. The spots were cut out, eluted with acid alcohol (25), and read in the spectrophotometer at 350 mμ. As no oligouronide standards were available, the results are presented as relative optical density values.

Chromatographic Materials and Apparatus

Cellulose phosphate was prepared from Solka floc by the method of Peterson and Sober (3) (0.45 mmole P/gm dry weight) for the early work and the large preparation described in this paper. The analytical chromatography reported here was done with Brown Company cellulose phosphate (0.9 mmole P/gm, Selectacel-P, available from Schleicher and Schuell) after adjustment to pH 2 in dilute NaCl, washing by decantation of the fines, and drying with acetone and air at room temperature.[3] Washing with ammonia, in an attempt to get rid of yellow-brown impurities, resulted in an irreversible change leading to columns that clogged completely with alkali. The impurities were not washed out up to pH 9 and so were permitted to remain and show up on the ultraviolet records. Columns 0.6 cm in diameter were packed with a suspension of 1 gm cellulose phosphate in about 100 ml of starting buffer (in a reservoir attached to the top of the column). Air pressure of about 20 cm Hg was used to pack the lower half of the column and then was increased to 50–60 cm Hg. The reservoir must be swirled periodically to prevent settling. The bed height with the Selectacel mentioned was about 27 cm, giving a bed volume of about 7 ml. If necessary, the top of the column can be compressed with a rod. If a sizable portion of the enzyme sample is insoluble at the starting pH, the top layer must be carefully stirred up to disperse the precipitate in the cellulose and prevent clogging.

The ultraviolet absorption of the effluent solution was monitored continuously with the aid of a colorimeter previously described (26) and a potentiometer recorder. Fractions were collected in 13×100 mm test tubes in a Research Specialties Company fraction collector with a timing unit set for 4 min. A Sigmamotor peristaltic pump provided a constant flow rate (about 0.5 ml/min), using $\frac{1}{32}$ in. ID Tygon tubing. Considerable difficulty was had with contamination of solutions with ultraviolet absorbing impurities from the Tygon, and this pump has since been replaced by a Beckman solution metering pump. The tubing was glass capillary where possible. Silicone, Teflon, and ether-extracted gum rubber tubing did not contaminate solutions. Capillary Teflon tubing connected the colorimeter effluent with the fraction collector. Citric acid also contained brown, ultraviolet-absorbing impurities, some brands more than others. These impurities were adsorbed on the cellulose phosphate and eluted in their turn. Most of this contamination was removed by

Note added in proof. The Selectacel-P, washed at pH 2, is usable for only 6–9 months; after this, columns clog when alkaline. Selectacel-P as received appears to be stable.

passing the stock solution through a cellulose phosphate column, but a small peak still appeared at about pH 5 in the blank runs.

The pH of the effluent fractions was determined in the 13-mm tubes with miniature (Leeds and Northrop) electrodes (at least every fifth tube was measured). The conductivity of the effluent solutions was determined with a commercial line operated bridge and an assembly small enough to fit into the 13-mm test tubes.

Analytical Chromatographic Method

The apparent second pK of cellulose phosphate depends on the ionic medium in which the titration is performed (3). Values obtained in this laboratory by titration of the free acid were about 7.4 in 0.01 M NaCl and 6.2 in 0.5 M NaCl. The apparent first pK's determined at the same time were 2.8 and 2.6, respectively, but must depend upon the ratio of cellulose phosphate to solution, since this is a strong acid ionization.

Gradient elution is done by the continual mixing of two solutions, either with the mixing chamber volume constant or by permitting the two reservoirs to come to the same level (27). With a constant-volume mixing chamber as used here, the effluent composition asymptotically approaches the composition of the reservoir solution. If instead of having a gradient of NaCl (for example) running into distilled water, one has a solution of a weak acid in the mixing chamber and that of a strong base in the reservoir, the pH of the effluent will be the result of both the titration and the dilution of the acid. The general form resembles the titration curve, but is asymmetric. In order to design a pH gradient, one must know the volume of alkali required to neutralize the acid in the mixing bottle. The results of an experiment with acetic acid were in accord with the usual gradient elution equation, and the volume of NaOH to give an arbitrary end point is proportional to the mixing bottle volume and to the logarithm of the initial mixing bottle concentration. In addition, the concentration of NaOH controls the form of the gradient.

The solutions used in this work were as follows: Starting buffer and mixing bottle contents (100 ml): 0.05 M citric acid, 0.05 M acetic acid, 0.05 M NaCl. Reservoir: 0.05 M NaOH. In this system, the loss of sodium ion from the mixing bottle is exactly compensated for by sodium from the reservoir. The dilution of chloride is compensated for by citrate and acetate due to the influx of hydroxide ion, which reacts with the organic acid present. Eventually the organic buffer is diluted and titrated so as to be ineffective, and the pH rises more rapidly. However, the buffering of the phosphate of the column intervenes and permits a longer range. By the time the column is titrated the mixing bottle contents are very alkaline and the break-through is precipitous.

Examples of the effluent pH obtained by gradient elution through cel-

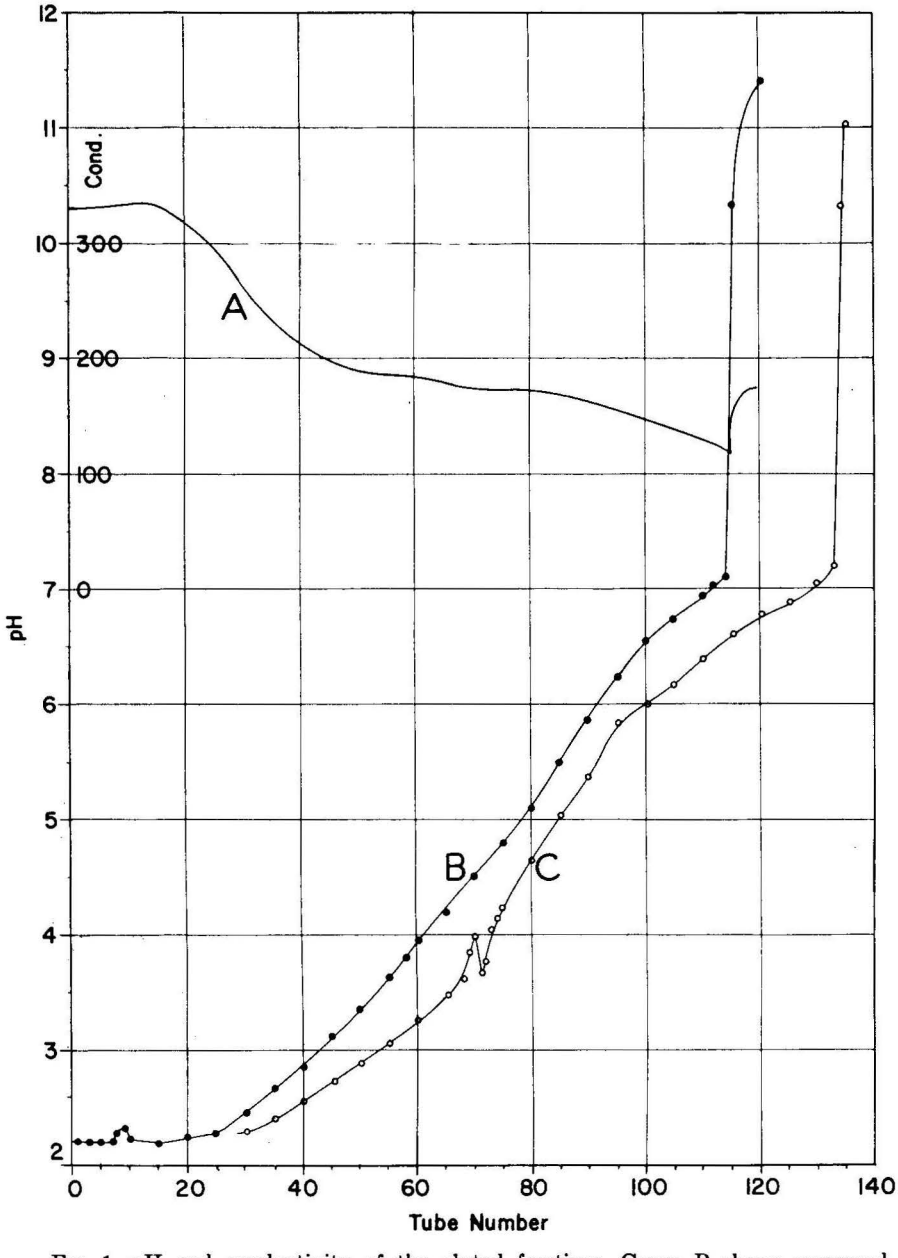


FIG. 1. pH and conductivity of the eluted fractions. Curve B shows measured pH's of fractions using gradient starting with 0.05 *M* each of citric acid, acetic acid, and NaCl. Gradient was started at fraction 15. Flow through column of cellulose phosphate plus sample was started at zero. Curve A shows conductivity in relative units on the same run. Curve C shows pH of similar run without acetic acid.

lulose phosphate columns are shown in Fig. 1. In these curves, citric acid is chiefly responsible for the trend from pH 2.2 to about 5.5 or 6.0, while cellulose phosphate controls the pH of the buffer above this. The trend in conductivity of the effluent is also shown. The initial drop in conductivity is due mainly to the replacement of chloride by citrate, but the gradual fall in conductivity from about pH 5.5 to the end point must be due to the removal of sodium ions by ion exchange, since the conductivity goes below the minimum values obtained without a column. This decrease in sodium concentration from 0.05 *N* to about 0.03 *N* was verified by flame photometry. Except for this loss due to ion exchange, the sodium concentration in the effluent is constant in the system described; the calculated ionic strength, however, is constant only to about pH 3.5, and then rises from the initial value of 0.05 to 0.08 or more. In addition to citric acid and NaCl, the initial solutions contained acetic acid, which had the effect of smoothing out a break in the effluent pH curve at pH 3.8. On one occasion when acetic acid was not being used and a large protein peak came at this point, the trend in pH temporarily reversed, as shown by the lower curve in Fig. 1. Repetition with acetic acid added showed no such inflection in the pH curve. In the portion of the curve controlled by phosphate, the progress of the highly alkaline region down the column could be seen as a colored band of impurity washed off the column. This band was about half way down the column when the effluent was at a pH of 6.5. It might be expected from the drop in conductivity that the effluent above pH 6.5 be poorly buffered; pH values in this range tended to rise upon standing overnight.

Preparation of Samples for Chromatography

The starting material was the Nutritional Biochemicals Corp. "Pectinase," which, according to the supplier, is derived from the fungus *Rhizopus tritici*.

On the basis of experiments by Kreisher (28) using gradient elution schemes similar to that described above, a stepwise elution sequence was devised to isolate the main polygalacturonase activity with the greatest purification and recovery. A column of 6 cm in diameter containing 50 gm of cellulose phosphate (0.45 mmole P/gm) was used with solutions at an ionic strength of 0.05. This was the minimum salt concentration which would dissociate polymeric complexes (28). Batches of 160 gm of Pectinase (723 gm total) were dissolved in 650 ml of water and the pH adjusted to 3.0 with acetic and hydrochloric acids. To this were added 1300 ml cold (—10°C) acetone and the precipitate centrifuged off at 0°C. The redissolved precipitate was again adjusted to pH 3.0 and the precipitation repeated. Subsequent operations were performed in the cold room.

The precipitate was taken up in 2 liters of pH 3.0 buffer (0.0033 *M* citrate, 0.04 *M* NaCl) and passed through the column which had been prewashed with the same buffer. The column was washed with this buffer (2 liters), and eluted with 4 liters each of 0.05 ionic strength citrate buffers at pH 3.5, 4.3, and 6.0 in this order, and finally, by 1 liter of 0.1 *M* ammonia. The ammonia eluate was neutralized and the pH 3.0, 3.5, and 4.0 and ammonia fractions were ultrafiltered by osmotically forced dialysis against Carbowax 6000 (2), kept frozen for a time and then all similar fractions pooled, precipitated with 2 vol of acetone (to remove most of the Carbowax), and freeze-dried. The pH 6.0 fractions were pooled and rerun through the column with the same sequence of buffers, and all but the pH 6.0 fraction added to their respective pool as above. The pH 6.0 fraction, containing nearly all the polygalacturonase, was concentrated on a large (12-in. diameter) pressure ultrafilter with a flat cellophane membrane. This concentration step was, however, unsatisfactory, due to the long time required. The concentrate was dissolved in water and freeze-dried.

RESULTS

The results of the batch separation are summarized in Table 1. When the eluates before ultrafiltration were assayed for enzyme content, the polygalacturonase activity (a viscometric method) in the pH 6 fraction

TABLE 1
FRACTIONATION OF *Rhizopus tritici* PECTINASE

Step or fraction	Wt. (gm)	N (%)	N (mg)	Recovered N (%)	Relative activity[b]		Tot. act.[b] (%)
					N basis	Wt. basis	
As recd.	723	0.66	4770	—	1.0	1.0	100
Acetone ppt.[a]	87	2.25	1950	—	2.5	8.5	—
Column eluates, ultrafiltered							
pH 3.0	11.78	3.93	450	(65%)	0.023	0.13	0.2
3.5	(0.003)	—	—	—	—	—	0.1
4.3	1.41	11.1	150	(22%)	0.90	14.4	2.8
6.0	0.40	8.8	36	(5.2%)	93.8	1270	69.0
NH_4OH	0.58	9.4	54	(7.8%)	0.87	13.2	1.0
Recovery	14.1	—	690	(100%)	—	—	72.6

[a] Calculated from other data.

[b] Arbitrary viscosity units for polygalacturonase determined before ultrafiltration.

was concentrated some 93-fold on a nitrogen basis and more than 1000-fold on a (final recovered) weight basis. Three-fourths of the initial activity was recovered and of this 94% was in the pH 6 fraction. Unfor-

tunately, two-thirds of this was lost in the concentration step. The characteristics of the polygalacturonases present were ascertained by the use of the reducing group method and paper chromatography of the products.

The initial rate of hydrolysis of pectate by the various fractions at several pH values is shown in Fig. 2. It is readily seen that more than

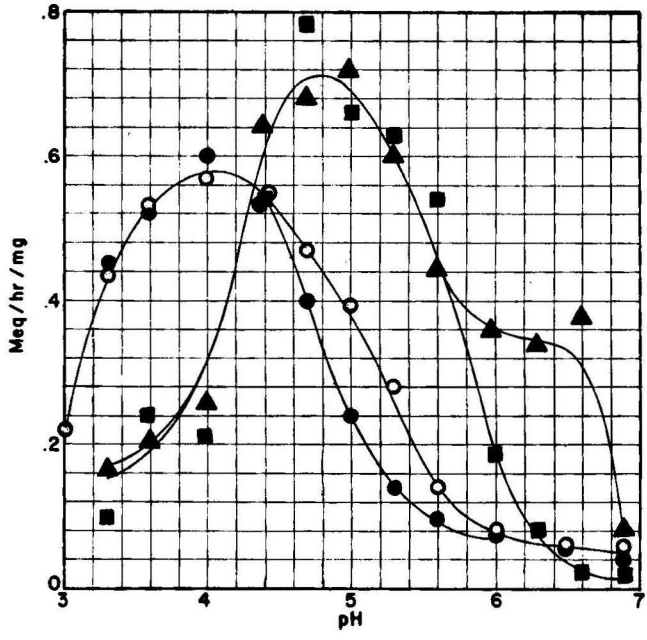


FIG. 2. Effect of pH upon rate of hydrolysis of pectate by the four large fractions as determined by production of reducing groups: filled circles, pH 3 fraction × 80; open circles, pH 4.3 fraction × 8; squares, pH 6 fraction × 1; triangles, ammonia fraction × 10.

one enzyme is present and that the pH 3 fraction and pH 6 fraction seem to be mainly enzymes with optima at pH 4 and 5, respectively. These two were submitted to simultaneous reducing group and chromatographic analysis, shown in Fig. 3. The production of mainly galacturonic acid and the high extent of hydrolysis indicate that the pH 3 fraction contained an exopolygalacturonase. The low extent of hydrolysis and the rapid production and lack of disappearance of oligouronides indicates that the pH 6 fraction contained essentially an endopolygalacturonase. The pH 4.3 fraction was a mixture of these two. The ammonia fraction, in addition to the endopolygalacturonase with pH 5 optimum, contained

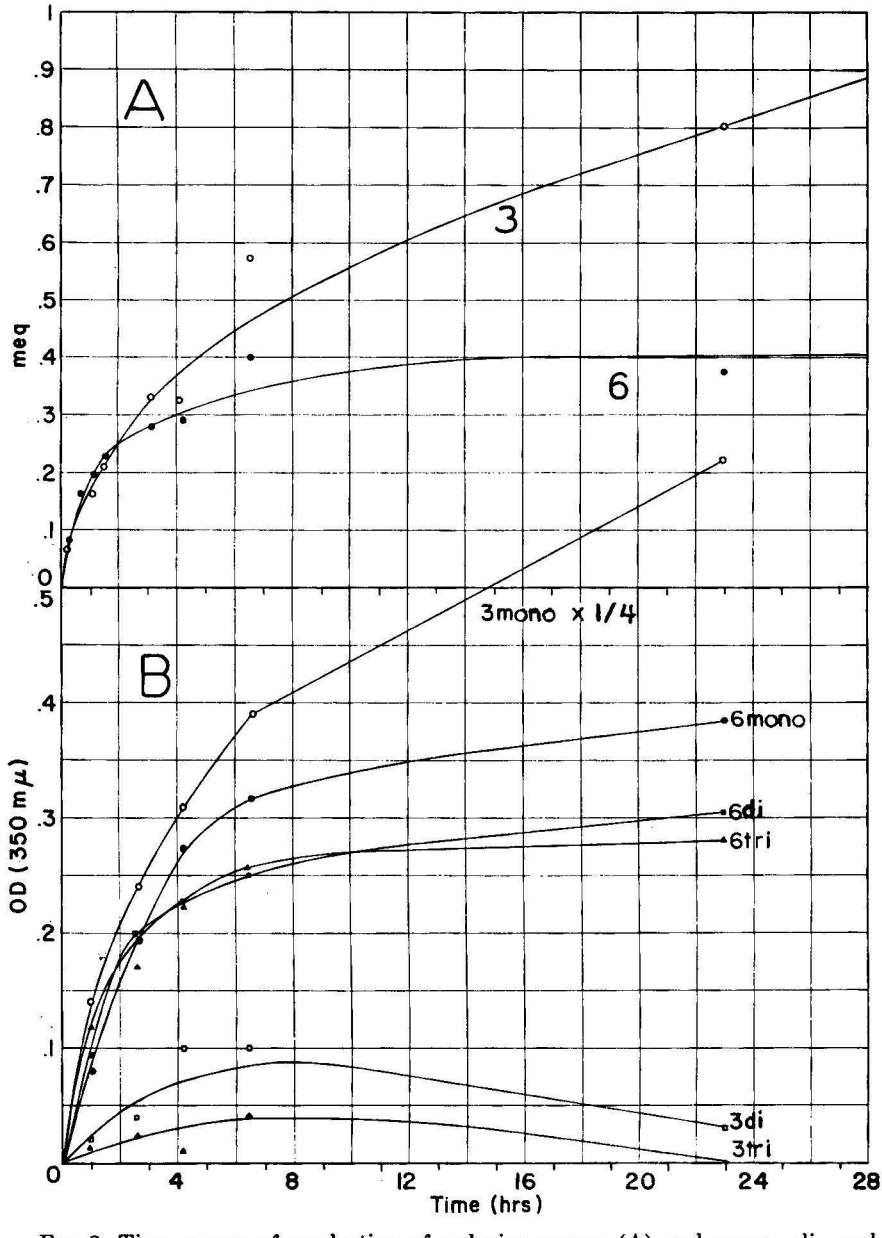


FIG. 3. Time course of production of reducing groups (A) and mono-, di-, and trigalacturonides (B), by the pH 3 and pH 6 fractions acting on pectate at pH 4.3. Filled symbols refer to the pH 6 fraction (0.0005%); open symbols refer to the pH 3 fraction (0.025%). The pH 3 monosaccharide curve has been multiplied by ¼ for convenience in plotting.

a minor fraction with a high pH optimum (pH 6.0–6.5), whose other characteristics remain uncertain.

The four main fractions were submitted to the gradient pH elution scheme outlined above, with concurrent ultraviolet recording and subsequent analysis for a variety of enzymes (Fig. 4). Samples applied to the column were 40 mg for the pH 3 fraction and 20 mg for the rest. These operations were all performed at room temperature.

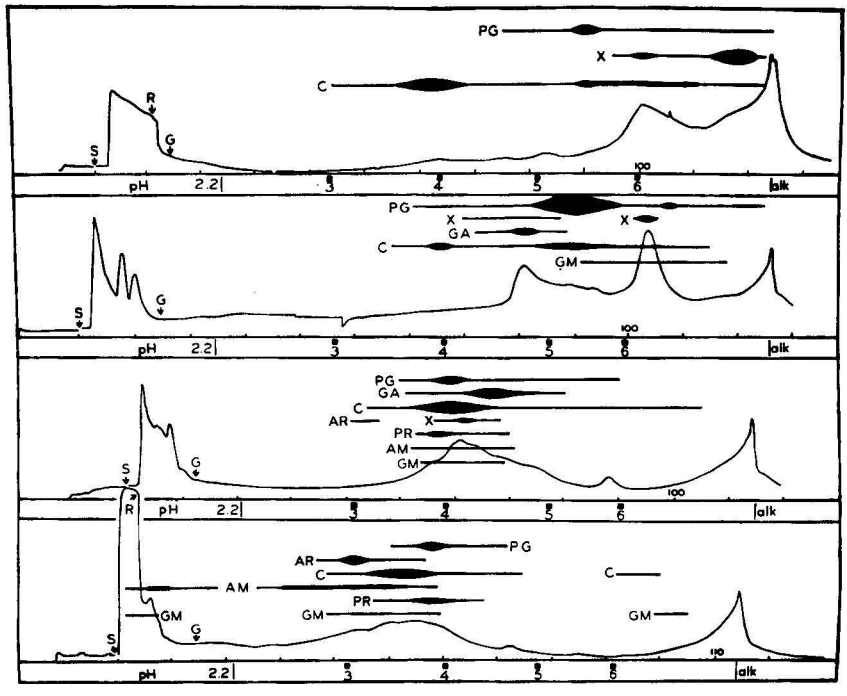


FIG. 4. Gradient pH elution of enzymes from cellulose phosphate columns. From the top down, the samples are: ammonia, pH 6, pH 4.3, and pH 3 fractions. Abscissas show numbers of eluate fractions (above the line) and measured pH of same (below the line). The continuous curve is a tracing of the recording of the ultraviolet colorimeter, with the ordinate in per cent absorption. S shows where column was inserted in the system with a brief disconnection. R shows reversal of flow in the colorimeter cell (see text). G shows where the inflowing gradient was started; the line at the right of "2.2" shows where the outflowing pH began to rise. The line to the left of "alk" shows the position of the sudden rise in pH. The enzymes (see text) are indicated semiquantitatively by the width of the lines superimposed above the colorimeter record, with the extent of detection by the methods used shown by the length of those lines. PG = polygalacturonase; X = xylanase; C = cellulase; GA = galactanase; GM = galactomannanase; AR = arabanase; PR = proteinase (gelatinase); AM = amylase.

It is evident, first of all, that a large amount of ultraviolet-absorbing material was not, or was only slightly, adsorbed on the cellulose phosphate. It is likely that at least some of this was concentrated from the reagents used in the batch separation. The pH 3 and ammonia fractions contained enough unadsorbed solute (polyethylene glycol from the ultrafiltration) to make a heavy layer in the colorimeter, and required the reversal of the inlet and outlet to remove this layer. A T-joint has since been inserted just before the absorption cell to be able to eliminate air bubbles in the cell and so permit flow from the top down. The final rise and peak on all the records also occurred with the blank runs and was due to colored material eluted from the cellulose phosphate.

Due to the large number present, the individual components overlapped considerably and the ultraviolet record shows little detail. In general, the gross fractions contained components which came off the column in the order to be expected from the pH at which they were eluted in preparation. Thus, almost all of the ultraviolet-absorbing material in the pH 3 fraction came off before that of the pH 6 fraction. The two large peaks in the pH 6 fraction caused some concern. The one at the higher pH was associated with black material insoluble at pH 2.2. The eluate fractions from this sample were analyzed for protein by the Folin-Lowry method in two separate experiments (Fig. 5; compare Fig. 4). This method is sensitive to peptide links as well as to phenols; while the ultraviolet recordings show any kind of aromatic grouping, but not the peptide links. The difference between the two figures (4 and 5) is plain, although the protein did show up in the recording.

The polygalacturonase assays on the same experiments shown in Fig. 4 are summarized in Fig. 6. Numerous assays having demonstrated that the pH values of the eluted fractions from a given sample were quite reproducibly correlated with the polygalacturonase eluted, and, since the flow rate was not controlled exactly, the figure has been drawn with the effluent pH as the abscissa instead of the tube number. One peak of activity appeared at pH 3.9–4.0. This must be an expolygalacturonase, as it is the only peak found in the pH 3 fraction. The major peak in the pH 6 and ammonia fractions was at an effluent pH of about 5.0, 5.3, or 5.5 when assayed with the cup-plate method at pH 3.5, 4.3, and 6.5, respectively. This is most clearly shown by the ammonia fraction (Fig. 7), and it is fairly clear that two enzymes are involved which have slightly different elution patterns and pH optima for activity. The upper part of Fig. 7 shows an experiment using the citric acid-NaCl mixture without acetic acid for elution, and the two peaks are quite evident. It seems likely that the shoulder on the pH-activity curve for the ammonia fraction (Fig. 2) is the result of the presence of the enzyme located by

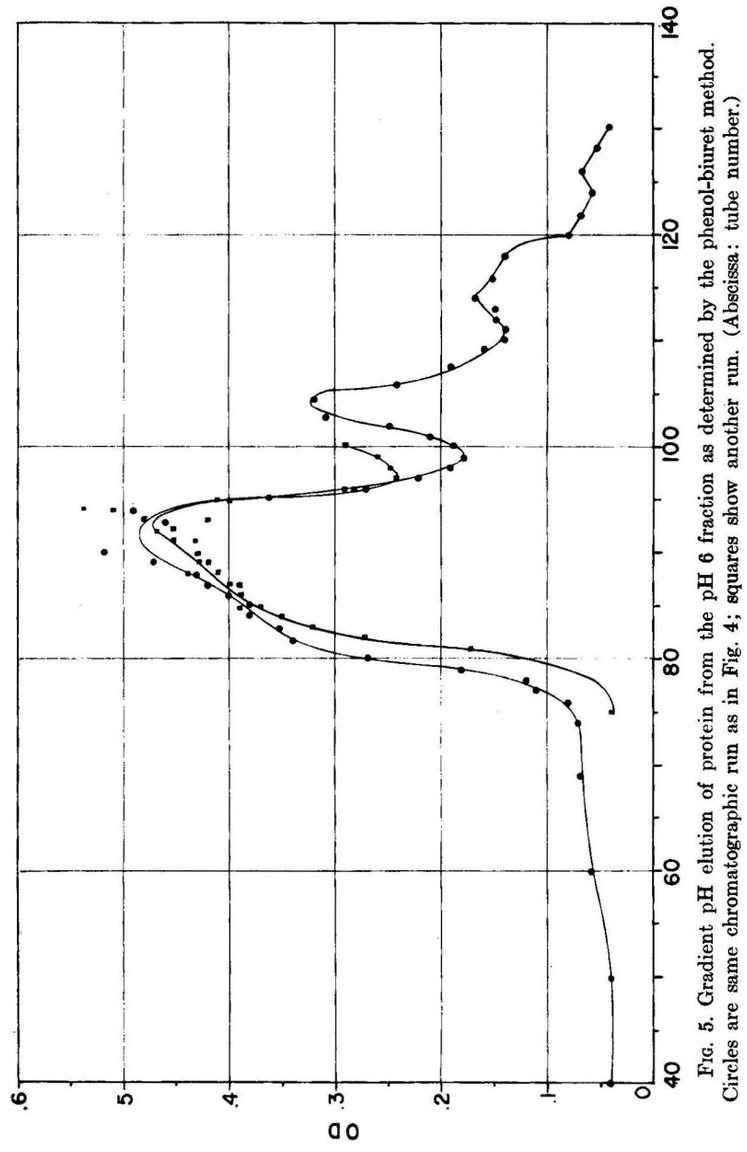


FIG. 5. Gradient pH elution of protein from the pH 6 fraction as determined by the phenol-biuret method. Circles are same chromatographic run as in Fig. 4; squares show another run. (Abscissa: tube number.)

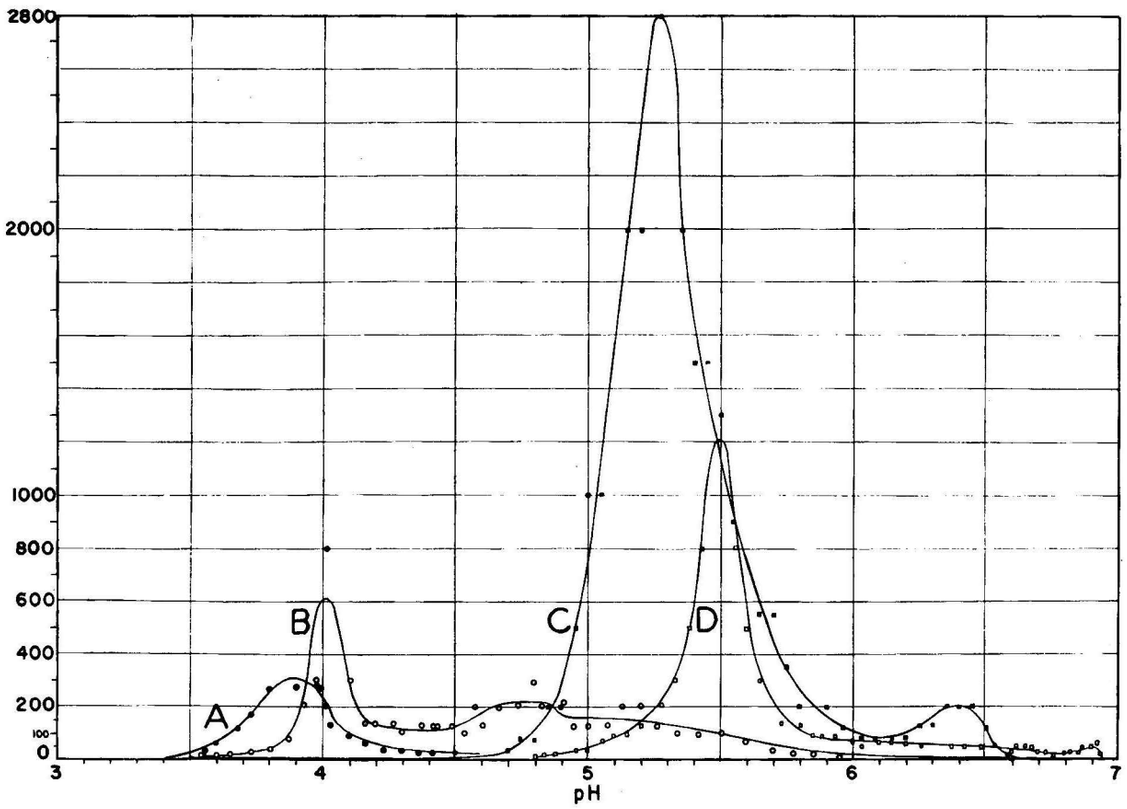


FIG. 6. Elution pattern of polygalacturonases as measured by cup-plate assay. Ordinate shows arbitrary enzyme units. Filled circles (A) show pH 3 fraction × 50; open circles (B) show pH 4.3 fraction × 10; filled squares (C) show pH 6.0 fraction × 1; open squares (D) show ammonia fraction × 10. All assayed at pH 4.3 except the ammonia fraction, done at pH 5.0 (see Fig. 7).

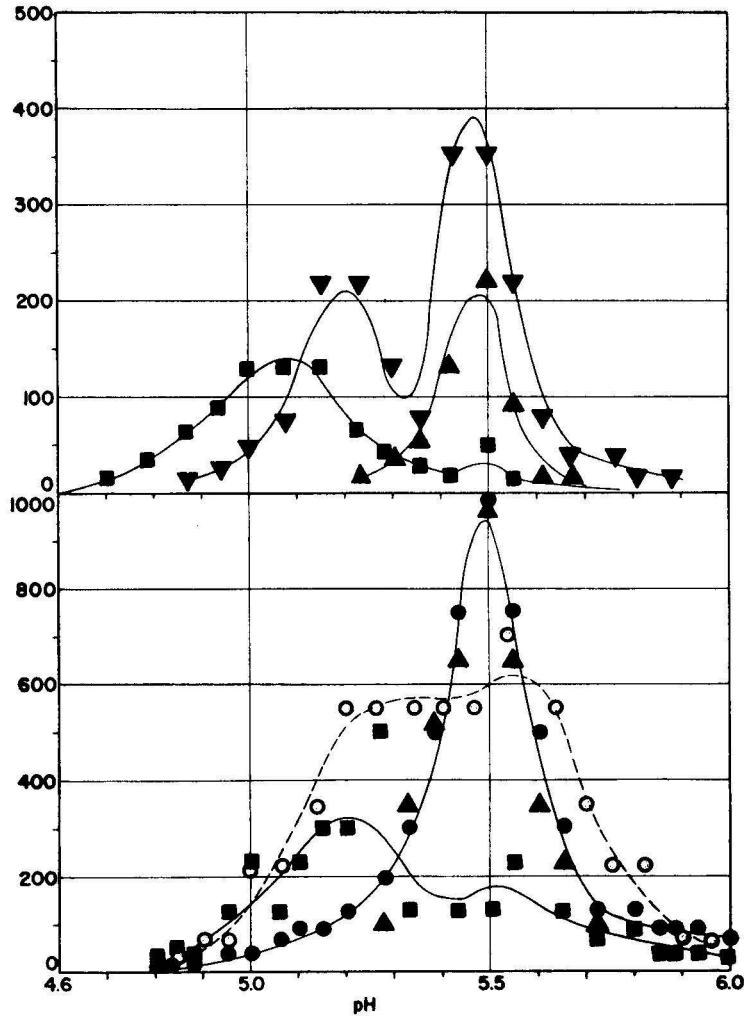


FIG. 7. Elution pattern of ammonia fraction as determined by cup-plate assay at several pH's. Upper and lower parts are different runs. Upper: assayed at pH 3.5 (squares) × 10; pH 4.3 (▼); pH 6.5 (▲) × 10. Lower, same run as shown in Figs. 4 and 6; pH 3.5 (■) × 10; pH 5 (●) × 10; pH 6.5 (▲) × 100. Dashed curve (○) shows pH 6 fraction assayed at pH 5, same run as shown in Figs. 4, 5, and 6. Compare assay at pH 4.3 in Fig. 6.

the cup-plate assay at pH 6.5 (Fig. 7). The minor peak on the pH 6 fraction is associated with the very large ultraviolet peak and may be spurious. There is, in addition, a suggestion of another component in the pH 4.3 fraction, at pH 4.8.

The assays for xylan hydrolysis showed two distinctly different enzymes. One, which liberated what appeared to be di-, tri-, and small amounts of higher saccharides of xylose and no monoxylose, was eluted at about pH 6.0 and above from the pH 6 and ammonia fractions. The other, which liberated free xylose without obvious oligosaccharides, was eluted at about pH 4.0 from the pH 4.3 fraction, and between pH 4 and 5 from the pH 6 fraction.

The assays for araban hydrolysis, liberating free arabinose from lupine pectate, showed an enzyme eluted at about pH 3.0 in both the pH 3 and 4.3 fractions. In the more active pH 3 fractions, this enzyme also liberated arabinose from the xylan sample. The assays for galactanase, using the lupine pectate, showed an enzyme eluted at about pH 4.1, 4.4, or 4.7 from the pH 3 (trace), 4.3, and 6 fractions, respectively.

Amylase appeared to be heterogeneous, being eluted in three zones. One was not adsorbed at pH 2.2 and appeared mainly in tubes 5–10 from the pH 3 fraction. The second was eluted between pH 2.5 and 3.6 from the pH 3 fraction; the third was eluted between pH 3.7 and 4.7 from the pH 4.3 fraction.

Enzyme(s) hydrolyzing guar gum were present in small amount, but due to the sensitivity of the test were readily found. In the pH 3 fraction, enzyme was eluted at pH 2.2 (unadsorbed), 2.8–4.0, and about 6.6. Enzyme appeared about pH 4.1 and from pH 5.3–6.8 from the pH 4.3 and 6 fractions, respectively.

Cellulase, determined by viscometry, appeared in two places. One was eluted at about pH 3.6 (pH 3 fraction), or about pH 4.0 (pH 4.3, 6, or ammonia fractions). The other was eluted above pH 5.

An enzyme hydrolyzing gelatin was eluted at about pH 3.8 from the pH 3 and 4.3 fractions.

DISCUSSION

It appears likely that some of the variation in the effluent pH at which an enzyme was found was not due to the presence of different enzymes, but rather that the presence of other eluted substances carried the enzyme off the column at a lower pH than would otherwise be the case—galactanase, for example. Arabanase, being eluted before the bulk of the protein, was not affected in this way.

The occurrence, in the original four fractions, of enzymes which chromatographed far outside the range of the bulk of the fraction is difficult to explain. Cellulase is the chief offender in this regard, but the galactomannanase in the pH 3 fraction also does not conform. The anomalous behavior of the cellulase in the ammonia fraction possibly may be due to adsorption on the cellulose proper (that is, not on the

phosphate) in the initial fractionation, since the ratio of protein to cellulose phosphate was about tenfold greater in the analysis than in the preparation.

The apparent occurrence of more than two enzymes hydrolyzing pectate deserves some comment. Endo- and exoenzymes have been well recognized (17), but Phaff and Demain (30) and Mill and Tuttobello (31) have claimed their preparations of endopolygalacturonase are homogeneous. On the other hand, Schubert (32) has shown that three or four distinct enzymes could be shown in preparations from *Aspergillus niger* by selective inactivation and adsorption. It is not clear, however, in what way these differ enzymically. In preparations from *Botryosphaeria ribis,* there were enzymes with two or three different pH optima acting on high-viscosity pectate as determined by a viscometric method (33).

Cellulase, too, has been highly purified in some cases, but crude preparations have been shown to be heterogeneous (34–36). Jermyn (35) found in *Aspergillus oryzae* at least eight components acting on glucosides. Reese and Levinson (36) proposed that enzymes acting on soluble cellulose derivatives (such as we used for assay) may not necessarily act on native cellulose. We have not tried our preparations on cotton fiber, but the four bulk fractions prepared in this study all acted upon cellophane.

Whistler and Masak (37) have reported on xylan-hydrolyzing enzymes derived from *Aspergillus foetidus,* finding both an endo- and an exoenzyme, which they partially separated.

SUMMARY

1. A method is described for the gradient elution of enzymes from a column of cellulose phosphate. By starting with 0.05 *M* each of citric acid, acetic acid, and sodium chloride in a constant volume mixing chamber, and 0.05 *N* NaOH in the reservoir, a pH gradient is produced. When this buffered solution passes through the column of cellulose phosphate, the pH of the effluent rises in a nearly linear manner from pH 2.2 to 7, whereupon the pH rises precipitously.

2. With this system of separation, the chromatographic behavior at room temperature is described of three polygalacturonases (one exo and two endo), two xylanases (exo and endo), two cellulases, an arabanase, a galactanase, a proteinase, and what appear to be three amylases and three enzymes hydrolyzing galactomannan. The enzymes were derived from a commercial "Pectinase" from *Rhizopus tritici.* Continuous recording of the ultraviolet absorption is also shown.

3. A batchwise separation of the crude *R. tritici* enzymes on a pre-

parative column of cellulose phosphate is described and the resulting four fractions analyzed by the above procedure. A purification of endopolygalacturonase activity of 93-fold on a nitrogen basis and 1000-fold on a solids basis was achieved in this preparative separation.

ACKNOWLEDGMENTS

The excellent technical assistance of Herbert G. Weaver and John L. Hess in portions of this work is gratefully noted.

REFERENCES

1. SOBER, H. A., AND PETERSON, E. A., *in* "Ion Exchangers in Organic and Biochemistry" (Calmon, C., and Kressman, T. R. E., eds.), p. 318. Interscience, New York, 1957.
2. TURBA, F., *Advan. Enzymol.* **22**, 417 (1960).
3. PETERSON, E. A., AND SOBER, H. A., *J. Am. Chem. Soc.* **78**, 751 (1956).
4. BROWN, G. L., AND MARTIN, A. V., *Nature* **176**, 971 (1955).
5. BROWN, G. L., AND MARTIN, A. V., *Symp. Soc. Exptl. Biol.* **12**, 6 (1958).
6. MARSHALL, M., METZENBERG, R. L., AND COHEN, P. P., *J. Biol. Chem.* **233**, 102 (1958).
7. METZENBERG, R. L., MARSHALL, M., AND COHEN, P. P., *J. Biol. Chem.* **233**, 1560 (1958).
8. MITZ, M. A., AND SCHLEUTER, R. J., *J. Am. Chem. Soc.* **81**, 4024 (1959).
9. ARTHUR, J. C., JR., AND MCLEMORE, T. A., *J. Am. Chem. Soc.* **78**, 4153 (1956).
10. AUDSLEY, A., JAMRACK, W. D., OLDBURY, A. E., AND WELLS, R. A., *Proc. U. N. Intern. Conf. Peaceful Uses At. Engery, 2nd, Geneva (1958)* **3**, 216 (1958).
11. HEAD, A. J., KEMBER, N. F., MILLER, R. P., AND WELLS, R. A., *J. Chem. Soc.* **1958**, 3418
12. HOFFPAUIR, C. L., AND GUTHRIE, J. D., *J. Biol. Chem.* **178**, 207 (1949).
13. JACUBOVIC, A. O., AND KNIGHT, C. S., *in* "Chromatographic and Electrophoretic Techniques," (Smith, I., ed.), p. 557. Heineman, London, 1960.
14. JURGENS, J. F., REID, J. D., AND GUTHRIE, J. D., *Textile Research J.* **18**, 42 (1948).
15. REID, J. D., MAZZENO, L. W., AND BURAS, E. M., *Ind. Eng. Chem.* **41**, 2831 (1949).
16. MCCLENDON, J. H., *Biochem. et Biophys. Acta* **48**, 398 (1961).
17. DEUEL, H., AND STUTZ, E., *Advan. Enzymol.* **20**, 341 (1958).
18. FISCHER, E. H., AND STEIN, E. A., *in* "The Enzymes" (Boyer, P. D., Lardy, H., and Myrbäck, K., eds.), 2nd Ed., Vol. 4, p. 301. Academic Press, New York, 1960.
19. LARNER, J., *in* "The Enzymes" (Boyer, P. D., Lardy, H., and Myrbäck, K., eds.), 2nd Ed., Vol. 4, p. 369. Academic Press, New York, 1960.
20. PIGMAN, W., *in* "The Enzymes" (Summer, J. B., and Myrbäck, K., eds.), 1st Ed., Vol. 1, part 2, p. 725. Academic Press, New York, 1951.
21. DINGLE, J., REID, W. W., and SOLOMONS, G. L., *J. Sci. Food Agr.* **4**, 149 (1953).
22. FINN, R. K., *Anal. Chem.* **31**, 975 (1959).
23. JANSEN, E. F., AND MACDONNELL, L. R., *Arch. Biochem.* **8**, 97 (1945).
24. LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L., AND RANDALL, R. J., *J. Biol. Chem.* **193**, 265 (1951).
25. WILSON, C. M., *Anal. Chem.* **31**, 1199 (1959).

26. McClendon, J. H., *Anal. Biochem.* **3,** 94 (1962).
27. Bock, R. M., and Ling, N. S., *Anal. Chem.* **26,** 1543 (1954).
28. Kreisher, J. H., Ph.D. Thesis, University of Delaware, 1961.
29. McClendon, J. H., and Somers, G. F., *Plant. Physiol.* **30,** 485 (1955).
30. Phaff, H. J., and Demain, A. L., *J. Biol. Chem.* **218,** 875 (1956).
31. Mill, P. J., and Tuttobello, R., *Biochem. J.* **79,** 57 (1961).
32. Schubert, E., *Biochem. Z.* **323,** 78 (1952).
33. McClendon, J. H., Somers, G. F., and Heuberger, J. W., *Phytopathol.* **50,** 258 (1960).
34. Grimes, R. M., Duncan, C. W., and Hoppert, C. A., *Arch. Biochem. Biophys.* **68,** 412 (1957).
35. Jermyn, M. A., *Australian J. Sci. Research* **B5,** 433 (1952).
36. Reese, E. T., and Levinson, H. S., *Physiol. Plantarum.* **5,** 345 (1952).
37. Whistler, R. L., and Masak, E., *J. Am. Chem. Soc.* **77,** 1241 (1955).

ANALYTICAL BIOCHEMISTRY 5, 313–320 (1963)

The Characterization of Estrogens

T. H. SIMPSON AND R. S. WRIGHT

From the Department of Scientific and Industrial Research, Torry Research Station, Aberdeen, Great Britain

Received July 23, 1962

INTRODUCTION

Naturally occurring estrogens are commonly identified by chromatography in a number of different solvent systems and by bioassay (1, 2); Kober reactions (3–5), the formation of sulfuric acid chromogens (6, 7), and related fluorimetric procedures (8, 9) are frequently adduced as supporting evidence. Less commonly, derivatives of the isolates are compared chromatographically with authentic estrogen derivatives (10, 11). When a sufficient quantity of the natural substance has been available, infrared absorption spectra (12, 13) and column chromatography (8) or countercurrent distribution (8, 14) of mixtures of the substance with isotopically labeled estrogen have been used to establish identity. The various procedures have recently been reviewed by O'Donnell and Preedy (15).

In the course of our work on the occurrence of estrogens in marine fauna, in which the concentrations of these compounds are frequently low (12, 16–19), it became clear that there was a need for some general procedure by which microgram quantities of estrogens could be adequately characterized. It seemed that adequate characterization might be based on the following criteria: (*a*) Paper chromatographic comparison of the natural estrogen with authentic material in several solvent systems. (*b*) Chromatographic comparison of at least two derivatives of the natural and authentic estrogen. The chromatographic systems should produce maximum separation of known estrogens and the derivatives should represent modifications of different reactive centers of the steroid molecule. In order to gain maximum sensitivity for the method, the sprays used to locate estrogens on the chromatograms should be nondestructive and the derivatives should be prepared sequentially.

By the method now described, it is possible, starting with less than 5 μg of estrogen, to chromatograph this in two separate solvent systems, to prepare in sequence three distinct functional derivatives, and to compare these with authentic materials.

METHOD

The phenolic steroids of a tissue or plasma extract are separated and purified by toluene-aqueous sodium hydroxide partition and subsequent chromatography by methods which have already been described (19). As an alternative to purification by paper chromatography, which may involve the running and elution of many papers, thin-layer chromatography on silicic acid-starch (95:5) using benzene-methanol (95:5) is recommended. It has the advantages of permitting greater loading and of rapidity of manipulation.

A portion of each isolate containing between 2 and 5 μg of estrogen is applied to Whatman No. 1 paper with the appropriate marker estrogen and irrigated with light petroleum-methanol (1) for sufficient time to ensure a movement of approximately 12–15 cm. After drying at room temperature the paper is drawn slowly and evenly through a freshly made solution of 2,7-dichlorofluorescein (0.001%) prepared by dissolving the reagent in a small volume of methanol and diluting with water, and viewed while wet under a glass shielded mercury vapor UV lamp having maximum transmission at 355 mμ. The dark, nonfluorescing zones are marked, and, after drying, extracted with methanol (3 portions, 1–2 ml each). The subsequent procedure to be followed is determined by the nature of the phenolic steroid suspected in the eluate.

If a nonalcoholic steroid, e.g., estrone, its 2-methoxy derivative, equilin, or equilenin is suspected, the eluate is evaporated *in vacuo* and acetylated with acetic anhydride and pyridine (1:1) overnight. The resulting ester is isolated by evaporation, chromatographed with authentic markers in the light petroleum-methanol system and located by use of the dichlorofluorescein reagent. The marked chromatogram is next hung, while still moist, in a closed tank containing concentrated ammonium hydroxide (d. 0.88). After 1 hr, the chromatogram is dried and the free phenolic steroids eluted from the marked areas with benzene-methanol (1:1). The eluates are then evaporated to low bulk and rechromatographed with appropriate markers in the toluene-propylene glycol system (20). After drying the paper at 100°C for 0.5 hr, the estrogens are located as their azo compounds, formed by drawing the paper successively through aqueous Fast Black Salt K (0.1%) and aqueous sodium carbonate (5%). The paper is then washed and is dried at room temperature; the azo compounds are eluted with benzene-methanol (1:1) and chromatographed with authentic Fast Black Salt K derivatives in toluene-light petroleum-ethanol-water (200:250:30:70) (1). Finally, the identity of tissue estrone is confirmed by reduction of its Fast Black Salt K azo derivative to the corresponding derivative of estradiol. The

chromatogram of the purified azo compound is drawn through a 1% aqueous solution of sodium borohydride, allowed to hang for 0.5 hr, washed with water until neutral, and then dried. The dye is then eluted with ether and chromatographed in the same system against authentic estradiol 17β Fast Black Salt K dye.

If an alcoholic steroid, e.g., estradiol, estriol, or a derivative of one of these, is suspected, the eluate is evaporated *in vacuo* and dissolved in formic acid (98%, 0.1 ml). The solution is allowed to stand for 4 hr and the formate isolated by evaporation. Alternatively, formylation may be achieved by hanging a chromatogram "spotted" with extract, over formic acid in a sealed tank for 15 hr. After chromatography, with appropriate authentic formates in the light petroleum-methanol system the esters are located by dichlorofluorescein and saponified on the paper by spraying with 4% aqueous sodium hydroxide and hanging at room temperature for 0.5 hr. The paper is next neutralized by hanging over concentrated hydrochloric acid and, after drying, the freed phenolic steroids eluted with benzene-methanol (1:1). The eluates are now chromatographed with authentic markers in either chloroform-formamide (21) or in toluene-propylene glycol (20); the former is the system of choice when estriol, the latter when estradiol or one of its derivatives, is suspected. The phenolic steroids are next converted to their Fast Black Salt K derivatives by the method already described and chromatographed against authentic compounds; the solvent system to be used when estriol is suspected is toluene-light petroleum-ethanol-water (200:100:30:70) (1). Finally, the identity of tissue estradiol is confirmed by oxidation of the Fast Black Salt K derivative, eluted from the last chromatogram to the corresponding derivative of estrone. The evaporated eluate is treated with chromium trioxide in pyridine (50 μl of 0.5% solution) at 37°C for 5 hr and the product isolated by the addition of methanol (0.25 ml) and water (1 ml) and subsequent extraction with ether. The extract is then chromatographed against authentic estrone Fast Black Salt K derivative in the system toluene-light petroleum-ethanol-water (200:250:30:70).

The following account of the identification of estrone and estradiol-17β in the ovaries of *Scyliorhinus caniculus* exemplifies the use of this method in the examination of tissue estrogens.

Dogfish ovaries (415 gm) were repeatedly extracted with chloroform-methanol (1:1) and the solvent evaporated *in vacuo,* leaving a fatty residue (78 gm). This was dissolved in toluene (3 liters, extracted with aqueous sodium hydroxide (4%; 6 $\times$ 400 ml), and the extract neutralized by addition of aqueous sulfuric acid (1:1). The solution yielded to ether a wax (177 mg) which, after further purification by partitioning between

toluene and sodium hydroxide, with subsequent neutralization and ether extraction, afforded a phenolic fraction weighing 98 mg.

This material was next divided into estrone- and estradiol-containing fractions by chromatography on Whatman No. 3 papers in the system light petroleum-methanol and these were further purified by chromatography on No. 1 papers in the same system.

One-half of the estrone fraction was chromatographed in the light petroleum-methanol system; after location by dichlorofluorescein it showed as a single spot having the same mobility as authentic estrone. It was eluted, acetylated, and rechromatographed, when it had the same R_f as estrone acetate. It was next saponified *in situ* by ammonia vapor, eluted, and chromatographed in the system toluene-propylene glycol, Fast Black Salt K being used as the locating reagent; R_f values of the natural and authentic estrone were again identical. The Fast Black Salt K azo derivative was eluted and rechromatographed in toluene-light petroleum-ethanol-water; the natural and authentic azo dyes showed identical mobilities. Finally the dye was reduced with sodium borohydride and eluted, yielding a dye which when rechromatographed showed the same mobility as authentic estradiol-17β Fast Black Salt K derivative. The amount of estrone in the extract was then estimated by the procedure previously used to determine estrone in the ovaries of cod (*Gadus callarias*) (19). The remaining half of the estrone fraction was divided into two equal portions which were then chromatographed in the systems light petroleum-methanol and toluene-propylene glycol; marker spots of accurately measured quantities (1, 1.5, 2.0, 2.5 and 3.0 μg) of authentic estrone were run on each chromatogram. Visual comparison of the Turnbull and of the Fast Black Salt K colors showed that the quantity of tissue estrone on each chromatogram was approximately 2.0 μg, corresponding to a concentration in the tissue of approximately 19 μg/kg.

The estradiol fraction was divided into two equal portions. One-half, after chromatography in light petroleum-methanol and location by dichlorofluorescein showed up as a single spot running level with authentic estradiol-17β. After being formylated *in situ*, it was eluted and rechromatographed in the same system; the natural and authentic estradiol formates had identical mobilities. After saponification and elution, the natural material was chromatographed in the system toluene-propylene glycol, the mobility again being identical with that of estradiol-17β. The Fast Black Salt K azo derivative, prepared on the paper, was eluted and chromatographed in toluene-light petroleum-ethanol-water; R_f values of natural and authentic compounds were again identical. Finally, the azo compound was oxidized with chromium trioxide in pyridine giving a product which on chromatography in the last men-

tioned system had the same mobility as authentic estrone Fast Black Salt K azo compound. The remaining half of the fraction was used for estimation of its content of estradiol-17β by the same procedure as that outlined for estrone. Comparison of the intensities of the Turnbull and of Fast Black Salt K colors with those produced from known quantities of authentic estradiol-17β indicated that the concentration of this estrogen in dogfish ovaries is approximately 19 μg/kg.

DISCUSSION

Although many reagents have been suggested (22) for the location of estrogens on paper chromatograms, only two, Turnbull's (23) and Folin and von Ciocalteu's (1) reagents are now widely used. Both these reagents oxidize the phenolic steroid to a mixture of unidentified products and are therefore unsuitable for use in a scheme of characterizing microgram quantities of estrogen where the reagent must satisfy the demands of being both sensitive and nondestructive.

The most likely reagent appeared to us to be some compound whose UV-activated fluorescence might be quenched by the ring A chromophore of estrogens. 2,7-Dichlorofluorescein has been found to be such a compound. When a chromatogram of estrogens is dipped into a very dilute solution of this reagent and viewed while wet in UV light, the phenols are readily seen as dark zones against a bright yellow fluorescing background. Experiments to determine the sensitivity of the reagent show that 0.5-μg quantities of estriol, estradiol-17β, and estrone occupying areas on the chromatogram of approximately 1 cm^2 are detectable. Although the spots are no longer visible when the chromatograms are dried, wetting with water restores the contrast. Acetates, formates, and methyl ethers of phenolic steroids are also detected by this reagent. The fact that dichlorofluorescein shows a UV absorption maximum at 280 mμ, very close to that of estrogens, probably explains its useful specificity as a locating reagent for these compounds; androgens, adrenocortical steroids, progestogens, and sterols are not detectable by this reagent. It is used in such low concentration (approximately 0.1 μg/cm^2 of paper) that it does not interfere with subsequent chemical transformations, viz., formate, acetate, or Fast Black Salt K derivative formation. Moreover it is so much more polar than the known estrogens and their derivatives that reagent eluted from one chromatogram remains on the origin of the subsequent chromatogram. Its disadvantage, however, is that it fails to locate estrogens chromatographed in impregnated systems such as the chloroform-formamide and toluene-propylene glycol systems, even when the chromatograms have been thoroughly dried before dipping into the fluorescent dye. The reason for this failure is not clear.

Acetates have most commonly been employed (10, 11) to characterize phenolic steroids. However, the fact that the acetates of estriol and estradiol-17β differ in R_f value, in the solvent systems recommended, much less than do the free phenols detracts from the value of acetylation as a means of characterizing these compounds. Formates seemed likely to prove more useful for this purpose.

A simple method of formylating hydroxy steroids on chromatograms by exposing these to formic acid vapor has been described by Edwards (24). By using this method, or alternatively, by formylating in solution, it has now been found that only alcoholic hydroxyl groups are esterified, estrone, 2-methoxyestrone, and equilin being recovered unchanged and estradiol-17α and 17β giving derivatives which coupled with neutral Fast Black Salt K without prior saponification. Model experiments on the formylation of estriol showed that, although a long reaction time furnishes the 16,17-diformate, reaction for shorter times yields a mixture in which a well-defined and readily separable monoformate fraction is the major constituent. In the chloroform-formamide system, the monoformate fraction can be separated into a major and a minor component; it is hoped to report the orientation of the two monoformates elsewhere. Mobilities of estrogen esters are given in Table 1.

TABLE 1

R VALUES, CALCULATED WITH RESPECT TO ESTRONE

Solvent system: light petroleum-methanol (1)	
Estradiol-17α formate	2.48
Estradiol-17β formate	2.67
Estriol 16,17-diformate	0.62
Estriol monoformate	0.15
2-Methoxyestradiol-17β formate	5.15
Estrone 3-acetate	4.70
2-Methoxyestrone 3-acetate	3.91
Equilin 3-acetate	4.25

Although the enzymic oxidation of estradiol-17β to estrone has been reported (25), attempts in this laboratory to accomplish the same conversion chemically have met with indifferent success. Chromium trioxide in pyridine, the best reagent, yielded only a small quantity of estrone and caused considerable destruction of material. It seemed likely that a partially hindered phenol might be oxidized more smoothly. This has now been realized, reaction of the Fast Black Salt K azo derivative of estradiol-17β with chromium trioxide in pyridine producing the corresponding derivative of estrone in good yield. Oxidation of 2-methoxy-

estradiol Fast Black Salt K derivative by the same method proved less satisfactory, the desired ketone being obtained in indifferent yield.

Estrone has been characterized by its reduction with aqueous sodium borohydride to estradiol-17β. It has now been found that the Fast Black Salt K derivative of estrone is also smoothly reduced to the azo derivative of estradiol-17β by this reagent, thus accommodating characterization by reduction into our scheme for the sequential identification of estrogens.

SUMMARY

The use of 2,7-dichlorofluorescein as a nondestructive locating reagent for phenolic steroids on chromatograms is described.

A procedure has been developed for the adequate characterization of estrogens from natural sources. Starting with less than 5 μg of steroid, this is chromatographed, esterified (formate or acetate), chromatographed again, and saponified. The freed steroid is then chromatographed in a second solvent system, converted to the azo compound derived from Fast Black Salt K, and eluted and the dye rechromatographed. Finally the azo dyes of estradiol and estrone are respectively oxidized by chromium trioxide in pyridine and reduced by aqueous sodium borohydride to the corresponding derivatives of estrone and estradiol-17β.

ACKNOWLEDGMENT

The work described in this paper forms part of the program of the Torry Research Station of the Department of Scientific and Industrial Research.

REFERENCES

1. Mitchell, F. L., and Davies, R. E., *Biochem. J.* **56**, 690 (1954).
2. Dao, T. L., *Endocrinology* **61**, 242 (1957).
3. Brown, J. B., *Biochem. J.* **60**, 185 (1955).
4. Short, R. V., *Mem. Soc. Endocrinol.* No. 8, 86 (1960).
5. Ittrich, G., *Z. physiol. Chem.* **312**, 1 (1958).
6. Bernstein, S., and Lenhard, R. H., *J. Org. Chem.* **18**, 1146 (1953).
7. Axelrod, L. R., *J. Biol. Chem.* **201**, 59 (1953).
8. Preedy, J. R. K., and Aitken, E. H. *J. Biol. Chem.* **236**, 1297, 1300 (1961).
9. Slaunwhite, W. R., Engel, L. L., Scott, J. F., and Ham, C. L., *J. Biol. Chem.* **201**, 615 (1953).
10. Savard, K., Andree, K., Brooksbank, B. W. L., Reyneri, G., Dorfmann, R. I., Heard, R. D. H., Jacobs, R., and Solomon, S. S., *J. Biol. Chem.* **231**, 765 (1958).
11. Short, R. V., *J. Endocrinol.* **20**, 147 (1960).
12. Wotiz, H. H., Botticelli, C. R., Hisaw, F. L., and Olsen, A. G., *Proc. Natl. Acad. Sci. U. S.* **46**, 580 (1960).
13. Oertel, G. W., West, C. D., and Eik-Nes, K. B., *J. Clin. Endocrinol. Metabolism* **19**, 1619 (1959).

14. GALLAGHER, T. F., KRAYCHY, S., FISHMAN, J., BROWN, J. B., AND MARRIAN, G. F., *J. Biol. Chem.* **233,** 1093 (1958).
15. O'DONNELL, V. J., AND PREEDY, J. R. K., "Hormones in Blood," p. 335. Academic Press, New York-London, 1961.
16. BOTICELLI, C. R., HISAW, F. L., WOTIZ, H. H., *Proc. Soc. Exptl. Biol. Med.* **103,** 875 (1960).
17. BOTICELLI, C. R., HISAW, F. L., AND WOTIZ, H. H., *Proc. Soc. Exptl. Biol. Med.* **106,** 887 (1961).
18. LISK, R. D., *Can. J. Biochem. Physiol.* **39,** 659 (1961).
19. GOTTFRIED, H., HUNT, S. V., SIMPSON, T. H., AND WRIGHT, R. S., *J. Endocrinol.* **24,** 425 (1962).
20. ZAFFARONI, A., BURTON, R. B., AND KEUTMANN, E. H., *J. Biol. Chem.* **188,** 763 (1951).
21. NEHER, R., AND WETTSTEIN, A., *J. Clin. Invest.* **35,** 800 (1956).
22. AXELROD, L. R., *J. Biol. Chem.* **201,** 59 (1953).
23. MEYER, A. S., *Biochim. et Biophys. Acta* **17,** 441 (1955).
24. EDWARDS, R. W. H., *J. Endocrinol.* **22,** xxvi (1961).
25. TALALAY, P., AND MARCUS, P. I., *J. Biol. Chem.* **218,** 675 (1956).

ANALYTICAL BIOCHEMISTRY 5, 321–329 (1963)

Electrophoresis of Histones and Histone Fractions on Polyacrylamide Gels[1]

H. C. McALLISTER, JR.,[2] Y. C. WAN, AND J. LOGAN IRVIN

From the Department of Biochemistry, University of North Carolina School of Medicine, Chapel Hill, North Carolina

Received July 27, 1962

INTRODUCTION

Increasing interest in the possible involvement of histones in genetic and metabolic functions of the cell nucleus has necessitated the development of fractionation techniques to facilitate the study of this heterogeneous class of proteins. Among the more often used methods is zone electrophoresis, the usual anticonvection medium being starch gel (1, 2). More recently the synthetic gel, polyacrylamide, has been used with remarkable success in the separation of some proteins (3, 4). It is now possible to cause polymerization of this gel at pH values as low as 2.3 (communication, Canalco, Inc.). This is especially desirable in working with histones, since aggregation is decreased and possible catheptic activity is minimized at low pH. The greatest advantage of the polyacrylamide system lies, however, in the extremely high resolution obtainable in relatively short runs. The separation of a few cationic proteins and polypeptides in this gel has already been reported (5, 6), and this work has stimulated the present authors to apply a modification of the technique to histone and histone fractions. This communication will describe some results of this study.

MATERIALS

All solutions were prepared from reagent-grade chemicals.

Buffered Temed catalyst: 1.15% in pH 2.9 acetate buffer, prepared by mixing 4.6 ml Temed (*N,N,N′,N′*-tetramethylethylenediamine) with 213 ml glacial HOAc[3] and 48 ml 1 *N* KOH and diluting to 400 ml with

[1] This investigation was supported (in part) by PHS research grant RG-8318 from the Division of General Medical Sciences, Public Health Service.

[2] Trainee, supported in part by PHS training grant 2G-404 (R1) from the Division of General Medical Sciences, Public Health Service.

[3] The following abbreviations are used: SGE, starch gel electrophoresis; PAGE, polyacrylamide gel electrophoresis; HOAc, acetic acid; RNase, ribonuclease; DMF. *N,N*-dimethylformamide.

distilled water. Stored in the cold, this solution is stable for at least one month.

Monomer solution: 60% acrylamide and 0.4% *N,N'*-methylenebisacrylamide, aqueous solution, stored at room temperature.

Ammonium persulfate catalyst: 2.8% aqueous solution, stored in the cold and prepared fresh weekly.

Electrophoresis buffer: 0.37 *M* glycine, adjusted to pH 4.0 with glacial HOAc.

Staining solution: 0.5% Amido Black in 7% HOAc.

Destaining solution: 6.0% acrylamide and 0.5 mg % riboflavin, aqueous solution, photopolymerized by exposure to a fluorescent light for 2 hr (or until the riboflavin is bleached). The viscous solution is then diluted 1:1 with 14% HOAc for use, and is stored in a brown glass bottle.

Crystalline pancreatic RNase was purchased from Sigma in high purity (5× recrystallized).

Histone fractions I and II (7) *and the "very lysine-rich" fraction* (8) were prepared from calf thymus histone according to the cited references.

Unfractionated histone was prepared (9) from calf thymus glands. The tissue was homogenized in 0.14 *M* NaCl-0.01 *M* sodium citrate, and the centrifuged sediment was treated three additional times in the same manner. Deoxypentose nucleoprotein was extracted from the residue for 12 hr at 5°C with 1 *M* NaCl and then precipitated by dilution into 6 vol of water. Purification was achieved by redissolving and reprecipitating two additional times in a similar manner. The purified deoxypentose nucleoprotein was then dissolved in 1 *M* NaCl, and 6 *N* HCl was added dropwise with stirring to a final concentration of 0.2 *N*. The precipitated deoxyribonucleic acid was washed and discarded, and the supernatant fluid, containing unfractionated histone, was dialyzed overnight against a large volume of cold distilled water. To avoid proteolytic action during dialysis, the pH of the histone solution was not permitted to rise above 2. The solution was then lyophilized to yield the unfractionated histone preparation, which was stored in a dry container at —10°C.

METHODS

The six-position electrophoresis apparatus employed in these experiments closely resembled that already described by Reisfeld *et al.* (6), but used as the gel containers 8-cm lengths of 11 i.d. glass tubing. The electrodes were of platinum, and a current-regulated DC source was provided by a commercial power supply. The gels were formed by first mixing 20 ml of buffered Temed catalyst with 10 ml of the monomer solution and 10 ml of 2.8% ammonium persulfate. The resulting solution was placed in the gel containers, which were already positioned in the

electrophoresis apparatus and stoppered on the bottom with inverted number 6 pharmaceutical "sleeve-type" stoppers. The monomer mixture was then gently overlaid with ¼ in. of distilled water, after which the polymerization occurred in about 45 min. The measured pH of the gel was 3. Since preliminary experiments in this laboratory with the 3-gel system (3, 10) revealed considerable holdback of histones in the sample and spacer gels, the protein (about 0.5 mg) was applied directly upon the gel, after removal of the water layer, in 0.2 ml of a 1:4 dilution of the electrophoresis buffer to which sucrose was added to a final concentration of 1 *M*. Undiluted buffer was then gently overlaid on the sample solution, which was stabilized by the dense sucrose. Alternatively, the solidified gels were overlaid directly with electrophoresis buffer after removal of the water layer, and the sucrose-containing sample solution was applied to the gel with a tuberculin syringe fitted with a long 20-gage needle. Electrophoresis was then commenced immediately (negative electrode in the bottom vessel) and allowed to continue at room temperature for 4.5 hr at a current of 15 ma per gel tube, which caused no excessive ohmic heating in gels of the diameter used. The gels were removed from their containers by rimming the tubes with a fine jet of water from a hypodermic needle, as described by Reisfeld *et al.* (6), and stained overnight in the staining solution. Destaining was accomplished electrically (6, 10) in destaining tubes prepared by removing the tips of 15-ml glass centrifuge tubes. The stained gel, wedged into the tapered tube by its own weight, was covered with the destaining solution, and this in turn was overlaid with 7% HOAc, which was also placed in the lower reservoir. A direct current of 20 ma per gel tube was then applied to the system (negative electrode in the upper vessel), and destaining was continued until removal of unbound dye was complete. The high viscosity of the destaining solution was necessary to minimize back diffusion of dye during electrical destaining.

A few experiments on the removal of unbound dye by diffusion into 7% HOAc seemed to indicate that no artifacts were introduced by electrical destaining.

The relative proportions of the electrophoretically separated histone components were estimated in a few samples of unfractionated histone preparations by determination of the relative amounts of dye bound to the various bands in the gels. For this purpose, to ensure saturation of the bands with dye, the gels were stained for 48 hr rather than the shorter interval used in qualitative experiments. After electrical removal of unbound dye from the gels, the stained bands were removed by slicing the gels with a razor blade, and the gel sections were separately homogenized in 3 ml of *N,N*-dimethylformamide (DMF) with a glass-glass or

Teflon-glass homogenizer. The homogenates were centrifuged at 1000 $\times$ g, and the optical densities of the clear supernatant extracts were determined in a spectrophotometer at 620 mμ, the absorption maximum for amido black in DMF. Blanks were prepared with DMF extracts of comparable sections of destained gels through which histones had not migrated. The percentage of the total bound dye present in each band (corrected for the corresponding blank) was calculated as equal to 100 times the optical density of the extract of a particular stained band divided by the sum of the optical densities of extracts of all bands in the gel. The percentage of total histones present in each band was assumed to be equal to the percentage of total bound dye found in that band.

Starch gel electrophoresis (SGE) was carried out in 16% gels in 0.01 N HCl (2).

RESULTS

The patterns obtained by this method are represented diagrammatically in Fig. 1. They were found to be quite reproducible in experiments from day to day as well as simultaneous, parallel runs. Several different preparations of unfractionated calf thymus histone showed almost identical patterns. The component bands in Fig. 1,C were found consistently and, for the purpose of this communication, have been designated by Roman numerals. Bands II and IV, found in all calf thymus histone preparations examined in this laboratory, stained more heavily than the other bands and hence provided easily recognized and very reproducible reference points in these patterns. The two bands are represented by areas of solid black in Fig. 1. The inclusion of a sample of unfractionated histone in each electrophoretic run of 6 samples facilitated the assignment of band identities in preparations of histone fractions. Band II was arbitrarily assigned a relative mobility (μ') of 1.00, and the calculations of μ' for other bands were made relative to the distance of migration of this reference band (an average of 3.8 cm in 4.5 hr at 15 ma). Under the experimental conditions described, band IV had a μ' of 0.76 $\pm$ 3% in all histone samples studied. The other bands which were seen consistently had average μ' values of 1.10, 0.87, 0.76, 0.68, 0.61, 0.37, 0.27, 0.16, and 0.01. A few preparations of unfractionated histones showed other bands, especially with μ' greater than 1.2, which were reproducible within the sample, but the number, mobilities, and depths of staining of these bands were not always the same in different preparations. Such a sample is shown in Fig. 1,E.

In Table 1 there are summarized the results of estimates, by determination of bound dye, of the percentages of various electrophoretically separated components of one preparation of unfractionated calf thymus

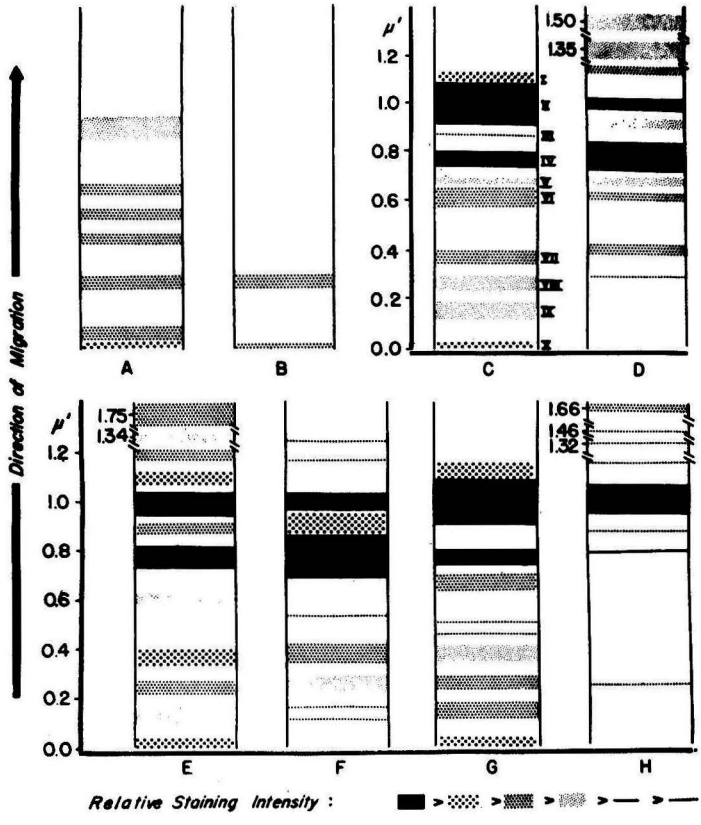


FIG. 1. Electrophoretic patterns of calf thymus histone and histone fractions. (A) Starch gel electrophoresis of unfractionated histone. (B) Starch gel electrophoresis of very lysine-rich histone. (C through H) Patterns representing electrophoresis in polyacrylamide gel: C, unfractionated histone; D, very lysine-rich histone; E, unfractionated histone showing several lightly staining bands not usually observed; F, histone II; G, histone I; H, RNase.

histones. These estimates were based upon the assumption that bound dye would be proportional to the quantities of proteins in the stained bands and that the intrinsic dye-binding capabilities of the various histone components would be equal. The former assumption seems reasonably secure, but the latter may not be strictly correct and will require re-examination when pure fractions become available for quantitative studies of dye-binding. In obtaining the data of Table 1, bands I, II, and III were not separated for determination of bound dye since the close proximity of these bands made separation by slicing an uncertain procedure. However, bands I and III are quantitatively minor

TABLE 1

ESTIMATION OF RELATIVE PROPORTIONS OF VARIOUS ELECTROPHORETIC COMPONENTS (BANDS) IN PREPARATIONS OF UNFRACTIONATED CALF THYMUS HISTONES

(Averages of 5 estimations on one preparation of unfractionated histones by determinations of bound dye)

Band number	Per cent of total histones in band
I + II + III	57.9
IV	13.3
V + VI	3.8
VII	4.8
VIII	4.9
IX	4.4
X	10.9

components in comparison with band II, which is the major component of calf thymus histones. Similarly bands V and VI were not separated for determination of bound dye. The data of Table 1 are in rough agreement with the subjective, visual estimate of relative intensities of staining of the various bands as indicated in Fig. 1 by degrees of shading of the bands. The agreement is not perfect to the extent that bands VI and VII generally appear visually to be slightly more intensely stained than bands VIII and IX, whereas the relative proportions of dye found in these bands (Table 1) were approximately the same. There are indications that these apparent discrepancies may be due to two factors: (*1*) Visual observation of relative intensities of staining of bands is a function of local concentrations of dye-protein complexes in the bands, whereas the dye extraction method determines total amounts of dye in the bands. (*2*) The absorption spectra of dye-protein complexes may vary slightly for different proteins and may differ from the absorption spectrum of extracted dye in DMF.

Since previous experiments in this laboratory with SGE of ethanol-fractionated histone I (relatively arginine-rich) and histone II (relatively lysine-rich) (7) have shown them to be heterogeneous in this medium (McClure, unpublished data) as well as in free solution (7, 9), these fractions were subjected to polyacrylamide gel electrophoresis (PAGE). Fig. 1,F and G shows the results of this study. In general, the patterns indicate gross heterogeneity in both fractions, but the patterns for these fractions differ from those of the unfractionated histone preparations. Specifically, in histone II, band X was missing; also the usually dark band II was much lighter, and band IV much darker, than in unfractionated histone. The pattern for histone I, on the other hand, was

characterized by a decrease in the intensity of band IV as compared with unfractionated histone, and, unlike histone II, band X was present.

Very lysine-rich histone (8), when subjected to SGE, showed a pattern identical to the one published in the latter communication, i.e., a single, slowly moving band (Fig. 1,B). Another aliquot of the same fraction when subjected to PAGE showed the heterogeneous pattern indicated in Fig. 1,D. Identical results were obtained with three preparations of very lysine-rich histone isolated from calf thymus.

A commercial sample of RNase (0.5 mg) when subjected to PAGE yielded a heavily staining band, with a mobility approximately equal to that of the reference band II of unfractionated histone, and a lightly staining band with μ' of 1.66. When 1.2 mg RNase was applied to the gel, additional lightly staining bands could be detected at the positions indicated in Fig. 1,H. Reisfeld *et al.* also have reported heterogeneity of commercial RNase (6).

DISCUSSION

Although electrophoresis of histones in the 3-gel polyacrylamide system of Ornstein and Davis (3, 10) and Reisfeld (6) was found to be unsatisfactory due to serious retention of protein in the sample and spacer gels, the single gel modification described here yielded excellent resolution of these proteins. With this technique, it is possible to demonstrate further separation of histone components which migrate as a single band in SGE. For example, the very lysine-rich histone fraction of Johns and Butler (8) migrated as a single band in SGE but separated into 9 bands during PAGE under the conditions described. Unfractionated histone, which showed 7 major bands in SGE, was resolved into 10 components by PAGE. It seems likely that a sieving effect of the polyacrylamide gel may contribute to the effectiveness of this gel in resolving the components of the histones. A detailed comparison of electrophoretic patterns of histones in starch gels with those in polyacrylamide gels is not possible at the present time since adequate comparison will be dependent upon the availability of histone fractions which are less heterogeneous than those which can be obtained by existing fractionation procedures. Electrophoresis in polyacrylamide gels should be useful in developing such procedures.

It is of interest that PAGE reveals the presence in the very lysine-rich histone fraction of minor components with mobilities greater than those of the major components of the usual preparations of unfractionated calf thymus histones (Fig. 1,D and C). However, such rapidly moving components have been found in occasional preparations of calf thymus histones (Fig. 1,E). There are several possible explanations for these

observations: (*1*) Since the very lysine-rich fraction of Johns and Butler is isolated from whole calf thymus by a direct procedure which does not involve prior isolation of deoxypentose nucleoprotein, the preparation may contain small amounts of basic proteins which are derived from the cytoplasm or other sources not usually associated with the deoxypentose nucleoprotein. (*2*) The minor components with high mobilities may be lost in the usual isolation of histones by way of the deoxypentose nucleoproteins. (*3*) The minor components may be derived from partial hydrolysis of other components during the isolation, which involves a prolonged contact with 1 *N* $HClO_4$.

It is of interest that crystalline pancreatic RNase contains components with mobilities in PAGE comparable to those of some of the components of calf thymus histone. Leslie (11) has reported that histones have RNase activity. We have found[4] that preparations of the very lysine-rich histones isolated by the direct method of Johns and Butler (8) have RNase activities with optima at approximately pH 5.5 and 7.5, and the specific RNase activities of these preparations are greater than those of unfractionated calf thymus histones. Preliminary attempts have been made to extract proteins from bands separated by PAGE of very lysine-rich histones for assay of RNase activities. RNase activity at pH 7.5 was found in extracts of bands with μ' of 1.55, 1.0, and 0.8, respectively, but the quantities of proteins extracted have been inadequate for accurate determination of specific RNase activities. Further work on this problem is in progress.

SUMMARY

Zone electrophoresis of histones in polyacrylamide gels provides resolution of components which is superior to that obtained with starch gels, and the reproducibility of the method is excellent. The method has been applied to a comparison of unfractionated calf thymus histones with various fractions obtained by the methods of Ui and Johns and Butler. The very lysine-rich histone fraction yielded 1 mobile band in starch gel and 9 bands in polyacrylamide gel. Crystalline pancreatic RNase yielded 2 major and 6 minor bands on electrophoresis in polyacrylamide gel, and the mobilities were comparable to those of some of the histone components. A method is described for estimation of relative amounts of electrophoretically separated components by determination of bound dye.

REFERENCES

1. NEELIN, J. M. AND NEELIN, E. M., *Can. J. Biochem. Physiol.* **38**, 355 (1960).
2. JOHNS, E. W., PHILLIPS, D. M. P., AND BUTLER, J. A. V., *Biochem. J.* **80**, 189 (1961).

[4] Moore, P. A., and Irvin, J. Logan, unpublished observations.

3. ORNSTEIN, L., "Disc Electrophoresis. Part I, Theory." Preprinted by Canalco and Distillation Products Industries, 1961.
4. RAYMOND, S., AND WANG, Y., *Anal. Biochem.* **1,** 391 (1960).
5. REISFELD, R. A., LEWIS, U. J., AND WILLIAMS, D. E., *Abstracts,* 141st Meeting, Am. Chem. Soc., p. 64C (1962).
6. REISFELD, R. A., LEWIS, U. J., AND WILLIAMS, D. E., *Nature* **195,** 281 (1962).
7. UI, N., *Biochim. et Biophys. Acta* **25,** 493 (1957).
8. JOHNS, E. W., AND BUTLER, J. A. V., *Biochem. J.* **82,** 15 (1962).
9. MCCLURE, C., Dissertation, Univ. of North Carolina, 1960; *Dissertation Abstr.* **21,** 2461 (1961).
10. DAVIS, B. J., "Disc Electrophoresis. Part II, Materials and Methods." Preprinted by Canalco and Distillation Products Industries, 1961.
11. LESLIE, I., *Nature* **189, 260** (1961).

ANALYTICAL BIOCHEMISTRY 5, 330–337 (1963)

Synthesis and Characterization of Sodium Cysteine-S-sulfate Monohydrate[1]

IRWIN H. SEGEL[2] AND MARVIN J. JOHNSON

From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

Received August 8, 1962

Cysteine-*S*-sulfate (cysteine-*S*-sulfonate, *S*-sulfocysteine) and thiosulfate have been suggested as intermediates in the conversion of inorganic sulfate to organic sulfur compounds by molds (1–6). This suggestion is based on growth studies with various mutant strains of *Neurospora* (1) and *Aspergillus* (2–4) and on the observation that a mutant strain of *Aspergillus* will accumulate trace amounts of cysteine-*S*-sulfate intracellularly (5). The cysteine-*S*-sulfate presumably is formed by the condensation of thiosulfate with serine (2–6). No data have been reported that support the enzymic reaction between thiosulfate and serine. DeMarco and co-workers (7) have demonstrated the copper- and pyridoxal-dependent cleavage of cysteine-*S*-sulfate to thiosulfate, ammonia, and pyruvate.

An easy method for the preparation of relatively large quantities of pure cysteine-*S*-sulfate is desirable in order to study the role of this compound in the sulfur metabolism of molds. The classical method of synthesis, by Clarke (8), is complicated and tedious. Furthermore, Clarke's method yields three crystalline products, one of which he characterized as monosodium cysteine-*S*-sulfate·1.5 H_2O. The other two crystalline products were not identified.

Kolthoff and Stricks (9) prepared trace amounts of cysteine-*S*-sulfate in ammoniacal solutions of cystine and sodium sulfite. The product was detected by polarographic techniques and was not isolated. DeMarco and co-workers have prepared sodium cysteine-*S*-sulfate by reacting cystine with sodium sulfate in NaOH (10) or ammonia (11) solution.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from E. R. Squibb and Sons, New Brunswick, New Jersey.

[2] National Science Foundation Predoctoral Research Fellow. Present address: Centre de Recherches Scientifiques Industrielles et Maritimes, Marseilles (6E), France.

The final product, however, was not extensively purified or characterized. Its behavior in chromatographic systems was the same as that of cysteine-*S*-sulfate prepared by Clarke's method; consequently, DeMarco and co-workers assumed their product was the sesquihydrate (personal communication). Nakamura (5) reported a synthesis of sodium cysteine-*S*-sulfate based on a modification of the method of Kolthoff and Stricks (9). The elemental analysis of Nakamura's material suggests that it is the anhydrous monosodium salt (personal communication).

This paper reports an improved method for the synthesis and isolation of large amounts of pure, crystalline sodium cysteine-*S*-sulfate monohydrate. The characterization of this compound is described.

MATERIALS AND METHODS

Crystalline cystine was obtained from Sigma Chemical Company. Sodium sulfite was a Baker and Adamson product and assayed a minimum of 98% Na_2SO_3. Elemental analysis of cysteine-*S*-sulfate for C, H, S, and Na were performed by Huffman Microanalytical Laboratories, Wheatridge, Colorado, and by W. Manser, Herrliberg, Switzerland. Infrared spectra were made on a Beckman IR 5 spectrophotometer. Paper electrophoresis was carried out on an E-C Apparatus Company paper electrophoresis apparatus.

EXPERIMENTAL PROCEDURES AND RESULTS

Synthesis of Cysteine-S-sulfate from Cystine and Sulfite

Cysteine-*S*-sulfate was prepared by aerating stoichiometric amounts of cystine and Na_2SO_3 in aqueous ammonia solution containing trace amounts of cupric chloride. The reactions involved are as follows:

$$2\ R{-}S{-}S{-}R + 2\ SO_3^{=} \rightarrow 2\ R{-}S{-}SO_3^{-} + 2\ RS^{-} \qquad (A)$$

$$2\ RS^{-} + \tfrac{1}{2}\ O_2 + H_2O \xrightarrow{Cu^{++}} R{-}S{-}S{-}R + 2\ OH^{-} \qquad (B)$$

$$\text{sum: } R{-}S{-}S{-}R + 2\ SO_3^{=} + \tfrac{1}{2}\ O_2 + H_2O \xrightarrow{Cu^{++}} 2\ R{-}S{-}SO_3^{-} + 2\ OH^{-}$$

Ten grams (41.7 mmoles) of L-cystine, 10.5 gm (83.4 mmoles) sodium sulfite, 30 ml of 15 *M* ammonia solution, and 55 ml of distilled water were mixed in a 500-ml Erlenmeyer flask (with indented sides for more efficient aeration). A few drops of $CuCl_2$ solution were added to make the reaction mixture about 2×10^{-3} *M* in Cu^{++}. The resulting brown color of the solution is caused by a Cu^{++}-NH_3-cysteine complex (12). The flask was placed on a rotary shaker operating at approximately 350 rpm and describing a 2-in. circle. Any equally efficient method of aeration may be used (13). The object of the aeration procedure is to reoxidize

the cysteine formed by reaction A. The oxidation reaction, B, is Cu^{++}-catalyzed.

After approximately 2 hr of shaking, the reaction had gone to completion, indicated by a change in color of the solution from brown to green. The time required for the color change, i.e., the time required for the reoxidation of cysteine formed by reaction A, depends on the efficiency of aeration. The exact color of the end point varies from green to blue depending on the concentration of the constituents.

At the end point the reaction mixture contained no detectable cystine as tested for by the nitroprusside-cyanide test. Acidification of the solution resulted in no odor of SO_2, indicating that no sulfite was present. However, an iodine titration indicated a total of 1.2 meq of reducing agent in the solution. A small amount of sulfate (3.4 mmoles) was detected gravimetrically as $BaSO_4$. The flask was allowed to shake another 4 hr to remove additional ammonia.

Enough Dowex 50 (H^+) (approximately 35 gm) was then added to the reaction mixture to bring it to pH 7.0. The resin was then removed by filtration and washed with water, and the filtrate and washings were combined. The solution (total volume 120 ml) was added, with constant stirring, to 15 vol of 95% ethanol. (Alternately, 25 vol of acetone may be used.) A white emulsion formed which upon violent stirring and scratching with a glass rod separated into an amorphous white precipitate and a cloudy supernatant fluid. The precipitate was removed with suction filtration and air-dried. The product, an off-white powder, weighed 17.6 gm.

The powder was recrystallized three times from aqueous ethanol in the following way: The powder was suspended in approximately 200 ml of 95% aqueous ethanol. The suspension was heated to boiling and water was added slowly, with constant stirring, until the powder dissolved. (A small amount of insoluble residue remained.) The resulting slightly turbid, amber solution was filtered hot and the clear filtrate slowly cooled to room temperature, then placed at 4°C overnight. The salt crystallized as clusters of white, crossed needles upon cooling. The crystals were removed by filtration with suction, washed with ethanol, and air-dried. The crystallization procedure was repeated until the crystals were pure white and the hot ethanol solutions were clear and colorless. The yields of air-dried product from three successive crystallizations were 12.1, 9.8, and 7.3 gm. The final yield was usually 35–40% of theory. No attempt was made to obtain second crops of crystals although the major loss in yield is in the recrystallization process.

Figure 1 shows the effect of varying the Cu^{++} concentration on the average rate of the reaction between cystine and sulfite. Equimolar

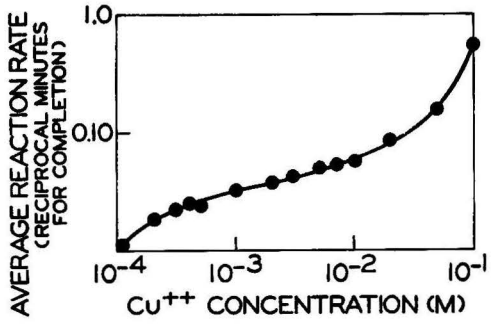


FIG. 1. Effect of Cu^{++} concentration on average reaction rate of cystine with Na_2SO_3. Seventy milliliters of 2.2 *M* ammonia solution containing 1.0 gm cystine and 1.05 gm Na_2SO_3 was placed in each of several smooth 500-ml Erlenmeyer flasks. Enough concentrated $CuCl_2$ solution was added to each flask to yield the indicated final Cu^{++} concentrations. The flasks were placed on a rotary shaker and aerated until the reaction had gone to completion as indicated by a change in color of the solution from brown to green or blue. The average rates are indicated as the reciprocal of the times taken to reach the end point. No corrections were made for loss of ammonia from solution during shaking.

concentrations of Co^{++} or Ni^{++} were much less effective than Cu^{++} in catalyzing the reaction.

Variations in the ammonia concentration had little effect on the rate of reaction. The amount of ammonia added to a reaction flask must be sufficient to dissolve all of the cystine. Prolonged aeration of the reaction mixture should be avoided. The solution should be aerated until 10–12 ml of 0.1 *N* H_2SO_4 is required to titrate 1.0 ml of the solution to the methyl red end point. In one experiment the aeration was continued for 20 hr after the end point, during which time the solution gradually turned from green to brown. The amount of reducing substances in the solution increased to a total of 7.4 meq. This reducing substance may be S_2O_3-formed by decomposition of cysteine-*S*-sulfate (7, 21). When such overaerated solutions were added to the ethanol or acetone in the subsequent isolation procedure an oil frequently formed.

CHARACTERIZATION OF THE PRODUCT

Water of Hydration. Clarke (8) characterized his product as monosodium cysteine-*S*-sulfate·$1.5H_2O$. The water of hydration was determined by heating the salt *in vacuo* at 105–110°C. However, heating our product at 96°C at atmospheric pressure resulted in a continual loss of weight and gradual browning. When the powder obtained from the acetone precipitation was heated at 10–15 mm Hg and 60° for 3 days the product lost 2.8% of its weight. The final crystalline product lost

only 0.28% of its weight when stored under the above conditions. The crystalline salt lost no weight when stored above P_2O_5 for 2 days. Since the water of hydration could not be determined by heating a known amount of our product to constant weight, it was calculated indirectly from elemental analyses, and from equivalent weight determinations.

Elemental Analysis. Analysis calculated for $C_3H_6O_5NS_2Na \cdot H_2O$: C, 14.93; H, 3.35; N, 5.81; S, 26.59; Na, 9.53. Found, C, 15.11; H, 3.32; N (Kjeldahl), 5.80; S, 26.47; Na, 9.50.

Acid Hydrolysis. The salt was hydrolyzed in strong HCl to inorganic sulfate and cysteine. The acid-labile sulfur was determined in the following way: Known amounts of the salt (250–300 mg) were dissolved in 5 ml of 2.5 *M* HCl containing 0.4 *M* $BaCl_2$. The solutions were heated at 96°C for 24 hr in sealed tubes. The $BaSO_4$ that formed was collected quantitatively, washed, dried, and weighed. Calculated, 13.30% acid-labile S. Found, 13.29.

The cysteine produced by acid hydrolysis was determined in the following way: 1 ml of a solution containing 4.82 mg (1 mmole) of the salt was added to 1.0 ml of 12 *N* HCl and the resulting solution placed in a boiling water bath. After 90 min the solution was cooled and diluted to 50 ml. The amount of cysteine formed upon hydrolysis was determined by the nitroprusside-cyanide method (14). Pure, known amounts of cysteine were treated in a similar manner and used as a standard. Found, 1.00 mmole.

Bromine Oxidation. The salt was oxidized with standard bromine water to inorganic sulfate and cysteic acid. The inorganic sulfate was identified by precipitation as $BaSO_4$; the cysteic acid was identified by paper eletcrophoresis at pH 8.1 in 0.2 *M* Tris buffer. Cysteic acid migrates, under these conditions, 55% as fast as inorganic sulfate. Cysteic acid was detected by ninhydrin spray. The reference inorganic sulfate used was radioactive and was detected with a thin-window counter. The theoretical bromine consumption is 6 equivalents; found, 6.01 equivalents.

Dichromate Oxidation. The salt was subjected to dichromate oxidation according to the method of Johnson (15). Cysteine was used as a reference compound since both cysteine and cysteine-*S*-sulfate should require the same number of equivalents of dichromate for oxidation. Cysteine requires 6 equivalents for oxidation to cysteic acid. However, the observed value was 8.30, indicating partial oxidation of cysteic acid. The observed value for cysteine-*S*-sulfate was 8.35.

Neutral Equivalent (Willstätter) Titration. Known amounts of the salt were titrated with standard alcoholic NaOH to the disodium salt (thymolphthalein end point). The salt was initially dissolved in an

amount of water such that the final ethanol concentration at the end point of the titration was at least 90%. The amount of water used was sufficient to prevent precipitation of the salt during the titration. The NaOH was standardized in a similar titration against glycine (recrystallized from aqueous ethanol). The calculated neutralization equivalent is 241.26; found, 240.

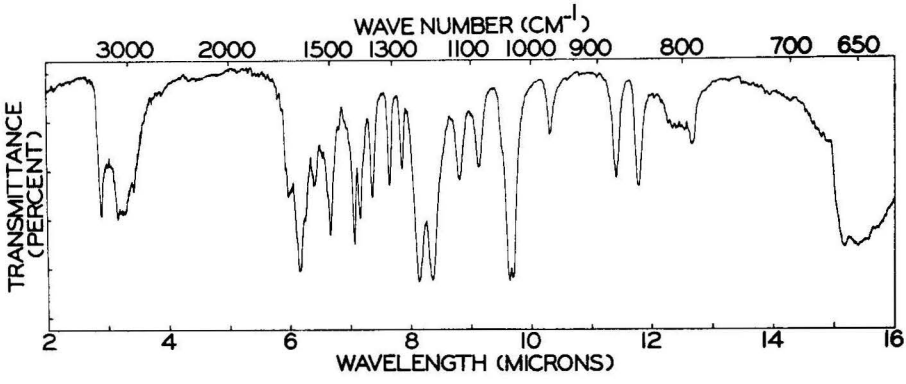


FIG. 2. Infrared spectrum of sodium cysteine-*S*-sulfate·H_2O in KBr. The KBr tablets were prepared at a pressure of 1000–2000 psi.

Infrared Spectrum. Figure 2 shows the infrared spectrum (in KBr) of the cysteine-*S*-sulfate preparation. DeMarco's preparation (recrystallized from aqueous ethanol as described above) and Nakamura's crystalline preparation gave exactly the same spectrum.

EQUILIBRIUM IN THE REACTION OF CYSTINE WITH SULFITE

In the preparation of cysteine-*S*-sulfate, reaction (A) is driven toward the right by the removal of cysteine by atmospheric oxygen in reaction (B). However, we have found that, if cysteine-*S*-sulfate is allowed to react with cysteine at pH 7, cystine and sulfite are produced. Here the reaction is the reverse of reaction (A):

$$R—S—SO_3^- + R—SH \rightarrow R—SS—R + HSO_3^- \qquad (C)$$

When the concentration of reactants is such that the amount of cystine formed exceeds its low solubility, the reaction is driven to the right by the precipitation of cystine.

The equilibrium point of this rapid, nonenzymic reaction was investigated in the following experiment: 2 mmoles of sodium cysteine-*S*-sulfate·H_2O were added to a solution of 2 mmoles of cysteine in 5.0 ml of 0.1 *M* phosphate buffer, pH 6.7. A copious precipitate of cystine

formed a few seconds after the cysteine-*S*-sulfate dissolved. The mixture was allowed to stand at 25°C for 20 min in a stoppered tube. The cystine was then removed by filtration, washed, dried, and weighed. The sulfite and residual cysteine in the filtrate and washings were titrated with standard iodine solution. Exactly 1.12 mmoles of cystine (and consequently 1.12 mmoles of sulfite) was formed in the reaction. The total iodine titration (3.07 meq) accounted for 1.12 mmoles (2.24 meq) of sulfite and 0.88 mmole (0.88 meq) of residual cysteine. That the inorganic reducing substance formed was sulfite and not thiosulfate could be shown by acidifying the incubation mixture with H_2SO_4 until the cystine precipitate dissolved; the resulting solution was clear and the odor of SO_2 was detected. Very small amounts (ca. 0.02 mmole) of thiosulfate would be detectable by the formation of a turbid suspension of colloidal sulfur upon acidification. Not cystine, cysteine, or sodium cysteine-*S*-sulfate liberates SO_2 when acidified. Control tubes containing 2 mmoles either of cysteine or of sodium cysteine-*S*-sulfate formed no trace of sulfite or cystine upon standing.

The equilibrium constant for reaction (C) can be estimated from the above data to be 3.45×10^{-3} (assuming a cystine solubility of 112 mg/liter):

$$K = \frac{(HSO_3^-)(\text{cysteine})}{(\text{cysteine-}S\text{-sulfate}^-)(\text{cysteine})} = \frac{(0.224)(4.67 \times 10^{-4})}{(0.176)(0.176)}$$
$$= 3.45 \times 10^{-3} \text{ (pH 6.7, 0.1 } M \text{ phosphate, 25°C)}$$

Cysteine-*S*-sulfate was also cleaved to sulfite and an insoluble R—S—S—R compound by reduced glutathione or NaHS. The nature of the disulfide precipitate formed in these reactions was not investigated.

Recently DeMarco and co-workers (20) reported the nonenzymic, thiol-stimulated exchange reaction between S^{35}-labeled cystine and unlabeled cysteine-*S*-sulfate:

$$R—S^*—S^*—R + R—S—SO_3H \xrightarrow{RHS} R—S^*—S—R + R—S^*—SO_3H \qquad (D)$$

This exchange can be explained by reactions (A) and (C).

Tracer experiments using S^{35}-labeled cystine, cysteine, cysteine-*S*-sulfate, or sulfite should be interpreted with care in light of the ease with which the sulfur atoms of these compounds can equilibrate nonenzymically.

SUMMARY

An improved method for the synthesis and isolation of large amounts of crystalline sodium cysteine-*S*-sulfate is described. The final product has been characterized as the monohydrate, monosodium salt by ele-

mental analysis and various equivalent weight determinations. The equilibrium constant for the reaction of cysteine-*S*-sulfate with cysteine, yielding cystine and inorganic sulfite, has been estimated.

ACKNOWLEDGMENTS

We would like to thank Professor Carlo DeMarco and Dr. Taro Nakamura for generous gifts of their sodium cysteine-*S*-sulfate preparations.

REFERENCES

1. Horowitz, N. H., *Advan. Genet.* **3,** 33 (1950).
2. Hockenhull, D. J. D., *Biochim. et Biophys. Acta* 3, 326 (1949).
3. Nakamura, T., and Sato, R., *Nature* **185,** 163 (1960).
4. Nakamura, T., *J. Gen. Microbiol.* **27,** 221 (1962).
5. Nakamura, T., and Sato, R., *Nature* **193,** 481 (1962).
6. Shepherd, C. J., *J. Gen. Microbiol.* **15,** 29 (1956).
7. DeMarco, C., Coletta, M., and Mari, S., *Ital. J. Biochem.* **10,** 446 (1961).
8. Clarke, H. T., *J. Biol. Chem.* **97,** 235 (1932).
9. Kolthoff, I. M., and Stricks, W., *J. Am. Chem. Soc.* **73,** 1728 (1951).
10. DeMarco, C., Coletta, M., Mondovi, B., and Cavallini, D., *Ital. J. Biochem.* **8,** 3 (1960).
11. Coletta, M., Benerecetti, S. A., and DeMarco, C., *Ital. J. Biochem.* **10,** 224 (1961).
12. Greenstein, J., and Winitz, M., "Chemistry of the Amino Acids," Vol. 1. Wiley, New York, 1961.
13. Smith, C. G., and Johnson, M. J., *J. Bacteriol.* **68,** 346 (1954).
14. Grunert, R. R., and Phillips, P. H., *Arch. Biochem. Biophys.* **30,** 217 (1951).
15. Johnson, M. J., *J. Biol. Chem.* **181,** 707 (1949).

ANALYTICAL BIOCHEMISTRY 5, 338–344 (1963)

On Phosphorimetry as Quantitative Microanalysis with Application to Some Substances of Biochemical Interest[1]

SIMON FREED AND MARY H. VISE

From the Chemistry Department, Brookhaven National Laboratory, Upton, Long Island, New York

Received August 28, 1962

Since fluorometry is widely employed as an analytical method of high sensitivity it is natural to inquire how the closely related method, phosphorimetry, compares with it. In a previous note (1) it was shown that phosphorescence of indole derivatives possessed discrete structures which permitted discrimination between substances that have indistinguishable single maxima in fluorescence at room temperature (see Fig. 1). In general one may expect better discrimination at lower temperatures because of intensification and sharpening of maxima and because of reduction of background. As a consequence there become available more definite analytical separation and detection of the emitting substances and determinations of greater sensitivity and precision. Figure 2 illustrates the higher degree of resolution obtainable at low temperature in the emission spectra of tryptophan. Fluorescence at −196°C exhibits two maxima rather than one at room temperature; the phosphorescence has even richer characteristic structure.

In addition to fluorescence, phosphorescence makes its appearance at low temperatures as high viscosity is acquired by the solution. Because of its much greater half-life, it can, as has long been known, be recorded separately (2). As with fluorescence, activation spectra are of value for analysis. It will be recalled that activation spectra of fluorescence are given by the progression of intensities (usually measured at the fixed wavelength of its maximum fluorescence) as the exciting radiation ranges over the spectrum that the substance absorbs. If in turn at discrete emission maxima, measurement of activation spectra are performed more than one activation spectrum may arise. Such occur from tryptophan, for example. Clearly this additional information obtainable at low temperature yields more discriminating analyses. Since the equipment required for fluorescence and phosphorescence differs little it is natural

[1] Research performed under the auspices of the U. S. Atomic Energy Commission.

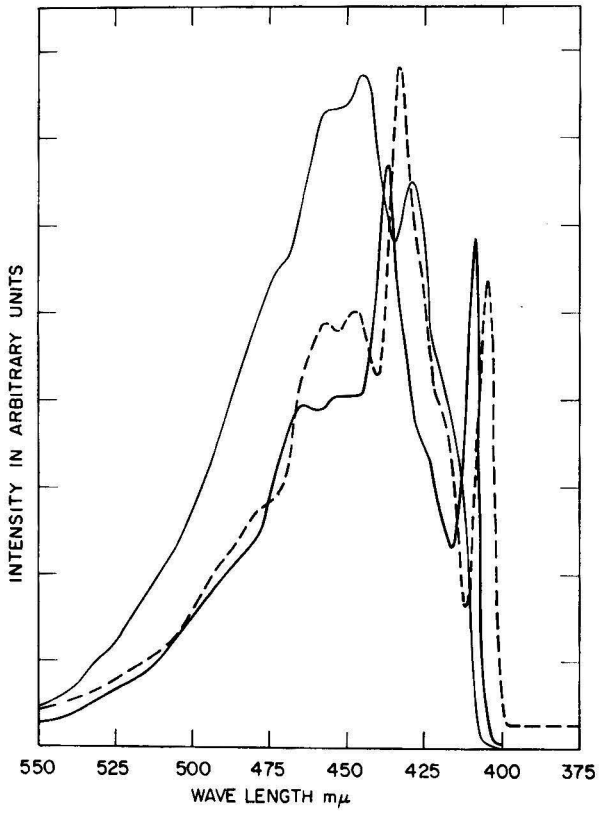


FIG. 1. Phosphorescence spectra of indole derivatives at 77°K. Tryptophan, heavy line. Indole, dashed line. Serotonin creatinine sulfate, thin line. Each in solvent, 9 parts methanol, 1 part ethanol. The indole was freshly sublimed. In the previous work cited (1), the phosphorescence curve of indole was affected by some impurity. The tryptophan and indole curves exhibit indistinguishable single peaks in fluorescence at room temperature.

to measure both spectra as well as their activation spectra at low temperatures practically at the same time. As the fluorescence spectrum is taken both kinds of spectra appear juxtaposed. Phosphorescence may be isolated from fluorescence by means of a phosphorimeter and then studied more favorably.

For purposes of analysis it is clearly desirable to have the substances in a single homogeneous phase. As the solution is cooled, separation into several phases would render reproducibility more difficult since the same solute in different phases may be expected to have somewhat different spectra. Not only would the over-all spectrum be more diffuse than if

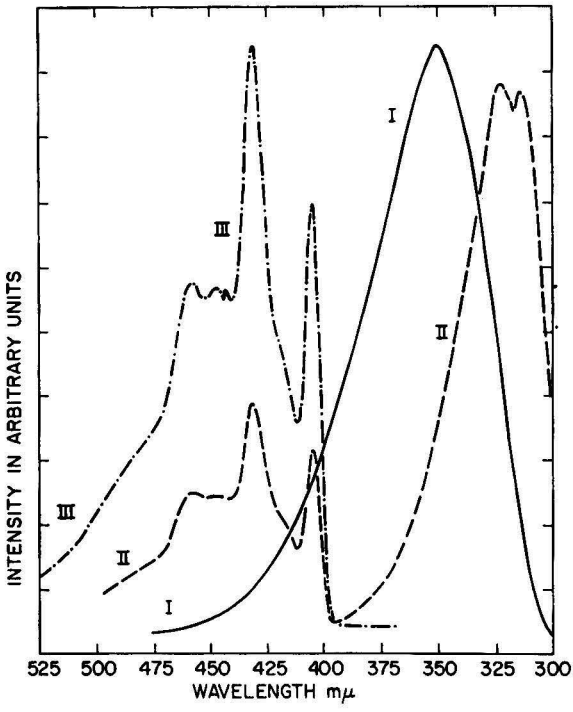


FIG. 2. Emission spectra of tryptophan at room temperature and 77°K. The three curves are on different scales of intensity. Unbroken curve I at the right represents the fluorescence spectrum at room temperature. The combined fluorescence and phosphorescence at 77°K is given by curve II (long dashes). The fluorescence at this temperature is resolved into two maxima at the right. The more elaborate structure at the left is the phosphorescence, which is also obtained with the phosphoroscope as curve III (dash point dash) at higher amplification.

the substance were in a single phase but the degree of diffuseness may vary with rate of cooling, precipitation, etc. This difficulty would be obviated if the solution persisted homogeneous as a glass at low temperatures.

The nature of the solute indicates the chemical characteristics required of a proper solvent. Multipolar substances such as amino acids, peptides, and nucleic acids require mixtures consisting mostly of polar solvents for the persistence of a single phase at low temperatures such as that of liquid nitrogen. In this report we shall consider a solvent consisting of 25% by volume water, 55% methanol, and 20% ethanol which becomes rigid between —150° and —160°C. At —160°, cracking of the organic glass occurred at times; at the temperature of liquid nitrogen (—196°C)

the glass cracked suddenly and violently into a few large fragments. Under these circumstances the intensities of emission spectra of the solution varied greatly merely upon thawing and refreezing the same solution. However rather reproducible intensities resulted if small chips of fused silica were added at the bottom of the sample tube. A variability of about $\pm 15\%$ usually remained after thawing and freezing. Presumably the sharp edges of the silica chips initiated cracks throughout the organic glass while it was still plastic, before excessive strains and pressures had developed. A further advantage contributed by the chips was the elimination of bursting of sample tubes which, before, was not infrequent. It would seem likely that chips would facilitate reproducibility in emission spectra at still lower temperatures and make possible further refinement for analysis. In this regard, it may be noted that the intensity of the phosphorescence from the glass at $-160°$ was less than that at $-196°$.

An internal standard or monitor was utilized to increase the precision and reproducibility. We shall illustrate with our specific analytical problem, namely, to determine the concentration of a tyrosine peptide, *N*-acetyl-L-tyrosine ethyl esters (ATEE), as well as that of an enzyme, chymotrypsin (or trypsin) which emits the phosphorescence of tryptophan.

All solutions and blanks contained the same concentration of a monitor, benzyl alcohol. The phosphorescence of the latter was always excited at 250 mμ. It has a maximum at 345 mμ free of overlap from the phosphorescence of tyrosine or tryptophan. The latter two are more effectively excited at 280 mμ, a frequency which produces negligible or easily corrected intensity in the phosphorescence of the monitor. Concentrations of the tyrosine peptide could be determined by comparing the intensity of its maximum at 395 mμ relative to that of a solution containing a known concentration of this peptide. Both these solutions contained also the same concentration of monitor so that the relative concentrations of the peptides of these solutions were given by the ratio of the heights of the maxima emitted by the peptides per unit height of the maxima of the monitor in their respective solutions. The same procedure was followed for the analyses of the enzymes emitting the tryptophan phosphorescence. Here the relative heights of the maxima at 455 mμ were measured as they proved to be free of overlap not only from the phosphorescence of benzyl alcohol but also of tyrosine.

To test the independence of emissions of the solutes ($2 \times 10^{-5}\,M$) in the presence of the monitor at $1.3 \times 10^{-4}\,M$, the latter concentration was doubled. The resulting intensities of phosphorescence are given in the Table 1, which shows that a doubling in the concentration of the monitor

TABLE 1

REPRODUCIBILITY AND CONSISTENCY OF PHOSPHORESCENCE SOLUTION OF *N*-ACETYL-L-TYROSINE ETHYL ESTER (2×10^{-5} *M*)

Group	Run No.	Exc. at 280 mμ, phosphorescence at 395 mμ, unmonitored, full scale, 3×10^{-9} amp	Exc. at 250 mμ, phosphorescence at 345 mμ of monitor, full scale, 3×10^{-10} amp	Ratio 395/345
		BENZYL ALCOHOL MONITOR (1.3×10^{-4} *M*)		
A	1	45.1	23.1	1.95
	2	44.7	24.0	1.86
	3	46.4	21.9	2.12
	4	45.5	21.9	2.08
		Av. 45.4 ± 0.52		2.00 ± 0.10
		MONITOR (2.5×10^{-4} *M*)		
B	1	45.8	44.9	1.02
	2	49.5	52.5	0.94
	3	52.6	47.5	1.11
	4	54.7	46.5	1.18
		Av. 50.7 ± 3.0		1.06 ± 0.08

SOLUTION OF α-CHYMOTRYPSIN (2×10^{-5} *M*)

Group	Run No.	Exc. at 280 mμ, phosphorescence at 455 mμ, unmonitored full scale, 10×10^{-9} amp	Exc. at 250 mμ, phosphorescence at 345 mμ of monitor, full scale 3×10^{-10} amp	Ratio 455/345
		MONITOR (1.3×10^{-4} *M*)		
C	1	21.7	15.1	1.43
	2	22.2	16.3	1.36
	3	16.3	11.2	1.46
	4	17.7	12.7	1.39
		Av. 19.5 ± 2.2		1.41 ± 0.03 (Monitored)
		MONITOR (2.6×10^{-4} *M*)		
D	1	28.2	36.1	0.78
	2	26.4	38.0	0.69
	3	22.5	29.8	0.76
	4	22.3	29.3	0.76
		Av. 24.9 ± 2.5		0.75 ± 0.03 (Monitored)

produced a halving in the ratio of the heights of the maxima of the solutes per unit height of the maxima of the monitor in their respective solutions. It was regarded as unnecessary to double similarly the concentrations of the peptide and of the enzyme since their concentrations being considerably less than that of the monitor would show if anything less deviation from linearity.[2] The effect of the monitor can be seen in the table. In groups A, B, C, D the average percentage deviations from the mean were respectively 1, 6, 11, and 10 in the unmonitored runs, while the corresponding monitored values were 5, 8, 2, and 4.

The light source employed for excitation was a high-pressure 1000-watt mercury arc (BH-6, General Electric Co.) Its intensity was monitored by a solution of benzyl alcohol (in the aqueous alcoholic solvent, free of air). The intensity of emission of this solution, sufficiently inert photochemically, was examined before and after each set of runs to monitor the constancy of the arc.

The phosphorimeter was of the classical Becquerel type consisting of a cylinder with a pair of slits at 180° rotating on a vertical axis on which were centered the sample tube in a Dewar flask, both of fused silica. This assembly was placed in between two monochromators (500-mm focal length, Bausch and Lomb Optical Co.), the first of which selected the wavelength from the arc for exciting the solution to emission. The latter radiation focused on the slit of the second monochromator at right angles to the first was analyzed by the rotation of its grating. At the exit slit was a photomultiplier tube (EMI Tube No. 6256B, EMI—U. S. Ltd., Westbury, L. I., N. Y.) whose output was amplified and recorded. The retardation was the time required by the rotating cylinder to make one quarter turn, for its slit to face the first monochromator and then face the second. With 3000 rpm of the motor, the retardation was 5 msec. A considerable reduction in speed did not affect the phosphorescence spectra, whose lifetimes were estimated as greater than a tenth of a second.

The sensitivity of phosphorimetry depends, of course, on the nature of the substances, on the intensity and constancy of the arc, gathering power of monochromators, etc. The sensitivity of phosphorimetry may be less or greater than that of fluorescence. At low temperature the latter is more intense than at room temperature.

The sensitivity may be greater not only because of the intrinsically

[2] *Note added in proof.* Recent calibrations showed that the peptide and the enzyme exhibited linearity between fluorescence intensity and concentration up to about $10^{-4}\,M$ and $5 \times 10^{-6}\,M$, respectively. These limits for linearity are probably also valid in phosphorescence. Analyses of higher concentrations than these require detailed calibrations of intensities with solutions of known concentrations.

stronger phosphorescence than fluorescence of some substances but also because the scattering of incident radiation which limits the precision of fluorescence is virtually absent in phosphorescence. This permits opening of the slits of the monochromators and consequent increased intensity of phosphorescence and further electronic amplification.

The chief experimental limitation of the sensitivity in phosphorescence is probably the purity of the solvents. In our present work the lack of constancy of the arc, even though monitored, remained a limitation on the reproducibility but 0.1 μg/ml ATEE could be readily measured. Neither the gratings nor the photomultiplier tube were calibrated for dependence of response upon wavelength.

SUMMARY

Because spectra of substances at low temperatures exhibit greater sharpness, resolution, and intensity and therefore also permit better discrimination between substances, spectrophosphorimetry at liquid nitrogen temperature was chosen for the quantitative analyses of an enzyme (α-chymotrypsin) and a peptide (N-acetyl-L-tyrosine ethyl ester). Such multipolar substances can be maintained in homogeneous solution at the temperature of liquid nitrogen by a mixture of water (25% by volume), methanol (55%), and ethanol (20%). At this temperature the solution forms a glass and fractures but with the aid of chips of fused silica and an internal standard (benzyl alcohol), a precision of 5 to 10% was reached.

REFERENCES

1. Freed, S., and Salmre, W., *Science* **128**, 1341 (1958).
2. Kiers, R. J., Britt, R. D., and Wentworth, W. E., *Anal. Chem.* **29**, 202 (1957).

ANALYTICAL BIOCHEMISTRY 5, 345–359 (1963)

Fluorescence of Magnesium-, Calcium-, and Zinc-8-quinolinol Complexes[1]

SHIZUO WATANABE,[2] WILLIAM FRANTZ,[3] AND DONNA TROTTIER[4]

From the Cardiovascular Research Institute, University of California Medical Center, San Francisco, California

Received July 16, 1962

INTRODUCTION

About ten years ago, R. Näsänen (1–3) made a series of extensive studies on the binding (or stability) constants of metal chelates of 8-quinolinol, using the light absorption method, the potentiometric method, and also the method of solubility equilibrium. For example, the pK values at zero ionic strength and 20°C were reported to be 4.74, 3.27, and 8.56 for Mg^{2+}, Ca^{2+}, and Zn^{2+} binding, respectively (3). In 1959, D. Schacter (4) reported that an ethanol solution of magnesium-8-quinolinol exhibited fluorescence with a peak intensity at 530 mμ. He showed that this fluorescence was dependent on pH and specific for Mg^{2+}. Among eleven other divalent cations he tested, Zn^{2+} was the only one that exhibited a significant intensity of fluorescence, i.e., approximately one-half that observed with Mg^{2+}. Ca^{2+} and Sr^{2+} exhibited negligible fluorescence and the other eight cations did not exhibit fluorescence. By utilizing these fluorescence characteristics, Schacter introduced a sensitive and specific method for magnesium determination in serum and urine.

In the present paper, the fluorescence of Mg^{2+}, Ca^{2+}, and Zn^{2+} chelates of 8-quinolinol is studied further in aqueous solutions (buffered with 0.1 M triethanolamine or 0.1 M tris(hydroxy)methylaminomethane) as well as in p-dioxane, which is found to be better than ethanol in increasing the sensitivity of the method; the results are analyzed in terms of the (relative) molar fluorescence and the binding constant.

[1] This work was supported by grants from the American Heart Association and U. S. Public Health Service (H6085).

[2] Established Investigator of the American Heart Association, Inc.

[3] The early part of this work was done while W. Frantz was on the Summer Research Fellowship of the American Physiological Society in 1960 at the Dartmouth Medical School, Hanover, New Hampshire. Present address: Department of Physiology, University of Michigan, Ann Arbor, Michigan.

[4] Dartmouth Medical School, Hanover, New Hampshire.

As for the sensitivity of the fluorescence method for Mg^{2+} determination, the molar fluorescence of Mg^{2+} chelate is shown to be 8×10^5 (practical units—see definition in the section *Materials and Methods*) in *p*-dioxane. Even in aqueous solutions, it is found to be about 7×10^4 and, accordingly, the sensitivity of this fluorescence method is still far superior to that of the light absorption method [the molar absorbancy = 2×10^3; cf. refs (2), (3), (5)], although the practical fluorescence unit could be compared to an absorbancy unit only in the practical purpose, depending on the commercially available instrument.

As to the specificity of this fluorescence method for Mg^{2+}, the molar fluorescence as well as the binding constant are found to make contributions to it. Ca^{2+}-8-quinolinol as well as Zn^{2+}-8-quinolinol is found to exhibit the fluorescence at 530 mμ upon activation at 360 mμ, but the molar fluorescence for both Ca^{2+} and Zn^{2+} chelates is found to be less than one-third of that for Mg^{2+} chelate. The pK values for metal binding with 8-quinolinol obtained by this fluorescence method are 4.4, 3.1, and about 8.1 at an ionic strength of approximately 0.1 and 25°C for Mg^{2+}, Ca^{2+}, and Zn^{2+}, respectively. These values are in good agreement with those reported by Näsänen *et al.* (see above). The analysis that is used in this paper is therefore justified and the fluorescence method for Mg^{2+} determination gains theoretical basis as firm as the light absorption method.

MATERIALS AND METHODS

An Aminco-Bowman spectrophotofluorometer equipped with a xenon lamp and a IP28 photomultiplier tube was used to measure the fluorescence. Two slits with 0.5-cm openings were used, one before the activation light reached the 1×1 cm sample cell and the other before the emitted light reached the second monochrometer. The fluorescence intensity was expressed by the reading on the per cent transmission scale of the photometer (practical units). Using a mercury lamp as a calibration standard for the light wavelength, the fluorescence peak and the activation peak were observed to be at 530 and 360 mμ, respectively. With Zn^{2+} chelate, a little deviation in wavelength of the peaks was observed (515 and 375 mμ) but all measurements were made of the fluorescence at 530 mμ with activation light of 360 mμ.

All chemicals used were commercially available and they were used without any further purification. 8-Quinolinol and magnesium nitrate ($6H_2O$) were Fisher certified reagents, and calcium chloride, zinc chloride, and magnesium chloride were Baker and Adamson standard purity reagents. Triethanolamine (TEA)[5] and tris(hydroxy)methyl-

[5] Abbreviations used are TEA for triethanolamine and Tris for tris(hydroxy)-methylaminomethane.

aminomethane (Tris) were Fisher certified reagents; they were used at concentrations of 0.1 *M* as buffers.

Sample solutions were kept in a constant-temperature bath of 25°, except for a few minutes for each measurement of the fluorescence intensity, when an aliquot volume (usually 3 ml) of sample solution was taken to the sample cell compartment which had no temperature control.

TREATMENT OF DATA

In the following discussion Mg^{2+} or Ca^{2+} will be denoted by M and undissociated 8-quinolinol by QH. The following equilibria are considered:

$$\begin{aligned} QH &\rightleftharpoons Q + H^+, & K_H \\ M + Q &\rightleftharpoons QM, & K \end{aligned}$$

In particular, it is considered that conditions are always such that the equilibrium, $M + 2\ OH^- = M(OH)_2$, can be ignored (see 6).

By combining Mass Law expressions, one obtains:

$$X/(M_0 - X)(X_0 - X) = K_H K/(K_H + [H^+]) \quad (1)$$

Here, the total concentrations of metal ions and 8-quinolinol are denoted by M_0 and X_0, respectively; the concentration of the complex QM by X; K_H and K are a dissociation constant and an association constant, respectively, for the equilibria considered above. At constant pH the expression on the right-hand side of Eq. (1) plays the role of an equilibrium constant which we shall call K'. Differentiating Eq. (1) with respect to M_0 and combining the result with Eq. (1) we obtain, at constant pH and X_0:

$$\partial X/\partial M_0 = (X_0 - X)^2/\{X/K' + (X_0 - X)/K' + (X_0 - X)^2\} \quad (2)$$

As M_0 is made very large, $X \to X_0$, and as M_0 is made very small, $X \to 0$, hence from Eq. (2) it follows that the $X(M_0)$ curve will be of a "saturation" type, i.e., at very large M_0 it will be flat (slope = 0), and near the origin its slope will be:

$$\lim_{X \to 0} (\partial X/\partial M_0) = X_0/\{1/K' + X_0\} \quad (3)$$

Actually we measure not X, but the fluorescence, f, arising from excitation of QM. If we assume that $f = \alpha X$ (where α is the "molar fluorescence" of QM; see also footnote 6), substituting this relation into Eq. (3) and taking the reciprocal of both sides, we obtain:

$$\beta \equiv \lim_{X \to 0} (\partial M_0/\partial f) = 1/\alpha + (1/\alpha)(1/K'X_0) \quad (4)$$

Equation (4) shows that a plot of the limiting slope, β, against $(1/X_0)$

is linear, that the intercept on the β axis ($1/X_0 = 0$) is $1/\alpha$, and that the slope of the line divided by this intercept is $1/K'$.

Recalling the definition of K' we may also re-express Eq. (4) as:

$$\beta \equiv (1/\alpha) + 1/(\alpha K X_0) + \{1/(\alpha K X_0)\}(1/K_H)[H^+] \quad (5)$$

Equation (5) shows that at constant X_0, β is linear with $[H^+]$. If I_H is the intercept on the $[H^+]$ axis ($\beta = 0$) of such a plot, then:

$$(-I_H) = K_H(1 + KX_0) \quad (6)$$

Accordingly:

if $KX_0 \ll 1$, then $(-I_H) = K_H$,
if $KX_0 = 1$, then $(-I_H) = K_H/2$,
and: if $KX_0 \gg 1$, then $(-I_H) = KK_HX_0$

Of course, it is also obvious from the definition of K' that, when $[H^+] \ll K_H$, $K' = K$, when $[H^+] = K_H$, $K' = K/2$, and when $[H^+] \gg K_H$, $K' = KK_H/[H^+]$.

RESULTS

Ia. Keeping a fixed concentration of 8-quinolinol ($X_0 = 1.92 \times 10^{-5}\ M$) and varying the total concentration of added Mg^{2+} (M_0), the intensity of fluorescence (f) at 530 mμ upon activation at 360 mμ was measured at various pH's (various curves of Fig. 1). The resulting data are closely fitted by theoretical curves (solid lines of Fig. 1) based on the foregoing analysis, and therefore they justify said analysis.

In curves such as those in Fig. 1, as M_0 is indefinitely increased $X \to X_0$ and $f \to f_{max}$, then from our assumptions it should be that $f_{max} = \alpha X_0$.

In Fig. 2 are shown plots of f_{max} vs. X_0 in two different solvents; in such plots the slope is α. The data are, in fact, linear plots provided $X_0 < 5 \times 10^{-5}\ M$. In an aqueous solution (0.1 M triethanolamine buffer, pH 9.45), $\alpha = 8 \times 10^4$; in an ethanolic solution, 80% v/v (Tris buffer, pH 9.5), $\alpha = 33 \times 10^4$. When X_0 exceeds ca. $5 \times 10^{-5}\ M$, the plots bend away from linearity; this is probably due to self-absorption by the increasing concentrations of the complex QM.[6]

b. Equation (1) is symmetrical as regards M_0 and X_0. Therefore if one starts with a fixed M_0 and increases X_0, or starts with a fixed X_0 and increases M_0, the f_{max} attained in either case should be the same provided the starting M_0 equalled the starting X_0. However, as shown in Fig. 3 (curves a and b), when we started with a fixed M_0 ($2 \times 10^{-5}\ M$)

[6] The fluorescence intensity is linearly with the concentration of fluorescing substance only when the concentration is very low.

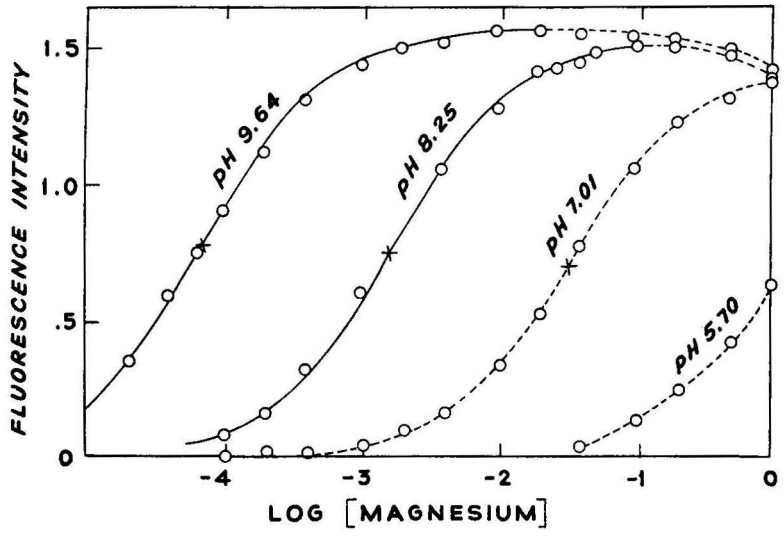


FIG. 1. Fluorescence as a function of Mg^{2+} concentration at various pH's. [8-quinolinol] $= 1.92 \times 10^{-5} M$, 0.1 M triethanolamine buffer, and 25°. Solid curves are calculated curves based on the analysis described in the text. Dashed curves are those tracing experimental results.

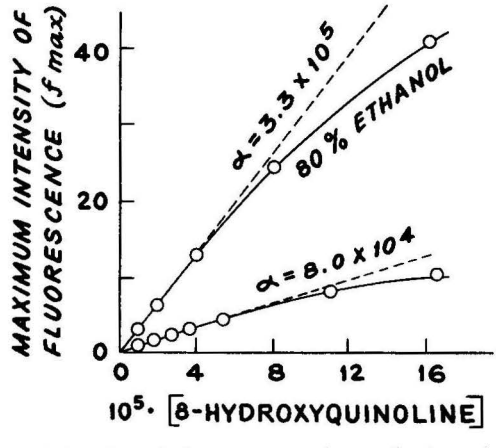


FIG. 2. Maximum intensity of fluorescence observed at various concentrations of 8-quinolinol at 25°. Upper curve: 80% (v/v) ethanol and pH 9.5 (0.1 M Tris buffer). Lower curve: aqueous solution and pH 9.45 (0.1 M triethanolamine buffer).

and increased X_0,[7] a lesser f_{max} was attained and, when the concentration X_0 exceeded about $10^{-3.0}$ M, the fluorescence intensity decreased with

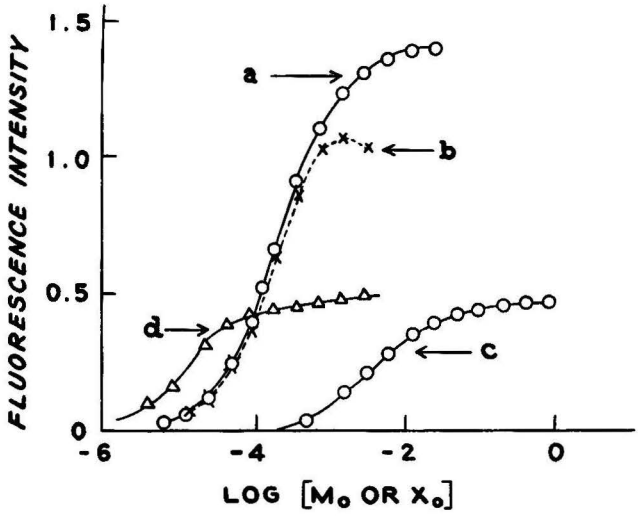


FIG. 3. Comparison of fluorescence intensity observed upon addition of increasing concentration of Mg^{2+} to 2×10^{-5} M 8-quinolinol with those of Ca^{2+} and Zn^{2+} and also with that observed upon addition of increasing concentration of 8-quinolinol to 2×10^{-5} M Mg^{2+}. Curve a: Mg^{2+} (M_0) was added to 2×10^{-5} M 8-quinolinol in 0.1 M triethanolamine buffer, pH 8.95. Curve b: 8-quinolinol (X_0) was added to 2×10^{-5} M Mg^{2+} in 0.1 M triethanolamine buffer, pH 8.95. Curve c: Ca^{2+} (M_0) was added to 2×10^{-5} M 8-quinolinol in 0.1 M triethanolamine buffer, pH 8.95. Curve d: Zn^{2+} (M_0) was added to 2×10^{-5} M 8-quinolinol in 0.1 M triethanolamine buffer, pH 7.4. Mg^{2+} (curve a), Ca^{2+} (curve c), and Zn^{2+} (curve d) $\equiv M_0$ were added to 2×10^{-5} M 8-quinolinol in 0.1 M triethanolamine buffer (pH 8.95: curves a and c and pH 7.4: curve d). 8-quinolinol ($\equiv X_0$) was added to 2×10^{-5} M Mg^{2+} in 0.1 M triethanolamine buffer, pH 8.95.

increasing X_0; this is probably not due to self-absorption of the formed complex QM since the concentration of QM in the presence of 2×10^{-5} M magnesium (M_0) cannot exceed 5×10^{-5} M, the concentration at which fluorescence of the complex began to show the self-absorption effect (See Fig. 2). This is also not due to the inadequacy of the considered equi-

[7] A solution of metal ion (Mg^{2+}, Ca^{2+}, or Zn^{2+}) without 8-quinolinol certainly does not exhibit fluorescence but 8-quinolinol solution did fluoresce without addition of metal ion. This fluorescence became significant at the concentration of 8-quinolinol approximately above 10^{-4} M, and was reduced only partially by addition of EDTA. Therefore, this fluorescence is not due to the metal contamination of 8-quinolinol reagent. In curve b of Fig. 3, the difference between the fluorescence intensity with and without addition of magnesium ion was taken as the fluorescence intensity.

libria, as will be indicated later in another type of very detailed experiments, but is probably due to absorption of both the activation light and fluorescence light by yellowish color of the 8-quinolinol medium which was visually noticeable at these higher concentrations such as $10^{-3.0}\,M$,[8] or above.

c. Bearing these complications in mind, we may estimate K'. For example, for curve a of Fig. 3, $X_0 = 2 \times 10^{-5}\,M$ and pH 8.95. In this case, the half-maximum fluorescence ($f_{max/2}$) is obtained when $[Mg^{2+}] = 10^{-3.67}\,M$. Such a condition is equivalent to setting $X = X_0/2$ in the left-hand side of Eq. (1), i.e., to having $K' = 1/(M_0 - X_0/2)$; thus from the values just cited, K' for $Mg^{2+} = 10^{3.67}$ liter mole^{-1} at pH 8.95. Likewise, K's for Ca^{2+} and for Zn^{2+} may be obtained from curves c and d; K' for $Ca^{2+} = 10^{2.47}$ liter mole^{-1} at pH 8.95 and K' for $Zn^{2+} = 1/(10^{-4.9} - 10^{-5.0}) \cong 10^{5.6}$ liter mole^{-1} at pH 7.4. With Zn^{2+}, however, precipitation took place even when this low pH was used. Although measurements with Zn^{2+} were done quickly before precipitation became visible, the fact that the plateau had not been reached with curve d reflects the precipitation. The K' value for Zn^{2+} cited above was therefore estimated on the assumption that the maximum fluorescence with Zn^{2+} is the same as that with Ca^{2+} (2.1×10^4). In order to avoid the precipitation with Zn^{2+}, the pH had to be lowered to less than 5. Using $0.1\,M$ acetic acid-tetramethylammonium hydroxide, the half-maximum fluorescence was observed when $[Zn^{2+}] = 10^{-3.1}\,M$ at pH 4.8, indicating K' for $Zn^{2+} = 10^{3.1}$ liter mole^{-1} at this pH. The maximum fluorescence observed at this pH was, however, very small—0.65×10^4 for $2 \times 10^{-5}\,M$ 8-quinolinol and accordingly $\alpha(Zn^{2+}\ \text{chelate}) = 3.3 \times 10^3$ at pH 4.8. Furthermore, Mg^{2+}, even in a concentration as high as $0.1\,M$, did not produce any fluorescence in the presence of $2 \times 10^{-5}\,M$ 8-quinolinol at this pH. Zn^{2+} chelate was therefore not studied further.

d. α (calculated from the slope of f_{max} vs. M_0 curves) and K' (calculated from "half-saturation" values as above) were measured in water-ethanol and water-*p*-dioxane solutions, at various pH's (Fig. 4). The results (see also Fig. 1) can be summarized by saying that K' is markedly reduced by pH but very little affected by adding *p*-dioxane; on the other hand, α is little affected by pH but markedly increased by *p*-dioxane. The effect of ethanol is qualitatively similar to that of *p*-dioxane.

IIa. The method of estimating K' by locating ($f_{max/2}$) is simple but it does not fit for estimation at lower pH's where a very high concentration of M_0 or X_0 is required to reach f_{max}; the method based on Eq. (4) or its analog, [$\partial X_0/\partial f$) vs. $1/M_0$] is in this sense superior. Besides,

[8] This yellowish color is already visually noticeable at around $5 \times 10^{-4}\,M$.

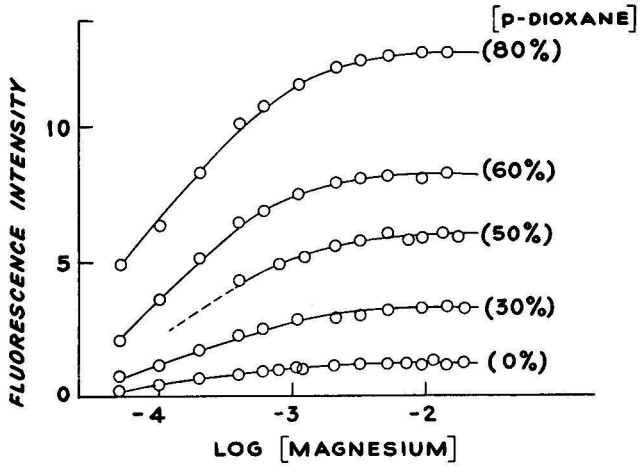


FIG. 4. Effect of *p*-dioxane concentration on fluorescence of Mg^{2+}-8-quinolinol. [8-quinolinol] $= 1.57 \times 10^{-5} M$ and pH $8.9 \sim 9.2$ ($0.1 M$ Tris buffer). The concentration of *p*-dioxane in % (v/v) is shown at the right-hand side of each curve.

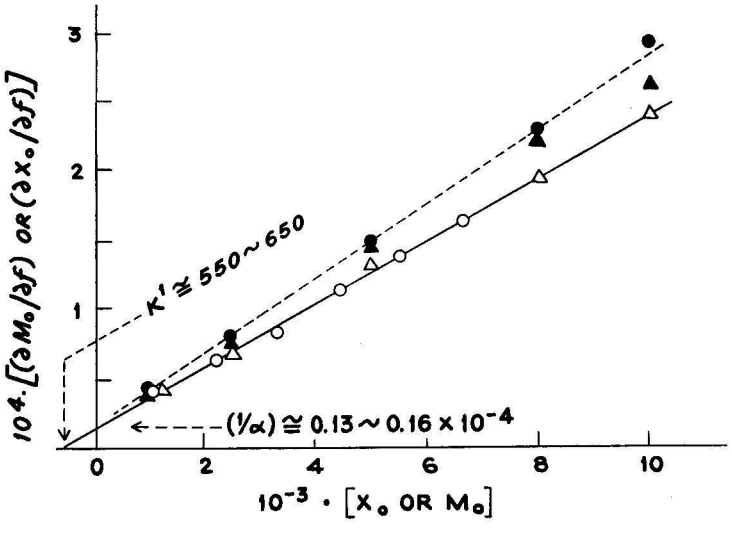


FIG. 5. Comparison of fluorescence increment upon addition of increasing concentration of Mg^{2+} to a fixed concentration of 8-quinolinol with that upon addition of 8-quinolinol to a fixed concentration of Mg^{2+}. $X_0 \equiv$ total concentration of 8-quinolinol and $M_0 \equiv$ total concentration of Mg^{2+}. ○, ●: $(\partial M_0/\partial f)$ versus $1/X_0$. △, ▲: $(\partial X_0/\partial f)$ versus $1/M_0$. ○, △: $0.1 M$ triethanolamine buffer, pH 8.2. ●, ▲: $0.1 M$ Tris buffer, pH 8.08.

$(\partial M_0/\partial f)$ or $(\partial X_0/\partial f)$ can be measured before the concentration of formed complex QM exceeds $5 \times 10^{-5}\ M$, thus eliminating one of the two complications attended in the former method. This second method is illustrated in Fig. 5 with measurements at pH $8.1 \sim 8.2$ using two different buffers (TEA and Tris), and with two symmetrical plots, i.e., $(\partial M_0/\partial f)$ vs. $1/X_0$ and $(\partial X_0/\partial f)$ vs. $1/M_0$. All four plots show a fairly good linear relation and have the same intercept on the vertical axis which gives $\alpha \cong 6.6 \times 10^4$. This α value is smaller than that obtained previously (8×10^4) from the results shown in Fig. 2. This may not be due to the relatively high concentrations of 8-quinolinol (10^{-3} to $10^{-4}\ M$) used for measurements of $(\partial M_0/\partial f)$ since measurements of $(\partial X_0/\partial f)$ were made with 8 quinolinol in concentrations less than $5 \times 10^{-5}\ M$ and, as already pointed out, the plot of $(\partial M_0/\partial f)$ vs. $1/X_0$ coincided with the plot of $(\partial X_0/\partial f)$ vs. $1/M_0$. That the difference between the two α values is, however, due to the difference in pH was indicated by the results shown in Table 1. The α value was obtained at various pH's from the intercept on the vertical axis in the plot of $(\partial X_0/\partial f)$ vs. $(1/M_0)$ and was observed to be dependent on pH.

TABLE 1
pH DEPENDENCE OF (RELATIVE) MOLAR FLUORESCENCE (α) OF Mg^{2+}-8-QUINOLINOL

pH	9.5	8.9	8.0	7.2
α	8.0	7.1	6.7	6.2

At 25° and in 0.1 M TEA buffer.

It should also be mentioned that the intercept on the horizontal axis in Fig. 5 gives the value for $(-K')$, 550 in Tris buffer (pH 8.1) and 650 in TEA (pH 8.2). Taking into account the difference in pH, the difference in K' between Tris and TEA buffers is almost negligible. The same situation will be seen later in Fig. 8b.

b. At a sufficiently low pH but not too low to avoid the participation of the ring ionization ($pK \cong 5.05$) and/or at a sufficiently low concentration of 8-quinolinol, an approximation to Eqs. (4) or (5) is $\beta \cong (1/\alpha K')(1/X_0)$. Such a case is illustrated in Fig 6, wherein β as a function of $1/X_0$ then passes through the origin and the reciprocal slope is $\alpha K'$; $\alpha K'$ for $Mg^{2+} \cong 3.2 \times 10^6$ liter mole^{-1} at pH 7.15 (empty circles) and $\alpha K'$ for $Ca^{2+} \cong 2.5 \times 10^6$ liter mole^{-1} at pH 8.87 (solid circles).

c. At high pH, e.g., 9.2, there arises a discrepancy between (∂X_0) and (∂M_0), as shown in Fig. 7, in the presence of 80% (v/v) ethanol. While the magnesium concentration is lower than approximately $5 \times 10^{-5}\ M$, a

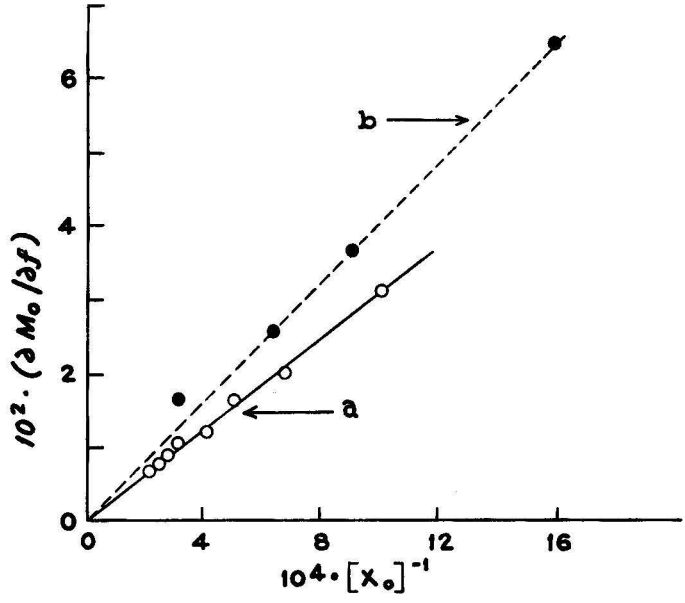


FIG. 6. Fluorescence increment per Mg^{2+} concentration and that per Ca^{2+} concentration at various concentrations of 8-quinolinol. $X_0 \equiv$ total concentration of 8-quinolinol. 0.1 *M* triethanolamine buffer. a: $M_0 \equiv$ total concentration of Mg^{2+}, pH 7.15. b: $M_0 \equiv$ total concentration of Ca^{2+}, pH 8.87.

plot of $(\partial X_0/\partial t)$ vs. $(1/M_0)$ shown in Fig. 7a provides the same values for K' and α as obtained from a plot of $(\partial M_0/\partial f)$ vs. $1/X_0$ shown in Fig. 7b (solid circles); $K' \cong 1.08 \times 10^4$ and $\alpha \cong 8 \times 10^5$. However, when the magnesium concentration higher than 5×10^{-5} *M* was used, a plot of $(\partial X_0/\partial f)$ vs. $(1/M_0)$ gave a lower value for α and a higher value for K': $\alpha \cong 3.3 \times 10^5$ and $K' \cong 3 \times 10^4$. This is possibly due to the existence of a species MQ_2 or M_2Q not taken into account in the foregoing analysis. It can be also seen in Fig. 7b that, when the concentration of 8-quinolinol becomes so high that the absorption effect of yellow colored media is expected (see Fig. 3), a plot of $(\partial M_0/\partial f)$ vs. $(1/X_0)$ shows deviation from linearity.

d. Taking account of (*1*) the light absorption effect by using 8-quinolinol in the concentration less than 10^{-3} *M* and (*2*) the effect of high pH's by using pH's lower than 9, and (*3*) the pH effect on α by using the *II*nd type of experimentation rather than the *I*st one, the K' values for Mg^{2+} were estimated at various pH's from the intercept on the horizontal axis in the plot of $(\partial M_0/\partial f)$ vs. $(1/X_0)$ and the results are summarized

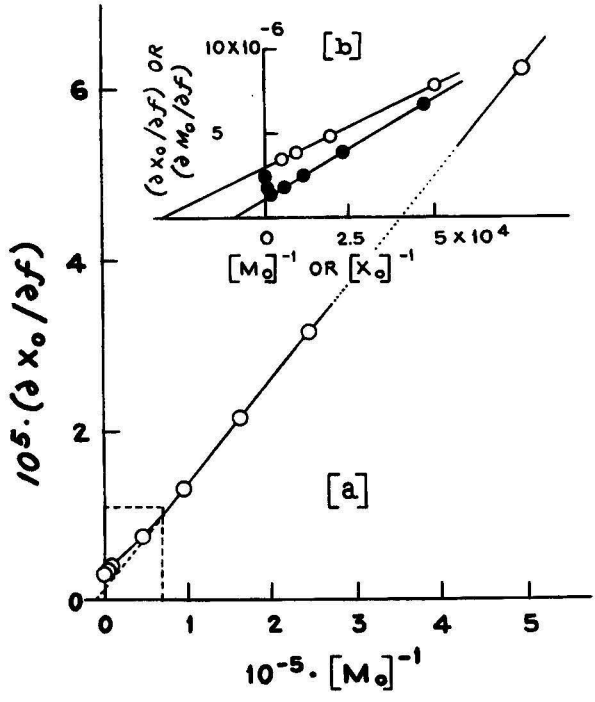


FIG. 7. Effect of high concentrations of Mg^{2+} and 8-quinolinol at high pH and in 80% (v/v) ethanol. $M_0 \equiv$ total concentration of Mg^{2+}, $X_0 \equiv$ total concentration of 8-quinolinol. pH 9.5 (0.1 M Tris buffer). The part of Fig. 7a framed by a dashed line is enlarged in 7b. ○: $(\partial X_0/\partial f)$ versus $1/M_0$, ●: $(\partial M_0/\partial f)$ versus $1/X_0$.

in Fig. 8a. As described in the foregoing analysis, the intercept on the pH axis gives the value for $(-\log KK_H)$: $\log KK_H \cong -5.3$.

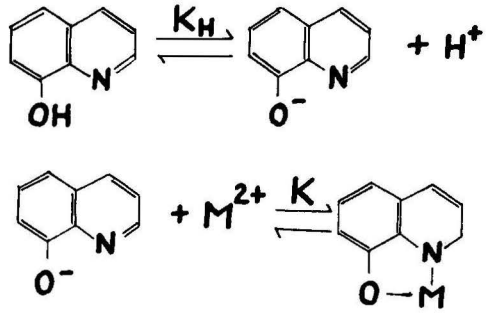
To obtain the KK_H value, there is another way of experimentation based on Eq. (6). By use of a fixed and high concentration of 8-quinolinol ($5 \times 10^{-4} M$) and a low pH region (<pH 9), $(\partial M_0/\partial f)$ was measured at various pH's; the results are plotted as a function of $[H^+]$ in Fig. 8b. The intercept at the $[H^+]$ axis gives a value for $(-KK_H X_0)$; $X_0 = 5 \times 10^{-4} M$ in this case and therefore $-5 \times 10^{-4} \times KK_H \cong -2.0 \times 10^{-9}$; hence $\log KK_H \cong -5.4$.

DISCUSSION

It is assumed in the first place that the fluorescence observed in a solution containing 8-quinolinol and metal ions is completely due to a complex formed from one mole to one mole binding of these two components (i.e., the 1:1 complex).

Experimental observations are made of the fluorescence intensity as a function of the metal ion concentration as well as that of the 8-quinolinol concentration.

The fluorescence is also studied as a function of pH. It is then shown that data obtained can be treated reasonably by adopting a well established reaction scheme for the binding of metal ions with 8-quinolinol:



For example, both a plot of $(\partial M_0/\partial f)$ versus $(1/X_0)$ and that of $(\partial X_0/\partial f)$ versus $(1/M_0)$ are in a linear relation as long as the concentrations X_0 and M_0 are relatively low and both of them give the same value for α or K as expected from the assumption and the adopted scheme. The relation of $(\partial M_0/\partial f)$ or $1/K$ to $[H^+]$ is shown (Fig. 8) to be also linear as expected from the adopted scheme and it gives the value for $KK_H(10^{-5.3})$. The value of K_H has been well established to be $10^{-9.9}$ at 20°C and zero ionic strength and $10^{-9.698}$ at 25° and ionic strength 0.09 by Näsänen *et al.* [(1), see also (7)]. It is therefore a reasonable approximation to take $10^{-9.7}$ as the value for K_H under the experimental conditions used in this paper, i.e., 25° and ionic strength about 0.1. The K value for Mg^{2+}-8-quinolinol is then calculated to be $10^{-5.3}/10^{-9.7} = 10^{4.4}$. With Ca^{2+}, the values for α and K' at pH 8.95 were estimated to be 2.1×10^4 and $10^{2.4}$, respectively, from curve c in Fig. 3. If $K_H = 10^{-9.7}$, the K for Ca^{2+}-8-quinolinol can be calculated to be $K = 10^{2.4}(1 + 10^{9.7-8.95}) = 10^{3.2}$. Furthermore, from slope b in Fig. 6, the value for $\alpha K'$ was found to be 2.5×10^6 at pH 8.87; if the α value obtained at pH 8.95 is adopted, K' is found to be $(2.5 \times 10^6)/(2.1 \times 10^4) = 10^{2.1}$ at pH 8.87 and accordingly $K \cong 10^{3.0}$. With Zn^{2+}, we have only approximate values because of the precipitation and low pH. From curve d in Fig. 3, α and K' were approximated to be 2.1×10^4 and $10^{5.6}$ at pH 7.4. From the latter, K for Zn^{2+}-8-quinolinol is estimated to be $10^{9.7-7.4} \times 10^{5.6} \cong 10^{7.9}$. Furthermore, K' for Zn^{2+} at pH 4.8 was approximately $10^{3.1}$ in acetate buffer although α at this pH was found to be very small

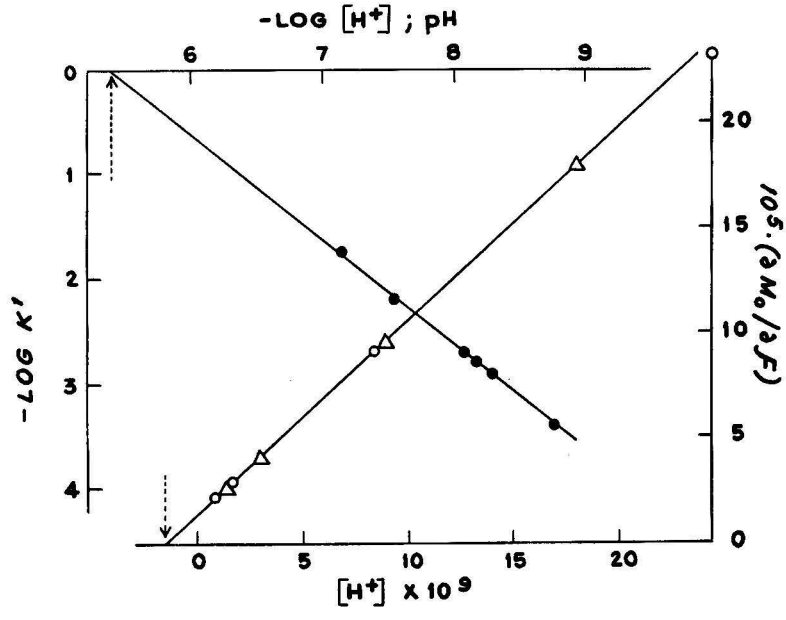
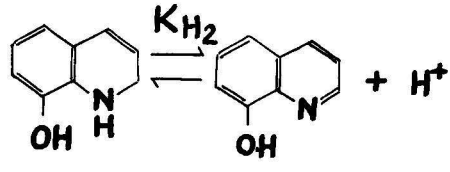


FIG. 8. pH dependence of apparent binding constant (K') for Mg^{2+} binding of 8-quinolinol and of the fluorescence increment at a fixed concentration of 8-quinolinol. A: upper left, $-\log K'$ versus pH (0.1 M triethanolamine buffer, ●). B: lower right, $(\partial M_0/\partial f)$ versus $[H^+]$ (0.1 M Tris buffer, ○; 0.1 M triethanolamine buffer, △).

(0.33×10^4). At this low pH, an ionization other than the phenol OH group has to be considered:



The dissociation constant of this ionization (K_{H_2}) was reported to be $10^{5.01}$ (1) at zero ionic strength and 20°. The observed K' should therefore be:

$$K' = K/(1 + [H^+]/K_H + [H^+]^2/[K_H K_{H_2}])$$

rather than:

$$K' = (KK_H)/(K_H + [H^+])$$

Approximating from the reported values (1) that $K_H \cong 10^{-9.7}$ and

$K_{H_1} \cong 10^{-4.8}$ under our experimental conditions (25°, ionic strength about 0.1), K is calculated to be:

$$K \cong 10^{3.1} \times 2 \times 10^{4.9} \cong 10^{8.3}$$

In Table 2, constants for metal binding of 8-quinolinol thus obtained by us are listed together with those established by Näsänen and Pettinen (3). If one considers the difference in experimental conditions noted in the table, agreement of these two sets of constants is very good.

TABLE 2
MOLAR FLUORESCENCE AND CONSTANTS FOR METAL ION BINDING WITH 8-QUINOLINOL

	Mg^{2+}	Ca^{2+}	Zn^{2+}
α	7.0 (pH 8.95)	2.1 (pH 8.95)	~2.1 (pH 7.4)
pK,[a] $\mu = 0$, 20°	4.74	3.27	8.56
pK, $\mu = 0.1$, 25°	4.4	3.0 ~ 3.2	7.9 ~ 8.3

[a] Näsänen and Pettinen (3).

As to the application of this fluorescence phenomenon to the magnesium determination, it should be mentioned that it is preferable from the viewpoint of sensitivity as well as specificity to use a high pH since K_H for the phenol OH group of 8-quinolinol is as high as 9.9 (1). Although there is an indication that complexes other than the 1:1 complex are formed at high pH, fluorescence at any fixed concentration of 8-quinolinol increases linearly with increasing Mg^{2+} concentration, and therefore the high pH does not disturb the Mg^{2+} determination. Furthermore, Zn^{2+} interferes with the Mg^{2+} determination more at lower than at higher pH's; the Zn^{2+} binding constant is so high, as compared with that of Mg^{2+}, that Zn^{2+} still exhibits fluorescence at lower pH's where Mg^{2+} cannot exhibit fluorescence, while at higher pH's Zn^{2+} is possibly removed by precipitation and the molar fluorescence of the Zn^{2+} complex seems to be less than one-third that of Mg^{2+} complex. That Ca^{2+} interference with the Mg^{2+} determination is very little is understandable from our result that not only the Ca^{2+}-binding constant is much smaller than that of Mg^{2+} but also the molar fluorescence of the Ca^{2+} complex is less than one-third of that of the Ca^{2+} complex.

SUMMARY

1. The study was made mainly in aqueous solutions of 0.1 M triethanolamine and tris(hydroxy)methylaminomethane on the fluorescence that was reported by Schacter in 1959 to be exhibited by an ethanolic solution of 8-quinolinol and metal ions (Mg^{2+}, Ca^{2+}, and Zn^{2+}). The

fluorescence was studied as a function of metal ion concentration, 8-quinolinol concentration, and also pH.

2. The molar fluorescence and the apparent binding constant of these metal complexes and 8-quinolinol were then estimated. The apparent constants thus estimated were found to be in good agreement with those established by Näsänen and Pettinen (1952) using the light absorption method, the potentiometric method, and the method of solubility equilibrium.

3. The sensitivity and specificity of the method for Mg^{2+} determination that was based on this fluorescence phenomenon were discussed in terms of the molar fluorescence and the binding constant.

ACKNOWLEDGMENT

The authors wish to thank Dr. Manuel F. Morales in this Institute for his constant encouragement and valuable discussion.

REFERENCES

1. NÄSÄNEN, R., LUMME, P., AND MUKULA, A-L., *Acta Chem. Scand.* **5**, 1199 (1951).
2. NÄSÄNEN, R., *Acta Chem. Scand.* **5**, 1293 (1951); see also **6**, 352 (1952).
3. NÄSÄNEN, R., AND PETTINEN, U., *Acta Chem. Scand.* **6**, 837 (1952).
4. SCHACTER, D., *J. Lab. Clin. Med.* **54**, 763 (1959).
5. BURTON, K., *Biochem. J.* **71**, 388 (1959).
6. STOCK, D. I., AND DAVIES, C. W., *Trans. Faraday Soc.* **44**, 856 (1948)
7. MARTELL, A. E., AND CALVIN, M., "Chemistry of the Metal Chelate Compounds." Prentice-Hall, New York, 1952.

ANALYTICAL BIOCHEMISTRY 5, 360–367 (1963)

SHORT COMMUNICATIONS

A Simple Estimation of Cathepsin C Using a New Chromogenic Substrate

Cathepsin C, a —SH activated intracellular peptidase first described by Fruton and his collaborators (1), has high specificity requirements. It only splits off N-terminal dipeptides from peptide chains or hydrolyzes dipeptide amides or esters if the penultimate amino acid is aromatic and has the L configuration (2, 3). The rate of splitting is decreased if the N-terminal amino acid, whose amino group must be unsubstituted, is bulky (2, 4). Typical, rapidly split cathepsin C substrates are thus Gly-Phe-amide, Gly-Phe-esters, and similar compounds. The enzyme also catalyzes the transfer of the dipeptidyl grouping of these substrates to suitable acceptors.

The estimation of cathepsin C is generally carried out by measuring the hydrolysis of Gly-Phe-amide or the formation of Gly-Phe-NHOH from Gly-Phe-amide and hydroxylamine (5). The first method, in which the ammonia produced is measured by the Conway procedure, is highly accurate and generally applicable. Difficulties are encountered only with tissue preparations containing small amounts of the enzyme and releasing ammonia by other reactions in the substrate blank. The method is, however, rather time- and substrate-consuming and unsuitable for the assay of a large number of, e.g., chromatographic fractions. The hydroxamate method is much more rapid, but only applicable in a very limited range of enzyme activity. It is, moreover, less accurate than the hydrolysis method. In connection with our work on the chromatographic purification of cathepsin C we therefore were in need of a method that combined rapidity and precision with sensitivity.

In view of the good results in other cases with peptide derivatives containing *p*-nitrophenol or *p*-nitroaniline as chromogenic substrates (6, 7[1]) Gly-Phe-*p*-nitrophenol and Gly-Phe-*p*-nitroanilide were synthesized and investigated as possible substrates.

[1] See also ref. (8), which appeared when this paper was in preparation.

The nitrophenyl ester was obtained by reacting Cbz-Gly-L-Phe and *p*-nitrophenol in the presence of dicyclohexylcarbodiimide and then reducing the substituted ester by the use of HBr in glacial acetic acid. It turned out to be unsuitable because of its extreme instability in water. It spontaneously hydrolyzes completely within several minutes, even at pH 5 and room temperature. This compound is also unstable toward alcohols and undergoes transesterification in alcohol-containing solvents, as during crystallization. Whether this surprising lability is a special property of dipeptide *p*-nitrophenyl esters—which might yield diketopiperazine intermediates—or is shared by other nitrophenyl esters containing a free amino group is unknown to us.

Gly-Phe-*p*-nitroanilide was prepared by condensation of Cbz-Gly-L-Phe and *p*-nitroaniline in the presence of dicyclohexylcarbodiimide, followed by decarbobenzoxylation of the intermediate using HBr in glacial acetic acid. A yellow oil was obtained, a small part of which could be crystallized from ethanol-ether. The yield of crystals was very poor (4%); the purity of the hygroscopic crystals was checked by spectrophotometric analysis. The crystalline compound has its maximum absorption at 315 mμ with a molar extinction coefficient of 12,500. These values agree well with those found for other *p*-nitroanilides (7). In contrast to the *p*-nitrophenyl ester, the anilide is completely stable in water, at least below pH 8. Cathepsin C and chymotrypsin split exactly 50%. Complete racemization must thus have occurred during preparation. Another synthetic route is under study.

The enzymic reaction was followed by determining the optical density increase at 410 mμ. At this wavelength the absorption of the substrate is negligible while the molar extinction of *p*-nitroaniline is about 9000. The method thus has a high degree of accuracy ($\pm$2%).

In the concentration range studied (2×10^{-4} to 1.5×10^{-3} *M* L isomer) the reaction rate was strictly proportional to the substrate concentration and remained so during the whole reaction course. The substrate concentrations must therefore be small compared to the Michaelis constant (K_m). It is also evident that the D isomer in these concentrations cannot be inhibitory. Otherwise, different first-order constants would have been obtained at different concentrations of the DL compound. As the solubility of the compound is limiting, much higher concentrations cannot be studied.

The first-order constant is proportional to the enzyme concentration. One cathepsin unit as defined by Fruton (1) corresponds to a first-order constant of 0.017 min^{-1} at pH 5.5 and 25°C. This value is not much different from what can be calculated for Gly-Phe-Am under these conditions.

The splitting of Gly-Phe-*p*-nitroanilide thus appears to be a rapid, sensitive, and convenient method for the estimation of cathepsin C and the DL compound can be employed.

R. J. PLANTA
M. GRUBER

Biochemisch Laboratorium, Rijksuniversiteit
Groningen, The Netherlands
Received August 28, 1962

REFERENCES

1. TALLAN, H. H., JONES, M. E., AND FRUTON, J. S., *J. Biol. Chem.* **194,** 793 (1952).
2. IZUMIYA, N., AND FRUTON, J. S., *J. Biol. Chem.* **218,** 59 (1956).
3. PLANTA, R. J., AND GRUBER, M., *Biochim. Biophys. Acta* **53,** 443 (1961).
4. PLANTA, R. J., Thesis, Groningen, 1962.
5. DE LA HABA, G., CAMMARATA, P. S., AND TIMASHEFF, S. N., *J. Biol. Chem.* **234,** 316 (1959).
6. MARTIN, C. J., GOLUBOW, J., AND AXELROD, A. E., *J. Biol. Chem.* **234,** 294, 1718 (1959).
7. ERLANGER, B. F., KOKOWSKY, N., AND COHEN, W., *Arch. Biochem. Biophys.* **95,** 271 (1961).
8. BUNDY, H. F., *Anal. Biochem.* **3,** 431 (1962).

Microanalytical Determination of Dihydroxy Aromatic Acids by Gas Chromatography[1]

The determination of aromatic acids by gas chromatography requires the preparation of volatile derivatives such as the methyl esters. This has been carried out satisfactorily by diazomethane methylation for such aromatic acids as hippuric, *p*-hydroxyphenylacetic, and homovanillic (1). The formation of methylated derivatives of dihydroxy aromatic acids such as 3,4-dihydroxybenzoic (protocatechuic), 3,4-dihydroxyphenylacetic (homoprotocatechuic), and 3,4-dihydroxymandelic acid by this procedure as well as methylation by BF_3-methanol and methonolic-HCl is unsatisfactory because of the formation of mixtures of derivatives (2). We have recently devised a method for the preparation of suitable volatile derivatives by acetylating the phenolic hydroxyl groups with acetic anhydride catalyzed by trifluoroacetic acid (3), followed by methylation of the carboxyl radical with diazomethane.

[1] Supported by a grant from the National Institutes of Health (B-3221) and the American Heart Association (61 G 57) and supported by the Florida, Orange County (Fla.), Broward County (Fla.), and the Suncoast Heart Associations.

A small amount of acid (10–15 mg) is dissolved in 1.0 ml of acetic anhydride and 0.5 ml of trifluoroacetic acid and heated in a 75°C water bath for 30 min in an unstoppered 9-ml glass vial. The mixture is then evaporated under a stream of air in a hood. This ordinarily requires about 10 mins. Finally, the mixture is treated with ethereal diazomethane dropwise until the evolution of nitrogen has ceased and yellow color persists. This mixture is then evaporated under air. The final product is dissolved in acetone or tetrahydrofuran for injection into the column. The derivatives were chromatographed on a 15% silicone column (Ge SE-30) on 110-120 mesh Chromosorb W (Analabs) at 190°C using a 6-ft 0.25-in. diameter U-tube and a Barber-Colman model 10 instrument. Operating conditions were HV 1100 volts, relative gain 3×10^{-7}, argon inlet pressure 25 psi, outlet flow 150 ml/min. The relative retention times and mass response of derivatives are given in Table 1. The sensitivity is

TABLE 1
RELATIVE RETENTION TIMES OF DERIVATIVES OF STANDARD AROMATIC ACIDS

Acid	Relative retention time of derivative[a]	Mass response ($mm^2/\mu g$)
Hippuric	1.00	—
Vanillic	1.68	50
Homovanillic	2.20	43
Protocatechuic	2.64	35
Homoprotocatechuic	3.44	41
Vanilmandelic	4.84	38
3,4-Dihydroxymandelic	7.44	20

[a] Approximate retention time of hippuric acid derivative (methyldiacetylglycine) 3.0 min.

such under these conditions that amounts less than 0.5–0.8 μg/mg creatinine cannot be detected. All of the aromatic acids examined in this table were found to have single peaks of the expected order of retention time with the exception of hippuric acid. When treated by this method hippuric acid yielded a single peak with a shorter retention time than that of methyl hippurate, which is produced by the reaction of diazomethane with hippuric acid alone. It was found that, under the conditions employed, hippuric acid transacylates to benzoic acid and diacetylglycine in the first reaction. These two products then yield methyl benzoate and methyldiacetylglycine, respectively. The former compound has such a short retention time on this column at this temperature that it is lost in the solvent overload. The second product has a retention time of about 3.0 min under the conditions described. The reaction must be carried out in anhydrous solvent although many different salts do not

interfere with the reaction. The method is also applicable to monohydroxy aromatic acids with phenolic hydroxyl radicals such as *m*-hydroxyphenylacetic acid and secondary alcohols such as phenyllactic acid.

Aromatic acids were isolated from urine by a modification (4) of the procedure of Shaw *et al.* (5). Over-all recoveries of added standards by this procedure (including extraction, acetylation, and methylation) were as follows: vanillic 87%; protocatechuic 88%; homoprotocatechuic acid 91%; vanilmandelic acid 92%; 3,4-dihydroxymandelic acid 84%; homovanillic acid 90%. Values for four normal children are given in Table 2.

TABLE 2
URINARY EXCRETION OF AROMATIC ACIDS BY NORMAL CHILDREN
(micrograms/milligram creatinine)

Acid	Mean (individual values)	
Vanillic	—	(4.5, 2.1, <0.5, <0.5)
Protocatechuic	<0.5	(<0.5, <0.5, <0.5, <0.5)
Homovanillic	6.8	(7.3, 8.6, 7.6, 3.5)
Homoprotocatechuic	<0.5	(<0.5, <0.5, <0.5, <0.5)
Vanilmandelic	4.7	(5.5, 0.9, 8.5, 3.7)
3,4-Dihydroxymandelic	<0.8	(<0.8, <0.8, <0.8, <0.8)

The determination of homoprotocatechuic acid is of diagnostic importance in children who are suspected of having neuroblastoma, a malignant tumor of childhood frequently associated with hypertension. Of 42 cases of this disease so far examined, 95% have been found to have an elevated urinary excretion of homovanillic acid (HVA) and/or vanilmandelic acid (VMA) (6). Since homoprotocatechuic acid may serve as an intermediate in the formation of HVA (7) and 3,4-dihydroxymandelic acid may serve as an intermediate in the formation of VMA (8), an elevated excretion of these intermediates might be expected in some cases. Only one case of neuroblastoma has been examined and demonstrated to excrete an excessive amount of homoprotocatechuic acid (9). We have examined 7 cases of neuroblastoma recently studied in this hospital. Of these, 2 had an elevated homoprotocatechuic acid level (420 and 350 μg/mg creatinine) but none had a detectable excretion of 3,4-dihydroxymandelic acid.

Figure 1 shows a chromatograph of a standard mixture of six acids listed in Table 1, together with an extract of urine from a child with neuroblastoma metastatic to bone [Case #3 in ref. (6)]. The large peaks corresponding to HVA, VMA, and homoprotocatechuic acid are seen. No detectable evidence of vanillic, protocatechuic or 3,4-dihydroxymandelic acid were observed in this patient's urine.

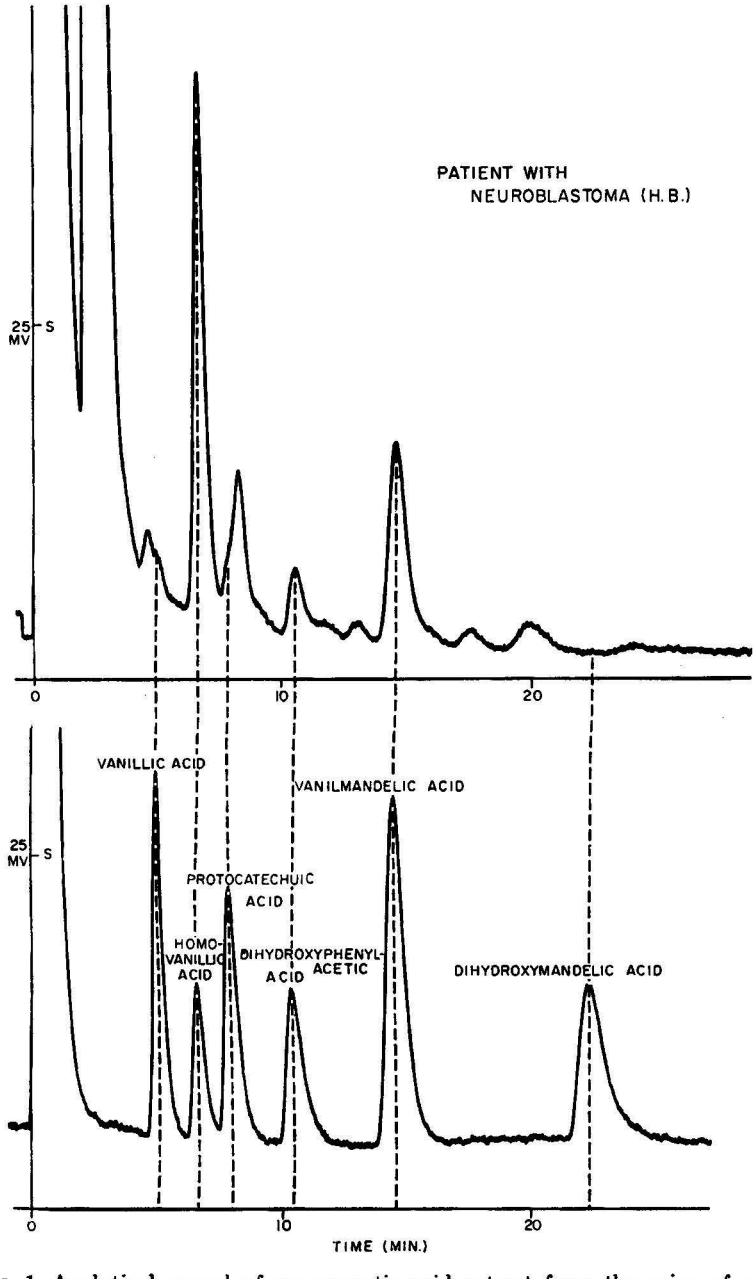


FIG. 1. Analytical record of an aromatic acid extract from the urine of a child with neuroblastoma (50 μg creatinine equivalent). S, solvent front.

REFERENCES

1. SWEELEY, C. C., AND WILLIAMS, C. M., *Anal. Biochem.* **2**, 83 (1961).
2. WILLIAMS, C. M., *Anal. Biochem.* **4**, 423 (1962).
3. TEDDER, J. M., *Chem. Revs.* **55**, 787 (1955).
4. WILLIAMS, C. M., MAURY, S., AND KIBLER, R. F., *J. Neurochem.* **6**, 254 (1961).
5. SHAW, K. N. F., MCMILLAN, A., AND ARMSTRONG, M. D., *J. Biol. Chem.* **226**, 255 (1957).
6. WILLIAMS, C. M., AND GREER, M., *J. Am. Med. Assoc.* **183**, 836 (1963).
7. WILLIAMS, C. M., BABUSCIO, A. A., AND WATSON, R., *Am. J. Physiol.* **199**, 722 (1960).
8. KOPIN, I. J., *Science*, **131**, 1372 (1960).
9. VON STUDNITZ, W., *Scand. J. Clin. Lab. Invest.* **12**, Suppl. 48 (1960).

CLYDE M. WILLIAMS
REID H. LEONARD

Department of Radiology
University of Florida College of Medicine
Gainesville, Florida, and
2544 Escambia Avenue, Pensacola, Florida
Received November 19, 1962

Convenient Apparatus for Protein Hydrolysis and Greaseless Freeze-Drying[1]

Hydrolysis of proteins for amino acid analysis, or other sealed tube procedures, may be conveniently carried out in the apparatus pictured in Fig. 1. The joints are O-ring joints, such as those supplied by Corning Glass Works or Kontes Glass Company. They are held together by a spherical-joint clamp. No loss of liquid has been observed at 110°C during 20-hr hydrolyses. For experiments in which evacuation is not required, the top tube may be sealed off before assembly. After the reaction is completed the tube may be readily opened without cutting the glass and the reaction mixture may be freeze-dried, dried on a rotary evaporator (using the adapter shown), or attached to other apparatus, fitted with a similar joint, for conversion to derivatives for gas chromatography, etc., all without transfers or the use of grease. We also use the O-ring joint in place of the standard-taper joint in the top of two-part freeze-drying flasks. Very high vacuum is maintained, and grease

[1] This investigation supported by U. S. P. H. S. Research Grant RG-9826.

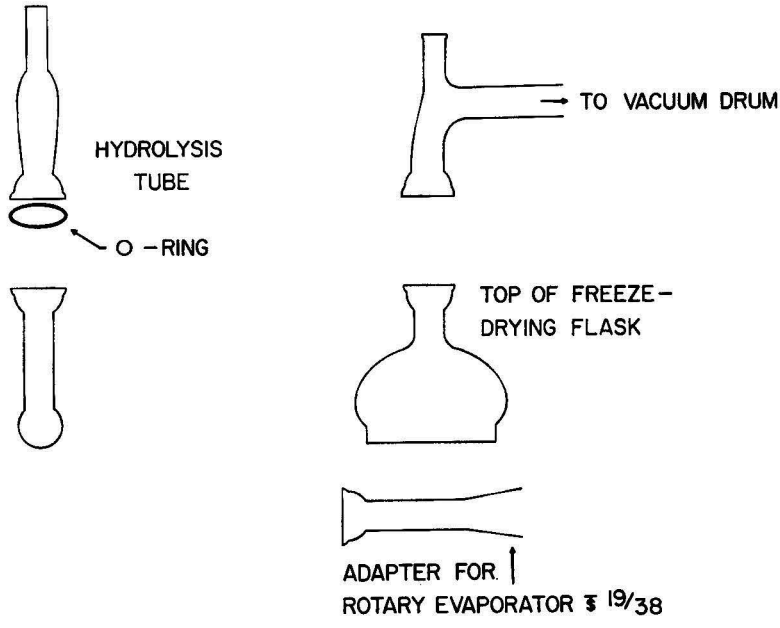


FIG. 1. Apparatus for hydrolysis and freeze-drying. Left side: top and bottom of hydrolysis tube; top may be sealed off. Right side: adapter with tubulature for serum stopper; adapter fits onto top of freeze-drying flask with O-ring and clamp. Right bottom: adapter for rotary evaporator.

is completely eliminated. Air may be slowly admitted by inserting a hypodermic needle through the serum stopper, which is inserted in the tubulation on the fitting, which is connected to the vacuum drum.

JAKE BELLO

Biophysics Department
Roswell Park Memorial Institute
Buffalo, New York
Received November 23, 1962

An Important Reprint

Now Available

Archives Italiennes de Biologie

Volumes 1-10, 1882-1888

Cloth bound set $225.00

Paper bound set 200.00

Single volumes, paper bound 20.00

ARCHIVES ITALIENNES DE BIOLOGIE, inaugurated in 1882 by A. Mosso in Turin and continued by V. Aducco in Pisa, stopped publication in 1935. Within half a century, the *Archives Italiennes de Biologie,* in 94 volumes, had provided the most important channel for the Italian contributions to the biological sciences.

ARCHIVES ITALIENNES DE BIOLOGIE, resumed in 1957, is now published in English and occasionally in French. They are devoted to the publication of original papers, which so far are predominantly neurophysiological in character, and of review articles.

Johnson Reprint Corporation

111 Fifth Avenue, New York 3, N. Y.

Johnson Reprint Company Ltd.

Berkeley Square House, London, W.1, England

Biological Transmission of Disease Agents

Symposium Held under the Auspices of the Entomological Society of America, Atlantic City, 1960

Edited by KARL MARAMOROSCH
Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York

March 1962, 192 pp., $7.00

The biological transmission of disease agents is of considerable interest and importance to workers in branches of biology, particularly to microbiologists, zoologists, virologists, plant pathologists, entomologists, veterinarians, and physicians. The chapters in this work, contributed by biologists of widely different interests and experience, cross the traditional and professional boundaries between workers engaged in plant and animal disease study.

CONTENTS

Agents of Plant Disease

Contributions by: L. M. BLACK, WILLIAM B. HEWITT, D. J. RASKI, K. SAKIMURA, JOHN T. SLYKHUIS, and EDWARD S. SYLVESTER

Agents of Human and Animal Diseases

Contributions by: DARRELL W. ANTHONY, HERBERT C. BARNETT, D. BLASKOVIC, A. W. LINDQUIST, CORNELIUS B. PHILIP, WILLIAM C. REEVES, J. REHACEK, R. TRAUB, and K. C. WILLETT A. W. LINDQUIST, CORNELIUS B. PHILIP, WILLIAM C. REEVES, J. REHÁCEK, R. TRAUB, and K. C. WILLETT.

ACADEMIC PRESS, New York and London

111 Fifth Avenue
New York 3, New York

Berkeley Square House
London, W.1, England

AP-874

ANALYTICAL BIOCHEMISTRY 5, 369–378 (1963)

Silicic Acid Column Chromatography for the Microdetermination of Cholesterol, Cholesterol Ester, and Phospholipid from Human Cerebrospinal Fluid

YUNG S. SHIN

From the Biochemistry Laboratory, St. Mary Mercy Hospital, Gary, Indiana

Received October 12, 1962

In recent years the silicic acid column has become an increasingly important tool in lipid chromatography (1–4) and the chromatographic microassay of human plasma lipids (5, 6). However, none of these microcolumn chromatographic methods could have been applied to the lipid fractionation of smaller amount of cerebrospinal fluids (CSF) because the lipid concentration in the fluid is about 500 times lower than that in the plasma. Microcolumn chromatography using aluminum oxide (7) or silicic acid (8) has been applied to the microestimation of free and esterified cholesterol from human CSF. But a minimum of 10 ml of the fluid has been required, and separation of phospholipid has not been described by these methods. Furthermore, it has been found that the resolution of lipids by these columns is unsatisfactory. The present report describes an improvement in the silicic acid microcolumn chromatography previously reported (5–10) that offers better separation of extremely minute quantities of free and esterified cholesterol as well as phospholipid using a solvent system of petroleum ether-ether (24:1, v/v), chloroform, and methanol. The method, which requires a minimum of 2 ml of the fluid, has been applied to a quantitative estimation of lipid fractions of human CSF.

METHODS

Reagents

Petroleum ether, purified by several treatments with concentrated sulfuric acid, followed by washing with water three times. After the water is removed with anhydrous $CaCl_2$, the petroleum ether is passed through a larger silicic acid column and distilled. The fraction boiling at 30–60°C is used.

Petroleum ether-ethyl ether: 24 vol of purified petroleum ether is

mixed with 1 vol of freshly redistilled ethyl ether, and freshly prepared before chromatography.

Chloroform, washed with 1 *N* hydrochloric acid twice, and with distilled water three times, filtered dried over anhydrous sodium sulfate, and distilled; 0.5% ethanol (v/v) is added as a preservative.

Silicic acid, specially prepared for the chromatography of lipid (minus 325 mesh, Bio-Rad Laboratories, Richmond, Calif.), is activated in the oven at 110° overnight and stored in the double desiccator.

$FeCl_3 \cdot 6H_2O$ color reagent: 4 ml of the stock solution (2.5% in 87% phosphoric acid) is diluted to 50 ml with concentrated sulfuric acid. The reagent is prepared weekly.

Glacial acetic acid, 99–100%, analytical grade (Merck).

Ammonium molybdate solution, 5% in distilled deionized water.

Fisk-SubbaRow reagent: 0.5 gm of purified 1-amino-2-naphthol-4-sulfonic acid is added with mechanical stirring to 200 ml of freshly prepared 15% sodium bisulfite (anhydrous), followed by 1.0 gm of anhydrous sodium sulfite. The solution is filtered, stored in a dark bottle, and prepared fresh weekly.

Lipid Extraction

The CSF is obtained by means of lumbar puncture, and the first 3 to 4 ml of the fluid is collected into a sterile screw-capped culture tube. The collected fluid is immediately stored in the refrigerator (4°) and determination is made within 48 hr. The spinal fluid which contains blood should be discarded; only clear fluid is subjected for lipid analysis. The fluid is centrifuged at 2500 rpm in a refrigerated centrifuge for 20 min; 2 to 3 ml of the clear supernatant fluid is transferred to a glass-stoppered centrifuge tube. A procedure slightly modified from that of Sperry and Brand (11) and Folch and Van Slyke (12) is used for extraction of a small quantity of lipid from the CSF. The fluid is incubated with an equal amount of methanol in a 55° water bath for 1 hr. During this period, the smaller amount of lipoprotein is flocculated and precipitated by warm alcohol; 4 to 6 ml of chloroform is then added to extract the lipid and to separate the lipid phase from the aqueous phase. The content is mixed for 1 min with a mixer (Vortex Jr.) and placed in the water bath for 1 hr with frequent mixing (at least a 1-min period of mixing for every 20 min). The tubes are centrifuged at 2500 rpm for 15 min. The upper phase is removed, and 0.5 ml of methanol is added to incorporate the remaining water. The contents are filtered through a 7-cm diameter Sharkskin fat-free filter paper (Schleicher & Schuell) into a plain 15-ml centrifuge tube. The original tube as well as the filter paper are rinsed with 1 ml of chloroform-methanol (2:1, v/v) solution.

The contents are taken to dryness under nitrogen at a maximum temperature of 60°.

Chromatography

A column is prepared as described in the previous paper (10) except that a piece of vinyl tubing[1] after softening with acetone is used instead of polyethylene tubing since the former holds the column and connector more firmly when it is dried. Preparation and purification of silicic acid (10) was not necessary when silicic acid specially prepared for lipid chromatography obtained from Bio-Rad Laboratories was used; 200 mg of activated silicic acid was used in this chromatography. A tube (Clay Adams, 100B) with attached hypodermic needle (size 18) is placed at the top of the column so that addition of solvents to the narrow column can be facilitated.

The column is prerinsed with 2 ml of petroleum ether. Then the lipid extract dissolved in 2 ml of petroleum ether is passed through the column at a flow rate of 1 drop/sec or every 2 sec. The centrifuge tube which contained lipid extract is rinsed with two 1-ml portions of petroleum ether and passed through the column. The cholesterol ester is eluted with 2.5 ml of petroleum ether-ether, and the tip of the column is rinsed with a few drops of petroleum ether. Free cholesterol is then eluted with 2 ml of chloroform. Since some of the phospholipids, e.g., sphingomyelin, may remain insoluble in petroleum ether (13), the centrifuge tube is again rinsed with two 2-ml portions of methanol and added to the column. The added methanol is passed through the column and the phospholipid is thus eluted into an ignition tube.[2] Also a column-passed reagent blank is similarly prepared. Application of impure solvents frequently caused slightly colored blank. If this occurs, all the solvents should be purified again. The column, after chromatography, is dried and the dried silicic acid is discarded. The column is refilled with activated silicic acid for each assay.

Colorimetric Measurements

Cholesterols are determined by the method of Shin and Lee (14), which is based on the Zak ferric chloride color reaction (15): 400 μl of glacial acetic acid and 350 μl of $FeCl_3 \cdot 6H_2O$ color reagent are added. The contents are mixed with the mixer for few seconds, and placed in the boiling water bath for 1 min. The cuvettes are removed and placed

[1] Lumen (3/16 in.), Puritan urinary drainage tube No. 316, Colonial Hospital Supply Co., Chicago, Ill.

[2] Pyrex ignition tube (15 × 125 mm) soaked in 50% nitric acid overnight, rinsed with deionized water 5 times, and dried.

in a cold water bath. The microspace cuvette adapters (Coleman 6-319) are inserted into the cuvettes and the color is read at 530 mμ with a Coleman Jr. spectrophotometer.

Lipid phosphorus is determined by the procedure previously described (16): 0.3 ml of 10 *N* sulfuric acid is added and the extract is placed in an oven at 230° for 10 min. After the tube is cooled, 1 drop of H_2O_2 is added and the tube is placed back in the oven for 5 min. Excess peroxide is removed by adding 1 drop of 5% urea solution and reheating for 5 min. When the tubes are cool, 1.2 ml of water and 400 μl of 5% ammonium molybdate solution are added to the digest. After mixing, 100 μl of Fisk-SubbaRow reagent is added and mixed well. The solution is placed in the boiling water bath for 7 min, and then cooled in an ice water bath. The color is measured at 830 mμ with a Beckman spectrophotometer using a 7 × 12 × 48 mm cuvette. In an alternative procedure for the Coleman Jr. spectrophotometer, 500 μl of water, 200 μl of 5% ammonium molybdate, and 50 μl of Fisk-SubbaRow reagent was used. The final color mixture is read at 700 mμ. The amount of phospholipid is calculated from the amount of lipid phosphorus multiplied by a factor of 25.

RESULTS

The distribution of esterified and free cholesterol and phospholipids in every 0.2-ml fraction of eluants has been determined to study the mode of the chromatographic separation of these lipid fractions (Fig. 1). The free and esterified cholesterol were identified by paper chromatography

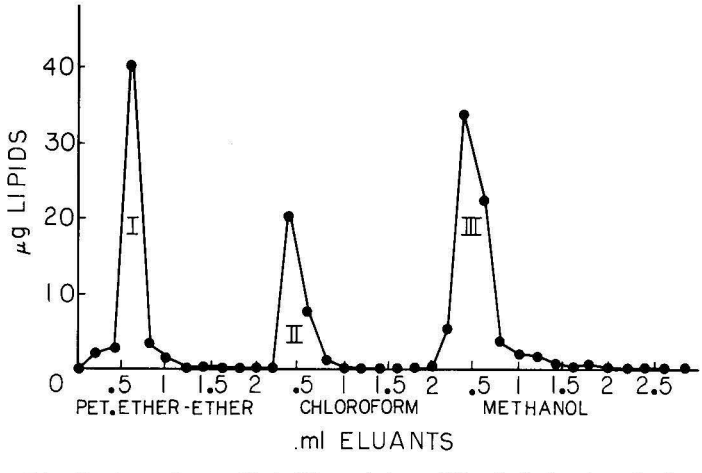


FIG. 1. Distribution of esterified (I) and free (II) cholesterol and phospholipid (III) in various fractions of eluants.

(17), and phospholipid was identified directly from the paper strip by ninhydrin for amino-containing phospholipids (18) and by phosphomolybdic acid-stannous chloride for choline-containing phospholipids (19). And a determination of phosphorus was made to estimate the quantity of phospholipid.

Recovery of column chromatography has been studied with standard solution of free cholesterol (Fisher) and purified cholesterol esters and phospholipids prepared from human serum. Essentially quantitative results were obtained, as shown in Table 1. Cholesterol stearate was pre-

TABLE 1
RECOVERY OF CHOLESTEROLS AND PHOSPHOLIPID

Material	Before column (μg)	After column (μg)	Recovery (%)
Free cholesterol	5	5.2	104.0
	10	9.8	98.1
	30	28.8	96.0
	60	57.4	95.7
Cholesteryl acetate	5	5.1	102.0
	60	55.7	92.8
Cholesteryl stearate	15.2	14.4	94.7
	30.4	28.3	93.1
Serum cholesterol esters	30.7	32.6	106.1
CSF cholesterol esters	7.6	8.2	109.2
	20.8	20.4	98.1
Serum phospholipid	5.4	5.1	94.5
	43.2	42.8	96.9
	172.8	164.0	94.9

pared by the method of Page and Rudy (20) and purified through a silicic acid column (5). Cholesterol esters from pooled human serum and CSF have been prepared after free cholesterol was precipitated by alcoholic digitonin solution (21) and purified through a silicic acid column (5). For the preparation of pure phospholipide, 20 ml of pooled human serum has been used. Phospholipid has been precipitated by acetone with smaller amount of saturated magnesium chloride in ethanol (22). Phospholipid is then re-extracted from the precipitate and purified by the method of Nelson and Freeman (4).

Wycoff and Parsons (5) reported that the first 2 ml of the eluant (4 ml of petroleum ether-chloroform, 1:1, v/v) contained most of the cholesterol ester. However, the next third milliliter of the eluant still contains trace amount of cholesterol ester while the last one-tenth of the eluant carried some free cholesterol. Various combinations of solvent

have been studied and the mode of lipid separation effected by such solvents has been studied. A solvent system of petroleum ether-ether (24:1, v/v) for the separation of cholesterol esters, chloroform for free cholesterol, and methanol for phospholipid has been found to be the most suitable for this particular type of column. Also, a comparison of this solvent system with the solvent system proposed by Wycoff and Parsons (5) and Papadopoulos *et al.* (8) has been made, and a better resolution of cholesterol esters from free cholesterol has been obtained (Fig. 2).

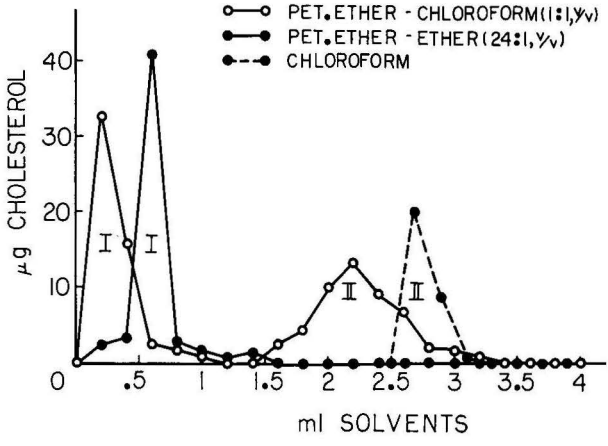


FIG. 2. Distribution of esterified (I) and free (II) cholesterol in various fractions of eluants.

Reproducibility of the column chromatography with free cholesterol, purified cholesterol esters, and phospholipid from human serum has been studied. The relative standard deviation for the determination of free cholesterol (19.9 μg), esterified cholesterol (21.3 μg), and phospholipid (44.6 μg) were 7.8, 7.9, and 5.4%, respectively.

The method has been applied for the determination of free cholesterol, esterified cholesterol, and phospholipid from human CSF. Since availability of CSF from normal healthy individuals was limited due to lack of volunteers, CSF obtained from hospitalized individuals and surgical patients prior to the spinal administration of anesthetics were studied. However, no patients who have any mental or neurological disorders were included in this study. The average value for free and esterified cholesterol and phospholipid concentration of human CSF from 41 individuals of both sexes and of age ranging from 7 to 83 years is presented in Table 2. Because the study will be continued extensively, a

TABLE 2
CHOLESTEROL AND PHOSPHOLIPID IM HUMAN CEREBROSPINAL FLUIDS[a]

Analysis for	Average	S.D.
Cholesterol (mg %)	0.463	0.154
Esterified (mg %)	0.258	0.101
Free (mg %)	0.205	0.079
% esterified	56.0	9.1
Phospholipid (mg %)	0.549	0.170
Cholesterol/phospholipid ratio	0.85	0.14

[a] Average of 41 non-neurological individuals.

confirmative result for the value for correlation with total protein and with other chemical components of the fluid, or with age and sex relationship, of cholesterol and phospholipide levels will appear in another publication.

DISCUSSION

When the separation of cholesterol from its ester involves a minute quantity, application of the digitonin precipitation technique for accurate microassay has been believed to be difficult (23). Direct determination of phosphorus from the lipid extracted by chloroform-methanol (7, 9) or by alkaline alcohol after precipitation of lipoprotein with trichloroacetic acid (24) was most commonly applied to the determination of phospholipids in CSF. Cohen (25) and recently Shin[3] found that almost all the phosphorus in human CSF is in free form, and phosphorus from extractable lipid consists only of 1 to 2% of the total CSF phosphorus. Therefore, a possibility of contamination of the phospholipid fraction with nonlipid phosphorus is very great. For this reason, the CSF lipid extract must be as free of nonlipid phosphorus as possible. A possible suggestion can be made that application of a chromatographic technique could be the most suitable way since the lipid separated by column is relatively free from nonlipid contaminants.

Microcolumn chromatography using alumina as an adsorbent (26) has been reported for the separation of relatively smaller amount of plasma lipids. And application of alumina column for the separation of CSF free and esterified cholesterol was made by Roboz *et al.* (7). However, the value of free cholesterol is significantly higher than that obtained by digitonin precipitation (Table 3). This higher value may possibly be due to the fact that cholesterol esters can be chemically

[3] Average of 124 determinations on free inorganic phosphorus and lipid phosphorus from normal human CSF indicates 1.38 and 0.022 mg %, respectively (to be published).

TABLE 3
COMPARISON OF CHOLESTEROL AND PHOSPHOLIPID LEVELS IN NORMAL HUMAN CEREBROSPINAL FLUID PREVIOUSLY DETERMINED BY DIFFERENT FRACTIONATION PROCEDURES

	Solvent fractionation		Chromatographic separation	
Cholesterol	Digitonin pptn. (29)		Aluminum oxide column (7)	Silicic acid column (8)
Total	0.395[a]		0.586	0.440
Free	0.122		0.315	0.210
Ester	0.273		0.269	0.230
% esterified	67.0		44.2	52.3
Phospholipid	TCA pptn. (29)	Chloroform-methanol extraction (7)	Aluminum oxide column	Silicic acid column (32)
Total	0.384	0.823	Not determined	0.480

[a] Mg/100 ml CSF.

altered by contact with aluminum oxide, which is shown to be highly active chemically, since a possibility exists that cholesterol ester may be hydrolyzed (27). A column chromatographic procedure using silicic acid, which is now considered a truly inert adsorbent, was recently employed for the lipid fractionation of a smaller amount of plasma (5, 6, 28). An attempt has been made by Papadopoulos *et al.* (8) to CSF free and esterified cholesterol using silicic acid column (6). The value reported by these investigators is in rough agreement with that of Tourtellotte *et al.* (Table 3). Since the chromatography requires 10 ml of the fluid, wide application of this technique to clinical laboratories is limited.

Tourtellotte (29) also reported that the average phospholipid value of human CSF is 0.384 mg %, which is much lower than the value reported by other investigators (Table 3), who have indicated that the falsely higher value reported elsewhere (23, 30, 31) may be due to contamination with inorganic phosphorus. Papadopoulos *et al.* (32) used 300 ml of pooled human CSF to obtain phospholipid in the chloroform extract, which is completely free of nonlipid phosphorus, through the silicic acid column. They reported a value of 0.48 mg %, in contrast to the value of Tourtellotte, and suggested that some of the lipids escaped during the precipitation procedure. The values reported by previous investigators vary to some extent, and the number of determination on human CSF has been too limited to evaluate statistically. Thus, a final conclusion has yet to be made. This is probably due to lack of adequate micromethods for the separation and determination of these lipid frac-

tions from a smaller amount of CSF. To date, no column chromatography which can separate free and esterified cholesterol as well as phospholipid from a single column using less than 10 ml of CSF has been reported. The method proposed here could be conveniently used for determination of these lipids in the clinical laboratory since the method requires only 2 ml of the fluid and is relatively simple and rapid.

SUMMARY

A chromatographic fractionation procedure has been devised for the separation of cholesterol, cholesterol ester, and phospholipide from a lipid mixture of 20 μg. A chromatographic determination of human CSF (non-neurological) total cholesterol, esterified cholesterol, and total phospholipid has been made. The values are 463 ± 154, 258 ± 101, and 549 ± 170 μg %, respectively. According to the chromatographic results, esterified cholesterol in human CSF comprises 56% of the total cholesterol.

REFERENCES

1. Fillerup, D. L., and Mead, J. F., *Proc. Soc. Exptl. Biol. Med.* **83,** 574 (1953).
2. Klein, P. D., and Janssen, E. T., *J. Biol. Chem.* **234,** 1417 (1959).
3. Freeman, N. K., Lindgren, F. T., Ng. Y. C., and Nichols, A. V., *J. Biol. Chem.* **227,** 449 (1957).
4. Nelson, G. J., and Freeman, N. K., *J. Biol. Chem.* **234,** 1375 (1959).
5. Wycoff, H. D., and Parsons, J., *Science* **125,** 347 (1957).
6. Lis, E. W., Tinoco, J., and Okey, R., *Anal. Biochem.* **2,** 100 (1961).
7. Roboz, E., Hess, W. C., Dinella, R. R., and Cevallos, W., *J. Lab. Clin. Med.* **52,** 158 (1958).
8. Papadopoulos, N., Cevallos, W., and Hess, W. C., *J. Neurochem.* **4,** 223 (1959).
9. Shin, Y. S., *Abstracts,* Div. Biol. Chem., 139th Meeting, Am. Chem. Soc., St. Louis, Mo., March, 1961.
10. Shin, Y. S., and Lee, J. C., *Clin. Chem.* **8,** 598 (1962).
11. Sperry, W. M., and Brand, F. C., *J. Biol. Chem.* **213,** 69 (1955).
12. Folch, J., and Van Slyke, D. D., *Proc. Soc. Exptl. Biol. Med.* **41,** 514 (1939).
13. Hevesy, G. C., and Hanh, L., *Kgl. Danske Videnskab. Selskab Biol. Med.* **15,** 60 (1940).
14. Shin, Y. S., and Lee, J. C., *Anal. Chem.* **33,** 1220 (1961).
15. Zak, B., Moss, N., Boyle, A. J., and Zlatkis, A., *Anal. Chem.* **31,** 950 (1959).
16. Shin, Y. S., *Anal. Chem.* **34,** 1164 (1962).
17. Quaife, M. L., Geyer, R. P., and Bollinger, H. R., *Anal. Chem.* **31,** 950 (1959).
18. Marinetti, G. V., Erbland, J., Albrecht, M., and Stotz, E., *Biochem. et Biophys. Acta* **26,** 130 (1957).
19. Chargaff, E., Levene, C., and Green, C., *J. Biol. Chem.* **175,** 67 (1948).
20. Page, I. H., and Rudy, H., *Biochem. Z.* **220,** 304 (1930).
21. Bloor, W. R., and Knudson, A., *J. Biol. Chem.* **27,** 107 (1916).
22. Sinclair, R. G., and Dolan, M., *J. Biol. Chem.* **142,** 659 (1942).
23. Rosenthal, H. L., and Jud, L., *J. Lab. Clin. Med.* **51,** 143 (1958).

24. TOURTELLOTTE, W. W., PARKER, F. M., AND DEJONG, R. N., *J. Lab. Clin. Med.* **52,** 491 (1958).
25. COHEN, H., *Quart. J. Med.* **17,** 289 (1924).
26. KERR, L. M. H., AND BAULDS, W. S., *Biochem. J.* **55,** 872 (1953).
27. TRAPPE, W., *Biochem. Z.* **306,** 316 (1940); *Biochem. J.* **305,** 150 (1940).
28. CREECH, B. G., AND SEWELL, B. W., *Anal. Biochem.* **3,** 119 (1962).
29. TOURTELLOTTE, W. W., RUSSELL, N., DEJONG, R. N., JANICH, S., AND GUSTAFSON, K., *Univ. Mich. Med. Bull.* **28,** 114 (1962).
30. ROEDER, F., *Z. ges. Neurol. Psychiat.* **168,** 519 (1940).
31. KNAUER, H., AND HEIDRICH, L., *Z. ges. Neurol. Psychiat.* **136,** 483 (1931).
32. PAPADOPOULOS, N. M., CEVALLOS, W., AND HESS, W. C., *A.M.A. Arch. Neurol.* **3, 677** (1960).

ANALYTICAL BIOCHEMISTRY 5, 379–387 (1963)

An Enzymic Method for the Quantitative Determination of Glycogen[1]

JOHN A. JOHNSON, JOEL D. NASH,
AND RAMON M. FUSARO

From the Division of Dermatology, University of Minnesota Hospitals, Minneapolis, Minnesota

Received July 23, 1962

The need arose in this laboratory for a convenient, reliable technique for determining glycogen. Several analytical procedures were examined closely in order to evaluate their merits and possible deficiencies. Of the numerous methods reported in the literature, acid hydrolysis followed by determination of the resulting glucose (1, 2), and the anthrone method (2–7), have gained wide recognition. The acid hydrolysis method has been combined with a number of chemical procedures for estimating glucose. Recently an enzymic method, which employs glucose oxidase and horseradish peroxidase, has been developed for the determination of glucose (8–11). This procedure is specific for glucose and yields highly reproducible results.

Of the presently available techniques for measuring glycogen, acid-hydrolysis coupled with the glucose oxidase procedure for determining glucose, appeared to be the best available method, but preliminary experiments in our laboratory with this technique demonstrated that recoveries of known amounts of glucose varied from 60 to 101%; recovery values for known quantities of glycogen exhibited even larger variation. The critical point in this technique was the neutralization of the acid hydrolyzate before the glucose determination. Apparently, local high concentration of alkali caused inconsistent loss of glucose. We resolved this difficulty by diluting the hydrolyzate with phosphate buffer, pH 7, of sufficient concentration to overcome the acid present. After this modification, recoveries were 94 to 96%. By including a glucose standard in the glycogen determinations and correcting for the glucose lost during hydrolysis, we were able to obtain consistent, reproducible results for known amounts of glycogen. We concluded that the acid hydrolysis method was useful for assay purposes. It was not suitable for studies in

[1] This investigation was supported (in part) by a PHS research grant, A-3964 (C2), from the Department of Health, Education, and Welfare, U. S. Public Health Service.

which the amount of glycogen available in test solutions was too small to permit dilution in the neutralization step.

The anthrone colorimetric procedure for determination of glycogen (2–7) was also examined. In this procedure hydrolysis of glycogen and development of color by the reaction of anthrone with glucose occurs simultaneously. This method was easy to employ, and standard curves approach linearity. The determination is conducted under strongly acidic conditions [e.g., 77% H_2SO_4 (5)]; these vigorous conditions cause occasional variations in color yield of replicate samples. Further, since cellulose is also hydrolyzed to glucose in this procedure, precautions must be taken to exclude lint, dust, paper fibers, etc. from the test solutions and reagents.

The following analytical procedures were also examined and were found to be unsuitable for our purposes: (A) *Iodine* (7, 12, 13): nonspecific; color yield varies with glycogen source. (B) *Phenol-sulfuric acid* (14): nonspecific for glycogen. (C) *Concanavalin A* (15, 16): not sensitive enough for micro amounts of glycogen. It was concluded after careful analysis of conventional methods for determining glycogen that mild hydrolysis of the glycogen, followed by glucose oxidase determination of the glucose product, would be a reliable quantitative procedure. In glucose studies using crude glucose oxidase, we observed that abnormally high results were obtained when glucose determinations were conducted in the presence of glycogen. These results indicated that a contaminating enzyme in the crude glucose oxidase was causing partial conversion of glycogen to glucose. The literature was searched in order to discover enzyme preparations exhibiting high amyloglucosidase activity. Enzyme preparations for converting grain starches to glucose are used extensively in the brewing and syrup industries (17–24). Because of the industrial applications of these enzyme mixtures, they are available commercially. Upon investigating several of these commercial products, we discovered that Diazyme (Miles Chemical Co.), an enzyme mixture derived from *Aspergillus niger*, converts glycogen to glucose in nearly quantitative yields. Moreover, the amount of glycogen released in a given incubation period is proportional to the original concentration of glycogen. Preliminary experiments indicated that the Diazyme mixture operates effectively in the same pH range as the glucose oxidase system. Solutions containing known amounts of glucose were subjected to the combined action of Diazyme, glucose oxidase, and horse-radish peroxidase. Plots of color yield versus concentration of glucose, yielded straight lines, thus indicating that the various enzymes present in the reaction mixture are compatible. Glycogen was then tested with the combined enzyme system, and color yield was proportional to original glycogen concentration.

REAGENTS

Phosphate Buffer. 0.1 *M*, pH 6.0; 0.5 *M*, pH 7.0.

Diazyme Solution. Two hundred milligrams of Diazyme is dissolved in 100 ml of phosphate buffer, pH 6.0, and filtered. This solution is stored at 4°C.

Glucose Oxidase Solution. Five hundred milligrams of crude glucose oxidase (Sigma Chemical Co.) is dissolved in 50 ml phosphate buffer, pH 6.0, and stored at —5°C.

Peroxidase Solution. Ten milligrams of horse-radish peroxidase (Sigma, Type I) is dissolved in 10 ml of phosphate buffer, pH 6.0, and stored at —5°C.

Dye Solution. Fifty milligrams of 3,3′-dimethoxybenzidine (Practical, Eastman Organic Chemicals Dept.) is dissolved in 10 ml 95% ethanol, and stored at —5°C.

Enzyme Mixture. Just before use, 2.5 ml of glucose oxidase solution, 2.5 ml of peroxidase, and 0.25 ml of dye are mixed and diluted to 25 ml with phosphate buffer, pH 6.0.

Glucostat Mixture (Worthington Biochemical Corporation). Dissolve contents of one chromogen vial and one glucostat vial in water and dilute to 50 ml with distilled water.

PROCEDURE

Color Yield vs. Glycogen Concentration

A reagent blank (distilled water) and three glycogen solutions (6, 12 and 18 μg/ml) are determined in triplicate. Twelve test tubes are set up, each containing 1 ml test solution, 1 ml Diazyme stock solution, and 1 ml enzyme mixture. The solutions are mixed and placed in a water bath incubator for 1 hr at 37°C. At the end of the incubation period, 0.5 ml 2 *N* HCl is added to each tube, and the optical densities of the solutions are read at 400 mμ in a Beckman DU spectrophotometer (1-cm path length) with distilled water in the reference cell. The experimental readings are then corrected for the average blank value.

Acid Hydrolysis Assay of Glycogen

Stock solutions of glycogen and glucose are made up to concentrations of 1 mg/ml with distilled water. One milliliter of 2 *N* H_2SO_4 is added to 1-ml aliquots of each of the stock solutions, and the resulting solutions are heated 2 hr at 105°C. The hydrolyzates are cooled in tap water and diluted to 50 ml with 0.5 *M* phosphate buffer, pH 7.0, to yield concentrations of 20 μg/ml of glucose and 22.2 μg/ml glucose equivalents in the test solutions. [A multiplication factor of 1.11 (4) was used to convert

glycogen to glucose equivalents.] Glucose standards of 8, 16, and 24 μg/ml are prepared from glucose stock solution by diluting the latter with appropriate quantities of $2N$ H_2SO_4 and $0.5M$ phosphate buffer, pH 7.0, to yield solutions of the same ionic strength as that of the test solutions. A blank solution is prepared by mixing 1 vol of $1N$ H_2SO_4 with 24 vol of $0.5M$ phosphate buffer, pH 7.0. One milliliter of glucostat mixture is added to triplicate 1-ml aliquots of blank, glycogen, and glucose hydrolyzates, and glucose standards. The solutions are incubated 1 hr at 37°C, after which 0.5 ml $2N$ HCl is added to each tube. Optical densities are read at 400 mμ. The observed assay values for glycogen are corrected for the amount of glucose lost from the glucose-hydrolysis solution.

Diazyme Assay of Glycogen Samples

Glycogen test solutions (24 μg glycogen/ml or 26.6 μg glucose equivalents/ml), and glucose standards (12, 24, and 36 μg/ml) are made up with distilled water. One milliliter of Diazyme solution and 1 ml of enzyme mixture are added to three 1-ml aliquots of each of the test solutions, the glucose standards, and a distilled water blank. After incubation for 1.5 hr, at 37°C, 0.5 ml $2N$ HCl is added to each solution, and the optical densities are read at 400 mμ. Observed glucose equivalents of the glycogen solutions are calculated on the basis of color yield of the glucose standards. Using the acid hydrolysis assay values of the glycogen samples as the criterion of purity of the test materials, per cent conversion of the glycogen test solutions to glucose is calculated.

RESULTS

Table 1 lists the results of nine separate experiments, each performed

TABLE 1
GLYCOGEN CONCENTRATION VS. COLOR YIELD

Glycogen (μg)	Optical densities[a] ± standard deviation (%)	
	Initial data	Corrected data
6	0.052 ± 6.9%	0.057 ± 4.0%
12	0.113 ± 6.5%	0.123 ± 2.7%
18	0.174 ± 5.6%	0.189 ± 3.1%

[a] After correction for blank readings of 0.051 ± 0.005.

in triplicate on solutions containing 6, 12, and 18 μg of human liver glycogen per milliliter. Standard deviations for this series of experiments were 6–7%. It was noted that color yield for given amounts of glycogen varied between experiments and was probably due to unavoidable varia-

tions in reaction conditions. When the observed experimental results are corrected for day-to-day color variation, standard deviations are 3–4% (Table 1). The corrected values are more representative of the reliability of our procedure, since glycogen in unknown test solutions is determined on the basis of color yield of glycogen standards subjected to identical conditions of enzyme strength and activity, incubation temperature, and duration, buffer pH, etc. Figure 1 illustrates that the Diazyme procedure gives color yields which are essentially proportional to glycogen concentration over a range of 6 to 18 μg/ml test solution (1.7–5.1 μg/ml final concentration).

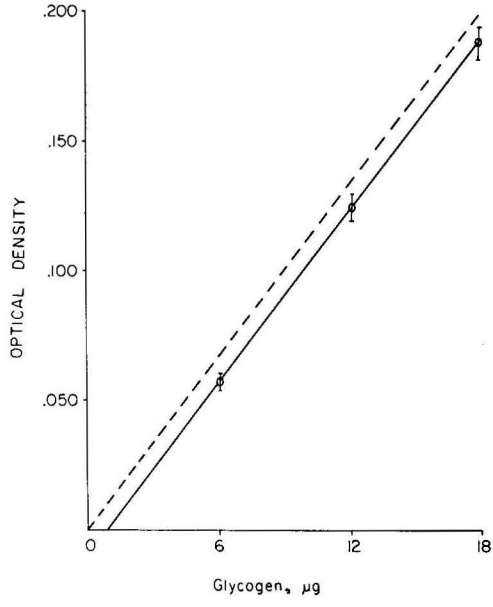


FIG. 1. Glycogen concentration versus optical density.

It should be noted that the standard curve does not pass through the origin. Numerous glucose oxidase determinations performed on glucose in this laboratory have invariably yielded standard curves which do pass through the origin. We initially thought that the anomalous position of the glycogen curve could be explained on the basis of abnormally high reagent blank. With respect to the experimental data illustrated in Fig. 1, a decrease of 0.009 in optical density of the blank (i.e., increase of 0.009 in the corrected optical densities) would transpose the curve so that it passes through the origin (broken line, Fig. 1). An investigation of change in optical density of reagent blank with time at 37°C, how-

ever, indicated that after 30 min the blank color increases at about the same rate as that of the glucose oxidase system alone. In other words, after a 30-min incubation, Diazyme does not contribute greatly to the optical density of the reagent blank. Furthermore, our experience with this preparation shows that Diazyme is very stable. A sample of the dry preparation has been stored in our laboratory at room temperature for over a year with no detectable loss in activity. Even aqueous stock solutions, barring contamination, are stable for several weeks at 4°C.

Diazyme is reported to contain α-amylase, amyloglucosidase, and maltase (25). Since these enzymes are capable of attacking various parts of the glycogen molecule, the kinetic behavior of the mixture is probably a complex function of the combined activities of the three enzymes. We therefore hesitate to apply rigorous kinetic theory to the glycogen hydrolysis reaction. One can, however, examine the reaction in a qualitative sense. At low substrate (glycogen) concentrations and under constant reaction conditions, the reaction is first order with respect to substrate, i.e., the velocity is proportional to substrate concentration. As substrate concentration is increased, a level (S_1) is reached at which the velocity is at a maximum, and the reaction becomes zero order; velocity is constant and independent of substrate concentration. Presumably the enzyme is saturated with substrate, and the velocity rate constant is determined by the velocity of some stage of the reaction other than the enzyme-substrate complexing step. As substrate is acted upon by the enzyme, the concentration of the former will decrease until it reaches the S_1 level, at which point the reaction will become first order. Thus, two intervals of substrate concentration must be considered: from zero to S_1, where the reaction is first order with respect to substrate; and above S_1, where the reaction is zero order. A kinetic study of the Diazyme reaction indicates that, at a glycogen concentration of 6 μg/ml incubation solution (the highest glycogen concentration employed in this study), the reaction is about 90% complete at 60 min and essentially complete at 90 min. Since the reaction does not go to completion under the conditions described in this report, an amount of substrate (S_2) will remain at the end of the incubation period. If the initial substrate concentration is high enough, the reaction will proceed at the rapid zero-order rate for a sufficient length of time that the reaction will be essentially complete at the end of the incubation period, i.e., S_2 will be insignificant compared to initial substrate level (S_0). As S_0 decreases, the zero-order reaction will proceed for a lesser fraction of the incubation time, the effect of the slower first-order reaction will become more significant, and the relative amount of S_2 will increase. Thus, as S_0 decreases, the ratio of S_2 to S_0 will increase and the difference between observed value (amount reacted,

or $S_0 - S_2$) and theoretical value (S_0) will increase. The net effect of the divergence between observed and theoretical values of substrate with decreasing S_0 will slant the substrate concentration curve downward as the curve approaches the origin, until S_0 equals S_1. In the interval between S_0 equals S_1 to S_0 equals zero, the reaction will be first order throughout the incubation period and the substrate concentration curve will be normal, i.e., a straight line passing through the origin. (We wish to point out that this interval is difficult to study with our method, since the glucose equivalent concentrations in this area appear to be below the level of sensitivity of the glucose oxidase system.) A possible explanation for the anomalous position of our glycogen curve is that the concentrations of our glycogen solutions are in the "downward-slanting" region of the curve, i.e., S_0 is greater than S_1, but not so great that the reaction proceeds at zero-order velocity throughout the incubation period.

We should point out that our description of the behavior of the portion of the glycogen curve above S_1 should represent a curved section, convex upward. However, if this curve is shallow, and if the range of test solutions encompasses only a small segment of the curve, a plot of glycogen concentration versus optical density will yield a straight line, within experimental error.

Since Diazyme consists of a mixture of enzymes, we are reluctant to attempt to evaluate rate constants (zero- and first-order) and S_1, in order to establish the validity of our above discussion. The important conclusion to be drawn from this discussion is that the investigator, in applying the method as described in this report, should be careful to include a series of glycogen standards in his determination which will accurately represent a sufficient portion of the glycogen concentration curve to include the quantities of glycogen expected in his test solutions. Although the experiments for determining the glycogen concentration curve were conducted in triplicate, we concluded at the end of the study that they could have been performed in duplicate with adequate assurance of reproducibility: of 108 observations (nine experiments, in triplicate, on a blank and three glycogen solutions), no result was omitted on the basis of wide disagreement with the other two readings of its triplicate set. This high degree of reliability, coupled with the ease with which the enzymic method can be employed, makes it an easy matter to include several standards in the determination.

A normal glycogen curve can be obtained by increasing the concentration of the glycogen solutions and incubating for 1.5 hr instead of 1 hr. We have performed several Diazyme determinations on glycogen solutions of 4, 8, 12, and 16 μg glycogen/ml incubation medium (the concentrations used in this study were 2, 4, and 6 μg/ml incubation medium).

Incubation time was 1.5 hr. A plot of glycogen versus optical density yielded a straight line passing through the origin and passing near (within 2% error) the points representing the three higher glycogen concentrations. The point representing the lowest concentration (4 μg/ml) lay below the curve by an interval equivalent to 5% of the observed optical density for that point. For the purposes of our study we felt that the economy in time afforded by a 1-hr incubation and the reproducibility of repeat experiments justified using the shorter incubation time. The glycogen concentrations employed by us represent the limit of sensitivity of our enzymic procedure. Preliminary experiments to date indicate that smaller quantities of glycogen may be determined by the application of micro volumetric techniques.

Since the procedure described in this report involves enzymic hydrolysis of glycogen, a species specificity may be encountered, i.e., glycogen from different animal sources may be converted to glucose in varying degree. In the interest of maximum reliability when applying this method, it is best to use as a reference standard glycogen isolated from the same species as the animal being studied. Since such a source of glycogen might not be readily available in all instances, we investigated the action of Diazyme on glycogen derived from two animal species. Accordingly, glycogen was isolated from rabbit liver and human liver, and purified by the method of Akher and Smith (26). These samples were assayed by the acid hydrolysis procedure described in this report. Four separate assays established that the rabbit liver and human liver samples were $96.4 \pm 1.0\%$ and $96.2 \pm 2.0\%$ pure, respectively (results are reported as assay value $\pm$ standard deviation). The glycogen samples were then subjected to the Diazyme method. Assay values for six independent determinations were $92.5 \pm 1.6\%$ (rabbit) and $89.0 \pm 1.1\%$ (human). When compared to the acid assays, these figures represent a conversion to glucose of 95.9% for the rabbit liver and 92.5% for the human liver glycogen. It is not known whether the difference in degree of conversion (3.4%) is significant, since the value lies within the observed limits of accuracy of the assays. The glycogen concentration in the assay experiments was 8 μg/ml incubation medium and incubation time was 1.5 hr. Therefore, the values quoted above should be an accurate measure of the degree of conversion to glucose effected by Diazyme on the two glycogen samples.

SUMMARY

A convenient enzymic method has been developed for the quantitative determination of glycogen. Using this method, we have obtained consistent, reproducible results for glycogen solutions in the concentration

range of 6 to 18 μg glycogen/ml of test solution. The enzyme mixture has been shown to effect 92–96% conversion of glycogen to glucose, and shows little species differentiation between rabbit liver and human liver glycogen.

ACKNOWLEDGMENTS

The authors are indebted to Dr. L. A. Underkofler, Miles Chemical Co., Clifton, New Jersey, for the gift of a generous amount of Diazyme. We also wish to thank Anita Young for technical assistance.

REFERENCES

1. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.* **100,** 485–91 (1933).
2. Fong, J., Schaffer, F. L., and Kirk, P. L., *Arch. Biochem. Biophys.* **45,** 319–26 (1953).
3. Kahan, J., *Arch. Biochem. Biophys.* **47,** 408–18 (1953).
4. Seifter, S., Dayton, S., Novic, B., and Muntwyler, E., *Arch. Biochem.* **25,** 191–200 (1950).
5. Templeton, M., *J. Histochem. Cytochem.* **9,** 670–2 (1961).
6. Trevelyan, W. E., and Harrison, J. S., *Biochem. J.* **50,** 298–303 (1952).
7. van der Vies, J., *Biochem. J.* **57,** 410–16 (1954).
8. Beach, E. F., and Turner, J. J., *Clin. Chem.* **4,** 462–75 (1958).
9. Huggett, A. St. G., and Nixon, D. A., *Lancet* **273,** 368–70 (1957).
10. Keilin, D., and Hartree, E. F., *Biochem. J.* **42,** 221–229, 230–8 (1948).
11. Saifer, A., and Gerstenfeld, S., *J. Lab. Clin. Med.* **51,** 448–60 (1958).
12. Morris, D. L., *J. Biol. Chem.* **166,** 199–203 (1946).
13. van Wagtendonk, W. J., Simonsen, D. H., and Hackett, Patricia L., *J. Biol. Chem.* **163,** 301–6 (1946).
14. Du Bois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F., *Anal. Chem.* **28,** 350–6 (1956).
15. Cifonelli, J. A., and Smith, F., *Anal. Chem.* **27,** 1639–41 (1955).
16. Montgomery, R., *Arch. Biochem. Biophys.* **67,** 378–86 (1957).
17. Beran, K., and Burger, M., Czech. Pat. 88,376 (Jan. 15, 1959); *Chem. Abstr.* **1960,** 7974a.
18. Okazaki, H. (to Sankyo Co.) Jap. Pat. 3038 (May 4, 1955); *Chem. Abstr.* **1957,** 13325a.
19. Nagai, Y., Jap. Pat. 7671 (Aug. 30, 1958); *Chem. Abstr.* **1960,** 4011b.
20. Langlois, D. P. (to A. E. Staley Mfg. Co.) U. S. Pat. 289,869 (June 23, 1959).
21. Langlois, D. P., and Turner, W. (to A. E. Staley Mfg. Co.), U. S. Pat. 2,893,921 (July 7, 1959).
22. Liggett, R. W., Mussulman, W. C., Rentshler, D. F., and Ziffer, J. (to A. E. Staley Mfg. Co.) U. S. Pat. 2,881,115 (Apr. 7, 1959).
23. Stone, I. (to Wallerstein Co., Inc.) U. S. Pat. 2,717,852 (Sept. 13, 1955).
24. USSR Pat. 119,852 (May 21, 1959); *Chem. Abstr.* **1960,** 3847a.
25. Pazur, J. H., and Ando, T., *J. Biol. Chem.* **234,** 1966–70 (1959).
26. Abdel-Akher, M., and Smith, F., *J. Am. Chem. Soc.* **73,** 994–6 (1951).

ANALYTICAL BIOCHEMISTRY 5, 388–392 (1963)

Detection of Bile Acids in Thin-Layer Chromatography[1]

DAVID KRITCHEVSKY, DOLORES S. MARTAK, AND GEORGE H. ROTHBLAT

From the Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania

Received August 14, 1962

INTRODUCTION

The detection of bile acids in thin-layer chromatography has been accomplished by means of the spray reagents previously used in paper chromatographic assay of these compounds, namely, phosphomolybdic acid (1) or antimony trichloride (2). Neher and Wettstein (3) reported on the use of an anisaldehyde spray reagent for detection of steroids in paper chromatography, but in the bile acids series they tested only cholic and deoxycholic acids. The present report represents the first thorough investigation of the use of the anisaldehyde spray reagent for bile acids. While both phosphomolybdic acid and antimony trichloride may give slightly different colors with different bile acids, the anisaldehyde reagent gives better color differentiation with the various bile acids than do either of the previously noted reagents, thus adding another parameter in studies requiring separation and identification of these compounds.

MATERIALS AND METHODS

The sources of the various compounds used are indicated in Table 1.

Thin-layer plates were prepared according to Stahl (4) with Silica Gel G (Merck), obtained from Brinkmann & Co., and a commercial apparatus (Desaga GmbH, Heidelberg, Germany). When chromatograms were run, 20 × 20 cm plates were used and the solvent front permitted to rise 135 mm from the origin. The chamber was saturated with the developing solution prior to running the chromatogram. The plates were not prewashed. All experiments were carried out at room temperature (23–5°C).

The aldehyde spray reagent was prepared immediately before use and was used within an hour of preparation. The reagent is composed of anisaldehyde 0.5 ml, glacial acetic acid 50 ml, and concentrated sulfuric acid 1 ml. After spraying, plates were heated in an oven at 125°C for

[1] This work was supported (in part) by grants from the National Institutes of Health, USPHS (H-3299) and the National Science Foundation (No. G-14241-R).

TABLE I
COLORS OBTAINED WITH VARIOUS BILE ACIDS (10 μG) WITH ANISALDEHYDE SPRAY REAGENT

Substituted cholanic acid	Color	
	Visible	Ultraviolet (3660 A)
— (cholanic)[a]	None	None
3α-Hydroxy (lithocholic)[c]	Blue gray–green gray	Yellow
3β-Hydroxy (isolithocholic)[c]	Blue	Yellow
3β-Hydroxy-Δ^5-cholenic[a]	Blue	Blue
12α-Hydroxy[e]	Lavender	Orange
3α,6α-Dihydroxy (hyodeoxycholic)[c]	Blue	Green
3α,6β-Dihydroxy[d]	Blue	Green
3α,7α-Dihydroxy (chenodeoxycholic)[b]	Blue	Green
3α,7β-Dihydroxy (ursodeoxycholic)[d]	Blue	Green
3α,12α-Dihydroxy (deoxycholic)[b]	Brown	Orange
3α,6α,7α-Trihydroxy (hyocholic)[c]	Olive	Yellow
3α,6β,7α-Trihydroxy[d]	Olive	Yellow
3α,6α,7β-Trihydroxy[d]	Lavender	Yellow
3α,6β,7β-Trihydroxy[d]	Pink	Yellow
3α,7α,12α-Trihydroxy (cholic)[a]	Purple	Orange
3α-Hydroxy-7-keto[e]	Pale tan	None
3α,7α-Dihydroxy-12-keto[e]	Gray green	Orange
3α-Hydroxy-7,12-diketo[e]	Pale tan	Lt. blue
3-Keto[e]	Brick red	Pink
3,7-Diketo[e]	Brick red	Pink
3,12-Diketo[e]	Brick red	Pink
3,7,12-Triketo (dehydrocholic)[a]	Orange	Pink
Taurocholic[a]	Purple	Yellow
Glycocholic[a]	Purple	Yellow

Source of bile acid: (*a*) Mann Research Laboratories, New York, N. Y. (*b*) Calbiochem, Los Angeles, Calif. (*c*) Light and Co., Ltd., Colnbrook, England. (*d*) Dr. W. L. Holmes. (*e*) Dr. J. Sjövall.

10 min. Colored spots were observed in visible and ultraviolet light (3660 A). No fluorescence was observed when the plates were viewed under short-wave (2537 A) ultraviolet light.

The sprayed plates are best examined 15 to 30 min after heating, at which time the color differences are most pronounced. Some of the colors begin to fade after 30 min.

RESULTS AND DISCUSSION

When the anisaldehyde reagent was used by Neher and Wettstein for detection of steroids in paper chromatography (3), they tested 100 μg quantities of cholic and deoxycholic acids and reported a blue color for both. The reagent has also been used for detection of saponins in paper chromatography (5).

Table 1 presents the spectrum of colors obtained with the anisaldehyde reagent when a variety of bile acids were tested. Of special interest are the different colors obtained with chenodeoxy- and deoxycholic acids, two compounds whose R_f values do not always permit unequivocal identification. The different colors obtained with isomers of hyocholic acid are also noteworthy. To test the sensitivity of this method, aliquots of 1, 5, 10, 25, and 50 μg each of the more common bile acids (cholic, deoxycholic, chenodeoxycholic, hyodeoxycholic, and lithocholic) were spotted on one plate and sprayed with the reagent. Even at a level of 1 μg of bile acid the colors were clear and identifiable. One other distinct advantage of the anisaldehyde spray reagent is lack of background color. The reagent reacts only with the test compounds and the resultant color stands out clearly from the white background.

In the use of similar reagents for detection of steroids the choice of aldehyde is not critical, since several different aldehydes may be used (6–8). We repeated the test on the common bile acids substituting salicylaldehyde, benzaldehyde, and furfuraldehyde for anisaldehyde, with results shown in Table 2. Only salicylaldehyde gave a spectrum of

TABLE 2
COLORS OBTAINED WITH BILE ACIDS USING VARIOUS ALDEHYDES IN THE SPRAY REAGENT[a]

Bile acid	Aldehyde			
	Anisaldehyde	Salicylaldehyde	Benzaldehyde	Furfuraldehyde
Lithocholic	Blue-green gray	Pink	Tan	Blue
Hyodeoxycholic	Blue	Purple	Brown	Blue
Chenodeoxycholic	Blue	Purple	Brown	Blue
Deoxycholic	Brown	Tan	Tan	Blue
Cholic	Purple	Brown	Tan	Blue

[a] Aldehyde 0.5 ml, glacial acetic acid 50 ml, concentrated sulfuric acid 1 ml.

colors. It is interesting to note the similarities and differences between different pairs of bile acids when anisaldehyde and salicylaldehyde were used. The use of other substituted benzaldehydes may give wider color differences between various bile acids but we have not investigated this area.

Concomitantly with our work on the color reagent we were testing various thin-layer chromatographic methods for separation of bile acids. During this time Gänshirt, Koss, and Morianz (9) reported several solvent systems useful for separation of some of the bile acids and their conjugates. Hofmann (10) also reported on two different solvent systems which can be used for thin-layer chromatography of bile acids.

We had devised a solvent system of the composition ether: petroleum ether: methanol: glacial acetic acid 70:30:8:1 which had given separation of bile acids similar to those obtained by the other workers (9, 10). For purposes of consistency we found that expression of the distances traveled by other bile acids in relation to the movement of cholic acid (R_c) was more satisfactory than R_f. (R_c is the ratio of the distance traveled by the bile acid in question to the distance traveled by cholic acid.) This system did not give sufficiently good separation of deoxycholic and chenodeoxycholic acids. Hamilton and Muldrey (11) reported that they had been able to separate these acids on silicate paper and Hamilton (12) has successfully used this solvent system in thin-layer chromatography. In our hands, the Hamilton solvent system (isooctane: isopropyl ether: glacial acetic acid 2:1:1) gave better separation of the common bile acids (Fig. 1) than did any of the other systems used. The

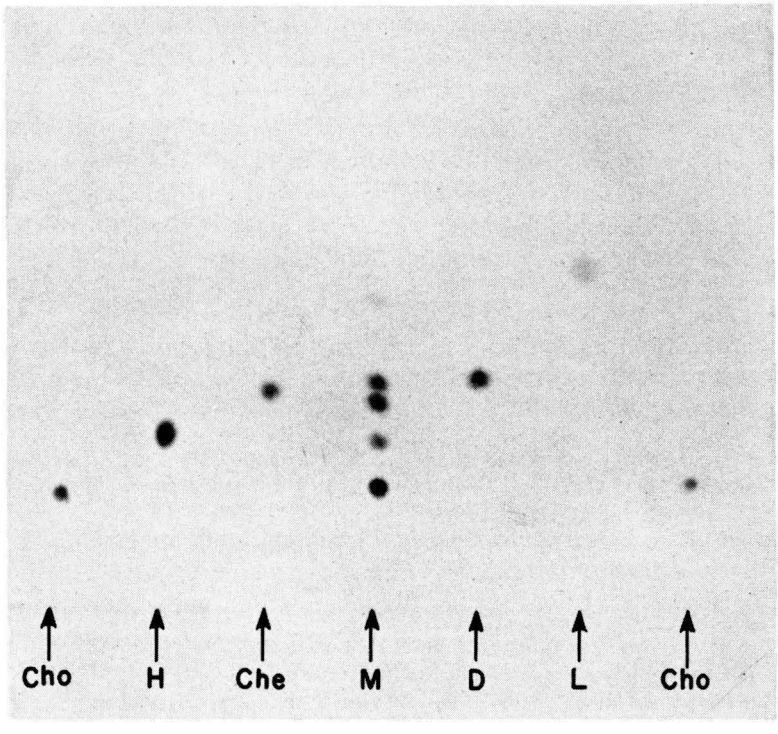


FIG. 1. Thin-layer chromatogram of bile acids. Solvent system: isooctane 50, isopropyl ether 25, acetic acid 25 (Hamilton, ref. 12). Silica gel G, 135 mm. Spray reagent: anisaldehyde 0.5 ml, concentrated H_2SO_4 1 ml, glacial HOAc 50 ml. Cho, cholic. H, hyodeoxycholic. Che, chenodeoxycholic. M, mixture. D, deoxycholic. L, lithocholic.

R_f and R_c values obtained using both our solvent system and Hamilton's are given in Table 3.

TABLE 3
AVERAGE R_f AND R_c VALUES[a] FOR VARIOUS BILE ACIDS[b]

Bile acid	Solvent I[c]		Solvent II[d]	
	R_f	R_c	R_f	R_c
Lithocholic	0.82 ± 0.03[e]	2.73 ± 0.09	0.47 ± 0.02	7.26 ± 0.20
Hyodeoxycholic	0.43 ± 0.01	1.40 ± 0.02	0.14 ± 0.01	2.18 ± 0.03
Chenodeoxycholic	0.55 ± 0.02	1.90 ± 0.04	0.21 ± 0.01	3.32 ± 0.04
Deoxycholic	0.60 ± 0.03	2.03 ± 0.04	0.26 ± 0.01	4.03 ± 0.08
Cholic	0.30 ± 0.01	—	0.07 ± 0.00	

[a] R_c = reference to cholic acid.
[b] Averages based on minimum of 6 determinations.
[c] Solvent I: ether 70, petroleum ether 30, methanol 8, acetic acid 1.
[d] Solvent II: isooctane 50, isopropyl ether 25, acetic acid 25 (Hamilton, ref. 12).
[e] Standard error of the mean.

SUMMARY

A spray reagent consisting of anisaldehyde (0.5 ml), glacial acetic acid (50 ml), and concentrated sulfuric acid (1 ml) is a sensitive means of detecting bile acids in thin-layer chromatography. Quantities as small as 1 μg may be easily detected. The bile acids give characteristic colors with this reagent, thus making identification easier.

ACKNOWLEDGMENT

We are indebted to Dr. James G. Hamilton for disclosing the solvent system he used for bile acid chromatography.

REFERENCES

1. KRITCHEVSKY, D., AND KIRK, M. R., *Arch. Biochem. Biophys.* **35**, 346 (1952).
2. SIPERSTEIN, M. D., HAROLD, F. M., CHAIKOFF, I. L., AND DAUBEN, W. G., *J. Biol. Chem.* **201**, 181 (1954).
3. NEHER, R., AND WETTSTEIN, A., *Helv. Chim. Acta* **34**, 2278 (1951).
4. STAHL, E., *Angew. Chem.* **73**, 646 (1961).
5. GÜNZEL, C., AND WEISS, F., *Pharmazie* **10**, 725 (1955).
6. HARDEN, A., AND ROBISON, R., *Biochem. J.* **17**, 115 (1923).
7. WOKER, G., AND ANTENER, I., *Helv. Chim. Acta* **21**, 1345 (1938).
8. SCHERRER, I., *Helv. Chim. Acta* **22**, 1329 (1939).
9. GÄNSHIRT, H., KOSS, F. W., AND MORIANZ, K., *Arzneimittel-Forsch.* **10**, 943 (1960).
10. HOFMANN, A., *J. Lipid Res.* **3**, 127 (1962).
11. HAMILTON, J. G., AND MULDREY, J. E., *J. Am. Oil Chemists Soc.* **38**, 582 (1961).
12. HAMILTON, J. G., private communication.

ANALYTICAL BIOCHEMISTRY 5, 393–415 (1963)

The Photometric Estimation of Luciferase in Luminous Bacterial Cells[1]

CHARLES H. BURNS, A. D. NEVILL, AND G. HOYT WHIPPLE

From the School of Public Health, The University of Michigan, Ann Arbor, Michigan

Received August 17, 1962

Recent studies have shown that the luminescence of luminous bacteria under certain conditions responds to ionizing radiation instantaneously (1) and, under other conditions, responds to doses as low as 500 rads (2). The intensity of the luminescence is proportional to the rate of the luciferase reaction taking place *in vivo*. Thus radiation effects both on the metabolic system supporting the luciferase reaction (3) and on the enzyme itself may contribute to the observed response. In order to evaluate the relative contributions of these two actions, it is necessary to establish a method for the assay of active luciferase in the cells. This paper describes an assay based on measurement of the luminous intensity of the luciferase reaction. Applications of this method will be found for irradiated cells (3) and for growing cells (4).

The procedure is derived from the work of Strehler and Cormier (5), McElroy *et al.* (6), and Strehler (7), who identified the reaction components required for maximum luminescence in cell-free extracts of *Photobacterium fischeri*. These components are reduced riboflavin phosphate (reduced flavin mononucleotide, $FMNH_2$), palmitic or other homologous aldehyde (5), oxygen, and the bacterial extract (luciferase). The cell-free extracts also contain an FMN-linked diphosphopyridine nucleotide (DPNH) oxidase which permits the substitution of a steady-state $FMNH_2$-generating system for the spontaneously autoxidizable $FMNH_2$. Nothing presently understood about the mechanism of the luciferase reaction suggests a method for measuring luciferase activity on a chemical basis.

The extracts referred to above were prepared by lysing the cells in deionized water and removing the cell debris by highspeed centrifugation to obtain an optically clear solution. The present assay was based on these crude extracts because no method of purification exists which permits the necessary total recovery of enzyme. When such extracts are

[1] This work was supported by Grant 2028A from the National Institutes of Health, for which we are deeply grateful.

used, however, the desired direct proportionality between enzyme concentration and enzyme activity (in this case the light intensity produced by the reaction) is not obtained with either the reaction initiated by $FMNH_2$ or that initiated by the combination of DPNH and FMN. Although it is possible to demonstrate a proportional relation in the case of the $FMNH_2$-initiated reaction using purified luciferase preparations (8), the reaction goes to completion in a matter of seconds, making the maximum intensity of the luminescence difficult to measure. The reaction initiated by DPNH plus FMN, on the other hand, approaches steady-state conditions lasting several minutes, but the maximum light intensity produced in this case is the result of two consecutive enzymic processes rather than one.

Despite the absence of direct proportionality between light intensity and enzyme concentration, they are found to follow a straight-line relationship for each reaction when plotted on log-log coordinates. This allows one to normalize different sets of values with a single constant and to observe departures from the normal relation.

Among the other requirements of a suitable assay procedure that have been met are the following: (*a*) assurance that the procedure extracts all of the luciferase available in the cells, (*b*) assurance that the deterioration of the enzyme and of the reaction components at 0°C (storage) and at 12 and 25° (reaction) is not a factor in the assay, (*c*) establishment of the optimum concentration of reaction components for luminescence, since FMN has been reported to be inhibiting at high concentrations (9), and (*d*) determination of a temperature for the $FMNH_2$-initiated reaction which permits thorough mixing of enzyme and substrates before maximum luminous intensity is reached.

METHODS

1. Materials and Equipment

The cell suspensions used in these studies were either growing cell suspensions which had reached maximum luminous intensity, or nonproliferating cell suspensions prepared from growing cell suspensions at maximum luminous intensity. The methods used to prepare these suspensions are similar to those used by Farghaly (10) and by Whipple (2).

The reaction components for the DPNH-FMN, or "slow" reaction, were prepared in individual stock solutions. Decanal (capric aldehyde) was chosen as the aldehyde because it is easily solubilized in water. Two working solutions were prepared from the four stock solutions: solution AB contained aldehyde and phosphate buffer; solution DF contained DPNH and FMN. Table 1 gives the pertinent information for the stock

TABLE 1

REACTION COMPONENTS AND THEIR CONCENTRATIONS AS USED IN STANDARD ASSAY PROCEDURES

Stock soln.	Component	M.W.	Concn. in stock soln. (*M*)	Ml stock soln. in 15 ml working soln.[a]	Concn. in working soln. (*M*)	Concn. in final reaction mix. (μmole/ml)
For both reactions:						
A	Decanal[b]	156	5.3×10^{-2} [c]	2.0	7.0×10^{-3}	1.2
	Tween-20	—	1.5%		0.2%	0.033%
B	Buffer:[d]			7.5		
	KH_2PO_4	136	0.27		1.35×10^{-1}	22.5
	Na_2HPO_4	142	0.27		1.35×10^{-1}	22.5
	NaCl	58.5	0.02		10^{-2}	1.7
For slow reaction:						
D	DPNH[e]	709	8×10^{-3} [f]	3.0	1.6×10^{-3}	0.27
F	FMN[g]	514	1.5×1^{-4} [h]	1.0	1.0×10^{-5}	0.0017
For fast reaction:						
F	FMN	514	1.5×10^{-4}	12.0[i]	1.2×10^{-4}	0.02

[a] Solutions A, B, and Tween-20 combined in working solution AB; solutions D and F combined in working solution DF. The remainder is distilled and deionized water (H_2O).

[b] Capric aldehyde, sp.gr. 0.828, Polak's Frutal Works, Middletown, N.Y.

[c] 0.2 ml purified liquid to 20 ml with aqueous 1.5% Tween-20.

[d] Buffer adopted from the work of McElroy and Green (8), final pH 6.8.

[e] Disodium salt, assay 80% or above, California Corporation for Biochemical Research.

[f] 70 mg/10 ml.

[g] Riboflavin 5₁-phosphate·$2H_2O$, Na salt; assay 96%; California Corporation for Biochemical Research.

[h] 80 mg/liter.

[i] This dilution is made before the solution is catalytically hydrogenated to form $FMNH_2$; this provides solution FH for the fast reaction.

solutions, the working solutions, and the final reaction mixture. Stock solutions were stored in light-tight containers at 3° for no longer than one month. Working solutions were prepared just before each set of assays and were stored in light-tight containers in an ice bath while the assays were performed.

For the $FMNH_2$ or "fast" reaction the aldehyde-buffer working solution was identical to solution AB used for the slow reaction. The $FMNH_2$ working solution (FH) was made by catalytic hydrogenation of a dilution of stock solution F described in Table 1. Figure 1 shows the apparatus used to prepare samples of $FMNH_2$ for injection into the reaction medium. This apparatus permits the rapid preparation of a large number of stable samples of reduced flavin (or other biochemicals)

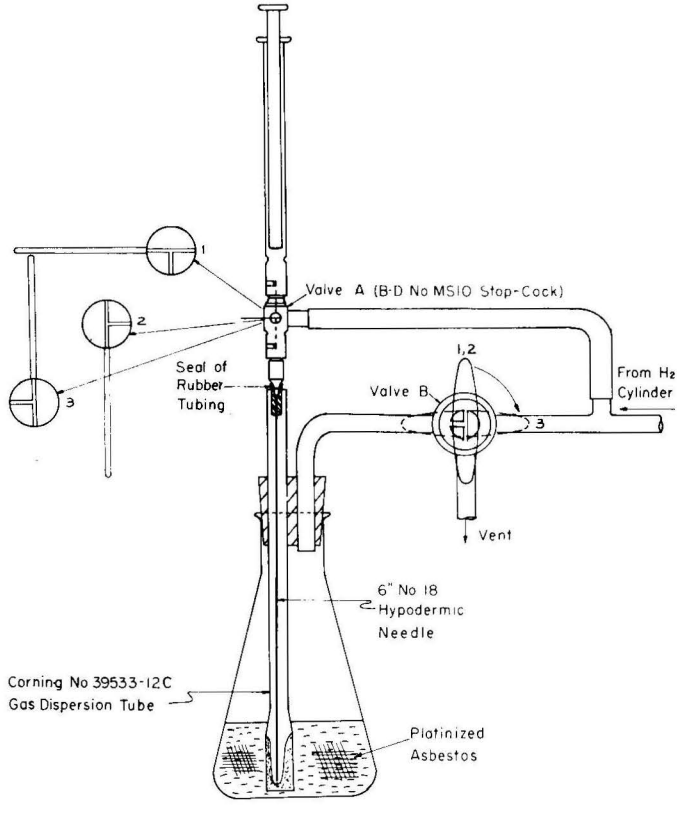


FIG. 1. Apparatus for preparing solution FH. With the three-way valves A and B in position 1, gaseous hydrogen at 5 psi is directed through valve A, down the hypodermic needle, through the sintered glass cylinder which disperses the gas, and into the FMN solution containing platinized asbestos. Excess gas escapes through valve B to the vent. The seal at the top of the dispersion tube may be loosened to expel trapped air. To collect a sample of $FMNH_2$, a hypodermic syringe of desired capacity is attached to the top of valve A and, while holding the plunger in place, the valve is turned to position 2. By removing and inserting the plunger, the syringe is dried and degassed. Valves A and B are then turned to position 3. This permits the hydrogen to be used as pressure to force the $FMNH_2$ into the sintered-glass cylinder and to filter out the platinized asbestos. The syringe plunger is held down while the solution of $FMNH_2$ rises up the hypodermic needle and into the syringe (the hydrogen initially in the needle will leak out through the dry syringe), whereupon the plunger is raised to collect a sample 0.2 ml in excess of the volume desired. Valves A and B are then returned to position 1; the syringe is removed, fixed with a 1-in. 24-gage needle, and depressed by 0.1 ml to expel air in the needle. The procedure may be repeated to prepare the desired number of samples in advance of their use. Immediately before each sample is injected into the reaction mixture, the 0.1 ml excess solution is expelled to remove oxidized $FMNH_2$ from the tip of the needle.

free of the platinized asbestos catalyst and in any volume desired. The operation of the device is explained in the figure caption.

Figure 2 shows the photometric apparatus used to measure the luminous intensity produced by the luciferase reaction. The reaction is carried

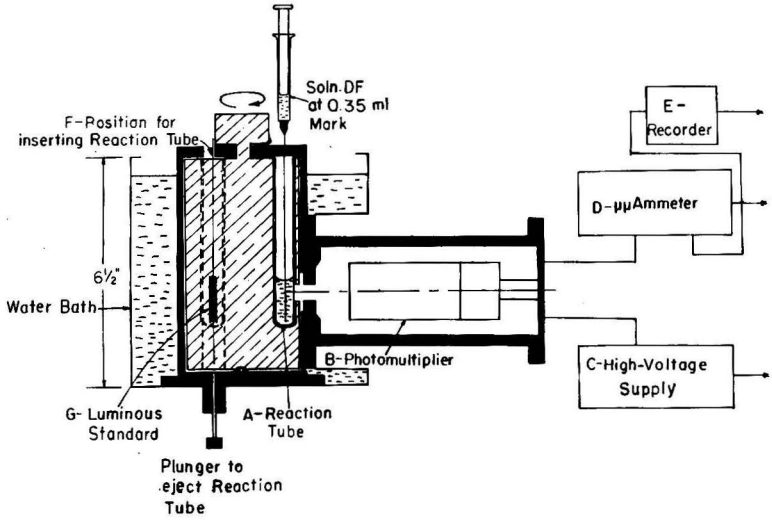


FIG. 2. Lateral cross section of photometer used for luciferase activity assays. Included in the sketch is a photometer tube containing the 2.0 ml of solution of CL plus 0.5 ml of solution AB. Solution DF is about to be added. See text for explanation of other details.

out in a tube (A). A fixed fraction of the light produced by the reaction falls on the photomultiplier tube (B). The photomultiplier is a Dumont No. 6292 and is powered by a Hammer Model N401 high-voltage supply (C). The photomultiplier current is measured by a Keithly Model 410 micromicroammeter (D). The voltage applied to the photomultiplier was approximately 1100 volts, and the photomultiplier output ranged from 0.01 to 10 mμ. When the photomultiplier dark current is balanced out, the remaining current is proportional to the intensity of the luminescence generated in tube A. The luminous standard (G in Fig. 2) consisted of a Lucite tube containing a strontium-90-activated phosphor.

The tube holder was a cylinder with three holes bored 120° apart. By revolving the cylinder, the reaction tube could be brought into position F, where it could be removed vertically. The reaction tubes were custom-made Pyrex test tubes selected for constant outside diameter (approximately 14 mm). Transmission measurements showed that variation in luminous intensity due to differences among the tubes was not

greater than ±2%. The cylinder housing was surrounded by a water bath to maintain constant temperature. Cooling coils fed by a commercial drinking water cooler were immersed in the bath to produce low temperatures for the fast reaction studies. A Texas Instrument Recorder (E) was used to trace the value of the luminous intensity throughout the course of the reaction.

2. Definitions

One Luminous Intensity Unit (LIU): The photometer current generated by a luminous standard designated in this laboratory as S-2.

Luciferase Activity: The maximum luminous intensity, expressed in LIU's, produced by the luciferase reaction when it is performed as described below.

One Luciferase Unit (LU): That amount of active enzyme per milliliter of aqueous cell extract which, when prepared and assayed according to the procedure specified below, produces a luciferase activity equal to 1 LIU. Cell suspension samples producing luminous activities other than 1 LIU, however, do not have proportionately different units of luciferase, as will be pointed out below.

3. Standard Assay Procedure Using the DPNH-FMN (Slow) Reaction

Samples of cell suspension are prepared for assay in the following manner: A 20.0-ml volume of suspension with a cell density between 0.5 and 20×10^8 cells/ml is centrifuged at $15{,}000 \times g$ for 3–5 min at 0°. The supernatant is discarded and in its place 5.0 ml ice-cold distilled-deionized water is introduced from a 5-ml hypodermic syringe. The syringe is fitted with a 6-in. 20-gage hypodermic needle; the mixture of cells and water is forced up and down the needle to disperse the lysing cells thoroughly. The lysate is then centrifuged at $25{,}000 \times g$ for 10 min at 0°. The supernatant or cell extract (CL) which becomes the assay sample, is transferred in two 2.0-ml portions to reaction tubes immersed in an ice bath. In dilution experiments, where less than 2.0 ml of CL is assayed, the difference is made up with H_2O or other diluent. The assay samples are stored for not longer than 1 hr.

The luciferase activity of the sample is determined as follows: A 1-ml hypodermic syringe fitted with a 6-in. 18-gage needle is filled to the 0.5-ml mark with solution AB, leaving no air space in the needle. Another 1-ml syringe is fitted with a 6-in. 16-gage needle closed at the end and having nine 0.25-mm diameter holes spaced along the last 2.0 cm. This is filled to the 0.35 ml mark with solution DF so that, with the 0.15 ml contained in the needle, the total contents are 0.50 ml. Both syringes are placed in the 25° water bath. At zero time, the reaction tube containing the 2.0-ml

sample of cell extract is transferred to the 25° water bath and agitated gently. During this interval the photometer dark current can be zeroed and the voltage supply can be adjusted to give a current of 1.00 μa with the luminous standard S-2. At 2.0 min the tube is removed from the water bath, wiped clean, and inserted rapidly into the photometer. The 0.5-ml volume of solution AB is then introduced under the surface of the sample. The syringe containing solution DF is placed in position as shown in Fig. 2. At 2.5 min, the contents of the syringe and the sample are quickly mixed by depressing the plunger, retracting it to flush out residual solution DF and depressing it again. Care is taken to avoid introducing air into the mixture because of the optical distortions produced by foam.

The maximum current occurring during the next 3 min is recorded. The maximum current in microamperes produced by the reaction is the luciferase activity in LIU, since the current produced by the luminous standard was set at 1 μa. The 5 ml of cell extract prepared from each cell suspension sample enables this assay to be run in duplicate.

4. Standard Assay Procedure Using the $FMNH_2$ (Fast) Reaction

Duplicate 2.0-ml aliquots of the cell extract are prepared for assay in the manner described above. The photometer bath temperature is lowered to 12° and maintained at this temperature thermostatically. Several 1-ml syringes, each containing approximately 0.8 ml $FMNH_2$ and fixed with a 1-in. 24-gage hypodermic needle, are prepared in advance by the procedure explained in Fig. 1, and are stored in the 12° water bath. Before zero time, 0.5 ml of solution AB is taken up in another syringe fitted with a 6-in. 18-gage needle and transferred to the water bath. At zero time the reaction tube containing the 2.0-ml assay sample is transferred to the 12° water bath and agitated gently. At 2.0 min the 0.5 ml of solution AB is introduced under the surface of the sample, and the reaction tube is then transferred to the photometer. A syringe containing $FMNH_2$ is quickly removed from the water bath and wiped dry, has its 24-gage needle exchanged for the 16-gage one used for solution DF above, is depressed to the 0.5-ml mark to fill the needle with $FMNH_2$, and is placed in position as shown in Fig. 2. At 2.5 min, the syringe plunger is quickly depressed. The maximum photometer current produced by this reaction is then observed.

5. Tests of Validity of Standard Assay Procedures

An early step in the standard procedure consists of removing by high-speed centrifugation the lysed cell debris, which renders the extract turbid and affects the apparent luminous intensity of the luciferase reaction.

Evidence that all the luciferase in the cell sample was present in the supernatant after this centrifugation was provided by (*a*) determining the period of time for lysis necessary for the resulting cell extract to acquire maximum luciferase activity, (*b*) resuspending the lysed cell debris after centrifugation and checking it for any residual luciferase activity, and (*c*) determining the effect of varying the volume of H_2O used to lyse a given number of cells on the luciferase activity of the resulting extract. The last test was carried out by preparing a concentrated lysing cell suspension (0.1 gm cell/ml) and making serial dilutions before centrifugation; the activities of the resulting extracts were compared with those prepared by centrifuging the concentrated lysing cell suspension first and making the same serial dilutions after centrifugation. The slow reaction was used to determine luciferase activity.

The stability of the enzyme in the cell extracts when stored at 0 and at 25° was determined by making serial dilution of a highly active extract to give concentrations in the ratio 4:2:1. These three solutions were kept at 0°. The initial luminous activities were determined on each of them using the slow reaction. One-third of each solution was then transferred to the 25° water bath and luminous activities were determined at approximately 15-min intervals thereafter. At approximately hourly intervals the luminous activity of the solutions stored at 0° was likewise determined.

The stability of FMN, DPNH, decanal, singly and in various combinations, was determined by appropriate combinations of stock solutions used to make the two working solutions. A single sample of cell extract sufficient for fifty luminous activity determinations was prepared and maintained in an ice bath. Four or five luminous activity determinations were made with each combination at intervals during a period of 2 hr.

The detergent Tween-20 was used to dissolve decanal in water. The optimum concentration of the detergent and decanal in solution AB and of DPNH and FMN in solution DF were determined individually by varying over a wide range the volume of the corresponding stock solution used to make up the working solution. For each working solution prepared, the slow reaction was run in duplicate on three dilutions of a cell extract.

The requirement of the fast reaction for rapid mixing of $FMNH_2$ and sample was met by the injection technique described above. The adequacy of the technique was checked by substituting methylene blue for solution FH and visually observing inhomogeneities in the resulting mixture. A satisfactory working temperature was found by determining the reaction temperature permitting the maximum to be reached in not less than 2 sec after the mixing of the reactants was complete. To deter-

mine the optimum requirement of $FMNH_2$ for the fast reaction, FMN solutions were made up with various concentrations from 25 to 300% of the stock FMN concentration. These were reduced and used directly as solution FH in the fast reaction, and the luminous activities were determined for each.

The relation between luciferase activity and enzyme concentration was established by making serial dilutions of various cell extracts and determining the luciferase activity of each dilution by the standard assay procedure. The diluents used were (*1*) H_2O, (*2*) a cell extract identical to that being diluted but whose luciferase activity had first been extinguished by heating at 50° for 30 min, and (*3*) a cell extract identical to that being diluted but whose luminous activity had been almost completely extinguished by exposure to 0.25 megarad of ionizing radiation. The relation between luminous activity and dilution was investigated by plotting the data on log-log scales.

RESULTS

1. Development of Luminescence in Individual Assays

Some typical curves showing the development of luminescence by the slow reaction induced by DPNH + FMN are shown by curves A to D in Fig. 3. The mixing of solution DF with the 2.5 ml of sample containing 0.5 ml of solution AB was accomplished in about 2 sec. The lag

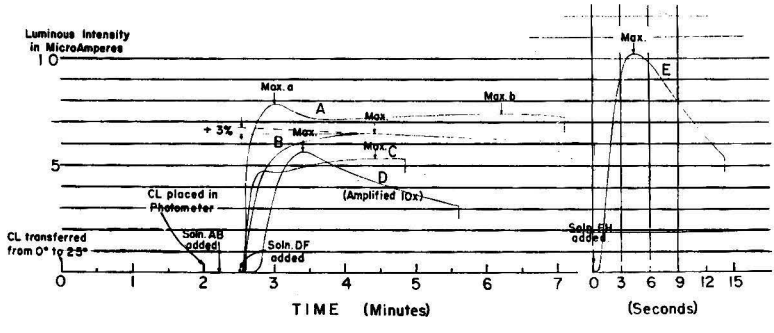


FIG. 3. Examples of types of tracings illustrating the course of *in vitro* luminous intensity with time generated by the slow reaction (curves A–D) and by the fast reaction (E). Zero seconds for curve E corresponds to the point 2.5 min on the left-hand scale with respect to sample warmup. Arrows denote position of maxima. Curve B was allowed to run out for 7 min to determine the rate of decline of luminous intensity with time. This decay curve is extrapolated back to the beginning of the curve to estimate a correction (+3%) of the observed maximum to compensate for loss of luciferase activity prior to attainment of maximum luminous intensity. See text for additional details.

between the completion of the mixing and the appearance of luminescence may be considered the time during which $FMNH_2$ is being generated by the oxidation of DPNH.

Once initiated, luminescence develops rapidly. Maximum luminescence may occur as early as 30 sec after the first appearance of luminescence (curves A and C) or it may be reached gradually after 2 min (curve B). Double maxima occasionally occur (curves A and C), possibly due to "burning off" of preformed $FMNH_2$-aldehyde-luciferase complex. The luminous intensity of curve D has been amplified 10-fold to show the behavior of a weak extract. By far the most frequent curve, and the one on which this assay is based, is that represented by curve B. The luminous intensity always reaches a maximum by 3.5 min of reaction (6 min after zero time), and declines thereafter. Agreement between successive duplicate samples is usually within $\pm 5\%$.

Curve E of Fig. 3 represents the time course of development of luminescence by the fast reaction at a reaction temperature of 12° when solution FH is added at time zero. The intensity reaches a maximum in 4–5 sec and declines without exhibiting a plateau. At room temperature, this reaction reaches a maximum in less than 0.5 sec, indicating that incomplete mixing of $FMNH_2$ and extracts is not the limiting factor.

2. Extraction of Luciferase from Bacterial Cells

Varying the length of the lysing period had no effect on the maximum luciferase activity of the resulting cell extract. This is shown by luciferase activities of 1.56 and 1.49 after 1 and 30 min, respectively, between the time of resuspending collected cells and start of centrifugation. Thus any luciferase bound to the insoluble cell material is so tightly bound as to be unaffected by variations in extraction time.

Resuspension of lysed cell debris collected by high-speed centrifugation with a minimum of H_2O yielded preparations with no detectable activity. On the basis of the dilution effect described below, this indicates that the cell debris together with adhering solution could not have contained more than 2–3% of the luciferase originally extracted.

Varying the volume of H_2O used to lyse a given number of cells gave the same result as adjusting to the same volume the concentrated extract after removal of cell debris. The failure to extract more enzyme by increasing the ratio of H_2O to cell mass indicates that all extractable luciferase has been obtained.

3. Stability of Luciferase in Samples Stored

The change of luminous activity with time for cell extract samples of three different initial luminous activities each stored at 0 and 25° was as

follows: storage at 0° for 3 hr did not cause detectable loss of activity, but samples stored overnight at this temperature showed some loss depending on activity. The most concentrated extract retained 95% of its activity overnight, while the most dilute retained only about 70%.

Storage at 25° results in rapid loss of activity. Dilution of the sample increases the rate of loss. However, the loss of activity in samples of low luminous activity is negligible over a 6-min period, the time required to perform the assay.

4. Optimum Combination and Requirement of Decanal, DPNH, and FMN for Maximum Luciferase Activity with the DPNH-FMN Reaction

Tests of different combinations of FMN, DPNH, and decanal in working solutions showed that decanal in combination with either FMN or DPNH resulted in lower luminous activities than when it was added separately or with the buffer. The combination of decanal with buffer, and of FMN with DPNH, appeared to result in no deterioration even when stored several days at 3°.

Figure 4 shows the effect of various concentrations of Tween-20 in the working solution on the luciferase activity of a given sample of cell extract. The presence of a low percentage (0.033% in the final reaction mixture) nearly doubles the luciferase activity whereas higher concentrations appear to interfere with the reaction.

Variation of the concentration of decanal in the reaction mixture for extracts of three different luciferase concentrations yielded the following results: Regardless of luciferase content, the 1.2 μmoles/ml decanal specified in the standard assay procedure is about twice the minimum for maximum activity. Increasing the concentration above the minimum decreases the maximum luminous activity slightly.

Variation of luciferase activity with concentration of DPNH in the reaction mixture for extracts of three different luciferase concentrations gave these results: The concentration chosen for use in the assay (0.27 μmoles/ml) is approximately the minimum for maximum activity. A large increase in DPNH concentration above this minimum may cause a slight increase in activity. The small rate of change of luminous activity with DPNH concentration in the range of the minimum does not appear to vary with the level of activity in the extracts.

Figure 5 shows the variation of luciferase activity with concentration of FMN in the reaction mixture for extracts of three different luciferase concentrations. All four curves confirm the existence of an optimum rather than a minimum concentration of FMN for maximum luminous activity. Concentrations above the optimum appear to be inhibitory. The higher the luciferase content of the sample, the more rapidly the

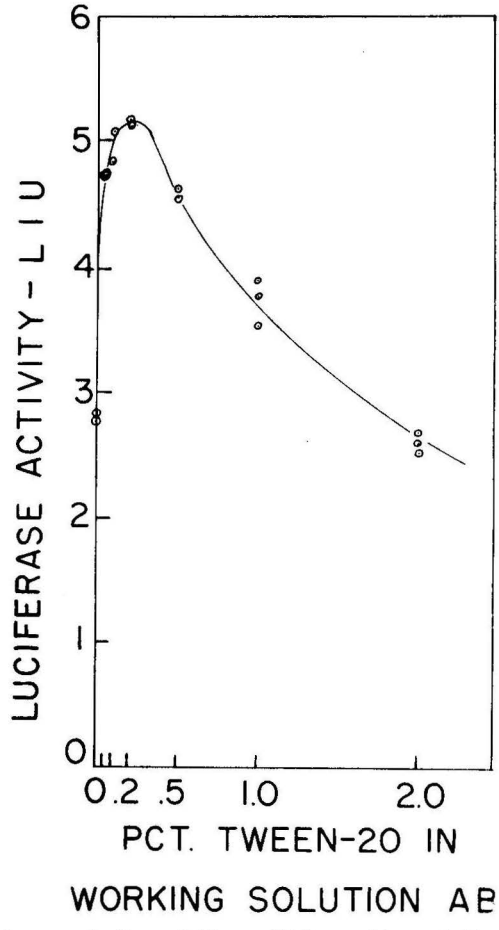


FIG. 4. Effect of concentration of Tween-20 in working solution AB on luciferase activity generated by the slow reaction.

curve rises and falls with increasing FMN concentration. The optimum concentration of FMN for the least active sample (curve D) was 1.0 μmole/ml; that for the sample of moderate activity (curve C), 1.9 μmoles/ml; and that for the most active sample (curves A and B), 2.1 μmoles/ml. Curves A and B were obtained with the same sample at different times. Since one concentration of FMN must be established for the assay, a concentration of 1.7 μmoles/ml was adopted (see Table 1).

Figure 5 shows that this concentration results in an error of —1.3% in the case of the most active sample and an error of +6% in the case of the sample of least activity.

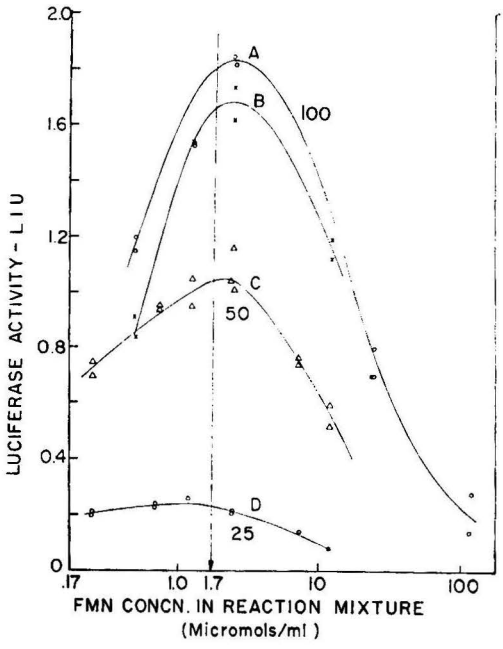


FIG. 5. Effect of concentration of FMN in reaction mixture on luciferase activity for a CL of high activity (100% of initial stock concentration used, curves A and B), of moderate activity (50% of concentration of stock CL, curve C), and of low activity (25% of concentration of stock CL, curve D). Curve B represents the same extract for curve A after storage at 3° overnight. The long vertical arrow represents the concentration adopted for the standard assay procedure.

5. Optimum Conditions for Maximum Luminous Activity with the $FMNH_2$ (Fast) Reaction

The tests with methylene blue showed that the best technique to achieve rapid mixing of reactants consisted of rapidly ejecting the syringe contents laterally outward into the 2.5 ml of reaction mixture through nine 0.25-mm holes spaced along the bottom 2.0 cm of a 6-in. 16-gage hypodermic needle whose end is closed off. A reaction temperature of 12° was found to slow the reaction sufficiently so that the maximum luminous intensity was not reached for approximately 2 sec following mixing of reactants.

Figure 6 shows the variation in luciferase activity with $FMNH_2$ concentration for the "fast" reaction carried out by the standard procedure. The results are similar to those obtained with various FMN concentrations in the slow reaction, except that the standard concentration of $FMNH_2$ found to be optimum with the fast reaction was 12× that of

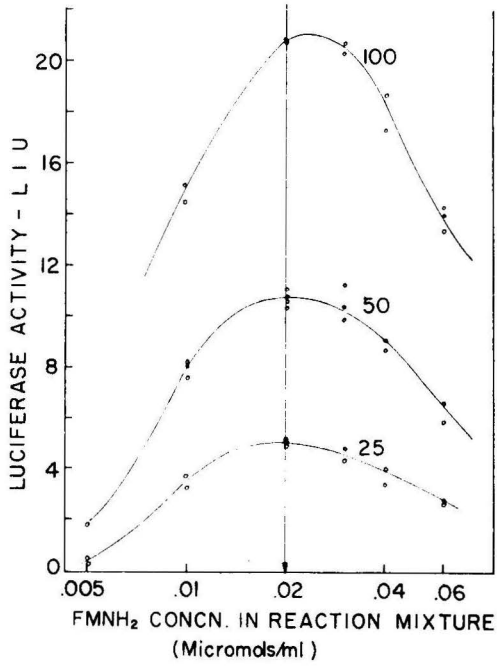


FIG. 6. Effect of concentration of reduced FMN ($FMNH_2$) in the reaction mixture on the luciferase activity of a CL of high activity (100% of initial stock concentration used), of moderate activity (50% of concentration of stock CL), and of low activity (25% of concentration of stock CL). The long vertical arrow represents the concentration adopted for the standard assay procedure.

FMN for the slow reaction. A slightly higher $FMNH_2$ concentration appears to be optimum for concentrated rather than for diluted enzyme preparation.

6. Relation between Luciferase Concentration and Luminous Activity with the Slow Reaction

Figure 7 shows the relation between luciferase concentration and luciferase activity graphed on log-log coordinates for a number of extracts prepared on various dates. The relationships appear to be linear on such plots down to low luciferase activities. The relationship between activity and concentration is therefore of the form $A = B(L)^k$, where A is luciferase activity, (L) is concentration of luciferase, B is a constant reflecting choice of units, and k is a constant governing the rate of change of luciferase activity with concentration. When luciferase activity is expressed in LIU and luciferase concentration in milliliters of luciferase

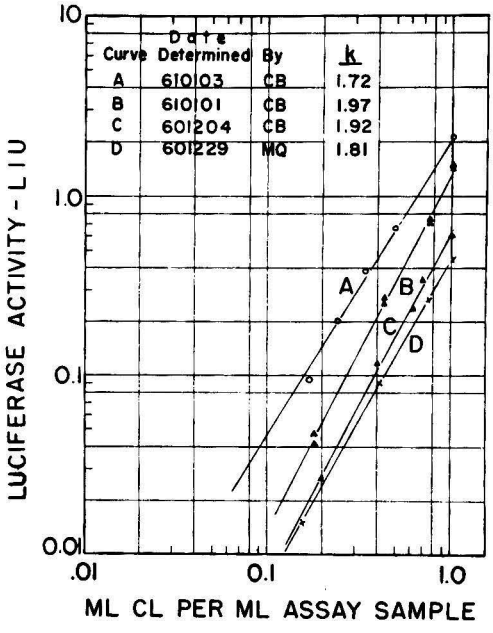


FIG. 7. Comparison of log luciferase activity–log enzyme concentration relation for cell extracts from various experiments. Diluent is distilled water in all cases. The values of k are those in $A = (E)^k$, where A = luciferase activity in LIU and (E) is concentration of enzyme preparation (CL) in the assay sample. The dates and initials signify that the four curves represent different cell extracts.

preparation (extract) per milliliter of assay sample, B becomes unity. The slope of the lines in Fig. 7 correspond to the value of k.

Figure 8 shows the effect of using different diluents on the log-log relationship described above. Curve A represents the normal case, in which H_2O was the diluent, as were those in Fig. 11. Curve B is the same extract diluted in the same manner using as diluent additional extract whose activity was extinguished by mild heating. This line was very nearly identical to that produced by using H_2O as diluent. Thus the effect of dilution with H_2O is not due to the dilution of any unknown factor in the cell extract which is involved in the luciferase reaction and which is stable to mild heating.

Curve C is that produced by using irradiated extract as diluent. A small residual activity remained in the diluent after it received 0.25 megarad. In the region of high extract concentration, where the activity was large compared to that of the diluent, the line is very nearly identical to lines A and B. At lower extract concentrations, the luminous intensity contributed by the luciferase in the diluted extract is so dimin-

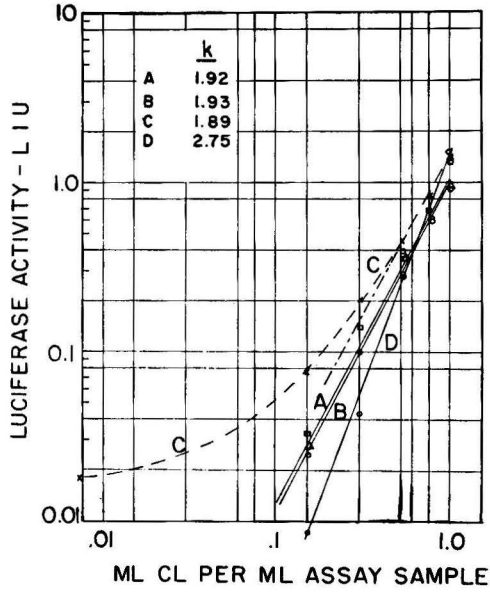


FIG. 8. Comparison of log luciferase activity–log enzyme concentration relationships for CL diluted with different diluents. Curve A, the diluent is DDW. Curve B, the diluent is a portion of the same CL previously heat-inactivated. Curve C, the diluent is a portion of the same CL whose luciferase activity was reduced to a low level by irradiation; k for this curve was obtained from the projection of its upper portion. Curve D represents the dilutions shown in curve A after 5 days of storage at 3°C. The significance of k is explained in Fig. 11.

ished that the amount present in the diluent itself begins to assert itself sufficiently to cause the line to deviate from linearity.

Curve D represents the same dilutions represented by curve A after additional portions of each dilution had been stored at 3° for five days. There is a marked change in slope, indicating that a particular value for the slope is useful only when the assays are completed within a few hours.

Figure 9 shows the effect of diluting with H_2O cell extracts prepared from bacterial suspensions which had been exposed to 0, 10, 20, and 30 kilorads of gamma radiation (3). There is a slight effect of irradiation on the slope of the log-log when compared to that of the controls. However, this deviation does not appear serious in the region where the relationships are used to estimate units of luciferase activity per milliliter of extract. It may be noted that extracts prepared from irradiated cells contained more rather than less luciferase activity. Further details of this investigation may be found by consulting reference (3).

The value of k shown in Figs. 7–9 and obtained in other experiments

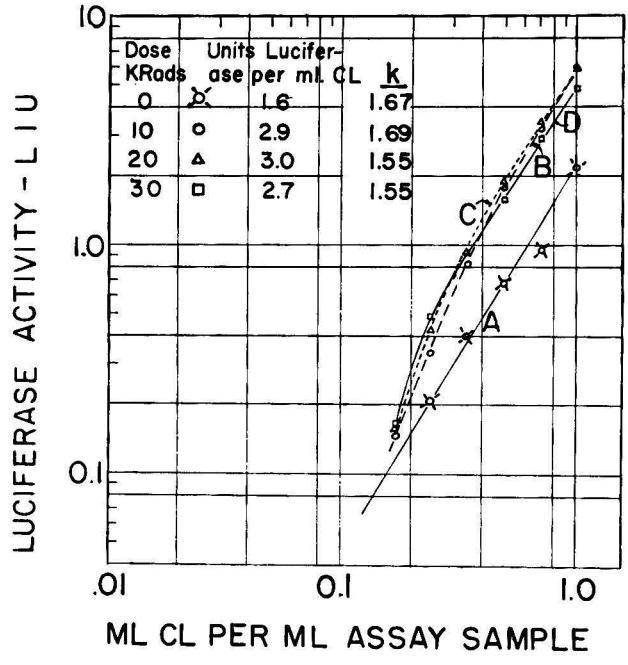


FIG. 9. Comparison of log luciferase activity–log enzyme concentration relation for CL's obtained from cell suspensions receiving 0 (curve A), 10 (curve B), 20 (curve C), and 30 kilorads of gamma radiation (curve D). The significance of k is explained in Fig. 11.

ranged from 1.55 to 2.75. The variation is least among various extracts prepared from the same cell suspension and assayed at the same time, as shown by the values of k for curves A, B, and C in Fig. 8 and for the linear portion of the four curves in Fig. 9. Irradiation of the cells before assay appears to have much less effect on the value of k (Fig. 9) than does storage of the extract for several days at 4° (curve D, Fig. 8). Extracts prepared from different cell suspensions, however, have a wide difference, as shown by an average of 1.62 for those in Fig. 9 to an average of 1.91 for those in Fig. 8.

7. *Luciferase Activity Determined by the Fast Reaction*

Figure 10 shows the variation of luciferase activity determined by the fast reaction at 12° with concentration of luciferase as determined by dilution of extracts prepared from two cell suspensions. The extract from one was assayed at successive daily intervals. When plotted on linear coordinates, a linear relation can be shown for the higher concentrations but not for the lower. An extrapolation of the linear relation for high concentrations does not pass through the origin. The intercept of the ex-

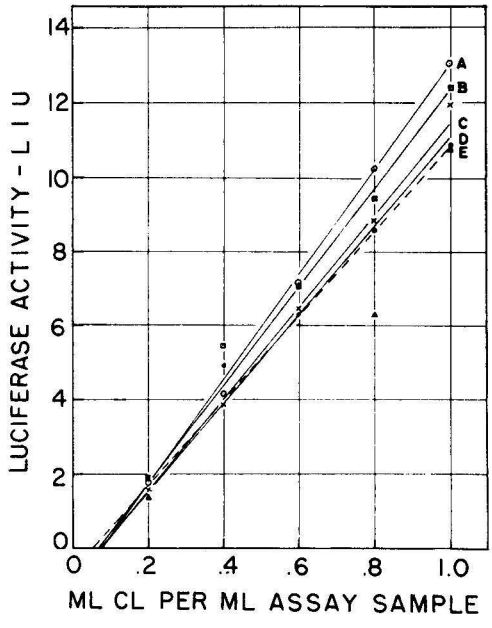


FIG. 10. Effect of dilution with DDW on luciferase activity of two CL's (curves A and E) prepared at different times as determined by the fast reaction. Curves B, C, and D represent the results with the CL used for A after storage under refrigeration for 1, 2, and 3 days, respectively. Storage does not change appreciably the intercept of the abscissa (0.075 ml CL/ml assay sample), whereas CL's prepared at another time do not have the same intercept (0.05 ml CL/ml assay sample).

trapolation on the concentration scale varies with the particular extract tested (5.2 to 7.5% in Fig. 10). The above activity-concentration relation would be applicable only for extracts containing more than 1 LU/ml.

Figure 11 shows the relationship between the luciferase activities generated by the two reactions at 12° using various dilutions of a cell extract. This relation, when plotted on log-log paper, is very close to that of the luciferase activities determined by the slow reaction plotted against luciferase concentration. The value of k is lower than the lowest value found for the slow reaction. The luciferase activity determined by the fast reaction varies from two to five times that by the slow reaction, depending on whether the luciferase content is high or low, respectively.

8. Determination of Units of Luciferase

In the conversion of luciferase activities determined by the slow reaction to units of luciferase per milliliter, it will be necessary to deter-

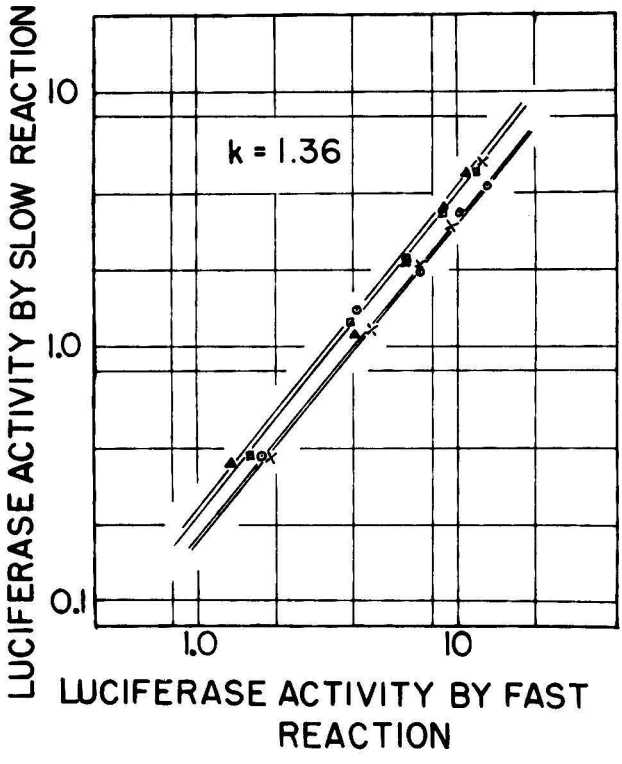


FIG. 11. Relationship between luciferase activities produced by the fast reaction and those produced by the slow reaction for a number of dilutions of a CL. Both reactions were carried out at 12°C. The value of k is the slope of the log-log plots and corresponds to k in $A = (E)^k$ in Figs. 7–9 and 11.

mine the value of k applying to extracts from each cell suspension assayed. This may be obtained by determining the luciferase activities, A_1 and $A_{1/2}$, of the undiluted and of a 1:2 dilution (with H_2O), respectively, of the fresh extract, in which case:

$$k = \frac{\log A_1 - \log A_{1/2}}{0.301}$$

The units of luciferase in this cell extract may then be calculated as follows:

$$\text{LU/ml} = \frac{1}{\text{antilog}\,(-\log A_1/k)}$$

If the values of k applicable to each of a series of extracts do not vary by more than $\pm 2.5\%$, a single average value of k may be used without

an error greater than ±2% in resulting values of luciferase concentration. This would permit luciferase activities to be determined graphically by use of a conversion line on log-log coordinates having a slope equal to k and passing through the point 1 LIU = 1 LU/ml. A ±2.5% error in the value of k will not be exceeded if the average deviations in the determination of I_1 and $I_{1/2}$ do not exceed ±1.6 and ±2.3%, respectively, for values of I_1 and $I_{1/2}$ in the neighborhood of 5 and 1.4 LIU, respectively.

The determination of luciferase concentration in units/ml from luciferase activity measured by the fast reaction also requires that two determinations be made on each cell extract. If one determination is made on an undiluted sample (A_1) and another on a 50% dilution ($A_{1/2}$), then the LU/ml may be calculated as follows:

$$\text{LU/ml (fast reaction)} = \frac{2A_1}{2 + \left(\frac{A_1 - 2A_{1/2}}{A_1 - A_{1/2}}\right)(A_1 - 1)}$$

Luciferase concentrations may be determined graphically from a conversion plot if it is found that the slope of the plot and its intercept with the ordinate referring to concentration of extract does not vary appreciably from one cell extract to another.

Table 2 shows the results of applying the standard assay procedure

TABLE 2
CORRELATION OF SAMPLE SIZE WITH AMOUNT OF LUCIFERASE FOUND

Vol. of sample of cell suspension	Luciferase units/sample	Luciferase units/ml cell suspension
5	1.8	0.36
10	3.5	0.35
15	5.8	0.39
20	7.6	0.34

using the slow reaction to various size samples of a single cell suspension. The relation between volume of sample and units of luciferase activity appears to be a reasonably constant one. It suggests that the average of duplicate determinations give a value of LU/10^9 cells to within ±10%.

DISCUSSION

The rapidity with which *Photobacterium fischeri* cells lyse when suspended in distilled and deionized water is probably a result of their being grown in media of high tonicity (equivalent to 0.6 M NaCl or

3.5%). Some lysis has been observed to occur with cells suspended in as much as 0.5% NaCl. The apparent absence of activity in the cell debris indicates that the enzyme passed quantitatively into the aqueous phase. High-speed centrifugation results in a water-clear extract which seldom exceeds 0.25% protein concentration. The enzyme appears sufficiently stable for the duration of an assay series when kept at ice temperature, and at 25° for the duration of a particular assay.

The significance of the unit of luciferase defined in this report depends upon the geometric specifications of the particular photometer housing used and on the particular luminous standard with which the luminous intensities are compared at the time. A chemiluminescent reaction whose quantitative chemical change is related to a known light output would be desirable to define a unit which would be applicable from one laboratory to another, as in the case of enzyme reactions measured on a chemical basis.

The "slow" reaction is more convenient to use as a basis for the assay because, at room temperature, the maximum luminous intensity prevails for a period longer than the response time of the photometer and the time needed to observe the ammeter reading. The luciferase activities determined in crude extracts by the "fast" reaction are not sufficiently proportional to concentration to offer an advantage over the "slow" reaction.

The fading of luminescence is characteristic of the *in vivo* as well as of the *in vitro* luciferase reaction. Part of this fading is believed to be associated with a progressive inactivation of the enzyme as it participates in the reaction (3). The fact that it occurs indicates the possibility that some luciferase activity may be lost before the maximum is reached (see curve B in Fig. 2). It is believed that this is a negligible source of error because the rate of decline following the maximum is sufficiently low to indicate little loss before the maximum is reached.

The optimum requirement of $FMNH_2$ in the fast reaction agrees with the minimum requirement for maximum activity in active preparations (0.2 μmole/ml) as shown by McElroy and Green (8); the concentration of DPNH used in the present procedure is about five times the minimum requirement (0.05 μmole/ml) demonstrated by Strehler and Cormier (9). The optimum concentration of aldehyde used in previous work was not specified, but is probably considerably lower than that used here.

What is known about the luciferase reaction indicates that it is rather complex. Because the oxidation of one molecule of $FMNH_2$ does not furnish the energy of one photon of visible light, two molecules are believed to be involved, with a luciferase-aldehyde complex functioning to

combine the energy of both. The aldehyde is believed to undergo peroxidation in this process. While oxygen reacts in some way with the luciferase-aldehyde-peroxide-$FMNH_2$ complex, it rapidly oxidizes available $FMNH_2$ and thereby competes with the light-emitting step. The reaction is further complicated by being inhibited by FMN, which presumably competes with the reduced form for available enzyme sites, or leads to increased formation of riboflavin, which is known to inhibit the reaction. However, since dilution of extracts with H_2O has the same effect on luciferase activity as does dilution with heat-inactivated extract, it is not likely that use of crude rather than purified extracts would make the assay more accurate, assuming no enzyme was lost in purification.

It is not surprising that the rate of the slow reaction is sharply diminished by dilution. Judging by the 2- to 5-fold greater maximum light intensity of the fast reaction over that of the slow reaction when both are carried out under the same conditions (Fig. 11), it is probable that, in the light-emitting step of the slow reaction, the substrate $FMNH_2$ is limiting. Dilution of the enzyme should thus cause more than a proportional decrease in maximum activity. What portion of the $FMNH_2$ generated is lost to autoxidation and how this portion varies with dilution are not known. The fact that the enzymes must presumably combine with more than one molecule of reduced flavin and the possibility that the DPNH oxidase which generates the $FMNH_2$ and the luciferase which consumes it may be part of a multienzyme, as suggested by McElroy and Green (8), adds to the difficulty of analyzing the effect of enzyme concentration on the luminous activity.

SUMMARY

The bacterial luciferase reaction carried out *in vitro* can be used as a basis for a semiquantitative assay of active luciferase in bacterial cells. Relations between maximum luminous intensity generated and the amount of enzyme present can be established for luciferase and for the combination of DPNH oixdase and luciferase. A definition is suggested for a unit of luciferase activity. It is believed that the assay procedure described can be made accurate to within $\pm 10\%$.

REFERENCES

1. Hug, O., and Wolff, I. *In* "Progress in Radiobiology," *Proc. 4th Intern. Conf. Radiobiol., Cambridge, England, August, 1955.* C. C Thomas, Springfield, Ill., 1956.
2. Whipple, G. H., *J. Cellular Comp. Physiol.* **43,** 415–423 (1954).
3. Burns, C. H., Nevill, A. D., and Whipple, G. H., *Science* **135,** 430–32 (1962).
4. Burns, C. H., Nevill, A. D., and Whipple, G. H., Luminescence and luciferase on *Photobacterium fischeri* cell suspensions (in preparation).

5. STREHLER, B. L., AND CORMIER, M. H., *J. Biol. Chem.* **211**, 213–225 (1954).
6. MCELROY, W. D., HASTINGS, J. W., SONNENFELD, V., AND COULOMBRE, J., *Science* **118**, 385–6 (1953).
7. STREHLER, B. L., *J. Am. Chem. Soc.* **75**, 1265 (1953).
8. MCELROY, W. D., AND GREEN, A. A., *Arch. Biochem. Biophys.* **56**, 240 (1955).
9. STRECHLER, B. L., AND CORMIER, M. J., *Arch. Biochem. Biophys.* **53**, 138 (1954).
10. FARGHALY, A. H., *J. Cellular Comp. Physiol.* **36**, 165 (1950).

ANALYTICAL BIOCHEMISTRY 5, 416–421 (1963)

Testosterone in Human Plasma[1]

ENRICO FORCHIELLI, GIUSEPPE SORCINI,[2]
MARY STYLIANOU NIGHTINGALE, NORMA BRUST,
AND RALPH I. DORFMAN

From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts

and

WILLIAM H. PERLOFF AND GERT JACOBSON

From The Division of Endocrinology and Human Reproduction of the Research Laboratories, Albert Einstein Medical Center, Philadelphia, Pennsylvania

Received August 28, 1962

In a preliminary report Finkelstein *et al.* described a method for the estimation of free testosterone in the peripheral plasma from normal men and women. Plasma from men and virilized females contained significantly more testosterone than plasma obtained from normal women. Since the appearance of the preliminary communication, Oertel (2) has reported on a method for measuring testosterone levels in plasma and Oertel and Kaiser (3) have measured levels of this androgen in the plasma of young male subjects. The present communication records improvements in the method and testosterone values in a larger series of normal men and women, hirsute and virilized women, and the influence of ovariectomy, adrenalectomy, wedge resection, prednisone, and human chorionic gonadotropin on plasma testosterone.

PREPARATION AND EXTRACTION OF PLASMA

Plasma was prepared from freshly drawn blood by centrifugation. Either citrate solution or heparin may be used as the anticoagulant. Plasma prepared from freshly drawn blood may be stored in the frozen state up to six months. For the analysis the plasma was divided into two equal portions, A and B. A tracer quantity of testosterone-C^{14} (about 2000 cpm, which corresponded to about 0.02 μg) which served as an internal recovery control was added to Portion A. Portion B served as the

[1] Supported in part by grants from Ruth Estrin Goldberg Memorial for Cancer Research, Mrs. Henry Krumb, and U. S. Public Health Service Grants CRTY-5001 and A-3341.

[2] Eli Lilly Fellow.

plasma blank and this was treated identically to A in all respects except that it was not incubated with the placental enzyme. Alcohol was added prior to the addition of the enzyme preparation and cofactors to denature the enzymes and inhibit any possible side reactions. The extraction and treatment of the plasma extract and isolation of the testosterone zone by paper chromatography was identical to that previously reported (1).

PREPARATION OF THE PLACENTAL ENZYME

A microsomal pellet containing the aromatizing enzyme system was prepared from human term placenta according to Ryan (4), resuspended in water, and lyophilized, and the dried powder was stored in a desiccator at —10°C. Five-tenths to 1 mg of this dried powder converts 1–2 μg of testosterone quantitatively to estrogenic material. This represents better than a 20-fold reduction in the weight of the enzyme preparation compared to that previously described (1).

Incubation, Extraction, and Treatment of the Extract

The eluate of the testosterone zone obtained from the paper chromatogram was concentrated to a small volume, transferred to 10-ml beakers, and evaporated to dryness. To this residue there was added 1.0 mg of the placental enzyme suspended in 0.5 ml of 0.1 *M* phosphate buffer at pH 7.0, together with 5 μmoles of glucose 6-phosphate, 100 μg of TPN, and 0.1 Kornberg unit of glucose 6-phosphate dehydrogenase in 0.5 ml 0.154 *M* KCl. The mixture was incubated for 1 hr at 37° in air.

The reaction was stopped by the addition of 5.0 ml of absolute ethanol and the insoluble material separated by centrifugation. The precipitate and residue remaining in the beaker was washed two times with 5-ml portions of absolute ethanol, followed by centrifugation each time. The combined alcoholic supernatant solutions were treated with 2–4 mg of sodium borohydride and allowed to stand at room temperature for 15 min. This step was introduced to reduce any estrone present to estradiol-17β, thus yielding a single uniform end product. The reaction mixture was concentrated to a small volume (1.0 ml) in a stream of nitrogen and diluted to 10 ml with water. After the addition of 2 to 3 drops of 1 *N* acetic acid (to neutralize any alkali formed from the decomposition of the borohydride) the mixture was extracted with 3–10 ml portions of freshly distilled benzene. The combined benzene extracts were washed with a small volume of water, dried over sodium sulfate, and concentrated to dryness. A partition into phenolic and neutral fractions was considered unnecessary since the small amounts of neutral material remaining after the enzyme reaction could be readily separated from the estradiol-17β by paper chromatography.

The chromatography and estimation of estradiol-17β was carried out as previously described (1). The number of C^{14} counts obtained in the estradiol-17β served as the index of recovery, which averaged 52.4% (range 40–64), and all values were corrected for losses on this basis.

RESULTS AND CONCLUSIONS

The plasma testosterone values in various subjects are presented in Table 1, and Table 2 reports the influence of various surgical procedures

TABLE 1
TESTOSTERONE IN HUMAN PLASMA

Subjects (age range)	No.	17-Ketosteroids per day	Mean testosterone, μg/100 ml (range)	
Normal men (23–74)	9	—	0.56 (0.1–0.98)	
Normal women (22–35)	10	—	0.12 (0.02–0.26)	
Hirsute women, polycystic ovaries (Stein-Leventhal type) (18–41)	6	12.2 (7–24)	0.33 (0.25–0.42)	
Hirsute women (diagnosis not established) (14–47)	4	13.7 (8–18)	0.25 (0.11–0.32)	
Virilized women, polycystic ovaries (Stein-Leventhal type) (14–36)	5	10.9 (6.4–20)	0.49 (0.30–0.74)	
Ovarian Hilus cell tumor (68)	1	2–4	2.0[a]	before surgery
			0.02	after surgery
			0.05	
			0.04	
Women with adrenal adenoma	4	1000	1.3[a]	
		250–1000	1.4	
		308	0.22	before surgery
			0.02	after surgery
		200–272	0.79[b]	

[a] These values were published previously (1).
[b] Also bilateral polycystic ovaries.

and treatments on plasma testosterone levels. Nine men varying in age from 23 to 74 years had plasma testosterone values ranging from 0.1 to 0.98 μg per 100 ml and a mean value of 0.56 μg. The corresponding plasma testosterone values obtained on ten normal women, 23 to 35 years of age, ranged from 0.02 to 0.26 with a mean of 0.12. Each of the female

TABLE 2
INFLUENCE OF SURGERY AND TREATMENT ON TESTOSTERONE IN PLASMA

Subjects (No.)	Treatment	Mean, testosterone μg/100 ml
Hirsute women (5)	Ovariectomy	0.11 (0.04–0.22)
Bilaterally adrenalectomized woman	25 mg cortisone plus 1 mg fluoxymestrone daily	0.08 0.09 (during treatment with 300 I.U. human chorionic gonadotropin)
Hirsute women (3)	Wedge resection	0.53 before 0.77 0.58 0.11 after 0.34 0.22
Hirsute women (4)	Prednisone treatment 10–20 mg/day	0.48 before 0.44 0.1 — 0.13 after 0.07 0.02 0.16

subjects had normal cycles and were not hirsute (Table 1). The same table presents the plasma levels of two groups of hirsute women. One group had proved polycystic ovaries and were classed as having the Stein-Leventhal syndrome. The mean 17-ketosteroid value for this group of six women, ranging in age from 18 to 41 years, was 12.2 (7–24) mg per twenty-four hours. The blood testosterone values ranged from 0.25 to 0.42 μg/100 ml, with a mean of 0.33 μg. The second group of 4 women were hirsute but the diagnosis was not established. The mean testosterone value for this group was 0.25 μg (0.11 to 0.32 μg) per 100 ml plasma and the mean 17-ketosteroid values were 13.7 mg per day.

Five subjects had the stigma of virilization and the blood testosterone values were considerably elevated. The mean value of 0.49 μg per 100 ml of plasma was quite similar to that found for the normal men. Of particular interest is the fact that the 17-ketosteroid mean value for this group was only 10.9 mg per day. Actually in only one patient who had a value of 20 mg per day were the 17-ketosteroids increased.

Plasma testosterone levels were studied in four women bearing virilizing adrenal adenomas. All had greatly elevated 17-ketosteroids and

three of the patients had very high testosterone levels. One subject had a value of 0.22 μg per 100 ml, which was in the normal range, which decreased to 0.02 μg after the tumor was removed. Of further interest was the fact that this latter subject, although severely hirsute, showed little or no evidence of virilism.

The presurgical value of 2 μg per 100 ml plasma for the patient with a hilus cell tumor had been reported previously. Postoperatively the values 0.02, 0.04, and 0.05 μg per 100 ml were found.

Ovariectomy of hirsute women produced a decrease in blood testosterone; the extent, however, varied from subject to subject. In one virilized Stein-Leventhal patient who had the excessive plasma testosterone level of 0.71 μg, bilateral ovariectomy decreased this value to 0.22. The mean value for all 5 ovariectomized women was 0.11 μg, which was not different from the plasma testosterone value of normal women.

Decreases in plasma testosterone were effected by wedge resection and by prednisolone treatment (Table 2). The surgical procedure caused a net decrease of 0.41 μg per 100 ml of plasma from a mean of 0.63 to 0.22 μg. Prednisolone effected a change from 0.34 μg to 0.07, indicating a net decrease of 0.27 μg of plasma testosterone.

In one hirsute ovariectomized patient not listed in Table 2, the plasma testosterone of 0.15 μg was decreased to 0.05 when the patient was treated daily with 8 mg of 6α-methylprednisolone. Daily treatment of this ovariectomized patient with the same dose of 6α-methylprednisolone plus 3000 I.U. of human chorionic gonadotropin (Follutein, Squibb) resulted in a plasma testosterone level of 0.01, indicating no significant change. This illustrates the relationship of the adrenal to testosterone formation.

Treatment of the adrenalectomized subject (Table 2) with 3000 I.U. of human chorionic gonadotropin once daily for three days produced no significant change in plasma testosterone level but did result in a definite rise in plasma estrogen levels. The pretreatment levels of estrone of 0.020 μg increased to 0.080 μg while the estradiol-17β level rose from <0.002 to 0.016 μg.

SUMMARY

A method is described for the estimation of free testosterone in human plasma. The mean value for normal men was 0.56 μg per 100 ml, for normal women 0.12 μg, for hirsute women with polycystic ovaries 0.33 μg, for virilized women with polycystic ovaries 0.49 μg. Both wedge resection and prednisone treatment significantly decreased the free plasma testosterone values.

ACKNOWLEDGMENTS

We are indebted to Drs. Irene T. Kline and Richmond Smith, Henry Ford Hospital, Detroit, Michigan, Dr. Manuel Neves e Castro, Lisbon, Portugal, Dr. T. B. Connor, University of Maryland School of Medicine, Baltimore, Dr. Isabel Taliaferro, Medical College Hospital, Medical College of Virginia, and Dr. Lloyd H. Smith, Jr., Massachusetts General Hospital, Boston, for their help in supplying us with plasma samples from patients.

REFERENCES

1. FINKELSTEIN, M., FORCHIELLI, E., AND DORFMAN, R. I., *J. Clin. Endocrinol. Metab.* **21,** 98 (1961).
2. OERTEL, G. W., *Acta Endocrinol.* **37,** 237 (1961).
3. OERTEL, G. W., AND KAISER, E., *Klin. Wochschr.* **39,** 1146 (1961).
4. RYAN, K. J., *J. Biol. Chem.* **234,** 268 (1959).

ANALYTICAL BIOCHEMISTRY 5, 422–425 (1963)

Determination of Plasma Estrone and Estradiol-17β[1]

SHOGO ICHII, ENRICO FORCHIELLI, WILLIAM H. PERLOFF, AND RALPH I. DORFMAN

From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts
and
The Albert Einstein Medical Center, Northern Division, Philadelphia, Pennsylvania

Received September 10, 1962

Methods have been available to estimate total estrogen levels in plasma hydrolyzed with acid of subjects in advanced stages of pregnancy (1, 3). However, very little is known of the levels of these substances in the plasma of nonpregnant women and men. Ittrich (4) has measured the total estrone, estradiol-17β and estriol in whole blood samples taken on various days of the cycle of a normally menstruating female. A similar study was made by Oertel (5). The first reported attempt to measure free circulating estrogens in normal as well as pregnant females was by Svendsen by means of a double isotope method (6). This communication deals with a method which permits the analysis of free estradiol-17β and estrone in the plasma of normal men and women.

METHOD

Plasma, after adding insignificant quantities of estrone-6,7-H^3 (8×10^{-4} μg containing 39,000 dpm), and estradiol-17β-6,7-H^3 (1×10^{-4} μg containing 40,000 dpm), was extracted with a mixture of chloroform:ether (1:3). The residue, after solvent distillation, was dissolved in 70% methanol, placed in a deep freeze overnight, centrifuged, and the supernatant partitioned against petroleum ether. The phenolic material of the 70% methanol fraction was subjected to paper chromatography in the Bush B_3 system. The zones containing estrone and estradiol-17β, after elution, were rechromatographed in the Bush A and B_1 system, respectively. The quantity of estrogens was determined by the phosphoric acid fluorescence method of Finkelstein (7). The fluorescence was measured in an Aminco-Bowman spectrophotofluorometer using 0.5 ml fused-quartz cuvettes. The radioactivity (for recovery correction) was deter-

[1] Supported in part by grants from Mrs. Henry Krumb, U. S. Public Health Service Grant No. H-1741, and American Cancer Society.

mined in a Packard Tri-Carb Model 314 scintillation counter from samples dissolved in 10 ml scintillation medium which consisted of 4 gm PPO [2,5-diphenyloxazole] and 100 mg of POPOP [1,4-bis-2-(5-phenyloxazolyl)benzene] dissolved in 1 liter of scintillation-grade toluene.

The average recovery of pure estradiol-17β and estrone added to plasma in amounts ranging from 0.01–0.06 μg was 62% (range 52–65.8%) as determined by fluorometry and 66% (58.3–71.6%) on the basis of recovered radioactivity.

RESULTS AND CONCLUSIONS

In women studied just at the end of the menstrual flow and at the midcycle about two and one-half times as much estrogen was found at the latter time. This corresponds to the cyclic variation observed in urinary estrogens [Brown *et al.* (8), Finkelstein *et al.* (9)]. The mean of the estrone values for women (0.032 μg) without considering cyclical variations, was about 50% greater than that found for men (0.023 μg). The mean of the estradiol-17β values for women was 10-fold greater (0.032 μg) than that observed for men (0.003 μg). Svendsen (6) determined the concentration of free estrone and estradiol-17β in the plasma of normal women by a double isotope method employing S^{35} and I^{131} with sensitivities of 0.0024 μg for esterone and 0.0014 μg for estradiol-17β. This investigator found slightly more estradiol-17β than estrone and the mean of a limited number of 5 samples indicated a value of 0.028 μg for estrone and 0.037 μg for estradiol-17β. These values are of the same order as those reported in Table 1.

The expected increase in both estrogens was found in the two pregnancy samples and on two samples from a patient who had a granulosa cell tumor. The adrenal venous plasma of a steer contained 0.100 μg of estrone and 0.028 μg of estradiol-17β per 100 ml while the jugular vein plasma sample had 0.020 μg of estrone and 0.032 μg of estradiol-17β. Plasma samples from two adrenal adenoma patients were studied, one with an elevated value for the estrogens and a second with normal values. The one patient with values in the normal range showed a decrease from 0.035 to 0.005 μg per 100 ml plasma for estrone and a decrease from 0.012 and 0.004 μg for estradiol-17β after the tumor was removed.

In seven subjects with hirsutism due to polycystic ovaries the mean of the estradiol-17β concentration was 0.021 μg (range 0.015–0.044), which did not seem to vary significantly from that found for the limited observations in normal women, but the estrone value of 0.070 μg for the hirsute women appeared to be significantly higher than that of normal women.

TABLE 1

CONCENTRATION OF ESTRONE AND ESTRADIOL-17β IN VARIOUS PLASMAS

Subjects or patients	Age (range)	Number of subjects	μg/100 ml	
			Estrone	Estradiol-17β
Normal women	18–33	9	0.032 (0.008–0.106)	0.032 (0.008–0.056)
	19–22	3	0.058 (0.032–0.106) midcycle	0.026 (0.013–0.023)
			0.023 (0.019–0.024) (day of end of menstrual flow)	0.010 (0.008–0.013)
Normal men	22–28	5	0.023 (0.010–0.036)	0.003 (0.002–0.006)
Pregnancy:				
7th month		1	1.03	0.55
9th month		1	0.57	0.98
Granulosa cell tumor	30	1	0.33–0.37	0.18–0.19
Adrenal adenoma			0.128	0.076
Hirsutism polycystic ovaries		7	0.070 (0.004–0.134)	0.021 (0.015–0.044)
Steer adrenal vein plasma		1	0.100	0.028
Jugular vein plasma		1	0.020	0.032
Women with ovarian dermoid cyst			0.998	0.085
Adrenal adenoma			0.035	0.012 before surgery
			0.005	0.004 after surgery

SUMMARY

As little as 0.002 μg of estrone and estradiol-17β may be determined using a combination of paper chromatographic separation, phosphoric acid fluorescence, and radioisotope-labeled estrogens added for recovery correction. The estrone and estradiol-17β concentration in plasma of normal menstruating women was 0.015 μg (0.008–0.028) and 0.031 μg (0.013–0.056) per 100 ml while in men's plasma the same constituents were 0.023 μg (0.010–0.036) to 0.003 μg (0.002–0.006) per 100 ml, respectively.

ACKNOWLEDGMENTS

The authors are indebted to Dr. M. T. Clegg of Davis, California, for the bovine plasma, to Doctors Irene T. Kline and Richmond Smith, of the Henry Ford Hospital, for the plasma samples on the granulosa cell tumor patient and one adrenal adenoma patient, and finally to Dr. Lloyd H. Smith, Jr., of Massachusetts General Hospital, for the plasma samples on the adrenal adenoma patient before and after surgery.

REFERENCES

1. SLAUNWHITE, R. W., JR., AND SANDBERG, A. V., *Proc. Soc. Exptl. Biol. Med.* **101**, 544 (1959).
2. OERTEL, G. W., WEST, C. D., AND EIK-NES, K. B., *J. Clin. Endocrinol. Metab.* **19**, 1619 (1959).
3. PREEDY, J. R. K., AND AITKEN, E. H., *J. Biol. Chem.* **236**, 1300 (1961).
4. ITTRICH, G., *Z. physiol. Chem.* **320**, 103 (1960).
5. OERTEL, G. W., *Klin. Wochschrift* **39**, 492 (1961).
6. SVENDSEN, R., *Acta Endocrinol.* **35**, 161 (1960).
7. FINKELSTEIN, M., *Acta Endocrinol.* **10**, 149 (1952).
8. BROWN, J. B., KLOPPER, A., AND LORAINE, J. A., *J. Endocrinol.* **17**, 401, 1958.
9. FINKELSTEIN, M., JEWELEWICZ, R., KLEIN, O., PFEIFFER, V., AND SODMORIAH-BECK, S., "Prenatal Care." P. Noordhoff, Groningen, The Netherlands, 1960.

ANALYTICAL BIOCHEMISTRY 5, 426–432 (1963)

Separation of Catecholamines and Their Metabolites[1]

D. T. MASUOKA, W. DRELL,[2] H. F. SCHOTT, A. F. ALCARAZ, AND E. C. JAMES

From the Psychopharmacology Research Laboratories, Veterans Administration Hospital, Sepulveda, California

Received September 11, 1962

Studies on the biosynthesis and metabolism of catecholamines in various tissues required a procedure for separating and measuring the radioactive precursors and metabolites. The ion-exchange chromatography method of Kirshner and Goodall (1) is excellent for the separation of adrenaline, noradrenaline, and dopamine, but the presence of *O*-methylated metabolites interferes with the resolution of the peaks. It was also desirable to separate and determine small quantities of 3,4-dihydroxyphenylalanine (dopa) in the presence of highly radioactive tyrosine.

Drell (to be published) in a study of various factors involved in the adsorption of catechol compounds on alumina found conditions under which catecholamines could be separated from catechol acids.

The procedure described below, a modification and extension of Drell's procedure (2), involves essentially the use of an alumina column to effect a partial separation of the solutes into three groups: noncatechols (such as tyrosine and 3-*O*-methylated metabolites of catecholamines), catecholamines, and catechol acids, followed by further resolution of each of these groups by means of a suitable resin column.

METHODS

Preparation of Alumina. 50–60 gm of aluminum oxide (Merck Reagent Chromatographic) was placed in a beaker containing 800–900 ml of 2 *N* HCl and heated on a steam bath for 2 hr with gentle stirring. The alumina then was washed with glass distilled water and the fines decanted at least 10 times. The damp alumina was dried in an oven at 200°C for 2–3 hr and stored immediately in a capped bottle. A glass tube of 1 cm i.d. and 18 cm long having a coarse fritted disk 1 cm from the bottom tip was used to form the column. After capping the bottom of the tube with parafilm, about 2 ml of 0.2 *M* ammonium acetate-acetic

[1] Supported by grants from the American Heart Association (60-G-27) and U. S. Public Health Service (MY-3663, B-1214).

[2] Established Investigator, American Heart Association, 1954–1959.

acid buffer, pH 6.1, was poured in, followed by 2 gm of dry alumina. The column was inverted many times to remove all bubbles. The parafilm cap was then removed and 50 ml of buffer was allowed to wash through the column.

Preparation of Amberlite CG-50 Resin. Amberlite CG-50, type 2 (200–400 mesh), obtained from Rohm and Haas Company, was prepared according to the procedure of Kirshner and Goodall (1). This involves washing, cycling through the sodium and acid forms as described by Hirs, Moore, and Stein (3), and finally conversion to the ammonium form. The resin was buffered at pH 6.1 for the separation of the catecholamines and at pH 6.5 for the 3-*O*-methylated metabolites with 0.2 *M* ammonium acetate-acetic acid buffer.

Preparation of Dowex 50 × 8. Dowex 50 in the hydrogen form (Bio-Rad Laboratories, AG 50W-×8, 200–400 mesh) was used. Approximately 8 gm of resin was suspended in 2 *N* HCl and poured into a 0.9 i.d. × 20 cm column. The bottom of the column was stoppered while the resin was allowed to settle. At least 300 ml of 0.5 *N* HCl was washed through the column overnight.

Preparation of Tissue Extract for Chromatography. In isolated tissue experiments, incubations were terminated by adding 2 drops of glacial acetic acid; 200 μg of each nonradioactive carrier was then added as required by the conditions of the experiment. The mixture was ground in a glass homogenizer, adjusted to pH 6.1 with 2 *N* ammonium hydroxide from a capillary pipet while stirring continuously, transferred to a conical centrifuge tube, and placed in a refrigerator for 15 to 30 min before centrifuging. The clear supernatant was saved, and the residue washed once or twice with 1–2 ml of 0.2 *M* ammonium acetate-acetic acid buffer, pH 6.1, by resuspending the residue, chilling, and centrifuging. The combined supernatant and washings were placed directly on the alumina column.

RESULTS AND DISCUSSION

A flowsheet of the procedure is presented in Fig. 1. Solutions containing 200 μg each of adrenaline, noradrenaline, dopamine, dopa, metanephrine, normetanephrine, and 3-methoxytyramine were used to develop the procedure. The mixture to be chromatographed was adjusted to pH 6.1 with 2 *N* ammonium hydroxide and added to the alumina column prepared according to the procedure above. When the level of the liquid dropped just to the top of the alumina, 50 ml of 0.5 *M* ammonium acetate-acetic acid buffer, pH 6.1, was allowed to wash through. The effluent and wash together contained the noncatechols, i.e., tyrosine and the *O*-methylated metabolites of the catecholamines (Alumina Effluent). This was followed

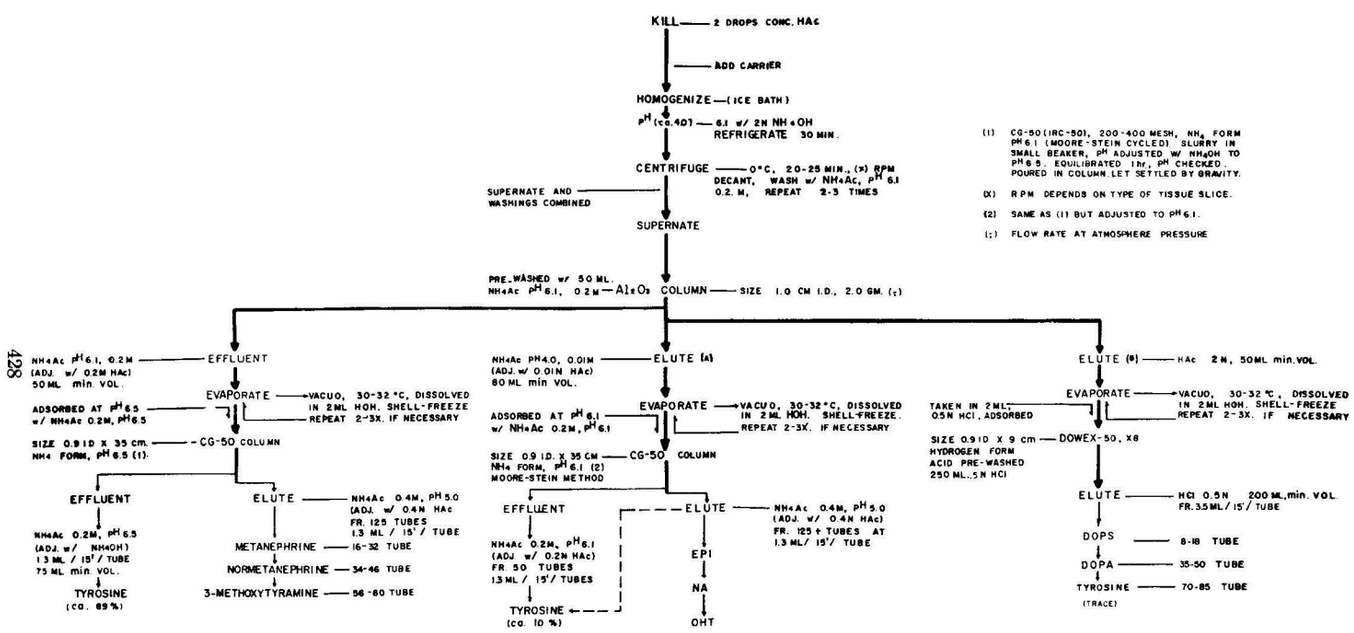


FIG. 1. Diagram of fractionation procedure for separating catecholamines, 3-*O*-methylated metabolites, and dopa. EPI, epinephrine or adrenaline. NA, noradrenaline. OHT, hydroxytyramine or dopamine. DOPA, 3,4-dihydroxyphenylalanine. DOPS, 3,4-dihydroxyphenylserine.

by 80 ml of 0.01 *M* ammonium acetate-acetic acid buffer, pH 4.0, to elute the catecholamines (Alumina Eluate 1). The alumina column was eluted finally with 50 ml of 2 *N* acetic acid to remove dopa and other catechol acids (Alumina Eluate 2). The flow rate was not controlled but allowed to drop freely at a rate of about 1 ml/min. The alumina effluent which contained the 3-*O*-methylated catecholamines was evaporated to dryness *in vacuo,* using a rotating evaporator and heating to not over 35°C. About 2 ml of water was added to dissolve the residue and evaporated off *in vacuo.* This procedure was repeated 2 to 3 times to completely remove the volatile ammonium acetate. It appeared necessary to remove the excess salts in order to obtain clean separation and sharp peaks from the subsequent resin column. The CG-50 resin, prepared as described above, was buffered at pH 6.5 with 0.2 *M* ammonium acetate-acetic acid solution. A slurry of the resin and buffer was poured into a glass column 0.9 cm i.d. × 55 cm with fritted disk until the height of the resin was 35 cm. The resin column was washed overnight with approximately 300 ml of the 0.2 *M* buffer. The final residue to be fractionated was dissolved in 2 to 4 ml of buffer, placed on the resin column, and chromatographed with 0.4 *M* ammonium acetate-acetic acid, pH 5.0.

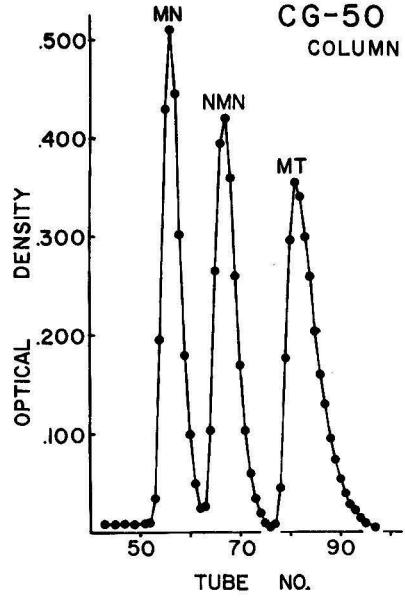


FIG. 2. Chromatographic fractionation of alumina effluent containing 200 μg each of metanephrine, normetanephrine, and 3-methoxytyramine. Optical density at 279 mμ.

Fractions of 1.3 ml were collected on an automatic fraction collector at the rate of 3.5–4 ml/hr by adjusting the level of the buffer reservoir. Optical density of the fractions was measured in a Beckman spectrophotometer at 279 mμ. Figure 2 shows the separation of 200 μg each of metanephrine, normetanephrine, and 3-methoxytyramine. Together with adrenaline, noradrenaline, and dopamine, these compounds were added to 70 mg of brain tissue, extracted as described above, and passed through an alumina column before placing on the CG-50 resin column.

Alumina Eluate 1, containing adrenaline, noradrenaline, and dopamine, was fractionated on the Amberlite CG-50 resin column buffered with 0.2 *M* ammonium acetate-acetic acid, as described above, which essentially is similar to the method of Kirshner and Goodall (1). The eluting buffer was 0.4 *M* ammonium acetate-acetic acid, pH 5.0; fractions of 1.3 ml were collected on an automatic fraction collector at a rate of 3.5–4 ml/hr. The optical density of the fractions were read as above at 279 mμ. Figure 3 illustrates the chromatographic separation on a CG-50 resin column similar to that of Kirshner and Goodall (1) of adrenaline, noradrenaline, and dopamine after preliminary separation

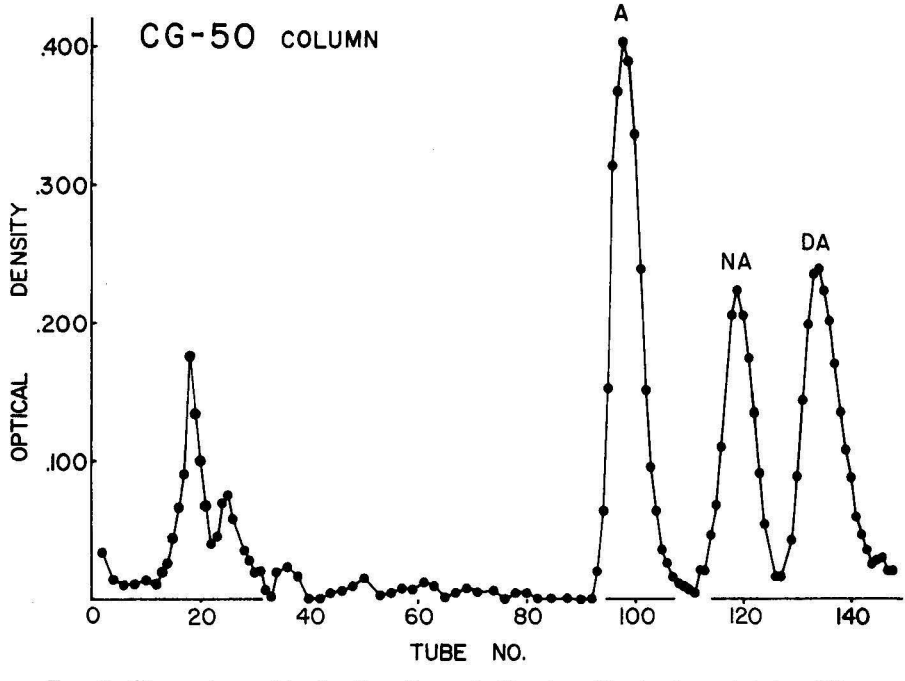


FIG. 3. Chromatographic fractionation of Alumina Eluate 1 containing 200 μg each of adrenaline, noradrenaline, and dopamine.

by alumina. Alumina Eluate 2 containing the catechol acids was evaporated to dryness *in vacuo* as described above. The residue, dissolved in 0.5 *N* HCl, was placed on the Dowex 50 × 8 resin column, previously prepared as described above, and eluted with 0.5 *N* HCl. Fractions of 1.3 ml were collected on an automatic fraction collector at a rate of 3.5–4 ml/hr.

Figure 4 shows the separation on Dowex 50 of an extract of bovine adrenal medulla to which dopa and *t*-3,4-dihydroxyphenylserine were

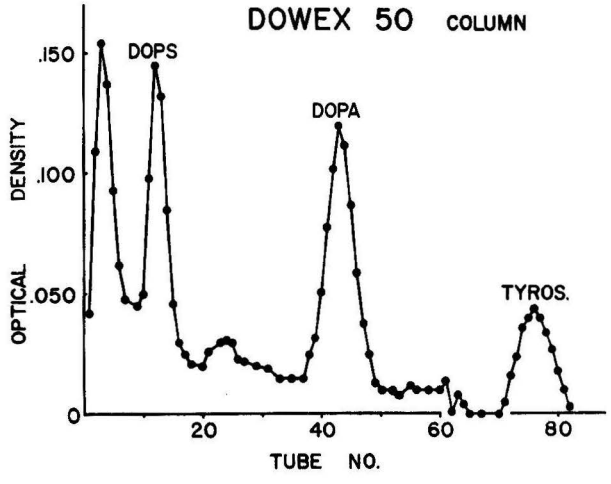


FIG. 4. Chromatographic fractionation of an extract of bovine adrenal medulla to which 200 μg each of dopa and *t*-3,4-dihydroxyphenylserine were added.

added. The endogenous catecholamines were removed by the alumina column and carrier tyrosine was added before the Dowex 50 resin column. Table 1 shows the recovery of pure standards with and without tissue after the various steps of the procedure.

In addition to the amines, the metabolites, 3,4-dihydroxymandelic acid (DOMA) and 3-methoxy-4-hydroxymandelic acid (MOMA), may be separated. After eluting the alumina column with 2 *N* acetic acid, 20 ml of 2 *N* HCl is passed through. Essentially all of the DOMA appears in the HCl eluate and the MOMA is found about equally distributed in the acetic acid and HCl fractions. The method which is described has been used, in part or in total, in this laboratory for a variety of studies involving mammalian brain tissue, thoracic sympathetic nerves, adrenal glands, and liver as well as octopus salivary glands. As shown in the table, recovery of added compounds was quite good when carried through the various steps of this procedure. In the use of this method for indi-

TABLE 1

PER CENT RECOVERY OF ADRENALINE (A), NORADRENALINE (NA), DOPAMINE (DA), METANEPHRINE (MN), NORMETANEPHRINE (NMN), 3-METHOXYTYRAMINE (MT), AND 3,4-DIHYDROXYPHENYLALANINE (DOPA) WITH AND WITHOUT THE PRESENCE OF BRAIN TISSUE

	Tissue extract on alumina			Ef. from alumina on CG-50, pH 6.5			El. 1 from alumina on CG-50, pH 6.1			El. 2 from alumina on Dowex 50
Sample used	Ef.	El. 1	El. 2	MN	NMN	MT	A	NA	DA	DOPA
MN, NMN, MT	100.5			102	87	96				
+ A, NA, DA		83								
Brain + MN, NMN, MT	99			98	89	97				
+ A, NA, DA		88								
MN, NMN, MT	97									
+ A, NA, DA		86					94	92	84	
Brain + MN + NMN + MT +	99.5									
A, NA, DA		88					70	81	82	
Brain + MN, NMN, MT +	98			100	89	96				
A, NA, DA		85					98	91	100	
DOPA			92							94
Brain + DOPA			100.5							83

vidual radioactive tracer experiments, it is advantageous to determine recovery of products by adding known carriers before extraction of the tissue and measuring their optical density readings after separation. The radioactivity of each fraction is counted separately rather than pooling the OD peaks as a further check on the identity of the material. Correlation of the radioactive peak to OD peak of carrier can be accomplished either by visual inspection or by statistical analysis.

SUMMARY

A method has been described for the separation and determination of 3,4-dihydroxyphenylalanine (dopa), adrenaline, noradrenaline, dopamine, metanephrine, normetanephrine, and 3-methoxytyramine in tissue by the use of alumina at pH 6.1, CG-50 and Dowex 50 × 8 resin columns. The greatest usefulness of this method is in radioactive tracer studies in which nonlabeled carriers are added before extraction and fractionation.

REFERENCES

1. KIRSHNER, N., AND GOODALL, M., *J. Biol. Chem.* **226**, 207 (1957).
2. DRELL, W., *Federation Proc.* **16**, 174 (1957).
3. HIRS, C. H. W., MOORE, S., AND STEIN, W. H., *J. Biol. Chem.* **200**, 493 (1953).

ANALYTICAL BIOCHEMISTRY 5, 433–442 (1963)

Anthrone as a Reagent for the Determination of *S*-Carboxymethylcysteine in Proteins

M. A. JERMYN

From the Division of Protein Chemistry, Wool Research Laboratories, C. S. I. R. O., Parkville (Melbourne), Victoria, Australia

Received September 14, 1962

Although protein is commonly stated to interfere in the determination of carbohydrate using sulfuric acid solutions of anthrone, such tryptophan-free proteins as insulin, and mixtures of amino acids containing no tryptophan, give little color during short heating periods with the reagent. The small amounts of nonspecific color can be still further reduced by the addition of phosphate ions to repress the catalytic effects of iron in the system. Tryptophan and tryptophan-containing proteins give a characteristic purple color ($\lambda_{max} = 548$ mμ) with anthrone in sulfuric acid. When the anthrone reagent was used in an attempt to detect possible carbohydrate in a high-sulfur protein fraction which contains very little tryptophan, from reduced and carboxymethylated wool (1), a bright orange-red color ($\lambda_{max} = 443$ and 542 mμ) was produced. This color was found to be specific to the *S*-carboxymethylcysteine present in the protein, and an identical color was given by the free amino acid. Even such closely related amino acids as lanthionine and *S*-1- and *S*-2-carboxethylcysteines [$-CH_2CH(NH_2)COOH$, $-CH(CH_3)COOH$, and $-CH_2CH_2COOH$, respectively, replacing $-CH_2COOH$] were found to produce no increase in optical density under the conditions of the test.

METHODS

All optical densities were measured using the Shimadzu QR-50 spectrophotometer.

Anthrone solutions were prepared by dissolving 20 mg in 100 ml of sulfuric acid of the required dilution at room temperature. They began to discolor noticeably after a few hours, and were used on the day of preparation. Solutions in H_2SO_4 concentrations between 70% (v/v) and 55% (v/v), which is approximately the lower limit of solubility, were prepared by dissolving the reagent in stronger sulfuric acid, diluting with water, and cooling rapidly. This method always leads to solutions of slightly higher optical density in the range 400–600 mμ than those

prepared directly. The concentration of anthrone used (0.02%, w/v) was that found optimal (2) for carbohydrate determination; the higher concentrations commonly employed for these determinations, e.g., 0.2% (3), lead to no improvement in color yields in either carbohydrate or amino acid determinations and have the disadvantage of a much more highly colored blank.

Sporadic contaminations, mostly due to detergent and dust-borne carbohydrate, are a frequent source of error with the reagent. It was found advantageous to carry out a dummy experiment with all flasks, test tubes, etc. to be used in analytical work, rinse them with distilled water, and subsequently keep them in a dust-free environment.

Reaction mixtures were prepared by mixing 9 ml of reagent solution with 1 ml of test solution. To obtain rapid and uniform mixing the reagent was blown in from a wide-nozzle pipet calibrated to deliver 9 ml on quick blowout.

REACTION WITH *S*-CARBOXYMETHYLCYSTEINE

When 1 ml of an aqueous solution containing 300 μg of *S*-carboxymethylcysteine was heated in a boiling water bath with 9 ml of anthrone solution in 70% (v/v) sulfuric acid, the absorption curves set out in Fig. 1 (A and B) were produced. The nature of the compound responsible for the color is unknown. The specificity of the reaction for *S*-carboxymethylcysteine and the existence of a lag period of approximately 7 min, identical for both free and protein-combined *S*-carboxymethylcysteine, raise the possibility that the initial reaction is some form of cyclization under the influence of hot 63% (v/v) sulfuric acid.

In general a heating time of 60 min was used for protein analyses therafter. Optical densities were measured at the 443 mμ peak since the 542 mμ peak nearly coincides with λ_{max} for tryptophan and the broad 530–580 mμ maximum of the unspecific protein reaction. Figure 2 shows that unspecific color development is in fact nearly zero at 443 mμ for insulin. It is uncertain that this conclusion would apply exactly to other proteins and also possible that the width of the nearby minimum (centered at 407 mμ) in the difference curve might be affected by the nature of the protein. It is therefore desirable to use the protein itself as its own blank. Whenever possible an equivalent amount of noncarboxymethylated protein has been used as the blank against which the color developed from the carboxymethylated protein is determined.

Under the above conditions increase in optical density at 443 mμ is linear with concentration of *S*-carboxymethylcysteine as the free amino acid up to 300 μg (Fig. 3). This increase is also linear with concentration of carboxymethylated protein up to an equivalent depth of color. No

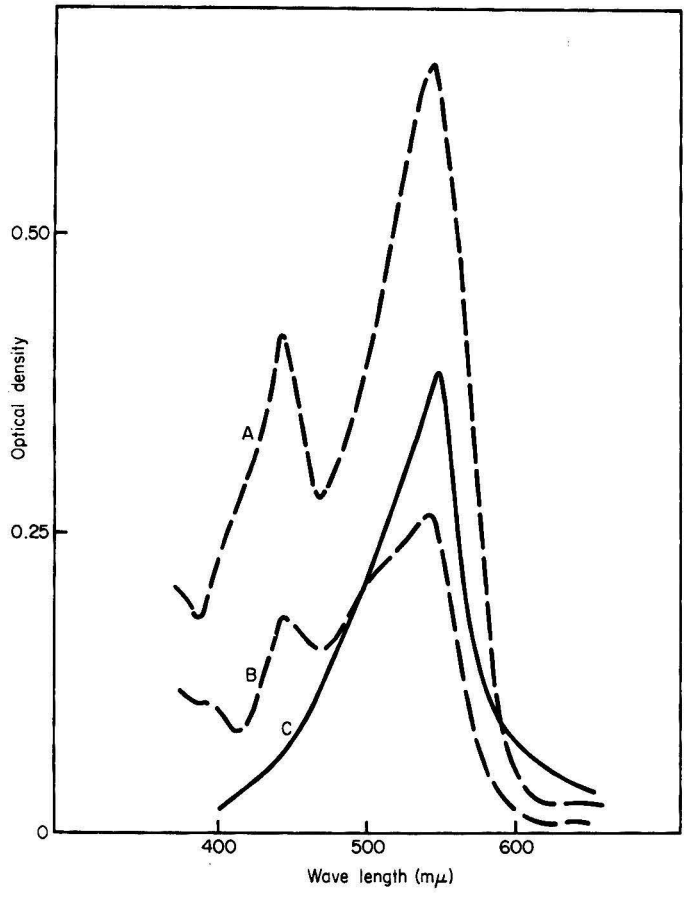


FIG. 1. Absorption spectra of *S*-carboxymethylcysteine and tryptophan treated with anthrone reagent. All curves were determined against a similarly treated blank containing 0.02% anthrone only. (A) 300 μg *S*-carboxymethyl cysteine heated 60 min at 100°C in 10 ml 63% (v/v) H_2SO_4 containing 0.02% anthrone. (B) 300 μg *S*-carboxymethylcysteine heated 30 min at 100°C in 10 ml 63% (v/v) H_2SO_4 containing 0.02% anthrone. (C) 40 μg DL-tryprophan after 30 min at room temperature in 10 ml 90% (v/v) H_2SO_4 containing 0.02% anthrone and 10^{-5} *M* in Fe^{+++}.

amino acid other than tryptophan gave a positive interference; a ten-fold molar excess of cysteine or cystine over *S*-carboxymethylcysteine reduced the optical density to about 70% of the expected figure, but equimolar amounts produced no observable effects.

The method may be illustrated by its application to a specimen of *S*-carboxymethyl serum albumin that had been prepared from Commonwealth Serum Laboratories crystalline bovine serum albumin by E. F.

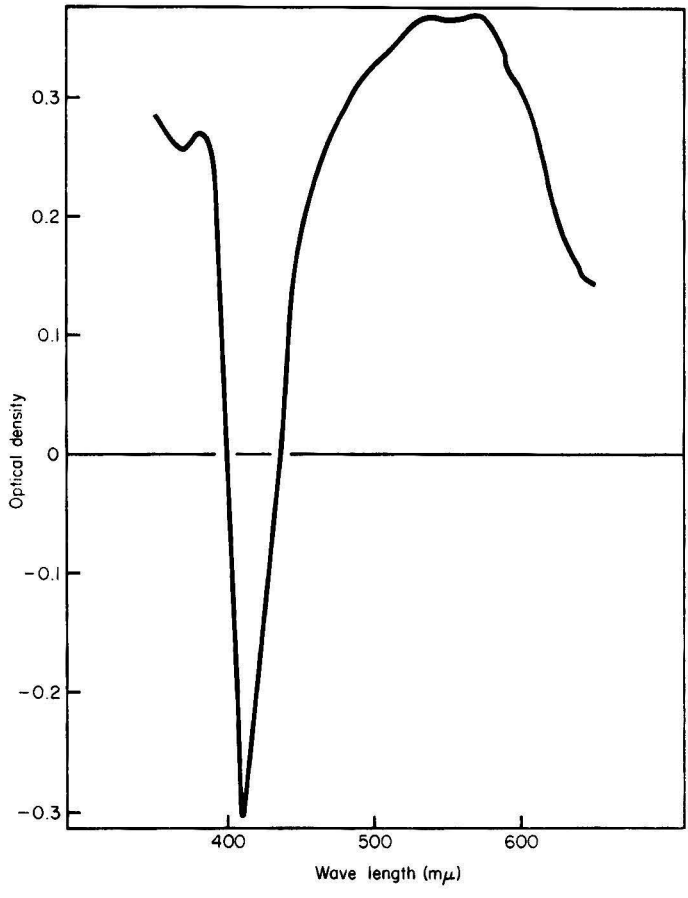


FIG. 2. Difference spectrum between 63% (v/v) H_2SO_4 containing 0.02% anthrone and 10^{-4} *M* in Fe^{+++}, heated 90 min at 100°C, and the same with the addition of 0.2 mg/ml insulin.

Woods of this laboratory by reduction with thioglycolate in 8 *M* urea, reaction with iodoacetate, and dialysis (4). The expected number of *S*-carboxymethylcysteine residues per gm-mole (71,000 gm) of carboxymethylated protein is 35. When a solution containing an amount of free tryptophan equivalent to that present in the serum albumin was used as a blank, the experimentally determined value of this number was 38.0 (mean of 3 determinations). If this blank was replaced by one containing an equivalent amount of noncarboxymethylated protein, the value from 5 successive determinations was 34.1, 34.7, 34.6, 33.6, 34.6 (mean 34.3). This result is of at least the same order of accuracy as that of any

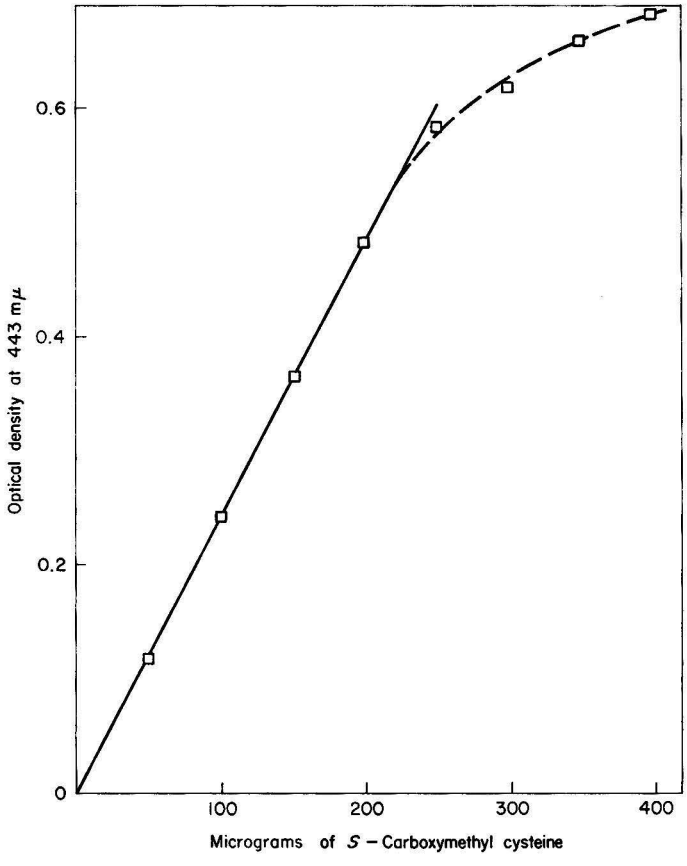


FIG. 3. Increase in optical density at 443 mμ over blank when 9 ml 70% (v/v) H_2SO_4, 0.02% in anthrone, is mixed with 1 ml of test solution and the mixture heated 60 min at 100°C.

other method of direct determination of the total disulfide plus sulfhydryl of serum albumin. Thus Leach (5) found 16.8–17.0 —SS— and 0.7 —SH groups in bovine serum albumin by a polarographic method.

The *S*-carboxymethylcysteine content of carboxymethylated bovine serum albumin may be taken as a known quantity. The amount present in other carboxymethylated proteins is highly uncertain and, as an example of this group, a sample of high-sulfur wool protein that had been analyzed for *S*-carboxymethylcysteine by A. Inglis and J. M. Gillespie of this laboratory using the Spinco automatic amino acid analyzer was taken for colorimetric analysis. Using protein solution (500 μg/ml) as the unknown and a solution of free *S*-carboxymethylcysteine (150 μg/

ml) as the standard, a content of *S*-carboxymethylcysteine in the protein of 1810 μmoles/gm was deduced. Considering that no correction could be made for the definite but uncertain low tryptophan content of the protein (the noncarboxymethylated protein, which would make the ideal blank, cannot be separated from wool), the agreement with the Spinco value (1740 μmoles/gm) was satisfactory. The latter value was highly uncertain since it had been obtained by a long extrapolation to allow for the considerable destruction of the amino acid during hydrolysis of this protein. The colorimetric value may actually be more reliable in such cases.

The reduction plus carboxymethylation step (4) is straightforward and quantitative; an alternative rapid one-step method has been described (6). The degree to which the colorimetric determination of *S*-carboxymethylcysteine can be turned into a simple routine determination of —SS— plus —SH appears therefore to be mainly a question of the degree to which the dialysis step can be reduced to routine. The method is also adaptable to use as a determination of sulfhydryl alone provided that the ratio of sulfhydryl to disulfide is unity or greater. The application to the routine determination of *S*-carboxymethylcysteine in protein fractionation, where relative rather than absolute values are required, is obvious.

REACTION WITH TRYPTOPHAN

The sulfuric acid concentration (63% v/v final) used in the *S*-carboxymethylcysteine determination was chosen as giving the best compromise between rapidity of color development on heating and the unwanted development of unspecific color due to the charring reaction of the protein. Since the principal limitation on the analytical use of the method was the color developed from tryptophan, the reaction with this amino acid was examined under a variety of conditions to see how far color development was reproducible. Unless this can be guaranteed, the use of high-tryptophan proteins as their own blanks could lead to serious errors.

When an attempt was made to develop the characteristic color from the tryptophan of proteins either (*i*) by mixing the protein solution with the anthrone reagent without further heating at high sulfuric acid concentrations or (*ii*) mixing and heating at lower sulfuric acid concentrations, it was found that color development was erratic and slow. One sample of crystalline lysozyme was found, however, that gave immediate color development under condition (*i*). Further investigation showed that this effect could be duplicated for all protein samples by incorporating Fe^{+++} in the reagent; $10^{-4}M$ (63% H_2SO_4) to $10^{-5}M$ (90% H_2SO_4)

appeared to be the effective concentration range. Under the conditions of Fig. 4, myoglobin, β-lactoglobulin, bovine serum albumin, insulin (zero),

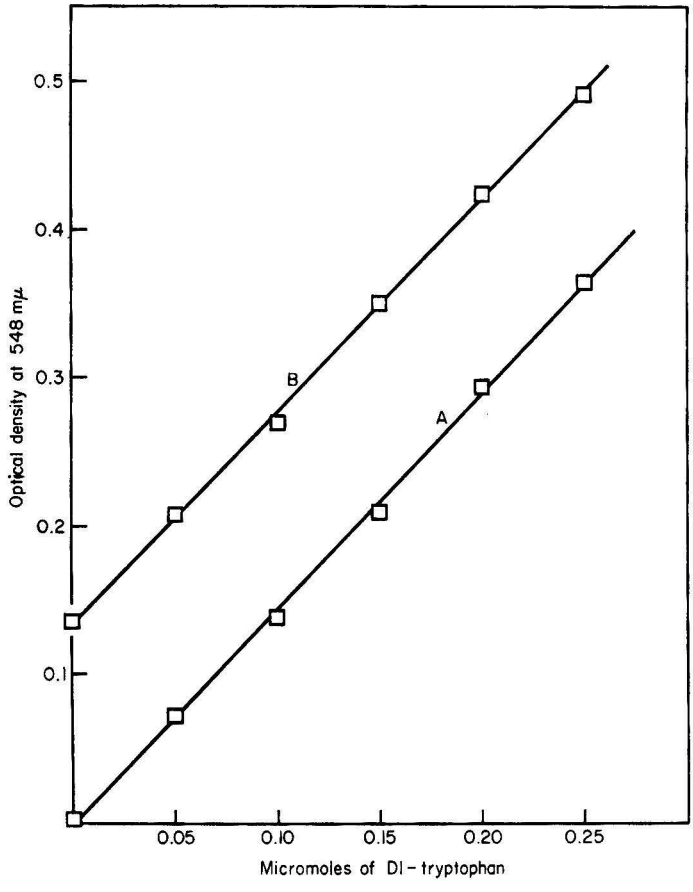


FIG. 4. Increase in optical density at 548 mμ over blank when 9 ml H_2SO_4, 0.02% in anthrone, is mixed with 1 ml test solution, 10^{-4} *M* in Fe^{+++}, and the reaction mixture allowed to stand 30 min at room temperature. Series A contains nothing in the test solution but indicated quantities of DL-tryptophan. Series B contains in addition in each tube 15 μmoles of an equimolar mixture of 20 other amino acids.

chymotrypsinogen, and bovine γ-globulin gave correct values for their already known tryptophan content against a DL-tryptophan standard. Ovalbumin and rennin gave high values and lysozyme and bovine fibrinogen gave low ones. Ribonuclease, using four separate samples of crystalline enzyme, gave a high unspecific color development under all conditions tried, although it contains no tryptophan.

The effect of Fe^{+++} could be reversed by 0.1 *M* H_3PO_4 in the reagent. There was now no immediate color development under condition (*i*) at any sulfuric acid concentration; in 90% (v/v) H_2SO_4, color developed slowly and could be read satisfactorily after 60–90 min. In 63% (v/v) H_2SO_4, the color was only developed to the same level after 30 min of heating at 100°C.

By using sulfuric acid concentration, the presence or absence of Fe^{+++} or H_3PO_4, and mixing with or without subsequent heating as reaction variables, a variety of conditions could be devised in which the color yield from proteins of known tryptophan content could be compared with that from a tryptophan standard. *Any protein would give a "correct" answer under some conditions; no conditions could be found under which all proteins gave a "correct" answer.* The reaction is therefore useless for tryptophan analysis. Heavy metal effects do not appear to have been generally examined as a possible source of variations in the chromogenic reactions between tryptophan and carbonyl compounds in acid solutions. Such effects, together with the inherently different reactivity of tryptophan in different pure proteins of known amino acid content that has been found here may afford some clue as to why the direct colorimetric determination of tryptophan in proteins has been in general so disappointing (7).

Since Fe^{+++} is a ubiquitous contaminant, its possible erratic presence is an obvious reason why tryptophan color development could vary between test sample and blank in the *S*-carboxymethylcysteine estimation. To deal with this situation, the two possible alternatives are to reinforce the Fe^{+++} effect by adding a standard concentration of iron, or to suppress it by adding H_3PO_4. The first of these alternatives is ruled out by the gross discoloration of protein solutions heated in 63% (v/v) H_2SO_4 in the presence of Fe^{+++}.

TABLE 1

COMPARATIVE COLOR YIELDS FROM *S*-CARBOXYMETHYL-L-CYSTEINE AND DL-TRYPTOPHAN

Heated 60 min at 100°C in 63% (v/v) H_2SO_4, 0.02% (w/v) in anthrone and 0.1 *M* in H_3PO_4.

Amino acid	Quantity (μmole)	Wavelength (mμ)	OD above blank
S-Carboxymethyl-L-cysteine	1.0	443	0.430
		542	0.651
DL-Tryptophan	0.25	548	0.402
L-Tryptophan in myoglobin	0.25	548	0.418
ovalbumin	0.25	548	0.558
chymotrypsinogen	0.25	548	0.204

The color yield from tryptophan alone and from proteins containing tryptophan was found to be reproducible on heating in 63% (v/v) H_2SO_4 containing 0.02% anthrone and 0.1 *M* in H_3PO_4, although only myoglobin, β-lactoglobulin, and bovine γ-globulin gave a "correct" result. Table 1 shows comparative color yields for *S*-carboxymethylcysteine and tryptophan under these conditions.

VALIDATION OF THE BLANK PROCEDURE

Noncarboxymethylated serum albumin is satisfactory as a blank for *S*-carboxymethylcysteine determination in the carboxymethylated protein. However it is low in tryptophan (2 equivalents per 69000 gm) and free of carbohydrate, and gives only a slight unspecific color reaction. Table 2 shows the effect of adding to both test and blank a protein high

TABLE 2

OPTICAL DENSITY DIFFERENCE AT 443 Mμ BETWEEN 1 MG DRY WEIGHT OF CARBOXYMETHYLATED BOVINE SERUM ALBUMIN TOGETHER WITH INDICATED ADDITIVE AND EQUIVALENT QUANTITY OF ORIGINAL SERUM ALBUMIN PLUS SAME QUANTITY OF ADDITIVE

Heated 60 min at 100°C in 63% (v/v) H_2SO_4, 0.02% (w/v) in anthrone and 0.1 *M* in H_3PO_4.

Additive	Amount (μg)	Difference in OD
None		0.211, 0.214
Crystalline		
lysozyme	1000	0.209, 0.217
ovalbumin	1000	0.213, 0.217
ribonuclease	1000	0.207, 0.213
Soluble starch	100	0.215, 0.218

in tryptophan (lysozyme), a protein containing both tryptophan and carbohydrate (ovalbumin), a protein with a high nonspecific color yield (ribonuclease), and a polysaccharide (soluble starch). The results show that the mean value determined for the *S*-carboxymethylcysteine content of serum albumin, is not significantly affected in any case. This result indicates that the procedure using the noncarboxymethylated protein as blank should be generally satisfactory.

SUMMARY

Anthrone reacts with *S*-carboxymethylcysteine in proteins on heating in 63% (v/v) sulfuric acid to give a specific chromogen. This can be made the basis of a method for determining total disulfide + sulfhydryl in proteins. The chromogenic reaction between anthrone and tryptophan

in sulfuric acid solutions appears to be iron catalyzed. If H_3PO_4 is added to the anthrone reagent to control this reaction, the color developed from the tryptophan present in proteins is reproducible under the conditions of *S*-carboxymethylcysteine determination, and the noncarboxymethylated protein can be used as the blank for this determination.

ACKNOWLEDGMENTS

I wish to thank Mr. J. M. Gillespie for a sample of high-sulfur wool protein and Dr. B. S. Harrap for a sample of carboxymethylated serum albumin.

REFERENCES

1. GILLESPIE, J. M., *Australian J. Biol. Sci.* **15**, 262 (1962).
2. JERMYN, M. A., *Nature* **162**, 38 (1956).
3. MILLER, G. L., SLATER, R., BIRZGALIS, R., AND BLUM, R., *Anal. Biochem.* **2**, 521 (1961).
4. HARRAP, B. S., AND WOODS, E. F., *Australian J. Biol. Sci.*, to be published.
5. LEACH, S. J., *Australian J. Chem.* **13**, 520 (1960).
6. THOMPSON, E. O. P., AND O'DONNELL, I. J. *Biochim. et Biophys. Acta* **53**, 447 (1961).
7. GREENSTEIN, J. P., AND WINITZ, M. *in* "Chemistry of the Amino Acids," p. 2322. Wiley, New York, 1961.

ANALYTICAL BIOCHEMISTRY 5, 443–451 (1963)

A Critical Study of Methods for the Determination of Nonprotein Nitrogen

PAMELA M. BELL

From the C. S. I. R. O. Wheat Research Unit, North Ryde, N. S. W., Australia

Received September 17, 1962

INTRODUCTION

The nonprotein nitrogen (NPN) fraction of living tissues has been arbitrarily defined as that group of nitrogen-containing compounds with a molecular weight less than 10,000 (1). Although quantitatively the fraction is usually only small, many of its constituents play an important role in cellular metabolism. The progress of studies on NPN fractions depends largely on the availability of reliable methods for the primary separation of protein from nonprotein nitrogen. Ideally, this requires a procedure which removes protein, and only proteins, according to the definition. Difficulties are encountered immediately this is applied, as even the simplest procedure for removing protein does not achieve separation purely on the basis of molecular size. Sources of error inherent in different methods include the hydrolysis of nitrogen-containing polymers, the adsorption of NPN compounds on to protein, and the anomalous behavior of some compounds with different protein precipitants.

Until now little comparative work on methods for determining NPN has been reported. The results of a comparative survey of some twenty methods on four different materials are given in this paper in an effort to put the choice of methods for preparing NPN on a more rational basis. The results of a study of protein-amino acid binding as a contributory cause to errors in the determination of NPN are also reported.

MATERIALS

Reagents. All reagents and solvents used were of analytical grade.

Serum. Fresh sheep's blood was clotted by mechanical agitation, squeezed through muslin, centrifuged, and freeze-dried to give a product with moisture content no greater than 5%. The frozen powder was stored at −15°C and reconstituted as required by making a 10% w/v solution in distilled water.

Milk. A 10% w/v solution of a commercial dried skim milk powder was made up as required. The same sample was used throughout to avoid variation between batches.

Bran Extract. Bran (50 gm) from a typical Australian wheat was extracted by stirring with 400, 300, and 200 ml of distilled water successively, centrifuging between each extraction. The supernatants were pooled and concentrated on a rotary film evaporator to about 200 ml.

Flour Extract. (*i*) For the comparative survey, 50 gm of a typical baker's flour (total N 2.07%) was extracted three times with 200 ml of distilled water by mixing for 10 min with a Vibromix vibratory mixer (A. G. für Chemie-Apparatebau, Zurich) and centrifuging the mixture. The pooled supernatants were concentrated on a rotary film evaporator to a final volume of about 100 ml and centrifuged again at $35,000 \times g$ for 30 min to give a clear solution. (*ii*) For the work on amino acid binding a similar extract was prepared more quickly by mixing a 1:2 flour water mixture of the same flour directly on a Vibromix for 10 min and then centrifuging at approximately $35,000 \times g$ at 5° for 30 min. This gave a clear, very pale solution with a nitrogen content of just over 1 mg/ml.

Storage of Extracts and Milk and Serum. The flour, bran, and skim milk powder were stored in airtight tins at room temperature, and the freeze-dried serum at —15°C, under which conditions no significant changes in the protein or nonprotein nitrogen were expected over the period of the experiments. The flour and bran extracts and reconstituted serum and milk were stored in a refrigerator at 5°C, fresh solutions being prepared before any detectable deterioration took place.

Isotopically Labeled Amino Acids. The following amino acids were obtained from The Radiochemical Centre, Amersham, Buckinghamshire, England: ^{14}C-DL-tryptophan, ^{14}C-DL-tyrosine, ^{14}C-DL-glutamic acid, ^{14}C-glycine (uniformly labeled), ^{35}S-(DL + meso)cystine, ^{35}S-DL-methionine.

METHODS

All analyses were carried out in duplicate.

Nitrogen

Nitrogen was estimated by a standard micro-Kjeldahl procedure using the digestion method of Bruel *et al.* (2). Ammonia was distilled into 2% boric acid and titrated with *N*/100 sulfuric acid. Slusanschi *et al.* (3) found that the presence of trichloroacetic acid (TCA), phosphotungstic acid, or metaphosphoric acid in deproteinized solutions had no measurable effect on the accuracy of the Kjeldahl method.

Methods for Obtaining NPN

In general the procedure shown for each method was that most used by other workers. Details are given below:

Dialysis. Visking 18/32 casing under standardized conditions was used. Five milliliters of sample was dialyzed against 45 ml of distilled water for 24 hr in stoppered cellulose nitrate 50-ml centrifuge tubes on a rocking dialyzer actuated by a synchronous motor in a cold room at 5°. This standardized procedure was used as a reference method throughout, it being assumed that dialysis equilibrium had been reached after 24 hr. In the light of work by Craig and others on the numerous factors affecting dialysis (4–6) this is clearly rather an arbitrary standard, but under the conditions specified it was felt that there were fewer theoretical objections to this method than to any other, such as precipitation by chemical or physical agents.

Heat. Samples were heated for 10 min in a boiling water bath and filtered.

Tungstic Acid. Van Slyke and Hawkin's modification (7) of the Folin-Wu method was used: to 1 part of sample (1:1 dilution in the case of serum) a freshly made mixture of 8 parts of $N/12$ sulfuric acid and 1 part of 10% sodium tungstate (w/v) was added, and the mixture shaken and filtered.

Copper Hydroxide. The method of Thomas (8) was used: to 5 parts of sample (1:1 dilution for serum), 1 part of 0.2 N sodium hydroxide and 1 of 0.2 N copper sulfate were added, followed by dropwise addition of 0.7 N copper sulfate with shaking until there was no further precipitate.

Ferric Oxide Hydrasol. The reagent was prepared by adding 5 ml of 5% (w/v) ferric chloride to 100 ml of boiling water. Precipitation of protein was carried out by dropwise addition of the ferric oxide hydrasol to the sample in a boiling water bath until there was no further precipitation (8, 9).

Lead Acetate. A 20% solution (w/v) of lead acetate [$Pb(C_2H_3O_2)_2 \cdot 3H_2O$] was added until there was no further precipitate (10).

Trichloroacetic Acid. Three concentrations (2.5, 5.0, and 10.0%) were tested by addition of equal volumes of cold 5, 10, or 20% TCA (w/v) to the sample. Precipitates were filtered or, in the case of flour extract, centrifuged and then filtered.

Phosphotungstic Acid. As for TCA.

Metaphosphoric Acid. Three parts of a 25% solution of metaphosphoric acid (w/v) was added to 25 parts of sample (1:5 dilution of serum, 1:2.5 of milk) (9).

Tannic Acid. A weight of tannic acid equal to the weight of protein in solution plus 0.4 mg/ml of sample was added as solid to the sample with vigorous stirring, the precipitate centrifuged off, and the supernatant filtered (11).

Sulfosalicylic Acid. Five per cent (w/v) sulfosalicylic acid solution was added to 4 ml of sample (1:1 dilution for serum) until there was no further precipitate (12).

Ethanol. Absolute alcohol was added to the sample to a final concentration of 50 or 70% (v/v). Serum was diluted 1:1.

Mercuric Chloride. The sample (1:1 dilution of serum) was mixed with equal parts of 5% hydrochloric acid and 5% mercuric chloride. Excess mercuric chloride was not removed with hydrogen sulfide; otherwise the method was that used by Hiller and Van Slyke (9).

Sevag Method. One volume of sample (1:1 dilution for serum) at pH 4.5 was shaken for 1 min with 0.25 vol of an 8:1 (v/v) mixture of chloroform and octyl alcohol and centrifuged, and the procedure repeated 6 times (13).

Phenol:Acetic Acid:Water. One gram of bran or flour, dry serum or milk was extracted 3 times with 10 ml of a 1:1:1 mixture of phenol: acetic acid:water by stirring and centrifuging. The supernatants were pooled (14).

General Procedure for Comparison of Methods

After precipitation by the method in question, the total volume of the supernatant or filtrate was measured, nitrogen estimations were carried out on duplicate aliquots, and 5 ml of the "soluble fraction" was dialyzed under the standard conditions. "NPN" or the total soluble nitrogen, was expressed as a percentage of the total nitrogen in the solution or extract. The "dialyzable NPN" of each fraction was expressed in the same way.

Experiments with Isotopically Labeled Amino Acids

Experimental Design. Stock solutions (0.5% w/v) of each amino acid were made up with the required specific activities by mixing labeled and unlabeled acid. Four milliliters of flour extract containing approximately 4 mg of nitrogen was mixed with 1 ml of the stock amino acid solution and protein removed by heat, TCA precipitation (final concentration 5%), or dialysis at pH 5, 7, or 9 using the techniques already described. Precipitates were centrifuged or filtered off and samples of each "protein" and "nonprotein" fraction plated in 1% (w/v) gelatin in 0.1 *M* sodium hydroxide. Samples were counted in a Philips end window counter (preset count unit type PW4052 coupled with a universal scaler type PW4032). Counts were corrected for background, and the amino acid nitrogen bound (from the difference "protein" minus "nonprotein" counts per minute and the known specific activities) was expressed as a percentage of the total amino acid plus protein nitrogen in the solution.

Further experiments with cystine binding were carried out by mixing the protein (1 ml of 0.1% solution) and amino acid (0.1 ml containing 3.6 μg cystine) in 0.1 *M* citrate-phosphate buffer at pH 5.0. For the experiments on the effect of pH, 0.1 *M* citrate-phosphate buffer over the range 4.5–7.5 by 0.5 of a unit steps was used. After incubation of the mixtures at 30° for 24 hr, 0.1-ml samples were pipetted directly on to filter paper disks and plunged into cold 10% TCA, then washed and dried as described by Mans and Novelli (15). Disks were counted in 5 ml of 0.4% PPO–0.01% POPOP toluene scintillation mixture in an Ecko liquid scintillation counter. The effect of pH, heating (10 min over 90°C), exhaustive dialysis, and sodium disulfite treatment (0.2 ml 10% $NaHSO_3$ for 10 min) on the TCA-precipitable ^{35}S-cystine under these conditions was studied. Background was determined on a filter paper disk carried through the washing procedure with the samples.

RESULTS

The results of the comparative study of NPN methods on flour, bran, serum, and milk are given in Table 1. The results obtained by dialysis on each fresh batch of the solutions and extracts are underlined for ease of comparison with the results following which were obtained by other methods on the same batch. Comparison of the diffusible nitrogen content of each NPN fraction with the NPN figure obtained by dialysis showed that relatively few fractions contained more diffusible nitrogen than the standard (Table 1, footnote *a*). These fractions were mainly those prepared by acid precipitation (e.g., TCA, and tungstic, sulfosalicylic, and metaphosphoric acids), suggesting that the increase in diffusible nitrogen results from hydrolysis of larger nitrogen-containing molecules. Apart from the other trends indicated in Table 1 (footnotes *b*, *c*, *d*), no consistent differences or similarities were observed relating method and results on one or all of the solutions.

Investigations of amino acid-protein binding using isotopically labeled amino acids gave the results shown in Table 2. It is clear that some binding does occur and, although quantitatively it appears small when expressed as a percentage of the total nitrogen, when expressed as a percentage of the amino acid nitrogen added it represents amounts varying from 2 to 18% of the "NPN fraction." Only slight binding appears to occur in the presence of TCA, but greater binding is observed during the precipitation of proteins by heat. The binding which occurs in dialysis experiments appears to be pH dependent. Further studies on the binding of cystine showed that the maximum binding observed, using the TCA precipitation technique, represented about 3 μmoles cystine bound per 100 gm flour. The bound cystine could be almost completely removed by

TABLE 1

TOTAL NITROGEN AND TOTAL DIFFUSIBLE NITROGEN OF NPN FRACTIONS OBTAINED BY A RANGE OF METHODS FOR SKIM MILK, SHEEP SERUM, WATER EXTRACT OF BRAN, AND WATER EXTRACT OF FLOUR

Method	Bran		Flour		Serum		Milk	
	NPN	NPN diffusible	NPN	NPN diffusible	NPN	NPN diffusible	NPN	NPN diffusible
Dialysis	9.4	9.4	1.4	1.4	6.0	6.0	4.6	4.6
Tungstic acid	8.6	8.6	5.0	3.2[a]	2.3	2.3	5.5	5.5[a]
Copper hydroxide	7.1	7.1	1.3[d]	0.8	4.9	1.5	6.3	3.0
Ferric oxide hydrasol	11.1	9.2	8.0	1.6[a]	3.4	1.6	9.2	4.4
Lead acetate	9.3	7.5	7.5	1.3	11.8[c]	0.5	2.4	1.7
Dialysis	9.9	9.9	1.8	1.8	6.3	6.3	4.7	4.7
5% TCA	13.6	10.9[a]	9.1	2.2[a]	2.5	2.4	7.1	4.6
10% TCA	12.2	10.7[a]	7.5	1.5	2.8	2.7	6.6	4.6
20% TCA	13.5	10.2[a]	9.5	2.3[a]	2.3	2.2	4.9	3.5
5% PTA	7.8	6.8	8.7	1.8	2.1	1.9	9.0	4.3
10% PTA	6.0	5.4	10.3	1.9[a]	2.9	1.1	4.0	3.6
20% PTA	5.4	3.6	9.2	1.1	2.3	1.4	3.8	2.4
Heat[b]	14.6	7.7	5.9	1.2	8.8	1.2	5.2	2.7
Dialysis	10.1	10.1	1.3	1.3	6.5	6.5	4.0	4.0
Metaphosphoric acid[b]	12.0	10.1	6.1	1.2	19.3	2.8	10.3	4.9[a]
Tannic acid	8.0	6.7	3.8	0.6	4.9	2.5	5.2	4.6[a]
5% sulfosalicylic acid	10.9	9.1	5.8	1.7[a]	2.4	2.4	8.6	4.3[a]
50% ethanol[b]	10.9	7.1	2.5	0.4	10.1	10.0[a]	5.8	1.4
70% ethanol	5.8	5.8	3.0	0.1	2.8	1.9	3.8	2.4
5% mercuric chloride	15.4	10.1	7.0	1.5	2.4	2.1	6.2	3.5
Sevag (chloroform and octyl alc.)	8.6	3.2	4.2	2.1	49.9	1.6	4.7	1.1
Extn. of dry material with 5% TCA	11.3	8.3	5.7	3.2	0.2	0.2	0.9	0.5
Phenol:acetic acid: water	9.6	2.5	4.9	0.4	5.4	0.2	6.7	3.0

NPN obtained by dialysis was used as reference standard. NPN values are given as percentages of N of extracts of bran or flour, or of total N of serum or milk, found in filtrates or dialyzates. NPN diffusible values were calculated from determinations of the dialyzable N of filtrates.

[a] Fractions which contain more diffusible NPN than the standard.

[b] Methods giving higher NPN figures than dialysis for all four solutions.

[c] The only serum NPN figure higher than the standard in addition to those mentioned in footnote *b*.

[d] The only flour NPN figure lower than the standard.

TABLE 2
BINDING OF PROTEIN AND ISOTOPICALLY LABELED AMINO ACIDS

Amino acid	Amino acid nitrogen bound				
	5% TCA (final concn.)	Heat	Dialysis at pH 5	Dialysis at pH 7	Dialysis at pH 9
Tryptophan	0.4, 0.4	3.0, —	4.2	0.0	1.0
Tyrosine	0.2, 0.3	0.6, 0.6	0.0	0.0	1.1
Glutamic acid	0.6, 0.5	2.1, 1.4	1.2	0.0	0.0
Glycine	0.4, 0.4	2.2, 1.9	0.5	0.0	1.3
Lysine	1.0, 0.7	1.5, 1.5	1.1	0.2	0.9
Methionine	0.3, 0.2	1.4, 1.1	0.0	0.2	0.3

Four milliliters of flour extract containing approximately 1 mg N/ml were mixed with 1 ml 0.5% (w/v) amino acid solution and subjected to TCA precipitation, heat coagulation, or dialysis.

Amino acid nitrogen bound in the "protein" fraction is expressed as percentage of total amino acid plus flour extract nitrogen.

treatment with sodium bisulfite and could be reduced by about 50% by boiling and by about 75% by exhaustive dialysis. Dialyzed flour extract bound less than the whole solution, and binding increased with pH to about 12 μmoles cystine per 100 gm of flour over the range pH 4.5 to 7.5.

DISCUSSION

The results of the comparison of methods for determining NPN (Table 1) demonstrate convincingly the dependence of NPN values on the method by which they are determined, and the lack of any significant relation between the results obtained by any one method on a range of materials, or by a range of methods on one material. The low results obtained with nearly all methods for serum confirm the efficacy of the methods commonly used in "deproteinizing" blood for clinical analysis. The high results on flour may reflect a lower content of diffusible nitrogen (by comparison with the other materials), but also the presence of proteins with exceptional solubility properties, such as a nondiffusible but TCA-soluble and nonheat-coagulable polysaccharide-protein complex which has been demonstrated in water extracts of flour (16). It can be seen that none of the methods studied completely avoid the main sources of error in determining NPN: hydrolysis of protein, nucleic acid, or other nitrogen-containing polymers; adsorption of nonprotein on to protein; or, for comparative purposes, the anomalous behavior of some proteins with different precipitants. Dialysis, or gel filtration, probably achieves separations most closely related to molecular size only, but even these techniques are subject to errors which have already been

mentioned. It has been emphasized that a method which proves satisfactory for one group or type of solution may give quite misleading results for another, for example, the use of ethanol with seed proteins. A general recommendation which can be made as a result of this work is the importance of realizing that such variations do occur, and that "NPN" obtained by one method must never be regarded as completely representing all that nonprotein nitrogen potentially present.

Rather, NPN must be defined by the method used for its preparation until more satisfactory methods are found for interpreting the theoretical definition in practical terms.

The study of amino acid-protein binding as a source of error in determining NPN showed that such reactions may affect the results in some cases. The type of reaction involved appears to vary, and insufficient work has been done to speculate on the nature of the binding observed, except in the case of cystine. Binding of cystine appears to involve a sulfhydryl exchange-type reaction, about half of which occurs on the diffusible portion of the flour extract. This agrees with the work of Stewart and Mauritzen (17) on the incorporation of ^{35}S-labeled cystine into flour-water doughs, but the degree of binding can hardly be regarded as significant in affecting the determination of NPN. The use of labeled amino acids in studying protein-nonprotein interactions should prove of value in further studies on the nature of such reactions. The contribution of any of the binding reactions demonstrated to anomalies in the determination of NPN would be insignificant when it is considered that the concentration of any single amino acid in a biological solution would be much less than that in the artificial systems studied. The possible composite effect of many such reactions with different amino acids may be significant, but cannot be estimated on the basis of the present work.

SUMMARY

Some twenty methods for the removal of protein from biological materials were used to prepare the nonprotein nitrogen fraction from skim milk, serum, a water extract of flour, and a water extract of bran. The results obtained by different methods on one solution or the same methods on different solutions were not entirely concordant and showed that NPN fractions obtained in these ways cannot validly be compared. Dialysis or related techniques appeared to achieve separations most closely related to NPN as theoretically defined. The binding of amino acids to protein was demonstrated using isotopically labeled amino acids in flour-protein solutions, but it was concluded that reactions of this type would not significantly affect the determination of NPN.

REFERENCES

1. SYNGE, R. L. M., *in* "Moderne Methoden der Pflanzenanalyse" (K. Paech and M. V. Tracey, eds.), Vol. IV, p. 1. Springer, Berlin, 1955.
2. BRUEL, D., HOLFER, H., LINDERSTRØM-LANG, K., AND ROZITS, K., *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **25**, 289 (1946).
3. SLUSANSCHI, H., SUTEANU, M., AND LOZINSCHI, A., *Chem. Abstr.* **54**, 22800d (1960).
4. CRAIG, L. C., AND KING, T. P., *J. Am. Chem. Soc.* **77**, 6620 (1955).
5. CRAIG, L. C., AND KING, T. P., *J. Am. Chem. Soc.* **78**, 4171 (1956).
6. CRAIG, L. C., KING, T. P., AND STRACHER, A., *J. Am. Chem. Soc.* **79**, 3729 (1957).
7. VAN SLYKE, D. D., AND HAWKINS, J. A., *J. Biol. Chem.* **79**, 739 (1928).
8. THOMAS, W., *Plant Physiol.* **2**, 55 (1927).
9. HILLER, A., AND VAN SLYKE, D. D., *J. Biol. Chem.* **53**, 253 (1922).
10. NEUBERGER, A., AND SANGER, F., *Biochem. J.* **36**, 662 (1942).
11. VOGELI, VON H., *Landwirtsch. Monatsh.* **1**, 71 (1956).
12. ASTRUP, T., AND OHLENSCHLAGER, V., *Biochem. J.* **42**, 211 (1948).
13. MARKHAM, R., *in* "Moderne Methoden der Pflanzenanalyse" (K. Paech and M. V. Tracey, eds.), Vol. IV, p. 250. Springer, Berlin, 1955.
14. SYNGE, R. L. M., AND YOUNGSON, M A., *Biochem. J.* **78**, 31P (1961).
15. MANS, R. J., AND NOVELLI, G. D., *Arch. Biochem. Biophys.* **94**, 48 (1961).
16. BELL, P. M., *J. Sci. Food Agric.* in press.
17. STEWART, P. R., AND MAURITZEN, L. M., *Nature* **197**, 48 (1963).

ANALYTICAL BIOCHEMISTRY 5, 452–457 (1963)

Increase in Sodium Contamination from Glass As a Function of Time after Cleaning[1]

FRANCES B. NESBETT AND ADELBERT AMES, III[2]

From the Department of Biological Chemistry, Harvard Medical School, and the Neurosurgical Service, Massachusetts General Hospital, Boston, Massachusetts

Received September 17, 1962

INTRODUCTION

Contamination has become an increasingly important problem as investigators have sought to perform accurate electrolyte analyses on smaller and smaller samples of biological material. [See Thiers (1) for an excellent review of this subject.] Glass presents a major hazard in this regard. In many instances it may be replaced by Vycor or plastic. But in the case of certain volumetric ware this may not be possible, and the investigator may be forced to use special cleaning and leaching techniques, with appropriate monitoring to make certain that the offending contaminants are indeed eliminated. Considerable information is available concerning the behavior of glass when exposed to various leaching solutions (e.g., 2, 3). However the authors are not aware of published information on the changes that take place in glass during dry storage after cleaning.

The data to be reported were obtained in a study ancillary to an investigation in which Na analyses were made on solutions containing as little as 5 μmoles/liter. In the course of these analyses it became apparent that Na contamination was occurring and that the pipets were the major source. The pipets were not at first suspected, as previous studies had shown them to be contamination-free when tested immediately following the rather extensive cleaning procedure used. The data presented below show that, although the pipets were indeed contamination-free immediately after cleaning, they became the source of increasingly serious contamination during subsequent storage in the dry state, in spite of being protected from air-borne contaminants.

[1] This investigation was supported by research grants (MH-K3-3769 and M-6011) from the National Institute of Mental Health, United States Public Health Service.

[2] Rockwell Levy Associate Biochemist in Neurosurgery at Massachusetts General Hospital.

METHODS

Two types of pipets were studied, both of which had been in routine use in the laboratory for several years. The "soft glass" type was represented by 0.5-ml Ostwald-Folin pipets, A. H. T. Co. specification, obtained through Arthur H. Thomas Co., Philadelphia, Pa. The "hard glass" type was represented by 1.0-ml volumetric pipets, Kimax brand, manufactured by Kimble Glass Co. from their borosilicate glass KG-33.

All pipets were subjected to a routine laboratory cleaning involving an overnight soak in hot detergent, a prolonged tap water rinse, a short soak in 2 *M* nitric acid, and a final rinse in distilled water, followed by several hours drying in a specially constructed tower heated by a 100-watt bulb at the bottom. Immediately before use in these tests, the pipets were given an additional cleaning which involved soaking for at least 30 min in a 2 *M* nitric acid bath followed by extensive rinsing with deionized water[3] and drying with acetone. Preliminary tests showed that air drying and acetone drying were equivalent in terms of the subsequent behavior of the pipets, the latter being adopted because of its speed. It was found necessary to treat the Fisher Certified Reagent Grade acetone, used for the drying, by a preliminary passage through a polyethylene column filled with Amberlite IR-120 cation-exchange resin in order to remove Na found to be present in the stock reagent at a concentration of about 2 μM. The Na in the untreated acetone was sufficient to cause appreciable contamination of the pipets dried with it.

The clean, dry pipets were carefully wrapped at both ends with two layers of Whatman No. 40 filter paper to prevent air-borne contamination and then stored at 24°, 102°, or —26°C for various times before testing. The pipets stored at 102° or at —26° were allowed to come to room temperature before testing. The pipets were macroscopically dry but were not stored over a desiccant.

The pipets were tested by measuring the amount of Na they contributed to a 3.6 m*M* solution of KCl or to deionized water when either of these fluids was drawn into the pipet, up to the mark, and held there for 1 min at room temperature. The testing fluid was delivered directly from the pipet into the flame photometer and compared to the same fluid delivered by a plastic pipet. The characteristics of the flame photometer have been described (4). In the present studies, Na was determined by the direct rather than by the internal standard method, as

[3] The deionized water used throughout this study was prepared by passing laboratory distilled water through a plastic cartridge containing a mixed-bed ion-exchange resin (Deminac brand, manufactured by Crystal Research Laboratories, Inc., Hartford, Conn.) directly into a plastic container.

it was undesirable to include Li in the solution used to test the pipets. With the standards and the instrument setting employed, the lowest detectable level of Na was approximately 0.1 μM or 2 parts per billion. Readings on Na at a concentration of 2.87 μM were reproducible to $\pm 2\%$ standard deviation.

RESULTS

The results of the studies in which the pipets were eluted with 3.6 mM KCl are shown in Fig. 1. When tested immediately after cleaning, neither

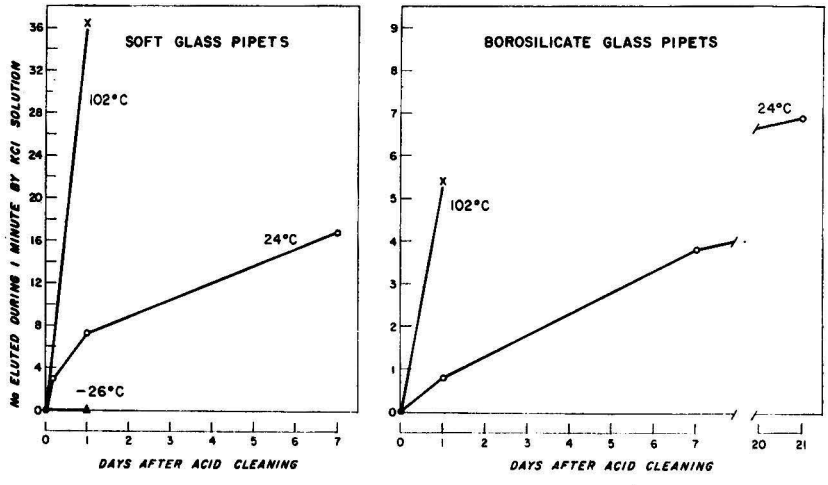


FIG. 1. Elution of Na (in μmoles/liter) from soft glass or borosilicate glass pipets when exposed to a 3.6 mM solution of KCl held in the pipets for 1 min at 24°C. Pipets had been cleaned previously by soaking in 2 M HNO_3 and then stored, dry, for varying times and at 3 different temperatures, as indicated. Each point represents 4 determinations.

type of pipet contributed a detectable amount of Na to the eluting fluid (i.e., Na contamination was less than 0.1 μmole/liter). However, after they had been stored, dry, at room temperature for only 4 hr, the soft glass pipets contributed 3 μmoles of Na per liter of fluid; and more and more Na become extractable, though at a decreasing rate, as the time of storage was extended. Tests on the borosilicate glass pipets resulted in a similar curve but at levels of about one-fifth of those observed with the soft glass.

The change taking place in the pipets during storage was very dependent on temperature. After storage for 1 day at 102°C, they contributed about 6 times as much Na to the eluting fluid as they did after

storage for the same interval at room temperature. When they had been stored for 1 day at —26°C, no measurable Na could be eluted, even from the soft glass pipets.

TABLE 1

NA ELUTED BY H_2O COMPARED WITH NA ELUTED BY KCL SOLUTION

Pipets were stored dry at 102°C for 24 hr following acid cleaning. Eluting solution was held in pipet for 1 min before testing.

Type of pipet	Sodium eluted, μmole/liter	
	By deionized H_2O	By 3.6 m*M* KCl soln.
Borosilicate glass, 1ml	1.9	6.7
	1.8	6.2
	1.8	5.2
	2.6	3.7
	—	—
	Av. 2.1	Av. 5.4
Soft glass, 0.5 ml	28	41
	29	35
	27	36
	—	33
	Av. 28	—
		Av. 36

The data presented in Table 1 provide an indication of the amount of variation between individual pipets when they were treated similarly. These data also show the relative effectiveness of deionized water compared with 3.6 m*M* KCl in eluting the Na. In the case of the borosilicate glass pipets, deionized water removed only about 40% as much Na as did the 3.6 m*M* KCl solution. In the case of the soft glass pipets, the water removed about 80% as much Na as the KCl solution. When the pipets were tested with the KCl solution immediately after being initially tested with water, an additional amount of Na was eluted, giving a total about equal to that recovered on an initial 1-min elution with the KCl solution. When pipets were subjected to successive elutions with KCl solution, the Na eluted fell off rapidly, so that after two such elutions the flame photometer reading was equal to that obtained when the test solution was delivered with a plastic pipet.

No systematic study was performed to determine whether the Na eluted was an original constituent of the glass or whether it had been adsorbed, sometime prior to the tests, during exposure to Na-containing solutions. In a few tests in which pipets were exposed for several minutes at neutral pH to high concentrations of Na and then rinsed with deionized

water, this did not significantly affect their behavior during subsequent elutions.

DISCUSSION

The data indicate that both the soft glass and the borosilicate glass pipets were modified by the cleaning procedure so that, immediately after cleaning, they released no detectable amounts of Na when eluted with deionized water or with 3.6 m*M* KCl solution. The fact that increasing amounts of Na could be eluted from these pipets as the time between cleaning and testing increased suggests that a progressive change was taking place in the glass surface surrounding the lumen. The rate of the change depended markedly on the temperature at which the pipets were stored.

Two explanations may be considered: (*1*) progressive change in the nature of the chemical binding of the Na in the glass surrounding the lumen; or (*2*) diffusion within the glass, which would bring to the surface Na ions that had been out of reach of the leaching solution previously used in the cleaning procedures. Studies by Johnson *et al.* (5) on Na diffusion in glass suggest that the latter is a likely possibility. Their data for soft glass, extrapolated to 50°C, give a value for the diffusion coefficient of Na of approximately 10^{-16} cm^2/sec.

If it is assumed that the eluted Na was an original constituent of the glass, it is possible to calculate the minimal volume of glass which would have had to have been extracted to account for the Na removed. In the case of the soft glass pipets, assuming a Na content of 9.6% by weight or 11 mmoles/cc, 7.7×10^{-4} mm^3 of glass containing the original amount of Na would have had to have been cleared to account for the Na eluted after 7 days of storage at room temperature. This volume would correspond to a layer about 10 A in depth surrounding the lumen of the pipet. In the case of the borosilicate glass pipets, assuming a Na content of 3% by weight or 2.9 mmoles/cc, similar calculations indicate that 13×10^{-4} mm^3 of glass, containing the original concentration of sodium, would have had to have been cleared to account for the Na removed after 7 days of storage at room temperature. This would also correspond to a layer of about 10 A in depth surrounding the lumen of these pipets. The correspondence in the depths calculated for the two types of pipets would appear to be largely fortuitous.

The greater effectiveness of the KCl solution, compared to deionized water, in eluting the Na, particularly in the case of the borosilicate glass pipets, suggests that this is in part an ion-exchange reaction. In view of the low concentrations involved, one cannot exclude the hydrogen ion from playing a role in this regard.

The relevance of these phenomena for the development of methods for microanalyses of Na is evident. If soft glass pipets are used and are dried in an oven after cleaning, Na contamination of the pipetted solution may be as high as 0.04 mmole/liter and may cause an appreciable error even in the analysis of rather more concentrated solutions. This difficulty may be met by subjecting the glassware to an acid rinse immediately before using or by substituting plastic for glass. In our laboratory the use of polypropylene pipets has proved to be a satisfactory solution to the problem.

SUMMARY

Pipets that had been leached with acid, rinsed with deionized water, dried, protected from air-borne contamination, and then stored for varying periods, were tested to determine their extractable Na. When tested immediately, they contributed no detectable Na to the eluting fluid (<0.1 μmole/liter Na). However, as the time between cleaning and testing was increased, the pipets contributed increasing amounts of Na to the eluting fluid—the amounts depending not only on the length of storage but also on the type of glass, the temperature at which it was stored, and the nature of the eluting fluid. The results are most easily interpreted as due to diffusion of Na ions within the glass, bringing them within reach of the previously leached lumenal surface.

Significant errors may occur in the analysis of dilute Na solutions unless glass pipets are used immediately after acid cleaning or substituted by plastic pipets.

REFERENCES

1. THEIRS, R. E., *Methods Biochem. Anal.* **5**, 273 (1957).
2. WICHERS, E., FINN, A. N., AND CLABAUGH, W. S., *Ind. Eng. Chem., Anal. Ed.* 13, 419 (1941).
3. LONG, A. O., AND WILLARD, J. E., *Ind. Eng. Chem., Anal. Ed.* **44**, 916 (1952).
4. AMES, A., AND NESBETT, F. B., *Anal. Biochem.* **1**, 1 (1960).
5. JOHNSON, J. R., BRISTOW, R. H., AND BLAU, H. H., *J. Am. Ceram. Soc.* **34**, 165 (1951).

ANALYTICAL BIOCHEMISTRY 4, 458–463 (1962)

SHORT COMMUNICATIONS

Liquid Scintillation Counting with Glass Fiber Paper[1]

The increased popularity of glass fiber paper chromatographic techniques has prompted us to evaluate its applicability to radioactive tracer studies. Wang and Jones (1) described a technique for measuring the radioactivity of the cellulose paper sections of chromatograms in which these were placed directly into the fluid and counted with a liquid scintillation counter. Geiger and Wright (2) noted that the pieces of paper could be placed in the counting vial at any angle without significantly affecting the results. The studies reported herein indicate that as many as 25 pieces of glass fiber paper can be assayed for radioactivity in one vial to yield quantitative results.

METHODS

Silica-gel impregnated glass fiber paper, prepared by the method of Hamilton *et al.* (3), was used throughout these studies. After separating cholesterol-4-C^{14} from a lipid mixture by the method of Swartwout *et al.* (4), one is able to locate the cholesterol by cutting a strip from each edge of the chromatogram; then these guide strips are sprayed with sulfuric acid, and charred by heating. The intensity of the charred cholesterol spot is directly related to the amount of cholesterol present; and by the use of a modified photometer the amount of cholesterol was determined in the unknown by calculating from a standard curve, which gives a straight line at the concentration employed. Furthermore, with the help of the two strips, one is able to locate the cholesterol on the uncharred part of the chromatogram and to punch out equal sized disks with a cork borer 12 mm in diameter. These concentration data of cholesterol-4-C^{14} then can be combined with the radioactivity data; thus one can calculate the specific activity (counts per minute per milligram) of the cholesterol or other radioactive compounds.

[1] Supported by the Division of Grants and Fellowships of the NIH, H-4150, 2G-648, HTS-5236, and Wesson Fund for Medical Research.

[2] Part of a dissertation submitted in partial fulfillment of the requirements for the Ph.D. Degree in Biochemistry, Tulane University of Louisiana, 1962.

RESULTS

The disks, as prepared above, were assayed by adding them either one at a time or five at a time to a vial containing 15 ml of scintillation fluid. In all instances the radioactivity (cpm) was directly proportional to the number of disks added to the vial. This proportional relationship was held when up to as many as 25 disks were used and when the total measured radioactivity varied from 100 to 40,000 cpm. These data are exemplified by Fig. 1. Similarly, studies were made in which fucose-1-C^{14}

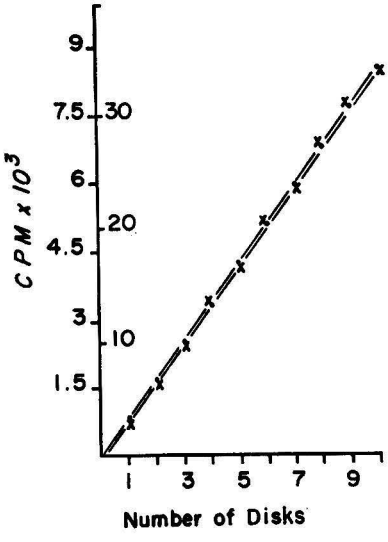


FIG. 1. Equal amounts of cholesterol-4-C^{14} were chromatographed from lipid mixtures and individual disks prepared (see text for details). The graph shows the results obtained when two different amounts of radioactivity were employed: 800 cpm per disk (×—×) and 3300 cpm per disk (·—·). Radioactivity is plotted against number of disks added to the vial.

was placed on glass paper and counted as above. In this instance a water-soluble compound was counted in a nonpolar scintillation fluid and, interestingly enough, the proportionality was observed between cpm and the number of disks added to the vial. This proportionality held regardless of whether the scintillation fluid was polar—POPOP [1,4-bis-2-(5-phenyloxazolyl)benzene] 0.2 gm, PPO [2,5-diphenyloxazole] 4.0 gm, ethylene glycol 20.0 gm, naphthalene 60.0 gm, methanol 100 ml, and dioxane 800 ml, or was nonpolar—POPOP 0.1 gm PPO 3.0 gm, ethanol 300 ml, and toluene 700 ml. The lines of proportionality coincide when the experiments were carried out using each of the two solvents listed

above. When a polar solvent was used, 96% of the fucose-1-C^{14} was eluted from the disks; however, only 25% was eluted from the disks when a nonpolar solvent was used in the scintillation fluid. This suggests that the radioactive material does not have to be eluted from the disks when as many as 25 of them are counted at one time in one vial.

In order to evaluate the possible quenching effect or light-absorbing properties of the glass fiber paper, 25 glass fiber disks were placed into a vial containing radioactivity which had been added in solution and there was no decrease in cpm (Table 1). Furthermore, up to 25 disks were added to a vial to which no radioactivity was added, which demonstrated that the glass fiber disks had no effect on the background.

TABLE 1

EFFECT OF ADDING GLASS FIBER DISKS TO VIALS OF SCINTILLATION FLUID ON MEASUREMENT OF RADIOACTIVITY (COUNTS PER MINUTE)

Cholesterol-C^{14} added to vial	Number of disks added to vial		
	0	10	25
0	80	87	90
2,510	2,560	2,550	2,490
6,100	6,110	6,210	6,150
23,200	23,250	23,190	23,290
31,800	31,790	31,860	31,830

If one compares the efficiency of counting C^{14} on cellulose paper with that of glass fiber paper (Fig. 2), it is observed that cellulose paper is only about 60% as efficient as glass fiber paper. In this experiment identical amounts of fucose-1-C^{14} were placed on each type of paper and the disks, prepared as above, were counted in a nonpolar scintillation fluid. The proportionality between radioactivity and number of disks added to the vial is equally good for both kinds of paper.

SUMMARY

The following conclusions appear warranted: Radioactivity is linearly proportional to the number of disks (up to 25) added to the counting vial over a range of 100 to 40,000 cpm. This relationship seems to hold for both lipid-soluble and water-soluble compounds. Glass fiber paper has a 60–70% higher counting efficiency than cellulose paper. The specific activity can be calculated by using the guide strip for determination of the amount of cholesterol and cholesterol esters; and, by using this data as well as the counts, one can calculate the specific activity of these compounds.

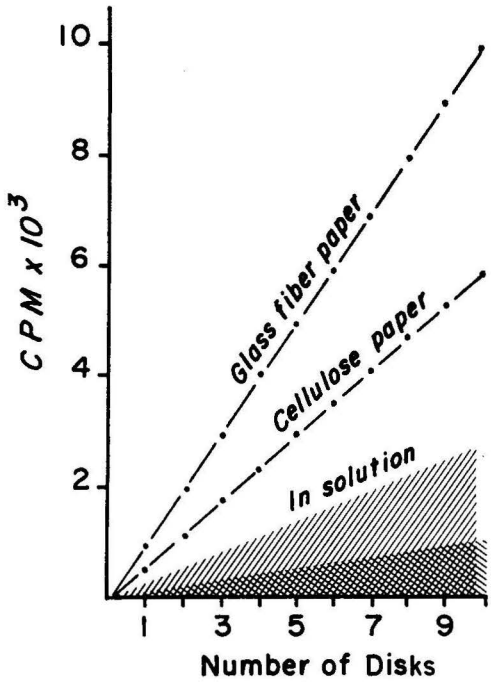


FIG. 2. Comparison of counting efficiency obtained when an equal amount of pure radioactive material (fucose-1-C^{14}) was adsorbed on glass fiber paper disks and cellulose paper disks. Radioactive material eluted from the glass fiber disks is shown by ////// and amount eluted from cellulose disks is shown by \\\\\\.

REFERENCES

1. WANG, C. H., AND JONES, D. E., *Biochem. Biophys. Res. Commun.* **1**, 203 (1959).
2. GEIGER, J. W., AND WRIGHT, L. D., *Biochem. Biophys. Res. Commun.* **2**, 282 (1960).
3. HAMILTON, J. G., SWARTWOUT, J. R., MILLER, O. N., AND MULDREY, J. E., *Biochem. Biophys. Res. Commun.* **5**, 226 (1961).
4. SWARTWOUT, J. R., DIECKERT, J. W., MILLER, O. N., AND HAMILTON, J. G., *J. Lipid Res.* **1**, 281 (1960).

KAROLY G. PINTER
JAMES G. HAMILTON
O. NEAL MILLER

Nutrition and Metabolism Laboratory of the Department of Medicine and the Department of Biochemistry,
Tulane University,
School of Medicine,
New Orleans, Louisiana
Received December 27, 1962

An Assembly for $C^{14}O_2$ Collection in Metabolic Studies for Liquid Scintillation Counting[1]

This report describes a modification of the Snyder-Godfrey (1) technique for the collection of $C^{14}O_2$ for subsequent scintillation counting. In the assembly to be described, Warburg flasks were replaced by 25-ml Erlenmeyer flasks as the main vessel. Several factors which cause some Warburg flasks to be less desirable than the Erlenmeyer flasks are: they are difficult to handle with accidental spillage of the acid from the side arm into the main compartment; removal of the vial from the Warburg flask may be difficult; and the use of Warburg flasks causes the cost of this method to be approximately \$7.25 per flask. All of these undesirable characteristics are eliminated and the cost is reduced to approximately \$0.80 per completed assembly.

The assembly used is diagrammatically shown in Fig. 1. The main vessel is a heavy-duty 25-ml Erlenmeyer flask (Corning No. 4980) into the top of which is placed a sleeve type of rubber stopper with a plug o.d.

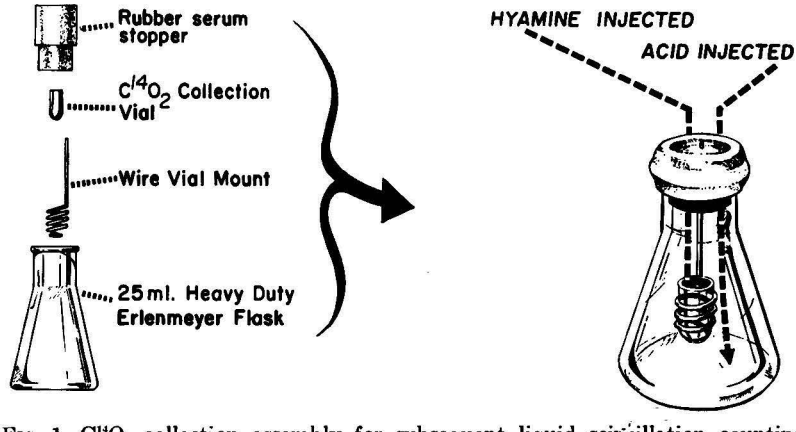


FIG. 1. $C^{14}O_2$ collection assembly for subsequent liquid scintillation counting.

of 13 mm. This stopper is used to close the system and to support the stainless-steel wire vial mount. Stainless-steel wire is used to prevent the mount from interacting with the chemicals, and is mounted by inserting the straight end of a 0.046-mm diameter stainless-steel wire through the inside of the serum stopper plug with an approximately 1-mm protrusion. Inserted into this formed wire mount is the $C^{14}O_2$ collection vial, obtained

[1] This investigation was supported by N. I. H. Grant H-7190.

by cutting the end of a Pyrex No. 9820 culture tube to a length of between 17 and 18 mm.

The complete assembly with the necessary media, homogenates, cells, or tissue slices can be sealed and gassed with the appropriate gas. Thirty-six assemblies can be incubated simultaneously in the standard Dubnoff metabolic shaker. At the end of the desired incubation interval 0.3 cc of 1 *M* Hyamine hydroxide (2), a high molecular weight quaternary amine for CO_2 trapping, is injected into the collection vial with a tuberculin syringe and the desired acid injected into the flask to terminate the reaction and evolve the $C^{14}O_2$ in the media. The flasks are allowed to continue shaking for an additional 2 hr to ensure complete trapping of $C^{14}O_2$ by the Hyamine. Following this incubation period the rubber stopper and assembly are removed and the collecting vial is transferred directly into the liquid scintillation counting vial containing 10 ml of 0.4% PPO (2,5-diphenyloxazole) and 0.01% POPOP [1,4-bis-2-(5-phenyloxazolyl)benzene] in toluene.

In order to confirm the efficiency of the materials and method employed by this procedure, amounts up to 6 ml in duplicate of $NaHC^{14}O_2$ with a specific activity of 6.7×10^4 cpm/ml were added to the 25-ml Erlenmeyer flasks. Then 0.3 ml Hyamine was injected into each collection vial followed by the injection of 0.3 ml 6 *N* H_2SO_4 into the $NaHC^{14}O_2$ solution for evolving the $C^{14}O_2$. After allowing 2 hr for the collection of $C^{14}O_2$ by the Hyamine, the vial from the flask was removed and placed in a counting vial containing the above liquid scintillation media for counting. Essentially 100% recovery of $C^{14}O_2$ was obtained. The glass collection vials again did not affect the counting characteristics of the C^{14} system (1).

SUMMARY

An inexpensive method for multiple determinations in metabolic studies of oxidation of C^{14} substrates for subsequent liquid scintillation counting is presented. As previously reported (1), complete transfer of $C^{14}O_2$ trapped in the collecting vial can be effected in a single operation.

REFERENCES

1. Snyder, F., and Godfrey, P., *J. Lipid Res.* **2**, 195 (1961).
2. Passman, J. M., Radin, N. S., and Cooper, J. A. D., *Anal. Chem.* **28**, 484 (1956).

Dianna Cuppy
Lamar Crevasse

Department of Medicine,
University of Florida,
School of Medicine,
Gainesville, Florida
Received January 11, 1963

ANALYTICAL BIOCHEMISTRY 5, 465–470 (1963)

Chromatographic Behavior on Ion-Exchange Paper of Bilirubin Conjugated with Sulfate and Glucuronide[1]

FRANCISCO R. VEGAS[2]

From the Department of Hematology, Laboratory Division, Montefiore Hospital, New York

Received June 6, 1962

The studies of Billing, Cole, and Lathe (1–3), Schmid (4), and Talafant (5) have shown that direct-reacting bilirubin is a polar and water-soluble conjugate of bilirubin. Furthermore, in addition to the glucuronide conjugate, there are other conjugates of bilirubin which are both polar and water soluble (3, 6). One of these, bilirubin sulfate, has been identified by the use of radioisotope and chemical methods (6, 7). However, a clear-cut resolution of the azo dipyrryl derivative of the bilirubin glucuronide and of the azo dipyrryl derivative of the natural bilirubin sulfate conjugates by partition chromatography has not been achieved to date. This paper will present a procedure for the identification of these two compounds.

MATERIALS AND METHODS

Synthetic "bilirubin sulfate" was prepared according to the method of Watson (8) and the identity of its azo dipyrryl derivative was confirmed by the partition chromatographic method of Schmid (4).

The azo dipyrryl derivative of free bilirubin was obtained by treating a chloroform solution of crystalline bilirubin (Pfanstiehl) with sulfanilic acid in 50% methanol (9). The solution was dried under vacuum at 40°C.

The azo derivative of bilirubin glucuronide was obtained from two sources: (*a*) from sera of jaundiced patients which was processed using the method of Schmid (4), and (*b*) from human bile (T-tube drainage) which was diazotized and purified according to Schmid (10). Both samples were chromatographed in the same manner (4).

The azo dipyrryl derivative from the conjugated bilirubin was eluted from the paper with *n*-butanol-water-glacial acetic acid 52:35:13

[1] This study was supported by Grant No. H-5415 of the National Heart Institute, National Institutes of Health, United States Public Health Service.

[2] Present address: Department of Laboratories, Jewish Chronic Disease Hospital, Brooklyn, New York.

(v/v/v). After drying all specimens were prepared for ascending ion-exchange paper chromatography by dissolving in 1 *N* acetic acid.

The samples were run on carboxymethylcellulose cation-exchange paper (Whatman CM 50), which was previously washed consecutively in the downward direction, with: (*1*) 0.1 *N* HCl for 24 hr, (*2*) water for 18 hr, (*3*) 0.1 *N* NaOH for 24 hr, and (*4*) water for 18 hr. These steps were carried out in a large chromatographic cabinet (Chromatocab). Following the last washing the paper was dried in a chromatography oven at 40°C for 1 hr. Ascending ion-exchange chromatography was achieved with *n*-butanol-pyridine-1 *N* ammonia 2:1:2 (v/v/v), pH 11.9 at room temperature.

RESULTS

When the acetic acid solutions of the azo compounds were run on the same strip, clear separation between them was obtained (Fig. 1). R_f for the azo dipyrryl derivative from synthetic bilirubin sulfate was 0.90, while that from the azo dipyrryl derivative of bilirubin glucuronide was 0.14. The azo dipyrryl compound of crystalline bilirubin ran at the solvent front. The azo dipyrryl glucuronide standards were tested first for the presence of alkali-stable pigment, either by alkaline hydrolysis, or by enzymic hydrolysis through the action of bacterial β-glucuronidase (Sigma) using 20 mg/mg at 37°C for 24 hr at pH 6.2 in 0.1 *M* acetate buffer (3, 12).

None of these samples demonstrated stable fractions when subjected to ascending partition chromatography (4). Watson (8) had previously shown that the azo dipyrryl derivative of synthetic "bilirubin sulfate" exhibits two chromatographic spots with R_f values of 0.30 and 0.22. Isselbacher and McCarthy (6) have pointed out that the lower spot is due not to an azo dipyrryl derivative but to the alteration of the bilirubin molecule during the course of synthesis. More recently Gregory and Watson (12) showed that this behavior is compatible with the formation of sulfate and sulfonate rather than to alteration of the bilirubin molecule.

We have observed, as did Watson (8), that treatment of bilirubin with the Liebermann-Burchard reagent results in a product which is water soluble, gives a "direct" Van der Bergh reaction, and exhibits an absorption maximum at 540 mμ. On ion-exchange paper our azo dipyrryl derivative of the synthetic bilirubin sulfate also gives two spots; a faint one (R_f 0.30) and the main fraction (R_f 0.90). In order to establish the identity of the main zone as azo dipyrryl sulfate, a further separation was performed. Both zones were eluted with acid-butanol mixture and the eluate was dried in vacuum, redissolved in acetate buffer, pH 4 (9),

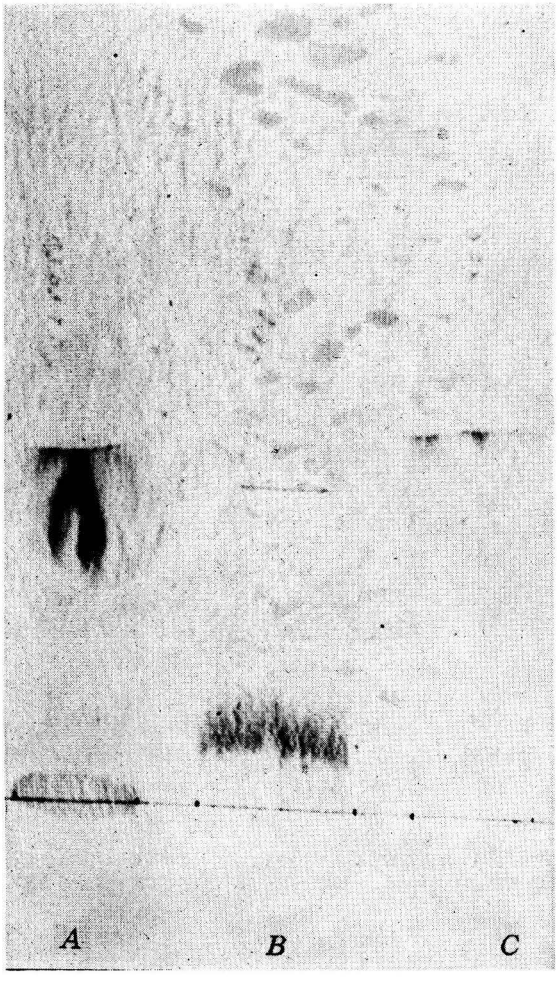


FIG. 1. Ascending ion-exchange paper chromatogram of azo dipyrryl derivatives: (A) Azo dipyrryl derivative of synthetic "bilirubin sulfate." (B) Azo dipyrryl glucuronide. (C) Azo dipyrryl derivative of free bilirubin.

and rechromatographed on an alumina oxide column. A brownish product descended with the effluent; the segment of column containing this coloration was cut off and discarded. In this way we eliminated the minor trailing spot, i.e., the azo derivative of the bilirubin sulfonate of Gregory and Watson (12), as well as other derivatives.

The upper part of the column was eluted with 0.1 *N* NaOH solution. The eluate was acidified and the pigment extracted by *n*-butanol. The butanol extract was dried in vacuum and redissolved in 1 *N* acetic acid.

Ascending ion-exchange paper chromatography was carried out again on Whatman CM paper. Only one spot with R_f 0.95 was found. The pigment was eluted from the paper with acid *n*-butanol, concentrated to dryness and redissolved in 1 *N* acetic acid. This material as well as the azo pigment of free bilirubin was chromatographed on Whatman No. 1 paper according to the method of Schmid (4). The azo pigment of free bilirubin had an R_f of 0.45 and the azo pigment eluted from the ion-exchange paper had an R_f of 0.35, comparable to the R_f described by Watson (8, 12) for the azo dipyrryl derivative of the synthetic bilirubin sulfate. It has been possible to show that this main fraction, after ion-exchange chromatography as previously described, contained sulfate. For this purpose the *n*-butanol extract containing the main zone was dried and dissolved in a small amount of 6 *N* HCl and heated at 100°C for 1 hr in order to hydrolyze the conjugate. The presence of sulfate ion was demonstrated by the method of Dodgson and Spencer (13).

Billing *et al.* (3), noted that conjugated bilirubin from bile was readily converted to free bilirubin in 0.06 *N* NaOH at room temperature. A small fraction, however, was stable to this treatment. Isselbacher and McCarthy (6) and Schoenfield *et al.* (7), have demonstrated that the stable fraction is mainly bilirubin sulfate. The azo pigment from bile was treated with 0.2 *N* NaOH at room temperature for 10 min, followed by acidification, extraction with acid *n*-butanol, and paper chromatography by the method of Schmid (4). Two spots were obtained, one near the origin (polar region) which was an alkali-stable pigment, and the other, the unconjugated bilirubin. The alkali-stable pigment was eluted from the paper with acid *n*-butanol, concentrated to dryness in vacuum, and redissolved in 1 *N* acetic acid. Ascending ion-exchange paper chromatography was carried on Whatman CM paper; only one spot was found with an R_f of 0.92. This spot was encountered in only two serum samples, both obtained during the obstructive phase of hepatitis. The findings in these two cases are still to be clarified.

DISCUSSION

It has been known since the studies of Billing *et al.* (3) that the azo pigment glucuronide is uncoupled by alkali. When a pyridine buffer at pH 11.9 is utilized during ion-exchange paper chromatography, no evidence of the breakdown of the azo pigment glucuronide is noted. This fact was readily demonstrated when the azo pigment glucuronide was eluted from the ion-exchange paper after chromatography. The azo pigment in acid *n*-butanol was concentrated to dryness in vacuum and redissolved in 1 *N* acetic acid. These concentrated acetic acid solutions

of azo pigment glucuronide and the azo derivative of free bilirubin were chromatographed on Whatman No. 1 paper according to the method of Schmid (4). The R_f of the azo pigment glucuronide eluted from the ion-exchange paper was 0.37, as was the R_f of the azo glucuronide obtained by the method of Billing (15). The R_f of the azo pigment of free bilirubin was 0.50. These findings would appear to substantiate the integrity of the azo pigment glucuronides in this buffer.

Despite possible structural differences (6, 8, 12) between the azo dipyrryl derivatives of synthetic "bilirubin sulfate" and the azo dipyrryl derivatives of the alkali-stable fraction of the naturally conjugated bilirubin fraction which is also a conjugated sulfate (6, 7), both have certain properties in common: (*a*) failure to react with hydroxylamine to form the corresponding hydroxamic acids (6, 12, 14) and (*b*) the apparent linkage of the sulfate to bilirubin molecule in both cases at the vinyl or hydroxyl position or being attached to one or more nitrogens in the pyrrole ring (6, 12, 14). Whatever the location of the sulfate ion, conjugation yields anionic complexes which behave similarly in ion-exchange paper chromatography. The azo derivative of free bilirubin also behaves as an anion in ion-exchanger paper chromatography.

Schachter (14) has demonstrated that the carboxyl group of the propionic acid of the bilirubin molecule is conjugated with one of the hydroxyl groups of glucuronic acid. This linkage also yields an anionic complex and its azo dipyrryl derivatives seem to have a reduced anionic function in ion-exchange paper chromatography in comparison with the azo dipyrryl derivatives of free bilirubin and the azo derivatives of synthetic and natural bilirubin sulfate.

Isselbacher and McCarthy (6) and Schoenfield *et al.* (7) have demonstrated that the azo dipyrryl derivatives of natural bilirubin sulfate migrate in the region of the more polar azo pigments in partition chromatography. It has been demonstrated (6, 7, 8, 12) that the azo dipyrryl derivatives of synthetic bilirubin sulfate have chromatographic properties similar to those of the azo bilirubin glucuronide. These observations confirm the impossibility of separating these two bilirubin conjugates by partition chromatography. Their resolution can be obtained, however, with the use of ion-exchange paper chromatography.

SUMMARY

Clear-cut resolution of the azo pigment of synthetic "bilirubin sulfate" and the azo derivative of the alkali-stable fraction of naturally conjugated bilirubin from that of azo glucuronide can be obtained by means of ion-exchange paper chromatography.

ACKNOWLEDGMENTS

The author wishes to thank Drs. Theodore H. Spaet and Jamshid Javid for advice and aid in the preparation of this manuscript.

REFERENCES

1. COLE, P. G., AND LATHE, G. H., *J. Clin. Path.* **6**, 99 (1953).
2. BILLING, B. H., AND LATHE, G. H., *Biochem. J.* **63**, 60 (1956).
3. BILLING, B. H., COLE, P. G., AND LATHE, G. H., *Biochem. J.* **65**, 774 (1957).
4. SCHMID, R., *Science* **124**, 76 (1956).
5. TALAFANT, E., *Collection Czech. Chem. Commun.* **22**, 661 (1957).
6. ISSELBACHER, K. J., AND MCCARTHY, E. A., *J. Clin. Invest.* **38**, 645 (1959).
7. SCHOENFIELD, L. J., BOLLMAN, J. L., AND HOFFMAN, H. N., II, *J. Clin. Invest.* **41**, 133 (1962).
8. WATSON, C. J., *Science* **128**, 142 (1958).
9. SCHACHTER, D., *J. Lab. Clin. Med.* **53**, 557 (1959).
10. SCHMID, R., *J. Biol. Chem.* **229**, 881 (1957).
11. WHATMAN, J., "Whatman Cellulose Ion-Exchangers," Technical Bulletin, 1031.
12. GREGORY, C. H., AND WATSON, C. J., *J. Lab. Clin. Med.* **60**, 17 (1962).
13. DODGSON, K. S., AND SPENCER, B., *Biochem. J.* **55**, 436 (1953).
14. SCHACHTER, D., *Science* **126**, 507 (1957).
15. BILLING, B. H., *J. Clin. Path.* **8**, 126 (1955).

ANALYTICAL BIOCHEMISTRY 5, 471–478 (1963)

A Computer Solution for Cation Analysis with Glass Electrodes[1]

SYDNEY M. FRIEDMAN AND F. K. BOWERS

From the Departments of Anatomy and Electrical Engineering, The University of British Columbia, Vancouver, Canada

Received August 17, 1962

The usefulness of cation-selective glass electrodes in ionic mixtures is limited by the ability of the glass to differentiate between the ions (1). In biological systems, the problem reduces to the fact that, while highly specific sodium glasses can be prepared, potassium-sensitive glasses are far less selective and respond to some extent to sodium as well (2). Under these circumstances, the resolution of sodium and potassium activities depends on the application of the equation of Eisenman *et al.* (1). Although it is probably quite feasible to shorten calculation with a nomogram, this is certainly not suitable for continuous monitoring. Since some of the more promising applications of these electrodes involve such continuous measurements, we have developed a simple analog computer solution to the equation. Although the special case featuring one pair of ions and specific range of values is described here, the solution is quite general and can be modified for any pair and any range.

METHODS

A. Theory

The potential measured by a conventional pH electrode assembly is determined by the H^+ activity of the solution to which the glass membrane is exposed:

$$E = E_0' + (RT/F) \ln (H^+)_a \tag{1}$$

where R, T, and F have their usual significance, E is the potential measured at the terminals, and E_0' is a constant to express the standard potential of the electrode.

When E is measured in two different solutions, a and b:

$$\Delta E = E_a - E_b = S \log (H^+)_a/(H^+)_b \text{ millivolts (mv)} \tag{2}$$

[1] This work was carried out with the aid of a grant from the British Columbia Heart Foundation.

where S is a constant obtained by calibration of the particular electrode being used, ideally 58 mv at 22°C.

In natural biological systems only H^+, Na^+, and K^+ affect the electrode potentials of our cation-selective glasses. Above pH 5, H^+ activity ceases to affect these glasses. The general equation of Eisenman *et al.* (1), as applied to a mixture of H^+, Na^+, and K^+ at a physiological pH is then as follows:

$$E = E_0' + RT/F \ln [(Na^+) + k_{NaK}(K^+)] \quad (3)$$

The constant k_{NaK} defines the sensitivity of the glass to K^+ relative to Na^+. If the activities of Na^+ and K^+ are written in the general form of A and B and the electrode selectivity constant as k, then when E is measured in two different solutions, a and b, of the same ions:

$$\Delta E = E_a - E_b = S \log (A + kB)_a/(A + kB)_b \quad (4)$$

Sodium-sensitive glasses currently in use are highly specific. Thus, for example, NAS_{11-18} has a k value of only 0.004 so that unless (K^+) exceeds approximately 30 meq/liter this cation will contribute negligibly to the electrode potential. Newer glasses are even more highly specific. For practical purposes then, Eq. (4) for a sodium electrode takes the same simple form as Eq. (2).

From these facts the computer equations can now be set out using the following additional terms:

E_A = output voltage of electrometer receiving the sodium electrode
E_B = output voltage of electrometer receiving the potassium electrode
E_A° = output voltage of E_A when A has a *standard value* here taken as equivalent to 175 meq/liter of Na as NaCl
E_B° = output voltage of E_B when A has the standard value and in the absence of B

Thus, for the sodium electrode, Eq. (4) becomes:

$$E_A - E_A^\circ/S_A = \log A/175 \quad (5)$$

In the computer we can generate a voltage $X_A = C(E_A - E_A^\circ)/S_A = C \log A/175$, where C is a suitable scaling factor; in our case $C = 500$. Accordingly, X_A is a logarithmic function of the Na concentration, A. To obtain a voltage varying linearly with A we employ a function generator to yield antilogs—thus, if the output voltage is arranged so that:

$$Y_A = 175(10^{X_A/C} - 1) \quad (6)$$

Then: $Y_A = A - 175$, as desired.

In similar fashion we also derive from the output of the potassium electrode a voltage linearly dependent on concentration:

$$X_B = C(E_B - E_B^\circ)/S_B = C \log (A + kB)/175$$
$$Y_B = 175(10^{X_B/C} - 1) = A + kB - 175 \quad (7)$$

The voltage $A - 175$ is directly available at one output and is conveniently handled as 1 volt = 1 meq/liter of (Na^+). This same voltage is subtracted from the second output to form kB, which is then calibrated and amplified to give a convenient output for B, e.g., 5 volts = 1 meq/liter of (K^+).

B. Circuit

The schematic diagram is shown in Fig. 1. In this study, the Donner

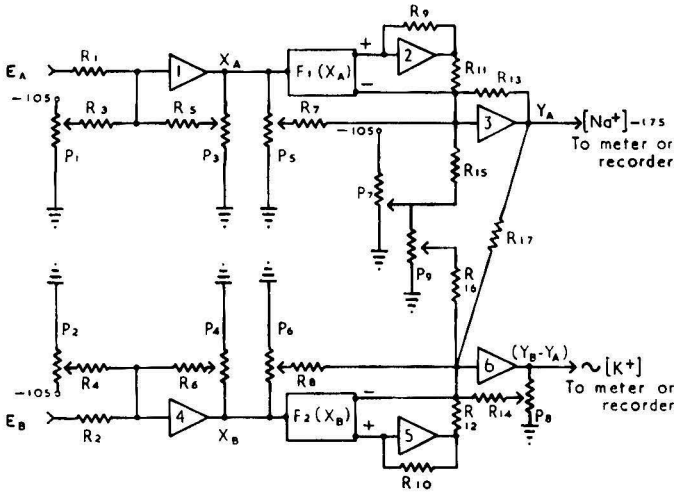


FIG. 1. Schematic diagram of computer circuit:

R1–2, 0.5 megohm	All resistors better than 1%
R3–6, 10 megohms	All potentiometers 100 K, 10-turn
R7–8, 1 megohm	R1–2 are chosen to suit voltages E_A *and*
R9–12, 0.1 megohm	E_B available from electrometer
R13–17, 1 megohm	amplifiers

model 3400 computer was used.

The first amplifier in each train, 1, 4, generates X_A or X_B and thus contains adjustments for the baseline, E_A° and E_B° and for the sensitivities S_A and S_B.

Potentiometer P5 and amplifiers 2, 3, together with the diode network of the first function generator, convert X_A into Y_A. Similarly, P6 and amplifiers 5, 6 are associated with the second function generator, which converts X_B into Y_B.

The output of amplifier 3, Y_A, is subtracted from amplifier 6 through

R17 so that the output of the latter is now proportional to $Y_B - Y_A$ and thus to B. Furthermore, the gain of this final potassium train amplifier is adjustable and is not normally the same as that of the final sodium amplifier so that the output voltage scale for B can be made convenient.

Through P7 we can add a variable voltage, A_0, to the output of amplifier 3 so that its output can be made $(A - 175) + A_0$, if this should prove convenient. The addition of A_0 should not affect the output of the potassium train and this is assured by R16, which adds to amplifier 6 a current equal and opposite to that coming by way of R15, R13, R17. Potentiometer 9 is needed to allow for variations of these four resistors from nominal values, and is locked when perfect compensation has been assured.

For the generation of antilogs of X_A and X_B two identical function generators are required. A Donner model 3750 generator containing 24 diode networks was split to yield the two generators, F1 and F2. (This is done by cutting the input line and the two output lines, leaving 12 diode networks attached to each half. A second set of terminals is then connected to the severed half.)

With 12 breakpoints in each generator a good straight-line approximation to the antilog can be produced, particularly as the range of $A + kB$ was limited to lie between 115 and 265.

The first function generator is set up by using standard procedures: Eq. (6) is evaluated for 14 equally spaced values of X_A from -91 to $+91$. Breakpoints are adjusted to lie on the 12 central values. The slope near $X_A = 0$ is adjusted with P5, while the 12 slope controls of the generator are used to give correct values of Y_A at the 12 outer tabulated values.

The second function generator is best adjusted by feeding the same input voltage into both generators. The second is then set up to produce zero output from amplifier 6 for all input voltages.

C. Calibration Procedure

All calibrating solutions should be buffered to the same pH. Tris [2-amino-2-(hydroxymethyl)-1,3-propanediol] is particularly useful for this.

1. Introduce solution I containing the *standard value* of Na (175 meq/liter NaCl). Adjust electrometer gains and biases to give preselected meter readings chosen to keep the anticipated range of operation on scale. These should not be altered unless computer is to be recalibrated. With P7 at zero, adjust P1 and P2 to yield zero from amplifiers 3 and 6.

2. Introduce solution II containing a different value of Na and no K,

e.g., 140/0. Adjust P3 and P4 so that outputs of sodium and potassium trains read correctly.

3. Introduce solution III containing a reasonable amount of K, e.g., 140/6. Adjust P8 for a convenient scale reading.

D. Electrodes

Hand-made, fully insulated conventional dip-type electrodes were used for the measurements described here. Sodium electrodes were made with NAS_{11-18} glass and potassium electrodes with NAS_{27-3} (2). In all cases the sensitive membrane was formed by a bulb of about 5-mm diameter blown on the end of an appropriate indifferent glass stem (General Electric Co. Pyrex-quartz series #GSC 3 for NAS_{11-18} and Corning Glass Works #0120 for NAS_{27-3}). The bulbs were filled with 0.1 *M* NaCl to a level sufficient fully to immerse a carefully prepared Ag—AgCl internal electrode. One standard calomel electrode served as common reference for both glass electrodes.

Grounding and shielding of a paired electrode assembly depends on the electrometers being used. For this study, a Master-Slave combination of 2 Cary Vibrating Reed Electrometers operating as grounded voltmeters (31 V mode) was used. This assembly permits stray interference to be handled by directly grounding the solution with a bare platinum wire. This ground, together with all other ground leads, was taken to a common point.

A volume of 2 ml of solution placed by syringe into a small tapered vessel of about 10-ml capacity was more than sufficient to cover the electrodes. Neither vessel nor electrodes were moved during the course of an experiment. Measurements were carried out at room temperature so that all solutions and all instrumental parts were at the same uniform temperature during a run. Individual runs usually lasted about 30 min and ambient temperature fluctuations within this period were negligible.

RESULTS

We have elsewhere described the precision of the cation-selective glass electrodes (3). In general, sodium and potassium electrodes discriminate changes of 0.2% or better. Here, however, we are primarily concerned with the ability of the computer assembly to interpret the electrode responses. In this operation, linearization is not absolute since the accuracy of the antilog function depends on the number of straight-line segments used to approximate a given range. Additionally, the computer contributes both to the basic noise level and to the drift of the instrument assembly. All of the tests were carried out using syringes to empty

and fill the electrode vessel. This is itself a fairly noisy procedure and accounts in large part for some of the random baseline shifts appearing in the figures.

Linearization of the electrode voltages and subtraction of the sodium train from the potassium train is quite satisfactorily achieved. In the example shown in Fig. 2A, the apparent K gave a false reading of at

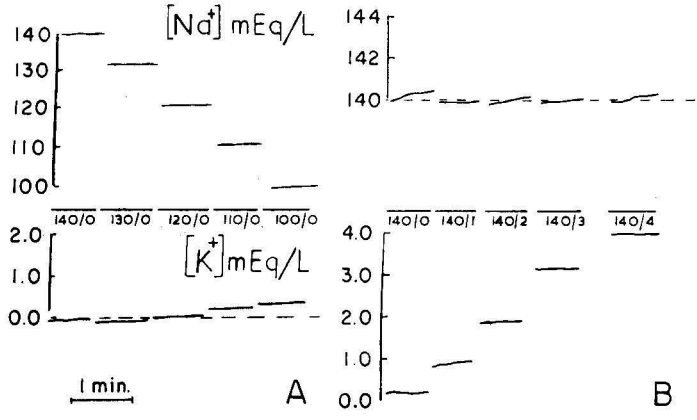


FIG. 2. (A) Response to changes of Na^+ concentration in 10 meq/liter steps. (B) Response to changes of K^+ concentration in 1 meq/liter steps. Ionic concentrations of solutions used are written along horizontal mid-line as Na/K. Two-minute equilibration time allowed between changes.

most 0.3 meq/liters in a 15-min run even though 5 different Na solutions covering a range of 40 meq/liters were introduced into the vessel. This K variation was partially random and partially due to slight imperfection in the tuning of P4. Similarly, in the example shown in Fig. 2B, the Na error was negligible, while a nearly perfect K response was recorded.

Precision and reproducibility are illustrated in Fig. 3. The results are almost as precise as those we had previously produced for sodium alone using the noiseless flowthrough arrangement for changing solutions.

Response time cannot be measured precisely with this assembly since the exchange of solutions by syringe produces a mechanical artifact. Additionally, of course, the shape, size, and glass thickness of the particular electrode determines its characteristic equilibration period. With these cautions in mind a reasonable estimate can be made from Fig. 4. The electrodes respond almost at once and well-made ones reach equilibrium in 10 to 20 sec. Since NAS_{11-18} also responds transiently to changes in K, however, at least one and preferably two minutes must be allowed for equilibration (3).

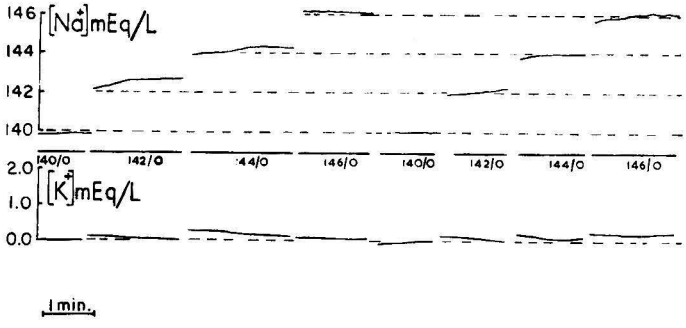


FIG. 3. Responses to stepwise change in Na^+. Ionic concentrations of solutions used are written along horizontal mid-line as Na/K. Values obtained (solid line oscillograph trace) are compared with theoretical (broken line). Two-minute equilibration time allowed between changes.

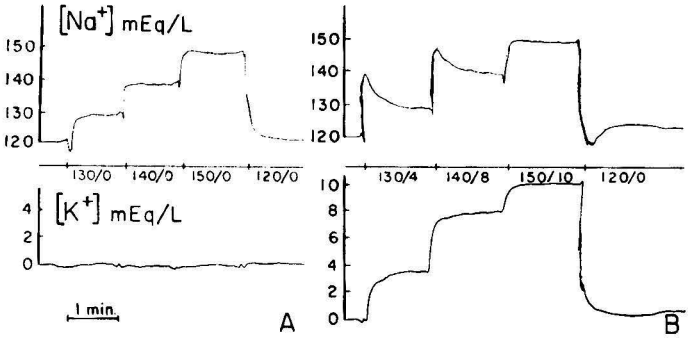


FIG. 4. (A) Response time for changes of Na^+ concentration. (B) Response time for simultaneous changes of Na^+ and K^+ concentrations. Ionic concentrations of solutions used are written along horizontal mid-line as Na/K.

Instrument drift is minimal despite the complexity of the apparatus. When noticeable unidirectional drift does occur it is almost always due to imperfections in the electrode itself. With well-made electrodes, steady drift need not exceed the equivalent of 1 meq/liter of Na or 1 volt/hr in the output of amplifier 3, for example, and may well be less. Accordingly, recalibration carried out after a few hours of continuous monitoring usually requires only minimal adjustment of the baseline. For intermittent operations such as that involved in single sample analysis even greater precision than illustrated here can be achieved by readjusting the baseline with one standard solution interposed between unknowns.

The efficiency of this computer solution evidently depends on the stability of the selectivity factor, k_{NaK}, of the K electrode. In our ex-

perience, this factor is, in fact, quite stable and any noticeable change marks the onset of electrode deterioration. NAS_{27-3} is a soft, easily attacked glass and electrodes made with it have a limited life span of the order of one to three months. The selectivity factor for this glass is around 5 and this is fortunately still sufficiently small to permit a reasonable range of K excursion to be handled by the computer. NAS_{11-18}, by contrast, is a hard, stable glass and electrodes made with it have an indefinite life span.

DISCUSSION

The computer solution for resolving the outputs of two interacting electrodes presented here can obviously be extended to a variety of cases. Experience with the assembly has demonstrated that the usefulness of cation-selective glass electrodes need not be handicapped by laborious calculation. Additionally, the information which we have gained from the assembly provides a natural base from which to design a circuit to handle this problem alone more simply and directly than can be accomplished by a multipurpose analog computer.

SUMMARY

Sodium and potassium activities can be read directly and continuously using an analog computer to handle the information obtained by cation-specific glass electrodes. The solution is quite general and can be modified to suit any similar electrode pair. The calculation involves the linearization of the exponential electrode response using a function generator adjusted to cover a preselected range of values. The assembly is capable of high precision and reproducibility.

REFERENCES

1. Eisenman, G., Rudin, D. O., and Casby, J. U., *Science* **126**, 831 (1957).
2. Friedman, S. M., *Methods Biochem. Anal.* **10**, 71 (1962).
3. Friedman, S. M., Jamieson, J. D., Nakashima, M., and Friedman, C. L., *Science* **130**, 1252 (1959).

ANALYTICAL BIOCHEMISTRY 5, 479–488 (1963)

Fluorometric Determination of Total Phospholipids in Rat Tissues[1]

ROBERT A. HARRIS AND DAVID GAMBAL

From the Veterinary Medical Research Institute, College of Veterinary Medicine, Iowa State University of Science and Technology, Ames, Iowa

Received September 5, 1962

INTRODUCTION

Microgram quantities of tissue phospholipids are commonly determined by the phosphorus analysis of Chen *et al.* (1), Bartlett (2), or Harris and Popat (3). These procedures require the digestion of organic material and the colorimetric determination of inorganic phosphate. An average molecular weight for the phospholipids is assumed for converting the inorganic phosphate to phospholipids.

Rhodamine 6G has been used for the fluorometric determination of detergents by Kondo (4) and for detecting phospholipids on paper chromatograms by Marinetti *et al.* (5). Small concentrations of acids in organic solvents were determined with rhodamine 6GX by Palit (6).

The purpose of this report is to describe a simple, rapid, and sensitive fluorometric procedure for determining quantitatively the total phospholipids in lipid extracts of various rat tissues and for monitoring column eluates for phospholipids.

EXPERIMENTAL

Materials

Solvents. Chloroform and methanol[2] were USP grade solvents and were mixed volume to volume. Chloroform for silicic acid chromatography was stored over anhydrous sodium sulfate. Practical-grade (99.5 + %)2,2,4-trimethylpentane[2] from Eastman Organic Chemicals was used without further purification.

[1] This project was supported in part by a grant from U. S. Public Health Service (GM07301-03, formerly RG-7301). Published as paper U. S. 627 of Veterinary Medical Research Institute, College of Veterinary Medicine, Iowa State University of Science and Technology, Ames, Iowa

[2] The following abbreviations are used: C:M, chloroform:methanol; TMP, 2,2,4-trimethylpentane.

Digestion Mixture. Reagent-grade perchloric, nitric, and sulfuric acids were mixed in the ratio 7:2:1 (v/v), respectively.

Silicic Acid. Prior to use, 3.5 gm silicic acid (Biochemical Research, especially prepared for the chromatography of lipids, 325 mesh), was washed with 15 ml each of methanol and benzene:Skelly B (15:85), and 30 ml chloroform.

Rhodamine 6G Reagent, 0.004% w/v. A chloroform solution for rhodamine 6G (Allied Chemical) was prepared daily and stored in the dark.

Fluorescein Standard, 0.02 μg/ml. An aqueous fluorescein solution was used as a reference for calibrating the photofluorometer.

Photofluorometers. A Coleman model 12 photofluorometer was used for the routine quantitative determination of the phospholipids. The excitation and emission spectra were determined using the Aminco-Keirs spectrophosphorimeter.

METHODS

Extraction of Tissue Lipids. Approximately 0.25 gm rat tissue was homogenized with 80 ml/gm of C:M (1:1) at 0°C in an Omni-Mixer, centrifuged, and the residue re-extracted with 30 ml/gm C:M (2:1). The extracts were combined and washed with 10 ml of 0.01 *M* magnesium chloride solution in glass-stoppered centrifuge tubes and centrifuged at 1500 rpm for 15 min; the chloroform layer was removed and dried over anhydrous sodium sulfate. Plasma (1 ml) was homogenized in a Tenbroeck grinder with 20 vol of C:M (1:3), heated to 60°C, and centrifuged; the residue was re-extracted with 10 vol of chloroform. The extracts were combined and treated like the tissue extracts. The lipid extracts were evaporated to dryness under partial vacuum and the residue was extracted with chloroform and diluted to 25 ml.

Development and Measurement of Fluorescence. Aliquots of the total lipid extracts representing approximately 1 mg tissue or 0.02 ml plasma were evaporated to dryness under nitrogen at 40°C. The tubes were cooled and 100 μl of rhodamine 6G reagent was added to each tube. The lipids were dissolved and the samples were diluted to 10 ml with trimethylpentane, mixed, and placed in a lightproof box. Two blanks, one containing no lipid and another containing lipid but no dye, were prepared simultaneously. The fluorescence was measured in calibrated cuvettes within 10 min using a Coleman photofluorometer with a primary filter (B_2) for an excitation wavelength of approximately 436 mμ and a secondary filter (PC-2) for measuring emission wavelengths longer than 530 mμ. The quantity of phospholipids was obtained from the standard curve of the mixed phospholipids for the specific tissue studied.

Standard Curve. The total mixed phospholipids were isolated from the

lipid extracts of the tissues as described by Borgström (7) and weighed; aliquots containing 10–60 μg were used for a standard curve as shown in Fig. 1. The fluorescence of the unknowns was determined and the quantity of the phospholipids was calculated from the standard curve prepared from the mixed phospholipids of the specific tissue.

Preparation of Pure Phospholipids. Cephalin was isolated from sheep brain (8) and purified by silicic acid chromatography. Lecithin was isolated from egg yolk (8) and purified by alumina chromatography as described by Rhodes *et al.* (9), and was chromatographically pure in the system described by Marinetti *et al.* (10).

Chemical Determination of Phospholipids. The lipid phosphorus was determined according to the procedure described by Chen *et al.* (1). The modified digestion mixture was used and 0.5% sodium hydroxide was added to the tubes after the digestion. Per cent phospholipids was calculated from the phosphorus content assuming an average molecular weight of 750 for the phospholipids.

RESULTS

Optimum Conditions

The volume of chloroform in 10 ml TMP can vary from 20–200 μl without affecting the fluorescence of the phospholipids, as reported in Table 1. The volume of chloroform routinely used was 100 μl/10 ml

TABLE 1
EFFECT OF CHLOROFORM ON FLUORESCENCE OF RHODAMINE 6G

Concentration (μl/10 ml TMP)	Fluorescent units[a]	
	Phospholipids[b]	Blank[c]
10.0	22.5	0.0
20.0	28.5	0.0
50.0	28.7	0.0
100.0	28.5	0.2
200.0	30.0	1.0
1000.0	52.0	24.5

[a] Average of duplicates.
[b] 62 μg phospholipids.
[c] The phospholipids were omitted.

TMP and the optimum amount of rhodamine 6G for these conditions was 2–4 μg, as shown in Table 2.

The fluorphor was photosensitive (Table 3). After a 30-min exposure to light, the fluorescence of the fluorphor was reduced approximately 7%, while the fluorphor prepared and stored in the dark lost only 3% of its

TABLE 2
EFFECT OF RHODAMINE 6G ON FLUORESCENCE OF PHOSPHOLIPIDS

Concentration (μg/100 μl $CHCl_3$)	Fluorescent units[a]
1.0	44.0
2.0	66.0
4.0	64.0
20.0	50.0
100.0	32.0

[a] 62 μg phospholipids. Average of duplicates.

TABLE 3
EFFECT OF LIGHT ON THE FLUORPHOR

Minutes[a]	Fluorescent units[b]	
	Light	Dark
0.2	83.7	83.7
5	82.6	83.4
10	81.5	83.4
15	80.2	82.8
30	77.7	81.6

[a] Reagent was added in an unlighted room and solutions were kept either in a light-proof box or under ordinary laboratory fluorescent lighting.
[b] Average of duplicates.

fluorescence. Therefore, the rhodamine 6G reagent was prepared and added to the samples in an unlighted room and the fluorescence of the samples was determined within 10 min.

Specificity for Phospholipids

The fluorphor was identified as a phospholipid-rhodamine 6G complex since only those lipids containing the acidic phosphate group fluoresced after interaction with the basic dye. This reaction is probably analogous to the determination of ionic organic compounds with oppositely charged ionic dyes (11). The fluorescence was directly proportional to the quantity of the mixed phospholipids, as shown in Fig. 1. Furthermore, the fluorescence of naturally occurring cephalin and lecithin or synthetic L-α-dipalmitoylcephalin[3] was proportional to their amounts (Fig. 2). The phospholipids of the testes were fractionated on silicic acid columns by increasing the amount of methanol in chloroform and collecting 3-ml fractions. Generally 6–18 tubes were collected for each concentration

[3] A generous gift from Dr. Erich Baer.

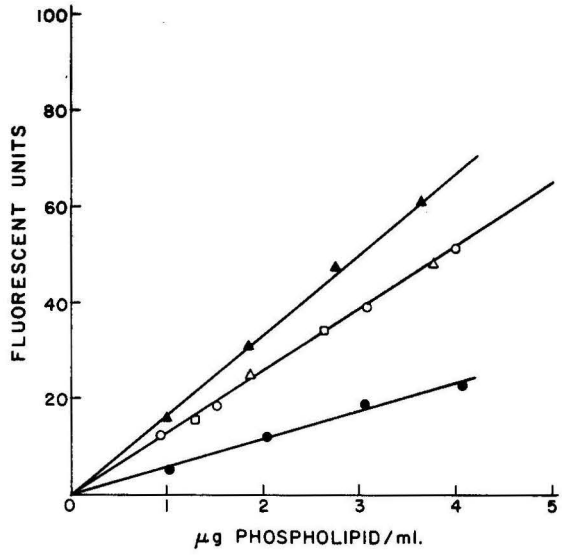


FIG. 1. Standard curves for total phospholipids of brain (▲), lung (□), testes (△), liver (○), and plasma (●).

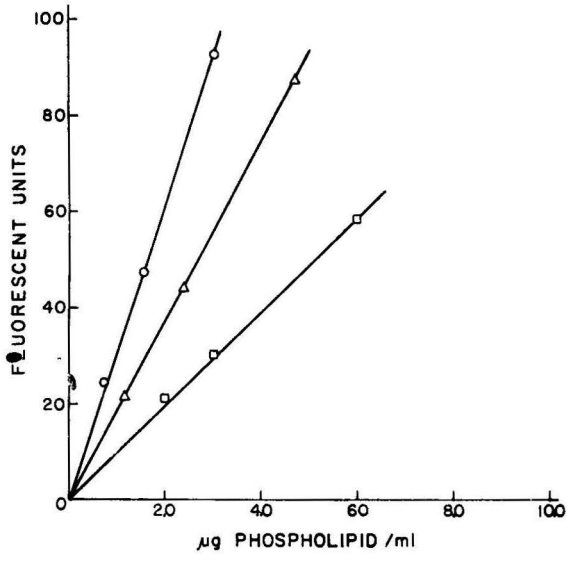


FIG. 2. Standard curves for naturally occurring lecithin (□), cephalin (○), and synthetic dipalmitoylcephalin (△).

of methanol in chloroform. The lipid phosphorus and the fluorescence of each fraction were determined; a relationship between fluorescence and lipid phosphorus is presented in Fig. 3. Only those fractions containing

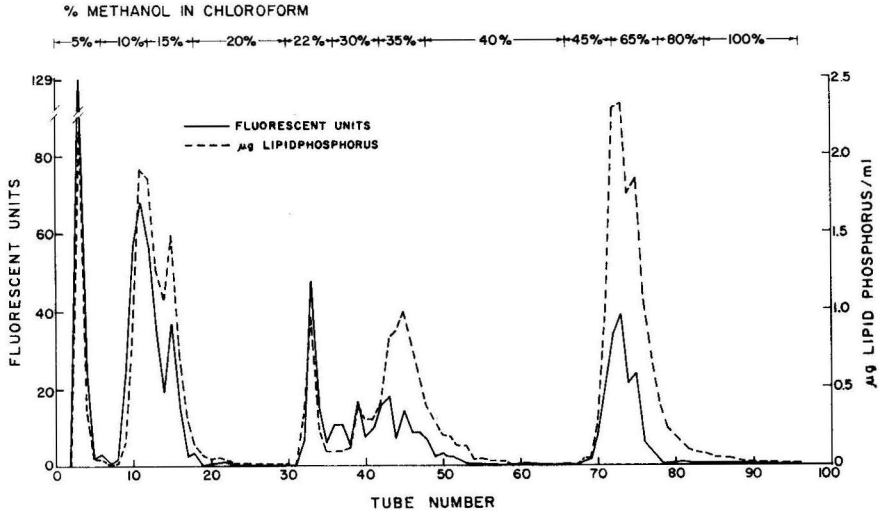


FIG. 3. Chromatography of rat testicular phospholipids on silicic acid column.

lipid phosphorus fluoresced and the fluorescence:lipid phosphorus ratio varied among the individual phospholipids.

The neutral lipid fraction containing free fatty acids, free and esterified cholesterol, mono-, di-, and triglycerides, waxes, and alcohols did not

TABLE 4
EFFECT OF NEUTRAL LIPIDS OF BRAIN AND LIVER ON FLUORESCENCE OF PHOSPHOLIPIDS

Lipid fraction	Per cent phospholipid (wet wt.)		
	Brain	Liver-1[d]	Liver-2[d]
Total lipids[a]	4.76 ± 0.08[e]	1.88 ± 0.02	1.83 ± 0.02
Total phospholipids[b]	5.12 ± 0.06	1.86 ± 0.04	1.90 ± 0.01
Neutral lipids[b]	0.02 ± 0.01	0.00	0.00
Total phospholipids plus neutral lipids[c]	5.26 ± 0.07	1.86 ± 0.01	1.88 ± 0.02

[a] Chloroform:methanol extract of tissue treated as previously described in text.
[b] Isolated by silicic acid chromatography.
[c] 40 μg phospholipids plus 52 μg neutral lipid fraction.
[d] 40 μg phospholipids plus 10–90 μg neutral lipids.
[e] Mean ± standard error.

fluoresce nor did it interfere with the fluorescence of the phospholipids (Table 4). The fluorescence of the total lipids, total phospholipids, or the recombination of the neutral lipid and phospholipids were identical. Cholesterol (7–500 μg) did not fluoresce under these conditions.

The total lipids or the total phospholipids of the tissues had excitation and emission maxima (uncorrected) at 540 and 560 mμ, respectively. Synthetic cephalin had an emission maximum at 556 mμ. The individual phospholipids had emission maxima between 556 and 568 mμ, with the ethanolamine containing phospholipids emitting at the lower wavelength.

Comparison of Methods

The amount of phospholipids in the liver, testes, lung, brain, and plasma of rats was determined by the proposed fluorometric procedure and the chemical determination of lipid phosphorus described by Chen *et al.* (1). In certain instances, the phospholipids were isolated by silicic acid chromatography and weighed. The results obtained by the chemical and fluorometric procedures compared favorably, as shown in Table 5.

TABLE 5
COMPARISON OF METHODS FOR PHOSPHOLIPID DETERMINATIONS

Tissue[a]	Per cent phospholipid (wet wt.)		
	Phosphorus determination	Fluorometric procedure	Silicic acid chromatography
Testes	1.07 ± 0.02[b]	1.23 ± 0.05	—
Lung	1.56 ± 0.09	1.50 ± 0.07	—
Liver	2.23 ± 0.08	2.04 ± 0.09	—
Brain	3.86 ± 0.13	4.70[c] ± 0.16	5.26 ± 0.16
	—	5.20[d] ± 0.12	—
Plasma	0.76	0.90	1.02

[a] Average of 10 rats.
[b] Standard deviation of the mean.
[c] Calculated from fluorescence of total lipids.
[d] Calculated from fluorescence of total phospholipids isolated by silicic acid chromatography.

The largest deviations occurred in the brain and plasma and in these cases the results obtained by the gravimetric and the fluorometric procedures compared more favorably than results obtained by chemical determination. The gravimetric procedure generally gave the highest results. Fluorescence of phospholipids from brain was greater after silicic acid chromatography than the fluorescence of the original total lipid extract (Table 4). Fluorescence of the phospholipids was not decreased

by addition of the neutral lipids; this enhancement of the fluorescence after chromatography was not observed in other tissues.

DISCUSSION

The fluorescence of the blank was negligible. Fluorescence of the phospholipids was constant between 20–200 μl of chloroform, as shown in Table 1. Larger quantities of chloroform were avoided because it increased fluorescence of the blank. However, fluorescence of the phospholipid and the blank was additive and could be employed. Traces of strong organic acids and polar solvents such as methanol also produced high blanks. Rhodamine 6G was extracted from the chloroform-trimethylpentane solution with water and produced erratic results; therefore a one-phase system was required.

A concentration of rhodamine 6G greater than 4 μg/100 μl chloroform greatly decreased the fluorescence (Table 2). The excess dye probably quenched fluorescence by energy transfer between the fluorphor and the excess dye. The optimum ratio of phospholipid to dye was 15–30 to 1 and this ratio should be maintained for maximum fluorescence. The fluorphor and the rhodamine 6G reagent were photosensitive; therefore the reagent was prepared daily and stored in the dark. Repeated excitation in the photofluorometer decreased fluorescence of the fluorphor.

Only the phosphorus-containing lipids in the lipid extracts formed the fluorphor with rhodamine 6G and fluorescence of the fluorphor was used for the quantitative determination of phospholipids in the presence of the neutral lipids, as shown in Table 4. Quantities of neutral lipids 9 times greater than normally found in tissues or 7–400 μg cholesterol did not fluoresce nor affect fluorescence of the phospholipids. The neutral lipid fraction of brain contained less than 1 μg of lipid phosphorus and had a slight fluorescence due to the phospholipids. These values are questionable because of the large dilution factor of 500 and this figure is the lower level of sensitivity of the chemical and fluorometric procedures. The neutral lipid fractions of liver, lung, and testes never fluoresced higher than the blank. The increased fluorescence of the brain lipids after chromatography may be due to a change in the ionic state of either the phospholipids or an inhibitor. The removal of an inhibitor seems unlikely since the fluorescence of the recombined neutral lipids and phospholipids was not decreased but was actually increased. Brain lipids may differ from the lipids of other tissues since silicic acid chromatography affected the fluorescence of brain lipids but not the lipids from other tissues.

Testicular phospholipids were fractionated on silicic acid columns by

increasing the amount of methanol in chloroform. The amount of lipid phosphorus and the fluorescence of each fraction were determined (Fig. 3). Tubes 3–5 and 32–35 were highly fluorescent, whereas other fractions had a lower fluorescence:lipid phosphorus ratio. These results suggest that the fluorescence was a function of the acidity of the phosphate group since peaks 4 and 33, which contained polyglycerol phosphatide and inositol phosphatide, respectively, gave the greatest fluorescence. The cephalin fraction (peak 11) gave a lesser fluorescence and the choline phosphatides (peaks 45 and 73) gave the least fluorescence. These results are in agreement with Fig. 2. The high fluorescence of brain lipids illustrated in Fig. 1 could be explained by the high content of cephalin in brain and the low response of plasma lipids could be explained by their high content of lecithin and sphingomyelin. Since the fluorescent intensities of cephalin and lecithin are different and their fatty acid composition is similar, the specific bases, either choline or ethanolamine, probably affected fluorescence indirectly by affecting ionization of the phosphate group. The choline-containing phospholipids had a lower fluorescence per unit of lipid phosphorus and fluoresced at longer wavelengths than cephalin. Synthetic L-α-dipalmitoylcephalin contained two saturated fatty acids and had a lower fluorescence than the naturally occurring cephalin, which has at least one unsaturated fatty acid; therefore the unsaturation of the fatty acids must also indirectly affect fluorescence. These factors could account for the variation in the slopes of the standard curves in Fig. 2. The phospholipid composition of the tissue should be known before the method is applied to the analysis of the total phospholipids since the difference in the molar response of the phospholipids to the dye is different. However, this technique is simple and rapid and has proved satisfactory for monitoring the column eluates for phospholipids (Fig. 3). In addition, the phospholipids with the greatest acidity had the highest fluorescence (tubes 4 and 33, which contain polyglycerol phosphatide and inositol phosphatide, respectively); therefore this technique may prove useful for identifying the phospholipids relative to their acidity.

Commercial products of L-α-dipalmitoyllecithin and -cephalin fluoresced at low concentrations but the fluorescence was quenched at higher concentrations, suggesting some impurity in the product. Marinetti (10) reported impurities in commercial products.

The results obtained by fluorometric procedure compared favorably with the results obtained by the chemical and chromatographic procedures described in Table 5 and the results presented in the literature (11). The normal range of phospholipids in the liver was 1.75–2.48% in

10 normal adult rats and the variation for 7 determinations on the same liver sample was 1.82–1.94%. Phospholipids could be stored at −15°C for one month without affecting their fluorescence.

SUMMARY

A simple, rapid, and sensitive fluorometric procedure for the quantitative determination of total phospholipids in lipid extracts of rat tissue and for monitoring column eluates for phospholipids is presented. It is based on the fluorescence of the phospholipid-rhodamine 6G complex formed by interacting the acidic phosphate group with the basic dye, and is specific for phospholipids. Cholesterol or neutral lipids from tissues do not affect the fluorescence in any manner. Standard curves of mixed phospholipids are prepared for each tissue investigated because each tissue has a different phospholipid composition and therefore a different fluorescent intensity. Total phospholipids were determined by the chemical, chromatographic, and fluorometric procedures and the results compared favorably.

REFERENCES

1. CHEN, P. S., JR., TORIBARA, T. Y., AND WARNER, H., *Anal. Chem.* **28,** 1756 (1956).
2. BARTLETT, G. R., *J. Biol. Chem.* **243,** 466 (1959).
3. HARRIS, M. D., AND POPAT, P., *J. Am. Oil Chemists' Soc.* **31,** 124 (1954).
4. KONDO, T., *Nippon Kagaku Zasshi* **77,** 1281 (1956); *Chem. Abstr.* **51,** 10187 (1957).
5. MARINETTI, G. V., ERBLAND, J., ALBRECHT, M., AND STOTZ, E., *Biochim. et Biophys. Acta* **30,** 543 (1958).
6. PALIT, S. R., *Anal. Chem.* **33,** 1445 (1961).
7. BORGSTRÖM, B., *Acta Physiol. Scand.* **25,** 101 (1952).
8. "Practical Physiological Chemistry" (P. B. Hawk, B. L. Oser, and W. H. Summerson, eds.), pp. 260–261. Blakiston, Philadelphia, 1951.
9. RHODES, D. N., AND LEA, C. H., *Biochem. J.* **65,** 526 (1957).
10. MARINETTI, G. V., *J. Lipid Res.* **3,** 1 (1962).
11. MUKERJEE, P., *Anal. Chem.* **28,** 870 (1956).
12. "Biochemists Handbook" (C. Long, ed.), pp. 644, 629, 862. Van Nostrand, Princeton, 1961.

ANALYTICAL BIOCHEMISTRY 5, 489–496 (1963)

Liquid Scintillation Counting: The Determination of Background Counts of Samples Containing Quenching Substances

B. SCALES

From the Pharmaceuticals Division, Imperial Chemical Industries Ltd., Alderley Park, Cheshire, England

Received September 17, 1962

INTRODUCTION

The determination of low levels of radioactivity in animal tissues following the administration of drugs labeled with C^{14} or H^3 is a frequently occurring problem. The direct assay of dissolved tissues by liquid scintillation counting using Herberg's (1) methods of sample preparation is convenient and reliable although steps must be taken to ensure that no volatile radioactive component is lost at any point during sample preparation. However, the colored materials from the dissolved tissue cause severe quenching, and the counting efficiency of each sample must be determined by the addition of an internal standard.

The variation in the degree of quenching of different samples also affects the background count, and for this reason, when low activities are being determined, it is usual to estimate the background count for each tissue by counting a duplicate sample, identical in all respects except that it lacks radioactivity. During the examination of many tissue samples by this method it became clear that the reproducibility of the background count from samples of the same tissue was occasionally very unsatisfactory, and it was evident that errors far outside the statistical errors could be unwittingly introduced by use of incorrectly estimated background counts.

This paper describes a method by which the background count of H^3- and C^{14}-containing tissue preparations can be determined without direct reference to duplicate preparations of inactive samples.

The principle of the method has already been mentioned by Domer and Hayes (2) in connection with the counting of suspensions of C^{14}-containing tissues, but these authors published no detailed account of its general applicability.

The present paper shows how the method can be extended to C^{14} and also to H^3 in solution, and attempts to give some idea of the errors in-

troduced when background determinations are carried out by this method.

MATERIALS AND METHODS

Chemicals and Phosphors. Sulfur-free toluene (May and Baker Ltd.) was used without further purification. Commercial dioxane was purified by Vogel's (3) method. 2,5-Diphenyloxazole (PPO), 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP), and a solution (1 *M*) of hydroxide of Hyamine 10-X in methyl alcohol were purchased from Packard Instrument Ltd., Wembley; naphthalene (scintillation grade) was obtained from Nash and Thompson Ltd., Surbiton, Surrey.

Two phosphor solutions were used in these studies: one, a solution of double the optimum concentration, contained PPO (9.0 gm/liter) and POPOP (0.20 gm/liter) in toluene. The other was a solution of naphthalene, PPO, and POPOP in a mixture of dioxane, methyl alcohol, and toluene (1)—hereafter called Diotol.

Preparation of Quenched Samples. CCl_4 (0–10 ml) or portions of a 1% solution of an alkyl ferrocene in toluene (0–10 ml) were added to vials containing 10.0 ml of the double-strength phosphor solution, and, where possible, the volume was made up to 20.0 ml with toluene.

Tissue samples were prepared by the two methods of Herberg (1) with slight modifications. In the first of these, samples of sheep or rat brain, liver, spleen, heart, kidney, fat, skin, muscle or marrow (not more than 0.35 gm) or plasma or whole blood (not more than 0.4 ml) were measured directly into the counting vials and were then gently shaken overnight in an oven at 45°C with 3.0 ml of a 1 *M* solution of hydroxide of Hyamine 10-X in methyl alcohol. After cooling to room temperature, hydrogen peroxide (30%, 0.05–0.25 ml) was added to the most highly colored samples, which were then left at room temperature for 15 min. Any excess hydrogen peroxide was destroyed by heating the samples at 45° for 15 min. Synthetic absolute ethyl alcohol (5.0 ml), double-strength phosphor (10.0 ml), and concentrated hydrochloric acid (0.30 ml) were added to the tissue solutions, which were then cooled to 0° prior to counting.

In the second method, whole tissue, blood, and plasma samples were dissolved by shaking at 45°C for 16 hr with ethanoïc KOH (3.0 ml, 0.5 *M*) and partially decolorized with hydrogen peroxide, as in the first method. When excess of hydrogen peroxide had been destroyed by further heating, the Diotol phosphor system (15 ml) was added, followed by concentrated hydrochloric acid (0.25 ml). The samples were thoroughly shaken and centrifuged at $500 \times g$ for 10 min at 0° to pack the precipitated potassium chloride firmly at the base of the vials.

Counting Equipment and Estimation of Radioactivity. All measurements were made with a Tri-Carb Model 314 EX, Liquid Scintillation Spectrometer, and the sample containers were standard 20-ml glass screwcap vials of low potassium content (Packard Instrument Ltd., Wembley).

Samples were assayed separately for C^{14} and H^3 in channel I at high-voltage taps 5 (1045 volts) and 8 (1280 volts), respectively. These high-voltage settings gave optimum counting conditions when the two isotopes were assayed in a simple toluene-phosphor system with the discriminators of channel I set at 10–100. The discriminators of channel II were set at 24.0–100 for C^{14} and 37.5–100 for H^3 counting; these settings were chosen so that for quenched samples the ratio of net counts in channel II to those in channel I was almost directly proportional to the counting efficiency (4).

Samples were counted with a refrigerator temperature of 0°C and all low activity samples were counted for 100 min. The counting efficiency of these samples was then determined by the use of radioactive standards. The samples were "spiked" by means of a micrometer syringe, with 0.100-ml portions of toluene solutions of either (1,2-H^3) hexadecane (nominally 10^5 dpm) or (carboxy-C^{14}) benzoic acid (nominally 10^4 dpm) and then counted for 10 min each.

RESULTS AND DISCUSSION

Background Variation and Quenching Agents

The background count obtained for any sample in a liquid scintillation counter can be considered as being made up of two components. The first of these is due to electronic noise; it can account for 20–100% of the total background count, and it is almost independent of the presence of the counting sample and its containing vial. The second component is due in part to cosmic rays which have penetrated the shielding and to disintegrations from K^{40} in the glass of the vial. The average energy of these particles is sufficient to cause the simultaneous formation of photoelectrons in both phototubes, and, after amplification, the resulting pulses will therefore pass through the coincidence circuit and the discriminator windows to be registered on the scalers.

The processes giving rise to the second component of the background count are presumably just as liable to quenching as are the processes which occur within the counting sample after the decay of a H^3 or C^{14} atom, and most of the variation is likely to be due to this component of the background count. Thus a sample with the least quenching would give the highest counting efficiency and background count, whereas a

highly quenched sample would have a low counting efficiency and a low background count, approaching the count due to electronic noise. Our investigations have shown these suppositions to be correct, in so much that there is a correlation between background count and counting efficiency, although it is far from linear (Figs. 1 and 2).

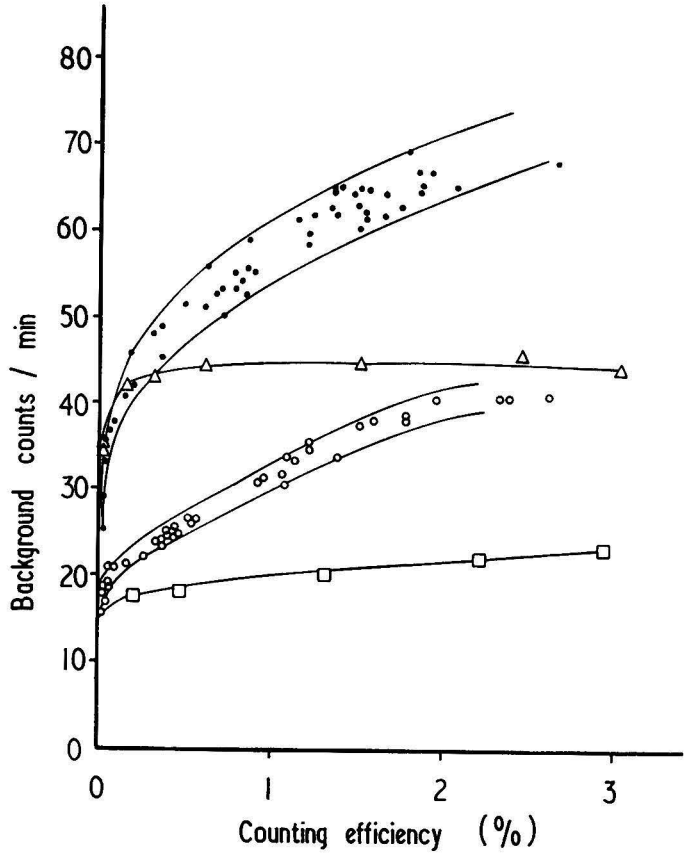


FIG. 1. Counting efficiency curves of quenched tritium-containing samples, showing variation of background count with counting efficiency: (△) CCl_4-quenched samples; (□) samples color-quenched with an alkyl ferrocene; (○) tissue samples prepared using hydroxide of Hyamine 10-X as a solubilizing agent; (●) tissue samples prepared using ethanolic KOH as a solubilizing agent.

Two distinct types of quenching agents exist: chemical and color. The chemical quencher acts by decreasing the efficiency with which energy is transferred from solvent to solute, and the color quencher, by absorption of light emitted from the phosphors. The curves in Figs. 1 and 2 show the different effects on H^3 and C^{14} counting efficiency of increasing the

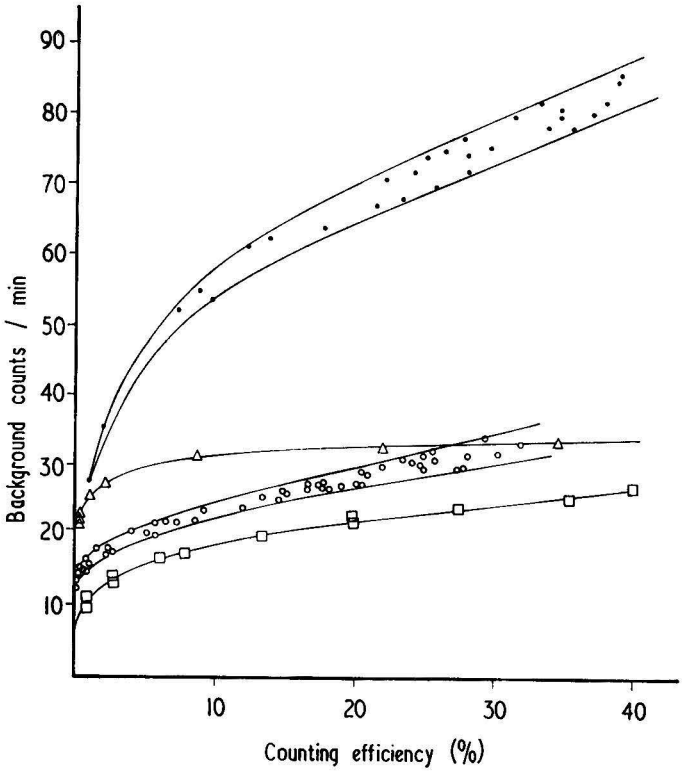


FIG. 2. Counting efficiency curves of quenched C^{14}-containing samples, showing variation of background count with counting efficiency: (△) CCl_4-quenched samples; (□) samples color-quenched with an alkyl ferrocene; (○) tissue samples prepared using hydroxide of Hyamine 10-X as a solubilizing agent; (●) tissue samples prepared using ethanolic KOH as a solubilizing agent.

concentrations of one substance which causes quenching by virtue of its color (an alkyl ferrocene), and of carbon tetrachloride which causes chemical quenching. There is little similarity between the curves obtained for the two types of quenchers although, for each one, the counting efficiency accurately reflects the background count. With the color quencher the background count falls rapidly as the counting efficiency declines, while with the chemical quenchers the background is not greatly reduced until the quenching (as determined by drop in counting efficiency) has become severe. Differences between the two types of quenchers are evident when counting efficiencies of quenched samples are determined by the shift in pulse height (4), but the exact cause of the difference is not known. Counting efficiences can usually be determined with much greater accuracy than the count rate of inactive samples, and the prin-

cipal errors in these curves should therefore be statistical errors dependent on the total number of counts obtained during each background determination. With the aid of a suitable calibration curve the background count of a weakly active sample containing only one quenching variable can therefore be determined from a knowledge of its counting efficiency to almost the accuracy of the calibration curve.

Figures 1 and 2 also contain curves obtained using two different methods of tissue preparation. Owing to the more variable quenching effect of substances derived from animal tissues, the scatter is greater than the expected statistical variation. However, the experimental points lie in a relatively narrow area, and the small uncertainty in the relationship between background count and efficiency is not serious.

The maximum amount of tissue and hydrogen peroxide added to the vials is governed by the necessity for maintaining a single-phase system, but variations in amount of either of these components below these limits only resulted in a combined change of efficiency and background count within the areas shown.

When inactive whole tissues were solubilized using hydroxide of Hyamine 10-X, and assayed under H^3- or C^{14}-counting conditions, the maximum error in estimating the background counts of active samples was no more than $\pm 4\%$ over the full range of background count, from a minimum of 15 cpm for C^{14} to a maximum of 41 cpm for H^3 (Figs. 1 and 2). The maximum H^3 background count obtained for tissue samples prepared in hydroxide of Hyamine 10-X closely approaches that obtained for unquenched samples prepared in a simple toluene-phosphor system; and a maximum background count of 34 cpm was obtained for similar samples when assayed under C^{14}-counting conditions.

When tissues were prepared by dissolution in ethanolic potassium hydroxide solution, there was an over-all increase in background count due to the presence of K^{40}. The background counts of C^{14} samples ranged from about 30 to 84 cpm and could be determined from the calibration curve with no more than a $\pm 4\%$ error. For H^3 samples, the background counts covered the range of about 30 to 70 cpm, but the scatter of points in the calibration curve was greater and the error on the estimated background count was $\pm 6\%$.

Summary of the Correction Method

1. A variety of different tissues of the type under investigation, but which are free from radioactivity, are dissolved and incorporated in the phosphor solution by the selected method. *2.* The rate of counting (background) is determined for each tissue-phosphor solution. The results may vary over a 2- to 3-fold range. *3.* A known amount of the appro-

priate radioactive element is added to each tissue-phosphor solution and the rate of counting is again measured. *4.* The counting efficiency is calculated for each sample from the difference in rate of counting between operations *3* and *2*. *5.* A calibration curve is plotted relating counting efficiency to background count. The points will show a limited scatter not due to statistical counting errors. *6.* Samples of similar tissues containing unknown amounts of radioactivity are dissolved, incorporated in phosphor solution, and counted under exactly similar conditions. *7.* Known amounts of the radioactive element are added to the test solutions and the counts are repeated. *8.* From the difference in the count rate of operations *7* and *6* the efficiency of counting is determined. *9.* By using the calibration curve the background corresponding to the determined efficiency is read off. *10.* This background count from operation *9* is subtracted from the count measured in operation *6* to give the count due to the radioactivity in the sample. *11.* The number of disintegrations per minute (dpm) in the sample is calculated from the count determined by operation *10* and the efficiency found in operation *8*.

This method of background determination has been applied to more than five hundred H^3- or C^{14}-containing tissue samples. Typical results for four C^{14}-containing tissues, prepared using hydroxide of Hyamine 10-X as the solubilizing agent, are shown in Table 1. The figures illus-

TABLE 1

INFLUENCE OF ESTIMATED BACKGROUND COUNT ON REPRODUCIBILITY OF CALCULATED DISINTEGRATION RATES

Determination of the disintegration rate of C^{14} in some tissue samples taken from sheep dosed with (β-C^{14})2-(β-methoxyethyl)pyridine at about 2.22×10^7 dpm/kg. Errors to 95% confidence limits are shown (±).

Tissue	Wet wt. (gm)	$(R_b) + (R_s)$ Total counting rate of sample (cpm)	Percentage counting efficiency	(R_b) Estimated background counting rate (cpm)	(R_s) Counting rate due to residual C^{14} (cpm)	Dpm/gm
Heart	0.3217	30.5	11.3	24	6.5 ± 1.5	179 ± 41
	0.3067	32.2	13.3	25	7.2 ± 1.5	177 ± 40
Liver	0.3415	31.7	6.5	21.5	10.2 ± 1.5	459 ± 69
	0.2941	42.6	14.1	25.5	17.1 ± 1.5	413 ± 37
Muscle	0.2842	38.6	21.3	28.5	10.1 ± 1.6	167 ± 27
	0.2794	33.6	16.4	26.5	7.1 ± 1.6	155 ± 36
Fat	0.3105	41.8	27.5	31.5	10.3 ± 1.7	121 ± 20
	0.3417	38.5	23.1	29.5	9.0 ± 1.7	114 ± 22

trate the very different counting rates and counting efficiencies often found for duplicate samples of the same tissue. When the suggested method of background determination, using the appropriate calibration

curve in Fig. 2, is applied to these figures, most of the apparent irregularities of the duplicate sample counts are smoothed out, and the reproducibility of the specific activities (dpm/gm) is highly satisfactory.

Because many different phosphor systems were being used while this work was in progress, it was convenient to employ the high-voltage settings which were optimum for counting H^3 and C^{14} in a simple toluene-phosphor system. It is possible that the errors in background estimation obtained by use of the calibration curves would have been smaller if the high voltage had been adjusted to balance-point conditions characteristic of the two systems used for the whole tissue preparations.

The results obtained with solutions of C^{14}-containing tissues bear a close similarity to those of Domer and Hayes (2), who used 0.1-gm suspensions of finely divided material for their investigations. H^3-containing material cannot be counted very satisfactory in suspension because of the high degree of self-absorption of the β-particles; counting efficiencies are therefore very low indeed, and when determined, as is customary, by the addition of internal standards, they are invariable falsely high. One advantage to be gained therefore by the use of tissue solutions, rather than suspensions, is that the suggested method of background determination becomes immediately applicable to both C^{14}- and H^3-containing materials.

SUMMARY

1. A method is described for the accurate estimation of the background count of samples containing quenching substances assayed by liquid scintillation counting techniques. It is derived by measurement of the counting efficiency of the active samples, and application of a correlation curve of background count versus efficiency, constructed by use of inactive samples.

2. The background count of C^{14}- and H^3-containing tissue samples, prepared using hydroxide of Hyamine 10-X, could be estimated with an accuracy of at least $\pm 4\%$.

3. The background count of tissue samples prepared by dissolution in ethanolic potassium hydroxide could be determined with an accuracy better than $\pm 4\%$ for C^{14}- and better than $\pm 6\%$ for H^3-containing tissues.

REFERENCES

1. Herberg, R. J., *Anal. Chem.* **32**, 42 (1960).
2. Domer, R. F., and Hayes, F. N., *Nucleonics* **18**, 100 (1960).
3. Vogel, A. I., "A Textbook of Practical Organic Chemistry," p. 175. Longmans, Green, London, 1951.
4. Baille, L. A., *Intern. J. Appl. Radiation Isotopes* **8**, 1 (1960).

ANALYTICAL BIOCHEMISTRY 5, 497–504 (1963)

Studies in Histochemistry, LXXI. Measurement of Protein in Millimicrogram Amounts by Quenching of Dye Fluorescence[1]

TOSISUKE HIRAOKA[2] AND DAVID GLICK

From the Division of Histochemistry, Department of Pathology, Stanford University Medical School, Palo Alto, California

Received September 20, 1962

In an earlier study (1), a dye-binding method for the determination of protein was described in which bromsulfalein was employed for colorimetric analysis with a sensitivity in the millimicrogram range of protein-nitrogen (P-N). This method, based on measurement of excess dye remaining after precipitation of dye-protein complex, has been used routinely in our laboratory over the past nine years for measurements on microtome sections of tissue, and more recently in application to measurement of precipitin reactions with μl-volumes of antiserum (2). The present investigation was undertaken to obtain increased sensitivity by the use of a fluorescent dye which would permit exploitation of the more sensitive measurement of fluorescence.

Preliminary experiments established that direct measurement of the loss of fluorescence, due to removal of a portion of the fluorescent dye by precipitation with protein, was a less sensitive procedure than measurement of the degree of quenching of the fluorescence caused by protein without precipitation. The most promising of a number of dyes tested proved to be eosin Y, and quenching of its fluorescence was used in a method for the determination of protein. The useful ranges of the methods, expressed in mμg P-N/μl original solution, are: bromsulfalein colorimetric method, 10–100 (0.2–2 mμg/μl final solution measured); and eosin Y fluorometric method, 0.1–20 (0.06–6). The precision of the methods is within a few per cent (standard error: colorimetric, 1.4%; fluorometric, 2.6%).

Betheil (3) employed a fluorometric method for determination of albumin in samples of human blood serum down to 5 μl. The analysis was

[1] This investigation was supported by research grants, No. HE 06716 and RG 9227, from the National Institutes of Health.

[2] On leave from the Botanical Institute, College of Science, Kyoto University, Kyoto, Japan.

based on the increase in the fluorescence of vasoflavine in alkaline solution due to the presence of the albumin. Other proteins in the serum had no influence. Analysis of the brain ganglioside, strandin, by fluorescence quenching of acridine dyes was reported by Harris *et al.* (4).

METHODS

An Aminco-Bowman recording spectrophotofluorometer with slit arrangement No. 5 was used for determination of activation and fluorescence spectra and for measurements of fluorescence intensity. In determinations of samples the instrument was adjusted with a standard quinine sulfate solution (845 mμg/ml 0.1 *N* H_2SO_4) to give 40% transmission at 450 mμ with a meter multiplier setting of 0.3 and excitation at 350 mμ. Per cent transmission times multiplier setting is a measure of fluorescence and is used for "fluorescence units." Three kinds of cuvettes were used according to sample volumes, rectangular quartz cuvettes of 1-cm light path for about 4 ml (used only for setting instrument with the quinine solution), quartz tubes of 7-mm bore for 0.6 ml, and Pyrex glass tubes of 3-mm bore for 40 μl. The latter required a special adapter not supplied with the instrument which, designed by L. J. Greenberg and D. Glick, consists of a blackened brass block (12 × 12 × 20 mm) with a 5-mm bore hole, to hold the sample tube, extending down from the top to within about 1 mm of the bottom. A hole, 2 mm in diameter, is drilled near the bottom (center of hole 4 mm from bottom) through each of two adjacent sides to the center hole. The exciting and fluorescent beams at 90° to one another pass through these holes. A rod (12 mm long, 3 mm in diameter) mounted on top of the block at one corner serves as a handle. For manipulations with μl volumes of liquid, constriction pipets and small reaction tubes (4 × 25 mm) were used. Vibration mixing was used for liquids in the small tubes.

The dyes, eosin B, erythrosin B, phloxine, and rose bengal, were found to have relatively weak fluorescence under the conditions required, but Mercurochrome and eosin Y were more suitable. Eosin Y was employed in the present work because it could be obtained commercially in a purer form.

For determination of optimal pH conditions, a series of solutions were prepared in which 0.4 ml of 1×10^{-5} *M* eosin Y was made up to 1 ml in each case with borax buffers (0.2 *M* borax-0.1 *N* HCl or NaOH for pH 9.2 to 11.6) and phosphate-citrate buffers (0.2 *M* Na_2HPO_4-0.1 *M* citric acid for pH 2.2 to 8.2).

In testing the method, the following purified proteins were used, bovine plasma albumin (Fraction V, Armour), porcine plasma (Fraction IV-3, 4, Armour), human anti-blood group A globulin (Ortho), and egg

albumin (Difco). In addition, tissues from albino rats were used. Fresh samples of the latter (0.2–0.5 gm) were treated with 10 ml of 1 *N* NaOH for 1 hr at room temperature, and any insoluble material was removed by centrifugation. The supernatant fluid was used without precipitation of the protein, or a portion (5 ml) was neutralized with 6 *N* HCl and 30% trichloroacetic acid (TCA) was added to yield a final concentration of 5%. The precipitated protein was centrifuged, washed twice with cold 5% TCA, and redissolved in 1 *N* NaOH, bringing the solution to the original 5-ml volume. The nitrogen content of this protein solution, as well as that of the others, was determined by Kjeldahl analysis. In some experiments, fresh frozen circular microtome sections (1 mm diameter, 4 or 16 μ thick) of rat liver were used.

RESULTS

Protein Effect on Activation and Fluorescence Spectra of Eosin Y as a Function of pH. From Fig. 1 it is apparent that maximum activation occurred at 518 mμ, and maximum fluorescence at 540 mμ with a pH of 3.1. Separate spectral curves of the fluorescence produced by activation at each of the peaks shown in curve 1 (259, 302, 342, 412, 490, and 518 mμ) established that, in all cases, maximal fluorescence occurred at 540 mμ. The activation and fluorescence peaks were greatly reduced in the presence of protein. A control experiment in which the dye was omitted from the solutions, with and without protein, revealed that activation at 518 mμ and measurement at 540 mμ gave negligible readings, indicating that no appreciable scattering of the activation beam occurred to influence the measurement.

In a preliminary experiment, the influence of pH on the fluorescence was found to be negligible between pH 5.1 and 11.6, the region of greatest fluorescence. However, as will be shown, the optimal pH for measurement of protein by fluorescence quenching is 3.1, and at this pH the fluorescence of the dye is only about 60% of the maximum at higher pH values.

For all of the experiments to follow, and for the method developed, the excitation and fluorescence wavelengths used for measurements were 518 and 540 mμ, respectively.

Comparison of Protein Quenching Effects at Various pH Values and Eosin Y Concentrations. From Table 1 it may be seen that the greatest quenching effect with bovine serum albumin was obtained at pH 3.1. A plot of loss of fluorescence against log of final protein concentration at each pH value showed that a linear relationship obtained over the following ranges (μg N/ml): 0.08–0.81, pH 2.1; 0.08–1.62, pH 3.1; 0.16–1.62, pH 4.1; 0.81–1.62, pH 6.1. The fact that the range of protein con-

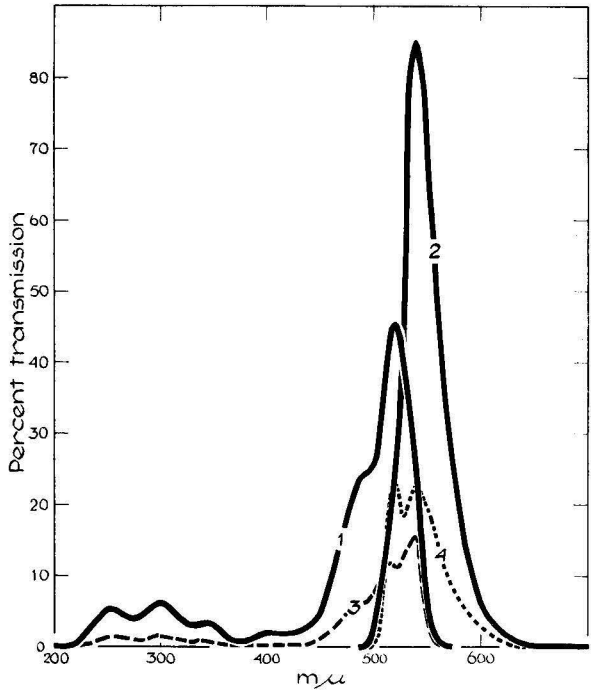


FIG. 1. Activation and fluorescence spectra of eosin Y at pH 3.1, and the quenching effect of protein. (1) Activation spectrum of eosin Y (0.6 ml 0.5 M NaCl + 0.4 ml 4×10^{-5} M dye in 0.06 M citric acid-0.12 M disodium phosphate), fluorescence recorded at 540 mμ. (2) Fluorescence spectrum of eosin Y (same as in curve 1) activated at 518 mμ. (3) Activation spectrum of eosin Y-protein (0.6 ml rat adrenal protein solution, 10 μg N/ml, in 0.5 M NaCl + 0.4 ml 4×10^{-5} M dye in buffer as for curve 1), fluorescence recorded at 540 mμ. (4) Fluorescence spectra of eosin Y-protein (same as in curve 3) activated at 518 mμ. Settings of fluorometer sensitivity and meter multiplier, 25 and 0.3, respectively (curves 1, 3), and 43.5 and 0.3 (curves 2, 4).

TABLE 1

LOSS OF FLUORESCENCE OF EOSIN Y IN BUFFERS OF VARIOUS pH VALUES AS A FUNCTION OF CONCENTRATION OF BOVINE SERUM ALBUMIN

(0.4 ml 4×10^{-6} M buffered eosin Y + 0.6 ml albumin in 1.0 M NaCl)

Final protein concn. (μg N/ml)	Decrease in fluorescence units[a]			
	pH 6.1	pH 4.1	pH 3.1	pH 2.1
0.02	0.0	0.0	0.1	0.0
0.08	0.0	0.0	0.3	0.1
0.16	0.0	0.1	0.6	0.2
0.81	0.3	1.0	1.5	0.3
1.62	0.4	1.4	1.9	0.3

[a] Per cent transmission × multiplier setting.

centrations over which useful measurements could be made was greatest at pH 3.1, as well as the greater magnitude of the quenching effect at this pH, led to adoption of pH 3.1 for the analytical method.

When different concentrations of the dye were made up in the buffer at pH 3.1, and the quenching effects of various concentrations of bovine serum albumin were measured, the greatest effect was obtained with the dye concentration of $4 \times 10^{-5} M$. For all of the dye concentrations, a linear relationship existed between loss of fluorescence and log of the final protein concentration over the range 0.08–1.62 μg N/ml.

Stability of the Buffered Eosin Y-Saline Solution and of the Protein Quenching Effect. A decay of the fluorescence as a function of time of standing (about 7.5% of the first hour) was found after dilution of the $1 \times 10^{-4} M$ dye to $4 \times 10^{-5} M$ with buffer (pH 3.1). When 4 vol of buffered dye solution, aged for 1 hr, was mixed with 6 vol of 0.5 M NaCl or protein-saline solutions (10 μg N/ml in 0.5 M NaCl) and the mixtures were allowed to stand for 1 hr, no significant change in fluorescence occurred with the NaCl alone or with egg albumin, but with adrenal protein the fluorescence value became approximately double. The variability

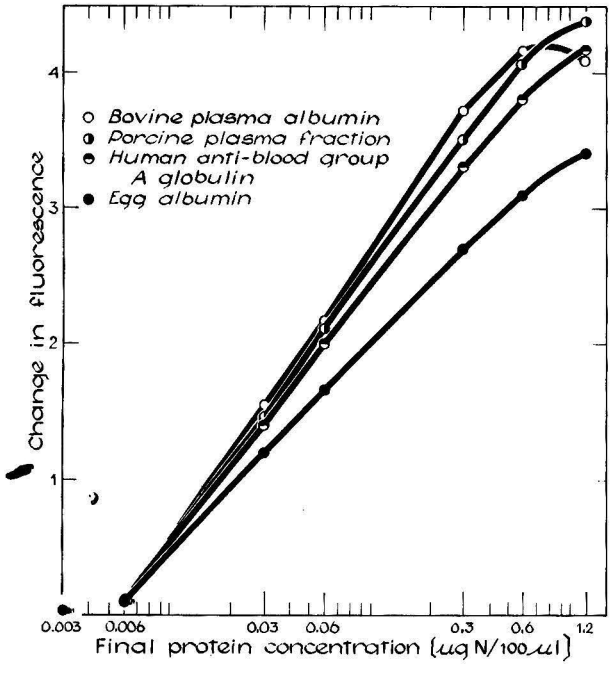


FIG. 2. Changes of fluorescence of eosin Y by partially purified proteins. Composition of solutions as for curve 3, Fig. 1. Fluorescence in units of per cent transmission × multiplier setting.

of these effects emphasizes the need for careful standardization of the procedure. The routine that was adopted was preparation of the buffered dye solution exactly 1 hr before use, and this solution was mixed with 0.5 *M* NaCl for a blank, and with protein in 0.5 *M* NaCl for analysis, exactly 10 min before measurement of fluorescence.

Comparison of Quenching Effects of Partially Purified Proteins and Proteins from Rat Organs. Relative effects of four different partially purified proteins and proteins from six rat organs on the quenching of the fluorescence of eosin Y are given in Figs. 2 and 3. The response il-

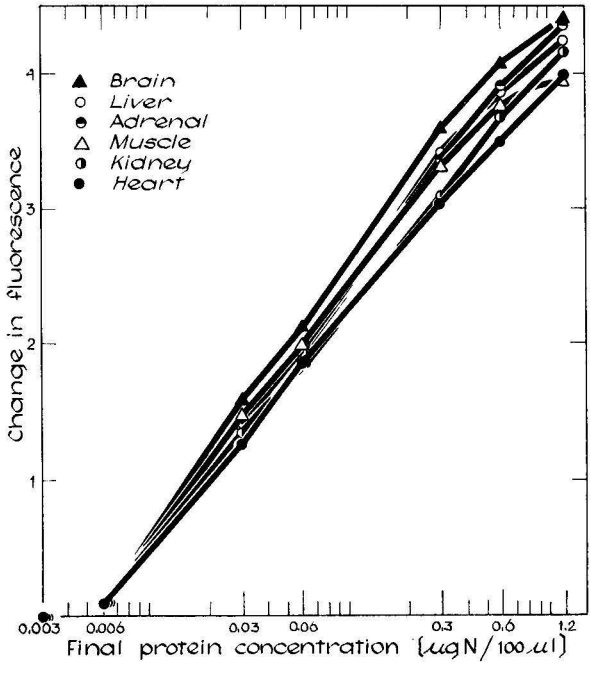


FIG. 3. Changes of fluorescence of eosin Y by proteins from rat organs. Composition of solutions as for curve 3, Fig. 1.

lustrated between protein concentrations of 0.006 to 0.6 μg N/100 μl, the relatively large magnitude of the response per unit of protein, and the nonspecific nature of the effect with the different proteins tested provide the basis of a useful analytical method.

The influence of TCA precipitation of the proteins from three rat organs on the quenching ability is apparent in Table 2. In most cases a slight increase in quenching was observed compared to the effect of the native protein.

TABLE 2

LOSS OF FLUORESCENCE OF BUFFERED EOSIN Y AS A FUNCTION OF CONCENTRATION OF NATIVE AND TCA-PRECIPITATED PROTEINS[a]

(4 vol buffered eosin Y, 4×10^{-5} *M*, pH 3.1, + 6 vol protein in 0.5 *M* NaCl)

Final protein-nitrogen concn. (μg/100 μl)	Decrease in fluorescence units					
	Adrenal		Liver		Kidney	
	Native	TCA ppt.	Native	TCA ppt.	Native	TCA ppt.
0.003	0.1	0.1	0.1	0.0	0.1	0.0
0.006	0.1	0.1	0.1	0.1	0.1	0.1
0.03	1.4	1.4	1.4	1.4	1.3	1.4
0.06	1.9	2.0	1.8	1.9	1.7	1.9
0.3	3.2	3.4	3.2	3.4	3.0	3.1
0.6	3.7	3.9	3.7	3.9	3.5	3.7
1.2	4.0	4.3	4.1	4.2	4.1	4.1

[a] Dissolved in 1.0 *N* NaOH, neutralized with 1.0 *N* HCl, and diluted with 0.5 *M* NaCl.

FINAL PROCEDURE FOR FLUOROMETRIC ANALYSIS OF PROTEIN

Reagents: 1.0 *N* NaOH, 1.0 *N* HCl, 0.1 *M* citric acid-0.2 *M* disodium phosphate buffer (pH 3.1), 4×10^{-5} *M* buffered eosin Y. (Prepare a stock soln. of 0.001 *M* eosin Y, Harleco, C.I. No. 768, 96% dye content, in water and store in a siliconed, glass-stoppered, glass bottle kept in a refrigerator. This solution is stable for 1 month. Dilute the stock solution to 1×10^{-4} *M*. Prepare the final solution by mixing 4 vol of the latter with 6 vol of the citric acid-phosphate buffer exactly 1 hr before adding to the protein solution in the analysis, step 3 in *Procedure.*)

Procedure:

1. Place sample (e.g., tissue section, 1-mm diameter, 4–16 μ thick) in bottom of reaction tube (15 mm long, 4-mm bore), add 40 μl of 1.0 *N* NaOH, mix, let stand at room temperature for 1 hr, and mix again. Centrifuge, if solution is not clear.
2. Neutralize a 10-μl aliquot with 10 μl of 1.0 *N* HCl, add 40 μl of the buffer (pH 3.1), and mix.
3. Add 40 μl of the buffered eosin Y and mix.
4. Transfer the clear liquid to the cuvette and measure the fluorescence at 540 mμ (activation at 518 mμ) exactly 10 min after addition of buffered eosin Y (step 3).
5. Run blank in which all steps are followed with omission of sample.
6. Subtract fluorescence value of unknown from that of blank to obtain quenching value and compare to standard curve.
7. Obtain standard curve by preparing a solution of the protein sample in 1.0 *N* NaOH; use an aliquot for measurement of Kjeldahl nitrogen

and another for the procedure (steps 2–6). Plot loss of fluorescence against log of protein-nitrogen. (The curve need not be redetermined as long as the same kind of sample is employed and the same batch of dye is used. Inclusion of the analysis of a standard protein solution of known nitrogen content would serve to standardize each series of determinations.)

Note: Figures 2 and 3 give standard curves for the protein materials indicated. In a test of the method, 5 circular sections of rat liver (1-mm diameter, 4 μ thick) were analyzed individually with a standard error of 2.6%.

SUMMARY

A study of conditions for the determination of protein, based on its effect in quenching the fluorescence of certain dyes, was made. A procedure was elaborated by which the quenching of the fluorescence of eosin Y provided a method for the determination of protein in the range of 0.1 to 20 mμg of protein-nitrogen per μl of sample solution (0.06–6 mμg/μl final solution measured) which gave a standard error of 2.6%.

ACKNOWLEDGMENT

The authors wish to thank Dr. Leonard J. Greenberg for his assistance in the course of this investigation.

REFERENCES

1. NAYYAR, S. N., AND GLICK, D., *J. Histochem. Cytochem.* **2**, 282 (1954).
2. GLICK, D., GOOD, R. A., GREENBERG, L. J., EDDY, J. J., AND DAY, N. K., *Science* **128**, 1625 (1958).
3. BETHEIL, J. J., *Anal. Chem.* **32**, 560 (1960).
4. HARRIS, A. F., SAIFER, A., AND WEINTRAUB, S. K., *Arch. Biochem. Biophys.* **95**, 106 (1961).

ANALYTICAL BIOCHEMISTRY 5, 505–514 (1963)

Fluorometric Determination of Oxytetracycline in Biological Material[1]

KENNETH H. IBSEN, RUSSELL L. SAUNDERS, AND MARSHALL R. URIST

From the Department of Surgery, Division of Orthopedics, University of California Medical Center, Los Angeles, California

Received September 20, 1962

INTRODUCTION

Oxytetracycline, like other forms of tetracycline, is deposited in bone, calcified cartilage, and extraskeletal sites of pathological calcifications (1, 2). The reaction is useful for morphological and physiological investigations of mineralized tissue in health and disease. To aid further physiological research, a sensitive and reliable quantitative chemical method of measurement of oxytetracycline is a necessity. The Kohn fluorescent method depends upon formation of a calcium-barbital-tetracycline complex, but it is not applicable to oxytetracycline (OTC) (3). The method to be described in this report is designed for OTC as well as other tetracyclines. The OTC is extracted from acidic aqueous solutions into amyl alcohol, and then from amyl alcohol into basic solutions; the concentration of OTC is proportional to the difference in fluorescence between a magnesium-OTC chelate, and the free compound, liberated by adding ethylene diaminetetraacetate to the solution.

APPARATUS AND MATERIALS

A Turner model 110 fluorometer was employed as equipped from the factory. The cuvettes used were either matched 15 × 75 mm Pyrex tubes (effective capacity 3.5–5.5 ml) or 12 × 75 Pyrex tubes (effective capacity 2.2–3.5 ml). Glassware was acid soaked and thoroughly rinsed before use. Before each experiment the cuvettes were filled with 7 *N* NH_4OH and matched using the widest light aperture.

No special purification of any of the reagent-grade chemicals employed was necessary. The OTC, purified by Chas. Pfizer and Co.,[2] gave only one fluorescing spot when chromatographed in the systems described by

[1] This work was aided by grants from the U. S. Public Health Service (No. A-3793); Squibb Institute for Medical Research; Easter Seal Foundation for Medical Research, National Society for Crippled Children and Adults; and Ayerst Laboratories, Inc.

[2] Chas. Pfizer and Company generously donated supplies of purified oxytetracycline.

Kelly and Buyske (4). The pH was determined using an ordinary glass electrode and no correction was made for any possible alkali ion effect at higher pH.

METHOD

Noncalcified Material

The sample containing 0.04–1 mμmoles of OTC is made to 5 ml with 10% trichloroacetic acid (TCA). The precipitated protein is removed by centrifugation and a 4-ml aliquot of the supernatant is pipetted into 4 ml of amyl alcohol in a 15-ml glass-stoppered centrifuge tube. The mixture is shaken for 2 min and then centrifuged for 5 min at 2000 rpm. Four milliliters of 7 *N* NH_4OH and up to 1.0 ml of hexane are added to 4 ml of the alcohol layer. The mixture is shaken and centrifuged as before. Four milliliters of the basic aqueous (lower) layer is pipetted into a 15 × 75 mm cuvette with the aid of a rubber bulb. A 0.5-ml portion of 8 m*M* $MgCl_2$ solution (4 μmoles) is carefully pipetted into each tube. The solutions are mixed by inverting the tubes against a piece of clean Parafilm and allowed to stand 15 min before being read at a suitable light aperture. To each tube 0.1 ml of a 0.1 *M* ethylene diaminetetraacetate (Versene) solution (10 μmoles) is added, using a 0.2-ml Warburg pipet. The solutions are mixed as before and allowed to stand at least 30 min before reading. They are then reread after a few minutes to ensure that stability is achieved. The difference between the Versene and the Mg readings is compared to the differences obtained with standards which have also been extracted. Standards and unknowns are read against blanks which have been subjected to the same treatment.

Calcified Materials

The material is ground into a powder using a hand coffee mill or a Plattner Diamond mortar. An aliquot containing a maximum of 100 mg of the powder is then extracted in a minimum of 5 cc of 10% TCA. Calcium analyses have shown that shaking the sample at room temperature for 1 hr is satisfactory, provided the material was well powdered. The sample is then handled as above except that the first amyl alcohol extract is back-extracted with an additional 4 cc of 10% TCA.

RESULTS

Activation and Fluorescence Spectra and Choice of Filters

Udenfriend *et al.* (5) have reported the activation maximum of OTC to be 390 mμ and the fluorescing maximum to be 520 mμ at pH 11. Preliminary investigation with the aid of an Aminco-Bowman spectro-photofluorometer confirmed the value of the fluorescing maximum and showed

the activation maximum to be in the range of 390 mμ but to be dependent upon the presence of metal ions and pH. Data previously reported (6) have shown that increasing pH, as well as metal ions, induced a bathochromic shift in the absorption maximum. As to be expected, the activation maximum appeared to parallel the changes in absorption. At pH 10.6 and in the presence of Versene, OTC is activated at 370–380 mμ, whereas the magnesium chelate is activated at about 405 mμ. Several possible filter combinations were compared. The Corning C.S. #7-60 filter, included in the kit provided by Turner, was used as a primary filter in the earlier experiments, and, therefore, it has been continued in use for the majority of the experiments. Comparable results are obtained with either the Corning C.S. #7-51 alone or a Corning C.S. #0-51 + 7-51 combination. The former raises the sensitivity slightly, while the latter may be of value in reducing background fluorescence. The Turner 405 filter reduces sensitivity seventeenfold and is inadequate.

The most specific secondary filter readily available is the Wratten 58; however, it is not stable. Consequently, a combined Wratten 2A + 12 was used. This results in a filter having a sharp cut off point at 500 mμ which passes almost all the light above 520 mμ.

pH

Figure 1 demonstrates the change in fluorescence which occurs when

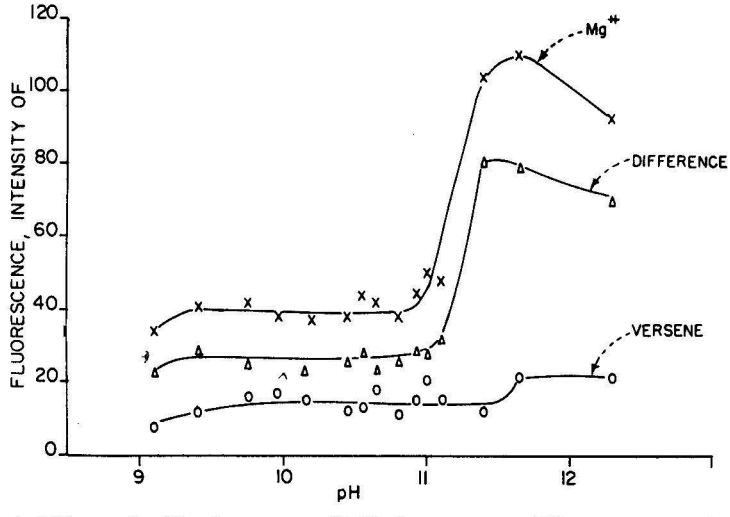


FIG. 1. Effect of pH change on OTC fluorescence. Filters employed were a Corning C.S. 7-60 primary and a combined Wratten 12 + 2A secondary. Readings were made using the largest light aperture. Concentrations: OTC, 22 μμ moles/ml; $MgCl_2$, 0.9 μmoles/ml; Versene, 2.2 μmoles/ml. Total volume was 4.5 ml.

the pH is varied from 9.2 to 12.3 in the presence of Mg^{++}, in the presence of Versene, and on the difference values. In each instance a plateau occurs between pH 9.5 and 10.9. Below pH 9, fluorescence falls off rapidly, and is zero at pH 6. At pH 7.4 the degree of fluorescence is only 10% of the fluorescence at pH 10.6 when measured in Versene. By using 7 *N* NH_4OH as buffer, it was possible to maintain the pH between 10.6–10.7 in all experiments. Furthermore, unlike other alkalies, NH_4OH was only slightly fluorescent.

Stability

The relative stability of OTC under the conditions to which it might be exposed during analysis are summarized in Table 1. It is very stable in 10% (0.62 *M*) TCA. In the presence of excess Versene, it is also stable

TABLE 1
STABILITY OF OTC UNDER VARIED CONDITIONS

Time (hr)	10% TCA, c	Per cent of zero time reading in: pH 10.6 $MgCl_2$ + Versene				pH 10.6 $MgCl_2$ only				pH 10.6 no addition			
		a	b	c	d	a	b	c	d	a	b	c	d
0	100	100	100	100	100	100	100	100	100	100	100	100	100
0.25	100	110	115	100	83	100	100	99	95	110	104	98	77
0.5	100	127	125	95	67	102	102	98	89	100	100	97	88
1	100	140	140	100	50	113	97	96	62	100	87	89	59
2	96	155	150	100	50	113	98	87	43	100	78	81	59
3	100	165	165	100	50	110	66	80	24	90	69	—	59
4	100	160	150	100	50	108	60	72	27	90	66	67	59
6.5	100	—	—	100	—	—	—	58	—	—	—	61	—
9	—	150	150	—	33	93	51	—	21	80	53	—	53
24	100	—	—	100	—	—	—	—	—	—	—	—	—

a = in dark. b = in deep shade (back of fume hood). c = room light under ordinary conditions. d = directly under and 2 ft. away from a fluorescent light source.

OTC concentration from 20–50 μmoles/ml in different experiments. Total volume was 4.5 ml. Samples were kept at room temperature. The laboratory was windowless; therefore, all light source is artificial. Primary filter was a Corning C.S. #7–60, while the secondary was a Wratten 12 + 2A combination.

at pH 10.6, provided it is protected from direct illumination. The increased fluorescence which can occur in the absence of bright light can be a troublesome phenomena. Because of this tendency for fluorescence to increase in excess Versene, a delay of at least one-half hour has been recommended prior to reading. Since the rate of change can vary in biological samples, it is advisable to make a subsequent reading to ensure that complete equilibration has occurred in all samples. At this high pH

there is an unmistakable tendency for light to accelerate decomposition. Care must, therefore, be exerted to avoid overillumination during analyses.

Effect of Ions

Table 2 compares the effect of increasing quantities of Ca^{++} and Mg^{++}

TABLE 2
EFFECT OF MAGNESIUM AND CALCIUM ON FLUORESCENCE

μmoles ion added	0	2.5	4	8	10	12
Fluorescence with Ca^{++}	25	—	26	28	39	40
Fluorescence with Mg^{++}	25	58	58	58	—	—

OTC concentration was 0.8 mμmoles/ml. Total volume was 4.5 ml. The next to smallest aperture was utilized. Primary filter was a Corning C.S. #7–60, while the secondary was a Wratten 12 + 2A combination.

on fluorescence. This ion effect is of a dual nature. It increases fluorescent intensity and causes a bathochromic shift in the absorption and activation spectrum. Therefore, in order truly to gage the ion effect, one needs to employ a fluorescence spectrophotometer. Cupric ion induces still higher fluorescent values than does Ca^{++} or Mg^{++}. However, the cupric ions also cause an instability which is so great that a proper reading cannot be obtained. The affect of Mn^{++} and Fe^{++} also could not be ascertained because these ions formed insoluble complexes. Aluminum ion reduces stability but enhances fluorescence only slightly, if at all, when measured with the 7-60, 12-2A filter combinations.

Extraction

Extraction of OTC from acid solutions into amyl alcohol is about 80% efficient, while the extraction back from amyl alcohol into basic aqueous solution is only 60% efficient, the total loss of OTC being approximately 50%. The distribution of OTC among the various phases is quite reproducible and the quantity of OTC recovered is directly proportional to the amount added in all concentrations studied (Fig. 2). When Mg^{++} is added to the basic solution prior to extraction, slightly lower recoveries of OTC occur. By saturating the 10% TCA solution with NaCl, about 10% more OTC could be extracted into the amyl alcohol. This procedure is, however, self-defeating, as NaCl will also induce the extraction of other fluorescing compounds from serum. Furthermore, the presence of salts makes it more difficult to separate the basic aqueous and alcohol layers. When such difficulty is encountered, it can be cir-

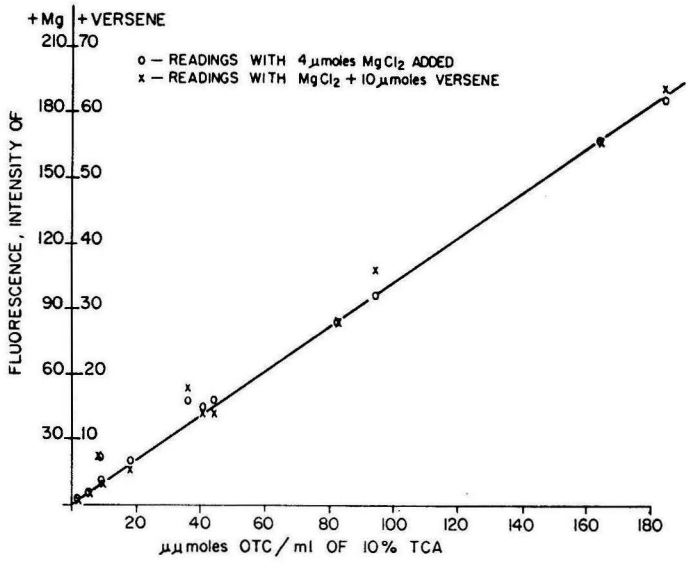


FIG. 2. Linearity of fluorometric response after extraction. Total volume initially was 5 ml of which a 4-ml aliquot was extracted. Details in text. Due to limitations in scale, readings were made at several apertures, but are reported in terms of maximum aperture (greatest sensitivity).

cumvented by adding hexane and decreasing the polarity of the organic phase. Up to 1 cc hexane does not affect the extraction of OTC.

The extractions remove much but not necessarily all other fluorescing materials. Because removal of fluorescing contaminants is variable, it is recommended that the change in fluorescence caused by converting the Mg chelate to the free form be used as an index of concentration. Under the conditions employed, this change in fluorescence appears to be specific for the tetracyclines. Despite the specificity of this change, extraction prior to determination is necessary to reduce background absorption, which interferes with the fluorescence of OTC, and to remove Ca^{++}, Mg^{++}, and phosphate ions. If the latter precipitate from solution, a portion of the OTC will be bound to the precipitate. When bone (up to 100 mg/5 cc has been tested) is being analyzed, two extractions are needed in order to reduce the calcium and phosphate ions to low enough values to prevent this precipitation. Each extraction removes approximately 90% of each ion.

Butanol can be substituted for amyl alcohol with about another 10% loss of OTC. Octanol extracts little OTC, and solvents that are more nonpolar are completely inadequate.

The time of shaking needed for maximum extraction to occur was tested by measuring the quantity of OTC extracted from each solvent at varied intervals. Two minutes of vigorous shaking is adequate.

Perchloric, hydrochloric, and tungstate-sulfuric acid extracts can be used as well as TCA. However, TCA and tungstate-sulfuric have the advantage of removing the greatest quantity of nonspecific fluorescent material. If tungstate-sulfuric extracts are used, an excess of sulfuric acid is needed to lower the pH.

Linearity

Figure 2 demonstrates that fluorescent intensity is proportional to the amount of OTC added to and extracted from 10% TCA. The values obtained with the double extraction technique are approximately 60% of values obtained with one extraction. Fluorescence is also completely linear under these conditions.

It is significant that the few instances in which deviation of the standard occurred the difference value would not deviate as much, if at all. This indicates that the deviation was due to small quantities of contamination and demonstrates the practical value of using change in fluorescence as a true measure of the OTC concentration.

TABLE 3
RECOVERY OF OTC FROM BIOLOGICAL SAMPLES

Tissue	OTC added	OTC recovered	Per cent recovered
	mμmole/ml	mμmole/ml	
Serum[a] (rabbit or human)	0	0	—
	0.064	0.062	97
	0.80	0.64	80
	2.00	2.04	104
	4.00	4.00	100
	8.00	8.20	103
	mμmole/gm	mμmole/gm	
Eggshell[b]	6.64	6.76	102
Bone[b] (rat humeri + radius)	0	0.016	—
	3.20	2.74	86
	8.82	9.20	104
(elk cortical)	0	0.14	—
	4.60	4.45	97

[a] The appropriate quantity of OTC was added to 0.5 ml serum, which was then deproteinized with 10% TCA, diluted as required, and subjected to the procedure described for noncalcified material in the text.

[b] The material was ground and an aliquot was added to a flask containing 10% TCA. The appropriate quantity of OTC was added and then analyzed for by the double extraction technique described for calcified material.

Recoveries

Table 3 shows that good recoveries were obtained from serum, bone, and egg shell. The OTC was added to the undiluted sample in TCA.

Other Tetracyclines

Although developed primarily for the determination of OTC, the efficiency of the method was tested for tetracycline, chlortetracycline, and 7-chloro-6-demethyltetracycline. Table 4 shows that the method is more

TABLE 4
FLUORESCENCE READINGS AND PER CENT EXTRACTION OF VARIOUS TETRACYCLINES

Compound	Reading[a] (50 μμmole/ml)			Per cent extracted
	Mg^{++}	Versene	Δ	
Tetracycline (Achromycin)[b]	143	16	127	86
7-Chloro-6-demethyltetracycline (Declomycin)[c]	140	10	130	74
Oxytetracycline (Terramycin)	88	28	60	50
Chlortetracycline (Aureomycin)[c]	25	5	20	77

[a] Without extraction; expressed in terms of widest aperture using the Corning C.S. #7–61, Wratten 12 + 2A filter combination.

[b] Purified product donated by Squibb and Sons.

[c] Commercial product, concentration estimated spectrophotometrically.

efficient for tetracycline and 7-chloro-6-demethyltetracycline than for OTC itself. Furthermore, it would be adequate for chlortetracycline under most circumstances. Udenfriend *et al.* (5) have shown that chlortetracycline has fluorescent characteristics which differ markedly from those of OTC and tetracycline. Therefore, determination of chlortetracycline might be improved by using a different filter combination.

DISCUSSION

As many as ten repeated analyses of identical serum samples have shown the mean deviation of the difference between the reading of the chelated and free form to be no more than ±1.5 calibration units, regardless of the actual concentration. As the instrument has a scale of 100 units, the difference reading must be greater than 15 in order to have a precision of ±10%, or smaller. If a 0.5-ml aliquot of serum were employed, this value would be given by a sample containing 180 μμmoles (0.09 μg) OTC/ml. This quantity is then the minimal amount which may be repeatedly measured with an expected mean deviation of less than ±10% and is well below the normal pharmacological level. We have obtained good recoveries with as little as 64 μμmoles OTC/ml

serum (Table 3), but cannot recommend this procedure for analysis of such small quantities.

Because the full range of the calibration scale is obtained by changing the size of the light aperture, it becomes possible to obtain a small reading with much greater quantities of OTC. If the concentration of an unknown sample is such that a smaller aperture must be used and the difference reading is therefore less than 15, the sample should be suitably diluted.

Even though samples of bone are extracted an additional time, the mean deviation of duplicate samples is still nearly ± 1.5 (7 identical samples gave a mean deviation of ± 1.4). A 100-mg aliquot of bone having a concentration of 1500 $\mu\mu$moles (0.75 μg) OTC/gm will give a difference reading of 15 and will, therefore, correspond to the minimal concentration of OTC in bone which can be analyzed with an expected mean deviation of $\pm 10\%$. Judging by the data of Buyske *et al.* (7), and by our own analyses, this should be less than the usual quantity of OTC found in bone after a pharmacological dose.

If necessary, smaller samples may be employed without loss of sensitivity by using smaller volumes and the 12×75 mm test tubes. Small increases in sensitivity can be obtained by substituting a Corning C.S. #7-51 filter for the 7-60, by using NaCl-saturated TCA, and by extracting the OTC into smaller than equal volumes of alcohol, and then into base. It is not recommended that dilution less than 1 to 10 be used for serum or that more than 100 mg of bone be dissolved in 5 ml of 10% TCA unless internal standards are employed.

It may be significant that the influence of divalent cations on fluorescence appears to be related to the affinity with which these ions bind OTC ($Cu > Mg > Ca$). Because of the bathochromic absorption shift also induced by these ions, a proper study of this phenomena must be made using a fluorescence spectrometer. Such a study is also complicated by instability induced by the more tightly bound ions.

SUMMARY

A specific sensitive fluorometric method for the analysis of oxytetracycline in biological samples is described. The method depends upon extraction of oxytetracycline from aqueous acid into amyl alcohol. The OTC in the amyl alcohol is then re-extracted with aqueous base. The concentration of oxytetracycline is proportional to the change in fluorescence caused by the addition of excess ethylene diaminetetraacetate to the Mg complex of oxytetracycline. The effect of time, pH, light, and metallic ions on fluorescence is described.

Comparison of the results obtained with oxytetracycline (Terramycin)

to those obtained with tetracycline (Achromycin) and 7-chloro-6-demethyltetracycline (Declomycin) show the method to be 3–4 times more sensitive toward the latter two. It appears to be about one-half as sensitive for the determination of chlortetracycline (Aureomycin).

REFERENCES

1. RALL, D. P., LOO, T. L., LANE, M., AND KELLY, M. G., *J. Natl. Cancer Inst.* **19,** 79 (1957).
2. HÄKKINEN, I. P. T., *Scand. J. Clin. Lab. Invest.* **11,** 301 (1959).
3. KOHN, K. W., *Anal. Chem.* **33,** 862 (1961).
4. KELLY, R. G., AND BUYSKE, D. A., *Antibiot. Chemotherapy* **10,** 604 (1960).
5. UDENFRIEND, S., DUGGAN, D. E., VASTA, B. M., AND BRODIE, B. B., *J. Pharmacol. Exptl. Therap.* **120,** 26 (1957).
6. IBSEN, K. H., AND URIST, M. R., *Proc. Soc. Exptl. Biol. Med.* **109,** 797 (1962).
7. BUYSKE, D. A., EISNER, H. J., AND KELLY, R. G., *J. Pharmacol. Exptl. Therap.* **130,** 150 (1960).

ANALYTICAL BIOCHEMISTRY 5, 515–522 (1963)

A Rapid Assay for Nucleotide Kinases Using C^{14}- or H^3-Labeled Nucleotides[1]

N. BURR FURLONG[2]

From the Biochemistry Department, The University of Texas, M. D. Anderson Hospital & Tumor Institute, Houston, Texas

Received September 24, 1962

INTRODUCTION

Nucleotide kinases catalyze the reaction between ATP[3] and a nucleotide to form the nucleoside di- or triphosphate and adenosine diphosphate. Lehman *et al.* (1) described an assay for nucleotide kinases in which the conversion of P^{32}-labeled nucleotides to products resistant to semen phosphatase and adsorbable on activated charcoal is measured. The phosphatase splits nucleoside monophosphates to nucleosides and inorganic P^{32}-phosphate, but does not hydrolyze the nucleoside polyphosphates formed in the kinase reaction. Activated charcoal adsorbs the nucleoside and the labeled nucleoside polyphosphates, but not the inorganic P^{32}-phosphate. Thus the radioactivity adsorbed to charcoal is proportional to the extent of conversion of nucleotide to nucleoside di- and triphosphates. Some recent studies in which the method has been employed are those of Kozinski and Bessman (2) and Kielley (3). The analysis is rapid and convenient, but is limited by the lack of commercial availability and short shelf-life of the P^{32}-nucleotides.

It has been found possible to adapt C^{14}- or H^3-labeled nucleotides to a similar analysis by adsorption of the labeled nucleoside di- and triphosphates on DEAE paper in the final step of the procedure. By using base-labeled compounds in the kinase reaction and, subsequently, treating the reaction mixture with semen phosphatase, the final products

[1] This investigation was supported in part by PHS Research Grant CA 04464 from the National Cancer Institute, Public Health Service, and an Institutional Grant G 18304 from the National Science Foundation.

[2] The author is grateful to The Public Health Service for a postdoctoral fellowship held during the period of this research.

[3] The following abbreviations are used: ATP, adenosine triphosphate; DEAE, diethylaminoethylcellulose; Tris-Cl, tris(hydroxymethyl)aminomethane hydrochloride; dAMP, deoxyadenylic acid; dCMP, deoxycytidylic acid; TMP, thymidylic acid; UMP, uridylic acid; TdR, thymidine; POP, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxazolyl)benzene; dATP, dCTP, and TTP, deoxyadenosine, deoxycytidine, and thymidine triphosphate, respectively.

consist of inorganic phosphate, labeled nucleoside, and labeled nucleoside polyphosphate. The labeled compounds are separated by adsorbing the nucleoside polyphosphate on DEAE paper disks and washing away the nonadsorbed nucleoside.

METHODS

Kinase Medium. In a total volume of 0.1 ml, in 6 × 50 mm culture tubes, the reaction mixture contains: ATP (0.67 μmole); $MgCl_2$ (0.8 μmole); Tris-Cl, pH 8.0 (4.3 μmoles); mercaptoethanol (0.1 μmole); C^{14}-nucleotide (55 mμmoles, 0.2 mc/mmole) or H^3-nucleotide (55 mμmoles, 2.5 mc/mmole); and kinase source (depending on the particular preparation, from 2 to 40 μl have been used).

Nucleotides which have been tested in this assay are dAMP-8-C^{14}, H^3-dCMP, dCMP-2-C^{14}, TMP-2-C^{14} (obtained from Schwarz BioResearch Inc.), UMP-6-C^{14} and UMP^{32} (kindly supplied by Dr. R. B. Hurlbert). Labeled deoxyguanylic acid has been unavailable commercially. The conversion of a nucleoside to nucleoside triphosphate has been assayed using T*d*R-2-C^{14} (obtained from New England Nuclear Corp.). In assaying for thymidylate kinase in crude extracts, the medium as described above is made 0.3 *M* in pH 9.2 glycine buffer. Incubation is carried out at 35°C.

Phosphatase Treatment. Aliquots of 25 μl of the kinase reaction mixture are removed at three times, for example, at 0, 10, and 30 min after the addition of the enzyme source, and placed in another set of 6 × 50 mm tubes containing 30 μl of 0.25 *M* pH 4.9 Na acetate buffer. The tubes are heated at 95°C for 3 min, to destroy enzyme activities, and chilled on ice. Then 5 μl (approximately 15 units) of human semen phosphatase (4) is added to each tube and they are placed at 35°C for 5 min. The tubes are centrifuged 2 min at approximately 1700 × *g* to clear the solution. (The handling of small volumes of liquid in narrow tubes is facilitated by the use of Lang-Levy type of micropipet such as those supplied by The Scientific Glass Apparatus Co.)

DEAE Disk Treatment. Two 25-μl aliquots of each solution after phosphatase treatment are transferred to duplicate sets of DEAE disks, 2 cm in diameter (or DEAE squares 2 × 2 cm). DEAE paper was obtained from H. Reeve Angel & Co. One set of disks is washed two times in a beaker of 95% ethanol at room temperature allowing approximately 10 ml of ethanol for each disk and 5 min of intermittent swirling for each wash. These disks are then blotted on paper towels and dried. The second set of disks is dried on a nonwettable surface without washing. The per cent conversion at each time point can be calculated directly from:

$$\frac{\text{(counts/minute) washed}}{\text{(counts/minute) unwashed}} \times 100$$

The conversion in mμmoles/hr is then derived from the original amount of nucleoside monophosphate.

Sources of Kinase. Three sources of kinase activity were tested: (*1*) An extract of *Escherichia coli* was prepared by disrupting a 10% suspension of *E. coli* in 0.25 *M* sucrose, 0.01 *M* $MgCl_2$, 0.01 *M* Tris-Cl, pH 7.4, and 0.004 *M* Na succinate in a French pressure cell operated at 8000 psi. The disrupted cellular material was centrifuged at 7000 × *g* for 20 min and the supernatant extract was used as enzyme source. (*2*) A rat liver fraction was prepared by homogenizing normal adult rat livers in a solution of 0.25 *M* sucrose-0.006 *M* KCl-0.01 *M* Tris-Cl, pH 8.0 (3 ml/gm tissue), then centrifuging at 100,000 × *g* for 1 hr. The supernatant solution (S-3) was used. (*3*) An S-3 fraction was prepared from the livers of rats taken 72 hr after partial hepatectomy. Protein determinations were by the method of Lowry *et al.* (5).

Radioactivity Measurements. Most of the determinations were made on a Nuclear-Chicago Model C115 counter; background counts averaged 1.9 cpm. The efficiency of counting C^{14} on paper disks as described is approximately 5%. Scintillation counting has also been employed by immersing the dried DEAE disk in 5 ml of 0.5% w/v POP, 0.01% w/v POPOP in reagent-grade toluene in standard counting vials. The counting efficiency for C^{14} on paper disks in a Packard Model 314 scintillation spectrometer is 25%, and for H^3, 5%. For the assay of tritiated compounds, an alternate counting method was also developed. The samples were placed on five-sided DEAE paper slips in the form of 1.7-cm squares with a triangular eluting point on one side. One edge of these slips was clamped between microscope slides and the slips were eluted with 1 *M* ammonium bicarbonate directly onto a planchet (0.3 ml eluant sufficed). The planchets were dried in a 60°C oven, thus decomposing the eluting salt. With this procedure, it was found possible to assay H^3 at 30% efficiency in a Tracerlab SC-50 windowless Geiger counter. The efficiency when counting H^3 is altered by the presence of salts or other solids on the disks and duplicate samples, one of which includes an internal counting standard, are recommended for greatest accuracy.

RESULTS

Elution of DEAE Disk. Various washing techniques were tested to achieve the greatest retention of the nucleoside polyphosphate in the DEAE disk with complete removal of the nucleoside. The results of one test are given in Table 1. Amounts of labeled dATP, dCTP, TTP, dAMP,

TABLE 1
A TEST OF WASHING PROCEDURES

Solutions of labeled compounds were placed on 2 × 2 cm squares of DEAE paper and dried. The amount of triphosphate, monophosphate, or thymidine placed on a square corresponded to the initial concentrations used in the kinase medium, equating the triphosphate to the ATP present. These were counted for total radioactivity. Then, 25 μl of 0.12 *M* acetate buffer was placed on each square and they were immediately washed in the given solvents, allowing 10 ml solvent for each disk. The wash was repeated in the same amount of fresh solvent after 5 min and the squares were then dried and recounted.

Compound	Per cent retained on DEAE squares		
	H_2O	Acetone	95% ethanol
dATP	92.2	96.5	100
dCTP	98.1	94.6	100
TTP	100	100	99.5
dAMP	78.3	76.8	86.3
dCMP	61.3	72.6	86.8
TMP	67.6	77.2	71.0
TdR	1.81	3.85	0.76

dCMP, TMP and TdR equivalent to those encountered in the procedure were placed on squares of DEAE in a solution resembling the phosphatase incubation medium. These squares were assayed to determine total radioactivity and then were washed in the solvent listed. Radioactivity was again determined and the per cent retained on the squares calculated. The use of 95% ethanol was found to result in the maximum triphosphate retention with almost complete removal of nucleoside.

Phosphatase Digestion. The semen phosphatase preparation was tested on ATP for the release of inorganic phosphate. When 1 ml of a solution 0.01 *M* in ATP was incubated with 50 μl of acid phosphatase in the medium specified above, only 0.06 μmole of phosphate was released in 5 min. This indicates that less than ¾% of the total triphosphate was hydrolyzed.

The completeness of the degradation of monophosphate is checked in each analysis by the radioactivity retained on the DEAE disks for the kinase sample taken at the 0 minute time point. These values did not exceed 2.5% of the total monophosphate present. There must be no delay in taking and processing this initial sample after the addition of enzyme to the kinase medium. Even at ice temperature, the rapid appearance of nucleoside polyphosphate is measurable with active kinase preparations.

Uses of the Modified Assay. The assay has been used in measuring the relative concentration of deoxynucleotide kinases in a variety of

tissue extracts. In the assay described above the concentration of labeled substrate is 0.55 m*M*, which is about eightfold higher than the concentration given by Lehman *et al.* (1). A low concentration of substrate makes possible the detection of very small amounts of enzymic activity, and is useful in the rapid assay of extracts relatively free of competing activities, such as preparations from *E. coli*. However, in the assay of extracts containing higher concentrations of the kinases or those which contain phosphatase or nucleotidase activities the substrate is inadequate to keep the enzyme saturated and the reaction rate is then no longer directly proportional to the amount of enzyme present. In these cases, the higher substrate concentration is recommended. Substrate concentrations up to 1.5 m*M* have been used successfully in these assays. Some of the difficulties which may be encountered are illustrated in the following assays.

An *E. coli* extract was incubated with 0.05 and 0.55 m*M* TMP as substrate. Conversion to thymidine polyphosphates was measured at 10 and 30 min; results are given in Table 2. At the lower substrate concen-

TABLE 2

EFFECT OF SUBSTRATE CONCENTRATION ON TMP KINASE ACTIVITY IN AN *E. coli* EXTRACT

Assays for TMP kinase were performed as described in the text with the addition of 10 μl of 3 *M* glycine buffer, pH 9.2, in the total volume of 0.1 ml. The enzyme source was 10 μl of an *E. coli* extract with total protein concentration of 6.2 mg/ml.

Initial substrate (mμmoles)	TMP converted (mμmoles)	
	In 10′	In 30′
5	3.0	3.9
55	6.2	16.0

tration it can be seen that the reaction is 60% complete at 10 min and 78% at 30 min. However, when a higher amount of substrate is used, approximate linearity of reaction rate is maintained for 30 min.

Table 3 lists the mμmoles of TMP converted at 5, 15, and 30 min by various amounts of regenerating rat liver S-3 using 0.05 m*M* substrate. At 5 min the reaction rate is proportional to the amount of enzyme added; however, the direct proportionality is lost by 15 min. Presumably by this time competing enzyme activities have altered the substrate or products.

The values at 30 min are included to illustrate another use of these assays. Quantitative assays of enzyme amount, such as discussed above, require that measurements be obtained before 10–20% of the nucleotide has reacted. However, it is of much practical interest to find the condi-

TABLE 3

TMP KINASE ASSAY USING DIFFERENT AMOUNTS OF REGENERATING RAT LIVER S-3

Assays for TMP kinase were performed as described in the text with the addition of 10 μl of 3 *M* glycine buffer, pH 9.2, in the total volume of 0.1 ml. The amount of substrate was 5 mμmoles. The enzyme source was regenerating rat liver S-3 with a protein concentration of 21 mg/ml.

Vol. of S-3 (μl)	TMP converted (mμmoles)		
	In 5′	In 15′	In 30′
5	0.13	0.44	0.65
15	0.41	0.94	1.35
50	1.47	2.37	2.82

tions which will bring about the maximum conversion of substrate to product with a given source of kinase, for example, in the preparation of nucleoside polyphosphates. From the data of Table 3, the maximum conversion of TMP is seen to be in the neighborhood of 60%. This conversion requires the use of a large amount of regenerating rat liver S-3.

Comparison of P^{32}- and C^{14}-Type Assays. A direct comparison of the modified kinase assay with the original was performed using 0.05 m*M* UMP^{32} in the assay described by Lehman *et al.* (1). Table 4 lists the

TABLE 4

COMPARISON OF "DEAE DISK" WITH "CHARCOAL" KINASE METHODS

The kinase assay using UMP-C^{14} was carried out as described in the text. The kinase assay using UMP^{32} followed the method described by Lehman *et al.* (1) except that 0.050 m*M* substrate was used. Samples were assayed at 1′, 3′, and 10′ after addition of the enzyme source. The enzyme source was 10 μl of adult rat liver S-3 with protein concentration of 23 mg/ml.

Assay	Substrate	Uridine polyphosphate formed (mμmoles)		
		In 1′	In 3′	In 10′
DEAE procedure	UMP-C^{14}	0.28	0.88	0.87
Charcoal procedure	UMP^{32}	0.29	0.75	0.80

mμmoles of nucleoside polyphosphate formed at corresponding stages of the assay as determined by each of these methods. The lower values seen in the "charcoal" analysis are believed to be due to the difficulty in completely transferring charcoal to the counting cup, and to losses of charcoal during the multiple centrifugations and decantations required. The assay indicates that the reaction had reached maximum conversion

of UMP before 3 min. The fact that this kinase reaction went no further toward completion than 17% indicates the presence of interfering enzymic activities in the liver S-3. This is the type of result which would be expected if the nucleotide substrate was being hydrolyzed by a phosphatase. Similar reaction kinetics have been observed when measuring dCMP kinase in rat liver S-3. The possibility of phosphatase interference in measuring kinase activities in extracts such as liver S-3 makes it mandatory to use a high substrate concentration and a short reaction time to accurately assess the actual kinase activity.

Comparison of DEAE Disk Procedure with Chromatography on DEAE Paper. Duplicate aliquots were taken at each time point. One set was analyzed as usual. The other set was spotted on 9×11 cm sheets of DEAE paper, then, 0.5 μmole each of nonlabeled nucleoside monophosphate and triphosphate were placed on each spot, and the chromatogram was developed with 0.25 *M* ammonium bicarbonate, as descending solvent, to within 1 cm of the bottom. The di- and triphosphates are not separated by this elution. After the sheet was dried, the spots were marked, cut out, and counted directly in scintillation solution. The set of samples on disks was counted similarly. The mμmoles converted to nucleoside polyphosphate were computed from the data.

TABLE 5

COMPARISON OF DEAE DISK PROCEDURE WITH CHROMATOGRAPHIC ANALYSES

The medium was prepared as described in the text except that the amount of substrate was 10 mμmoles in a total volume of 0.2 ml. Duplicate aliquots were removed at each time point and one aliquot was assayed as described in the *Methods* section, the other aliquot was analyzed by chromatography on DEAE paper as described in the text. The enzyme source was an *E. coli* extract containing 6.0 mg protein/ml: 5 μl was used in the AMP kinase assays and 75 μl in the TMP kinase assays. In addition, the TMP kinase medium was made 0.22 *M* in glycine buffer pH 9.2.

Type of assay	Substrate	Nucleoside polyphosphate formed (mμmoles)		
		0′	5′	15′
Disk	dAMP-C^{14}	0.10	3.2	4.6
Chrom.	dAMP-C^{14}	0.11	3.5	4.3
Disk	TMP-C^{14}	0.26	2.7	2.7
Chrom.	TMP-C^{14}	0.45	3.0	2.6

The results of this analysis are shown in Table 5. The agreement between the disk method and the chromatographic analysis is very close. There is almost complete conversion of dAMP to deoxyadenosine polyphosphate in 15 min. TMP kinase reaches a maximum conversion near

60% in about 5 min with the amount of enzyme solution used in this assay.

SUMMARY

A rapid quantitative procedure for the analysis of nucleotide kinases utilizing C^{14}- or H^3-labeled nucleoside monophosphates as substrates has been developed. After residual monophosphate is selectively destroyed by a phosphatase, the procedure measures the labeled nucleoside di- and triphosphates which are retained on a DEAE paper disk washed in 95% ethanol.

ACKNOWLEDGMENTS

The author is indebted to Miss Nancy Lund and Mr. Ariel Thomann for assistance in the development of this procedure. The author is also grateful to Mrs. Gerry Willard for the observation that TMP kinase shows higher activity in glycine buffer.

REFERENCES

1. LEHMAN, I. R., BESSMAN, M. J., SIMMS, E. S., AND KORNBERG, A. *J. Biol. Chem.* **233**, 163 (1958).
2. KOZINSKI, A. J., AND BESSMAN, M. J., *J. Mol. Biol.* **3**, 746 (1961).
3. KIELLEY, R. K., *Biochem. et Biophys. Acta* **53**, 150 (1961).
4. WITTENBERG, J., AND KORNBERG, A., *J. Biol. Chem.* **202**, 431 (1953).
5. LOWRY, O. H., ROSEBROUGH, N. F., FARR, A. L., AND RANDALL, R. J., *J. Biol. Chem.* **193**, 265 (1951).

ANALYTICAL BIOCHEMISTRY 5, 523–530 (1963)

Quantitative Analysis of Fecal Bile Acids by Gas-Liquid Chromatography[1]

M. MAKITA[2] AND W. W. WELLS[3]

From the Biochemistry Department, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania

Received September 27, 1962

The analysis of bile acids by spectrophotometric methods is often associated with significant errors from contaminating compounds (1, 2). Furthermore, the response of dihydroxy- and trihydroxycholanic acids to various colorimetric and spectrophotometric methods is variable (3). Partition chromatography procedures (4) are lengthy and, in the case of fecal extracts, may be hindered by the presence of microbial hydroxy fatty acids (5).

Vanden Heuvel *et al.* (6) reported that gas-liquid chromatography was useful in the separation of bile acid methyl esters and Blomstrand (7) subjected the methyl esters of human biliary bile acids to gas-liquid chromatography. Sjövall *et al.* (8) have reported the chromatography of substituted cholanic acids on polar phenyl silicone phases, and Holmes *et al.* (9) and Bloomfield (10) have studied model conditions for bile acid analysis by gas-liquid chromatography.

In connection with our studies of cholesterol metabolism, a method for the quantitative analysis of fecal neutral sterol trimethylsilyl ethers on a polar nitryl silicone liquid phase was reported (11). Gas chromatographic separation of the trimethylsilyl ethers of a wide variety of methyl cholanates on the nitryl silicone phase used in the former study was found to be inadequate. Polar phases (e.g., ethylene glycol polysuccinate), commonly employed for fatty acid methyl esters, were unstable at temperatures required to elute the methyl cholanate trimethylsilyl ethers. However, a polar phase, Hi Eff-8B,[4] which was stable up to 280°C, was successful in separating the major fecal bile acids. This re-

[1] This investigation was supported by a research grant (H-2458, C4) from the National Institutes of Health, U. S. Public Health Service.

[2] On leave of absence, School of Pharmacy, Osaka University.

[3] This work was carried out during the tenure of an Established Investigatorship of the American Heart Association.

[4] Hi Eff-8B is cyclohexanedimethanol polysuccinate, Applied Science Laboratories, Inc., State College, Pennsylvania.

port describes the quantitative determination of fecal bile acids in the rat.

METHODS AND MATERIALS

Table 1 lists the origin and properties of the methyl cholanates em-

TABLE 1
RETENTION TIMES OF VARIOUS SUBSTITUTED METHYL CHOLANYL TRIMETHYLSILYL ETHERS[a]

Compound (Source[b])	M.p.[c] (°C)	Relative[d] retention time[e]	
		245°C, 15 psi	280°C, 40 psi
Methyl cholanate (a)	—[f]	1.00	—
3α-Hydroxy (b)	127–9	1.77	—
3β-Hydroxy (c)	113–5	1.42	—
3α,6α-Dihydroxy (d)	113–5	1.64	—
3α,7α-Dihydroxy (e)	65–71	1.40	—
3α,7β-Dihydroxy (e)	150–2	2.04	—
3α,12α-Dihydroxy (b)	78–86	1.30	—
3α,6α,7α-Trihydroxy (f)	—[f]	1.31	—
3α,6α,7β-Trihydroxy (f)	—[f]	2.61	—
3α,6β,7α-Trihydroxy (f)	—[f]	0.93	—
3α,6β,7β-Trihydroxy (f)	—[f]	1.58	—
3α,7α,12α-Trihydroxy (f)	155–6	0.97	—
3-Keto (c)	116–7	5.54	—
3α-Hydroxy-7-keto (c)	—[f]	5.45	—
3α-Hydroxy-12-keto (c)	112–4	5.16	1.35
3α,7α-Dihydroxy-12-keto (c)	156–8	4.01	1.00
3α,12α-Dihydroxy-7-keto (c)	152–4	4.10	1.00
3α-Hydroxy-7,12-diketo (f)	—[f]	—	3.20
3,7-Diketo (c)	158–61	—	4.47
3,12-Diketo (c)	126–8	—	4.44

[a] Bile acid derivatives are prepared as described in the text.

[b] Sources are: (a) Mann Research Laboratories, Inc. (b) Nutritional Biochemicals Corporation. (c) Prepared by the authors. (d) Dr. C. Sweeley, (e) California Corporation for Biochemical Research. (f) Dr. S. L. Hsia.

[c] Melting points are those of the corresponding substituted methyl cholanates.

[d] Relative to methyl cholanate at 245°C, 15 psi (10.4 min), and to 7-ketomethyl deoxycholate di(trimethylsilyl) ether at 280°C, 40 psi (4.9 min).

[e] Column conditions: 0.5% Hi Eff-8B (cyclohexanedimethanol polysuccinate, Applied Science, Inc.) on 80–100 mesh Gas-Chrom P; 9 ft × 3 mm, temperature and pressure as indicated.

[f] Melting points not obtained due to insufficient material.

ployed as standards in this study. In some cases, samples were obtained from commercial sources as noted or synthesized in our laboratory according to published methods. We are indebted to Dr. S. L. Hsia, St.

Louis University, and Dr. C. C. Sweeley for authentic samples of bile acids, as noted in Table 1.

Gas-Liquid Chromatography

The support (Gas-Chrom P,[5] 80–100 mesh) was coated with 0.5% of Hi Eff-8B (cyclohexanedimethanol polysuccinate) by techniques previously described (11, 12). A Barber-Colman instrument equipped with an argon ionization detector (A4183) was employed. Flash heater temperature was 290°C, column temperature 245°, detector temperature 257°, argon pressure 15 psi. The electrometer gain was 30 and detector voltage was 750. With a 9 ft $\times$ 3 mm glass U-column, the number of theoretical plates for 3α,7α,12α-trimethylsilyl triether of methyl cholanate was 2500.[6] Appropriate solutions of either single or mixed standard solutions of the various substituted methyl cholanyl trimethyl silyl ethers were prepared and injections of 0.2–5.0 μl were made with a 10-μl Hamilton syringe (No. 701, Hamilton Co., Whittier, California). For quantitative work, the areas[7] under each peak were determined by a Keuffel and Esser Co. compensating polar planimeter and the area plotted against the mass based on the corresponding weight of the original free bile acid.

EXPERIMENTAL

Preparation of Bile Acid Methyl Esters

Methyl esters of bile acids (standards or naturally occurring) were prepared according to the micro diazomethane procedure of Schlenk *et al.* (13). In general, the synthetic or isolated acids were dried in a ground-glass stoppered 12-ml heavy-walled centrifuge tube and dissolved in 0.3–0.4 ml of methanol and 10 volumes of anhydrous diethyl ether. The diazomethane was generated as described (13) and the excess diazomethane removed by ebullition with carrier nitrogen. The solvent was then removed in a warm water bath by a stream of nitrogen. The methyl esters of authentic bile acids were recrystallized and the melting points compared favorably to those in the literature (Table 1).

Preparation of Trimethylsilyl Ethers of Methyl Cholanates

Pure methyl cholanates or methylated fecal bile acid fractions were dissolved in anhydrous pyridine (distilled over BaO) (ca. 1 ml/10 mg).

[5] Applied Science Laboratories, Inc.

[6] Number of theoretical plates $= (x/y)^2(16)$, where $x =$ distance from injection to peak and $y =$ width of peak base.

[7] One unit on vernier = 0.01 sq in.

To this solution was added 0.1 ml of hexamethyldisilazane[8] and 0.03 ml of trimethylchlorosilane.[9] After standing a minimum of 10 min at room temperature, direct injections of appropriate aliquots of the reaction mixture into the gas chromatograph were made. Samples standing for several days were discarded, since evidence of ether instability was observed after prolonged storage.

Quantitative Response

Preliminary study of the retention times of the common bile acids found in the feces of rats on a standard semisynthetic diet revealed peaks which corresponded to significant amounts of deoxycholic, hyodeoxycholic, lithocholic, and 12-ketolithocholic acids, and a moderate peak having the same retention time as epilithocholic acid. Minor quantities of at least 9 other peaks (Fig. 2, X_1–X_9) were detected. Standard solutions of the trimethylsilyl ethers of methyl deoxycholic, hyodeoxycholic, lithocholic, and 12-ketolithocholic acids were prepared as previously described. Detector response was linear over a range of 0.5–2.0 μg (Fig. 1), and the molar response was variable, requiring calculation of the individual compounds.

Retention Times of Various Methyl Cholanyl Trimethylsilyl Ethers

Inspection of the retention times of the trimethylsilyl ethers of methyl cholanates (Table 1) discloses an inverse relationship of retention time and the number of substituent hydroxyl groups in most cases. In the muricholic acid series, the 7β and 6α oriented hydroxyl groups exerted a delaying influence on retention time. A further example of the effect of the 7β-hydroxyl group can be seen in the case of chenodeoxycholic vs. ursodeoxycholic acids. Keto groups resulted in a significant increase in the retention time of the methyl cholanates. Bile acids with more than one keto group could be detected and quantitated by increasing the temperature and pressure to 280°C and 40 psi, respectively. The preliminary finding that fecal extracts from rats fed a standard semisynthetic diet contained insignificant amounts of bile acids with more than one keto group led to the more common use of the moderate temperature (245°C) and pressure (15 psi).

Fecal Bile Acids

The fecal bile acids of several species have been studied in our laboratory. In this report, however, the quantitative data will be derived exclusively from the rat fed a specific diet. In a typical analysis, 4 adult

[8] Peninsular Chemresearch, Inc., Gainesville, Florida.

[9] General Electric Company, Silicone Products Department, Waterford, New York.

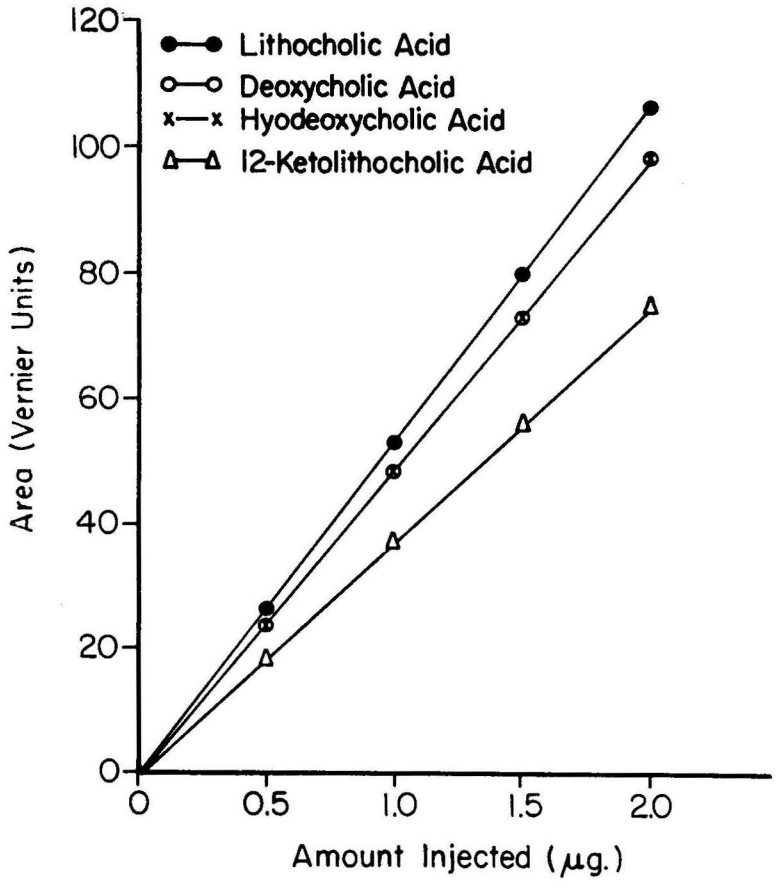


FIG. 1. Relationship of varying amounts of trimethylsilyl ether of methyl lithocholate, deoxycholate, hyodeoxycholate, and 12-ketolithocholate to area recorded. The abscissa is expressed as μg of free bile acids. The ordinate refers to planimeter area (see footnote 7). Column and conditions as for Table 1.

male rats of the Holtzman strain (250 gm) were fed a semisynthetic diet (11) for 4 days. Fecal pellets were collected for the next 5 days while the animals consumed the diet *ad libitum*. The combined pellets were extracted with acetone:absolute ethanol (1:1) for 24 hr in a Soxhlet apparatus. The extract was transferred to a 100-ml volumetric flask. This extract was designated F.E. (see Table 2) for the analysis and recovery study. An aliquot of 5 ml was transferred to a 250-ml round-bottom flask and the solvents were evaporated *in vacuo*. For each sample containing up to 10 mg of total bile acids, 5 ml of 1.25 *N* NaOH was added to the residue. The mixture was then autoclaved for 3 hr at 120°C and

TABLE 2

ANALYSIS AND RECOVERY OF VARIOUS BILE ACIDS[a] FROM RAT FECES BY GAS-LIQUID CHROMATOGRAPHY OF THEIR METHYL ESTER-TRIMETHYLSILYL ETHERS

Material	Dexoycholic		Hyodeoxycholic		Lithocholic		12-Keto-lithocholic	
	Added (mg)	Found (mg)	Added (mg)	Found (mg)	Added (mg)	Found (mg)	Added (mg)	Found (mg)
5 ml F.E.[b]								
Sample 1	—	0.57	—	1.00	—	0.32	—	0.28
2	—	0.55	—	0.98	—	0.32	—	0.25
3	—	0.59	—	1.02	—	0.34	—	0.28
Mean		0.57		1.00		0.33		0.27
Sample 4	0.99	1.60	1.00	2.00	0.99	1.34	1.02	1.25
5	0.99	1.54	1.00	1.91	0.99	1.27	1.02	1.17
6	0.99	1.46	1.00	1.85	0.99	1.30	1.02	1.25
Mean		1.53		1.92		1.30		1.22
Average recovery (%)		97		92		98		93

[a] The values represent weight of bile acid calculated from the methyl ester-trimethylsilyl ethers.

[b] F.E. signifies fecal extract (see text for description); 5 ml is equivalent to 1 rat-day.

15 psi. The flask was cooled to room temperature and the saponified mixture transferred with 5–10 ml of water to a 250-ml separatory funnel. The mixture was extracted 4 times with 3 volumes of ether and the combined ether extracts were washed with several 1–2 ml portions of water. The ether fraction may be used to determine neutral sterols (11). The aqueous phase with water wash added from the last procedure was acidified to pH 1–2 with about 1.25 ml of 6 *N* HCl. The bile acids were then extracted 4 times with 3 volumes of diethyl ether. The combined ether extracts were washed with small (1–3 ml) quantities of water until the water was free of Cl ions ($AgNO_3$ test). The ether was evaporated from a round-bottom flask and the residue dissolved in a mixture of 30 ml of 70% ethanol and 30 ml of Skellysolve B (previously equilibrated). The bile acids were partitioned through a 3-funnel countercurrent distribution, and the combined 70% ethanol fraction was dried in a 12-ml heavy-walled ground-glass stoppered centrifuge tube. The procedure for methylation and ether formation has been described above. Three 5-ml aliquots of the fecal extract were each separately analyzed for deoxycholic, hyodeoxycholic, lithocholic, and 12-ketolithocholic acids. Average daily excretions of 0.57, 1.00, 0.33, and 0.27 mg, respectively, were found. Discounting peak X_4, these values are generally in good agreement or lower than the daily excretion of bile acids reported by others (14). One explanation for values higher than those reported here

is the possibility of contamination by hydroxy fatty acids in methods employing titration of the total bile acid fraction (15). For recovery studies, known amounts of each of the 4 bile acids (as their methyl esters) were added to each of 3 F.E. aliquots (5 ml). The average recovery of the bile acids varied from 92 to 98%. A comparison of the chromatographic record of some standard trimethylsilyl ethers of substituted methyl cholanates with that of a treated aliquot of rat feces bile acid fraction (Fig. 2) tentatively identified the major components as

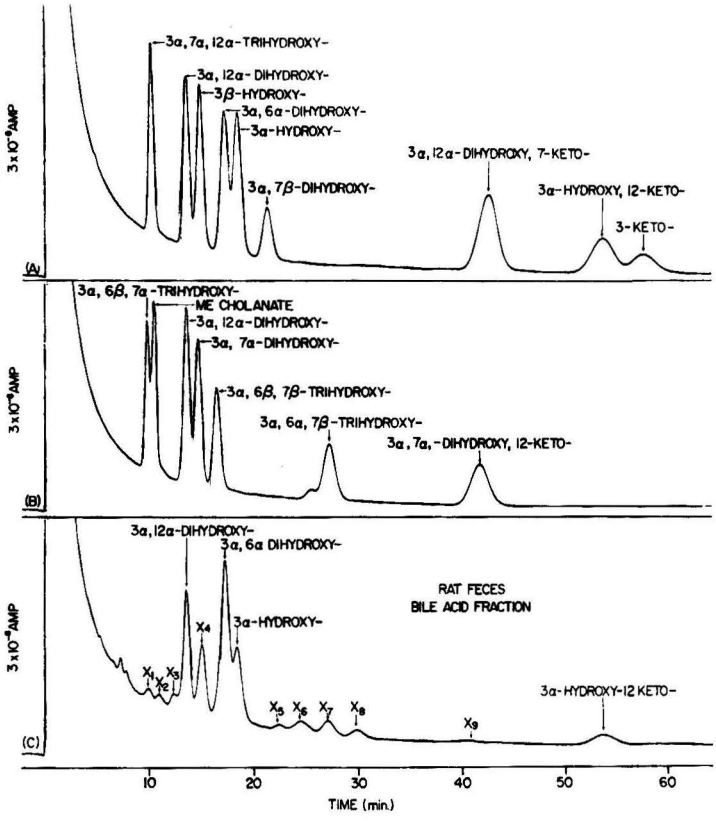


FIG. 2. Analytical records obtained in gas-liquid chromatography of: (A, B) trimethylsilyl ethers of methyl cholanates substituted as noted above; (C) rat feces bile acid fraction treated with diazomethane and hexamethyldisilazane and trimethylchlorosilane in pyridine. Column and conditions as for Table 1.

previously noted, with the exception of X_4, whose positive identification requires further investigation.

DISCUSSION

The column used in this study was stable over a 4-week period of continual use. The advantage of pyridine as catalyst and solvent for the ether formation over our previously recommended solvent, tetrahydrofuran, was notable. The use of dry pyridine (longer shelf life) resulted in complete ether formation of all hydroxyl groups under mild conditions and reduced the minimum reaction time to 10 min [vs. 2 hr (11)]. The application of this procedure to physiological and nutritional studies of sterol and bile acid metabolism is under investigation.

SUMMARY

1. A method has been developed for the quantitative determination of fecal bile acids of the rat by gas-liquid chromatography of their methyl ester trimethylsilyl ethers on a 0.5% cyclohexanedimethanol polysuccinate phase supported on Gas-Chrom P. Recoveries of added amounts of pure bile acids were shown to vary from 92 to 98%.

2. The principal bile acids found in the feces of rats fed a standard semisynthetic diet were tentatively identified as deoxycholic, hyodeoxycholic, lithocholic, and 12 ketolithocholic, and the daily excretion by adult male rats was 0.57, 1.00, 0.33, and 0.27 mg, respectively. Several bile acids of questionable identity were present in minor quantities.

REFERENCES

1. KIER, L., *J. Lab. Clin. Med.* **40**, 755 (1952).
2. MOSBACH, E. H., KALINSKY, J. H., HALPERN, E., AND KENDALL, F. E., *Arch. Biochem. Biophys.* **51**, 402 (1954).
3. MACINTYRE, I., AND WOOTTON, I. D. P., *Ann. Rev. Biochem.* **29**, 635 (1960).
4. SJÖVALL, J., *Acta Physiol. Scand.* **29**, 232 (1953).
5. JAMES, A. T., WEBB, J. P. W., AND KELLOCK, T. D., *Biochem. J.* **78**, 333 (1961).
6. VANDEN HEUVEL, W. J. A., SWEELEY, C. C., HORNING, E. C., *Biochem. Biophys. Res. Commun.* **3**, 33 (1960).
7. BLOOMSTRAND, R., *Proc. Soc. Exptl. Biol. Med.* **107**, 126 (1961).
8. SJÖVALL, J., MELONI, C. R., AND TURNER, D. A., *J. Lipid Res.* **2**, 317 (1961).
9. HOLMES, W. L., AND STACK, E., *Biochim. et Biophys. Acta* **56**, 163 (1962).
10. BLOOMFIELD, D. K., *Anal. Chem.* **34**, 737 (1962).
11. WELLS, W. W., AND MAKITA, M., *Anal. Biochem.* **4**, 204 (1962).
12. HORNING, E. C., MOSCATELLI, E. A., AND SWEELEY, C. C., *Chem. & Ind. (London)* **1959**, 751.
13. SCHLENK, H., AND GELLERMAN, J. L., *Anal. Chem.* **32**, 1412 (1960).
14. PORTMAN, O. W., *Am. J. Clin. Nutr.* **8**, 462 (1960).
15. GAYLOR, J. L., HARDY, R. W. F., AND BAUMANN, C. A., *J. Nutr.* **70**, 293 (1960).

ANALYTICAL BIOCHEMISTRY 5, 531–541 (1963)

A Specific Assay for Tocopherols in Plant Tissue[1]

R. A. DILLEY AND F. L. CRANE

From the Department of Biological Sciences, Purdue University, Lafayette, Indiana

Received September 27, 1962

Numerous methods for analysis of α-tocopherol in plant material have been described. No attempt will be made here to review them, as excellent reviews are available (1, 2).

The most common method of measuring tocopherol is the colorimetric Emmerie-Engel assay. Booth (3) recently pointed out the many sources of spurious results encountered when this method is used, making it highly nonspecific and rather difficult to employ with confidence.

In an attempt to obtain greater specificity for tocopherol analysis we have developed an assay partly from previously described methods and partly from new innovations. In principle, the method is as follows:

1. Saponification in the presence of pyrogallol or direct solvent extraction (acetone).
2. Paper or thin-layer preparative strip chromatography to separate the tocopherol from other compounds.
3. Gold chloride oxidation of the eluted tocopherol to the tocopheryl-quinone and spectrophotometric assay of the quinone form by reading the drop in absorbance upon reduction, by added borohydride, of the quinone to the hydroquinone.

MATERIALS

Tocopherols (α, β, γ, and δ) were obtained from Eastman Distillation Products Industries as a generous gift from Dr. S. R. Ames. α-Tocopheryl-quinone was obtained from the same source, as was the isooctane and n-heptane (both spectral grade). Gold chloride as $HAuCl_4 \cdot 3H_2O$ was obtained from Baker Chemical Co. Paraffin oil, viscosity 335/350, was obtained from Fisher Scientific Co. Silica gel G for thin-layer chromatography and the apparatus for preparing the chromatogram plates were obtained from Brinkmann Instruments Inc. All other chemicals were reagent grade.

[1] This work was supported in part by grant number RG-8025 (C 1) from the U. S. Public Health Service and grant number G 16083 from the National Science Foundation.

The reagents used to detect tocopherol spots on chromatograms were made up as follows:

The $FeCl_3$-α,α'-dipyridyl reagent is composed of 1 part 0.2% w/v $FeCl_3$ in ethanol, 1 part 0.5% w/v α,α'-dipyridyl in ethanol, and 2 parts water made up just prior to use and kept in the dark. This reagent gives a red color at room temperature when it comes in contact with any compound capable of reducing Fe^{+++} to Fe^{++} (4).

The neotetrazolium chloride reagent is made according to Lester and Ramasarma (5) with 0.25% w/v neotetrazolium chloride in 0.25 *M* potassium phosphate, pH 7.0. This is suitable for papers only, since after spraying the paper must be put in a 105–110°C oven for several minutes.

Readings were taken with a Beckman model D.U. spectrophotometer.

METHODS

Extraction of Neutral Lipids from Plant Tissue

Saponification. Saponification in the presence of pyrogallol (to protect tocopherol from oxidative destruction) is a standard procedure (6, 7). We recommend the following modification to the procedure: (*1*) To a homogenate of plant tissue add ethanol to make the final slurry 66% in ethanol. Reflux for 15 min. Cool slightly. (*2*) Add 15% KOH weight per volume of original tissue homogenate and 10% pyrogallol weight per volume of original homogenate. Reflux again for 10 min to effect the saponification. The cooled mixture is extracted repeatedly with *n*-heptane or petroleum ether, the organic phase washed three times with water to remove salts, and its volume reduced to dryness at 50°C; the residue is taken up in *n*-heptane. This organic phase is now ready for strip chromatography.

Direct Solvent Extraction. This less severe procedure, when carried out at temperatures near 0°C, will allow recovery of other neutral lipids such as plastoquinone and vitamin K, which are partly or wholly destroyed by saponification. We find acetone to be the most useful solvent for extraction. We recommend an initial 80% acetone extraction of homogenized tissue at about 0°C for 4 hr. The mixture should be filtered or centrifuged and the precipitate extracted again overnight at about 0°C with 100% acetone. The combined acetone supernatants may be reduced to near dryness, taken up in a suitable volume of *n*-heptane, isooctane, or petroleum ether, and washed with water to remove traces of salt. This organic phase, reduced to a small volume, is ready for the chromatography step.

Preparative Strip Chromatography

Paper Method. This is suitable for saponified extracts but not for

direct extraction material since the chlorophyll causes streaking on the paper when enough extract is applied to recover sufficient tocopherol. We have used the method of Brown (8) with petroleum jelly-impregnated paper, developed with ethanol/water 3:1 v/v in an ascending flow. The paper is prepared by dipping a sheet of Whatman No. 3 paper in a 3% v/v solution of paraffin oil (described in *Materials*) in chloroform and is air-dried. The extract is applied as a strip along the origin with a portion of the paper at one side used for spotting a pure tocopherol sample, tocopherol plus extract, and an extract sample. After developing in ascending flow for 2.5 hr these spots are sprayed with a suitable reagent to locate the tocopherol. Either the $FeCl_3$-α,α'-dipyridyl reagent or the neotetrazolium chloride reagent is suitable.

Thin-Layer Chromatography Method. This is suitable for either type of extract and has many other advantages over the paper method, such as its faster rate of development, greater capacity of crude extract per unit length of strip, and the characteristic of allowing a smaller amount of compound to give a visible spot when developed with the color-developing reagent. For details of the technique of preparing the plates see Technical Bulletin No. 20, March, 1962, of Brinkmann Instruments Inc., 115 Cutter Mill Road, Great Neck, New York. The stationary phase is silica gel G and the mobile phase is benzene in an ascending flow. This method was previously used by Seher (9) for identification of tocopherols. We have modified it slightly for use as a preparative technique. As much as 12 mg of chlorophyll has been applied along a 14-cm strip of the origin and there was no streaking to interfere with the separation and recovery of the tocopherol.

The thin-layer plates are coated with silica gel G (Brinkmann), dried in a 105–110°C oven for 30 min, and stored in an 80° oven until used.

For application of the extract to the plate, a 50- or 100-λ micropipet is useful. With care, the contents of the pipet can be applied along the origin as one moves the pipet smoothly along, close enough to the plate so that the liquid drop touches the plate (the tip of the pipet must not touch the silica gel as it will gouge out a strip of the adsorbent). Enough extract to give about 10 to 100 μg of tocopherol is sufficient. As before, three check spots of pure tocopherol, extract, and tocopherol plus extract may be applied, to be later developed with the $FeCl_3$-α,α'-dipyridyl reagent. The portion of the chromatogram above the strip should be covered with a paper towel while spraying the check spots. Upon development of the check spots, the corresponding tocopherol area over the strip is scraped off and placed in 10 ml of methanol. After several minutes the solution is centrifuged (a clinical-type centrifuge is satisfactory), the supernatant decanted, and the precipitate stirred up with another 10 ml methanol and centrifuged again. The combined supernatants are reduced

to dryness and taken up in 4 ml ethanol. One milliliter of this solution is used for the oxidation step.

Gold Chloride Oxidation of Tocopherol to the Tocopherylquinone

Oxidation of tocopherol to the tocopherylquinone by gold chloride was first described by Karrer and Geiger (10) and studied by Tishler and Wendler (11). According to the above workers the reaction is a two-electron reduction of Au^{++} to metallic Au.

To 1 or 2 ml of ethanolic solution containing 5 to 100 μg tocopherol add 0.2 ml 20% w/v aqueous gold chloride. This keeps the equivalents of gold 200 times or more in excess of the tocopherol. When using the paper chromatographic method for isolating tocopherol there may be turbidity in the oxidation mixture (due to paraffin) unless about 2 ml of ethanol is added.

The reaction is allowed to proceed for 25 min in the dark, at which time 15 ml isooctane or *n*-heptane (spectral grade) is added and the combined mixture swirled *gently* in a separatory funnel. Three washes with distilled water (20 ml each) are performed with gentle swirling. If the mixture is shaken vigorously there may be some gold trapped in the organic phase which can interfere with the spectrophotometric assay.

The organic phase is evaporated to dryness and taken up in ethanol or 4:1 ethanol/isooctane (if paraffin appears in the evaporating flask) to a volume of 3 or 4 ml. The sample is now ready for the spectrophotometric assay.

Spectrophotometric Assay of Tocopherylquinone

The absorbance of an aliquot of the above solution is read at 262 mμ in ethanol before and after adding 0.02 ml of a 20% w/v solution of KBH_4 in 50% aqueous ethanol. From the ΔA (A = absorbance), the concentration of tocopherylquinone can be calculated by the formula:

$$\frac{\Delta A}{E^{1\%}_{1cm}\ \text{ox-red}\ 262\ m\mu} \times 10 = \text{mg } \alpha\text{-tocopherylquinone/ml}$$

The $E^{1\%}_{1cm}$ ox-red 262 mμ in ethanol for α-tocopherylquinone is 397. When the results are to be given in terms of the chroman form, i.e., α-tocopherol, the $\Delta E^{1\%}_{1cm}$ should be 414 (cf. *Discussion*).

Stepwise Detailed Assay for α-Tocopherol in Spinach Chloroplasts

Saponification. Twenty milliliters of chloroplast suspension containing 1.574 mg/ml chlorophyll was added to 51 ml of 95% ethanol and refluxed for 15 min. After brief cooling, 3.76 ml of 80% aqueous KOH and 2 gm of pyrogallol were added to the flask, refluxed again for 10 min,

and cooled. The soap was extracted six times with approximately 150 ml *n*-heptane. The organic phase was washed three times with water, dried over Na_2SO_4 and evaporated to dryness; the residue was taken up in 8 ml ethanol + 2 ml isooctane.

Paper Chromatography. Whatman No. 3 paper 11.5 by 18 in. was treated with 3% v/v paraffin oil in $CHCl_3$; 0.625 ml of the above extract was applied along 11 in. of the long dimension of the paper. Pure α-tocopherol (30 μg), 20 μl of the extract, and 30 μg of α-tocopherol plus 20 μl of extract were applied as three separate spots adjacent to the strip of extract. The paper, stapled into a cylinder, was placed in a chromatography jar containing 3:1 v/v ethanol/water in the bottom and allowed to develop for 2.5 hr. The three check spots were sprayed with the neotetrazolium reagent as explained above and placed in 110°C heat for 2 min to allow the spots to develop. A strip was then cut from the remaining portion of the paper corresponding to the position of the α-tocopherol on the check paper. A corresponding blank was cut from the paper above the solvent front, and both were eluted with acetone. The eluates were evaporated to dryness and taken up in 8 ml ethanol and 2 ml isooctane (to solubilize the paraffin). The solutions may be centrifuged in a clinical centrifuge to bring down any paper fibers. Since the sample is usually diluted 2-fold with ethanol prior to the spectrophotometer reading, the use of 4:1 ethanol/isooctane in the above step makes a negligible difference in the absorbance change.

Gold Chloride Oxidation. The oxidation was carried out as explained above.

Spectrophotometric Measurement of α-Tocopherylquinone. This was carried out as outlined above.

Direct Extraction. A different batch of spinach chloroplasts was used for this assay. To a volume of 113 ml of chloroplasts containing 0.19 mg chlorophyll/ml was added 450 ml acetone to make the final mixture 80% in acetone. This mixture was left for 4 hr at 4°C, and then filtered; 300 ml acetone was added back to the residue. This was left overnight at 4°C and filtered. The combined acetone extracts were reduced in volume to about 50 ml, added to 150 ml *n*-heptane, and shaken in a separatory funnel. This heptane extraction was repeated 6 times until the aqueous phase contained a barely noticeable green color. Adding a pinch of NaCl helped break the emulsion which tended to form. The combined heptane extract was reduced to 13 ml volume *in vacuo* at 50°C.

To a thin-layer chromatogram plate prepared as explained above with silica gel G, 0.8 ml of the above solution was applied as a strip along the origin. Check spots were applied along one side as explained above. The plate was developed by ascending flow with benzene. After 1 hr the

solvent front had moved ¾ the distance up the plate, at which time the plate was removed and the check spots sprayed with the $FeCl_3$-α,α'-dipyridyl reagent, whereupon the α-tocopherol spots became visible. The corresponding α-tocopherol strip was scraped off the plate and eluted with methanol as described above. A blank plate developed with benzene, from which a corresponding silica gel strip was scraped off, served as a control.

The oxidation treatment was the same as for the sample prepared from the paper chromatogram, except that pure ethanol was used rather than 4:1 v/v ethanol/isooctane.

RESULTS AND DISCUSSION

The determination of the extinction coefficient for the oxidized minus reduced spectra of α-tocopherylquinone at 262 mμ in ethanol ($\Delta E^{1\%}_{1cm}$) was carried out using a sample of the pure compound and borohydride reduction. With the value of 412 for $E^{1\%}_{1cm}$, as determined from gravimetric and spectral measurements, $\Delta E^{1\%}_{1cm}$ was found to be 397 ± 2. The value of 412 for $E^{1\%}_{1cm}$ 262 mμ in ethanol is in the proper range for this compound. Tishler and Wendler assign the value of 420 for $E^{1\%}_{1cm}$ 262 mμ in petroleum ether (11); the value in ethanol would be slightly lower than this.

When $\Delta E^{1\%}_{1cm}$ ox-red for α-tocopherylquinone is adjusted to the molecular weight of α-tocopherol, the resultant $\Delta E^{1\%}_{1cm}$ is 414. This is the value to be used in calculating the amount of α-tocopherol in a sample assayed by this method.

The chromatographic pattern of tocopherols in the paper and thin-layer systems has been reported previously (8, 9). The R_f values of α-, β-, γ-, and δ-tocopherols as determined by us are given in Table 1. It is seen that β- and γ-tocopherols are not resolved by these systems. Green (7) has described a system for separating these isomers with two-dimensional paper chromatography.

Experiments were carried out to determine the per cent recovery of pure α-tocopherol from the paper and thin-layer chromatographic separations and the gold chloride oxidation steps. Table 2 gives the results of using the procedures as explained in the *Methods* section. The amounts of pure α-tocopherol applied to each chromatogram, the amount measured by the assay, and per cent recovery are listed. For the paper chromatographic technique 86 and 87% recoveries were obtained in two separate runs, and for the thin-layer technique 86 and 89% recoveries were obtained.

If saponification is used as the extraction method a certain loss of tocopherol is to be expected due to the rather harsh conditions. To deter-

TABLE 1

CHROMATOGRAPHIC DATA OF α-, β-, γ-, AND δ-TOCOPHEROLS, VARIOUS QUINONES, AND β-CAROTENE WITH THE PAPER AND THIN-LAYER CHROMATOGRAPHIC SYSTEMS

Compound	R_f [a]	
	Thin-layer	Paper
α-Tocopherol	0.52	0.47
β-Tocopherol	0.37	0.70
γ-Tocopherol	0.37	0.71
δ-Tocopherol	0.24	0.86
Plastoquinone A[b]	1	0
Plastoquinone B	1	0
Plastoquinone C	0	<0.47
Coenzyme Q_{10}	1	0
β-Carotene	1	0
Vitamin K_1	1	0

[a] The systems are as described under *Methods*.

[b] For the definition of these compounds see ref. (12).

mine the probable maximum destruction due to this treatment, a sample of pure α-tocopherol (25.1 mg) was saponified for 15 min in dim light in the presence of 20 ml ethanol, 3.48 gm KOH (15% w/v), 2.32 gm pyrogallol (10% w/v), and 8 ml H_2O. The mixture was cooled and extracted five times with 150 ml *n*-heptane; the organic phase was washed three times with water, evaporated to dryness, and taken up in 5 ml ethanol. On the basis of absorbance at 292 mμ there was 22.8 mg α-tocopherol or a 91% recovery. Bouman *et al.* (13) also reported a 91% recovery of α-tocopherol from saponification carried out in the dark and in the presence of pyrogallol.

An experiment was conducted to determine the recovery of α-tocopherol added to a spinach chloroplast assay procedure. Spinach chloroplasts

TABLE 2

RECOVERY OF α-TOCOPHEROL FROM PAPER CHROMATOGRAPHY, THIN-LAYER CHROMATOGRAPHY, AND GOLD CHLORIDE OXIDATION

Chromatographic system	Sample	α-Tocopherol applied to chromatogram (mg)	α-Tocopherol obtained by gold chloride assay (mg)	Per cent recovery
Paper	1	1.93	1.67	86
	2	1.93	1.68	87
Thin-layer	1	0.659	0.585	89
	2	0.330	0.284	86

The procedure was as described under *Methods*, starting with the chromatography step.

were prepared in the usual fashion (14) and suspended in 0.5 *M* sucrose, buffered at pH 8.0. The chlorophyll content was 1.57 mg/ml, determined by Arnon's method (15). Two samples of 20 ml each, to one of which was added 13.18 mg α-tocopherol, were carried through the assay procedure explained in the *Methods* section, using the paper chromatographic separation. Table 3 shows the results of the assay. It is seen that 76 and

TABLE 3

ASSAY OF SPINACH CHLOROPLASTS FOR α-TOCOPHEROL AND RECOVERY OF ADDED α-TOCOPHEROL

Sample	α-Tocopherol added (mg)	α-Tocopherol measured by assay (mg)	Per cent recovery of added α-tocopherol
1	None	0.45	
2	13.18	10.4	76
3	13.18	10.8	79

79% recoveries were realized in the two assays. This is in the range one would expect since by saponification 9% α-tocopherol is lost and in the remainder of the steps 14% of the α-tocopherol is lost (see Table 2), and the total recovery would be the product of the individual recoveries, i.e., $0.91 \times 0.86 = 0.78$.

As a measure of the reproducibility of the procedure, a sample of saponified spinach whole leaf tissue was assayed four times using the paper chromatographic separation. For this experiment 235 gm fresh weight of spinach leaf tissue was treated according to the technique described in the *Methods* section. Table 4 gives the results of these assays.

TABLE 4

REPRODUCIBILITY OF THE ASSAY

Sample	α-Tocopherol as measured by assay (mg)
1	6.40
2	6.18
3	6.11
4	5.98
Average	6.18 ± 0.2, or ±3%

The amount of α-tocopherol measured was 6.2 ± 0.2 mg or ±3%. From these data we conclude that, in our hands, this assay will measure 78% ± 3% of the α-tocopherol in a given sample of plant tissue in the saponification procedure and 87% ± 3% when direct cold solvent extrac-

tion is used. The corresponding correction factor is used to adjust our results to arrive at the best estimate of the tocopherol content in a tissue sample.

Comparisons were made between α-tocopherol values measured by this

TABLE 5

α-TOCOPHEROL IN LEAVES OF VARIOUS PLANT SPECIES

Species	mg α-tocopherol / gm fresh weight	mg α-tocopherol / gm dry weight
Spinach (*Spinacia oleracea*)	0.032	1.0
Lilac (*Syringa vulgaris*)	0.38	3.2
Bush bean (*Phaseolus vulgaris* var. tendergreen)	0.047	0.33
Runner bean[a] (*Phaseolus coccineus*)		
Young leaves	0.025	0.17
Old leaves	0.080	0.53

[a] Data from ref. (16).

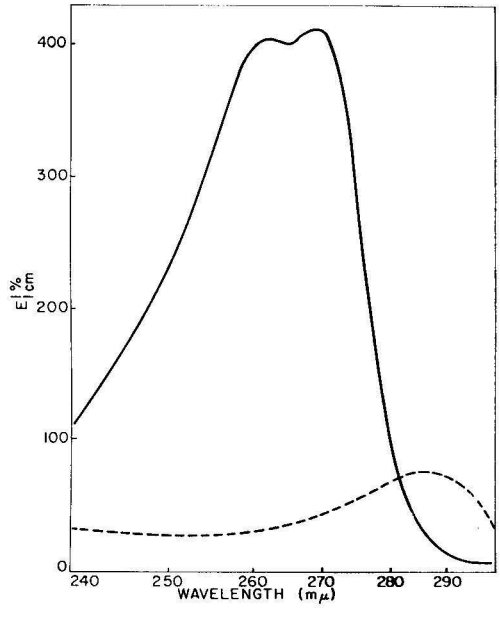


FIG. 1. UV adsorption spectra of oxidized-reduced form of α-tocopherylquinone in ethanol. The upper curve represents the oxidized form and the lower (broken) curve, the reduced form. The ordinate is in units of the absorbance of a 1% solution with an optical path length of 1 cm, i.e., $E^{1\%}_{1cm}$. The abscissa gives the wavelength in mμ.

method (using saponification and paper chromatographic separation) in spinach, lilac, and bush bean leaves. A value for the α-tocopherol content of runner bean taken from the literature (16) is compared to our values for bean and found to agree quite well. Table 5 gives these data.

This method may be used to differentiate between α-tocopherol, β- and γ-tocopherols combined, and δ-tocopherol. The data presented in this paper deals only with α-tocopherol, but one may adapt the assay for the other classes of tocopherols. Figures 1 and 2 show the spectra of α- and

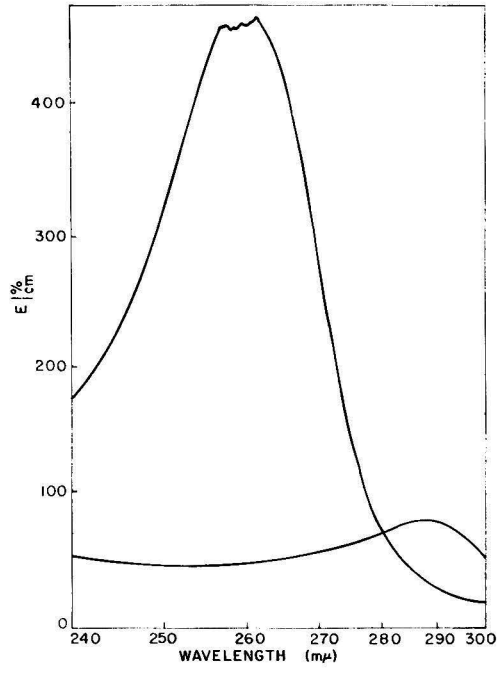


FIG. 2. UV absorption spectra of the oxidized-reduced form of γ-tocopherylquinone in ethanol. The coordinates are in the same units as Fig. 1. $\Delta E^{1\%}_{1cm}$ oxidized-reduced at 262 mμ is 412 for γ-tocopherylquinone.

γ-tocopherylquinones oxidized and reduced, with the $\Delta E^{1\%}_{1cm}$ value for γ-tocopherol given in the legend.

The specificity of the method lies mainly in the fact that upon oxidation the $E^{1\%}_{1cm}$ 262 mμ increases from less than 10 for α-tocopherol to 412 for the quinone form and upon reduction the value falls to about 30. It is the drop in absorbance upon reduction ($\Delta E^{1\%}_{1cm}$ 262 mμ ox-red in ethanol is 397 ± 2) of the quinone form which is used as the measure of α-tocopherol.

SUMMARY

A method for the assay of α-tocopherol in plant material (adaptable for assay of β- and γ-tocopherols combined and δ-tocopherol) is presented that is more specific than assays presently available and just as sensitive.

The method employs either saponification or direct cold solvent extraction and either paper or thin-layer chromatographic separation. The thin-layer method has distinct advantages which are discussed.

Data are given which show the level of recovery expected in the various stages of the procedure. The over-all recovery for saponification and either chromatographic means is $78 \pm 3\%$ while the direct extraction and either chromatographic means yields about $87 \pm 3\%$.

This method has been used to assay several plant species; the results are shown to agree quite well with previously reported values.

ACKNOWLEDGMENT

The technical assistance of Mrs. Carolyn McNeely is gratefully acknowledged.

REFERENCES

1. LEHMAN, R. W., *Methods Biochem. Anal.* **2**, 153 (1955).
2. Report Prepared by the Vitamin E Panel, *Analyst* **84**, 356 (1959).
3. BOOTH, V. H., *Anal. Chem.* **33**, 1224 (1961).
4. EMMERIE, A., AND ENGEL, C., *Rec. trav. chim.* **57**, 1351 (1938).
5. LESTER, R. L., AND RAMASARMA, T., *J. Biol. Chem.* **234**, 672 (1959).
6. LIPS, H. J., *J. Am. Oil Chemists' Soc.* **33**, 426 (1956).
7. GREEN, J., *J. Sci. Food Agr.* **9**, 801 (1958).
8. BROWN, E., *Biochem. J.* **51**, 237 (1952).
9. SEHER, A., *Mikrochim. Acta* **308** (1961).
10. KARRER, P., AND GEIGER, A., *Helv. Chim. Acta* **23**, 455 (1940).
11. TISHLER, M., AND WENDLER, N. L., *J. Am. Chem. Soc.* **63**, 1532 (1941).
12. DILLEY, R. A., KEGEL, L. P., AND CRANE, F. L., *Plant Physiol.* **37**, Suppl. xi (1962).
13. BOUMAN, J., AND SLATER, E. C., *Biochem. et Biophys. Acta.* **26**, 624 (1957).
14. CRANE, F. L., *Plant Physiol.* **34**, 128 (1959).
15. ARNON, D. I., *Plant Physiol.* **24**, 1 (1949).
16. BOOTH, V. H., AND HOBSON-FROHOCH, A., *J. Sci. Food Agr.* **12**, 251 (1961).

ANALYTICAL BIOCHEMISTRY 5, 542–547 (1963)

Electrochromatographic Separation of Inorganic Phosphate, Adenosine Monophosphate, Adenosine Diphosphate, and Adenosine Triphosphate[1,2]

T. R. SATO, J. F. THOMSON, AND W. F. DANFORTH[3]

From the Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois

Received October 1, 1962

For the investigation of enzymically catalyzed phosphate-adenosine triphosphate exchange, a precise analytical procedure for the separation of phosphate, adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP) was required. To avoid some of the disadvantages inherent in other methods, as reviewed by Klowwen (1), we have devised a sensitive and reliable electrochromatographic procedure. This method, which resembles those reported by Schaefer (2) and by von Korff (3), depends upon the differential electrical migration of the phosphorus compounds in the presence of zinc ions in a carefully buffered solution. In addition to improving the separability of the phosphorus compounds, the zinc ions stabilize the ADP and the ATP against hydrolysis (4–8).

METHODS

Background or Buffer Solutions

An effective background or buffer solution, which determines the separability of the several species, was selected by systematic variation of buffers and of stabilizing salts as shown in the experimental section. The solution eventually chosen contained citric acid (0.035 *M*) plus sodium citrate (0.0148 *M*) as buffer, pH 3.8, and zinc acetate (0.003 *M*) to improve separability and stability of the phosphorus compounds. It satisfied several requirements: effective separation of the phosphorus com-

[1] This work performed under the auspices of the U. S. Atomic Energy Commission.

[2] Taken from a thesis submitted by T. R. Sato to the Department of Biology, Illinois Institute of Technology, in partial fulfillment of the requirements for the Doctor of Philosophy degree.

[3] Department of Biology, Illinois Institute of Technology, Chicago, Illinois.

pounds, formation of well-defined zones, suitable pH and electrical conductivity, and noninterference with detection tests for the separated species.

Papers

The buffer solutions were stabilized with commercial filter paper made of wood pulp (Eaton-Dikeman Co., Grade 301, thickness 0.076 cm; Whatman filter papers No. 3 MM, No. 1, and No. 4). Several strips (2.54-cm width) were washed with 1 *N* nitric acid for one day and then with distilled water for four days. The washed strips were separated and then dried in air on clean sheets of polyethylene (7).

Materials

Nonradioactive AMP, ADP, and ATP were obtained from Pabst Laboratories. P^{32}-labeled ADP and ATP were prepared by enzymic exchange using rat liver mitochondria as described by Colowick and Kaplan (9). The AMP was not labeled by this method. $H_3P^{32}O_4$ was obtained from Oak Ridge National Laboratories. The radioactivity served to locate the several species separated by electrochromatography. Mitochondria were prepared from rat liver according to the method of Schneider and Hogeboom (10) using 0.25 *M* sucrose.

Migration Apparatus

Migrations at a moderate potential (about 5–10 volts/cm) were carried out in strips of the acid-washed paper 2.54 cm wide and 91.44 cm long. These strips were placed on a sheet of polyethylene across a water-cooled flat plate which was constructed by soldering copper tubing to one side of a copper plate 0.32 cm thick. For the migration studies, the strips of paper were first moistened with the buffer solutions. Aqueous solutions of the phosphorus compounds (about 5–10 μl of about 0.001 *M* "reaction mixture") were placed in small zones near the cathode end of the moist paper. The strips of paper were then covered with polyethylene sheets, and direct current potential, either 5 volts/cm for 17 hr or 10–15 volts/cm for 2–4 hr, was applied. After the migration, the ends of the strips of paper were cut off, and the strips of paper were uncovered and allowed to dry in air.

PROCEDURE

For the isolation of the radioactive ADP and ATP formed by the exchange reaction, the electrochromatographic method was employed. The exchange reaction was carried out in 15-ml centrifuge tubes and the experiment was terminated by the addition of 0.1 vol of cold 1 *N*

perchloric acid (or equivalent) with chilling. The precipitated protein was centrifuged, and the cold extract was neutralized immediately with 1 *N* potassium hydroxide to pH 6 to 7, using phenol red as an internal indicator. The mixture was chilled almost to the freezing point to bring about the precipitation of potassium perchlorate, which was removed by centrifugation. The supernatant solution was immediately submitted to electrical migration in the citrate-zinc acetate solution described above.

For detection and location of the radioactive compounds separated by the electrical migration, radioautographs were made with Kodak no screen X-ray film. Both the radioactive and the nonradioactive nucleotides, especially the AMP, could be detected with ultraviolet light by noting the dark spots on the fluorescing background of the paper. Phosphoric acid was located by its blue color after treatment of the paper with molybdic acid and exposure to ultraviolet light (11). The quantity of P^{32} incorporated in the compounds on the electrochromatograms was measured with an automatic chromatogram scanner, Vanguard 880,[4] provided with a windowless, gas-flow tube and a precision rate counter, the output of which was taken to a recorder having a chart speed synchronized with that of the scanner mechanism. The strips were pulled under a slit of aperture 0.16 or 0.63 cm at rates ranging from 0.63 to 5.08 cm/min. Proper selection of conditions in this range made possible reproducible determinations of amounts of P^{32} in spots containing as few as 100 or as many as 2×10^6 total counts per minute. The area under the curve for each radioactive compound was integrated by the automatic data system of the Vanguard 880. Each recorded peak was above the background level. Conversion to the quantity of P^{32} was effected by comparison with peaks for known standards of P^{32} compounds electrochromatographed and scanned at the same time (see legend for Fig. 2).

RESULTS AND DISCUSSION

The variation of the electrical migration rate of orthophosphate, AMP, ADP, and ATP in citric-citrate electrolyte with concentration of zinc acetate at pH 3.8 is illustrated by Fig. 1. As indicated above, sequence and separability were established with authentic nucleotide phosphorus compounds which were submitted to electrical migration and located by ultraviolet light.

Zones of ATP and orthophosphate migrated at the same rate in the absence of zinc ions. With optimum concentration of zinc ions (0.003–0.004 *M*), the ATP zone was well separated from the radioactive inor-

[4] Vanguard Instrument Company, P. O. Box 244, La Grange, Illinois.

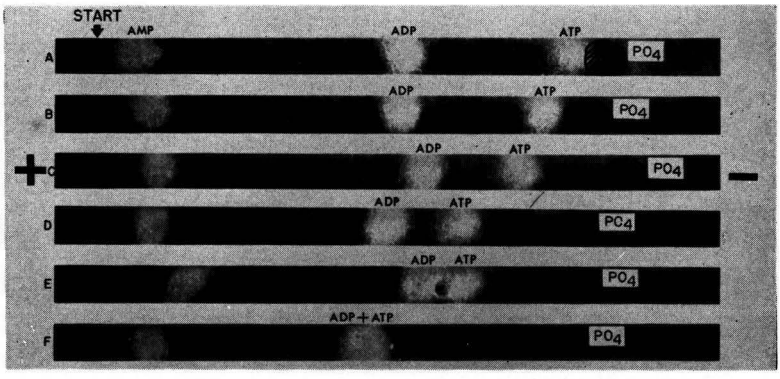


FIG. 1. Photograph of an ultraviolet contact print of electrochromatogram showing migration of the adenosine phosphate ions, AMP, ADP, ATP, and radioactive orthophosphate, $H_3P^{32}O_4$ in the citric acid-citrate electrolyte solution plus various concentrations of zinc ions. Each of the narrow paper strips contained 10 μl of radioactive phosphate plus nonradioactive AMP, ADP, and ATP; the background solution consisted of 0.035 *M* citric acid—0.0148 *M* citrate solution plus various concentrations of zinc acetate adjusted to final pH 3.8. Zinc ion concentration: A, none; B, 0.001 *M*; C, 0.003 *M*; D, 0.004 *M*; E, 0.005 *M*; F, 0.01 *M*. Migration time, 4 hr; potential, 1000 volts/100 cm. The distance between letters A and E is 18 cm.

ganic phosphate zone, located by autoradiography. The rate of migration of ATP was determined primarily by the concentration of zinc ions; it decreased progressively with the addition of 0.001, 0.003, 0.004, 0.005, and 0.010 *M* zinc ions. The addition of the zinc ions to the citric-citrate electrolyte had only a slight effect on the rate of migration of AMP, ADP, and orthophosphate. For effective separations, a critical concentration of zinc salt is required to cause the ATP to migrate more slowly than phosphate but faster than ADP.

The effect of varying the pH of the background electrolyte from pH 3.0 to 5.5 is illustrated in Fig. 2. In these experiments the zinc ion concentration was constant at 0.003 *M*. A pH of 3.0 (A, Fig. 2) provided a separation of ADP plus ATP from PO_4, but no separation of ADP and ATP. A pH of 3.8 (B, Fig. 2) provided an excellent separation of the four species. At pH values of 4.5 and greater (C, D, E, Fig. 2), there was excellent separation of ADP and ATP but no separation of ATP and PO_4. The most effective separations are obtained at pH 3.8.

The definition of the zones in the electrochromatogram is indicated by the radioautograph reproduced as Fig. 2. The sharpness of the zones in a chromatogram made under favorable conditions (B, Fig. 2) was confirmed by scanning the paper with a strip counter constructed by Kisieleski and Smetana (12) as shown by the tracing in Fig. 3.

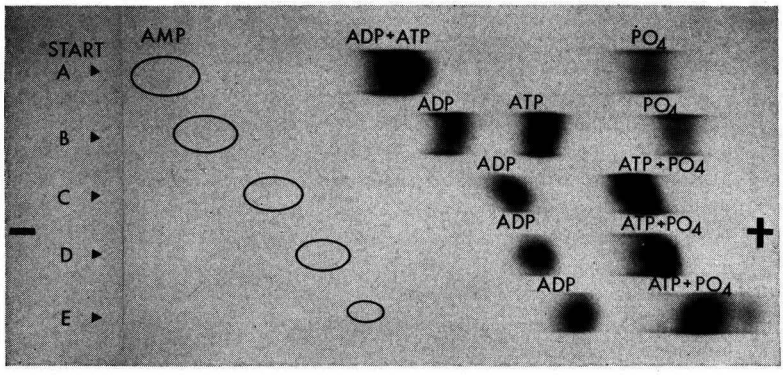


FIG. 2. Effect of pH upon separation of AMP, ADP, ATP, and phosphate ions (PO_4) from the ATP-PO_4 exchange reaction mixture. Migration in 0.035 *M* citric acid—0.01458 *M* sodium citrate electrolyte plus 0.003 *M* zinc acetate. pH values: A, 3.0; B, 3.8; C, 4.5; D, 5.0; E, 5.5. Each zone contained 10 μl of a solution of radioactive ADP, ATP, and PO_4 and nonradioactive AMP. ADP added, 154 cps; found, 151. ATP added, 212 cps; found, 208. PO_4 added, 116 cps; found, 112. Potential, 1000 volts/80 cm; migration time, 4 hr. The distance between the letters A and E is 18 cm.

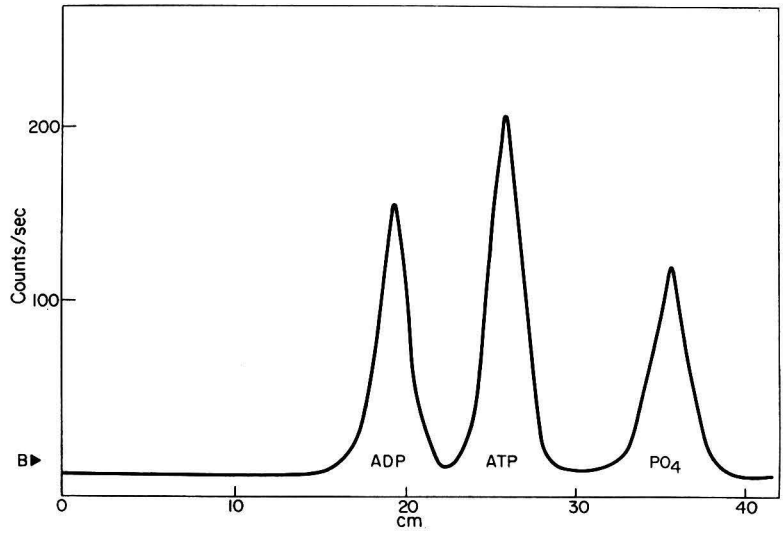


FIG. 3. Curve representing distribution of radioactive species in an electrochromatogram formed by migration of the ATP-PO_4 exchange reaction mixture at pH 3.8. The electrochromatogram was the same one that provided radioautograph B shown in Fig. 2. See text for methods.

The results summarized in Figs. 1–3 show that electrochromatography under carefully controlled conditions is an effective technique for the separation of mixtures of H_3PO_4, AMP, ADP, and ATP. Since separations were equally good with pure compounds or with mitochondrial extracts, the method should be suitable for studies on biological material.

SUMMARY

Electrochromatography with carefully adjusted electrolyte solutions (0.003 *M* zinc acetate, 0.035 *M* citric acid, and 0.0148 *M* sodium citrate) at pH 3.8 is an effective technique for the separation of mixtures of phosphoric acid, adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate.

ACKNOWLEDGMENT

We wish to express our gratitude to Dr. H. H. Strain for reading this manuscript and for his comments.

REFERENCES

1. KLOWWEN, H. M., *J. Chromatog.* **7**, 216 (1962).
2. SCHAEFER, J., *Arch. Exptl. Pathol. Pharmacol.* **236**, 483 (1959).
3. VON KORFF, R. W., *Anal. Biochem.* **3**, 244 (1962).
4. BELL, R. N., *Ind. Eng. Chem., Anal. Ed.* **19**, 97 (1947).
5. BRITZKE, E. V., AND DRAGUNOV, S. S., *J. Chem. Ind.* (*U.S.S.R.*) **4**, 49 (1927).
6. GERBER, A. B., AND MILES, F. T., *Ind. Eng. Chem., Anal. Ed.* **10**, 519 (1958).
7. SATO, T. R., *Anal. Chem.* **31**, 841 (1959).
8. STRAIN, H. H., AND SATO, T. R., U. S. Atomic Energy Comm. TID-7512 1956, pp. 175–182.
9. COLOWICK, S. P., AND KAPLAN, N. O., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. IV, p. 853. Academic Press, New York, 1957.
10. SCHNEIDER, W. C., AND HOGEBOOM, G. H., *J. Biol. Chem.* **183**, 123 (1950).
11. BANDURSKI, R. S., AND AXELROD, B., *J. Biol. Chem.* **193**, 405 (1951).
12. KISIELESKI, W. E., AND SMETANA, F., *Atompraxis* **4**, 261 (1958).

ANALYTICAL BIOCHEMISTRY 5, 548–554 (1963)

Rapid Enzyme Assay Technique Utilizing Radioactive Substrate, Ion-Exchange Paper, and Liquid Scintillation Counting[1]

JOHN R. SHERMAN[2]

From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

Received October 5, 1962

INTRODUCTION

This communication presents a rapid and sensitive enzyme assay technique. The assay technique consists of determining a labeled anionic product as the radioactivity remaining on anion-exchange paper as measured by direct liquid scintillation counting of the paper after elution of the labeled nonionic substrate from the paper with water. Phosphorylations catalyzed by galactokinase and hexokinase are employed as examples.

METHODS AND RESULTS

Counting Procedures. Dry DEAE-cellulose paper strips (Whatman No. DE20, free base form) containing applied C^{14}-labeled compounds were placed in standard liquid scintillation counting vials containing PPO-dimethyl POPOP-toluene solvent consisting of 4 gm of 2,5-diphenyloxazole and 100 mg 1,4-bis-2-(4-methyl-5-phenyloxalzolyl)benzene per liter of reagent-grade toluene, and counted at tap 2–80 (875 volts) and window settings 10–50–100 volts in a Packard 314E Tri-Carb Liquid Scintillation Spectrometer. All scintilaltion cpm given in this paper (except where otherwise noted) were those observed in the 10–50 volt window. For C^{14}-labeled compounds on DEAE-cellulose paper the counting rate was not significantly dependent on the orientation of the strip and no photoluminescent effects were noted.

Counting Proportionality and Counting Efficiency. Radioactivity measured in the scintillation counter was observed to be proportional to the

[1] Supported by grants from the National Institutes of Health, the National Science Foundation, and Graduate School of the University of Wisconsin.

[2] This investigation was carried out during the tenure of a Predoctoral Fellowship (GF-13,799) from the Division of General Medical Sciences, U. S. Public Health Service.

amount of C^{14}-labeled material applied to the DEAE-cellulose paper (Fig. 1).

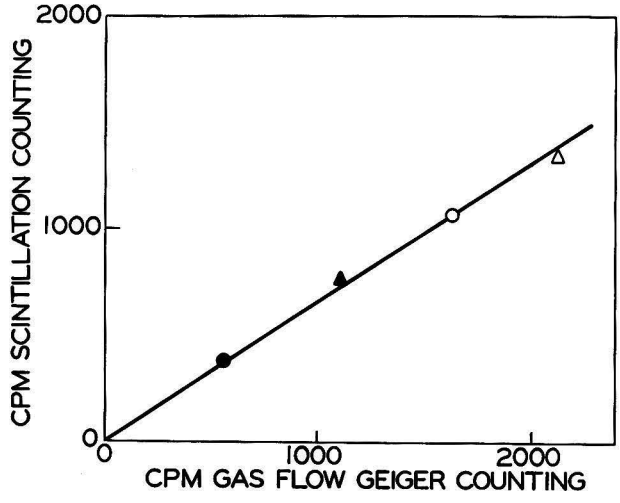


FIG. 1. Counting proportionality and relative counting efficiencies. Aliquots (5 ●, 10 ▲, 15 ○, and 20 μl △) of C^{14}-gal-1-P [prepared enzymically, Sherman and Adler (1)] were pipetted on DEAE-cellulose paper, which was then dried and counted as in routine counting procedure. These same volumes were also dried on aluminum planchets and counted at negligible thickness with a Nuclear Chicago D-47 windowless gas-flow Geiger counter. The slope of the line is 0.67. The same slope was observed when the experiment was repeated with C^{14}-gal and with benzoic acid-7-C^{14}.

D-Galactose-1-C^{14} (=C^{14}-gal), D-galactose-1-P-C^{14} (=C^{14}-gal-1-P), and benzoic acid-7-C^{14} were observed to give identical slopes when scintillation cpm were plotted against Geiger cpm by the procedures of Fig. 1. These compounds, therefore, are counted with the same efficiency on DEAE-cellulose paper by scintillation counting. (It is assumed that C^{14}-labeled compounds have the same counting efficiency at negligible thickness when counted in the windowless gas-flow Geiger counter under identical geometric conditions.) Standard benzoic acid-7-C^{14} on DEAE-cellulose paper gave a 58% counting efficiency in the 10–100 volt window and a 48% counting efficiency in the 10–50 volt window. From the foregoing discussion, it follows that C^{14}-gal and C^{14}-gal-1-P are also counted with these efficiencies. These absolute efficiencies compare favorably with the 57% efficiency observed by Wang and Jones (2) for glucose-U-C^{14} on Whatman No. 1 paper.

When the strips containing C^{14}-gal-1-P, C^{14}-glucose, or benzoic acid-7-

C^{14} were removed from the vials after counting, only a small increase in counting rate over the normal background counting rate was observed.[3] Therefore, virtually all of the radioactivity of these compounds remain adsorbed on the DEAE-cellulose paper.

Enzyme Proportionality. The C^{14} product remaining on DEAE-cellulose paper after elution of the substrate with water was related to enzyme concentration (galactokinase and hexokinase), as shown in Fig. 2. The

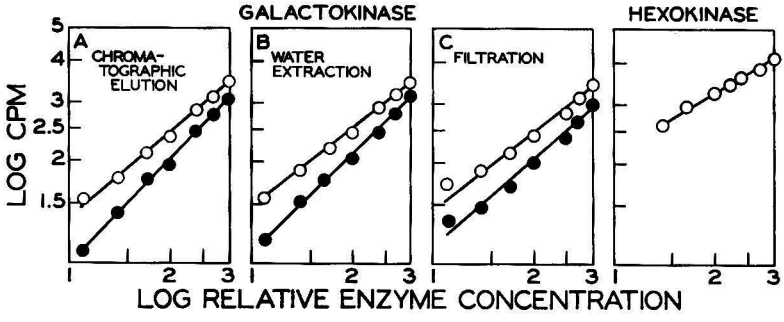


FIG. 2. Galactokinase and hexokinase proportionality. The galactokinase reaction mixture contained 100 mμmoles C^{14}-gal (8170 scintillation cpm or 3160 Geiger cpm per 30 μl), 150 mμmoles ATP, 400 mμmoles $MgCl_2$, 320 mμmoles NaF, 1 μmole 2-mercaptoethanol, 10 μmoles Tris-HCl buffer at pH 7.8, and galactokinase purified from *E. coli* [Sherman and Adler (1)], total volume 100 μl. Dilutions of enzyme for assay were made with 0.02 *M* potassium phosphate buffer containing 0.01 *M* mercaptoacetic acid, 0.001 *M* EDTA, and 100 μg bovine serum albumin per ml, final pH 7.2. After incubation at 35°C for 25 min, 30-μl aliquots of reaction mixture were pipetted (with Carlsberg micropipets) in an area 6 to 8 cm from an end of a 1.5 × 14 cm strip of dry DEAE-cellulose paper (this stops the reaction), in an area 2 cm from the bottom of a 1.5 × 8 cm dry strip of the same type paper, and in a circle of diameter 2.2 cm at the center of a circular disk (diameter 4.7 cm) of the same type dry paper. (*Note:* It is not necessary for the reaction spot to dry before commencing elution procedures described below.)

A. *Chromatography.* The 1.5 × 14 cm strips were eluted with deionized water by descending chromatography until the "front" reached the bottom of the strips. This required about 20–25 min at room temperature. The strips were then cut 5 cm from the bottom (to remove the area containing C^{14}-gal), and then the strips were dried with a hair dryer (drying time is about 10 min). The area containing the original spot was cut from the dried strip and counted by two methods: (1) windowless gas flow Geiger counting on aluminum planchets (●), (2) liquid scintillation counting (○).

B. *Water Extraction.* The 1.5 × 8 cm strips were greatly "swirled" for 1 min in a

[3] This counting rate increase can be eliminated by filtration of lint from the solvent. For example, when solvent from a vial with a counting rate of 26 cpm was filtered through Whatman No. 2 filter paper, the "background" decreased to 15 cpm. The solvent and vials, therefore, can be used repeatedly. This has also been noted by Davidson (3).

three methods of substrate elution [chromatographic (A), water extraction (B), and filtration (C)] gave almost identical results. Chromatographic elution requires little manual effort and many assays can be performed simultaneously by using a train of paper strips. Filtration is the most rapid separation method, requiring only seconds to separate substrate from product.

The amount of galactose phosphorylated is directly proportional to the amount of galactokinase added up to about 25% utilization of galactose.

The time course of a galactokinase reaction is shown in Fig. 3.

Chromatographic Separation of C^{14}-gal from C^{14}-gal-1-P on DEAE-Cellulose Paper. Elution of C^{14}-gal from the area of the reaction mixture "spot" on DEAE-cellulose paper is shown in Fig. 4. C^{14}-gal-1-P remains adsorbed to the original spot. Product characterization and stoichiometry are given elsewhere (1).

DISCUSSION

This assay technique should be generally applicable to measuring enzymes which catalyze the conversion of a radioactive, uncharged material into one that is negatively charged, for example, enzymes that catalyze certain phosphorylations, sulfations, or oxidations to carboxylic acids. Since the radioactive eluent from the paper strips can be easily

---

600-ml beaker containing deionized water. This extraction process was repeated three times with fresh beakers of water. The strips were then dried with a hair dryer, and the area counting the spots removed and counted as with (A).

C. *Filtration.* The disks were washed with several 150-ml volumes of deionized water employing a Millipore filtration apparatus. The disks were then dried with a hair dryer, and the area containing the original spot was removed and counted as with (A).

The hexokinase reaction mixture was the same as above except for the appropriate substitutions of D-glucose-U-C^{14} and yeast hexokinase (crystalline, from C. F. Boehringer und Soehne GmBH, Mannheim) for D-galactose-1-C^{14} and galactokinase, respectively, and the total volume was 50 μl containing 20,600 scintillation cpm per 40 μl. Dilutions of enzyme were made as above. After incubation for 5 min at 37°C, 40-μl aliquots were spotted in DEAE-cellulose paper as noted with (A) above. Chromatographic elution and scintillation counting were performed as described above. When D-glucose-1-H^3 (New England Nuclear Corp.) was used as substrate, the orientation of the paper affected the counting of the H^3 product. Maximum variation of a single determination from the average of several determinations made at random paper strip orientations was about $\pm$8–10%. For precise work with tritium it is necessary, therefore, to remove the H^3 product from the paper with a suitable solvent prior to quantitation or to achieve reproducible geometry of the paper strips (3) or to count the strips several times at random orientations and use the maximum values or the average values so obtained.

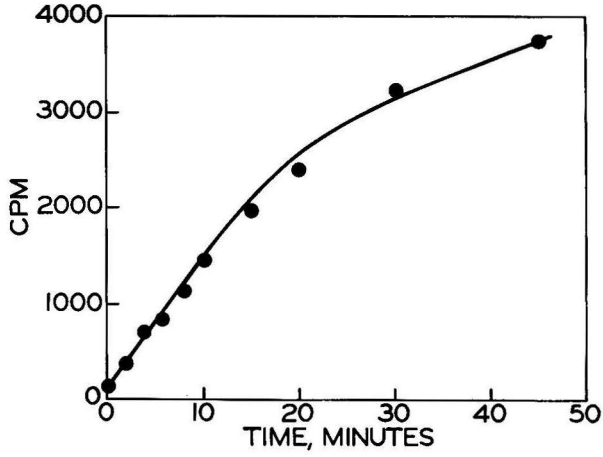


FIG. 3. Galactokinase reaction time course. A 0.5-ml galactokinase reaction mixture (see Fig. 2) containing 6536 cpm per 40 μl was incubated at 37°C with purified *Escherichia coli* galactokinase. At the times indicated 40-μl aliquots of reaction mixture were spotted on DEAE-cellulose paper. Chromatographic product separation and counting were performed as noted in Fig. 2.

	SCINT. CPM	GEIGER CPM SIDE OF SPOTTING	GEIGER CPM OPPOSITE TO SIDE OF SPOTTING	TYPE OF ACTIVITY
×××	966	370	387	C^{14}-GAL-1-P
×××	3956	1401	1503	C^{14}-GAL-1-P
×××	1100	491	444	C^{14}-GAL-1-P
	0	6	2	–
	0	3	0	–
	0	3	0	–
	107	58	58	C^{14} -GAL
	4050	1712	1578	C^{14} -GAL

← DIRECTION OF ELUTION — DEAE-CELLULOSE PAPER

FIG. 4. Separation of C^{14}-gal from C^{14}-gal-1-P on DEAE-cellulose paper. A 50-μl galactokinase reaction mixture like that described with Fig. 2 containing 10,600 scintillation cpm or 3930 Geiger cpm per 40 μl was incubated for 30 min at 37°C after which a 40-μl aliquot was spotted in the area (1.5 × 2 cm) indicated by cross hatching above. The strip was eluted with deionized water by descending chromatography until the "front" reached the bottom of the strip. Then the strip was dried with a hair dryer, and cut into areas indicated by the dashed lines. Both sides of the paper were counted on aluminum planchets in a windowless gas-flow Geiger counter; then the strips were counted in the scintillation counter by the methods described in the text. The cpm corresponding to a particular area of the strip are given parallel to that area in the above figure. Only part of the strip is shown; its full dimensions are 1.5 × 14 cm.

collected and measured, this technique should also prove applicable to such reactions as those catalyzed by phosphatases, etc., where the product is an uncharged material and the reactant is negatively charged. Some charged compounds can be separated from one another on DEAE-cellulose paper by utilizing a proper solvent or by electrophoretic means. Quantitation of such materials (when labeled) can then be made by the scintillation procedures of this paper, although an internal standard may be required if the solvent is not volatile (3). The commercial availability of cation-exchange paper should permit the measurement by the techniques described here of enzymes which catalyze the conversion of uncharged materials into cationic materials.

The data of Fig. 4, where both sides of the paper strips were counted by Geiger counting, indicate that the radioactivity distributes itself fairly uniformly in the paper. Thus, windowless gas-flow Geiger counting can give satisfactory results by this technique; however, the Geiger counting efficiency is only about 38% of that observed with liquid scintillation counting in the 10–50 volt window.

Dowex 2 paper (Schleicher and Schuell No. 2493) has disadvantages when compared with DEAE-cellulose paper (4); for example, its scintillation counting efficiency was lower and the radioactivity did not distribute itself uniformly in the paper.

SUMMARY

A rapid enzyme assay technique is described in which a labeled anionic product is determined as the radioactivity remaining on anion-exchange paper as measured by direct liquid scintillation counting of the paper after elution of the labeled nonionic substrate from the paper with water by one of three methods: (A) descending chromatography, (B) water extraction, or (C) direct elution employing a Millipore filtration apparatus.

Reactions catalyzed by galactokinase and hexokinase were employed as examples. The technique generally should be applicable to reactions catalyzed by enzymes converting uncharged materials into anionic materials. The technique should also prove useful in enzymic reactions in which the product can be separated readily from the reactant on anion-exchang paper by means of a suitable solvent or by electrophoretic means.

Benzoic acid-7-C^{14}, galactose-1-C^{14}, and galactose-1-P, C^{14} were counted with an efficiency of about 58% on DEAE-cellulose paper by liquid scintillation counting. Windowless gas-flow Geiger counting of radioactivity of these compounds on DEAE-cellulose paper gave satisfactory results but the efficiency of counting was only about 18%.

REFERENCES

1. SHERMAN, J. R., AND ADLER, J., *J. Biol. Chem.* **238,** 873 (1963); *Federation Proc.* **21,** 268 (1962).
2. WANG, C. H., AND JONES, D. E., *Biochem. Biophys. Res. Communs.* **1,** 203 (1959).
3. DAVIDSON, E. A., *Packard Tech. Bull.* No. 4 (revised Jan., 1962), Packard Instrument Company, Inc., Box 428, LaGrange, Illinois.
4. SHERMAN, J. R., M. S. Thesis, University of Wisconsin, 1962.

Erratum

Volume **5**, Number 3, in the Short Communication entitled "A New Method for Microanalysis of Blood Galactose" by Derek Watson, pp. 260–262:

Page 260, paragraph 1, line 6 should read: "75 and 48%, respectively, . . ."

paragraph 2, lines 2 and 3 should read: ". . . with 1.7 ml glucose oxidase solution containing 44 ml water, 1 ml Fermcozyme and 5 ml *M* acetate buffer pH 5.6."

AUTHOR INDEX

A

Alcaras, A. F., 426
Ames, Adelbert III, 452
Annunziata, Rita, 291
Arroyo, E., 158
Avena, Remedios M., 265

B

Babson, Arthur L., 86
Balint, John A., 143
Barnes, Allen C., 99
Bell, Pamela M., 443
Bello, Jake, 366
Blum, Amos E., 70
Blum, J., 158
Bowers, F. K., 471
Brakke, Myron K., 271
Broquist, H. P., 255
Brust, Norma, 416
Burchill, P. I., 1
Burns, Charles H., 393

C

Campbell, William, 86
Cannon, Paul L., Jr., 208
Cantoni, G. L., 258
Charlwood, P. A., 226
Clayton, R. A., 1
Cohen, Richard, 99
Crane, F. L., 471
Crevasse, Lamar, 462
Cuppy, Dianna, 462

D

Danforth, W. F., 542
D'Arconte, Lucius, 195
Davis, Joseph R., 64
Dilley, R. A., 531
Dorfman, Ralph I., 416, 422
Drell, W., 426

E

Ellison, Solon A., 263

F

Filipowicz, B., 187
Flavin, Martin, 60
Forchielli, Enrico, 416, 422
Frantz, William, 345
Freed, Simon, 338
Friedman, Sydney M., 471
Furlong, N. Burr, 515
Fusaro, Ramon M., 379

G

Gambal, David, 479
Glick, David, 497
Gross, Maria, 187
Gruber, M., 360
Guilbault, George G., 208

H

Hamilton, James G., 458
Harris, Robert A., 479
Hiraoka, Tosisuke, 497
Horn, Millard J., 70
Huckabay, William B., 28

I

Ibsen, Kenneth H., 505
Ichii, Shogo, 422
Irvin, J. Logan, 321

J

Jacobson, Gert, 416
James, E. C., 426
Jermyn, M. A., 433
Johnson, John A., 379
Johnson, Marvin J., 330
Jones, G. E. Seegar, 99

K

Karickhoff, Carl W., 93
Katz, Sam, 7
Kobayashi, Yutaka, 284
Koechlin, Bernard A., 195
Kramer, David N., 208
Kreisher, J. H., 295
Kritchevsky, David, 88, 388
Kumar, Soma, 265

L

Larson, R. L., 255
Leonard, Reid H., 362

LEPOUTRE, L., 149
LINDE, RICHARD E., 246
LIPSETT, MORTIMER B., 217
LONBERG-HOLM, K. K., 11

M

MCALLISTER, H. C., JR., 321
MCCLENDON, J. H., 295
MAKITA, M., 523
MARTAK, DOLORES S., 388
MASUOKA, D. T., 426
MEHL, JOHN W., 170
MILCH, S., 158
MILLER, O. NEAL, 458
MORRIS, ROBERT N., 64
MOSBACH, E. H., 158
MOSES, V., 11

N

NASH, JOEL D., 379
NEELY, LAVALLE, 133
NELSON, GARY J., 116
NESBETT, FRANCES B., 452
NEVILL, A. D., 393
NIGHTINGALE, MARY STYLIANOU, 416
NORDIN, P., 175

O

OTTOLENGHI, ABRAMO C., 37

P

PERLOFF, WILLIAM H., 416, 422
PERRI, V., 179
PESCH, LEROY A., 291
PINTER, KAROLY G., 458
PLANTA, R. J., 360

Q

QUAY, W. B., 51

R

RAMSEY, HAROLD A., 83
REYNOLDS, EDWARD S., 246
RINDI, G., 179
RIVLIN, RICHARD S., 267
ROSENKRANZ, HERBERT S., 263
ROTHBLAT, GEORGE H., 388

S

SARLOS, I. J., 99
SATO, T. R., 542
SAUNDERS, RUSSELL L., 505
SCALES, B., 489
SCHEIG, ROBERT LEE, 291
SCHILLING, E. D., 1
SCHOTT, H. F., 426
SEGEL, IRWIN H., 330
SHAPIRO, IRWIN L., 88
SHERMAN, JOHN R., 548
SHIN, YUNG S., 369
SIMPSON, T. H., 313
SKOCZYLAS, BOGNA, 187
SLAUNWHITE, W. R., JR., 133
SORCINI, GUISEPPE, 416
SPITZER, HUGH L., 143
STEWART, L., 175
STOCKX, J., 149
STOWE, BRUCE B., 107

T

THOMSON, J. F., 542
TROTTIER, DONNA, 345
TURNER, DAVID A., 99

U

UDENFRIEND, SIDNEY, 258
UPDEGRAFF, DAVID M., 28

V

VANDENDRIESSCHE, L., 149
VEGAS, FRANCISCO R., 465
VISE, MARY H., 338
VON SCHUCHIN, SUSANNE, 93

W

WAN, Y. C., 321
WARREN, HELEN W., 70
WATANABE, SHIZUO, 345
WATSON, DEREK, 260
WELLS, W. W., 523
WHIPPLE, G. HOYT, 393
WILLIAMS, CLYDE M., 362
WILLIAMSON, ALAN R., 47
WILSON, HILDEGARD, 217, 267
WRIGHT, R. S., 313

Z

ZALTZMAN-NIRENBERG, PEROLA, 258
ZAMENHOF, STEPHEN, 47

SUBJECT INDEX

A

Acetyl hydroxamate, paper chromatographic separation, KUMAR AND AVENA, 265

Adenine compounds, rapid colorimetric determination, DAVIS AND MORRIS, 64

Adenosine diphosphate, electrochromatographic separation, SATO *et al.*, 542

Adenosine monophosphate, electrochromatographic separation, SATO *et al.*, 542

Adenosine triphosphate, electrochromatographical separation, SATO *et al.*, 542

Alanine, in proteins and foods, microbiological determination, WARREN *et al.*, 70

o-Aminobenzaldehyde, color reaction with reduced pyridine nucleotides, LARSON AND BROQUIST, 255

Anthrone, for determination of *S*-carboxymethylcysteine in proteins, JERMYN, 433

B

Bacterial membrane substance, lipid phosphorus as a measure of, KOLB *et al.*, 78

Bile acids, detection in thin-layer chromatography, KRITCHEVSKY *et al.*, 388

Bilirubin, conjugated with sulfate and glucuronide, chromatographic behavior on ion exchange paper, VEGAS, 465

Blood galactose, microanalysis, WATSON, 260

Bromine-82, for estrogen analysis, SLAUNWHITE AND NEELY, 133

C

Calcium, in tissues, colorimetric determination with murexide, REYNOLDS AND LINDE, 246

Calcium-8-quinolinol, fluorescence of, WATANABE *et al.*, 345

C^{14}-carboxyl indoleacetic acid, synthesis, STOWE, 107

Carbon-14 determination, low-level, by improved Schöniger combustion and ionization chamber, VON SCHUCHING AND KARICKHOFF, 93

C^{14}-labeled nucleotides, for assay of nucleotide kinases, FURLONG, 515

C^{14}-nitrile indoleacetonitrile, synthesis, STOWE, 107

$C^{14}O_2$, assembly for collection of, in liquid scintillation, CUPPY AND CREVASSE, 462

S-carboxymethylcysteine, in proteins, determination with anthrone, JERMYN, 433

Catecholamines, separation, MASUOKA *et al.*, 426

Cathepsin C, estimation, PLANTA AND GRUBER, 360

Cation analysis, with glass electrodes, computer solution, FRIEDMAN AND BOWERS, 471

Cesium chloride density gradient centrifugation, apparatus for sampling, ELLISON AND ROSENKRANZ, 263

Chlordiazepoxide (Librium), spectrofluorometric microdetermination in plasma, KOECHLIN AND D'ARCONTE, 195

Cholesterol, from human cerebrospinal fluid, microdetermination by silicic acid column chromatography, SHIN, 369

Cholesterol-C^{14} Digitonide, radioassay, SHAPIRO AND KRITCHEVSKY, 88

Cholesterol ester, from human cerebrospinal fluid, microdetermination by silicic acid column chromatography, SHIN, 369

Chromatography of lipids, concave gradients applied to, NELSON, 116

Coprecipitation, influence on quantitative estimations of ribonuclease activities, STOCKX AND VANDENDRIESSCHE, 149

Cuvette, rinsing device, BABSON AND CAMPBELL, 86

Carbon dioxide, rapid micro gas analysis system for, UPDEGRAFF AND HUCKABAY, 28

D

Density gradient columns, photometric scanning, BRAKKE, 271

Density gradient ultracentrifugation, use of radioactivity labeled marker proteins, CHARLWOOD, 226

Dihydrocholesterol, in tissues, new determination, MOSBACH *et al.*, 158

8-dimethylamino-6-hydroxypurine, fluorometric assay in presence of guanine, UDENFRIEND *et al.*, 258

Differential extractions for spectrophotofluorometric measurement of 5-hydroxy- and 5-methoxyindoles, QUAY, 51

Dihydroxy aromatic acids, microanalytical determination by gas chromatography, WILLIAMS AND LEONARD, 362

Dye fluorescence, quenching for protein measurement, HIRAOKA AND GLICK, 497

E

Egg yolk turbidimetry, for phospholipase C determination, OTTOLENGHI, 37

Electrochromatographic separation of Pi, AMP, ADP, ATP, SATO *et al.*, 542

Enzyme assay technique using radioactive substrate, ion-exchange paper and liquid scintillation counting, SHERMAN, 548

Enzyme kinetics, determination by electrochemical method, GUILBAULT *et al.*, 208

Estradiol-17β, determination, ICHII *et al.*, 422

Estrogens, analysis with bromine-82, SLAUNWHITE AND NEELY, 133

Estrogens, characterization, SIMPSON AND WRIGHT, 313

N-ethyl maleimide, labeled, purification, FLAVIN, 61

Exponential gradients, device for generating, KATZ, 7

F

Fecal bile acids, analysis by gas-liquid chromatography, MAKITA AND WELLS, 523

Fluorometric determination of phospholipids in rat tissues, HARRIS AND GAMBAL, 479

Fraction collector, MEHL, 170

G

Galactosamine, detection and rapid differentiation, WILLIAMSON AND ZAMENHOF, 47

Galactosamine uronic acid, detection and rapid differentiation, WILLIAMSON AND ZAMENHOF, 47

Gas analysis, micro, rapid system for, UPDEGRAFF AND HUCKABAY, 28

Gas chromatography, for quantitative analysis of fecal bile acids, MAKITA AND WELLS, 523

Gas chromatography, for microanalytical determination of dihydroxy aromatic acids, WILLIAMS AND LEONARD, 362

Gas chromatography determination of urinary pregnanediol, TURNER *et al.*, 99

Glucosamine, detection and rapid differentiation, WILLIAMS AND ZAMENHOF, 47

Glucosamine uronic acid, detection and rapid differentiation, WILLIAMSON AND ZAMENHOF, 47

Glycogen, enzymic determination, JOHNSON *et al.*, 379

Gradient gradients production apparatus, application to chromatography of lipids, NELSON, 116

Gradients, device for generating, zero, linear or exponential types, KATZ, 7

H

H^3-labeled, nucleotides, for assay of nucleotide kinases, FURLONG, 515

Histidine decarboxylase activity, determination by liquid scintillation counting of $C^{14}O_2$, KOBAYASHI, 284

Histones, electrophoresis on polyacrylamide gels, MCALLISTER *et al.*, 321

Hydrocarbon gases, rapid micro gas analysis system for, UPDEGRAFF AND HUCKABAY, 28

Hydrogen, rapid micro gas analysis system for, UPDEGRAFF AND HUCKABAY, 28

5-hydroxyindoles, differential extractions for spectrophotofluorometric measurement, QUAY, 51
β-hydroxybutyryl hydroxamates, paper chromatographic separation, KUMAR AND AVENA, 265

I

Indoleacetic acid with C^{14}-carboxyl, synthesis of, STOWE, 107
Indoleacetonitrile with C^{14}-nitrile, synthesis of, STOWE, 107
Inorganic phosphate, electrochromatographic separation, SATO *et al.*, 542
Ionization chamber and Schöniger combustion, for low-level carbon-14 determination, VON SCHUCHING AND KARICKHOFF, 93

L

Linear gradients, device for generating, KATZ, 7
Lipid phosphorus, microdetermination as measure of bacterial membrane substance, KOLB *et al.*, 78
Liquid scintillation counting, using glass fiber paper, PINTER *et al.*, 458
Liquid scintillation counting, background counts containing quenching substances, SCALES, 489
Liquid scintillation counting for enzyme assay, SHERMAN, 548
Liquid scintillation counting of $C^{14}O_2$ for determination of histidine decarboxylase activity, KOBAYASHI, 284
Liquid scintillation phosphor, method for separation from polar steroids, RIVLIN AND WILSON, 267
Luciferase in bacterial cells, photometric estimation, BURNS *et al.*, 393

M

Magnesium-8-quinolinol, fluorescence of, WATANABE *et al.*, 345
Metabolite of lactam character, spectrofluorometric microdetermination in plasma, KOECHLIN AND D'ARCONTE, 195
5-methoxyindoles, differential extractions for spectrophotofluorometric measurement, QUAY, 51
Murexide, for colorimetric determination of calcium in tissues, REYNOLDS AND LINDE, 246

N

Ninhydrin, anomalous reactions of, SCHILLING *et al.*, 1
Nitrogen and phosphorus, in nucleoproteins, micromethod for simultaneous determination of, FILIPOWICZ *et al.*, 187
Nonprotein nitrogen, determination, BELL, 443
Nucleotide kinases, assay using C^{14}- or H^3-labeled nucleotides, FURLONG, 515

O

Oligosaccharides, degree of polymerization, STEWART AND NORDIN, 175
Oxygen, rapid micro gas analysis system for, UPDEGRAFF AND HUCKABAY, 28
Oxytetracycline, in biological material, fluorometric determination, IBSEN *et al.*, 505

P

Paper chromatograms, two-dimensional, semiautomatic device for measurement of radioactivity, MOSES AND LONBERG-HOLM, 11
Periodate oxidation, use for urine corticoid analysis, WILSON AND LIPSETT, 217
Phosphatidyl serine, quantitative column separation, SPITZER AND BALINT, 143
Phosphatidyl ethanolamine, quantitative column separation, SPITZER AND BALINT, 143
Phospholipase C, determination, by egg yolk turbidimetry, OTTOLENGHI, 37
Phospholipid, from human cerebrospinal fluid, microdetermination by silicic acid column chromatography, SHIN, 369
Phospholipids, in rat tissues, fluorometric determination, HARRIS AND GAMBAL, 479
Phosphorimetry, as quantitative microanalysis, FREED AND VISE, 338
Phosphorus and nitrogen, in nucleoproteins, micromethod for simultaneous

determination of, FILIPOWICZ *et al.*, 187
Plasma estrone, determination, ICHII *et al.*, 422
Polar steroids, separation from the liquid scintillation phosphor, RIVLIN AND WILSON, 267
Polyacrylamide gels, for electrophoresis of histones, MCALLISTER *et al.*, 321
Polyethylene powder, for chromatography of thiamine and pyrithiamine, RINDI AND PERRI, 179
Polysaccharide hydrolases, fungal, chromatography of, MCCLENDON AND KREISHER, 295
Protein, millimicrogram amounts, measurement of by quenching of dye fluorescence, HIRAOKA AND GLICK, 497
Protein hydrolysis and freeze-drying, BELLO, 366
Pyridine nucleotides, separation by thin-layer chromatography, SCHEIG *et al.*, 291
Pyrithiamine, in biological materials, separation and determination by chromatography, RINDI AND PERRI, 179

R

Radioactively labeled marker proteins, in density gradient ultracentrifugation, CHARLWOOD, 226
Radioactivity measurement on two-dimensional paper chromatograms, semiautomatic device for, MOSES AND LONBERG-HOLM, 11
Reduced pyridine nucleotides, color reaction with *o*-aminobenzaldehyde, LARSON AND BROQUIST, 255
Ribonuclease activities, influence of coprecipitation, on quantitative estimations of, STOCKX AND VANDENDREISSCHE, 149

S

Schöniger combustion and ionization chamber, for low-level carbon-14 determination, VON SUCHUCHING AND KARICKHOFF, 93
Silic acid column chromatography, for microdetermination of cholesterol, cholesterol ester, and phospholipid, SHIN, 369
Sodium contamination, from glass, NESBETT AND AMES, 452
Sodium cysteine-*S*-sulfate monohydrate, synthesis and characterization, SEGEL AND JOHNSON, 330
Starch gel electrophoresis, thin-layer, on glass slides, RAMSEY, 83

T

Testosterone in human plasma, FORCHIELLI *et al.*, 416
Thiamine, in biological materials, separation and determination by chromatography, RINDI AND PERRI, 179
Thin-layer chromatography, for detection of bile acids, KRITCHEVSKY *et al.*, 388
Thin-layer chromatography, separation of pyridine nucleotides, SCHEIG *et al.*, 291
Thin-layer starch gel electrophoresis on glass slides, RAMSEY, 83
Thiocholine iodine esters, kinetics of enzymic hydrolysis of, by electrochemical method, GUILBAULT *et al.*, 208
Tocopherols, in plant tissue, assay, DILLEY AND CRANE, 531

U

Urinary pregnanediol, determination by gas chromatography, TURNER *et al.*, 99
Urine corticoids, analysis with periodate oxidation, WILSON AND LIPSETT, 217

Z

Zero gradients, device for generating, KATZ, 7
Zinc-8-quinolinol, fluorescence of, WATANABE, 345