Standard Methods of Clinical Chemistry

VOLUME 7

By The American Association of Clinical Chemists

Editor-in-Chief
GERALD R. COOPER

Series Editor
J. STANTON KING, JR.

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MIRIAM REINER—A BIOGRAPHICAL SKETCH

Uniquely, Dr. Miriam Reiner has been associated with methodology research and analytical services in clinical chemistry all of her professional life. She has exhibited intense interest in promoting quality performance in clinical chemistry laboratories, and in trying to develop new procedures which in turn will open up new horizons for clinicians. Her work on bilirubin and total protein determinations, electrophoresis, blood gases and electrolytes, kidney stones, and sugar metabolism anomalies has contributed greatly to improved performance in the clinical laboratory. She has been particularly sensitive to the need for knowledge about individual and group variation for each procedure in her laboratory.

Dr. Reiner has been a part of each of the phases in the development of clinical chemistry as a profession in the United States. She was an associate of Harry Sobotka, working first on methodology research and later assuming the responsibility to run the Clinical Chemistry Laboratory at Mt. Sinai Hospital in New York City. For about 20 years, she collaborated with physicians such as Kurt Stern, I. Snapper, and Bela Shick on the use of clinical chemistry in internal medical and pediatric research.

In 1951, Dr. Reiner became head of the Section of Clinical Chemistry of the D. C. General Hospital in Washington. Like many other clinical chemistry laboratories across the country, her laboratory experienced remarkable growth in services, introduction of automation, development and application of micromethods, institution of control systems as an essential component, and emergence of clinical chemistry as a responsible profession.

"Mim," as Dr. Reiner is known to her friends and colleagues, was intimately associated with the founding efforts of the American Association of Clinical Chemists, the journal *Clinical Chemistry,* and the volumes of *Standard Methods of Clinical Chemistry.* In 1948, a group of nine New York City chemists, including Dr. Reiner, along with groups from Philadelphia and Boston, originated the American Association of Clinical Chemists, and within a year the organization had 200 members from all parts of the United States of America. She was instrumental in the development of *Clinical Chemistry,* which started as a newsletter, later became a bimonthly, and then a monthly, journal.

She was the first editor of *Standard Methods of Clinical Chemistry,* which was begun in response to the urgent concern of the American Association of Clinical Chemists for doing something about the standardization of methods. Volume 1 was published in 1953 to try to make avail-

able the most frequently used determinations, tested not only in the laboratory of the submitter but also checked in other laboratories, to ensure their working under all conditions.

The Editorial Staff of Volume 7 wishes to show its great respect and the deep appreciation of all members of the American Association of Clinical Chemists to Miriam Reiner by dedicating this volume to her.

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PREFACE

The members of the editorial staff, the Submitters, and the Checkers of Volume 7 have worked closely together to present reliable, frequently used methods. These methods have been studied and evaluated in detail to determine the best technique, the limitations and advantages, and the effective means of control in each case. Several methods were explored to determine how an automated or radioisotope procedure can best be studied and evaluated for use in future volumes. Subjects on standardization and control systems were also included, because these are essentials for effective operation of any clinical chemistry laboratory. Special efforts were made to include documentation of specificity, precision, and potential clinical usefulness of the different methods.

Several methods were chosen because they provide dependable performance even though they are more time-consuming than some in common use. Other methods were selected for study because of potential usefulness in new areas being encouraged in the clinical laboratory, such as clinical laboratory toxicology, chemical tests used in control of therapy, radioisotope hormone assays, and physical measurements.

The problems that arise from the lack of specificity among procedures are becoming more acute in the clinical laboratories. Automation is accomplishing higher within-day precision, but not necessarily contributing to determination of "true" values. Commercial diagnostic product systems are often limited in application because of the lack of specificity. The greatly increasing workload in clinical laboratories is prompting the use of relatively nonspecific procedures in order to be able to get the work done. Many of the problems encountered in automation, commercial diagnostic product systems, and routine procedures used in the clinical laboratory are caused by the use of relatively nonspecific chemical procedures. A continuing effort therefore must be maintained to study the chemical bases, improve technique, confirm reliability, and find better ways to calibrate the commonly used analytical procedures in clinical chemistry. This latter was the goal of the Editors, Submitters, and Checkers of Volume 7.

The Editorial Committee wishes to express its appreciation and the thanks of the members of the American Association of Clinical Chemists to all of the Submitters and Checkers for the contributions they have made to Volume 7.

GERALD R. COOPER, *Editor-in-Chief*

Atlanta, Georgia
April, 1972

CONTENTS

III. MINERALS, HEAVY METALS

IV. PROTEINS

V. TOXICOLOGY

VI. VITAMINS AND HORMONES

I. ENZYMES

MEASUREMENT OF 5′-NUCLEOTIDASE ACTIVITY IN SERUM

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Introduction

5′-Nucleotidase (5-ribonucleotide phosphohydrolase, EC 3.1.3.5) is a phosphohydrolase that specifically catalyzes hydrolysis of 5′-nucleotides. This ubiquitous enzyme, widely distributed in human and animal tissues, is also found in plants and bacteria (1). Its activity is increased in the presence of Mg^{2+} or Mn^{2+} and strongly inhibited by Ni^{2+} (2). In the presence of Mg^{2+}, α-amino acids are activating agents (3).

In 1954, Dixon and Purdom (4) found 5′-nucleotidase activity in human serum and reported an abnormally high activity in the serum of patients with hepatobiliary disease, but normal activity in patients with osteoblastic osseous disease. Nonspecific alkaline phosphatase will also hydrolyze 5′-nucleotides, and in the method of Dixon and Purdom, as well as in subsequent procedures (5–7), attempts have been made by various means to correct for the effect of the nonspecific alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1). Assays have been made with both β-glycerophosphate and adenosine-5′-phosphate as substrate, with the assumption that the rate of hydrolysis of the two substrates by the two enzymes was the same (4), attempted inhibition of phosphatase with EDTA (1.5 mmol/liter) (5) or high concentrations of Mg^{2+} (6), and inhibition of the 5′-nucleotidase with Ni^{2+} (7). Of these procedures, the most satisfactory is the use of Ni^{2+} (7–9). It has recently been proposed that alkaline phosphatase activity can be decreased by adding a second substrate such as phenylphosphate or β-glycerophosphate for which it has a greater affinity. This has been the basis for several proposed assay methods for 5′-nucleotidase (10–12).

Principle

The 5′-nucleotide used in the assay is adenosine-5′-phosphate (AMP) and the reaction proceeds as follows:

$$\text{Adenosine-5′-phosphate} + H_2O \xrightarrow{\text{5-nucleotidase}} \text{adenosine} + \text{phosphate}$$

Adenosine-5′-phosphate (5 mmol/liter) in sodium diethyl barbiturate buffer, pH 7.5, is incubated at 37°C both in the presence and absence of Ni^{2+} (10 mmol/liter). At zero time and at 60 min, aliquots are taken, deproteinized with trichloroacetic acid, and inorganic phosphorus determined in aliquots of the filtrate. The difference in the amount of phosphorus liberated in the presence and absence of Ni^{2+} is an index of the 5′-nucleotidase activity.

Units

Units of activity are defined in terms of the difference between the number of nanomoles of inorganic phosphorus liberated per minute by 1 ml of serum per milliliter of reaction mixture in the presence of 10 mmol of Ni^{2+} per liter and in its absence. This is equivalent to micromoles of product formed per minute per liter of serum.

Reagents

1. *Sodium diethyl barbituric acid buffer, 40 mmol/liter, pH 7.50.* Dissolve 8.2476 g of sodium diethyl barbiturate (Veronal) in 800 ml of water in a 1-liter volumetric flask. Adjust the pH electrometrically to 7.50 with 10N HCl, and dilute to the mark with water. Layer with toluene and store at room temperature.

2. *Manganese sulfate, 20 mmol/liter.* Dissolve 0.8450 g of $MnSO_4 \cdot H_2O$ in water and dilute to 250 ml.

3. *Adenosine-5′-phosphate solution, 50 mmol/liter, pH 7.50.* Dissolve 9.1300 g of adenosine-5′-monophosphoric acid (Sigma Type IV) (Sigma Chemical Co., St. Louis, Mo. 63178) in about 200 ml of water in a 500-ml volumetric flask. Adjust the pH electrometrically to 7.50 with 10N NaOH (about 6.2 ml) and dilute to the mark.

4. *Nickel chloride solution, 0.1 mol/liter.* Dissolve 4.7540 g of $NiCl_2$ in water and dilute to 200 ml with water.

5. *Substrate mixture with nickel chloride.* Prepared freshly before use. The volumes listed are multiplied by the number of determinations to

be done to yield the total volume. $NiCl_2$ (0.1 mol/liter), 0.5 ml; buffer, 3.25 ml; $MnSO_4$ (20 mmol/liter), 0.25 ml; and AMP (50 mmol/liter), 0.5 ml.

6. *Substrate mixture without nickel chloride.* Prepared freshly before use. The following volumes are multiplied by the number of determinations to be done to yield the total volume: buffer, 3.75 ml; $MnSO_4$ (20 mmol/liter), 0.25 ml; and AMP (50 mmol/liter), 0.5 ml.

7. *Stock phosphorus standard, 400 μg of inorganic phosphorus per milliliter.* Dissolve 0.880 g of pure dry monopotassium phosphate (KH_2PO_4) in distilled water, in a 500-ml volumetric flask, and dilute to the mark with water. Add 0.2 ml of chloroform as a preservative.

8. *Working phosphorus standards, 80 and 120 μg of inorganic phosphorus per milliliter.* Into each of two 100-ml volumetric flasks, pipet 20 ml and 30 ml of the stock phosphorus standard solution and 1 ml of 10N H_2SO_4 as preservative. Dilute to the mark with water. Because the standards are taken through the entire procedure, they are diluted 10-fold during the protein-free filtrate step, and then final concentration is 8 and 12 μg of inorganic phosphorus per milliliter.

9. *Trichloroacetic acid stock solution (227 g/liter).* Dissolve the contents of a 1-lb bottle of trichloroacetic acid in water, and dilute to 2 liters.

10. *Trichloroacetic acid, 100 g/liter.* Dilute 440 ml of stock trichloroacetic acid to 1 liter.

11. *Molybdate reagent.* Dissolve 25 g of ammonium molybdate·$4H_2O$ in water (heat but do not boil). Transfer the solution quantitatively to a 1-liter flask containing 500 ml of 10.0N H_2SO_4, and dilute to 1 liter with water.

12. *Aminonaphtholsulfonic acid reagent.* Dissolve 146 g of sodium metabisulfite ($Na_2S_2O_5$) and 5 g of anhydrous sodium sulfite in about 800 ml of water. Heat to about 50°C. Add 2.5 g of 1-amino-2-naphthol-4-sulfonic acid and shake until dissolved. Dilute to 1 liter. Filter and store in an amber-colored bottle, away from light.

Procedure

1. Transfer 4.5 ml of substrate mixture containing Ni^{2+} into four tubes and 4.5 ml of substrate mixture without Ni^{2+} into four other tubes.

2. Place the tubes in a water bath at 37°C for 5 min, for temperature equilibration.

3. To one tube with nickel and one without, add 0.5 ml of water (reagent blank), to another such set of tubes add 0.5 ml of serum, to a third set add 0.5 ml of the 80 μg/ml inorganic phosphorus working

standard, and to the fourth set 0.5 ml of the 120 μg/ml inorganic phosphorus working standard. Mix. Stopper and incubate at 37°C for exactly 60 min (use a stopwatch).

4. Exactly 60 min after the enzyme reaction was started, add 5 ml of the 100 g/liter trichloroacetic acid solution to the blank, standards, and serum assay tubes.

5. Mix and filter through Whatman #42 filter paper.

NOTE: Checker B. C. centrifuges at this point.

6. Transfer 2 ml of filtrate into the tube.

7. Add 6.6 ml of water.

8. To all tubes add 1 ml of molybdate reagent. Mix.

9. To all tubes add 0.4 ml of aminonaphtholsulfonic acid reagent and mix.

10. After 10 min read the absorbance (A) of the solutions in a suitable colorimeter or spectrophotometer at a wavelength of 660 nm. (We used an Evelyn or Klett photoelectric colorimeter or a Coleman Jr. spectrophotometer.) For routine assay of large numbers of sera, the Evelyn colorimeter and matched cuvets have been used.

11. If an automatic pipetter is used, all of the color development reagents may be added in one step. We used an Oxford pipetter, adjusted to deliver 8.0 ml. Just before use, sufficient reagent containing 6.6 parts of water, 1 part of molybdate reagent, and 0.4 parts of aminonaphtholsulfonic acid reagent is prepared and mixed well.

Calculations

$$(1)\quad F_1 = \frac{\frac{A_{8\mu g\ std}}{8} + \frac{A_{12\mu g\ std}}{12}}{2}$$

where F_1 is the absorbance per microgram of phosphorus.

$$(2)\quad \frac{A\ (\text{without } Ni^{2+}) - A\ (\text{with } Ni^{2+})}{F_1} = \text{micrograms of phosphorus hydrolyzed by}$$

5′-nucleotidase/0.1 ml of serum per ml reaction mixture.

$$(3)\quad \text{Nucleotidase activity (units)} = \frac{(\mu g\ P_i \text{ hydrolyzed by 5′-nucleotidase}) \cdot 10{,}000}{60 \times 31}$$

$$F_2 = \frac{10{,}000}{60 \times 31} = 5.38$$

$$(4)\quad \text{Nucleotidase activity (units)} = \frac{A\ (\text{without } Ni^{2+}) - A\ (\text{with } Ni^{2+})}{F_1} \times 5.38$$

one unit = 1 μmole of substrate utilized/minute per liter of serum

Normal Values and Precision

The normal range for serum 5′-nucleotidase that we find (95% confidence limits) is 3.2–11.6 units. A similar upper limit has been obtained by one of the Checkers (J. L.) The precision of a serum quality-control sample analyzed on 15 successive days was 16.1 units (SD, 1.8 units). The analytical value for the tube with nickel was 7.33 ± 0.36 μg of P_i and for the tube without nickel, 4.30 ± 0.80 μg of P_i.

Discussion

Numerous methods have been described for measuring serum 5′-nucleotidase activity (4–7). In the method of Dixon and Purdom (4) the extent of hydrolysis of β-glycerophosphate (1.47 mmol/liter) by the enzyme in the serum sample, at pH 7.5, is subtracted from the extent of similar hydrolysis of AMP (2.3 mol/liter) at the same pH. This procedure is based on the assumption that the rates of hydrolysis of the two substrates by the two enzymes, alkaline phosphatase and 5′-nucleotidase, are identical. Indeed, the ratio of activities at pH 7.5 with β-glycerophosphate and AMP as substrates is 1.0 for phosphatase derived from human intestine, but it is about 1.5 for phosphatase from human bone (8). In another method, 0.3 ml of $MgSO_4$ (0.4 mol/liter) added to the reaction mixture proposed by Dixon and Purdom was stated to completely inhibit the alkaline phosphatase activity at pH 7.5 and 9.3 (6). Actually this concentration of magnesium exerts an activating or slight inhibitory effect at pH 9.3 to 9.5 on bone or intestinal alkaline phosphatase, depending upon the substrate, the source of enzyme, and the method used for its preparation (8). In addition, concentrations of Mg^{2+} as great as 0.4 mol/liter do not inhibit the action at pH 7.5 of bone phosphatase on β-glycerophosphate, and under similar conditions human intestinal phosphatase is inhibited about 40% (8).

The nickel inhibition method as proposed by Campbell (7) and used in this report (9) is based on an assumption that Ni^{2+} inhibits 5′-nucleotidase but not alkaline phosphatases. This assumption is not completely justified. At pH 7.5, with 5 millimolar AMP as substrate, we observed the following inhibitions of purified alkaline phosphatases by 10 millimolar nickel: intestine, 0%; kidney, 0%; bone, 70%; and liver, 70% (8, 13). The activity of bone phosphatase at pH 7.5, the optimal pH for 5′-nucleotidase activity, is 4% of that observed at the optimal pH for phosphatase, 9.5 (8). The implication of these findings is that in a patient with a supranormal alkaline phosphatase activity of 100 units, all of which came from bone, 4 units of activity would be observed, at pH