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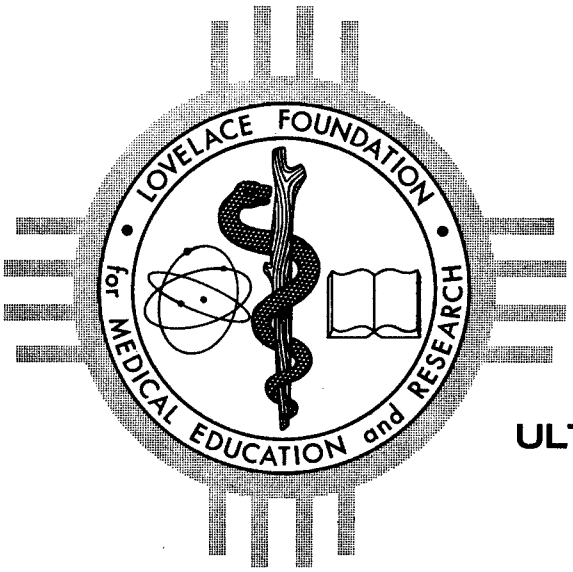

ULTRAMICRO METHODS IN BIOCHEMISTRY
III. THE DETERMINATION OF SERUM CHOLESTEROL
IV. THE DETERMINATION OF GLUCOSE

Albuquerque, New Mexico

by

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AND
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by
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ABSTRACT

Ultramicro procedures for the determination of serum cholesterol and of serum, plasma, or whole blood glucose are described. The chromogenic reaction of cholesterol with ferric iron was adapted to an ultramicro method. The results obtained with this procedure are compared to those obtained with a macro analysis which applies the chromogenic reaction of cholesterol with acetic anhydride and sulfuric acid in the presence of p-toluene sulfonic acid. Statistical evaluations of the method to determine reproducibility and variance are presented. The reliability of this method in the presence of elevated serum bilirubin has been verified. One determination done in duplicate requires 20 µl of serum.

The color reaction obtained when glucose reacts with β-glucose oxidase in the presence of peroxidase and o-anisidine was evaluated for ultramicro analysis. The results obtained are compared with those given by the Technicon AutoAnalyzer procedure which utilizes the reduction of ferricyanide by glucose. Statistical evaluation of the precision of the method and variance analysis are presented, and the precautions necessary for reliable determination of glucose are emphasized. One determination done in duplicate requires 10 µl of whole blood, serum, or plasma.

ACKNOWLEDGMENTS

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ULTRAMICRO METHODS IN BIOCHEMISTRY

III. THE DETERMINATION OF SERUM CHOLESTEROL

INTRODUCTION

A program designed to adapt procedures for the analysis of blood and serum to an ultramicro scale was described in a previous communication (1). The following report details the development of an ultramicro method for the determination of serum cholesterol.

Among the commonly used procedures for the determination of serum cholesterol are those which utilize the Lieberman-Burchard color reaction preceded by extraction of the cholesterol into an organic solvent (2, 3, 4, 5). One procedure applies this color reaction without preliminary extraction of the serum cholesterol (6). A second approach to cholesterol determination employs a color reaction with ferric iron (7). This method has been criticized on the ground that it gives erroneously high values in the presence of elevated levels of serum bilirubin (8). A modification described by Chiamori and Henry (9) is reported to eliminate the interference of bilirubin. Numerous other modifications of these basic procedures have been suggested (10, 11, 12) in attempts to simplify the analysis without loss of reliability.

The methods which utilize the color reaction with ferric iron have been reported to be very sensitive, to be less influenced by temperature and reaction time (13), and to give equivalent reactions with free and esterified cholesterol (9). They also require fewer manipulations and would be less likely to introduce technical errors during the analysis. The Chiamori and Henry (9) modification of the Zak (11) procedure was chosen for adaptation to the ultramicro determination of total serum cholesterol.

PROCEDURE

A. Instrumentation

In the original investigations of this method, absorbance of the final colored solution was measured either with the Coleman[1] Model 6 spectrophotometer equipped with an ultramicro cuvette, or the Spinco[2] spectrocolorimeter. The viscous nature of the final reaction mixture introduced a difficult problem in filling the ultramicro cuvettes. Filling could be accomplished satisfactorily, but the process required a considerable amount of time. Introduction of the adapter for 10x75-mm round cuvettes for the Spinco spectrocolorimeter eliminated this problem. The cuvettes also could be used for reaction tubes, thus removing the necessity for one transfer of solution. These cuvettes with adapter were used with the Spinco spectrocolorimeter in the procedure described below.

B. Reagents

1. Glacial acetic acid. Glacial acetic acid, Fisher Scientific Company, No. A-969 was found to be satisfactory without further purification.

2. Sulfuric acid. Reagent grade concentrated sulfuric acid.

3. Ferric chloride reagent. Dissolve 250 mg of reagent grade ferric chloride ($FeCl_3 . 6H_2O$) in glacial acetic acid and dilute to 500 ml.

4. Cholesterol standard (200 mg per 100 ml). Use reagent grade cholesterol which has been recrystallized from glacial acetic acid. Dissolve 200 mg of recrystallized cholesterol in glacial acetic acid and dilute to 100 ml.

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[1] Coleman Instruments, Inc., Maywood, Illinois.

[2] Beckman/Spinco Division, Palo Alto, California.

C. Procedure

1. To glass tubes with a minimum capacity of 2 ml add 1 ml of ferric chloride reagent. Prepare tubes in duplicate for blanks, standard, and serum.

2. Into appropriately labeled tubes add from a Sanz pipette (1) 10-μl samples of blank (distilled water), standard or unknown (serum), and mix thoroughly with the Vortex mixer.

3. Allow the tubes to stand at room temperature for four minutes. Place the tubes in a water bath or heating block at 40°C for two minutes. This step precipitates the protein. The time periods indicated should be observed fairly closely, but they are not highly critical.

4. After the tubes have cooled to room temperature, centrifuge them at 500 g for five minutes.

5. Transfer 0.5 ml of the supernatant solution to clean, 10x75-mm Spinco cuvettes. Either a Sanz, polyethylene pipette or a standard glass Oswald or transfer pipette may be used at this step.

6. Add 0.3 ml of concentrated sulfuric acid from a 2-ml burette and mix thoroughly.

7. Immediately after mixing place the tubes in a water bath at 70°C for three minutes. Timing at this step must be precise.

8. After exactly three minutes in the bath, remove the tubes and allow them to cool for 10 minutes.

9. Centrifuge the cuvettes for five minutes at approximately 500 g. This step eliminates density currents which may otherwise cause unreliable readings.

10. Read the absorbance of blanks, standards, and unknowns at 545 mμ against water as a reference solution.

D. Calculation

$$\frac{\text{Absorbance of unknown} - \text{absorbance of blank}}{\text{Absorbance of standard} - \text{absorbance of blank}} \times 200 =$$

mg cholesterol per 100 ml.

RESULTS

A. Standardization

Solutions of cholesterol in glacial acetic acid ranging from 100 to 600 mg per 100 ml were prepared. These standards were carried through the procedure as described and the results are shown in Figure 1. A linear relationship between absorbance and concentration was obtained up to a value of 600 mg per 100 ml. Most serum cholesterol levels encountered will fall within this range.

B. Comparison of Methods

The results obtained with this method were compared to those obtained in a clinical chemistry laboratory[1] with the macro method of Pearson, Stern, and McGavack (6). The results are listed in Table 1. Good agreement between the two methods was obtained.

C. Precision of the Method

Thirty determinations were performed on one serum sample during the course of one week. The mean value was 198 mg per 100 ml with a standard deviation of $\pm$ 3.8 mg per 100 ml.

Five different serum samples were analyzed in triplicate. The specimens varied from 108 to 325 mg per 100 ml in cholesterol concentration and the standard deviation was $\pm$ 1.6 mg per 100 ml as calculated by the formula for variance analysis:

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[1]Division of Biochemistry, Department of Laboratories, The Lovelace Clinic, Albuquerque, New Mexico.

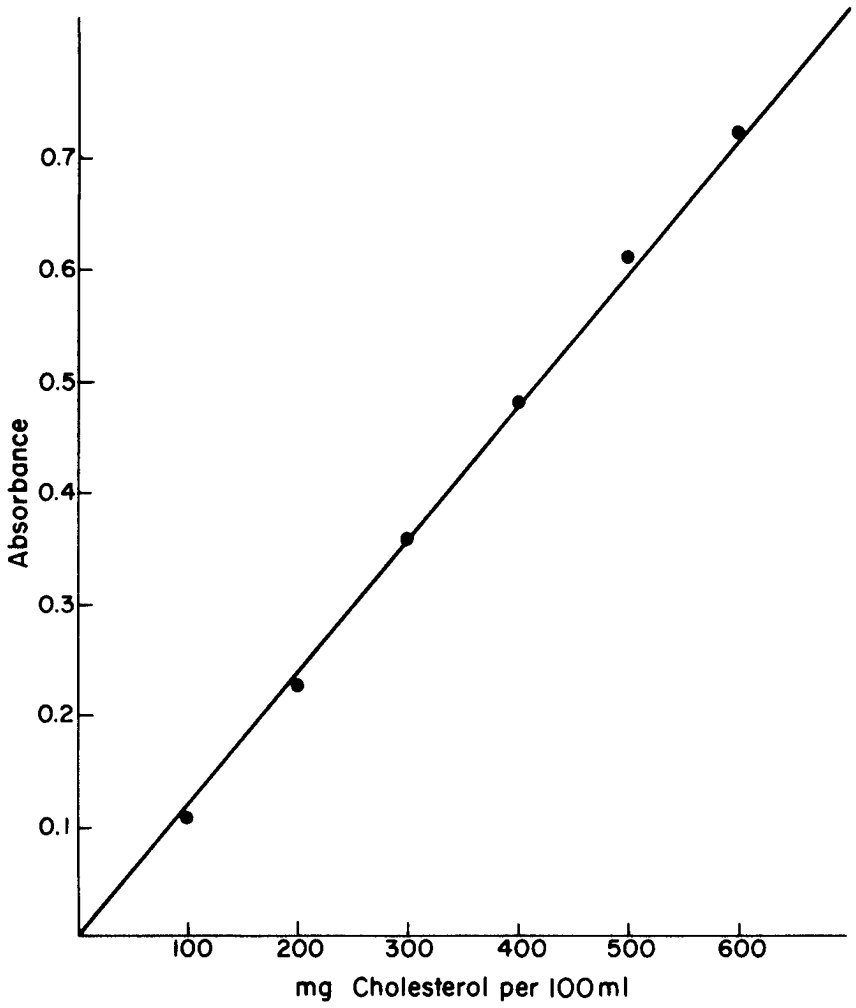

Fig. 1 Standard Curve for Cholesterol

TABLE 1

COMPARISON OF THE ULTRAMICRO METHOD

WITH THE METHOD OF PEARSON, STERN, AND McGAVACK (6)

	Cholesterol concentration *	
Sample	Ultramicro	Pearson et al.
1	198	200
2	180	177
3	294	289
4	243	238
5	236	232
6	286	286
7	317	310

* mg per 100 ml serum

$$SD = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n - 1}}$$

D. Effect of Bilirubin

The claim of Chiamori and Henry (9) that their modification of the Zlatkis, Zak, and Boyle (7) procedure eliminated interference by elevated serum bilirubin was also investigated. Seven serum samples were divided into two parts. One part was diluted with an equal quantity of albumisol (a 5 percent buffered, human albumin solution prepared by Merck, Sharp, and Dohme). The other part was diluted with an equal volume of a bilirubin standard (1) which contained 10 mg bilirubin per 100 ml prepared in an aliquot of the same lot of albumisol. This gave a final bilirubin concentration of 5 mg per 100 ml plus whatever bilirubin was in the original serum. Cholesterol determinations were done and the results are presented in Table 2. At the concentrations investigated, bilirubin does not significantly affect the results obtained by this method.

DISCUSSION

A. Reaction Tubes

One of the basic pieces of equipment of the Spinco ultramicro system is the 400- µl polyethylene reaction- or test-tube. Initial attempts to adapt cholesterol procedures to the ultramicro scale utilized these tubes. The methods of Leffler (12), Zak (11), and Chiamori and Henry (9) were investigated. Poor reproducibility and low results were obtained with all three procedures. The precipitating and extracting techniques, as well as the time and temperature of color development, were varied. None of these modifications corrected the low values or improved the reproducibility.

TABLE 2

EFFECT OF BILIRUBIN CONCENTRATION

ON THE CHOLESTEROL DETERMINATION DESCRIBED

	Cholesterol concentration*	
Sample	With bilirubin	Without bilirubin
1	165	168
2	180	179
3	124	121
4	183	179
5	116	116
6	175	175
7	139	136

* mg per 100 ml

The procedure of Chiamori and Henry (9) as described was then investigated. On the macro scale, this procedure was reproducible and accurate. Glass tubes were used in the macro procedure, so it was decided to use them in the ultramicro procedure. By the use of 10-μl serum sample and a total reaction mixture of 0.8 ml, sufficient sample was obtained for use in the 10x75-mm cuvette. The low values encountered when smaller volumes were used in polyethylene reaction tubes were not encountered and good reproducibility was obtained.

B. Reaction Temperature

In the method of Chiamori and Henry (9), as soon as the serum is mixed with the ferric chloride reagent, the tube is placed in a 60°C water bath for two minutes. The temperature of 40°C used in the procedure herein described was found to be adequate for cholesterol analyses. Recent investigations have revealed that this step may be eliminated without affecting the reliability of the method. Consideration must also be given to control of the temperature of the reaction when sulfuric acid is mixed with acetic acid-ferric chloride solution. It soon became apparent that a uniform reaction temperature was required for all of the tubes if good reproducibility was to be obtained. Consequently, the standard treatment of the reaction mixture in a 70°C water bath was adopted because this was approximately the temperature reached when the sulfuric acid was mixed with the ferric chloride-acetic acid extract in the macro procedure. Reaction temperatures of 110°C have also been used and have been found to be satisfactory.

C. Control Serum

The routine use of a control serum or a previously analyzed pooled serum with each set of determinations is recommended. Errors due to reagent deterioration or improper technique which may otherwise go unnoticed can be detected by their use.

SUMMARY

An ultramicro adaptation of the Zak (11) method for the serum cholesterol determination as modified by Chiamori and Henry (9) is described. Ten microliters of serum are required for a single determination. The determination is not affected by bilirubin concentrations up to 5 mg per 100 ml. Good reproducibility may be obtained with this method, a thoroughly reliable procedure for the determination of serum cholesterol.

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ULTRAMICRO METHODS IN BIOCHEMISTRY

IV. THE DETERMINATION OF GLUCOSE

INTRODUCTION

The commonly used methods for the determination of blood glucose are modifications of the original methods which utilized the reduction of the cupric or ferric ions by glucose at elevated temperatures, followed by a colorimetric or titrimetric estimation of the amount of reduction accomplished (1, 2). The facts that these reactions are non-specific and that a certain amount of reduction results from the presence in blood of non-sugar reducing substances are understood when these procedures are employed. The requirement for the use of elevated temperatures adds some technical problems when these methods are modified for ultramicro analysis. Both of these difficulties were overcome when Keston (3) introduced the reaction of glucose with β-glucose oxidase as a means for the determination of glucose. Only glucose and 2-deoxyglucose react with the enzyme, the latter at about 12 per cent (4) of the rate of glucose. Furthermore, 2-deoxyglucose is not present in blood in a significant amount. This reaction does not require elevated temperatures, and is quite adaptable to ultramicro analysis. Teller (5) and Washako and Rice (6) described a serum glucose procedure using glucose oxidase, and ultramicro adaptations have also been developed (7, 8). The following report is an evaluation of the use of β-glucose oxidase for the ultramicro determination of serum glucose.

PROCEDURE

A. Equipment

Both the Coleman Model 6[1] spectrophotometer equipped with a

[1] Coleman Instruments, Inc., Maywood, Illinois.

micro cuvette and the Spinco spectrocolorimeter[1] were used successfully in this method. All reactions were carried out in 400-μl polyethylene test tubes. A Vortex mixer is required. All solution measurements were made with the Sanz (9) polyethylene pipette.

B. Reagents

1. Zinc sulfate (1.07%). Dissolve 2 gm of zinc sulfate ($ZnSO_4 \cdot 7H_2O$), reagent grade, in distilled water and make up to 100 ml.

2. Sodium hydroxide (0.12 N). Dissolve 2.4 gm of sodium hydroxide (NaOH), reagent grade, in distilled water and make up to 500 ml. One part of the zinc sulfate solution should be equivalent to one part of the sodium hydroxide reagent. Titrate the zinc sulfate with sodium hydroxide using a phenophthalein indicator and dilute the stronger solution to equal the weaker solution.

3. Glucose oxidase-peroxidase-chromogen. The enzyme mixture under the trade name "Glucostat" was obtained from Worthington Biochemical Corporation, Freehold, New Jersey.

To approximately 50 ml of 0.9 per cent sodium chloride in a 100-ml graduated cylinder add the contents of the chromogen vial and the enzyme vial after they have been dissolved in 0.9 per cent sodium chloride. Rinse the vials two or three times with the sodium chloride solution and add the rinses to the cylinder. Make the volume up to 80 ml with 0.9 per cent sodium chloride. Store the enzyme-chromogen mixture in brown bottles in 10-ml portions in the freezing compartment of the refrigerator. The frozen mixture is good for at least one week.

4. Stock glucose standard (1000 mg/100 ml). Dissolve 1 gm of dry glucose, reagent grade, in distilled water and make up to 100 ml.

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[1]Spinco Division of Beckman Instruments, Inc., Palo Alto, California.

Add 0.1 gm of benzoic acid. Keep in the refrigerator.

5. Working standards

100 mg/100 ml. Dilute 1.0 ml of stock standard to 10 ml with distilled water.

200 mg/100 ml. Dilute 2.0 ml of stock standard to 10 ml with distilled water.

6. Hydrochloric acid (4.0 N, approximate). Dissolve 350 ml of concentrated hydrochloric acid in water and dilute to 1 liter.

C. Procedure

1. To a 400-μl polyethylene micro test tube add 100μl of zinc sulfate solution. Prepare tubes for blank, standard, and unknown. Measure 5μl of blank (water), standard, or unknown into the appropriate tubes so that the sample mixes with the zinc sulfate solution. Then add 100μ l of sodium hydroxide. Use both a 100-mg and a 200-mg per 100 ml glucose standard with each set of determinations.

2. Mix samples thoroughly (Vortex mixer) and centrifuge for one minute.

3. Transfer 100μl of the clear supernatant solution to a clean polyethylene micro test tube and, noting the exact time, add 250μl of Glucostat reagent and mix.

4. Exactly 30 minutes later add 10μl of 4 N hydrochloric acid and mix thoroughly to stop the reaction.

5. Read the blanks, standards, and unknowns in a micro cuvette against water at 410 mμ.

D. Calculation

$$\frac{\text{Absorbance of unknown - Absorbance of blank}}{\text{Absorbance of standard - Absorbance of blank}} \times \text{concentration of standard} =$$

mg glucose/100 ml

RESULTS

A. Standard Curve

A standard curve is shown in Figure 1 with glucose concentrations ranging up to 300 mg per 100 ml. Curve 1 was obtained by performing the reaction at 37°C. Curve 2 represents values from reactions conducted at room temperature. The values for Curve 3 were obtained by conducting the reaction at room temperature and, after the addition of 4.0 N hydrochloric acid, diluting the solution with an equal volume of Glucostat reagent. All three curves show that there is a linear relationship between absorbance and concentration over the ranges studied.

B. Dilution of Final Reaction Mixture

It would be advantageous to be able to dilute the final reaction mixture of a sample in which the color is too strong for accurate measurement of absorbance. It is evident from Curve 3 of Figure 1 that a careful 1:1 dilution of the completed standard reaction mixture with Glucostat reagent will give results with one-half the slope of the undiluted standard curve. Table 1 compares results obtained on whole blood with the procedure as described, to the results obtained on the second aliquot of the same blood, but after the final color solution had been diluted with an equal volume of Glucostat reagent before measurement of absorbance. The results show good agreement. It is, therefore, feasible with careful technique to dilute an aliquot from a completed reaction without sacrificing accuracy.

C. Standard Deviations

Thirty determinations were done on the same blood sample which had been preserved with sodium fluoride. The mean value was 90.7 mg per 100 ml of whole blood and the standard deviation was $\pm$ 1.6

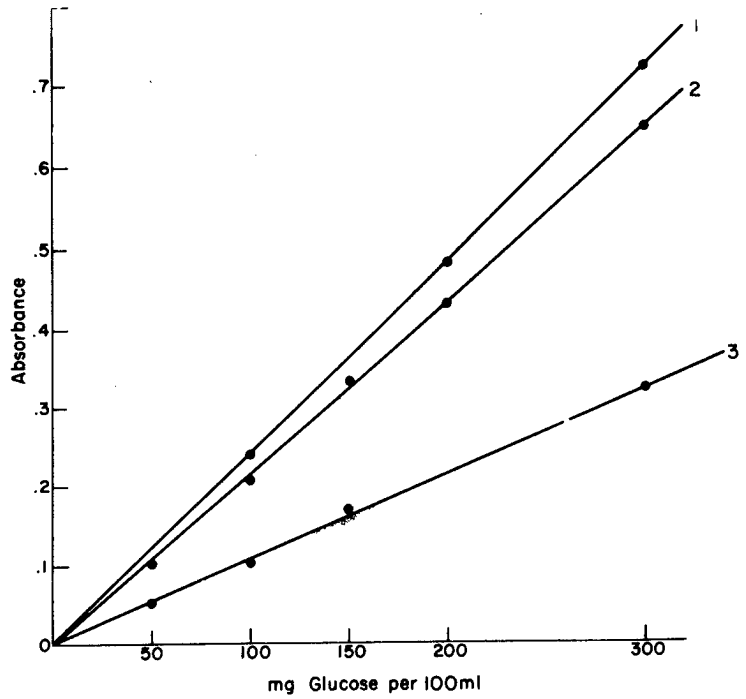

Fig. 1 Standard Curves for Glucose

Curve 1. Reaction carried out at 37°C.

Curve 2. Reaction conducted at room temperature.

Curve 3. After completion of the reaction at room temperature, the colored solution was diluted with an equal volume of glucostat reagent.

TABLE 1

COMPARISON OF DILUTED TO UNDILUTED REACTION MIXTURE

Sample	Diluted* mg/100 ml	Undiluted mg/100 ml
1	155	157
2	90	90
3	97	95
4	102	100
5	198	195
6	70	72
7	226	225
8	95	100
9	195	197
10	85	83
11	105	107
12	76	75

*The reaction was carried to completion and stopped with 4 N HCl. One aliquot was determined as described in "Procedure." Another aliquot was diluted 1:1 with Glucostat, then determined and the result multiplied by two.

mg per 100 ml.

Five different blood samples were analyzed in triplicate and the standard deviation determined by the following formula for variance analysis:

$$SD = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n - 1}}$$

The samples ranged from 86 to 170 mg per 100 ml of blood and the standard deviation was $\pm$ 1.8 mg per 100 ml.

D. Comparison of the Glucose Oxidase Method to the AutoAnalyzer Method

Results of plasma glucose determination obtained with the ultramicro method were compared to results obtained by reduction of ferricyanide in the Technicon AutoAnalyzer.[1] Three separate series were run, the first with the original standard dialysis membrane and a 6 mm flow cell, and the last two with the 15 mm flow cell and the thinner "C" membrane. With the first series, consisting of 12 specimens, the results with the AutoAnalyzer averaged 4 mg per 100 ml higher than those obtained with the ultramicro Glucostat method. The second series (22 specimens) averaged 13 mg per 100 ml higher with the AutoAnalyzer, and the third series (20 specimens) averaged 17 mg per 100 ml higher with the Auto Analyzer.

The analysis of non-sugar reducing substance by the AutoAnalyzer with a "C" dialysis membrane was also investigated. Seven serum samples were incubated with washed yeast for three hours at 37°C. After centrifuging to remove the yeast, the serum was analyzed with the Auto Analyzer and also by means of the ultramicro glucose oxidase method. The results with the AutoAnalyzer, uncorrected, ranged between 10 and 23 mg per 100 ml. All of the specimens except one contained less than 5 mg per 100 ml by the glucose oxidase method. The exception contained 6 mg glucose per 100 ml by analysis. It is doubtful that any value

[1] Technicon Instruments, Inc., Chauncey, New York.

below 5 mg per 100 ml by this method is sufficiently valid to use as a correction factor. The treatment with yeast seemed to have been adequate to remove glucose. The values obtained probably represent a fairly close approximation to the non-glucose reducing substance in human plasma which is able to pass through the AutoAnalyzer "C" membrane, and which contributes to the analytical results referred to glucose as a standard.

DISCUSSION

A. Temperature of Reaction

Standard curves carried out at 37°C and at room temperature are shown in Figure 1, Curves 1 and 2, respectively. Although the slope is a little steeper for the reaction at 37°C, no distinct advantage was gained by conducting the reaction at the higher temperature.

B. Time of Reaction

Some procedures based on the action of glucose with glucose oxidase suggest a reaction time of 10 minutes. The time adopted for the method herein reported was 30 minutes. The reaction is approaching completion after 30 minutes and a small error in time is not as critical as at the 10-minute time period. Since the reaction has proceeded longer at the 30-minute period, it gives greater absorbance for the same concentration. The advantages of better reproducibility and higher absorbance values appeared to outweigh the disadvantage of increased reaction time. The longer incubation time, therefore, was chosen.

C. Use of Precipitating Reagents

The glucose oxidase method, because of its specificity, can be used directly with serum free from hemolysis and with a low bilirubin content. Both hemolysis and elevated bilirubin are encountered with sufficient frequency that precipitation of the protein is advisable. Therefore, this step was made a routine part of the procedure.

A Folin and Wu filtrate is not suitable for the glucose oxidase procedure. When compared with a zinc sulfate-sodium hydroxide filtrate,

the Folin-Wu filtrate gave erratic results.

D. Inclusion of the Second Standard

The concentration of glucose in commercial laboratory control sera appears to be rather labile. Values agreeing with the assayed results can be obtained only a few hours after the serum has been reconstituted. If a control serum is available which is freshly reconstituted, it can be used satisfactorily as a check on the procedure. However, it has been found in this laboratory to be more economical and of sufficient accuracy to include a second standard as a check on the procedure rather than use a control serum with each determination.

E. Time Period Between Addition of Glucostat Reagent to Samples

In order to insure precision of timing for the completion of the reaction, it is necessary to allow a time period between individual tubes when the reaction is begun. The most convenient time period between samples for the addition of Glucostat reagent was found in this laboratory to be one minute. This gives adequate time for correction of minor problems in pipetting which may arise.

F. Choice of Acid for Stopping the Reaction

Both hydrochloric acid and sulfuric acid (6) have been used to stop the reaction. The amount of hydrochloric acid used in this procedure is sufficient to stop the reaction and a yellow color is produced. Higher concentrations of sulfuric acid produce a blue-purplish color which has greater absorbancy than the color produced with hydrochloric acid, but its use was found to be impractical when applied to ultramicro analysis. It produced a viscous solution which was conducive to the formation of density currents when the mixture was introduced into the micro cuvettes.

SUMMARY

An ultramicro procedure for the determination of glucose by the glucose-oxidase procedure has been modified and described. A 5-μl sample is required. Standard curves determined at room temperature

and at 37°C have been presented. The precision of the method is satisfactory. The results obtained with this method have been compared with those obtained with the AutoAnalyzer.

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