Analysis on Heat Coagulated Blood and Serum

V. Determination of Glucose

Morris London and Jesse H. Marymont, Jr.*

A method for the determination of serum glucose employing a heat clot technic for the elimination of protein and other interfering substances is described. Serum (0.10 ml.) and absolute methyl alcohol (0.09 ml. delivered as six drops) are mixed in a shell vial and a clot is formed by immersion of the vial in a 60° water bath for 4 min. The clot is overlaid with 2.0 ml. of water and the container is placed in a 37° water bath for 45 min.; 0.50 ml. of the extract is added to 6.0 ml. of a glucose oxidase reagent and the solution placed in a 37° water bath for 30 min. Two drops of 4N HCl is added and the absorbance at 420 mμ measured. The serum glucose is calculated in the usual manner by comparing the absorbance of the unknown with that of a standard. The method is simple, reliable, and well suited for routine use in the clinical laboratory.

In a recent series of papers (1-4), heat coagulation and extraction was shown to be a simple and reliable technic for obtaining some of the low molecular weight constituents of serum (urea, uric acid, and inorganic phosphate) in protein-free solution. The method eliminates the use of reagents for protein precipitation which represent potential sources of error in analysis. The constituents to be measured must be stable during the heat coagulation process. Glucose is unstable at 100° in serum, and cannot be quantitatively analyzed by the method as originally described.

The present paper describes a procedure in which absolute methyl alcohol is added to the serum sample lowering the temperature necessary for heat coagulation of proteins permitting formation of a firm clot at 60°. The alcohol volatilizes during and immediately after the heat-clotting process, and is not present in the extract. Glucose is

From the Division of Laboratories, North Shore Hospital, Manhasset, N. Y.
Received for publication Mar. 7, 1963.
*Present address: Department of Laboratories, Wesley Medical Center, Wichita 14, Kansa.

942
stable at 60° under the conditions established. The method is accurate,
simple, and reproducible for the measurement of serum glucose.

Reagents

1. *Glucose oxidase reagent*  Prepare as recommended by manu-
   facturer (Glucostat, Worthington Biochemical Corp., Freehold, N. J.).

2. *Stock glucose standard* (1 ml. = 1.0 mg. glucose)  Dissolve 100
   mg. of glucose in sufficient 0.2% (w/v) benzoic acid solution to make
   100 ml. of solution.

3. *Dilute glucose standard* (1 ml. = 0.05 mg. glucose).  Dilute
   1.0 ml. of stock standard to 20.0 ml. with water. Prepare fresh daily.

Method

Place 0.10 ml. of serum in the bottom of a 25 × 95-mm. shell vial and
add 6 drops of absolute methyl alcohol (0.09 ml.). A Pasteur pipet
with a rubber bulb was used for this addition. Mix by gentle rotation.
Immerse the vial in a water bath at 60° for 4 min. Overlay the clot with
2.0 ml. of water and place in a water bath at 37° for 45 min. Transfer
0.50 ml. of extract to a 19 × 105 mm. Coleman cuvet. Simultaneously
set up a blank and standard by substituting 0.50 ml. of water and 0.50
ml. of dilute standard for the extract in two similar cuvets. Add 6.0 ml.
of glucose oxidase reagent to all tubes, mix, and incubate in a water
bath at 37° for 30 min. Add 2 drops of 4N HCl and mix well. Measure
the absorbance of the unknown and standard in a Coleman Jr. spectro-
photometer at 420 mμ after setting the instrument to zero with the
blank.

\[
\text{Serum glucose (mg./100 ml.)} = \frac{\text{absorbance of unknown}}{\text{absorbance of standard}} \times 100 \times \frac{21}{20}
\]

Results

In Fig. 1 serum glucose values obtained with the enzymic method
using a Somogyi deproteinization technic (5) are compared with those
found by the heat coagulation technic described. They are similar
within the limits of experimental error.

Under the conditions established, a firm coagulum forms after about
2 min. of heating. Serum glucose is stable for at least 5 min. at 60°, but
is unstable at 100° as shown in Fig. 2. A coagulation time of 4 min. was
selected in order to insure volatilization of all methanol present.

The rate of extraction of glucose from the coagulum is a function
of both the extraction temperature and the clot thickness. In Fig. 3 glucose extraction is shown as a function of time under the conditions described. Extraction is virtually complete after 30 min. but a time of 45 min. was used to allow for slight variations in clot thickness and ex-

traction temperature. At temperatures above 37° the evaporation of water from the extract can be appreciable, and vials should be stoppered. Since the sugar is not stable at 60° for 6 min. (see Fig. 2), extraction cannot be accomplished at the same temperature as coagulation.

**Fig. 1.** Comparison of serum glucose values obtained by glucose oxidase method after removal of proteins by method of Somogyi, and by heat coagulation and extraction.

**Fig. 2.** Effect of heating of serum on subsequent glucose recovery.
Recovery studies were performed by the addition of known amounts of glucose to sera from hospitalized patients. The results of 7 such analyses are shown in Table 1. Recoveries varied from 93 to 104%, with an average of 99%. The data indicate excellent correlation between theoretical and experimental values—further evidence that glucose is stable during the heat coagulation process.

Bilirubin interferes with the glucose oxidase method by competing with the chromogen for the hydrogen peroxide formed. Evaluation of the partition of total bilirubin between clot and extract was accomplished by measuring the concentration of the pigment in extracts obtained from a group of sera* whose total and conjugated bilirubin levels were known. Determinations were made by the method of Malloy and Evelyn (6). The results of 9 such analyses are shown in Table 1.

---

*Sera with elevated bilirubin levels were kindly supplied by Mr. Benjamin Fingerhut, Kings County Hospital, Brooklyn, N. Y.
Table 2. Partition of Bilirubin between Clot and Extract at 37°

<table>
<thead>
<tr>
<th>Serum Bilirubin concentration (mg./100 ml.)</th>
<th>Extract* Total</th>
<th>Clot† Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>Direct 3.3</td>
<td></td>
</tr>
<tr>
<td>18.2</td>
<td>10.8</td>
<td>14.9</td>
</tr>
<tr>
<td>19.4</td>
<td>10.2</td>
<td>16.4</td>
</tr>
<tr>
<td>14.8</td>
<td>8.8</td>
<td>12.4</td>
</tr>
<tr>
<td>16.0</td>
<td>9.5</td>
<td>10.8</td>
</tr>
<tr>
<td>18.8</td>
<td>10.8</td>
<td>15.8</td>
</tr>
<tr>
<td>11.8</td>
<td>4.8</td>
<td>10.6</td>
</tr>
<tr>
<td>15.6</td>
<td>8.5</td>
<td>13.2</td>
</tr>
<tr>
<td>19.4</td>
<td>10.5</td>
<td>16.1</td>
</tr>
<tr>
<td>8.6</td>
<td>5.8</td>
<td>5.6</td>
</tr>
</tbody>
</table>

*Corrected values. The concentrations shown are those theoretically obtained if the extract volumes are concentrated to the original serum volumes.
†These represent the differences between the concentrations of total bilirubin in serum and in extract.

2. It is evident that in the heat clot method most of the bilirubin remains with the clot. The interference of bilirubin in the extract from highly icteric sera is negligible.

Hemolysis interferes with the glucose oxidase method in two ways. The catalase from the erythrocytes results in breakdown of hydrogen peroxide tending to falsely lower sugar values. The hemoglobin molecule in acid solution has an absorption at 420 mµ, and tends to elevate glucose values falsely. To evaluate the effect of hemolysis on the heat clot method for serum glucose, analyses were performed on a group of 9 sera to which sufficient hemolyzed erythrocytes had been added to result in a hemoglobin concentration of 0.75 gm./100 ml. (equivalent to 5% hemolysis of a sample with a hemoglobin concentration of 15 gm./100 ml.). The glucose in each serum was measured, using the glucose oxidase reagent in 5 different ways. The results are tabulated in Table 3. In each case color development was for 30 min. in a water bath at 37°; 5.0 ml. of glucose oxidase reagent was used for each determination.

Column 1 of Table 3 shows results obtained with 0.50-ml. aliquots of Somogyi filtrates. Reactions were stopped with 2 drops of 4N HCl and absorbances were measured at 420 mµ.

Column 2 shows results obtained by direct addition of 0.025-ml. aliquots of serum. Reactions were stopped and absorbances measured as described for Column 1.
Table 3. Effect of 5% Hemolysis on Glucose Values Measured with Glucose Oxidase Reagent 5 Different Ways

<table>
<thead>
<tr>
<th>Glucose (mg./100 ml.)</th>
<th>Direct</th>
<th>Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somogyi 420 µ</td>
<td>530 µ</td>
<td>420 µ</td>
</tr>
<tr>
<td>127</td>
<td>140</td>
<td>117</td>
</tr>
<tr>
<td>80</td>
<td>92</td>
<td>71</td>
</tr>
<tr>
<td>127</td>
<td>138</td>
<td>108</td>
</tr>
<tr>
<td>67</td>
<td>91</td>
<td>61</td>
</tr>
<tr>
<td>93</td>
<td>116</td>
<td>85</td>
</tr>
<tr>
<td>107</td>
<td>127</td>
<td>94</td>
</tr>
<tr>
<td>97</td>
<td>128</td>
<td>95</td>
</tr>
<tr>
<td>71</td>
<td>89</td>
<td>63</td>
</tr>
<tr>
<td>95</td>
<td>118</td>
<td>89</td>
</tr>
<tr>
<td><strong>MEAN:</strong></td>
<td>96</td>
<td>87</td>
</tr>
</tbody>
</table>

Column 3 also shows results obtained by direct addition of 0.025-ml. aliquots of serum, but reactions were stopped with 1.5 ml. of 50% sulfuric acid and absorbances measured at 530 mµ, as described by McComb and Yushok (7).

Column 4 shows results obtained with 0.50-ml. aliquots of a 1/21 heat clot extract. Reactions were stopped and absorbances measured as described for Column 1.

Column 5 also shows results obtained with 0.50-ml. aliquots of a 1/21 heat clot extract, but with reactions stopped and absorbances measured as described for Column 3.

**Discussion**

Methyl alcohol, when added to serum, lowers the temperature necessary for the coagulation of proteins (8). The methanol is completely volatilized during the heating period, and its use permits the formation of a firm serum clot under conditions which do not result in the destruction of glucose. The alcohol itself is a protein precipitant, and does cause the partial precipitation of the serum proteins. However, the separation of protein from solution is incomplete without subsequent heating.

The boiling point of methanol (64.5°) is only slightly above the temperature used for heat coagulation of serum (60°), and the alcohol rapidly evaporates during the clotting procedure. As mentioned, although a firm clot is formed after 2 min. of heating, a coagulation time
of 4 min. is recommended to insure complete evaporation of the methanol.

The glucose oxidase reagent is a buffered mixture of glucose oxidase, horse-radish peroxidase, and o-dianisidine. The enzyme mediates the aerobic oxidation of glucose to gluconic acid with the formation of hydrogen peroxide; the latter reacts with o-dianisidine to produce an orange color, the intensity of which is proportional to the amount of glucose present. If methanol were not completely removed from the system during coagulation of proteins, it would be present in the extract, and compete with the chromogen for hydrogen peroxide with the formation of formaldehyde. This would result in falsely low values of serum glucose.

During the extraction process, glucose is divided between the clot and supernatant water in direct proportion to the volume of each present. In the procedure described the methanol is volatilized during the clotting phase and does not enter into the calculations. With 0.10 ml. of serum and 2.0 ml. of water, a total of 20/21 of the serum glucose is extracted into the supernatant. The factor 21/20 in the calculations compensates for glucose remaining in the clot, and must be appropriately altered if the ratio of serum to water is changed.

From the data in Table 3, obtained from serum samples with 5% hemolysis, several conclusions can be reached.

Values obtained by the direct addition of hemolyzed serum to glucose oxidase reagent are not accurate. In this system hemoglobin tends to elevate values while the catalase present tends to depress them. The direction of the final error depends on which exerts the greater effect. Hemoglobin has intense absorption at 420 m\(\mu\), and measurements at this wavelength tend to be too high. At 530 m\(\mu\) the catalase action usually predominates, and values may be depressed.

Values obtained with heat clot extracts do not show interference due to catalase, presumably because the enzyme is denatured during the clotting process. Measurements made at 420 m\(\mu\) are elevated due to small amounts of colored hemoglobin degradation products in the extracts. The errors are small, however, and are considerably less than with the direct method. Measurements made at 530 m\(\mu\) are in excellent agreement with those obtained with Somogyi filtrates, and demonstrate that the heat clot method is applicable with moderately hemolyzed specimens.

The use of 1.5 ml. of 50% sulfuric acid to stop the color reaction is suitable as a routine procedure. It necessitates the handling of large
amounts of 18N acid, however, and for this reason alone we do not prefer it. It is somewhat more sensitive than the method employing the more dilute acid system, and may be of value for ultramicro technics.

We envision the heat clot as a protein mesh in which the high molecular weight constituents of serum are trapped. The size of the mesh is a function of the protein concentration, and it partially determines which substances remain in the clot and which diffuse into the extract. By adding 22% bovine albumin to the serum sample before coagulation the mesh can be made tighter. When this is done to a specimen with extreme hemolysis, for example, the amount of colored hemoglobin degradation products extracted is reduced though the extraction of glucose is not impaired. This technic for controlling the makeup of a serum extract has not yet been utilized, but it may have some, as yet unforeseen, applications which will widen the usefulness of the heat clot method.

References