THE BIURET REACTION IN THE DETERMINATION OF SERUM PROTEINS

II. MEASUREMENTS MADE BY A DUBOSCQ COLORIMETER COMPARED WITH VALUES OBTAINED BY THE KJELDAHL PROCEDURE

BY HOWARD W. ROBINSON AND CORINNE G. HOGDEN

(From the Children's Hospital Research Foundation and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati)

(Received for publication, June 22, 1940)

From the results reported in Paper I of this series (1) it is evident that the biuret reaction may be used for the estimation of serum proteins with a high degree of accuracy when the optical density values at wave-length 560 m\(\mu\) are obtained with a spectrophotometer, and when the color solutions are prepared by the introduction of 20 per cent copper sulfate to protein solutions in which the concentration of alkali is 3 per cent. However, optical instruments which permit the selection of narrow ranges of the spectrum are not available in many laboratories, but in such places the Duboscq type of colorimeter is still widely used for colorimetric methods. The many requests for the application of the information obtained in our work (1) to a very simple procedure that might be used on small samples of sera with the common laboratory equipment led us to determine the reliability of a method under such conditions. The results are such that we believe the method should be called to the attention of many laboratory workers who are anxious to secure reliable protein determinations with the least time and equipment.

The method to be presented is similar to that suggested by Fine (2) in which serum diluted with physiological saline was used as a standard for colorimetric comparison. Fine's statement that the standard solution could be kept for 6 months if preserved with chloroform has been doubted by many workers, because, in their experience, the solutions became turbid in a very short time.
The problem of a suitable standard was solved as far as we were concerned by the use of rabbit sera. Stock solutions prepared from human or dog sera became turbid in some cases after standing for only a week, but in no instance has a dilution of rabbit serum deteriorated so rapidly. Two dilutions of rabbit sera stored under conditions described below have remained clear and given constant biuret colors for more than a year. From our previous work we feel that the use of a 20 per cent copper sulfate solution should be much better than the 5 per cent solution as employed by Fine. The precipitation of the protein by trichloroacetic acid as suggested by Fine is recommended for the production of clear color solutions. The addition of a dilute solution of copper sulfate to alkaline serum solutions proposed by Kingsley (3) has never given us the clear reproducible solutions that are possible when colors are developed in alkaline solutions of the precipitated protein. With the Kingsley method we have obtained results comparable to those shown in his paper only under the most rigorous control of the time of reading after production of colors. In these experiments we never used ether to clear the solutions because none of the sera was jaundiced or lipemic.

Method

Reagents—
0.9 per cent sodium chloride solution.
10 per cent trichloroacetic acid solution.
3 per cent sodium hydroxide solution.
20 per cent copper sulfate (CuSO₄·5H₂O) solution.

Preparation of Standard—10 cc. of blood serum from a normal rabbit with a total serum protein of about 6 gm. per 100 cc. are diluted to exactly 250 cc. with 0.9 per cent sodium chloride solution. Total nitrogen concentration of this sample is determined by the micro-Kjeldahl procedure described previously by Robinson, Price, and Cullen (4). Non-protein nitrogen is determined at the same time on a sample of the serum. The protein content is obtained by subtracting the non-protein nitrogen from the total nitrogen, and converting the protein nitrogen to protein with the factor 6.25. The concentration of protein in the diluted serum should be approximately 0.25 per cent. This stock solution is distributed in 50 cc. containers, preserved with a crystal of thymol, and stored in a refrigerator when not in use. This diluted rabbit
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serum standard usually has a very faint opalescence which interferes in no way with the final color and which remains stable for at least 6 months. Any increase in turbidity or any flocculation has been regarded with suspicion and such standards should be discarded for new ones.

Total Serum Protein—0.2 cc. of serum is measured with a “contain” pipette into a 15 cc. graduated centrifuge tube, the pipette being washed several times with saline solution. If duplicate determinations are desired, one may dilute 0.5 cc. of serum to 10 cc. with saline and then measure two portions of 4 cc. each with a transfer pipette into the centrifuge tubes.

The volume of the diluted serum is brought to about 5 cc. with saline and an equal amount of 10 per cent trichloroacetic acid is added. The contents are well mixed with a fine glass rod which is rinsed with a little acid when it is removed. Centrifuge the mixture for 5 to 10 minutes, or until the supernatant fluid is clear and can be poured off without disturbing the precipitate. Invert the tube and allow to drain well on a filter paper.

The protein precipitate is mixed with a drop of 3 per cent sodium hydroxide by means of a fine stirring rod until a smooth paste is formed. A few cc. of sodium hydroxide are then added to complete solution of the protein. The mixture must be inspected for small gel-like particles which indicate that the protein is not yet entirely dissolved. After the volume is brought to about 9 cc. with the alkali, 0.25 cc. of 20 per cent copper sulfate is added and the final volume adjusted to 10 cc. with the alkali. Close the tube with a clean rubber stopper, and shake the contents vigorously for a minute in order to develop the biuret color.

After standing for 10 minutes or longer the color solutions are centrifuged at high speed for about 1/2 hour in order to remove the excess cupric hydroxide. The supernatant liquid is carefully drawn off with a pipette, and compared in a Duboscq colorimeter with a standard color solution. The standard is prepared simultaneously with the unknown by using 5 cc. of standard stock solution in a manner identical with that described for the unknown. If the standard is set at 20 mm. the calculation is

\[
\frac{20.0}{\text{Reading of unknown}} \times \frac{\text{gm. protein in standard}}{\text{cc. serum in unknown}} \times 100 = \text{gm. protein per 100 cc. serum}
\]
Biuret Reaction for Proteins. II

Serum Albumin—30 parts of 22 per cent sodium sulfate are added to 1 part of serum in order to precipitate the globulin (Howe (5)). The mixtures are filtered or centrifuged at the end of 4 hours in the manner described by us (6). 7 cc. of filtrate are measured into a 15 cc. centrifuge tube and the protein is precipitated with trichloroacetic acid. The procedure is in every detail identical with that described for total serum proteins. However, in order to obtain more equal color comparison, it is preferable to use 4 cc. of the stock rabbit serum solution instead of 5 cc. for the standard color. The calculation of the serum albumin is

\[
\frac{20.0}{\text{Reading of unknown}} \times \text{gm. protein in standard} \times \frac{31}{7} \times 100
\]

= gm. albumin per 100 cc. serum

Results

60 determinations of the total serum protein and forty-six determinations of the serum albumin were made by this method on

\[
\text{TABLE I}
\]

Deviations of Serum Protein Determinations by Biuret Colorimetric Method from Values Calculated from Nitrogen Determinations by Kjeldahl Method

<table>
<thead>
<tr>
<th>No. of subjects</th>
<th>No. of determinations</th>
<th>Range of values of protein concentration</th>
<th>Range of deviations between the two methods</th>
<th>Standard deviation(^*) (\sqrt{\frac{\Sigma d^2}{N-1}})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total serum proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>18</td>
<td>26</td>
<td>3.2-8.4</td>
<td>-0.6 to +0.6</td>
</tr>
<tr>
<td>Dog</td>
<td>10</td>
<td>20</td>
<td>4.7-7.5</td>
<td>-0.3 &quot; +0.5</td>
</tr>
<tr>
<td>Rabbit</td>
<td>8</td>
<td>14</td>
<td>5.3-6.5</td>
<td>-0.2 &quot; +0.4</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Serum albumins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>20</td>
<td>25</td>
<td>1.9-5.6</td>
<td>-0.3 to +0.3</td>
</tr>
<tr>
<td>Dog</td>
<td>9</td>
<td>14</td>
<td>2.5-3.7</td>
<td>-0.2 &quot; +0.2</td>
</tr>
<tr>
<td>Rabbit</td>
<td>4</td>
<td>7</td>
<td>3.4-4.9</td>
<td>-0.1 &quot; +0.3</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>46</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\* d is the deviation between the two methods.
human, dog, and rabbit sera and compared with the protein values calculated from nitrogen determinations by the Kjeldahl method. The human subjects consisted of normal persons and of sick patients. Twenty-two determinations (eleven total serum proteins and eleven serum albumins) of the latter group were on the blood sera of children with the nephrotic syndrome. The dogs and rabbits were apparently normal animals which had not been used for other experimental procedures. The results are summarized in Table I. The greatest deviation between the Kjeldahl and biuret method obtained at any time was 0.6 gm. of protein per 100 cc. of serum. The mean of the deviations for total serum proteins was $-0.028$ gm. per 100 cc. of serum and for serum albumin was $-0.021$. These small differences between the two methods were not statistically significant.

**SUMMARY**

1. A method is described in detail for the determination of serum proteins by the biuret reaction in which the color comparisons are made with the Duboscq colorimeter. The suitability of a dilute rabbit serum saline solution as a standard is discussed.

2. Results by this method show satisfactory agreement with those obtained from Kjeldahl determinations, so that the use of this procedure is warranted for the reliable estimation of serum proteins when the amount, time, and equipment are limited.

**BIBLIOGRAPHY**

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