DETERMINATION OF PROTEIN IN URINE BY THE BIURET METHOD

By ALMA HILLER, ROGER L. GREIF, AND WILLIAM W. BECKMAN

With the Technical Assistance of JOHN PLAZIN

(From the Hospital of The Rockefeller Institute for Medical Research, New York)

(Received for publication, July 15, 1948)

A method for the determination of protein in urine by means of the biuret reaction was described by Hiller (1) and by Hiller, McIntosh, and Van Slyke (2), in which pure biuret prepared by Kahlbaum was used as a standard for visual colorimetry. Since Hiller (1) the biuret method has been used for the determination of urinary proteins by Price (3) and by Lehmann (4), and has been widely used in many forms and modifications for the determination of plasma proteins. A review of the literature on the biuret method will not be given here, but may be found in the papers of Küntzel and Dröschler (5) and of Robinson and Hogden (6). The latter workers studied the conditions necessary for the production of a stable color which bears a quantitative relationship to the protein concentration and also published transmission curves for the biuret color.

The present paper describes a photometric method for the determination of urinary protein in which the entire procedure is carried out in a single test-tube, which serves as a cuvette for the photoelectric spectrophotometer. The method can be used even when the urine contains Evans blue excreted after blood volume determinations. Since pure preparations of biuret are not now obtainable on the market, solutions of urinary proteins, of concentration determined by accurate Kjeldahl analysis, are used to prepare standard optical density curves, and the use of a chrome-alum solution for checking the curves is detailed.

METHOD

Apparatus

Cuvettes, 15 by 100 mm. (outer measurements). Test-tubes, thick wall, without lip, from the Arthur H. Thomas Company, No. 9446, make good inexpensive cuvettes. After cleaning with dichromate cleaning mixture the tubes are tested for uniformity by reading the optical density of a solution containing approximately 15 gm. of CuSO₄·5H₂O per 100 ml. in each tube in a spectrophotometer at a wave-length of 560 mμ. Tubes which read within an optical density variation of ±0.0015 of the average are chosen as cuvettes. These are then etched with numbers and calibrated.
to contain 10 ml. The tubes must be carefully handled to prevent scratch-
ing. Before each series of readings in the spectrophotometer, finger-marks
must be removed by wiping the outside of the tube first with a damp, then
with a dry towel. They must be cleaned immediately after use. A good
cleaning procedure is to remove precipitates by rinsing and shaking with
water; then small amounts of copper sulfate which adhere to the upper part
of the inner walls of the tubes are removed with dilute hydrochloric acid,
approximately 1 N. The tubes are then rinsed five times with tap water
and three or four times with distilled water and inverted in a test-tube rack
to drain onto a towel. Only the tubes used for blank determinations need
be dry. The tubes used for protein analysis can be used without drying.

Rubber stoppers, solid No. 0, to fit cuvettes.

Centrifuge metal shields 3½ inches long and ¾ inch in diameter will fit the
size of cuvettes used. With an eight place head and three or four place
trunnion carriers a large number of tubes can be centrifuged at one time.

Photoelectric spectrophotometer. The Coleman junior model was used,
but any type can be used, with a cuvette holder 3 inches in length. If the
holder is too wide for the cuvettes, a ring of hard rubber can be fitted into
the top of the holder to keep the cuvettes in a vertical position.

A 5 ml. burette marked at 0.25 ml. intervals to deliver the copper sulfate
solution.

A 25 ml. burette to deliver trichloroacetic acid.

Dispenser for sodium hydroxide. An aspirator bottle or a separatory
funnel type of vessel, equipped with a soda lime tube and an outlet tube
with a small opening for delivery of small drops.

Reagents

Trichloroacetic acid, 10 per cent solution. Keep in the refrigerator
when not in use.

Copper sulfate, 20 gm. of CuSO₄·5H₂O per 100 ml.

Sodium hydroxide, reagent grade, 3 per cent solution. This solution is
stored in the dispenser described under “Apparatus,” and should not be
allowed to age beyond 2 weeks, as the solution on standing over a longer
period of time in glass gives higher blanks and lower protein values than
when freshly made (see Table III).

Procedure

If the urine contains a precipitate, it should be filtered before analysis.

Precipitation of Protein—Enough urine to contain between 5 and 20 mg.
of protein is pipetted into the bottom of each cuvette. The samples of
albuminous urine usually range from 0.5 to 5 ml. to contain the desired
amount of protein. If the concentration of protein is higher than 30 mg.
per liter, the urine is diluted with water so that a sample will contain between 5 and 20 mg. of protein. To the urine in each cuvette an equal volume of 10 per cent trichloroacetic acid is added. If the total volume of urine plus trichloroacetic acid is 6 ml. or less, mixing can be accomplished by shaking the cuvette. If the volume is greater than 6 ml., a thin stirring rod should be used to get complete mixture, with care not to scratch the wall of the cuvette. The rod is washed down with a few drops of water. The cuvette is allowed to stand 10 minutes, then centrifuged at about 2500 R.P.M. for 10 minutes. The supernatant solution is decanted and the cuvette is inverted against a towel or filter paper for a moment to drain.

**Dissolving Protein Precipitate**—The protein precipitate is dissolved by adding 2 ml. of 3 per cent sodium hydroxide. The cuvettes are shaken occasionally until solution is complete, but shaking must be gentle to avoid formation of foam. If cuvettes containing appreciable amounts of protein are allowed to stand for a time without occasional shaking, undissolved protein at the bottom of the cuvette will form a clear gel and may be overlooked, as it is difficult to see and requires a longer time to dissolve than does the white precipitate. If the cuvette is examined with a window or light as background, while whirling gently, a spiral of dissolving protein can be seen rising from the bottom of the cuvette. Solution is complete when there are no transparent strands on shaking the solution. More 3 per cent sodium hydroxide solution is then added with occasional shaking, until the volume in the cuvette reaches the 10 ml. mark.

Two dry cuvettes are filled to the 10 ml. mark with 3 per cent sodium hydroxide to serve as blanks.

**Setting Zero Point of Photometer**—The zero optical density point is set before each reading with the holder in place, but without a cuvette (air zero).

**Blanks**—The density of the 3 per cent NaOH in the cuvettes is read as $D_{B1}$, Blank 1.

**Reading of Optical Density, $D_1$, Due to Pigments in Urine Carried Down by Precipitate of Protein**—The tubes containing redissolved protein are wiped and the optical density is read as $D_1$ at wave-length 560 m.$\mu$.

**Development of Biuret Color and Reading of Optical Density, $D_2$**—After $D_1$ readings have been made, 0.25 ml. of 20 per cent copper sulfate is added from the 5 ml. marked burette to each protein solution and blank. Each tube is stoppered as soon as the copper sulfate has been added, and is immediately shaken vigorously about 15 times. Unless shaking is prompt, clumps of copper hydroxide may stick to the sides. Stoppers are removed and washed. The tubes are allowed to stand 10 minutes for the biuret color to develop, and are then centrifuged for 4 minutes at about 2500 R.P.M. (Blanks should not stand longer than 10 minutes before centrifuga-
tion as they tend to be lowered on standing.) They are then wiped clean
and the optical density is read at 560 mμ, with the zero point set as de-
scribed above. The readings of the protein solutions are recorded as \( D_2 \)
and the blanks are \( D_{B2} \), Blank 2. Tubes should be washed immediately
after use as described under "Apparatus."

**Calculation**

\[
D_1 = \text{optical density reading of 10 ml. of protein solution}
\]

\[
D_{B1} = \text{optical density reading of solvent (3 per cent NaOH) = Blank 1}
\]

\[
D_2 = \text{optical density reading of reagent blank (NaOH + CuSO}_4\text{) = Blank 2}
\]

\[
D_P = \text{due to biuret color formed from protein in a volume of 10 ml.}
\]

\[
(1) \quad D_P = 1.025 \left( D_2 - D_{B2} \right) - \left( D_1 - D_{B1} \right)
\]

The mg. of protein in the sample analyzed are read from a curve con-
structed for this purpose, relating \( D_P \) to mg. of protein for the specific set
of cuvettes and spectrophotometer used.

\[
\text{Mg. protein per liter urine} = \frac{\text{mg. protein in sample analyzed} \times 1000}{\text{ml. urine in sample taken}}
\]

**Construction of Curve Relating Optical Density to Mg. of Protein in Sample
Analysed**—The relationship between optical density reading and protein
concentration depends on the specific set of cuvettes and on the spectro-
photometer used. It is therefore imperative that a curve be constructed
for each set of apparatus.

Several urine samples containing protein can be used for this purpose.
The urines are analyzed for total nitrogen and non-protein nitrogen by the
macro-Kjeldahl procedure of Hiller, Plazin, and Van Slyke (7), who found
that the best results of analyses of plasma protein were obtained when
mercury was used as catalyst. For determination of non-protein nitrogen
the protein is precipitated under the same conditions as for the biuret
method; equal volumes of urine and 10 per cent trichloroacetic acid are
mixed, let stand 10 minutes or longer, then centrifuged. Aliquot por-
tions of the supernatant solution are taken for analysis.

The calculation for the protein content of the urines is

\[
\text{Protein per 100 ml.} = 6.25 \left[ \left( \text{total N per 100 ml.} \right) - \left( \text{non-protein N per 100 ml.} \right) \right]
\]

Of each urine analyzed three or four samples of various size are chosen,
containing amounts of protein ranging from about 3 to 15 mg. These are
analyzed in triplicate by the biuret method, and the \( D_P \) values calculated
by Equation 1 are plotted against the mg. of protein in the samples taken
for analysis. A straight line curve can thus be constructed for calculations.
Such a curve was checked at frequent intervals and was found to remain constant over a period of 6 months.

**EXPERIMENTAL**

*Choice of Wave-Length for Reading Biuret Color*—Optical densities of the biuret color prepared from a sample of urine protein were read at various wave-lengths in the Coleman junior photoelectric spectrophotometer. A curve constructed from these data showed a maximum optical density at wave-lengths between 550 and 570 m\(\mu\), and was almost identical with the curve of Robinson and Hogden (6). A wave-length of 560 m\(\mu\) was chosen.

*Reproducibility of Results by Biuret Method*—Urine samples were chosen which contained varying amounts of protein, between 3 and 19 mg.

**Table I**

<table>
<thead>
<tr>
<th>Urine sample (ml.)</th>
<th>Protein found in sample; mean of 20 determinations (mg.)</th>
<th>Standard deviation from mean (mg.)</th>
<th>per cent of mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>3.105</td>
<td>±0.065</td>
<td>±2.09</td>
</tr>
<tr>
<td>1</td>
<td>6.328</td>
<td>±0.077</td>
<td>±1.22</td>
</tr>
<tr>
<td>2</td>
<td>12.798</td>
<td>±0.126</td>
<td>±0.99</td>
</tr>
<tr>
<td>3</td>
<td>19.030</td>
<td>±0.171</td>
<td>±0.90</td>
</tr>
</tbody>
</table>

Twenty analyses were performed on each sample chosen. The results are shown in Table I.

*Stability of Biuret Color*—On six urine specimens which were analyzed by the biuret method, optical density readings were taken within \(\frac{1}{2}\) hour after development of the biuret color. The cuvettes were stoppered and let stand at room temperature, 22°, in daylight but not in direct sunlight, and readings were repeated at 3 and 4 hours after color development. The results, recorded in Table II, show no appreciable change in optical density over a period of 4 hours.

*Effect of Age of 3 Per Cent Sodium Hydroxide Solution on Results Obtained by Biuret Method*—3 per cent sodium hydroxide solutions were protected from atmospheric CO\(_2\) and were used after standing in glass containers for intervals up to 90 days, as indicated in Table III. For the analyses a solution of serum albumin containing 3.9 mg. of protein per ml. was used. Samples of 1 ml. were analyzed in triplicate for each sodium hydroxide solution. All the analyses in Table III were done on the same day. Reagent blanks were determined for each solution used. The sodium hydrox-
ide solutions which were kept for 42 to 90 days all gave higher reagent blanks. The results shown in Table III indicate that the 3 per cent so-

Table II

<table>
<thead>
<tr>
<th>Urine No.</th>
<th>Optical density readings; time after biuret color development</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within 1/2 hr.</td>
</tr>
<tr>
<td>Water</td>
<td>0.050</td>
</tr>
<tr>
<td>Reagent blanks</td>
<td>0.081</td>
</tr>
<tr>
<td>152</td>
<td>0.441</td>
</tr>
<tr>
<td>153</td>
<td>0.391</td>
</tr>
<tr>
<td>154</td>
<td>0.233</td>
</tr>
<tr>
<td>155</td>
<td>0.349</td>
</tr>
<tr>
<td>156</td>
<td>0.300</td>
</tr>
<tr>
<td>164</td>
<td>0.590</td>
</tr>
</tbody>
</table>

Table III

<table>
<thead>
<tr>
<th>Age of NaOH</th>
<th>Protein found in sample</th>
<th>Deviation from protein found with freshly made NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>days</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>0</td>
<td>3.9</td>
<td>-0.1</td>
</tr>
<tr>
<td>1</td>
<td>3.8</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>3.9</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>3.9</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>3.8</td>
<td>-0.1</td>
</tr>
<tr>
<td>5</td>
<td>4.0</td>
<td>+0.1</td>
</tr>
<tr>
<td>6</td>
<td>3.7</td>
<td>-0.2</td>
</tr>
<tr>
<td>7</td>
<td>4.0</td>
<td>+0.1</td>
</tr>
<tr>
<td>13</td>
<td>3.8</td>
<td>-0.1</td>
</tr>
<tr>
<td>17</td>
<td>3.7</td>
<td>-0.2</td>
</tr>
<tr>
<td>19</td>
<td>3.7</td>
<td>-0.2</td>
</tr>
<tr>
<td>42</td>
<td>3.6</td>
<td>-0.3</td>
</tr>
<tr>
<td>49</td>
<td>3.7</td>
<td>-0.2</td>
</tr>
<tr>
<td>60</td>
<td>3.6</td>
<td>-0.3</td>
</tr>
<tr>
<td>90</td>
<td>3.7</td>
<td>-0.2</td>
</tr>
</tbody>
</table>

dium hydroxide solution may be used for a period of 2 weeks without appreciably affecting the accuracy of the procedure.

Correction for Urinary Pigments in Biuret Method—When protein is precipitated from a highly concentrated urine, some of the pigment is carried down with the precipitate. When such a precipitate is dissolved in alkali,
the solution is sufficiently colored to give a slight optical density reading at the wave-length at which the biuret color is read.

To determine the amount of error introduced into the biuret method by this color, a serum albumin solution containing 10.6 mg. of protein per ml. was analyzed before and after addition of a dilute normal, straw-colored urine and of a concentrated normal, deeply colored urine. The results were calculated with and without the correction for the color of the protein solution (optical density readings \( D_1 \) in the method). Column 4 of Table IV shows that with varying amounts of protein in the sample the corrected readings indicate, within the limits of error of the method, the amount of protein present. When the calculations are made without correcting for pigment (Columns 5 and 6, Table IV), the results are increased to a degree beyond the experimental error of the method. This error becomes relatively greater when smaller amounts of protein are analyzed.

The dye, Evans blue (T-1824), when injected into patients with proteinuria for the determination of plasma volume, is excreted bound to the urinary protein. A number of urines containing T-1824 were analyzed for protein by macro-Kjeldahl determination and by the biuret method. Results by the biuret method were calculated with and without the correction for dye (included in the \( D_1 \) readings). Table V shows that results calculated with the correction check with the results by Kjeldahl analysis within the limits of error, whereas the uncorrected photometric results are higher.

**Table IV**

<table>
<thead>
<tr>
<th>Normal urine</th>
<th>Serum albumin solution</th>
<th>Protein in sample calculated from added albumin</th>
<th>Protein in sample by biuret method</th>
<th>Protein uncorrected, in per cent of corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>Sample taken (ml.)</td>
<td></td>
<td>Corrected for pigment (mg.)</td>
<td>Uncorrected for pigment (mg.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(4)</td>
<td>(5)</td>
</tr>
<tr>
<td>Dark</td>
<td>3</td>
<td>0.25</td>
<td>10.6</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.65</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.3</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10.6</td>
<td>10.6</td>
</tr>
<tr>
<td>Light</td>
<td>4</td>
<td>1</td>
<td>10.6</td>
<td>10.6</td>
</tr>
</tbody>
</table>

Comparison of Determinations of Urine Protein by Macro-Kjeldahl, by Biuret Method, and by Sedimentation Method of Shevky and Stafford (8) As Modified by MacKay (9)—Twelve urines were analyzed for protein (1) by
the macro-Kjeldahl procedure described for construction of the curve for conversion of optical density to protein content, (2) by the biuret method, and (3) by the sedimentation method of Shevky and Stafford (8) as modi-

\[\text{TABLE V}\]

**Analyses of Urine Containing Evans Blue (T-1884) by Biuret Method**

<table>
<thead>
<tr>
<th>Urine specimen</th>
<th>Protein per liter</th>
<th>Biuret method</th>
<th>Biuret, per cent of Kjeldahl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm.</td>
<td>Corrected for dye</td>
<td>Uncorrected for dye</td>
</tr>
<tr>
<td>B-251</td>
<td>13.8</td>
<td>14.0</td>
<td>14.5</td>
</tr>
<tr>
<td>B-147</td>
<td>33.0</td>
<td>33.0</td>
<td>33.5</td>
</tr>
<tr>
<td>Se</td>
<td>10.5</td>
<td>10.4</td>
<td>10.8</td>
</tr>
<tr>
<td>C</td>
<td>17.5</td>
<td>18.0</td>
<td>18.2</td>
</tr>
<tr>
<td>M-173</td>
<td>25.2</td>
<td>28.0</td>
<td>28.6</td>
</tr>
<tr>
<td>B-171</td>
<td>41.5</td>
<td>41.0</td>
<td>42.0</td>
</tr>
<tr>
<td>M-177</td>
<td>20.7</td>
<td>21.0</td>
<td>21.4</td>
</tr>
</tbody>
</table>

\[\text{TABLE VI}\]

**Comparison of Determinations of Urine Proteins by Macro-Kjeldahl, by Biuret Method, and by Method of Shevky and Stafford**

<table>
<thead>
<tr>
<th>Urine specimen</th>
<th>Protein per liter</th>
<th>Per cent deviation from Kjeldahl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm.</td>
<td>Biuret method</td>
</tr>
<tr>
<td>B-147</td>
<td>33.0</td>
<td>33.0</td>
</tr>
<tr>
<td>B-251</td>
<td>13.8</td>
<td>14.0</td>
</tr>
<tr>
<td>B-171</td>
<td>41.5</td>
<td>41.0</td>
</tr>
<tr>
<td>M-173</td>
<td>28.2</td>
<td>28.0</td>
</tr>
<tr>
<td>N-177</td>
<td>20.7</td>
<td>21.0</td>
</tr>
<tr>
<td>R</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>W</td>
<td>6.3</td>
<td>6.3</td>
</tr>
<tr>
<td>O'F</td>
<td>25.6</td>
<td>25.2</td>
</tr>
<tr>
<td>Sp</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Rα</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>C</td>
<td>17.5</td>
<td>18.0</td>
</tr>
<tr>
<td>Se</td>
<td>10.5</td>
<td>10.4</td>
</tr>
</tbody>
</table>

fied by MacKay (9). In Table VI the results by the biuret method are seen to check with those by macro-Kjeldahl analysis within the limits of error. The results by the sedimentation method show deviations from the Kjeldahl, ranging from -3.5 to +51.8 per cent.

*Procedures for Frequent Checking of Calculation Curve—To find a pro-
procedure for checking the calculation curve at frequent intervals a search
was made for compounds which either give a biuret reaction or which give
an optical density curve similar to that of the biuret color.

Chromic ammonium sulfate, $\text{Cr}_2(\text{SO}_4)_3(\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$, was chosen
because in aqueous solution its transmission curve is near that of the biuret
color, with a minimum transmission between wave-lengths of 570 and 590
$\mu\text{m}$. Aqueous solutions of 0.5 and 0.25 per cent were prepared. These
solutions showed a color instability when first made up. A daily increase
was found in the optical density readings until the 4th day, but thereafter
the readings remained unchanged when made at intervals up to 1 year.
The optical density readings of the two solutions were located on the cal-
culation curve and checked at intervals. Two preparations of chromic
ammonium sulfate, one Baker’s “analyzed,” one a C.P. product obtained
from the Fisher Scientific Company, gave the same results.

SUMMARY

A biuret method for the determination of urinary protein is described
in which the entire procedure is carried out in a single test-tube which
serves as a cuvette for the photoelectric spectrophotometer. Results
agree with those by an accurate macro-Kjeldahl method (digestion with
mercury catalyst), the standard deviation from the Kjeldahl values be-
ing of the order of $\pm 1\%$ per cent when the urine samples contained 6 to
19 mg. of protein.

BIBLIOGRAPHY

chemistry; Methods, Baltimore, 682 (1932).
DETERMINATION OF PROTEIN IN URINE BY THE BIURET METHOD
Alma Hiller, Roger L. Greif, William W. Beckman and With the technical assistance of John Plazin


Access the most updated version of this article at http://www.jbc.org/content/176/3/1421.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/176/3/1421.citation.full.html#ref-list-1