New Biuret Reagent for the Determination of Proteins in Cerebrospinal Fluid

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The biuret reaction has become an important analytic procedure for rapid and accurate determinations of protein concentrations in biologic fluids. Since the initial reports of Riegler (1), Autenrieth and Mink (2), and Autenrieth (3), who showed that proteins react with alkaline copper solutions to yield a violet-blue color, many modifications have appeared to improve the stability of alkaline copper solutions and to define optimal conditions for the reaction. These studies have resulted in biuret procedures for serum protein that yield results equivalent to those obtained by micro-Kjeldahl analysis (4–9).

In applying the biuret procedure (10) to cerebrospinal fluid protein determinations, we observed that some undiluted fluids become sufficiently turbid to invalidate the analyses. Initial experiments indicated that the turbidity could be prevented by the addition of a chelating agent, disodium ethylenediamine tetraacetate (EDTA) to the biuret reagent. The results of this study and the development of a new copper reagent form the basis of this report.

MATERIALS AND METHODS

Biuret Reagent

The reagent described by Fister (10) was used during the initial phases of this study.

Micro-Kjeldahl Determinations

These were performed by the procedure of Pregl with selenium oxychloride as catalyst. The analyses were corrected for nonprotein nitrogen determined on trichloroacetic acid filtrates.
Standard Protein

Stock Solution: Human serum albumin (American Red Cross) is diluted with physiologic saline to contain approximately 50 mg. per ml. as determined by Kjeldahl analysis. Albumin standard solutions, preserved with chloroform or thymol, are stable at 4° for about 1 month. Solutions kept frozen at -20° are stable indefinitely.

Working Solution: Dilute 5 ml. albumin stock solution to 100 ml. with physiologic saline. This solution is stable for about 1 month in the refrigerator when preserved with chloroform. A slight turbidity may develop which does not interfere.

Analytic Procedure

Place 2 ml. saline, albumin working standard, and cerebrospinal fluid respectively in 3 test tubes. Add 3 ml. biuret reagent and mix by inversion. After 20-30 minutes at room temperature determine the absorbence in a suitable photoelectric colorimeter adjusted to 0 absorbence with the saline-biuret reagent blank.

The Klett-Summerson and Sheard Sanford Colorimeters with filters transmitting at 540 or 550 mhydration have proved to be satisfactory in this laboratory.

EXPERIMENTAL

Effect of EDTA on Development of Turbidity

The inclusion of graded amounts of EDTA to saline-biuret blank solutions results in a linear decrease in absorbence until a constant minimal absorbence is obtained at concentrations of EDTA greater than 15 mg. per cuvette (Fig. 1). In the presence of cerebrospinal fluid, the decrease in absorbence parallels blank absorbence when the EDTA concentration is greater than 5 mg. per cuvette. However, in the absence of EDTA or at concentrations of EDTA lower than 5 mg. per cuvette, the absorbence of solutions containing cerebrospinal fluid is disproportionately high and does not parallel the absorbence of the blank solutions. In the absence of EDTA, many of these solutions appear visibly turbid. The optimal quantity of EDTA was, therefore, arbitrarily selected as 18 mg. per cuvette.

1 Commercially available standardized human serum albumin (Pro-Sol) distributed by Standard Scientific Supply Corporation, New York, N. Y., has been found to be a satisfactory standard.
Fig. 1. The effect of disodium ethylenediamine tetraacetic acid (EDTA) on absorbence. Curve A, cerebrospinal fluid. Curve B, blank solutions.

Cause of Turbidity

The alkaline earth elements calcium and magnesium appeared to be the most likely substances present in body fluids to cause turbidity under the alkaline conditions of the biuret reaction.

In order to test this assumption more completely, known quantities of calcium were added to a series of albumin standards. The protein solutions were then tested by the analytic procedure and the absorbences obtained with the biuret reagent (10) compared with that of the reagent containing an optimal amount of EDTA as determined above.

It is apparent that small amounts of calcium result in increased absorbence, which is effectively prevented by the addition of EDTA (Fig. 2).

Sensitivity of Reagents

Standard curves prepared with the reagent alone or containing optimal quantities of EDTA are shown in Fig. 3. It can be seen that the presence of EDTA does not alter the proportional development of color. Although EDTA decreases the sensitivity of the reaction, the range of concentration over which the response is linear is not appreciably altered. The range is sufficient to include the majority of protein concentrations usually encountered in cerebrospinal fluids.

*Dilute albumin standards were found to be free of calcium.*
Fig. 2. The effect of calcium concentration on the biuret reaction. Curve A, biuret reagent alone. Curve B, biuret reagent containing the optimal amount of EDTA.

Fig. 3. Relationship between protein concentration and absorbance. Curve A, biuret reagent alone. Curve B, biuret reagent containing the optimal amount of EDTA.
Absorption Spectra

Absorption spectra of albumin solutions treated with biuret reagent alone or with biuret reagent containing an optimal amount of EDTA were obtained with a Beckman DU spectrophotometer. The shape of the spectra were similar with both reagents, exhibiting a broad absorption curve with peak absorbences at 550 mμ. Maximum absorbence, however, was depressed in the presence of EDTA as was to be expected from the decreased sensitivity of the reaction.

New Biuret Reagent

With the above information available, a new biuret reagent was prepared as follows:

Dissolve 1.50 Gm. Cu SO₄·5H₂O in about 500 ml. water. Add 6.0 Gm. disodium ethylenediamine tetracetate (EDTA) and 1.0 Gm. KI and dissolve. Add 300 ml. 2.50 N NaOH, mix well, and dilute to 1 L. The reagent is stable when stored at room temperature in polyethylene containers.

RESULTS

A series of protein determinations was carried out on cerebrospinal fluid with various biuret reagents and these were compared with values obtained by micro-Kjeldahl analysis. In order to obtain sufficient material for all comparative analyses, cerebrospinal fluids obtained by cisternal puncture at autopsy within 3 hours after death were used. These samples were preserved with chloroform and refrigerated until all analyses could be completed.

Table 1. Comparison of Protein Concentration in Cerebrospinal Fluids Obtained by Various Procedures

<table>
<thead>
<tr>
<th>Sample</th>
<th>Kjeldahl</th>
<th>Fister (10)</th>
<th>Fister (10) + added EDTA</th>
<th>New</th>
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<tr>
<td>1</td>
<td>37</td>
<td>61</td>
<td>40</td>
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<tr>
<td>9</td>
<td>48</td>
<td>73</td>
<td>57</td>
<td>54</td>
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<tr>
<td>Average (mg./100 ml.)</td>
<td>90</td>
<td>101</td>
<td>92</td>
<td>89</td>
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</table>
The values obtained by the micro-Kjeldahl method and the new biuret reagent are in good agreement as shown in Table 1. On the other hand, it is evident that the original biuret reagent (10) yields significantly greater values than those obtained by either the micro-Kjeldahl or the new reagent. It is also apparent that the addition of EDTA to the original biuret reagent yields values comparable to those obtained by Kjeldahl analysis or by the new reagent.

**DISCUSSION**

The biuret reaction for the determination of proteins of biologic interest would appear to be the method of choice when all factors of accuracy and time are considered. However, the necessary precision and accuracy of the method can only be obtained by rigid control of conditions for the reaction, as is evident from the many modifications which have appeared in the literature. The present modification appears to be most useful when protein concentrations of less than 300 mg. per 100 ml. are to be determined. At such low protein concentrations a small degree of turbidity may yield values that are significantly too high. The use of the new EDTA reagent offers no real advantage for the determination of serum proteins since dilution of these materials prior to analysis decreases the calcium concentration to levels which do not seriously interfere. However, the lower blank absorbence obtained with the new reagent may be desirable. When applied to determinations of serum proteins the new reagent yields results comparable to Kjeldahl analysis.

Occasional observations have been made on the reduction of the copper salt of the reagent in the presence of biologic fluids, as has previously been observed by Weichselbaum (8). The reduction of copper, to yield a pale green-to-orange colloidal suspension, occurred only when the biuret reaction tubes had remained at room temperature for over 40 minutes. A second determination on the same material failed to result in reduction of the copper. On one occasion during the past 12 months, reduction of the reagent occurred with the standard albumin solution and again the reduction was not apparent until the solution had stood for 40–60 minutes. Since the reduction is not consistent or reproducible, we believe that the effect may be due to errors in the cleansing of glassware, pipets, etc., but we have not investigated the effect further.

**SUMMARY**

A new biuret reagent is described which contains disodium ethylenediamine tetraacetate as the copper stabilizing agent. The reagent effec-
tively prevents the formation of cloudiness in determinations of cerebrospinal fluid due, in part, to the precipitation of calcium in alkaline solution. Results obtained with the new reagent compare favorably with those obtained by Kjeldahl analysis.

REFERENCES

3. Autenrieth, W., München. med. Wchnschr. 64, 241 (1917).