THE BIURET REACTION OF PROTEINS IN THE PRESENCE OF ETHYLENE GLYCOL

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The biuret method offers some advantages as a simple, rapid photometric method for the determination of proteins. In the methods which have been proposed, precipitation of cupric hydroxide gives rise to certain difficulties (1, 2). It occurred to us that the precipitates might be avoided by the addition of a reagent which would form a compound with copper which was soluble in alkali, but of such a nature that the copper could still react with protein. Ethylene glycol has been examined and found suitable, and with the use of this substance it is possible to prepare a single, alkaline copper solution which may be added to the protein for the development of the biuret color.

Absorption Spectra—The absorption spectrum of the complex of cupric ion and ethylene glycol is shown in Curve B of Fig. 1. The concentration of ethylene glycol was 8.0 ml. per 100 ml., and that of sodium hydroxide was 2.5 gm. per 100 ml. (hereafter concentrations expressed in these terms will be referred to as per cent). The values of \( E_{\%} \) are calculated in terms of the total copper concentration, and were obtained with solutions in which the concentration ranged from 0.001 to 0.12 per cent. One maximum is observed at 630 \( \mu \) (\( E_{\%} = 4.75 \)) and another at 297 \( \mu \) (\( E_{\%} = 64.3 \)), with a minimum in the region from 380 to 430 \( \mu \).

The absorption spectrum of the egg albumin-copper complex is shown in Curve A of Fig. 1, and was determined by subtracting the blank due to the copper and ethylene glycol reagent and that due to the protein in the ultraviolet region. The values of \( E_{\%} \) are calculated in terms of the protein concentration, and maxima are found at 545 \( \mu \) (\( E_{\%} = 2.58 \)) and at 360 \( \mu \) (\( E_{\%} = 11.3 \)), with a minimum at 430 \( \mu \). The position of the maximum reported by Sizer (3) is 552 \( \mu \). The position of the maximum found for mixed serum proteins in the present study is

\[ 1 \text{ The notation is that commonly used, according to which } \log \frac{I_0}{I} = E_{\%} \text{, } cd, \text{ where } I_0/I \text{ represents the original intensity divided by the intensity of the transmitted light, } c \text{ is the concentration in gm. per } 100 \text{ ml., and } d \text{ is the depth of the solution in cm.} \]
also essentially the same as for egg albumin, whereas the absorption
maximum reported by Robinson and Hogden (2) is 560 m

The displacement of the maximum by protein makes it possible to correct
the values obtained in the visible region for the absorption due to copper
not combined with protein. The absorption in alkaline mixtures con-
taining copper, ethylene glycol, and protein at 750 m may be taken as
due to the copper-ethylene glycol complex. \( E_{1 \text{ cm}}^{\%} \) is 2.0 at this wave-
length. At 545 m, the value of \( E_{1 \text{ cm}}^{\%} \) for copper-ethylene glycol is
3.1. The value for the absorption due to the protein-copper complex
at 545 m would be obtained by subtracting 1.57 times the optical density

Fig. 1. Curve A, the absorption spectrum of the egg albumin-Cu complex. Values
for the scale of extinction are on the right, and are calculated in terms of the protein
concentration. The values above 430 m were obtained with 0.134 and 0.235 per cent
protein. The upper branch of the curve below 430 m was obtained with 0.0134 per
cent and the lower branch with 0.0335 per cent protein. Curve B, the absorption
spectrum of copper sulfate and ethylene glycol in alkaline solution. The extinction
is calculated in terms of the copper concentration, and is referred to the scale on the
left.

at 750 m from the optical density at 545 m. Similar corrections may
be made at other wave-lengths.

When such a correction is made for the absorption due to the copper
which is not combined with protein, the relationship between optical
density and protein concentration is still not strictly linear. In Table 1,
the results obtained with egg albumin, and absorption at 530 and 750 m,
are given (a wave-length of 545 m would have been somewhat more suitable
than 530 m). The values of \( E_{1 \text{ cm}}^{\%} \) are seen to decrease somewhat as
the concentration increases. This may actually be a deviation from
Beer's law, or may depend, at least in part, upon an inadequate correction
when the blank amounts to as much as 50 per cent of the total reading.
The curve for the absorption of the protein-copper complex given in Fig.
1 would therefore be somewhat different for different concentrations. The values used were the averages for concentrations of 0.134 and 0.235 per cent protein in the region from 350 to 750 m. The difference between the values of $E_{1/\text{cm.}}^{1\%}$ is in the neighborhood of 3 per cent at these two protein concentrations.

In the spectral region below 350 m, the correction for uncombined copper cannot be so readily made, since both the copper and protein concentrations are diminished to bring the readings into a suitable range. The values which have been plotted were obtained by subtracting the optical densities of alkaline copper-ethylene glycol and of alkaline protein solutions of the concentrations used in the biuret reaction. The upper branch of Curve A was obtained with 0.0134 per cent egg albumin and the lower branch with 0.0335 per cent egg albumin.

**Effect of Copper Concentration**—The concentration of copper needed to produce maximum color development is dependent upon the concentration of protein. In Fig. 2 the optical density is shown as a function of the copper concentration for three concentrations of human plasma protein. It will be seen that the absorption is independent of protein concentration when the copper concentration is low. This indicates that all of the copper is bound to protein, and permits us to calculate the value of $E_{1/\text{cm.}}^{1\%}$ referred to the concentration of copper in the protein-copper complex. This value is $18.8$ at 545 m. The concentration of copper needed to give an excess for plasma protein concentrations up to about 0.15 per cent is approximately 0.05 per cent.

### Table I

**Relation between Egg Albumin Concentration and Extinction Coefficient or Colorimeter Readings**

<table>
<thead>
<tr>
<th>Protein (gm. per 100 ml.)</th>
<th>Optical density at 1 cm.</th>
<th>$E_{1/\text{cm.}}^{1%}$ at 530 m</th>
<th>Colorimeter reading, Filter $54$</th>
<th>Increase in colorimeter reading for 1 per cent protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>530 m</td>
<td>750 m</td>
<td>Due to biuret color, 530 m</td>
<td>530 m</td>
</tr>
<tr>
<td>0.004</td>
<td>0.210</td>
<td>0.063</td>
<td>0.093</td>
<td>2.77</td>
</tr>
<tr>
<td>0.067</td>
<td>0.287</td>
<td>0.079</td>
<td>0.175</td>
<td>2.61</td>
</tr>
<tr>
<td>0.101</td>
<td>0.373</td>
<td>0.078</td>
<td>0.263</td>
<td>2.61</td>
</tr>
<tr>
<td>0.134</td>
<td>0.445</td>
<td>0.074</td>
<td>0.340</td>
<td>2.54</td>
</tr>
<tr>
<td>0.168</td>
<td>0.522</td>
<td>0.073</td>
<td>0.419</td>
<td>2.55</td>
</tr>
<tr>
<td>0.201</td>
<td>0.598</td>
<td>0.068</td>
<td>0.502</td>
<td>2.56</td>
</tr>
<tr>
<td>0.235</td>
<td>0.671</td>
<td>0.065</td>
<td>0.579</td>
<td>2.47</td>
</tr>
<tr>
<td>0.269</td>
<td>0.735</td>
<td>0.061</td>
<td>0.649</td>
<td>2.41</td>
</tr>
</tbody>
</table>
Stability of Protein-Copper Complex—As in the studies previously reported (2), we have found the color to be stable, but that it requires some time to develop fully. When egg albumin is used, about 90 minutes must be allowed for development of the color. After this time, the color is constant for at least 20 hours. The situation is complicated in the case of plasma, since a turbidity develops in about 1 hour. As this turbidity also develops in plasma treated with the same concentration of alkali, there does not seem to be any way of entirely eliminating the changes with time in the case of plasma, and a comparison with the results obtained on egg albumin is correspondingly inexact.

Use of Modified Biuret Method in Determination of Protein Concentrations—The relation between protein concentration and optical density has already been noted in the case of egg albumin. In those experiments, and in the ones to be presented, the final concentration of copper was 0.043 per cent, the ethylene glycol concentration was 8 volumes per 100, and the sodium hydroxide concentration was 4.3 per cent. Table I also gives the results obtained with solutions of egg albumin with the Klett photoelectric colorimeter. The readings were all made at 2 hours. Table II gives the values for human plasma and trichloroacetic acid precipitates of plasma, for which the readings were made at 30 to 40 minutes. During the period between 25 and 45 minutes, the changes in readings are relatively small, and reasonably consistent values can be obtained at definite times.

It will be seen that the optical density is not strictly proportional to the protein concentration. $E_{1\text{cm}}%$ decreases somewhat with increasing protein concentration, although the correction for the decreased copper
available for combination with ethylene glycol has been made. This would again indicate some deviation from Beer's law. It will also be noted that the values for plasma protein are about 15 per cent lower than those for egg albumin at equal concentrations, although this may be in part due to incomplete development of color in the case of plasma. The albumin and globulin fractions of plasma have not been compared in this investigation, but it does not seem likely that they will differ more in this method than in previous methods (2). At any rate, no obvious difficulties are encountered when this procedure is applied to the filtrate from the globulin precipitation by the Howe method, and sodium sulfate does not alter the color intensity. Even ammonium ion has relatively little effect, and may be neglected when the ammonia nitrogen does not exceed the protein nitrogen.

Inspection of the absorption curves in Fig. 1 shows that the sensitivity of the method might be increased by using the near ultraviolet. For this purpose, we have chosen to use a wave-length somewhat longer than that of the region of maximum absorption, since the correction due to residual copper will be reduced more than the absorption due to the protein-copper complex. Corrections due to other absorbing materials which might be present in solutions other than those of purified proteins will also generally be smaller. The results of some measurements made on egg albumin at 320 mμ are given in Table III. The copper concentration was decreased to 0.0047 per cent and the ethylene glycol to 4 volumes per 100 for these measurements. The sensitivity of the method is increased

<table>
<thead>
<tr>
<th>Protein (mg. per 100 ml.)</th>
<th>1 cm., at 530 mμ</th>
<th>Increase in colorimeter reading for 1 per cent protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E_{1%}^1 cm.</td>
<td>Plasma</td>
</tr>
<tr>
<td>0.050</td>
<td>2.28</td>
<td>2.15</td>
</tr>
<tr>
<td>0.050</td>
<td>2.11</td>
<td></td>
</tr>
<tr>
<td>0.099</td>
<td>2.22</td>
<td>2.07</td>
</tr>
<tr>
<td>0.100</td>
<td>2.24</td>
<td></td>
</tr>
<tr>
<td>0.149</td>
<td>2.08</td>
<td></td>
</tr>
<tr>
<td>0.150</td>
<td>2.21</td>
<td></td>
</tr>
<tr>
<td>0.201</td>
<td>2.15</td>
<td></td>
</tr>
<tr>
<td>0.251</td>
<td>2.11</td>
<td></td>
</tr>
</tbody>
</table>
between 3 and 4 times over that in the visible region. The same increase in sensitivity may be achieved in the case of plasma, although this advantage will be somewhat offset by the greater influence of turbidity at these wave-lengths.

EXPERIMENTAL

Measurements of Optical Density—The instrument used was a Beckman quartz photoelectric spectrophotometer. The instrument had been recently rechecked by the manufacturer, and readings have been made on potassium chromate at regular intervals. The wave-length settings have always checked to within 1 μm, and the density readings to within 1 per cent. The tungsten lamp was used as a light source throughout, and the band width varied from about 2 μm in the visible to about 4 μm at the shortest wave-lengths.

<table>
<thead>
<tr>
<th>Protein mg. per 100 ml.</th>
<th>Optical density, 1 cm., 320 μm</th>
<th>Protein-Cu E1% 1 cm., 320 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Protein in alkali</td>
</tr>
<tr>
<td>0</td>
<td>0.091</td>
<td>0.005</td>
</tr>
<tr>
<td>6.7</td>
<td>0.162</td>
<td>0.011</td>
</tr>
<tr>
<td>13.4</td>
<td>0.225</td>
<td>0.016</td>
</tr>
<tr>
<td>20.1</td>
<td>0.286</td>
<td>0.022</td>
</tr>
<tr>
<td>26.8</td>
<td>0.348</td>
<td>0.027</td>
</tr>
<tr>
<td>33.5</td>
<td>0.413</td>
<td>0.027</td>
</tr>
</tbody>
</table>

The photoelectric colorimeter was a Klett-Summerson, and was used with the manufacturer's Filter 54, having a transmission centered at 540 μm.

Protein Solutions—The egg albumin was prepared from frozen egg white, by precipitation with ammonium sulfate. The initial precipitate of egg albumin was not crystalline, but the material was obtained crystalline in the subsequent three precipitations. The ammonium sulfate was removed by dialysis against running water for 48 hours and against 20 volumes of distilled water for 24 hours. The residual ammonia nitrogen was 0.25 per cent. The concentration of protein was determined by drying to constant weight at 105°.

The human plasma was a commercial product, supplied through the courtesy of the Hyland Laboratories. The protein concentration was obtained from the dry weight of the heat-coagulated material after two washings with 50 per cent alcohol, one with 95 per cent alcohol, and one
with ether. These values were checked by obtaining the nitrogen precipitated by 7.5 per cent trichloroacetic acid and multiplying by 6.25.

Combined Copper Reagent—In some of the earlier experiments, it was noted that solutions containing sodium hydroxide, copper sulfate, and ethylene glycol developed a marked turbidity and eventually deposited a red precipitate. This process was accelerated by light or heat, and was also observed in samples containing plasma. It seems likely that reducing impurities were present in the ethylene glycol, and that the high concentrations of glucose in the commercial plasma were responsible for additional reduction of the copper. The effect of reduction in the blanks can be eliminated by mixing the combined reagent and heating it to complete the reaction. A satisfactory reagent for work in the visible region may be prepared by mixing 100 ml. of ethylene glycol, 40 ml. of 60 per cent NaOH, and 50 ml. of 4 per cent CuSO₄·5H₂O. This solution is diluted to about 400 ml. and heated until precipitation is complete. After the cuprous hydroxide is filtered off, sufficient sodium hydroxide is added to make the final concentration 10 to 11 per cent. This reagent appears to be stable for at least several months. We have used 10 ml. in a total volume of 25 ml. for protein determinations.

It is obvious that reagents of a different composition may prove more useful in special cases. For the work in the ultraviolet region, a dilute copper reagent was made in a similar way, except that the final ethylene glycol concentration was decreased to 1 volume in 10, and only enough copper was added to give a very faint blue color after the mixture had been heated. The final concentration of sodium hydroxide was also lowered to 6.3 per cent. This reagent contained about 0.012 per cent copper, and was used in the amount of 10 ml. in a final volume of 25 ml.

In both of the reagents suggested, there is obviously a considerable excess of ethylene glycol, and additional copper can be added without precipitation. The concentration of alkali may also be varied over a rather wide range, and we have observed no differences in the absorption of the copper-protein complex with sodium hydroxide concentrations between 2 and 6 per cent in the final solutions. However, the higher concentrations of alkali increase the rate of development of turbidity in the plasma protein solutions. We feel that a final concentration of alkali between 2 and 4 per cent is most satisfactory. It is probably the safest procedure to place solutions containing plasma in the dark while the biuret color is allowed to develop. The reducing effect of glucose in plasma may thus be avoided.

DISCUSSION

The introduction of an additional component into the biuret reaction is obviously objectionable on the grounds that interactions of an unknown
nature may be expected, and the interpretation of the results may be further complicated. The finding that the value of $E_{1\%cm.}$ is somewhat dependent upon concentration, even at protein concentrations at which the copper is still in excess, is not in agreement with the observation of Robinson and Hogden (2) on filtered solutions which do not contain ethylene glycol. The influence of an inadequate correction for the absorption of copper not combined with protein may have to be considered as a factor in the present study. The difference between positions reported for the absorption maximum can be more readily interpreted in terms of the uncombined copper present in the solutions obtained with the usual procedure. Since the absorption of this copper is farther toward the red end of the spectrum, but overlaps the absorption of the protein-copper complex, the combined absorption curve will be broadened and shifted toward the longer wave-lengths.

In our own experience, we have found the use of ethylene glycol justified, and we believe that the possibility of avoiding precipitation of cupric hydroxide or elimination of the filtration outweighs the disadvantages. It may also be possible to apply this method to a more detailed study of the reaction between cupric ion and proteins and related compounds. The dissociation constant of the complex and the relation between the absorption in the near ultraviolet and visible regions would be of some interest.

The purchase of the spectrophotometer was made possible by a grant from the Rockefeller Foundation.

SUMMARY

1. The biuret reaction is modified by the introduction of ethylene glycol, which prevents the precipitation of cupric hydroxide. A single reagent may be employed, which gives optically clear solutions when mixed with protein.

2. Deviations from Beer's law, which may be as large as 10 per cent for a 4-fold change in protein concentration, have been observed under these conditions. Such deviations can readily be corrected for, and do not seriously impair the usefulness of the method.

3. The sensitivity of the biuret method may be further increased by making use of the greater absorption of the protein-copper complex in the region around 320 m$\mu$.

BIBLIOGRAPHY

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