The Assay of Urinary Peptides Using a Biuret Reagent

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It has been pointed out previously that the method for the assay of urinary peptides using the biuret reagent is a much simpler procedure than others in current use (1). However, a disturbing difficulty with this method has been the lack of reproducibility of the biuret reagent (1). This resulted in sporadic errors approximately 10 per cent and in a feeling of uncertainty regarding the method itself. A study was made, therefore, of the reproducibility of this reagent. In addition, considerably more information on interfering substances was gathered, and a study was made of the stability of peptides in urine specimens.

MATERIALS AND METHOD

Reagents

Sodium tungstate, 10 per cent aqueous solution of reagent grade or Folin-specified sodium tungstate (Na₂WO₄·2H₂O). The reagent is stored in an alkali-resistant container, such as a polyethylene bottle.

Sulfuric acid, 2/3 N, standardized.

Folin Permutit.

Biuret reagent: to about 250 ml. of water in a liter volumetric flask, the following substances are added, dissolving each before the next is added: 19.0 Gm. of sodium potassium tartrate (Rochelle salt), 20.0 Gm. NaOH, 5.50 Gm. CuSO₄·5H₂O, and 1.0 Gm. of KI. Water is added to the mark and mixed. The reagent is stored in an alkali-re-
sistant bottle and is stable until a black or reddish precipitate appears. It is advisable to construct a new standard curve each time new reagent is prepared.

**PROCEDURE**

The bottom of a collection vessel is completely covered with toluene. A 24-hour specimen of urine is collected and its volume measured to the nearest milliliter. After 20 ml. is poured into a small flask, 2.0 Gm. of Folin Permutit is added, and the mixture is shaken intermittently for 5 minutes. The sample is centrifuged, and 3.00 ml. of the supernatant fluid and 5.0 ml. of water are measured into a 15 ml. centrifuge tube. A blank is prepared by adding 8 ml. of water to a similar tube. Then 1.00 ml. of 10 per cent sodium tungstate is added and mixed. This is followed by 1.00 ml. of 2/3N H₂SO₄, after which the sample is shaken vigorously. After standing 5 minutes the samples are centrifuged if necessary. A 5.00 ml. aliquot of the clear supernatant fluid is measured into a 15 ml. centrifuge tube. This is followed by 5.0 ml. of the biuret reagent, after which the solutions are mixed. After being allowed to stand exactly 25 minutes, the samples are centrifuged for precisely 5 minutes at approximately 2500 rpm.

The absorbance of the clear solution is read in a photoelectric colorimeter at 405 mμ, using the blank for the zero setting. If the absorbance reads less than 0.05 optical density units on the Coleman Junior spectrophotometer, the determination must be repeated using 8 ml. of urine instead of the 3 ml. urine with 5 ml. water. (The Coleman Junior spectrophotometer and 19 × 150 mm. cuvets were used for all colorimetric measurements.) If this absorbance reading is greater than 0.3, the determination must be repeated using less than 3 ml. urine plus enough water to total 8 ml. of solution.

**Calculation**

From the standard curve the absorbance is converted to Gm. of peptide, as glutathione (GSH), per 100 ml. of diluted urine. Then,

\[
\text{Gm. per 100 ml.} \times \frac{8}{3} \times \frac{\text{(urine volume in ml.)}}{100} =
\]

Gm. of peptide (as GSH) per 24 hrs.

If other than 3 ml. Permutit-treated urine is used, this volume in ml. is substituted for the denominator 3 in the dilution factor \(\frac{8}{3}\).
**Standard Curve**

Some GSH is pulverized and dried to constant weight at 105-115°. Then 0.100 Gm. is dissolved in 25 ml. of water. This solution should be used within 24 hours. Volumes of GSH solution and water as shown in Table 1 are measured into small flasks. To each flask is added 1.00 ml. of 10 per cent sodium tungstate and the contents are mixed. (Mixing takes place after the addition of each successive reagent.) Then 1.00 ml. of 2/3 NH₄SO₄ is added. A 5.00 ml. aliquot of each solution is pipetted into test tubes, followed by 5.0 ml. of biuret reagent. After being left standing exactly 30 minutes the solutions are read in a colorimeter at 405 mμ using Standard No. 1 for the zero setting. Readings are plotted against Equivalent Diluted Urine Concentrations. The plot absorbance versus concentration made on arithmetic graph paper should be straight, with a slope of slightly greater than 1.0.

**EXPERIMENTAL BIURET REAGENT**

The optimum concentration of copper was determined in the following manner. Biuret reagents were prepared in which the concentration of CuSO₄·5H₂O varied from 0.1 to 0.8 per cent. In all of these reagents the ratio of NaOH to CuSO₄·5H₂O was constant at 3:1, the ratio of the Rochelle salt to CuSO₄·5H₂O was kept at 4:1, and the concentration of KI was 0.1 per cent. Test samples were 5 different concentrations of water solutions of GSH. In Fig. 1 are plotted the average calculated differences in optical density resulting from changes in copper concentration. It can be seen that the smallest effect of a change in copper concentration occurs in two areas, (1) 0.5 to 0.6 and (2) from 0.65 to 0.8. Since greater concentrations of copper lead to increased blanks and a reduced sensitivity, lower concentrations are to be preferred. Thus, a concentration of 0.55 per cent

<table>
<thead>
<tr>
<th>Table 1. Volumes for Standardization</th>
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<tr>
<td><strong>GSH solution</strong> (ml.)</td>
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<tr>
<td>------------------------</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>2.00</td>
</tr>
<tr>
<td>5.00</td>
</tr>
<tr>
<td>8.0</td>
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</table>
CuSO₄·5H₂O was adopted as optimal rather than the 0.50 per cent previously used (1).

The optimum concentrations of tartrate, sodium hydroxide, and potassium iodide were determined following the same general pattern described above for copper. However, as test substances urine from normal patients and from burned patients was used, as well as water solutions of GSH.

For tartrate, the lowest ratio of tartrate to copper in an area of minimum effect on results was chosen as optimal, since higher ratios tend to inhibit color development (2). Thus, the concentration of Rochelle salt was changed from 4.5 per cent to 1.9 per cent (equivalent to a change in ratio of from 9:1 to 3.5:1.)

The effect of NaOH on results was minimal over the range of 1.5 to 3.5 per cent. Since high concentrations of NaOH tend to make the reagent unstable (2) it was felt that a concentration of 2.0 per cent would be optimal (the concentration had been 0.5 per cent).

The KI concentration was varied from 0.05 to 0.55 per cent with essentially no effect on the results. We therefore chose a concentration of 0.1 per cent since it was the concentration used in this series of
experiments and because ‘‘... this amount of iodide has no detectable effect upon the rate, degree, or quality of biuret color production’’ (2) (the concentration of KI previously used was 0.5 per cent).

WAVE LENGTH OF CHOICE

Using a biuret reagent prepared as above, spectral-density (S-D) curves were obtained on 7 normal and 6 pathologic (burns) human urines. These curves fall into a pattern dependent on the peptide concentration regardless of the source’s being normal or pathologic. The peaks vary from the 410-415 mμ range for high peptide concentrations to 400 or possibly less at low concentrations. Illustrations of this shift are given in Fig. 2. For reasons to be mentioned later, optical density readings will normally be 0.3 or less, at which levels a wavelength close to 400 mμ is to be preferred. To avoid a setting at the extreme limit of our instrument, 405 mμ was felt to be the wavelength of choice. S-D curves were prepared on several sera (diluted with water) and compared with those obtained on urine. Curves on three sera were parallel, so just one was taken as illustrative (Fig. 3). This curve is completely different from the ones obtained on urine, from which it can be concluded that in urine we are indeed measuring something quite different from plasma proteins.

BEER-LAMBERT CORRELATION

As discussed elsewhere (1), a pure compound could not be used for the correlation of the color reaction with the Beer-Lambert law, since we are actually measuring various mixtures of peptides differing both in size and configuration. Consequently, step dilutions of a number of different urines were used as an expedient. Dilutions were made with water such that each urine was available as 20, 40, 60, 80, and 100 per cent of its original strength. The color obtained from urines on 5 normal adults yielded straight lines up to an optical density of 0.30. Of 5 urines from patients convalescing from burns, 3 yielded straight lines up to an optical density of 0.30 and 2 did not. Those that did not were in a low normal range. Typical curves are plotted in Fig. 4, where it can be seen that urines in the low normal range, from recuperating burn patients, may yield a reading falsely low by 10 to 20 per cent.

PRECISION

Over a period of approximately a month, 25 urines were assayed in duplicate to determine the precision of the method. The statistical
Fig. 3. A spectral-density curve on diluted serum when color is formed with the biuret reagent as described.

Fig. 2. Spectral-density curves from urines with different peptide concentrations.
Fig. 4. The correlation between step dilutions of urine and the absorbance of the color they form with the biuret reagent. The dash part of a line indicates a change in slope. The three curves illustrate the three types observed: (a) the upper curve illustrates the change in slope that occurs beyond an optical density reading of 0.30; (b) the center curve is an example of the linearity usually seen when all readings are below 0.30; and (c) the lower curve is an example of the nonlinearity sometimes found on urine from convalescing burn patients with a normal peptide excretion.

The procedure described by Mainland (3) was used with the following significant results, all in terms of optical density:

Standard deviation = ± 0.00296
Coefficient of variation = 1.68 per cent.

Since twice the standard deviation is 0.0059, readings using the Coleman instrument should not be estimated beyond the closest thousandth, and reports are not significant beyond 1 part in 100.

INTERFERING SUBSTANCES

Creatinine

A water solution of 250 mg. per 100 ml. yielded a reading of 0.005. Creatinine was then added to 7 urines (of unknown initial concentra-
tion) so as to increase the concentration by 240 mg. per 100 ml. The average increase in absorbance was 0.007.

**Uric Acid**

Addition of uric acid to urine showed no interference up to an added increment of 280 mg. per 100 ml. over the initial (unknown) concentration. The average increase in absorbance for 6 urines at this concentration was 0.002.

**Ammonia**

Water solutions of ammonia in the form of NH₄Cl showed no interference up to concentrations of 500 mg. per 100 ml. as NH₄⁺. At this level absorbances of 0.004 were obtained.

**Glycine**

Glycine was added to 4 normal urines (of unknown initial concentration) so as to increase the concentration by 50 mg. per 100 ml. The average increase in absorbance was 0.004. By increasing this increment to 100 mg. per 100 ml. the absorbance showed an overall average increase of 0.005. In one instance, in which glycine was added to urine from a burned patient, an orange precipitate formed on addition of the biuret reagent.

**Hippuric Acid**

Hippuric acid does not interfere at least up to concentrations of 280 mg. per 100 ml.

**Glucose**

Water solutions of glucose ranging from 20 to 1000 mg. per 100 ml. were prepared. A sample of the results of using our procedure on these solutions is given in Table 2. At concentrations approximating 3.0 Gm. per 100 ml., an orange precipitate may appear. More commonly, however, the biuret reagent will simply become light blue. At concentrations approximating 8.0 Gm. per 100 ml. a yellow precipitate will frequently form.

**Oxalic Acid**

The addition of 100 mg. per 100 ml. of oxalic acid to each of 4 normal urines (of unknown initial concentration) produced an average decrease in absorbance of 0.010.
Table 2. Effect of Glucose on Absorbance

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<thead>
<tr>
<th>Glucose (mg./100 cc.)</th>
<th>Optical density</th>
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<tbody>
<tr>
<td>20</td>
<td>0.002</td>
</tr>
<tr>
<td>60</td>
<td>0.004</td>
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<tr>
<td>200</td>
<td>0.008</td>
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<tr>
<td>600</td>
<td>0.032</td>
</tr>
<tr>
<td>1000</td>
<td>0.075</td>
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Folin Permuit

Excesses of permuit are to be avoided, since addition of 6 Gm. rather than 2 Gm. to 20 ml. of urine have sometimes increased and sometimes decreased readings significantly.

Stability of Sample

Aliquots of fresh, pooled, normal urines were handled in four ways: (1) kept at room temperature, (2) refrigerated, (3) covered with toluene, and (4) had 0.1 Gm. of thymol added per 100 ml. of sample. Peptide assays were made at intervals from 2 to 163 hours. An increase of approximately 15 per cent is found within 19 hours at room temperature. Refrigeration, toluene, and thymol are adequate preservatives up to 48 hours. By the sixth day (139 hours) all samples showed a substantial increase in peptide values.

Discussion

Refrigeration, toluene, or thymol have been described as adequate preservatives, and one of these should be used to avoid falsely elevated values, which will occur even during a 24-hour collection period. On the other hand, acetic acid, which is sometimes used as a urine preservative, almost completely inhibits color development (1). We have found toluene to be the most convenient preservative to use.

It is important to centrifuge after 25 minutes of color development whether or not turbidity is apparent, since slight suspensions are frequently masked by the biuret reagent peptide color.

We know from a comparison of the S-D curves on serum and urine that serum proteins that may have found their way into the urine are not a serious contaminant in this procedure. This is not unexpected since all but a very slight trace of these proteins would be precipitated by the tungstic acid. Nor is it surprising that the curves for proteins and peptides are different. It is well known that peptides of different lengths frequently yield different colors with the same biuret reagent.
Poroshin (4) and Plekhan (5) in the U.S.S.R. have reported studies using this property (among others) to analyze simple mixtures of peptides.

As previously mentioned (1), erratic, unusable standard curves were occasionally encountered with the biuret reagent used in the past, which necessitated remaking the reagent. The acceptable curves were reproducible but not quite straight (1). Using the reagent recommended in this report with readings made at 405 m, the standard curve is straight with a slope of approximately 1.04. Our experience to date has shown this slope to vary only within the limits of 1.02-1.05.

The evidence presented indicates that this method is ordinarily safe from interference by most of the nitrogenous and reducing substances tested. Before oxalic acid will interfere, it must be present in concentrations approximating 40 times normal; creatinine, uric acid, ammonia, and hippuric acid do not interfere until their concentration is near 5 times normal. However, assuming glycine to be representative of free amino acids, interference from this source (most likely due to chelate formations with copper) may be noted at concentrations approximating the upper limit of normal. Glucose may show some interference (presumably by reducing the cupric ion) even at normal levels.

Where interference does occur it is in the direction of an increase, except for oxalic acid, which in sufficient concentration results in a decrease. Excess Folin Permutit may result in a decrease or increase. Experiments on urea and Alconox (a laboratory detergent) previously reported (1) were not repeated. Those results showed that urea does not interfere until approximately 3 times the normal concentration is present, and Alconox will increase readings if present in amounts approximating 0.5 mg. or more.

**SUMMARY**

The components of a biuret reagent have been investigated to minimize variations in readings resulting from small variations in composition. When this biuret reagent is used for the assay of urinary peptides, readings should be made at 405 m. There is a limited but adequate straight-line relationship between concentration and absorbance on normal urine. Urine from convalescing burn patients containing normal concentrations of peptides may read 10-20 per cent low.
This method has a coefficient of variation of 1.68 per cent. Under ordinary circumstances there will be no interference from oxalic acid, creatinine, uric acid, ammonia, or hippuric acid. Amino acids and glucose may result in false elevations of readings. Samples must be preserved by refrigeration or with toluene or thymol, and when so treated are stable up to 48 hours for peptide assay.

REFERENCES