A MICROCOLORIMETRIC DETERMINATION OF CREATINE IN URINE BY THE JAFFE REACTION*

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WITH THE TECHNICAL ASSISTANCE OF GLORIA KURZMANN

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In 1945, a method was described by Bonsnes and the writer (1) for the determination of urinary creatine which involved only the addition of picric acid to dilute urine solutions and heating in a boiling water bath. An 80 per cent conversion of creatine to creatinine was obtained. The method has now been modified to achieve 100 per cent conversion, on the basis of Clark and Thompson’s recent studies (2) on the effect of pH on the quantitative conversion of creatine to creatinine. They have shown that 80 per cent of creatine is converted at pH 1, 85 per cent at pH 1.5, 98 to 100 per cent at pH 2 to 2.5, and that with increasing pH the per cent conversion is considerably decreased, falling below 50 per cent at pH 4.5. In the light of Clark and Thompson’s observations, the pH at the start and finish of the heating procedure employed in the original method of Bonsnes and Taussky was reinvestigated. It was found to be slightly more acid than requisite to achieve complete conversion of creatine to creatinine. By increasing the initial total volume it was possible to obtain a mixture of the appropriate pH range for complete conversion. Optimal conditions were established for the quantitative conversion of 10 to 80 γ of creatine, a convenient range for a wide variety of urine concentrations. Under these experimental conditions glucose, which normally interferes with the determination of creatine by the Benedict method (3) does not do so in amounts up to 60 gm. per liter of urine. Preliminary reaction of the urine with iodine was introduced to eliminate the interference of acetone, acetoacetic ester, and ascorbic acid.

EXPERIMENTAL

Reagents—
Creatinine stock solution. Creatinine is dried at 105° to constant weight.

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0.500 gm. of creatinine is dissolved and diluted to 1 liter with 0.1 N hydrochloric acid. This solution contains 500 γ of creatinine per cc.

**Creatine stock solution.** Creatine monohydrate is dried in a desiccator over calcium chloride to remove moisture or heated at 120° to the anhydrous state until the weight is constant. 0.660 gm. of creatine monohydrate or 0.580 gm. of anhydrous creatine is dissolved and diluted to 1 liter with water (not stable). Either of these solutions contains 500 γ in terms of creatinine per cc.

**Picric acid, 0.04 N.** A saturated solution of picric acid is prepared by adding about 16 gm. of picric acid to 1 liter of water in a 2 liter flask. This solution is then heated to about 80°, when complete solution takes place. After cooling to room temperature excess picric acid crystallizes. About 690 cc. of this saturated solution are diluted to 1 liter and titrated with 0.1 N NaOH, with phenolphthalein as indicator.

**Sodium hydroxide, 0.75 N.** 42 cc. of NaOH (1:1 by weight) are diluted to 1 liter and titrated with 1.0 N HCl with a mixture of bromocresol green and methyl red as indicator.

**Iodine reagent, 0.05 N.** It is not necessary to standardize this solution. 6.4 gm. of pure iodine plus 13 gm. of potassium iodide are transferred to a liter volumetric flask. About 40 cc. of water are added, and the flask is shaken until all of the iodine is dissolved. When this is accomplished, the solution is diluted to the mark.

**Chloroform,** analytical reagent.

**Method**

Urine is first treated with the iodine reagent. Following this preliminary step, equal amounts of diluted urine samples are pipetted directly into colorimeter tubes for the determination of preformed creatinine and into special tubes for the determination of total creatinine. Creatine is converted to creatinine in a picric acid medium by heating in a boiling water bath. The final Jaffe reaction is carried out simultaneously for the preformed and total creatinine. The orange-red color develops maximally within 20 minutes and is stable for about 1 hour. The intensity of the color is determined in a Klett-Summerson photoelectric colorimeter with a No. 54 filter. The range of sensitivity is from 10 to 80 γ. The final color does not obey Beer's law; hence a calibration graph has to be constructed.

**Procedure for Determination of Creatine and Creatinine in Urine**—The pH of the urine specimen is adjusted to approximately 6 ± 0.5 (with pH paper) by adding concentrated HCl or 20 per cent NaOH dropwise.

**Preliminary Treatment and Extraction**—3 cc. of urine are pipetted into a 10 cc. glass-stoppered graduated cylinder. Iodine reagent is added dropwise until a definite change in color persists. Usually 4 to 5 drops are
sufficient, but in the presence of large amounts of reducing substances slightly more will be required; an excess of 1 or 2 drops is not harmful. After standing for 2 minutes to insure excess of iodine, the volume is adjusted to 6 cc. with water. 6 cc. of chloroform are added, and the graduate is stoppered and shaken to extract excess iodine and reaction products. Separation of the two phases takes place readily, with a more or less pronounced emulsion interface. 2 cc. of the upper aqueous phase are pipetted into a 100 cc. volumetric flask and diluted to the mark. This represents a urine dilution of 1:100, a convenient dilution factor for the usual range of urinary creatinine and creatine values of 24 hour specimens. This dilution can be changed for more dilute or concentrated specimens.

Determination of Preformed Creatinine—3 cc. of the above are pipetted directly into colorimeter tubes, 1 cc. of 0.04 N picric acid and 1 cc. of 0.75 N NaOH are added, and the tubes are well shaken. After 20 minutes the intensity of the color is read in the Klett-Summerson colorimeter with filter No. 54.

Conversion of Creatine to Creatinine—3 cc. of the 1:100 dilution are pipetted into heavy walled 12 cc. centrifuge tubes graduated at 4 cc. 1 cc. of 0.04 N picric acid and 3 cc. of water are added, and the tubes are shaken to insure proper mixing. They are inserted into a constant level boiling water bath deep enough to cover the surface of the liquid in the tubes. It was found very helpful to put a small rubber ring, the shape of a garden hose washer, around each tube to be able to suspend it in the stand in the water bath; furthermore, this serves to cover completely the opening for the tube in the stand and thus prevents steam from escaping and condensing on the wall of the tube. After about 1½ hours the volume in the tubes will have decreased to below 4 cc.; however, boiling down to as little as 2 cc. is not harmful. After cooling and readjusting of the volume to the 4 cc. mark, 1 cc. of 0.75 N NaOH is added and the tubes are well mixed. After 20 minutes the contents of these tubes are transferred to colorimeter tubes. The intensity of the color is read in the Klett-Summerson colorimeter with a No. 54 filter as for the preformed creatinine.

Analysis of Standard Solutions—Appropriate dilutions are prepared from the stock solutions of creatine and creatinine to give concentrations of 10 to 80 μ per 3 cc. These dilutions are then carried through the procedure as described for diluted urine. The preliminary treatment of the standard solutions with iodine can be omitted, as the results were found to be identical with or without this step in the procedure. A calibration graph is prepared. With each series of analyses a blank and at least one standard, usually 30 μ, are analyzed.

1 Available from E. Machlett and Son, 220 East 23rd Street, New York 10, New York.
DET E RMINATION OF CREATINE

Calculation of Results for Urine

Preformed Creatinine—
\[
\frac{\gamma_{\text{found (from calibration graph)}}}{30 \text{ (for 1:100)}} \times \frac{V}{24 \text{ hrs.}} = \text{mg. preformed creatinine per 24 hrs.}
\]

Total Creatinine—
\[
\frac{\gamma_{\text{found (from calibration graph)}}}{30 \text{ (for 1:100)}} \times \frac{V}{24 \text{ hrs.}} = \text{mg. total creatinine per 24 hrs.}
\]

Creatine Expressed As Creatinine—Mg. of total creatinine less mg. of preformed creatinine = mg. of creatine per 24 hours.

DISCUSSION

Stability of Creatine and Creatinine in Stock Solutions—The acidified creatinine solution is stable for at least 6 months. Creatine solutions and mixtures of creatine plus creatinine were prepared as aqueous as well as solutions acidified with hydrochloric, sulfuric, and acetic acids; these solutions were kept at room temperature and refrigerated for 1 week. The spontaneous per cent conversion of creatine to creatinine in these various solutions was as follows: in the aqueous mixture about 5 per cent, in hydrochloric and sulfuric acids about 10 per cent, and in acetic acid about 30 per cent.

Influence of Acidity—Clark and Thompson pointed out that conversion of creatine to creatinine is complete at pH 2 to 2.5. In the original conversion procedure of Bonsnes and Taussky there were in each tube 3 cc. of diluted urine plus 1 cc. of 0.04 N picric acid, a total volume of 4 cc., which was then reduced to 3 cc. in a boiling water bath. Measurement of the pH of the initial total volume of 4 cc. showed it to be 2.1 and, when boiled down to about 3 cc., 1.9, a range of pH at which conversion is not complete according to Clark and Thompson. By increasing the initial total volume of 4 cc. to 7 cc. with the addition of distilled water, the mixture was brought into the pH range at which conversion is complete. In retrospect it is apparent that Folin (4, 5), who introduced the use of picric acid alone for the conversion of very small amounts of creatine to creatinine in urine samples, achieved the necessary condition for its complete conversion by the use of a great dilution factor. He also pointed out that, when larger amounts of urine samples were analyzed, this method was not accurate because of the release of large amounts of ammonia. Hence it is evident that Folin was aware of the influence of acidity on the conversion of creatine to creatinine, although he did not investigate this factor more specifically.

In Fig. 1 is illustrated the relationship of pH and picric acid (from satu-
rated to 0.004 N). Saturated picric acid has a pH of 1.4, 0.04 N pH 1.5, and with further dilution the pH continues to rise. A dilution of 1 part of picric acid with 6 parts of water gives pH 2.4. This is the starting point of the boiling procedure as indicated in Fig. 1. When the solution is boiled down to below 4 cc., pH of 2.1 is reached.

Fig. 1. Relationship of pH and picric acid concentration. On the left, the ordinate represents the normality of diluted picric acid; on the right, the dilution of 1 cc. of 0.04 N picric acid with different amounts of distilled water. The starting point of the boiling procedure (pH 2.4) is represented by the upper solid circle; the lower solid circle indicates a volume of 4 cc., pH 2.1.

Fig. 2. Per cent conversion of creatine to creatinine as a function of the initial dilution.

Fig. 2 shows the per cent conversion of creatine to creatinine as a function of the initial dilution. A total initial volume of 1.5 cc. results in 25
per cent conversion, 2 cc. in 70 per cent, 5 cc. in 90 per cent. However, from 7 cc. and above, creatine is completely converted into creatinine.

Comparison of Creatine in Urine by Micromethod and Benedict’s Method—Table I provides a comparison of values obtained by the present micromethod with an aliquot of 0.06 cc. of urine and by the Benedict method with an aliquot of 1.0 cc. of a 24 hour specimen.

Table II and III show recovery experiments carried out with the addition of creatine and creatinine to urine.

Interfering Substances—Many investigators have studied the specificity of the Jaffe reaction (6-13). A number of preliminary treatments were suggested to eliminate or reduce the effect of interfering substances. Gaeb-

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Table I

<table>
<thead>
<tr>
<th>Urine No.</th>
<th>Creatine added</th>
<th>Micromethod</th>
<th>Benedict</th>
<th>Difference</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>mg. per 24 hrs.</td>
<td>mg. per 24 hrs.</td>
<td>per cent</td>
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<tr>
<td>1</td>
<td>205</td>
<td>205</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>615</td>
<td>605</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>437</td>
<td>406</td>
<td>-6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>115</td>
<td>105</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>758</td>
<td>795</td>
<td>-5</td>
<td></td>
</tr>
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<td>6</td>
<td>815</td>
<td>775</td>
<td>5</td>
<td></td>
</tr>
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<td>7</td>
<td>548</td>
<td>573</td>
<td>-5</td>
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</tr>
<tr>
<td>8</td>
<td>130</td>
<td>150</td>
<td>-13</td>
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Table II

<table>
<thead>
<tr>
<th>Urine No.</th>
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<th>Creatine + creatinine</th>
<th>Creatine recovered</th>
<th>per cent</th>
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<tr>
<td>1</td>
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<td>1</td>
<td>6</td>
<td>58.0</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>64.0</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>1</td>
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<td>76.0</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>14.7</td>
<td>95</td>
<td></td>
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<td>2</td>
<td>1.5</td>
<td>16.2</td>
<td>100</td>
<td></td>
</tr>
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<td>4.5</td>
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<td>95</td>
<td></td>
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<td>6.0</td>
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<td>105</td>
<td></td>
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</table>
H. H. Taukky

Ler and Keltch (14), Borsook (15), Fisher and Wilhelmi (16), and Hare (17), used Lloyd's reagent, an aluminum silicate, to adsorb creatinine and thereby separate it from non-specific chromogenic substances. Miller and Dubos (18) prepared a specific enzyme capable of decomposing creatinine. However, when creatinine is determined in normal urine before and after these special procedures, the values are essentially the same (17, 19, 20). Among the most commonly known interfering substances are glucose, acetone, and acetoacetic ester. Glucose as such does not interfere, because it has a much slower reaction velocity in the cold, but, after hydrolysis with HCl, urea and glucose combine to form products which then give a color with alkaline picrate. However, under the milder condition employed here for the conversion of creatine to creatinine, glucose up to 60 gm. per liter of urine does not interfere with the determination of creatine.

Table III
Recovery of Added Creatinine to Urine

<table>
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<tr>
<th>Creatinine added</th>
<th>Creatinine found</th>
<th>Creatinine recovered</th>
</tr>
</thead>
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<td>35.5</td>
<td>100</td>
</tr>
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<td>38.5</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>41.5</td>
<td>103</td>
</tr>
<tr>
<td>9</td>
<td>44.8</td>
<td>100</td>
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<tr>
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<td>47.5</td>
<td>98</td>
</tr>
<tr>
<td>30</td>
<td>65.0</td>
<td></td>
</tr>
</tbody>
</table>

In the investigation of a number of other interfering substances, it was noted that ascorbic acid gave a strongly positive Jaffe reaction. The well known reducing properties of ascorbic acid suggested the investigation of the value of a number of oxidizing agents for eliminating this interference. Iodine proved the most satisfactory, as it not only oxidized ascorbic acid but also eliminated the interference of acetoacetic ester and acetone. Per-manganate also eliminated the interference of ascorbic acid, but unlike iodine, tended to oxidize glucose which then resulted in a marked augmentation of the final color development. Therefore, the preliminary treatment with iodine and extraction with chloroform were adopted to eliminate unknown interfering substances, including large amounts of ascorbic acid as well as acetone and acetoacetic ester. Ascorbic acid up to 3 gm., acetoacetic ester up to 16 gm., and acetone and glucose up to 50 gm. per liter of urine do not interfere.

Thymol and toluene when in contact with urine for 24 hours did not change the results for creatine or creatinine. A number of other substances were investigated for their interference with the determination of
creatinine and creatine. The following did not interfere when added in amounts of 1 mg. (equivalent to 16 gm. per liter) to 20 γ of the sum of creatine plus creatinine or to urine aliquots of the same order of magnitude: alanine, cysteine, glycine, histidine, leucine, glycogen, inulin, urea, acetoacetic ester; the acids, acetylsalicylic, citric, gluconic, glutaric, hippuric, lithium lactate, malic, malonic, oxalic, succinic, and uric; D-arabinose, L-arabinose, D-galactose, lactose, and D-xyllose. 500 γ (8 gm. per liter) of fructose and 200 γ (4 gm. per liter) of Versene do not interfere. 100 γ (1.6 gm. per liter) of the following substances do not interfere: arginine, glutathione, guanidine, methionine, and the acids, benzoic, glutamic, maleic, oxaloacetic, and p-aminohippuric. 60 γ (800 mg. per liter) of pyruvate, 30 γ (400 mg. per liter) of α-ketoglutaric acid, and 20 γ (250 mg. per liter) of glycocyamine do not increase the colorimeter readings for creatine and creatinine. Creatine phosphate is completely split under the conditions of our procedure; hence, the analytical results for creatine will include whatever creatine phosphate may be present.

The improved specificity of this method has led us to apply the above procedure to serum, with results to be described elsewhere.

**SUMMARY**

A micromethod has been presented for the determination of creatine in small amounts of urine. The method is based on the complete conversion of creatine to creatinine in a boiling water bath with the sole addition of diluted picric acid. The interference of large amounts of acetone, acetoacetic ester, ascorbic acid, and glucose has been eliminated. The range of sensitivity of the method is from 10 to 80 γ. Comparisons with Benedict’s method and recovery experiments are in good agreement.

I wish to thank Dr. Ephraim Shorr for his advice and encouragement during this study.

**BIBLIOGRAPHY**

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