THE DETERMINATION OF CREATININE WITH SODIUM 3,5-DINITROBENZOATE

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The reaction of picric acid with creatinine in alkaline solution (Jaffe's reaction), which is the basis of the Folin method (1) for the determination of creatinine, has been thought to be either a reduction of the acid or else the formation of a red tautomer of creatinine picrate. Greenwald (2) obtained evidence that the tautomerism involves picric acid as well as creatinine, and that other nitro compounds give no color under the conditions used (2–4). The reaction, therefore, has been thought to be peculiar to this one acid. However, similar rearrangements probably account for the color in alkaline solution of many nitro compounds, such, for instance, as trinitrobenzoic acid, which dissolves in sodium carbonate to give a pale yellow solution of the sodium salt, but gives a deep red color with excess of sodium hydroxide. Any nitro compound which behaves in this way toward alkali is obviously unsuitable for use in place of picric acid in the Folin procedure.

It has been found in this laboratory that 3,5-dinitrobenzoic acid gives a brilliant garnet-red with creatinine in alkaline solution and can be used for its determination. Under similar conditions, the acid alone gives no red. We have repeatedly purified the acid by recrystallization from alcohol and obtained a product which did not change in its color-producing qualities as purification proceeded. The chromogenic property reached a maximum with the maximum purity. On the other hand, one batch of the crude acid, as purchased, contained a yellow substance which influenced the reaction in such a way that the color obtained was very pale and very quickly destroyed. Greenwald and Gross (4), who have
overlooked the color which is given by the acid, may have done so because of the presence of this impurity in their solutions.

The behavior of the new reagent toward creatinine of urine and of blood has been compared with that of picrate, and its use has been found to be advantageous. When pure, the reagent gives a very pale yellow solution of the sodium salt, which color is negligible when amounts of creatinine greater than 0.025 mg. are determined. With amounts greater than this, the color is easily read in a colorimeter and can be attributed entirely to the creatinine without detectable error. Furthermore, the reagent develops less extraneous color than picrate with certain substances such as glucose and acetoacetic acid, which frequently occur in urine and blood. Consequently, the values obtained by analysis of such solutions are more trustworthy than those obtained with the Folin procedure.

EXPERIMENTAL

Purification of 3,5-Dinitrobenzoic Acid

The acid, purchased from the Eastman Kodak Company, melted from 200–204°. Of this impure acid, 50 gm. were dissolved in 100 cc. of boiling 80 per cent alcohol. The solution was filtered and cooled to about 5°, whereupon crystals formed within a few minutes. After about ½ hour at 5°, the crystals were filtered off and were washed with 50 per cent alcohol. For use, in this instance, a second recrystallization was necessary in order to remove the yellow impurity more completely. The purified acid was very pale yellow in color and melted at 204–204.5° (capillary tube).

Preparation of Reagent (6 Per Cent Sodium Dinitrobenzoate)

The acid is not readily soluble in water, but dissolves in sodium carbonate solution with evolution of carbon dioxide. 30 gm. of the acid were suspended in 420 cc. of water, and 80 cc. of 10 per cent sodium carbonate were added. When no more of the acid dissolved, the solution was filtered and was ready for use.

The reagent was nearly colorless, but several preparations were comparable in color to a solution containing 1 cc. of saturated picric acid (room temperature) in 5 liters of water. A large part of the acid may be recovered by adding HCl to the solution after the determination. Recrystallization from alcohol in the presence of
norit to remove adsorbed colored material yields a product suitable for further use.

**Determination of Creatinine in Urine**

**Reagents**—
- 6 per cent sodium 3,5-dinitrobenzoate.
- 5 per cent sodium hydroxide.
- Standard solution of creatinine in 0.1 N HCl.

**Procedure**—To 1 cc. of creatinine standard (≈ 1 mg. of creatinine in 0.1 N HCl) in a tube graduated at 25 cc., and to 1 cc. of urine in another, are added 20 cc. of 6 per cent sodium dinitrobenzoate (graduated cylinder)\(^1\) and 2 cc. of 5 per cent sodium hydroxide. The solutions, diluted at once to 25 cc., are mixed, and, after 10 minutes, comparisons are made in a colorimeter. The calculation is as usual. The reactions with the standard and the unknown must be carried on within 5 minutes of each other and the comparison should be made within 30 minutes after completion of the color development. If solutions with less color are desired, dilution may be made to 50 cc. without decrease of stability of color. If diluted to 100 cc., 5 per cent fading occurs in 5 minutes.

**Observations on Color**—The reaction of 3,5-dinitrobenzoate with creatinine corresponds to that of picrate in that a large excess of the reagent must be used for full color development. With the amount stated, the color develops rapidly (10 minutes) from violet to garnet, which remains for 5 minutes, and then fades slowly (1 hour) through crimson to reddish brown. The hue and intensity of the color depend upon the purity of the 3,5-dinitrobenzoate, the color with impure reagent being definitely inferior. With less than 20 cc. of 6 per cent dinitrobenzoate, complete color is not evolved and the changes in intensity and hue are more rapid. With 2 cc. of 5 per cent NaOH, a pink color (indicator effect) may be formed with some preparations of dinitrobenzoate in the absence of creatinine, although with the pure reagent such color is

\(^1\) 15 cc. of 3 per cent sodium dinitrobenzoate and 2 cc. of 5 per cent sodium hydroxide may be used, in which case the color is less deep by about 25 per cent and is less stable. It is desirable that the standard and the unknown be made simultaneously and that the reagent be measured with a pipette.
Creatinine Determination

inappreciable. This indicator color may be removed by the careful addition of $\text{KH}_2\text{PO}_4$ without destroying the color due to creatinine. A $0.2\text{ M}$ solution of $\text{KH}_2\text{PO}_4$ (27.2 gm. per liter) is convenient, and the amount to be added is determined readily with a control tube.

Fading is more rapid than with picrate and depends to a large extent upon the alkalinity of the solution. The color is destroyed at once by acid and returns when the solution is made alkaline. With less alkali than is prescribed, the color is not proportional to the creatinine content; with more alkali, the reaction is too rapid. Under the conditions given (2 cc. of 5 per cent NaOH and 20 cc. of 6 per cent dinitrobenzoate) there is an interval of about 5 minutes after the maximum color development when fading is not noticeable. This constancy of color may be attributed to equal rates of color development and of fading.

Table I

**Determination of Creatinine in Water Solution**

<table>
<thead>
<tr>
<th>Present</th>
<th>0.60</th>
<th>0.80</th>
<th>1.20</th>
<th>1.40</th>
<th>1.60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found</td>
<td>0.60</td>
<td>0.83</td>
<td>1.22</td>
<td>1.42</td>
<td>1.60</td>
</tr>
</tbody>
</table>

Each solution was compared against the same standard which contained 1.0 mg. of creatinine.

The sensitivity of the reagent was tested toward a number of substances, several of which are known to interfere with the color in the picrate procedure. No color is developed with glucose in 10 per cent solution, nor with creatine, arginine, methylguanidine, $\text{as}$-dimethylguanidine, guanidine, fructose, and cystine. Less color is given than by picrate with acetone and acetoacetic acid, but the reagent is similarly sensitive toward uric acid, furfural, and formaldehyde. Amounts of uric acid 10 times greater than are present in normal urine do not interfere in the determination of creatinine.

That the color obtained is proportional to the amount of creatinine is illustrated by the data of Table I, in which water solutions of creatinine were analyzed.

In order to determine whether or not the values for urine are the
same as those given by picrate, determinations with the two reagents were made. Purified picric acid was used throughout. The values obtained with picrate are slightly lower than those with dinitrobenzoate, but the differences are not significant. The figures given (Table II) are typical of a number of analyses which were made.

**Table II**

*Comparison of Picric Acid and 3,5-Dinitrobenzoic Acid for Analysis of Urine for Creatinine*

The values are given in mg. per cc.

<table>
<thead>
<tr>
<th>Urine No.</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Picric acid</td>
<td>2.54</td>
<td>1.72</td>
<td>1.21</td>
<td>0.84</td>
<td>1.57</td>
<td>0.99</td>
</tr>
<tr>
<td>Dinitro</td>
<td>2.58</td>
<td>1.83</td>
<td>1.24</td>
<td>0.85</td>
<td>1.62</td>
<td>1.02</td>
</tr>
</tbody>
</table>

Urine I represents urine collected after ingestion of creatinine; Urine II, 10 cc. of Urine I + 5 cc. of water; Urine III, 5 cc. of Urine I + 5 cc. of water + 5 cc. of creatinine solution (≈ 5 mg. of creatinine); Urines IV, V, and VI were normal urines. Urine I was modified as indicated in order to demonstrate that no substance other than creatinine of the urine influenced the color.

**Determination of "Apparent Creatinine" of Blood**

**Reagents**—
Saturated solution of sodium 3,5-dinitrobenzoate, made from 20 gm. of dinitrobenzoic acid, 150 cc. of water, and 50 cc. of 10 per cent sodium carbonate.

10 per cent sodium hydroxide.

Standard solution of creatinine in 0.1 N HCl.

**Procedure**—To 10 cc. of tungstic acid filtrate (Folin-Wu) in a test-tube and to 0.01 or 0.005 mg. of creatinine in 10 cc. of solution in another are added 3 cc. portions of saturated sodium dinitrobenzoate and 0.5 cc. of 10 per cent sodium hydroxide. The tubes are shaken and allowed to stand for 10 minutes. Within the following 10 minute interval the solutions are compared in a colorimeter. The readings are referred to a graph (see below) from which the creatinine content of the filtrate is established.

**Construction of Graph of Creatinine Values**—When determinations of creatinine are made upon amounts smaller than 0.03 mg. per 10 cc. of solution, the color obtained is not proportional to the
Creatinine Determination

creatinine content. A similar statement is true for the picric acid procedure (5). Before accurate values are obtained it is necessary that solutions containing from 0.003 to 0.03 mg. portions of creatinine per 10 cc. be carried through the above reaction and compared one with another. Graphs correlating the colorimeter readings with creatinine concentrations can then be made. These graphs show that high values for creatinine of blood filtrates result when calculations are made in the usual way.

Observations Regarding Color—Because of the small amount of creatinine in filtrates from normal blood, concentrated reagents are necessary to give sufficient color for use in a colorimeter. The 3 cc. of saturated dinitrobenzoate are most convenient, but deeper colors may be obtained by dissolving 0.3 gm. of dry sodium dinitrobenzoate in the 10 cc. of filtrate. Fading is rapid, and the time interval between completion of the color development and comparison must be correspondingly short. Conditions for greater stability of color were sought for without success.

Comparison of Picrate and Dinitrobenzoate Procedures for Creatinine of Blood

Whereas the picrate values for creatinine of retention bloods usually are acceptable, those reported in bloods with normal or low creatinine content are subject to serious doubt (5–7). The color due to the picrate alone is enormously deep as compared with that due to the creatinine, and there seem to be substances in blood other than creatinine, which react with the picrate to produce extraneous color. The first of these objections does not apply to determinations with dinitrobenzoate, inasmuch as the color obtained with a filtrate is much deeper than with the reagents alone. The second objection applies much less to the dinitrobenzoate than to the picrate procedure, as the reagent is more specific in its reaction, and as the interfering brown color from the filtrate can be judged with more accuracy.

For comparison of the two procedures (8), tungstic acid filtrates were used. Each filtrate represented mixed blood from three or more patients, all with normal or slightly high urea nitrogen. They were analyzed by both procedures; the figures are recorded in Table III. The picrate values, except for two, are higher by about 0.4 to 1.0 mg.
In order to establish the more accurate values, filtrates were analyzed by both procedures, and then the creatinine of similar portions of filtrate was removed by adsorption upon Lloyd's reagent and determined after liberation from the adsorbent by use of MgO, as described by Gaebler (5). The analyses are recorded in Table IV.

The values for adsorbed creatinine, as determined by the two reagents, agreed within the experimental error of the methods and were essentially the same as those obtained by use of dinitro-

### Table III

<table>
<thead>
<tr>
<th>Filtrate No.</th>
<th>Picrate mg. per 100 cc.</th>
<th>Dinitrobenzoate mg. per 100 cc.</th>
<th>Filtrate No.</th>
<th>Picrate mg. per 100 cc.</th>
<th>Dinitrobenzoate mg. per 100 cc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.2</td>
<td>0.6</td>
<td>11</td>
<td>1.0</td>
<td>0.38</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>0.5</td>
<td>12</td>
<td>0.6</td>
<td>0.1*</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>1.0</td>
<td>13</td>
<td>2.1</td>
<td>1.6†</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>0.4</td>
<td>14</td>
<td>1.1</td>
<td>0.1*</td>
</tr>
<tr>
<td>5</td>
<td>0.9</td>
<td>0.5</td>
<td>15</td>
<td>1.03</td>
<td>0.70</td>
</tr>
<tr>
<td>6</td>
<td>1.2</td>
<td>0.6</td>
<td>16</td>
<td>0.95</td>
<td>0.30</td>
</tr>
<tr>
<td>7</td>
<td>1.0</td>
<td>0.5</td>
<td>17</td>
<td>1.08</td>
<td>0.50</td>
</tr>
<tr>
<td>8</td>
<td>1.0</td>
<td>0.44</td>
<td>18</td>
<td>1.48</td>
<td>1.0</td>
</tr>
<tr>
<td>9</td>
<td>0.98</td>
<td>0.32</td>
<td>19</td>
<td>1.54</td>
<td>0.9</td>
</tr>
<tr>
<td>10</td>
<td>1.2</td>
<td>0.8</td>
<td>20</td>
<td>1.1</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Values for both procedures were determined from graphs. Bloods had been drawn 4 or 5 hours before analysis.

* Filtrates 12 and 14 were matched against a blank; the creatinine color was doubtful.

† A blood with high creatinine content is included in Filtrate 13.

Creatinine 3,5-dinitrobenzoate may be prepared by mixing hot alcohol solutions of creatinine and of 3,5-dinitrobenzoic acid. The salt separates almost immediately, and may be filtered off with nearly quantitative yield when the solution has cooled. After recrystallization from hot water (80 cc. per gm.) or from 50 per
cent alcohol (1.6 gm. per 100 cc.) it is white, crystalline, and anhydrous. It decomposes with effervescence at a temperature which seems to depend on the rate of heating, usually between 230–240°. The creatinine content, determined colorimetrically, is 35 per cent. The calculated value is 34.7 per cent. The salt is not readily soluble in water (about 0.035 gm. in 100 cc. at 20°), but dissolves when sodium bicarbonate is added. Owing to the instability of creatinine in alkaline solution, the salt is not recommended as a standard for creatinine determinations.

Table IV
Creatinine of Filtrates before and after Adsorption upon Lloyd's Reagent

The values are given in mg. per 100 cc.

<table>
<thead>
<tr>
<th>Filtrate No.</th>
<th>Without adsorption</th>
<th>Adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dinitrobenzoate</td>
<td>Picrate</td>
</tr>
<tr>
<td>1</td>
<td>0.6</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>0.3</td>
<td>0.9</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>0.45</td>
<td>1.1</td>
</tr>
<tr>
<td>5</td>
<td>0.7</td>
<td>1.2</td>
</tr>
<tr>
<td>6</td>
<td>0.65</td>
<td>1.3</td>
</tr>
<tr>
<td>7</td>
<td>0.52</td>
<td>0.96</td>
</tr>
<tr>
<td>8</td>
<td>0.52</td>
<td>1.2</td>
</tr>
<tr>
<td>9</td>
<td>0.42</td>
<td>1.1</td>
</tr>
<tr>
<td>10</td>
<td>0.5</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Creatinine in amounts up to 0.3 mg. per 10 cc. in pure solution was accounted for quantitatively by the procedures used.

A study was made of the conversion of creatine into creatinine by heating in a water bath with 3,5-dinitrobenzoic acid, which then was used for the development of color in situ. The conversion requires heating for 3 hours with excess of the acid, and therefore offers no advantage over the well known procedure with hydrochloric acid. The extraneous color produced as a result of the heating with urine is negligible, but the excess acid dissolves slowly in sodium carbonate so that the development of color must be delayed longer than is desirable.
SUMMARY

Procedures are described for the determination of creatinine of urine and of blood with sodium 3,5-dinitrobenzoate and alkali. With this reagent the extraneous color is negligible when amounts of creatinine greater than 0.02 mg. per 10 cc. are determined. The color is more easy to read in a colorimeter than that obtained in the Folin picrate procedure. The reagent is more specific toward creatinine than is picrate, and with tungstic acid blood filtrates the values are practically identical to those obtained after adsorption of the creatinine upon Lloyd's reagent.

Addendum—The authors had not seen the article by Benedict and Behre (9) on the use of 3,5-dinitrobenzoic acid for the determination of creatinine until after the proof of this paper had been corrected. Our work, therefore, should be considered as supplementing theirs.

BIBLIOGRAPHY

THE DETERMINATION OF CREATININE WITH SODIUM 3,5-DINITROBENZOATE
Wilson D. Langley and Margaret Evans