Colorimetric Determination and Distribution of Urinary Creatinine and Creatine

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"Apparent" creatinine and "total" creatinine levels in urine can be estimated to within 2 per cent accuracy by means of the Jaffe colorimetric reaction, provided that certain requirements for the colorimetric reaction itself are met, and provided that appropriate conditions for the conversion of creatine to creatinine are used. The amount of noncreatinine Jaffe-positive material appears to be negligible in most urines, but it can be evaluated with paper chromatography and ion-exchange technics.

More than 88 per cent of normal subjects excrete 300-900 mg. creatinine per 100 lb. of total body weight per day, with the average at 600 mg. Normal males excrete creatinine at an average rate of 875 mg.

Creatine excretion in normal subjects ranges more widely. From 0-800 mg. per 100 pounds of total body weight per day is excreted (as creatinine), with the average at 275 mg., regardless of sex or age.

Certain malabsorption syndromes (cystic fibrosis and celiac disease) include distinct creatinurias.

Comparative constancy in the rate of urinary excretion of the metabolic waste-product creatinine from normal individuals was first observed by Folin in 1905 (1). Subsequently, it was observed that creatinine excretion per unit body weight per day may be fairly individualistic (2, 3), and that creatinine is accompanied by creatine. The latter is regarded as a metabolizable compound that is concentrated principally in muscle, presumably as creatine phosphate, and that in the free state can be converted irreversibly to creatinine by means of acid digestion.

The brilliant reddish-orange reduction product (Jaffe color)
formed from creatinine with alkaline picrate has served for many years as the basis of a sensitive colorimetric determination of "apparent" and "total" creatinine, where "apparent" creatinine is defined as the sum of all Jaffe-positive compounds, whereas "total" creatinine includes converted creatine. Creatine itself is negative to the Jaffe reaction.

Wide differences in creatinine and creatine levels for the same urine specimen have suggested that neither the conditions for maximum and reproducible Jaffe-color development, nor those for the conversion of creatine to creatinine, have been thoroughly specified. The Jaffe reaction is known to be complex (4). Ordinarily, "apparent creatinine" is determined directly by a colorimetric procedure, developed originally by Folin and refined by Bonsnes and Taussky (5), in which the optical density of an alkaline picrate solution at 520 m\(\mu\) is determined after 15 min. or more. Creatine levels are generally expressed as the difference between "total creatinine" and "apparent creatinine," although Barrett's alpha-naphthol and diacetyl reaction (6) or the alkaline ferricyanide-nitroprusside stain for paper chromatograms (7) have also been used for direct determination. In an effort to eliminate the contribution of noncreatine Jaffe-positive compounds, such as glycocyamine (Fig. 1) or various keto compounds, creatinine has been absorbed on Lloyd's Reagent (aluminum silicate) and eluted with dilute acids (8, 9). A lack of specificity with the use of this technic has been noted (10).

In the present study, the factors that contribute to maximum precision of the Jaffe reaction have been reassessed. With paper-chromatographic (11) and ion-exchange technics as a guide, a refined colorimetric procedure has been developed to determine creatinine and creatine excretion rates in single 24-hour urine specimens (voided) from 52 normal children and adults and from a selected pathologic series.
Materials and Methods

Reagents
Crystalline creatinine and crystalline creatine hydrate (Eastman) were used without further purification. The latter was found to contain less than 0.6% creatinine. Picric acid (Baker; 10% water as stabilizer) was dissolved by warming to make a 0.92% aqueous solution.

Urine Collection
Twenty-four-hour normal human urine samples were preserved shortly after collection in polyethylene bottles at below-freezing temperatures. The volumes collected per 100 pounds of body weight in normal persons, 1-76 years of age, ranged from 500 to 2000 ml./day, and averaged ca. 1000 ml. Diets were not controlled.

Colorimetry
The following conditions were finally adopted. One milliliter of an appropriate dilution (for initial evaluation, 1/20 ml.) of native urine or of standard creatinine solution, such that the sample contained 10-40 mg. of creatinine per milliliter, is pipetted into 18 X 150-mm. matched test tubes, followed by 1 ml. 0.92% picric acid and 4.0 ml. of 0.1N sodium hydroxide (or 5 ml. of the premixture). After 45 min. at room temperature, the optical density at 490 m of a Coleman Junior Spectrophotometer Model 6A is read with a creatinine-free blank prepared with one ml. of water.

Paper Chromatography
Suitable volumes of urine, or of standards, were applied in 5-μl. (maximum) portions to the base line (1½ in. from the bottom) of Whatman No. 1 paper at intervals of ¾-7½ inches. The chromatograms were developed overnight by the ascending technic in covered cylindrical battery jars with 100 ml. of Ethyl Cellosolve: 3N NH₄OH, (4:1). The solvent front generally advanced about 12 in. from the base line. About 10 μg. of creatinine could be seen as an intense absorption spot when the air-dried chromatogram was viewed by transmitted ultraviolet light (bactericidal lamp with visible cut-off filter)

*Optimum wave length should be evaluated separately for each instrument. With the instrument used in the present study, the maximum occurred at an indicated wave length of 500 μm. The Jaffe colors formed after 45 min. are stable for at least 3 hours.
at $R_f$ 0.43-0.46. It was preferred to spray the chromatogram with a mixture of 1% picric acid: 0.5N NaOH, 1:1, in order to develop the characteristic bright-red spots for creatinine, clearly visible on the yellow background. The spots reached maximum intensity after about $\frac{1}{2}$ hour at room temperature. The colors so developed are stable for several weeks. Reproducibility of such "apparent creatinine" assays was within about 10%.

**Ion-Exchange Absorption**

About 5 ml. urine, dissolved in 25 ml. water, was passed through a Dowex-50(H) cation exchanger, 150 mm. $\times$ 7 mm. in diameter (16 mEq.). The column was washed with water until the effluents were neutral; the effluents were then discarded. Creatinine, amino acids, urea, and other absorbed compounds were then eluted from the column with 50 ml. normal NH$_4$OH, followed by 25 ml. water. The eluate was then evaporated to dryness in vacuo at 50-55°, and the solid residue redissolved in water as required.

Recovery experiments showed that 82 per cent of creatinine, and 77 per cent of creatine, could be recovered under the specified conditions. The comparatively strong basicity of these compounds (i.e., the presence of the guanido group) probably accounts for such non-quantitative recoveries in both cases, because most amino acids can be recovered under the same conditions in better than 90 per cent yields (arginine, only 75 per cent). It is probable that a somewhat greater volume or concentration of ammonia is necessary for quantitative recoveries.

As evaluated by paper chromatography, the specimen eluates were wholly free of Jaffe-positive compounds other than creatinine.

**Conversion of Creatine to Creatinine**

The following conditions were finally adopted for quantitative conversion. To 0.1-0.5 ml. of urine in a small evaporating dish was added 2 ml. of 6N HCl. The mixture was evaporated to dryness at 100° on a steam bath (15-20 min.), and the dry residue, free of insolubles, was redissolved in 5-10 ml. water, and 1-ml. samples used for the "total creatinine" assay. Within experimental error (2%), creatine hydrate (in the range 100-300 $\mu$g.) was shown to be converted irreversibly to creatinine. The conditions originally used by Folin (2) are not recommended, principally because the ratio of acid to urine is too low.
Results

**Colorimetry**

Fig. 2 shows the absorption spectrum of the alkaline picrate creatinine complex as evaluated by a Cary Continuous Ultraviolet Spectrophotometer (1 cm. cells) for 80 μg. creatinine per milliliter and 45 min. reaction time. The intense absorption of the reagent makes mandatory the selection of 490 mμ for colorimetry rather than the observed maximum of 484 mμ. As is illustrated in Fig. 3, optimum color development is achieved with 4 ml. of 0.07-0.1N NaOH added to 1 ml. sample and 1 ml. of 0.92% picric acid. The rate of color development in Fig. 4 for 80 μg./ml. of creatinine indicates that the reaction is 99.9% complete in 45 min., with a half-time of 4.3 min., whereas after 15 min. the reaction is only 90% complete. With respect to the concentration of picric acid, 1 ml. of picric acid of various concentrations was added to 80 μg. of creatinine contained in 5 ml. of 0.1N NaOH, and reaction half-times were observed of 7, 12, 21, 35, and 60 min. for 0.46%, 0.23%, 0.14%, 0.07%, and 0.02% initial concentration of picric acid, respectively. Consequently, the highest
practical value (0.92%) was selected. The fact that the reaction half-times do not follow a simple relationship to concentration illustrates the complexity of the reaction. Indeed, the composition of the color is unknown, and evidently is not due to picramic acid, as has been suggested, since there is a marked dissimilarity of the absorption spectra.

As may be seen in Fig. 5, the optical density ($D_{490}$) vs. concentration ($C$) of creatinine is not a linear curve. It is typically exponential, of the form:

$$D_{490} = A [1 - \exp(-bC)]$$

with $A = 1.20$, $b = 0.020$, and $C$ in micrograms per milliliter, using $18 \times 150$ mm. cuvets with the Coleman Junior Spectrophotometer and 45 min. reaction time. Points on the curve generally are reproducible to within 2%, but the absence of a linear relationship is not understood.

Because of limitations of the spectrophotometer used, it was found
best to adjust concentrations of unknowns such that they contained 10–40 μg. creatinine per milliliter. Serial dilutions of urines repeatedly provided linear plots of “apparent” creatinine vs. concentration of sample.

**Apparent Creatinine and Creatinine**

Appropriate volumes of urine, to contain about 7 μg. of “apparent” creatinine, were paper-chromatographed in parallel with standards. As may be seen in Fig. 6, not more than a few per cent of Jaffe-positive material, other than creatinine, is present in any of 15 urines. Barely perceptible Jaffe-positive spots of R, 0.13, 0.23, and 0.30 accompany the intense creatinine spot at R, 0.45.

The creatinine in three normal adult urines was absorbed on Dowex-50(H), eluted with ammonia as described, and the creatinine contents (corrected for non-quantitative recovery) compared with the original “apparent creatinine” levels. Within experimental error, it was found that the contribution by noncreatinine Jaffe-positive compounds was negligible.

**Urinary Creatine**

Although creatine does not give a color with the Jaffe reagent, it may be converted irreversibly to creatinine under acidic conditions. Reported studies have shown that complete conversion for low concentrations of creatine is effected at 26° in more than 350 hours in normal HCl and within 24 hours at 60° in normal HCl, but within 3 hours at 98° in 0.5N HCl (2). In the present study, the rates of conversion of creatine hydrate to creatinine at 100° as a function of hydrochloric acid concentration were evaluated. In confirmation of the results of Edgar and Wakefield (12), the rates of conversion (given below) have been found not only to exhibit excellent first-order kinetics (high ratio of acid to creatine) at each concentration of acid, but also are strictly proportional to the “activity” (13) of the hydrochloric acid. (The activity coefficient of 6N HCl at room temperature is approximately 4.0.) The observed half-times are in excellent agreement with the values calculated from the data of Edgar and Wakefield, obtained in 0.38N HCl (Table 1).

The conversion of creatine to creatinine evidently is a simple acid-catalyzed bimolecular reaction that has an activation energy of 20.2 kcal. per mol. (12). Conversion was demonstrated to be complete in about 8 min. at 100° in 6N HCl, in keeping with the rule that
Table 1. **Half-Times of Conversion of Creatine to Creatinine at 100°**

<table>
<thead>
<tr>
<th>Normality of HCl</th>
<th>Observed half-time (min.)</th>
<th>Calculated* half-time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 (water, pH 5.5)</td>
<td>ca. 250</td>
<td>...</td>
</tr>
<tr>
<td>0.09</td>
<td>105</td>
<td>105</td>
</tr>
<tr>
<td>0.95</td>
<td>11.0</td>
<td>10.0</td>
</tr>
<tr>
<td>5.4</td>
<td>ca. 0.8</td>
<td>ca. 0.6</td>
</tr>
</tbody>
</table>

*From reference 12.

A first-order reaction is 99.9 per cent complete at 10 times its half-time.

Extrapolation of these data to 0.01N HCl at 37° suggests that not more than 10% creatine is converted to creatinine in 100 hours and, in view of the fact that the acidity of most urines is rarely lower than pH 5, a 10 per cent conversion in 4 days at body temperature must represent a generous upper limit. The 15-min. conversion time (2) for 0.5N HCl at 117° is erroneous; 55 min. seems more appropriate. Evidently creatine is a comparatively stable compound.

Routine quantitative conversion of creatine to creatinine (see under Materials and Methods) is achieved when a creatine-rich sample is adjusted to 6N HCl (constant-boiling) and subsequently evaporated to dryness with the aid of a steam bath in 15–20 min.

**Creatinine and Creatine Contents of Normal Urines**

"Apparent" creatinine excretion rates, expressed in this study as milligrams per 100 lb. per day (45.4 kg.) as found in single 24-hour urine specimens collected from 35 normal healthy children, ages 1–12, as well as those collected from 17 adults, ages 13–76, are listed in Table 2 together with creatine excretion rates. It is assumed that converted creatinine arises only from creatine. Acid digestion appears to be without effect upon the noncreatinine Jaffe-positive material, as judged by the unaltered appearance of paper chromatographs.

<table>
<thead>
<tr>
<th>Normal children (35)</th>
<th>Creatinine (mg./cwt./day)</th>
<th>Creatinine (as creatinine) mg.</th>
<th>S.D.</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>550</td>
<td>280</td>
<td>165</td>
<td></td>
<td></td>
</tr>
<tr>
<td>875</td>
<td>240</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>640</td>
<td>270</td>
<td>225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVERAGE (52)</td>
<td>610</td>
<td>275</td>
<td>185</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. **Creatine and Creatinine Excretion (mg./cwt./day) in Normal Urine**
**Fig. 5.** Optical density versus creatinine concentration. For explanation see text.

**Fig. 6.** Paper chromatogram in Jaffe stain of urinary creatinine. There is about 7 µg. creatinine in each sample. The standard creatinine samples are labeled S. The cellosolve: 3N NH₄OH dimension is vertical. B, C, E, L, M, N are from normals. A, G, H, K are from cases of cystic fibrosis. J, Q, R are from cases of generalized aminoaciduria.
grams after acid digestion. In order to avoid confusion, creatine levels are reported as creatinine equivalents.

The distribution for creatinine (Fig. 7) is comparatively sharp. When the median is at 600 mg. per 100 lb. per day, a range of ± 300 includes all but 2 cases (or 3.8 per cent) who excrete less than 300 mg. and 4 cases (or 7.7 per cent) who excrete more than 900 mg., disregarding differences in age or sex. Creatine excretions (Fig. 8) have a different and broader distribution.

**Creatinine and Creatine Excretion in Pathologic Conditions**

The creatinine and creatine excretion rates of selected subjects are listed in Table 3.

The creatinine excretion rates in these cases appears to be normal. On the other hand, creatine excretion rates for patients with cystic

![Fig. 7. Frequency distribution of urinary creatinine for 52 normal health subjects: children (35), women (10), men (7).](image)

![Fig. 8. Frequency distribution of urinary creatine excretion, expressed as creatinine, for 52 normal healthy subjects, with age or sex disregarded.](image)
Table 3. Urinary Creatinine and Creatine in Malabsorption Diseases

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. subjects</th>
<th>No. observations</th>
<th>Creatinine (mg./cwt./day)</th>
<th>Creatine (mg./cwt./day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystic fibrosis</td>
<td>11</td>
<td>12</td>
<td>480</td>
<td>580</td>
</tr>
<tr>
<td>Celiac disease</td>
<td>12</td>
<td>17</td>
<td>430</td>
<td>425</td>
</tr>
<tr>
<td>Pancreatic insufficiency (not due to cystic fibrosis)</td>
<td>1</td>
<td>10</td>
<td>660</td>
<td>810</td>
</tr>
<tr>
<td>Wilson's disease</td>
<td>4</td>
<td>7</td>
<td>550</td>
<td>160</td>
</tr>
<tr>
<td>Phenylpyruvic oligophrenia</td>
<td>9</td>
<td>11</td>
<td>470</td>
<td>245</td>
</tr>
</tbody>
</table>

fibrosis, celiac disease, and a single case of pancreatic insufficiency, are decidedly greater than normal (average, 275 mg.).

A spot check of 152 pathologic specimens of all kinds, 1/10 of which were repeats from the same subjects, indicated that in only 14 per cent creatinine excretion rates were lower than 300 mg. creatinine per 100 lb. per day, whereas in 5 per cent the excretion rates were higher than 900 mg. Creatinine levels apparently do not reflect the bulk of pathologic disorders.

Discussion

It has been fairly well established that the excretion rate of creatinine is fairly constant in normal humans, being reasonably independent of minor variations in diet and in rates of exercise (1, 2). Creatinine presumably originates as the result of metabolic reactions of creatine phosphate in muscle, and the assumption of the constancy of excretion per unit body weight per day is not entirely valid, as is indicated by the present study. Confirmed in this study is the tendency for adult human males to excrete creatinine at a higher rate than do normal women (Table 1 and Fig. 7), as well as the tendency for children to approximate adult creatinine excretion rates. The creatinine excretion rates, as determined in the present study, average approximately 80 per cent of the values in bulk of previous measurements. Averages from pathologic cases have shown a slight tendency to depart downward from the "normal" range, but in general the bulk of the excretion patterns for most disorders fall within the "normal" range.

Although no attempt has been made to evaluate systematically the effect, the available data suggest that variations of creatinine per unit body weight per day for a group of selected normal individuals roughly parallels the daily variations found in specimens from individuals. This impression is, of course, at variance with the findings of Folin and others (2), and can be corrected only by systematic
evaluation. Our data do suggest, however, that creatinine excretion of any given individual cannot be considered abnormal unless the average daily excretion of the individual (from 10 specimens, for example) is found to fall outside of the normal range.

On the other hand, the excretion of creatine from normal individuals follows a wider distribution than is apparent for creatinine, but in conflict with previous findings the average diurnal excretion in milligrams per 100 lb. per day (0–800 mg.; average, 275) is independent of age and weight for normal individuals between 1 and 76 years (Table 2). The creatine-creatinine ratio for normal individuals was found to be 0.45 in this study, whereas the average reported by Hunter (2) for 40 cases of all ages is about 0.37.

For reasons that are not clearly understood, creatinuria is known to occur in advanced cases of malnutrition (2), and has been reported (14, 15) for several advanced cases of cystic fibrosis. Tocopherol administration is reported to reduce effectively the creatinuria in cystic fibrosis, but was not effective in two cases of biliary atresia (14).

In this study, the ratio of creatine-creatinine for malabsorption syndromes are: cystic fibrosis of the pancreas, 1.2; celiac disease, 1.0; pancreatic insufficiency (a single case), 1.2. Whether these creatinurias are due to a failure to metabolize exogenous creatine, to increased ingestion rates of creatine, or possibly even to accelerated creatine biosynthesis, is not known.

References