Creatine and Creatine Kinase Measurement*

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Many methods for measuring creatine are known, the principal ones being direct colorimetric assay (1), indirect colorimetric assay (2), and destruction with bacterial adaptive enzymes (3). However, none of these methods are specific nor are they sensitive enough to detect very small amounts of creatine. These assays have been used to examine urine and serum for creatine content, but, because of their limitations, there is little agreement about creatine concentrations in these fluids.

It was felt that any study involving creatine would be considerably facilitated by an improved means of determination. In recent years, enzymatic assays have been increasingly used for the measurement of biological materials (4, 5). This report describes an enzymatic assay for the determination of creatine and its application to creatine measurement in serum and urine.

EXPERIMENTAL AND RESULTS

Determination of Creatine—In the attempt to devise an enzymatic assay for creatine the choice of enzyme is limited since creatine is the substrate of only one well characterized enzyme, creatine kinase.

By coupling creatine kinase (Reaction 1) with pyruvate kinase (Reaction 2) and lactic dehydrogenase (Reaction 3), one obtains a summed reaction (Reaction 4) in which the conversion of creatine to creatine phosphate is accompanied by the oxidation of an equimolar amount of DPNH.

\[
\begin{align*}
\text{Creatine} + \text{ATP} &\rightleftharpoons \text{creatine phosphate} + \text{ADP} \\
\text{Phosphoenolpyruvate} + \text{ADP} &\rightleftharpoons \text{pyruvate} + \text{ATP} \\
\text{Pyruvate} + \text{DPNH} + \text{H}^+ &\rightleftharpoons \text{lactate} + \text{DPN}^+ \\
\text{Creatine} + \text{phosphoenolpyruvate} + \text{DPNH} + \text{H}^+ &\rightleftharpoons \text{creatine phosphate} + \text{lactate} + \text{DPN}^+ + \text{H}_2\text{O}
\end{align*}
\]

Under the conditions used Reaction 4 proceeds virtually to completion and the change in absorbancy at 340 μm becomes a direct measure of the amount of creatine in the assay. Fig. 1 illustrates a typical example of a creatine determination. The system is assembled with the exception of creatine kinase. By waiting until there is no further change in absorbancy at 340 μm any ADP or pyruvate present in the system will be consumed. The creatine kinase is then added and the optical density changes are measured until they stop or become linear with respect to time.

The lower baseline is extrapolated to the time of creatine kinase addition to correct for any ATPase or DPNH oxidase activity present in this enzyme. The creatine kinase dependent change in absorbance is taken as a measure of the creatine. In this instance, the change is equivalent to 0.200 μmole of DPN+ as expected for the 0.200 μmole of creatine added to this assay.

The proportionality between absorbancy change and creatine content was examined. Over a 20-fold range of creatine concentrations, (0.44 to 8.8 μg) there is a linear relationship between the change in absorbancy and creatine concentration. It is of interest that at these low concentrations of creatine the kinetics are those of a unimolecular reaction and, hence, the time necessary to achieve completion (99% conversion of creatine to creatine phosphate) is independent of creatine concentration.

Specificity of Assay—In order to gain some insight into the feasibility of applying the assay to biological systems a number of creatine analogues, which might be expected in natural mate-
In order to determine the affinity of creatine kinase for glyco-
cyamine, the reaction rate was measured with various sub-
strate concentrations. Due to the limited solubility of glyco-
cyamine, saturation of creatine kinase could not be obtained.
It was not possible to determine the $K_m$ for glyco-
cyamine. However for purposes of comparison it may be noted that at a
substrate concentration of 2.40 μmoles per ml, the rate of reac-
tion is 240 times greater with creatine than with glyco-
cyamine.

Glyco-cyamine added to the assay system is able to react with
creatine kinase in a noncompetitive manner due to the large
amount of available enzyme present. Furthermore, the glyco-
cyamine rate is linear over the time of creatine determination,
since, at concentrations comparable to creatine it reacts so slowly
its concentration does not perceptibly change while the creatine
is reacting.

Experiments were performed in which equal amounts and 10-
fold excesses of glyco-cyamine were added to the creatine assay.
As can be seen from Table I, the presence of an equal amount
of glyco-cyamine introduces a 2% error. The error becomes more
serious with a 10 fold excess of glyco-cyamine. Fortunately this
situation is readily detected during the determination as a con-
tinued linear decrease in absorbancy after the rapid initial fall
due to creatine.

**Effect of glycocyamine on creatine determination**

Conditions were the same as those of Fig. 1; creatine and glyco-
cyamine were added before the addition of creatine kinase. Total
volume was 1.0 ml in quartz cells, light path = 1.0 cm.

**TABLE I**

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatine</td>
<td>0.030</td>
</tr>
<tr>
<td>Glycocyamine</td>
<td>0.030</td>
</tr>
<tr>
<td>6-minute value</td>
<td></td>
</tr>
<tr>
<td>0.030</td>
<td>0.170</td>
</tr>
<tr>
<td>0.030</td>
<td>0.180</td>
</tr>
<tr>
<td>0.030</td>
<td>0.206</td>
</tr>
</tbody>
</table>

*Completion time for creatine determination; see Fig. 1.*

**Effect of glycocyamine on creatine determination**

Conditions were the same as those of Fig. 1; creatine and glyco-
cyamine were added before the addition of creatine kinase. Total
volume was 1.0 ml in quartz cells, light path = 1.0 cm.

**TABLE II**

**Analytical composition of enzymatically synthesized
 glycocyamine phosphate**

The reaction mixture contained 200 μmoles of phosphoenol-
pyruvate, 21.0 μmoles of ADP, 2100 μmoles of glycocyamine, 20.0
μmoles of MgCl₂, 2.0 mg of pyruvate kinase, and 25.0 mg of crea-
tine kinase. Total volume was 60 ml, the temperature was 25°C,
and the pH adjusted initially to 7.80 with KHCO₃. Hydrolysis of
the isolated calcium salt was accomplished by heating for 2
minutes at 100°C in 1 N HCl.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Before hydrolysis</th>
<th>After hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycocyamine</td>
<td>0.05</td>
<td>3.12</td>
</tr>
<tr>
<td>Inorganic phosphate</td>
<td>0.00</td>
<td>2.93</td>
</tr>
</tbody>
</table>

Bound phosphate
Bound glycocyamine = 0.95

In addition, the assay time and kinetic curves were the same as for pure
creatine solutions.

However, the creatine values in Table III are chiefly contrib-
ted to by artifactitious creatine that arises from the large
amounts of creatine in the urine. Storage of urine, both in the
bladder and during the 24-hour collection period, is responsible
for introducing this artifact. In order to obtain the true creatine
content of urine entering the bladder from the kidneys the fol-
lower experiment was carried out:

Three normal men voided completely at a given time. Four
hours later, they again voided and aliquots of the urine were

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Inorganic phosphate.
measured for pH, creatine, and creatinine. Samples were also incubated in stoppered vessels under toluene at 38°. At various intervals aliquots were removed for pH and creatine determinations. The results are shown in Fig. 2. Extrapolation of the curves to the mean time between the two voidings gives the value for the creatine entering the bladder from the kidneys. The error in creatine determinations of fresh urine, due to bladder incubation, varies and is from 10 to 30% in the present study of urine specimens from men. Anderson et al. (12) have reported 24-hour urinary creatine values for men similar to those of Table III. These workers do not give any information about the artifactitious creatine in such urine specimens.

Comparison of the rates of creatine formation from urinary creatinine (Fig. 2) with those obtained from buffered solutions of pure creatinine at various pH values showed that the urinary rate is about 50% greater. The basis for this phenomenon is not apparent at the present time.

Creatine Determination in Serum and Red Blood Cells—Whereas creatine determinations could be performed directly on urine this was not possible in the case of serum. Creatine was removed from 10.0 ml of rabbit serum by passage of the serum over 1.0 ml of a column of Dowex 50-H+. This step was required to separate the creatine from the large amount of pyruvate present in the serum as this amount will oxidize all the DPNH in the assay system. The creatine was eluted from the column with 5 N NH₂OH and the solution evaporated to dryness. This creatine was then measured after dissolving it in a known volume of water. All of the creatine was removed, as repassage of the same serum over the column did not yield any more creatine. In addition, passage of a known amount of creatine onto this column with subsequent elution gave full recovery of the creatine without conversion to creatinine. The serum contained 13.8 mg per liter of creatine.

Rat red blood cells, 0.50 ml, were hemolyzed in 2.0 ml of distilled water at 0°. A 1.0 ml aliquot was diluted 4-fold and centrifuged at 10,000 X g for 20 minutes. The supernatant was hydrolyzed to convert any creatine phosphate to creatine and passed over a column of Dowex 50-H⁺, as described above. The red cells contained 15.3 mg per liter of creatine.

**TABLE III**

*Urinary creatine*

Conditions were the same as Fig. 1, except: total volume was 1.0 ml in quartz cuvettes, light path = 1.0 cm.

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Total</th>
<th>Body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mg/kg</td>
</tr>
<tr>
<td>19</td>
<td>34.4</td>
<td>0.42</td>
</tr>
<tr>
<td>28</td>
<td>10.1</td>
<td>0.24</td>
</tr>
<tr>
<td>31</td>
<td>26.6</td>
<td>0.42</td>
</tr>
<tr>
<td>10</td>
<td>22.9</td>
<td>0.33</td>
</tr>
<tr>
<td>2</td>
<td>14.6</td>
<td>0.22</td>
</tr>
<tr>
<td>8</td>
<td>21.8</td>
<td>0.27</td>
</tr>
<tr>
<td>6</td>
<td>27.4</td>
<td>0.43</td>
</tr>
<tr>
<td>29</td>
<td>24.0</td>
<td>0.33</td>
</tr>
<tr>
<td>9</td>
<td>18.1</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Mean .................. 0.32 ± 0.09 Men

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Total</th>
<th>Body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>64.1</td>
<td>1.08</td>
</tr>
<tr>
<td>34</td>
<td>103.2</td>
<td>1.81</td>
</tr>
<tr>
<td>16</td>
<td>59.5</td>
<td>0.90</td>
</tr>
<tr>
<td>5</td>
<td>24.6</td>
<td>0.44</td>
</tr>
<tr>
<td>23</td>
<td>32.3</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Mean .................. 0.95 ± 0.36 Women

**TABLE IV**

*Creatine kinase activity of tissues*

Conditions were the same as shown in Fig. 1 except: 12.0 µmoles of creatine, pH 9.0, were added to the assay system. The pH remained constant throughout the experiment and the total volume was 1.0 ml in quartz cells, light path = 1.0 cm.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Soluble protein activity†</th>
<th>Δ absorbance/min./mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrocnemius</td>
<td>4.800</td>
<td></td>
</tr>
<tr>
<td>Diaphragm</td>
<td>3.750</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>1.840</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>0.400</td>
<td></td>
</tr>
<tr>
<td>Large intestine</td>
<td>0.059</td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>0.027</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>Serum, human</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* This amount of creatine is not saturating.
† Measured spectrophotometrically in the supernatant fraction.
of DPNH then becomes dependent upon the amount of creatine kinase present (Fig. 3). The reaction rate is linear with time until most of the DPNH is consumed.

**Creatine Kinase in Tissue**—A variety of rat tissues were examined for creatine kinase activity in order to test the applicability of the creatine kinase assay. A rat weighing 150 g was killed by decapitation and samples of various tissues were excised. These were placed in 1 M glycine, pH 9.0, at 0°C and homogenized in a Vertis-45 at 45,000 r.p.m. for 20 seconds. Centrifugation at 18,000 × g for 20 minutes reduced DPNH oxidase activity. The supernatant was examined for creatine kinase activity and the results are listed in Table IV. The enzyme was not detected in serum although full activity of added creatine kinase was recovered.

**DISCUSSION**

The enzymatic determination of biological substances is often very sensitive and specific. However, inhibition by diverse biological materials and side reactions, by endogenous enzymes, are potential disadvantages. These complications can usually be detected but not always removed, thereby limiting the practicality of the assay. The present method for creatine and creatine kinase measurement has been tested on various biological materials in order to determine its usefulness.

It was shown that glycocyamine could interfere with creatine determinations if present in tissue at a concentration 10 times that of the creatine in the sample. However, glycocyamine is not found in normal tissues in sufficient concentration to cause any interference (18).

In experiments with serum it was found that serum contains sufficient pyruvate to oxidize all of the DPNH in the assay system. Instead of adding more DPNH to the assay, creatine was separated from the pyruvate by ion exchange chromatography. This step is quantitative and also provides a means of concentrating the creatine from serum.

The ATPase and DPNH oxidase activities of tissue can be removed by various protein precipitants. In determining creatine kinase activity of tissue this step is obviously not feasible but the creatine kinase rate is then corrected for the usually small background rate of the side reactions.

Several interesting features of creatine and creatine kinase distribution in the body tissues are brought out in this study. For example, it is often stated that creatine does not occur in urine from normal adults, particularly men (14). Consequently, the “creatinuria” associated with muscular diseases is assumed to be a distinct qualitative change from normal. However, the previous studies were based upon other assay methods which, because of their limitations, could not detect the small amounts of creatine that normally occur in urine. Furthermore, the artifact produced by conversion of creatine to creatine during urinary storage in the bladder could not have been demonstrated by the older assays. Thus, the finding of small but definite amounts of creatine in urine from men and women indicates that the creatinuria of muscular disease is not a distinct qualitative event but only a quantitative change from the normal. Furthermore, the 3-fold difference in creatinuria in men and women will become even more striking when allowance is made for creatine conversion to creatine which occurs in the bladder, since the added artifactual creatine is a constant subtracted from both means.

In the past, creatine kinase activity has been determined by a less sensitive, complicated, and indirect assay method which is not directly applicable to tissue (9). By contrast, the present method is simple and direct, and furthermore, has the advantage of the use of zero order kinetics instead of the second order kinetics of the older assay.

Another spectrophotometric method for determining creatine kinase has been reported by Oliver (18), who also measured creatine kinase activity in tissues. The distribution of this enzyme in rat tissues was similar to that reported in the present study.

The present demonstration of phosphoglycocyamine formation is a measure of the sensitivity of the enzymatic assay. Furthermore, the simple, direct applicability of this assay suggests that it can be used for many practical operations. For example, muscular disease is not usually discovered until its manifestations become clinically obvious. However, it may be possible to detect various myopathies in their early, subclinical stage by this sensitive test for urinary creatine. Any deviation from the normal excretion, especially in men, might provide an early clue to the diagnosis of muscular pathology.

During the past 50 years creatine has been the subject of many investigations. A great deal of this work was conflicting and produced extensive controversy. The chief handicap appears to have been the lack of a direct, specific and sensitive method for measuring creatine. It is felt that the enzymatic assay fulfills these criteria and that further investigations will be aided by its use.

**SUMMARY**

An enzymatic method for creatine and creatine kinase determination is presented which is of greater specificity and sensitivity than that of other methods in common use.

This method has been applied to the estimation of creatine in urine and serum and to creatine kinase determinations in tissue. In contrast to previous studies, creatinuria has been shown to be of general occurrence in adults.

The sensitivity of this method is emphasized by the detection of phosphoglycocyamine synthesis by creatine kinase. The enzymatic preparation of this phosphaen is described.

**REFERENCES**


2. **FOLIN, O., Z. physiol. Chem., 41, 223 (1904).**


5. **MALEY, F., AND OCHOA, S., J. Biol. Chem., 235, 1538 (1958).**

6. **WARBURG, O., AND CHRISTIAN, W., Biochem. Z., 311, 384 (1914).**


