Fluorometric Measurement of Creatine Kinase Activity

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The amount of creatine liberated in the creatine kinase reaction is conveniently measured by formation of a fluophor with ninhydrin in strongly alkaline solution. The method is rapid and sensitive. The incubation conditions developed are more nearly optimal than those in previous methods.

Serum creatine kinase activity has been measured by determining the amount of creatine liberated in the following reaction: ADP + creatine phosphate $\rightarrow$ ATP + creatine. The Voges-Proskauer reaction, production of a color by reaction of creatine with diacetyl and $\alpha$-naphthol, has been used to measure enzymatically released creatine (1-5). The presence of a sulfhydryl compound in the incubation mixture, necessary for the maintenance of a linear reaction rate, interferes with subsequent color development (2, 3, 6). This difficulty has been overcome by addition of the sulfhydryl reagent, $p$-chloromercuribenzoate, and by prolonging the reaction time to 60 min. (2). In 1960, Conn reported a procedure for the determination of creatine, based upon the production of a fluophor with ninhydrin in strongly alkaline solution (7). His observations, that the method is less sensitive to sulfhydryl interference than is the Voges-Proskauer reaction, and that creatine phosphate is non-fluorogenic, suggested that the procedure might be readily adaptable to measurement of creatine kinase activity. This report describes such an application.

Materials and Methods

Creatine hydrate was purchased from Mann Research Laboratories, New York, N. Y. and purified by recrystallization from warm water. ADP sodium, Tris (Trizma Base), and phosphocreatine sodium (creatinine-free) were purchased from Sigma Chemical Co., St. Louis, Mo.; imidazole, triketohydrindene hydrate, and 2-mercaptoethanol, from

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Eastman Organic Chemicals, Rochester, N. Y.; frozen, pooled, non-hemolyzed rabbit serum, from Pel-Freez Biologicals, Rogers, Ark.; creatine kinase, from Nutritional Biochemicals; dichromate test-grade acetic acid, from Merck & Co.; and other chemicals from Fisher Scientific Co.

Fluorescence was measured on a fluorometer* with a No. 110-812 primary filter (peak transmission at 405 m\(\mu\)) and a No. 110-817 secondary filter (passing wavelengths longer than 485 m\(\mu\)). Most specimens were read with a No. 3 slit and 3X range setting.

Reagents

1. Buffer  Dissolve 13.6 gm. of imidazole and 4.3 gm. of Mg(C\(_2\)H\(_3\)O\(_2\))\(_2\)\(\cdot\)4H\(_2\)O in 800 ml. of water. Adjust to a pH of 6.8 at 25\(^\circ\) with glacial acetic acid. Dilute to 1 L. and mix. Store at room temperature.

2. Substrate. Dissolve 95 mg. of phosphocreatine, sodium salt, in 2.5 ml. of buffer and 7.5 ml. of water. Pipet 0.2-ml. aliquots into 16- \(\times\) 100-mm. tubes, stopper, and store in a freezer.

3. ADP solution  Dissolve 0.266 gm. of ADP, sodium salt, in 25 ml. of buffer and sufficient water to make 50 ml. Store frozen in 1- to 2-ml. aliquots.

4. Potassium hydroxide  40% (w/v) solution.

5. Barium hydroxide  0.3N.

6. Zinc sulfate  5% (w/v) solution.

7. Ninhydrin  Prepare a 1% (w/v) solution of triketohydrindene hydrate in 95% ethanol. Store at room temperature in an amber bottle.

8. Standard  Dissolve 0.1492 gm. of creatine hydrate in sufficient water to make 500 ml. Store in the refrigerator. This solution loses about 1% of its creatine content, measured fluorometrically, per week. Stock and recrystallized creatine hydrate produced the same amount of fluorescence.

9. Mercaptoethanol solution  Add 0.1 ml. of 2-mercaptoethanol to 50 ml. of buffer. Prepare immediately before use.

All reagents except the standard and mercaptoethanol solution are stable for at least 1 month.

Proposed Method

1. Add 0.2 ml. of mercaptoethanol solution to substrate tubes, labeled RB (reagent blank), St (Standard), and X (serum).

2. Add 0.1 ml. of water, standard, and serum, to RB, St, and X, respectively; mix and place in 25\(^\circ\) bath for at least 5 min.

3. Pipet 0.2 ml. of ADP solution into all tubes at 30-sec. intervals; mix, and incubate for exactly 10 min.

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4. Stop reaction by addition of 2 ml. of 0.3N barium hydroxide.
5. Add 5 ml. of water to all tubes.
6. Add 2 ml. of 5% zinc sulfate to each tube, shaking vigorously immediately after each addition; let stand for 10 min.
7. Reshake all tubes and centrifuge for 5 min.
8. Pipet 4 ml. of each supernatant into 18-× 100-mm. tubes.
9. Add 1.5 ml. of 95% ethanol and 2 ml. of 1% ninhydrin to each and mix.
10. Starting with RB, add 0.5 ml. of 40% KOH to each tube, mix, and transfer to fluorometer cuvets.
11. Between 5 and 15 min. after the addition of KOH, set RB to 0% F and measure the fluorescence of St and X.
12. Calculation:

\[
\frac{\% F_X \times 200}{\% F_{St}} = \text{units} \times (10^3 \times \text{muoles creatine/ml. serum/min.})
\]

If activity is over 1200 U., repeat assay using diluted serum.

Notes
1. Hemolysis does not interfere (2).
2. Fluorescence reaches a peak about 5 min. after the addition of KOH, and fades slowly thereafter (7). Therefore, for maximum precision, add KOH at 30-sec. intervals and read fluorescence at constant reaction time.
3. If test is delayed, store serums in the freezer (2).*

Results and Discussion

In Conn's procedure (7), equal volumes of ethanolic solutions of ninhydrin and KOH are added to the creatine-containing solution, while in the proposed method, 1.5 ml. of 95% ethanol and 0.5 ml. of 40% aqueous KOH are substituted for 2 ml. of 10% ethanolic KOH. While this change results in a 6% loss of fluorescence, the disadvantage is more than overcome by the ease of preparation and greater stability of the aqueous alkali. No difference in the rate of fading of fluorescence was noted.

Linearity of the combined deproteinization and fluorescence reactions was next examined. Standards corresponding to creatine kinase activities, up to 5000 U., were prepared by addition of creatine to substrate and mercaptoethanol solution and treatment with deproteinizing reagents, as in the proposed method. Aliquots (4 ml.) of the supernatants

*The stability of the enzyme in frozen specimens has been questioned (14). We have observed no loss of activity after at least 1 week at -20°, in serum specimens from normal, postmyocardial-infarction, and muscular-dystrophic subjects.
were subjected to the modified Conn reaction. Results indicated linearity of fluorescence to about 1650 U. (Fig. 1). The slope of the linear portion of the curve is 90% of that obtained with pure aqueous creatine standards. Slit width and range setting were changed whenever F exceeded 100%; the factor is the ratio of %F of the last tube readable under old instrument settings to its %F under new settings. Addition of ADP to standards prior to deproteinizing reagents is without effect on fluorescence. Fluorescence of reagent blanks prepared with Sigma phosphocreatine is consistently low.

The ability of barium hydroxide to stop the enzymatic activity was evaluated. For this experiment, a highly active enzyme source was provided by rabbit serum, containing added creatine kinase and assaying 1940 U. by the proposed method. Identical incubation mixtures were treated with (1) 2 ml. of 0.3N barium hydroxide, (2) 2 ml. of 5% zinc sulfate, (3) 2 ml. each of 0.3N barium hydroxide and 5% zinc sulfate, and (4) 0.3 ml. of 0.03M p-chloromercuribenzoate. Tubes were stoppered and allowed to remain at 25° for 1 hr. before completion of deproteinization. Serum blanks (SB) were prepared in which ADP was replaced by water. Upon the development of fluorescence in the supernatants, no significant differences in net creatine content (X − SB) were observed. However, nonenzymatic hydrolysis of creatine phosphate was appreciable when zinc sulfate was used to stop the reaction. Chappell and Perry (3) utilized an organomercury inhibitor to stop enzyme activity; under
their conditions, the Somogyi deproteinizing reagents, alone, were not completely inhibitory.

The range of linearity of enzymatic activity was determined by measuring creatine kinase activity of rabbit serum by the proposed method, except that the incubation time was varied from 0 to 80 min. Release of creatine proceeded linearly to about $1200 \times 10^{-3} \mu$ mole/ml. of serum, which corresponds to a 23% conversion of creatine phosphate (Fig. 2).

![Graph](image)

**Fig. 2.** Creatine kinase activity of rabbit serum as a function of time.

In selecting a buffer, we first obtained a pH-activity curve with Tris-HCl, using the incubation conditions of Hughes (2) coupled with the proposed deproteinization and fluorescence procedures. The enzyme source was serum from a patient who had had a myocardial infarction. The pH of buffer solutions at 25°C covered the range of 6.5–8.6, corresponding to an incubation mixture pH range of 6.88–8.00 at 38.5°C. In disagreement with the findings of Hughes, we observed no maximum with a pH-7.4 buffer; activity was highest with the pH-6.5 buffer. When maleate buffers (pH 5–7.6 at 25°C) were substituted for Tris, maximum
activity was observed at a pH (incubation mixture at 39°) between 6.4 and 6.5. With imidazole buffers (pH, 5.7–7.2 at 25°), maximum activity observed at a pH (incubation mixture at 25°) between 6.7 and 6.8. Imidazole was selected because the pH of maximum activity nearly coincides with the pK of the buffer system, low fluorescence of blanks was obtained, and activity is sufficient to permit incubation at 25°. With the use of incubation-mixture buffer concentrations of 0.05, 0.1, and 0.3M, equal activities were observed at the two lowest concentrations, while a 14% decrease was noted at the highest concentration.

For determining optimal concentrations of components of the incubation mixture, initial concentrations chosen represent a consensus of published methods (1–5, 8–10). Use of chloride and sulfate in preparation of reagents was avoided, since those anions have been shown to inhibit creatine kinase activity (11). Initial and final concentrations selected are shown in Table 1. Substances were tested in the sequence shown; adjustments to optimal concentrations were made as the experiment progressed. Buffer concentration (0.1M), buffer pH (6.8), and incubation temperature (25°) were held constant. Fresh, randomly selected human serums were pooled and used as the enzyme source. Thio- glycolic acid was also tested and found to be slightly less stimulatory than 2-mercaptoethanol. Omission of 2-mercaptoethanol resulted in a 79% loss of activity, but increases to 10 times the original concentration resulted in no further stimulation. Because of the complexity of the reaction, it is quite possible that with 0.0075M creatine phosphate as a starting point, further adjustments in concentrations of the other constituents might produce still higher activities.

Creatine kinase levels of serum from patients who had had myocardial infarctions were measured at 6 temperatures between 20 and 38°. From an Arrhenius plot of data by the method of least squares, the following equation was obtained: \( \log_{10}k = -3004/T^\circ + 10.086 \), where \( k \) is the ratio of the reaction velocity at \( T^\circ \) (Absolute) to the velocity at 25° C. \( Q_{10} \) (25–35°) is 2.16. The ratio of activity at 37° to that at 25° is 2.49.

**Table 1. Initial and Final (Optimal) Concentrations of Components of Incubation Mixture**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Initial</th>
<th>Range (M)</th>
<th>Final</th>
<th>Activity increase initial to final (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>.003</td>
<td>.001–.006</td>
<td>.003*</td>
<td>0</td>
</tr>
<tr>
<td>Magnesium acetate</td>
<td>.004</td>
<td>0–.012</td>
<td>.010</td>
<td>19</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>.004</td>
<td>0–.040</td>
<td>.008</td>
<td>0</td>
</tr>
<tr>
<td>Creatine phosphate</td>
<td>.003</td>
<td>.001–.015</td>
<td>.0075</td>
<td>21</td>
</tr>
</tbody>
</table>

*Higher concentrations were inhibitory.
Human and rabbit serum specimens were incubated for 10 min. with the use of reagents and conditions described by Hughes (2) and as proposed. Deproteinization and fluorescence development, in both cases, were performed according to our procedure. Creatine kinase activities of the human and rabbit sera were 64 and 526 U., respectively, by the proposed method; activities were 60 and 492 U., respectively, with Hughes’ incubation conditions. Considering that Hughes incubated the reaction at 37°, the gain in sensitivity achieved in the proposed method is more than twofold.

Normal creatine kinase activities were calculated by the N.E.D. method (12), from analysis of sera obtained from 18 female student nurses and 22 male nonprofessional blood donors. Mean activities were 25.8 and 27.2 U. for females and males, respectively; 95% limits were 13–52 for females and 13–57 for males. One apparently normal 23-year-old male, not included in this series, had an activity of 222 U.; questioning revealed that he had played basketball for 2 hr. on the previous evening. Creatine kinase elevations resulting from strenuous muscular activity have been noted by Colombo et al. (13). Serum blanks (ADP omitted) were included for all of the normal subjects, but excluded from the above calculations. Mean and maximum blanks were 3.1 and 7.0 U., respectively. Higher blanks have not been observed in sera from patients after myocardial infarctions, in which creatine kinase activities range up to 1180 U. Therefore, in agreement with Duma and Siegel (5), we recommend that serum blanks be omitted.

Precision was measured in the normal range by duplicate analyses of 21 serums. Separate aliquots of each serum were frozen immediately after removal from the clot. Activities of the 2 aliquots were determined separately. The mean and the standard deviation were 28.9 ± 4.1 U. From separate analyses of 6 aliquots of rabbit serum, the mean and the standard deviation were found to be 648 ± 27 U. The routine use of frozen rabbit serum to monitor precision and to detect loss of substrate is strongly recommended.

Our unit of enzyme activity, \(10^3 \times \mu\text{mole/ml./min.}\), was chosen because it is more nearly consistent with recommendations of the International Union of Biochemistry than some other units (2, 5), and because it permits reporting of clinical results in whole numbers, as suggested by Hess and MacDonald (14).

References
