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 determinaton of creatine and its application to creatine measurement in serum and urine.

EXPERIMENTAL

Materials and Methods

Protein was determined by the method of Warburg and Christian (6). Inorganic phosphate was measured by a modified Fiske-SubbaRow method with ascorbic acid as a reducing agent (7). Glycocyamine was measured by the diacetyl-α-naphthol method of Eggleton et al. (1). Creatine phosphate was hydrolyzed by the method of Barker and Ennor (8). Absorbancy measurements were made in a Beckman model DU spectrophotometer.

Creatine and glycocyamine were obtained from the California Corporation for Biochemical Research. Crystalline lactic dehydrogenase was obtained from C. F. Boehringer and Sons, Mannheim, Germany, and did not have pyruvate kinase, creatine kinase, or ATPase activities. The specific activity was 27 μmoles per minute per mg of protein, as measured in the creatine assay system with a saturating amount of pyruvate.

Crystalline pyruvate kinase was a gift of Dr. A. Tietz and did not have any lactic dehydrogenase, creatine kinase, or ATPase activities. The specific activity was 21 μmoles per minute per mg of protein as measured in the creatine assay system with a saturating amount of ADP.
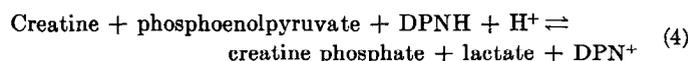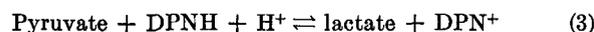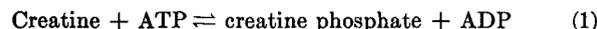
Creatine kinase was crystallized from rabbit muscle by the short method of Kuby et al. (9), and did not have pyruvate kinase or lactic dehydrogenase activities. Slight residual ATPase activity remained after two crystallizations. The specific

activity was 130 μmoles per minute per mg of protein, measured as described in the results.

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.

$$\text{Creatine} + \text{ATP} \rightleftharpoons \text{creatine phosphate} + \text{ADP} \quad (1)$$

$$\text{Phosphoenolpyruvate} + \text{ADP} \rightleftharpoons \text{pyruvate} + \text{ATP} \quad (2)$$

$$\text{Pyruvate} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{lactate} + \text{DPN}^+ \quad (3)$$

$$\text{Creatine} + \text{phosphoenolpyruvate} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{creatine phosphate} + \text{lactate} + \text{DPN}^+ \quad (4)$$

Under the conditions used Reaction 4 proceeds virtually to completion and the change in absorbancy at 340 mμ 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 mμ 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-
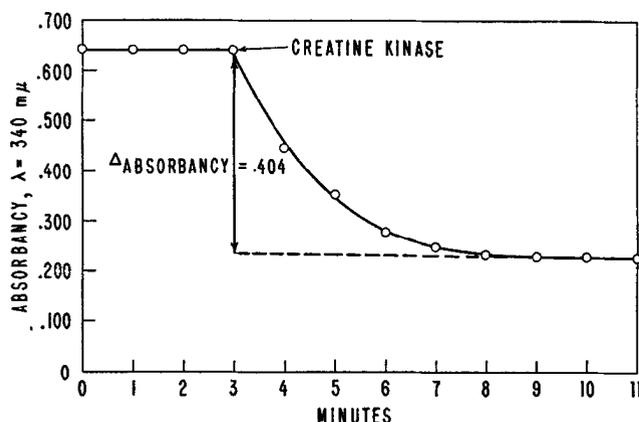
FIG. 1. The determination of creatine. The following components were present in the reaction mixture: 150 μmoles of glycine buffer, pH 9.0; 1.00 μmole of $MgCl_2$; 0.32 μmole of DPNH; 1.00 μmole of ATP; 2.00 μmoles of phosphoenolpyruvate; 0.200 μmole of creatine; 0.024 mg of lactic dehydrogenase; 0.060 mg of pyruvate kinase; and 1.00 mg of creatine kinase. The total volume was 3.0 ml in quartz cells, light path = 1 cm.

TABLE I

Effect of glycocyamine on creatine determination

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

Creatine	Glycocyamine	8-minute value*
μmole	*μmole*	Δ *absorbancy*
0.030	0	0.176
0	0.030	0.004
0.030	0.030	0.180
0	0.300	0.036
0.030	0.300	0.206

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

TABLE II

Analytical composition of enzymatically synthesized glycocyamine phosphate

The reaction mixture contained 200 μmoles of phosphoenolpyruvate, 21.0 μmoles of ADP, 2100 μmoles of glycocyamine, 20.0 μmoles of $MgCl_2$, 2.0 mg of pyruvate kinase, and 25.0 mg of creatine kinase. Total volume was 60 ml, the temperature was 25°, and the pH adjusted initially to 7.80 with $KHCO_3$. Hydrolysis of the isolated calcium salt was accomplished by heating for 2 minutes at 100° in 1 N HCl.

Constituent	Before hydrolysis	After hydrolysis
	μmole/mg	*μmoles/mg*
Glycocyamine........	0.05	3.12
Inorganic phosphate..	0.00	2.93

$$\frac{\text{Bound phosphate}}{\text{Bound glycocyamine}} = 0.95$$

rials, were evaluated for their capacity to interfere with the assay. They were tested for the ability to act as substrates and become phosphorylated or to act as inhibitors. Creatinine, arginine, citrulline, ureidosuccinate, canavanine, and glycocyamidine were without effect on the assay. Only glycocyamine interfered with creatine determination. This effect was more extensively investigated.

In order to determine the affinity of creatine kinase for glycocyamine, the reaction rate was measured with various substrate concentrations. Due to the limited solubility of glycocyamine, saturation of creatine kinase could not be obtained. It was not possible to determine the K_m for glycocyamine. However for purposes of comparison it may be noted that at a substrate concentration of 2.40 μmoles per ml, the rate of reaction is 240 times greater with creatine than with glycocyamine.

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

Enzymatic Preparation of Phosphoglycocyamine—Although ATP formation from synthetic glycocyamine phosphate has been shown (10), the reverse reaction had not been demonstrated previously (10, 11).

In order to prove that glycocyamine phosphate formation was indeed catalyzed by creatine kinase, a large scale incubation was set up and glycocyamine phosphate was isolated from the reaction mixture according to the procedure of Fawaz and Seraidarian (10). This resulted in a calcium salt of 60% purity containing almost equal amounts of bound phosphate and bound glycocyamine (Table II).

Creatine in Urine—In order to explore further the usefulness of the assay it was applied to the measurement of the creatine in urine. In preliminary experiments it was found that, apart from the necessity of adjusting the urine to pH 9.0 and centrifugation of the insoluble material, no other treatment of the urine was required.

Urines from normal men and women were examined for creatine content; 24-hour samples were collected under toluene to inhibit bacterial growth. Urine specimens were adjusted to pH 9.0 with 18 N KOH and kept at 0° to limit conversion of creatinine to creatine. Used for each sample was 0.10 ml of urine, and the total creatine was computed from the measured 24-hour volume (Table III).

No inhibitors of the assay were present in the urine as shown by increasing the urine samples to 2-fold and 4-fold, with resultant 2-fold and 4-fold increase in creatine values. 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 contributed to by artifactitious creatine that arises from the large amounts of creatinine 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 following 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

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 NH₄OH 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 × 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.

Determination of Creatine Kinase—It is also possible to measure creatine kinase activity with the same assay system simply by adding a saturating amount of creatine. The rate of oxidation
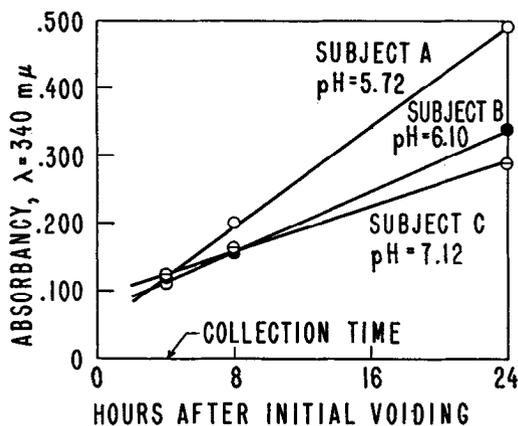

FIG. 2. The effect of urine incubation upon the urinary creatine content. The experimental conditions are described in the text. The pH remained constant throughout the experiment and the sample was 0.30 ml measured in 1.0 ml total assay volume. Quartz cells, light path = 1.0 cm.
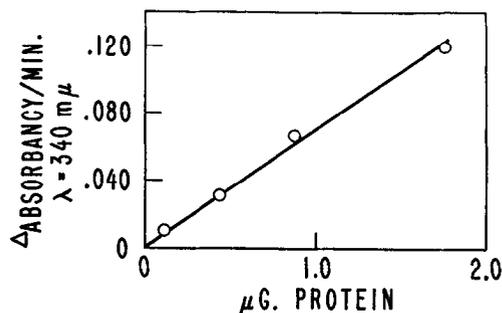

FIG. 3. The creatine kinase standard curve. The conditions were the same as Fig. 1 except: 100 μmoles of creatine, pH 9.0, were added to the assay system and the total volume was 1.0 ml in quartz cells, light path = 1.0 cm.

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.

Subject No.	Total	Body weight
	mg	*mg/kg*
19	34.4	0.42
28	19.1	0.24
31	26.6	0.42
10	22.9	0.33
2	14.6	0.22
8	21.8	0.27
6	27.4	0.43
29	24.0	0.33
9	18.1	0.20
Mean		0.32 ± 0.09 Men
20	64.1	1.08
34	103.2	1.81
16	59.5	0.90
5	24.6	0.44
23	32.3	0.52
Mean		0.95 ± 0.55 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.

Tissue	Soluble protein activity†
	Δ absorbancy/min./mg
Gastrocnemius	4.800
Diaphragm	3.750
Brain	1.540
Heart	0.400
Large intestine	0.059
Small intestine	0.023
Lung	0.011
Kidney	0.008
Serum, human	0

* 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° 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 (13).

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 creatinine to creatine during urine 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 creatinine conversion to creatine which occurs in the bladder, since the added artifactitious 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 (15), 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, expecially 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 phosphagen is described.

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