# Creatine

Erich Bernt, Hans-Ulrich Bergmeyer and Hans Möllering

Creatine, methylguanidinoacetic acid, is chiefly found in the muscles of different organisms. It constitutes 0.3 – 0.5 % of the fresh weight. It also occurs in blood, in brain, in certain transudates and in the thyroid glands. The present methods of determining creatine in body fluids and in muscle depend on direct or indirect colorimetric measurements; these methods are not specific and are not very sensitive.

Creatine can be phosphorylated with ATP and creatine phosphokinase (CPK) to creatine phosphate. This specific reaction is the basis for the following description of the measurement of creatine in serum, which is based in turn on the work of *Tanzer* and *Gilvarg*[1].

## Principle

Creatine phosphokinase (CPK) catalyses the reaction:

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

The ADP is phosphorylated by phosphoenolpyruvate (PEP) and pyruvic kinase (PK):

(2) $$\text{ADP} + \text{PEP} \longrightarrow \text{ATP} + \text{pyruvate}$$

The pyruvate which is formed is reduced by reduced diphosphopyridine nucleotide (DPNH) and lactic dehydrogenase (LDH):

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

The decrease in DPNH concentration, measured by the change in optical density at 340 or 366 mμ, is proportional to the amount of creatine.

The equilibrium of equation (1) depends to a great extent on the pH; in the weakly acid range it is in favour of creatine and in the weakly alkaline range it is in favour of creatine phosphate. The measurements are therefore carried out at pH 9. The turnover number of the enzyme is relatively low (25000 moles creatine/mole enzyme/min. at 38°C). Relatively large amounts of enzyme (*ca.* 3 mg./assay mixture) are therefore required to enable the reaction to proceed at a suitable rate. This is particularly necessary with serum, because certain constituents of serum inhibit the enzyme. So far it has not proved possible to eliminate this inhibition.

## Reagents

1. Triethanolamine hydrochloride
2. Potassium carbonate, A. R.
3. Sodium hydrogen carbonate, A. R.
4. Magnesium chloride, $MgCl_2 \cdot 6H_2O$, A. R.
5. Perchloric acid, A. R., sp. gr. 1.67, 70 % (w/w)
6. Phosphoenolpyruvate, PEP
   tricyclohexylammonium salt; commercial preparation, see p. 1024.
7. Adenosine triphosphate, ATP
   disodium salt, ATP-$Na_2H_2 \cdot 3H_2O$; commercial preparation, see p. 1006.
8. Reduced diphosphopyridine nucleotide, DPNH
   sodium salt, DPNH-$Na_2$; commercial preparation, see p. 1011.
9. Creatine phosphokinase, CPK
   from rabbit skeletal muscle, lyophilized; specific activity at least 10 units/mg. according to *Racker et al.* *). Commercial preparation, see p. 973.

*) Definition of a unit, see p. 32, 33.

1) *M. L. Tanzer* and *C. Gilvarg*, J. biol. Chemistry *234*, 3201 [1959].

10. Lactic dehydrogenase, LDH
crystalline, from rabbit skeletal muscle, suspension in 2.2 M ammonium sulphate solution, pH *ca.* 7. Specific activity at least 360 units/mg. according to *Racker et al.* *).

11. Pyruvic kinase, PK
crystalline, from rabbit skeletal muscle, suspension in 2.1 M ammonium sulphate solution, pH *ca.* 6. Specific activity at least 90 units/mg. according to *Racker et al.* *). Commercial preparation, see p. 997.

**Purity of the reagents**

The LDH and PK preparations must be free from creatine phosphokinase, also all three enzyme preparations must not contain more than 0.001% ATPase and myokinase (relative to the CPK activity). The ATP and PEP must be reasonably free from ADP and pyruvate respectively.

## Preparations of Solutions

Prepare all solutions with fresh, doubly distilled water. To prevent bacterial contamination, sterilize the containers.

I. Triethanolamine-$K_2CO_3$ solution (0.43 M triethanolamine; 0.54 M $K_2CO_3$):
Dissolve 2.0 g. triethanolamine hydrochloride and 1.9 g. $K_2CO_3$ in doubly distilled water and make up to 25 ml.

II. Phosphoenolpyruvate-magnesium chloride solution ($1 \times 10^{-2}$ M PEP; 0.4 M $MgCl_2$):
Dissolve 14 mg. PEP (tricyclohexylammonium salt) and 300 mg. $MgCl_2 \cdot 6H_2O$ in 3.0 ml. doubly distilled water.

III. Sodium hydrogen carbonate (5% w/v):
Dissolve 5 g. $NaHCO_3$ in doubly distilled water and make up to 100 ml.

IV. Reduced diphosphopyridine nucleotide-adenosine triphosphate solution ($5 \times 10^{-3}$ M β-DPNH; $2.5 \times 10^{-2}$ M ATP):
Dissolve 16 mg. DPNH-$Na_2$ and 30 mg. ATP-$Na_2H_2 \cdot 3H_2O$ in 2 ml. $NaHCO_3$ solution (III).

V. Lactic dehydrogenase-pyruvic kinase, LDH-PK (1 mg. of each protein/ml.):
Dilute the stock suspensions with 2.1 ammonium sulphate solution and mix equal parts.

VI. Creatine phosphokinase, CPK (60 mg. protein/ml.):
Dissolve 60 mg. of lyophilized protein in 1 ml. of a 1 : 10 dilution of $NaHCO_3$ solution (III). Prepare the solution freshly each day.

VII. Perchloric acid (*ca.* 6% w/w):
Dilute 5.2 ml. 70% $HClO_4$ to 100 ml. with doubly distilled water.

**Stability of the solutions**

Store all solutions and suspensions, stoppered, in a refrigerator at 0 to 4° C. In this state they keep for several weeks. Prepare the PEP-magnesium solution and the DPNH-ATP solution freshly each week. Solutions I and VII are stable indefinitely.

## Procedure

**Deproteinization**

Use only fresh serum free from haemolysis.

Pipette successively into a centrifuge tube 5 ml. ice-cold perchloric acid solution (VII) and 5 ml. serum, mix thoroughly with a thin glass rod and centrifuge for 15 min. at 3000 r.p.m.

To 4 ml. of the supernatant add 1.1 ml. solution I, and after allowing to stand for 15 min. in an ice bath, filter off the precipitate of potassium perchlorate. After equilibrating at *ca.* 25°C, take 2.00 ml. of this solution, which is buffered at *ca.* pH 9, for the assay.

**Spectrophotometric measurements**

Wavelength: 340 or 366 mμ; light path: 1 cm.; final volume: 2.35 ml.; room temperature. Pipette successively into the cuvette:

2.00 ml. deproteinized sample (neutralized and buffered)
0.15 ml. PEP-magnesium solution (II)
0.10 ml. DPNH-ATP solution (IV)
0.05 ml. LDH-PK suspension (V).

Mix thoroughly with a thin glass rod and observe the optical density change until constant (10—15 min.). Then read the optical density $E_1$.

Mix in

0.05 ml. CPK solution (VI).

After 50, 55 and 60 min. read the optical density; by extrapolation of these values to the time of the addition of the CPK (refer to p. 39) determine the final optical density $E_2$. $E_1 - E_2 = \Delta E$ is used for the calculations.

**Calculations**

Under the given conditions the reaction proceeds stoichiometrically according to equations (1), (2) and (3). According to the extinction coefficients of DPNH, with a 1 cm. light path, an optical density change of 0.100 corresponds to 0.016 μmoles DPNH/ml. at 340 mμ and 0.030 μmoles DPNH/ml. at 366 mμ, and therefore to the same amount of creatine. Or with a final volume in the cuvette of 2.35 ml., an optical density change of 0.100 corresponds to 0.0376 μmoles = 4.93 μg. creatine at 340 mμ; 0.071 μmoles = 9.3 μg. creatine at 366 mμ.

To obtain the creatine content per ml. serum it is necessary to allow for the dilution on deproteinization (1 + 1) and on precipitation of the perchlorate (4 + 1.1) and also for the amount taken for assay (2 ml.) by multiplying by $2 \times (5.1/4) \times 0.5 = 1.275$.

Therefore for measurements at 340 mμ

$\Delta E \times 62.9$ = μg. creatine/ml. serum
$\Delta E \times 6.29$ = mg.% creatine in serum

at 366 mμ

$\Delta E \times 118.5$ = μg. creatine/ml. serum
$\Delta E \times 11.85$ = mg.% creatine in serum

## Sources of Error

Insufficient purity of the reagents (see p. 408) especially of the enzymes, leads to creatine values which are too high. If the PEP and ATP contain too much pyruvate and ADP respectively, then too much DPNH will be consumed before the start of the creatine reaction. In this case more DPNH must be added before the addition of CPK.

If the activity of the CPK preparation is too low this results in low values for the creatine; with 1.5 mg. of the enzyme only 80% of the added creatine is recovered. Solutions of CPK are inactivated < pH 7, therefore high dilutions in distilled water are to be avoided.

The removal of pyruvate from the sample with ion exchange resins[1] before the enzymatic determination is unnecessary; it can easily lead to loss of creatine.

## Specificity

Creatine phosphokinase is specific for creatine and adenosine-5′-triphosphate.

Creatinine, arginine, citrulline, ureidosuccinate, canavanine and glycocyamidine do not effect the determination. Glycocyamine reacts with creatine phosphokinase, but only at a $^1/_{40}$ of the rate of creatine.

Neither adenosine-5′-diphosphate nor inosine-5′-triphosphate act as phosphate donors.

## Details for Measurement in Tissues

With homogenates it is important to make sure that the filtrate after deproteinization is free from perchlorate and is adjusted to pH 9. Homogenates of, for example, skeletal muscle of the mouse contain so much creatine (*ca.* 0.6%) that only a 0.5% homogenate can be analysed. In this case, the amounts of triethanolamine-$K_2CO_3$ solution (I) given for serum are no longer sufficient to attain pH 9. Use more of solution I and allow for this in the calculations.