Enzymatic Assay of CREATINE PHOSPHOKINASE (EC 2.7.3.2)

PRINCIPLE:

Phosphocreatine + ADP $\xrightarrow{\text{Creatine Phosphokinase}}$ Creatine + ATP

D-Glucose + ATP $\xrightarrow{\text{Hexokinase}}$ Glucose 6-Phosphate + ADP

Glucose 6-Phosphate + ß-NADP $\xrightarrow{\text{G-6-PDH}}$ 6-PG + ß-NADPH

Abbreviations used:
ADP = Adenosine 5'-Diphosphate
ATP = Adenosine 5'-Triphosphate
G-6-PDH = Glucose-6-Phosphate Dehydrogenase
ß-NADP = ß-Nicotinamide Adenine Dinucleotide Phosphate, Oxidized Form
ß-NADPH = ß-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form
6-PG = 6-Phospho-D-Gluconate

CONDITIONS: T = 30°C, pH = 7.4, A_{340nm}, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 250 mM Glycylglycine Buffer with 0.10% (w/v) Bovine Serum Albumin, pH 7.4 at 30°C.
(Prepare 100 ml in deionized water using Glycylglycine, Free Base, Sigma Prod. No. G-1002, and Albumin Bovine Serum, Sigma Prod. No. A-4503. Adjust to pH 7.4 at 30°C with 1 M NaOH.)

B. 400 mM Phosphocreatine Solution
(Prepare 10 ml in deionized water using Phosphocreatine, Disodium Salt Hydrate, Sigma Prod. No. P-6502.)

C. 40 mM Adenosine 5'-Diphosphate Solution (ADP)
(Prepare 10 ml in deionized water using Adenosine 5'-Diphosphate, Sodium Salt, Sigma Prod. No. A-8146.)

REAGENTS: (continued)

D. 1000 mM D-Glucose Solution
(Prepare 10 ml in deionized water using ß-D(+)Glucose, Sigma Prod. No. G-5250.)

E. 20 mM ß-Nicotinamide Adenine Dinucleotide Phosphate Solution (ß-NADP)
(Dissolve the contents of one 30 mg vial of ß-Nicotinamide Adenine Dinucleotide Phosphate, Sodium Salt, Sigma Stock No. 240-330, in the appropriate volume of deionized water.)

F. 300 mM Magnesium Acetate
(Prepare 5 ml in deionized water using Magnesium Acetate, Tetrahydrate, Sigma Prod. No. M-0631.)

G. Hexokinase Solution
(Immediately before use, prepare a solution containing 300 units/ml of Hexokinase, Sigma Prod. No. H-4502, in cold deionized water.)

H. Glucose-6-Phosphate Dehydrogenase Enzyme Solution (G-6-PDH)
(Immediately before use, prepare a solution containing 10 units/ml of Glucose-6-Phosphate Dehydrogenase, Sigma Prod. No. G-6378, in cold deionized water.)

I. Creatine Phosphokinase Enzyme Solution[1] (Creat PPK)
(Immediately before use, prepare a solution containing 0.3 unit/ml of Creatine Phosphokinase in cold Reagent A.)

PROCEDURE:

Prepare a reaction cocktail by pipetting (in milliliters) the following reagents into a suitable container:

Deionized Water	36.00
Reagent A (Buffer)	10.00
Reagent B (Phosphocreatine)	2.00
Reagent C (ADP)	2.00
Reagent D (D-Glucose)	2.00
Reagent E (ß-NADP)	1.20
Reagent F (Magnesium Acetate)	0.80

Mix and adjust to pH 7.4 at 30°C with 1 M NaOH.

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PROCEDURE: (continued)

Pipette (in milliliters) the following reagents into suitable cuvettes:

	Test	Blank
Reaction Cocktail	2.70	2.70
Reagent G (Hexokinase)	0.10	0.10
Reagent H (G-6-PDH)	0.10	0.10

Mix by inversion and equilibrate to 30°C. Monitor the A_{340nm} until constant, using a suitably thermostatted spectrophotometer. Then add:

Reagent A (Buffer)	------	0.10
Reagent I (Creat PPK)	0.10	---

Immediately mix by inversion and record the increase in A_{340nm} for approximately 5 minutes. Obtain the ΔA_{340nm}/minute using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{340nm}/\text{min Test} - \Delta A_{340nm}/\text{min Blank})(3)(df)}{(6.22)(0.1)}$$

3.0 = Total volume (in milliliters) of assay
df = Dilution factor
6.22 = Millimolar extinction coefficient of ß-NADPH at 340nm
0.1 = Volume (in milliliters) of enzyme used

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

UNIT DEFINITION:

One unit will transfer 1.0 µmole of phosphate from phosphocreatine to ADP per minute at pH 7.4 at 30°C.

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FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mix, the final concentrations are 50 mM glycylglycine, 0.020% (w/v) bovine serum albumin, 13 mM phosphocreatine, 1.3 mM adenosine 5'-diphosphate, 33 mM D-glucose, 0.40 mM ß-nicotine adenine dinucleotide phosphate, oxidized form, 4.0 mM magnesium acetate, 30 units hexokinase, 1.0 unit glucose-6-phosphate dehydrogenase and 0.03 unit creatine phosphokinase.

REFERENCES:

Noda, L., Nihei, T. and Morales, M.F. (1960) *J. Biol. Chem.* **235**, 2830-2834

Forster, G., Bernt, E. and Bergmeyer, H.U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.U. ed) Volume II, 2nd ed., 789-793, Academic Press, Inc., New York, NY

NOTES:

1. Ammonium sulfate and chloride are strong inhibitors of Creatine Phosphokinase.

2. This assay is based on the cited reference.

3. Hexokinase Unit Definition: One unit will phosphorylate 1.0 µmole of D-glucose per minute at pH 7.6 at 25°C.

4. Glucose-6-Phosphate Dehydrogenase Unit Definition: One unit will oxidize 1.0 µmole of D-glucose-6-phosphate to 6-phospho-D-gluconate per minute in the presence of NADP at pH 7.4 at 25°C.

5. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.