Creatine Phosphate

Walther Lamprecht and Philipp Stein

The need for a specific and reliable*) method for the determination of creatine phosphate (CP) in cell and tissue extracts is met by the enzymatic assay with creatine phosphokinase[2] (CPK, ATP-creatine transphosphorylase). This assay is based on the enzymatic determination of the adenosine triphosphate (ATP) formed in the creatine phosphokinase reaction by means of hexokinase and glucose-6-phosphate dehydrogenase (see p. 543). Creatine phosphate can therefore be determined together with ATP and glucose-6-phosphate or hexose monophosphate in a combined assay.

Principle

The enzyme creatine phosphokinase (CPK) catalyses the transfer of phosphate from creatine phosphate to adenosine diphosphate (ADP) (equation 1). In the presence of hexokinase (HK), the ATP formed phosphorylates glucose to glucose-6-phosphate (G-6-P) with the regeneration of ADP (equation 2). Glucose-6-phosphate dehydrogenase (G6P-DH) catalyses the oxidation of G-6-P with triphosphopyridine nucleotide (TPN) (equation 3). In the over-all reaction (equation 4) 1 mole of TPNH is formed for each mole of creatine phosphate. The increase in optical density at 366 mμ due to the formation of TPNH is measured.

(1) $$\text{Creatine phosphate} + \text{ADP} \overset{Mg^{2+}}{\rightleftharpoons} \text{creatine} + \text{ATP}$$

(2) $$\text{Glucose} + \text{ATP} \overset{Mg^{2+}}{\rightleftharpoons} \text{glucose-6-phosphate} + \text{ADP}$$

(3) $$\text{Glucose-6-phosphate} + \text{TPN}^+ \rightleftharpoons \text{6-phosphogluconate} + \text{TPNH} + \text{H}^+$$

(4) $$\text{Creatine phosphate} + \text{glucose} + \text{TPN}^+ \rightleftharpoons \text{creatine} + \text{6-phosphogluconate} + \text{TPNH} + \text{H}^+$$

With sufficient ADP and Mg^{2+}, creatine phosphokinase reacts quantitatively with creatine phosphate. Under optimum conditions 1 mole of enzyme (about 80000 g.) forms 150000 moles ATP/min. The Michaelis constants[3] at 38°C and pH 6–7 are: 5×10^{-3} M for CP and 1×10^{-3} M **) for ADP. The $\Delta F'$ for reaction (1) from left to right is *ca.* 3 kcal. at pH 7.5[2].

The pH optimum of creatine phosphokinase for the formation of ATP is between 6 and 7.

The CPK reaction is activated by divalent metal ions, especially those of the alkaline earths. The activation increases with increase in atomic weight. Mg^{2+} and Mn^{2+} are activators, while Zn^{2+} or Cu^{2+} inhibit. The activation is optimal when the Mg^{2+} concentration is approximately equal to the ADP concentration.

The equilibria of the hexokinase and glucose-6-phosphate dehydrogenase reactions (equations 2 and 3) lie completely to the left when they are coupled together (further details, see p. 544). With a suitable excess of ADP, glucose and TPN, and in the presence of sufficient Mg^{2+} (to activate the CPK and HK reactions) the three coupled enzyme reactions proceed rapidly and quantitatively.

---

*) A comparison of the results by the colorimetric methods for the determination of CP in tissue extracts indicated not only large variations in the individual values, but also there was not even approximate agreement between the over-all results[1].

**) In a two substrate reaction the values for K_M are dependent on the concentration of the other substrates[4].

1) *G. Michal* and *W. Lamprecht*, Hoppe-Seylers Z. physiol. Chem. *324*, 170 [1961].
2) *S. A. Kuby* et al., J. biol. Chemistry *209*, 191 [1954]; *210*, 65, 83 [1954].
3) *L. Noda, S. A. Kuby* and *H. Lardy* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, vol. II, p. 605.
4) *G. Michal*, Diploma Thesis, Technische Hochschule Munich, Germany, 1957.

Reagents

1. Triethanolamine hydrochloride, A. R.
2. Triphosphopyridine nucleotide, TPN
   sodium salt, TPN-NaH_2. Commercial preparation, see p. 1029.
3. Magnesium chloride, A. R., $MgCl_2 \cdot 6\ H_2O$
4. Glucose, A. R., $C_6H_{12}O_6 \cdot H_2O$
5. Perchloric acid, A. R., sp. gr. 1.67 (*ca.* 70% w/w) or sp. gr. 1.54 (*ca.* 60% w/w)
6. Potassium carbonate, A. R., K_2CO_3
7. Methyl orange
8. Adenosine diphosphate, ADP
   sodium salt, ADP-Na_3. Commercial preparation, see p. 1004.
9. Glucose-6-phosphate dehydrogenase, G6P-DH
   from yeast, suspension in 3.3 M ammonium sulphate solution (pH *ca.* 6) or lyophilized preparation. Commercial preparation, see p. 975.
10. Hexokinase, HK
    from yeast, lyophilized or suspended in ammonium sulphate solution. Commercial preparation, see p. 983.
11. Creatine phosphokinase, CPK
    from rabbit muscle, lyophilized preparation. Commercial preparation, see p. 973.

Purity of the enzyme preparations

Creatine phosphokinase of the required purity is available from C. F. Boehringer & Soehne, Mannheim-Waldhof, Germany. Its preparation from rabbit muscle is relatively simple[3], but the further purification is difficult. The specific activity should be at least 100 units/mg. (defined according to *Bücher et al.*[5]). Relative to the CPK specific activity, the preparation must contain less than 0.01% myokinase, ATPase, glucose-6-phosphatase, 6-phosphogluconic dehydrogenase, TPNH oxidase, phosphohexoisomerase, hexokinase or glutathione reductase. For the purity of the hexokinase and glucose-6-phosphate dehydrogenase preparations see the details given in the Section "Determination of ATP with HK and G6P-DH", p. 544.

Preparation of Solutions (for *ca.* 20 determinations)

I. Triethanolamine buffer (0.05 M; pH 7.5—7.6):
   Dissolve 4.65 g. triethanolamine hydrochloride in 200 ml. doubly distilled water, add 11 ml. 1 N NaOH and dilute to 500 ml. with doubly distilled water.

II. Triphosphopyridine nucleotide (*ca.* 7×10^{-3} M β-TPN):
   Dissolve 7.5 mg. TPN-NaH_2 in distilled water and make up to 1.5 ml.

III. Magnesium chloride (0.1 M):
   Dissolve 2.03 g. $MgCl_2 \cdot 6\ H_2O$ in doubly distilled water and make up to 100 ml.

IV. Glucose (0.5 M):
   Dissolve 9.91 g. glucose ($C_6H_{12}O_6 \cdot H_2O$) in doubly distilled water and make up to 100 ml.

V. Perchloric acid (6% w/v):
   Dilute 7.8 ml. 70% $HClO_4$ or 9.7 ml. 60% $HClO_4$ to 100 ml. with doubly distilled water.

[5] *G. Beisenherz, H. J. Boltze, Th. Bücher, R. Czok, K. H. Garbade, E. Meyer-Arendt* and *G. Pfleiderer*, Z. Naturforsch. *8b*, 555 [1953].

VI. Potassium carbonate (*ca.* 5 M):
Dissolve 69 g. K_2CO_3 in 100 ml. doubly distilled water.

VII. Methyl orange indicator:
Dissolve 50 mg. methyl orange in 100 ml. doubly distilled water.

VIII. Adenosine diphosphate, ADP (*ca.* 5 mg./ml.):
Dissolve 10 mg. ADP-Na_3 in 2 ml. doubly distilled water.

IX. Glucose-6-phosphate dehydrogenase, G6P-DH:
Dissolve 10—15 mg. lyophilized enzyme in 1 ml. doubly distilled water or dilute 0.3 ml. enzyme suspension to 1.5 ml. with doubly distilled water (→ 0.2 mg. protein/ml.).

X. Hexokinase, HK (1—10 mg. protein/ml.):
Dissolve 2—20 mg. lyophilized enzyme in 2 ml. doubly distilled water or dilute the enzyme suspension with doubly distilled water.

XI. Creatine phosphokinase, CPK (1—1.5 mg. protein/ml.):
Dissolve 1—1.5 mg. lyophilized enzyme in 1 ml. doubly distilled water.

Stability of the solutions

Store all solutions, stoppered, at 1—4° C. Prepare the TPN and glucose solutions freshly each week, the enzyme solutions from lyophilized powders daily, and the G6P-DH suspension diluted with water every 2—3 days.

Procedure

Experimental material

Freeze tissue samples within a fraction of a second [6]. (Use "jaws" made from aluminium or light alloy blocks, which have been pre-cooled in liquid air). Creatine phosphate is exceedingly labile in the presence of creatine phosphokinase *).

Deproteinization

See "Determination of ATP with Hexokinase and Glucose-6-phosphate Dehydrogenase" (p. 546).

Spectrophotometric measurements

Preliminary remarks: The volume of the sample (V_5) taken for the assay is so arranged that the optical density change is 0.025—0.150 and the reaction is complete in 30 min.

Method: Wavelength: 366 mμ; light path in assay (a): 1 cm., in assay (b): 2 cm.; final volume (Vc) in assay (a): 3.19 ml., in assay (b): 5.19 ml. Measure against a control cuvette containing 4 ml. buffer (solution I).

Assay (a): Determination of CP in the absence of ATP and hexose monophosphates. Suitable for the assay of the purity of CP preparations and determination of CP isolated or separated by electrophoresis or by paper or column chromatography.

*) For this reason CP assays on rat heart muscle give erroneous results, if, as is usual, the thorax of the narcotized animal is opened. Interruption of respiration causes an instantaneous decrease in the CP content of the heart muscle [7].

[6] *A. Wollenberger, O. Ristau* and *G. Schaffer*, Pflügers Arch. ges. Physiol. Menschen Tiere *270*, 399 [1960].

[7] *D. Michel, W. Lamprecht, Ph. Stein* and *J. Eisenburg*, Verh. dtsch. Ges. Kreislaufforsch. *27*, 247 [1961].

Pipette into the cuvette:

deproteinized sample	0.10 ml.	
buffer (solution I)	2.40 ml.	
TPN solution (II)	0.10 ml.	
$MgCl_2$ solution (III)	0.10 ml.	
ADP solution (VIII)	0.10 ml.	3 min.; E_1
G6P-DH solution (IX)	0.02 ml.	5 min.; E_2 ($G6P\text{-}DH_1$)
G6P-DH solution (IX)	0.02 ml.	1 min.; E_3 ($G6P\text{-}DH_2$)
HK solution (X)	0.05 ml.	15 min.; E_4 (E_{ATP}, $\Delta E_{ATP/ADP}$)
HK solution (X)	0.05 ml.	1 min.; E_5 (E_{HK})
glucose solution (IV)	0.20 ml.	$^1/_2$ min.; E_6 ($E_{glucose}$)
CPK solution (XI)	0.05 ml.	15–25 min.; E_7 (E_{CP}, ΔE_{CP})
CPK solution (XI)	0.05 ml.	1 min.; E_8 (E_{CPK})

Read the initial optical density of the mixture before the addition of the enzymes over a period of 1–3 min. (E_1). Pipette the stated amounts of the enzymes onto a small plastic spoon*) and mix into the solution in the experimental cuvette. After the first addition of enzyme (0.02 ml. G6P-DH soln.) follow the change in optical density for 5 min. (E_2), then mix a further 0.02 ml. G6P-DH solution into the cuvette and after 1 min. read the optical density (E_3). The optical density change $E_3 - E_2$ (*i.e.* optical density change $E_{G6P\text{-}DH_2}$ due to the addition of G6P-DH) should be the same as the optical density change $E_2 - E_1 = E_{G6P\text{-}DH_1}$.

After the addition of 0.05 ml. HK solution a change in optical density occurs which varies with purity of the ADP preparation; after 15 min. the optical density is constant (E_4). The addition of a further 0.05 ml. HK solution gives the optical density change due to the HK solution. After 1 min. record the optical density (E_5). Subtract $E_5 - E_4 = E_{HK}$ from the difference $E_4 - E_3 = E_{ATP}$:

$(E_4 - E_3) - (E_5 - E_4) = E_{ATP} - E_{HK} = \Delta E_{ATP/ADP}$ serves to calculate the ATP content of the ADP preparation.

After the addition of the glucose solution a small decrease in optical density occurs (due to dilution of the assay mixture), which is complete in less than $^1/_2$ min. (E_6).

The addition of 0.05 ml. CPK solution starts the reaction with CP; it is usually complete in 15–25 min. The change in optical density is followed until constant (E_7). A further 0.05 ml. CPK solution gives the optical density change due to the CPK solution. Read the optical density after 1 min. (E_8).

Subtract $E_8 - E_7 = E_{CPK}$ from the difference of $E_7 - E_6 = E_{CP}$:
$(E_7 - E_6) - (E_8 - E_7) = E_{CP} - E_{CPK} = \Delta E_{CP}$ is used for the calculations.

*) Small polyethylene rods (diameter *ca.* 3 mm.); analogous to the well-known glass spoons which can be made in the laboratory. In contrast to glass spoons, they do not scratch or damage the walls of the cuvettes (see Fig. 3, p. 17).

Assay (b): Determination of CP in the presence of ATP and hexose monophosphates and of ATP and hexose monophosphates in the presence of CP. Suitable for the analysis of tissues and organs.

Pipette into the cuvette:

deproteinized sample	0.10 ml.	
buffer (solution I)	4.00 ml.	
TPN solution (II)	0.10 ml.	
$MgCl_2$ solution (III)	0.35 ml.	3 min.; E_1
G6P-DH solution (IX)	0.02 ml.	5 min.; E_2 (E_{HMP})
G6P-DH solution (IX)	0.02 ml.	1 min.; E_3 ($E_{G6P\text{-}DH}$; ΔE_{HMP})
glucose solution (IV)	0.40 ml.	$^1/_2$ min.; E_4 ($E_{glucose}$)
HK solution (X)	0.05 ml.	15 min.; E_5 (E_{ATP})
HK solution (X)	0.05 ml.	1 min.; E_6 (E_{HK}; ΔE_{ATP})
ADP solution (VIII)	0.05 ml.	5 min.; E_7 ($E_{ATP/ADP}$)
CPK solution (XI)	0.05 ml.	15—25 min.; E_8 (E_{CP})
CPK solution (XI)	0.05 ml.	1 min.; E_9 (E_{CPK}; ΔE_{CP})

Read the initial optical density of the mixture before the addition of the enzymes over a period of 1—3 min. (E_1). Pipette the stated amounts of the enzyme solutions onto a small plastic spoon *) and mix into the solution in the experimental cuvette. After the first enzyme addition (0.02 ml. G6P-DH soln.) follow the change in optical density for 5 min. (E_2), then mix a further 0.02 ml. G6P-DH solution into the cuvette and after 1 min. read the optical density (E_3). Subtract the optical density difference $E_3 - E_2$ (optical density change due to the addition of the G6P-DH) from the difference of $E_2 - E_1 = E_{HMP}$:

$(E_2 - E_1) - (E_3 - E_2) = E_{HMP} - E_{G6P\text{-}DH} = \Delta E_{HMP}$ is the optical density difference corresponding to the HMP or G-6-P content of the sample (the latter only with highly purified G6P-DH preparations).

After the addition of the glucose solution observe the decrease in optical density (due to dilution of the assay mixture) for not longer than 30 sec. and read E_4 **).

The addition of 0.05 ml. HK solution starts the reaction with ATP and it is usually complete in about 12 min. Follow the change in the optical density for 15 min. until constant (E_5) ***).

*) See the footnote on p. 613.

**) Since the G6P-DH preparation is contaminated with hexokinase (in spite of this being less than 0.01 %), the addition of glucose results in a small but significant reaction of ATP (*ca.* $\Delta E = 0.003$/min.). This change in optical density is only observed after 3—5 min., therefore the HK should be added soon after the glucose, and certainly not later than 1 min. For the same reason, the glucose is not added until after the G6P-DH.

***) Often after 20—25 min. a decrease in optical density is observed (TPNH oxidase?), which, however, does not interfere with the measurements of ΔE_{ATP}. Nevertheless, in such cases the enzyme preparations (usually the HK preparation) are unsuitable for the determination of CP.

Addition of a further 0.05 ml. HK solution gives the change in optical density due to the HK solution. After 1 min. read the optical density (E_6).

Subtract $E_6 - E_5 = E_{HK}$ from the difference of $E_5 - E_4 = E_{ATP}$:
$(E_5 - E_4) - (E_6 - E_5) = E_{ATP} - E_{HK} = \Delta E_{ATP}$ is used to calculate the ATP content of the sample.

After the addition of 0.05 ml. ADP solution a change in optical density usually occurs (depending on the purity of the ADP preparation). After 5 min. read the optical density (E_7). $(E_7 - E_6) = \Delta E_{ATP/ADP}$ is the optical density difference which corresponds approximately to the ATP content of the ADP preparation.

The reaction with CP is started by the addition of 0.05 ml. CPK solution; it is usually complete in 15–25 min. The change in optical density is observed for 30 min. (E_8). A further 0.05 ml. CPK solution gives the optical density change due to the addition of the enzyme solution; read the optical density after 1 min. (E_9).

Subtract $E_9 - E_8 = E_{CPK}$ from the difference of $E_8 - E_7 = E_{CP}$:
$(E_8 - E_7) - (E_9 - E_8) = E_{CP} - E_{CPK} = \Delta E_{CP}$ is the optical density difference corresponding to the CP content of the sample.

Calculations

Under the stated conditions the reactions proceed stoichiometrically. Double the amount of deproteinized sample gives double the value for ΔE_{CP} (and ΔE_{HMP} and ΔE_{ATP}) within the limits of accuracy of the measurements.

The CP content of a tissue sample is calculated from ΔE_{CP} as follows:

$$\frac{\Delta E_{CP} \times V_C \times V_2 \times V_4}{\varepsilon \times d \times V_1 \times V_3 \times V_5} = \mu\text{moles CP/g. or ml. tissue}$$

where

V_C = final volume in the cuvette after the last addition of enzyme [ml.]
V_1 = weight [g.] or volume [ml.] of the tissue sample
$V_2 = V_1 +$ [g.] or [ml.] perchloric acid used for deproteinization
V_3 = volume of the perchloric acid extract before neutralization [ml.]
$V_4 = V_3 +$ ml. K_2CO_3 required for neutralization
V_5 = volume of the deproteinized sample in the cuvette [ml.]
ε = extinction coefficient of TPN; $\varepsilon_{366} = 3.3$ cm.^{2}/μmole
d = light path [cm.]

A water content of 75% is assumed for tissue (liver, muscle, heart). According to p. 546, 3.25 ml. perchloric acid solution (V) are required to deproteinize 1 g. tissue. If it is wished to express the results in μmoles/g. tissue, then it is necessary to insert V_1 in g., V_2 in g. tissue + 1.035 × ml. perchloric acid (1.035 = specific gravity of 6% perchloric acid) in the calculations.

If it is wished to express the results as μmoles CP/ml. tissue, then the weight of the tissue sample V_1 [g.] is divided by the specific gravity of the tissue and the volume [ml.] of perchloric acid is added to V_2. To obtain the μg. CP/g. tissue, the μmoles CP/g. tissue must be multiplied by the molecular weight of CP (211.08).

For corrections for the blood content of the tissue and for the intercellular space see "Determination of ATP with Hexokinase and Glucose-6-phosphate Dehydrogenase", p. 549.

Sources of Error

1. Practically all the sources of error can be traced to excessive contamination of the hexokinase or glucose-6-phosphate dehydrogenase preparations (especially with TPNH oxidase and GSSG reduc-

tase) or to a large amount of HK in the glucose-6-phosphate dehydrogenase. Many of the commercial preparations of yeast HK vary considerably in their purity. It is recommended that each new preparation should be carefully tested with crystalline ATP.

2. In the presence of large amounts of PO_4^{3-} (*e.g.* in the analysis of deproteinized incubation mixtures which contain Krebs-Ringer phosphate saline) turbidity occurs in the cuvette, often only during the assay, due to the precipitation of fine crystals of magnesium-ammonium phosphate (simulating an increase in optical density).

3. Enzyme solutions which have been diluted with water and aged for several days are unsuitable.

4. If the optical density changes do not come to a stop even after a long time ("drift"), in spite of the enzymes being of the required purity, then the course of the optical density changes should be plotted graphically. Extrapolation gives the correct values (refer to p. 39).

Specificity

Creatine phosphokinase specifically catalyses the reaction:

$$\text{CP} + \text{ADP} \rightleftharpoons \text{creatine} + \text{ATP}.$$

ADP cannot replace ATP in the reverse reaction. Inosine phosphates are not active as phosphate acceptors or donors. The compounds related to creatine, such as creatinine, arginine, *etc.*, are not phosphorylated. Glycocyamine can replace creatine in the reverse reaction, but only when present in ten-fold excess[8].

Other Methods for the Determination of Creatine Phosphate

So far no other method for the enzymatic determination of CP has been described. The method described here has proved of value in studies on heart metabolism[9]. Enzymatic spectrophotometric determinations of high-energy phosphates by difference was proposed by *Slater*[10] and *Kratzing* and *Narayanaswami*[11].

Non-enzymatic assay methods for CP depend mainly on the determination of the phosphate group. The most important is the colorimetric assay with reduced phosphomolybdic acid[12–16]. CP assays in which the creatine moiety is determined employ either the Jaffe reaction[17,18] or the colour reaction with diacetyl-α-naphthol[19–21].

In many cases the CP is separated chromatographically or electrophoretically before the colorimetric determination[22–27].

---

8) *M. L. Tanzer* and *C. Gilvarg*, J. biol. Chemistry *234*, 3201 [1959].
9) *Ph. Stein*, Diploma Thesis, Technische Hochschule Munich, Germany, 1960.
10) *E. C. Slater*, Biochem. J. *50*, vii [1951].
11) *C. C. Kratzing* and *A. Narayanaswami*, Biochem. J. *54*, 317 [1953].
12) *C. H. Fiske* and *J. Subbarow*, J. biol. Chemistry *81*, 629 [1929].
13) *O. H. Lowry* and *J. A. Lopez*, J. biol. Chemistry *162*, 421 [1945].
14) *B. E. Wahler* and *A. Wollenberger*, Biochem. Z. *329*, 508 [1958].
15) *J. Berenblum* and *E. Chain*, Biochem. J. *32*, 295 [1938].
16) *J. B. Martin* and *D. M. Doty*, Analytic. Chem. *21*, 965 [1949].
17) *H. H. Taussky*, J. biol. Chemistry *208*, 835 [1954].
18) *H. Mc. Ilwain*, *H. L. Buchel* and *J. D. Ceshire*, Biochem. J. *48*, 12 [1951].
19) *D. Eggleton*, *S. R. Elsden* and *N. Gough*, Biochem. J. *37*, 526 [1943].
20) *A. H. Ennor* and *H. Stocken*, Biochem. J. *42*, 557 [1948].
21) *A. H. Ennor* and *H. Rosenberg*, Biochem. J. *51*, 606 [1952].
22) *A. Fleckenstein* and *E. Gerlach*, Naunyn-Schmiedebergs Arch. exp. Pathol. Pharmakol. *219*, 531 [1953].
23) *A. Fleckenstein* and *J. Janke*, Pflügers Arch. ges. Physiol. Menschen Tiere *258*, 177 [1953].
24) *A. Martonosi*, Biochem. biophys. Res. Commun. *2*, Nr. 1, S. 12 [1960].
25) *P. C. Caldwell*, Biochem. J. *55*, 458 [1955].
26) *W. Thorn*, *W. Isselhard* and *K. Irmsher*, Biochem. Z. *330*, 385 [1958].
27) *E. Gerlach* and *J. Janke*, Biochem. Z. *330*, 565 [1958].