Adenosine-5′-diphosphate and Adenosine-5′-monophosphate

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Principle

Adenosine-5′-diphosphate (ADP) is phosphorylated with phosphoenolpyruvate and pyruvate kinase (PK). The pyruvate formed is reduced with DPNH and lactic dehydrogenase (LDH). Adenosine-5′-monophosphate (AMP) can also be phosphorylated with ATP and myokinase (MK) and the resulting two equivalents of ADP can be determined as described. Consequently it is possible to measure individually pyruvate, ADP and AMP in a single operation[1].

(1) $$AMP + ATP \xrightleftharpoons{MK,\ Mg^{2+}} 2\ ADP$$

(2) $$2\ ADP + 2\ phosphoenolpyruvate \xrightleftharpoons{PK,\ Mg^{2+},\ K^{+}} 2\ ATP + 2\ pyruvate$$

Indicator reaction:

(3) $$2\ Pyruvate + 2\ DPNH + 2\ H^{+} \xrightleftharpoons{LDH} 2\ lactate + 2\ DPN^{+}$$

The equilibrium of reaction (3) is sufficiently far to the right[2] ($K \approx 10^4$ [l./mole][2]) so as to reduce all the pyruvate. The equilibrium of reaction (2) ($K = 2 \times 10^3$ at 30°C[3,4]) is sufficiently far to the right to overcome the equilibrium of the ADP dismutation (*ca.* 66% to the left[5,6]) and allow all the AMP to react.

Reagents *)

1. Perchloric acid, A. R.; sp. gr. 1.67; *ca.* 70% (w/w)
2. Potassium carbonate, K_2CO_3, A. R.
3. Potassium hydroxide, A. R., 2 N
4. Triethanolamine, freshly distilled b. p. 277–279°C/150 mm.
5. Magnesium sulphate, $MgSO_4 \cdot 7H_2O$, A. R.
6. Potassium chloride, KCl, A. R.
7. Ethylene-diamine-tetra-acetic acid, EDTA
   disodium salt, EDTA-$Na_2H_2 \cdot 2H_2O$, *e.g.* Titriplex III **)
8. Hydrochloric acid, 2 N, A. R.
9. Sodium hydroxide, 2 N, A. R.
10. Phosphoenolpyruvate, PEP
    crystalline tricyclohexylammonium salt; commercial preparation, see p. 1024.
11. Reduced diphosphopyridine nucleotide, DPNH
    disodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
12. Adenosine-5′-triphosphate, ATP
    crystalline disodium salt, ATP-$Na_2H_2 \cdot 3H_2O$; commercial preparation, see p. 1006.
13. Adenosine-5′-monophosphate, AMP
    disodium salt, AMP-Na_2; commercial preparation, see p. 1005.

*) Complete reagent kits are available commercially, see p. 1035.
**) from E. Merck, Darmstadt (Germany).

1) *H. Adam*, Ph. D.-Thesis, Universität Marburg/L., 1955.
2) *F. Kubowitz* and *P. Ott*, Biochem. Z. *314*, 94 [1943].
3) *E. Negelein*, unpublished; cf. *F. Kubowitz* and *P. Ott*, Biochem. Z. *317*, 193 [1944].
4) *O. Meyerhof* and *P. Oesper*, J. biol. Chemistry *179*, 1371 [1949].
5) *H. M. Kalckar*, J. biol. Chemistry *148*, 127 [1943].
6) *L. V. Eggleston* and *R. Hems*, Biochem. J. *52*, 156 [1952].

14. Lactic dehydrogenase, LDH
crystalline, from skeletal muscle, suspended in 2.1 M ammonium sulphate solution (pH 5.5); 10 mg. protein/ml. Commercial preparation, see p. 986.

15. Pyruvate kinase, PK
crystalline, from skeletal muscle, suspended in 1.95 M ammonium sulphate solution (pH 5.5); 5 mg. protein/ml. Commercial preparation, see p. 997.

16. Myokinase, MK
from skeletal muscle, suspended in 3.3 M ammonium sulphate solution (pH ≈ 6); 5 mg. protein/ml. Commercial preparation, see p. 989.

Purity of the enzyme preparations

The LDH should have a specific activity of at least 20000 units*)/mg. and be free from MK and PK (*i.e.* less than 0.01% with respect to the LDH activity). The specific activity of the PK should be at least 2500 units*)/mg. and that of the MK at least 2000 units*)/mg. Within the limits given above PK should be free from MK, and MK should be free from ATPase.

Preparation of Solutions

I. Triethanolamine buffer (5×10^{-2} M; pH 7.55):
Dissolve 7.46 g. triethanolamine in *ca.* 700 ml. doubly distilled water, adjust to pH 7.55 with *ca.* 15 ml. 2 N HCl and dilute with doubly distilled water to 1000 ml.

II. Magnesium sulphate (0.5 M):
Dissolve 6.02 g. $MgSO_4 \cdot 7H_2O$ in doubly distilled water and make up to 100 ml.

III. Potassium chloride (2 M):
Dissolve 14.91 g. KCl in doubly distilled water and make up to 100 ml.

IV. Ethylene-diamine-tetra-acetate (100 mg./ml.):
Dissolve 10 g. EDTA-$Na_2H_2 \cdot 2H_2O$ in doubly distilled water, neutralize with 2 N NaOH and dilute with doubly distilled water to 100 ml.

V. Phosphoenolpyruvate (*ca.* 4×10^{-2} M PEP):
Dissolve 100 mg. PEP (crystalline tricyclohexylammonium salt) in doubly distilled water and make up to 5 ml. Determine the PEP content of the solution enzymatically (see p. 224).

VI. Reduced diphosphopyridine nucleotide (*ca.* 10^{-2} M β-DPNH):
Dissolve 20 mg. DPNH-Na_2 in 2 ml. doubly distilled water. Determine the DPNH content of the solution enzymatically (see p. 531).

VII. Adenosine triphosphate (*ca.* 10^{-2} M ATP):
Dissolve 10 mg. ATP-$Na_2H_2 \cdot 3H_2O$ in 2 ml. doubly distilled water.

VIII. Adenosine monophosphate (*ca.* 10^{-2} M AMP):
Dissolve 10 mg. AMP-Na_2 in 2 ml. doubly distilled water.

IX. Lactic dehydrogenase, LDH (0.1 mg. protein/ml.):
Dilute 0.01 ml. of a crystalline suspension (10 mg. protein/ml.) to 1 ml. with 2.25 M ammonium sulphate solution (pH 6.5).

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*) According to *Th. Bücher*[7] one unit is the amount of enzyme dissolved in 1 ml. which decreases the optical density of DPNH by 0.100 in 100 seconds at 366 mμ with a 1 cm light path.

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

X. Pyruvate kinase, PK (0.5 mg. protein/ml.):
Dilute 0.1 ml. of a crystalline suspension (5 mg. protein/ml.) to 1 ml. with 2.1 M ammonium sulphate solution (pH 5.5).

XI. Myokinase, MK (0.25 mg. protein/ml.):
Dilute 0.05 ml. of a crystalline suspension (5 mg. protein/ml.) to 1 ml. with 3.3 M ammonium sulphate solution (pH 6).

For deproteinization:

2 N KOH; 3.75 M K_2CO_3; perchloric acid solutions Xa and Xb on p. 541

Procedure

Deproteinization

Since hydrolysis of ATP must be avoided during extraction of ADP and AMP from tissues, refer to the determination of adenosine-5′-triphosphate with phosphoglycerate kinase (p. 539) for all details connected with the preparation of the extract.

Spectrophotometric measurements

Wavelength: 340 or 366 mμ; light path: 1 cm.; final volume: 2.0 ml.; room temperature.

Prepare the following reaction mixture*) and neutralize:

0.036 ml. magnesium sulphate solution (II)
0.076 ml. potassium chloride solution (III)
0.004 ml. EDTA solution (IV)
0.040 ml. PEP solution (V)
0.030 ml. DPNH solution (VI)
0.007 ml. ATP solution (VII)

or a multiple of the individual volumes.

Pipette successively into the cuvettes:

deproteinized sample (extract)
and buffer (I) to 2.00 ml.
0.140 ml. reaction mixture.

Mix in (with a small glass or plastic rod flattened and bent at one end)

0.020 ml. LDH suspension (IX).

Follow the optical density until no further change occurs (3—5 min.), read optical density E_1. Mix in

0.020 ml. PK suspension (X).

Take readings until reaction stops (6—9 min.) and then measure optical density E_2. If the reaction does not come to a complete stop, extrapolate from the point of PK addition: the change in optical density/min. (from the linear part of the curve) multiplied by the number of minutes since addition of PK, gives the optical density to be subtracted from the final optical density E_2.

*) As DPNH preparations occasionally contain some AMP (just as ATP preparations contain some ADP and phosphoenolpyruvate solutions some pyruvate), it is necessary to carry out a blank determination for all three substrates on the salt-substrate buffer mixture. This composite mixture should not be kept for longer than 6 hours without carrying out a new blank determination.

Immediately mix in

0.018 ml. MK suspension (XI).

Take readings until the reaction stops (10—13 min.) and then measure optical density E_3. If the reaction does not stop, extrapolate as described above.

To test the correct functioning of the system mix in

ca. 0.01 ml. AMP solution (VIII).

A renewed reaction should occur immediately (Fig. 1). The optical density differences $E_1-E_2 = \Delta E_{ADP}$ and $E_2-E_3 = \Delta E_{AMP}$ are used for the calculations.

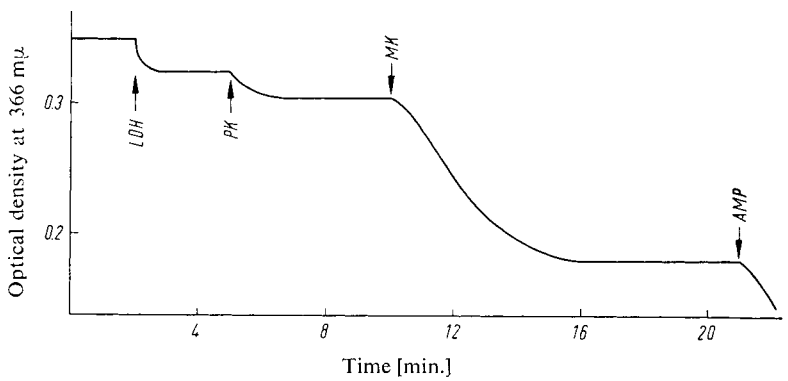

Fig. 1. Determination of pyruvate, ADP and AMP in the same mixture; decrease in optical density at 366 mμ.
LDH = lactic dehydrogenase, PK = pyruvate kinase, MK = myokinase, AMP = adenosine-5′-monophosphate as control.

Calculations

Extinction coefficient for DPNH (25° C):

$$\varepsilon_{340} = 6.22 \text{ [cm.}^2\text{/}\mu\text{mole]}$$
$$\varepsilon_{366} = 3.30 \text{ [cm.}^2\text{/}\mu\text{mole]}$$

$$\frac{\Delta E_{ADP} \times V_A \times V_E}{\varepsilon \times d \times V_P} = \mu\text{moles ADP in extract}$$

and

$$\frac{\Delta E_{AMP} \times V_A \times V_E}{2 \times \varepsilon \times d \times V_P} = \mu\text{moles AMP in extract}$$

where $\Delta E_{ADP} = E_1-E_2$
$\Delta E_{AMP} = E_2-E_3$
V_A = volume of test mixture in the cuvette (2.0 ml.)
V_E = total volume of extract [ml.]
V_P = volume of extract added to the cuvette [ml.]
ε = extinction coefficient [cm.^{2}/μmole]
d = light path of the cuvette [1 cm.]

for measurements at 366 mμ under the stated conditions it follows that:

$$\frac{\Delta E_{ADP} \times 2 \times V_E}{3.30 \times 1 \times V_P} = \Delta E_{ADP} \times 0.606 \times \frac{V_E}{V_P} = \mu\text{moles ADP in extract}$$

and

$$\Delta E_{AMP} \times 0.303 \times \frac{V_E}{V_P}\ \mu\text{moles AMP in extract.}$$

These values divided by the fresh weight of tissue taken give the μmoles ADP or AMP per g. tissue.

The results are reproducible to ±1.5% and agree with UV absorption, phosphate and ribose determinations. As little as 10^{-8} moles of nucleotide can be measured with this accuracy. Micro-cuvette-allow the determination of 10^{-9} moles of nucleotide.

Specificity

IDP, GDP, UDP or CDP are quantitatively converted, but at different rates (refer to Fig. 2). The time course of the reactions with IDP, GDP or UDP cannot be differentiated from that with ADP; only with CDP is a correction by extrapolation possible.

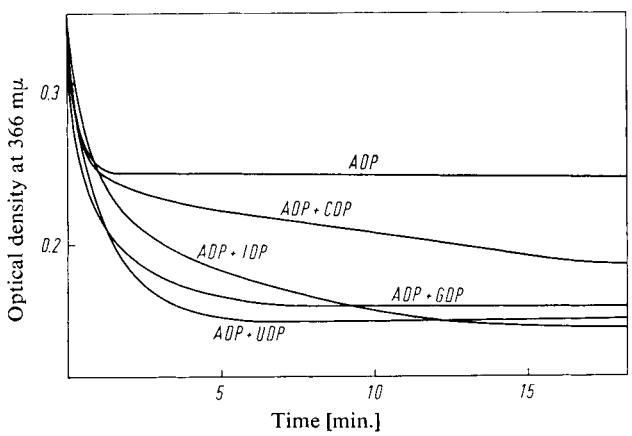

Fig. 2. Determination of ADP in the presence of about equimolar concentration of IDP, GDP, UDP or CDP; decrease in optical density at 366 mμ after addition of pyruvate kinase.