D-Glycerate

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D-Glycerate can be determined with D-glycerate kinase from rat liver in a coupled spectrophotometric assay[1].

Principle (refer to[2,3])

Adenosine triphosphate (ATP) and D-glycerate kinase phosphorylate D-glycerate to give 2-phosphoglycerate (equation 1)[4], which is converted by enolase to phosphoenolpyruvate (equation 2). This is split by pyruvic kinase to give pyruvate (equation 3). The pyruvate is reduced by DPNH according to equation (4); the decrease in the optical density due to the oxidation of DPNH serves as a measure of the amount of D-glycerate which has reacted. As the equilibrium of reaction (4) is far to the right D-glycerate reacts quantitatively.

(1) D-Glycerate + ATP $\xrightarrow{Mg^{2+}}$ 2-phosphoglycerate + ADP

(2) 2-Phosphoglycerate $\overset{Mg^{2+}}{\rightleftharpoons}$ phosphoenolpyruvate

(3) Phosphoenolpyruvate + ADP $\overset{K^+ Mg^{2+}}{\rightleftharpoons}$ pyruvate + ATP

(4) Pyruvate + DPNH + H^+ $\rightleftharpoons$ L-lactate + DPN^+

Balance: D-Glycerate + DPNH + H^+ $\longrightarrow$ L-lactate + DPN^+

Reagents

1. Tris-hydroxymethyl-aminomethane, tris
2. Hydrochloric acid, A. R., 2 N
3. Magnesium sulphate, A. R., $MgSO_4 \cdot 7H_2O$
4. Potassium chloride, A. R.
5. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
6. Adenosine triphosphate, ATP
   crystalline sodium salt, ATP-$Na_2H_2 \cdot 3H_2O$. Commercial preparation, see p. 1006.
7. Lactic dehydrogenase, LDH
   from skeletal muscle (rabbit), crystalline suspension in 2.2 M ammonium sulphate solution; commercial preparation, see p. 986.
8. Pyruvic kinase, PK
   from skeletal muscle (rabbit), crystalline suspension in 2.1 M ammonium sulphate solution; commercial preparation, see p. 997.

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1) *O. Warburg:* Wasserstoffübertragende Fermente. Dr. Werner Saenger GmbH, Berlin 1948.
2) *H. Holzer* and *A. Holldorf*, Biochem. Z. *329*, 283 [1957].
3) *R. Kattermann, U. Dold* and *H. Holzer*, Biochem. Z. *334*, 218 [1961].
4) *W. Lamprecht, T. Diamantstein, F. Heinz* and *P. Balde*, Hoppe-Seylers Z. physiol. Chem. *316*, 97 [1959].

9. Enolase
   from skeletal muscle (rabbit), crystalline suspension in 2.6 M ammonium sulphate solution; commercial preparation, see p. 973.

10. D-Glycerate kinase
    Preparation of the enzyme, see p. 223. Enzyme suspension in 1.2 M ammonium sulphate solution, containing 2–4 mg. protein/ml.; specific activity at least 500 units *)/mg.

Preparation of Solutions

I. Tris buffer (0.2 M; pH 7.4):
   Dissolve 2.43 g. tris-hydroxymethyl-aminomethane in *ca.* 50 ml. doubly distilled water, adjust to pH 7.4 (glass electrode) with *ca.* 8 ml. 2 N HCl; after equilibration of the temperature dilute to 100 ml. and check pH again.

II. Magnesium sulphate (0.5 M):
   Dissolve 1.23 g. $MgSO_4 \cdot 7H_2O$ in doubly distilled water and make up to 10 ml.

III. Potassium chloride (0.5 M):
   Dissolve 0.37 g. KCl in doubly distilled water and make up to 10 ml.

IV. Reduced diphosphopyridine nucleotide (*ca.* 10^{-2} M β-DPNH):
   Dissolve 10 mg. DPNH-Na_2 in 1 ml. doubly distilled water.

V. Adenosine triphosphate (0.2 M ATP):
   Dissolve 119 mg. ATP-$Na_2H_2 \cdot 3H_2O$ in 1.0 ml. doubly distilled water.

VI. Lactic dehydrogenase, LDH (5 mg. protein/ml.):
   If necessary, dilute commercial preparations with 2.2 M ammonium sulphate solution.

VII. Pyruvic kinase, PK (2 mg. protein/ml.):
   If necessary, dilute commercial preparations with 2.1 M ammonium sulphate solution.

VIII. Enolase (2 mg. protein/ml.):
   If necessary, dilute commercial preparations with 2.6 M ammonium sulphate solution.

IX. D-Glycerate kinase (3 mg. protein/ml.):
   If necessary, dilute the enzyme suspension obtained according to [3] with 1.2 M ammonium sulphate solution.

Procedure

For preparation and extraction of samples (blood, tissue, *etc.*), see determination of pyruvate with lactic dehydrogenase p. 254.

Spectrophotometric measurements

Wavelength: 366 mμ; glass cuvettes, light path: 2.0 cm.; final volume 4.0 ml.; measure against a water blank. The light path and final volume may be altered in order to increase the sensitivity of the assay. Allow buffer and solution for assay to warm to room temperature.

*) A unit is the amount of enzyme which causes an optical density decrease of 0.001 per minute at 366 mμ in an assay mixture of 3.0 ml. and with a light path of 1 cm.

Pipette successively into the cuvettes:

Experimental cuvette	*Control cuvette*
1.70 ml. buffer (solution I)	1.70 ml. buffer (solution I)
0.06 ml. $MgSO_4$ solution (II)	0.06 ml. $MgSO_4$ solution (II)
0.06 ml. KCl solution (III)	0.06 ml. KCl solution (III)
0.06 ml. DPNH solution (IV)	0.06 ml. DPNH solution *) (IV)
0.03 ml. ATP solution (V)	0.03 ml. ATP solution (V)
0.02 ml. LDH suspension (VI)	0.02 ml. LDH suspension (VI)
0.02 ml. PK suspension (VII)	0.02 ml. PK suspension (VII)
0.03 ml. D-glycerate kinase suspension (IX)	0.03 ml. D-glycerate kinase suspension (IX)
sample + water to 3.98 ml.	water to 3.98 ml.

Read the optical densities E_1 of both cuvettes. If the change in optical density in both cuvettes is not greater than 0.001 to 0.002 per 30 sec., then start the reaction by mixing into both cuvettes

0.02 ml. enolase suspension (VIII).

Follow the optical densities E_2 of both cuvettes until no significant change in either cuvette is observed (usually 20—30 min.).

Calculate the difference $E_1 - E_2$ for the experimental and control cuvettes:

$$\Delta E_{exp.} \text{ and } \Delta E_{con.}$$

$\Delta E_{con.}$ is the correction for unspecific optical density changes due to addition of the enzyme and for trivial side reactions caused by contaminants. $\Delta E = \Delta E_{exp.} - \Delta E_{con.}$ is the optical density change caused by the reaction of D-glycerate.

If in control experiments with known amounts of D-glycerate it is found that LDH, PK, and GK contain no enolase, then enolase can be added first and the reaction started with D-glycerate kinase.

Calculations

$$\frac{\Delta E \times V}{3.3 \times d} = \mu\text{moles D-glycerate/cuvette}$$

Where

ΔE = optical density change

V = cuvette contents in ml.

d = light path in cm.

3.3 = extinction coefficient [cm.²/μmole] for DPNH at 366 mμ.

Further Determinations

If the sample contains other metabolites besides D-glycerate, which can react in this assay system, they may be determined in the same mixture. The most important is pyruvate whose reduction to lactate (see p. 253) is brought about by first adding lactic dehydrogenase to the cuvette. Then the other enzymes and ATP are added, thus phosphoenolpyruvate (see p. 229) and 2-phosphoglycerate (see p. 229) can also be estimated. In this case the assay for D-glycerate is started with D-glycerate kinase instead of enolase.

*) The absorption due to the sample containing the glycerate in the experimental cuvette can be compensated for in the control cuvette by the addition of more DPNH.

Appendix

Isolation of D-glycerate kinase

D-Glycerate kinase preparations for the assay described here can be obtained in 2—3 days from an acetone powder of rat liver.

Extract 5 g. acetone-dried powder with 100 ml. tris buffer (solution I), centrifuge and fractionate the supernatant ("crude extract") with saturated ammonium sulphate solution. Dialyse the enzyme solution for 2 hours and add calcium phosphate gel. After eluting the enzyme from the gel with 0.033 M phosphate buffer (pH 7.4) dialyse again. Fractionate the red enzyme solution on a DEAE-cellulose column with increasing concentrations of phosphate buffer (pH 7.4). D-Glycerate kinase appears on elution with 0.2 M phosphate buffer (pH 7.4). Combine the fractions containing the enzyme and bring to 55% saturation with saturated ammonium sulphate solution. After centrifuging at 20000 g dissolve the enzyme in a little 0.01 M tris buffer (pH 7.4) and add ammonium sulphate to 20% saturation. The purification at this stage is 25 to 30-fold; the activity of the enzyme when stored at −15°C decreases by *ca.* 50% in 3 months. The purification of the enzyme from horse liver[5] or from a rat liver mitochondrial fraction[4] has been described. It can also be purified about 6-fold from baker's yeast by acid precipitation[6].

[5] *A. Ichihara* and *D. M. Greenberg*, J. biol. Chemistry *225*, 949 [1957].
[6] *S. Black* and *N. G. Wright*, J. biol. Chemistry *221*, 171 [1956].