D-Ribulose-1,5-diphosphate

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Principle

The estimation of ribulose diphosphate is based on the following reactions[1]

(1) Ribulose-1,5-diphosphate + CO_2 ⟶ 3-phosphoglycerate

(2) 3-Phosphoglycerate + ATP ⇌ 1,3-diphosphoglycerate + ADP

(3) 1,3-Diphosphoglycerate + DPNH + H^+ ⇌ glyceraldehyde-3-phosphate + phosphate + DPN^+

(4) Glyceraldehyde-3-phosphate ⇌ dihydroxyacetone phosphate

(5) Dihydroxyacetone phosphate + DPNH + H^+ ⇌ α-glycerophosphate + DPN^+

Reaction (1) is catalysed by ribulose diphosphate carboxylase, reaction (2) by phosphoglycerate kinase, reaction (3) by glyceraldehyde-3-phosphate dehydrogenase, reaction (4) by triose phosphate isomerase, and reaction (5) by α-glycerophosphate dehydrogenase. Since the cleavage of ribulose-1,5-diphosphate to two molecules of 3-phosphoglycerate is irreversible, and since the conversion of 3-phosphoglycerate to α-glycerophosphate is virtually quantitative, four molecules of DPNH are oxidized for each molecule of ribulose diphosphate present.

Reagents

1. Tris-hydroxymethyl-aminomethane, tris
2. Hydrochloric acid, 2N
3. Ethylene-diamine-tetra-acetic acid, EDTA
   disodium salt, EDTA-$Na_2 \cdot 2H_2O$
4. Adenosine triphosphate, ATP
   disodium salt, ATP-$Na_2H_2 \cdot 3H_2O$; commercial preparation, see p. 1006.
5. Magnesium chloride, $MgCl_2 \cdot 6H_2O$
6. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
7. Triosephosphate isomerase/α-glycerophosphate dehydrogenase, TIM/GDH
   mixed crystalline suspension in $(NH_4)_2SO_4$ solution. Commercial preparation, see p. 999.
8. Glyceraldehyde-3-phosphate dehydrogenase, GAPDH
   crystalline suspension in $(NH_4)_2SO_4$ solution. Commercial preparation, see p. 979.
9. Phosphoglycerate kinase, PGK
   crystalline suspension in $(NH_4)_2SO_4$ solution. Commercial preparation, see p.994.
10. Ribulose diphosphate carboxylase
    from spinach leaves; simplified preparation, see[2].

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[1] More sensitive modification of the previously described method of *E. Racker*, Arch. Biochem. Biophysics *69*, 300 [1957].

Purity of reagents and enzymes

Preparations of ATP should not contain 3-phosphoglycerate or fructose-1,6-diphosphate. Ribulose diphosphate carboxylase must be free from phosphoribulokinase. Preparations which fulfil the following conditions are suitable:

a) In the complete assay mixture no oxidation of DPNH takes place in the absence of ribulose diphosphate.

b) The reaction with known amounts of ribulose diphosphate is completed in less than ten minutes.

Preparation of Solutions

I. Tris buffer (1 M; pH 7.4):
Dissolve 12.11 g. tris-hydroxymethyl-aminomethane in *ca.* 50 ml. distilled water, adjust to pH 7.4 with 42.5 ml. 2 N HCl and dilute with distilled water to 100 ml. Check pH value (glass electrode).

II. Adenosine triphosphate (0.1 M ATP):
Dissolve 121 mg. ATP-$Na_2H_2 \cdot 3H_2O$ in 2 ml. distilled water.

III. Magnesium chloride (0.1 M):
Dissolve 203 mg. $MgCl_2 \cdot 6H_2O$ in 10 ml. distilled water.

IV. Reduced diphosphopyridine nucleotide (*ca.* 0.004 M β-DPNH; pH 9):
Dissolve 7 mg. DPNH-Na_2 in 2 ml. distilled water, adjust with alkali to about pH 9.

V. Triosephosphate isomerase/α-glycerophosphate dehydrogenase, TIM/GDH (500 μg. protein/ml.):
Before use dilute 0.1 ml. crystalline suspension to 0.4 ml. with tris buffer (solution I).

VI. Glyceraldehyde-3-phosphate dehydrogenase/phosphoglycerate kinase, GAPDH/PGK (250 units*) of each/ml.):
Before use measure the activity of the glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase suspensions and dilute to 500 units/ml. with 0.005 M EDTA solution (pH 7.4). Mix equal parts of the diluted suspensions.

VII. Ribulose diphosphate carboxylase (40 units*)/ml.):
Dilute the enzyme solution prepared according to[2] with tris buffer (solution I), containing 0.002 M EDTA, to give 40 units/ml.

Stability of the solutions

The DPNH solution is stable for several weeks in the frozen state. Store the commercial enzyme preparations as undiluted suspensions at 2° C. They are stable in this state for several months. Ribulose diphosphate carboxylase preparations are not usually very stable, but the enzyme prepared according to[2] can be stored for several months.

Procedure

Spectrophotometric measurements

Wavelength: 340 mμ; light path: 1 cm.; final volume: 1 ml.; Read the experimental cuvette against control cuvette.

To two quartz cuvettes add sufficient distilled water to bring the final volume of the test mixture to 1 ml. Then add:

*) A unit is the amount of enzyme which converts 1 μmole of substrate in 1 min.

[2] *E. Racker* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1961, Vol. V, p. 266.

Experimental cuvette	*Control cuvette*
neutralized sample (containing 0.003–0.02 μmoles ribulose diphosphate)	distilled water
0.10 ml. buffer (soln. I)	0.10 ml. buffer (soln. I)
0.05 ml. ATP soln. (II)	0.05 ml. ATP soln. (II)
0.05 ml. $MgCl_2$ soln. (III)	0.05 ml. $MgCl_2$ soln. (III)
0.03 ml. DPNH soln. (IV)	0.03 ml. distilled water
0.05 ml. TIM/GDH susp. (V)	0.05 ml. TIM/GDH susp. (V)

Measure optical density E_1 at 340 mμ, then to both cuvettes add

0.02 ml. GAPDH/PGK suspension (VI).

On completion of the reaction measure optical density E_2 at 340 mμ. The decrease in optical density $E_1 - E_2$ corresponds to the 3-phosphoglycerate content of the sample. Then to both cuvettes add

0.02 ml. ribulose diphosphate carboxylase solution (VII)

and after completion of the reaction measure optical density E_3 at 340 mμ.

Calculations

A decrease in optical density of 6.22 corresponds to the oxidation of 1 μmole DPNH. Four moles of DPNH are oxidized for each mole of ribulose diphosphate cleaved. The ribulose diphosphate content of the test mixture is calculated from the formula:

$$\frac{0.98\ E_2 - E_3}{4 \times 6.22} = \text{μmoles ribulose diphosphate/ml. test mixture}$$

0.98 is the correction factor for the 2% dilution of the solution on addition of 0.02 ml. ribulose diphosphate carboxylase.

Sources of Error

The impurities mentioned above which may occur in commercial samples of ATP, seriously interfere with the assay. Ribulose diphosphate carboxylase must be completely free from phosphoribulokinase otherwise ribose-5-phosphate and ribulose-5-phosphate are determined together with ribulose diphosphate.