D-Ribulose

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D-Ribulose has been determined by the cysteine-carbazole method[1], but this is not specific[2,3]. By comparison, the high degree of specificity of the ribitol dehydrogenase from *Aerobacter aerogenes* allows the estimation of D-ribulose in the presence of other sugars[4,5].

Principle

Ribitol dehydrogenase catalyses the reversible oxidation of ribitol to D-ribulose in the presence of diphosphopyridine nucleotide (DPN):

(1) $$\text{Ribitol} + \text{DPN}^+ \rightleftharpoons \text{D-ribulose} + \text{DPNH} + \text{H}^+.$$

The apparent equilibrium constant for this reaction is 7.17×10^{-3} at pH 8.0 and 28° C[4]. With excess DPNH the D-ribulose is virtually quantitatively converted to ribitol with the simultaneous oxidation of an equivalent amount of DPNH.

Reagents

1. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
2. Tris-hydroxymethyl-aminomethane, tris
3. Ribitol dehydrogenase, RDH
   for isolation, see p. 184.

Preparation of Solutions (for *ca.* 6 determinations)

I. Reduced diphosphopyridine nucleotide (*ca.* 10^{-3} M β-DPNH):
Dissolve 7.82 mg. DPNH-Na_2 in doubly distilled water and dilute to 10 ml.

II. Tris buffer (1.0 M; pH 7.4):
Dissolve 12.11 g. tris-hydroxymethyl-aminomethane in *ca.* 50 ml. doubly distilled water, adjust to pH 7.4 (glass electrode) with 42.5 ml. 2 N HCl and dilute to 100 ml.

III. Tris buffer (1.0 M; pH 8.5):
Dissolve 12.11 g. tris-hydroxymethyl-aminomethane in *ca.* 70 ml. doubly distilled water, adjust to pH 8.5 (glass electrode) with 15.0 ml. 2 N HCl and dilute to 100 ml.

IV. Ribitol dehydrogenase, RDH (*ca.* 1 mg. protein/ml.):
The enzyme purified as described on p. 184 is obtained as a solution containing approximately 1 mg. protein/ml. after elution from the calcium phosphate gel. This solution contains about 5 RDH units*)/0.1 ml.

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*) An RDH unit is the amount of enzyme which in a mixture of 4 μmoles DPN, 4 μmoles ribitol, 400 μmoles tris buffer (pH 7.4) and a total volume of 2.3 ml., increases the optical density at 340 mμ by 0.100 in 2 min. at 28° C.

1) *Z. Dische* and *E. Borenfreund*, J. biol Chemistry *192*, 583 [1951].
2) *G. Ashwell* and *J. Hickman*, J. biol. Chemistry *226*, 65 [1957].
3) *S. S. Cohen*, J. biol. Chemistry *201*, 71 [1953].
4) *R. C. Nordlie* and *H. J. Fromm*, J. biol. Chemistry *234*, 2523 [1959].
5) *H. J. Fromm*, J. biol. Chemistry *233*, 1049 [1958].

Stability of the solutions

Prepare the DPNH solution freshly each week and store in frozen state. The tris buffer is stable indefinitely at 4°C. The RDH solution keeps for longer than a month at 3°C. Repeated freezing and thawing leads to considerable loss of activity.

Procedure

Spectrophotometric measurements

Wavelength: 340 mμ; quartz cuvettes, light path: 1 cm.; final volume: 3 ml.

A control cuvette contains tris buffer (pH 8.5) instead of enzyme solution. The small absorption due to the enzyme solution is determined separately.

Prepare the following mixture for the experimental and control cuvettes:

3.0 ml. sample (*ca.* 0.1 μmole D-ribulose/ml.)
1.5 ml. DPNH solution (I)
3.0 ml. tris buffer (solution II)
1.2 ml. doubly distilled water.

Mix and allow to come to room temperature (*ca.* 25°C). Pipette into

Experimental cuvette	*Control cuvette*
2.9 ml. above mixture	2.9 ml. above mixture
0.1 ml. RDH solution (IV)	0.1 ml. tris buffer (solution III)

and mix well. Wait for the completion of the reaction (40 to 60 min.), then read the optical density E_1 (control cuvette against experimental cuvette).

The optical density of the enzyme solution (E_e) is determined for each enzyme preparation by measuring the absorption of 0.1 ml. enzyme solution + 2.9 ml. doubly distilled water against 0.1 ml. tris buffer (pH 8.5) + 2.9 ml. doubly distilled water.

The sum of $E_1 + E_e$ gives the optical density change ΔE_{340}, corresponding to the DPNH oxidized.

Calculations

The reaction proceeds stoichiometrically. For each mole of D-ribulose reduced one mole of DPNH is oxidized to DPN[5]. The molar extinction coefficient of DPNH at 340 mμ and at the pH of the measurements is 6.22 cm²/μmole. Under the conditions of the method, more than 99% of D-ribulose is converted to ribitol.

The D-ribulose concentration in the sample is calculated from the formula:

$$\mu\text{moles D-ribulose/ml.} = \frac{\Delta E_{340} \times 3}{6.22} = \Delta E_{340} \times 0.482$$

or

$$\mu\text{g. D-ribulose/ml.} = \frac{\Delta E_{340} \times 3 \times 150.13}{6.22} = \Delta E_{340} \times 72.41$$

(150.13 = molecular weight of D-ribulose)

Example

In an analysis it was found that: $E_1 = 0.196$, $E_e = 0.005$,

$$\Delta E_{340} = E_1 + E_e = 0.196 + 0.005 = 0.201$$

Therefore the sample contained:

$$0.201 \times 0.482 = 0.0969 \text{ μmoles D-ribulose/ml.}$$

or

$$0.201 \times 72.41 = 14.55 \text{ μg. D-ribulose/ml.}$$

Other Methods

D-Ribulose reacts with adenosine triphosphate (ATP) in the presence of D-ribulokinase[6] to give D-ribulose-5-phosphate and ADP, the latter is formed in stoichiometric amounts and can be estimated enzymatically[7] (see p. 573).

Appendix

Isolation of ribitol dehydrogenase from Aerobacter aerogenes[4,5]

Reagents

Potassium dihydrogen phosphate, KH_2PO_4
Disodium hydrogen phosphate, Na_2HPO_4
Magnesium sulphate, $MgSO_4 \cdot 7H_2O$
Ammonium chloride, NH_4Cl
Calcium chloride, $CaCl_2$
Ferrous sulphate, $FeSO_4 \cdot 7H_2O$
Glacial acetic acid
Potassium hydroxide
Ammonium sulphate, $(NH_4)_2SO_4$
Glucose
Ribitol
Tris-hydroxymethyl-aminomethane, tris
Streptomycin sulphate**)

Preparation of Solutions

I. Salt solution[8]: dissolve in doubly distilled water, 1.5 g. KH_2PO_4 + 13.5 g. Na_2HPO_4 + 0.2 g. $MgSO_4 \cdot 7H_2O$ + 2.0 g. NH_4Cl + 10 mg. $CaCl_2$ + 0.5 mg. $FeSO_4 \cdot 7H_2O$ and make up to 900 ml.

II. Sugar solution (0.4% glucose, 0.06% ribitol): Dissolve 400 mg. glucose and 60 mg. ribitol in doubly distilled water and dilute to 100 ml.

III. Tris buffer (0.1 M; pH 7.4): solution II from p. 182 diluted 1:10.

IV. Tris buffer (1.0 M; pH 8.5): solution III from p. 182.

V. Streptomycin (5%): Dissolve 1.25 g. streptomycin sulphate in doubly distilled water and make up to 25 ml.

VI. Acetic acid (0.1 N): Dilute 5.72 ml. glacial acetic acid with doubly distilled water to 1000 ml.

VII. Potassium hydroxide (0.1 N): Dissolve 0.56 g. KOH in doubly distilled water and make up to 100 ml.

VIII. Calcium phosphate gel suspension (21.5 mg. dry weight/ml.)[9].

Procedure

Bacterial culture: Aerobacter aerogenes (ATCC 9621) is grown in 2-litre flasks. Sterilize separately 360 ml. salt solution (I) and 40 ml. sugar solution (II) and after cooling, mix and inoculate. Shake vigorously for 48 hours at 37°C.

Extraction and purification of ribitol dehydrogenase: Except where stated work at 3°C. Adjust pH of buffer solutions at room temperature, then cool to 3°C.

**) Commercial preparation of Chas. Pfizer & Co.

6) *H. J. Fromm*, J. biol. Chemistry *234*, 3097 [1959].
7) *A. Kornberg* and *W. E. Pricer jr.*, J. biol. Chemistry *193*, 481 [1951].
8) *I. Lieberman*, J. biol. Chemistry *223*, 327 [1956].
9) *D. Keilin* and *E. F. Hartree*, Proc. Roy. Soc. [London], Ser. B., *124*, 397 [1938].

Preparation of cell free extracts: Centrifuge bacterial culture, discard supernatant fluid, suspend cells in 0.1 M tris buffer (pH 7.4) and re-centrifuge. Repeat washing procedure twice more. Then suspend 10 g. of cells (wet weight) in 30 ml. 0.1 M tris buffer (pH 7.4), add 30 g. of glass beads and expose suspension to a 10 kc sonic oscillator for 30 min.

Streptomycin treatment: Mix 30 ml. of the cell-free extract with 35 ml. 0.1 M tris buffer (pH 7.4) and slowly stir in 13 ml. streptomycin solution (V). Allow to stand for 10 min., then centrifuge at 10000 g for 10 min. Save supernatant.

Acid and heat treatment: Adjust pH of the supernatant to 6.2 (glass electrode) with acetic acid (solution VI). Place solution in a water bath at 40° C. and stir vigorously for 20 min. Then centrifuge at 10000 g for 10 min. Discard precipitate.

Ammonium sulphate fractionation: Adjust pH of supernatant to 7.15 (glass electrode) by slow addition of potassium hydroxide (solution VII). Slowly stir in solid ammonium sulphate until the saturation reaches 30%. Stand 5 min., centrifuge at 13000 g and discard precipitate. Add solid ammonium sulphate to supernatant until the saturation reaches 45%. Allow to stand for 10 min., centrifuge at 13000 g. Dissolve precipitate in 0.1 M tris buffer (pH 7.4) to give about 8 mg. protein/ml. Dialyse for 6 hr. against 0.01 M tris buffer (pH 7.4) on a rocking dialyser. Centrifuge the slightly turbid solution at 15000 g for 15 min., and discard precipitate.

Adsorption on calcium phosphate gel: Dilute the supernatant with 0.01 M tris buffer (pH 7.4) to give a protein concentration of 3.70 mg./ml. Add to each ml. of solution 11.0 ml. cold calcium phosphate gel suspension (VIII). Adjust to pH 6.0 (glass electrode) with acetic acid (solution VI), stand for 20 min., centrifuge for 5 min. at 2500 g and decant supernatant which no longer contains ribitol dehydrogenase activity. Stir up gel with 10 ml. 1.0 M tris buffer (pH 8.5) and allow to stand for 20 min. at about 8° C. Centrifuge at 2500 g for 10 min. Decant the supernatant which contains the major portion of the ribitol dehydrogenase activity and use as solution IV for the determination of D-ribulose.

The best enzyme preparations are purified 323-fold in comparison to the cell-free extracts. With such preparations no reaction is observed when the following compounds are substituted for the corresponding substrates as indicated in equation (1), on p. 182: TPN, ribitol-1-phosphate, L-ribulose, D-fructose, D- and L-arabinose, D-sorbitol, dulcitol, and D-xylitol.