D-Sedoheptulose-1,7-diphosphate

Determination with Aldolase

Bernard L. Horecker

Compounds which yield triose phosphate can be determined with α-glycerophosphate dehydrogenase by means of the original spectrophotometric procedure of *Warburg* and *Christian*[1]. *Racker*[2] was the first to use this method for the measurement of fructose-1,6-diphosphate (see p. 246). It is also suitable for the determination of sedoheptulose-1,7-diphosphate[3].

Principle

Aldolase catalyses the reaction:

(1) D-Sedoheptulose-1,7-diphosphate $\rightleftharpoons$ dihydroxyacetone phosphate + D-erythrose-4-phosphate

The dihydroxyacetone phosphate formed oxidizes reduced diphosphopyridine nucleotide (DPNH) with the aid of α-glycerophosphate dehydrogenase (GDH):

(2) Dihydroxyacetone phosphate + DPNH + H^+ $\rightleftharpoons$ α-glycerophosphate + DPN^+

Since fructose-1,6-diphosphate also reacts with aldolase to give dihydroxyacetone phosphate, it must be determined separately if it is present in the sample together with sedoheptulose-1,7-diphosphate. This determination is carried out with glyceraldehyde-3-phosphate dehydrogenase, which at low enzyme concentrations only oxidizes glyceraldehyde-3-phosphate and not D-erythrose-4-phosphate:

(3) D-Fructose-1,6-diphosphate $\rightleftharpoons$ dihydroxyacetone phosphate + D-glyceraldehyde-3-phosphate

(4) D-Glyceraldehyde-3-phosphate + DPN^+ $\xrightarrow{AsO_4^{2-}}$ 3-phosphoglycerate + DPNH + H^+

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4
2. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 2H_2O$
3. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
4. α-Glycerophosphate dehydrogenase, GDH
   from skeletal muscle. Crystalline suspension in ammonium sulphate solution. Commercial preparation, see p. 981.
5. Aldolase
   from skeletal muscle. Crystalline suspension in ammonium sulphate solution. Commercial preparation, see p. 970.

Preparation of Solutions

I. Phosphate buffer (0.1 M; pH 7.5):
   Dissolve 0.90 g. KH_2PO_4 and 6.00 g. $Na_2HPO_4 \cdot 2H_2O$ in distilled water and make up to 400 ml.

II. Reduced diphosphopyridine nucleotide (*ca.* 1.5×10^{-3} M β-DPNH):
   Dissolve 10 mg. DPNH-Na_2 in distilled water and make up to 5 ml.

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1) *O. Warburg* and *W. Christian*, Biochem. Z. *303*, 40 [1939].
2) *E. Racker*, J. biol. Chemistry *167*, 843 [1947].
3) *B. L. Horecker, P. Z. Smyrniotis, H. H. Hiatt* and *P. A. Marks, 212*, 827 [1955].

III. α-Glycerophosphate dehydrogenase, GDH (12 mg. protein/ml.):
Dilute the ammonium sulphate suspension with distilled water.

IV. Aldolase (2 mg. protein/ml.):
Dilute the ammonium sulphate suspension with distilled water.

Stability of the solutions

The DPNH solution is stable at −16°C for several weeks. The dilute enzyme solutions should also be stored at −16°C, but the crystalline suspensions in ammonium sulphate solution should be stored at 2°C.

Procedure

Experimental material

Deproteinize the sample solution with perchloric acid as described in the chapter "Pyruvate" (p. 254). Or to inactivate interfering enzymes, adjust to between pH 6 and 6.5 and boil for 1 min. in a 12 ml. conical centrifuge tube. Centrifuge off the coagulated protein and use a portion of the supernatant for the determination.

Spectrophotometric measurements

Preliminary remarks: The amount of DPNH should be in excess of the sedoheptulose-1,7-phosphate, but not sufficient to make the initial optical density too high for accurate measurement of the optical density changes. A concentration of 0.07 μmoles DPNH/ml. is sufficient. This gives an optical density of about 0.430 at 340 mμ. From time to time it is necessary to check whether the two enzymes oxidize DPNH in the absence of substrate. If this is the case, then the experimental results must be suitably corrected.

Method: Wavelength: 340 mμ; light path: 1 cm.; final volume: 1.0 ml.

Pipette into the cuvettes:

Experimental cuvette	*Control cuvette*
0.71 ml. distilled water	0.73 ml. distilled water
0.20 ml. buffer (solution I)	0.20 ml. buffer (solution I)
0.05 ml. DPNH solution (II)	0.05 ml. DPNH solution (II)
0.02 ml. aldolase solution (IV)	0.02 ml. aldolase solution (IV)
0.02 ml. sample	

Take readings of the initial optical density E_1 of both cuvettes against water for several minutes. Mix into both cuvettes

0.01 ml. GDH solution (III).

Read the optical density every 2−3 min. until it is constant (after 5−10 min.). Record the final optical density E_2. Use the differences ΔE_{sample} and $\Delta E_{control}$ for the calculations (each $\Delta E = E_1 - E_2$).

Calculations

Under the conditions of the method sedoheptulose-1,7-phosphate is quantitatively converted to dihydroxyacetone phosphate. Therefore the formation of 1 μmole DPN is equivalent to 1 μmole sedoheptulose-1,7-phosphate and it follows that:

$$\frac{\Delta E_{sample} - \Delta E_{control}}{6.22 \times 0.02} = \mu\text{moles sedoheptulose-1,7-phosphate/ml. sample}$$

where 6.22 = extinction coefficient (cm.²/μmole) for DPNH at 340 mμ
0.02 = ml. sample in the assay mixture.

Sources of Error

The fructose-1,6-diphosphate content of the sample must be determined separately with DPN and glyceraldehyde-3-phosphate dehydrogenase (refer to p. 246). The same holds if the sample contains glyceraldehyde-3-phosphate. If the presence of dihydroxyacetone phosphate is suspected in the sample then a third cuvette should be prepared containing the sample but no aldolase. The ΔE_{sample} should be corrected for any decrease in optical density occurring in this third cuvette.

Appendix

Isolation of sedoheptulose-1,7-diphosphatase [1]

Autolyse 450 g. dry baker's yeast (Fleischmann) with 240 ml. toluene and filter. To every 100 ml. of the filtrate add 22.6 g. $(NH_4)_2SO_4$, centrifuge at 0° C and 13000 g and discard the precipitate. To every 100 ml. of the supernatant add 12.0 g. $(NH_4)_2SO_4$, centrifuge as above and dissolve the precipitate in 10 ml. distilled water.

A quantitative recovery of about 360 mg. protein, having an activity of 0.66 units/mg., is obtained from 450 g. dried yeast.

To remove traces of transketolase dialyse against 0.6% EDTA solution (pH 7.8) for 40 hours.

Determination with Sedoheptulose-1,7-diphosphatase

Efraim Racker

Principle

The determination of sedoheptulose-1,7-diphosphate is based on the reaction:

(1) Sedoheptulose-1,7-diphosphate ⟶ sedoheptulose-7-phosphate + phosphate

The reaction is catalysed by a specific sedoheptulose diphosphatase from yeast [1]. The sedoheptulose-7-phosphate formed is determined enzymatically (see p. 107) or the inorganic phosphate is estimated colorimetrically [2]. The second method is described here.

Reagents

1. Tris-hydroxymethyl-aminomethane, tris
2. Trichloroacetic acid, A. R.
3. Sedoheptulose-1,7-diphosphate, SDP
   barium salt. Prepared from fructose-6-phosphate and fructose-1,6-diphosphate according to [1].
4. Ammonium molybdate, $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$
5. Sodium sulphite, Na_2SO_3
6. Sodium hydrogen sulphite, $NaHSO_3$
7. Hydrochloric acid, A. R., 5 N
8. Sulphuric acid, A. R., conc.
9. Potassium dihydrogen phosphate, KH_2PO_4

[1] *E. Racker* and *E. A. R. Schroeder*, Arch. Biochem. Biophysics *74*, 326 [1958].
[2] *K. Lohmann* and *L. Jendrassik*, Biochem. Z. *178*, 419 [1926].

10. 1-Amino-2-naphthol-4-sulphonic acid
The preparation must be pure. Otherwise purify as follows: dissolve 150 g. $NaHSO_3$ and 10 g. Na_2SO_3 in 1000 ml. distilled water at 90° C, then dissolve 15 g. of the sulphonic acid in this solution and filter hot. After cooling the filtrate, add 10 ml. conc. HCl, filter off the precipitate, wash with 300 ml. distilled water and then with ethanol until the filtrate is colourless. Dry the residue in the dark, powder and store in a brown bottle.

11. Sedoheptulose-1,7-diphosphatase
isolated from baker's yeast according to[1]; see p. 116.

Purity of the enzyme preparation

If inorganic phosphate is determined, it is essential that the sedoheptulose-1,7-diphosphatase preparation contains no fructose-1,6-diphosphatase nor any unspecific phosphatases. This is tested by incubation of the preparation with fructose-1,6-diphosphate.

If the enzymatic assay of sedoheptulose-7-phosphate is used, contamination with fructose-1,6-diphosphatase does not interfere, but presence of unspecific phosphatases which cleave sedoheptulose-1,7-diphosphate at C-7 lead to low values. However, even rather crude preparations of sedoheptulose-1,7-diphosphatase (obtained according to[1]) are suitable.

Preparation of Solutions

I. Tris buffer (1 M; pH 7.2):
Dissolve 12.11 g. tris-hydroxymethyl-aminomethane in 50 ml. distilled water, adjust to pH 7.2 with *ca.* 18 ml. 5 N HCl and dilute to 100 ml. with distilled water.

II. Trichloroacetic acid (10% w/v):
Dissolve 10 g. trichloroacetic acid in distilled water and make up to 100 ml.

III. Sedoheptulose-1,7-diphosphate standard solution (7×10^{-3} M SDP):
Dissolve 44.94 mg. barium salt in 5 ml. distilled water, remove the Ba^{2+} with Dowex 50 (Na^+-form) and dilute to 10 ml. with distilled water.

IV. Molybdate (*ca.* 2×10^{-3} M):
Dissolve 2.5 g. $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$ in 500 ml. distilled water. Carefully mix 14 ml. conc. H_2SO_4 with 200 ml. distilled water, add the dilute acid solution to the molybdate solution and dilute to 1000 ml. with distilled water.

V. Reducing agent:
Dissolve 5.7 g. $NaHSO_3$ and 0.2 g. Na_2SO_3 in 50 ml. distilled water. Dissolve 0.1 g. 1-amino-2-naphthol-4-sulphonic acid in this solution and dilute to 100 ml. with distilled water.

VI. Phosphate standard solution (5×10^{-4} M):
Dissolve 68 mg. KH_2PO_4 in 500 ml. distilled water, carefully add 10 ml. conc. H_2SO_4 and dilute to 1000 ml. with distilled water.

VII. Sedoheptulose-1,7-diphosphatase (6 units*)/ml.):
If necessary, dilute the enzyme preparation obtained according to[1] with distilled water.

Stability of the solutions

All solutions, except for III, V and VII keep at room temperature practically indefinitely. The sedoheptulose-1,7-diphosphate standard solution should be stored at −20° C. It keeps for several weeks

*)A unit is the amount of enzyme which converts 1 μmole of substrate in 1 min. (refer to p. 32).

in this state. The reducing agent (V) should be stored in the dark at room temperature, in small bottles which are completely filled. The contents of an opened bottle are only usable for a week. The sedoheptulose-1,7-diphosphatase keeps for several years at −20° C.

Procedure

Preliminary remarks: If the sedoheptulose-7-phosphate is to be determined enzymatically, then it is necessary to deproteinize the sample with perchloric acid (see p. 109). In the determination of inorganic phosphate the deproteinization is carried out with trichloroacetic acid after the incubation (see "Enzymatic reaction").

Enzymatic reaction

The method is controlled with at least one standard which contains a known amount of SDP. Pipette into centrifuge tubes:

	Experimental	*Control*	*SDP Standard*
buffer (solution I)	0.01 ml.	0.01 ml.	0.01 ml.
enzyme solution (VII)	0.05 ml.	0.05 ml.	0.05 ml.
sample	+	+	−
SDP standard solution (III)	−	−	0.01 ml.
distilled water	to 0.1 ml.	to 0.1 ml.	to 0.1 ml.

Add

0.1 ml. trichloroacetic acid solution (II)

immediately to the control tube, and to the other tubes after

30 min. incubation at 37° C (water bath).

Centrifuge, wash the precipitates with

0.5 ml. distilled water

and again centrifuge. Combine the supernatants and discard the precipitates.

Phosphate estimation

The phosphate content of the supernatants is determined colorimetrically according to[2] Wavelength: 660 or 700 mμ.

Pipette into test tubes:

Experimental, Control and SDP standard	*Phosphate standard*
0.70 ml. molybdate solution (IV)	0.70 ml. molybdate solution (IV)
0.15 ml. supernatant	0.15 ml. phosphate standard solution (VI).

When all the tubes are prepared, mix into each tube

0.15 ml. reducing agent (V)

and note the time. Allow sufficient time between each pipetting of the reducing agent, so that later a colorimetric measurement can be carried out in this time interval. Allow each tube to stand for between 15 and 60 min. at room temperature (there must be the same time interval between the addition of the reducing agent and the colorimetric measurements) and then read the optical densities.

Calculations

1 μmole of phosphate is formed for each μmole of sedoheptulose-1,7-diphosphate. Therefore:

$$\frac{E_E - E_C}{E_S} \times [P] \times 4.7 = \mu\text{moles sedoheptulose-1,7-diphosphate/enzymatic reaction mixture.}$$

where

E_E = optical density of the solution from the experimental tube

E_C = optical density of the solution from the control tube

E_S = optical density of the phosphate standard

[P] = μmoles phosphate in the standard tube (here: 0.08 μmoles)

4.7 = conversion of the ml. supernatant in the phosphate determination mixture to the volume of the whole supernatant.

Appendix

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To remove traces of transketolase, dialyse for 40 hours against 0.6% EDTA solution (pH 7.8).