D-1,3-Diphosphoglycerate

Erwin Negelein

The spectrophotometric determination of D-1,3-diphosphoglycerate was described in 1939 by *Negelein* and *Brömel*[1].

Principle

The following reversible reaction is used for the determination of D-1,3-diphosphoglycerate:

(1) Glyceraldehyde-3-phosphate + phosphate + DPN^+ $\rightleftharpoons$ 1,3-diphosphoglycerate + DPNH + H^+

This reaction proceeds virtually quantitatively from right to left if the solutions used are free from inorganic phosphate. About a 10% excess of DPNH is sufficient at pH 7.9. The reaction is catalysed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). 1 Mole of D-1,3-diphosphoglycerate oxidizes 1 mole of DPNH. The decrease of DPNH is measured spectrophotometrically. Sufficient enzyme is taken so that the reaction is complete within a few minutes.

The reaction is not inhibited by approximately equal amounts of strychnine, therefore the crystalline strychnine salt[1] of 1,3-diphosphoglycerate can also be determined in this way.

Reagents

1. Sodium pyrophosphate, $Na_4P_2O_7 \cdot 10H_2O$, A. R.
2. Hydrochloric acid, A. R., 1 N
3. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
4. D-Glyceraldehyde-3-phosphate dehydrogenase, GAPDH
   crystalline, from yeast or skeletal muscle. Crystalline suspension in 2.4 M ammonium sulphate solution. Commercial preparation, see p. 979.

   Purity of the enzyme preparation

   The GAPDH preparation should be recrystallized and must satisfy the usual requirements for enzymatic assays.

Preparation of Solutions

Use fresh, preferably doubly distilled water from a glass apparatus.

I. Pyrophosphate buffer (0.10 M; pH 7.9):
   Dissolve 4.47 g. $Na_4P_2O_7 \cdot 10\ H_2O$ in about 90 ml. distilled water, add 6.0 ml. 1 N HCl, and dilute with distilled water to 100 ml.

II. Reduced diphosphopyridine nucleotide (*ca.* 2.2×10^{-3} M β-DPNH):
   Dissolve 4.1 mg. DPNH-Na_2 in distilled water and make up to 2.0 ml.

III. D-Glyceraldehyde-3-phosphate dehydrogenase, GAPDH (1 mg. protein/ml.):
   Dilute the crystalline suspension with distilled water to *ca.* 1 mg. protein/ml.

---

[1] *E. Negelein* and *H. Brömel*, Biochem. Z. *303*, 132 [1939]; *E. Negelein* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 216.

Stability of the solutions

The pyrophosphate buffer should be free of orthophosphate and therefore must not be kept for more than a few days. The DPNH solution should also be stored only for a few days at 0°C. The enzyme solution is prepared freshly each day and kept at 0°C.

Procedure

Stability of 1,3-diphosphoglycerate

1,3-Diphosphoglycerate is a labile compound which spontaneously decomposes to give 3-phosphoglycerate and inorganic phosphate. No conditions are known under which 1,3-diphosphoglycerate is stable for a long time. The rate of decomposition depends on the temperature and on the pH of the solution. In aqueous solution at pH 7.2 and 38°C the half-life is 27 minutes, *i.e.* 2.6% of the compound decomposes per minute. The rate of decomposition is lowest in aqueous solutions of slightly alkaline reaction, above pH 7 to pH 9; under these conditions approximately 6% decomposes in 24 hours at 0°C. The compound is slightly more stable when it is stored as a frozen, slightly alkaline solution. A loss of 3% was found under these conditions after 24 hours. The substance is not stable in the dry state; the neutral and the acid Na-salt, amorphous Ca-salt and the crystalline strychnine salt all decompose. Molybdate greatly accelerates the hydrolysis in acid solution, so that in colorimetric phosphate estimations the labile phosphate group is split off and is determined as inorganic phosphate.

Experimental material

In the living cell the concentration of 1,3-diphosphoglycerate is so low under physiological conditions, that its estimation is hardly possible. According to *Bücher*[2] the equilibrium of the reaction forming 1,3-diphosphoglycerate (glyceraldehyde-3-phosphate dehydrogenase) and the subsequent phosphate transfer reaction (phosphoglycerate kinase) result in a low stationary concentration of 1,3-diphosphoglycerate. Even in the cell the rate of the 1,3-diphosphoglycerate consuming reaction is greater than that of the producing reaction, because of the concentration and activity of the phosphoglycerate kinase. Therefore the only experimental material likely to be encountered are samples from *in vitro* experiments with purified D-1,3-diphosphoglycerate.

The forward reaction of the phosphorylating oxidation reaction (1) can be used for the preparation of D-1,3-diphosphoglycerate. When reaction (1) is coupled with reaction (2) it proceeds virtually quantitatively from left to right in the presence of a small, catalytic amount of DPN[1].

(2) $$\text{DPNH} + \text{H}^+ + \text{acetaldehyde} \rightleftharpoons \text{DPN}^+ + \text{ethanol}.$$

The DPNH formed according to (1) is re-oxidized to DPN and the over-all balance of the reactions is

(3) $$\text{D-Glyceraldehyde-3-phosphate} + \text{phosphate} + \text{acetaldehyde} \rightleftharpoons \text{D-1,3-diphosphoglycerate} + \text{ethanol}.$$

Diphosphoglycerate can be separated from the other reactants and the enzymes by precipitation with acetone at pH 2.1. The reaction mixture is acidified to pH 2.1 with 1 N H_2SO_4 and quickly poured into 10 volumes cold acetone. The precipitate is centrifuged in the cold, washed once with cold acetone and dried in a vacuum desiccator. As the 1,3-di-

[2] *Th. Bücher*, Biochim. biophysica Acta *1*, 292 [1947].

phosphoglycerate is not stable in the dry state, dissolve it in a few ml. of cold water as soon as it is dry. The insoluble, denatured protein is filtered off and the clear acid solution neutralized with 1 N NaOH.

From a preparation starting with 4.4×10^{-4} moles D-glyceraldehyde-3-phosphate, 3.6×10^{-4} moles 1,3-phosphoglycerate were obtained[1] (yield: 80 to 85%). 1,3-Diphosphoglycerate can not be isolated when it is present in too low a concentration. A better deproteinizing agent is not known. The solution to be analysed must be free from interfering enzymes and from deproteinizing agents. The solution should be made only weakly acid during the deproteinization because of the lability of 1,3-diphosphoglycerate.

Spectrophotometric measurements

Wavelength: 340 or 334 mμ; silica cells, light path 0.5 cm.*); final volume: 2.5 ml.; read against the control cuvette.

Pipette successively into the cuvettes:

Experimental cuvette	*Control cuvette*
0.83 ml. pyrophosphate buffer (soln. I)	0.83 ml. pyrophosphate buffer (soln. I)
0.20 ml. DPNH solution (II)	1.67 ml. distilled water
1.24 ml. distilled water	
0.20 ml. of the sample to be analysed, containing not more than 0.4 μmoles 1,3-diphosphoglycerate.	

Measure optical density E_1. To the experimental cuvette add

0.03 ml. GAPDH solution (III),

mix and follow the course of the reaction on the spectrophotometer scale. The amount of protein taken is so adjusted that the reaction comes to a stop within a short period of time. Read the final optical density E_2. After suitable correction for the dilution due to the enzyme addition, the DPNH oxidized is calculated from the decrease in optical density $\Delta E = E_1 - E_2$.

If more than 90% of the DPNH is oxidized then the determination must be repeated with a smaller amount of the 1,3-diphosphoglycerate solution. A cuvette with a larger light path should be used if the 1,3-diphosphoglycerate concentration is low.

Calculations

Under the stated conditions the reaction is virtually quantitative, even when 90% of the DPNH has been oxidized. The concentration of D-1,3-diphosphoglycerate in the sample is

$$c = \frac{\Delta E}{\varepsilon \times d} \times \frac{V}{a} \text{ μmoles/ml.}$$

where

c = concentration of D-1,3-diphosphoglycerate in the sample [μmoles/ml.]
ΔE = decrease in optical density ($E_1 - E_2$)
ε = extinction coefficient [cm.²/μmole]
d = light path [cm.]
V = volume of fluid in the test cuvette [ml.]
a = volume of sample added to the test cuvette [ml.]

*) For measurements at 366 mμ use a light path of 1 cm. and the same assay mixture.

The extinction coefficient ε is 5.90 $cm.^2/\mu mole$ at 334 mμ.
6.22 $cm.^2/\mu mole$ at 340 mμ.
3.30 $cm.^2/\mu mole$ at 366 mμ.

Example

Wavelength: 334 mμ; ε = 5.90 $cm.^2/\mu mole$; a = 0.20 ml.; V = 2.50 ml. and d = 0.5 cm. The measured ΔE = 0.436. The ΔE has to be corrected owing to the dilution of the test solution by addition of 0.03 ml. GAPDH solution:

$$\Delta E_{corrected} = 0.436 \times \frac{2.47}{2.50} = 0.431.$$

The sample contains:

$$c = \frac{0.431}{5.90 \times 0.50} \times \frac{2.50}{0.20} = 1.83 \text{ } \mu\text{moles D-1,3-diphosphoglycerate/ml.}$$