# D-6-Phosphogluconate

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## Principle

6-Phosphogluconic dehydrogenase (6-PG-DH) catalyses the oxidation of 6-phosphogluconate (gluconic acid-6-phosphate) by triphosphopyridine nucleotide (TPN):

(1) 6-Phosphogluconate + $TPN^+$ $\rightleftharpoons$ $CO_2$ + ribulose-5-phosphate + TPNH + $H^+$

The equilibrium of the reaction lies far to the right, so that with a slight excess of TPN and at pH 7–8, a quantitative oxidation of 6-phosphogluconate is obtained[1]. The reaction proceeds very quickly. The increase of optical density at 334, 340 or 366 mμ due to the reduction of TPN is a measure of the reaction.

As it is not possible at present*) to obtain a 6-phosphogluconic dehydrogenase preparation of sufficient purity (in particular, free from hexokinase and glucose-6-phosphate dehydrogenase), the enzymatic determination of 6-phosphogluconate can only be carried out on solutions which do not contain either glucose or glucose-6-phosphate. The method therefore cannot be used for tissue extracts.

## Reagents

1. Triethanolamine hydrochloride
2. Sodium hydroxide, A. R., 2 N
3. Magnesium chloride, A. R., $MgCl_2 \cdot 6\ H_2O$
4. Triphosphopyridine nucleotide, TPN
   monosodium salt, TPN-$NaH_2$; commercial preparation, see p. 1029.
5. 6-Phosphogluconic dehydrogenase, 6-PG-DH
   from yeast, commercial preparation, see p. 993.

**Purity of the enzyme preparation**

6-Phosphogluconic dehydrogenase is prepared from brewer's yeast according to[1]. From 120 g. dried yeast about $6.2 \times 10^3$ units**) 6-PG-DH is obtained with a specific activity of 54 units/mg. The preparation can be freeze-dried without any great loss of activity (yield *ca.* 80%) and in this state it is stable for months. The preparation is contaminated with about 20% glucose-6-phosphate dehydrogenase and about 5% hexokinase (relative to the 6-PG-DH activity).

## Preparation of Solutions

Prepare all solutions with fresh, doubly distilled water.

I. Triethanolamine buffer (0.4 M; pH 7.6):
   Dissolve 18.6 g. triethanolamine hydrochloride in about 200 ml. doubly distilled water, add 18 ml. 2 N NaOH and dilute to 250 ml.

II. Magnesium chloride (0.1 M):
   Dissolve 2 g. $MgCl_2 \cdot 6\ H_2O$ in doubly distilled water and make up to 100 ml.

III. Triphosphopyridine nucleotide (*ca.* $2 \times 10^{-2}$ M β-TPN):
   Dissolve 20 mg. TPN-$NaH_2$ in 1 ml. doubly distilled water.

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*) The enzyme has now been crystallized: *S. Pontremdi, A. de Flora, E. Grazi, G. Mangiarotti, A. Bonsignore* and *B. L. Horecker*, J. biol. Chemistry *236*, 2975 [1961].

**) According to *Bücher et al.* Definition, see p. 33.

1) *B. L. Horecker* and *P. Z. Smyrniotis*, J. biol. Chemistry *193*, 371 [1951].

IV. 6-Phosphogluconic dehydrogenase, 6-PG-DH (*ca.* 800 units*)/ ml.):
Take up the lyophilized preparation (about 16 mg. protein) in 1 ml. M/25 glycylglycine buffer pH 7.5 and remove the insoluble material by centrifuging in the cold.

**Stability of the solutions**

Store all the solutions, stoppered, in a refrigerator at 0—4°C. The enzyme solution is stable only for a few days. Preferably prepare the daily requirement of the enzyme by dissolving the lyophilized preparation freshly each time.

## Procedure

**Spectrophotometric measurements**

*Preliminary remarks:* The concentration of 6-phosphogluconate in the assay mixture should not exceed $1 \times 10^{-7}$ moles/ml. A blank can usually be omitted. The sample must be free from glucose-6-phosphate and fructose-6-phosphate, and its pH should be between 5 and 9.

*Method:* Wavelength: 340, 334 or 366 mμ; light path: 1 cm.; final volume: 1.08 ml. Read against the control cuvette. Pipette successively into the cuvettes:

| *Experimental cuvette* | *Control cuvette* |
|---|---|
| up to 0.7 ml. sample | 2 ml. buffer (solution I) |
| make up to 1 ml. with buffer (solution I) | |
| 0.01 ml. TPN solution (III) | |
| 0.05 ml. $MgCl_2$ solution (II). | |

Mix thoroughly, bring the contents of the cuvettes to room temperature and read the optical density $E_1$ twice with an interval of 3 min. Mix into the experimental cuvette

0.02 ml. 6-PG-DH solution (IV).

When the optical density no longer increases (5—10 min. after the addition of the enzyme, depending on the 6-phosphogluconate concentration) read the optical density $E_2$ twice with an interval of 3 min. The changes within 3 min. in the initial optical density $E_1$ and the final optical density $E_2$ are usually small in comparison to difference in optical density $\Delta E = E_2 - E_1$ and can be neglected. If necessary, apply a correction (see p. 39). The correct functioning of the assay can easily be checked, if on completion of the reaction 0.010 ml. $2 \times 10^{-3}$ M 6-phosphogluconate solution is added to the experimental cuvette. The increase in optical density should be complete in 3—5 min. Read the optical density $E_3$ twice within 3 min. and calculate the difference $\Delta E' = E_3 - E_2$. $\Delta E'$ should be 0.115 at 340 mμ, 0.112 at 334 mμ and 0.061 at 366 mμ.

**Calculations**

Under the stated conditions 6-phosphogluconate is quantitatively oxidized, so that the 6-phosphogluconate content of the sample can be calculated from the optical density difference $\Delta E$:

$$\frac{\Delta E \times V}{\varepsilon \times d \times V_s} = \mu\text{moles 6-phosphogluconate/ml. sample.}$$

where $V$ = assay volume in ml.
$V_s$ = volume of the sample in ml.
$d$ = light path of the cuvette in cm.
$E$ = optical density difference $[E_2 - E_1]$
$\varepsilon$ = extinction coefficient [cm.$^2$/μmole].

*) See footnote on p. 143.

If the measurements are made at 366 mμ then the contents of the cuvette must be equilibrated at 25° C, because the extinction coefficient is dependent on temperature at this wavelength[2].

**Example**

A 6-phosphogluconate solution (0.4 ml.) was tested and the measurements were made at 340 mμ:

| | |
|---|---|
| Before addition of 6-PG-DH | 0 min. $E_1 = 0.068$ |
| | 3 min. $E'_1 = 0.068$ |
| After addition of 6-PG-DH | 0 min. $E_2 = 0.493$ |
| | 3 min. $E_2' = 0.493$ |

$$\Delta E = E_2 - E_1 = 0.425$$

$$\frac{0.425 \times 1.08}{6.28 \times 1 \times 0.4} = 0.183 \text{ μmoles 6-phosphogluconate/ml. sample}$$

## Specificity

In the absence of glucose, glucose-6-phosphate and fructose-6-phosphate the assay is specific for 6-phosphogluconate. Gluconate does not react.

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[2] *H. J. Hohorst*, Biochem. Z. *328*, 509 [1957].