D-Glucose-6-phosphate and D-Fructose-6-phosphate

Determination with Glucose-6-phosphate Dehydrogenase and Phosphoglucose Isomerase

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Principle

Glucose-6-phosphate dehydrogenase (G6P-DH, Zwischenferment) catalyses the oxidation of glucose-6-phosphate (G-6-P) by triphosphopyridine nucleotide (TPN).

(1) Glucose-6-phosphate + TPN^+ $\rightleftharpoons$ 6-phosphogluconolactone + TPNH + H^+

According to[1] the equilibrium constant is $K_c = 6.0 \times 10^{-7}$ moles/l. (28°C). At pH 7.6 K_c is 2.4×10^1 moles/l., so that with a suitable excess of TPN (about five times the G-6-P concentration) the oxidation of G-6-P is virtually quantitative[2]. The reaction is measured by the increase in optical density at 340 or 334 (also 366) mμ when the TPN is reduced.
Phosphoglucose isomerase (PGI) catalyses the reaction

(2) Fructose-6-phosphate $\rightleftharpoons$ glucose-6-phosphate

The equlibrium constant is $K_c \approx 2$. By coupling reaction (2) with reaction (1) fructose-6-phosphate (F-6-P) can be made to react almost quantitatively in the presence of excess TPN.

Reagents

1. Potassium carbonate, K_2CO_3, A. R.
2. Methyl orange indicator
3. Perchloric acid, A. R.; sp. gr. 1.67; *ca.* 70% (w/w)
4. Triethanolamine hydrochloride
5. Sodium hydroxide, A. R., 2 N
6. Magnesium chloride, $MgCl_2 \cdot 6H_2O$, A. R.
7. Triphosphopyridine nucleotide, TPN
   sodium salt, TPN-NaH_2. Commercial preparation, see p. 1029.
8. Glucose-6-phosphate dehydrogenase, G6P-DH, Zwischenferment
   from yeast, suspension in 3.3 M ammonium sulphate solution. Commercial preparation, see p. 974.
9. Phosphoglucose isomerase, PGI
   from yeast, crystalline suspension[3] in 2.4 M ammonium sulphate solution. Commercial preparation, see p. 993.

Purity of the enzyme preparations

The G6P-DH preparation should have a specific activity of about 4000 units/mg. (according to *Bücher**)) or about 70 units/mg. (according to *Racker**)). Contamination of the preparation by hexokinase should not exceed 0.2%, by 6-phosphogluconic dehydrogenase 0.01%, by phosphoglucose isomerase 0.05% and by glutathione reductase 0.5% (relative to the activity of the G6P-DH preparation). Also the enzyme must be practically free from flavin enzymes (see under "Sources of Error").

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*) Definition of units, see p. 33.

1) *L. Glaser* and *D. H. Brown*, J. biol. Chemistry *216*, 67 [1955].
2) *A. J. Kornberg*, J. biol. Chemistry, *182*, 805 [1950].
3) *H. Klotsch* and *H.-U. Bergmeyer*, Angew. Chem. *72*, 920 [1960].

Phosphoglucose isomerase should have a specific activity of *ca.* 10^4 units/mg. (according to *Bücher**). It should be practically free of 6-phosphogluconic dehydrogenase, phosphoglucomutase, glutathione reductase and flavin enzymes (TPNH oxidase).

Preparation of Solutions

Prepare all solutions with fresh, doubly distilled water.

I. Potassium carbonate (*ca.* 5 M):
Dissolve *ca.* 69 g. K_2CO_3 in distilled water and make up to 100 ml.

II. Methyl orange indicator:
Dissolve *ca.* 50 mg. methyl orange in distilled water and make up to 100 ml.

III. Perchloric acid (*ca.* 6% w/v):
Dilute *ca.* 7.7 ml. $HClO_4$ (sp. gr. 1.67) to 150 ml. with distilled water.

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

V. Magnesium chloride (0.5 M):
Dissolve 10 g. $MgCl_2 \cdot 6H_2O$ in distilled water and make up to 100 ml.

VI. Triphosphopyridine nucleotide (*ca.* 2×10^{-2} M β-TPN):
Dissolve 20 mg. TPN-NaH_2 in 1 ml. distilled water.

VII. Glucose-6-phosphate dehydrogenase, G6P-DH (*ca.* 0.25 mg. protein/ml.):
Dilute the enzyme suspension containing about 5 mg. protein/ml. in 3.3 M ammonium sulphate solution with distilled water.

VIII. Phosphoglucose isomerase, PGI (*ca.* 0.1 mg. protein/ml.):
Dilute the crystalline suspension containing about 10 mg. protein/ml. in 2.4 M ammonium sulphate solution with distilled water.

Stability of the solutions

Store all solutions, stoppered, in a refrigerator. Under these conditions even the TPN solution and the enzyme suspensions are stable for several weeks.

Procedure

Experimental material

Obtain blood without constriction of the vein and immediately deproteinize. Quickly inactivate tissue samples, preferably by the "quick-freeze" method[4] (see p. 47).

Deproteinization and extraction

Preliminary remarks: Add perchloric acid to deproteinize samples. Since glucose-6-phosphate and fructose-6-phosphate are intracellular metabolites[4] (for example, they are absent from plasma), a thorough extraction of the tissue is essential for their quantitative analysis. Extract tissue twice with $HClO_4$ solution and to simplify the calculations always work so that the ratio of volume of extract to tissue weight is 8 : 1. Under these conditions the error due to retention of the compounds in the precipitate is not more than 3—4% and can usually be neglected.

*) Definition of units, see p. 33.

[4] *H. J. Hohorst, F. H. Kreutz* and *Th. Bücher*, Biochem. Z. *332*, 18 [1959].

Method: Weigh a centrifuge tube containing a glass rod and 5 ml. perchloric acid solution (III). Add about 1 g. of the sample: allow blood to flow directly from the cannula, and powder frozen tissue[4] (see also p. 48) before adding. Quickly mix and re-weigh. Homogenize suspension and centrifuge for 10 min. at 3000 g. Carefully decant the supernatant, stir the sediment with 1 ml. perchloric acid solution (III) + 1 ml. doubly distilled water and centrifuge again. Combine the supernatant fluids, measure the volume and dilute with doubly distilled water to 8 ml./g. sample.

Neutralization: Cool 8 ml. of extract in ice, and while stirring vigorously with a magnetic stirrer, pipette in 0.02 ml. indicator solution (II) followed by about 0.1 ml. carbonate solution (I) from a 0.2 ml. capillary pipette. When the CO_2 evolution has almost ceased add more carbonate solution until the mixture is salmon pink (pH *ca.* 3.5); this requires approximately another 0.18 ml. carbonate solution. Allow the neutralized extract to stand about 10 min. in ice water, decant or pipette off the supernatant from the precipitated perchlorate and use a portion for the determination.

Spectrophotometric measurements

Preliminary remarks: Calculation of the results is simplified if the same ratio of total volume to sample volume is always chosen. A control can usually be omitted. The glucose-6-phosphate concentration should not exceed 10^{-7} moles/ml. assay mixture when carrying out measurements on tissue extracts (see under "Sources of Error").

Method:

Wavelength: 340 or 334 mμ; light path: 1 cm.; final volume: 1.025 ml. Measure against the control cuvette.

Experimental cuvette	*Control cuvette*
0.5 ml. buffer (solution IV)	2 ml. buffer (solution IV)
0.5 ml. deproteinized extract	
0.01 ml. TPN solution (VI)	
0.01 ml. $MgCl_2$ solution (V)	

Wavelength: 366 mμ; light path: 2 cm.; final volume 2.55 ml. Measure against the control cuvette.

Experimental cuvette	*Control cuvette*
1.0 ml. buffer (solution IV)	2.5 ml. buffer (solution IV)
1.5 ml. deproteinized extract	
0.02 ml. TPN solution (VI)	
0.02 ml. $MgCl_2$ solution (V)	

Mix the cuvette contents thoroughly, allow to warm to room temperature, read the optical density E_1 and then again after a 3 min. interval. Using a small glass spatula mix

0.005 or 0.010 *) ml. G6P-DH solution (VII)

into the experimental cuvette. After completion of the reaction (3—5 min. after addition of enzyme, according to the G-6-P concentration) read the optical density E_2 and then again 3 min. later. The changes in optical density occurring within 3 min. are usually small in

*) For measurements at 366 mμ.

comparison to the optical density difference $\Delta E = E_2 - E_1$ and can be neglected. Otherwise a correction must be applied (see p. 39).

After measuring E_2, to determine fructose-6-phosphate, mix

0.01 ml. PGI solution (VIII)

into the experimental cuvette and on completion of the reaction (3–5 min.) read the optical density E_3 twice or more times at 3 min. intervals.

To test if the assay is working correctly, on completion of the reaction mix successively into the experimental cuvette

0.01 ml. 2×10^{-3} M G-6-P and F-6-P solution.

The increases in optical density should be complete after 3–5 min. Read the optical densities E_4 and E_5 twice within 3 min. and calculate the differences $\Delta E'_{G\text{-}6\text{-}P} = E_4 - E_3$ and $\Delta E'_{F\text{-}6\text{-}P} = E_5 - E_4$. $\Delta E'$ should be 0.121 at 340 mμ and 0.052 at 366 mμ.

Because of their low concentration G-6-P and F-6-P can only be measured in blood extracts at 340 mμ or 334 mμ and with a light path of 5 cm. Final volume: 4.57 ml.

Experimental cuvette	*Control cuvette*
2.0 ml. buffer (solution IV)	5 ml. buffer (solution IV)
2.5 ml. deproteinized extract	
0.02 ml. TPN solution (VI)	
0.04 ml. $MgCl_2$ solution (V)	

Start the reactions as described above by addition of 0.01 ml. G6P-DH and PGI solution.

Calculations

Glucose-6-phosphate and fructose-6-phosphate are quantitatively converted under the given conditions. Their concentrations in the sample are calculated from the optical density differences ΔE:

$$\frac{\Delta E \times \text{dil.}}{\varepsilon \times d} = \mu\text{moles G-6-P or F-6-P/g. tissue}$$

where

ΔE = optical density difference ($E_2 - E_1$ for G-6-P, $E_3 - E_2$ for F-6-P).
dil. = total dilution of the sample in the assay (that is 17 : 1 at 334 or 340 mμ; 14.1 : 1 at 366 mμ).
ε = extinction coefficient [cm.²/μmole]
d = light path [cm.]

With constant dilution ratios the equation simplifies to

$$\Delta E \times F = \mu\text{moles G-6-P or F-6-P/g. tissue} \quad \left(F = \frac{\text{dil.}}{\varepsilon \times d}\right)$$

Substitute for F the following values:

Tissue extracts, 334 mμ: F = 2.80
340 mμ: F = 2.71
366 mμ: F = 2.14

Blood extracts, 340 mμ, 5 cm. cuvette: F = 0.485.

At 366 mμ ε is slightly dependent on the temperature[5]. Therefore allow cuvettes to reach room temperature before commencing measurements. The values for F given here are for 25° C.

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

Example

The volume of a neutralized extract of 1.084 g. of rat liver was 9.0 ml. The measurements were carried out at 366 mμ (d = 2 cm., 25° C):

Before addition of G6P-DH	0 min.	$E_1 = 0.138$
	3 min.	$E'_1 = 0.140$
After addition of G6P-DH	0 min.	$E_2 = 0.303$
	3 min.	$E'_2 = 0.305$

then $\Delta E = E'_2 - E'_1 = 0.305 - 0.140 = 0.165$

$0.165 \times 2.14 = 0.353$ μmoles G-6-P/g. tissue

Further Determinations

Other metabolites can be determined in the same assay mixture by the addition of specific enzymes and coenzymes, *e.g.* adenosine triphosphate (ATP) by addition of glucose and hexokinase or conversely glucose by the addition of ATP and hexokinase[4] (refer to p. 543 or 117).

Sources of Error

If the reaction takes longer than 10 min. to complete the enzyme preparations have lost activity and therefore larger amounts should be taken or they should be replaced.

If the G6P-DH preparation contains appreciable amounts of enzymes oxidizing TPNH (see under "Purity of the enzyme preparations") the optical density will reach a maximum and then decrease. The oxidation of TPNH by flavin enzymes can be obviated by passing oxygen-free nitrogen through the cuvette[4,6].

Specificity

The assay with G6P-DH is specific for glucose-6-phosphate; in particular glucose, 6-phosphogluconate and (in the absence of PGI) fructose-6-phosphate do not react. Interference from glucosamine-6-phosphate[7] need not be considered in blood, liver and muscle extracts.

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[6] *H. Börning, K. Stade, H. Frunder* and *G. Richter*, Hoppe-Seylers Z. physiol. Chem. *310*, 232 [1958].
[7] *D. H. Brown*, Biochim. biophysica Acta *7*, 487 [1951].