Glucose-6-phosphatase

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Glucose-6-phosphatase (G-6-Pase) occurs in mammalian liver[1–3] and kidney[2], and in avian liver[4]. Preparations from some other mammalian organs catalyse the hydrolysis of glucose-6-phosphate (G-6-P) under the conditions of the G-6-Pase assay, but this activity appears to be due to phosphatases with low substrate specificity (*e.g.* acid or alkaline phosphatases[2,5]). G-6-Pase is not found in foetal mammalian liver but appears shortly after birth[6,7]. Hydrolysis of G-6-P by preparations from tadpole liver has been reported, but no pH optimum was given for the enzymatic activity[8]. Similarly, liver from mature frogs and fish catalyses the hydrolysis of G-6-P, but in both cases the pH optima are similar to those of acid and alkaline phosphatases[3].

G-6-Pase occurs in the liver microsomes and its activity can be used to follow the preparation of these particles during tissue fractionation studies[9,10]. The enzyme requires no cofactors or activators. It has been solubilized and partially purified[11]. G-6-Pase is not completely specific for G-6-P. It does not catalyse the hydrolysis of glucose-1-phosphate, fructose-1,6-diphosphate, 6-phosphogluconate or β-glycerophosphate. Some phosphate esters, for example, fructose-6-phosphate, are hydrolysed at relatively slow rates[5,12,13]. The G-6-Pase activity can be determined by measuring the amount of glucose or inorganic phosphate formed on incubation with G-6-P[14].

Principle

Glucose-6-phosphatase catalyses the reaction:

(1) $$\text{Glucose-6-phosphate} + H_2O \longrightarrow \text{glucose} + \text{phosphate}$$

The rate of the reaction is measured by the increase of inorganic phosphate with time.

Optimum Conditions for Measurements

G-6-Pase has a broad pH optimum between 6.0 and 7.0[1–3,14]. The activity is usually measured at pH 6.5 to minimize the interference from alkaline phosphatases[9]. The substrate concentration in the assay mixture should be at least 0.03 M[15,16].

Reagents

1. Citric acid, $C_6H_8O_7 \cdot H_2O$
   or
   Sodium cacodylate, $Na(CH_3)_2AsO_2 \cdot 3H_2O$

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2. Glucose-6-phosphate, G-6-P
   barium salt, $C_6H_{11}O_9PBa \cdot 7H_2O$; commercial preparation, see p. 1017.
3. Sodium sulphate, Na_2SO_4, or potassium sulphate, K_2SO_4
4. Trichloroacetic acid
5. Ammonium molybdate, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$
6. Sodium hydrogen sulphite, $NaHSO_3$
7. Sodium sulphite, Na_2SO_3
8. 1-Amino-2-naphthol-4-sulphonic acid
   The preparation must be pure. If not, purify as follows: Dissolve 150 g. $NaHSO_4$ and 10 g. Na_2SO_3 in 1000 ml. distilled water at 90°C. Dissolve 15 g. of the sulphonic acid in this mixture, filter hot and add 10 ml. conc. HCl to the filtrate when cool. Filter off the precipitate, wash with 300 ml. distilled water and then wash with ethanol until the filtrate is colourless. Dry the residue in the dark, powder and store in a dark bottle.
9. Potassium dihydrogen phosphate, KH_2PO_4

Preparation of Solutions

I. Buffer

Several buffers have been recommended for the assay of G-6-Pase, including citrate[3, 17], cacodylate[9, 11], maleate[12, 15] and histidine[13, 18] buffers. The first two have been used most extensively and appear to be equally reliable.

a) Citrate buffer (0.1 M; pH 6.5):
   Dissolve 2.101 g. citric acid ($C_6H_8O_7 \cdot H_2O$) in 50 to 75 ml. distilled water, adjust to pH 6.5 with 30% (w/v) NaOH or KOH and dilute to 100 ml. with distilled water.

b) Cacodylate buffer (0.1 M; pH 6.5):
   Dissolve 2.14 g. sodium cacodylate $\cdot 3H_2O$ in 50 to 75 ml. distilled water, adjust the pH to 6.5 with 5 N HCl and dilute to 100 ml. with distilled water.

II. Glucose-6-phosphate (0.08 M G-6-P):
Suspend 417 mg. G-6-P-Ba salt $\cdot 7H_2O$ in 2 to 3 ml. distilled water. Dissolve by addition of the minimum amount of 1 N HCl. Add 114 mg. Na_2SO_4 or 139 mg. K_2SO_4. Mix thoroughly, centrifuge and discard the precipitate of $BaSO_4$. Carefully add a drop of Na_2SO_4 solution to the supernatant; no precipitate should form. Adjust the pH to 6.5 with 30% (w/v) NaOH or KOH and dilute to 10 ml. with distilled water.

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

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

V. Reducing agent (*ca.* 4.2×10^{-2} M 1-amino-2-naphthol-4-sulphonic acid; *ca.* 0.56 M SO_3^{2-}):
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 mixture and dilute to 100 ml. with distilled water.

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

Stability of the solutions

After addition of toluene the citrate buffer can be stored at 0 to 4°C for at least two weeks and is usable so long as no bacterial growth has occurred.

The G-6-P solution should be distributed in several test tubes, each containing about the amount required for a day's determinations, and should be stored in the frozen state. The solution can be stored overnight at 0 to 4°C, but it is an excellent growth medium for certain moulds which produce phosphatases.

The trichloroacetic acid, molybdate and phosphate solutions are stable indefinitely at room temperature[19]. The reducing agent should be stored in the dark in small bottles which are completely filled. Do not use the contents of an opened bottle for longer than a week.

Procedure

Experimental material

Chill liver or kidney in an ice bath immediately after removing from the animal. Homogenize 250 mg. tissue with 9.75 ml. buffer (solution I a or b) in a *Potter-Elvehjem* homogenizer (see p. 49) and then filter through cheesecloth. The homogenate contains 2.5 mg. tissue/0.1 ml. Homogenates can be kept for at least 1 hour in an ice bath without loss of activity.

Enzymatic reaction

Place two test tubes containing G-6-P solution (II) and buffer (solution I a or b) in a water bath at 37°C. For each sample prepare a tissue control (tube 1) and for each series a reagent control (tube 2).

Pipette into centrifuge tubes:

Experimental	*Control 1*	*Control 2*
0.1 ml. filtered homogenate	0.1 ml. filtered homogenate	0.1 ml. buffer (soln. I)

Place the tubes in a water bath at 37°C, after *ca.* 5 min. mix in

0.1 ml. G-6-P soln. (II, 37°C)	0.1 ml. buffer (soln. I, 37°C)	0.1 ml. G-6-P soln. (II, 37°C)

and note the time of each addition. Incubate the tubes for exactly 15 min. at 37°C and then add

2 ml. trichloroacetic acid solution (III)

to each tube. Centrifuge and use the clear supernatant for the phosphate determination.

Phosphate determination

The phosphate content of the supernatant is determined colorimetrically by the method of *Fiske* and *Subarrow*[19]. Wavelength: 660 or 700 mμ.

[19] *C. H. Fiske* and *P. Subbarow*, J. biol. Chemistry *66*, 375 [1925].

Pipette into test tubes:

Experimental and controls	*Standard*
5 ml. molybdate solution (IV)	5 ml. molybdate solution (IV)
1 ml. supernatant	1 ml. phosphate standard solution (VI)

When all the tubes are prepared, mix in

1 ml. reducing agent (V)

to each tube and note the time. Allow sufficient time between the additions of the reducing agent, so that the colorimetric measurement on each tube can be made at the same length of time after the addition. Allow each tube to stand at room temperature for at least 15 min. and not more than 60 min. and then read the optical density. Zero the instrument with the tube prepared from control 2.

Calculations

$$\frac{E_E - E_{C1}}{E_S} \times [P] \times 2.2 = \mu\text{moles phosphate liberated in the enzymatic reaction}$$

where

E_E = optical density of the experimental tube
E_{C1} = optical density of the control tube 1
E_S = optical density of the standard tube
[P] = μmoles phosphate in the standard tube (0.5 μmoles)
2.2 = volume of the enzymatic reaction mixture after addition of trichloroacetic acid solution [ml.]

To convert to μmoles phosphate/min./g. tissue multiply by $\frac{1000}{15 \times 2.5}$

where

15 = period of the enzymatic reaction [min.]
2.5 = mg. tissue in the enzymatic reaction mixture.
1000 = conversion from mg. to g.

Example

Under the conditions described above the following values were measured in the phosphate determination with a liver sample: $E_E = 0.277$; $E_{C1} = 0.030$; $E_S = 0.385$. The standard contained 0.500 μmoles phosphate. Therefore:

$$\frac{0.277 - 0.030}{0.385} \times 0.5 \times 2.2 = 0.705\ \mu\text{moles phosphate in the enzymatic reaction mixture}$$

$$= 0.705\ \mu\text{moles phosphate/15 min./2.5 mg. liver}$$

$$= 0.705 \times \frac{1000}{15 \times 2.5} = 18.8\ \mu\text{moles phosphate/min./g. liver}$$

Values for normal rats are between 13 and 15 μmoles phosphate/min./g. liver. Values far below or up to double these values may be obtained (see below) according to the condition or type of animal.

Stability of the Enzyme in the Sample

Liver homogenates can be kept in an ice bath for 4 hours without loss of activity[4], but longer storage leads to a loss[3]. Freezing does not inactivate the enzyme, but repeated freezing and thawing does destroy its activity[5]. Liver, frozen soon after removal, can be stored at −18°C for several months without decrease in the G-6-Pase activity[17]. This also applies to homogenates which are frozen immediately after their preparation[3].

Sources of Error

The hydrolysis of G-6-P is also catalysed by unspecific phosphatases. The most important is the alkaline phosphatase of intestine, whose pH optimum is between 9 and 10[2], but which is also active at pH 6.5. In contrast to G-6-Pase it is activated by Mg ions. G-6-Pase is inhibited by molybdate, fluoride and arsenate, but not by Be which inhibits many other phosphatases[14]. Activation by Mg, inhibition by Be and the position of the pH optimum can therefore serve to distinguish G-6-Pase from other phosphatases.

Detergents and surface-active agents rapidly inactivate G-6-Pase[13, 18, 20], but their effect depends on the concentration. Low concentrations may activate slightly, but higher concentrations (*e.g.* films of detergent left in glassware due to insufficient rinsing) completely inhibit the enzyme. The enzyme is also inactivated by incubation at 37°C for 15 min. at pH 5[5].

Factors Influencing the Activity

Patients with glycogen-storage disease (*von Gierke*'s disease) frequently have a very low concentration of G-6-Pase in the liver[17]. In diabetic animals liver G-6-Pase is greatly elevated[14, 21], while kidney G-6-Pase is slightly raised[22]. After treatment with insulin the values return to normal. The activity of liver G-6-Pase depends on the diet. The activity is increased in rats which receive large amounts of fructose. If fat or protein are substituted for most of the starch in the diet, then there is a temporary elevation of activity (a few days)[22–24].

Hormones also affect the activity of the liver enzyme. Rats treated with cortisone[25] or thyroxine[26] show a raised G-6-Pase activity. Hypophysectomy reduces the activity and this can be restored by injection of cortisone and thyroxine, but not by either of these hormones alone[16].

The activity of the enzyme expressed per gram liver increases during starvation[27], presumably because the enzyme is conserved while other tissue constituents are lost. If the activity is calculated per gram body weight, then the change is small[22, 28]. To distinguish apparent from true changes, activity should always be expressed per gram liver and per gram body weight (or per gram original body weight).

The G-6-Pase activity of rat liver decreases when the animals are fed a protein-free diet[29] or are infused continuously with glucose[30]. After removal of the pancreas the glucose infusion has no effect. Glucose inhibits G-6-Pase *in vitro*[12, 31, 32], but there is no connection between the *in vitro* and *in vivo* effects of excess glucose. Apart from the inhibitors mentioned above the enzyme is also inhibited by silicic acid[33].

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