Glucose-6-phosphate Dehydrogenase
(Zwischenferment)

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Glucose-6-phosphate dehydrogenase (G6P-DH) was first isolated from erythrocytes and from fermenting yeast by *O. Warburg* et al.[1-5], who carried out an extensive purification and characterization of the enzyme. It has been demonstrated in practically all animal tissues and in microorganisms. Blood cells[6-11], adipose tissue[12] and the lactating mammary gland[13,14] are especially rich sources of the enzyme. Less occurs in liver, pancreas, kidney, lung, brain and gastric mucosa, while only traces are found in skeletal and heart muscle and virtually none in serum[12]. Some human and animal tumours contain a high concentration of this enzyme[12,15-17].

There are two methods for the determination of the activity of the enzyme:

1. Manometric measurement of the oxygen uptake of cytolysates on addition of G-6-P, TPN^+ and old yellow enzyme (FMN).
2. Spectrophotometric determination of the rate of TPNH formation.

Principle

G6P-DH catalyses the reaction:

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

The rate of formation of TPNH is a measure of the enzyme activity and it can be followed by means of the increase in absorption at 340 or 366 mμ[3].

Optimum Conditions for Measurements

The optimum pH of the G6P-DH reaction is 8.3 for the enzyme from yeast or blood cells (Fig. 1). Between pH 7.4 and 8.6 there is little change in the enzyme activity. The measurements are made at pH 7.5, because this is nearest to physiological conditions and allows comparison to be made with other enzyme activities which are usually measured at this pH.

The use of phosphate buffer should be avoided because 0.1 M phosphate completely inhibits the enzyme[4]; 0.05 M triethanolamine buffer (pH 7.5) containing 0.005 M ethylene-diamine-tetra-acetate has proved best. Activity measurements in cytolysates and cell homogenates are not very accurate, because the presence of 6-phosphogluconic dehydrogenase (additional TPN reduction)

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1) *O. Warburg* and *W. Christian*, Biochem. Z. *242*, 206 [1931].
2) *O. Warburg* and *W. Christian*, Biochem. Z. *254*, 438 [1932].
3) *O. Warburg*, *W. Christian* and *A. Griese*, Biochem. Z. *282*, 157 [1935].
4) *E. Negelein* and *E. Haas*, Biochem. Z. *282*, 206 [1935].
5) *E. Negelein* and *W. Gerischer*, Biochem. Z. *284*, 289 [1936].
6) *H. D. Waller*, *G. W. Löhr* and *M. Tabatabai*, Klin. Wschr. *1957*, 1022.
7) *G. W. Löhr* and *H. D. Waller*, Klin. Wschr. *1958*, 865.
8) *G. W. Löhr* and *H. D. Waller*, Klin. Wschr. *1959*, 833.
9) *H. D. Waller*, *G. W. Löhr*, *F. Grignani* and *R. Gross*, Thromb. Diath. haem. *3*, 520 [1959].
10) *G. W. Löhr*, *H. D. Waller* and *H. E. Bock*, Verh. dtsch. Ges. inn. Med. *66*, 1045 [1960].
11) *G. W. Löhr* and *H. D. Waller*, Dtsch. med. Wschr. *1961*, 27, 87.
12) *E. Schmidt* and *F. W. Schmidt*, Klin. Wschr. *1960*, 957.
13) *G. E. Glock* and *P. McLean*, Biochim. biophysica Acta *15*, 590 [1953].
14) *G. E. Glock* and *P. McLean*, Biochem. J. *56*, 171 [1954].
15) *A. Delbrück*, *H. Schimassek*, *K. Bartsch* and *Th. Bücher*, Biochem. Z. *331*, 297 [1959].
16) *Th. Bücher* and *M. Klingenberg*, Angew. Chem. *70*, 552 [1958].
17) *H. J. Hohorst* in *H. Wilmanns*: Chemotherapie der Tumoren. Schattauer-Verlag, Stuttgart 1960.

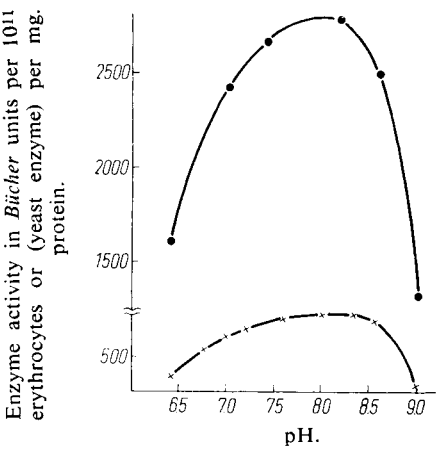

Fig. 1. The dependence of the activity of G6P-DH from yeast (— • — • —) and erythrocytes (— × — × —) on pH.

results in too high an activity being obtained. TPNH oxidizing reactions (glutathione reductase, methaemoglobin reductase and other flavoproteins) tend to compensate for this; nevertheless, the measurement of G6P-DH activity in biological material is liable to error. A more accurate, but more complicated method of assay is to remove the interfering 6-phosphogluconic dehydrogenase by adsorption on $Ca_3(PO_4)_2$ gel[18]. Magnesium ions activate the G6P-DH reaction in glycylglycine buffer, but not in phosphate or triethanolamine buffer[18].

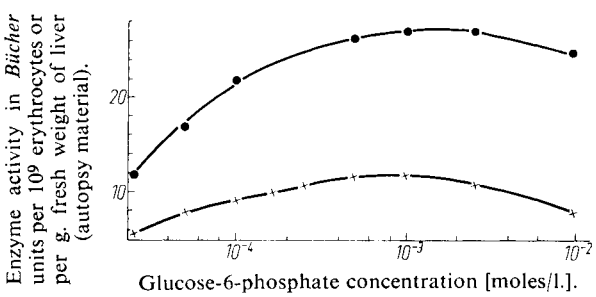

Fig. 2. The dependence of the G6P-DH activity in human liver homogenates (— • — • —) and in erythrocyte haemolysates (— × — × —) on the glucose-6-phosphate concentration. 0.05 M triethanolamine buffer, pH 7.5; 25° C; 5×10^{-4} M TPN.

The substrate affinity of G6P-DH from different types of cells for glucose-6-phosphate and TPN is shown in Table 1.

Table 1. Michaelis constants of G6P-DH from different sources

Source of enzyme	Michaelis constant for G-6-P	TPN
Yeast	6.9×10^{-5} M	5.0×10^{-5} M
Erythrocytes (human)	7.4×10^{-6} M	1.2×10^{-5} M
Leucocytes (human)	5.6×10^{-6} M	1.3×10^{-5} M
Paramyeloblasts (acute leukaemia, human)	1.8×10^{-5} M	9.1×10^{-6} M
Lymphocytes (chronic lymphadenoma, human)	2.3×10^{-5} M	1.2×10^{-5} M
Liver (human)	3.3×10^{-5} M	2.3×10^{-5} M

Measurements are made on tissue samples with 6.7×10^{-4} M G-6-P and 5×10^{-4} M TPN, which are optimum concentrations for the enzyme from erythrocytes (refer to Fig. 2).

[18] *A. Kornberg* and *B. L. Horecker* in *S. P. Colowick* and *N. O. Kaplan*: Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 323.

Reagents

For preparation of the sample

1. Triethanolamine hydrochloride
2. Ethylene-diamine-tetra-acetic acid, EDTA
   disodium salt, EDTA-$Na_2H_2 \cdot 2H_2O$
3. Ethylene-diamine-tetra-acetic acid,
   magnesium-dipotassium salt, EDTA-$MgK_2 \cdot 2H_2O$
4. Sodium citrate, A. R.
5. Sodium chloride, A. R.
6. Potassium hydroxide, A. R., 1 N
7. Sodium hydroxide, A. R., 0.1 N
8. Ammonium chloride, A. R., anhydrous
9. Potassium dihydrogen phosphate, A. R., KH_2PO_4
10. Disodium hydrogen phosphate, A. R., $Na_2HPO_4 \cdot 2H_2O$
11. Potassium chloride, A. R.
12. Magnesium chloride, A. R.
13. Sodium hydrogen carbonate, $NaHCO_3$
14. Glucose
15. Digitonin
16. Silicone oil *)
17. Toluene

For measurements **)

No. 1, 2, 7 and 13. Also:

18. Glucose-6-phosphate, G-6-P
    disodium salt, G-6-P-Na_2; commercial preparation, see p. 1017.
19. Triphosphopyridine nucleotide, TPN
    sodium salt, TPN-NaH_2; commercial preparation, see p. 1029.

Preparation of Solutions

Prepare all solutions with glass distilled water.

For preparation of the sample

For erythrocytes:

I. Citrate (3.8% w/v):
   Dissolve 3.8 g. sodium citrate in distilled water and make up to 100 ml.

II. Physiological saline (0.9% w/v):
   Dissolve 9 g. NaCl in distilled water and make up to 1000 ml.

III. Triethanolamine buffer (0.05 M; pH 7.5):
   Dissolve 0.93 g. triethanolamine hydrochloride and 0.2 g. EDTA-$Na_2H_2 \cdot 2H_2O$ in *ca.* 50 ml. distilled water, adjust to pH 7.5 with 0.1 N NaOH and dilute to 100 ml. with distilled water.

*) e.g. Silicone "Wacker S.W. 60", Wacker-Chemie, GmbH, Munich, Germany.
**) Complete reagent kits are available commercially, see p. 1036.

IV. Digitonin solution (saturated):
Add about 1 g. digitonin to 100 ml. distilled water, shake well and filter off precipitate.

Additional for leucocytes

V. EDTA-MgK_2 (0.115 M; pH 7.4):
Dissolve 4.917 g. EDTA-$MgK_2 \cdot 2H_2O$ in a little distilled water, adjust to pH 7.4 with 1 N KOH and dilute to 100 ml. with distilled water.

VI. Ammonium chloride (0.87% w/v):
Dissolve 8.7 g. NH_4Cl, anhydrous, in distilled water and make up to 1000 ml.

VII. Phosphate buffer-NaCl mixture (1.33×10^{-3} M phosphate; 0.88% NaCl; pH 7.4):
Mix 28 ml. of a solution containing 9.078 g. KH_2PO_4/1000 ml. and 72 ml. of a solution containing 11.876 g. $Na_2HPO_4 \cdot 2H_2O$/1000 ml. To 20 ml. of this mixture add 4.27 g. EDTA-$MgK_2 \cdot 2H_2O$ and dilute to 1000 ml. with 0.9% NaCl solution (II).

VIII. Tyrode solution, calcium-free:
Dissolve 8.00 g. NaCl; 0.20 g. KCl; 0.10 g. $MgCl_2 \cdot 6H_2O$; 0.05 g. $Na_2HPO_4 \cdot 2H_2O$; 1.00 g. glucose; 1.00 g. $NaHCO_3$ in distilled water and make up to 1000 ml. To prevent bacterial growth add a few drops of toluene and store in a dark bottle.

Additional to solutions I–IV for platelets:

IX. EDTA-Na_2H_2 (1% w/v, EDTA-$Na_2H_2 \cdot 2H_2O$):
Dissolve 1 g. EDTA-$Na_2H_2 \cdot 2H_2O$ in 100 ml. physiological saline (II).

For liver homogenates only:

X. Physiological saline containing 6.6×10^{-4} M EDTA:
Dissolve 0.25 g. EDTA-$Na_2H_2 \cdot 2H_2O$ in physiological saline and make up to 1000 ml.

For measurements

Solution I. and

XI. Glucose-6-phosphate (*ca.* 4×10^{-2} M G-6-P):
Dissolve 130 mg. G-6-P-Na_2 in 10 ml. distilled water.

XII. Triphosphopyridine nucleotide (*ca.* 3×10^{-2} M β-TPN):
Dissolve 25 mg. TPN-NaH_2 in 1.0 ml. 1% $NaHCO_3$ solution.

Stability of the solutions

All solutions should be stored, stoppered, in a refrigerator at 0–4° C. The TPN and glucose-6-phosphate solutions are stable for 2–3 weeks under these conditions, but in the frozen state they keep considerably longer.

Procedure

Preliminary treatment of the sample

Use only fresh serum, absolutely free from haemolysis. Blood cells are rich in G6P-DH.

Erythrocytes

Take 0.5 ml. venous blood into a syringe containing 0.5 ml. citrate solution (I), centrifuge at *ca.* 1000 g on a bench centrifuge and wash the cells twice with 5 ml. physiological saline (II). Suspend the sediment in 1 ml. physiological saline, mix well by rotation and count the erythrocytes twice in a Zeiss-Thoma counting chamber (about 2×10^6/mm.^{3}).

Haemolysis: In a centrifuge tube mix

1.0 ml. erythrocyte suspension
1.0 ml. distilled water
0.7 ml. triethanolamine buffer (solution III)
0.3 ml. digitonin solution (IV).

Allow to stand for 15 min. in a refrigerator at 4°C, then centrifuge (15 min. at 1000 g) and discard the insoluble material.

Leucocytes

Isolation of leucocytes: In a boiling tube mix

45 ml. venous blood
5 ml. EDTA-MgK_2 solution (V).

To sediment the erythrocytes allow to stand in long, inclined test tubes for 30 min. in an incubator at 37°C. Carefully suck off the supernatant containing the leucocytes from the erythrocyte layer with a bulb pipette and transfer to a 10 ml. centrifuge tube.

To remove the erythrocytes: Mix

1 vol. leucocyte-containing supernatant
3 vol. ammonium chloride solution (VI).

Allow to stand exactly for 5 min. and then centrifuge off the leucocytes at low speed (100 to 200 g) for 3 min. Carefully suck off the supernatant and discard (it contains haemolysed erythrocytes and platelets).

Wash the sediment three times with phosphate buffer-NaCl mixture (VII), centrifuging each time at low speed (100 g) in the cold. After the last washing suspend the sediment in 5 ml. ice-cold Tyrode solution (VIII).

Carry out a leucocyte count (four times) in a Zeiss-Thoma counting chamber. The cell count should be above 10000/mm^3.

Cytolysis: In a centrifuge tube mix

5 ml. leucocyte suspension
2 ml. distilled water
2 ml. 0.05 M triethanolamine buffer (solution III)
1 ml. digitonin solution (IV).

Allow to stand for 60 min. in a refrigerator at 0 to 4°C and then centrifuge off (15 min. at 3000 g) the insoluble material in the cold. Discard the sediment.

Platelets[9]

Take 18.00 ml. of venous blood, using a siliconized V2A canula and a graduated, siliconized syringe which contains 2.00 ml. EDTA-Na solution (IX). Use only siliconized tubes, pipettes, *etc.* for all operations. Immediately centrifuge the blood in pre-cooled, 8 ml. centrifuge tubes for 10 min. at 4°C and 100 g. Carefully pipette off the supernatant plasma containing the platelets, combine in pre-cooled centrifuge tubes and centrifuge for 15 min. at 4°C and 1040 g. Decant the supernatant plasma which contains few platelets, add to the sediment 6 ml. physiological saline (II), carefully stir with a wooden rod and then centrifuge at 4°C and 1040 g. Suspend the platelets in 2 ml. physiological saline (II). Carry out a double platelet

count with a phase contrast microscope according to the technique of *Feissly* and *Ludin*[19] (*ca.* 2×10^6 platelets/mm.^{3}).

Lysis: In a centrifuge tube mix

2.0 ml. platelet suspension
1.0 ml. distilled water
0.7 ml. triethanolamine buffer (solution III)
0.3 ml. digitonin solution (IV).

Allow to stand for 60 min. in a refrigerator at 4° C, centrifuge off (15 min. at 1000 g and 4° C) the insoluble material. Discard the sediment.

Liver tissue[20]

Immediately blot liver samples obtained by puncture (at least 10 mg. wet weight) on filter paper to remove most of the blood and then weigh on a torsion balance. Add the tissue and EDTA-physiological saline (X) (0.04 ml./mg. wet weight) to an ice-cold *Potter-Elvehjem* homogenizer (refer to p. 49) and homogenize for exactly 2 min. in an ice bath (stopwatch). Centrifuge for 20 min. at 0 to 1.5° C and 15000 r.p.m. Decant the clear supernatant. The time between the liver puncture and the start of the centrifugation should not be more than 5 min.

The accuracy of the determination of activity in liver tissue can be increased if the haemoglobin content of the supernatant is estimated and on the basis of this estimation the additional G6P-DH due to the blood cells is calculated. This value is subtracted from the total G6P-DH activity of the liver homogenate.

Spectrophotometric measurements

Wavelength: 340 or 366 mμ; light path: 1 cm.; temperature: 25° C (constant temperature cuvette chamber); final volume 3.0 ml.

The optical density increase should not be more than 0.030/min. (measured at 366 mμ); if necessary, take less or dilute the sample.

Serum

Pipette successively into the cuvette:

1.90 ml. triethanolamine buffer (solution III)
1.00 ml. serum
0.05 ml. TPN solution (XII).

Mix with a glass rod flattened at one end. Keep at 25° C for 5 min., mix in

0.05 ml. G-6-P solution (XI),

wait for an optical density increase of about 0.020, start a stopwatch and read the optical density at 2 min. intervals for 10 min. Calculate the mean optical density change/min. ΔE/min. is used for the calculations.

Erythrocytes

Because of the high absorption of the sample due to the haemoglobin a blank cuvette must be used: replace the TPN solution by buffer solution (III), otherwise as for the experimental cuvette. Read against this blank.

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[19] *R. Feissly* and *H. Lüdin*, Helv. Physiol. Arch. *7*, 9 [1949].
[20] *E. Schmidt, F. W. Schmidt* and *E. Wildhirt*, Klin. Wschr. *1958*, 171.

Pipette successively into the cuvette:

2.85 ml. triethanolamine buffer (solution III)
0.05 ml. erythrocyte haemolysate
0.05 ml. TPN solution (XII).

Proceed as described under "Serum".

Leucocytes

Prepare a blank cuvette as described under "Erythrocytes". Read against this blank.

Pipette successively into the cuvette:

2.70 ml. triethanolamine buffer (solution III)
0.20 ml. leucocyte cytolysate
0.05 ml. TPN solution (XII).

Proceed as described under "Serum".

Platelets

Prepare a blank cuvette as described under "Erythrocytes". Read against this blank.

Pipette successively into the cuvette:

2.40 ml. triethanolamine buffer (solution III)
0.50 ml. lysed platelets
0.05 ml. TPN solution (XII).

Proceed as described under "Serum".

Liver tissue

Prepare a blank cuvette as described under "Erythrocytes". Measure against this blank.

Pipette successively into the cuvette:

2.40 ml. triethanolamine buffer (solution III)
0.50 ml. supernatant of the liver homogenate
0.05 ml. TPN solution (XII).

Proceed as described under "Serum".

Calculations

The usual definition of 1 unit of enzyme activity according to the American literature[21] is the amount of enzyme in 1 ml. sample (serum, haemolysate, cytolysate or supernatant of a tissue homogenate), which at 25° C in a 3 ml. assay mixture changes the optical density of TPNH (DPNH) at 340 mμ by 0.001 in 1 min.

Serum

Consequently with 1 ml. serum in a 3 ml. assay mixture

$$\frac{\Delta E_{340}/\text{min.}}{0.001} = (\Delta E_{340}/\text{min}) \times 1000 = \text{G6P-DH units/ml serum.}$$

Erythrocytes

0.05 ml. haemolysate is taken for the determination of G6P-DH activity in erythrocytes, which on haemolysis is diluted three-fold. Therefore the total dilution factor is $3 \times 20 = 60$. It follows that:

$$(\Delta E_{340}/\text{min}) \times 60000 = \text{G6P-DH units/ml. erythrocyte suspension.}$$

If the units/ml. erythrocyte suspension are divided by the erythrocyte count/ml. and then multiplied by 10^9, the G6P-DH activity is obtained in units/10^9 erythrocytes.

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[21] *J. S. LaDue, F. Wróblewski* and *A. Karmen*, Science [Washington] *120*, 497 [1954].

Leucocytes

Dilution factor: $2 \times 5 = 10$

$$(\Delta E_{340}/\text{min}) \times 10000 = \text{G6P-DH units/ml. leucocyte suspension.}$$

For conversion to the number of cells, see "Erythrocytes".

Platelets

Dilution factor = 4

$$(\Delta E_{340}/\text{min}) \times 4000 = \text{G6P-DH units/ml. platelet suspension.}$$

For conversion to the number of platelets, see "Erythrocytes".

Liver tissue

The G6P-DH activity in liver is related either to mg. soluble protein (determined by the biuret method) in the homogenate supernatant or to g. fresh weight.

If measurements are carried out at 366 mμ then the values obtained by the equations given above must be multiplied by 1.89 (= ratio of the extinction coefficients of TPNH at 340 and 366 mμ.).

Conversion to other units

According to *Bücher*[22] a unit is the amount of enzyme contained in 1 ml., which at 25°C and with a 1 cm. light path, changes the optical density of TPNH (DPNH) at 366 mμ by 0.100 in a 100 sec.

Therefore for 1 unit according to *Bücher*:

$$\Delta E_{366} = 0.100/100 \text{ sec.}$$
$$\Delta E_{366} = 0.060/\text{min.}$$
$$\Delta E_{340} = 0.113/\text{min.}$$

with a 3 ml. assay volume $\Delta E_{340} = 0.0370/\text{min.}$

Therefore 1 *Bücher* unit corresponds to 37.7 Wróblewski units (definition, see p. 33, 840) or to a substrate change of 1.09 μmoles/hour/ml. mixture.

Normal Values

Material	*Wróblewski* units	*Bücher* units
Serum	0–0.4/ml.	0–0.01/ml.
Erythrocytes	272 ± 26/10^9 cells	7.2 ± 0.7/10^9 cells
Granulocytes	19000 ± 6850/10^9 cells	506 ± 182/10^9 cells
Lymphocytes	6970 ± 415/10^9 cells	185 ± 11/10^9 cells
Platelets	151 ± 15/10^9 cells	4 ± 0.4/10^9 cells
Liver	1770 ± 640/g. fresh weight	47 ± 17/g. fresh weight

Interference by Pharmacological Agents

G6P-DH is inhibited by primaquine[11] and other 8-aminoquinolines (antimalaria agents) in millimolar concentration, as well as by phenylhydrazine. Nevertheless, the therapeutic concentration of these substances is more than ten-fold lower and therefore they have no significant effect on the measurements.

Stability of the Enzyme in the Sample

In the living organism the half-life of G6P-DH in erythrocytes in 60 days[8]. Half the enzyme activity is lost in 2 days from digitonin haemolysates in triethanolamine buffer (pH 7.5). The G6P-DH from erythrocytes has a half-life of only <1 day in serum. On account of the instability of the enzyme the determination of G6P-DH activity in haemolysates, cytolysates, serum and plasma should be carried out within a few hours of their preparation.

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[22] *G. Beisenherz, H. J. Boltze, Th. Bücher, R. Czok, K. H. Garbade, E. Meyer-Arendt* and *G. Pfleiderer*, Z. Naturforsch. *8b*, 555 [1953].