Glycogen

Determination as D-Glucose with Hexokinase, Pyruvic Kinase and Lactic Dehydrogenase

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Glycogen is usually determined by hydrolysis to glucose which is then estimated chemically. A new departure is the enzymatic determination of the glucose liberated. In principle, all methods for the enzymatic determination of glucose should be applicable, but the method described here has already proved itself. Interference occurs in the enzymatic determination of D-(+)-glucose with hexokinase and glucose-6-phosphate dehydrogenase (see p. 117), if the glucose-6-phosphate dehydrogenase preparation contains 6-phosphogluconic dehydrogenase; pure preparations are difficult to prepare and therefore commercial preparations are expensive. For the quantitative determination of glucose in blood and other large series of tests, the following method[1] is advantageous because of the cheaper reagents required.

Principle

(1) D-Glucose + ATP*) $\xrightarrow[Mg^{2+}]{HK}$ D-glucose-6-phosphate + ADP

(2) ADP + PEP $\xrightarrow[Mg^{2+},K^{+}]{PK}$ ATP + pyruvate

(3) Pyruvate + DPNH + H^{+} $\xrightleftharpoons{LDH}$ lactate + DPN^{+}

In this series of reactions, glucose is phosphorylated with ATP and stoichiometric amounts of ADP are formed. The ADP is converted with PEP in the auxiliary reaction (2) to ATP and pyruvate, the latter being determined by means of the decrease in optical density on oxidation of DPNH to DPN (indicator reaction 3). Owing to the favourable Michaelis and equilibrium constants all the reactions proceed quantitatively from left to right.

Reagents

1. Ethanol, 96% (w/v)
2. Sulphuric acid, A. R., 2 N
3. Sodium hydroxide, A. R., 2 N
4. Potassium hydroxide, A. R., 30% (w/v)
5. Trichloroacetic acid, A. R.
6. Triethanolamine hydrochloride, A. R.
7. Phosphoenolpyruvate, PEP
   tricyclohexylammonium salt; commercial preparation, see p. 1024.
8. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, $DPNH\text{-}Na_2$; commercial preparation, see p. 1011.
9. Potassium chloride, A. R.
10. Magnesium sulphate, A. R., $MgSO_4 \cdot 7H_2O$

*) Abbreviations: ATP, ADP, AMP = adenosine tri-, di-, and monophosphate; DPN = diphosphopyridine nucleotide; DPNH = reduced diphosphopyridine nucleotide; PEP = phosphoenolpyruvate; HK = hexokinase; PK = pyruvic kinase; LDH = lactic dehydrogenase.

1) *G. Pfleiderer* and *L. Grein*, Biochem. Z. *328*, 499 [1957].

11. Adenosine triphosphate, ATP
crystalline disodium salt, ATP-$Na_2H_2 \cdot 3\,H_2O$, commercial preparation, see p. 1006.

12. Lactic dehydrogenase, LDH
crystalline, from heart or skeletal muscle. Commercial preparation from rabbit muscle (crystalline suspension in 2.2 M ammonium sulphate solution), see p. 986.

13. Pyruvic kinase, PK
crystalline, from rabbit muscle, suspension in 2.1 M ammonium sulphate solution. Commercial preparation, see p. 997.

14. Hexokinase, HK
from yeast, as dry powder or crystalline suspension in 3.0 M ammonium sulphate solution. Commercial preparation, see p. 983.

Purity of the enzyme preparations

LDH, PK and HK preparations from Boehringer & Soehne GmbH, Mannheim (Germany), satisfy the requirements for purity. HK preparations are often contaminated with myokinase; they are unsuitable.

Preparation of Solutions

I. Trichloroacetic acid (20% w/v):
Dissolve 20 g. trichloroacetic acid, A. R., in doubly distilled water and make up to 100 ml.

II. Triethanolamine buffer (0.1 M; pH 7.6):
Dissolve 18.6 g. triethanolamine hydrochloride in about 800 ml. doubly distilled water, adjust pH to 7.6 with *ca.* 22 ml. 2 N NaOH and dilute to 1000 ml. with doubly distilled water.

III. Phosphoenolpyruvate (*ca.* 3×10^{-2} M PEP):
Dissolve 100 mg. PEP (tricyclohexylammonium salt) in doubly distilled water and make up to 7 ml.

IV. Reduced diphosphopyridine nucleotide (*ca.* 1.2×10^{-2} M β-DPNH):
Dissolve 50 mg. DPNH-Na_2 in 5 ml. doubly distilled water.

V. Potassium chloride (2 M):
Dissolve 14.9 g. KCl in doubly distilled water and make up to 100 ml.

VI. Magnesium sulphate (0.5 M):
Dissolve 12.3 g. $MgSO_4 \cdot 7\,H_2O$ in doubly distilled water and make up to 100 ml.

VII. Adenosine triphosphate (*ca.* 3×10^{-2} M ATP):
Dissolve 100 mg. ATP-$Na_2H_2 \cdot 3\,H_2O$ in 5 ml. doubly distilled water.

VIII. Lactic dehydrogenase, LDH (*ca.* 5 mg. protein/ml.):
If necessary, dilute the stock suspension with *ca.* 2.1 M ammonium sulphate solution.

IX. Pyruvic kinase, PK (*ca.* 5 mg. protein/ml.):
If necessary, dilute the stock suspension with 2.1 M ammonium sulphate solution.

X. Hexokinase, HK (2 mg. protein/ml.):
Dissolve 10 mg. dry powder in buffer (solution II) and make up to 5 ml. Dilute suspensions of highly purified HK with 3.0 M ammonium sulphate solution.

Stability of the solutions

The solutions of DPNH, ATP and PEP are stable indefinitely in the frozen state. The enzyme suspensions keep for several months at 0 to 4° C without loss of activity.

Procedure

Preliminary treatment of the experimental material

Digestion of the tissue, isolation of the glycogen and hydrolysis to glucose[2]:

In a centrifuge tube graduated at 10 ml., mix

2 ml. 30% KOH

1 ml. sample (homogenized tissue, tissue extract or deproteinized solution),

heat for 15 min. in a boiling water bath and add

ca. 3.5 ml. ethanol.

Just bring to the boil and then cool to room temperature. Centrifuge off the precipitate containing the glycogen and wash with about

3 ml. ethanol.

Remove traces of ethanol from the precipitate by heating on a water bath, add

2 ml. 2 N H_2SO_4

heat for 120 min. in a boiling water bath. The glycogen is hydrolysed to glucose. Cool to room temperature, neutralize (pH 5–7) with

2 N NaOH

and dilute to 10 ml. with distilled water. Use 0.1 ml. of this solution for the glucose determination.

Deproteinization of blood

Take blood from the finger tip of a fasting subject and immediately deproteinize. Into a centrifuge tube pipette

0.8 ml. water

0.1 ml. blood

mix well, add

0.1 ml. trichloroacetic acid solution (I),

centrifuge for a few minutes in a bench centrifuge. Use the supernatant directly for the measurements. The buffer is sufficient to neutralize the excess acid.

Spectrophotometric measurements

Wavelength: 340 or 366 mμ; light path: 1 cm.; final volume: 3.10 ml.; room temperature. Read against a water blank.

Pipette successively into the cuvette:

2.53 ml. buffer (solution II)
0.04 ml. PEP solution (III)
0.06 ml. DPNH solution (IV)
0.10 ml. KCl solution (V)
0.10 ml. $MgSO_4$ solution (VI)
0.10 ml. ATP solution (VII)
0.01 ml. LDH suspension (VIII)
0.01 ml. PK suspension (IX)
0.05 ml. HK solution or suspension (X)

[2] *C. Good, H. Kramer* and *M. Somogyi*, J. biol. Chemistry *100*, 485 [1933].

Mix, allow to stand 5 min. Any ADP and pyruvate, which may be present as impurities in the solutions, react during this period. Read optical density E_1. Start the glucose determination by mixing in

0.10 ml. sample.

The reaction is complete in *ca.* 10 min. Read optical density E_2. Correct for any further small decrease in optical density by extrapolation from the time of the start of the reaction (see p. 39).

Calculations

Correction is made for the dilution on addition of 0.10 ml. sample:

$$E_1 \times \frac{3.0}{3.1} = E_{1\,corr.} = 0.97\,E_1$$

This is used in calculating the results.

$$\Delta E = E_{1corr.} - E_2$$

$$\frac{\Delta E \times 180}{6.22 \times 10^3} = \text{mg. glucose/ml. of reaction mixture}$$

180 = molecular weight of glucose.

6.22×10^3 = extinction coefficient of DPNH [cm.²/mmole] at 340 mμ. The value is 3.3×10^3 at 366 mμ. If the results are multiplied by 3.10, this gives the amount of glucose in the reaction mixture or in 0.1 ml. sample. To convert to mg.% glucose the results must be multiplied by the dilution factor on deproteinization (10 : 1) and then related to 100 ml. blood.

$$\frac{\Delta E \times 180 \times 3.1}{6.22 \times 10^3} \times \frac{10}{1} \times 1000 = \frac{\Delta E \times 10 \times 588}{6.22} = \text{mg.\% glucose in blood.}$$

The glucose values are practically identical with the glycogen values. Hydrolysis of pure glycogen preparations gives values of 90 to 95% glucose[3].

Specificity and Sources of Error

In the presence of ATP, hexokinase phosphorylates not only glucose, but also fructose, glucosamine and mannose[4,5]. These compounds are unlikely to interfere with the determination of glucose, since according to the literature, they usually only occur in a bound form or in very low concentrations (fructose up to 4 mg.%). The specificity of the glycogen determination is very high, because the glycogen is previously purified and separated from compounds of low molecular weight.

High glucose values will be obtained if the tissue extracts contain much ADP. In such cases, the order of addition of sample and hexokinase to the reaction mixture is interchanged, so that ADP can react according to equations (2) and (3), prior to the actual determination. The reaction is then started with pure hexokinase (free from myokinase)[6].

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3) *G. Pfleiderer* and *L. Grein*, unpublished.
4) *M. R. McDonald*, J. gen. Physiol. *29*, 393 [1946].
5) *D. H. Brown*, Biochim. biophysica Acta *7*, 487 [1951].
6) *W. Thorn*, *W. Isselhard* and *B. Müldener*, Biochem. Z. *331*, 545 [1959].