L-(−)-Glycerol-1-phosphate

Determination with Glycerol-1-phosphate Dehydrogenase

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Principle

Glycerol-1-phosphate dehydrogenase *) (GDH) catalyses the oxidation of L-(−)-glycerol-1-phosphate by diphosphopyridine nucleotide (DPN):

(1) L-(−)-Glycerol-1-phosphate + DPN^+ $\rightleftharpoons$ dihydroxyacetone phosphate + DPNH + H^+

The equilibrium of the reaction, which lies far to the left, has a constant K_c of 5.8×10^{-12} moles/l.[1] at 25° C. The oxidation of L-(−)-glycerol-1-phosphate with DPN is virtually quantitative if the reaction products are removed from the mixture. Protons are bound by the use of an alkaline reaction medium and dihydroxyacetone phosphate (DAP) is trapped as the hydrazone. The basic equation for the spectrophotometric assay of L-(−)-glycerol-1-phosphate is:

(2) L-(−)-Glycerol-1-phosphate + DPN^+ + hydrazine $\xrightarrow[pH\,9.5]{GDH}$ DAP hydrazone + DPNH + H_3O^+

Relatively high concentrations of DPN and GDH are necessary to obtain a quantitative and sufficiently fast reaction (see under "Sources of Error"). The course of the reaction is followed spectrophotometrically by the increase in optical density due to the formation of DPNH.

Reagents

1. Hydrazine sulphate, A. R.
2. Glycine, A. R.
3. Sodium hydroxide, A. R., 2 N.
4. Potassium carbonate, K_2CO_3, A. R.
5. Methyl orange indicator
6. Perchloric acid, A. R., sp. gr. 1.67; *ca.* 70% (w/w)
7. Ethylene-diamine-tetra-acetic acid, EDTA
   disodium salt, EDTA-$Na_2H_2 \cdot 2H_2O$ (Titriplex III, Trilon B, Versene)
8. Diphosphopyridine nucleotide, DPN
   free acid. Commercial preparation, see p. 1010. Only preparations containing at least 75% β-DPN are suitable.
9. Glycerol-1-phosphate dehydrogenase, GDH
   crystalline, from skeletal muscle, suspension in 2.0 M ammonium sulphate solution; commercial preparation, see p. 981.

Purity of the enzyme preparation

The GDH preparation should have a specific activity of at least 2000 units/mg. (according to *Bücher* **) or 36 units/mg. (according to *Racker* **). Contamination by lactic dehydrogenase or malic dehydrogenase should not exceed 0.05% (relative to the GDH activity).

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*) Synonym: α-glycerophosphate dehydrogenase.
**) Definition of units, see p. 32 and 33.
1) *H. J. Hohorst*, Ph. D.-Thesis, Universität Marburg, 1960.

Preparation of Solutions (for *ca.* 20 determinations)

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. Hydrazine-glycine buffer (0.4 M hydrazine; 1 M glycine; pH 9.5):
Suspend 7.5 g. glycine, 5.2 g. hydrazine sulphate and 0.2 g. EDTA-$Na_2H_2 \cdot 2H_2O$ in a little distilled water, add 51 ml. 2 N NaOH and dilute to 100 ml. with distilled water.

V. Diphosphopyridine nucleotide (*ca.* 5×10^{-2} M β-DPN):
Dissolve 40 mg. DPN in distilled water and make up to 1 ml.

VI. Glycerol-1-phosphate dehydrogenase, GDH (*ca.* 6 mg. protein/ml.):
Dilute the enzyme suspension containing *ca.* 10 mg. protein/ml. in 2.0 M ammonium sulphate solution with distilled water.

Stability of the solutions

Store all solutions, stoppered, in a refrigerator at 0–4°C. The DPN solution is stable for several weeks and need not be neutralized because of the high buffering capacity of the hydrazine-glycine buffer. The hydrazine-glycine buffer is only stable for a week; it is better to prepare a stock solution containing hydrazine sulphate, glycine and disodium EDTA. This is stable virtually indefinitely and small portions can be adjusted to pH 9.5 as required.

Procedure

Experimental material

Freeze tissue samples within a fraction of a second[2] and do not allow to thaw until ready to deproteinize.

Deproteinization

Preliminary remarks: Add perchloric acid to deproteinize the sample. There are two possible methods of extraction: single extraction and calculation of the volume of the extract on the basis of an assumed, mean water content of the sample (see p. 254), or repeated and therefore quantitative extraction of the tissue. The first method is suitable when only glycerol-1-phosphate is to be determined and when the tissue is easily disintegrated, *e.g.* liver. The second method is preferable when other metabolites are to be determined in the same extract. With a single extraction the ratio of the volume of extract to the tissue weight should be 4 : 1. If the tissue is assumed to have a water content of 75%, then

6.5 ml. perchloric acid solution (III)

are added to 2 g. tissue. With repeated extraction the ratio of the volume of extract to the tissue weight should be 8 : 1. Usually it is sufficient to extract twice and to dilute the extract to the corresponding volume to weight ratio of 8 : 1. In this case the error due to retention of the compound in the precipitate is not more than 3–4%.

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

Method:

Single extraction: Weigh a centrifuge tube containing a glass rod and 5 ml. perchloric acid solution (III). Add about 2 g. tissue (frozen powder)[2], mix quickly and re-weigh. From the increase in weight (= weight of tissue) calculate the total volume of perchloric acid required (see above) and then add the requisite amount of the perchloric acid solution (III) to the 5 ml. already present. Mix the suspension thoroughly. Grind lumps of tissue on the walls of the tube with a glass rod and then centrifuge at 3000 g for 5 min. Transfer the supernatant to a cooled 10 ml. flask for neutralization.

Repeated extraction: Weigh a centrifuge tube containing a glass rod and 5 ml. perchloric acid solution (III). Add about 1 g. of tissue (frozen powder), mix quickly and re-weigh. If necessary homogenize the material. Centrifuge for about 5 min. at 3000 g. Decant the supernatant, mix the precipitate with 1 ml. perchloric acid solution (III) + 1 ml. distilled water and re-centrifuge. Combine the supernatants, measure the volume and make up to 8 ml./g. sample with distilled water.

Neutralization: Pipette 0.02 ml. indicator solution (II) into 8 ml. of tissue extract and, while stirring vigorously with a magnetic stirrer and cooling with ice, add from a 1 ml. graduated pipette 0.1 ml. carbonate solution (I). Wait until the CO_2 evolution has practically ceased and then add more carbonate solution until the mixture is salmon-pink (*ca.* pH 3.5). A total of about 0.16 ml. carbonate solution is required. Allow to stand for 10 min. in an ice bath, decant or pipette off the supernatant from the precipitated perchlorate and use a portion for the determination.

Spectrophotometric measurements

Preliminary remarks: The ratio of the total volume to the sample volume should not exceed 2 : 1 so that the hydrazine-glycine buffer is not diluted too much. It is convenient always to use the same dilution ratio so that, in calculating the results, the optical density differences need only be multiplied by a constant factor. A control or blank cuvette can usually be omitted, therefore the measurements are made against air or water (see under "Sources of Error").

Method: Wavelength: 340 or 334 mμ; light path: 1 cm.; final volume: 1.01 ml.

Experimental cuvette	*Control cuvette*
0.45 ml. hydrazine-glycine buffer (IV) 0.05 ml. DPN solution (V) 0.50 ml. deproteinized extract	water or in special cases (see under "Sources of Error") as for experimental cuvette.

Wavelength: 366 mμ; light path: 2 cm.; final volume: 2.02 ml.

Experimental cuvette	*Control cuvette*
0.9 ml. hydrazine-glycine buffer (IV) 0.1 ml. DPN solution (V) 1.0 ml. deproteinized extract	water or in special cases as for the experimental cuvette.

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

0.01 ml. *) GDH suspension (VI).

*) Or 0.02 ml. for an assay volume of 2.02 ml.

On completion of the reaction (10—20 min. after the enzyme addition, depending on the glycerol-1-phosphate concentration) read the optical density E_2 twice with an interval of 3 min. The changes in the initial optical density E_1 and in the final optical density E_2 during the 3 min. period are usually small in comparison to the optical density difference $\Delta E = E_2 - E_1$ and can be neglected. The ΔE at 340 mμ should not exceed 1.0 (corresponding $\Delta E_{366} \approx 0.53$). If E_2 is still increasing 20 min. after the addition of the enzyme, while the initial optical density E_1 was constant for at least 3 min., then providing no other interfering substances are present (see below), the activity of the glycerol-1-phosphate dehydrogenase is probably too low.

To check that the assay is working correctly, mix 0.01 ml. 0.002 M L-(—)-glycerol-1-phosphate into the experimental cuvette on completion of the reaction. The increase in optical density should be complete in 10—20 min. Read the optical density E_3 twice within 3 min. and calculate the difference $\Delta E' = E_3 - E_2$. Under the stated conditions $\Delta E'$ should be 0.123 at 340 mμ and 0.065 at 366 mμ.

Calculations

L-(—)-Glycerol-1-phosphate reacts quantitatively, so that the amount in the sample can be calculated from the optical density difference:

$$\frac{\Delta E \times \text{dil.}}{\varepsilon \times d} = \mu\text{moles glycerol-1-P/g. tissue}$$

where

ΔE = optical density difference ($E_2 - E_1$)
dil. = total dilution of the sample
ε = extinction coefficient [cm.²/μmole]
d = light path [cm.]

With constant dilution ratios the equation simplifies to

$$\Delta E \times F = \mu\text{moles glycerol-1-P/g. tissue} \left(F = \frac{\text{dil.}}{\varepsilon \times d} \right)$$

If the ratio of volume of extract to tissue weight is 4 : 1, then:

volume of the neutralized extract to weight of tissue is 4.1 : 1, dilution of extract in assay is 2.02 : 1 and the total dilution is 8.28 : 1.

Giving values for F at 334 mμ: 1.36
340 mμ: 1.32
366 mμ: 1.25

If the ratio of volume of extract to tisssue weight is 8 : 1, then:

volume of the neutralized extract to weight of tissue is 8.2 : 1, dilution of the extract in the assay is 2.02 : 1 and the total dilution is 16.5 : 1.

Giving values for F at: 334 mμ: 2.72
340 mμ: 2.64
366 mμ: 2.50

At 366 mμ the extinction coefficient is dependent on the temperature (see p. 27). The values given here for F_{366} are for 25° C.

Example

1.276 g. tissue (rat liver) was added to 5 ml. perchloric acid. After extracting twice, the volume of fluid was 7.6 ml. and this was made up to 10.2 ml. with 2.6 ml. perchloric acid (volume of extract: weight of tissue = 8 : 1) and neutralized.

Measurements at 334 mμ; light path, 1 cm.; against control cuvette containing water.

Before addition of GDH 0 min. $E_1 = 0.148$
3 min. $E_1 = 0.150$

After addition of GDH 10 min. $E_2 = 0.241$
13 min. $E_2 = 0.243$

$\Delta E = E_2 - E_1 = 0.241 - 0.148 = 0.093$

$0.093 \times 2.72 = 0.253$ μmoles glycerol-1-P/g. tissue.

Further Determinations

Other metabolites can be determined in the same assay mixture by the addition of specific enzymes [2], *e.g.* L-(+)-lactate and L-(−)-malate.

Sources of Error

1. A constant end-point is not reached within 30 min. if the activity of the glycerol-1-phosphate dehydrogenase is too low. Check the activity of the enzyme and, if necessary, use larger amounts of enzyme or a fresh preparation.

2. The initial optical density is not constant if: a) the cuvette contents were not brought to room temperature before beginning the measurements, b) the hydrazine-glycine buffer is more than 8 days old, c) the DPN preparation is impure or d) a change occurs in the absorption due to the tissue extract. In the last case, measure against a control cuvette, which contains the same solutions as the experimental cuvette, but to which no enzyme is added.

3. The addition of the enzyme causes a sharp change in optical density. If the optical density increases then the enzyme usually has too high an absorption and therefore a new enzyme preparation should be used. If the optical density decreases then the hydrazine-glycine buffer is too alkaline. At pH >9.6 the initial optical density of the assay mixture is higher. Addition of the enzyme includes addition of ammonium sulphate which lowers the pH and so causes a sharp decrease in optical density.

4. The optical density reaches a maximum and then falls (especially at higher temperatures, *e.g.* 37° C), because of the autoxidation of DPNH [3]. The remedy is to evacuate the experimental cuvette.

Specificity

The assay is specific for L-(−)-glycerol-1-phosphate. The dextrorotatory isomer does not react. The racemate only reacts to 50% of the amount present. Glycerol-2-phosphate (β-glycerophosphate), phosphoserine, glycerylphosphorylcholine and glycerylphosphorylcolamine do not react [4].

[3] *H. J. Hohorst*, Biochem. Z. *328*, 509 [1957].
[4] *H. J. Hohorst*, Diploma-Thesis, Universität Marburg, 1959.