D-(—)-β-Hydroxybutyrate

Dermot H. Williamson and Jane Mellanby

Principle

D-(−)-β-Hydroxybutyric dehydrogenase catalyses the reaction:

(1) D-(−)-β-Hydroxybutyrate + DPN^+ ⇌ acetoacetate + DPNH + H^+

The equilibrium constant K $[H^+]$ is 1.45×10^{-9} at 25°C[1]. Hence at pH 8.5 40% of the D-(−)-β-hydroxybutyrate is oxidized to acetoacetate. In the presence of hydrazine, the acetoacetate is removed in the form of its hydrazone and the reaction proceeds quantitatively from left to right. The increase of optical density at 340 mμ due to the formation of DPNH is a measure of the reaction.

Reagents

1. Tris-hydroxymethyl-amino-methane, tris
2. Hydrochloric acid, 1 N and 0.2 N
3. Perchloric acid, A. R., sp. gr. 1.54; *ca.* 50% (w/w)
4. Potassium hydroxide
5. Universal indicator *)
6. Hydrazine hydrate (99—100%)
7. Diphosphopyridine nucleotide, DPN
   free acid; commercial preparation, see p. 1010.
8. DL-β-Hydroxybutyric acid
   sodium salt; commercial preparation, see p. 1021.
9. D-(−)-β-Hydroxybutyric dehydrogenase
   from *Rhodopseudomonas spheroides*. For a description of the purification, see p. 457.

Purity of the enzyme preparation

The enzyme preparation obtained according to p. 451 is still contaminated with malic dehydrogenase and traces of a polyol dehydrogenase. Malate therefore interferes with the estimation of D-(−)-β-hydroxybutyrate. Mannitol and sorbitol, which are usually absent from animal tissues or if present occur in low concentrations, are slowly oxidized in the presence of DPN.

Preparation of Solutions

I. Tris buffer (0.1 M; pH 8.5):
Dissolve 1.21 g. tris-hydroxymethyl-aminomethane in 50 ml. distilled water, add 14.3 ml. 0.2 N HCl and make up to 100 ml. with distilled water. Check pH (glass electrode).

II. Hydrazine buffer (pH 8.5):
Mix 1 ml. of hydrazine hydrate and 5 ml. 1 N HCl and dilute to 20 ml. with distilled water.

III. Perchloric acid (*ca.* 30% w/v):
Dilute 40 ml. 60% $HClO_4$ to 120 ml. with distilled water.

IV. Potassium hydroxide, (*ca.* 20% w/v):
Dissolve 20 g. KOH distilled water and make up to 100 ml.

*) Commercial preparation from British Drug Houses Ltd., Poole, England.

[1] *H. A. Krebs, J. Mellanby* and *D. H. Williamson*, Biochem. J. *82*, 96 [1962].

V. Diphosphopyridine nucleotide (*ca.* 1.3×10^{-2} M β-DPN):
Dissolve 20 mg. DPN in 2 ml. distilled water.

VI. D-(−)-β-Hydroxybutyric dehydrogenase (*ca.* 1000 units/ml. *):
Prepare the solution according to the method on p. 457. If necessary, dilute the solution with 0.01 M phosphate (pH 7.6) to give *ca.* 1000 units/ml.

Stability of the solutions

Store the DPN solution at −15° C. The enzyme solution is stable for at least a month when stored at 2−4° C. Prepare the hydrazine solution freshly each day. Keep the other solutions at room temperature in stoppered bottles.

Procedure

Experimental material

The method has been used for the determination of D-(−)-β-hydroxybutyrate in whole blood, serum and the medium in which tissue slices have been incubated. D-(−)-β-Hydroxybutyrate can be determined in tissue homogenates if no appreciable amounts of malate **, sorbitol*** or mannitol are present.

Deproteinization

Refer to "Acetoacetate" p. 455.

Spectrophotometric measurements

Wavelength: 340 mμ; light path: 1 cm.; final volume: 3.1 ml. Read against the control cuvette.

For each series of measurements prepare a cuvette containing 0.20 μmoles D-(−)-β-hydroxybutyrate (= 50.4 μg. DL-Na-β-hydroxybutyrate). If sufficient cuvettes are available up to 12 samples can be analysed simultaneously.

Pipette into the cuvettes:

Experimental cuvette	*Control cuvette*
1.0 ml. hydrazine hydrate buffer (solution II)	1.0 ml. hydrazine hydrate buffer (solution II)
0.5 ml. tris buffer (solution I)	0.5 ml. tris buffer (solution I)
1.5 ml. of sample (containing 0.05−0.20 μmoles D-(−)-β-hydroxybutyrate)	1.5 ml. distilled water
0.1 ml. DPN solution (V)	0.1 ml. DPN solution (V).

Mix well and read the optical density at 340 mμ at two minute intervals until a constant reading is obtained: E_1. Mix into both cuvettes

0.025 ml. of D-(−)-β-hydroxybutyric dehydrogenase solution (VI)

and read the optical density at 340 mμ at 10 minute intervals until the reaction has stopped (*ca.* 40−60 min.). Final optical density E_2.

*) A unit is the amount of enzyme which causes a decrease in optical density of 0.010/min. at 340 mμ in the following assay mixture: 100 μmoles tris buffer (pH 7.4), 0.5 μmoles DPNH and 10 μmoles acetoacetate in a final volume of 3 ml.

**) Determination, see p. 328.

***) Determination, see p. 167.

Calculations

Under the conditions described here at least 98 % of the D-(−)-β-hydroxybutyrate is oxidized with the stoichiometric formation of an equivalent amount of DPNH.

Therefore it follows that:

$$\frac{\Delta E \times 3.1}{6.22} = 0.498 \times \Delta E = \mu\text{moles D-(−)-β-hydroxybutyrate/reaction mixture}$$

or

$$\frac{\Delta E \times 3.1 \times 104}{6.22} = 51.9 \times \Delta E = \mu\text{g. D-(−)-β-hydroxybutyric acid/reaction mixture}$$

where

$\Delta E = E_2 - E_1$

3.1 = assay volume [ml.]

6.22 = extinction coefficient of DPNH at 340 mμ [cm.^{2}/μmole] [2)]

104 = molecular weight of β-hydroxybutyric acid.

Sources of Error

Malate, sorbitol and mannitol interfere with the assay, if the enzyme preparation contains malic and polyol dehydrogenase. Lactate interferes if the enzyme preparation contains lactic dehydrogenase.

[2)] *B. L. Horecker* and *A. Kornberg*, J. biol. Chemistry *175*, 385 [1948].