Acetoacetate

Jane Mellanby and Dermot H. Williamson

In 1937, *Green* and collaborators[1] described an insoluble enzyme from pig heart muscle which catalysed the reversible oxidation of D-(−)-β-hydroxybutyrate. The discovery that cell-free extracts of certain bacteria which accumulate poly-β-hydroxybutyrate contain a soluble and stable D-(−)-β-hydroxybutyric dehydrogenase[2-4] has led to the partial purification of this enzyme and its use as an analytical reagent[4].

Principle

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

(1) D-(−)-β-Hydroxybutyrate + DPN^+ $\rightleftharpoons$ acetoacetate + DPNH + H^+

The equilibrium constant of this reaction $K[H^+]$ is 1.45×10^{-9} at 25° C[4a]. At pH 7.0 and with a suitable excess of DPNH, at least 98% of the acetoacetate is reduced to D-(−)-β-hydroxybutyrate with the simultaneous oxidation of an equivalent amount of DPNH. The decrease of optical density at 340 mμ due to the oxidation of DPNH is measured.

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
2. Dipotassium hydrogen phosphate, K_2HPO_4, A. R.
3. Perchloric acid, A. R., sp. gr. 1.54; *ca.* 60% (w/w)
4. Potassium hydroxide
5. Universal Indicator *)
6. Acetoacetic acid
   hydrolyse freshly distilled ethyl acetoacetate[5], neutralize the product with 1 N NaOH and standardize manometrically[6].
7. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
8. D-(−)-β-Hydroxybutyric dehydrogenase
   from *Rhodopseudomonas spheroides*. For a description of the purification, see Appendix, p. 457.

Purity of the enzyme preparation

The enzyme preparation obtained according to p. 457 is still contaminated with malic dehydrogenase and traces of a polyol dehydrogenase. Oxaloacetate therefore interferes with the estimation of acetoacetate, but can be removed by preliminary incubation with malic dehydrogenase. However, owing to the very low concentration of oxaloacetate in animal tissues and its instability, this procedure is usually unnecessary. The polyol dehydrogenase slowly reduces D-fructose in the presence of DPNH.

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

1) *D. E. Green, J. G. Dewan* and *L. F. Leloir*, Biochem. J. *31*, 934 [1937].
2) *R. Gavard, D. Combre* and *A. Tuffet*, C. R. hebd. Séances Acad. Sci. *251*, 1931 [1960].
3) *M. Doudoroff, J. M. Merrick* and *R. Contopoulou*, Fed. Proc. *20*, 272 [1961].
4) *D. H. Williamson, J. Mellanby* and *H. A. Krebs*, Biochem. J. *82*, 90 [1962].
4a) *H. A. Krebs, J. Mellanby* and *D. H. Williamson*, Biochem. J. *82*, 96 [1962].
5) *G. Ljunggren*, Biochem. Z. *145*, 422 [1924].
6) *N. L. Edson*, Biochem. J. *29*, 2082 [1935].

Preparation of Solutions

I. Phosphate buffer (0.1 M; pH 7.0):
   a) Dissolve 13.6 g. KH_2PO_4 in 1000 ml. distilled water
   b) Dissolve 17.4 g. K_2HPO_4 in 1000 ml. distilled water.
   Mix solutions a) and b) in the ratio of 39 : 61 parts by volume. Check the pH (glass electrode).

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

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

IV. Reduced diphosphopyridine nucleotide (*ca.* 6×10^{-3} M β-DPNH):
   Dissolve 10 mg. DPNH-Na_2 in 2 ml. distilled water. Adjust the concentration of the solution so that 0.1 ml. diluted to 3.1 ml. gives an optical density of 0.9 to 1.0 at 340 mμ with a 1 cm. light path.

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

Stability of the solutions

Store the DPNH solution at −15°C. The enzyme solution is stable for at least a month at 2−4°C. Keep the other reagents at room temperature in stoppered bottles.

Procedure

Experimental material

The method has been used for the determination of acetoacetate in blood, serum, and the medium in which tissue slices have been incubated.

Chill blood samples immediately after collection and deproteinize as soon as possible to minimize the non-enzymatic decarboxylation of acetoacetate[4,7] which is greatly accelerated by blood (*e.g.* if blood containing 1 μmole acetoacetate/ml. is incubated at 37°C for 1 hour, only 60% of the keto acid is recovered).

Deproteinization

The samples are treated with perchloric acid, the precipitated protein centrifuged off and the excess perchloric acid removed as the insoluble potassium salt. The deproteinization of blood is given as an example:

Pipette into a 15 ml. conical centrifuge tube

3 ml. perchloric acid solution (II).

Cool in an ice bath and add

3 ml. blood (cooled to 2−4°C).

*) A unit is the amount of enzyme which causes a decrease in optical density of 0.010/min. at 340 mμ in the following system: 100 μmoles tris buffer, (pH 7.4), 0.5 μmoles DPNH and 10 μmoles acetoacetate in a final volume of 3.0 ml.

7) *Di A. Rossi*, Arch. Sci. biol. [Bologna] *24*, 73 [1938].

Mix thoroughly with a thin glass rod, allow to stand for 10 min. in an ice bath, then centrifuge for 10 min. at 3000 g. Pour off the supernatant fluid and measure the volume. Mix into the supernatant

0.005 ml. Universal Indicator

and slowly add

KOH solution (III)

until the colour changes from red to green or blue-green (pH 7—8). A total of 1.8 to 2 ml. KOH are required. Note the exact amount. Allow to stand for approximately 30 min. in the ice bath and then centrifuge for 10 min. at 3000 g. Decant the supernatant and use this for the determination of acetoacetate.

Spectrophotometric measurements

Wavelength: 340 mμ; light path: 1 cm.; final volume: 3.1 ml. Read against a blank cuvette containing 3.1 ml. water. For each series of measurements prepare a cuvette containing 0.20 μmoles acetoacetate (20.4 μg.). If sufficient cuvettes are available, up to 12 samples can be analysed simultaneously.

Pipette into the cuvettes:

Experimental cuvette	*Control cuvette*
1.0 ml. phosphate buffer (solution I)	1.0 ml. phosphate buffer (solution I)
2.0 ml. sample (containing 0.05—0.2 μmoles acetoacetate)	2.0 ml. distilled water
0.1 ml. DPNH solution (IV)	0.1 ml. DPNH solution (IV).

Mix well and read the optical density E_1. Mix into all the cuvettes (including the blank cuvette)

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

and read the optical density at 5 min. intervals until the reaction has stopped (*ca.* 20 min.) or until the optical density change is the same in the experimental and control cuvette. Final optical density is E_2.

Calculations

Under the assay conditions, at least 98% of the acetoacetate is reduced to D-(−)-β-hydroxybutyrate with stoichiometric formation of an equivalent amount of DPN.

Therefore it follows that:

$$\frac{(\Delta E_E - \Delta E_C) \times 3.1}{6.22} = 0.498 \times (\Delta E_E - \Delta E_C) = \mu\text{moles acetoacetate/assay mixture}$$

or

$$\frac{(\Delta E_E - \Delta E_C) \times 3.1 \times 102}{6.22} = 50.8 \times (\Delta E_E - \Delta E_C) = \mu\text{g. acetoacetic acid/assay mixture}$$

where

$\Delta E_E = E_1 - E_2$ in the experimental cuvette

$\Delta E_C = E_1 - E_2$ in the control cuvette

3.1 = volume of the assay mixture [ml.]

6.22 = extinction coefficient[8] of DPNH at 340 mμ [cm.²/μmole]

102 = molecular weight of acetoacetic acid.

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

Example

The following values were found:

Experimental cuvette: $E_1 = 0.950$; $E_2 = 0.830$; $\Delta E_E = 0.120$

Control cuvette: $E_1 = 0.945$; $E_2 = 0.935$; $\Delta E_C = 0.010$

$\Delta E_E - \Delta E_C = 0.120 - 0.010 = 0.110$

The experimental cuvette therefore contained

$0.110 \times 0.498 = 0.0548$ μmoles acetoacetate

or

$0.110 \times 50.8 = 5.59$ μg. acetoacetic acid

Sources of Error

Oxaloacetate and D-fructose interfere with the determination of acetoacetate. Oxaloacetate can be removed by preliminary incubation of the sample with malic dehydrogenase. If D-fructose is present, there is a considerable delay before a stable end-point is reached. Since the reduction of fructose proceeds much more slowly than the reduction of acetoacetate, interference from fructose can be corrected for by extrapolation. In the analysis of the experimental material listed on p. 455 no appreciable interference from these substances has been encountered. Pyruvate interferes if the enzyme preparation contains lactic dehydrogenase.

Other Determinations

Under the assay conditions described above, pyruvate, α-oxoglutarate and oxaloacetate can be estimated by the addition of lactic dehydrogenase, glutamic dehydrogenase and malic dehydrogenase respectively. D-(−)-β-Hydroxybutyric dehydrogenase can also be used for the determination of D-(−)-β-hydroxybutyrate (see p. 459).

Appendix

Purification of D-(−)β-Hydroxybutyric Dehydrogenase

Reagents

Potassium dihydrogen phosphate, KH_2PO_4
Dipotassium hydrogen phosphate, K_2HPO_4
Magnesium sulphate, $MgSO_4 \cdot 7\ H_2O$
Manganese sulphate, $MnSO_4 \cdot 4\ H_2O$
Diammonium hydrogen phosphate, $(NH_4)_2HPO_4$
Ammonium sulphate, $(NH_4)_2SO_4$, A. R.
Calcium chloride, $CaCl_2$
Sodium acetate $\cdot 3\ H_2O$
Ferric citrate
Nicotinic acid
Thiamine
Biotin
Sodium glutamate
Ethyl alcohol
Ethylene-diamine-tetra-acetic acid (EDTA) disodium salt, EDTA-$Na_2H_2 \cdot 2\ H_2O$
Magnesium acetate $\cdot 4\ H_2O$

Preparation of solutions

I. Phosphate buffer (0.05 M; pH 7.4; 0.01 M Mg acetate): Dissolve 6.8 g. KH_2PO_4 in distilled water and make up to 1000 ml. Dissolve 8.7 g. K_2HPO_4 in distilled water and make up to 1000 ml. Mix the solutions in the ratio of 19 : 81 parts by volume. Dissolve 1.07 g. Mg acetate $\cdot 4\ H_2O$ in 500 ml. of the buffer.

II. Phosphate buffer (0.01 M; pH 7.4): Prepare buffer as above omitting Mg acetate and dilute 100 ml. to 500 ml. with distilled water.

III. Ammonium sulphate (saturated at 0°C; pH 7.7; 0.001 M EDTA): Mix 730 g. $(NH_4)_2SO_4$ and 372 mg. of EDTA-$Na_2H_2 \cdot 2H_2O$ with 1000 ml. distilled water. Dissolve the ammonium sulphate by warming to *ca.* 40°C and then cool to room temperature. Adjust to pH 7.7 by addition of *ca.* 7 ml. of conc. NH_4OH (sp. gr. 0.88). Check the pH on a sample diluted 1 : 10 with distilled water (glass electrode).

Procedure

Bacteria: Rhodopseudomonas spheroides (NCIB *) 8253).

Culture: Dissolve in 8000 ml. distilled water: 40 g. KH_2PO_4 + 40 g. K_2HPO_4 + 1.6 g. $MgSO_4 \cdot 7\ H_2O$ + 6.4 g. $(NH_4)_2HPO_4$ + 16 g. Na glutamate + 24 g. Na acetate·3 H_2O + 320 mg. $CaCl_2$ + 240 mg. ferric citrate + 8 mg. $MnSO_4 \cdot 4\ H_2O$ + 8 mg. nicotinic acid + 8 mg. thiamine + 0.8 mg. biotin. Sterilize the medium, cool and inoculate with 250 ml. of a 24 hour culture. Incubate at 30° C for 48 hours with continuous aeration.

Cell-free extract: Collect the cells by centrifuging, wash twice with a total volume of 2000 ml. 0.01 M phosphate buffer (solution II) and suspend in 150 ml. of this buffer. Sonicate the cells at 19 to 20 kc/sec. for 15 min. Centrifuge for 10 min. at 0° C and 5000 g. Discard the precipitate.

1st Ammonium sulphate fractionation: At 2° C add to every 100 ml. of the cell-free supernatant 66 ml. $(NH_4)_2SO_4$ solution (III) over a period of 15 min. Remove the precipitate by centrifuging for 10 min. at 10000 g. To the supernatant add a further 120 ml. $(NH_4)_2SO_4$ solution (III) for each 100 ml. of the original extract and leave the mixture to stand for one hour. Centrifuge for 10 min. at 10000 g and discard the supernatant.

Alcohol fractionation: Dissolve the precipitate in 0.05 M phosphate buffer (solution I) and adjust the protein concentration to between 4 and 5 mg./ml. Centrifuge off the precipitate which forms. Cool the supernatant, contained in a beaker, to −2° C in a dry ice-acetone bath. Stir continuously with a magnetic stirrer and introduce ethanol (43 ml./100 ml.), cooled to −15° C, beneath the surface of the liquid. Centrifuge for 15 min. at 10000 g and discard the precipitate.

2nd Ammonium sulphate fractionation: At 2° C add to every 100 ml. of the supernatant 186 ml. $(NH_4)_2SO_4$ solution (III). Centrifuge off the precipitate and discard. To the supernatant add a further 114 ml. $(NH_4)_2SO_4$ solution (III) per 100 ml. of the supernatant from the alcohol fractionation and leave the mixture to stand overnight at 2° C. Centrifuge at 10000 g for 20 min. and dissolve the precipitate in 5 ml. 0.01 M phosphate buffer (solution II) for every 100 ml. of the original cell-free extract.

The enzyme solution is used in the determination of acetoacetate (p. 454) and D-(−)-β-hydroxybutyrate (p. 459).

*) National Collection of Industrial Bacteria. Address: Torry Research Station, Aberdeen, Scotland.