L-(+)-β-Hydroxybutyryl Coenzyme A

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Principle

L-(+)-β-Hydroxybutyryl-CoA, which is an intermediate in the degradation of fatty acids[1], is oxidized to acetoacetyl-CoA by diphosphopyridine nucleotide (DPN) in the presence of β-hydroxyacyl dehydrogenase. This reaction is the reverse of that used to estimate acetoacetyl-CoA (see p. 425).

$$\text{(1)}\quad \underset{\underset{\text{OH}}{|}}{\text{CH}_3\text{–CH–CH}_2\text{–CO}\sim\text{SCoA}} + \text{DPN}^+ \rightleftharpoons \underset{\underset{\text{O}}{\|}}{\text{CH}_3\text{–C–CH}_2\text{–CO}\sim\text{SCoA}} + \text{DPNH} + \text{H}^+$$

As with all pyridine nucleotide-dependent reactions the equilibrium is dependent on pH and by use of a more alkaline medium is displaced towards the right. Above pH 8.5 the equilibrium is further displaced because of enol dissociation. This allows the quantitative oxidation of β-hydroxybutyryl-CoA. The reaction is measured by the absorption at 340 mμ of the reduced diphosphopyridine nucleotide (DPNH) formed.

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
2. Potassium hydrogen carbonate, $KHCO_3$, A. R.
3. Potassium hydroxide, A. R.
4. Perchloric acid, A. R., sp. gr. 1.67, *ca.* 70% (w/w)
5. Hydrochloric acid, conc., *ca.* 37% (w/w), A. R.
6. Tris-hydroxymethyl-aminomethane, tris
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.
9. β-Hydroxyacyl dehydrogenase, HOADH
   purified from sheep liver according to[2] or crystalline from pig heart according to[3] (see also p. 425.); commercial preparation, see p. 984.

Preparation of Solutions

Prepare all solutions with metal-free, distilled water.

I. Potassium dihydrogen phosphate (0.2 M):
Dissolve 2.722 g. KH_2PO_4 in distilled water and make up to 100 ml.

II. Potassium hydroxide (*ca.* 8 N):
Dissolve 45 g. KOH in distilled water with cooling and make up to 100 ml.

III. Potassium hydrogen carbonate (*ca.* 1 M):
Dissolve 10 g. $KHCO_3$ in distilled water and make up to 100 ml.

IV. Tris buffer (*ca.* 0.5 M; pH 9.5):
Dissolve 12.1 g. tris in 150 ml. distilled water, add 0.35 ml. conc. HCl and dilute to 200 ml. with distilled water.

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[1] *F. Lynen*, Fed. Proc. *12*, 683 [1953].
[2] *F. Lynen* and *O. Wieland* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. I, p. 566.
[3] *J. R. Stern*, Biochim. biophysica Acta *26*, 448 [1957].

V. Perchloric acid (*ca.* 4 M):
Dilute 35 ml. 70% $HClO_4$ to 100 ml. with distilled water.

VI. Ethylene-diamine-tetra-acetate (0.1 M):
Dissolve 1.86 g. EDTA-$Na_2H_2 \cdot H_2O$ in distilled water and make up to 50 ml.

VII. Diphosphopyridine nucleotide (*ca.* 0.01 M β-DPN):
Dissolve 7.4 mg. DPN in about 0.5 ml. distilled water, neutralize with a few drops of potassium hydrogen carbonate solution (III) and dilute to 1 ml. with distilled water.

VIII. β-Hydroxyacyl dehydrogenase, HOADH (3 mg. protein/ml.):
Dilute with water containing EDTA (0.05 ml. solution VI/ml.).

Stability of the solutions

β-Hydroxyacyl dehydrogenase, see p. 426. β-Hydroxybutyryl-CoA has a stability similar to acetoacetyl-CoA (p. 426), but is more easily hydrolysed by alkali. Solutions of DPN are preferably stored in a deep-freeze, but are also stable for several weeks at 0° C. The other solutions should be stored in a refrigerator to prevent bacterial growth and under these conditions are stable indefinitely. Polyethylene bottles should be used for the buffer and the alkaline solutions.

Procedure

Extraction and deproteinization of the sample

Refer to the chapter on acetyl coenzyme A (p. 420). Solutions I—III and V are required.

Spectrophotometric measurements

Preliminary remarks: Rapid hydrolysis of acyl mercaptans occurs at the pH of the assay mixture and therefore highly active enzyme preparations must be used so that the oxidation is complete in 1 to 2 minutes. The β-hydroxybutyryl-CoA is added last to minimize the time of contact with alkaline medium and the reaction is started as soon as possible. At the end of the reaction it is necessary to test with indicator paper, whether the pH has fallen below 9. If this is the case, then the solution of the CoA derivative is too strongly buffered. So by a preliminary test the amount of 0.1 N KOH required to maintain the correct pH in the reaction mixture is determined and this amount of KOH is added before the enzyme.

Method: Wavelength: 340, 334 or 366 mμ; light path: 1 cm.; room temperature; final volume: 2 ml. Read against a water blank or air.

Pipette successively into the cuvette:

tris buffer (solution IV)	0.70 ml.	
EDTA solution (VI)	0.04 ml.	
DPN solution (VII)	0.05 ml.	
sample solution	up to 1.20 ml.	(containing 0.015—0.2 μmoles β-hydroxybutyryl-CoA)
distilled water	to 1.99 ml.	

Mix well, read the initial optical density E_1, then start the reaction by addition of

0.01 ml. HOADH solution (VIII, *ca.* 200 units according to [2]).

When the increase in optical density stops (optical density is constant for 1 minute) read the final optical density E_2.

Calculations

With an assay volume of 2 ml., a 1 cm. light path and measurements at 340 mμ:

$$0.322 \times \Delta E \times \frac{V}{v} = \text{μmoles L-(+)-β-hydroxybutyryl-CoA in the whole sample}$$

measurements at 334 mμ:

$$0.341 \times \Delta E \times \frac{V}{v} = \text{μmoles L-(+)-β-hydroxybutyryl-CoA in the whole sample}$$

measurements at 366 mμ:

$$0.607 \times \Delta E \times \frac{V}{v} = \text{μmoles L-(+)-β-hydroxybutyryl-CoA in the whole sample}$$

where

V = volume of the sample in ml.
v = portion of this solution taken for assay in ml.
$\Delta E = E_2 - E_1$
ε = extinction coefficient of DPNH; the values are for 340 mμ: 6.22 cm.²/μmole; 334 mμ: 5.87; 366 mμ: 3.30 (see p. 27).

Example

Coenzyme A (10 μmoles) was converted to β-hydroxybutyryl-CoA with β-butyrolactone[4]. The volume of the neutral solution was 3.2 ml. and 0.05 ml. was used for the determination. Wavelength: 366 mμ. $E_1 = 0.117$. 190 units HOADH were added. After 2 min.: $E_2 = 0.243$. $\Delta E = 0.126$. Final pH was 9.2 to 9.3.

$$0.607 \times 0.126 \times \frac{3.2}{0.05} = 4.90 \text{ μmoles L-(+)-β-hydroxybutyryl-CoA in the whole sample}$$

With 0.08 ml. β-hydroxybutyryl-CoA solution $E_1 = 0.121$; $E_2 = 0.322$; $\Delta E = 0.201$.

$$0.607 \times 0.201 \times \frac{3.2}{0.08} = 4.88 \text{ μmoles L-(+)-β-hydroxybutyryl-CoA in the whole sample}$$

Sources of Error

Contamination of the β-hydroxyacyl dehydrogenase with β-ketoacylthiolase favours the determination of β-hydroxybutyryl-CoA, because the combination of the two enzymes allows the assay to be carried out at less alkaline pH values (about pH 8), owing to the position of the equilibrium of the β-ketoacylthiolase reaction. The addition of a mercaptan (coenzyme A, pantetheine, *N,S*-diacetylcysteamine) in stoichiometric amounts is a prerequisite.
Several β-hydroxybutyryl thiolesters can interfere and the higher homologues of β-hydroxybutyryl-CoA are also active (see "Specificity").

Specificity

β-Hydroxyacyl dehydrogenase is not very specific with regard to the mercaptan component. As well as β-hydroxybutyryl-CoA, the β-hydroxybutyryl derivatives of pantetheine phosphate, pantetheine, *N,S*-diacetylcysteamine and several other mercaptans (refer to[4]) are also oxidized. The CoA derivatives of all the higher homologues of L-(+)-β-hydroxybutyric acid, up to about C_{20}, also react (refer to p. 428). There is nevertheless, a high specificity with regard to the β-hydroxyacylmercaptan grouping $R-CH(OH)-CH_2-CO-S-R'$: neither α-hydroxyacyl derivatives, nor the free acids or primary and secondary alcohols are oxidized. The selectivity of the enzyme for stereoisomers is just as rigorous. Only the CoA derivatives of the L-(+)-β-hydroxyacids are oxidized[5] and only this isomer is formed on reduction of acetoacetyl-CoA. This explains why only 50% of

[4] *K. Decker:* Die aktivierte Essigsäure. Ferd. Enke, Stuttgart 1959.
[5] *A. L. Lehninger* and *G. D. Greville*, Biochim. biophysica Acta *12*, 188 [1953].

the β-hydroxybutyryl-CoA prepared from CoA and β-butyrolactone reacts (refer to "Example"). Although HOADH from sheep liver is specific for DPN, *Stern*[3] found that the enzyme from pig heart can also use TPN as hydrogen acceptor (the relative rates with DPN and TPN are 10 : 1).

Other Methods for the Determination of β-Hydroxybutyryl-CoA

The determination can be made more sensitive if β-hydroxyacyl dehydrogenase, β-ketoacylthiolase, L-malic dehydrogenase and condensing enzyme are combined[6]: for each mole L-(+)-β-hydroxybutyryl-CoA 3 moles DPN are reduced:

(2) L-(+)-β-Hydroxybutyryl-CoA + 2 L-malate + 3 DPN^+ + $2H_2O$ ⟶
2 citrate + CoASH + 3 DPNH + 5 H^+

Usually the greater expenditure on enzymes is scarcely justified by the advantage gained. The same is true for a combined test in which *p*-nitroaniline (refer to p. 419) is acetylated in the presence of HOADH, β-ketoacylthiolase, arylamine transacetylase, DPN and catalytic amounts of CoA (or pantetheine):

(3) L-(+)-β-Hydroxybutyryl-CoA + 2 *p*-nitroaniline + DPN^+ ⟶
2 *p*-nitroacetanilide + CoASH + DPNH + H^+

When no interfering substances, especially thiolesters, are present then non-enzymatic assay methods can be used (p. 424).

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[6] *J. R. Stern, A. Del Campillo* and *A. L. Lehninger*, J. Amer. chem. Soc. *77*, 1073 [1955].