Acetoacetyl Coenzyme A

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Principle

β-Hydroxyacyl dehydrogenase (HOADH) catalyses the following step in the fatty acid cycle[1]:

$$(1)\quad H_3C-\underset{\underset{OH}{|}}{CH}-CH_2-CO\sim SCoA + DPN^+ \rightleftharpoons H_3C-\overset{\overset{O}{\|}}{C}-CH_2-CO\sim SCoA + DPNH + H^+$$

The reaction is pyridine nucleotide-dependent and therefore can be measured spectrophotometrically. The equilibrium constant[2] K is 1.9×10^{-10} moles/l. at 25°C. The position of the equilibrium allows quantitative reduction of acetoacetyl-CoA with reduced diphosphopyridine nucleotide (DPNH) at pH values up to about 7. The decrease in absorption at 340 mμ due to the oxidation of DPNH serves as a measure of the reaction.

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
2. Disodium hydrogen phosphate, $Na_2HPO_4\cdot2H_2O$, A. R.
3. Potassium hydrogen carbonate, $KHCO_3$, A. R.
4. Potassium hydroxide, A. R.
5. Perchloric acid, sp. gr. 1.67, *ca.* 70% (w/w), A. R.
6. Ethylene-diamine-tetra-acetic acid, EDTA
   disodium salt, EDTA-$Na_2H_2\cdot2H_2O$ (Titriplex III, Trilon B, Versene).
7. Reduced diphosphopyridine nucleotide, DPNH
   sodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
8. β-Hydroxyacyl dehydrogenase, HOADH
   from sheep liver; the enzyme purified according to *Lynen* and *Wieland*[2] contains 7800 units*/ mg. protein and is free from β-ketoacylthiolase. The preparation of a crystalline β-hydroxyacyl dehydrogenase from pig heart has been described by *Stern*[3]. Crystalline 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. Phosphate buffer (0.2 M; pH 6.8):
   Dissolve 1.361 g. KH_2PO_4 and 1.781 g. $Na_2HPO_4\cdot2H_2O$ in distilled water and make up to 100 ml.

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

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*) A unit is the amount of enzyme which at 20° C, in an assay volume of 2 ml., decreases the optical density of DPNH at 366 mμ by 0.001/min. (light path 1 cm.)[2].

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 1955, Vol. I, p. 566.
3) *J. R. Stern*, Biochim. biophysica Acta *26*, 448 [1957].

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

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-$Na_2H_2 \cdot 2H_2O$ in distilled water and make up to 50 ml.

VII. Reduced diphosphopyridine nucleotide (*ca.* 0.01 M β-DPNH):
Dissolve 9.3 mg. DPNH-Na_2 in 1 ml. distilled water.

VIII. β-Hydroxyacyl dehydrogenase, HOADH (*ca.* 3 mg. protein/ml.):
Dilute the stock suspension with phosphate buffer (solution II), containing 0.005 M EDTA, to 3 mg. protein/ml.

Stability of the solutions

Solutions of β-hydroxyacyl dehydrogenase are stable for long periods at 0°C. Repeated freezing and thawing leads to a rapid decrease in activity. Solutions of acetoacetyl-CoA should be between pH 4 and 6; they are stable for several months in the frozen state. Acetoacetyl-CoA is hydrolysed by alkali just as rapidly as acetyl-CoA and like the latter it is sensitive to the prolonged action of strong acid. The presence of high concentrations of mercaptans, especially cysteine, should be avoided at pH values greater than 6.5 (thiol exchange).

The DPNH solution should be freshly prepared each week and should always be stored at 0°C. All other solutions are stable indefinitely, but in order to avoid bacterial contamination they should be stored in a refrigerator. Polyethylene bottles should be used for the buffer and alkaline solutions.

Procedure

Extraction and deproteinization of sample

See chapter on acetyl coenzyme A (p. 420). Solutions III and V are needed. Use freeze-drying instead of phenol extraction to concentrate solutions.

Spectrophotometric measurements

Wavelength: 340, 334 or 366 mμ; light path: 1 cm.; final volume: 2 ml.; room temperature. Measure against the control cuvette.

Pipette successively into the cuvettes:

	Experimental	*Control*
phosphate buffer (solution II)	0.50 ml.	0.50 ml.
sample	up to 1.30 ml.	—
EDTA solution (VI)	0.05 ml.	0.05 ml.
DPNH solution (VII)	0.05 ml.	0.01 ml.
distilled water	to 1.99 ml.	to 1.99 ml.

Mix well, measure the optical density E_1. Start the reaction by mixing into both cuvettes

0.005 ml. HOADH solution (VIII) (*ca.* 100 units).

After about 3—4 min. read the optical density E_2 which should be constant.

Note: The DPNH concentration is so arranged that the optical density at 366 mμ lies on the most accurate range of the spectrophotometer. If measurements are made at 340 or 334 mμ then only 0.03 ml. DPNH solution is pipetted into the experimental cuvette. The

addition of DPNH to the control cuvette ensures that even when $E_2 = 0$ there is still DPNH present in the experimental cuvette. It is recommended that the amount of acetoacetyl-CoA be so arranged that the optical density difference $E_1 - E_2$ does not exceed 0.500.

Calculations

For measurements at 366 mμ with an assay volume of 2 ml. and 1 cm. light path it follows that:

$$0.607 \times \Delta E \times \frac{V}{v} = \mu\text{moles acetoacetyl-CoA in the whole sample.}$$

For measurements at 340 mμ:

$$0.322 \times \Delta E \times \frac{V}{v} = \mu\text{moles acetoacetyl-CoA in the whole sample.}$$

For measurements at 334 mμ:

$$0.341 \times \Delta E \times \frac{V}{v} = \mu\text{moles acetoacetyl-CoA in the whole sample}$$

where

V = volume of whole sample of acetoacetyl-CoA in ml.;
v = portion of the acetoacetyl-CoA sample taken for assay in ml.;
$\Delta E = E_1 - E_2$
ε = extinction coefficient for DPNH, the values [cm.²/μmole] are 6.22 at 340 mμ; 5.87 at 334 mμ; 3.3 at 366 mμ (see p. 27).

Example

Coenzyme A (10 μmoles) was converted to acetoacetyl-CoA by the diketene method[4]; volume of the whole sample was 2.5 ml. and 0.02 ml. were taken for assay. Wavelength 366 mμ; $E_1 = 0.774$: $E_2 = 0.638$: $\Delta E = 0.136$.

$$0.607 \times 0.136 \times \frac{2.5}{0.02} = 10.3\ \mu\text{moles acetoacetyl-CoA in the whole sample.}$$

The following values were obtained with 0.03 ml.: $E_1 = 0.782$; $E_2 = 0.582$; $\Delta E = 0.200$.

$$0.607 \times 0.200 \times \frac{2.5}{0.03} = 10.1\ \mu\text{moles acetoacetyl-CoA in the whole sample.}$$

Sources of Error

The purest preparations of β-hydroxyacyl dehydrogenase are practically free from β-ketoacylthiolase. If this is not the case then acetyl-CoA will react according to the over-all equilibrium of both reactions[4] and lead to high values. For the same reason acetoacetyl-CoA cannot be determined in the presence of free coenzyme A, pantetheine, pantetheine phosphate or *N*-acetylcysteamine, using an enzyme preparation containing thiolase. The *S*-acetoacetyl derivatives of these mercaptans and the higher homologues of acetoacetyl-CoA are reduced by β-hydroxyacyl dehydrogenase and DPNH (see "Specificity").

Specificity

β-Hydroxyacyl dehydrogenase does not show a very marked specificity with regard to either the mercaptan or β-ketoacyl grouping. As well as the CoA derivative of acetoacetic acid ($K_M = 0.4 \times 10^{-4}$ M), the thiol ester of pantetheine phosphate ($K_M = 2.2 \times 10^{-4}$ M), pantetheine ($K_M = 0.9 \times 10^{-4}$ M) or *N*-acetylcysteamine ($K_M = 90 \times 10^{-4}$ M) are also reduced by DPNH[4]. The K_M

[4] *K. Decker:* Die aktivierte Essigsäure. Ferd. Enke, Stuttgart 1959.

values are for the enzyme obtained from sheep liver; β-hydroxyacyl dehydrogenase from pig liver shows only insignificant differences with respect to the K_M values, but with the turnover number there are small differences which are nevertheless important.

It is commonly agreed that only one β-hydroxyacyl dehydrogenase exists; that is to say the enzyme reacts with the β-hydroxy or β-ketoacyl-CoA derivatives of every chain length up to about C_{20}. A differentiation of chain length is therefore not possible with this enzyme. Other carbonyl compounds, for example α-keto acid derivatives, aldehydes, ketones and β-keto acids not combined with CoA do not react. The reversible reduction of free acetoacetate to D-(−)-β-hydroxybutyric acid is catalysed by another enzyme[5] (see p. 454).

Other Methods for the Determination of Acetoacetyl Coenzyme A

Acetoacetyl CoA is split by β-ketoacylthiolase according to the equation:

(2) $$\text{Acetoacetyl-CoA} + \text{CoASH} \rightleftharpoons 2\ \text{acetyl-CoA}$$

The pH-dependent UV-absorption of acetoacetyl-CoA at 300 mμ, which can be increased by Mg^{2+}, may be used to measure the reaction[4,6]. Another possibility for measuring acetoacetyl-CoA is to combine reaction (2) with arylamine transacetylase[7] (see p. 424) or with the condensing enzyme[8,9] (see p. 448). The purification procedure for β-ketothiolase[8,10] has been shown to have poor reproducibility and therefore this enzyme is not recommended for the routine determination of acetoacetyl-CoA.

Acetoacetyl-CoA can be estimated relatively quickly and simply by a non-enzymic method dependent on its UV-absorption[4]. The hydroxamic acid and the nitroprusside reaction (refer to p. 424) are not applicable to β-ketoacyl-CoA derivatives.

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5) *A. L. Lehninger* and *G. D. Greville*, Biochim. biophysica Acta *12*, 188 [1953].
6) *J. R. Stern*, J. biol. Chemistry *221*, 33 [1956].
7) *F. Lynen*, *K. Decker*, *O. Wieland* and *D. Reinwein* in *G. Popják* and *E. LeBreton:* Biochemical Problems of Lipids. Butterworths, London 1956.
8) *D. S. Goldman*, J. biol. Chemistry *208*, 345 [1954].
9) *J. R. Stern*, *M. J. Coon* and *A. Del Campillo*, J. biol. Chemistry *221*, 1 [1956].
10) *J. R. Stern* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 581.