Coenzyme A

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Coenzyme A (CoA) participates in many important biochemical reactions[1], sometimes as the free form, but mainly as acyl-CoA. Some of these reactions are suitable for the determination of CoA. In the catalytic assays (refer to p. 7) the CoA is regenerated during the reaction and therefore the rate of the reaction is a measure of the CoA present[2-6]. These methods require a CoA standard preparation of known purity, which is not easily obtained. For this reason, methods of estimation in which a final value is obtained are generally preferred (refer to p. 4).

The methods described there do not give values which are in complete agreement, since their specificity is different.

I. The assay with β-hydroxyacyl-CoA dehydrogenase (HOADH) estimates CoA-SH, dephospho-CoA, pantetheine, *N*-(acetyl-β-alanyl)-cysteamine, *N*-acetylcysteamine and other compounds. The assay can be so arranged that the oxidized derivatives (R—S—S—R) can also be estimated.

II. The assay with phosphotransacetylase (PTA) only estimates CoA-SH. The rate of the reaction with dephospho-CoA is negligible.

III. The assay with thiokinase (TK) estimates CoA-SH and dephospho-CoA. Pantetheine does not react.

IV. The assay with the citrate cleavage enzyme (CCE) estimates only CoA-SH. The reaction with dephospho-CoA is eliminated by extrapolation.

The difference in the assay methods is seen clearly if the analytical results for a highly purified CoA preparation (A) and partially decomposed preparation (B) (due to long storage at raised temperature with access to moisture) are compared:

Assay	Preparation A		Preparation B	
	Total CoA	CoA-SH	Total CoA	CoA-SH
with HOADH	83.1%	78.8%	67%	64.6%
with PTA		77.7%		18.1%
with TK		79.0%		36.5%
with CCE		78.0%		17.8%

For preparation A all the values for CoA-SH are similar. From this it can be concluded that hardly any break-down products of CoA are present. With the partially decomposed preparation B there are large differences between the results. The values with PTA and CCE agree within the limits of accuracy of the methods, which is to be expected from the virtually similar specificity of the two methods. The lower specificity of the assays with TK and HOADH is stressed by the higher values obtained with these two methods.

In practice the PTA assay is preferred to the CCE assay because of the greater stability of the enzyme. However, too high a phosphate and sodium ion concentration in the sample (*e.g.* in tissue extracts) can interfere with the PTA assay (see p. 518 and 520). Both assays show that the amount of enzymatically active CoA in CoA preparations decreases relatively rapidly on storage.

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1) *F. Lynen* and *K. Decker*, Ergebn. Physiol., biol. Chem. u. exp. Pharmakol. *49*, 327 [1957].
2) *N. O. Kaplan* and *F. Lipmann*, J. biol. Chemistry *174*, 37 [1948].
3) *G. D. Novelli* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 916.
4) *F. Lipmann* and *L. C. Tuttle*, J. biol. Chemistry *159*, 21 [1945]; cf.[10].
5) *E. R. Stadtman* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 596.
6) *R. W. v. Korff*, J. biol. Chemistry *200*, 401 [1953].

In the TK assay the compound measured has a very high specific extinction. This is especially advantageous if very low concentrations have to be measured, for example, in tissues. Apart from CoA, only dephospho-CoA and possibly 4′-phosphopantetheine react.

The HOADH assay has the advantage that it allows the separate determination of CoA-SH, oxidized CoA and their derivatives. It estimates certain compounds, which can serve as precursors of CoA, and is also suitable as an assay for all potentially CoA-active compounds.

I. Determination of CoA-SH and CoA-S-S-CoA with β-Hydroxyacyl-CoA Dehydrogenase

Principle

β-Hydroxyacyl-CoA dehydrogenase (HOADH) catalyses the reduction of acetoacetyl-CoA to β-hydroxybutyryl-CoA with reduced diphosphopyridine nucleotide (DPNH)[7]:

(1) $$CH_3-CO-CH_2-CO-S-CoA + DPNH + H^+ \rightleftharpoons CH_3-CHOH-CH_2-CO-S-CoA + DPN^+$$

The equilibrium of the reaction at pH < 7.5 lies almost completely to the right, the equilibrium constant

(2) $$K = \frac{[CH_3-CHOH-CH_2-CO-S-CoA][DPN^+]}{[CH_3-CO-CH_2-CO-S-CoA][DPNH^+][H^+]} = 5.25 \times 10^9 \text{ [l./mole] at } 25^\circ C$$ [8].

The decrease of optical density at 340 or 366 mμ due to the oxidation of DPNH is measured.
To determine CoA-SH it is converted to acetoacetyl-CoA with diketene[9]:

(3) $$\begin{matrix} CH_2=C-O \\ \quad\quad | \quad | \\ \quad H_2C-C=O \end{matrix} + \text{CoA-SH} \longrightarrow CH_3-CO-CH_2-CO-S-CoA$$

and the product is estimated with HOADH according to equation (1).

To determine CoA−S−S−CoA, it is first reduced with thioglycollic acid[10] at pH 9 and the resulting CoA-SH is then determined as already described. The reduction is only quantitative with a large excess of thioglycollic acid.

(4) $$R-SH + CoA-S-S-CoA \rightleftharpoons R-S-S-CoA + CoA\text{-}SH$$

(5) $$R-S-S-CoA + R-SH \rightleftharpoons R-S-S-R + CoA\text{-}SH$$

In a mixture of CoA-SH and CoA−S−S−CoA, the total amount of CoA can be determined after reduction of the sample, while the amount of CoA-SH can be obtained in a second assay in which the reduction step is omitted. The difference gives the amount of CoA−S−S−CoA.

Reagents

1. Sodium pyrophosphate, A. R., $Na_4P_2O_7 \cdot 10H_2O$
2. Hydrochloric acid, A. R.; sp. gr. 1.19; *ca.* 36% (w/w)
3. Diketene *), b. p. 68°C/92 mm.
   The preparation becomes yellow on storage. It should be distilled, stored in a refrigerator and used within 14 days.

*) *e.g.* from Dr. Th. Schuchardt, Munich, Germany.

7) *F. Lynen*, Fed. Proc. *12*, 683 [1953].
8) *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.
9) *K. Decker* and *F. Lynen*, 3rd Congr. Intern. Biochim., Commun. 36, Brussels 1955.
10) *K. Decker:* Die aktivierte Essigsäure. F. Enke, Stuttgart 1959.

4. Thioglycollic acid, about 80% pure
5. Potassium hydroxide, A. R.
6. Reduced diphosphopyridine nucleotide, DPNH
   sodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
7. Sodium hydrogen carbonate, *ca.* 1% (w/v)
8. β-Hydroxyacyl-CoA dehydrogenase, HOADH
   crystalline suspension in 2.8 M ammonium sulphate (pH 6.0). Commercial preparation, see p. 984.

Purity of the enzyme preparation

The enzyme preparation should have a specific activity of at least 5 units*)/mg. and should contain $< 1\%$ malic dehydrogenase or lactic dehydrogenase (relative to the specific activity of the HOADH). Crotonase, butyryl-CoA dehydrogenase and deacylases should not be detectable in the HOADH preparation.

Preparation of Solutions

I. Hydrochloric acid (*ca.* 1 N):
   Dilute 83 ml. HCl (sp. gr. 1.19) to 1000 ml. with doubly distilled water.

II. Pyrophosphate buffer (0.1 M; pH 7.3):
   Dissolve 44.61 g. $Na_4P_2O_7 \cdot 10\,H_2O$ in *ca.* 750 ml. doubly distilled water, add about 82 ml. 1 N HCl, check the pH with a glass electrode and dilute to 1000 ml. with doubly distilled water.

III. Potassium hydroxide (2 N):
   Dissolve 11.22 g. KOH in doubly distilled water and make up to 100 ml.

IV. Reduced diphosphopyridine nucleotide (*ca.* 0.013 M β-DPNH):
   Dissolve 10.0 mg. DPNH-Na_2 in 1 ml. 1% (w/v) $NaHCO_3$ solution.

V. β-Hydroxyacyl-CoA dehydrogenase, HOADH (5 mg. protein/ml.):
   If necessary, dilute the stock suspension with 2.8 M ammonium sulphate solution (pH 6).

Stability of the solutions

The buffer solution (II) is stable at room temperature; growth of micro-organisms can be retarded by storage in a refrigerator. Store the DPNH solution in a refrigerator and prepare freshly every two days. The HOADH suspension is stable for several months at 0 to 4°C.

Procedure

Experimental material

CoA solutions of any purity can be analysed. Adjust acid solutions (*e.g.* after deproteinization of tissue samples with perchloric acid, see p. 254) to pH 4 to 6 by the addition of 5 M K_2CO_3 with constant stirring. Avoid adding an excess of K_2CO_3 since CoA is unstable in alkaline solution.

Preliminary treatment of sample

a) For the determination of total CoA:
Dissolve the CoA preparation in doubly distilled water to give about 1 mg./ml. or dilute CoA solutions accordingly. Cool 10 ml. of this solution in a test tube to 0°C, add

0.01 ml. thioglycollic acid

*) according to *E. Racker et al.*, Arch. Biochem. Biophysics *74*, 306 [1958]; definition: μmoles substrate/min.

and adjust to pH 9.0 (indicator paper or glass electrode) with

ca. 0.2 ml. 2 N KOH.

Allow to stand for 15 min. in an ice bath. Add

0.01 ml. diketene

and mix vigorously for 3 min. at 0°C. The drops of diketene dissolve during this period. Check the pH every minute and maintain at 7.4 (indicator paper or glass electrode).

b) For the determination of CoA-SH:
Dissolve CoA preparations in doubly distilled water to give about 1 mg./ml., or dilute CoA solutions accordingly. Cool 10 ml. of this solution in a test tube to 0°C and adjust to pH 8.0 (indicator paper or glass electrode) with

ca. 0.15 ml. 2 N KOH.

Add

0.01 ml. diketene,

mix for 3 min. at 0°C and maintain the pH at 7.4 during the mixing.

Spectrophotometric measurements

Wavelength: 340 or 366 mμ; light path: 1 cm.; final volume: 3.0 ml.; room temperature. Measure against a cuvette containing water.

Pipette successively into the cuvette:

2.84 ml. buffer solution (II)
0.10 ml. sample after preliminary treatment
0.05 ml. DPNH solution (IV).

Mix with a thin stirring rod and read the optical density E_0. Mix in

0.01 ml. HOADH suspension (V)

and follow the decrease in optical density until no further change occurs (2 to 3 min.). Read the optical density E_1. To measure the small absorption due to the HOADH, again add

0.01 ml. HOADH suspension (V)

and read the optical density E_2. $E_2 - E_1 = \Delta E_{HOADH}$. The value $\Delta E = E_0 - E_1 + \Delta E_{HOADH}$ is used for the calculations.

Calculations

According to p. 37

$$\frac{\Delta E \times V}{\varepsilon \times d \times v} = \mu\text{moles CoA/ml. sample}$$

where

ΔE = optical density difference
V = volume of the assay mixture [ml.]
v = volume of sample in the assay mixture [ml.]
d = light path of the cuvette [cm.]
ε = extinction coefficient of DPNH (for 340 mμ: 6.22 cm².$/\mu$mole; for 366 mμ: 3.30 cm.²$/\mu$mole).

Therefore at 340 mμ:

$$\frac{\Delta E \times 3}{6.22 \times 0.1} = \Delta E \times 4.823 = \mu\text{moles CoA-SH/ml. treated sample}$$

at 366 mμ:

$$\frac{\Delta E \times 3}{3.30 \times 0.1} = \Delta E \times 9.10 = \mu\text{moles CoA-SH/ml. treated sample}$$

To convert to μg. CoA-SH the results must be multiplied by the molecular weight of CoA-SH (767.6). To obtain the concentration of CoA in the untreated sample it is necessary to multiply by the dilution factor due to the addition of diketene, thioglycollic acid and KOH.

Example

A commercial CoA preparation (9.85 mg.) was dissolved in distilled water and made up to 10 ml. After preliminary treatment for the determination of total CoA (a) and CoA-SH (b) the volumes of the solutions were:

a) 10.30 ml. b) 10.20 ml.

The following optical densities were measured at 366 mμ:

	a) (Total CoA)	*b)* (CoA-SH)
E_0	0.300	0.300
E_1	0.202	0.204
E_2	0.207	0.209
	$\Delta E_{HOADH} = E_2 - E_1 = 0.005$	
$\Delta E = E_0 - E_1 + \Delta E_{HOADH}$	= 0.103	0.101

The total CoA: $0.103 \times 9.10 \times 767 = 718$ μg./ml. treated sample

CoA-SH: $0.101 \times 9.10 \times 767 = 704$ μg./ml. treated sample

The dilution factors due to the additions in the preliminary treatment of the samples were:

a) 10.30/10 = 1.03 b) 10.20/10 = 1.02

The untreated sample therefore contained:

Total CoA:	740 μg./ml.
CoA-SH:	718 μg./ml.
Difference (CoA—S—S—CoA):	22 μg./ml.

As the sample solution contained 985 μg./ml., the total CoA content of the sample was:

$$\frac{740 \times 100}{985} = 75.1\,\%$$

Specificity and Sources of Error

For the enzyme preparation obtained from pig heart *Decker*[10] gives the following turnover numbers *): for the acetoacetyl derivatives of CoA 20900, dephospho-CoA 13200, pantetheine 11700, *N*-(acetyl-β-alanyl)-cysteamine 4630 and *N*-acetylcysteamine 3900. Similar turnover numbers were measured for several synthetic model substances (2′-deoxy-bisnorpanthetheine derivatives). Therefore in the assay with HOADH several of the break-down products of CoA are estimated.

*) Moles of substrate reacting/min./100000 g. protein.

II. Determination of CoA-SH with Phosphotransacetylase

In the presence of arsenate, CoA has been estimated with phosphotransacetylase (PTA) in a catalytic assay[1]. The determination of CoA with this enzyme by the end-point method (see p. 4) was developed by *E. R. Stadtman*[2].

Principle

The enzyme PTA catalyses the reversible transfer of acyl groups between phosphate and CoA[1,3]:

(1) $$CoA\text{-}SH + CH_3CO{-}OPO_3H_2 \rightleftharpoons CoA\text{-}S{-}COCH_3 + H_3PO_4$$

Acetyl phosphate reacts with CoA with the formation of acetyl-CoA and free phosphoric acid. The equilibrium lies to the right at pH 8.0 and 28°C[4]:

(2) $$K' = \frac{[Ac-CoA]\,[phosphate]}{[acetyl\ phosphate]\,[CoA]} = 74.$$

With an excess of acetyl phosphate the reaction is practically quantitative. Acetyl-CoA absorbs more strongly at 233 mμ than CoA[5]. As *W. Seubert*[2a] has shown with the model compound *S*-acetyl-*N*-succinylcysteamine, the extinction difference at 233 mμ is $\Delta\varepsilon_{233} = 4.44 \times 10^6$ cm.²/mole. The increase in optical density at 233 mμ is measured. Only CoA-SH is estimated, since no reducing reagent is added.

Reagents

1. Hydrochloric acid, A. R.; sp. gr. 1.19; *ca.* 36% (w/w)
2. Tris-hydroxymethyl-aminomethane, tris
3. Acetyl phosphate, Li_2 salt, $C_2H_3O_4PLi_2$ *)
4. Phosphotransacetylase, PTA
   from *Cl. kluyveri*; crystalline suspension in 3.0 M ammonium sulphate solution (pH 6.0); commercial preparation, see p. 996.

   Purity of the enzyme preparation

   The enzyme preparation should have a specific activity of at least 450 units**)/mg. Deacylases, thiolase and phosphatases should not be detectable in the preparation. The activity with dephospho-CoA as substrate should not exceed 1.0% of the activity with CoA.

Preparation of Solutions

I. Hydrochloric acid (*ca.* 1 N):
Dilute 83 ml. HCl (sp. gr. 1.19) to 1000 ml. with doubly distilled water.

II. Tris buffer (0.1 M; pH 7.6):
Dissolve 12.1 g. tris-hydroxymethyl-aminomethane in *ca.* 500 ml. doubly distilled water, adjust to pH 7.6 (glass electrode) with about 70 ml. 1 N HCl (solution I) and dilute to 1000 ml. with doubly distilled water.

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*) *e.g.* from Serva-Entwicklungslabor, Heidelberg, Germany.

**) according to *E. Racker et al.*, Arch. Biochem. Biophysics *74*, 306 [1958]; definition: μmoles substrate/min.

1) *E. R. Stadtman, G. D. Novelli* and *F. Lipmann*, J. biol. Chemistry *191*, 365 [1951].

2) *E. R. Stadtman*, unpublished.

2a) *W. Seubert*, personal communication.

3) *H. Chantrenne* and *F. Lipmann*, J. biol. Chemistry *187*, 757 [1950].

4) *E. R. Stadtman* in *S. P. Colowick*, and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 596.

5) *E. R. Stadtman*, J. cell. comp. Physiol. *41*, 89 [1953].

III. Acetyl phosphate (0.1 M):
Dissolve 15.2 mg. acetyl phosphate, Li_2 salt, in doubly distilled water and make up to 1 ml.

IV. Phosphotransacetylase, PTA (0.1 mg. protein/ml.):
If necessary, adjust the enzyme solution to a concentration of 1 mg. protein/ml. by dilution with 3.0 M ammonium sulphate solution (pH 6.0). Dilute the required amount of this solution freshly each day by addition of 9 volumes of 3.0 M ammonium sulphate solution (pH 6.0).

Stability of the solutions

The tris buffer (solution II) for this determination must be stored in a refrigerator. Decant the required amount each day. It can be left at room temperature during the day. The acetyl phosphate solution (III) must be kept cool during the analysis and should be prepared freshly every 2 days. The enzyme suspension is stable for months at 0 to 4°C in a concentration of 1 mg. protein/ml.

Procedure

Experimental material

Solutions of any purity can be analysed, providing that they allow sufficient light to be transmitted at 233 mμ and their inorganic phosphate and, more especially, Na^+ content is low (concentrations in the assay mixture: $< 10^{-4}$ M HPO_4^{2-}, $< 10^{-2}$ M Na^+). Crude tissue extracts are therefore usually not analysed with PTA. Also samples with a high acetyl-CoA content (*e.g.* yeast extracts) should not be assayed with PTA because the reaction does not go to completion. In such cases, use the CCE or TK assay.

Dissolve the CoA preparation in doubly distilled water to give *ca.* 1 mg./ml. or dilute CoA solutions accordingly.

Spectrophotometric measurements

Wavelength: 233 mμ; light path: 1 cm. (silica cuvettes); final volume: 3.0 ml.; room temperature. Measure against a cuvette containing tris buffer (solution II). If the sample is strongly coloured, add 0.2 ml. sample to this blank cuvette.
Pipette successively into the cuvette:

2.69 ml. tris buffer (solution II)
0.10 ml. acetyl phosphate solution (III)
0.20 ml. sample.

Mix with a thin stirring rod and read the optical density E_0. Mix in

0.01 ml. PTA suspension (IV),

follow the increase in optical density until the end of the reaction (3 to 5 min.) and read the optical density E_1. To measure the absorption of the enzyme, once again mix in

0.01 ml. PTA suspension (IV)

and read the optical density E_2. $E_2 - E_1 = \Delta E_{PTA}$ lies between 0 and 0.010. The value $\Delta E = E_1 - E_0 - \Delta E_{PTA}$ is used for the calculations.

Calculations

According to the general equation on p. 37:

$$\frac{\Delta E \times V}{\Delta \varepsilon \times d \times v} = \mu\text{moles CoA/ml. sample}$$

where

ΔE = optical density difference
V = volume of the assay mixture [ml.]
v = volume of sample taken for the assay [ml.]
d = light path of the cuvette [cm.]
$\Delta \varepsilon$ = coefficient of the difference in extinction between CoA and acetyl-CoA (4.44 cm.^{2}/μmole at 233 mμ).

The optical density difference corrected for the absorption due to the enzyme $\Delta E = E_1 - E_0 - \Delta E_{PTA}$ is inserted in the above formula:

$$\frac{\Delta E \times 3}{4.44 \times 0.2} = \Delta E \times 3.38 = \mu\text{moles CoA-SH/ml. of the solution analysed.}$$

To convert the results to μg. CoA-SH/ml. it is necessary to multiply by the molecular weight of CoA-SH (767.6).

Example

A CoA preparation (9.86 mg.) was dissolved in distilled water and made up to 10 ml. The following optical densities were measured: $E_0 = 0.236$; E_1 (after 4 min.) $= 0.543$; $E_2 = 0.552$; $\Delta E_{PTA} = 0.009$. Therefore $\Delta E = E_1 - E_0 - \Delta E_{PTA} = 0.298$.
$0.298 \times 3.38 \times 767 = 774$ μg. CoA-SH/ml. sample.

Taking into account the weight of sample, the CoA-SH content of the preparation is 78.6%.

Specificity and Sources of Error

PTA from *Cl. kluyveri* is specific for CoA-SH. In the absence of reducing substances PTA does not react with oxidized CoA[6], nor with deamino-CoA (*N. O. Kaplan*, cited in [4]). According to[7] it also does not react with dephosphorylated derivatives of CoA, such as dephospho-CoA. However, we have observed[6] with our highly purified and crystalline enzyme[8] (specific activity 1 200 units/mg.) a clearly perceptible reaction with dephospho-CoA, when large amounts of enzyme are used (0.01 mg./assay mixture). This has already been reported with less purified enzyme preparations[8a]. Nevertheless, with the use of the small amounts of enzyme described under "Spectrophotometric measurements" this interference can be disregarded. It is probable that the slight activity with dephospho-CoA is due to the enzyme not being completely specific, rather than to the presence of a contaminant, since during the purification of the enzyme the relative activity with dephospho-CoA does not decrease.

Preparations of the enzyme from other micro-organisms can also react with pantetheine[9].

The presence of large amounts of glutathione (GSH) in the assay mixture interferes because of a non-enzymatic transfer of acyl groups between acetyl-CoA and GSH[10]. At pH 7.6 (as in the assay described here) this reaction does not proceed very rapidly[6]. According to[10] the rate is optimal at

6) *G. Michal*, unpublished.
7) *T. P. Wang*, *L. Shuster* and *N. O. Kaplan*, J. Amer. chem. Soc. *74*, 3204 [1952].
8) *H. U. Bergmeyer*, *H. Klotzsch* and *G. Lang*, Angew. Chem. *72*, 807 [1961].
8a) *T. P. Wang* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. II, p. 649.
9) *G. M. Brown*, Fed. Proc. *16*, 159 [1957].
10) *E. R. Stadtman*, J. biol. Chemistry *196*, 535 [1952].

pH 8.1 to 9.0, while at pH < 7.0 the rate is practically zero. Even when glutathione is added to the assay mixture in the form of impure CoA solutions or tissues extracts, the error due to this non-enzymatic reaction can be disregarded because of the short reaction times.

The enzyme requires K^+ or NH_4^+ ions for full activity[11]; usually the ammonium sulphate content of the enzyme suspension is sufficient. Na^+ or Li^+ ions inhibit, but in the presence of NH_4 ions this effect is reduced[11]. The Li^+ content of the acetyl phosphate solution does not interfere to any significant extent.

High concentrations of inorganic phosphate and acetyl-CoA in the assay mixture lead to low values[10]. The extent of this interference can be estimated from the position of the equilibrium (see p. 517) and presumably can be reduced by increasing the acetyl phosphate concentration.

III. Determination of CoA-SH with Thiokinase

Principle

Thiokinase (TK) from beef liver[1] (the so-called "fatty acid activating enzyme" of *Mahler et al.*) catalyses the formation of acyl-CoA:

(I) $$R-COO^- + ATP^{4-} + CoA\text{-}SH \rightleftharpoons R-CO-S-CoA + AMP^{2-} + HP_2O_7^{3-}$$

Saturated fatty acids with a chain length of from C_3 to C_{14} can be activated; the unsaturated derivatives react at practically the same rate. Substituted acids react more slowly. The equilibrium constant of the reaction[1], measured with oenanthic acid, is

$$K' = \frac{[AMP^{2-}]\,[HP_2O_7^{3-}]\,[RCO-S-CoA]}{[ATP^{4-}]\,[CoA-SH]\,[RCOO^-]} = 1.11 \text{ to } 1.48 \text{ at pH 8 and } 38°C.$$

With an excess of ATP and $RCOO^-$ the reaction proceeds virtually quantitatively from left to right. *Wakil* and *Hübscher*[2] used the reaction with sorbic acid to determine CoA by an end-point method. Sorbyl-CoA has a strong absorption peak at *ca.* 300 mμ due to conjugated chromophore groups, and this makes it suitable for measurements of the activation reaction[2]. *Wakil* and *Hübscher* found an extinction coefficient for sorbyl-CoA of $\varepsilon_{300} = 19 \times 10^6$ cm.²/mole. According to our measurements this value is too low[3]. With *S*-sorbyl-*N*-acetylcysteamine as a model substance, we found that $\varepsilon_{300} = 23.53 \times 10^6$ cm.²/mole at pH 7.0 and 27°C (after correction for the individual absorption of the components).

The increase of optical density at 300 mμ is measured. As the assay mixture contains no reducing reagents only CoA-SH is estimated.

Reagents

1. Hydrochloric acid, A. R.; sp. gr. 1.19; *ca.* 36% (w/w)
2. Sodium hydroxide, A. R.
3. Tris-hydroxymethyl-aminomethane, tris
4. Magnesium chloride, A. R., $MgCl_2 \cdot 6\,H_2O$
5. Sorbic acid, $CH_3-CH=CH-CH=CH-COOH$
6. Adenosine triphosphate, ATP
   crystalline disodium salt, ATP-$Na_2H_2 \cdot 3\,H_2O$; commercial preparation, see p. 1006.

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[11] *E. R. Stadtman*, J. biol. Chemistry *196*, 527 [1952].
[1] *H. R. Mahler, S. J. Wakil* and *R. M. Bock*, J. biol. Chemistry *204*, 453 [1953].
[2] *S. J. Wakil* and *G. Hübscher*, J. biol. Chemistry *235*, 1554 [1960].
[3] *G. Michal* and *H.-U. Bergmeyer*, Biochim. Biophysica Acta *67*, 599 [1963].

7. Thiokinase, TK
from beef liver [1], 20 mg. protein/ml. Suspension in 2.0 M ammonium sulphate solution (pH 6.0). Preparation, see Appendix, p. 523.

Purity of the enzyme preparation
The enzyme preparation should have a specific activity of at least 0.4 units *)/mg. It should be free from deacylases, crotonase and phosphatases.

Preparation of Solutions

I. Hydrochloric acid (*ca.* 1 N):
Dilute 83 ml. HCl (sp. gr. 1.19) to 1000 ml. with doubly distilled water.

II. Sodium hydroxide (*ca.* 1 N):
Dissolve 40 g. NaOH in doubly distilled water and make up to 1000 ml.

III. Tris buffer (0.1 M; pH 7.6):
Dissolve 12.1 g. tris-hydroxymethyl-aminomethane in *ca.* 500 ml. doubly distilled water, adjust to pH 7.6 (glass electrode) with *ca.* 70 ml. 1 N HCl (solution I) and dilute to 1000 ml. with doubly distilled water.

IV. Magnesium chloride (0.1 M):
Dissolve 203 mg. $MgCl_2 \cdot 6\ H_2O$ in doubly distilled water and make up to 10 ml.

V. Na sorbate (0.1 M):
Add 1 ml. 1 N NaOH (solution II) to 112 mg. sorbic acid and dilute to 10 ml. with doubly distilled water. If necessary, filter.

VI. Adenosine triphosphate (0.1 M ATP):
Dissolve 60.5 mg. ATP-$Na_2H_2 \cdot 3\ H_2O$ in doubly distilled water and make up to 1 ml.

VII. Thiokinase, TK (20 mg. protein/ml.):
Dilute the stock suspension with 2.0 M ammonium sulphate solution (pH 6.0).

Stability of the solutions
Store the tris buffer (solution III) in a refrigerator and decant the daily requirement of this buffer. It can remain at room temperature during the day.
Keep solutions IV to VII cool, even during the working period. Prepare the sorbate and ATP solutions (V and VI) freshly every two days. The enzyme suspension (VII) is stable for several weeks at 0 to 4°C.

Procedure

Experimental material
Solutions of any purity can be analysed. Adjust acid solutions (*e.g.* tissue samples after deproteinization with perchloric acid, see p. 254) to pH 4 to 6 by addition of 5 M K_2CO_3 solution with thorough stirring. An alkaline pH is to be avoided, since the CoA is unstable in alkali. Weigh out CoA preparations to give a solution containing *ca.* 1 mg./ml. or dilute CoA solutions accordingly.

Spectrophotometric measurements
Wavelength: 300 mμ; light path: 1 cm. (silica cuvettes); final volume: 3.0 ml.; room temperature. Measure against a cuvette containing 2.70 ml. tris buffer (solution III) + 0.30 ml. Na sorbate (solution V). If the sample is strongly coloured, add 0.05 ml. sample to the blank cuvette.

*) according to *E. Racker et al.*, Arch. Biochem. Biophysics *74*, 306 [1958]; definition: μmoles substrate/min.

Pipette successively into the cuvette:

2.36 ml. tris buffer (solution III)
0.10 ml. $MgCl_2$ solution (IV)
0.30 ml. Na sorbate solution (V)
0.15 ml. ATP solution (VI)
0.05 ml. sample.

Mix with a thin stirring rod and read the optical density E_0. Mix in

0.04 ml. TK suspension (VII),

follow the increase in optical density until the reaction is complete (20 to 30 min.) and read the optical density E_1. To measure the absorption due to the enzyme, again mix in

0.04 ml. TK suspension (VII)

and read the optical density E_2. $E_2 - E_1 = \Delta E_{TK}$ lies between 0.040 and 0.070. The value $\Delta E = E_1 - E_0 - \Delta E_{TK}$ is used for the calculations.

Calculations

According to the general equation on p. 37:

$$\frac{\Delta E \times V}{\varepsilon \times d \times v} = \mu\text{moles CoA/ml. sample}$$

where

ΔE = optical density difference
V = volume of the assay mixture [ml.]
v = volume of sample taken for the assay [ml.]
d = light path of the cuvette [cm.]
ε = specific extinction of sorbyl-CoA (23.53×10^6 cm.^{2}/μmole at 300 mμ).

By insertion of the optical density difference corrected for the absorption due to the enzyme $\Delta E = E_1 - E_0 - \Delta E_{TK}$

$$\frac{\Delta E \times 3.0}{23.53 \times 0.05} = \Delta E \times 2.55 = \mu\text{moles CoA-SH/ml. of the solution analysed.}$$

To convert to μg. CoA-SH/ml. solution, it is necessary to multiply by the molecular weight of CoA-SH (767.6).

Example

A CoA preparation (8.00 mg.) was dissolved in doubly distilled water and made up to 10 ml. The following optical densities were measured: $E_0 = 0.007$; $E_1 = 0.411$; $E_2 = 0.475$. Therefore $E_1 - E_0 = 0.404$; $\Delta E_{TK} = 0.064$ and $\Delta E = 0.340$.

The sample therefore contains $0.340 \times 2.55 \times 767 = 664$ μg. CoA-SH/ml. The CoA-SH content of the preparation is $\frac{664}{800} \times 100 = 83\%$.

Specificity and Sources of Error

The method is specific for CoA-SH. In contrast to the original description [2], we have omitted glutathione (GSH) from the assay mixture[3], since the glutathione accepts acyl groups from sorbyl-CoA non-enzymatically, so that more acyl thioester is formed than corresponds to the amount of CoA added (refer to[4]). The pH has been reduced to 7.6, so therefore the GSH content of the sample does not interfere (the non-enzymatic formation of acyl-GSH is very slow[4] at pH values below 8). In contrast to pH 9, the final optical density at pH 7.6 is constant. Oxidized CoA does not react.

[4] *E. R. Stadtman*, J. biol. Chemistry *196*, 535 [1952].

According to[1] the enzyme does not react directly with GSH, synthetic DL-thiotic acid, cysteine or pantetheine. However, we found[5] that dephospho-CoA reacts at a similar rate to CoA-SH. It is therefore not possible to estimate these two compounds separately with the assay described here. The concentrations of ATP, sorbate and Mg^{2+} must not be lower than those stated, otherwise the reaction proceeds too slowly.

Appendix

Purification of thiokinase[1]

Homogenize beef liver in 8.5% sucrose solution (containing 0.5% KH_2PO_4), maintaining the pH at 7.0 with 6 N KOH. Centrifuge (2300 g), filter the supernatant through cheese cloth and stir into 0.9% KCl solution. Centrifuge (15000 g), suspend the precipitate (mitochondria) in 0.9% KCl, and stir into 10 volumes of acetone at −15°C. Suspend the dried precipitate in 0.02 M K_2HPO_4 solution and stir vigorously for 1 hour at 0°C. Centrifuge (2300 g), add 20 g. $(NH_4)_2SO_4$/100 ml. supernatant and discard the precipitate. Add a further 15 g. $(NH_4)_2SO_4$/100 ml. supernatant, centrifuge off the precipitate and dissolve in 0.02 M $KHCO_3$ solution (15 mg. protein/ml.). Fractionate the solution with $(NH_4)_2SO_4$: discard the protein precipitating between 0 and 20 g. $(NH_4)_2SO_4$/100 ml. Collect the protein precipitating between 20 and 24 g. $(NH_4)_2SO_4$/100 ml. (fraction A), 24 and 28 g. (fraction B) and 28 and 31 g. (fraction C). Combine fractions A and C and re-fractionate as already described. Combine the protein precipitating between 24 and 28 g. $(NH_4)_2SO_4$/100 ml., dissolve in 0.02 M $KHCO_3$ solution and dialyse against 0.02 M $KHCO_3$ solution. Stir in 8 mg. Cγ-alumina gel/10 mg. protein and centrifuge immediately. Add 35 g. $(NH_4)_2SO_4$/100 ml. supernatant and adjust to pH 7.0 with NH_4OH. Collect the precipitate and dissolve in 0.05 M $KHCO_3$ solution, dialyse against 0.05 M $KHCO_3$ solution and then fractionate with saturated $(NH_4)_2SO_4$ solution (pH 8.0). Collect four or five fractions between 30 and 50% saturation, combine the most active fractions and suspend in half-saturated $(NH_4)_2SO_4$ solution.

The enzyme is then about 12-fold purified. The activity is determined by the decrease of CoA-SH, which is measured by SH estimation with Na nitroprusside[1] or better still by an assay based on the CoA determination described above[6]. The activity of the enzyme increases with increasing alkalinity between pH 7.0 and 10.5[1].

IV. Determination of CoA-SH with the Citrate Cleavage Enzyme

The citrate cleavage enzyme (CCE) was discovered by *Srere* and *Lipmann*[1] and further studied by *Srere*[2], who described an optical assay for measurement of the activity of the enzyme[2]. The determination of CoA with this enzyme is based on the same principle.

Principle

The enzyme CCE catalyses the cleavage of citrate which requires CoA and ATP:

(1) Citrate + ATP + CoA-SH ⇌ acetyl-S-CoA + oxaloacetate + ADP + H_3PO_4

This reaction clearly differs from that catalysed by the condensing enzyme; it is possible to effect a considerable separation of the two enzymes[2].

The position of the equilibrium of reaction (1) has not yet been measured. Ignoring the influence of the ratio ATP/ADP the formation of citrate is strongly exergonic (*e.g.* with the condensing enzyme, *Stern, Ochoa* and *Lynen*[3] give the $\Delta F'$ as −7.72 kcal. at pH 7.2 and 22°C). It is assumed that the

[5] *G. Michal*, unpublished.
[6] *W. Seubert*, personal communication.

[1] *P. A. Srere* and *F. Lipmann*, J. Amer. chem. Soc. *75*, 4874 [1953].
[2] *P. A. Srere*, J. biol. Chemistry *234*, 2544 [1959].
[3] *J. R. Stern, S. Ochoa* and *F. Lynen*, J. biol. Chemistry *198*, 313 [1952].

energy liberated on hydrolysis of ATP is sufficient to compensate for this amount of energy. In order to follow reaction (1) spectrophotometrically it is coupled with a DPNH-dependent reaction:

(2) $$\text{Oxaloacetate} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{malate} + \text{DPN}^+$$

The equilibrium constant for this reaction[3-5]

$$K' = \frac{[\text{oxaloacetate}]\,[\text{DPNH}]}{[\text{L-malate}]\,[\text{DPN}^+]} = 2.33 \times 10^{-5} \text{ at pH 7.2 and 22°C.}$$

The oxaloacetate formed in reaction (1) is continually removed, thereby ensuring a quantitative conversion in the direction of citrate cleavage. The decrease in optical density at 340 or 366 mμ due to the oxidation of DPNH is a measure of the over-all reaction.

As the assay mixture contains no reducing substances (apart from DPNH) only CoA is estimated.

Reagents

1. Hydrochloric acid, A. R.; sp. gr. 1.19; *ca.* 36% (w/w)
2. Tris-hydroxymethyl-aminomethane, tris
3. Magnesium chloride, A. R., $MgCl_2 \cdot 6\,H_2O$
4. Sodium citrate, A. R., $Na_3C_6H_5O_7 \cdot 5^1/_2\,H_2O$
5. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
6. Sodium hydrogen carbonate, *ca.* 1% (w/v) solution
7. Malic dehydrogenase, MDH
   crystalline suspension in 2.8 M ammonium sulphate solution (pH 6.0); commercial preparation, see p. 988.
8. Adenosine triphosphate, ATP
   crystalline disodium salt, ATP-$Na_2H_2 \cdot 3\,H_2O$; commercial preparation, see p. 1006.
9. Citrate cleavage enzyme, CCE
   from chicken liver[2]; for preparation, see the Appendix, p. 527.

Purity of the enzyme preparations

The CCE preparation should have a specific activity of at least 1.5 units *)/mg. It should be free from DPNH oxidase and phosphatases. The MDH should have a specific activity of at least 300 units *)/mg. (measured in 0.1 M phosphate buffer, see p. 988). The preparation should contain $< 0.01\%$ LDH and DPNH oxidase (relative to the MDH activity). Phosphatases should not be detectable.

Preparation of Solutions

I. Hydrochloric acid (*ca.* 1 N):
Dilute 83 ml. HCl (sp. gr. 1.19) to 1000 ml. with doubly distilled water.

II. Tris buffer (0.1 M; pH 7.6):
Dissolve 12.1 g. tris-hydroxymethyl-aminomethane in *ca.* 500 ml. doubly distilled water, adjust to pH 7.6 (glass electrode) with *ca.* 70 ml. 1 N HCl and dilute to 1000 ml. with doubly distilled water.

III. Magnesium chloride (0.1 M):
Dissolve 203 mg. $MgCl_2 \cdot 6\,H_2O$ with doubly distilled water and make up to 10 ml.

*) according to *E. Racker et al.*, Arch. Biochem. Biophysics *74*, 306 [1958]; defintion: μmoles substrate/min.

4) *F. Schlenk, H. Hellström* and *H. von Euler*, Ber. dtsch. chem. Ges. *71*, 1471 [1938].

5) *K. Burton* and *T. H. Wilson*, Biochem. J. *54*, 86 [1953].

IV. Sodium citrate (*ca.* 0.28 M):
Dissolve 1.0 g. $Na_3C_6H_5O_7 \cdot 5^1/_2\ H_2O$ in doubly distilled water and make up to 10 ml.
V. Reduced diphosphopyridine nucleotide (*ca.* 0.013 M β-DPNH):
Dissolve 10.0 mg. DPNH-Na_2 in 1% $NaHCO_3$ solution and make up to 1 ml.
VI. Malic dehydrogenase, MDH (10 mg. protein/ml.):
Dilute the stock suspension with 2.8 M ammonium sulphate solution (pH 6.0).
VII. Adenosine triphosphate (0.1 M ATP):
Dissolve 60.5 mg. ATP-$Na_2H_2 \cdot 3\ H_2O$ in distilled water and make up to 1 ml.
VIII. Citrate cleavage enzyme (20 mg. protein/ml.):
Dilute the stock solution with 0.1 M citrate buffer (pH 7.0).

Stability of the solutions

Store the tris buffer (solution II) in a refrigerator and only decant the daily requirement of this buffer. It can be left at room temperature during the day.
The solutions III to VII should be kept cold, even during the assay. Prepare the DPNH and ATP solutions (V and VII) freshly every 2 days. The MDH solution (VI) is stable at 0 to 4° C for several months. The CCE solution (VIII) is usable for not longer than 1 week.

Procedure

Experimental material

Solutions of any purity can be analysed. Adjust acid solutions (*e.g.* after deproteinization with perchloric acid, see p. 254) to pH 4 to 6 by addition of 5 M K_2CO_3 solution with efficient stirring. Alkaline pH is to be avoided, since the CoA is unstable in alkali. Weigh out CoA preparations so as to give a solution containing *ca.* 1 mg/ml. or dilute CoA solutions accordingly.

Spectrophotometric measurements

Wavelength: 340 or 366 mμ; light path: 1 cm.; final volume: 3.005 ml.; room temperature. Measure against the control cuvette.
Pipette successively into the cuvettes:

	Experimental	*Control*
tris buffer (solution II)	2.02 ml.	2.22 ml.
$MgCl_2$ solution (III)	0.30 ml.	0.30 ml.
sodium citrate solution (IV)	0.20 ml.	0.20 ml.
DPNH solution (V)	0.06 ml.	0.06 ml.
MDH solution (VI)	0.005 ml.	0.005 ml.
ATP solution (VII)	0.20 ml.	0.20 ml.
sample	0.20 ml.	—

Mix with a thin stirring rod, adjust the spectrophotometer so that the optical density of the control cuvette is 0.300. Read the optical density of the experimental cuvette (E_0). Mix into both cuvettes

0.02 ml. CCE solution (VIII).

Read the optical density every two minutes, while maintaining the control cuvette at E = 0.300. After several minutes ΔE/min. is constant and is between 0.001 and 0.005/min. Plot the optical density reading against time and extrapolate to the time of the CCE addition (E_1). A separate determination of the absorption due to the enzyme is unnecessary as the control cuvette also contains enzyme. The value $\Delta E = E_0 - E_1$ is used for the calculations.

Calculations

According to the general formula on p. 37:

$$\frac{\Delta E \times V}{\varepsilon \times d \times v} = \mu\text{moles CoA/ml. sample}$$

where

ΔE = optical density difference
V = assay volume [ml.]
v = volume of sample in the assay [ml.]
d = light path of the cuvette [cm.]
ε = extinction coefficient of DPNH (for 340 mμ: 6.22 cm.^{2}/μmole; for 366 mμ: 3.30 cm.^{2}/μmole).

Therefore for measurements at 340 mμ:

$$\frac{\Delta E \times 3.005}{6.22 \times 0.2} = \Delta E \times 2.41 = \mu\text{moles CoA-SH/ml. sample}$$

for measurements at 366 mμ:

$$\frac{\Delta E \times 3.005}{3.30 \times 0.2} = \Delta E \times 4.55 = \mu\text{moles CoA-SH/ml. sample.}$$

To convert to μg. CoA-SH the result must be multiplied by the molecular weight of CoA-SH (767.6).

Example

A CoA preparation (10.0 mg.) was dissolved in doubly distilled water and made up to 10.0 ml. The following optical densities were measured at 366 mμ (experimental against blank cuvette = 0.300):

Before the addition of CCE:		0.325
After the addition of CCE:	1 min.	0.273
	3 min.	0.200
	5 min.	0.125
	7 min.	0.104
	9 min.	0.099
	11 min.	0.096
	13 min.	0.094
	15 min.	0.093
	17 min.	0.092
	19 min.	0.091
	21 min.	0.090

Extrapolation to the time of the CCE addition gives $E_1 = 0.101$. $\Delta E = E_0 - E_1 = 0.325 - 0.101 = 0.224$.

$0.224 \times 4.55 \times 767.6 = 781$ μg. CoA-SH/ml. sample. Therefore the CoA-SH content of the preparation is $\frac{781}{1000} \times 100 = 78.1\%$.

Specificity and Sources of Error

The citrate cleavage enzyme is specific for CoA-SH. With dephospho-CoA as substrate only a small constant decrease in optical density could be observed. This did not exceed the optical density decrease which occurs in the assay with impure CoA preparations[6] (ΔE/min. *ca.* 0.002), and any error due to this decrease is eliminated by the extrapolation method.

[6] *G. Michal*, unpublished.

The enzyme does not react with CoA—S—S—CoA[6]; glutathione or other reducing compounds are omitted from the reaction mixture, in order to exclude the reduction of CoA—S—S—CoA. With the method described here the assay must be started with the enzyme (not with ATP[2]), since impure CoA preparations may contain ATP.

Appendix

Preparation of the citrate cleavage enzyme[2]

Homogenize chicken liver with 10 volumes 20% ethanol (0.4 M with respect to KCl), centrifuge at high speed and filter through muslin. Add $^1/_{17}$ volume of 1 M acetate buffer (pH 5.4) to the supernatant and centrifuge again at high speed. Add $^1/_{100}$ volume of 0.1 M zinc acetate solution to the supernatant, stir for 30 min. and centrifuge at high speed. Extract the precipitate with 0.5 M KCl solution in a glass homogenizer, centrifuge at high speed and store the supernatant. Re-extract the precipitate and centrifuge. Combine the supernatants, dilute with distilled water to 0.1 M KCl, centrifuge and fractionate the supernatant with ethanol. Dissolve the protein precipitating between 7 and 12% ethanol in distilled water and fractionate this solution with $(NH_4)_2SO_4$. Adsorb the protein precipitating between 30 and 40% saturation on Cγ-alumina gel (1 mg./mg. protein) at pH 5.9 and after 15 min. elute with 0.1 M citrate buffer (pH 7.0). The purification at this stage is about 100-fold.

The CoA assay described here is suitable for the determination of the enzyme activity.