# Butyryl-CoA and the CoA Derivatives of the Higher Saturated Fatty Acids

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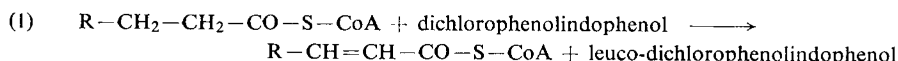
The determination of the activity of dehydrogenases with redox dyes as hydrogen acceptors was developed by *Thunberg*[1]. This method can also be used for the determination of the CoA derivatives of the higher, saturated fatty acids by means of the "general acyl-CoA dehydrogenase" (GAD) and the "electron-transferring flavoprotein" (ETF).

## Principle

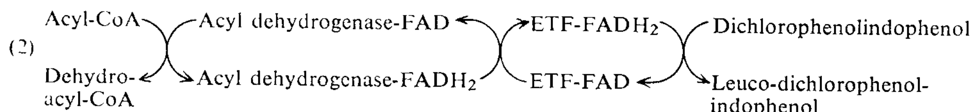
GAD and ETF catalyse the transport of hydrogen from acyl-CoA to dichlorophenolindophenol with the formation of $\alpha,\beta$-dehydroacyl-CoA and leuco-dichlorophenolindophenol:



$$(1)\quad R-CH_2-CH_2-CO-S-CoA + \text{dichlorophenolindophenol} \longrightarrow R-CH=CH-CO-S-CoA + \text{leuco-dichlorophenolindophenol}$$

The redox potentials for the systems butyryl-CoA/crotonyl-CoA ($R = CH_3$) and dichlorophenol-indophenol/leuco-dichlorophenolindophenol are $E'_0 = -0.015$ V[2] and $E'_0 = +0.22$ V respectively. The reaction goes virtually to completion. With an excess of dichlorophenolindophenol, acyl-CoA is quantitatively converted to dehydroacyl-CoA; the amount of acyl-CoA added can be determined by the decolorization of the dye.

According to *Crane* et al.[3] liver contains three acyl dehydrogenases, which have their maximum activity with butyryl-CoA, capryl-CoA and lauryl-CoA. From the acyl dehydrogenase the hydrogen is transferred to the flavine adenine dinucleotide (FAD) of the ETF[4]:




For the determination of acyl-CoA, ETF and an acyl dehydrogenase that reacts with the CoA derivative to be determined must be available. Especially suitable is GAD, which reacts with the CoA derivatives of the $C_4$ to $C_{16}$ fatty acids.

## Reagents

1. Potassium dihydrogen phosphate, A. R., $KH_2PO_4$
2. Disodium hydrogen phosphate, A. R., $Na_2HPO_4 \cdot 2H_2O$
3. 2,6-Dichlorophenolindophenol, sodium salt
4. Iodine, sublimed
5. Potassium iodide
6. "Electron-transferring flavoprotein", ETF
   from pig liver mitochondria, for isolation, see p. 436.
7. "General acyl-CoA dehydrogenase", GAD
   from pig liver mitochondria, for isolation, see p. 436.

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[1] *C. Oppenheimer* and *L. Pinaussen*: Die Methodik der Fermente. Georg Thieme, Leipzig 1929, Vol. III, p. 8.
[2] *G. Hauge*, J. Amer. chem. Soc. *78*, 5266 [1956].
[3] *F. L. Crane, S. Mii, J. G. Hauge, D. E. Green* and *H. Beinert*, J. biol. Chemistry *218*, 701 [1956].
[4] *F. L. Crane* and *H. Beinert*, J. biol. Chemistry *218*, 717 [1956].

#### Purity of the enzyme preparations

The ETF preparation should have a specific activity of 3.6 units *)/mg. The GAD should be purified until it has a specific activity of 5.6 units/mg. (measured with butyryl-CoA as substrate, method see[3]).

## Preparation of Solutions

I. Phosphate buffer (M/15; pH 7.0):
Dissolve 7.12 g. $Na_2HPO_4 \cdot 2H_2O$ and 3.54 g. $KH_2PO_4$ in distilled water and make up to 1000 ml.

II. 2,6-Dichlorophenolindophenol (0.3 mg./ml.; pH 7.0):
Dissolve 3.0 mg. Na salt of the dye in buffer (solution I) and make up to 10 ml.

III. Iodine-potassium iodide ($10^{-2}$ N iodine):
Dissolve 1.8 g. KI in a little distilled water in a 1000 ml. volumetric flask, add 1.27 g. iodine, stopper flask and shake until all the iodine is dissolved. If necessary, add some more KI crystals. Dilute with distilled water to 1000 ml. Store the solution in a brown bottle.

IV. "Electron-transferring flavoprotein", ETF (12 mg. protein/ml.):
Dilute the enzyme suspension prepared according to p. 436 to 12 mg./ml. with phosphate buffer (solution I).

V. "General acyl-CoA dehydrogenase", GAD (10 mg. protein/ml.):
Dilute the enzyme suspension prepared according to p. 436 (30 to 40 mg. protein/ml.) to 10 mg./ml. with phosphate buffer (solution I).

#### Stability of the solutions

Prepare the dichlorophenolindophenol solution freshly each week. The enzyme preparations are stable for about 6 months at $-15°C$.

The CoA derivatives of the saturated fatty acids are stable almost indefinitely when stored as dry powders in a desiccator at 0° C. The stability of aqueous solutions of these sulphydryl compounds is related to the stability of the thioester bond. The thioester bond of the *S*-acyl-*N*-acetylcysteamine derivatives of the lower fatty acids is hydrolysed in a few minutes by 0.1 N KOH[8]. The stability of the thioester bond increases with increase in the chain length of the fatty acid. Between pH 3 and 4 the thioester bond is very stable. Acetyl-CoA can be heated for fifteen minutes at 100°C at pH 3 to 4 without appreciable decomposition[5]. Aqueous solutions of the CoA derivatives should therefore be stored at slightly acid pH and at low temperatures (0 to $-15°C$).

## Procedure

#### Experimental material

If the samples contain large amounts of reducing agents they must be oxidized with iodine before the determination. To avoid an excess of iodine, first determine the requisite amount of iodine in a portion of the sample and then titrate the rest of the sample, except for a small portion, to a permanent yellow colour. Then decolorize by the addition of the small portion of the sample which has not been titrated. Reducing compounds present in small amounts are oxidized with an excess of dichlorophenolindophenol before the start of the determination.

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*) According to[3] 1 unit is the amount of enzyme in a volume of 0.5 ml., which decreases the optical density of 2,6-dichlorophenolindophenol at 600 mμ by 1000 in 1 min.

5) *E. Stadtman*, J. biol. Chemistry *196*, 535 [1952].

**Spectrophotometric measurements**

Wavelength: 600 mμ; light path: 1 cm.; final volume: 1.02 ml. Measure against the control cuvette.

Pipette successively into the cuvettes:

| *Experimental cuvette* | *Control cuvette* |
|---|---|
| 0.93 – 0.94 ml. buffer (solution I) | 0.95 ml. buffer (solution I) |
| 0.05 ml. dichlorophenolindophenol | 0.05 ml. dichlorophenolindophenol |
| 0.01 – 0.02 ml. sample | |

Allow to stand for about 5 min. until a constant optical density $E_1$ is obtained. Mix into both cuvettes

0.01 ml. ETF suspension (IV)
0.01 ml. GAD suspension (V)

and follow the fall in optical density with time in the experimental cuvette. Set the absorption of the control cuvette to a constant value during the assay. When the optical density of the experimental cuvette becomes constant (*ca.* 5 min.) read $E_2$. $\Delta E = E_1 - E_2$ is used for the calculations.

**Calculations**

The acyl-CoA content of the sample is obtained according to the formula (refer to p. 37):

$$\frac{\Delta E \times V}{\varepsilon \times d} = \mu\text{moles acyl-CoA/reaction mixture}$$

where

$\Delta E$ = measured decrease in optical density
$V$ = final volume in the cuvette in ml.
$\varepsilon$ = extinction coefficient (15.6 cm.$^2$/μmole *) at 600 mμ, 14.3 cm.$^2$/μmole at 578 mμ)
$d$ = light path in cm.

## Sources of Error

CoA samples used for the preparation of acyl derivatives by treatment with acid anhydrides must be checked for their glutathione content. Any glutathione will react with the acid anhydride to give *S*-acyl-glutathione derivatives and subsequently these will be hydrolysed to glutathione by deacylases present in the enzyme preparations. This will lead to high values for the acyl-CoA since the liberated glutathione will decolorize some of the dichlorophenolindophenol.

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*) The data for the extinction coefficient of dichlorophenolindophenol vary between 16.0 and 19.0 cm.$^2$/μmole[3,6–8]. The extinction coefficient given here was obtained by means of the purified CoA derivatives of butyric, caproic, caprylic, capric and palmitic acids[8,9]. No contaminants absorbing at 260 mμ could be detected chromatographically in these preparations; for the ratio of the *S*-acyl : adenine : phosphate content values between 1 : 1 : 2.99 and 1 : 1.03 : 3.16 were obtained[8]. Independent of the chain length of the fatty acid residue there was a straight line relationship between the adenine content of the CoA derivatives and the $\Delta E$ values obtained by the enzymatic method of determination.

6) *R. E. Basford* and *F. M. Huennekens*, J. Amer. chem. Soc. *77*, 3871 [1955].
7) *D. A. Green*, *S. Mii* and *H. R. Mahler*, J. biol. Chemistry *206*, 7 [1954].
8) *W. Seubert*, Ph. D. Thesis, Universität München [1955].
9) *W. Seubert*, Biochem. Prep. *7*, 80 [1959].

## Appendix

### Isolation of the "General acyl-CoA dehydrogenase" (GAD) and the "Electron-transferring flavoprotein" (ETF)

The activity of both enzymes is measured with the same method as described for the determination of the acyl-CoA derivatives. The measure of the enzyme activity is the rate of decolorization of the dye. Since a combination of both enzymes is required for the transport of the substrate hydrogen to an acceptor, only the activity of the enzyme which limits the rate of the over-all reaction can be properly measured. For example, in order to follow the purification of GAD, ETF must be added in excess for the assay of activity. Similarly, the same applies to the isolation of ETF. Therefore it is necessary to first purify one of the enzymes "blind".

*"Electron-transferring flavoprotein" (ETF)*[4]

Extract 100 g. acetone-powder of pig liver mitochondria with tris-acetate buffer (pH 7.5) and precipitate the inactive protein in the extract with Zn lactate. By addition of solid ammonium sulphate to the filtrate from this precipitation obtain four protein fractions at 35, 50, 65 and 85% saturation. Combine the fractions between 50 and 65% and 65 and 85% saturation, dialyse and then precipitate inactive protein by the addition of Zn lactate. Fractionate the filtrate from this precipitation with alcohol at $-15^{\circ}$C (10, 16 and 40% alcohol). ETF occurs in the third fraction (16–40%). After dialysis of the protein solution fractionate with solid ammonium sulphate. Fractionate the 65–90% fraction at pH 8.1 with ammonium sulphate. A preparation suitable for the determination is obtained between 70 and 75% saturation.

*"General acyl-CoA dehydrogenase" (GAD)*[3]

Extract 300 g. acetone-powder of pig liver mitochondria with phosphate buffer and fractionate the extract after centrifugation with solid ammonium sulphate. Dialyse the precipitate obtained between 55 and 65% saturation and then heat for 2 min. at 57°C. Centrifuge off denatured protein and precipitate inactive protein by addition of Zn lactate to the supernatant. Fractionate with alcohol and then fractionate the precipitate obtained between 18 and 23% alcohol with ammonium sulphate at pH 8.1.