# EFFECTS OF ETHANOL AND PROTEIN DILUTION ON MICROSOMAL PALMITOYL-CoA HYDROLASE ACTIVITY ASSAYED BY DIFFERENT METHODS

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## 1. Introduction

In the determination of palmitoyl-CoA hydrolase activity, we have used both a spectrophotometrical assay, where 5,5′-dithiobis-(2-nitrobenzoic acid) is used to follow the CoASH release from palmitoyl-CoA, and a radiochemical assay to measure the release of [1-$^{14}$C]palmitate from [1-$^{14}$C]palmitoyl-CoA [1]. In rats fed a normal diet, microsomal palmitoyl-CoA hydrolase had spec. act. 38–46 mmol . mg protein$^{-1}$ . min$^{-1}$ [1–3], in agreement with [4] and slightly higher than in [5]. A spec. act. ~150 nmol . mg protein$^{-1}$ . min$^{-1}$ in normal fed rats and ~205 nmol/mg protein/min in clofibrate fed rats was found [6]; much higher than found in [7]. However, another assay buffer and aldrithiol, instead of 5,5′-dithiobis-(2-nitrobenzoic acid), to follow CoASH release, were used in [6].

These results show that the radiochemical assay method gave values similar to the spectrophotometrical method when 5,5-dithiobis-(2-nitrobenzoid acid) was used to follow the CoASH release. Using aldrithiol to follow the CoASH release, the palmitoyl-CoA hydrolase activity increased mainly due to the addition of the liberated CoASH from the reaction:

Palmitoyl-CoA + ethanol → ethyl palmitate + CoASH

which is active in the rat liver microsomal fraction [8–10]. In addition, dilution of the microsomal protein prior to measuring the hydrolysis of palmitoyl-CoA increased the hydrolase activity. This phenomenon was not found in other cellular subfractions and is possibly explained by the effect of microsomal aggregation or disaggregation.

*Abbreviations:* Hepes, *N*-2-hydroxyethylpiperazine *N*′-2-ethanesulfonic acid

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## 2. Experimental

### 2.1. *Materials*

[1-$^{14}$C]Palmitoyl-CoA was purchased from New England Nuclear, Boston, MA. Palmitoyl-CoA, 5,5′-dithiobis-(2-nitrobenzoic acid) and Hepes were purchased from Sigma Chemical Co., St Louis, MO. Aldrithiol was a gift from Dr Osmundsen, Institute of Medical Biochemistry, Oslo. All other chemicals were of the highest commercially available purity.

### 2.2. *Rats, diets and preparation of subcellular fractions*

Male Wistar rats were randomly selected for a control group and a clofibrate treated group. The control group was given ordinary, commercial food as in [11] and the clofibrate treated group was given the same food containing 0.3% (w/w) clofibrate. Both groups were fed 10–14 days before they were killed.

The subfractions of liver homogenates were prepared essentially as in [12] with some modifications. Livers were homogenized in ice-cold sucrose solution (0.25 M sucrose and 10 mM Hepes buffer (pH 7.2)) and the following differential centrifugation fractions were obtained: the precipitate from 1200 × *g*, 5 min (the nuclear fraction), the precipitate between 1200 × *g*, 5 min and 6000 × *g*, 10 min (the mitochondrial fraction), the precipitate between 6000 × *g*, 10 min and 25 000 × *g*, 10 min (the light mitochondrial fraction), the precipitate between 25 000 × *g*, 10 min and 48 000 × *g*, 60 min (the

microsomal fraction) and the supernatant after 48 000 × *g*, 60 min (the particle-free supernatant).

2.3. *Enzyme assays and other analytical methods*

Palmitoyl-CoA hydrolase (EC 3.1.2.2) activity was assayed both radiochemically and spectrophotometrically with 5,5-dithiobis-(2-nitrobenzoic acid) as in [1]. With the radiochemical assay method, the mixture contained in final vol. 0.25 ml: 20 mM Hepes buffer (pH 7.4) or 20 mM potassium phosphate buffer (pH 7.2), protein, with and without ethanol (2.2% (w/w)) and [1-$^{14}$C]palmitoyl-CoA concentration as indicated in tables 1,2 and fig.1.

Hydrolysis of palmitoyl-CoA by the spectrophotometrical assay methods at 324 nm and 412 nm, using, respectively, 0.5 mM aldrithiol and 0.3 mM 5,5-dithiobis-(2-nitrobenzoic acid) to follow the CoA-SH release, was determined in the same buffers as described for the radiochemical assay method. The final volume was 1 ml and the incubation temperature was as indicated in tables 1,2 or fig.1. In some experiments aldrithiol (5 mg/ml) was solubilized in ethanol and diluted to 0.5 mM final conc. containing 2.2% ethanol. The amount of ethyl palmitate was measured as in [10] where the heptane layer was washed twice with 0.05 N NaOH in 50% ethanol. All spectrophotometric measurements were performed with a Schimadzu recording spectrophotometer MPS 5000. Radioactivity was counted in a Packard Tricarb Liquid Scintillation Spectrometer (Model 3385). Protein was determined using the Folin-Ciocalteu reagent [13].

## 3. Results and discussion

In [1–3,14] the determination of palmitoyl-CoA hydrolase was carried out at optimal substrate concentrations and within the linear range of enzyme concentration.

Table 1 shows, however, that dilution of the microsomal protein of rats fed a diet containing clofibrate increased the hydrolase activity ~1.6–1.8-times. The same effect was observed of rats fed a normal diet (not shown). This effect of dilution was not dependent on the protein content or substrate concentrations as shown in table 1.

In addition, the hydrolase activity of the undiluted microsomes was not increased by increasing the substrate concentrations. Thus, the data in table 1 suggest that a dilution of an inhibitor, or structural changes of the microsomal membranes/vesicles might have occured [7].

The aggregation phenomenon of rat microsomes has been reported [5] and suggested that palmitoyl-CoA hydrolase was located on the luminal surface of microsomal vesicles. The dilution effect was not observed with the particle-free supernatant or the mitochondrial fraction. Thus, aggregation/disaggregation of the microsomes may explain the lower specific activity in the undiluted microsomes than in the diluted microsomes.

Table 1 shows that ethanol decreased the rate of palmitoyl-CoA hydrolysis in the diluted and the undiluted microsomal preparations. The presence of

Table 1
Effect of ethanol and protein dilution of microsomal palmitoyl-CoA hydrolase activity

| Palmitoyl-CoA (μM) | Protein (μg) | Protein diln. (1:4) | Ethanol (2.2%) | Palmitoyl-CoA hydrolase act. (nmol . mg protein$^{-1}$ . min$^{-1}$) | Ethylpalmitate formation (nmol . min$^{-1}$ . mg protein$^{-1}$) |
|---|---|---|---|---|---|
| 50 | 76 | – | – | 58 | 0 |
| 300 | 76 | – | – | 62 | 0 |
| 50 | 76 | – | + | 46 | 8 |
| 50 | 19 | + | – | 94 | 0 |
| 100 | 76 | + | – | 90 | 0 |
| 50 | 19 | + | + | 73 | 19 |

Microsomes prepared from rats fed a diet containing clofibrate were used. The protein content was ~16 mg/ml. The hydrolase activity and the ethyl palmitate formation were measured radiochemically in 20 mM Hepes buffer (pH 7.4) (see section 2) with addition of the components as indicated. The protein was diluted 4 times in 20 mM Hepes buffer (pH 7.2). Incubation temperature was 35°C

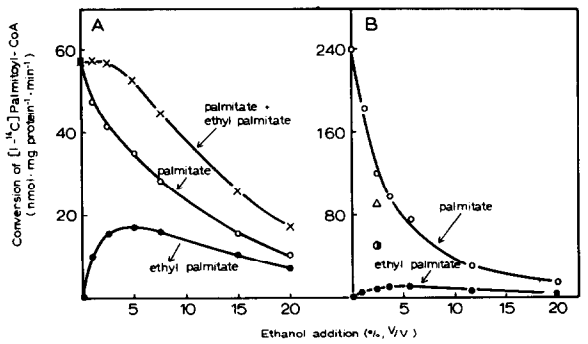


Fig.1. Conversion of [1-$^{14}$C]palmitoyl-CoA into [1-$^{14}$C]-palmitate and ethyl [1-$^{14}$C]palmitate. Palmitate and ethyl palmitate formation of: (A) 20 μg microsomal proteins and 50 μM palmitoyl-CoA; (B) 3 μg purified microsomal palmitoyl-CoA hydrolase [3] and 25 μM palmitoyl-CoA. The ethanol concentrations were as indicated. Incubations were performed at 30°C for 3 min (A) and 2 min (B). (B) (△, ●) hydrolase activity with 50 μM and 75 μM palmitoyl-CoA, respectively.

ethanol resulted in formation of ethyl palmitate (table 1, fig.1) in agreement with [8–10]. In addition, the activity of palmitoyl-CoA hydrolase + formation of ethyl palmitate with added ethanol up to ~3% (v/v) (fig.1) was similar to the hydrolase activity found in the absence of ethanol (table 1). Furthermore, the purified microsomal palmitoyl-CoA hydrolase [3] is not a general esterase, using ethanol instead of water, as ethyl palmitate formation was only ~5% of the hydrolase activity (fig.1B). This result indicates that the palmitoyl-CoA hydrolase is different from the enzyme which forms ethyl palmitate. The ethanol inhibition of the palmitoyl-CoA hydrolysis seemed to act directly on the enzyme, not as a function of the ratio palmitoyl-CoA/ ethanol, as increasing the palmitoyl-CoA concentration could not reverse the hydrolase activity in the presence of ethanol (fig.1B). Thus, by the spectrophotometrical assay with aldrithiol (containing 2.2% (w/w) ethanol), an overestimation of the hydrolase activity may take place as the CoA-SH release may be due to both of the two reactions:

$$\text{Palmitoyl-CoA} + H_2O \rightarrow \text{palmitate} + \text{CoASH} \quad (1)$$

$$\text{Palmitoyl-CoA} + \text{ethanol} \rightarrow \text{ethyl palmitate} + \text{CoASH} \quad (2)$$

The palmitoyl-CoA hydrolase activity obtained with the different assay methods with various amounts of substrate is shown in table 2. The radiochemical method and the spectrophotometrical assay method using 5,5′-dithiobis-(2-nitrobenzoic acid) gave similar values in both Hepes buffer and potassium phosphate buffer. The spectrophotometrical assay method using aldrithiol to follow the CoASH release in the presence and absence of ethanol differed from the other methods. In potassium buffer, the palmitoyl-CoA hydrolase activity was lower compared with the radiochemical assay method, while in the Hepes buffer, the palmitoyl-CoA hydrolase activity was increased. The highest activity was found when aldrithiol was dissolved in ethanol, which can be explained by the

Table 2
Palmitoyl-CoA hydrolase activity (nmol . min$^{-1}$ . mg protein$^{-1}$) assayed by different methods

| Palmitoyl-CoA (μM) | Spectrophotometrically | | | Radiochemically | |
|---|---|---|---|---|---|
| | With aldrithiol | | With DTNB[a] | Hepes buffer | Phosphate buffer |
| | Hepes buffer | Phosphate buffer | Hepes buffer | | |
| 40 | 97 ( 95)[b] | 67 | 83 | 83 | 83 |
| 50 | 103 (140)[b] | 71 | 91 | 91 | 95 |
| 75 | 111 (165)[b] | 75 | 95 | 83 | 87 |
| 100 | 107 (160)[b] | 52 | 91 | 72 | 80 |

[a] 5,5′-Dithiobis-(2-nitrobenzoic acid)
[b] Aldrithiol solubilized in ethanol

Diluted microsomal protein (~20 μg) (1:4) from rats treated with clofibrate, were used in all experiments. The different assay methods were as section 2. Incubation temperature was 35°C

CoASH release of the reaction described in (1) and (2).

In summary the results here presented show that spectrophotometrical determination of palmitoyl-CoA hydrolase activity in the microsomal fraction of rat liver, can be artifactual since SH-trapping reagents react with all reduced CoASH released from other enzymatic reactions, especially in the presence of ethanol.

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