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Deacylation of Acetyl-Coenzyme A and Acetylcarnitine by Liver Preparations

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The breakdown of acetylcarnitine catalysed by extracts of rat and sheep liver was completely abolished by Sephadex G-25 gel filtration, whereas the hydrolysis of acetyl-CoA was unaffected. Acetyl-CoA and CoA acted catalytically in restoring the ability of Sephadex-treated extracts to break down acetylcarnitine, which was therefore not due to an acetylcarnitine hydrolase but to the sequential action of carnitine acetyltransferase and acetyl-CoA hydrolase. Some 75% of the acetyl-CoA hydrolase activity of sheep liver was localized in the mitochondrial fraction. Two distinct acetyl-CoA hydrolases were partially purified from extracts of sheep liver mitochondria. Both enzymes hydrolysed other short-chain acyl-CoA compounds and succinyl-CoA (3-carboxypropionyl-CoA), but with one acetyl-CoA was the preferred substrate.

The deacylation of both acetyl-CoA and acetylcarnitine could lead to the formation of acetate *in vivo*, and significant amounts of acetate have been shown to be released from the liver in rats (Seufert, *et al.*, 1974), lactating cows (Baird *et al.*, 1975) and sheep (Costa *et al.*, 1976). In the last paper the acetyl-CoA-cleavage activity of liver extracts *in vitro*, prepared from biopsy samples, was sufficient to account for the rates of liver acetate production *in vivo*.

It has been suggested that acetylcarnitine is deacylated in liver by a specific acetylcarnitine hydrolase (Costa & Snoswell, 1975*a*) and that the breakdown of acetyl-CoA in cell-free preparation is due to the combined action of carnitine acetyltransferase (EC 2.3.1.7) and the acetylcarnitine hydrolase (Costa & Snoswell, 1975*b*). By contrast a number of workers had previously suggested that an acetyl-CoA hydrolase (EC 3.1.2.1), originally classified on the basis of a very brief report in a paper dealing with the deacylation of succinyl-CoA (3-carboxypropionyl-CoA) in pig liver (Gergely *et al.*, 1952), was the main enzyme responsible for the deacylation of acetyl-CoA. This enzyme was reported to be predominantly found in the mitochondria and widely distributed in bovine (Quraishi & Cook, 1972) and in rat and sheep tissues (Knowles *et al.*, 1974), with highest activity in the liver. A particularly rich source is the brown fat of the hamster (Bernson, 1976).

In view of the significant rates of production of acetate by the liver under some physiological conditions, it seemed important to investigate the enzymic deacylation of both acetyl-CoA and acetylcarnitine in more detail in order to understand the basis of this acetate production.

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Materials and Methods

Tissue preparations

Rat liver homogenates (20%, w/v) were prepared in 25mM-sucrose containing 2.3mM-potassium phosphate buffer (pH 7.4) and 0.1% Triton X-100 by the method of Costa & Snoswell (1975*a*). These homogenates were frozen in liquid N_2, thawed and centrifuged at 10000*g* for 5min, and the supernatant was used to examine the hydrolysis of acetylcarnitine and acetyl-CoA. Where indicated, 2ml samples of supernatant were passed down columns (1.9cm × 15cm) of Sephadex G-25 (medium grade) equilibrated in 25mM-phosphate buffer (pH 7.4). Only the first few ml of eluate containing protein were used in subsequent assays.

Sheep liver, kindly provided by Mr. D. W. Pethick of the A.R.C. Institute of Animal Physiology, Babraham, was collected immediately after slaughter. Homogenates (40%, w/v) were prepared in 0.3M-sucrose/10mM-4-morpholinepropanesulphonic acid/1mM-EGTA adjusted to pH 7.2, by using an electric blender. The homogenate was centrifuged at 500*g* for 10min, and the supernatant filtered through muslin and re-centrifuged at 15000*g* for 10min. The pellet was resuspended in 3vol. of homogenizing buffer and re-centrifuged at 15000*g* for a further 10min. The washed mitochondria were suspended in

a minimum volume of homogenizing buffer and stored at −20°C.

Protein concentrations were measured by a modification of the method of Gornall *et al.* (1949).

Assay of acetylcarnitine hydrolysis

Extracts of rat liver described above were incubated in a mixture containing 100mM-Tris/HCl buffer (pH 8.0) and 10mM-acetyl-L-carnitine in a volume of 1.0ml at 37°C for 20min. The reaction was stopped by the addition of 0.2ml of 15% (w/v) $HClO_4$. L-Carnitine was measured in the neutralized supernatant by using 4,4′-dithiobispyridine in the assay system described by Ramsay & Tubbs (1975).

Assay of acetyl-CoA hydrolysis

At first sight the assay of acetyl-CoA hydrolase presents no difficulty, since the liberated CoA can react with chromophoric reagents such as 5,5′-dithiobis-(2-nitrobenzoic acid) or 4,4-dithiobispyridine. However, tissues contain very large amounts of 3-oxoacyl-CoA thiolases (EC 2.3.1.9 and EC 2.1.3.16) (Middleton, 1975), which can form CoA by the reaction:

$$2\ \text{Acetyl-CoA} \rightleftharpoons \text{acetoacetyl-CoA} + \text{CoA}$$

Although the equilibrium is very unfavourable, a CoA-trapping system can cause the continuous consumption of acetyl-CoA, as mentioned by Kohlhaw & Tan-Wilson (1977). It was also pointed out by Middleton (1974) that under appropriate conditions thiolases can rapidly liberate CoA from acetyl-CoA. In liver mitochondrial extracts this interference by thiolase might be exacerbated by the presence of 3-hydroxy-3-methylglutaryl-CoA synthase (EC 4.1.3.5) and 3-hydroxy-3-methylglutaryl-CoA lyase (EC 4.1.3.4), which together in effect deacylate acetoacetyl-CoA. The thiolase interference is avoided by allowing the acetyl-CoA hydrolase reaction to proceed in the absence of a CoA trap, since the formation of acetoacetyl-CoA will be very small (and transient if a hydrolase is also liberating CoA). For this reason we have used an incubated system and subsequent CoA measurement as outlined below for the experiments described in this paper. A direct spectrophotometric assay involving continuous reaction of CoA with 4,4′-dithiobispyridine was only used for the preliminary screening of fractions during enzyme purification. The discontinuous assay was also used for CoA derivatives other than acetyl-CoA, although in these cases thiolase would probably not have caused any interference.

In the routine assay 4mM-acetyl-CoA, 100mM-Tris/HCl buffer (pH 8.0), 10mM-KCl and enzyme were incubated in a volume of 0.1ml for 5min at 30°C. After 2.5 and 5min 30μl samples were removed and placed in cuvettes containing 2.0ml of 100mM-potassium phosphate buffer (pH 7.2), 1mM-EGTA and 125μM-4,4′-dithiobispyridine. The increase in A_{324} was followed for 2min and then extrapolated back to the time of adding the sample to the cuvette, so permitting calculation of the CoA released during the incubation. The rate of CoA release was linear with time for 5min. One unit of enzyme activity is the amount that caused the release of 1μmol of CoA/min under the conditions described. Various concentrations of acetyl-CoA and other acyl-CoA derivatives were used in kinetic experiments.

Preparation and assay of acyl-CoA compounds

Acetyl-CoA, propionyl-CoA, butyryl-CoA and succinyl-CoA were prepared from free CoA and the acid anhydrides, and decanoyl-CoA, lauroyl-CoA and palmitoyl-CoA were prepared from free CoA and the corresponding acyl thioglycollates (Chase & Tubbs, 1972).

The concentrations of acetyl-, propionyl, butyryl- and decanoyl-CoA were determined by reaction with 4,4′-dithiobispyridine at 324nm, after the addition of carnitine acetyltransferase in the presence of excess of carnitine. The concentrations of lauroyl-CoA and palmitoyl-CoA were determined in a similar system but containing ox liver carnitine palmitoyltransferase (prepared by Dr. M. R. Edwards in this Department) in place of carnitine acetyltransferase. Succinyl-CoA was measured by determining the increase in free CoA after alkaline hydrolysis.

Enzymes and chemicals

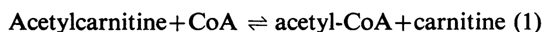
Carnitine acetyltransferase and CoA were obtained from the Boehringer Corp. (London), London W.5, U.K. 4-4′-Dithiobispyridine (4-Aldrithiol) was obtained from the Aldrich Chemical Co., Wembley, Middx., U.K., and L-carnitine was generously supplied by Otsuka Pharmaceutical Co., Tokushima, Japan.

Results

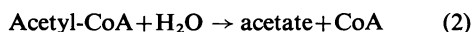
Deacylation of acetylcarnitine by rat liver homogenates

Costa & Snoswell (1975*a*) suggested that the breakdown of acetylcarnitine was catalysed by a specific acetylcarnitine hydrolase. However, in the present work it was found that passage of a high-speed supernatant of either rat (Table 1) or sheep liver homogenate through a column of Sephadex G-25 completely abolished the breakdown of acetylcarnitine, but had no effect on the hydrolysis of acetyl-CoA (cf. Seufert *et al.*, 1976). These results were in direct contrast with those reported by Costa & Snoswell (1975*a*). Further, the addition of small amounts of either CoA or acetyl-CoA to the Sephadex-treated extracts of rat liver acted catalyti-

cally and led to the extensive release of carnitine (Table 1). CoA, acetyl-CoA and carnitine were not detected in the Sephadex-treated extracts. These findings suggested that the breakdown of acetylcarnitine is due to the coupled reactions:



$$\text{Acetylcarnitine} + \text{CoA} \rightleftharpoons \text{acetyl-CoA} + \text{carnitine} \quad (1)$$



$$\text{Acetyl-CoA} + H_2O \rightarrow \text{acetate} + \text{CoA} \quad (2)$$

catalysed respectively by carnitine acetyltransferase and an acetyl-CoA hydrolase.

Purification of mitochondrial acetyl-CoA hydrolases from sheep liver mitochondria

Initial experiments indicated that some 75% of the acetyl-CoA hydrolase activity in sheep liver was found in the mitochondrial fraction, in agreement with Knowles *et al.* (1974). The results in Table 2 show that the specific activity of the mitochondrial fraction was 8-fold greater than that of the initial liver homogenate, and a preparation of sheep liver mitochondria was used as the starting material for enzyme purification.

Frozen mitochondrial suspension (51 ml, about 40 mg of protein/ml) was thawed and mixed with an equal volume of 50 mM-potassium phosphate buffer (pH 7.5). The mixture was then sonicated with an MSE Soniprobe at an amplitude of 12 μm peak-to-peak for three 20 s periods with cooling in an ice bath in between. The preparation was then centrifuged at 106000 *g* for 30 min.

The supernatant was adjusted to 40% saturation by the addition of 242 g of solid $(NH_4)_2SO_4$/litre and after 30 min was centrifuged at 30000 *g* for 10 min. The supernatant was then adjusted to 70% saturation by the further addition of solid $(NH_4)_2SO_4$ (205 g/litre) and centrifuged as above. The pellet was dissolved in 5 mM-phosphate (pH 7.5): this, and all buffers used in later steps, contained 10% (v/v) glycerol and also 0.5 mM-dithiothreitol, the omission of which led to rapid inactivation.

The solution was then desalted by passage down a column of Sephadex G-25 (medium grade) previously equilibrated in 5 mM-potassium phosphate buffer (pH 7.5), and applied to a column (11.5 cm × 6.5 cm) of DEAE-cellulose previously equilibrated in the same buffer at 5°C. The column was washed with 1 bed vol. of the same buffer and then a linear gradient of phosphate to 50 mM applied (total volume 1 litre).

One peak of acetyl-CoA hydrolase activity was eluted with 5 mM-phosphate from this DEAE-cellulose column, immediately after the main peak of protein (Fig. 1). A second peak, completely separated from the first, was eluted when the phosphate concentration reached about 25 mM. Most of

Table 1. *Deacylation of acetylcarnitine by preparations of rat liver*

Homogenates of rat liver were prepared in hypo-osmotic sucrose containing Triton X-100 as described in the text and, where appropriate, were treated with Sephadex G-25. Release of carnitine from 10 mM-acetylcarnitine was measured in a 1 ml incubation system as described in the text.

Treatment/addition	Carnitine released (nmol)
Untreated extract (10.7 mg protein)	540
Sephadex-treated extract (5.1 mg of protein)	None
Sephadex-treated extract + 20 nmol of acetyl-CoA	316
Sephadex-treated extract + 19 nmol of CoA	354

Table 2. *Purification of acetyl-CoA hydrolases from sheep liver mitochondria*

Homogenates of sheep liver, the isolation of mitochondria and the purification and assay of acetyl-CoA hydrolase are described in the text.

Fraction	Volume (ml)	Acetyl-CoA hydrolase (units/ml)	Protein (mg/ml)	Specific activity (units/mg of protein)	Purification (fold)
Crude homogenate	410	0.340	52	0.0065	—
Mitochondrial extract	69	1.56	30	0.052	8
40–70%-satd.-$(NH_4)_2SO_4$ fraction	10	8.5	62	0.136	21
Peak 1 from DEAE-cellulose column (pooled)	2.5	8.2	16	0.51	78
Peak 2 from DEAE-cellulose column (pooled)	2.6	5.8	12	0.48	74

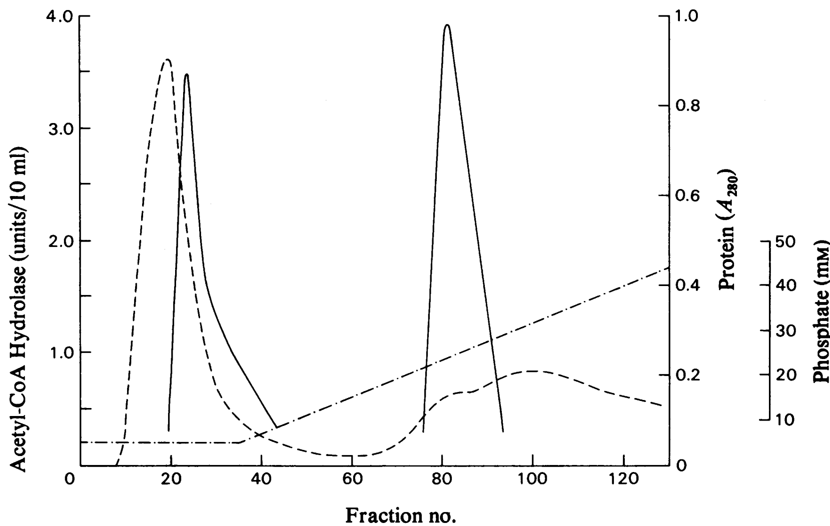


Fig. 1. *Elution profile of acetyl-CoA hydrolase activity and protein from DEAE-cellulose*
A DEAE-cellulose column (11 cm × 6.5 cm diam) previously equilibrated in 5mM-phosphate buffer (pH 7.5) containing 10% glycerol and 0.5mM-dithiothreitol was loaded with about 600mg of protein; for details see the text. The column was washed with 1 bed volume of this buffer and then a linear gradient to 50mM-phosphate was applied: 10ml fractions were collected. Fractions 21–40 and 77–90 were pooled separately. ——, Acetyl-CoA hydrolase activity; ----, protein (A_{280}); –·–·–·, phosphate concentration.

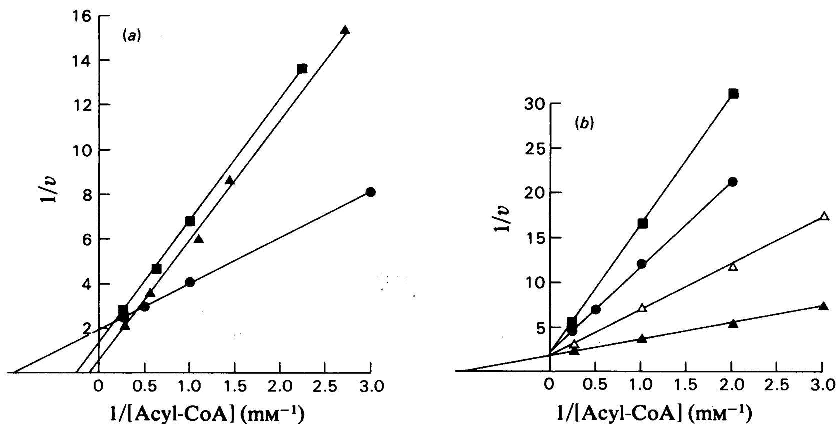


Fig. 2. *Utilization of various acyl-CoA compounds by the first* (*a*) *and second* (*b*) *peaks of acetyl-CoA hydrolase activity isolated from the DEAE-cellulose column* (*see Fig.* 1)
Hydrolase activity was measured as described for acetyl-CoA in the text. For succinyl-CoA, correction was made for the non-enzymic rate of hydrolysis. *v* is expressed as μmol of CoA released/min per mg of protein. ●, Propionyl-CoA; ▲, acetyl-CoA; ■, succinyl-CoA, △, acetyl-CoA in the presence of 0.8mM-CoA (*b* only).

the protein adsorbed on the column was not eluted with 50mM-phosphate and required higher concentrations for elution, but no further acetyl-CoA hydrolase activity was obtained by this treatment.

The fractions containing the two peaks of acetyl-CoA hydrolase activity were pooled separately and precipitated by adding solid $(NH_4)_2SO_4$ (529g/litre). After centrifugation at 30000*g* for 10min the pellets were dissolved in 5mM-phosphate buffer (pH 7.5) containing 40% (v/v) glycerol and 0.5mM-dithio-

threitol. This concentration step caused some loss of activity, especially for the second peak. Both peaks still contained thiolase activity, with acetoacetyl-CoA as substrate, but in the first peak the specific activity was decreased some 6-fold compared with the original mitochondrial extract and in the second peak by 20-fold.

Properties of the two peaks from the DEAE-cellulose column showing acetyl-CoA hydrolase activity

The material in the first peak eluted from the DEAE-cellulose column hydrolysed propionyl-CoA more rapidly than acetyl-CoA at low substrate concentrations (Fig. 2*a*). Activity with succinyl-CoA was similar to that with acetyl-CoA. Butyryl-CoA was hydrolysed at a similar rate to propionyl-CoA, and decanoyl-CoA, lauroyl-CoA and palmitoyl-CoA were scarcely hydrolysed when tested at concentrations of 0.5–4 mM.

By contrast the material in the second peak from the DEAE-cellulose column showed its highest activity with acetyl-CoA (Fig. 2*b*): the K_m value, about 1 mM, was considerably lower than that for the first enzyme. Propionyl-CoA, butyryl-CoA and succinyl-CoA showed higher K_m values than acetyl-CoA. Free CoA was a competitive inhibitor (Fig. 2*b*), as it also was with the first enzyme (results not shown). Decanoyl-CoA was a very poor substrate, and lauroyl-CoA and palmitoyl-CoA were not appreciably utilized.

Discussion

In the present work we have not detected acetylcarnitine hydrolase activity in either rat or sheep liver, but have found that extracts of mitochondria from sheep liver contain at least two distinct enzymes that hydrolyse acetyl-CoA and other acyl-CoA compounds. Prass *et al.* (1977) have extensively purified an acetyl-CoA hydrolase from rat liver cytosol which is cold-labile and activated by ATP. An acyl-CoA hydrolase has been partially purified from pig heart mitochondria (Lee, 1977): this enzyme was activated by Ca^{2+} and was more active with decanoyl-CoA than with acetyl-CoA. Neither of the sheep liver hydrolases described here was activated by ATP or Ca^{2+}, and both were relatively inactive with decanoyl-CoA, were stable to storage at −20°C, and had much higher K_m values than the very active enzyme from hamster brown-fat mitochondria (Bernson, 1976). It appears that there are several enzymes capable of hydrolysing acetyl-CoA.

The K_m values for the sheep liver mitochondrial hydrolases are relatively high with respect to acetyl-CoA and the other acyl-CoA compounds utilized, but the mitochondrial concentration of acetyl-CoA can reach more than 1 mM (e.g. Garland *et al.*, 1965): the same is true for succinyl-CoA (Smith *et al.*, 1974). The physiological function of the acetyl-CoA hydrolases is obscure, but presumably one or more of them is responsible for the acetate formation by the liver referred to in the introduction. Their competition with other enzymes utilizing acetyl-CoA remains to be elucidated.

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