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Acetyl-Coenzyme A Deacylase Activity in Liver is not an Artifact

SUBCELLULAR DISTRIBUTION AND SUBSTRATE SPECIFICITY OF ACETYL-COENZYME A DEACYLASE ACTIVITIES IN RAT LIVER

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Whole liver and isolated liver mitochondria are able to release free acetate, especially under conditions of increased fatty acid oxidation. In the present paper it is shown that rat liver contains acetyl-CoA deacylase (EC 3.1.2.1) activity (0.72 μmol/min per g wet wt. of liver at 30°C and 0.5 mM-acetyl-CoA). At 0.5 mM-acetyl-CoA 73% of total enzyme activity was found in the mitochondria, 8% in the lysosomal fraction and 19% in the postmicrosomal supernatant. Mitochondrial subfractionation shows that mitochondrial acetyl-CoA deacylase activity is restricted to the matrix space. Mitochondrial acetyl-CoA deacylase showed almost no activity with either butyryl- or hexanoyl-CoA. Acetyl-CoA hydrolase activity from purified rat liver lysosomes exhibited a very low affinity for acetyl-CoA (apparent $K_m > 15$ mM compared with an apparent K_m value of 0.5 mM for the mitochondrial enzyme) and reacted at about the same rate with acetyl-, n-butyryl- and hexanoyl-CoA. We could not confirm the findings of Costa & Snoswell [(1975) *Biochem. J.* **152**, 167–172] according to which mitochondrial acetyl-CoA deacylase was considered to be an artifact resulting from the combined actions of acetyl-CoA–L-carnitine acetyltransferase (EC 2.3.1.7) and acetylcarnitine hydrolase. The results are in line with the concept that free acetate released by the liver under physiological conditions stems from the intramitochondrial deacylation of acetyl-CoA.

In experiments with isolated perfused rat livers and rat liver slices, a significant net production of free acetate from short- and long-chain fatty acids in addition to ketone-body formation has been shown (Seufert *et al.*, 1974; Knowles *et al.*, 1974; Frölich & Wieland, 1975). Under conditions of enhanced β-oxidation of fatty acids the relative increase of acetate release is even higher than that of ketone-body release (Seufert *et al.*, 1974). A formation of free acetate by rat liver tumour cells has been observed by Lynen *et al.* (1959) and Hepp *et al.* (1966). Knowles *et al.* (1974) described a substantial formation of acetate from fatty acids and pyruvate by livers from non-herbivorous and herbivorous animals. Costa *et al.* (1976) calculated on the basis of transhepatic catheterization measurements that in lactating ewes the hepatic acetate release accounted for 70% of the fatty acids taken up by the liver. Conflicting opinions exist about the mechanism and intracellular localization of the ethanol-independent formation of acetate in liver. Hepp *et al.* (1966) and Knowles *et al.* (1974) described a hepatic acetyl-CoA-deacylating activity. However, both groups used a rather non-specific assay system in which the free CoA generated in the acetyl-CoA deacylase reaction was determined colorimetrically, a method which in our hands gives far too high and also inconsistent values. Moreover, Costa & Snoswell (1975) claimed to have presented evidence for hepatic acetyl-CoA deacylase activity being an artifact resulting from a combined action of acetyl-CoA–L-carnitine acetyltransferase at the inner mitochondrial membrane and an acetylcarnitine deacylase at the outer mitochondrial membrane.

Walter & Söling (1976) have shown that under special experimental conditions a transfer of acetyl groups across the mitochondrial membrane other than by the citrate pathway can occur. However, it could not be decided from their experiments whether the transport of the C_2 units across the inner mitochondrial membrane occurred in the form of free acetate or of acetylcarnitine. It was shown by Hoffmann & Weiss (1978) and by our own group (Seufert *et al.*, 1977) that isolated rat liver mitochondria can release a considerable portion of the intramitochondrially produced C_2 units as free acetate.

The following experiments were undertaken to examine the possible existence of acetyl-CoA

deacylase activity in rat liver and to look for its intracellular distribution and substrate specificity. According to the results obtained, rat liver contains a specific acetyl-CoA deacylase that is almost exclusively located in the mitochondrial matrix space. Some of the results have been presented at the 10th International Congress of Biochemistry, Hamburg, 1976 (Seufert *et al.*, 1976).

Experimental

Tissue fractionation

Male Wistar rats (180–200g) from the Zentralinstitut für Versuchstierzucht, Hannover-Linden, Germany, which had been kept on a standard laboratory diet (Altromin-R; Fa. Altrogge, Lage/Lippe, Germany), were killed by decapitation and the livers were removed and washed in buffer A. Buffer A was prepared as follows (final concentrations): sucrose, 250mM; triethanolamine/HCl, 3.4mM, pH7.6; EGTA, 1mM; dithioerythritol, 2mM. Subcellular fractionation was carried out as described by de Duve *et al.* (1955).

One group of animals was pretreated with Triton WR-1339 as described by Wattiaux *et al.* (1963) to achieve a better separation of mitochondria and lysosomes. After subcellular fractionation by the method of de Duve *et al.* (1955) the combined mitochondrial and lysosomal fractions were further separated on a discontinuous sucrose gradient (Vignais & Nachbaur, 1968). The sucrose solutions contained 20mM-glycylglycine buffer, adjusted to pH7.4 with 2M-KOH, and 2mM-dithioerythritol.

Subfractionation of mitochondria was carried out by a slight modification of the digitonin method of Schnaitman & Greenawalt (1968). The dry digitonin powder was dissolved in 3ml of buffer A and slowly added (2 or 1.7mg per 10mg of mitochondrial protein) to 2ml of the mitochondrial suspension over 5min. After 15min 7ml of buffer A was added, followed by centrifugation for 10min at 11000*g*. The pellet containing the particles consisting of matrix space and the inner membrane (mitoplasts) was washed with 2×10ml of buffer A. For subfractionation the mitoplasts were suspended in buffer A (approx. 5mg of protein/ml) and sonicated for 4×15s at setting 8–10A with a Branson sonifier at 0°C. After sonication the inner-membrane fraction was sedimented by centrifugation (60min×100000*g*). The supernatant was considered to be representative of the matrix space. Nuclei were isolated as described by Widnell & Tata (1964).

Determination of enzyme activities

Determination of acetyl-CoA deacylase activity. Since the acetyl-CoA deacylase assays described in the literature (Hepp *et al.*, 1966; Knowles *et al.*, 1974; Costa & Snoswell, 1975) proved to be insufficient, an isotope assay was developed based on the liberation of [1-^{14}C]acetate from [1-^{14}C]acetyl-CoA. The assay mixture contained in a final volume of 0.5ml (final concentrations): Tris/HCl, pH7.4, 100mM; $MgCl_2$, 1mM; [^{3}H]acetate (sp. radioactivity 10mCi/mmol), 4×10^5d.p.m.; [1-^{14}C]acetyl-CoA (sp. radioactivity 0.3mCi/mmol) as indicated. The temperature was 30°C. The reaction was initiated by addition of the enzyme. After 5min the reaction was stopped with 0.1ml of 5M-HCl. Thereafter, 2ml of a charcoal suspension (5g/100ml) was added. After vigorous shaking for 2min the mixture was loaded on a Selecta no. 595 filter paper (Schleicher und Schüll, Dassel, Germany). The charcoal was treated with 5ml of methanol/water (3:7, v/v). From the eluate 5ml was transferred to a plastic counting vial and 10ml of Instagel was added. The samples were counted for ^{3}H and ^{14}C radioactivity in a Packard 3380 Tri-Carb liquid-scintillation spectrometer equipped with the triple-A attachment for automatic quench correction. The recovery of [^{3}H]acetate was used to correct for loss of [^{14}C]acetate, which was in the range 30–50%. The adsorption of [1-^{14}C]acetyl-CoA on the charcoal was more than 99% complete for acetyl-CoA concentrations between 0.1 and 20mM. The results were corrected by use of a control incubation in which the reaction was started with a heat-inactivated (1min at 90°C) enzyme. The test was linear with respect to time and protein concentration as indicated in Fig. 1.

Identical results were obtained when the separation of [1-^{14}C]acetate and [1-^{14}C]acetyl-CoA was carried out on a column (0.5cm×14cm) of Dowex 1 (X8; formate form; 200–400 mesh) by elution with a linear gradient (0–0.4M-formic acid) and a subse-

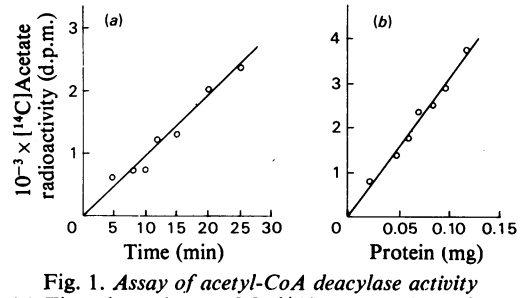

Fig. 1. *Assay of acetyl-CoA deacylase activity*
(*a*) Time-dependence of [1-^{14}C]acetate release from [1-^{14}C]acetyl-CoA. Acetyl-CoA deacylase activity from rat liver mitochondrial matrix space (14μg of protein) was used as enzyme source. For details of the assay procedure see the Experimental section. (*b*) Dependence of [1-^{14}C]acetate formation from [1-^{14}C]acetyl-CoA by mitochondrial acetyl-CoA deacylase on the concentration of protein in the assay mixture.

quent second linear gradient (0.4M-formic acid to 2M-ammonium formate).

In some experiments acetyl-CoA deacylase activity was determined by a slight modification of the optical assay method of Robinson *et al.* (1976). In this assay system enzyme activity is measured by following the increase in A_{412} when free CoA generated during deacylation of acetyl-CoA reacts with 5,5′-dithiobis-(2-nitro-benzoic acid). The assay system contained in a final volume of 1ml (final concentration): Tris/HCl, pH7.6, 0.05M; 5,5′-dithiobis-(2-nitrobenzoic acid), 100μM; acetyl-CoA, 0.5mM. The reaction was started by the addition of enzyme. The reaction temperature was 30°C. The same assay system was also used for the determination of n-butyryl- and hexanoyl-CoA hydrolase activity.

Determination of marker enzyme activities. Lactate dehydrogenase (EC 1.1.1.27) was measured as described by Bergmeyer & Bernt (1974), adenylate kinase (EC 2.7.4.3) as described by Bergmeyer (1974) and glutamate dehydrogenase (EC 1.4.1.3) as described by Söling *et al.* (1973). Monoamine oxidase (EC 1.4.3.4) was assayed at 25°C and 250nm by the method of Tabor *et al.* (1954) with benzylamine as substrate, by using an Aminco DW 2 dual-wavelength spectrophotometer in the split-beam mode. Glucose 6-phosphatase (EC 3.1.3.9) was measured as described by Baginski *et al.* (1974); succinate dehydrogenase (EC 1.3.99.1) activity was determined at 25°C by following the reduction of cytochrome *c* in the system described by Brdiczka *et al.* (1968). Acid phosphatase (EC 3.1.3.2) activity was measured as described by Andersch & Szczypinski (1947) and *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30) as described by Barrett (1972).

Protein was determined by the biuret method of Gornall *et al.* (1949) or with the Folin method of Lowry *et al.* (1951). Bovine serum albumin was used as standard in both methods.

Materials

Biochemicals were purchased from Boehringer Mannheim Corp., Mannheim-Waldhof, Germany, and digitonin, Dowex 1 (X8, 200–400 mesh) and Triton WR-1339 were from Serva, Heidelberg, Germany. Before use digitonin was freshly recrystallized from ethanol (60°C). Sephadex G-25 (medium grade) was obtained from Deutsche Pharmacia G.m.b.H., Frankfurt/Main, Germany, and 5,5′-dithiobis-(2-nitrobenzoic acid) was from Sigma Chemical Co., München, Germany. All other chemicals, including n-butyric anhydride and n-hexanoic anhydride, came from E. Merck A.G., Darmstadt, Germany. Selecta filter paper no. 595 was purchased from Schleicher und Schüll.

[1-^{14}C]Acetyl-CoA (sp. radioactivity 60mCi/mmol) and [^{3}H]acetate (sp. radioactivity 2–5Ci/mmol) were purchased from the Amersham Buchler Co., Braunschweig, Germany. [^{3}H]Acetate was purified before use by ion-exchange chromatography on Dowex 1 (X8, 200–400 mesh, formate form; 0.5cm×14cm). The purified [^{3}H]acetate was eluted with a linear (0–0.4M) formic acid gradient.

The purity of [1-^{14}C]acetyl-CoA was examined by its conversion into citrate with citrate synthase (EC 4.1.3.7). The citrate was separated by chromatography on a Dowex 1 (X8) column in the same way as for radioactive acetate, but after the first linear gradient (0–0.4M-formic acid) citrate was eluted with a second linear gradient formed from 100ml of 0.4M-formic acid and 100ml of 0.4M-ammonium formate. The citrate contained at least 98% of the initial amount of [1-^{14}C]acetyl-CoA. A ready-made scintillation 'cocktail' (Instagel) was supplied by Packard Instruments, Frankfurt/Main, Germany.

n-Butyryl-CoA and n-hexanoyl-CoA were prepared as described by Simon & Shemin (1953). Acyl-CoA was determined by the method of Decker (1959) by measuring the absorption change at 233nm after alkaline hydrolysis.

Results

At an acetyl-CoA concentration of 0.5mM, the total acetyl-CoA deacylase activity was of the order of 0.7μmol/min per g wet wt. of liver. When the acetyl-CoA concentration was raised to 2.5mM, acetyl-CoA deacylase activity was doubled (Table 1). This increase was due mainly to an increased activity of the lysosome fraction (Table 1).

On fractionation of rat liver by differential centrifugation, acetyl-CoA deacylase activity was recovered predominantly in the mitochondrial fractions and the cytosol (Table 1). The two different acetyl-CoA concentrations were used in the acetyl-CoA deacylase assay to discriminate between acetyl-CoA deacylases with different affinities. Acetyl-CoA deacylase activity in the nuclear fraction was most probably due to contamination with other cell constituents and with unbroken cells, as can be deduced from the high activities of glutamate dehydrogenase, acid phosphatase and glucose 6-phosphatase. When the nuclei were further purified by a sucrose-density-centrifugation step as described by Widnell & Tata (1964), no acetyl-CoA deacylase activity could be detected any more.

An almost complete separation of mitochondria and lysosomes could be achieved by further sucrose-density-gradient centrifugation (Vignais & Nachbaur, 1968) of the M+L fraction from livers of Triton WR-1339-pretreated rats (Table 2). The relative specific activities of glutamate dehydrogenase and acetyl-CoA deacylase in the lysosomal and the cytosolic fraction differ greatly under these experimental conditions. The acetyl-CoA deacylase activity

Table 1. *Subcellular distribution of acetyl-CoA deacylase activity in livers from fed rats after differential centrifugation (de Duve et al., 1955)*

Acetyl-CoA deacylase activity was tested at 2.5mM- (*a*) and 0.5mM- (*b*) acetyl-CoA respectively. Abbreviations: H, total homogenate; N, nuclear fraction; M, mitochondria; L, lysosomes; P, microsomal fraction; S, cytosol. Results given are mean values±S.D. for three experiments.

		Enzyme activities (μmol/min per g wet wt. of liver)					
Fraction	Protein (mg/g wet wt. of liver)	Acetyl-CoA deacylase (*a*)	Acetyl-CoA deacylase (*b*)	Glutamate dehydrogenase	Acid phosphatase	Glucose 6-phosphatase	Lactate dehydrogenase
H	183±25	1.50±0.03	0.72±0.01	226±16	10.0±0.2	6.9±1.2	284±5
	Protein or enzyme activity as percentage of amount found in total homogenate (H)						
H	100	100	100	100	100	100	100
N	32±3	26.8±8.4	31.1±5.8	46.9±9.1	29.3±1.2	27.2±1.1	10.2±0.3
M	16±2	29.1±11.9	25.1±5.0	42.2±6.1	18.8±2.8	12.2±2.8	2.4±0.4
L	6±1	12.7±2.2	5.5±1.0	1.6±0.1	15.1±2.5	10.8±3.2	2.2±0.7
P	8±1	1.3±0.4	1.3±0.2	1.0±0.2	4.7±1.2	42.6±5.7	4.9±2.0
S	37±2	30.7±12.3	26.9±9.5	0.06±0.02	15.0±1.3	2.6±0.4	73.8±6.4
Recovery	99±6	99±35	90±20	92±8	83±3	95±10	93±4.8

Table 2. *Subcellular distribution of acetyl-CoA deacylase (tested at 0.5mM-acetyl-CoA) in livers from fed rats that had been treated with Triton WR-1339*

The particulate fractions were separated as described by de Duve *et al.* (1955). The M+L fraction separated by the method of de Duve *et al.* (1955) was further separated into a mitochondrial (M) and a lysosomal (L) fraction as described by Vignais & Nachbaur (1968). Abbreviations: n.d., not detectable; otherwise as given in the legend to Table 1. Results are mean values±S.D. for four experiments.

		Enzyme activities (μmol/min per g wet wt. of liver)						
Fraction	Protein (mg/g wet wt. of liver)	Acetyl-CoA deacylase	Glutamate dehydrogenase	Succinate dehydrogenase	Acid phosphatase	*N*-Acetyl-glucosaminidase	Glucose 6-phosphatase	Lactate dehydrogenase
H	177±5	0.93±0.16	253±10	7.0±1.7	12.5±1.0	5.6±0.6	7.5±2.5	293±15
	Protein or enzyme activity as percentage of amount found in total homogenate (H)							
H	100	100	100	100	100	100	100	100
N	34±3	25.0±2.5	39.6±3.2	26.4±5.4	25.8±3.4	23.2±4.9	27.6±8.0	12.4±1.0
M+L	19±1	34.5±6.8	48.1±1.9	60.5±7.9	35.5±3.2	50.2±4.9	19.2±6.5	3.3±0.8
M	13±1	30.7±3.2	36.3±1.9	53.2±6.9	3.1±0.6	1.3±0.2	8.5±3.5	1.0±0.1
L	1±3	2.2±0.5	0.14±0.05	0.24±0.10	25.2±4.1	23.2±1.3	1.0±0.5	0.04±0.03
P	10±1	1.8±0.2	2.0±0.2	0.66±0.15	12.5±1.2	8.1±0.4	46.4±14	4.5±1.5
S	38±3	17.8±2.5	0.10±0.05	n.d.	21.1±2.5	3.7±2.0	3.2±0.7	73.3±5.6
Recovery in N+M, L, P, S	101±2	80±8	90±1	88±13	95±8	85±11	96±29	94±3
Recovery in N, M, L, P, S	96±2.6	77±7	79±2	80±4	88±9	60±5	87±23	91±4

in both compartments can only partly be explained by contamination with mitochondrial enzymes. From the data given in Table 2 the absolute distribution of acetyl-CoA deacylase activity among the different compartments was calculated. This was carried out by assuming that glutamate dehydrogenase, *N*-acetylglucosaminidase, glucose 6-phosphatase and lactate dehydrogenase occur exclusively in the mitochondrial, lysosomal, microsomal and cytosolic compartments respectively, and that according to our measurements no acetyl-CoA deacylase occurs in the nuclear fraction. When tested at 0.5μM-acetyl-CoA 73% of the total enzyme activity was recovered in the mitochondria, 19% in the cytosol and 8% in the lysosomes.

The results of subfractionation of isolated rat liver mitochondria are given in Tables 3–5. When the digitonin treatment was carried out with 2mg of

digitonin/10mg of mitochondrial protein (Table 3), the first supernatant contained the total activity of adenylate kinase, a marker for the intermembrane space, and most of the activity of monoamine oxidase, a marker for the outer mitochondrial membrane. Under these fractionation conditions the first supernatant represents the intermembrane space and the outer membrane. The glutamate dehydrogenase activity found in the first supernatant indicates some damage to mitoplasts. Nearly all activity of succinate dehydrogenase, which is representative of the inner mitochondrial membrane, was found in the mitoplasts. When mitoplasts were further fractionated by sonication and high-speed centrifugation (Table 4) the succinate dehydrogenase activity was detected in the inner-membrane fraction.

The acid phosphatase activity found in the mitochondrial preparation was due to contamination with lysosomes (Tables 1 and 2), as is indicated by the fact that 98.3% of this activity could be removed by treatment with digitonin (Table 3). After digitonin treatment (2mg of digitonin/10mg of mitochondrial protein) 36.6% of acetyl-CoA deacylase was found in the first supernatant and 63.4% in the mitoplasts (Table 3). Most of the acetyl-CoA deacylase activity of the first supernatant originates from damaged mitoplasts, as indicated by the release of glutamate dehydrogenase activity into the first supernatant (Table 3). Part of acetyl-CoA deacylase activity in the first supernatant is also due to contaminating lysosomes, as indicated by the acid phosphatase activity in the first post-digitonin supernatant. The possibility that acetyl-CoA deacylase in the first post-digitonin supernatant resided in the outer-membrane fraction was further excluded by experiments in which a slightly lower digitonin/protein

Table 3. *Acetyl-CoA deacylase in subfractionated rat liver mitochondria*

Subfractionation was as described by Schnaitman & Greenawalt (1968) with 2mg of digitonin/10mg of mitochondrial protein. Specific enzyme activities are expressed as nmol/min per mg of protein. Results are mean values±S.D. from three experiments. Abbreviation: n.d., non-detectable. Values in parentheses refer to activity as percentage of total activity in first supernatant+mitoplasts.

		Enzyme activities (nmol of substrate converted/min per mg of protein)					
	Protein (mg/ml)	Acetyl-CoA deacylase	Acid phosphatase	Monoamine oxidase	Adenylate kinase	Glutamate dehydrogenase	Succinate dehydrogenase
Mitochondria	40	5.3±1.1	118±10	7.7±1.7	663±134	2070±140	70±10
First supernatant	3.0±0.5	7.3±2.5	224±57	14.9±7.3	1360±200	890±340	12±4
		(36)	(98)	(93)	(100)	(22)	(10)
Mitoplasts	7.0±0.3	13.2±3.7	4.5±2.0	1.2±1.0	n.d.	3080±600	110±20
		(64)	(2)	(7)	(0)	(78)	(90)

Table 4. *Distribution of acetyl-CoA deacylase in rat liver mitochondria and mitoplasts*

Subfractionation of mitochondria as described by Schnaitman & Greenawalt (1968) was carried out with 2mg of digitonin/10mg of mitochondrial protein. Mitoplasts were further fractionated by sonication and centrifugation (see the Experimental section). Results are from a single experiment. Note that monoamine oxidase activity is mainly in the first supernatant. Values in parentheses refer to activity as percentage of total activity in first supernatant+matrix space+inner membrane. Abbreviation: n.d., not detectable.

		Enzyme activities (nmol of substrate converted/min per mg of protein)					
Fraction	Protein (mg/ml)	Acetyl-CoA deacylase	Acid phosphatase	Monoamine oxidase	Adenylate kinase	Glutamate dehydrogenase	Succinate dehydrogenase
Mitochondria	28	4.6	43.2	7.0	1150	1480	59
First supernatant	1.5	7.0	95.3	17.0	5450	1320	18
		(37)	(87)	(81)	(100)	(22)	(11)
Matrix space	2.2	11.2	1.7	n.d.	n.d.	4350	2
		(59)	(1)	(0)	(0)	(71)	(2)
Inner membranes	2.0	0.8	13.0	4.0	n.d.	440	137
		(4)	(12)	(9)	(0)	(7)	(87)
	Recovery in first supernatant+matrix space+inner membranes as percentage of amount in whole mitochondria						
Recovery	74	107	96	92	147	102	52

Table 5. *Distribution of acetyl-CoA deacylase in rat liver mitochondria and mitoplasts*

Conditions were the same as those of Table 4, except that only 1.7mg of digitonin/mg of mitochondrial protein was used. Results are from a single experiment. Note that monoamine oxidase activity under these conditions is recovered mainly in the inner-membrane fraction, although adenylate kinase can be found almost quantitatively in the first supernatant, as in the experiments shown in Tables 3 and 4. Values in parentheses refer to activity as percentage of total activity in first supernatant+matrix space+inner membranes.

		Enzyme activities (nmol of substrate converted/min per mg of protein)					
Fraction	Protein (mg/ml)	Acetyl-CoA deacylase	Acid phosphatase	Monoamine oxidase	Adenylate kinase	Glutamate dehydrogenase	Succinate dehydrogenase
Mitochondria	18	3.1	27.2	4.2	1750	1940	33
First supernatant	1.7	6.6	95.3	4.3	7200	1180	14
		(33)	(87)	(23)	(99)	(13)	(9)
Matrix space	3.2	11.7	1.7	0.6	2	6650	4
		(58)	(1)	(4)	(<1)	(74)	(2)
Inner membranes	4.9	1.8	13.0	13.4	n.d.	1120	140
		(9)	(12)	(13)	(0)	(13)	(89)
		Recovery in first supernatant+matrix space+inner membranes as percentage of amount found in whole mitochondria					
Recovery	61	141	88	83	104	98	85

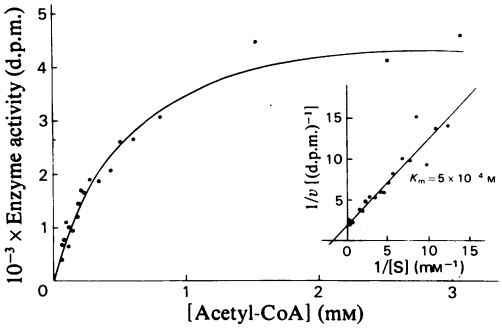

Fig. 2. *Dependence of the reaction velocity of mitochondrial acetyl-CoA deacylase from rat liver on the concentration of acetyl-CoA*

The assay conditions were as described in the Experimental section. The assay mixture contained 57μg of mitochondrial protein. The mitochondria were prepared from Triton WR-1339-pretreated rats (see the Experimental section) and solubilized by addition of 0.1% (w/v) sodium deoxycholate. The insert shows a double-reciprocal plot of the data.

ratio was used (Table 5), which was still sufficiently high to remove the contents of the intermembrane space (as indicated by the release of adenylate kinase), but left most of the outer membrane attached to the mitoplasts, as indicated by the appearance of most of the monoamine oxidase activity in the mitoplast pellet.

Since the distribution of acetyl-CoA deacylase between the first post-digitonin supernatant and the mitoplasts did not significantly vary when different digitonin/protein ratios were used (compare Tables 4 and 5), acetyl-CoA deacylase activity in the first supernatant after digitonin treatment cannot come from the outer mitochondrial membrane and, since the acetyl-CoA deacylase activity in the first post-digitonin supernatant results almost completely from lysosomal contamination, the true mitochondrial acetyl-CoA deacylase activity must reside nearly exclusively in the mitochondrial matrix space.

The distribution of acetyl-CoA deacylase activity among the various subfractions of mitochondria was determined in nine experiments. The mean specific activities were 4.0±0.3, 5.5+0.5, 11.9+0.9 and 1.2±0.2nmol/min per mg of protein for total mitochondria, first supernatant, matrix space and inner mitochondrial membranes respectively. Taking the sum of acetyl-CoA deacylase activities in the first supernatant+matrix space+inner membranes as 100%, the following distribution was obtained: first supernatant 34±3%, matrix space 59±2%, inner mitochondrial membranes 7±2%.

Acetyl-CoA deacylase activity of highly purified liver mitochondria from Triton WR-1339-injected rats showed a dependence on the acetyl-CoA concentration that followed Michaelis–Menten kinetics, with an apparent K_m value for acetyl-CoA of 0.5mM (Fig. 2). Acetyl-CoA-deacylating activity in purified lysosomes from Triton WR-1339-pretreated rats showed a much lower affinity for acetyl-CoA (Fig. 3), with an apparent K_m of about 15mM. This made it likely that lysosomal acetyl-CoA deacylase activity represents enzyme protein(s) different from mitochondrial acetyl-CoA deacylase. In accordance with this assumption is the finding that lysosomal

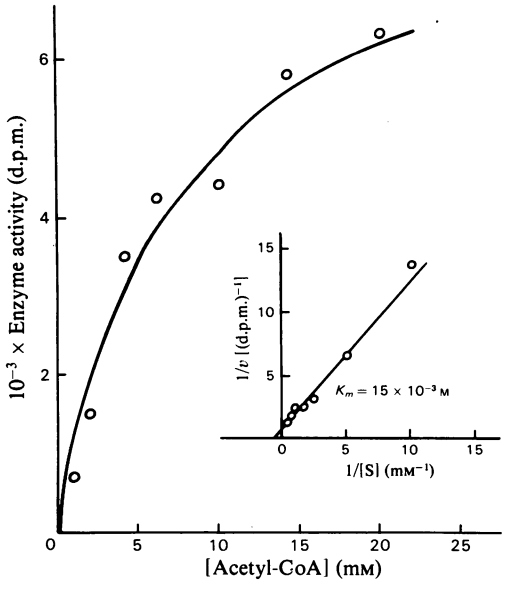

Fig. 3. *Dependence of the reaction velocity of lysosomal acetyl-CoA-deacylating activity from rat liver on the concentration of acetyl-CoA*

The assay conditions were as described in the Experimental section. The assay mixture contained 28 μg of lysosomal protein. Lysosomes were prepared from Triton WR-1339-pretreated rats (see the Experimental section) and solubilized by addition of 0.1% sodium deoxycholate. The insert shows a double-reciprocal plot of the data.

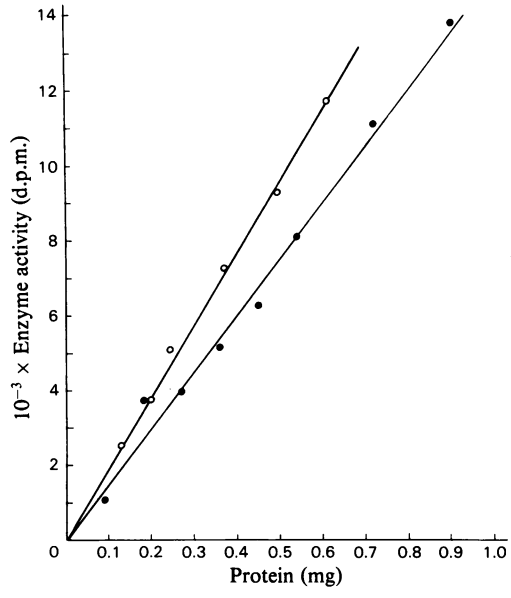

Fig. 4. *Effects of gel filtration on mitochondrial acetyl-CoA deacylase activity*

Mitochondria purified from livers of Triton WR-1339-treated rats were sonicated and then centrifuged at 100000*g* for 60min. A 2ml portion of the supernatant (25mg of protein in 2ml of 25mM-phosphate/2mM-dithioerythritol, pH7.4) was chromatographed on a column (2cm × 30cm) of Sephadex G-25 (medium grade) that had been equilibrated with the same phosphate buffer. Corresponding amounts of gel-filtered (○) and unfiltered (●) mitochondrial protein were measured for acetyl-CoA deacylase activity.

Table 6. *Acetyl-CoA deacylase activity with substrates of different chain length in mitochondria and lysosomes from rats*

The animals had been treated before the experiment with Triton WR-1339 to separate mitochondria and lysosomes (see the Experimental section). Enzyme activity was tested in the optical assay system (see the Experimental section) at 0.5mM substrate concentration (means ± S.E.M., $n = 3$).

	Acetyl-CoA deacylase activity (nmol/mg of protein per min)	
Substrate	Mitochondria	Lysosomes
Acetyl-CoA	14.2 ± 2.7	6.2 ± 0.9
n-Butyryl-CoA	2.0 ± 0.7	5.8 ± 0.6
n-Hexanoyl-CoA	1.1 ± 0.4	6.1 ± 0.7

extracts hydrolysed n-butyryl- and hexanoyl-CoA at almost the same rate as acetyl-CoA when tested at a concentration of 0.5mM, whereas a mitochondrial extract when tested under the same conditions with n-butyryl- and hexanoyl-CoA exhibited only 14 and 8% respectively of the activity observed with acetyl-CoA as substrate (Table 6). Costa & Snoswell (1975) have claimed that mitochondrial acetyl-CoA deacylase activity is mimicked by the combined action of acetyl-CoA–L-carnitine acetyltransferase and acetylcarnitine hydrolase. Therefore we have treated purified rat liver mitochondria with 0.1% deoxycholate and filtered the extract through a Sephadex G-25 column (2cm × 30cm) for removal of free L-carnitine and other low-molecular-weight compounds. In contrast with the results of Costa & Snoswell (1975), gel filtration did not increase, but rather abolished, mitochondrial acetyl-CoA deacylase activity (Fig. 4), indicating that acetyl-CoA deacylation occurs without any involvement of carnitine.

Discussion

We (Seufert *et al.*, 1977) have described the formation of free acetate in addition to citrate by isolated rat liver mitochondria, and Hoffmann & Weiss (1978), who used a system in which acetate released by iso-

lated rat liver mitochondria was continuously removed by an enzymic trapping system, have reported rates of formation of free acetate as high as 0.47 and 0.54 nmol/unit of glutamate dehydrogenase/min with pyruvate and hexanoate as substrate respectively. These values can be calculated from the data of Hoffmann & Weiss (1978) under the assumption of a specific activity of glutamate dehydrogenase of 5.7 units/mg of mitrochondrial protein. In accordance with these results and with previous reports (Seufert *et al.*, 1974) dealing with hepatic net production of free acetate we could demonstrate acetyl-CoA deacylase activity in rat liver. However, the activity of this enzyme in total liver extract was considerably lower than the activity reported by Knowles *et al.* (1974). This discrepancy is most likely to result from two reasons: (1) Knowles *et al.* (1974) used an assay system that was much less specific (colorimetric determination of liberated CoA); (2) Knowles *et al.* (1974) used an acetyl-CoA concentration as high as 4 mM. At this concentration a considerable part of the total acetyl-CoA deacylase activity in liver represents lysosomal hydrolytic activity.

Knowles *et al.* (1974) found most of the acetyl-CoA deacylase activity in the 'P' fraction [= particulate fraction according to Pette (1966)]. This is in accordance with our findings. However, they were not aware of the fact that their particulate fraction represented not only mitochondrial but also lysosomal acetyl-CoA deacylase activity. Since their way of extracting the enzymes from the 'P' fraction released proteins not only from mitochondria, but also from lysosomes, peroxisomes and microsomal fraction, the concomitant liberation of acetyl-CoA deacylase activity together with mitochondrial marker enzymes cannot be taken as proof for the intramitochondrial location of acetyl-CoA deacylase, especially in view of the fact that no marker enzyme for the other particulate fractions had been measured.

We have now clearly separated specific acetyl-CoA deacylase activity located in the mitochondria from unspecific acyl-CoA deacylase activity located in the lysosomal fraction.

Since the specific acetyl-CoA deacylase activity is located almost exclusively in the mitochondrial matrix space (see Tables 4 and 5), it seems most likely that under physiological conditions hepatic formation of free acetate occurs in this compartment. This fits our expectation, as acetyl-CoA formation occurs mainly in the mitochondrial matrix, and the concentrations of acetyl-CoA in the mitochondria (0.86–2.74 mM) were 10.8–35.4 times those in the cytosol (Siess *et al.*, 1976).

The above values for intramitochondrial concentrations of acetyl-CoA are well within the range of the apparent K_m for acetyl-CoA reported in the present paper for mitochondrial acetyl-CoA deacylase.

Although we found a rather high specificity for the hepatic mitochondrial enzyme for acetyl-CoA, Robinson *et al.* (1976) described a short-chain acyl-CoA deacylase in rat brain mitochondria that hydrolysed not only acetyl-CoA, but also propionyl-, butyryl-, acetoacetyl-, succinyl-, malonyl- and octanoyl-CoA, with K_m values for all substrates below 0.1 mM. Unfortunately, the original experimental results were not presented in their paper.

We could not confirm the findings of Costa & Snoswell (1975) according to which mitochondrial acetyl-CoA deacylase activity was considered to be an artifact resulting from the combined actions of an acetyl-CoA–L-carnitine acetyltransferase at the inner mitochondrial membrane and an acetylcarnitine hydrolase at the outer mitochondrial membrane. Removal of low-molecular-weight compounds by gel filtration did not diminish acetyl-CoA deacylase activity of broken mitochondria (Fig. 4). The concept of Costa & Snoswell (1975) would also be incompatible with the exclusive occurrence of acetyl-CoA deacylase in the matrix space that has been shown in the present paper (Tables 4 and 5). Furthermore, a combination of outer and inner mitochondrial membranes had only negligible acetyl-CoA deacylase activity in our hands. Finally, we could clearly separate acetyl-CoA–L-carnitine acetyltransferase activity from acetyl-CoA deacylase activity during purification of acetyl-CoA deacylase from ox liver mitochondria (K. Koppe & C. D. Seufert, unpublished work).

Recently, Snoswell & Tubbs (1978) reported also on the existence of acetyl-CoA deacylase activity in mitochondria from sheep and rat liver. Since acetyl-CoA deacylase activity was not abolished by gel filtration, the authors concluded that mitochondrial acetyl-CoA deacylase represents enzymic activity that is distinct from acetylcarnitine hydrolase.

Acetyl-CoA deacylase activity in highly purified rat liver lysosomes apparently represents a rather unspecific acetyl-CoA hydrolase activity, as indicated by the substantial hydrolysis of n-butyryl-CoA and n-hexanoyl-CoA, and by the extremely low affinity for acetyl-CoA. This makes it unlikely that lysosomes play a significant role in the hepatic formation of free acetate under physiological conditions. Acetyl-CoA deacylase activity in the cytosol does not seem to result from mitochondrial contamination in view of the low glutamate dehydrogenase activity in these fractions (Table 1). However, the mitochondrial contamination of the cytosolic fraction may have been underestimated to some extent, as some of the glutamate dehydrogenase released from damaged mitochondria could have been adsorbed by mitochondrial membranes, as has been described by Walter & Anabitarte (1971). Any further progress

concerning the characteristics and the metabolic role of mitochondrial acetyl-CoA deacylase in liver requires its purification, especially since nothing is known yet about a possible control of hepatic mitochondrial acetyl-CoA deacylase activity by its products or by other metabolites. The finding by Bernson (1976) that acetyl-CoA deacylase from hamster brown adipose tissue can be regulated *in vitro* by ADP, NADH and CoA warrants further research along these lines.

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References

Andersch, M. A. & Szczypinski, A. J. (1947) *Am. J. Clin. Pathol.* **17**, 571–574

Baginski, E. S., Foa, P. P. & Zak, B. (1974) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H. U., ed.), pp. 909–913, Verlag Chemie, Weinheim

Barrett, A. J. (1972) in *Lysosomes: A Laboratory Handbook* (Dingle, J. T., ed.), pp. 46–135, North-Holland Publishing Co., Amsterdam

Bergmeyer, H. U. (1974) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H. U., ed.), pp. 517–521, Verlag Chemie, Weinheim

Bergmeyer, H. U. & Bernt, E. (1974) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H. U., ed.), pp. 607–612, Verlag Chemie, Weinheim

Bernson, V. S. M. (1976) *Eur. J. Biochem.* **67**, 403–410

Brdiczka, D., Pette, D., Brunner, G. & Miller, F. (1968) *Eur. J. Biochem.* **5**, 294–304

Costa, N. D. & Snoswell, A. M. (1975) *Biochem. J.* **152**, 167–172

Costa, N. D., McIntosh, G. H. & Snoswell, A. M. (1976) *Aust. J. Biol. Sci.* **29**, 33–42

Decker, U. (1959) in *Die Aktivierte Essigsäure: Das Coenzym A und seine Acylderivate im Stoffwechsel der Zelle*, pp. 84–89, Verlag Ferdinand Enke, Stuttgart

de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617

Fröhlich, J. & Wieland, O. (1975) *Z. Klin. Chem. Klin. Biochem.* **13**, 241

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766

Hepp, D. Prüsse, E., Weiss, H. & Wieland, O. (1966) *Biochem. Z.* **344**, 87–102

Hoffmann, G. & Weiss, L. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 733–740

Knowles, S. E., Jarrett, I. G., Filsell, O. H. & Ballard, F. J. (1974) *Biochem. J.* **142**, 401–411

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Lynen, F., Srere, P. A. & Seubert, W. (1959) *Biochim. Biophys. Acta* **33**, 313–319

Pette, D. (1966) in *Regulation of Metabolic Processes in Mitochondria* (Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C., eds.), pp. 28–50, Elsevier Publishing Co., Amsterdam

Robinson, J. B. Jr., Mahan, D. E. & Koeppe, R. E. (1976) *Biochem. Biophys. Res. Commun.* **71**, 959–965

Schnaitman, C. & Greenawalt, J. W. (1968) *J. Cell Biol.* **38**, 158–175

Seufert, C. D., Graf, M., Janson, G., Kuhn, A. & Söling, H. D. (1974) *Biochem. Biophys. Res. Commun.* **57**, 901–909

Seufert, C. D., Grigat, K. P., Koppe, K. & Söling, H. D. (1976) *Abstr. Int. Congr. Biochem. 10th* p. 244

Seufert, C. D., Grigat, K. P., Koppe, K., Bufe, N. & Söling, H. D. (1977) *Diabetologia* **13**, 431

Siess, E., Brocks, D. & Wieland, O. H. (1976) *FEBS Lett.* **69**, 265–271

Simon, E. J. & Shemin, D. J. (1953) *J. Am. Chem. Soc.* **75**, 2520

Snoswell, A. M. & Tubbs, P. K. (1978) *Biochem. J.* **171**, 299–303

Söling, H. D., Kleineke, J., Willms, B., Janson, G. & Kuhn, A. (1973) *Eur. J. Biochem.* **37**, 233–243

Tabor, C. W., Tabor H. & Rosenthal, S. M. (1954) *J. Biol. Chem.* **208**, 645–661

Vignais, P. M. & Nachbaur, J. (1968) *Biochem. Biophys. Res. Commun.* **33**, 307–314

Walter, P. & Anabitarte, M. (1971) *FEBS Lett.* **12**, 289–292

Walter, U. & Söling, H. D. (1976) *FEBS Lett.* **63**, 260–266

Wattiaux, R., Wibo, M. & Baudhuin, P. (1963) *Lysosomes Ciba Found. Symp. 1963* 176–200

Widnell, L. L. & Tata, J. R. (1964) *Biochem. J.* **92**, 313–317