MAMMALIAN FATTY ACID ACTIVATION

Studies on substrate specificity and subcellular localization

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CONTENTS

Paper IV

P.H.E. Groot, The activation of short-chain fatty acids by the soluble fraction of guinea-pig heart and liver mitochondria: the search for a distinct propionyl-CoA synthetase. (1975) Biochim. Biophys. Acta 380, 12-20.

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INTRODUCTION

In this thesis studies are described on mammalian fatty acid: CoA ligases (AMP forming), enzymes which catalyze the following general reaction:

$$RCOO^- + ATP^{4-} + CoASH \overset{Mg^{2+}}{\rightleftharpoons} RCOSCoA + AMP^{2-} + PP_i^{3-} \quad (1)$$

Other names used for these enzymes in the literature are fatty acid thiokinases or fatty acyl-CoA synthetases. In this work we will use the recommended name (fatty) acyl-CoA synthetase[1].

Several different types of fatty acyl-CoA synthetases, all catalyzing acyl-CoA formation according to reaction (1), have been described. These enzymes differ in specificity towards the substrate fatty acids.

Although the equilibrium constant of reaction (1) is near unity, which indicates full reversibility, acyl-CoA formation is favoured by a rapid metabolism of the reaction products pyrophosphate, AMP and acyl-CoA.

In contrast to the free fatty acid, fatty acyl-CoA is very reactive by the presence of the energy-rich thioester bond. That is why reaction (1) is also known as the fatty acid activation reaction. It fulfills a central role in fatty acid metabolism in practically all organisms.

Although mammalian fatty acid activation has been studied during the last two decades intensively, several questions still remained. Three general questions can be formulated:

1. What is the fatty acid substrate specificity of the different mammalian acyl-CoA synthetases and for which acids is the affinity high enough to guarantee substantial activation by these enzymes in vivo.

2. What is the tissue distribution and the subcellular localization of the different mammalian acyl-CoA synthetases.
3. What is the function of the different mammalian acyl-CoA synthetases and how are they regulated.

Our studies, presented in this thesis, are mainly concerned with question 1. Some studies concerning question 2, carried out in co-operation with Dr. H.R. Scholte, are also presented.

In Appendix Paper I by Groot, Scholte and Hülsmann[2], a review of the literature on mammalian fatty acid activation is given. In this paper our experiments on mammalian acyl-CoA synthetases are discussed in connection with results from other studies. The introduction, given here, is very limited. For a more complete introduction into the field of mammalian acyl-CoA synthetases we will refer to Appendix Paper I.

Information about the molecular and enzymatic properties of fatty acyl-CoA synthetases can also be found in a recent paper of Londesborough and Webster[3]. Other reviews in this field are given in the reference list of Appendix Paper I.

CHAPTER I

SCOPE OF THE PRESENT STUDY

Because of their central role in fatty acid metabolism, knowledge of the properties of the different types of mammalian acyl-CoA synthetases seems to be of great importance. Studies on fatty acid activation in mammalian tissues are complicated by the existence of several types of acyl-CoA synthetases with often overlapping fatty acid specificities. At least four different mammalian acyl-CoA synthetases (AMP forming) can be distinguished. These enzymes have been purified and their existence is generally accepted. This group includes:

1. acetyl-CoA synthetase (EC 6.2.1.1). This enzyme has been purified from beef heart[4-8]. Both acetate and propionate are accepted as substrate, although acetate is the favoured substrate (see Appendix Paper I Table 1).
2. butyryl-CoA synthetase. This enzyme has also been purified from beef heart and separated from acetyl-CoA synthetase[9]. Propionate up to heptanoate are substrates. Butyrate is the favoured substrate.
3. medium-chain acyl-CoA synthetase. This enzyme, purified from beef-[10], human-[11], pig-[12] and rabbit liver[13], performs a very broad substrate specificity towards carboxylic acids. Butyrate up to laurate, several branched-, hydroxy- and unsaturated fatty acids in the C_4-C_{12} range, and many aromatic carboxylic acids are all accepted as substrates. The highest $\underline{V}$ and lowest $\underline{K}_m$ were found for heptanoate and octanoate respectively[10].

4. palmitoyl-CoA synthetase or long-chain acyl-CoA synthetase (EC 6.2.1.3). This enzyme has been partially purified from rat-liver microsomes[14-16]. High rates of acyl-CoA formation were found with laurate up to stearate with an optimum for palmitate. Oleate and linoleate are also good substrates for this enzyme. Low rates of acyl-CoA formation were found with butyrate and lignocerate (C_{24}).

Several other types of mammalian acyl-CoA synthetases have been proposed but their existence has been less well established. This group includes a lauroyl-CoA synthetase[17-20], a propionyl-CoA synthetase[21,22], a salicylate activating enzyme[23] and a long-chain acyl-CoA synthetase specific for cis-unsaturated long-chain fatty acids[24]. In connection with our work, several of these enzymes will be discussed in more detail later in this section.

The subcellular localization of the four generally accepted acyl-CoA synthetases is not the same. Palmitoyl-CoA synthetase or better palmitoyl-CoA synthetase activity was found to be associated both with the microsomal membranes and the mitochondria in liver[19,25,26] and kidney[19] of the rat. In heart and skeletal muscle of the rat most of the palmitate activation was found in the mitochondria[19,27]. The intramitochondrial localization of the palmitoyl-CoA synthetase activity has been extensively studied in rat-liver (for references consult Appendix Paper I, section V,B). The bulk of activity was found to be associated with the outer membrane; a minor contribution (about 10% of the mitochondrial palmitate activation) was localized in the inner membrane-matrix compartment of rat-liver mitochondria[28]. This latter activity could explain the carnitine-independent long-chain fatty acid oxidation by liver mitochondria, observed in many other studies (for references see Appendix Paper I, section V,B).

We studied this minor palmitoyl-CoA synthetase activity in rat-liver mitochondria in more detail (Groot et al.[29] Appendix Paper III) and were able to demonstrate that this activity can be ascribed to an overlap in activity towards long-chain fatty acids of the medium-chain acyl-CoA synthetase, present in the matrix fraction of (rat) liver mitochondria. The fatty acid substrate specificity of at least rat-liver medium-chain acyl-CoA synthetase is therefore broader than reported by Mahler et al.[10] for the beef-liver enzyme (see above). The $\underline{K}_{\underline{m}}$ for palmitate of the rat-liver medium-chain acyl-CoA synthetase turned out to be high (23 μM) compared with the values found in rat liver microsomes (1.25 μM)[30] using a similar assay and we concluded[29] that the role of medium-chain acyl-CoA synthetase in palmitate metabolism of the liver will be very modest (Appendix Paper III). The subcellular localization of palmitoyl-CoA synthetase is discussed in more detail in Appendix Paper I, section V,B.

The existence of a medium-chain acyl-CoA synthetase has only been unambiguously demonstrated in liver mitochondria, from which it has been purified. The presence of such an enzyme in heart- and kidney mitochondria of the rat had been suggested by Aas[19]. The hindleg muscles of the rat, in which white muscle fibers dominate, are not equipped with this enzyme. Hence, Aas[19] could only find a sluggish rate of octanoate activation in a homogenate of these muscles. We have looked for the presence of this enzyme in a more reddish type of skeletal muscle, the musculus masseter of the rat. Red type skeletal muscles are more equipped for long-lasting aerobic metabolism[31] and their acyl-CoA synthetase outfit might therefore resemble that of heart muscle. In fatty acid oxidation experiments with isolated M. masseter mitochondria it was found that octanoate, the favoured substrate of medium-chain acyl-CoA synthetase[10], could only be oxidized in the presence of L-carnitine (Groot and Hülsmann[32], Appen-

dix Paper II). This result excluded the presence of a medium-chain acyl-CoA synthetase in the matrix fraction of these mitochondria. In a further study of the fatty acid activation by these mitochondria it was found that palmitate and octanoate are competitive substrates for one and the same acyl-CoA synthetase, which is localized on the outer membrane. The affinity for palmitate of this enzyme was much higher than for octanoate, which resulted in a strong inhibition of octanoate activation at low concentrations of palmitate. Our studies also questioned the existence of a mitochondrial outer membrane lauroyl-CoA synthetase in addition to a palmitoyl-CoA synthetase, as supposed by others[7-20] (Appendix Paper II). The substrate specificity of long-chain acyl-CoA synthetase will be discussed in more detail in Appendix Paper I, section IV A 3.

Short-chain (C_2-C_4) fatty acid activation in mammalian tissues was found both in the cytosol and in the mitochondria[19,33-38]. Inside the mitochondria short-chain acyl-CoA synthetases were localized in the matrix compartment[19,22,33]. At least two different short-chain acyl-CoA synthetases can be involved in short-chain fatty acid activation: acetyl-CoA synthetase and butyryl-CoA synthetase. Both enzymes are able to activate propionate. However, the velocities of activation with this substrate were only 30% of the velocities found with the favoured substrates which are acetate and butyrate respectively[6,9]. Medium-chain acyl-CoA synthetase can also contribute to the butyrate activation[10] ($\underline{K}_m$ butyrate = 1.59 mM) and even to the acetate activation ($\underline{K}_m$ acetate = 20 mM)[39]. All short- and medium-chain acyl-CoA synthetases, discussed here, were derived from mitochondrial preparations which were obtained from heart (acetyl- and butyryl-CoA synthetase) or liver (medium-chain acyl-CoA synthetase). Scholte et al.[22] observed that the rate of propionate activation in mitochondria obtained from several mammalian tissues is often too high compared with the acetyl- and butyryl-CoA synthetase activities, to be

explained by contributions of only acetyl- and butyryl-CoA synthetases. In mitochondria isolated from guinea-pig heart, the rate of propionate activation could even exceed the sum of the rates of acetate plus butyrate activation. From these results and from differences in effects of different buffers on the rates of acetate- en propionate activation, Scholte et al. concluded the existence of a distinct propionyl-CoA synthetase. From similar evidence Cook et al.[21] proposed a propionyl-CoA synthetase for sheep-lung.

We have checked this hypothesis in a soluble fraction obtained from guinea-pig heart mitochondria. Using substrate competition experiments as a tool to decide whether two fatty acids are activated by one or by two acyl-CoA synthetases, we were able to demonstrate that the high propionate activation in this fraction can still be explained by the sum of the contributions of an acetyl- and a butyryl-CoA synthetase.
We concluded[40] that a distinct propionyl-CoA synthetase is absent in guinea-pig heart mitochondria (Appendix Paper I, section IV A 1 c, Appendix Paper IV).
Similar kinetic studies, initiated by the appearance of a paper of Ash and Baird[41], in which a distinct propionyl-CoA synthetase was proposed for beef-liver mitochondria, were performed in a soluble fraction of guinea-pig-liver mitochondria. From the low K_m for propionate (0.6 mM) compared with that for acetate (11.3 mM) and butyrate (5.4 mM) and some fatty acid substrate competition studies, we concluded the presence of a distinct propionyl-CoA synthetase in guinea-pig-liver mitochondria (Appendix Paper I, section A 1 c, Appendix Paper IV).

A full characterization of substrate specificity of this propionyl-CoA synthetase was hampered by the presence of the medium-chain acyl-CoA synthetase in the soluble fraction of guinea-pig-liver mitochondria. Therefore a

purification of propionyl-CoA synthetase from guinea-pig-liver mitochondria was started*. The medium-chain acyl-CoA synthetase could be separated from the propionyl-CoA synthetase by ion-exchange chromatography on phosphocellulose at pH 6.5, using a linear KCl gradient in 10 mM potassium phosphate buffer (Chapter II, Fig. 1).
A third acyl-CoA synthetase, shown to be identical in substrate specificity with the salicylate activating enzyme, partially purified by Killenberg et al.[23] from beef-liver mitochondria, was copurified with the propionyl-CoA synthetase (Chapter II, Fig. 1). The latter two acyl-CoA synthetases could be separated on DEAE-Sephadex by elution with 100 mM tris-HCl of pH 8.0 (Chapter II, Fig. 2).

Compared with the soluble fraction of guinea-pig-liver mitochondria, a 30-fold purification of both propionyl-CoA synthetase and the salicylate activating enzyme was obtained (Chapter II, Table I). Purified propionyl-CoA synthetase was shown to be free of other short-chain fatty acid activating enzymes, which was concluded from fatty acid substrate competition studies (Chapter II, Figs. 3-5, Table IV). The following kinetic parameters were obtained: acetate ($\underline{V}$ = 72%, $\underline{K}_m$ = 32 mM) propionate ($\underline{V}$ = 100%, $\underline{K}_m$ = 0.43 mM) butyrate ($\underline{V}$ = 44%, $\underline{K}_m$ = 4.6 mM).
No activity could be detected with hexanoate and octanoate. From our studies we concluded that guinea-pig-liver mitochondria are equipped with three soluble acyl-CoA synthetases: propionyl-CoA synthetase, medium-chain acyl-CoA synthetase and the salicylate activating enzyme. Acetyl-CoA synthetase and butyryl-CoA synthetase are absent in these liver mitochondria.

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* Recently we became aware of the thesis of Latimer[42], dealing with the purification of a distinct propionyl-CoA synthetase from sheep-liver mitochondria. His studies will be discussed in Chapter II.

The distribution of short-chain acyl-CoA synthetases in other organs of the rat and guinea-pig was studied in co-operation with Dr. H.R. Scholte (Appendix Paper I, section V A, Groot et al.[43], Scholte and Groot[44]). This study had two main purposes:

1. How is short-chain fatty acid activation distributed between the cytosol and the mitochondria in different tissues.
2. Which types of short-chain acyl-CoA synthetases are found in different tissues.

Especially the literature on subcellular distribution of short-chain acyl-CoA synthetases is very confusing as will be discussed in Appendix Paper I, section V A and VI. From our studies[43,44] we concluded that all tissues studied are equipped with at least two mitochondrial short-chain acyl-CoA synthetases (Appendix Paper I, section V A and VI). Mitochondrial acetyl- and butyryl-CoA synthetase are present in heart, kidney, skeletal muscle, adipose tissue, small intestinal epithelium and lactating mammary gland: A mitochondrial propionyl-CoA synthetase is present in liver and probably also in adipose tissue. In lactating mammary gland, small intestinal epithelium, adipose tissue and liver we obtained evidence for a cytosolic acetyl-CoA synthetase. All short-chain acyl-CoA synthetase activity found in the cytosol of heart, kidney and skeletal muscle could be explained by leakage of short-chain acyl-CoA synthetases from the matrix mitochondrialis of damaged mitochondria into the supernatant fraction.

In Table I and II are summarized the results of our studies on fatty acid substrate specificity and of the localization of mammalian acyl-CoA synthetases. Several results taken from the literature are incorporated in these tables.

One can speculate about the function of all these enzymes. Mitochondrial acetyl- and butyryl-CoA synthetases may be involved in the oxidation of acetate and butyrate

TABLE I

SUBSTRATE SPECIFICITY TOWARDS FATTY ACIDS AND KINETIC DATA OF MAMMALIAN ACYL-CoA SYNTHETASES

Fatty[1] acid	acetyl-CoA synth.				propionyl-CoA synth.				butyryl-CoA synth.			medium-chain acyl-CoA synth.				palmitoyl-CoA synth.				salicylate act.enzyme			
	K_m mM		v^2 %		K_m mM		v^2 %		K_m mM		v^2 %	K_m mM		v^2 %		K_m mM		v^2 %		K_m mM		v^2 %	
C_2	0.7	(0.8)	100	(100)	32		72	(5)	-	-	(0)			0.2								0	
C_3	~7	(11)	100	(30)	0.43	(0.12)	100	(100)	~7	-	(30)			3								16	
C_4	-	-	-	(0)	4.6	-	44	(12)	0.5	(1.5)	(100)		(1.6)	35	(26)				-	~5		74	
C_6					-		0	(8)	-	-	(60)		-	100		-	(0.02)		(0.3)			100	100
C_7							-	-		-	(10)	-	(0.4)	-	(100)	-		-	-			-	
C_8							0	(4)		-	(0)	0.008	(0.15)	58	(43)	0.2	(0.011)	29	(7)			6	
C_{12}							-					-	-	14	-	-	(0.0046)	100	(100)				
C_{16}												0.023		1	0	0.007	(0.0012)	100	(91)				
benzoate							0					-		14	-							122	32
sali-cylate							0					-		0.02	-						(0.0014)	8	3
legend reference	a	b	a	c	d	e	d	e	a	f	f	g	h	k	h	l	m	l	m	n	o	n	p

The values that are taken from the literature are in parentheses.
1) chain-length of saturated straight-chain fatty acid, 2) observed velocities of acyl-CoA formation plotted as percentage of the velocity found with the favoured fatty acid. When a real $\underline{V}$ has been determined the value has been underlined.
a) guinea-pig heart mitochondria, Groot[40], Appendix Paper IV, b) purified beef-heart enzyme, Campagnari and Webster[6], c) purified beef-heart enzyme, Webster et al.[9], v determined at 10 mM fatty acid concentration, d) purified guinea-pig liver enzyme, this thesis Chapter II, e) purified sheep-liver enzyme, Latimer[42], v determined at 3.3 mM fatty acid concentration, f) purified beef-heart enzyme, Webster et al.[9], v determined at 16.8 mM fatty acid concentration, g) partially purified enzyme from rat-liver mitochondria, Groot et al.[29], Appendix Paper III, h) purified beef-liver enzyme, Mahler et al.[10], k) partially purified guinea-pig liver enzyme, this thesis Chapter II, v determined at different carboxylic acid concentrations, l) skeletal muscle mitochondria of the rat, Groot and Hülsmann[32], Appendix Paper II, palmitate and laurate were complexed with bovine serum albumin, m) rat-liver microsomes, Suzue and Marcel[30], uncomplexed fatty acids were used, n) purified guinea-pig liver enzyme, this thesis Chapter II, v values determined at different carboxylic acid concentrations, o) beef-liver enzyme, Londesborough and Webster[3], p) beef-liver enzyme, Killenberg et al.[23], v determined at 2 mM (C_6 and benzoate) or 0.5 mM (salicylate).

TABLE II

ORGAN- AND SUBCELLULAR LOCALIZATION OF ACYL-CoA SYNTHETASES.

Organ	Subcellular fraction			
	mitochondria		microsomes	cytosol
	outer membr.	matrix		
Liver	C_{16}[a]	C_3,Sal,C_8[b]	C_{16}[c]	C_2[d]
Heart	C_{16}[e]	C_2,C_4[f],C_8[g]	C_{16}[h]	-[f]
Kidney	C_{16}[k]	C_2,C_4[f],C_8[l]	C_{16}[k]	-[f]
Skeletal muscle	C_{16}[m]	C_2,C_4[f]	-[k]	-[f]
Adipose tissue	C_{16}[n]	C_2,C_3,C_4[f]	C_{16}[n]	C_2[d]
Lactating mammary gland	C_{16}[o]	C_2,C_4[f]	C_{16}[o]	C_2[f]
Small intestinal epithelium	C_{16}[f]	C_2,C_4[f]	C_{16}[p]	C_2[f]

C_2, C_3, C_4, C_8 and C_{16} stand for the presence of acetyl-, propionyl-, butyryl-, medium-chain acyl-, and palmitoyl-CoA synthetase respectively. Sal. indicates the presence of the salicylate activating enzyme; - = no acyl-CoA synthetase present.
a) Norum et al.[45], b) this thesis, Chapter II, c) Farstad et al[25], d) Barth et al.[34]; Groot et al.[2], Appendix Paper I; Scholte and Groot[44], e) Scholte[46], f) Groot et al.[2], Appendix Paper I, Scholte and Groot[44], g) may be present, Aas[19], h) low activity according to Aas[19] and De Jong and Hülsmann[27], k) Aas[19], l) may be present, Aas[19], Kellerman[13], m) Pande and Blanchaer[47]; Groot and Hülsmann, Appendix Paper II, n) practical all activity is microsomal, Lippel et al.[48], o) Scholte and Groot, unpublished results, p) De Jong and Hülsmann[27].

respectively. In herbivores, especailly ruminants, short-chain fatty acids are produced in large amounts by microbial fermentation of cellulose in the alimentory tract. In other animals acetate may be formed during alcohol oxidation or as a product of several intracellular reactions (see Appendix Paper I, section VII A). Involvement of both enzymes in propionate metabolism is questionable, since both enzymes have a high $\underset{\sim}{K_m}$ for propionate. Propionate may be metabolized in the liver mitochondria after activation by propionyl-CoA synthetase. In this organ, propionate can be used for glucose synthesis. It may be of interest to note that kidney mitochondria are not equipped with a propionyl-CoA synthetase (Appendix Paper I, section V A; Groot et al.[43]; Scholte and Groot[44]). However, although kidney is an organ with gluconeogenic properties, propionate may not occur there in significant amounts, since the bulk of intestinal propionate enters the body through the portal vein so that propionate may mainly be removed by the liver. The role of a cytosolic acetyl-CoA synthetase is unknown but its localization in tissues performing active de novo fatty acid synthesis suggests a role of this enzyme in supplying acetyl-CoA for this process.

Medium-chain acyl-CoA synthetase may be involved in the oxidation of medium-chain fatty acids in liver. Its activity towards several aromatic carboxylic acids suggests an additional role in the excretion of unoxidizable acids from vegetable sources (conjugation with glycine after activation). The salicylate activating enzyme may also be involved in this process (see Chapter II).

The role of palmitoyl-CoA synthetase, the central enzyme in long-chain fatty acid metabolism, is obvious. It will provide long-chain acyl-CoA esters for β-oxidation and for synthetic processes. Whether the microsomal- and the mitochondrial palmitoyl-CoA synthetases are specialized in one of these processes is not completely understood and will be discussed in more detail in Appendix Paper I, section VII, c.

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CHAPTER II

THE PURIFICATION OF PROPIONYL-CoA SYNTHETASE

This chapter begins with the experimental procedure leading to a 30-fold purification of guinea-pig liver propionyl-CoA synthetase. The different steps will be discussed afterwards. Two other acyl-CoA synthetases have been shown to contaminate the crude starting fraction: medium-chain acyl-CoA synthetase[1] and the salicylate activating enzyme[2]. Their behaviour during the purification was monitored by measurements of the octanoyl- and salicyloyl-CoA synthetase activities respectively.

PURIFICATION PROCEDURES:

The livers of 4 white guinea-pigs (500-700 g body weight) were weighed after removal of the gallbladders. They were cut in small pieces in ice-cold isolation medium (0.25 M sucrose - 1 mM Tris-HCl of pH 7.5 at $0^{o}C$). 80-90 g of liver tissue was used in most purifications. A 15% w/v homogenate was prepared in isolation medium using a Potter-Elvehjem homogenizer. A mitochondrial fraction without contaminating lysosomes was isolated exactly as described by Loewenstein et al.[3] for rat-liver. The mitochondria were washed 4 times in 320 ml isolation medium, suspended in about 60 ml of ATP-containing salt medium (80 mM Tris-HCl of pH 7.5, 40 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 0.2 mM dithiotreitol) and stored at $-20^{o}C$. The next day the mitochondria were disrupted by thawing, followed by ultrasonic treatment. Membranes were spun down (60 min at 300,000 x $\underline{g_{max}}$) and the supernatant was collected.

700-900 mg of soluble mitochondrial protein was obtained in this procedure and used as the starting material for the purification of propionyl-CoA synthetase.
The specific activities of propionate-, octanoate- and salicylate activation in this fraction were 114 ± 15 mU/mg protein (4), 72 ± 4 mU/mg protein (4) and 2.3 ± 0.3 mU/mg protein (3) respectively (mean ± S.E.M. and number of experiments in parentheses). (In one experiment in which brown instead of white guinea-pigs were used, we observed a 4 times lower propionyl-CoA synthetase activity. Octanoyl- and salicyl-CoA synthetase activities were in the normal range. In the further purification of this propionyl-CoA synthetase normal purification factors were found).

The soluble mitochondrial fraction was adjusted to pH 8 and a saturated ammonium sulphate solution of pH 8 was added to a final concentration of 25 g/100 ml. The temperature was kept at 0°C. The precipitate was spun down (10 min at 30,000 x g_{max}) and discarded. The supernatant solution was brought to an ammonium sulphate concentration of 32 g/100 ml and the precipitate was collected as before. The supernatant was discarded and the precipitate was dissolved in about 20 ml of the ATP-containing salt medium. About 50% of the propionyl-CoA synthetase activity was recovered in this fraction; the specific activity increased about 2.5 times (Table I). 30-40% of the octanoyl-CoA synthetase activity (which we used as a marker activity for medium-chain acyl-CoA synthetase) and salicyloyl-CoA synthetase activity were recovered in the same fraction. The ammonium sulphate fraction was dialyzed overnight against 10 mM potassium phosphate buffer of pH 6.5, containing 0.2 mM dithiotreitol and 0.1 mM EDTA, and subsequently cleared by centrifugation (Table I, 1st dialysis, stage 3). After dilution with phosphate buffer to 100 ml, the fraction was applied to a phosphocellulose column (12 x 2.5 cm) filled with Whatman Phosphocellulose P11,

which was equilibrated with the same buffer. The flow-rate was adjusted to 50 ml/h and the column was washed with phosphate buffer until no more protein was eluted (about 100 ml). More than 90% of the octanoyl- and 100% of the propionyl- and salicyloyl-CoA synthetase activities were bound to the phosphocellulose under these conditions. A linear KCl gradient in phosphate buffer was applied and fractions of 11 ml were collected. All fractions were assayed for C_3-, C_8- and salicyloyl-CoA synthetase activities. The octanoyl-CoA synthetase activity was eluted first (at 100 mM KCl), followed by the elution of both propionyl- and salicyloyl-CoA synthetase activities (at 190 mM KCl). The elution pattern is given in Fig. 1. The recoveries of activities in this step were generally higher than 90%. The 4 to 5 fractions with the highest rate of propionate activation (and also of salicylate activation) were pooled (Table I, stage 4) and protein precipitated by adding a saturated solution of ammonium sulphate (final concentration 38 g/100 ml). This fraction was used in the subsequent purification. Octanoyl-CoA synthetase was precipitated in a similar way and preserved for further studies (see Table V). The acyl-CoA synthetase activities in both precipitates remained rather constant during one month at 4°C.

The precipitated propionyl- and salicyloyl-CoA synthetases were collected by centrifugation and dissolved in a few ml Tris-HCl buffer (100 mM Tris-HCl, 0.2 mM dithiotreitol, 0.1 mM EDTA, pH 8.0 at 0°C). After overnight dialysis against the same buffer, denaturated protein was removed by centrifugation (Table I, 2nd dialysis, stage 5). The dialysate (about 6 ml) was applied to a DEAE-Sephadex column (35 x 2.5 cm, containing 8 g DEAE-Sephadex A-50, Pharmacia) equilibrated with the same Tris-HCl buffer. Elution was performed under starting conditions at a flow-rate of 20 ml/h and fractions of 5.5. ml were collected. Propionyl- and salicyloyl-CoA synthetase acti-

TABLE I

THE PURIFICATION OF PROPIONYL-CoA SYNTHETASE AND THE SALICYLATE ACTIVATING ENZYME FROM A SOLUBLE FRACTION OF GUINEA-PIG-LIVER MITOCHONDRIA.

Stage of purification	Vol.	Total prot.	Propionate activation			Salicylate activation		
			spec.act.	tot.act.	recovery	spec.act.	tot.act.	recovery
	ml	mg	mU/mg	U	%	mU/mg	U	%
1 soluble mitochondrial fraction	63	707	130	92	100	1.98	1.4	100
2 25-32g $(NH_4)_2SO_4$/100 ml	20	153	300	46	50	3.2	0.49	35
3 1st dialysis	22	120	292	35.3	38.4	3.3	0.4	29
4 phosphocellulose	44	9.9	1480	14.6	15.9	22	0.22	16
5 2nd dialysis	6.1	6.7	1030	6.9	7.5	18	0.12	8.6
6 DEAE-Sephadex fraction I	50	0.7	4140	2.9	3.2	-	-	-
DEAE-Sephadex fraction II	61	0.55	-	-	-	62	0.034	2.4

Conditions for the purification are given in the text. Propionyl-CoA synthetase was determined as described by Groot[4] (Appendix Paper IV), using an incubation mixture containing 40 mM tricine-KOH, 10 mM $MgCl_2$, 8 mM ATP, 1.2 mM EDTA, 5 mM phosphoenolpyruvate, 1.35 mM coenzyme A, 8 U/ml of both adenylate kinase and pyruvate kinase, 1.5 U/ml inorganic pyrophosphatase, 2 mM [1-^{14}C]-propionate (0.2 Ci/mole), 5 mM L-carnitine and 4 U/ml carnitine acetyltransferase. The pH of the final mixture was adjusted to 8. The incubation volume was 0.25 ml, the reaction time 10 min at 37°C. Salicyloyl-CoA synthetase was determined according to Killenberg et al.[2] with some modifications. The incubation mixture was similar as that used in the propionyl-CoA synthetase assay except that carnitine, carnitine acetyltransferase and propionate were omitted and 1 mM [carboxyl-^{14}C]-salicylate (0.5 Ci/mole) was added. The incubation volume was 0.25 ml and the reaction time was 10 or 20 min at 37°C. The reaction was stopped with 0.05 ml 2 M $HClO_4$ and unreacted radiosalicylate was removed by 5 subsequent ether extractions. The total waterphase, containing radiosalicyloyl-CoA, was transferred in a counting vial. After the addition of 10 ml Insta-gel, radioactivity was determined by liquid scintillation spectrophotometry.

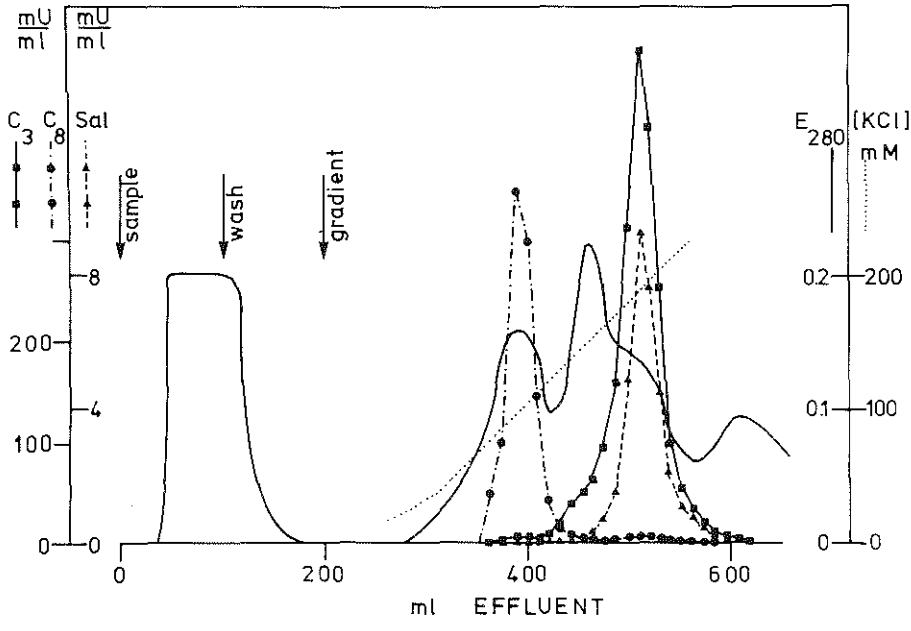


FIG. 1

THE SEPARATION OF MEDIUM-CHAIN ACYL-CoA SYNTHETASE FROM PROPIONYL-CoA SYNTHETASE PLUS THE SALICYLATE ACTIVATING ENZYME BY PHOSPHOCELLULOSE CHROMATOGRAPHY.

Conditions of the phosphocellulose chromatography are given in the text. ■—■, propionyl-CoA synthetase activities; ●-·-·-●, octanoyl-CoA synthetase activities; ▲---▲, salicyloyl-CoA synthetase activities; ———, E_{280}; ....., [KCl] Octanoyl- and propionyl-CoA synthetase activities were determined as shown in Table I at 0.5 and 2 mM fatty acid respectively. Details of the separation of octanoylcarnitine from octanoate are given by Groot and Hülsmann[5] (Appendix Paper II Method II). Salicyloyl-CoA synthetase was determined exactly as shown in Table I.

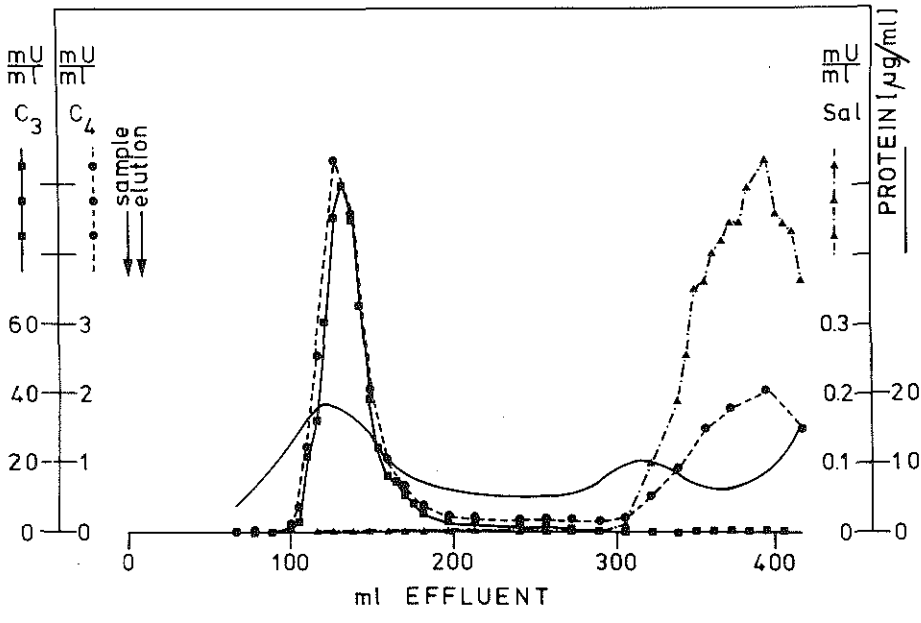


FIG. 2

THE SEPARATION OF PROPIONYL-CoA SYNTHETASE FROM THE SALICYLATE ACTIVATING ENZYME BY DEAE-SEPHADEX CHROMATOGRAPHY.

Conditions of the DEAE-Sephadex chromatography are given in the text. ■——■, propionyl-CoA synthetase activities; ●-·-·-●, butyryl-CoA synthetase activities; ▲---▲, salicyloyl-CoA synthetase activities; ——— , protein.
Protein was measured after precipitation with trichloroacetic acid by a micro-adaptation of the method of Lowry et al.[6].
Propionyl- and butyryl-CoA synthetase activities were determined as given in Table I at 2 mM fatty acid. Salicyloyl-CoA synthetase activities were determined as given in Table I. Not all salicyloyl-CoA synthetase activity had left the column in this experiment.

vities were measured in these fractions. The elution profile is given in Fig. 2.
Propionyl-CoA synthetase and the salicylate activating enzyme were completely separated. Butyrate is activated by both enzymes (which will be discussed later in this chapter). Fractions with the highest rate of propionate activation were pooled (Table I, DEAE-Sephadex fraction I, stage 6). Salicyloyl-CoA synthetase was treated in a similar way (stage 6, fraction II). During the complete purification procedure both propionate- and salicylate activating enzymes were about 30 times purified (Table I). Recoveries, however, were low (about 3%). The purified propionyl-CoA synthetase has shown to be free of other short-chain fatty acid activating enzymes and was used in a substrate specificity study.

The purification of propionyl-CoA synthetase was difficult, due to the instability of the enzyme. Especially the presence of proteolytic activity interfered heavily. For that reason we isolated lysosomal-poor mitochondria for the purification. Because sulfhydryl compounds may have a stabilizing effect on acyl-CoA synthetases[7-10], we added 0.2 mM dithiotreitol to all our media. When fractions had to be preserved for some time, protein was precipitated with ammonium sulphate and stored at 4°C. In the phosphocellulose poolfraction (Table I, stage 4), stored under these conditions during 1 month, 40% losses in specific activities of propionyl- and salicyloyl-CoA synthetase activities were observed.

When we started our purification of propionyl-CoA synthetase, we were only aware of the presence of one other acyl-CoA synthetase in the soluble fraction of guinea-pig liver mitochondria: the medium-chain acyl-CoA synthetase. After the successful separation of both enzymes by phosphocellulose chromatography we have studied the substrate specificity of partially purified propionyl-CoA synthetase. The kinetic parameters, obtained in this

TABLE II

KINETIC PARAMETERS OF PARTIALLY PURIFIED PROPIONYL-CoA SYNTHETASE AFTER PHOSPHOCELLULOSE CHROMATOGRAPHY.

Activated fatty acid	K_m mM	V %
Acetate	35	68
Propionate	0.35,0.47	100
Butyrate	5.0	85
Hexanoate		43
Octanoate		4

The phosphocellulose poolfraction of propionyl-CoA synthetase (Table I, stage 4) was used in all experiments. Acyl-CoA synthetase activities were determined under the conditions described in Table I. 6-9 different fatty acid concentrations, each in duplicate, were used in these studies (acetate, 2-80 mM; propionate, 0.2-40 mM; butyrate, 1-50 mM and hexanoate or octanoate, 1-25 mM). For the C_6 and C_8 activations strong substrate inhibitions interfered with the K_m and V determinations. In this table V is plotted as the percentage of the V for propionate (1180 mU/mg protein). The $^1/v$ versus $^1/S$ plots of acetate and butyrate activations were strictly linear. Plots for propionate activation were strictly linear below 10 mM propionate (these values were used for K_m and V determination). However, at high concentrations of propionate unexpected high velocities were observed.

study are given in Table II. To test whether the observed C_2-C_8 acyl-CoA formation can be ascribed entirely to propionyl-CoA synthetase, we tested the influence of propionate addition on the kinetics of acetate, butyrate and octanoate activation. A simple competitive inhibition by propionate can be expected in that case and the K_i for propionate inhibition should be identical to the K_m for pro-

pionate activation (0.4 mM, see Table II)[11]. Although this could be demonstrated in experiments in which the influence of propionate on acetate activation was tested (results not shown), such a simple inhibition of butyrate activation by propionate was not observed. Only small inhibitory effects were found, while the fact that the K_m for propionate is only one tenth the K_m for butyrate (Table II) predicts a strong inhibition when both fatty acids are activated by one single protein. We concluded from these results the contribution of a second acyl-CoA synthetase to butyrate activation. This second enzyme might even activate propionate at a high K_m which can be conclude from the non-linear kinetics of propionate activation above 10 mM propionate (see legend Table II).

A reinvestigation of the literature revealed the salicylate activating enzyme as a possible candidate. This enzyme, observed by Killenberg et al.[2] in beef-liver mitochondria, has been separated from the medium-chain acyl-CoA synthetase. Although this salicylate activating enzyme had a high affinity for salicylate (K_m for salicylate was 0.0014 mM)[12] the velocities of hexanoate and benzoate activation were much higher than for salicylate activation. We tested the influence of salicylate and benzoate on butyryl- and propionyl-CoA formation and observed strong inhibitory effects on butyrate activation (90% inhibition under the conditions shown in Table III). The inhibition of propionate activation was only moderate (23%). The small inhibitory effects of propionate on butyrate activation, and vice versa, are also shown in this table. Participation of the salicylate activating enzyme during butyrate activation is very likely from these experiments. Moreover, during other purification attempts in which the salicyloyl-CoA synthetase activity was measured directly, a copurification of propionyl- and salicyloyl-CoA synthetase activity could be demonstrated (Table I and Fig. 1). In the following step in the purification procedure the DEAE-

TABLE III

THE INFLUENCE OF A SECOND CARBOXYLIC ACID ON PROPIONATE- AND BUTYRATE ACTIVATION MEASURED WITH PROPIONYL-CoA SYNTHETASE AFTER PHOSPHOCELLULOSE CHROMATOGRAPHY.

Carboxylic acid added (unlabelled)	% inhibition propionate activation	% inhibition butyrate activation
-	0	0
Salicylate	23	91
Benzoate	23	90
Hexanoate	20	80
Butyrate	15	
Propionate		27

The phosphocellulose poolfraction of propionyl-CoA synthetase (Table I, stage 4) was used in all experiments. Propionyl- and butyryl-CoA synthetase activities were measured at 5 mM fatty acid concentration. The added unlabelled carboxylic acids were present at 5 mM. The specific activities of propionate and butyrate activation in the absence of other carboxylic acids were 727 and 663 mU/mg protein respectively.

Sephadex chromatography, both enzymes could be separated and in Fig. 2 it can be seen that butyrate is activated by both propionyl-CoA synthetase and the salicylate activating enzyme. Both acyl-CoA synthetases were precipitated by adding a saturated ammonium sulphate solution (final concentration 38 g/100 ml) and preserved in this form at 4^{o}C. The substrate specificity of propionyl-CoA synthetase was studied by substrate competition to prove the involvement of only one acyl-CoA synthetase in this stage of purification. Results of these studies are given in Table IV and Figs. 3-5. In these figures it is clearly shown

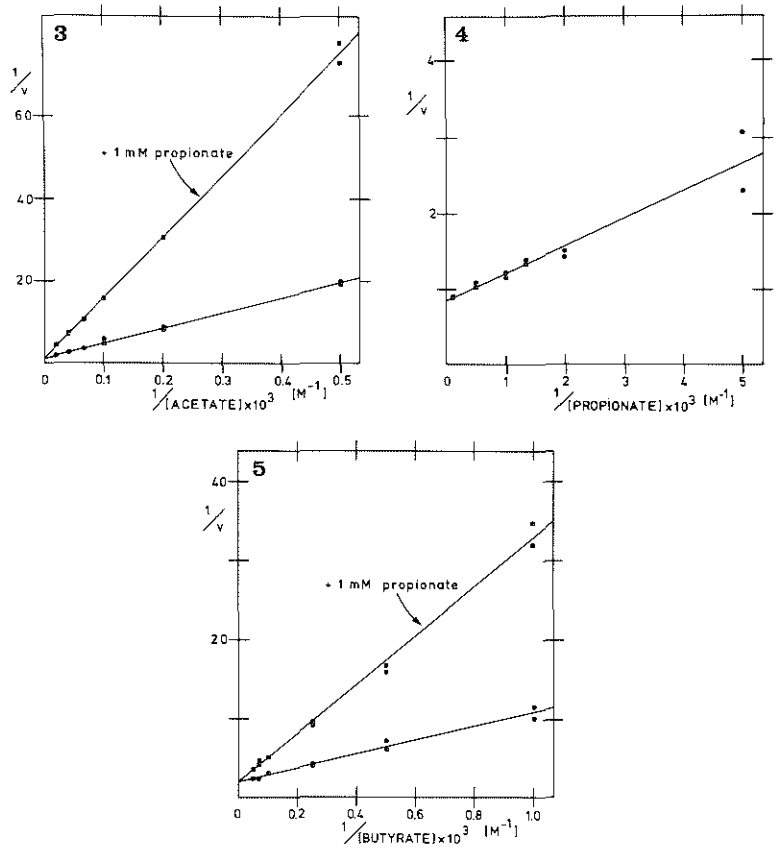
TABLE IV

KINETIC PARAMETERS OF PURIFIED PROPIONYL-CoA SYNTHETASE AFTER DEAE-SEPHADEX CHROMATOGRAPHY.

Activation tested ([1-^{14}C] labelled)	in the presence of (unlabelled)	K_m mM	K'_m mM	K_i mM	V %
Acetate	-	32			72
Acetate	1 mM propionate		121	0.35	71
Propionate	-	0.43			100
Butyrate	-	4.6			44
Butyrate	1 mM propionate		17	0.38	46

The propionyl-CoA synthetase poolfraction, after DEAE-Sephadex chromatography (Table I, stage 6, fraction I), was used in all experiments. Short-chain fatty acid activation was determined as shown in Table I. Where indicated, 1 mM unlabelled propionate was present. K_m, K_i and V values were calculated from the data given in Figs. 3, 4 and 5, using the least square method. V values are given as percentage of the V found for propionate activation.

that both acetate and butyrate activation are competitively inhibited by the presence of propionate. The calculated K_i values for propionate inhibition (0.35 and 0.38 mM respectively, Table IV) are in good agreement with the K_m value for propionate activation (0.43 mM). These results then strongly suggest that acetate, propionate and butyrate are activated by a single protein (compare Dixon and Webb[11]). Propionyl-CoA synthetase thus has a low K_m for propionate (0.43 mM) while the K_m's for acetate and butyrate are relatively high (32 and 4.6 mM respectively). The involvement of propionyl-CoA synthetase in the metabolism of acetate and butyrate is therefore doubtful. The role of



FIGS. 3-5
KINETICS OF PURIFIED PROPIONYL-CoA SYNTHETASE AFTER DEAE-SEPHADEX CHROMATOGRAPHY

Lineweaver-Burk plots of acetate, propionate and butyrate activation by the DEAE-Sephadex purified propionyl-CoA synthetase (Table I, stage 6, fraction I) and the influence of 1 mM unlabelled propionate on acetate and butyrate activation. For details consult Table IV and the text. v is given in U/mg protein.

liver propionyl-CoA synthetase is discussed in Appendix Paper I, section VII A.

Our preparation of propionyl-CoA synthetase is free of inorganic pyrophosphatase activity. A low myokinase activity, however, could still be demonstrated, interfering with our studies on the stoicheometry of the propionate activation. However, a 1:1 stoicheometry in propionyl-CoA and pyrophosphate formation could be demonstrated (results not shown) which makes reaction (1) (Chapter I, Introduction) very likely.

This is not the first time that a distinct propionyl-CoA synthetase is purified. Recently we became aware of the thesis of Latimer[13] concerning the purification of such an enzyme from sheep-liver mitochondria by completely different methods. His enzyme preparation was able to catalyze C_2 to C_8 activation with optimal activity towards propionate (see Chapter I, Table I) and may be contaminated with other acyl-CoA synthetase.

Our experiments have indicated the existence of three acyl-CoA synthetases in the soluble fraction of guinea-pig-liver mitochondria. All three enzymes were completely separated in this study. In Table V we have compared the substrate specificities of these enzymes, as measured in the highest purified enzyme fractions.

Medium-chain acyl-CoA synthetase displays the broadest substrate specificity. Even palmitate is accepted as a substrate. This is in agreement with our studies with rat-liver mitochondria (Groot et al.[10], Appendix Paper III). We found an optimal activity of this enzyme with hexanoate. The activation rate with heptanoate, not determined in the present study, will be slightly higher[1]. Substantial rates were also found with benzoate (Table V). The absence of activity towards salicylate, as reported by Schachter and Taggart[14] and by Killenberg et al.[2], could be confirmed in the present study.

TABLE V

THE CARBOXYLIC ACID SPECIFICITIES OF MEDIUM-CHAIN ACYL-CoA SYNTHETASE, PROPIONYL-CoA SYNTHETASE, AND THE SALICYLATE ACTIVATING ENZYME PURIFIED FROM GUINEA-PIG-LIVER MITOCHONDRIA.

activation tested	observed acyl-CoA synthetase activity: medium-chain acyl-CoA synth. %	propionyl-CoA synth. %	salicylate act. enzyme %
acetate	0.2	11	0
propionate	3	100	16
butyrate	35	19	74
hexanoate	100	0	100
octanoate	58	0	6
laurate	14	-	-
myristate	5	-	-
palmitate	1	-	-
benzoate	14	0	122
salicylate	0.02	0	8

The poolfraction of octanoyl-CoA synthetase activity obtained by phosphocellular chromatography (medium-chain acyl-CoA synthetase, see Fig. 1) and poolfractions I and II obtained after DEAE-Sephadex chromatography (containing propionyl-CoA synthetase and the salicylate activating enzyme respectively, Table I, stage 6) were used in these studies. C_2 to C_8 and salicylate activation were determined as given in Table I. Benzoate activation was tested in the same way as the activation of salicylate. The activations of C_{12}, C_{14} and C_{16} were tested as described by Groot and Hülsmann[5] (Appendix Paper II, Method I). The incubation mixture, however, was adapted to our short-chain fatty acid activation assay (mixture shown in Table I in which L-carnitine plus carnitine acetyltransferase are replaced by L-[Me-^{3}H]carnitine plus carnitine palmitoyltransferase 80 mU/ml assay volume). The

following fatty acid concentrations were used: C_2, C_3 and C_4 5 mM; C_6 2 mM (medium-chain acyl-CoA synthetase) or 5 mM; C_8 1 mM (medium-chain acyl-CoA synthetase) or 5 mM; C_{12}, C_{14} and C_{16} 1 mM, complexed with bovine serum albumin (0.143 mM). Benzoate and salicylate were used in final concentrations of 1 mM. Velocities of acyl-CoA formation were plotted as percentages of the velocities found with the favoured fatty acids which were 1000 mU/mg (C_6 activation by medium-chain acyl-CoA synthetase) 4000 mU/mg (C_3 activation by propionyl-CoA synthetase) and 720 mU/mg (C_6 activation by salicylate activating enzyme).

Propionyl-CoA synthetase shows activity with C_2, C_3 and C_4. No activity could be demonstrated with C_6 and C_8, in contrast to what has been reported by Latimer[12]. His enzyme preparation therefore, may be contaminated to some extent with other acyl-CoA synthetases.

In our studies hexanoate was found to be the favoured fatty acid of the salicylate activating enzyme. An even higher rate of acyl-CoA formation was found with benzoate (Table V). In guinea-pig-liver mitochondria the salicylate activating enzyme will give a somewhat higher contribution to the total benzoate activation than the medium-chain acyl-CoA synthetase, as can be calculated from our results. Benzoyl-CoA can be conjugated with glycine (yielding hippurate) by the action of glycine acyltransferase (EC 2.3.1.13), an enzyme which was found in liver[14] and kidney[15] mitochondria. The conjugated aromatic acids can be excreted by the kidneys into the urine. Since a similar conjugation reaction can also occur with salicyl-CoA[16], the physiological role of the salicylate activating enzyme may be associated with theses processes.

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SUMMARY

This thesis is dealing with two aspects of the ATP-dependent fatty acid activation in mammalian tissues: a) the fatty acid substrate specificity of the different type of acyl-CoA synthetases, b) the tissue- and intracellular localization of acyl-CoA synthetases. From our work in this field the following conclusions can be drawn:

1. The innermembrane-matrix space of rat-liver mitochondria is not equipped with a specific palmitoyl-CoA synthetase. The palmitate activation found for this compartment can be ascribed to the matrix medium-chain acyl-CoA synthetase. The high K_m of this enzyme for palmitate suggests that the contribution of the medium-chain acyl-CoA synthetase in long-chain fatty acid metabolism of the liver is very modest.

2. A matrix medium-chain acyl-CoA synthetase, as present in liver mitochondria, was not found in rat skeletal muscle. Octanoate, however, can be activated by an acyl-CoA synthetase localized on the mitochondrial outer membrane. Octanoate and palmitate were shown to be competing for activation. Therefore we concluded that medium- and long-chain fatty acids in muscle are activated by one acyl-CoA synthetase; palmitoyl-CoA or long-chain acyl-CoA synthetase.

3. Guinea-pig-liver mitochondria are equipped with a short-chain acyl-CoA synthetase with high preference for the substrate propionate. This propionyl-CoA synthetase has been purified and its fatty acid substrate specificity studied. The following kinetic parameters were found: acetate, V = 72%, K_m = 32 mM; propionate, V = 100%, K_m = 0.43 mM; butyrate, V = 44%,

K_m = 4.6 mM. Hexanoate and octanoate were not accepted as substrate.
In addition to this propionyl-CoA synthetase, two other acyl-CoA synthetases were found to be present in a soluble fraction of guinea-pig-liver mitochondria: a medium-chain acyl-CoA synthetase and a salicylate activating enzyme. Substrate specificity studies of the latter two enzymes are also presented in this thesis.

4. The subcellular localization of short-chain acyl-CoA synthetases in tissues of rat and guinea-pig was studied. It was concluded that the total short-chain fatty acid activation (catalyzed by an acetyl- and a butyryl-CoA synthetase) in heart, kidney and skeletal muscle is localized in the matrix mitochondrialis. In liver, adipose tissue, small intestinal epithelium and lactating mammary gland a cytosolic acetyl-CoA synthetase was found. The latter three tissues are also equipped with mitochondrial acetyl- and butyryl-CoA synthetases. However, liver contains a mitochondrial propionyl-CoA synthetase and a similar enzyme may be present in adipose tissue mitochondria.

SAMENVATTING

In dit proefschrift wordt voornamelijk aandacht besteed aan twee aspecten van de ATP-afhankelijke vetzuuractivering in zoogdierweefsels: a) de substraatspecificiteit voor vetzuren van de verschillende vetzuur activerende enzymen; b) de weefsel- en subcellulaire localisaties van vetzuur activerende enzymen.
Ons onderzoek heeft geleid tot de volgende conclusies:

1. In de binnenmembraan-matrixruimte van rattelevermitochondriën bevindt zich geen specifiek palmitoyl-CoA synthetase. De palmitaatactivering in dit compartiment kan worden toegeschreven aan het in de matrix gelocaliseerde midketen vetzuur activerende enzym. De hoge K_m voor palmitaat van dit enzym (23 µM) doet vermoeden dat deze activiteit nauwelijks een bijdrage kan leveren tot het langketen vetzuurmetabolisme in de lever.

2. In skeletspieren van de rat blijkt een midketen vetzuur activeringsenzym in de matrix mitochondrialis niet voor te komen en worden midketen vetzuren op het mitochondriale buitenmembraan geactiveerd. In deze activeringsreactie bleken octanoaat en palmitaat competitieve substraten te zijn, waaruit geconcludeerd werd dat mid- en langketen vetzuren door één acyl-CoA synthetase worden geactiveerd, namelijk het palmitoyl-CoA synthetase ofte wel langketen acyl-CoA synthetase.

3. In de cavialevermitochondriën bevindt zich een oplosbaar kortketen vetzuur activerend enzym met een duidelijke voorkeur voor propionaat. Dit propionyl-CoA synthetase werd gezuiverd en de substraatspeci-

ficiteit onderzocht. De volgende kinetische constanten werden gevonden: acetaat V = 72%, K_m = 32 mM; propionaat V = 100%, K_m = 0.43 mM; butyraat V = 44%, K_m = 4.6 mM. Hexanoaat en octanoaat bleken niet door dit enzym geactiveerd te kunnen worden.
Naast dit propionyl-CoA synthetase komen in de oplosbare fractie van cavialevermitochondriën nog een midketen vetzuur activerend en een salicylaat activerend enzym voor. De substraatspecificiteit van deze laatste twee acyl-CoA synthetasen werd tevens onderzocht.

4. Met betrekking tot de subcellulaire localisatie van kortketen vetzuur activerende enzymen in ratte- en caviaweefsels kon geconcludeerd worden dat in hart, nier en skeletspier de totale kortketen vetzuuractivering (gekatalyseerd door een acetyl- en een butyryl-CoA synthetase) in de matrix mitochondrialis gelocaliseerd is. In lever, vetweefsel, dunne darm epitheel en de lacterende borstklier bevindt zich een cytoplasmatisch acetyl-CoA synthetase. De laatste drie weefsels bevatten tevens mitochondriale acetyl- en butyryl-CoA synthetasen. Deze enzymen bleken in lever afwezig te zijn. Levermitochondriën echter bevatten een propionyl-CoA synthetase. Een zelfde enzym is mogelijk ook aanwezig in de mitochondriën van vetweefsel.

CURRICULUM VITAE

(op verzoek van de Universiteit)

De schrijver van dit proefschrift behaalde in 1963 het eindexamen HBS-B aan het Marnix College te Ede en begon in ditzelfde jaar aan een studie scheikunde (letter f) aan de Universiteit van Amsterdam. Het kandidaatsexamen werd afgelegd in 1967, het doctoraalexamen (hoofdvak: biochemie; bijvak: klinische chemie; speciale richting: organische chemie) werd afgelegd in 1971. In juni 1971 trad hij in dienst van de toenmalige Medische Faculteit Rotterdam en voerde hij op de afdeling biochemie I (hoofd: Prof. Dr. W.C. Hülsmann) de experimenten uit die in dit proefschrift beschreven zijn.

APPENDIX PAPERS

FATTY ACID ACTIVATION: SPECIFICITY, LOCALIZATION AND FUNCTION

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I. INTRODUCTION

Most cells are able to metabolize fatty acids. The most important pathways are:

1. β-oxidation to acetyl-CoA
2. esterification with glycerol-phosphate, glycerol-derivatives, sphingosine, glycol, long-chain alcohols etc.
3. chain-elongation
4. desaturation
5. α and ω oxidation.

The pK_a value of unbranched, unsubstituted monocarboxylic fatty acids at 37°C varies between 4.8 and 5.0 Accordingly, fatty acid molecules, present in real solution, will be practically completely ionized at physiological pH values. The carboxylate ion, however, is chemically unreactive towards nucleophilic substitution. Esters, thioesters and acid anhydrides, in which both the carboxylate resonance and the negative charge on the carboxyl group have disappeared, are more reactive. The most important activation of fatty acids is the esterification with coenzyme A and this activation will be discussed in this paper. The direct acyl activation (the formation of acyl-phosphate), important in some microorganisms, will not be discussed (see Jencks, 1962). The formation of the energy-rich thioesterbond between the fatty acid anion and coenzyme A is achieved by ATP hydrolysis according to the following overall reaction

$$RCOO^- + ATP^{4-} + CoASH \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} RCOSCoA + PP_i^{3-} + AMP^{2-} \quad (1)$$

(Lipmann et al., 1952; Kornberg and Pricer, 1953; Mahler et al., 1953; Beinert et al., 1953; Jones et al., 1953;

Eisenberg, 1955; Berg, 1956; Samuel et al., 1970; Bar-Tana et al., 1971.)

Reaction (1) is catalyzed by acid: CoA ligases (AMP forming) (EC 6.2.1.1-3) also called acyl-CoA synthetases or fatty acid thiokinases (recommended name: acyl-CoA synthetases). At physiological pH, the equilibrium constant of reaction (1) is near unity which indicates a ready reversibility (Mahler et al., 1953; Jones, 1953; Hele, 1954). Pyrophosphate hydrolysis catalyzed by inorganic pyrophosphatase (EC 3.6.1.1) will displace the equilibrium in the direction of acyl-CoA formation.

Acyl-CoA synthetases are found in many types of cells including bacteria (Massaro and Lennarz, 1965; Overath et al., 1969; Weeks et al., 1969; Samuel et al., 1970), molds (Eisenberg, 1955), yeasts (Lipmann et al., 1952; Berg, 1956), higher plants (Millerd and Bonner, 1954; Young and Anderson, 1974 a) and in many mammalian cells. This paper will predominantly deal with the properties of mammalian acyl-CoA synthetases. The subcellular localization and organ distribution in mammalian tissues will be discussed in Sections V and VI respectively.

Many different acyl-CoA synthetases have been described. The enzymes differ in fatty acid substrate specificity as will be discussed in Section IV, A. In addition to the ATP-dependent fatty acid activation, Rossi and Gibson (1964) described a mammalian acyl-CoA synthetase, specific for GTP, which catalyzed the activation of fatty acids according to

$$RCOO^- + GTP^{4-} + CoASH \rightleftharpoons RCOSCoA + GDP^{3-} + P_i^{2-} \quad (2)$$

The importance and even the existence of GTP-dependent acyl-CoA synthetases has been questioned by others (Pande and Mead, 1968 b; Hittelman et al., 1969; Van Tol et al., 1969; Lippel and Beattie, 1970) as will be discussed in Section IV, B. Unless indicated by the additions, GTP

dependent or GDP forming, fatty acid activation or acyl-CoA synthetase will stand for the ATP dependent processes.

II ASSAYS FOR ACYL-CoA SYNTHETASES

A. MEASUREMENT OF ACYLHYDROXAMATE FORMATION

Hydroxylamine splits acyl-CoA in CoA and hydroxamic acid (Jones et al., 1953; Kornberg and Pricer, 1953). With $FeCl_3$ a purple ferric hydroxamic acid complex is formed, with an $\underline{A}_{540\ nm} = 1\ mM^{-1}cm^{-1}$ (Lipmann and Tuttle, 1945). The high concentration of hydroxylamine, needed for the assay, has been shown to inhibit purified acid:CoA ligase (Massaro and Lennarz, 1965). In crude active preparations, however, the method has been found satisfactory.

Sladek et al. (1970) improved the sensitivity of the methode by preparation and separation of $[^{14}C]$-acetohydroxamate from $[^{14}C]$-acetate. The separation was performed by the retention of the unreacted radioacetate on a Dowex 2 X-8 column by isoionic exchange chromatography. The method was also applied to propionate.

B. MEASUREMENT OF -SH DISAPPEARANCE

The disappearance of the CoA sulfhydryl group during the activation was measured with nitroprusside (Grunert and Phillips, 1951) and with 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959), with $\underline{A}_{520\ nm}$ of 6.3 $mM^{-1}cm^{-1}$, and $\underline{A}_{412\ nm}$ of 13.6 $mM^{-1}cm^{-1}$, respectively. The former reaction product is less stable than the latter.

C. ASSAY OF THE FORMED ACYL-CoA WITH ACYLACCEPTOR AND AUXILLIARY ENZYME(S)

1. With choline and choline acetyltransferase (EC 2.3.1.6) (Nachmansohn and Machado, 1943; Nachmansohn

and Berman, 1946). Tencati and Rosenberg (1973) improved the sensitivity of this method by the conversion of [^{3}H]-acetyl-CoA into [^{3}H]-acetylcholine, and separated this compound from the unreacted radioacetate by column chromatography on Dowex-50.

2. With an arylamine and arylamine acetyltransferase (EC 2.3.1.5) (Lipmann, 1945; Kaplan and Lipmann, 1948; Lynen et al., 1951; Handschuhmacher et al., 1951; Tabor et al., 1953).
3. With oxaloacetate and citrate synthase (EC 4.1.3.7) (Stern et al., 1951; Beinert et al., 1953). With malate, NAD^+ and malate dehydrogenase (EC 1.1.1.37) as oxaloacetate generating system, special attention must be paid to the proportionality of acetyl-CoA and NADH formation (Pearson, 1965). A radioassay was developed by Prinz et al. (1966). [4-^{14}C]-oxaloacetate was here generated from 4-^{14}C-aspartate and α-ketoglutarate by aspartate aminotransferase (EC 2.6.1.1). The radioactive citrate was separated by electrophoresis. Methods C1-C3 have the highest velocity with acetyl-CoA synthetase. The systems are less or not reactive with higher acyl-CoA synthetases.
4. With carnitine and carnitine acyltransferases (EC 2.3.1.7 or 21). This method has the advantage that it is not restricted to acetate activation. The method was developed by Farstad et al. (1967) for the determination of palmitoyl-CoA synthetase. It is applicable for activation from C_{10}-$C_{\geq 22}$. The acyl-CoA formed is converted to acylcarnitine by the use of labelled carnitine and partially purified carnitine palmitoyltransferase (EC 2.3.1.21). [^{14}C]- or [^{3}H]-acylcarnitine is separated from radioactive carnitine by extraction with butanol.

   Aas and Bremer (1968) adapted the method for lower fatty acids. Now carnitine acetyltransferase (EC 2.3.1.7) plus carnitine octanoyltransferase, both

present in the commercial preparation (Solberg, 1971) was added. The separation was carried out by thin layer chromatography. The results with C_2 were unreliable (Aas and Bremer, 1968; Aas, 1971 a).

We modified this method by the use of [^{14}C]-fatty acids and unlabeled L-carnitine. The carnitine esters were separated from the unreacted [^{14}C]-fatty acids by column chromatography on Dowex 50W (200-500 Mesh) (Groot and Hülsmann, 1973). This modification was also suitable for C_2 activation and for competition experiments, and was used in some new experiments reported in the present work.

D. DIRECT ASSAY OF ACYL-CoA

Yates et al. (1966) assayed palmitoyl-CoA synthetase by measuring the conversion of CoA into its acid insoluble form. Higher sensitivity was obtained by measurement of radioactive acyl-CoA. Several methods can be used for the separation of the product from the radioactive fatty acid. Stacey et al. (1964) removed unreacted fatty acids (C_2-C_8) by steam volatilization. Schubert (1965) removed C_2 by evaporation to dryness. All fatty acids can be removed by their solubility in ether (Galton and Fraser, 1969; Samuel and Ailhaud, 1969; Samuel et al., 1970). We also used this method, suitable for the activation of very small amounts of fatty acids, and adapted the procedure of Marcel and Suzue (1972). For details consult Groot et al. (1974).

Acyl-CoA and labeled fatty acids (C_{2-4}) were separated by Reijnierse (1973) by column chromatography on Dowex 1 X-4, and by Huang (1970) (C_2) by chromatography on Gelman chromatography media.

E. SPECTROPHOTOMETRIC MEASUREMENT OF THE ESTER FORMATION

Acyl-CoA esters have two absorption bands. One due to adenine with a peak at 260 nm, and another at 232

nm (for saturated fatty acids) due to the thiolester bond of 16.4 and 4.5 $mM^{-1}cm^{-1}$, respectively (Stadtman, 1957). The increase of the absorbancy at 232 has successfully been used for acid:CoA ligase determination (Beinert et al., 1953; Cha and Parks, 1964), especially for more purified enzyme preparations.

Since most spectrophotometers had lamps with a low emission at 232 nm, fatty acids were used with conjugated double bonds. These shift the absorbancy to longer wavelengths: 263 nm for crotonoyl-CoA (Lynen and Ochoa, 1953), 303 nm for sorboyl-CoA (Wakil and Hübscher, 1960), 376 nm for 2,4,6,8 decatetraenoyl-CoA (Yates and Garland, 1967; Garland et al., 1970).

F. ASSAY OF PARTIAL AND REVERSE REACTIONS

ATP formation from acetyl-CoA and PPi, and ^{32}P incorporation from radiopyrophosphate (in the presence of acetate) and other exchange reactions were measured by Berg (1956), Eisenberg (1957), Bar-Tana and Shapiro (1964). Bar-Tana et al. (1971) measured [^{14}C]-palmitate formation from radioactive palmitoyl-CoA.

General remarks

In choosing a method for the determination of acid:CoA ligase it is important to take full account of the strong inhibition of the acid:CoA ligases by the products acyl-CoA, PP_i and AMP (see Section IV) and the possible presence of disturbing enzymes like acyl-CoA hydrolases (EC 3.1.2.1,2), acylcarnitine hydrolase (EC 3.1.1.28), ATP-ases (EC 3.6.1.3,8), lipases (EC 3.1.1.3), transacylases, and also of enzymes which destruct CoA (Skrede, 1973). Since the activation energy is quite high (about $\sim$20 Kcal. $mole^{-1}$), it is advisable to measure the reaction at 37°C (de Jong, 1971).

III. REACTION MECHANISM

The mechanism most widely accepted is given by Berg (1956) as proposed for acetate activation by yeast acetyl-CoA synthetase (EC 6.2.1.1). Acyl-AMP is a reaction intermediate and the full reaction is represented by:

$$H^+ + RCOO^- + ATP^{4-} + E \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} E.RCO\ AMP^- + PP_i{}^{3-} \qquad (3)$$

$$E.RCO\ AMP^- + CoASH \rightleftharpoons E + RCOSCoA + AMP^{2-} + H^+ \qquad (4)$$

It is based on the following observations: a) exchange of $^{32}PP_i$ and ATP requires acetate and not CoASH. b) acethydroxamic acid is formed from ATP, acetate and hydroxylamine in the absence of CoASH. c) synthetic acetyl-AMP is converted into ATP in the presence of PP_i and into acetyl-CoA in the presence of CoASH. d) exchange of ^{14}C-labeled AMP and ATP requires acetate and CoASH. e) exchange of ^{14}C-labeled acetate and acetyl-CoA requires AMP and PP_i (Berg, 1956). Mg^{2+} ions are required in partial reaction (3) but not in (4). Biosynthesis of acetyl-AMP, a requirement to accept this reaction scheme, could not be demonstrated in this and other studies (Lee Peng, 1956; Jencks and Lipmann, 1957; Tolbert and Huennekens, 1956; Whitehouse et al., 1957; Eisenberg, 1957). Acetyl-AMP synthesis was performed by Webster and Campagnari (1962) and Webster (1963) using substrate quantities of enzyme. The biosynthesis of acyl-AMP from acetate or butyrate and ATP required Mg^{2+} ions; acyl-AMP formation from acetyl-CoA or butyryl-CoA and AMP turned out to be Mg^{2+} independent. These results are suggestive for the correctness of Berg's mechanism.

Butyrate activation by medium-chain acyl-CoA synthetase

purified from beef liver was studied in more detail by Bar-Tana (Bar-Tana et al., 1968; Bar-Tana and Rose, 1968 a and b). The initial-velocity pattern of the overall reaction was found to correspond to the Bi Uni Uni Bi Ping Pong model of Cleland (1963 a, b, c) for reactions involving three substrates in agreement with the reaction scheme of Berg (1956). Sigmoidal shaped substrate saturation curves were found in measurements of the overall reaction for both ATP (at low concentration of CoA) and CoA (at low concentration of ATP). The curves tended to a hyperbolic form at more saturating concentrations of the second substrate. Two interchangeable conformational states of the enzyme were assumed to exist: state R, having high affinity for CoA and ATP and catalyzing preferentially butyryl-AMP dependent CoA disappearance (reaction 4) and state T favoured by the presence of PP_i which catalyzed preferentially the ATP formation of butyryl-AMP and PP_i (reverse reaction 3) (Bar-Tana and Rose, 1968 a). A side fraction, obtained in the purification procedure (Bar-Tana et al., 1968), performed different kinetics of butyrate activation (Bar-Tana and Rose, 1968 b) but probably represents a modified native enzyme.

The mechanism of long-chain fatty acid activation has been studied by Vignais and Zabin (1958) and by Bar-Tana and coworkers (Bar-Tana and Shapiro, 1964; Bar-Tana et al., 1971; Bar-Tana et al., 1972; Bar-Tana et al., 1973 a and b; Brandes et al., 1973) and is still under study. Although ATP formation and palmitoyl-CoA formation from synthetic palmitoyl-AMP could be demonstrated both in liver microsomes of rat and guinea-pig (Vignais and Zabin, 1958; Bar-Tana and Shapiro, 1964; Bar-Tana et al., 1972) no palmitoylhydroxamate was formed from palmitate and ATP unless CoA was present (Bar-Tana and Shapiro, 1964). Moreover, during purification of rat liver microsomal palmitoyl-CoA synthetase, the ability to catalyze partial reactions (3) and (4) was lost (Bar-Tana et al.,

1972). A kinetic analysis with this purified enzyme (Bar-Tana et al., 1973 a) showed a Bi Uni Uni Bi Ping Pong reaction sequence (Cleland, 1963 a, b, c) in accordance with Berg's reaction mechanism. A binding of equimolar amounts of palmitate and AMP to this enzyme could be demonstrated after an incubation with palmitate and ATP. This complex was stabilized by high concentrations of ATP and reacted with coenzyme A to form palmitoyl-CoA and AMP (Bar-Tana et al., 1973 b). Two structures of the complex were considered: A) enzyme-palmitoyl-AMP and B) palmitate-enzyme-AMP. The transfer of [^{18}O] label from palmitate to AMP (Bar-Tana et al., 1973 a) is in favour of proposal A. The absence of partial reactions (3) and (4) with the purified enzyme (Bar-Tana et al., 1972) and the transfer of ^{3}H label from the enzyme-[^{3}H]-palmitate-AMP complex into the heptane layer in a Dole extraction, supports proposal B (Bar-Tana et al., 1973 b). The real structure of the complex and therefore the reaction mechanism has still to be elucidated. The occurrence of the partial reactions (3) and (4) in crude rat liver microsomes can probably be ascribed to a second long-chain acyl-CoA synthetase, only present in liver microsomes (Brandes et al., 1973). A partial separation of both activities has been accomplished. The question whether the two catalytic activities reside in two different proteins or whether a conformational change of palmitoyl-CoA synthetase, occurring during purification making the protein inaccessable to palmitoyl-AMP, remains to be answered (Brandes et al., 1973).

IV. SUBSTRATE SPECIFICITY OF ACYL-CoA SYNTHETASES

In this survey we confine ourselves to monocarboxylic fatty acids. The activation of dicarboxylic acids proceeds

via a same mechanism (Pettersen, 1973). In plants and microorganisms distinct enzymes for the activation of oxalate (Giovanelli, 1966) malonate (Hayaishi, 1953; Wolfe et al., 1954; Reuser and Postma, 1973) and succinate (Palmer and Wedding, 1966; Gibson et al., 1967) have been described. Malonate activation in mammalian tissues (Nakada et al., 1957) is probably due to the action of acetyl-CoA synthetase (EC 6.2.1.1) (Scholte et al., 1971). The enzyme succinyl-CoA synthetase (GDP forming) (EC 6.2.1.4) will not be discussed in this chapter since it catalyzes in vivo a reversed activation reaction. In Section IV, A, short-, medium- and long-chain acyl-CoA synthetases (AMP forming) (EC 6.2.1.1-3) will be considered; in Section IV, B, acyl-CoA synthetase (GDP forming) is reviewed.

A. SPECIFICITY TOWARDS FATTY ACIDS

1. SHORT-CHAIN ACYL-CoA SYNTHETASES (AMP FORMING)

In this section we shall discuss the enzymes, performing maximal activity towards acetate, propionate or butyrate*.

a. Acetyl-CoA synthetase (EC 6.2.1.1)

The existence of acetyl-CoA synthetase, an enzyme with the lowest K_m and highest V_{max} towards acetate,

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* In this section we shall also discuss the butyryl-CoA synthetase purified by Webster et al. (1965) from beef heart. This enzyme is distinctly different from the medium-chain acyl-CoA synthetase purified by Mahler et al. (1953) from beef liver, which enzyme has obtained the confusing recommended name butyryl-CoA synthetase (EC 6.2.1.2) (see Florkin and Stotz, 1973). In this review we will use the name butyryl-CoA synthetase only for enzymes with properties identical to those of the enzyme purified by Webster while enzymes of the beef liver type will be called medium-chain acyl-CoA synthetase.

TABLE I
SUBSTRATE SPECIFICITY AND KINETIC PARAMETERS OF PURIFIED SHORT-CHAIN ACYL-CoA SYNTHETASES.

Enzymes and authors	enzyme source	Kinetic parameters							
		V_{max} (in %)				K_m (in mM)			
		C_2	C_3	C_4	C_5	C_2	C_3	C_4	C_5
Acetyl-CoA synthetases									
Beinert et al. (1953)	beef heart	100	50	0					
Hele (1954)	beef heart	100	100			1.42	5.0		
Millerd and Bonner (1954)	spinach leaves	100	0	84*					
Eisenberg (1955)	Rhodospirillum rubrum	100	90	0					
Berg (1956)	yeast	100	30	0					
Campagnari and Webster (1963)	beef heart	100	30	0		0.79	11.0		
Ohmann (1964)	Euglena gracilis	100				1.2			
Huang and Stumpf (1970)	potato tuber	100	low	very low		0.2			
Propionyl-CoA synthetases									
Latimer (1967)	sheep liver	5**	100**	12**			0.12		
Young and Anderson (1974 a)	pinus radiata	100	94	93	25	4.7	0.21	0.33	2.1
Butyryl-CoA synthetases									
Webster et al. (1965)	beef heart	0	30	100	80			1.5	

* probably two enzymes
** measured at 3.33 mM

is well documented as shown in Table I. Acetyl-CoA formation according to reaction (1) is catalyzed optimally between pH 7.2 and 8.5 (Millerd and Bonner, 1954; Eisenberg, 1955; Webster and Campagnari, 1962; De Vincenti and Klein, 1970; Huang and Stumpf, 1970).

Kinetic parameters. Reported K_m's for acetate vary between 0.2 and 1.5 mM (Table I). Low K_m's for ATP are reported for the enzymes present in potato tuber: 0.038 mM (Huang and Stumpf, 1970) and rat brain: 0.027 mM (Tencati and Rosenberg, 1973), higher values were found in yeast (1-10 mM; De Vincenti and Klein, 1970), Rhodospirillum rubrum (about 1 mM; Eisenberg, 1955) and beef heart (0.9-1.8 mM; Campagnari and Webster, 1963; Webster, 1969). Similar differences were found in K_m's for coenzyme A and Mg^{++} ions: 0.0167 mM and 0.25-0.40 mM respectively in potato tuber, 0.019 mM and 0.22 mM in rat brain, 0.1 mM and 1.5 mM in Rhodospirillum rubrum and 0.4-1.4 mM and 1.4 mM in beef heart.

Substrate specificity. Practically all acetyl-CoA synthetases purified are able to activate propionate. The K_m values, however, are much higher than for acetate activation (see Table I). Butyrate is practically not activated. Some activity towards acrylate (Campagnari and Webster, 1963) and fluoroacetate (Brady, 1955; Marcus and Elliott, 1956; Huang and Stumpf, 1970) has been described.

Molecular weight. Molecular weights of 59,500 (Huang and Stumpf, 1970) and 57,000 (Londesborough et al., 1973) have been described for enzymes purified from potato tuber and beef heart respectively. The yeast enzyme is of larger size, 130,000 (De Vincenti and Klein, 1970) and probably composed of 4 subunits.

Stabilizers, inhibitors and stimulators. Plant and mammalian acetyl-CoA synthetases are stabilized by sulfhydryl reagents (Huang and Stumpf, 1970; Webster, 1969). Enzymatic thiol groups, however, seem not to be involved in the catalysis (Londesborough et al., 1973). AMP and PP_i, products of the forward reaction, inhibit the acetyl-CoA formation (Eisenberg, 1955; Huang and Stumpf, 1970). High concentrations of the substrates of the forward reaction may also be inhibitory (Campagnari and Webster, 1963).

Many acetyl-CoA synthetases are stimulated by high concentrations of K^+, NH_4^+ and Ru^+ and inhibited by Na^+ and Li^+ (Von Korff, 1953; Campagnari and Webster, 1963; De Vincenti and Klein, 1970; Tencati and Rosenberg, 1973). A detailed analysis by Webster and coworkers (Campagnari and Webster, 1963; Webster, 1963; Webster, 1965 a and b; Webster, 1966; Webster, 1967; Webster, 1969) showed that the beef heart enzyme displayed a rather complex cation requirement. A double requirement for divalent cations in the overall reaction was shown (Webster, 1965 b; Webster, 1967; Webster, 1969). Metal ions of one group (Mg^{++}, Mn^{++}, Fe^{++}, Co^{++} and Ca^{++}) are required only in the first partial reaction (reaction (3)) and have high K_m's near that of ATP (0.9 mM). The second divalent cation requirement for acetyl-CoA synthetase can be shown only after bound metal has been removed from the enzyme. Under these conditions, the overall partial reactions are stimulated by Ni^{++}, Cd^{++}, Fe^{++} and Ca^{++} in concentrations only slightly exceeding that of the enzyme. Monovalent cations are also absolutely required for both the overall and the partial reactions (Webster, 1966; Webster, 1967; Webster, 1969). Rb^{++}, NH_4^+, $Tris^+$, K^+, Na^+ and Li^+ ions are all satisfactory (K_m's between 1 and 3 mM). High concentrations

of Na^+ and Li^+, however, are inhibitory while 10-200 mM of the other ions stimulate maximally.

b. Butyryl-CoA synthetase

A butyryl-CoA synthetase, an enzyme with the highest V_{max} towards butyrate, has been purified from beef heart mitochondria by Webster et al. (1965). This enzyme could be separated from acetyl-CoA synthetase on TEAE cellulose. The butyryl-CoA synthesis according to reaction (1) is catalyzed optimally at pH values between 8 and 9. Straight chain saturated fatty acids with 3 to 7 C atoms are activated. Insignificant activity was found with pyruvate, malate, acrylate, fumarate, succinate, α or β-hydroxybutyrate, α-ketobutyrate, acetoacetate, γ-aminobutyrate, benzoate, phenylacetate, L-phenylalanine, L-tyrosine and with L-tryptophan as substrate. K_m values of 1.5, 1.8, 3 and 0.85 mM were reported for butyrate, Mg^{2+}, ATP and CoA respectively (Webster et al., 1965). The enzyme differs from the medium-chain acyl-CoA synthetase of Mahler et al. (1953) in several aspects including substrate specificity and sensitivity towards pyrophosphate inhibition (see Section IV, A, 2). A similar butyryl-CoA synthetase is probably present in bovine rumen epithelium (Cook et al., 1969; Ash and Baird, 1973) and other mammalian tissues except liver (see below).

c. Propionyl-CoA synthetase

Propionyl-CoA synthetase, an enzyme with the lowest K_m and highest V_{max} towards propionate, has recently been purified from seeds of pinus radiata (Young and Anderson, 1974 a) and characterized (Young and Anderson, 1974 b) (Table I). The mechanism of propionate activation was found to be in agreement with the scheme of Berg.

TABLE II

KINETIC PROPERTIES OF ACETATE, PROPIONATE AND BUTYRATE ACTIVATION BY A MATRIX FRACTION OF GUINEA-PIG HEART MITOCHONDRIA MEASURED AT 40 mM TRICINE-KOH AND 300 m M TRIS-HCl.

Activation tested	Tricine-KOH		Tris-HCl	
	V *)	K_m **)	V *)	K_m **)
Acetate	1105	0.7	637	0.8
Propionate	900	6.4	892	7.9
Butyrate	170	0.3	143	0.8

*) in nmole [1-^{14}C]-acylcarnitine formed/min.mg protein
**) in mM

Short-chain acyl-CoA synthetases (AMP forming) were determined with a radioassay as described by Groot et al. (1974). The incubation mixture (0.25 ml) consisted of: 10 mM $MgCl_2$, 8 mM ATP, 1.2 mM EDTA, 0.3 mM KCN, 3 µg/ml oligomycine, 5 mM L-carnitine, 4 U/ml carnitine acetyltransferase, 5 mM phosphoenolpyruvate, 1.35 mM CoA and 8 U/ml adenylate kinase and pyruvate kinase. The mixture was buffered either with 40 mM Tricine-KOH or 300 mM Tris-HCl (pH = 8). [1-^{14}C] labeled fatty acids were added to a final concentration of 0.5-80 mM. The reaction temperature was 37^{0}C.

Distinct propionyl-CoA synthetases in mammalian tissues have been proposed for sheep lung (Cook et al., 1969) and for guinea-pig heart mitochondria (Scholte et al., 1971), proposals based on the high propionate activation compared with acetate and butyrate activation in these tissues. The short-chain fatty acid activation in a matrix fraction of guinea-pig heart mitochondria, the fraction in which all short-chain fatty acid activation is found in this tissue (Scholte et al., 1971; Section V, A), was further studied by us. Kinetic parameters of C_2, C_3 and C_4 activation, determined in two different assay buffers, are given in Table II. Remarkable differences in the V_{max} were found when acetate activation was determined in 40 mM Tricine-KOH instead of 300 mM Tris-HCl, differences that were not seen for propionate activation. In this difference between acetate and propionate activation, additional evidence was thought to be found to propose the presence of a distinct propionyl-CoA synthetase in guinea-pig heart (Scholte et al., 1971). This conclusion, however, has turned out to be too preliminary.

Fatty acid competition experiments, a useful tool to decide whether the activation of two fatty acids is catalyzed by one or two enzymes (Samuel et al., 1970; Suzue and Marcel, 1972 a and b; Marcel and Suzue, 1972; Groot and Hülsmann, 1973; Groot et al., 1974; Young and Anderson, 1974 b) indicated that both in 40 mM Tricine-KOH and in 300 mM Tris-HCl, propionate activation in the matrix fraction of guinea-pig heart mitochondria can be explained by a major contribution (about 80%) of acetyl-CoA synthetase and a minor contribution of either butyryl- or medium-chain acyl-CoA synthetase. Results of our competition studies are given in Fig. 1 and 2 and in Table III. Acetate activation is competitively inhibited by propio-

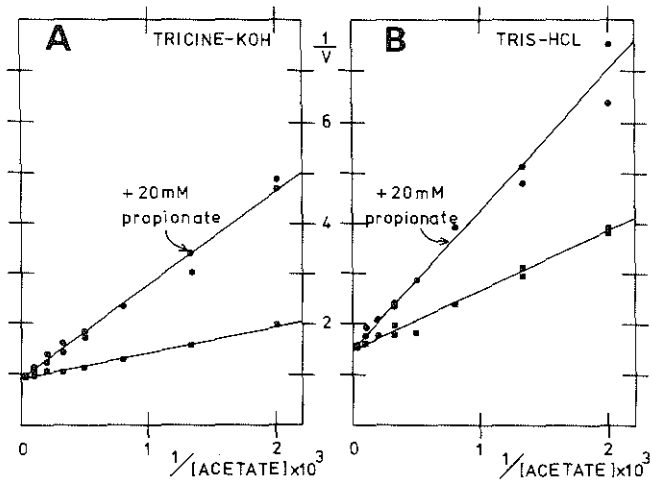

FIG. 1

ACETATE ACTIVATION BY A MATRIX FRACTION OF GUINEA-PIG HEART MITOCHONDRIA AND THE EFFECT OF PROPIONATE.

For details see Table II.

A) Lineweaver-Burk plot of acetate activation measured in 40 mM Tricine-KOH. ■——■ [1-^{14}C]-acetate present. ●——● [1-^{14}C]-acetate and 20 mM propionate present.
B) identical to A), however, 300 mM Tris-HCl instead of 40 mM Tricine-KOH.

nate (Figs. 1 A and B) and the calculated K_i values for propionate (7.5 mM in Tricine-KOH and 4.5 in Tris-HCl) are in close agreement with the K_m values for propionate given in Table II, a result to be expected when both fatty acids are activated by the same enzyme (Dixon and Webb, 1964). The reverse experiments (Fig. 2) show that at least at the higher propionate concentrations, propionate activation is competitively inhibited by acetate. The hyperbolic Lineweaver-Burk plots, obtained in the presence of acetate, are probably explained by a minor contribution of a butyryl-CoA synthetase to the propionate activation. The latter en-

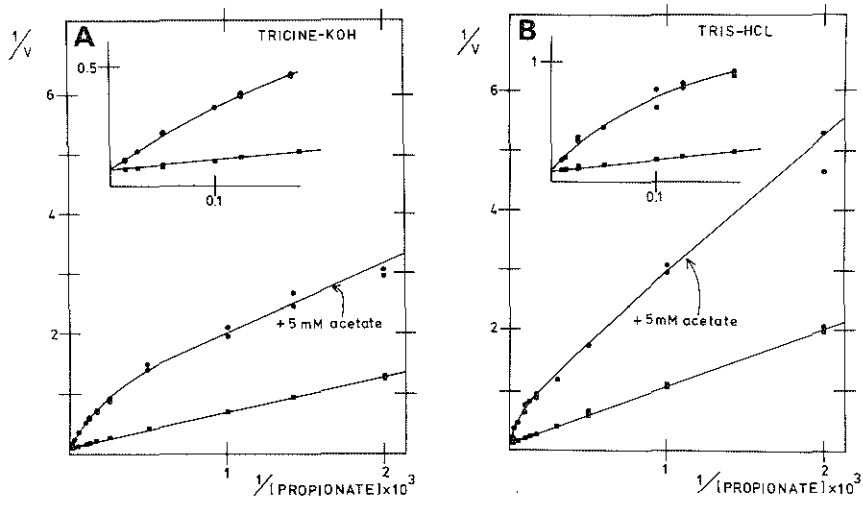

FIG. 2

PROPIONATE ACTIVATION BY A MATRIX FRACTION OF GUINEA-PIG HEART MITOCHONDRIA AND THE EFFECT OF ACETATE.

For details see Table II.

A) Lineweaver-Burk plot of propionate activation measured in 40 mM Tricine-KOH. ■——■ [1-^{14}C]-propionate present. ●——● [1-^{14}C]-propionate and 5 mM acetate present.
B) identical to A), however, 300 mM Tris-HCl instead of 40 mM Tricine-KOH.

zyme is not inhibited by acetate (Webster et al., 1965). This supposition is substantiated by competition experiments shown in Table III. Propionate activation, tested at 20 mM propionate, is decreased to 23-24% when 20 mM acetate is present. The remaining activity is further diminished to 7-10% when 20 mM butyrate is also present. Although the different effects of the assay buffers on the V_{max} of acetate and propionate activation are not fully understood, we must conclude from these studies that a

TABLE III

INFLUENCE OF ACETATE AND BUTYRATE ON PROPIONATE ACTIVATION IN A MATRIX FRACTION OF GUINEA-PIG HEART MITOCHONDRIA.

Activation tested ([1-^{14}C]-labeled substrate)	In the presence of (unlabeled)	acyl-CoA synthetase activity			
		Tricine-KOH		Tris-HCl	
		spec. act.*)	% inhibn.**)	spec.act.*)	% inhibn.**)
Acetate	-	1003	-	494	-
Propionate	-	698	-	615	-
Butyrate	-	189	-	192	-
Propionate	Acetate	167	76	139	77
Propionate	Butyrate	573	18	553	10
Propionate	Acetate and butyrate	73	90	41	93

*) in nmole [1-^{14}C]-acylcarnitine formed/min.mg protein. **) % inhibition.
For details see Table II. Each fatty acid was present in a final concentration of 20 mM.

distinct propionyl-CoA synthetase is absent in guinea-pig heart mitochondria and that the propionate activation found in these organelles can be ascribed to a major contribution (about 80% of acetyl-CoA synthetase and a minor contribution (about 20%) of an enzyme which has more affinity for butyrate than for propionate and which probably represents a butyryl-CoA synthetase as purified by Webster et al. (1965) from beef heart mitochondria.

We also studied the short-chain fatty acid activation in matrix fraction obtained from guinea-pig liver mitochondria. The kinetic parameters of C_2, C_3 and C_4 activation in this fraction are given in Table IV. The K_m for propionate in this liver fraction turned out to be very low (0.6 mM) when compared with the value obtained in the same fraction in heart (6.4 mM) while the K_m values for both acetate and butyrate

TABLE IV
KINETIC PROPERTIES OF ACETATE, PROPIONATE, AND BUTYRATE ACTIVATION BY A MATRIX OF GUINEA-PIG LIVER MITOCHONDRIA.

Activation tested	V *)	K_m **)
Acetate	28	11.3
Propionate	137	0.6
Butyrate	248	5.4

*) in nmole [1-^{14}C]-acylcarnitine formed/min.mg protein
**) in mM.

For details see Table II. Acyl-CoA synthetase activity was measured in 40 mM Tricine-KOH.

TABLE V

ACTATE, PROPIONATE AND BUTYRATE ACTIVATION IN A MATRIX FRACTION OF GUINEA-PIG LIVER MITOCHONDRIA: THE INFLUENCE OF THE ADDITION OF A SECOND FATTY ACID.

Activation tested ([1-^{14}C]-labeled substrate	In the presence of (unlabeled)	acyl-CoA synthetase activity			
		Tricine-KOH		Tris-HCl	
		spec. act.*)	% inhibn.**)	spec. act.*)	% inhibn.**)
Acetate	-	27	-	16	-
Propionate	-	144	-	116	-
Butyrate	-	142	-	103	-
Acetate	Propionate	0	100	0	100
Propionate	Acetate	144	0	112	4
Propionate	Butyrate	108	25	91	22
Propionate	Octanoate	115	20	84	28
Butyrate	Propionate	101	29	80	20
Butyrate	Octanoate	28	80	18	83
Butyrate	Propionate and octanoate	7	95	5	95

*) in nmole [1-^{14}C]-acylcarnitine formed/min.mg protein
**) % inhibition
For details see Table II. Each fatty acid was present in a final concentration of 20 mM.

activation in liver (11.3 and 5.4 mM, respectively) are 15 times higher than the corresponding values in heart (0.7 and 0.3 mM, respectively). These results suggest the presence of a distinct propionyl-CoA synthetase in guinea-pig liver mitochondria. Fatty acid competitition experiments, shown in Table V, substantiate this suggestion. The complete inhibition of acetate activation by propionate together with the absence of any inhibitory effect of acetate on propionate activation indicates that all acetate activation in this guinea-pig liver fraction can be ascribed to the propionyl-CoA synthetase. Butyrate activation, however, is less inhibited by propionate than would be expected from a calculation using the K_m values given in Table IV and assuming that all propionate and butyrate activation is catalyzed by one and the same enzyme. A medium-chain acyl-CoA synthetase, as found in beef liver mitochondria (Mahler *et al.*, 1953), contributing to the butyrate activation, could explain the difference. In Table V is shown that octanoate, the preferred substrate of medium-chain acyl-CoA synthetase from beef liver (see Section IV, A, 2) strongly inhibits the butyrate activation while the effect on propionate activation is only moderate. In the presence of both propionate and octanoate practically all butyrate activation has disappeared. We concluded from these experiments (Groot, 1974) that two acyl-CoA synthetases are present in the matrix fraction of guinea-pig liver mitochondria: a) a propionyl-CoA synthetase, active with at least C_2, C_3 and C_4 with a high preference for propionate (see K_m values Table IV) and 2) a medium-chain acyl-CoA synthetase. A butyryl-CoA synthetase as found by Webster *et al.* (1965) in beef heart seems to be absent in guinea-pig liver mitochondria but present in guinea-pig

heart mitochondria (compare the K_m values for butyrate in Tables II and IV). Our experiments in guinea-pig liver complement the experiments of Ash and Baird (1973) in beef liver. In this study, discussed in more detail in Section VII, similar indications were found for a distinct propionyl-CoA synthetase in beef liver. Recent studies by one of us (P.H.E.G.) have shown that the guinea-pig liver propionyl-CoA synthetase can be completely separated from the medium-chain acyl-CoA synthetase by phosphocellulose chromatography. A salicylate activating enzyme as described by Killenberg et al. (1971) is copurified with the propionyl-CoA synthetase. The latter two enzymes can be separated by DEAE-Sephadex chromatography and the purified propionyl-CoA synthetase is now being characterized.
Recently we became aware of the thesis of Latimer (1967) concerning purified propionyl-CoA synthetase from sheep liver with K_m values for C_3, CoA and ATP of 0.12, 0.70 and 0.43 mM, respectively.

2. MEDIUM-CHAIN ACYL-CoA SYNTHETASE

An enzyme catalyzing the activation of fatty acids of intermediate chain-length has been purified 10 times from acetone powder extract from beef liver particles (Mahler et al., 1953). The enzyme displayed a rather broad substrate specificity. Saturated straight-chain fatty acids from C_4 to C_{12} are activated. Maximum activity was found with C_7 but the K_m for octanoate turned out to be the lowest (0.15 mM). Other K_m values are in the range between 1.5×10^{-4} and 1.6×10^{-3} mM (butyrate 1.59×10^{-3}). Similar enzyme preparations have been obtained from pig liver particles (Jencks and Lipmann, 1957), pig kidney and rabbit liver (Kellerman, 1958), human liver and kidney (Moldave and

Meister, 1957) and rat liver (Lehninger and Greville, 1953).

A large group of other carboxylic acids can also be activated by medium-chain acyl-CoA synthetase. This group included several branched and unsaturated medium-chain fatty acids, hydroxy-substituted fatty acids including D and L 3-hydroxybutyrate, benzoate, phenylacetate, 2,4-dichlorophenoxyacetate, p-aminobenzoate, benemid, phenylpropionate, p-aminophenylacetate, cinnamate, α-picolinate and nicotinate (Mahler et al., 1953; Schachter and Taggart, 1954). When used in high concentrations, acetate and propionate are also activated (Jencks and Lipmann, 1957). Pent-4-enoic acid, an interesting hypoglycemic drug, is probably also activated by this enzyme, which can be concluded from the strong inhibiting effect of octanoate on pent-4-enoic acid activation (Fukami and Williamson, 1971; Holland and Sherratt, 1973; Holland et al., 1973). Beef liver may contain more medium-chain acyl-CoA synthetases. Two medium-chain acyl-CoA synthetases could be separated by Killenberg et al. (1971). Both enzymes were able to activate medium-chain fatty acid but only one enzyme was able to activate salicylate while the other (a "Mahler enzyme") did not. The two medium-chain acyl-CoA synthetases obtained by Bar-Tana et al. (1968) seem to be interconvertible (Bar-Tana and Rose, 1968) and represent two forms of the "Mahler enzyme". Evidence for a phosphorylation (to the active form) and dephosphorylation (to the inactive form) of medium-chain acyl-CoA synthetase has been presented by Harel et al. (1961). In addition to the mammalian enzymes, medium-chain acyl-CoA synthetases have been described for bacteria (Samuel et al., 1970) and higher plants (Millerd and Bonner, 1954).

The activity of beef liver medium-chain acyl-CoA synthetase increases with an increase in pH from 6.5

enzymes. Some results of substrate specificity studies taken from the literature are given in Table VI.

Big differences in K_m values for the fatty acids are reported (e.g. K_m values for oleate from 1.39 μM (Marcel and Suzue, 1972) up to 4000 μM (Borgstrøm and Wheeldon, 1961) are given for the liver microsomal enzyme). These differences can be ascribed to difference in the assay methods used. The presence of albumine which strongly binds long-chain fatty acids (Goodman, 1958; Reynolds et al., 1968; Spector et al., 1969; Spector et al., 1971) and the binding of fatty acids to other proteins or lipid components in the assay system, strongly interferes with K_m determinations. Most reliable kinetic parameters are probably given in the studies of Marcel and Suzue (1972) and Suzue and Marcel (1972 a and b) in which a very sensitive radioassay was used, worked out by Samuel et al. (1970) and based on the insolubility of acyl-CoA in diethyl ether (see Section II). K_m values for long-chain fatty acids were found to be 1 to 3 μM, using rat liver microsomes as enzyme source. Similar values were found by Pande (1972) using a similar assay. The dependency of the K_m on protein concentration and assay volume, reported in the study, may be counteracted by the use of silanized reaction tubes and the presence of triton WR 1339 in the assay, as used by Suzue and Marcel (1972 b). Differences in the K_m for coenzyme A, between straight-chain saturated fatty acids (33-39 μM for the activation of C_{16}, C_{18} and C_{22}) and unsaturated fatty acids (156-333 μM for oleate, linolate, linoleate and arachidonate) found in rat liver microsomes by Pande and Mead (1968 a), led to the suggestion of the existence of two long-chain acyl-CoA synthetases, one for saturated and one for cis-unsaturated fatty acids. A similar suggestion has

been given by Lippel (1971 a, 1973 a) who also proposed two long-chain acyl-CoA synthetases in rat liver microsomes: one for the activation of saturated and trans-monounsaturated fatty acids and a second one for the activation of cis-mono-, di-, and polyunsaturated fatty acids. These proposals were disproved by Marcel and Suzue (1972). From fatty acid competition studies with rat liver microsomes they concluded that palmitate, palmitoleate, oleate, linoleate and linolenate are all activated by the same long-chain acyl-CoA synthetase. The big difference in K_m for coenzyme A between palmitate and oleate activation as reported by Pande and Mead (1968 a) was not reproduced in this study (K_m values for CoA of 7.2 and 9.5 µM were found respectively).

In Fig. 3 the result of our competition studies between oleate (C_{18}:1, Δ9 cis) and elaidate (C_{18}:1, Δ9 trans) are shown using a similar assay as used by Marcel and Suzue (1972). It can be seen that these cis- and trans-monounsaturated fatty acids behave as mutual competitors in the activation which disproves Lippel's proposal. From these experiments it can be concluded that at least in rat liver microsomes all C_{16} and C_{18} fatty acids, in spite of their configuration, are activated by the same acyl-CoA synthetase. The influence of the position of double bonds on K_m and V_{max} in the activation of cis, cis octadecadienoic acids has been studied by Suzue and Marcel (1972 a). The activation of cis- and trans-isomers of octadecanoic acid was studied by Lippel *et al.* (1973 a and b). Although the V_{max} for acids with a Δ9 double band is somewhat lower than for their isomers (Suzue and Marcel, 1973 a; Lippel *et al.*, 1973 a and b), the K_m for Δ9 unsaturated fatty acids turned out to be the lowest (Suzue and Marcel, 1972 a). The activation of branched long-chain fatty acids

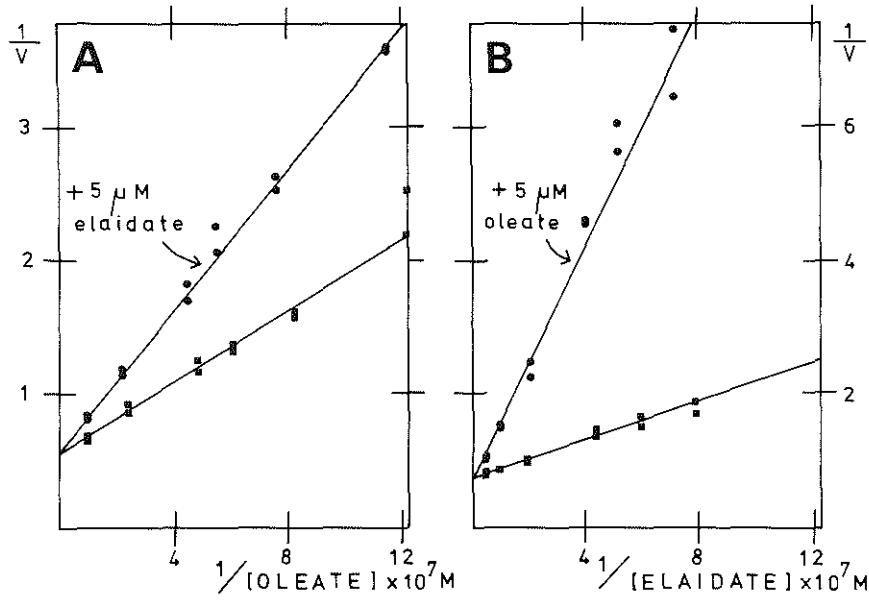

FIG. 3

OLEATE AND ELAIDATE ACTIVATION BY RAT-LIVER MICROSOMES.

[1-^{14}C]-oleate (Fig. 3 A) and [9,10-^{3}H]-elaidate (Fig. 3 B) activations were tested according to Marcel and Suzue (1972) as described by Groot et al. (1974).

A) [1-^{14}C]-oleate activation ■——■ ; [1-^{14}C]-oleate activation in the presence of 5 µM unlabeled elaidate ●——● (K_m oleate = 2.7 µM; V_{max} = 189 mU/mg protein).

B) [9,10-^{3}H]-elaidate activation ■——■ ; [9,10-^{3}H]-elaidate activation in the presence of 5 µM unlabeled oleate ●——● (K_m elaidate = 2.2 µM; V_{max} = 142 mU/mg protein).

(Lippel, 1973 b) and hexadecadienoic acids (Pettersen, 1973) by rat liver microsomes and by rat liver mitochondria, has been reported.

From substrate specificity studies, performed with microsomes or mitochondria from several rat tissues, Aas (1971 a and b) suggested the existence of two long-chain acyl-CoA

synthetases, one with optimal activity towards laurate and one with optimal activity towards palmitate (see also Table VI). Similar ideas were brought forward by Brindley and Hübscher (1966), Hübscher (1970) and by Pande (1972) and were substantiated by Farstad et al. (1973), who demonstrated, using the same assay as Aas, the presence of only one peak at C_{16} in human thrombocytes. Competition experiments performed by Suzue and Marcel (1972 b) in rat liver microsomes, however, showed that octanoate and palmitate are activated by the same enzyme which excludes the existence of a distinct lauryl-CoA synthetase in this fraction. In rat skeletal muscle mitochondria, in which a matrix octanoate activation is absent, we were also able to demonstrate that, although two activity peaks were present (at C_{12} and C_{16}), octanoate is activated by the same acyl-CoA synthetase as palmitate (Groot and Hülsmann, 1973). The affinity of this mitochondrial enzyme towards palmitate was much higher than towards octanoate, which was concluded from the very strong inhibition of octanoate activation by palmitate. In summary, fatty acid competition studies performed in both microsomal and mitochondrial fractions of rat tissues do not give any support for the presence of more than one long-chain acyl-CoA synthetase in each fraction. Whether the mitochondrial and microsomal enzymes are immunologically different has to be tested.

b. K_m values of other reactants

The reported K_m values for ATP in palmitate activation by rat liver microsomes vary between 0.29 and 4 mM (Pande and Mead, 1968 a; Van Tol and Hülsmann, 1970; Bar-Tana et al., 1971; Pande, 1972). A K_m of 3.23 mM has been reported for the purified rat liver

microsomal enzyme (Bar Tana et al., 1972). A strong influence of the salt concentration during the assay on the K_m for ATP has been reported (Pande, 1972), which probably explains the wide range of reported values. A similar explanation can be given for the differences in K_m values for CoA (3.4-52 µM for palmitate activation by rat liver microsomes, Pande and Mead, 1968 a; Bar-Tana et al., 1971; Marcel and Suzue, 1972; Pande, 1972).

c. pH optimum and molecular weight

The pH optimum of long-chain fatty acid activation in rat liver microsomes (Pande and Mead, 1968 a; Pande, 1972; Lippel et al., 1973 a), guinea-pig liver microsomes (Kornberg and Pricer, 1953) and rat gut microsomes (Senior and Isselbacher, 1960) was found to be between 6.5 and 8. With the purified rat liver microsomal enzyme, a sharp pH optimum at pH 9 was obtained (Bar-Tana et al., 1971). The molecular weight of this purified enzyme was estimated (250,000). This values, however, may be an overestimation due to the binding of an unknown amount of detergent to the enzyme (Bar-Tana et al., 1971).

d. Stabilizers, inhibitors and stimulators

Palmitate activation by long-chain acyl-CoA synthetase is inhibited by the products of the reaction palmitoyl-CoA and AMP. The inhibition by palmitoyl-CoA is competitive towards CoA. A practically identical K_i for palmitoyl-CoA (4 µM) and K_m for CoA (3.3 µM) have been reported by Pande (1973) using rat liver microsomes. AMP inhibition of palmitate activation by both rat liver microsomes and mitochondria is competitive towards ATP (Van Tol and Hülsmann, 1970) although a mixed type of inhibition has also been re-

ported (Pande and Mead, 1968 a). The K_i for AMP, however, (0.2 mM) is lower than the K_m for ATP (0.4-0.7 mM, Van Tol and Hülsmann, 1970). Adenosine, a physiological breakdown product of AMP, is still a more potent competitive inhibitor (De Jong and Hülsmann, 1970 b; Van Tol and Hülsmann, 1970; De Jong, 1971). K_i values of 0.1 mM have been reported. Rat liver mitochondrial palmitate activation is further inhibited by α-bromopalmitate (Mahadevan and Sauer, 1971), atractyloside (Alexandre et al., 1969; Skrede and Bremer, 1970; Pande, 1973), bongkrekic acid (Batenburg, 1974) and several detergents including sodium desoxycholate, triton X-100 and sodium taurocholate (Pande and Mead, 1968 a). α-Bromopalmitate inhibition has also been reported for palmitate activation in rat liver microsomes (Pande et al., 1971).

Both microsomal and mitochondrial long-chain fatty acid activation are stimulated by the presence of high concentrations of several salts (Aas, 1970; Pande, 1972; Groot and Hülsmann, 1973). Both activities are also strongly stimulated by the addition of the particle-free supernatant from several rat tissues (Farstad et al., 1967; Farstad, 1967 and 1968). However, salt stimulation and particle-free supernatant stimulation do not seem to be additive (Aas,1970; Groot and Hülsmann, 1973). Particle-free supernatant stimulation of long-chain fatty acid activation, which is only seen when ATP and Mg^{2+} ions are present in equimolar amounts (Aas, 1970), has been ascribed to a protein or a protein bound factor (Farstad et al., 1967; Farstad, 1967 and 1968). This factor has been partially purified (Aas, 1970). Preincubation of rat liver microsomes in the presence of 5 mM $MgCl_2$ decreased the rate of palmitate activation, an inactivation which can be pre-

vented by the presence of ATP (Farstad, 1968). Inactivated enzyme, however, is partially reactivated by an incubation with particle-free supernatant factor and ATP. A phosphorylation of long-chain acyl-CoA synthetase to an active enzyme by a kinase present in the particle-free supernatant has been proposed (Farstad, 1968). These stimulations are probably not unique for membrane-bound acyl-CoA synthetases. Octanoate activation by a matrix preparation of rat heart mitochondria is also highly stimulated by the addition of a muscle particle-free supernatant (Scholte, unpublished). Particle-free supernatant stimulation of palmitate activation by the rat liver microsomal enzyme could not be detected by others (Bar-Tana, 1971; Lippel, 1971 b). These experiments, however, were performed under conditions (high salt or a low ATP/Mg^{++} ratio) where no stimulation can be observed (Aas, 1970). Further experiments have to be performed to elucidate the significance of the particle-free supernatant stimulation. Long-chain fatty acid activation in rat gut and rat liver microsomes is also stimulated by lecithin (Pande and Mead, 1968 a; Pande, 1972; Rodgers et al., 1972) and albumin (Rodgers et al., 1972), a stimulation which can probably be ascribed to a prevention of detergent like effects of free fatty acids on the activation enzyme by a reversible binding to the additions made. Mitochondrial long-chain acyl-CoA synthetase is very sensitive towards proteolytic attack. Rapid inactivation of the enzyme in the presence of Nagarse (subtilisin, EC 3.4.21.14) has been described (De Jong and Hülsmann, 1970 a and b; Pande and Blanchaer, 1970; Van Tol and Hülsmann, 1970; De Jong, 1971; Hülsmann et al., 1971; Groot et al., 1974). During palmitate activation (ATP, CoA and palmitate present) a protection against Nagarse was observed (De Jong,

1971). The stabilizing effect of ATP on long-chain acyl-CoA synthetase, described by many groups (Farstad, 1968; Aas, 1972; Pande, 1972; Sánchez et al., 1973) can probably also be explained by a similar protection against endogenous proteolytic activities.

B. SPECIFICITY TOWARDS NUCLEOTIDES

1. ACYL-CoA SYNTHETASES (AMP FORMING)

Purified mammalian acyl-CoA synthetases (AMP forming) are very specific in their nucleotide requirement (Campagnari and Webster, 1963; Webster et al., 1965; Webster, 1969; Bar-Tana et al., 1971). Only ATP and in some cases dATP is accepted. A very low activity with CTP and other ribonucleotides has been described for the bacterial enzymes (Massaro and Lennarz, 1965; Samuel et al., 1970). Activation of long-chain fatty acids, in rat liver mitochondria (Wojtczak et al., 1965) and medium-chain fatty acids, in bovine brain mitochondria (Beattie and Basford, 1966), by high-energy non-phosphorylated intermediates of oxidative phosphorylation as energy source has been proposed. These proposals, however, were rejected by Van den Bergh (1965 a, 1966) and Van den Bergh et al. (1969), who demonstrated the involvement of nucleoside triphosphates, formed by substrate level phosphorylation linked to the α-oxoglutarate oxidation step of the Krebs cycle.

2. ACYL-CoA SYNTHETASES (GDP FORMING)

In addition to acyl-CoA synthetases (AMP forming), a second group of mammalian fatty acid activating enzymes has been described: acyl-CoA synthetases (GDP forming), which catalyze the following reversible reaction:

$$RCOO^- + GTP^{4-} + CoASH \xrightleftharpoons{Mg^{2+}} RCOSCoA + GDP^{3-} + P_i^{2-} \quad (2)$$

(Rossi and Gibson, 1964; Allman et al., 1966; Rossi et al., 1966; Sartorelli et al., 1966; Galzigna et al., 1967; Rossi et al., 1967; Rossi et al., 1968; Rossi et al., 1970; Rossi and Carignani, 1971). Acyl-CoA synthetases (GDP forming) seem to be present in several mammalian tissues and have been isolated from beef liver (Rossi and Gibson, 1964), rat kidney (Rossi et al., 1968) and rat liver (Galzigna et al., 1967) mitochondria. ITP but not UTP, CTP nor ATP can replace GTP (Rossi and Gibson, 1964; Galizigna et al., 1967). Fatty acids with chain length from C_4 to C_{12} are activated by the beef liver enzyme. The purified rat liver enzyme activates C_4 to C_{16}. The absence of activity towards long-chain fatty acids of the beef liver enzyme has been ascribed to the use of organic solvents in the isolation procedure. A lipid constituent of the enzyme, probably lecithine, which influences the substrate specificity, is removed in this procedure (Sartorelli et al., 1966). Rat liver acyl:CoA ligase (GDP forming) has been extensively studied (Sartorelli et al., 1966; Galzigna et al., 1967; Rossi et al., 1970). The lowest K_m was found with octanoate (0.2 mM). The K_m for palmitate turned out to be much higher (3.1 mM). The enzyme is, like all other GTP-dependent acyl-CoA synthetases, strongly inhibited by F^- ions and inorganic phosphate (Galzigna et al., 1967). In the presence of 16 mM phosphate buffer, GTP-dependent fatty acid activation can be ignored (Rossi et al., 1967). The enzyme also catalyzes arsenolysis of acyl-CoA esters in the absence of GDP and inorganic phosphate (Galzigna et al., 1967; Rossi et al., 1968). The partially purified enzyme is inhibited by atractyloside (Allman et al., 1966). In more recent work is has been shown that a cofactor, probably 4'-phosphopantetheine, has to be bound to the enzyme to perform catalytic activity (Rossi et al., 1970). Acyl-CoA synthetase (GDP forming) is localized in the mitochondria in

the innermembrane-matrix compartment (Van den Bergh, 1967 b and c; Galzigna et al., 1967; Garland et al., 1970; Haddock et al., 1970; Lippel and Beattie, 1970). When tested with medium-chain fatty acids as substrates, a matrix localization was found (Haddock et al., 1970). However, when palmitate was used as substrate, an equal distribution between the innermembrane and matrix fractions was observed (Lippel and Beattie, 1970). A recent study of Rossi and Carignani (1971) explains this discrepancy. Two acyl-CoA synthetases (GDP forming) were reported in rat liver mitochondria, one firmly bound in a lipoprotein complex to the innermembrane and specific for long-chain fatty acids and a second one specific for short- and medium-chain fatty acids which is located in the matrix mitochondrialis.

The importance of GTP-dependent fatty acid activation is questionable. The activity of these enzymes is very low when compared with ATP-dependent fatty acid activation. Moreover, the findings of Rossi's group in rat liver mitochondria (GTP-dependent oleate activation at about 25% of the rate of ATP-dependent oleate activation (Galzigna et al., 1967) could not be confirmed by other laboratories (GTP-dependent, but not necessarily GTP-specific, palmitate activation turned out to be 4% of the rate of ATP-dependent palmitate activation (Van Tol et al., 1969; Lippel and Beattie, 1970)). This was reported to be due to a difference in rat strain used (Rossi et al., 1970). No or very low long-chain acyl-CoA synthetase activity with GTP substituted for ATP was further found in bovine brain mitochondria (Beattie and Basford, 1966), rat liver (De Jong and Hülsmann, 1970 b), heart (Van den Bergh, 1967 a; Pande and Mead, 1968 b), kidney (Van den Bergh, 1967; Pande and Mead, 1968 b), intestine (Pande and Mead, 1968 b), adipose tissue (Hittelman et al., 1969; Drahota et al., 1970; Lippel et al, 1971) and other rat organs (Pande and Mead,

1968 b). Arguments for the operation of a long-chain acyl-CoA synthetase (GDP forming) have thought to be found in fatty acid oxidation studies. The carnitine-independent long-chain fatty acid oxidation by uncoupled rat liver mitochondria, in which no ATP is formed by oxidative phosphorylation, has been ascribed to an acyl-CoA synthetase (GDP forming) initiated fatty acid oxidation (GTP generated from the succinyl-CoA ⟶ succinate step of the Krebs cycle) (Van den Bergh, 1965 b, 1966, 1967 a and b).
The inhibition of this fatty acid oxidation by inorganic phosphate, thought to be diagnostic for the involvement of an acyl-CoA synthetase (GDP forming), could also be explained by a phosphate-induced leakage of Krebs cycle intermediates out of the matrix compartment of the mitochondria (Van Tol et al., 1969; De Jong et al., 1969). Moreover, recently Batenburg and Van den Bergh (1972 and 1973) have shown that during carnitine-independent long-chain fatty acid oxidation in rat liver mitochondria pyrophosphate is accumulated when the mitochondrial pyrophosphatase is inhibited by fluoride. This was seen in both coupled and uncoupled mitochondria. As pyrophosphate is the product of the acyl-CoA synthetase (AMP forming) and not of acyl-CoA synthetase (GDP forming) catalyzed fatty acid activation, it must be concluded that the uncoupled mitochondria are able to activate long-chain fatty acids with ATP generated from GTP by the action of nucleosidediphosphate kinase (EC 2.7.4.6). Long-chain acyl-CoA may be formed by the action of medium-chain acyl-CoA synthetase (AMP forming) (Groot et al., 1974; see Section V, B). Taking these results together, it can be concluded that for the explanation of the properties of the carnitine-independent long-chain fatty acid oxidation by uncoupled rat liver mitochondria no long-chain acyl-CoA synthetase (GDP forming) has to be assumed.

The practical absence of GTP-dependent fatty acid activation in many tissues except in some rat strains does not indicate that its role in fatty acid metabolism is very important.

V THE SUBCELLULAR LOCALIZATION OF ACYL-CoA SYNTHETASES

A. SHORT- AND MEDIUM-CHAIN ACYL-CoA SYNTHETASES

The subcellular localization of ATP-dependent acetate, propionate, butyrate and octanoate activation has been studied in many mammalian tissues. Generally the cell fractionation technique (De Duve et al., 1955), or the sequential extraction procedure (Klingenberg and Pfaff, 1966; Pette, 1966) were used. The interpretation of the results of these studies can be influenced by the marker enzymes used. In all studies (Aas and Bremer, 1968; Aas and Bremer, 1969; Aas, 1971 a; Scholte et al., 1971; Barth et al., 1971; Barth et al., 1972 a; Quraishi and Cook, 1972; Ballard, 1972; Tencati and Rosenberg, 1973; Goodridge, 1973; Batenburg, 1974) performed with a variety of mammalian tissues, at least part of the acetate, propionate or butyrate activation was found to be localized in the mitochondria.

Experiments performed with isolated mitochondria indicate that the short-chain fatty acid activation is latent i.e. mitochondria have to be disrupted to expose the activity (Aas and Bremer, 1968; Aas, 1971 a). Oxidation experiments, performed with isolated rat heart mitochondria, further show that short-chain fatty acids can be oxidized in the absence of carnitine (Fritz et al., 1962) which indicates that the activation must be localized beyond the carnitine barrier (Fritz and Yue, 1963; Bremer, 1963), which is the innermembrane. Mito-

chondrial sublocalization studies performed by Aas (1971 a) in rat liver and by Scholte et al. (1971) in guinea-pig heart have shown that the ATP-dependent short-chain fatty acid activation is localized in the matrix mitochondrialis.

Short-chain fatty acid activation, especially of acetate, has also been reported to be present in the cytoplasmic fractions of liver (Kornacker and Lowenstein, 1965; Aas and Bremer, 1968; Ballard, 1972; Barth et al., 1971 and 1972 a; Murthy and Steiner, 1972; Goodridge, 1973), kidney (Barth et al., 1971 and 1972 a; Quraishi and Cook, 1972), heart (Barth et al., 1971 and 1972 a; Ballard, 1972; Quraishi and Cook, 1972) and other organs like epididymal fat, mammary gland, skeletal muscle and brain (Barth et al., 1971 and 1972 a; Ballard, 1972; Quraishi and Cook, 1972; Tencati and Rosenberg, 1973) of several species. 80-95% of the total acetate activation has been reported to be present in the rat liver cytosol (Barth et al., 1971; Ballard, 1972), 13-50% was found in the rat heart cytosol. Scholte et al. (1971), however, reported that all acetate activation found in the guinea-pig heart cytosolic fraction could be ascribed to leakage of the enzyme from damaged mitochondria and concluded that all acetate activation (and also propionate and butyrate activation) is localized in the matrix mitochondrialis. In this study, propionyl-CoA carboxylase (EC 6.4.1.3) was used to calculate the leakage of the mitochondrial matrix enzymes into the cytosolic fraction. It is a good marker enzyme, because it is like the short-chain acyl-CoA synthetases, very loosely bound to the inner side of the mitochondrial innermembrane (Scholte et al., 1971). The marker enzyme used in other studies, glutamate dehydrogenase (Aas and Bremer, 1968; Barth et al., 1971; Goodridge, 1973) is more tightly bound to the innermembrane (Scholte, 1969) which implies that the mitochondrial matrix leakage is underestimated. Therefore,

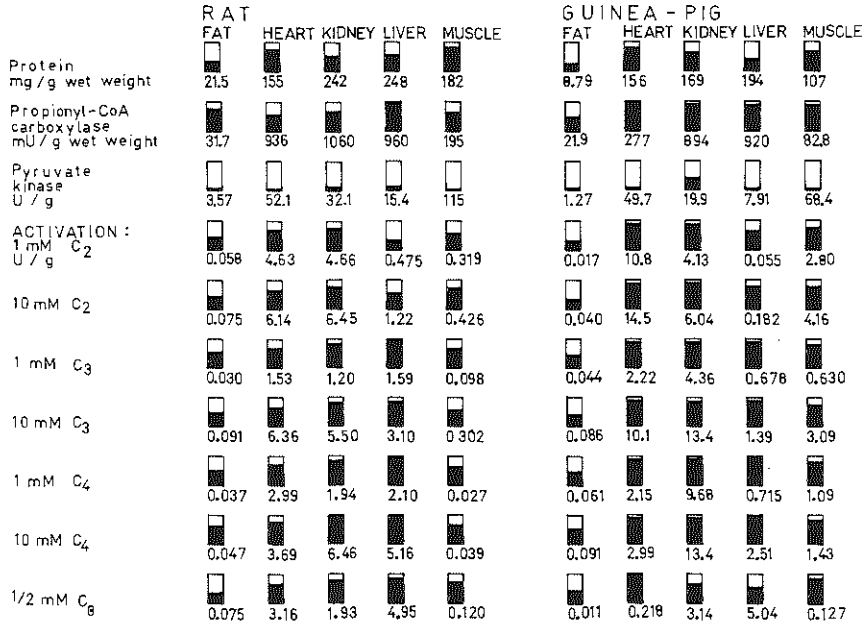

FIG 4

THE SUBCELLULAR LOCALIZATION OF SHORT-CHAIN FATTY ACID ACTIVATION IN RAT AND GUINEA-PIG TISSUES.

10% w/v homogenates were prepared in a medium of 0.25 M sucrose, 10 mM Tricine-KOH, 1 mM EDTA, 0.2 mM dithiothreitol (pH = 7.4) by means of an electrically driven Potter-Elvehjem homogenizer, and centrifuged for 10 min at 30,000 x g_{max}. After separation of the supernatants, the sediments were resuspended in medium. In both fractions propionyl-CoA carboxylase was determined as a mitochondrial matrix marker enzyme (at 25°C, Scholte et al. (1973), minus the ATP regenerating system) and pyruvate kinase as a cytoplasmic marker enzyme (at 25°C, ref. in Scholte et al. (1973)). 0.8 mM fructose 1,6-diphosphate was added in the assays of fat, kidney and liver fractions.
C_2, C_3, C_4 and C_8 activations were measured at the indicated concentrations of fatty acid according to the procedure described by Groot et al. (1974), after preincubation of the fractions with 0.025 % Lubrol WX for 12 min at 0°C. The fractionated fat and muscle were epididymal fat pad and M.masseter, respectively. The floating fat was removed from the supernatant. The distribution of activities (as part of total activity = whole rectangle) between the particulate fraction (black area) and supernatant (open area) is shown.

we reexamined the distribution of short- and medium-chain fatty acid activation (C_2, C_3, C_4 and C_8) in different rat and guinea-pig tissues and compared the distribution between a particulate fraction (10 min at 30,000 x g_{max}) and its supernatant with the distribution of propionyl-CoA carboxylase (a mitochondrial matrix marker) and pyruvate kinase (EC 2.7.1.40) (a good cytoplasmic marker in the presence of 0.2 mM dithiotreitol (Scholte, 1973 a). To obtain additional information about the K_m values for the fatty acids, the activation experiments were carried out at low (1 mM) and at high (10 mM) concentrations. The results of these studies are given in Fig. 4. In practically all tissues tested, more than 90% of the cytoplasma marker pyruvate kinase is found in the supernatant fraction. Appreciable amounts of mitochondria are damaged during the homogenization of rat heart, skeletal muscle and kidney and of guinea-pig epididymal fat which can be concluded from the propionyl-CoA synthetase activity which has escaped from the mitochondria to the supernatant. Mitochondria from other tissues are far less damaged. The percentage of the total tissue activity of C_2, C_3 or C_4 activation, found in the supernatant of kidney, heart and skeletal muscle of both rat and guinea-pig does not exceed the percentage of propionyl-CoA carboxylase in this fraction. It can therefore be concluded that kidney, heart and skeletal muscle of both species are not equipped with cytoplasmic short-chain acyl-CoA synthetases. All C_2, C_3 and C_4 activation present, must be localized in the matrix mitochondrialis in these tissues.

In liver and epididymal fat tissue, however, a different pattern is found. 42-67% (rat) and 21-39% (guinea-pig) of the acetate activation (tested at 10 and 1 mM respectively) is found in the supernatant, while only 5% (rat) and 11% (guinea-pig) propionyl-CoA synthetase is found in this fraction. In epididymal fat about 30% (rat)

and 17% (guinea-pig) of the total acetate activation, after correction for mitochondrial leakage, can be ascribed to a cytoplasmic enzyme. Some C_3 but practically no C_4 activation in liver and epididymal fat can be ascribed to a cytoplasmic short-chain acyl-CoA synthetase. The activities of the cytoplasmic acetyl-CoA synthetases are low, especially in the guinea-pig. In several tissues somewhat more C_8 activation is found in the supernatant than can be explained by mitochondrial leakage. In these supernatant fractions (10 min at 30,000 x g_{max}) appreciable amounts of microsomes may be present, and the microsomal long-chain acyl-CoA synthetase will contribute to the activation of octanoate.

Rough estimates of K_m values for short-chain fatty acids, calculated from the activities at 1 and 10 mM, suggest the presence of an acetyl-CoA synthetase (K_m for C_2 <1 mM, K_m for C_3 >5 mM) in the cytoplasma of liver and adipose tissue of both rat and guinea-pig. In recent studies (not shown) we also found such an enzyme in rat small intestinal epithelium and lactating mammary gland (Scholte and Groot, 1975). K_m values for C_2, C_3 and C_4 in the particulate fraction of both rat and guinea pig liver are similar to those obtained in a mitochondrial matrix fraction of guinea-pig liver (see Table IV). In kidney, heart and skeletal muscle the K_m values for both C_2 and C_4 are low (<1 mM) but high for C_3 (>5 mM), indicating the presence of acetyl-CoA and butyryl-CoA synthetases and the absence of a distinct propionyl-CoA synthetase, which is in agreement with our study in guinea-pig heart mitochondria (Table II).

In Saccharomyces cerevisiae acetyl-CoA synthetase has a bimodel distribution, depending upon the age of the culture. After 24 h of aerobic growth, the enzyme was found principally in the light particulate fraction supposed to contain the microsomes, while after 48 h of incubation the enzyme sedimented with the mitochondria.

The activity in the supernatant was very low (Klein and Jahnke, 1971). Chloramphenicol prevented the accumulation of the enzyme in the mitochondria. The enzyme remained on the microsomes (Klein and Jahnke, 1968).

In Tetrahymena pyriformis most of acetyl-CoA synthetase is localized in the peroxysomes, probably due to the specific functions of these organelles in the protozoan (Levy, 1970). Localization studies of medium-chain acyl-CoA synthetase (AMP forming) are somewhat complicated by the overlapping activities of both long-chain acyl-CoA synthetases (Suzue and Marcel, 1972; Groot and Hülsmann, 1973) and butyryl-CoA synthetase (Webster et al., 1965) towards medium-chain fatty acids (C_5-C_{10}). The enzyme has been purified from the post-nuclear particulate fraction of liver (Mahler et al., 1953; Jencks and Lipmann, 1957; Moldave and Meister, 1957; Kellermann, 1961) which suggests a mitochondrial localization. Subfractionation studies of Aas and Bremer (1968) and Aas (1971 a) in rat liver, heart and kidney give further support to this supposition. The activation of medium-chain fatty acids on the rat liver microsomes (Suzue and Marcel, 1972) and on the mitochondrial outermembrane (Groot and Hülsmann, 1973) is strongly inhibited by palmitate and could be ascribed to overlapping activity of long-chain acyl-CoA synthetase towards medium-chain fatty acids. Studies with isolated rat liver mitochondria indicate that most of the hexanoate and octanoate activation is latent (Aas, 1971 a). This finding is in agreement with mitochondrial subfractionation studies (Haddock et al., 1970; Aas, 1971 a; Groot et al., 1974; Batenburg, 1974) in which it was shown that medium-chain acyl-CoA synthetase is localized in the matrix mitochondrialis.

B. LONG-CHAIN ACYL-CoA SYNTHETASE

The first studies on the subcellular localization of long-chain acyl-CoA synthetase have been carried out in

mammalian small intestinal mucosa (Senior and Isselbacher, 1960; Ailhaud et al., 1963; Johnston and Bearden, 1962; Hübscher et al., 1963) and the enzyme was found to be localized predominantly in the microsomal fraction. This result has been confirmed by De Jong and Hülsmann (1970 a). The activity found in this study to be associated with the mitochondrial fraction could be explained by microsomal contamination. Fractionation studies, however, performed in rat liver (Farstad et al., 1967; Van Tol and Hülsmann, 1969; Lippel et al., 1970; De Jong and Hülsmann, 1970 a; Aas, 1971 a) and in guinea-pig liver (Borgstrøm and Wheeldon, 1962) have shown that in addition to the active microsomal long-chain acyl-CoA synthetase, mitochondria contribute 30-50% to the overall activity. A similar mitochondrial contribution is found in rat kidney (Aas, 1971 a) while in tissues in which an endoplasmatic reticulum is not well developed, like heart and skeletal muscle, most of the long-chain acyl-CoA synthetase is found to be associated with the mitochondria (De Jong and Hülsmann, 1970 a; Aas, 1971 a). The absence of long-chain acyl-CoA synthetase activity in rat small intestinal mitochondria (De Jong and Hülsmann, 1970 a) does not fit this concept and can probably be explained by a selective loss of the outermembrane from the mitochondria to the microsomal fraction during the rather rough homogenization procedure. Preliminary experiments in our laboratory indicate that about 30% of the palmitate activation present in a homogenate of isolated rat small intestinal villus cells can be ascribed to the mitochondria after a correction for the cross-contamination with microsomes (marker enzymes: monoamine oxidase for the mitochondrial outermembrane and rotenone insensitive NADPH-cytochrome c reductase for the microsomes). In conclusion, long-chain acyl-CoA synthetase seems to be localized both on the mitochondria and on the mem-

branes found in the microsomal fraction. The distribution of activity between these two fractions probably depends on the relative amounts of mitochondria and microsomal membranes present in a tissue. Other localizations of long-chain acyl-CoA synthetase, however, have also been described. A high percentage of the overall activity in rat liver has been reported to be present in the nuclear fraction (Creasey, 1962; Pande and Mead, 1968 a) and to be purified in a plasma membrane preparation obtained from this nuclear fraction (Pande and Mead, 1968 a). A plasma membrane long-chain acyl-CoA synthetase has also been reported in recent studies of Batenburg (1974) and Van Golde et al. (1974) in which use was made of a mitochondrial inner- instead of an outermembrane marker enzyme. This activity could explain the preferential incorporation of [^{14}C]-label into the plasma membrane fraction of isolated rat liver hepatocytes after a short incubation in the presence of [^{14}C]-palmitate (Wright and Green, 1971). A role of this enzyme in the uptake and transport of long-chain fatty acid by the cell has been proposed (Pande and Mead, 1968 a; Wright and Green, 1971). The presence of a nuclear and/or plasma membrane bound long-chain acyl-CoA synthetase has recently been questioned by Lippel et al. (1970) and Lippel and Blythe (1972). In their studies in rat liver, practically all nuclear and plasma membrane associated activity could be explained by contamination with microsomes and mitochondrial outermembranes. This leaves two main sites of long-chain acyl-CoA synthetase activity in the mammalian cell: the mitochondria and the membranes found in the microsomal fraction.

The intramitochondrial localization of long-chain acyl-CoA synthetase in rat liver mitochondria has been studied using the subfractionation techniques worked out by Parsons et al. (1966), Sottocasa et al. (1967) and Schnaitman and Greenawalt (1968). The general con-

clusion of these subfractionation studies (Norum et al., 1966; Garland et al., 1969; Van den Bergh et al., 1969; Lippel and Beattie, 1970; Van Tol and Hülsmann, 1970; Haddock et al., 1970; Aas, 1971 a) is that the bulk of long-chain acyl-CoA synthetase activity (AMP forming) in rat liver mitochondria is localized on the outermembrane. A minor activity, however, is found in the innermembrane matrix fraction. Subfractionation studies by Van Tol and Hülsmann (1970) of rat liver mitochondria, in which the outermembrane-bound long-chain acyl-CoA synthetase (AMP forming) has been destroyed by a preincubation of the mitochondria with the protease Nagarse (De Jong and Hülsmann, 1970 a and b; Pande and Blanchaer, 1970), showed that about 90% of the mitochondrial long-chain synthetase activity is bound to the outermembrane. The remaining 10% is localized in the innermembrane matrix compartment. Further studies of Groot et al. (1974) have shown that the Nagarse resistent long-chain acyl-CoA synthetase is localized in the matrix fraction of rat liver mitochondria. We were unable to demonstrate neither long- nor medium-chain acyl-CoA synthetase (AMP forming) in association with the innermembrane as reported by Van den Bergh et al. (1969) and Rossi et al. (1971), in agreement with studies of Aas (1971 a) and Batenburg (1974). The matrix long-chain acyl-CoA synthetase activity was found to be purified together with the octanoyl-CoA synthetase activity and substrate competition experiments performed by Groot et al. (1974) have shown that octanoate and palmitate are activated by the same enzyme. The K_m for octanoate (7.6 µM) turned out to be much lower than the K_m for palmitate (23 µM) which indicates that the matrix long-chain fatty acid activation in liver mitochondria can be ascribed to the medium-chain acyl-CoA synthetase. The overlapping activity of medium-chain acyl-CoA synthetase in the matrix compartment towards long-chain

fatty acids can explain the carnitine-independent long-chain fatty acid oxidation in rat liver mitochondria found by others (Van den Bergh, 1965 b, 1966, 1967 b and c; Rossi et al., 1967; De Jong and Hülsmann, 1970 b; Skrede and Bremer, 1970; Van Tol and Hülsmann, 1970; De Jong, 1971; Batenburg and Van den Bergh, 1972 and 1973). Fatty acid oxidation experiments, however, have led to more complicated compartmentation models for long-chain acyl-CoA synthetase in rat liver mitochondria (Van den Bergh, 1967 b and c; Garland and Yates, 1967; Garland et al., 1969). In addition to the outermembrane-bound enzyme and the activity in the matrix-compartment, a third ATP-dependent long-chain acyl-CoA synthetase was proposed, localized between the (functionally defined) atractyloside barrier and the carnitine barrier (Van den Bergh, 1967 b and c). However, direct effects of atractyloside on both outermembrane long-chain acyl-CoA synthetase (Alexandre et al., 1969; Skrede and Bremer, 1970) and carnitine palmitoyl transferase (Skrede and Bremer, 1970) in addition to the well known inhibitory effects of atractyloside on the adenine nucleotide translocator, could explain Van den Bergh's experiments without the assumption of a third long-chain acyl-CoA synthetase. At the present time the results of fatty acid oxidation studies with rat liver mitochondria are in agreement with the observed localization of the ATP-dependent long-chain fatty acid activation.

In rat heart mitochondria, in contrast to liver mitochondria, palmitate oxidation is totally carnitine-dependent (Fritz et al., 1962; De Jong and Hülsmann, 1970 b; De Jong, 1971). This is in agreement with mitochondrial subfractionation studies of Scholte (1973 b). In contrast to liver mitochondria, all long-chain acyl-CoA synthetase (AMP forming) was found to be localized on the mitochondrial outermembrane. Heart mitochondria are probably not equipped with the rather broad-

specific medium-chain acyl-CoA synthetase as purified from beef liver by Mahler et al. (1953), but contain a butyryl-CoA synthetase as purified from beef heart by Webster et al. (1965), an enzyme which does not display any activity towards long-chain fatty acids.

VI ORGAN DISTRIBUTION OF ACYL-CoA SYNTHETASES

A systematic study of the organ distribution of ATP-dependent fatty acid activation in the rat, has been carried out by Aas (1971 a). The relation between chain-length of saturated, straight-chain fatty acids and their activation in different rat organ homogenates, is given in Fig. 5. This figure is taken from the paper of Aas (1971 a). Long-chain fatty acids are activated in all tissues tested. Aas (1971 a) reports that oleate activation is usually identical to stearate activation while linoleate and linolenate are activated with half that velocity. Marcel and Suzue (1972) and Suzue and Marcel (1972), however, using a more reliable assay for kinetic studies, found somewhat higher activation rates for linoleate and linolenate than for oleate and stearate in rat liver microsomes. The specific activities (in U/g wet weight) for palmitate activation given in Fig. 5 are in close agreement with the values given by Pande and Mead (1968 b) in their distribution study of rat tissues. However, they reported lower values for testes (0.03 U/g wet weight). Their value in intestine (0.02 U/g wet weight) is 100 times lower than those measured in our laboratory in homogenates of isolated small intestinal villus cells (2.1 U/g wet weight) probably due to proteolysis (Scholte and Groot, 1975). Long-chain fatty acid activation is high in tissues performing active fatty acid oxidation (liver, heart) or triglyceride synthesis (epididymal fat, small intestinal mucosa). Epididymal fat tissue has the highest specific activity for

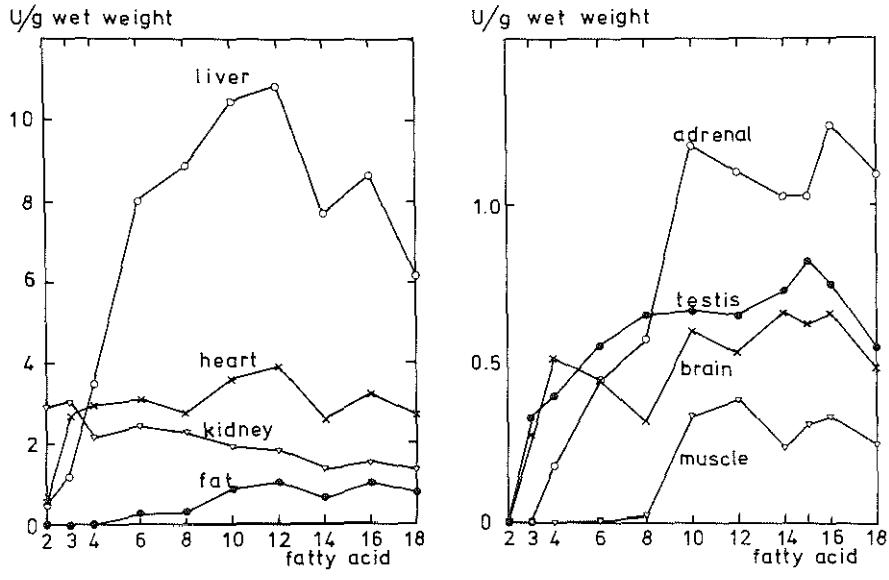

FIG. 5
THE ACTIVATION OF SATURATED STRAIGHT-CHAIN FATTY ACIDS BY HOMOGENATES OF SEVERAL ORGANS FROM THE RAT.

Abscissa: fatty acid chain length. Ordinates: specific activity of fatty acid activation in μmoles/g wet weight. min. The activations were tested at 10 mM (C_2-C_4), 7.5 mM (C_5 and C_6), 3.75 mM (C_7), 1.5 mM (C_8 and C_9) and 1 mM (>C_9) fatty acid concentration. The incubation mixture contained 8.3 mg/ml albumin. For details see Aas (1971 a). (Reproduced from Aas (1971 a) with the kind permission of the author and of Biochimica et Biophysica Acta.)

long-chain fatty acid activation on protein basis.

In rat and human skeletal muscle, in which practically all long-chain acyl-CoA synthetase is associated with the mitochondria (Aas, 1971; Scholte and Busch, 1974) interesting differences are found between red and white muscle. Mitochondria, isolated from a red skeletal muscle of the rabbit, perform a two times higher palmitate activation than mitochondria from white muscle (18.9 and 7.8 mU/mg

mitochondrial protein, respectively; Pande and Blanchaer, 1971). Red skeletal muscles, equipped with an enzymatic apparatus to perform aerobic metabolism (Pette, 1966) during long-lasting slow activity, also contain a higher concentration of mitochondria (see De Haan et al., 1973) which magnifies the difference in long-chain acyl-CoA synthetase activity between red and white muscle. Palmitate activation in mitochondria, isolated from both types of muscle, only slightly exceeds the maximal rate of β-oxidation (Pande, 1971), which stresses the importance of the mitochondrial long-chain fatty acid activation in muscle to initiate fatty acid oxidation. Physical training, which induces an increase in oxidative capacity of skeletal muscle (Holloszy, 1967; Fage et al., 1968; Holloszy et al., 1970; Molé et al., 1971; Baldwin et al., 1972) leads to an increase of palmitoyl-CoA synthetase activity in the trained muscle. A more than two fold increase was found on tissue weight basis in the hind limb muscles of rats, trained for several months on treadmill (Molé et al., 1971). This increase in palmitate activation can be ascribed to both enhanced mitochondrial concentration and increased specific activity of the mitochondria.

Interesting differences in long-chain acyl-CoA synthetase activity in different parts of rat small intestinal mucosa have also been described. The specific activity of palmitate activation in microsomal fractions of scrapings of mucosa from upper- and lower jejunum and upper- and lower ileum decreases from 119-21 mU/mg microsomal protein (Rodgers and Bochenek, 1970). Acyl-CoA monoglyceride acyltransferase activity parallels the palmitate activation and the activity of both enzymes probably reflects the importance of the different parts of small intestine in lipid absorption.

Palmitate activation has also been measured in human liver, adipose tissue, blood platelets and leucocytes (Farstad and Sanders, 1971; Farstad et al., 1973; Scholte

and Busch, 1974). The activity found in human liver (7.5 mU/g wet weight) is about half the activity found in the rat. In human adipose tissue, similar values were found as in the rat. Long-chain acyl-CoA synthetase in human liver, platelets and leucocytes is, like in rat, shared between the mitochondrial and microsomal fractions.

Medium-chain fatty acids are activated in all rat tissues tested. However, low activation rates were found in epididymal fat and skeletal muscle. Medium-chain fatty acids can be activated both by long-chain acyl-CoA synthetase and by butyryl-CoA synthetase, the enzyme purified by Webster et al. (1965) from beef heart. The observed C_5-C_{10} activation in different tissue therefore, does not indicate the presence of a separate medium-chain acyl-CoA synthetase, an enzyme with the lowest K_m for octanoate as purified by Mahler et al. (1953) from beef liver. Medium-chain acyl-CoA synthetase is present in rat liver mitochondria (Groot et al., 1974). Its presence in other tissues, however, is uncertain. Kidney and heart probably contain a butyryl-CoA synthetase instead. The octanoate activation, found in skeletal muscle mitochondria of the rat, could be ascribed to long-chain acyl-CoA synthetase (Groot and Hülsmann, 1973).

Short-chain acyl-CoA synthetases are found in all rat organs tested. Here again, very low C_2, C_3 and C_4 activation rates were reported in epididymal fat and skeletal muscle (Aas, 1971 a). As shown in Fig. 4, however, we were able to find appreciable amounts of both acetate (0.43 U/g wet weight) and propionate activation rates (0.30 U/g wet weight) in rat musculus masseter. Even ten fold higher activation rates were found in M. masseter of the guinea-pig. These values are much higher than the corresponding values in guinea-pig liver. Similarly, a high rate of acetate activation (2.2 U/g wet weight) was found in M. masseter of the sheep (Ballard, 1972), although in other muscles of the sheep much lower values were found (0.04-

0.08 U/g wet weight). Short-chain fatty acids can be produced by the fermentation of starch and cellulose in the rumen (sheep, cow) or coecum (guinea-pig) (Hagen and Robinson, 1953; Henning and Hird, 1970). They can contribute to 70% of the caloric requirements in ruminants (Bergman et al., 1965). Although the high acetate activation rate in M. masseter of sheep and guinea-pig might suggest adaptation to the presence of higher amounts of volatile fatty acids in the alimentary tract of those animals, the relatively low rate of acetate activation in other muscles of sheep, quite identical to those found in rat leg muscles (0.07 U/g wet weight, Ballard, 1972), is noteworthy. A similar comparison is also possible for liver short-chain fatty acid activation. Ash and Baird (1973) reported for beef liver the following short-chain fatty acid activation rates: C_2, 0.83; C_3, 3.88 and C_4, 2.88 U/g wet weight. When these values are compared with those obtained by us in guinea-pig (0.18, 1.39 and 2.51 U/g wet weight, respectively), all measured at 10 mM fatty acid concentration, no pronounced differences can be concluded. These findings contradict an adaptation of short-chain fatty acid activation to the enhanced importance of short-chain fatty acids in the metabolism of ruminants.

In conclusion (see also Section V, A): liver, small intestinal epithelium, adipose tissue and lactating mammary gland contain a cytosolic acetyl-CoA synthetase. Mitochondrial acetyl- and butyryl-CoA synthetases are present in kidney, heart, skeletal muscle, adipose tissue, small intestinal epithelium and lactating mammary gland. A mitochondrial propionyl-CoA synthetase is present in liver and probably also in adipose tissue.

VII FUNCTION AND REGULATION OF ACYL-CoA SYNTHETASES

A. SHORT-CHAIN ACYL-CoA SYNTHETASES

It was mentioned earlier that short-chain fatty acids are important substrates in the energy metabolism of ruminants. (For production rates of volatile fatty acids in sheep rumen, consult Annison et al. (1967).) The concentration of short-chain fatty acids in the rumen content varies with the nutritional state. A total concentration of 47-94 mM was found in sheep, fed with hay (Annison et al., 1957). Acetate, propionate and butyrate contributed for 64, 30 and 5 molecular % to the overall short-chain fatty acid concentration in the sheep rumen content. Butyrate, however, is absent in the portal blood of these sheep. Here acetate and propionate contribute to about 80 and 17 molecular % respectively to the overall short-chain fatty acid concentration of 0.98-1.78 mM. After passage through the liver, propionate had also disappeared from the blood. In carotic blood only acetate is left (0.51-0.92 mM). These results indicate that butyrate is mainly metabolized in the rumen epithelium to produce ketone bodies (Pennington, 1952; Annison et al., 1957) while propionate is mainly metabolized in the liver of sheep (Annison et al., 1957; Bergman and Wolff, 1971), where it can be used as substrate for gluconeogenesis. Little acetate is metabolized by the liver (Bergman and Wolff, 1971). This fatty acid is probably oxidized in heart, skeletal muscle and adipose tissue (for discussion see Ballard, 1972). The importance of the specificity of the different short-chain acyl-CoA synthetases in the regulation of short-chain fatty acid metabolism in different ruminant tissues is recently discussed in the

paper of Ash and Baird (1973). Using total homogenates of bovine liver or rumen epithelium, they studied the influence of another short-chain fatty acid on the activation of acetate, propionate and butyrate. Butyrate activation in rumen epithelium turned out to be most active (3.9 U/g wet weight) and this activation was not influenced by the presence of equimolar concentration of either acetate or propionate. Propionate activation (1.3 U/g wet weight) was strongly inhibited by butyrate while the low acetate activation (0.23 U/g wet weight) is only slightly influenced by propionate or butyrate. These effects, which are ascribed to competition between fatty acids for the activation enzyme could explain the preference of rumen epithelium for butyrate. A butyryl-CoA synthetase and an acetyl-CoA synthetase seem to be present in this tissue. Propionate activation in bovine liver (3.88 U/g wet weight) was not influenced by acetate and only slightly by butyrate, while the low acetate activation in this tissue (0.83 U/g wet weight) was rather strongly inhibited by equimolar concentrations of both butyrate and propionate. Butyrate activation too (2.88 U/g wet weight) was rather strongly inhibited by propionate. The presence of a distinct propionyl-CoA synthetase in bovine liver was proposed. We confirmed this for rat liver (see Section IV, A). These results can explain the preference of liver for propionate when both propionate and acetate are present.

These results obtained with ruminants, suggest some organ specialization in the metabolism of short-chain fatty acids which give rise to differences in the enzymatic outfit to activate these fatty acids. The function of an active system for short-chain fatty acid activation in non-ruminants is less well understood. Although short-chain fatty acids are also produced by microbial fermentation in coecum and colon of non-rumi-

nants, their contribution to the caloric intake of the animals will be small (for references about herbivorous, non-ruminal animals see Henning and Hird, 1970 and 1972). Another source of exogeneous acetate is alcohol. Alcohol can be formed by fermentation in the alimentary tract of non-ruminants (Krebs and Perkins, 1970) or it can be consumed. But here again, the contribution to the overall caloric intake does not seem to be very important. Nevertheless, significant amounts of acetate are present in the blood of humans and other non-herbivores, even after fasting (Ballard, 1972). The turnover of this acetate is at least as fast as in ruminants, which must lead to the conclusion that this acetate is derived from truly endogenous sources (for references see Ballard, 1972). It is not well understood which compounds and which organs contribute to the free acetate in the blood of non-ruminants. It may be produced by the action of acetyl-CoA hydrolase (EC 3.1.2.1), arylesterase (EC 3.1.1.2), acetylesterase (EC 3.1.1.6) and acetylcholinesterase (EC 3.1.1.7).

Recent perfusion studies of Seufert et al. (1974) performed with livers from fed, fasted and diabetic rats, suggest that acetate is one of the (normal) products during hexanoate oxidation in this tissue.

The small differences in the specific activities of short-chain fatty acid activation in tissues of ruminants and non-ruminants (Section VI) suggest that short-chain acyl-CoA synthetases are constitutive enzymes, i.e. that their synthesis is not strongly regulated by the amounts of short-chain fatty acids that have to be metabolized. However, some influence of the diet on short-chain fatty acid activation in the rat has been reported (Aas and Daae, 1971; Barth et al., 1972 a). Aas and Daae reported a somewhat lower rate of acetate activation both in liver and in heart after 48 h fasting (-36% calculated on body weight basis). A

fat-rich diet did not significantly influence acetate activation either in liver or in heart, while glucose feeding after 48 h fasting, conditions which stimulate lipogenesis, resulted in a slightly higher acetate activation in heart but not in liver, when compared with 48 h fasting. Barth et al. (1972 a) reported stronger effects. Changes in cytoplasmic and mitochondrial acetate activation were both considered in this study. Fasting of the rats for 48 h resulted in a 50% lower specific activity on protein basis of cytoplasmic acetate activation in both liver and epididymal fat tissue. Refeeding a 70% carbohydrate diet, which induces lipogenesis, resulted in a 1.7 and 5.5 fold increase in cytoplasmic acetate activation in liver and epididymal fat tissue, respectively. Mitochondrial acetate activation in liver, kidney, heart and epididymal fat and cytoplasmic acetate activation in kidney and heart were neither influenced by fasting nor by carbohydrate refeeding. We would like to stress again that in our opinion in kidney and heart no cytoplasmic acetate activation is present while in liver and epididymal fat the rate of cytoplasmic acetate activation is modest. Discrepancies between our results and those of others may be ascribed to the use of unsuitable mitochondrial matrix markers (see Section V, A).

We studied C_2, C_3 and C_4 activation in liver, heart, ileum and jejunum of a normal fed rat and compared those values with those of a fed germ-free animal, in which no short-chain fatty acids can be produced in the alimentary tract. The influence of acetate drinking (100 ml 0.1 M sodium acetate in 3 days) by a normal rat was also tested. The results of these experiments are given in Table VII. In each experiment only one animal was used, so that the results should be interpretated with reserve. Neither the absence of intestinal bacteria, nor acetate feeding strongly influenced short-chain fatty acid activation in all tissues tested.

TABLE VII

ACETATE, PROPIONATE AND BUTYRATE ACTIVATION BY 10 MIN 40,800 x g_{max} SUPERNATANTS OF SONICATED HOMOGENATES OF LIVER, HEART AND ISOLATED INTESTINAL CELLS OF THE RAT

	Normal fed rat				Germ-free, fed rat				Acetate-fed rat			
(mU/mg)	C_2	C_3	C_4	glutamate dehydro-genase	C_2	C_3	C_4	glutamate dehydro-genase	C_2	C_3	C_4	glutamate dehydro-genase
Liver	4.3	10.3	14.6	1780	3.2	6.3	11.9	1680	4.1	10.1	25.6	1280
Heart	9.5	66.7	43.4	149	9.4	43.5	29.9	145	13.8	53.0	51.8	137
Ileum	7.4	15.5	9.8	511	9.8	15.5	8.7	623	8.6	9.7	4.8	736
Jejunum	3.0	8.9	6.1	386	4.5	13.3	9.7	319	4.7	10.0	6.4	306

After 8 h without food and H_2O, the last rat was fed during 70 h with 100 ml 0.1 M sucrose containing 0.1 M acetic acid brought to pH 7.0 with NaOH. The fatty acid concentration in the assays were 10 mM. The activities are expressed in mU per mg supernatant protein. For details of the assay consult Groot et al. (1974). Glutamate dehydrogenase was determined according to Schmidt (1970) at 30°C. The suspensions (2 ml) were sonicated for 3 min with 21 kHz, amplitude 2 µm peak to peak, at 0°C.

In conclusion: with the exception of cytoplasmic acetyl-CoA synthetase in liver and epididymal fat tissue, the synthesis of short-chain acyl-CoA synthetases does not seem to be regulated by the amount of available short-chain fatty acids. Mitochondrial acetyl- and butyryl-CoA synthetases may have their function in providing intramitochondrial acetyl- and butyryl-CoA for further metabolism (oxidation, ketone-body synthesis, etc.). The presence of a special propionyl-CoA synthetase in liver mitochondria permits propionate activation, which is not influenced by the possible presence of acetate in this tissue and enables the animal to use propionate for glucose synthesis. The role of the cytoplasmic acetyl-CoA synthetase in liver and epididymal fat requires special comment. According to Barth et al. (1972 a) cytoplasmic acetyl-CoA synthetase in both tissues is influenced by the diet parallel to citrate cleavage enzyme (EC 4.1.3.8) which suggests a role in lipogenesis. A role in the transport of acetyl-groups from the mitochondrial matrix compartment to the cytoplasma has been considered (Kornacker and Lowenstein, 1965). According to this concept, acetyl-CoA formed during pyruvate oxidation is hydrolyzed to free acetate by a mitochondrial acetyl-CoA hydrolase. This acetate can leave the mitochondrion and can be reactivated by the cytoplasmic acetyl-CoA synthetase to be used in de novo fatty acid synthesis or extramitochondrial mevalonate synthesis (Barth et al., 1972 b). How important this mechanism is for making acetyl-CoA available to the cytosol, when compared with the citrate cleavage enzyme or the carnitine acetyltransferase transport system, remains an open question. Also the possibility of direct activation of acetate from the blood has to be considered.

B. MEDIUM-CHAIN ACYL-CoA SYNTHETASE

As discussed in the preceeding sections, medium-chain acyl-CoA synthetase has only been demonstrated unequivocally in liver mitochondria. The function of such an active medium-chain fatty acid activation is not fully understood. The enzyme may of course activate medium-chain fatty acids consumed with the food. Its localization in liver would be very appropionate because medium-chain fatty acids are transported, after their absorption in the small intestine, as free fatty acids by the portal blood. Medium-chain fatty acids, however, are not abundantly present in natural food. Milk is probably the richest natural source of medium-chain fatty acids. Medium-chain fatty acids are practically not incorporated into triglycerides and phospholipids (except in mammary gland) and a rapid oxidation or elongation of these fatty acids in the liver mitochondria is probably desired.

Medium-chain acyl-CoA synthetase, however, has a very broad substrate specificity (see Section IV, A, 2). Not only aliphatic fatty acids but also many other acids, including several aromatic compounds, can be activated. A role of the medium-chain acyl-CoA synthetase in the detoxification of unoxidizable acids by activation and subsequent conjugation to e.g. glycine has probably to be considered as well.

We found that the activity of medium-chain acyl-CoA synthetase in rat liver mitochondria was not influenced by feeding a diet containing large amounts of medium-chain triglycerides (MCT). Specific activities of octanoate activation measured in mitochondria of livers of MCT fed and control rats are given in Table VIII. We also measured octanoate activation in mitochondria which are pre-incubated with the protease Nagarse. By this procedure, the outermembrane-bound long-chain acyl-CoA synthetase is destroyed (De Jong and Hülsmann,

TABLE VIII

THE EFFECT OF MEDIUM-CHAIN TRIGLYCERIDE (MCT) FEEDING ON OCTANOATE ACTIVATION BY RAT LIVER FRACTIONS

Fraction	Octanoate activation (mU/mg protein ± S.D.)	
	Control diet	MCT diet
Homogenates	30.7 ± 4.2 (3)	28.4 ± 1.0 (3)
Mitochondria	44.8 ± 5.1 (3)	43.7 ± 5.7 (3)
Nagarse-treated mitochondria	30.0 ± 4.2 (3)	27.2 ± 6.2 (3)

Rats (about 300 g body weight) were fed ad libitum with a liquid diet containing either 3.0 g protein, 15.8 g carbohydrates and 9.0 g corn oil per 100 ml (control diet) or 3.6 g protein, 14.6 g carbohydrates and 9.0 g fat composed of 40 cal % MCT and 60 cal % corn oil per 199 ml (MCT diet). The diets are isocaloric (160 Kcal/100 ml). The duration of the feeding period was 7 days (mean caloric intake: 89 Kcal/24 h. The body weight remained constant during this period. Rats were sacrified, the liver removed, weighed and homogenized. The details of isolation of mitochondria the incubation of isolated mitochondria with Nagarse and of octanoate activation are given by Groot et al. (1974). The liver weights of MCT fed rat (3.58 ± 0.10 g/100 g body weight) were higher than those of the control animals (3.19 ± 0.24 g/100 g body weight).

1970 a; Pande and Blanchaer, 1970) and octanoate activation then is identical with the medium-chain acyl-CoA synthetase activity. The specific activity of octanoate activation in both diet groups were similar, suggesting that the liver medium-chain acyl-CoA synthetase is a constitutive enzyme.

C. LONG-CHAIN ACYL-CoA SYNTHETASE

The function of long-chain acyl-CoA synthetase seems rather obvious. It has to supply long-chain acyl-CoA esters to be used in synthetic processes (as triglyceride and phospholipid synthesis, chain-elongation) or in oxidative processes (as β-oxidation, desaturation). Long-chain acyl-CoA synthetase is localized both on the outer side of the mitochondrial outermembrane and on the membranes of the endoplasmatic reticulum. This microsomal long-chain acyl-CoA synthetase may be part of a multi-enzyme complex. Evidence for this is found in the work of Rao and Johnston (1966), who purified a triglyceride synthetase complex from hamster small intestinal mucosal microsomes, containing acyl-CoA synthetase, acyl-CoA: acylglycerol-O-acyltransferase (EC 2.3.1.22) and acyl-CoA: 1,2-diacylglycerol-O-acyltransferase (EC 2.3.1.20). Such a multi-enzyme complex would facilitate the transport of the rather hydrophobic intermediates in triglyceride synthesis. The existence of other multi-enzyme complexes, containing other pools of microsomal long-chain acyl-CoA synthetase, has to be considered (Brindley, 1973). Transport of long-chain acyl-CoA may, however, be facilitated by the cytoplasmic Z-protein, described recently by Mishkin and Turcotte (1974), a protein with a high affinity for long-chain acyl-CoA. Z-protein is present in many mammalian tissues.

Two processes, consuming long-chain acyl-CoA, are quantitative the most important ones: β-oxidation and esterification with α-glycerolphosphate. Can long-chain acyl-CoA synthetase be the rate-limiting step in these processes? The rate-controlling factors in long-chain fatty acid oxidation were studied by Pande (1971) using isolated mitochondria from rat heart and liver and from rabbit red and white skeletal muscle. In liver and heart mitochondria, the optimal palmitate activation

rate was found to be much higher than the optimal rates of β-oxidation (7 and 2 times, respectively). In red and white skeletal muscle mitochondria palmitate activation only slightly exceeded the optimal rate of β-oxidation. This indicates that palmitate activation is not necessarily rate-limiting in fatty acid oxidation in these tissues. This result is in agreement with studies from our laboratory (Van Tol and Hülsmann, 1970; De Jong, 1971; Groot and Hülsmann, 1973).

The rate-limiting steps in glyceride esterification were recently studied by Brindley and coworkers (Brindley, 1973; Sánchez et al., 1973; Lloyd-Davies and Brindley, 1973). In guinea-pig small intestinal mucosa microsomes, palmitate activation may be rate-limiting in phosphatidic acid synthesis by the sn-glycerol-3-phosphate acylation pathway (Brindley, 1973). In rat liver microsomes (Lloyd-Davies and Brindley, 1973; Van Tol, 1974), rat liver mitochondria (Sánchez et al., 1973; Van Tol, 1974) and homogenates of liver, heart and epididymal fat of the rat (Aas and Daae, 1971) rates of palmitate activation exceed the rates of sn-glycerol-3-phosphate acylation several times (4-30 x).

In Escherichia coli, long-chain acyl-CoA synthetase has been shown to be an inducible enzyme (Overath et al., 1969; Weeks et al., 1969). Variations in long-chain acyl-CoA synthetase activity in tissues of rats, fed with different diets, are only very moderate. Fasting (24-28 h) of the rat had practically no effect on palmitate activation in liver, heart and adipose tissue (Aas and Daae, 1971; Lippel, 1971 b; Lippel, 1972). In rat small intestinal mucosa a small decrease in activity was found after 14 h fasting (-20%; Mc Manus and Isselbacher, 1970) and a large decrease (-81%; Scholte and Groot, 1975) was found after 48 h fasting. Fat feeding or fat refeeding after fasting also had no effect on palmitate activation in liver, heart and epididymal fat

tissue of the rat, while glucose feeding or glucose refeeding may lead to a somewhat lower palmitate activation rate in liver (Aas and Daae, 1971; Lippel, 1972). These results suggest that long-chain acyl-CoA synthetase is a constitutive enzyme in mammals.

Regulation of long-chain acyl-CoA synthetase by a stimulating factor present in the cytoplasma of many mammalian tissues (see Section IV, A, 3), has been considered (Farstad, 1968). This factor, which was supposed to be a long-chain acyl-CoA synthetase kinase, was reported to be more active in fasted rat liver than in livers of fed animals and could stimulate long-chain acyl-CoA synthetase in vivo to metabolize the larger supply of long-chain fatty acids, derived from the fat stores (Farstad, 1968). Effects of the stimulating factor, however, were found to be rather variable (Aas, 1971 a) and depended on the assay conditions (Aas, 1970; Groot and Hülsmann, 1973). This hampered further studies about the possible regulatory role of this factor. Other factors, perhaps involved in the regulation of fatty acid activation, are the concentration of long-chain fatty acids, CoA and long-chain acyl-CoA. The relation between the concentration and the rate of utilization of long-chain fatty acids can be found in the fundamental work of Spector about transport into, and utilization of long-chain fatty acids by, animal cells (see Spector, 1968). The intracellular concentration of fatty acids in ascites tumor cells turned out to be dependent on the unbound free fatty acid concentration in the incubation medium. This free concentration was determined by the molar ratio ($= \bar{V}$) of free fatty acid and albumin in the incubation medium. The transport of free fatty acids into the tumor cell was so fast, that the intracellular concentration of free fatty acids was not influenced by the rate of fatty acid utilization. Both esterification and oxidation of free fatty acids by the tumor cells

turned out to be dependent on the intracellular concentration of the fatty acids and therefore on the $\bar{V}$ in the incubation medium (Spector, 1968). These results suggest that under these conditions the rate of fatty acid utilization is limited by the level of the intracellular free fatty acid and in this way on the degree of saturation of the fatty acid activation reaction. These results can probably be extended to whole tissues as recently discussed by Spector (1971) at least when the values of $\bar{V}$ are low. Recent studies of Oram et al. (1973) indicate that at low values of $\bar{V}$ (0-1.3), either fatty acid uptake or fatty acid activation is rate-limiting in palmitate utilization by the isolated perfused rat heart. Under these conditions the rate of fatty acid uptake was proportional to the concentration of palmitate in the perfusion fluid. At the higher values of $\bar{V}$ (1.3-2.6) long-chain acyl-CoA, long-chain acylcarnitine, acetyl-CoA and acetylcarnitine accumulated in the tissue while the tissue levels of free CoA and carnitine were drastically lowered. The rate of fatty acid uptake under abundant fatty acid supply is practically constant and limited by the rate of acetyl-CoA oxidation. The rate of palmitate activation may be depressed to a level, which can be utilized in the subsequent (rate-limiting) steps in fatty acid utilization, by the lower concentration of cytoplasmic CoA (Oram et al., 1973) or as pointed out by Pande (1973) by a lower ratio of free CoA over palmitoyl-CoA.

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THE ACTIVATION AND OXIDATION OF OCTANOATE AND PALMITATE BY RAT SKELETAL MUSCLE MITOCHONDRIA*

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SUMMARY

1. Rat skeletal muscle mitochondria can oxidize octanoate just as well as palmitate. The oxidation of both fatty acids is strongly carnitine dependent, which indicates that the fatty acid CoA ester formation (fatty acid activation) is localized on the outer membrane.

2. The palmitoyl-CoA synthetase and octanoyl-CoA synthetase activities were measured in these mitochondria. Palmitoyl-CoA synthetase was inhibited by octanoate while the octanoyl-CoA synthetase was inhibited by palmitate. Both inhibitions seem to be of the competitive type. These results suggest that both fatty acids are activated by the same enzyme.

3. Octanoyl-CoA synthetase in rat skeletal muscle mitochondria was strongly (3 to 4 times) stimulated by the addition of skeletal muscle cytosol or by the addition of a high concentration of salt.

INTRODUCTION

Recent experiments on fatty acid activation in mammalian tissues have shown that probably six different enzymes are involved in the fatty acid CoA ester formation, each enzyme with a different chain-length specificity. We can now distinguish: (a) acetyl-CoA synthetase (acetate: CoA ligase (AMP), EC 6.2.1.1) described by Lipmann *et al.*[1] and purified by Campagnari *et al.*[2], (b) propionyl-CoA synthetase, described by Scholte *et al.*[3], (c) butyryl-CoA synthetase, purified by Webster *et al.*[4], (d) heptanoyl-CoA synthetase (acid: CoA ligase (AMP), EC 6.2.1.2), purified by Mahler and Wakil[5], (e) palmitoyl-CoA synthetase (acid:CoA ligase (AMP), EC 6.2.1.3) described by Kornberg and Pricer[6], purified by Bar-Tana *et al.*[7], and (f) lauroyl-CoA synthetase as is suggested by Aas[8] and by Pande[9]. All these enzymes have a broader substrate specificity than their names suggest and catalyse the ATP-dependent acyl-CoA formation.

The short- and medium-chain fatty acid activating enzymes (C_2, C_3, C_4 and C_7) are localized in the matrix mitochondrialis. Long-chain fatty acid activating enzymes

* Part of this work was presented at the 15th International Conference on the Biochemistry of Lipids, held from August 28th–September 1st (The Hague, The Netherlands).

(C_{12} and C_{16}) are membrane bound and are found on the mitochondrial outer membrane and on the microsomal membranes.

Aas[8] has shown that in the heart, liver and kidney of the rat, at least five of the fatty acid activating enzymes are present (C_2, C_4, C_7, C_{12} and C_{16}) but that skeletal muscle and epididymal fat tissue probably only contain the two long-chain fatty acid activating enzymes. In those studies predominantly white muscle fibres (rat hindleg muscles) were studied. Red-type skeletal muscle, however, is more equipped for aerobic metabolism, probably reflected in the possession of a complete set of fatty acid activating enzymes in the mitochondria. We isolated mitochondria not only from white but also from a red-type skeletal muscle. Although both types of mitochondria were found to be able to activate octanoate at a very high rate, no matrix medium-chain activating enzyme could be detected. The present paper will describe the properties of this octanoate activation and compare them with those of palmitate activation.

MATERIALS AND METHODS

Reagents

Nagarse was purchased from Serva Entwicklungslabor, Heidelberg, Germany. Soybean trypsin inhibitor, 3 times crystallized, was supplied by Calbiochem, Lucerne, Switzerland.

Dowex 50W, 50/100 mesh, and fatty acid-poor bovine serum albumin were purchased from Fluka, Buchs, Switzerland.

[1-^{14}C]Palmitate, [1-^{14}C]octanoate, [9,10-^{3}H]palmitate and DL-[*Me*-^{14}C]carnitine were supplied by the Radiochemical Centre, Amersham, England.

Labeled fatty acids were diluted with the unlabeled compounds (British Drug Houses) and used in a specific activity of 0.5 Ci/mole. Labeled DL-carnitine was diluted with L-carnitine (Koch-Light Laboratories, Colnbrook, England) to a specific activity of 0.05 Ci/mole (based on L-carnitine).

Oligomycin was purchased from Sigma Chemical Co., St. Louis, Mo.

Nucleotides, enzymes and coenzymes were supplied by C. F. Boehringer and Sons, Mannheim, Germany.

Preparations

Male Wistar rats, weighing about 250 g, were used for all preparations. They had free access to food and water until they were killed. Rats were killed, after ether anesthesia, by decapitation and muscle tissue was directly isolated and transferred to cold 0.25 M sucrose.

Mitochondria from musculus masseter or musculus vastus were isolated in the following procedure. All steps were carried out at 3 ± 1 °C. Muscle tissue, about 1 g, was chopped with a McIlwain tissue slicer in two perpendicular directions (0.1 mm distance between the cuts) and transferred to 10 ml cold isolation medium as described by Chappell and Perry[10] and modified by us (100 mM KCl, 50 mM Tris–HCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA and 0.5 mg albumin/ml, pH 7.5). 1 mg of trypsin was added per g tissue and the muscle tissue was incubated for 7 min at 4 °C under magnetic stirring. Trypsin was then inactivated by an excess of trypsin inhibitor and the volume was made 40 ml by the addition of isolation medium. No indications could

be obtained that such a controlled trypsin incubation leads to destruction of fatty acyl-CoA synthetase activity in the mitochondria.

Homogenization was accomplished with a Potter-Elvehjem homogenizer with a teflon pestle (3 strokes) and the homogenate was filtered through two layers of nylon gauze.

Nuclei were sedimented (5 min at $600 \times g_{max}$) and the crude mitochondrial pellets were sedimented from the supernatant (10 min at $6000 \times g_{max}$). Mitochondria were washed three times in isolation medium and the final pellet was suspended in an appropriate volume of isolation medium without albumin. About 5 mg mitochondrial protein was isolated per g muscle tissue.

Particle-free supernatant was isolated from musculus masseter without the use of trypsin. Chopped tissue was directly homogenized (20 strokes) in about 10 ml albumin-free isolation medium/g tissue and particles were spun down (60 min at $300\,000 \times g_{max}$). The clear supernatant was used in the experiments.

Sonicates of mitochondria were prepared at 0–5 °C with a MSE 100-W Ultrasonic Desintegrator (Measuring and Scientific Equipment, London) operated at 20 kcycles/s (amplitude 2 μm, peak to peak) for 60 s/ml of suspension. The protein concentration during sonication was 1–5 mg/ml.

Carnitine palmitoyltransferase (palmitoyl-CoA:carnitine *O*-palmitoyltransferase, EC 2.3.1.–) was purified from calf liver mitochondria as described by Farstad *et al.*[11], with the only modification that the final enzyme preparation was centrifuged for 2 h at $105\,000 \times g_{max}$. This step leads to a lowering of the palmitoyl-CoA hydrolase activity (Jansen, H., unpublished). Our final preparation was practically free from hydrolase activity and of octanoyl- and palmitoyl-CoA synthetase activities.

Methods

Oxygen uptake was determined polarographically or manometrically as indicated.

Long-chain acyl-CoA synthetase was measured in two ways. In most experiments a modification of the method of Farstad *et al.*[11] was used (Method I). The reaction was carried out at 37 °C in a reaction volume of 0.25 ml. The incubation medium contained 40 mM KCl, 5 mM $MgCl_2$, 80 mM Tris–HCl, 0.5 mM EDTA, 0.3 mM KCN. 0.4 mM coenzyme A, 5 mM ATP, 5 mM phosphoenolpyruvate, pyruvate kinase and myokinase 8 units/ml each, 3 μg/ml oligomycin, 80 munits/ml carnitine palmitoyltransferase, 5 mM [*Me*-^{14}C]carnitine (specific activity of L-carnitine 0.05 Ci/mole) and 0.5 mM of the potassium salts of either palmitate, myristate or laurate (all complexed with bovine serum albumin in a molar ratio of 7:1). The reaction was started by the addition of sonicated mitochondria and terminated after 10 or 20 min by the addition of 0.02 ml concentrated HCl. Palmitoylcarnitine was extracted with 1.5 ml butanol after the addition of 1 ml water. The butanol phase was washed with 1 ml water, saturated with butanol and 0.5 ml was transferred to a counting vial together with 2.5 ml ethanol and 10 ml toluene (containing 4 g PPO and 0.1 g POPOP per l). Radioactivity was measured in a Packard Tri-Carb (model 544) liquid scintillation counter and dpm calculated with external standardization. Not more than 0.05 mg mitochondrial protein was added and under these conditions palmitoylcarnitine formation was linear with protein concentration and incubation time. Incubations were run in duplicate.

Octanoyl-CoA synthetase and in some experiments palmitoyl-CoA synthetase were determined with a new method (Method II), developed in our laboratory (Scholte, H. R., unpublished) for short-chain fatty acid activation and modified by us for medium- and long-chain fatty acid activation. The principle of this method is the same as that of Method I. In this method, however, labeled fatty acids were used instead of labeled carnitine and the fatty acylcarnitine ester formed was separated from the free fatty acid by Dowex-50W ion-exchange chromatography. The incubation was carried out under identical conditions as described in Method I. However, unlabeled L-carnitine and [^{14}C]- or [^{3}H]-labeled fatty acids were used. When octanoate activation was determined carnitine acetyltransferase (4 units/ml) was used instead of carnitine palmitoyltransferase. Incubations were terminated by the addition of 1.2 ml ethanol and denaturated protein was removed by centrifugation. 1 ml supernatant was transferred to a Dowex column. We used 2 ml pipets for this purpose, filled with 0.15 g Dowex 50W (H^+). Carnitine and fatty acylcarnitine esters were quantitatively bound under these conditions, while the labeled fatty acid is not bound. The fatty acid was washed away 3 times with 2 ml ethanol. The fatty acylcarnitine ester was eluted with 2 ml conc. HCl/butanol (50/50, v/v) and a 1-ml aliquot was transferred to a counting vial and mixed with 10 ml Insta-gel (Packard), 2.5 ml ethanol and 0.5 ml 10 M NaOH. After sedimentation of the NaCl formed, the radioactivity in the vials was counted. Columns were regenerated with an additional amount of conc. HCl and, after washing with distilled water, were ready for use. This method appeared to be very reproducible. The trap for acyl-CoA in our assay was very effective. No acyl-CoA is accumulated during the incubation which was checked by thin-layer chromatography. We checked the product formation as a function of the incubation time and the amount of added mitochondrial protein. Octanoylcarnitine formation was at least linear during 20 min with 0.15 mg mitochondrial protein. For palmitate activation these values were 20 min and 0.06 mg, respectively. Fatty acid activation was always determined in freshly isolated mitochondria.

Protein was determined with the biuret method or according to the method of Lowry *et al.*[12].

RESULTS AND DISCUSSION

Oxidation experiments

It can be seen from Fig. 1 that mitochondria isolated from masseter muscle show a sluggish rate of endogenous respiration in the presence of added coenzyme A and ATP, which is not stimulated by the addition of either octanoate or palmitate. However, addition of carnitine vigorously stimulates respiration. If carnitine is added prior to the fatty acids, fatty-acid dependent stimulation of respiration may be observed (results not shown). The fact that 2,4-dinitrophenol stimulates respiration (Fig. 1) indicates that the mitochondria have intact oxidative phosphorylation and suggests that in the absence of an uncoupler of oxidative phosphorylation fatty acid oxidation may be limited by the availability of ADP. Malate was added to provide optimal concentrations of citric acid cycle intermediates[13]. In these experiments fatty acid activation, and/or β-oxidation under state 3 conditions, were rate limiting since the addition of 10 mM pyruvate instead of fatty acid in the presence of dinitrophenol resulted in a 2.6 times higher rate of O_2 uptake (not shown). It has been shown by

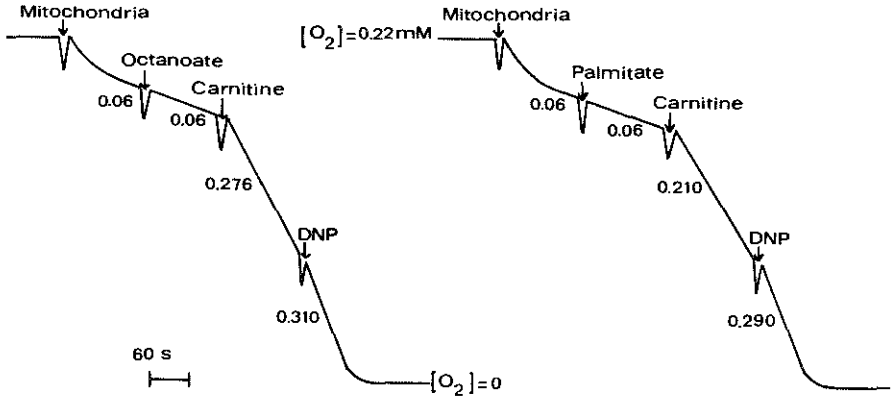

Fig. 1. Polarographic demonstration of carnitine-dependent octanoate and palmitate oxidation by intact masseter muscle mitochondria. The reaction volume was 2.1 ml and the standard reaction medium contained: 25 mM sucrose, 1 mM L-malate, 5 mM ATP, 10 mM potassium phosphate buffer, 5 mM $MgCl_2$, 30 mM KCl, 1 mM EDTA, 75 mM Tris–HCl buffer, 1 mM NAD^+, 0.025 mM cytochrome *c* and 0.1 mM coenzyme A. The pH was 7.5 and the reaction temperature 37 °C. 0.7 mg musculus masseter mitochondria were used in each experiment. Additions were: 0.5 mM sodium octanoate, 0.1 mM potassium palmitate (complexed to bovine serum albumin in a 7:1 molar ratio), 2 mM L-carnitine and 0.1 mM 2,4-dinitrophenol (DNP). The values given in the figures refer to oxygen consumption in μatoms O/min per mg mitochondrial protein (10 mM pyruvate added instead of fatty acid (dinitrophenol present) resulted in an oxygen consumption of 0.79 μatoms O/min per mg protein, not shown).

Pande[14] that in rabbit skeletal muscle mitochondria the citric acid cycle is not rate limiting in the oxidation of palmitate. The fact that the oxidation of octanoate in rat masseter muscle mitochondria is fully carnitine dependent can also be seen from Fig. 2, in which $^{14}CO_2$ formation was measured during the oxidation of [1-^{14}C]octanoate. These experiments then lead to the conclusion that octanoate is activated in rat skeletal muscle mitochondria and that the activation occurs outside the carnitine barrier[15,16], possibly on the mitochondrial outer membrane. It is unlikely that significant octanoyl-CoA synthetase is present in the matrix mitochondrialis, as is the case in heart mitochondria[8], and has leaked from the skeletal muscle mitochondria during the isolation of these particles. In the first place carnitine exerts a strong stimulation on fatty acid oxidation and, secondly, pyruvate oxidation leads to high rates of oxidation (0.79 μatoms O/min per mg protein or 532 μl O_2/h per mg protein), suggesting intactness of the mitochondria. That octanoate, like palmitate, is activated on the mitochondrial outermembrane, is strongly suggested by experiments in which Nagarse (EC 3.4.4.16) treatment of the isolated skeletal muscle mitochondria leads to a complete loss of both octanoate and palmitate oxidation (results not shown). Therefore the effect of Nagarse on octanoyl-CoA synthetase is the same as that on palmitoyl-CoA synthetase in heart[17]- and skeletal muscle[18] mitochondria. The Nagarse treatment was carried out as described in ref. 17. Since Nagarse does not penetrate the mitochondrial membranes, it probably attacks enzymes only when localized on the outside of the mitochondrial outer membrane[19]. In the following experiments the activation of octanoate and palmitate by skeletal muscle mitochondria was studied more directly.

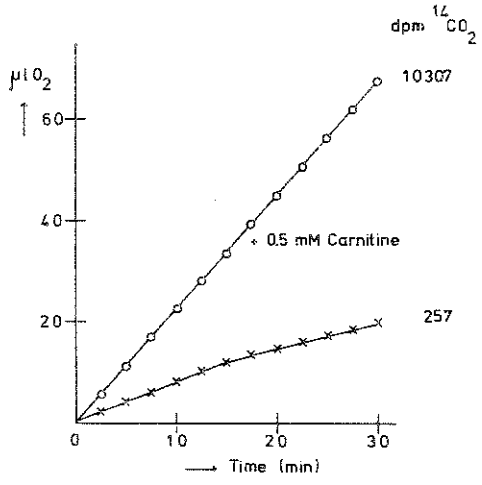

Fig. 2. The oxidation of [1-^{14}C]octanoate by intact masseter muscle mitochondria. Oxygen consumption was measured with a differential respirometer at 37 °C. Reaction vessels contained 3 ml of the same reaction mixture as given in Fig. 1. [1-^{14}C]Octanoate, specific activity 0.5 Ci/mole, was present in a concentration of 0.5 mM. 1.51 mg mitochondrial protein was present in each incubation. CO_2 was trapped with a KOH-soaked paper present in the center well. Where indicated, L-carnitine was present in a concentration of 0.5 mM. The reaction was started by the addition of the mitochondria and oxygen consumption was monitored every 2 min. After 30 min the reaction was terminated with 6 μmoles *p*-chloromercuribenzene sulfonic acid and after 2 h equilibration the KOH-soaked paper was transferred to a counting vial with 10 ml Insta-gel and counted. The values given in this figure refer to dpm $^{14}CO_2$ evolved, corrected for the binding of evaporated [1-^{14}C]octanoate (about 200 dpm). Oxygen consumption was not corrected for endogenous respiration.

Activation experiments

From our oxidation experiments it must be concluded that octanoate, like palmitate, can be activated by masseter muscle mitochondria. Both synthetase activities were measured. In Fig. 3 the effect of increasing fatty acid concentration on both synthetase activities is given. These curves permit a calculation of apparent K_m and V values. For the octanoate activation an apparent K_m of 0.2 mM was found. For palmitate activation a much lower value of 8 μM was calculated. The V was higher for palmitate activation (33 nmoles/min per mg protein) than for octanoate activation (17 nmoles/min per mg protein). High octanoate concentrations were quite inhibitory. Optimal octanoate activation (12 nmoles/min per mg protein) was found at 0.5 mM. In all our experiments we used palmitate complexed to bovine serum albumin (7:1 molar ratio). Because less palmitate will be available for the acyl-CoA synthetase when palmitate is bound to bovine serum albumin, the apparent K_m of 8 μM is overestimated.

With freshly isolated mitochondria the same v/S curve for palmitate activation was found when Method II was used. This indicates that no measurable dilution of [1-^{14}C]palmitate with endogenous palmitate takes place. For palmitoyl-CoA synthetase in rat liver microsomes an apparent K_m for palmitate of only 1–3 μM was found (refs 9, 20); in those experiments, however, palmitate was not complexed with albumin.

The specific activity for palmitate activation in muscle mitochondria found by us

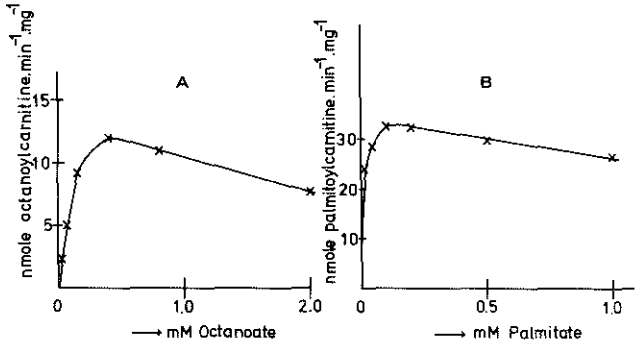

Fig. 3. The rate of fatty acid activation in masseter muscle mitochondria in relation to the concentration of fatty acid (octanoate or palmitate) used. (A) shows octanoate activation, which was measured according to Method II (0.07 mg mitochondrial protein was used). (B) shows palmitate activation, which was measured according to Method I (0.0154 mg mitochondrial protein was used). For further details see Methods.

for rat is somewhat higher than the value found by Pande and Blanchaer[21] for rabbit, in which the values for white muscle-type and red muscle-type were 7.8 and 18.9 nmoles/min per mg protein, respectively. We could not confirm this result in the rat. Mitochondria isolated from the musculus vastus, which contains more white fibers than the masseter muscle, as judged by the 2 times higher specific activity of glycerol-3-phosphate dehydrogenase[22] in the mitochondrial fraction, were just as active as masseter muscle mitochondria in palmitate and octanoate activation (not shown).

An interesting question is whether octanoate and palmitate are activated by one or separate enzymes on the mitochondrial outer membrane. For that reason substrate competition between [9,10-^{3}H]palmitate and [1-^{14}C]octanoate in the activation assay (Method II) was studied. Table I clearly shows that octanoate activation is strongly inhibited by palmitate. This inhibition seems to be competitive because raising the octanoate concentration from 0.4 to 2.0 mM in the presence of 0.2 mM

TABLE I

INFLUENCE OF PALMITATE ON OCTANOATE ACTIVATION IN MASSETER MUSCLE MITOCHONDRIA

Octanoate and palmitate activation were determined according to Method II. The incubation medium contained both carnitine palmitoyltransferase and carnitine acetyltransferase, in concentrations of 80 munits/ml and 4 units/ml, respectively. Incubations were carried out with 0.03 mg mitochondrial protein. Where indicated (9,10-^{3}H]palmitate (complexed to bovine serum albumin in a 7:1 molar ratio) and [1-^{14}C]octanoate were present in a final concentration as indicated. The specific activities of both fatty acids were 0.5 Ci/mole. The incubation time was 10 min. Activities are given in nmoles acylcarnitine formed/min per mg mitochondrial protein. For further details see Methods.

Fatty acid	*Specific activity of the activation of*	
	C_8	C_{16}
0.4 mM octanoate	16.4	—
2.0 mM octanoate	8.9	—
0.2 mM palmitate	—	26.2
0.4 mM octanoate + 0.2 mM palmitate	0.8	26.5
2.0 mM octanoate + 0.2 palmitate	1.7	24.7

palmitate leads to a 2 times higher octanoate activation while the same rise in the absence of palmitate leads to a decrease in octanoate activation (*cf.* Fig. 3A). On the other hand, when the concentration of octanoate is increased, the rate of palmitate activation declines, although slightly. These effects have been shown repeatedly and therefore suggest that both octanoate and palmitate are activated by one enzyme. This is substantiated by experiments shown in Fig. 4, in which a Dixon plot is presented of an experiment in which both palmitate and octanoate concentrations were varied. From this plot it can be calculated that the K_i of octanoate for palmitate activation is about 0.2 mM. This value is the same as the apparent K_m for octanoate, a result that would be expected when octanoate and palmitate are activated by the same enzyme. The strong inhibition of octanoate activation by palmitate probably explains the discrepancy of our results with those of Aas[8], who found no appreciable octanoate activation in skeletal muscle. Even when tissue constituents are slightly aged, phospholipase action may give rise to sufficient long-chain fatty acids so that medium-chain fatty acid activation is heavily depressed.

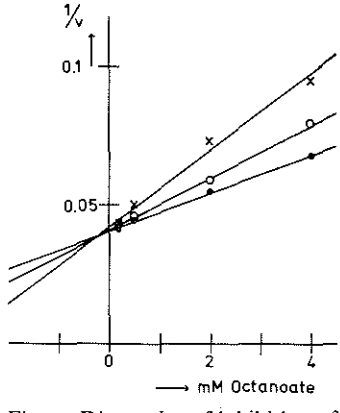

Fig. 4. Dixon plot of inhibition of palmitoyl-CoA synthetase by octanoate in a sonicate of masseter muscle mitochondria. Palmitoyl-CoA synthetase activity was determined according to Method II. 0.013 mg mitochondrial protein was present in each incubation and the reaction time was 10 min. All incubations were run in duplicate. [1-^{14}C]Palmitate, specific activity 0.5 Ci/mole was used in these experiments. ×——×, 0.1 mM palmitate; ○——○, 0.2 mM palmitate and ●——●, 0.4 mM palmitate (complexed to bovine serum albumin in a 7:1 molar ratio). The lines were constructed with the least-squares method.

Aas[8] reported in his extensive study on the organ- and subcellular distribution of fatty acid activating enzymes in the rat that probably two membrane-bound fatty acid activating enzymes in the rat are responsible for long-chain fatty acid activation. one with an optimal activity with C_{12}, and one with an optimal activity with C_{16}. This hypothesis was supported by the distribution pattern of acyl-CoA synthetases found in microsomes and mitochondrial outer membranes purified from rat liver. These results were supported by Pande[9], who found in rat liver microsomes, peak activities with C_{12} and C_{16}. We could confirm these results with masseter muscle mitochondria (Table II). In view of the competition between palmitate and octanoate in the activation reaction, our interpretation is different. We think that only one long-chain fatty acid activating enzyme is present on the mitochondrial outer membrane and that the

TABLE II

ACTIVATION OF FATTY ACIDS OF DIFFERENT CHAIN LENGTHS BY A SONICATE OF MASSETER MUSCLE MITOCHONDRIA

Octanoate activation was determined with Method II. All other fatty acid activations were measured with Method I. Laurate, myristate and palmitate were complexed with bovine serum albumin (7:1 molar ratio). The sonicated mitochondria were present in amounts of 0.055 mg protein. Incubation at 37 °C for 10 min. For further details see Methods.

Fatty acid	*Concentration (mM)*	*Fatty acid activation (nmoles/min per mg protein)*
Octanoate	0.5	10.0
Laurate	0.5	35.0
Myristate	0.5	19.5
Palmitate	0.5	35.5

"gap" at myristate is caused by differences in physical properties of the fatty acids. For instance, the surface activity of myristate is two times higher than that of laurate and three times that of palmitate[23]. In microsomes different peaks in the chain-length specificity pattern of long-chain acyl-CoA synthetase may also be due to the operation of one enzyme. Evidence for this may be found in the recent work of Suzue and Marcel[24], who found that in rat liver microsomes octanoate activation may also be strongly inhibited by palmitate, on the basis of which they concluded the involvement of one enzyme.

Stimulation of octanoate and palmitate activation in rat skeletal mitochondria

During our study on the subcellular localization of octanoyl-CoA and palmitoyl-CoA synthetase in rat masseter muscle, a very low recovery for the activation of octanoate was found. Similar results were described by Farstad *et al.*[11] in a study on palmitoyl-CoA synthetase in rat liver.

These authors discovered a factor, present in the cytosol of liver (but also in other tissues) which stimulated the long-chain fatty acid activation in the particulate fractions. This factor was heat labile, non-dialysable and ammonium sulfate precipitable and was stimulated optimally when Mg^{2+} and ATP were present in equimolecular amounts[11,25,26]. Stimulation was also seen in the absence of this factor, when long-chain fatty acid activation was determined at high concentrations of salts[26]. Under these conditions the stimulating effect of the cytosolic factor was only very modest.

We tested the influence of masseter muscle cytosol on octanoate and palmitate activation by masseter muscle mitochondria. The results of these experiments are given in Table IV. Octanoate activation is clearly stimulated by the addition of cytosolic protein, while the effect on palmitate activation is only modest. It can be seen that bovine serum albumin cannot replace cytosol in its stimulatory action. The stimulation by cytosol is abolished when it is heated at 100 °C or even only at 60 °C for 10 min. Dialysis of the cytosol does not remove the stimulating factor.

We tested the influence of increasing amounts of cytosolic protein on octanoate and palmitate activation. When the ratio cytosolic protein over mitochondrial protein was about 10, octanoate activation was stimulated 4-fold (experiments not shown).

Stimulation of fatty acid activation is also observed, when relatively high

TABLE III

STIMULATION OF ACYL-CoA SYNTHETASE IN SONICATED MASSETER MUSCLE MITOCHONDRIA BY MASSETER MUSCLE CYTOSOL

Mitochondria and cytosol were isolated as described under Preparations. Octanoyl-CoA synthesis was determined according to Method II, palmitoyl-CoA synthesis according to Method I. The amounts of mitochondrial protein used and the additions made are indicated. In Expt 1 an aliquot of the cytosol was heated for 10 min at 100 °C, in Expt 2 for 10 min at 60 °C (denaturated protein was not removed). The dialysed cytosol used in Expt 2 was dialysed against 20 mM Tris–HCl (pH 7.5) for 20 h and clarified by centrifugation. The incubation time was 10 min at 37 °C. All values are corrected for the (very low) acyl-CoA synthetase activity present in the cytosol.

Mitochondrial protein (mg)	*Additions*	*Activation**	
		C_8	C_{16}
Expt 1			
0.07	none	10.4	
0.07	0.62 mg cytosol	20.0	
0.07	0.62 mg boiled cytosol	11.5	
0.07	0.62 mg bovine serum albumin	11.9	
0.0233	none		35.9
0.0233	0.21 mg cytosol		40.2
0.0233	0.21 mg boiled cytosol		32.9
0.0233	0.21 mg bovine serum albumin		26.7
Expt 2			
0.045	none	13.2	
0.045	0.42 mg cytosol	36.7	
0.045	0.33 mg dialysed cytosol	26.1	
0.045	0.42 mg heated cytosol	16.5	

* nmoles/min per mg mitochondrial protein at 37 °C.

TABLE IV

STIMULATION OF PALMITATE ACTIVATION IN MASSETER MUSCLE MITOCHONDRIA WHEN ASSAYED AT LOW SALT CONCENTRATIONS

Palmitate activation was assayed according to Method I, with the exception that Tris–HCl concentration in the incubation medium was lowered to 20 mM and KCl was omitted (except where shown to be added). 0.052 mg mitochondrial protein was used and where indicated 0.34 mg cytosolic protein. For the contribution of the latter to palmitate activation (low activity) was corrected. Incubation (37 °C) was carried out for 10 min. For other details see Methods.

Additions	*Palmitate activation (nmoles min per mg mitochondrial protein)*
None	17.9
Cytosol	28.7
KCl (final concn 200 mM)	26.6
Cytosol + KCl	25.1

concentrations of salt (KCl or $(NH_4)_2SO_4$) are added instead of cytosol. The effect of increasing KCl concentrations on the activation of palmitate and octanoate are shown in Fig. 5. Maximal (2-fold) stimulation of octanoate activation is reached at 200 mM added KCl. The stimulations of octanoate activation by the cytosolic factor and by high salt were not additive. The effect of KCl addition on palmitate activation at 200 mM added KCl is extremely small, as can be seen. Aas[26] has already found that

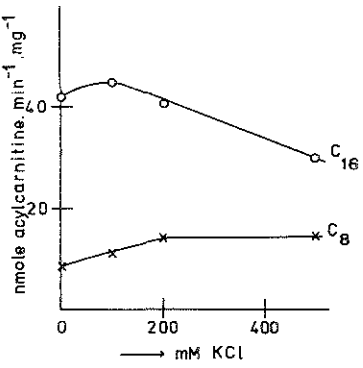

Fig. 5. The effect of KCl addition on octanoyl- and palmitoyl-CoA synthetase activities in sonicated masseter muscle mitochondria. Palmitoyl-CoA synthesis was determined according to Method I, octanoyl-CoA synthesis according to Method II. 0.035 mg mitochondrial protein was present in each incubation. The incubation time was 10 min. The mitochondrial preparations used in these 2 series of experiments were different. ×——×, octanoyl-CoA synthetase; ○——○, palmitoyl-CoA synthetase.

the supernatant effect on long-chain fatty acid activation in liver is higher at low salt concentrations. This led us to test whether cytosol has also a more pronounced effect on palmitate activation in skeletal muscle mitochondria at low salt concentrations. It can be seen from Table IV that, at low salt concentration, the rate of palmitate activation is low when compared with the values given earlier in this paper. Indeed, both supernatant and KCl then stimulate more effectively; again here, the effects are not additive. This might indicate a similar mode of activation. Both salt and cytosol have a much clearer effect on octanoate than on palmitate activation. We considered the possibility that the stimulation of octanoate activation is caused by a binding of endogenous long-chain fatty acids in the mitochondrial preparation by a protein present in the cytosol. Such a protein, the Z protein, has been described by Mishkin *et al.*[27], and is present in skeletal muscle. However, since the strong long-chain fatty acid binding protein bovine serum albumin could not replace cytosol in stimulating octanoate activation and, moreover, since the cytosol also stimulated palmitate activation to a small extent, an alternative explanation is more likely. Perhaps both activations of octanoate and palmitate by the outer membrane acyl-CoA synthetase are stimulated by a supernatant protein kinase, as suggested for palmitate activation by Farstad[25]. However, the distinct differences between the effect of cytosol on octanoate and palmitate activation are not in favour of this concept. It is clear that more work is required to elucidate the nature of the stimulating factor(s).

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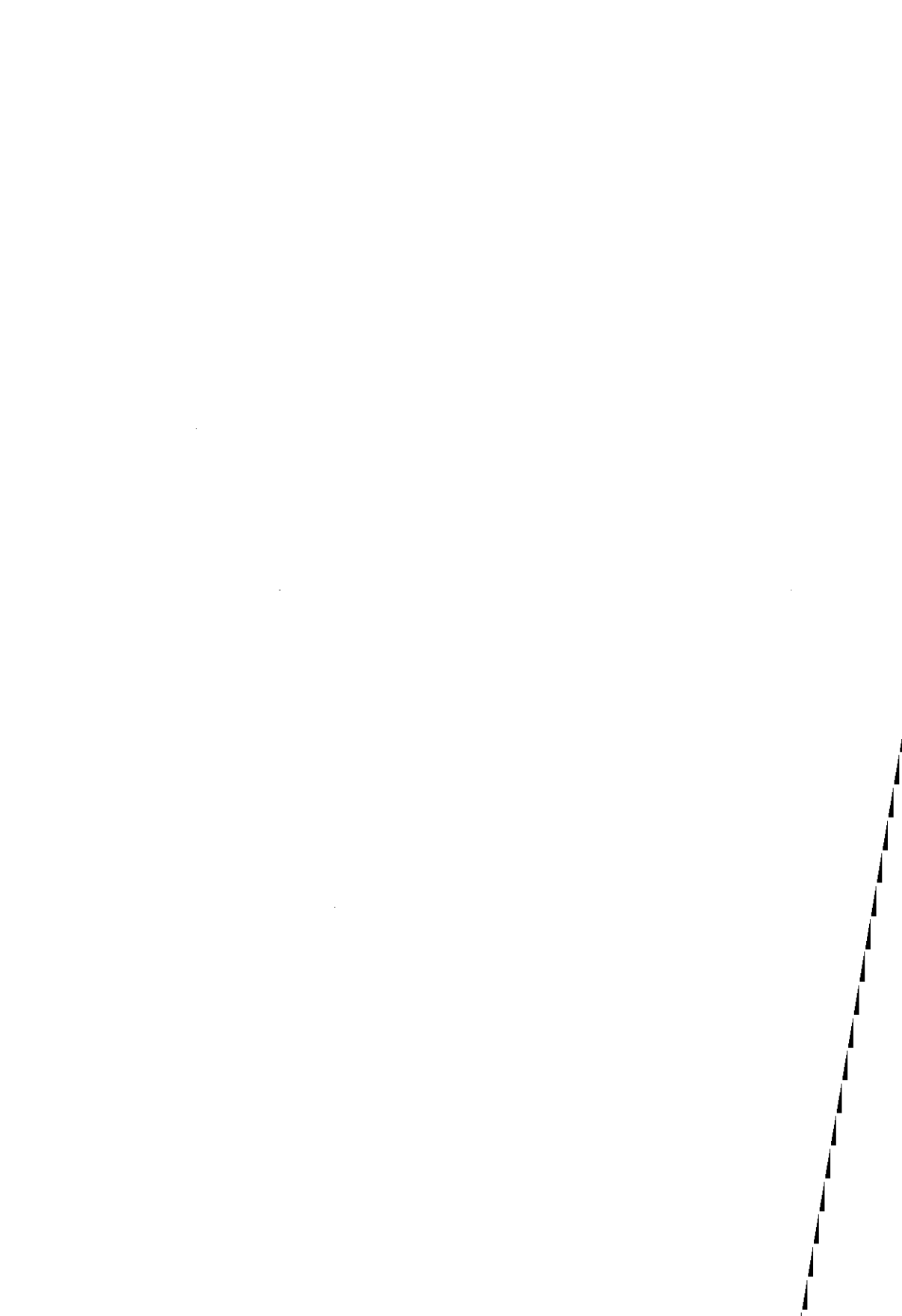

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IDENTIFICATION OF THE PALMITOYL-CoA SYNTHETASE PRESENT IN THE INNER MEMBRANE-MATRIX FRACTION OF RAT LIVER MITOCHONDRIA

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SUMMARY

1. The palmitoyl-CoA synthetase (palmitate:CoA ligase (AMP)) present in the inner membrane-matrix fraction of rat liver mitochondria was studied after the outer membrane acyl-CoA synthetase (acid:CoA ligase (AMP), EC 6.2.1.3) had been destroyed by the protease Nagarse.

2. When Nagarse-treated liver mitochondria are subfractionated in a membraneous and a soluble fraction, both palmitoyl-CoA synthetase and octanoyl-CoA synthetase (acid:CoA ligase (AMP), EC 6.2.1.2) are distributed between these fractions as malate dehydrogenase. This indicates that both acyl-CoA synthetase activities are localized in the matrix mitochondrialis.

3. When this mitochondrial soluble fraction is submitted to Sephadex G-100 chromatography, both palmitoyl-CoA synthetase and octanoyl-CoA synthetase activities reside in one single peak with a calculated molecular weight of 47000. For acetyl-CoA synthetase (acetate:CoA ligase (AMP), EC 6.2.1.1.), also present in this soluble fraction, a molecular weight of 62000 was calculated.

4. Kinetic experiments performed with fractions purified by Sephadex chromatography clearly showed that palmitate is activated by the same enzyme as octanoate. This indicates that no special palmitoyl-CoA synthetase is present in the inner membrane-matrix fraction of rat liver mitochondria.

INTRODUCTION

The localization of the long-chain fatty acid activation in the rat liver mitochondria has been subject of many studies [1–6]. The results, however, are not without controversion. From the mitochondrial subfractionation studies, carried out by Norum and coworkers [1], it was concluded that the bulk of palmitoyl-CoA synthetase in rat liver mitochondria is bound to the outer membrane. This result was confirmed by Van den Bergh [2], Lippel and Beattie [3], Van Tol and Hülsmann [4] and by Aas [5]. It is in agreement with latency studies of Garland et al [6] and of Aas and Bremer [7].

The recent subfractionation studies of Rossi et al. [8], in which it was found

that palmitoyl-CoA synthetase (AMP) resides for 20% in the outer membrane-, for 72% in the inner membrane- and for 6% in the soluble fraction, are difficult to interpret because no marker enzymes were included in this study. It has been demonstrated, however, that rat liver mitochondria are able to oxidize palmitate in the absence of carnitine [4, 9–13] and also when the fatty acid activation in the outer-membrane is blocked by removal of the extra-mitochondrial coenzyme A [14]. This indicates that palmitate activation must also be present inside the carnitine barrier, which is localized in the inner membrane. It has been described that long-chain fatty acids can be activated by a GTP-dependent acyl-CoA synthetase present in the matrix fraction of rat liver mitochondria [10]. In this reaction GTP is hydrolysed to GDP and inorganic phosphate. However, carnitine-independent palmitate oxidation is also seen when the GTP-dependent enzyme is completely inhibited by the presence of inorganic phosphate [4, 9, 11, 12].

Recently Batenburg and Van den Bergh [13] demonstrated that during palmitate oxidation in the absence of carnitine, inorganic pyrophosphate accumulates when the mitochondrial pyrophosphatase is inhibited by fluoride, whether oxidative phosphorylation is uncoupled or not. This indicates that under both conditions an ATP-dependent acyl-CoA synthetase is operative.

More evidence for a dual localization of the ATP-dependent long-chain acyl-CoA synthetase in rat liver mitochondria is given by the subfractionation studies of Van Tol and Hülsmann [4], who used the protease Nagarse to destroy the outer membrane-bound activity [15, 16]. From their results it can be concluded that 90–95% of the palmitoyl-CoA synthetase present in rat liver mitochondria, is bound to the outer membrane while the remaining activity is found in the inner membrane-matrix compartment.

The palmitoyl-CoA synthetase found in the inner-membrane-matrix compartment was inhibited by octanoate in a non-competitive way, which suggested that this enzyme is different from the medium-chain acyl-CoA synthetase, present in the matrix fraction.

We have studied this palmitoyl-CoA synthetase further. Although the activity is low, when compared with the microsomal- and outer membrane-bound activities, it is of interest because of its localization inside the carnitine barrier. Moreover, palmitoyl-CoA synthetase is present in the liver cell in such an excess, that the relatively small inner membrane-matrix activity is still high enough to allow optimal palmitate oxidation in isolated mitochondria [4, 11, 12].

MATERIALS AND METHODS

Reagents

Nagarse was purchased from Serva Entwicklungslabor, Heidelberg, Germany.

Dowex-50 W, 50–100 mesh and fatty acid-poor bovine serum albumin were purchased from Fluka, Buchs, Switzerland.

Sephadex G-100 was supplied by Pharmacia, Uppsala, Sweden.

[1-^{14}C]Palmitate, [1-^{14}C]octanoate and [1-^{14}C]acetate were supplied by the Radiochemical Centre, Amersham, England, and [2,3-$^{3}H_2$]octanoate by Schwarz-Mann, Orangeburg, N.Y.

L-[*Me*-^{3}H]Carnitine was synthetized according to Stokke and Bremer [17].

The labelled fatty acids were diluted with the unlabelled compounds (British Drug Houses Ltd, Poole, England) to specific activities of 0.5 Ci/mole or 8 Ci/mole. Labelled carnitine was diluted with L-carnitine (Koch–Light Laboratories, Colnbrook, England) to a specific activity of 0.28 Ci/mole.

Oligomycin was purchased from Sigma Chemical Co., St. Louis, Mo.

Inorganic pyrophosphatase was supplied by Worthington Biochemical Co., Freehold, N.J.

Carnitine acetyltransferase (from pigeon breast muscle), alkaline phosphatase (from calf intestine), cytochrome *c* (from horse heart), creatine kinase (from rabbit muscle) and all other enzymes, coenzymes and substrates were purchased from C.F. Boehringer and Sons, Mannheim, Germany.

Preparations

Male Wistar rats, weighing about 250 g, were used for all preparations. They had free access to food and water until they were killed. Rats were killed after ether anaesthesia by decapitation. The liver was immediately removed and chilled in ice-cold isolation medium. All the following steps were carried out at 2 ± 2 °C. In the experiments in which Nagarse-treated rat liver mitochondria were used (see Results, Fig. 1 and Table I) the procedure was as follows: The liver was cut in small pieces and homogenized in 0.25 M sucrose – 0.01 M glucose in a Potter–Elvehjem homogenizer. Nuclei were spun down (5 min at $800 \times g$) and mitochondria were sedimented from the supernatant (10 min at $5090 \times g$). The mitochondria were washed twice and finally suspended in 8 ml isolation medium. Nagarse was added and mitochondria were incubated for 30 min at 0 °C. The protease treatment was terminated by a 10-fold dilution with 0.25 M sucrose containing 1% (w/v) bovine serum albumin and the Nagarse was subsequently removed by four washing steps, three in sucrose–albumin and one in a salt medium, 40 mM KCl, 80 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM ATP and 0.2 mM dithiothreitol, which was used in the subsequent procedure. ATP and dithiothreitol were added to stabilize the different acyl-CoA synthetases. Finally, the mitochondria were suspended in 7 ml salt medium. The preparation of a membraneous and a soluble fraction was as follows: Mitochondria were disrupted by freezing (in solid CO_2/acetone) and subsequent thawing (in tap water). Membranes were spun down (60 min at $300000 \times g$), washed once in salt medium, and sedimented again. Both supernatants were combined.

This soluble fraction will contain as well inter-membrane space proteins as mitochondrial matrix proteins. Subfractionation studies with Nagarse-treated rat liver mitochondria, carried out by Van Tol and Hülsmann [4], showed, however, that about 90% of the Nagarse-resistant palmitoyl-CoA synthetase is localized in the inner membrane-matrix compartment. The top fraction of the sucrose gradients, on which their subfractionation was carried out, which should contain the inter-membrane proteins, always contained less than 5% of the palmitoyl-CoA synthetase present in Nagarse-treated rat liver mitochondria (Van Tol, A., personal communication). This makes a localization of palmitoyl-CoA synthetase in the inter-membrane space unlikely. Moreover, palmitate oxidation in Nagarse-treated rat liver mitochondria was found to be carnitine independent [12], a result which would be expected when a palmitoyl-CoA synthetase is localized inside the inner membrane. Therefore, we will refer to the soluble fraction as matrix fraction.

In the experiments, in which the matrix fraction was subjected to Sephadex G-100 chromatography, we modified the procedure. Because these experiments are rather time consuming, minimal contamination with Nagarse (and probably other proteolytic enzymes) leads to gradual destruction of acyl-CoA synthetase activities and to bad recoveries. In these experiments lysosomal-poor mitochondria were isolated according to Loewenstein et al. [18]. These mitochondria were washed four times in isolation medium (0.25 M sucrose, 0.001 M Tris–HCl, pH 7.5) and finally suspended in our salt medium as before (7 ml per liver used). Mitochondria were disrupted by five times freezing and thawing and a crude mitochondrial matrix fraction was isolated as before. The outer membrane contamination in this fraction was very low, as judged by the low monoamine oxidase activity. To exclude any contamination with the outer membrane-bound acyl-CoA synthetase, not destroyed in these mitochondria, the following procedure was introduced.

To the crude matrix fraction, a saturated solution of $(NH_4)_2SO_4$ was added to a final concentration of 22 g/100 ml. The pH was kept near 8 by adding 1 M NH_4OH. After 15 min stirring at 0 °C the precipitate was collected (10 min at $12000 \times g$) and suspended in salt medium. Outer membrane fragments were quantitatively recovered in this fraction, as concluded from monoamine oxidase measurements. The supernatant of this precipitation step was brought on an $(NH_4)_2SO_4$ concentration of 50 g/100 ml and the precipitate was collected as before and suspended in salt medium (second $(NH_4)_2SO_4$ precipitate). The palmitoyl- and octanoyl-CoA synthetase activities, present in a crude matrix fraction of Nagarse-treated mitochondria, were quantitatively recovered in this fraction.

The second $(NH_4)_2SO_4$ precipitate, suspended in salt medium, was applied to a Sephadex G-100 column (1.5 cm × 90 cm), previously equilibrated with salt medium without ATP. The elution was carried out with the same medium and fractions of 0.9 ml were collected. Fractions showing palmitoyl- and octanoyl-CoA synthetase activity (see Results, Fig. 2, Fractions 59–86) were pooled and protein was precipitated by adding $(NH_4)_2SO_4$ to 50 g/100 ml. When stored in precipitated form at 4 °C, all acyl-CoA synthetases tested were stable for at least one month. This Sephadex fraction was used in our kinetic studies (see Results, Fig. 3). Carnitine palmitoyltransferase (palmitoyl-CoA:L-carnitine-*O*-palmitoyltransferase, EC 2.3.1.21) was purified from calf liver, as described by Farstad et al. [19] with some modifications, described before [20].

When enzyme assays were to be carried out with whole mitochondria, these organels were sonicated with MS 100-W Ultrasonic Desintegrator (Measuring and Scientific Equipment, London), operated at 20 kcycles/s (amplitude 2 μm, peak to peak) for 60 s/ml. The protein concentration during sonication was 1–5 mg/ml.

METHODS

Monoamine oxidase (Monoamine:oxygen oxidoreductase (deaminating), EC 1.4.3.4) was assayed according to Aas [5] with tyramine as the substrate at 37 °C (pH 7.95).

Malate dehydrogenase (L-Malate:NAD oxidoreductase, EC 1.1.1.37) was assayed according to Bergmeyer [21], except that 1 μM rotenone was added to the incubation medium (30 °C).

Cytochrome *c* oxidase (Ferrocytochrome *c*:oxygen oxidoreductase, EC

1.9.3.1) was measured polarographically by measuring the oxygen consumption with a "Clark" oxygen electrode, as described by Schnaitman et al. [22] (30 °C).

The proteins used for calibrating the Sephadex column for molecular weight determination, carnitine acetyltransferase (acetyl-CoA:L-carnitine-*O*-acetyltransferase, EC 2.3.1.7), alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) and creatine kinase (ATP:creatine phosphotransferase, EC 2.7.3.2), were all determined to standard enzymatic methods [21].

Fatty acyl-CoA synthetases were measured in three ways. In Methods I and II the reaction product acyl-CoA was converted by carnitine acyl transferases and carnitine into the acyl-carnitine esters, which were determined. In both methods the incubation medium contained 40 mM KCl, 5 mM $MgCl_2$, 80 mM Tris–HCl, 5 mM ATP, 0.3 mM KCN, 0.5 mM EDTA, 0.4 mM coenzyme A, 3 μg/ml oligomycin, 1.0 unit/ml inorganic pyrophosphatase, 5 mM phosphoenolpyruvate and myokinase and pyruvate kinase, 8 units/ml each. The pH was 7.5 and the reaction volume was 0.25 ml. In Method I, used for the fatty acids C_{12}–C_{16}, extra additions were 5 mM L-[*Me*-^{3}H]carnitine, 80 units/l carnitine palmitoyltransferase and 0.5 mM fatty acids (complexed in a 7:1 molar ratio to bovine serum albumin). The reaction was started by the addition of the enzyme source and terminated after 10 min by 0.02 ml concentrated HCl (reaction temperature 37 °C). Long-chain acyl-carnitine was extracted with butanol, as was described previously [19, 20]. In Method II, used for the fatty acids C_2–C_8, the extra additions were 5 mM L-carnitine, 4 units/ml carnitine acetyltransferase and [^{14}C]- or [^{3}H]-labelled fatty acid in a concentration of 10 mM (C_2–C_4, spec. act. 0.2 Ci/mole) or 0.5 mM (C_8, spec. act. 0.5 Ci/mole). The reaction was started by the addition of the enzyme source and terminated after 10 min by 1.2 ml ethanol (temperature 37 °C). The acylcarnitine ester was separated from the fatty acid on Dowex-50 W, as described previously [20]. Incubations were run at least in duplicate and the product formation was under the conditions shown always linear with the incubation time and with the amount of protein tested.

In our kinetic studies (Fig. 3) a direct assay (Method III) was used. This method is based on the insolubility of esters of medium- and long-chain fatty acids and coenzyme A in diethyl ether [23, 24]. Because this method was used to assay more purified fractions, the incubation medium was simplified and consisted of: 40 mM KCl, 80 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM ATP, 0.4 mM coenzyme A, 1.0 unit/ml inorganic pyrophosphatase and 0.8 mg/ml Triton WR-1339. The potassium salts of [1-^{14}C]palmitate or [2,3-$^3H_2$]octanoate (spec. act. 8 Ci/mole) were added from warm 0.25 mM stock solutions (pH 8) to final concentrations varying between 4 and 40 μM. The incubation was carried out in silanized tubes [24] in a volume of 0.25 ml at 37 °C. The reaction was started by the addition of the enzyme source and terminated after 2 min by the addition of 0.6 ml 0.5 M H_2SO_4 (octanoate activation) or 0.2 ml methanol + 0.6 ml water (palmitate activation). The unreacted fatty acid was removed by four extractions with 5 ml diethyl ether. Because long-chain fatty acyl-CoA is quite insoluble in water at low pH, it was essential to centrifuge between each extraction. The complete water-phase was transferred to a counting vial, mixed with 10 ml Insta-gel (Packard) and counted. Triton WR-1339, included in the assay mixture to ensure homogenous dispersion of the fatty acids [24], slightly stimulated both octanoate and palmitate activation. The reproducibility of this method was excellent and, under the conditions tested, product formation was

strictly linear with time and with (enzyme) protein. Protein was determined according to Lowry et al. [25].

RESULTS

When rat liver mitochondria are exposed to increasing amounts of the protease Nagarse, a sharp decrease in palmitoyl-CoA synthetase activity is observed (Fig. 1). However, when a level of 5% of the initial value is reached, no further decrease is shown. Before the assay, mitochondria were carefully washed to remove Nagarse, because in sonicated mitochondria in the presence of Nagarse, a further decrease in palmitoyl-CoA synthetase activity is observed (results not shown). The mitochondrial inner membrane stays intact during this procedure, which can be concluded from the unchanged activity of the matrix enzyme malate dehydrogenase.

This experiment is in agreement with former work from our laboratory [4], in which it was shown that the Nagarse-resistant palmitoyl-CoA synthetase in rat liver mitochondria is localized in the inner membrane-matrix compartment and, therefore, is inaccessable to proteolytic attack. The localization of this palmitoyl-CoA synthetase activity in the inner membrane-matrix compartment, was investigated in rat liver mitochondria, in which the outer membrane-bound palmitoyl-CoA synthetase was destroyed by an incubation with Nagarse. Under the Nagarse incubation conditions, used in this experiment, palmitoyl-CoA synthetase activity is reduced to the 5% level of the initial value (compare Fig. 1). Because octanoate, when activated on the mitochondrial outer membrane, is activated by the same acyl-CoA synthetase as palmitate [20], the outer membrane octanoyl-CoA synthetase activity will be destroyed simultaneously.

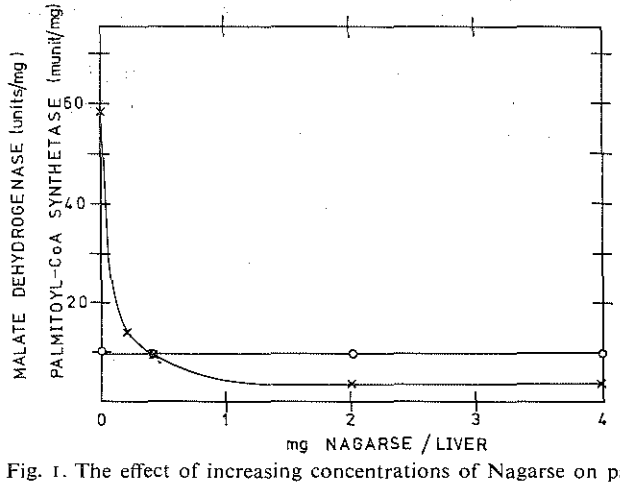

Fig. 1. The effect of increasing concentrations of Nagarse on palmitoyl-CoA synthetase activity in rat liver mitochondria. Mitochondria isolated from two livers (about 20 g) were isolated and suspended in 15 ml sucrose – 10 mM glucose. Batches of 3 ml were incubated for 30 min at 0 °C with increasing concentrations of Nagarse and subsequently washed, as described under Methods. The final mitochondrial pellet was suspended in salt medium, sonicated, and palmitoyl-CoA synthetase (Method I) and malate dehydrogenase were determined. The amount of Nagarse added, given on the abscissa, is recalculated on the base of mitochondria isolated from one liver. x—x, palmitoyl-CoA synthetase in units/g protein; ○—○, malate dehydrogenase in units/mg protein.

TABLE I

THE PARTITION OF PALMITOYL- AND OCTANOYL-CoA SYNTHETASE AND MARKER ENZYMES BETWEEN THE MEMBRANEOUS AND SOLUBLE FRACTION OF NAGARSE-TREATED RAT LIVER MITOCHONDRIA

Mitochondria, isolated from one rat liver, were incubated with 2 mg Nagarse and washed afterwards, as described under METHODS. These mitochondria, suspended in 7 ml salt medium, were disrupted by two times freezing and thawing and separated in a membraneous and a soluble fraction. The membranes were washed once in salt medium. Enzymatic activities were determined in the subfractions and compared with the activities in unfractionated, Nagarse-treated mitochondria. Palmitoyl-CoA synthetase was determined according to Method I and octanoyl-CoA synthetase according to Method II.

	Absolute values	Percentage values		
Fraction:	Mitochondria	Membranes	Soluble	Recovery
Protein (mg)	30	49.5	37.3	86.8
Monoamine oxidase (munits)	606	100	0.7	100.7
Malate dehydrogenase (units)	438	8.85	73.1	81.95
Cytochrome *c* oxidase (units)	27.75	81.4	0.0	81.4
Octanoyl-CoA synthetase (munits)	1830	11.6	68.6	80.2
Palmitoyl-CoA synthetase (munits)	117	11.45	80.3	91.75

Table I shows the distribution of palmitoyl-CoA synthetase and octanoyl-CoA synthetase between the membraneous and soluble fraction in Nagarse-treated rat liver mitochondria. Some marker enzymes were also tested. Both palmitoyl- and octanoyl-CoA synthetase activities were equally distributed as the mitochondrial matrix enzyme malate dehydrogenase. The soluble fraction in this subfractionation, however, will contain both inter membrane and matrix proteins. Because Van Tol and Hülsmann [4] have shown, that the Nagarse-resistant palmitoyl-CoA synthetase in rat liver mitochondria is localized in the inner membrane-matrix compartment, which excludes a localization in the inter membrane space, we concluded that this palmitoyl-CoA synthetase is a soluble enzyme and localized in the matrix mitochondrialis. The localization of octanoyl-CoA synthetase in the matrix mitochondrialis has already been shown by Aas [5].

We have tried to separate the palmitoyl- and octanoyl-CoA synthetase activities by Sephadex G-100 gel chromatography, a procedure which would give additional information on the molecular weight(s) of the enzyme(s). In these time-consuming experiments, minimal contamination of Nagarse and probably other proteolytic enzymes lead to bad recoveries of fatty acyl-CoA synthetase activities in the column fractions. For this reason the procedure to isolate a mitochondrial matrix fraction was changed (see Methods) and the proteins, precipitating between the addition of 22 and 50 g $(NH_4)_2SO_4$/100 ml matrix fraction, were collected. In this fraction practically all palmitoyl- and octanoyl-CoA synthetase activity was recovered, when a matrix fraction of Nagarse-treated mitochondria was used (93 and 107%, respectively, at recoveries of 94 and 108%). This $(NH_4)_2SO_4$ fraction was applied to the Sephadex column. The results of this gel chromatography are given in Fig. 2. Palmitoyl-, lauroyl- and octanoyl-CoA synthetase activities were eluted in identical peaks (concluded from the constant ratio C_8/C_{16} and C_8/C_{12} activation, which were 10 and 2.5, respectively) after the main protein peak had been eluted from the column. From

the elution pattern of proteins of known molecular weight, under the assumption that there exists a linear relation between the logarithm of the molecular weight and the elution volume, the molecular weight of these enzyme(s) was determined to be 47000.

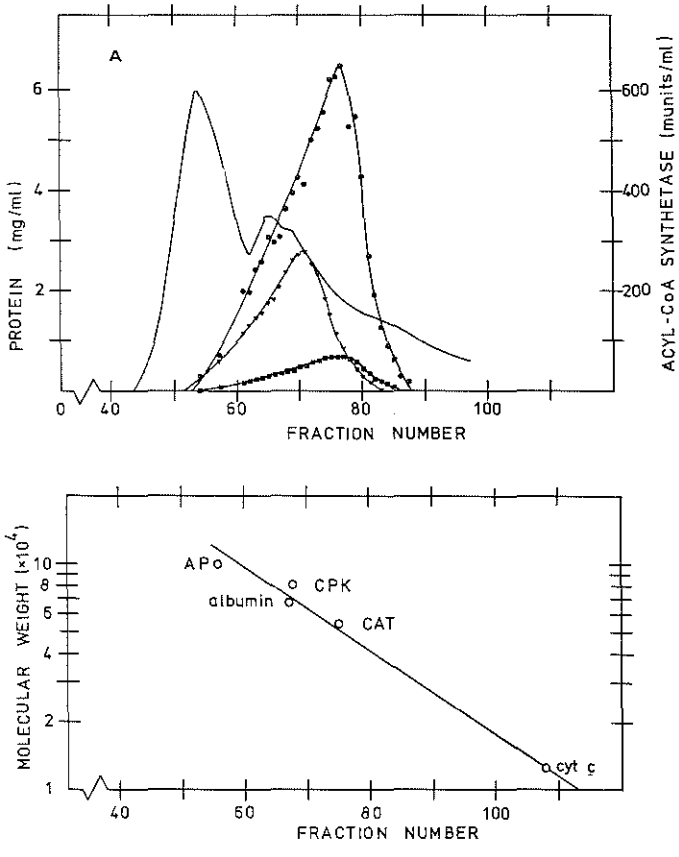

Fig. 2. The Sephadex G-100 elution profile of fatty acyl-CoA synthetases present in the soluble (matrix) fraction of rat liver mitochondria in relation to proteins of known molecular weight. (A) A 22–50 g/100 ml $(NH_4)_2SO_4$ precipitate from a soluble fraction of lysosomal-poor rat liver mitochondria (from five rat livers) was prepared according to Methods. 142 mg of this precipitate, mixed with 0.8 mg cytochrome *c* (as internal marker for the reproducibility of the gel filtration), was dissolved in 2.6 ml salt medium and applied to the Sephadex G-100 column (1.5 cm × 90 cm). The column was eluted with salt medium without ATP. Fractions of 0.9 ml were collected and C_2, C_8, C_{12} and C_{16} activations were determined (C_2 and C_8 with Method II and C_{12} and C_{16} with Method I). Recoveries were 100.5, 85.5, 88.5 and 118% for C_2, C_8, C_{12} and C_{16}, respectively. For reasons of clarity C_{12} activation is not included in this Figure. —, protein in mg/ml; ▼—▼, C_2 activation; ●—●, C_8 activation and ■—■, C_{16} activation, all in units/l. (B) 2.6 ml salt medium, containing 0.8 mg cytochrome *c* (from horse heart, mol. wt 12400 [25]), 0.1 mg carnitine acetyltransferase (from pigeon breast muscle, 80 units/mg, mol.wt 55000 [27]), 1 mg bovine serum albumin, complexed with a very small amount of [1-^{14}C]palmitate (mol.wt 67000 [26]), 1 mg creatine phosphokinase (from rabbit muscle, 25 units/mg, mol. wt 81000 [28]) and 0.1 mg alkaline phosphatase (from calf intestine, 350 units/mg, mol. wt 100000 [29]) were applied to the Sephadex column under the same conditions as in (A). Cytochrome *c* was measured by the absorbance at 412 nm, albumin by counting the radioactivity in each fraction and carnitine acetyltransferase (CAT), creatine phosphokinase (CPK) and alkaline phosphatase (AP) according to standard methods.

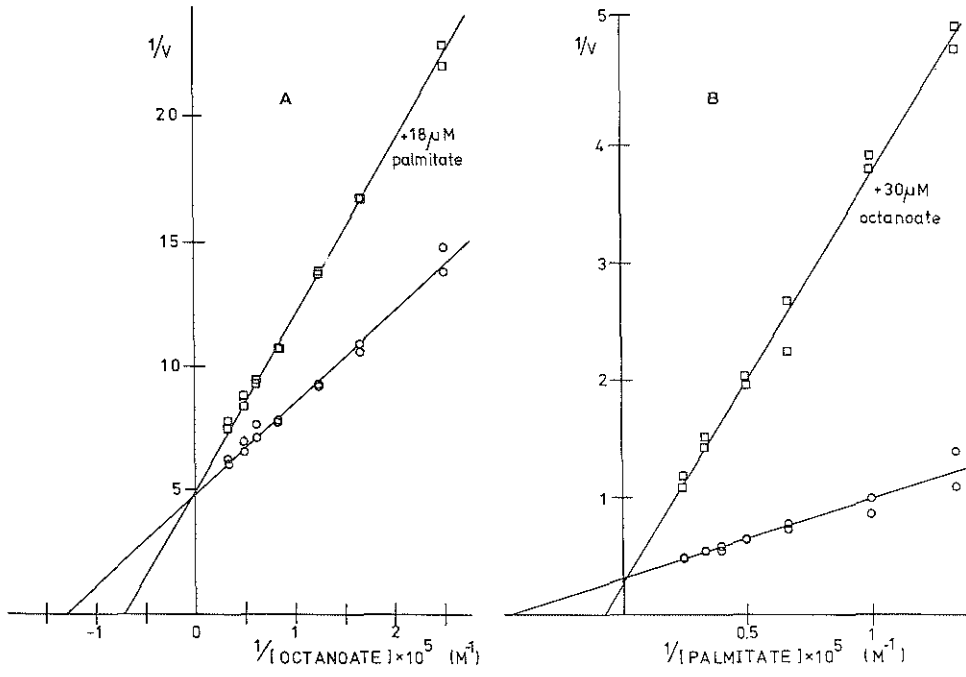

Fig. 3. Kinetic properties of the octanoyl- and palmitoyl-CoA synthetase, present in the pooled Sephadex fraction (Fractions 59–86, Fig. 2). Octanoyl- and palmitoyl-CoA synthetase activities were measured according to the direct assay method III. (A) Lineweaver–Burk plot (substrate [2,3-$^3H_2$]-octanoate, spec. act. 8 Ci/mole) for the octanoate activation. 0.00115 mg protein was present in each incubation. ○—○, octanoate activation in the absence of palmitate; □—□, octanoate activation in the presence of 18 μM unlabelled palmitate. (B) Lineweaver–Burk plot (substrate [1-^{14}C]palmitate, spec. act. 8 Ci/mole) for the palmitate activation. 0.00575 mg protein was present in each incubation. ○—○, palmitate activation in the absence of octanoate; □—□, palmitate activation in the presence of 30 μM octanoate. The lines were constructed with the least-squares method. Kinetic values, calculated from both figures, are given in Table II.

Acetyl-CoA synthetase, also present in this matrix fraction of rat liver mitochondria [5], was eluted before. The calculated molecular weight of this enzyme was 62000. Propionyl-CoA synthetase and butyryl-CoA synthetase, however, were eluted in very broadpeaks (mol. wt between 45000 and 75000), probably due to the involvement of more than one activating enzyme (results not shown).

To elucidate the question, if palmitate is activated by the octanoyl-CoA synthetase or by a distinct enzyme with similar molecular weight, kinetic experiments were performed with the pooled active fractions from the Sephadex column. In these experiments we used the direct assay method for fatty acyl-CoA synthetase (method III), successfully used in similar studies by Samuel et al. [23] and by Suzue and Marcel [24, 30]. This method permits an accurate measurement of medium- and long-chain acyl-CoA synthetase activities at very low fatty acid concentration (below the critical micellar concentration). Results of these experiments are given in Fig. 3 and Table II. The K_m values for octanoate and palmitate are 7.62 and 22.42 μM, respectively. Octanoate activation is competitively inhibited by palmitate, while palmitate activation is competitively inhibited by octanoate. The calculated K_i values for these

TABLE II

KINETIC PROPERTIES OF THE OCTANOYL- AND PALMITOYL-CoA SYNTHETASE IN THE SEPHADEX G-100 POOL FRACTION

Kinetic properties were calculated from the Lineweaver–Burk plots, given in Fig. 3. When octanoyl-CoA synthetase and palmitoyl-CoA synthetase were measured (Methods II and I, respectively) velocities of 223 units/g and 23.5 units/g were found, respectively.

Octanoyl-CoA synthetase	
K_m for octanoate (μM)	7.62
K_i for palmitate (μM)	22.42
V (units/g)	198
Palmitoyl-CoA synthetase	
K_m for palmitate (μM)	22.92
K_i for octanoate (μM)	5.67
V (units/g)	33.4

inhibitions were 22.96 and 5.67 μM, respectively. These values are in excellent agreement with the K_m values, a result that would be expected when both fatty acids are activated by the same enzyme. From this experiment we concluded that in this fraction octanoate and palmitate are activated by one and the same acyl-CoA synthetase.

DISCUSSION

When rat liver mitochondria, from which the outer-membrane-linked acyl-CoA synthetase activity is removed by preincubation with the protease Nagarse, are subfractionated in a membraneous and a soluble fraction, both octanoyl- and palmitoyl-CoA synthetase are found in the soluble (matrix) fraction. Both activities were quantitatively recovered in a 22–50 g/100 ml $(NH_4)_2SO_4$ precipitate of the soluble fraction and both activities showed the same behaviour on Sephadex G-100. Substrate competition experiments, performed with the active fractions of the Sephadex gel filtration experiment, clearly showed that palmitate and octanoate are activated by the same enzyme. From these results we conclude that palmitate, when activated in the inner membrane-matrix compartment of rat liver mitochondria, is activated by the medium-chain acyl-CoA synthetase (acid:CoA ligase, EC 6.2.1.2), described and purified from beef liver by Mahler and Wakil [31]. The molecular weight of this medium-chain acyl-CoA synthetase was determined from its elution profile on Sephadex G-100 and was found to be about 47000. Mahler and Wakil [31] reported a molecular weight in the range of 30000–60000 for the enzyme purified from beef liver. The mitochondrial acetyl-CoA synthetase was found to be of bigger size (mol wt 62000). A similar molecular weight (57000 $\pm$ 3500) was recently reported by Londesborough et al. [32] for the enzyme purified from ox heart mitochondria.

Van den Bergh [2] reported an active medium-chain acyl-CoA synthetase in the inner membrane fraction of rat liver mitochondria. This activity is possibly due to contamination or adherance of matrix proteins, since at least under our conditions, all octanoyl-CoA synthetase present in the membraneous fraction of Nagarse-treated liver mitochondria could be explained by contamination with matrix material (Table I). Our results are in agreement with the subfractionation studies of Aas [5]. The palmitoyl-CoA synthetase activity, found by us (8.4 units/g protein) in the soluble (matrix) fraction of Nagarse-treated mitochondria, is much higher than that

reported by Aas [5] (0.9 unit/g protein). However, his suggestion that this activity could be due to the medium-chain acyl-CoA synthetase appears to be correct, according to our present study. The value, presented by us, is perhaps still underestimated, because it became evident that the free palmitate concentration, present in our assay method I, is still suboptimal. This is illustrated by the higher V for palmitate activation, calculated from the kinetic experiments given in Fig. 3 and Table II (33.4 units/g protein) in comparison with the value of 23.5 units/g protein, which was measured with assay method I. Using the association constants for the binding of palmitate to bovine serum albumin, as given by Spector et al. [33], it can be calculated that the free palmitate concentration in our assay Method I (0.5 mM palmitate complexed in a 7:1 molar ratio to albumin) is about 20 μM, a value of the same magnitude as the K_m for palmitate.

In an earlier report from our laboratory [4] it was found that the palmitoyl-CoA synthetase present in Nagarse-treated mitochondria is non-competitively inhibited by octanoate. This led to the suggestion, that a distinct palmitoyl-CoA synthetase in the inner membrane-matrix fraction of rat liver mitochondria could be present, which motivated the present study. The kinetic experiments in the earlier study were carried out with sonicated Nagarse-treated mitochondria, using the hydroxamate assay method of Pande and Mead [34]. We think that this method was probably not reliable for these kinetic studies, because very high palmitate concentrations have to be used (higher than the critical micellar concentration). The method used in the present study has many advantages. Fatty acids can be used at micromolar concentrations (below the critical micellar concentration) and a product formation of 0.1 nmole is enough for accurate activity determination. The amount of enzyme protein to be added was in the microgram range and because the enzyme appeared to be soluble, a membrane-free enzyme source could be used. The palmitoyl-CoA synthetase activity present in the matrix fraction of rat liver mitochondria, is low compared with the outer membrane and microsomal activities. The localization inside the carnitine barrier, however, has made it subject of many studies, especially fatty acid oxidation studies with isolated mitochondria. It has been shown [4, 11, 12] that Nagarse-treated rat liver mitochondria, in the presence of ADP (State 3), are still able to oxidize palmitate at maximal rates. This indicates that under these conditions, the matrix palmitoyl-CoA synthetase activity can completely support the demand of palmitate activation. The importance of palmitate activation in the matrix, however, is questionable. The K_m for palmitate of the matrix enzyme is high (23 μM), when compared with the K_m for the microsomal enzyme (1–3 μM) [24, 30], determined with the same assay method.

The concentration of free palmitate inside the liver cell is unknown. This concentration, however, will be in equilibrium with the concentration of free palmitate in the blood [35] (free means not bound to albumin). Under physiological conditions the molar ratio between palmitate and albumin in the blood will not exceed a value of 3. It can be calculated [33] that at this molar ratio, the concentration of free palmitate in the blood will be about 1 μM. This would indicate that the concentration of free palmitate in the liver cell will be lower than 1 μM. This concentration is so low, when compared with the K_m for palmitate of the matrix palmitoyl-CoA synthetase activity, that the importance of this activity is questionable.

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THE ACTIVATION OF SHORT-CHAIN FATTY ACIDS BY THE SOLUBLE FRACTION OF GUINEA-PIG HEART AND LIVER MITOCHONDRIA

THE SEARCH FOR A DISTINCT PROPIONYL-CoA SYNTHETASE

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Summary

1. The ATP dependent acetyl-, propionyl- and butyryl-CoA synthetase activities were measured in the soluble fraction of both guinea-pig heart and liver mitochondria.

2. When measured in 300 mM Tris—HCl, the V of propionate activation in heart (=892 munits/mg protein) is much higher than the V of acetate (=637 munits/mg protein) and butyrate activation (=143 munits/mg protein. Fatty acid competition experiments, however, clearly show that most of the propionate activation (K_m = 7.94 mM) is caused by the acetyl-CoA synthetase (EC 6.2.1.1) (K_m for acetate = 0.8 mM), while the remaining activity is probably caused by a butyryl-CoA synthetase (K_m for butyrate = 0.83 mM). This indicates that in guinea-pig heart the presence of a distinct propionyl-CoA synthetase is very unlikely.

3. In liver a completely different pattern of short-chain fatty acid activation is found: low acetate activation and moderate propionate and butyrate activation. Substrate competition experiments and kinetics of fatty acid activation indicate that in this tissue a distinct propionyl-CoA synthetase is present with high affinity for propionate (K_m = 0.6 mM) and some affinity towards acetate and butyrate (K_m values respectively 11 mM and 5.4 mM).

Introduction

Although the short-chain fatty acid activation (ATP dependent) has been the subject of many studies, espectially in ruminant tissues, the number of acyl-CoA synthetases involved is still an open question. Acetyl-CoA synthetase (EC 6.2.1.1), the best studied enzyme, has been purified from many sources in-

cluding yeast, bacteria, moulds, plants, birds and mammalian tissues (for references see Campagnari and Webster [1]). Hele [2] purified this enzyme from beef heart and found practically identical acyl-CoA synthetase activity with acetate and with propionate. The K_m for propionate, however, was higher than the K_m for acetate (5.0 and 1.45 mM respectively).

Two short-chain acyl-CoA synthetases were purified from beef heart mitochondria by Webster and coworkers [1,3]: an acetyl-CoA synthetase, activating acetate and propionate (K_m values were 0.79 and 11 mM respectively), and a butyryl-CoA synthetase, activating C_3 to C_7 (K_m for butyrate 1.5 mM). The activity of both enzymes towards propionate was 30% of that found for the favoured substrate. Short-chain fatty acid activation in sheep tissues had been investigated by Cook and coworkers [4]. From substrate specificity studies in mitochondrial extracts they found some evidence for the presence of a distinct propionyl-CoA synthetase in sheep lung.

Scholte et al. [5] studied the intracellular distribution of the short-chain acyl-CoA synthetase activities in guinea-pig heart and concluded that both acetate and propionate activation in this tissue are localized in the mitochondrial matrix fraction. The propionate activation in this tissue was too high to be explained by the sum of the activities of acetyl-CoA and butyryl-CoA synthetases, each having 30% of the maximal activity with propionate [1,3]. Moreover, acetate activation was doubled, when the assay was carried out in 40 mM tricine—KOH instead of 300 mM Tris—HCl, whereas propionate activation was not. From these results, Scholte et al. [5] concluded, that in guinea-pig heart mitochondria a distinct propionyl-CoA synthetase is present. Because of our interest in the function and properties of the different acyl-CoA synthetases in mammalian tissues, we investigated this further.

In former work from our laboratory [6,7] and from others [8—10] it was shown that substrate competition experiments are very useful to answer the question whether one or more enzymes are involved in the activation of fatty acids of different chainlength. This paper will communicate these fatty acid competition studies.

Materials and Methods

Reagents

[1-^{14}C]-labeled fatty acids were supplied by the Radiochemical Centre (Amersham, England), mixed with the free acids (British Drug Houses Ltd.) and neutralized with KOH. The final specific activity was about 0.2 Ci/mole. Acetyl- and propionyl-CoA, a gift of Dr H.R. Scholte, were synthetized from the fatty acid thiophenol esters. All other reagents were as described earlier [6,7].

Preparations

White guinea-pigs (weight 300—700 g) were used for all preparations. Heart mitochondria were prepared in a salt medium, described by Chappel and Perry [11] (100 mM KCl, 50 mM Tris—HCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA and 0.5 mg/ml bovine serum albumin, pH = 7.5). A 5% w/v homogenate was prepared using a Potter—Elvehjem homogenizer with a Teflon pestle.

Nuclei were spun down (5 min at 480 × g_{max}) and from the supernatant the mitochondria were sedimented (10 min at 4500 × g_{max}). The mitochondria were washed twice and finally suspended in a few ml of a salt medium (40 mM KCl, 80 mM Tris—HCl, 1 mM $MgCl_2$, 1 mM ATP and 0.2 mM dithiothreitol, pH = 7.5) [7]. Mitochondria were disrupted by ultrasound and membrane fragments were spun down (60 min at 300 000 × g_{max}). The remaining supernatant, the mitochondrial soluble fraction, was preserved in batches at —70° C. Under these conditions, short-chain fatty acid activation was stable for at least 3 months. Lysosomal-poor guinea-pig liver mitochondria were prepared as described previously for rat liver mitochondria [7]. A soluble fraction of these mitochondria was prepared as described for heart mitochondria.

Methods

Short-chain acyl-CoA synthetase was measured in a coupled assay in which the acyl-CoA synthetase dependent acylcarnitine production was measured. This method, worked out by Scholte in our laboratory, has been described in detail previously (Groot et al., Method II, see refs 6,7). In order to compare our results in guinea-pig with those of Scholte et al. [5], the incubation mixture was adapted and consisted of: 10 mM $MgCl_2$, 8 mM ATP, 1.2 mM EDTA, 0.3 mM KCN, 3 μg/ml oligomycin, 5 mM carnitine, 4 U/ml carnitine acetyltransferase, 5 mM phosphoenolpyruvate, 1.35 mM coenzyme A and 8 U/ml adenylate kinase and pyruvate kinase. The mixture was buffered either with 40 mM tricine—KOH or with 300 mM Tris—HCl (pH = 8.0). [1-^{14}C]-labeled fatty acids were present in the concentrations given in the legends (see Result section). The final reaction volume was 0.25 ml, the temperature 37° C. The reaction was started by the addition of the acyl-CoA synthetase source and terminated after 10 min by the addition of 1.2 ml ethanol. The reaction product, acylcarnitine, was separated from the fatty acid on Dowex 50 W. The activity of the added acyl-CoA synthetase in the assay never exceeded 10 mU. Under these conditions, product formation is linear with time of incubation and with the protein concentration.

To test the possible influence of acyl-CoA hydrolase activity on our acyl-CoA synthetase assay, acyl-CoA hydrolase was tested under conditions approaching the conditions of the synthetase assay. The incubation volume was 1 ml and consisted of: 40 mM tricine—KOH, 10 mM $MgCl_2$, 8 mM ATP, 1.2 mM EDTA and 0.3 mM KCN (pH = 8.0). The CoA esters of acetate and propionate were added in a final concentration of 1 mM. The reaction temperature was 37° C. The reaction was started by the addition of an aliquot of the soluble fraction of guinea-pig heart or liver mitochondria and was followed either continuously in the presence of 0.1 mM 5,5′-dithio-*bis*-(3-nitro-benzoic acid) (DTNB) at 412 nm or discontinuously. In the latter case DTNB was added after termination of the incubation by heating at 100° C followed by cooling to room temperature.

Results

Studies with guinea-pig heart mitochondria

In Table I, the kinetic parameters are given for the activation of C_2, C_3

TABLE I

KINETIC PROPERTIES OF ACETATE-, PROPIONATE- AND BUTYRATE ACTIVATION BY THE SOLUBLE FRACTION OF GUINEA-PIG HEART MITOCHONDRIA, MEASURED AT 40 mM TRICINE—KOH AND AT 300 mM TRIS—HCl

The soluble fraction of guinea-pig heart mitochondria was prepared according to Methods and frozen at -70°C in batches. All kinetic experiments were performed with the same soluble fraction. Acyl-CoA synthetase activity was measured either in 40 mM tricine—KOH or in 300 mM Tris—HCl according to Methods. 0.0105 mg protein was added in all incubations. The kinetic parameters of C_2 and C_3 activation are the mean of two separate experiments (see also Figs. 1 and 2). V is measured in nmole acylcarnitine formed/min. mg protein. K_m is measured in mM.

Activation tested	Tricine—KOH		Tris—HCl	
	V	K_m	V	K_m
Acetate	1105	0.71	637	0.80
Propionate	900	6.39	892	7.94
Butyrate	170	0.30	143	0.83

and C_4 by the soluble fraction of guinea-pig heart mitochondria. In guinea-pig heart, this fraction will contain all the short-chain acyl-CoA synthetase activity, for Scholte et al. [5] localized all the C_2 and C_3 activation in the matrix fraction of these mitochondria. Measurements were carried out at low (40 mM tricine—KOH) and high (300 mM Tris—HCl) buffer concentrations. The V for acetate activation is strongly influenced by the buffer used, while for propionate and butyrate activations this is not the case (Table I). In both buffers, the K_m values for acetate and butyrate activations are quite low (0.3—0.8 mM) when compared with propionate activation (6—8 mM). The mutual ratio of the V values for C_2, C_3 and C_4 activation in both buffers is in good agreement with the results of Scholte et al. [5] who measured the acyl-CoA synthetase in the same fraction by means of the coenzyme A disappearance under similar conditions. Propionate activation, especially at 300 mM Tris—HCl, is much higher than the sum of 30% of the V for acetate plus 30% of the V for butyrate, the activities towards propionate of acetyl- and butyryl-CoA synthetase, as given by Webster and coworkers [1,3]. This phenomenon together with the different behaviour of the V of acetate and propionate activation by change of the assay buffer (see Table I), made Scholte et al. [5] suppose that propionate is activated by a distinct propionyl-CoA synthetase.

We checked this hypothesis by substrate competition experiments. The results of these studies are given in the form of Lineweaver-Burk plots, shown in Figs. 1 and 2. Because butyrate activation is very low when compared with acetate and propionate activation, we tested the influence of acetate on propionate activation and vice versa. In both buffers tested, acetate activation is competitively inhibited by the presence of propionate (Fig. 1). The observed inhibition was independent of the reaction time which indicates that the acid itself and not a product of propionate activation inhibits the acetate activation (results not shown). The calculated K_i values for the inhibitor propionate (7.4 mM in tricine—KOH and 4.5 mM in Tris—HCl) are of the same magnitude as the K_m values for propionate activation, given in Table I, a result that would be expected when both fatty acids are activated by the same acyl-CoA synthetase.

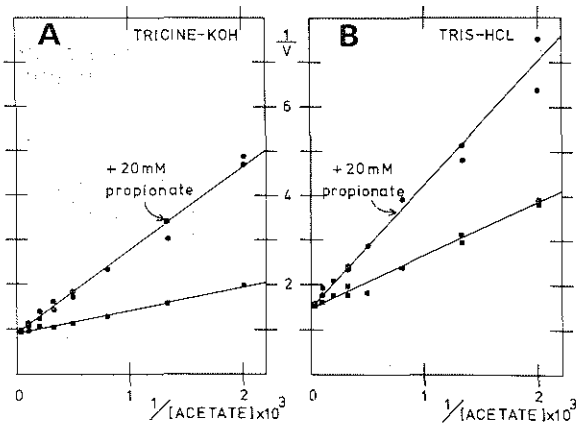

Fig. 1. Acetate activation by the soluble fraction of guinea-pig heart mitochondria and the effect of propionate. For details see Table I and Methods. A, Lineweaver-Burk plot of acetate activation measured in 40 mM tricine—KOH. ■———■, [1-^{14}C] acetate present; ●———●, [1-^{14}C] acetate and 20 mM propionate present. B, identical to A, however 300 mM Tris—HCl instead of 40 mM tricine—KOH.

The reverse experiment is given in Fig. 2. At least at high propionate concentrations, a pattern of competitive inhibition of propionate activation by acetate is found (see insert Fig. 2). The hyperbolic $1/v$ against $1/[S]$ relation found in the presence of acetate, however, indicates that more than one enzyme is involved in propionate activation, one enzyme with the highest V which is competitively inhibited by acetate and one enzyme which is probably not profoundly influenced by acetate. The straight $1/v$ vs $1/[S]$ relation which is found in the absence of acetate indicates that the K_m for propionate of both enzymes is not very different. All inhibitions were again independent of the

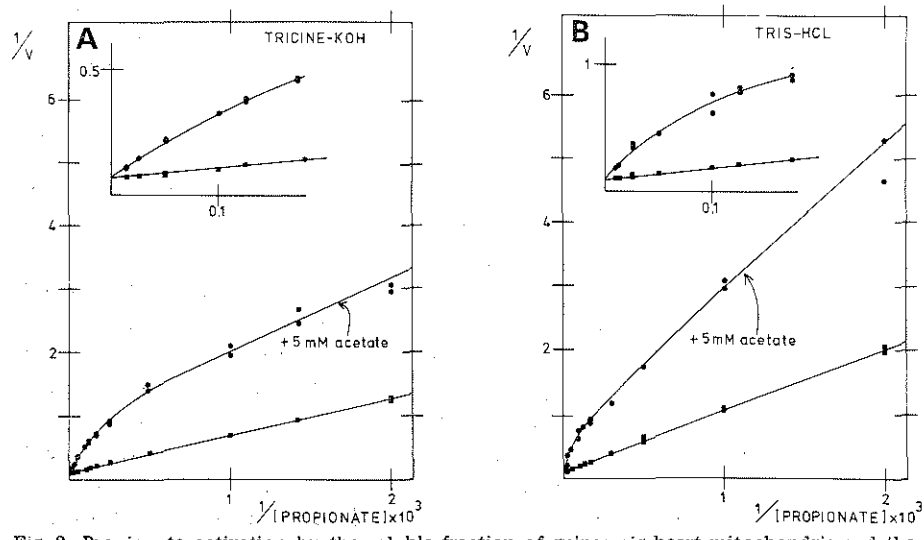

Fig. 2. Propionate activation by the soluble fraction of guinea-pig heart mitochondria and the effect of acetate. For details see Table I and Methods. A, Lineweaver-Burk plot of propionate activation measured in 40 mM tricine—KOH. ■———■, [1-^{14}C] propionate present; ●———●, [1-^{14}C] propionate and 5 mM acetate present. B, identical to A, however 300 mM Tris—HCl instead of 40 mM tricine—KOH.

TABLE II

INFLUENCE OF ACETATE AND BUTYRATE ON PROPIONATE ACTIVATION IN THE SOLUBLE FRACTION OF GUINEA-PIG HEART MITOCHONDRIA

Short-chain acyl-CoA synthetase activity was determined either in 40 mM tricine—KOH or in 300 mM Tris—HCl. Each fatty acid, indicated in the table below, was present in a final concentration of 20 mM. 0.0105 mg protein was present in all incubations. Specific activity is measured in nmole acylcarnitine formed/min. mg protein.

Activation tested ([1-^{14}C]-labeled substrate)	In the presence of (unlabeled)	Acyl-CoA synthetase activity			
		Tricine—KOH		Tris—HCl	
		Spec. act.	% Inhibition	Spec. act.	% Inhibition
Acetate	—	1003	—	494	—
Propionate	—	698	—	615	—
Butyrate	—	189	—	192	—
Propionate	Acetate	167	76	139	77
Propionate	Butyrate	573	18	553	10
Propionate	Acetate and butyrate	73	90	41	93

reaction time. Our interpretation of these results is that most of the propionate activation in guinea-pig heart can be ascribed to the acetyl-CoA synthetase, while a minor contribution is given by a second acyl-CoA synthetase. Table II shows that a good candidate for this second enzyme is butyryl-CoA synthetase. When tested in a final concentration of 20 mM, propionate activation in this guinea-pig heart fraction is 10—18% inhibited by butyrate, while 20 mM acetate inhibits about 77% of the propionate activation. In the presence of both acetate and butyrate, practically no propionate activation is left. These results exclude the presence of a distinct propionyl-CoA synthetase in this fraction. The major contribution to propionate activation is given by acetyl-CoA synthetase (K_m for acetate 0.7—0.8 mM and K_m for propionate 6.4—8.0 mM), while a minor contribution is given by a second acyl-CoA synthetase (K_m for butyrate 0.3—0.8 mM, depending on the assay buffer, and K_m for propionate 6.4—8.0 mM). This second acyl-CoA synthetase is probably identical in its substrate specificity to the butyryl-CoA synthetase, isolated from beef heart by Campagnari and Webster [1].

Studies with guinea-pig liver mitochondria

During the present work a paper by Ash and Baird [12] on short-chain fatty acid activation in bovine rumen and liver tissue appeared. Fatty acid activation competition experiments, performed in that study, led to the assumption of a distinct propionyl-CoA synthetase in bovine liver. Our studies on short-chain fatty acid activation in guinea-pig liver will be reported now. The kinetic parameters of C_2, C_3 and C_4 activation in the soluble fraction of guinea-pig liver mitochondria are given in Table III. Both V values and K_m values are completely different from those in heart. The lowest K_m was found for propionate (about 10 times lower than in heart), while the K_m values for acetate and butyrate activation are at least 10 times higher than those obtained for heart.

TABLE III

KINETIC PROPERTIES OF ACETATE-, PROPIONATE- AND BUTYRATE ACTIVATION BY THE SOLUBLE FRACTION OF GUINEA-PIG LIVER MITOCHONDRIA, MEASURED IN 40 mM TRICINE—KOH

For details see Methods and Table I. Acyl-CoA synthetase activity was measured in 40 mM tricine—KOH. 0.0425 mg protein was present in all incubations. V is measured in nmole acylcarnitine formed/min.mg protein. K_m is measured in mM.

Activation tested	V	K_m
Acetate	28	11.3
Propionate	137	0.6
Butyrate	248	5.4

Substrate competition experiments performed with the liver preparation are given in Table IV. Acetate activation is completely inhibited by propionate when both fatty acids are present at 20 mM, while propionate activation is unaffected by acetate. Propionate activation is about 25% inhibited by the presence of butyrate, while in the reverse experiment a practically identical inhibition of butyrate activation by propionate was found. Butyrate activation, however, is strongly (80%) inhibited by 20 mM octanoate, while propionate activation is only slightly influenced by octanoate (about 25% inhibition). The presence of both propionate and octanoate reduces butyrate activation to a very low level (95% inhibition). These results strongly indicate the presence of two acyl-CoA synthetases in guinea-pig liver mitochondria: (1) a distinct

TABLE IV

ACETATE-, PROPIONATE- AND BUTYRATE ACTIVATION IN A SOLUBLE FRACTION OF GUINEA-PIG LIVER MITOCHONDRIA: THE INFLUENCE OF THE ADDITION OF A SECOND FATTY ACID

Short-chain acyl-CoA synthetase activity was determined either in 40 mM tricine—KOH or in 300 mM Tris—HCl according to Methods. Each fatty acid, as indicated in the Table below, was present in a final concentration of 20 mM. 0.0425 mg protein was present in all the incubations. Specific activity is measured in nmole acylcarnitine formed/min.mg protein.

Activation tested ([1-^{14}C]-labeled substrate)	In the presence of (unlabeled)	Acyl-CoA synthetase activity			
		Tricine—KOH		Tris—HCl	
		Spec. act.	% Inhibition	Spec. act.	% Inhibition
Acetate	—	27	—	16	—
Propionate	—	144	—	116	—
Butyrate	—	142	—	103	—
Acetate	Propionate	0	100	0	100
Propionate	Acetate	144	0	112	4
Propionate	Butyrate	108	25	91	22
Propionate	Octanoate	115	20	84	28
Butyrate	Propionate	101	29	80	20
Butyrate	Octanoate	28	80	18	83
Butyrate	Propionate and octanoate	7	95	5	95

propionyl-CoA synthetase which preferentially activates propionate (K_m = 0.6 mM) and with at least some activity towards acetate (K_m = 11 mM) and butyrate (K_m is about 5 mM). (2) a medium-chain acyl-CoA synthetase, as described by Mahler et al. [13] for beef liver, which has C_7 and C_8 as preferred substrates but which has overlapping activity towards shorter fatty acids as butyrate.

Measurements of acyl-CoA synthetase can be underestimated by interference of acyl-CoA hydrolase, an activity also present in the mitochondrial matrix fraction of most tissues [14]. Although in our assay, in which short-chain acyl-CoA is transferred into the acylcarnitine ester, the effect of acyl-CoA hydrolase will be less prominent than in a direct assay, we tested short-chain acyl-CoA hydrolase in the soluble fraction of both guinea-pig heart and liver mitochondria. When acyl-CoA hydrolase was tested in the presence of 0.1 mM DTNB (see Methods), liver hydrolysed C_2-CoA and C_3-CoA quite rapidly (both about 200 nmoles/min. mg protein), while the rates in heart were somewhat lower (C_2-CoA hydrolase: 66 mU/mg, C_3-CoA hydrolase: 107 mU/mg). However, when measurements were performed in the absence of DTNB, a condition more closely resembling the conditions of the acyl-CoA synthetase assay, much lower acyl-CoA hydrolase activities were found although measurements under these conditions showed a bad time and protein dependency. In liver, the values were at least 10 times lower, while in heart, in the absence of DTNB during the incubation, no measurable acyl-CoA hydrolase activity could be detected. This effect is probably caused by a strong inhibition of short-chain acyl-CoA hydrolase by its reaction product coenzyme A. Our results, however, do not indicate a strong interference of acyl-CoA hydrolase in our acyl-CoA synthetase assay.

Discussion

Studies on fatty acid activation are always complicated by the presence of several acyl-CoA synthetases with often overlapping substrate specificities. In most studies, only the maximal velocity of fatty acid activation is considered. Different fatty acids, however, which are activated by the same acyl-CoA synthetase, compete with each other for the activation enzyme. Such competitions are likely to occur in vivo. A high V of activation of a specific fatty acid does not guarantee rapid metabolism. K_m values for the fatty acids of the different acyl-CoA synthetases present are also important. The object of the present study was to establish the possible presence of a distinct propionyl-CoA synthetase in guinea-pig heart and liver mitochondria. In guinea-pig heart we were able to demonstrate that most of the propionyl-CoA synthetase activity, present in the soluble fraction of the mitochondria, can be ascribed to acetyl-CoA synthetase. The K_m for propionate of this enzyme turned out to be about 10 times higher than for acetate. Propionate activation was not only inhibited by acetate but also by butyrate. These inhibitions were additive (see Table II). However, the K_m for butyrate activation in heart is very low (0.3 mM), when compared with the value found in liver (5.4 mM) under identical assay conditions. Therefore we think that in heart two short-chain acyl-CoA synthetases are present: (1) acetyl-CoA synthetase, activating acetate and propionate and

(2) a butyryl-CoA synthetase activating at least butyrate and propionate. The latter enzyme is probably similar in substrate specificity to the butyryl-CoA synthetase, purified from beef heart by Campagnari and Webster [1]. The remarkable difference, however, between acetate and propionate activation, when assayed in 40 mM tricine—KOH instead of 300 mM Tris—HCl, as discovered by Scholte et al. [5], while most of the propionate activation is caused by acetyl-CoA synthetase, is not understood.

In contrast to heart, propionate is activated by the soluble fraction of liver mitochondria with a much lower K_m than acetate and butyrate. Competition experiments in this tissue indicate that acetate is activated by the same enzyme as propionate. At least part of the butyrate activation can also be ascribed to this enzyme, which can be concluded from the mutual inhibition of propionate and butyrate in the activation reaction. Butyrate activation, however, is strongly inhibited by octanoate while propionate activation is only slightly influenced. This indicates that butyrate is predominantly activated by a medium-chain acyl-CoA synthetase, as described by Mahler et al. [13], an enzyme with the lowest K_m for octanoate. Our results strongly suggest that in guinea-pig liver a short-chain acyl-CoA synthetase is present, which preferentially activates propionate.

Although short-chain fatty acid metabolism in guinea-pig will be less important than in ruminants, it has been shown that the large caecum of these animals contains high concentrations of short-chain fatty acids (46—99 μmole/g wet weight). The fermentation of cellulose in this part of the intestine [15,16], produces mostly acetate (±90%) and propionate (±10%). Small intestine, caecum and colon of the guinea-pig all have a large capacity to absorb these short-chain fatty acids [15]. When these fatty acids reach the portal blood, the liver is a good candidate to metabolize propionate where this fatty acid can be used for gluconeogenesis, while the heart is a good candidate to metabolize acetate.

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