HEPATIC FATTY ACID OXIDATION

Activity, localization and function of some enzymes involved

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE MEDISCHE FACULTEIT TE ROTTERDAM,
OP GEZAG VAN DE DECAAN PROF. D.C. DEN HAAN,
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ARIE VAN TOL

GEBOREN TE ABBENES IN 1942

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
CoA	Coenzyme A (reduced)
DNP	2,4-dinitrophenol
dpm	Disintegrations/min
EDTA	Ethylenediaminetetraacetate
$FADH_2$	Flavine-adenine dinucleotide (reduced)
g_{max}	Maximal relative centrifugal force
GDP	Guanosine 5'-diphosphate
GTP	Guanosine 5'-triphosphate
h	Hour
K_m	Michaelis constant
K_i	Inhibitor constant
min	minute
NAD^+	Nicotinamide-adenine dinucleotide (oxidized)
NADH	Nicotinamide-adenine dinucleotide (reduced)
P_i	Inorganic orthophosphate
PP_i	Inorganic pyrophosphate
RCOCoA	Acyl-CoA
RCOOH	Carboxylic acid
Tris	Tris(hydroxymethyl)aminomethane
TSH	Thyroid-stimulating hormone

LIST OF ENZYMES*

Number	Systematic name	Trivial name
1.6.2	NADPH: ferricytochrome *c* (b_5) oxidoreductase	NADPH-cytochrome *c* reductase
1.4.3.4	Monoamine: oxygen oxidoreductase (deaminating)	Monoamine oxidase
1.9.3.1	Ferrocytochrome *c*: oxygen oxidoreductase	Cytochrome *c* oxidase
2.3.1	Palmitoyl-CoA: carnitine *O*-palmitoyltransferase	Carnitine palmitoyltransferase
3.1.1.1	Carboxylic-ester hydrolase	Carboxylesterase
3.1.3.9	D-glucose-6-phosphate phospho-hydrolase	Glucose-6-phosphatase
3.1.3.11	D-fructose-1,6-diphosphate 1-phosphohydrolase	Fructose-1,6-diphosphatase
3.4.4.16	Subtilopeptidase A	Nagarse
4.1.3.7	Citrate oxaloacetate-lyase (CoA-acetylating)	Citrate synthase
6.2.1.3	Acid: CoA ligase (AMP)	Long-chain acyl-CoA synthetase

* The numbers and systematic names are given according to the recommendations of the Commission of Enzymes of the International Union of Biochemistry.

CHAPTER I

INTRODUCTION

This chapter is not intended to give a complete review of fatty acid metabolism. Only those aspects are discussed, that are necessary for a general understanding of the experiments described in this thesis.

Fatty acid oxidation is an important pathway for energy production in mammals and birds[1]. In animal tissues the enzymes of fatty acid oxidation are located in the mitochondrion[2,3,4]. Recent reports suggest that this is not the case in Castor bean endosperm. In this tissue the enzymes of β-oxidation are localized in a very fragile cell organelle, called the glyoxysome[5].

Fatty acids are transported in the blood complexed to albumin[6,7], or in esterified form as triglycerides and phospholipids, complexed to protein (lipoproteins[8,9]). Lipoproteins are synthesized in the liver and in the intestinal epithelium (chylomicrons[9]). Before entering the cell these triglycerides are generally hydrolyzed by lipoprotein lipase, an enzyme activated by heparin and probably present in the endothelial cells of the capillary wall[10].

From the foregoing it is evident that fatty acid presented to the cell for further metabolism is in the form of "free" fatty acid.

Fatty acids cannot participate in any reaction of intermediary metabolism, before they have been "activated" to their thioester with CoA. This reaction is necessary for triglyceride and phospholipid biosynthesis, for acyl interchange between complex lipids, for chain-elongation reactions and also for oxidative degradation of fatty acids.

After being activated to acyl-CoA, and, if necessary, transported to the cellular compartment of β-oxidation (see Chapter II), fatty acid can be oxidized stepwise to acetyl-CoA by the β-oxidation system. The discovery that the product of β-oxidation is a C_2 fragment by F. KNOOP in 1904 preceded the elucidation of the nature of this fragment by almost half a century. This elucidation had to await the discovery of CoA. In 1948 LIPMANN[11] suggested that an acetylated CoA might be the unknown C_2 fragment and a few years later acetyl-CoA was isolated from yeast by LYNEN *et al.*[12].

Acetyl-CoA can be oxidized to CO_2 by the reactions of the citric acid cycle. In liver, acetyl-CoA is also converted to ketone bodies (acetoacetate, β-hydroxybutyrate and to a small extent acetone), under certain conditions. Acetoacetate and β-hydroxybutyrate, which cannot be oxidized completely to CO_2 in liver, are transported in the blood from the liver to other tissues. Muscle and kidney cortex can oxidize acetoacetate, even in the presence of glucose[13,14]. Recent reports indicate that immature brain, and adult brain after prolonged starvation, also can oxidize β-hydroxybutyrate and acetoacetate[15,16,17].

The reduced hydrogen acceptors of the β-oxidation system and of the enzymes of the citric acid cycle (NADH and $FADH_2$) are oxidized by the enzymes of the respiratory chain, so that oxygen becomes the terminal electron acceptor. In "coupled" mitochondria this process of electron transfer in the respiratory chain is accompanied by the synthesis of ATP from ADP and P_i.

High rates of fatty acid oxidation, accompanied by ketogenesis, are observed under conditions of increased supply of fatty acids to the liver, in combination with a decreased intracellular glucose level. These states include diabetes, hyperthyroidism, pregnancy, obesity, starvation and fat feeding. Under these conditions elevated plasma fatty acid levels as well as glucose intolerance can be observed[18-22]. Fatty acid uptake by isolated perfused liver is proportional to the concentration from 0.1 to at least 1.8 mM[23]; this covers the *in vivo* range (*cf.* ref. 24). The uptake of fatty acids seems to be a passive process and is not affected by fasting[23,25,26], glucose[25] or glucagon[23]. From this it seems likely that the rate of fatty acid oxidation and ketogenesis is to be regulated by the supply of glucose and/or fatty acids. Release of fatty acids from adipose tissue is decreased by insulin[7]. At first it was thought that this effect of insulin was due to

an increased esterification[27], but the situation was found to be more complex. A rapid release of fatty acids from adipose tissue was demonstrated *e.g.* after addition of adrenaline, nor-adrenaline, ACTH, glucagon or TSH[28]. These lipolytic hormones stimulate the "hormone sensitive lipase" by increasing the level of 3',5'-(cyclic)-AMP[29-31]. It has been shown that the stimulating effect of low concentrations of catecholamines, ACTH or glucagon is counteracted by extremely low concentrations of insulin[32]. Insulin has been found to lower the level of 3',5'-(cyclic)-AMP in adipose tissue[30].

It can be concluded that the regulations of glucose and fatty acid metabolism are closely interrelated. In adipose tissue, a lack of insuline (*plus* glucose) results in fatty acid release. In muscle, glycolysis is inhibited during fatty acid oxidation, probably by way of citrate inhibition of phosphofructokinase[33,34]. In liver, glycolysis is inhibited and gluconeogenesis is stimulated during long-chain fatty acid oxidation[35-39]. The close relationship between ketogenesis and gluconeogenesis has been pointed out by KREBS[14].

From the foregoing it is clear that the liver plays an important role in glucose and fatty acid metabolism. Hepatic long-chain fatty acid oxidation, the subject of the present study, is therefore of major importance in mammalian intermediary metabolism. Some of the reactions occurring in fatty acid oxidation will be discussed in detail in Chapter II. Several excellent reviews on fatty acid catabolism have been published. The reader is referred to those by GREVILLE AND TUBBS[40] and by BRESSLER[41].

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CHAPTER II

SOME ASPECTS OF FATTY ACID OXIDATION BY ISOLATED RAT-LIVER MITOCHONDRIA

Long-chain fatty acid activation

Long-chain fatty acids can be activated in rat liver by long-chain acyl-CoA synthetase, which catalyzes reaction (1)

$$RCOOH + ATP + CoA \rightleftharpoons RCOCoA + AMP + PP_i \qquad (1)$$

The enzyme was first demonstrated by KORNBERG AND PRICER[1] in liver microsomes. The overall reaction is identical with that of acetate activation, described by LIPMANN *et al.*[2], and with those of butyrate[3,4] and octanoate activation[4,5]. The activation of short- and medium-chain fatty acids involves an enzyme-bound acyladenylate intermediate[6-9].

The subcellular localization of ATP-dependent long-chain fatty acid activation has been extensively studied[10-14]. Most of these studies have been performed with rat liver, and it is generally agreed that in this tissue the enzyme is located both in mitochondria and microsomes. From our own observations we concluded that mitochondria and microsomes exhibit about equal activities[12] (Appendix, Paper II). The distribution between mitochondria and microsomes is organ-specific and linked with the function of the various organs[13]. The high activity of long-chain fatty acid activation reported to be present in the plasma membrane of the rat-liver cell[11] could not be found, using the estimation method described in ref. 12 (Ap-

pendix, Paper II). Recently LIPPEL *et al.*[14], measuring palmitoyl-hydroxamate formation, also could not detect any significant activity in the plasma-membrane fraction. In studying the intracellular localization of ATP-dependent palmitoyl-CoA synthetase, good recoveries are obtained after separation of the tissue homogenate into the subcellular fractions[12] (Appendix, Paper II). The activity of fatty acid activation measured in this study is higher than published before, especially in the mitochondria. For these high activities the addition of a protein factor, described by FARSTAD *et al.*[10] as occurring in the particle-free supernatant, to the particulate ATP-dependent long-chain acyl-CoA synthetase was not required (see also ref. 15).

From studies on the intramitochondrial localization of ATP-dependent palmitoyl-CoA synthetase it can be concluded that the bulk of the mitochondrial palmitate activation is located in the mitochondrial outer membrane[16-18] (see Appendix, Paper IV). In addition it is clearly demonstrated that some activity also resides in the inner membrane-matrix compartment of rat-liver mitochondria[18] (Appendix, Paper IV). In this study the destructive effect of Nagarse (subtilopeptidase A), described by DE JONG AND HÜLSMANN[13] and by PANDE AND BLANCHAER[19], was used as a tool in enzyme localization. When a rat-liver homogenate is treated with Nagarse some enzymes are destroyed, while others are not. The sensitivity of a certain enzyme to this treatment probably depends in the first place on the accessibility to Nagarse of the enzyme. Thus, monoamine oxidase, localized on the inside of the mitochondrial outer membrane[20] (which is impermeable to macromolecules[21]), is not sensitive to Nagarse treatment[13,19]. Other enzymes, localized in the inner membrane – matrix compartment of intact mitochondria, such as cytochrome *c* oxidase[13,18], mitochondrial carnitine palmitoyltransferase[19] (see also Table I) and one of the ATP-dependent palmitate activating enzymes[18] are also Nagarse-resistent, because of their inaccessibility to Nagarse. The acyl-CoA synthetase located in the outer mitochondrial membrane, however, is very sensitive to the Nagarse treatment[18] (Appendix, Paper IV). It has been suggested therefore, that this enzyme is located on the outside of the mitochondrial outer membrane[19]. Another enzyme localized in the mitochondrial outer membrane, rotenone-insensitive NADH-cytochrome *c* reductase, was found to be partially sensitive[18]. Of the microsomal

TABLE I

INFLUENCE OF NAGARSE TREATMENT AND FASTING (48 h) ON CARNITINE PALMITOYLTRANSFERASE ACTIVITY IN SUBCELLULAR FRACTIONS FROM RAT LIVER

The enzyme activity was measured using the CoA-dependent isotope-exchange method described by NORUM[125]. For the isolation of the subcellular fractions M (mitochondria) and P (microsomes) see ref. 13. S refers to the final supernatant, after centrifuging down the microsomes. Where indicated, the 10% rat-liver homogenate, in 0.25 M sucrose-0.01 M Tris-HCl (pH 7.4), was stirred with 0.2 mg/ml Nagarse (subtilopeptidase A) for 30 min at 0°, before the subcellular fractions were isolated. Mitochondria and microsomes were in contact with Nagarse for the same period of time (90 min). For further details of the enzyme assay see ref. 12 (Appendix, Paper II). The enzyme activity is given as butanol-extractable dpm/mg protein/min.

Fraction	Palmitoyl-[^{3}H]-carnitine formation			
	Control rats		Fasted rats	
	- Nagarse	+ Nagarse	- Nagarse	+ Nagarse
M	2322	2105	1815	1535
P	84	40	325	75
S	30	20	33	23

enzymes tested so far, palmitoyl-CoA synthetase[13] and the microsomal carnitine palmitoyltransferase (Table I) are sensitive to Nagarse treatment, whereas glucose-6-phosphatase and carboxylesterase are not[13]. In this connection it may be of interest to note that SABATINI *et al.*[22] have shown that glucose-6-phosphatase is localized at the inside of the endoplasmic reticulum. TAKESUE AND OMURA[23] have shown that (microsomal) NADPH-cytochrome *c* reductase is solubilized during Nagarse treatment. After centrifugation of the microsomal particles, the enzyme activity remains in the supernatant. This may indicate that the accessibility of the different microsomal enzymes to Nagarse is not the same. Another possibility is that some enzymes, because of their specific protein structure or protein-membrane interaction, are less sensitive to Nagarse than others. Although it has been reported that Nagarse is an unspecific proteolytic enzyme[24], it can be concluded from the work discussed above that some enzymes (*e.g.* NADPH-cytochrome *c* raductase) are

not attacked by Nagarse, not even after solubilization. This suggests very strongly that protein structure is also an important factor.

By comparing the enzymic properties of untreated mitochondria with Nagarse-treated rat-liver mitochondria it is possible to study differences between the ATP-dependent palmitate activating enzymes localized in the outer membrane and in the inner membrane-matrix compartment, respectively. It was found that the enzymes differ significantly with respect to their K_m's for palmitate and ATP and with respect to their K_i's for AMP and adenosine. These parameters are also compared with those of the microsomal ATP-dependent long-chain acyl-CoA synthetase (see also refs. 11 and 25). The inhibitions by AMP and adenosine are both competitive with respect to ATP. It appears that the enzyme localized in the inner-membrane matrix compartment can activate fatty acids even when the ATP/AMP ratio is relatively low[18] (Appendix, Paper IV). There is evidence that this ratio can be low in the matrix space of rat-liver mitochondria during fatty acid oxidation[26-28]. The enzyme localized in the mitochondrial outer membrane is likely to be inhibited if the extramitochondrial ATP/AMP ratio is low. The dual localization described above provides an explanation for the carnitine-independent long-chain fatty acid oxidation by isolated rat-liver mitochondria[18,29,30]. By comparison of the kinetics of fatty acid activation and oxidation it is concluded that, in the absence of carnitine, the long-chain fatty acids oxidized are activated by the ATP-dependent long-chain acyl-CoA synthetase localized in the inner membrane-matrix compartment. In the presence of carnitine both fatty acid-activating systems may contribute to fatty acid activation, necessary for oxidation[18] (Appendix, Paper IV). From the observation that the inner membrane-matrix enzyme is inhibited by octanoate in a non-competitive way with respect to palmitate[18], it can be concluded that the enzyme differs from the short- and medium-chain ATP-dependent fatty acid activating enzymes, reported by AAS AND BREMER[31] to be localized in the matrix of rat-liver mitochondria. It appears therefore that the inner membrane-matrix compartment contains three different ATP-dependent acyl-CoA synthetases: a short-, a medium-, and a long-chain acyl-CoA synthetase.

In addition to fatty acid activation for oxidative purposes, the different enzymes probably activate fatty acids for synthetic pathways. Chain-elongation of long-chain fatty acids has been described

in microsomes[32] and in both mitochondrial outer[33] and inner membranes[33,34]. The acylation of α-glycerophosphate occurs in the microsomes[35,36] and in the mitochondrial outer membrane[36,37], with only minor, if any, activity in the inner membrane-matrix fraction. Acylation of lysophosphatidic acid takes place in mitochondria[38,39], microsomes[39,40] and the soluble fraction of the rat-liver cell[41]. Intramitochondrially there is a difference in the predominant end product of acylation between the outer and inner membranes. NACHBAUR *et al*[42] found that incorporation into phosphatidylethanolamine is more active than into phosphatidylcholine. The specificity of the reacylation systems for phosphatidylethanolamine is more striking in the inner than in the outer membrane of mitochondria. Phospholipase activity (at pH 8.0), which is necessary for the incorporation of fatty acids into phospholipids under these conditions, is highest in the outer membrane.

ROSSI AND GIBSON[43] have reported the existence of a GTP-dependent fatty acid-activating enzyme in liver mitochondria, catalyzing reaction (2)

$$\text{RCOOH} + \text{GTP} + \text{CoA} \rightleftharpoons \text{RCOCoA} + \text{GDP} + \text{P}_\text{i} \qquad (2)$$

This enzyme has been purified from rat-liver mitochondria[44], and it has been shown to activate fatty acids of various chain lengths. The K_m for long-chain fatty acid of the purified enzyme is extremely high (2-3 mM) and the enzyme is strongly inhibited by P_i and fluoride. Lecithin and 4'-phosphopantetheine are required for maximal activity[45]. Because of the very high K_m for palmitate and the inhibition by low concentrations of inorganic phosphate it is not likely that this enzyme plays an important role in fatty acid activation. Although direct localization studies have never been carried out, it is assumed that the enzyme is localized in the inner membrane-matrix compartment of the mitochondrion[46]. It has been suggested that this synthetase could operate in the reverse direction *e.g.* by reducing the concentration of acyl-CoA and increasing the mitochondrial CoA pool[44]. Its possible involvement in the synthesis of GTP for intramitochondrial reactions may be mentioned. Although a preferential inactivation of the GTP-dependent acyl-CoA synthetase cannot be totally excluded (*cf.* ref. 45), it is clear from the experiments described in ref. 47 (Appendix, Paper I) that, because of the very high

activity of the ATP-dependent acyl-CoA synthetase, the GTP-dependent acyl-CoA synthetase does not contribute significantly to total mitochondrial fatty acid activation from a quantitative point of view. It will be shown below that the inhibition of fatty acid oxidation by P_i and fluoride, in the presence of DNP, does not necessarily imply the operation of the GTP-dependent fatty acid activation.

Influence of inorganic phosphate on the operation of the citric acid cycle

From studies on the oxidation of β-hydroxybutyrate and acetoacetate by heart sarcosomes it became evident that P_i in the presence of DNP inhibits the oxidation of these substrates[48,49] by stimulation of reaction (3):

$$\text{Succinyl-CoA} + \text{GDP} + P_i \rightleftharpoons \text{succinate} + \text{GTP} + \text{CoA} \quad (3)$$

Succinyl-CoA is necessary for the activation of acetoacetate to acetoacetyl-CoA, prior to oxidation. In the presence of DNP, the addition of P_i causes a drop in the concentration of succinyl-CoA[49].

Reaction (3), however, is also thought to be necessary to provide GTP (and ATP[50]) for fatty acid activation in the presence of DNP in both liver and heart. Yet inhibition by P_i of fatty acid oxidation by rat-liver mitochondria in the presence of DNP has been observed[44,47,51,52]. This phenomenon has been considered to be "diagnostic" for the operation of the GTP-dependent fatty acid-activating enzyme described above[44,51,52]. However, it was found that the oxidation of pyruvate or palmitoylcarnitine, substrates which do not need activation prior to oxidation, are also inhibited by P_i in the presence of DNP[47] (Appendix, Paper I). This inhibition can be relieved by malate. If the oxidation is carried out in the presence of malate no inhibition by P_i is observed. In Table II the results of a manometric experiment are given. Fluoride inhibits the oxidation of pyruvate to the same extent as P_i. The additions of both malate and P_i, however, are necessary to overcome the inhibition by fluoride completely. In the absence of added P_i, fluoride causes an accumulation of α-oxoglutarate (formed from added malate). It is concluded that fluoride inhibits the citric acid cycle by inhibition of the oxidation of α-oxoglutarate, probably by removing P_i necessary for reaction (3) (*cf.* refs. 55 and 56). The inhibition of the citric acid cycle by P_i and

TABLE II

INHIBITION OF PYRUVATE OXIDATION IN INTACT RAT-LIVER MITOCHONDRIA

Oxygen uptake was measured with differential manometers. The reaction medium contained 25 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 40 mM Tris-HCl, 75 mM sucrose, 0.1 mM DNP, 10 mM pyruvate, 7.5 mg of mitochondrial protein and, where indicated, 10 mM potassium-(L)-malate, 15 mM potassium fluoride or 15 mM P. The centre wells of the manometer vessels were provided with KOH and a filter paper. The reaction volume was 1 ml, the temperature 25° and the pH 7.5; readings were taken at regular intervals. After 53 min of incubation the reactions were stopped with perchloric acid (final concn. 4%); After centrifugation the supernatants were neutralized with KOH. $KClO_4$ was removed by centrifugation in the cold. Acetoacetate and α-oxoglutarate were estimated as described by MELLANBY AND WILLIAMSON[53] and BERGMEYER AND BERNT[54], respectively.

Additions	without L-malate		10 mM L-malate	
	oxygen uptake*) (μAt)	α-oxoglutarate accumulation (μmoles)	oxygen uptake*) (μAt)	α-oxoglutarate accumulation (μmoles)
none	13	0.1	17	0.2
15 mM P_i	5	0.1	18	0.2
15 mM KF	4	0.1	12	1.1
15 mM KF + 15 mM P_i	5	0.0	19	0.2

*) The oxygen uptake (in μ atoms) was corrected for the amount of acetoacetate formed (2 μatoms O for each μmole of acetoacetate), so that the oxygen uptake becomes a more direct measure for the contribution of the citric acid cycle in pyruvate oxidation.

fluoride causes a decrease in GTP (and ATP) production *via* substrate-level phosphorylation, therefore limiting the energy required for fatty acid activation. In addition, the oxygen uptake is decreased because the citric acid cycle cannot operate at full capacity. DE JONG *et al.*[57] found that P_i exerts its action under these conditions by causing a loss of citrate and malate from the mitochondria, probably by the exchange mechanisms described by CHAPPELL AND HAARHOF[58] and by MEYER *et al.*[59].

The use of glucose-6-phosphatase and NADPH-cytochrome *c* reductase as marker enzymes in the study of intramitochondrial enzyme localization

Mitochondria contain two membranes, an outer and a folded

inner membrane, giving rise to the cristae[60,61]. The methods used for the subfractionation of mitochondria are the following: swelling in hypotonic media[62-64], sonication[65-67], freezing and thawing[67], and treatments with digitonin[68-70], organic solvents[71], detergents[72] and phospholipase[66,71,72]. After these treatments the outer and inner membranes are separated by density-gradient centrifugation or by differential centrifugation. A modification of the method of PARSONS *et al.*[62,73] consists of the following procedure. After swelling of the mitochondria in 20 mM phosphate buffer the membranes are separated in a continuous sucrose density gradient[12] (Appendix, Paper II). This method yields pure outer membranes, while the inner membrane remains intact. The soluble matrix enzymes are not released form the inner membrane-matrix compartment[74] (Appendix, Paper III).

Whatever method is used for the separation of outer and inner mitochondrial membranes, it is very important to keep in mind that the starting material, in most cases washed mitochondria, may be contaminated with other subcellular fractions. Consideration has been given for example to contamination with microsomes[74,75] and with lysosomes[76]. The identification of the separately isolated membranes has been based on morphological criteria (see, *e.g.*, ref. 73). It was subsequently found that the isolated membranes contain characteristic enzyme activities. The use of "marker" enzymes to identify subcellular fractions has proved to be a fast and in most cases reliable tool in studies of enzyme localization. In some cases histochemical methods at the electron-microscopical level were developed to study enzyme localization. With this technique it was shown that monoamine oxidase is probably localized on the inside of the mitochondrial outer membrane[20]. This is in agreement with the observed insensitivity for Nagarse treatment[13,19], which has been mentioned earlier.

From ref. 74 (Appendix, Paper III) it is evident that it is not always justified to conclude from the observed specific activity of a microsomal enzyme in isolated mitochondrial outer membranes that this microsomal enzyme is also a true constituent of the outer membrane. If mitochondria are isolated by differential centrifugation, they still contain significant amounts of microsomal enzymes[12,62,74]. From the data given in refs. 12 and 74 (Appendix, Papers II and III) it can be calculated, using the method described by

HULSMANS[77], that 11% of the protein of the twice-washed mitochondrial fraction is microsomal protein (if glucose-6-phosphatase and cytochrome *c* oxidase are used as marker enzymes for the microsomes and mitochondria respectively). In the gradient used for separation of the outer and inner mitochondrial membranes, the contaminating microsomal membranes do not give the same pattern of sedimentation as the mitochondrial outer membranes. It is concluded that at least the bulk of the glucose-6-phosphatase and the NADPH-cytochrome *c* reductase activity found in isolated mitochondria is derived from contaminating microsomal membranes and not from mitochondrial outer membranes (*cf.* ref. 78). The increase in specific activity of the microsomal enzymes in isolated mitochondrial outer membranes, when compared with isolated intact mitochondria[78], can be explained by the observation that mitochondrial outer membranes and microsomal membranes have about the same density[74] (Appendix, Paper III). The observation that different microsomal markers behave differently in sucrose density gradient centrifugation can be explained by the heterogenous distribution of these markers between microsomal subfractions[74,79-81] (see Appendix, Paper III). If a 900 x g_{max} supernatant of a rat-liver homogenate is centrifuged in a continuous sucrose density gradient, about 40% of the glucose-6-phosphatase activity sediments together with the mitochondria[12] (Appendix, Paper II). About 20% of the glucose-6-phosphatase activity of a 27000 x g_{max} supernatant of a rat-liver homogenate (not containing any mitochondria), centrifuged in the same way, also sediments at the same density as isolated mitochondria[74]. This explains why isolated mitochondria are contaminated rather specifically with glucose-6-phosphatase, when compared with other microsomal enzymes, *e.g.* NADPH-cytochrome *c* reductase (Appendix, Paper III). It is concluded that glucose-6-phosphatase and NADPH-cytochrome *c* reductase can be used as microsomal marker enzymes in studies on intramitochondrial enzyme localization.

Carnitine palmitoyltransferase and the effect of carnitine on long-chain fatty acid oxidation

FRITZ[82,83] showed that carnitine stimulates the oxidation of fatty acids. The enzyme involved in this stimulation (carnitine palmitoyltransferase) was discovered simultaneously by BREMER[84] and by FRITZ AND YUE[85]. Carnitine palmitoyltransferase catalyzes the

reversible reaction (4)

$$\text{Palmitoyl-CoA} + \text{carnitine} \rightleftharpoons \text{Palmitoylcarnitine} + \text{CoA} \quad (4)$$

By studying the kinetics of the enzyme solubilized from calf-liver mitochondria, BREMER AND NORUM[86] found that palmitoyl-CoA is not only a substrate, but also a strong inhibitor of the reaction running from left to right. This inhibition is competitive with respect to the second substrate carnitine. In addition they found that this competitive inhibition is prevented by other detergents (including fatty acids[87]).

The enzyme from rat liver shows the same kinetics when tested in intact mitochondria, mitochondrial particles, or in the soluble fraction of sonicated mitochondria. However, the expected inhibition of palmitoyl-CoA oxidation (carnitine present) at high palmitoyl-CoA concentrations was not be observed by BREMER AND NORUM[88] in intact mitochondria. Fig. 1 shows an experiment in which the oxidations of palmitoyl-CoA and of palmitoylcarnitine are compared at different substrate concentrations. It can be seen that the oxidation of palmitoyl-CoA is inhibited at high substrate concentrations, whereas the oxidation of palmitoylcarnitine is not. The discrepancy with the observations of BREMER AND NORUM[88] is probably due to the difference in oxidation conditions. Under their conditions palmitoyl-CoA is converted to β-hydroxybutyrate and acetoacetate (malonate is present), while in the experiment shown in Fig. 1 palmitoyl-CoA is oxidized at least partly to CO_2 (ADP, P_i and malate are present[89,90]); see also ref. 91; Appendix, Paper V). Therefore, under the latter conditions, palmitoyl-CoA may inhibit not only carnitine palmitoyltransferase, but also some reaction of the citric acid cycle. Indeed, reports are found in the literature that palmitoyl-CoA can inhibit isolated citrate synthase[92,93], although this may not occur *in vivo* (see *e.g.* refs. 94 and 95). Alternatively, palmitoyl-CoA could inhibit the citric acid cycle activity by altering mitochondrial structure through its detergent action. A preliminary experiment showed that isolated rat-liver mitochondria swell more readily when palmitoyl-CoA is used instead of an equivalent amount of palmitoylcarnitine (not shown). The oxidation of pyruvate or palmitoylcarnitine is also inhibited by palmitoyl-CoA under the conditions used in the experiments illustrated in Fig. 1 (not shown). Therefore,

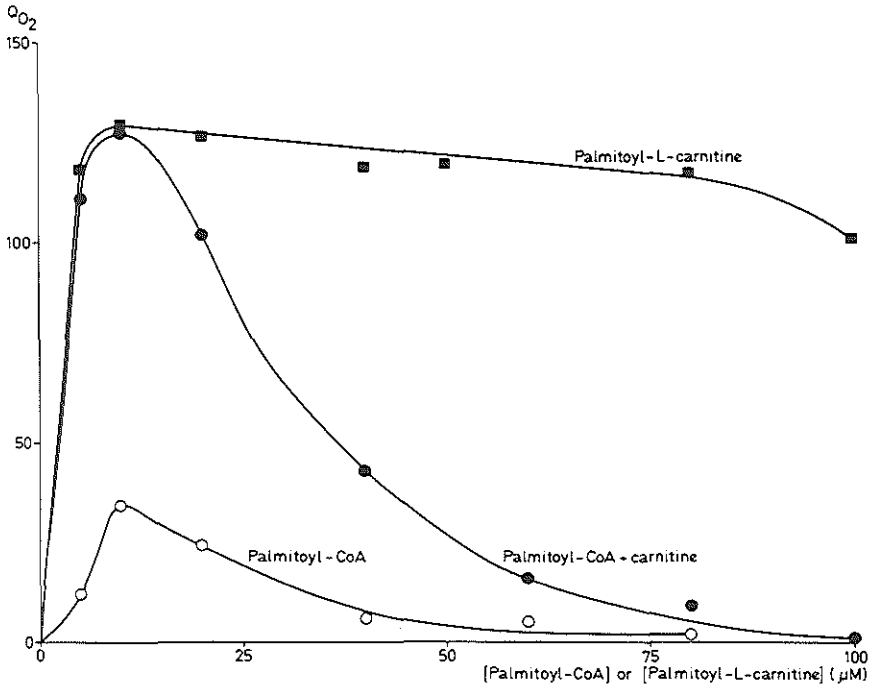

Fig. 1. Palmitoyl-CoA and palmitoyl-L-carnitine oxidation by isolated rat-liver mitochondria at different substrate concentrations. Incubations were carried out with 3.1 mg of mitochondrial protein, under state 3 conditions in the presence of added malate. For details of the incubation conditions see ref. 18 (Appendix, Paper IV). Where indicated L-carnitine was added (final concentration 0.5 mM). The oxygen uptake (Q_{O_2}) was measured with the "Clark" oxygen electrode and is given as μl O_2/mg protein/h. The Q_{O_2}'s are corrected for the oxygen uptake in the absence of added substrate.

accumulation of acylcarnitine is less harmful to mitochondria than the accumulation of acyl-CoA.

A carnitine acetyltransferase, specific for short-chain acyl-CoA or acylcarnitine, has been crystallized from pigeon liver[96]. Recently, evidence has been presented by BRDZICKA *et al.*[97] that this enzyme has a dual localization in mitochondria isolated from rat liver and pig kidney. Because of the rather confusing literature on the localization of carnitine palmitoyltransferase[84,98,99] a reinvestigation of the subcellular localization in rat liver seemed worthwhile. It was shown that the enzyme has a dual localization, since it is present in both the mitochondrial inner membrane-matrix compartment and

the microsomes[12] (Appendix, Paper II). Because the reaction catalyzed by carnitine palmitoyltransferase is shown to be readily reversible[86], it is concluded that long-chain acylcarnitine can be formed and used both inside and outside the mitochondria. In agreement with NORUM *et al*[100] it was found that the mitochondrial enzyme is recovered almost quantitatively in the inner membrane-matrix fraction after the separation of the mitochondrial outer membrane from the inner membrane-matrix. Recently this finding was confirmed by HADDOCK *et al.*[101].

From experiments on the effect of fasting on the activity of carnitine palmitoyltransferase it can be concluded that the microsomal enzyme is activated after a 48 h fast, while the mitochondrial enzyme is not [Tables I and III; compare also ref. 12 (Appendix, Paper II) and ref. 98]. NORUM[98] found that carnitine palmitoyltransferase is activated, especially in the extramitochondrial compartment, in livers from fasted, diabetic and fat-fed rats. Table III shows

TABLE III

EFFECT OF FASTING (48 h) AND THYROXINE TREATMENT ON CARNITINE PALMITOYLTRANSFERASE ACTIVITY IN FRACTIONS FROM RAT LIVER

The enzyme activity was assayed using the CoA-dependent isotope-exchange reaction described by NORUM[125]. Where indicated the animals were given daily intraperitoneally injections of 150 μg of thyroxine/100 g body weight for 14 days. For the isolation of the subcellular fractions and further details see ref. 12 (Appendix, Paper II). The enzyme activity is given as butanol-extractable dpm/amount of supernatant corresponding to 1 g of liver/min.

Fraction	Palmitoyl-[^{3}H]-carnitine formation			
	Control rats	Fasted rats	Thyroxine treated rats	
			Expt. I	Expt. II
900 x g max supernatant	29850	49817	48947	53792
12000 x g max supernatant	2520	10468	10273	10373

that the same is true for hyperthyroid rats. NORUM[98] concluded that the activation of the extramitochondrial carnitine palmitoyltransferase after fasting and fat-feeding or in diabetes is independent of *de novo* protein synthesis. If this conclusion is correct, the activation may be exerted by fatty acids[87]. It is known that the fatty acid concentration in the plasma, and therefore also in the liver, is increased under the conditions mentioned[102-104]. As increased levels of plasma free fatty acids give rise to increased fatty acid oxidation and ketogenesis, it can be concluded that the activity of microsomal carnitine palmitoyltransferase correlates with the rate of fatty acid oxidation and/or ketogenesis.

The concept that carnitine palmitoyltransferase plays a role in fatty acid oxidation is generally accepted[105,106]. Acyl-CoA cannot penetrate the mitochondrial inner membrane[46], so that acyl-CoA formed outside this membrane has to be converted to acylcarnitine, which can penetrate the inner mitochondrial membrane. This conversion is catalyzed by the carnitine acyltransferases[106-109]. Before oxidation of the acyl moiety, acylcarnitine has to be converted back to acyl-CoA, because it is only in this form that activated fatty acids can be oxidized by the β-oxidation system. YATES AND GARLAND[110] found that the carnitine palmitoyltransferase is partially latent to the substrate palmitoyl-CoA and concluded that there are two carnitine palmitoyltransferases in rat-liver mitochondria; one on each side of the barrier for acyl-CoA (the "carnitine" barrier). Also in further studies, which make use of the inhibitor 2-bromostearoyl-CoA[111], evidence is given in favour of a dual localization of carnitine palmitoyltransferase in rat-liver mitochondria[111-113]. The enzyme supposed to be located outside the "carnitine barrier" (transferase I) has about 20% of the activity of the enzyme localized inside this barrier (transferase II). Therefore transferase I may be rate limiting for palmitoyl transfer. The reported differences between the transferases I and II with respect to the K_m's for carnitine[110,112] could not be found by BREMER AND NORUM[88].

It is tempting to correlate the microsomal carnitine palmitoyltransferase with the formation of acylcarnitine from acyl-CoA (transferase I) and the mitochondrial inner membrane-matrix enzyme with the reformation of acyl-CoA from acylcarnitine (transferase II). As was calculated above, isolated mitochondria contain a considerable amount of microsomal protein, so that it can be expected that a cer-

tain amount of microsomal carnitine palmitoyltransferase is present in isolated mitochondria. Whether the "external" transferase proposed by GARLAND *et al.*[112] is identical with the microsomal transferase[12] (Appendix, Paper II) cannot be concluded at the present time. Further experiments with Nagarse could provide some information since the microsomal activity, increased by fasting, is largely sensitive to Nagarse (see Table I). It is clear however that, if not identical, these two carnitine palmitoyltransferases are localized in the same functional compartment of the rat-liver cell and therefore can cooperate in the formation of acylcarnitine from acyl-CoA. The rate of oxidation of palmitoyl-CoA in the presence of carnitine is proportional to the palmitoylcarnitine level, as long as this level is low[88]. Despite the observation that, in isolated mitochondria, the capacity for palmitoylcarnitine formation is much higher than the capacity for palmitoylcarnitine oxidation[12,88], it is quite possible that in the intact cell, because of the high intracellular protein concentration, the effective palmitoyl-CoA concentration is so low that carnitine palmitoyltransferase cannot operate at maximal capacity, as it can in isolated mitochondria. In that case the microsomal carnitine palmitoyltransferase could contribute to carnitine-dependent fatty acid oxidation in the intact rat-liver cell. Under conditions of enhanced fatty acid oxidation elevated levels of both acyl-CoA and acylcarnitine are found in rat liver[114-117]. Microsomal long-chain acyl-CoA synthetase is inhibited strongly by palmitoyl-CoA[118]. Therefore another possible function of the microsomal carnitine palmitoyltransferase could be the prevention of excessive accumulation of long-chain acyl-CoA under conditions of fatty acid mobilization, when triglyceride (lipoprotein) synthesis and therefore fatty acid activation, must proceed rapidly.

The solubilized and purified carnitine palmitoyltransferase from calf-liver mitochondria is not sensitive to Nagarse treatment (Drs. J.W. DE JONG, personal communication). It was investigated whether differences between the mitochondrial and the microsomal enzymes exist with respect to Nagarse sensitivity. Mitochondria and microsomes from rat liver were treated with Nagarse under comparable conditions. From Table I it can be seen that the microsomal carnitine palmitoyltransferase is sensitive to Nagarse treatment, whereas the mitochondrial enzyme is not. It is also shown that fasting increases the specific activity in the microsomal fraction but not in the

mitochondrial fraction. The experiments shown in this Table give additional evidence for the existence of a microsomal carnitine palmitoyltransferase, which is increased after a 48-h fast (*cf.* ref. 12, Appendix, Paper II).

Regulation of fatty acid oxidation and ketogenesis

In ref. 18 (Appendix, Paper IV) conditions are given under which the oxidation of palmitate and oleate by isolated rat-liver mitochondria gives an optimal rate of oxygen uptake in the absence of added carnitine and CoA. Under these conditions (state 3 oxidation in the presence of malate), fatty acids are oxidized almost completely to CO_2 (*cf.* refs. 89 and 90). However, in the absence of added ADP and in the presence of ATP, carnitine and CoA, most of the fatty acid molecules are converted to ketone bodies[91] (Appendix, Paper V), even in the presence of added malate. As can be seen from reactions (5) – (7) much less O_2 is consumed and much less ATP is produced (per molecule of palmitate metabolized) during ketogenesis than during the complete oxidation of fatty acids to CO_2, or that a high rate of O_2 uptake

$$1 \text{ palmitic acid} + 23\ O_2 + 129 \text{ ADP} + 129\ P_i \longrightarrow 16\ CO_2 + 129 \text{ ATP} + 145\ H_2O \quad (5)$$

$$1 \text{ palmitic acid} + 7\ O_2 + 33 \text{ ADP} + 33\ P_i \longrightarrow 4 \text{ acetoacetate} + 33 \text{ ATP} + 37\ H_2O \quad (6)$$

$$1 \text{ palmitic acid} + 5\ O_2 + 21 \text{ ADP} + 21\ P_i \longrightarrow 4\ \beta\text{-OH-butyrate} + 21 \text{ ATP} + 21\ H_2O \quad (7)$$

during ketogenesis requires a higher rate of acyl-CoA formation than a similar rate of respiration during the complete oxidation to CO_2. Since the capacity of the ATP-dependent palmitoyl-CoA synthetase localized in the inner membrane-matrix compartment is limited, a contribution from the outer-membrane palmitoyl-CoA synthetase is required during ketogenesis. This enzyme can react rapidly only if CoA is added (*cf.* ref. 119), and if the transport of activated fatty

acid (acylcarnitine) from the site of activation to the site of β-oxidation is sufficiently rapid. It can, therefore, be understood that carnitine and CoA are absolutely required for optimal rates of ketogenesis, whereas CO_2 production can proceed at an optimal rate in the absence of carnitine and added CoA[91] (Appendix, Paper V). In fact it has been found that ketogenesis is strongly stimulated and CO_2 production is strongly inhibited by the addition of carnitine *plus* CoA, both in the presence or absence of malate[91] (Appendix, Paper V). The rate of ketogenesis correlates very closely with the observed β-hydroxybutyrate/acetoacetate ratio. As this ratio is a measure of the intramitochondrial NADH/NAD^+ ratio[120], it is clear that carnitine *plus* CoA, by increasing the rate of fatty acid oxidation, increases the intramitochondrial NADH/NAD^+ ratio and therefore decreases the intramitochondrial oxaloacetate concentration. This results in a decreased citrate synthesis and therefore inhibition of CO_2 production by way of the citric acid cycle[91] (Appendix, Paper V). In addition, it is quite possible that the high NADH/NAD^+ ratio results in a direct inhibition of the isocitrate dehydrogenase reaction[121]. The inhibition of the citric acid cycle activity will contribute to the rise in the acetyl-CoA concentration, allowing rapid ketogenesis.

Like all oxidative processes, fatty acid oxidation is controlled primarily by the availability of ADP and/or P_i. In this context a rather unique property of long-chain fatty acid ("free" as well as "activated") may be mentioned. In contrast to other substrates these fatty acids are able to induce ATPase activity when added to isolated mitochondria[55,122,123]. With respect to the degree of uncoupling, unsaturated fatty acids are much more active than saturated fatty acids[124,126]. It seems possible therefore that the control of fatty acid oxidation by ADP and P_i is abolished at very high fatty acid concentrations. This mechanism may play a role in thermogenesis by brown adipose tissue[127-129]. However, when an isolated rat liver is perfused with oleate no such ATPase is operating[130].

As was pointed out in Chapter I, the release of fatty acids from adipose tissue and the hydrolysis of triglycerides in various other tissues is controlled by hormones. These hormones, by determining the amount of fatty acid presented to the liver, probably play a major role in the regulation of fatty acid oxidation.

In addition to the fatty acid concentration, the extramitochon-

drial ATP/AMP ratio and the extramitochondrial concentrations of CoA and carnitine could influence the rate of fatty acid oxidation and especially of ketogenesis, by influencing the activity of the acyl-CoA synthetase localized in the outer mitochondrial membrane and the activity of carnitine palmitoyltransferase[18,91] (Appendix, Papers IV and V).

From several lines of evidence it is clear that the tissue contents of CoA and carnitine are important factors in the regulation of fatty acid oxidation. After administration of hypoglycin (L-α-amino-β-methylenecyclopropanepropionic acid) or 4-pentenoic acid the tissue becomes depleted of CoA and carnitine, because of the accumulation of CoA and carnitine derivatives of metabolites of these agents (which cannot be oxidized). This leads to subsequent impairment of processes catalyzed by CoA and/or carnitine. The impaired rate of long-chain fatty acid oxidation is restored to normal after the addition of carnitine and CoA[131-134]. Under other conditions of carnitine deficiency, *e.g.* in the newborn rat[132], in rats treated with diphtheria toxin[135-137] and in choline-deficient rats[138], the rate of oxidation of long-chain fatty acids is impaired, whereas that of short-chain fatty acids, which are oxidized independent of carnitine, is normal. In most of these cases there is an intracellular accumulation of triglycerides, that can be cured by the addition of exogenous carnitine. It may be of interest to note that in livers of fed rats about 25% of the total carnitine is in the form of propionylcarnitine[139]. If propionylcarnitine is in equilibrium with propionyl-CoA through the carnitine acetyltransferase reaction[97], both CoA and carnitine are partially not available for CoA- and carnitine-requiring processes. Therefore it is possible that in fed rats the conditions for carnitine-dependent fatty acid oxidation and especially for ketogenesis are not optimal (*cf.* ref. 91; Appendix, Paper V). The opposite is true for fasted rat liver. Propionylcarnitine (propionyl-CoA) is virtually absent[139] and therefore more carnitine (CoA) is available for carnitine-dependent fatty acid oxidation and ketogenesis (*cf.* ref. 91; Appendix, Paper V). Moreover, during fasting the extramitochondrial ATP/AMP ratio is probably high (in this state the activity of fructose 1,6-diphosphatase, which is inhibited by AMP[140,141], is high). These factors make it possible for the palmitoyl-CoA synthetase in the outer mitochondrial membrane to operate. Because of the high activity of this enzyme sufficient fatty acids can be activated, even

for rapid ketogenesis. Long-chain fatty acid oxidation will be carnitine-dependent under these conditions.

It should be noted that the extramitochondrial carnitine palmitoyltransferase, possibly one of the rate-limiting enzymes in ketogenesis, is activated in fasted rat liver. This reaction is the only one of the initial reactions of fatty acid oxidation of which the activity is enhanced after a 48-h fast. The mitochondrial palmitoyl-CoA synthetase, of which the bulk is localized in the outer membrane, is not activated (unpublished observation) and neither is the mitochondrial carnitine palmitoyltransferase (Table I).

It is likely then, that, in hepatic fatty acid oxidation, metabolic control is mediated primarily by variation of the concentration of the substrates fatty acid, CoA and carnitine. The long-chain fatty acid concentration may regulate directly the activity of the carnitine palmitoyltransferase localized in the microsomes (see ref. 87).

The function of the very active ATP-dependent long-chain acyl-CoA synthetases, localized in the outer mitochondrial membrane and in the microsomes, is clear, since high activities of fatty acid activation are required during rapid fatty acid mobilization, which leads to ketosis and lipoprotein synthesis. It is not known at the present time what can be the physiological significance of an additional, relatively inactive, long-chain acyl-CoA synthetase localized inside the "carnitine barrier". It may be speculated that under certain physiological conditions the outer-membrane enzyme is inhibited, *e.g.* if the ATP/AMP ratio or the ATP/adenosine ratio is extremely low. Evidence has been presented that the transport of activated fatty acids into the mitochondrial inner membrane by way of the carnitine palmitoyltransferase reaction cannot operate at optimal rates in fetal and neonatal rats, because carnitine and carnitine palmitoyltransferase activity are practically absent[132,142]. Under these conditions it is still possible to activate a limited amount of fatty acid for oxidative and for synthetic reactions in the inner membrane-matrix compartment.

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SUMMARY

1. ATP-dependent long-chain acyl-CoA synthetase has a very high activity in isolated rat-liver mitochondria, ranging from 70-130 nmoles/mg protein/min at 37°.
2. The intracellular localization of ATP-dependent long-chain acyl-CoA synthetase in rat liver is bimodal: mitochondrial and microsomal. The activity in these particles is about equal.
3. The intramitochondrial localization of ATP-dependent long-chain acyl-CoA synthetase in rat liver is bimodal; about 90% of the activity is located in the mitochondrial outer membrane and about 10% in the inner membrane-matrix compartment.
4. The two mitochondrial fatty acid-activating enzymes differ with respect to their K_m's for fatty acid and ATP and with respect to their K_i's for AMP and adenosine. These parameters are compared with those of the microsomal long-chain fatty acid-activating enzyme. The inhibitions by AMP and adenosine are of the competitive type with respect to ATP. The inner membrane-matrix enzyme, in contrast to the outer-membrane and microsomal enzymes, is strongly inhibited by octanoate. This inhibition is non-competitive with respect to palmitate.
5. Comparison of the kinetics of the two mitochondrial ATP-dependent long-chain fatty acid-activating enzymes with the kinetics of long-chain fatty acid oxidation shows, that during fatty acid oxidation at low concentrations of palmitate or oleate, in the presence of carnitine, the outer-membrane acyl-CoA synthetase is operating. In the absence of carnitine and at relatively high concentrations of long-chain fatty acid, the fatty acids oxidized are activated in the inner membrane-matrix compartment of the mitochondrion.

6. The intracellular localization of carnitine palmitoyltransferase is bimodal; the enzyme activity resides both in the mitochondrial inner membrane-matrix compartment and in the microsomal fraction of the rat-liver cell. Fasting increases only the microsomal enzyme activity. Thyroxine treatment also increases carnitine palmitoyltransferase activity, especially in the extramitochondrial compartment.
7. Glucose-6-phosphatase and NADPH-cytochrome *c* reductase are distributed heterogeneously over microsomal subfractions and can both be used as microsomal marker enzymes. Isolated rat-liver mitochondria are contaminated with microsomes, with a relatively high glucose-6-phosphatase activity.
8. The oxidation of palmitate, palmitoylcarnitine or pyruvate by isolated rat-liver mitochondria, in the presence of dinitrophenol, is inhibited by inorganic phosphate. Because this inhibition can be relieved by malate, it is concluded that the inhibition occurs at the level of the citric acid cycle. Fluoride also inhibits the oxidation of pyruvate in the presence of dinitrophenol. Malate and inorganic phosphate are both required to overcome the inhibition by fluoride completely. In the presence of malate and fluoride, α-oxoglutarate accumulates.
9. Ketogenesis occurs if the intramitochondrial NADH/NAD^+ ratio is high. The addition of carnitine, and especially carnitine *plus* CoA, to rat-liver mitochondria oxidizing palmitate increases this ratio, by increasing the palmitoyl-CoA oxidation. Under conditions of ketogenesis, the citric acid cycle is inhibited, by a decrease of the oxaloacetate concentration.
10. The activity of the extramitochondrial carnitine palmitoyltransferase may also contribute to the regulation of fatty acid metabolism.

SAMENVATTING

1. De activering van palmitaat met ATP en CoA in geïsoleerde rattelevermitochondriën is zeer snel, variërend van 70-130 nmolen/mg eiwit/minuut bij 37°.
2. Het ATP-afhankelijke palmitoyl-CoA synthetase heeft een dubbele localisatie in de rattelevercel. De enzymactiviteit is ongeveer gelijk verdeeld over mitochondriën en microsomen.
3. Het ATP-afhankelijke palmitoyl-CoA synthetase heeft een dubbele localisatie in rattelevermitochondriën; ongeveer 90% van de activiteit is gelocaliseerd in het mitochondriale buitenmembraan en ongeveer 10% in het binnenmembraan-matrix compartiment.
4. De twee mitochondriale palmitoyl-CoA synthetasen verschillen met betrekking tot hun K_m's voor vetzuur en ATP en met betrekking tot hun K_i's voor AMP en adenosine. Deze parameters worden vergeleken met die van het microsomale palmitoyl-CoA synthetase. De remmingen door AMP en adenosine zijn competitief met betrekking tot ATP. Het enzym in het binnenmembraan-matrix compartiment, in tegenstelling tot het buitenmembraan enzym en het microsomale enzym, wordt sterk geremd door octanoaat. Deze remming is niet-competitief met betrekking tot palmitaat.
5. Vergelijking van de kinetica van de mitochondriale ATP-afhankelijke palmitoyl-CoA synthetasen met de kinetica van de palmitaat (oleaat) oxydatie laat zien, dat, tijdens vetzuuroxydatie bij lage concentraties van palmitaat of oleaat en in aanwezigheid van carnitine, de te oxyderen vetzuren geactiveerd worden in het mitochondriale buitenmembraan. In afwezigheid van carnitine en bij relatief hoge palmitaat of oleaat concentraties worden de te oxyderen vetzuren geactiveerd in het binnenmembraan-matrix compartiment.

6. Het carnitine palmitoyltransferase heeft een dubbele localisatie in de rattelevercel; de enzymactiviteit bevindt zich in het mitochondriale binnenmembraan-matrix compartiment en in de microsomen fractie. Vasten stimuleert alleen de microsomale activiteit. Thyroxine behandeling stimuleert ook de carnitine palmitoyltransferase activiteit, vooral in het extramitochondriale compartiment.
7. Glucose-6-fosfatase en NADPH-cytochroom *c* reductase zijn heterogeen verdeeld over microsomale subfracties en kunnen beide gebruikt worden als microsomale referentie enzymen. Geïsoleerde rattelevermitochondriën zijn verontreinigd met die microsomen, die een relatief hoge glucose-6-fosfatase activiteit bezitten.
8. De oxydatie van palmitaat, palmitoylcarnitine of pyruvaat door geïsoleerde rattelevermitochondriën wordt, in aanwezigheid van dinitrofenol, geremd door anorganisch fosfaat. Omdat de remmingen opgeheven kunnen worden door malaat, wordt geconcludeerd dat de remmingen optreden in de citroenzuurcyclus. Fluoride remt ook de oxydatie van pyruvaat in aanwezigheid van dinitrofenol. Malaat en fosfaat zijn beide nodig om de remming door fluoride geheel op te heffen. In aanwezigheid van malaat en fluoride hoopt α-oxoglutaraat op.
9. Ketonlichamen worden gevormd indien de intramitochondriale $NADH/NAD^+$ verhouding hoog is. De toevoeging van carnitine, en vooral van carnitine *plus* CoA, aan rattelevermitochondriën tijdens palmitaat oxydatie verhoogt deze verhouding, doordat meer palmitoyl-CoA geoxydeerd wordt. Tijdens de synthese van ketonlichamen is de citroenzuurcyclus geremd, door een verlaging van de oxaalacetaat concentratie.
10. De activiteit van het extramitochondriale carnitine palmitoyltransferase zou ook kunnen bijdragen tot de regulatie van het vetzuurmetabolisme.

CURRICULUM VITAE

(OP VERZOEK VAN DE MEDISCHE FACULTEIT)

In 1960 begon de schrijver van dit proefschrift met de studie der scheikunde aan de Rijksuniversiteit te Leiden. Het kandidaatsexamen werd afgelegd in 1964; het doctoraalexamen, met als hoofdvak Biochemie, in 1966. Hierna volgde een aanstelling als wetenschappelijk medewerker bij de afdeling Biochemie van de Medische Faculteit Rotterdam. Op deze afdeling werd het in dit proefschrift beschreven onderzoek verricht.

APPENDIX

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SHORT COMMUNICATIONS

BBA 53200

On fatty acid activation in rat liver mitochondria

Two systems for the activation of fatty acids in liver mitochondria have been described, one catalyzed by ATP-dependent fatty-acid-activating enzymes[1] (acid: CoA ligase (AMP), EC 6.2.1.2 and EC 6.2.1.3) and a GTP-linked fatty-acid-activating system, discovered by ROSSI AND GIBSON[2].

The purpose of the present investigation was to evaluate the relative contributions of the two fatty-acid-activating systems. Rat liver mitochondria, isolated in 0.25 M sucrose, were suspended in 0.1 M Tris–HCl (pH 7.4), containing 2 mM reduced glutathione and 1 mM EDTA, prior to sonication for 60 sec at 3 A (Branson, Model S75) ar 0–4°. The sonicate was centrifuged for 10 min at 9500 × *g*, and the pellet was discarded. The supernatant was passed through a Sephadex G-25 column equilibrated with 0.1 M KCl before being tested for its ability to activate palmitate, oleate or octanoate.

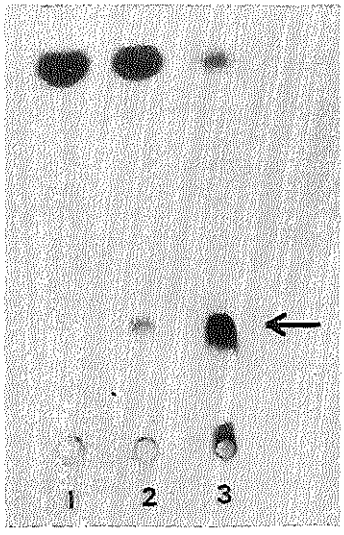

Fig. 1. Autoradiographic demonstration of palmitate activation. The reaction medium contained 100 mM Tris–HCL, 0.25 mM [1-^{14}C]palmitic acid (specific activity, 0.2 μC/μmole) complexed with 0.035 mM bovine serum albumin, 3 μg oligomycin, 10 mM DL-carnitine, 0.5 mM CoA, 2 mM $MgCl_2$, 30 mM KCl, 0.32 mg of purified palmitoyl-CoA:carnitine palmitoyltransferase and 0.2 mg of mitochondrial protein. The enzyme fractions were prepared as described in the text. Additions were in (2) 2 mM GTP and in (3) 2 mM ATP. The reaction volume was 1 ml, the temperature 37°, and the pH 7.4. After 10 min of incubation the reaction was stopped with HCl and the incubation medium extracted with *n*-butanol, as described by FARSTAD, BREMER AND NORUM[3]. 0.05 ml of each extract was applied to a thin-layer plate (silica gel). The chromatogram was developed with chloroform-methanol–water (70:30:5, v/v/v). Reference samples of [^{14}C]palmitate, [^{14}C]palmitoylcarnitine and [^{14}C]palmitoyl-CoA were chromatographed in the same way (not shown), indicating that the thick spots at the upper end of the autoradiogram are palmitate, those indicated by the arrow palmitoylcarnitine, and those just above the starting points palmitoyl-CoA. Agfa-Gevaert-Osray film was used (exposure time, 17 h).

The acyl-CoA formed was trapped as acylcarnitine by acyl-CoA:carnitine transferase, which is present in the sonicate. Addition of purified palmitoyl-CoA: carnitine palmitoyltransferase, prepared as described by FARSTAD *et al.*[3], enhanced the amount of acylcarnitine formed by about 50%, so that it was added routinely.

It can be seen from Fig. 1 that the addition of GTP or ATP is required to activate palmitic acid, as judged by the formation of [^{14}C]palmitoyl-CoA and [^{14}C]-palmitoylcarnitine, detected by autoradiography of thin-layer chromatograms. It can also be seen that ATP is a much more efficient source of energy than GTP. By scraping the radioactive materials from the thin-layer plates and measuring the radio-activity by liquid scintillation spectrophotometry, it was calculated that the specific activity (μmoles/mg protein per h) of the ATP-dependent reaction was 2.9 and that of the GTP-dependent reaction was 0.1.

When in other experiments palmitate was replaced by octanoate or oleate, the "GTP-system" had 3–6% of the activity of the "ATP-system".

Reports from the literature[4,5], on the other hand, suggested to us that in intact liver mitochondria, the "GTP-system" is about as active as the "ATP-system". In these studies fatty acids are oxidized by intact rat liver mitochondria in the presence of dinitrophenol and the absence of (inhibitory) P_i. The addition of dinitrophenol is required to stimulate the citric acid cycle by which, *via* oxidation of α-oxoglutarate, GTP can be generated. Under these conditions ATP is also formed[6]. Moreover the

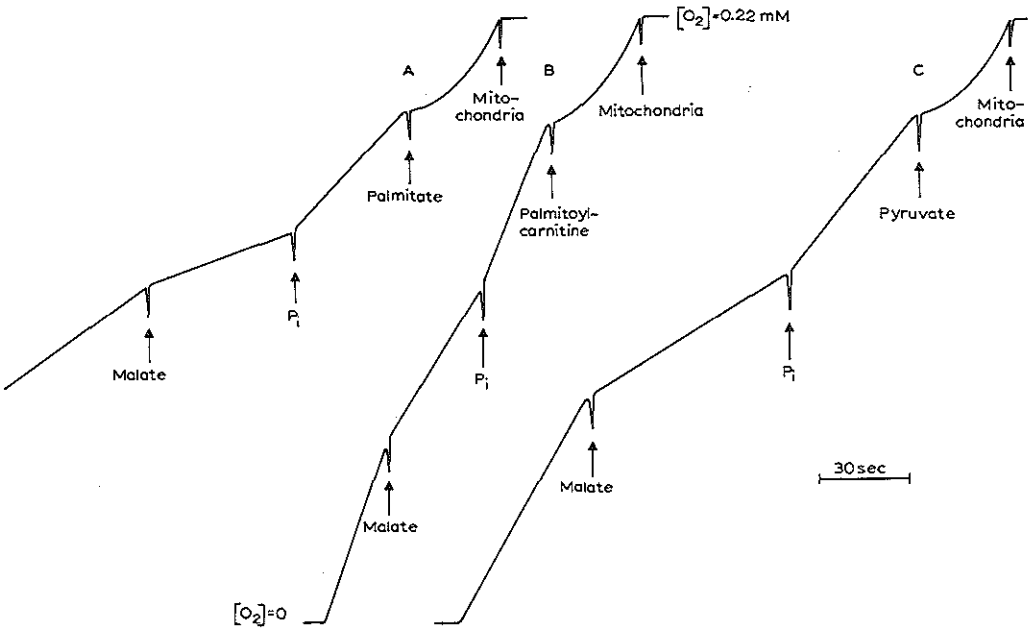

Fig. 2. Palmitate, palmitoylcarnitine and pyruvate oxidation, as measured with the Clark "oxygen electrode". The reaction medium contained 32 mM KCl, 4.5 mM $MgCl_2$, 73 mM Tris–HCl, 1 mM EDTA, 0.1 mM dinitrophenol, 12 mM sucrose and 4.4 mg of mitochondrial protein. 0.12 mM palmitic acid complexed with 0.015 mM bovine serum albumin, 0.24 mM DL-palmitoylcarnitine and 5 mM pyruvate were added as indicated in Expts. A, B and C, respectively. Other additions were, where indicated, 17 mM potassium phosphate and 4.5 mM DL-malate. The reaction volume was 2.2 ml, the temperature 37°, and the pH 7.5.

ADP present in the reaction medium[5] contributes to the formation of ATP through the myokinase reaction[6].

In the presence of dinitrophenol then, the "ATP-system" for fatty acid activation might still operate in addition to the "GTP-system". The inhibition of fatty acid oxidation by P_i in the presence of dinitrophenol, used as an argument in favor of the operation of the GTP-dependent fatty acid activation[4,5] in intact mitochondria, will therefore be analyzed further.

It can be seen from Fig. 2A that we can confirm the inhibitory action of P_i on palmitate oxidation in the presence of dinitrophenol. It may also be noted, however, that malate partially relieves the inhibition. This indicated to us that P_i at least partially inhibits the citric acid cycle. In agreement with this interpretation is the observation that substrates which do not need activation prior to oxidation, such as palmitoylcarnitine (Fig. 2B) or pyruvate (Fig. 2C), also show P_i-inhibited respiration which can be overcome by malate.

It may be concluded, then, that P_i inhibition of fatty acid oxidation does not necessarily mean the operation of the GTP-dependent fatty-acid-activation system in intact mitochondria.

We do not conclude from our data obtained with sonicated mitochondria (Fig. 1) that the "GTP-system" does not contribute significantly to fatty acid activation, since preferential inactivation of the "GTP-system" during isolation cannot be excluded. The "ATP-system", however, is so active (2.9 μmoles/mg protein per h) that in intact mitochondria, palmitate oxidation with a Q_{O_2} of 1478 could be expected if the activation reaction were rate limiting (in practice we never found Q_{O_2}'s for palmitoylcarnitine oxidation exceeding 150).

Future experiments will be required to elucidate the role of substrate-linked phosphorylation in fatty acid oxidation.

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THE LOCALIZATION OF PALMITOYL-CoA:CARNITINE PALMITOYLTRANSFERASE IN RAT LIVER

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SUMMARY

1. The distribution of palmitoyl-CoA:carnitine palmitoyltransferase has been studied in subcellular fractions of rat liver. By using two different estimations for the enzyme activity and by differential centrifugation and linear sucrose density gradient centrifugation, the enzyme is shown to be localized both in mitochondria and microsomes.

2. The mitochondrial palmitoyl-CoA:carnitine palmitoyltransferase is localized in the inner membrane *plus* matrix fraction.

3. During palmitate oxidation by isolated mitochondria, in the presence of a physiological concentration of carnitine, palmitoylcarnitine accumulates. From this and experiments with sonicated mitochondria, it is concluded that the capacities of long-chain fatty acid activation and of palmitoyl-CoA:carnitine palmitoyltransferase *in vitro* by far exceed the capacity of fatty acid oxidation.

INTRODUCTION

The long-chain fatty acyl-CoA synthetase (acid:CoA ligase (AMP), EC 6.2.1.3) was first demonstrated by KORNBERG AND PRICER[1] in rat-liver microsomes. In more recent investigations, palmitoyl-CoA synthetase has been demonstrated by different methods in both mitochondria and microsomes. A thorough study on the intracellular distribution of the enzyme, with the use of marker enzymes for the different subfractions, was made by FARSTAD *et al.*[2]. They reported that the microsomes have 70 % and the mitochondria 30 % of the activity present in rat-liver cells.

On the other hand, the localization of the palmitoyl-CoA:carnitine palmitoyltransferase (trivial name, carnitine palmitoyltransferase) is reported to be 100 % mitochondrial[3] and probably solely in the inner mitochondrial membrane[4].

The concept that long-chain fatty acids are partly oxidized in a carnitine-dependent fashion is generally accepted. It is assumed that acyl-CoA cannot penetrate the inner mitochondrial membrane[5]. Therefore acyl-CoA formed at the site of the endoplasmic reticulum or the mitochondrial outer membrane[2,4] is transported as acylcarnitine through the mitochondrial inner membrane, prior to oxidation of the acyl moiety[6,7]. In this scheme the transport of activated fatty acids is thought to function from the outside to the inside of mitochondria. Rephrasing this scheme,

acylcarnitine is a storage form of activated long-chain fatty acid, which can pass through the mitochondrial inner membrane. In principle the possibility exists that acylcarnitine is not only used for intramitochondrial but also for extramitochondrial reactions. Support for this idea came from the observation that the capacity of mitochondrial fatty acid activation exceeds by far that for fatty acid oxidation[8]. If this excess of activated fatty acid partially exists as acylcarnitine, it must be converted back to the CoA-derivative before utilization. As mentioned already, mitochondria contain carnitine palmitoyltransferase. The literature on the existence of carnitine palmitoyltransferase in the microsomes is rather confusing[3,9,10]. We therefore reinvestigated the distribution of carnitine palmitoyltransferase with the use of marker enzymes. The present paper demonstrates that the carnitine palmitoyltransferase is localized not only in the mitochondria but also in the microsomes.

MATERIALS AND METHODS

Oligomycin and kynuramine were purchased from Sigma Chemical Co. (St. Louis, Mo.), crystalline bovine serum albumin (Fraction V) from Sigma or from Pentex (Kankakee, Ill.), and palmitic acid, sucrose and ascorbic acid from the British Drug Houses.

DL-[*Me*-^{3}H]Carnitine, with a specific activity of 75 μC/μmole, was a gift of Dr. J. Bremer from the Oslo University and L-carnitine was a gift from Otsuka Pharmaceutical Factory (Osaka, Japan). The radioactive carnitine was diluted with L-carnitine to a specific activity of 0.95 or 0.032 μC/μmole L-carnitine. The radioactive fatty acids were purchased from the Radiochemical Centre (Amersham, England) and nucleotides, phosphoenolpyruvate, cytochrome *c*, pyruvate kinase (EC 2.7.1.40) and adenylate kinase (EC 2.7.4.3) from C. F. Boehringer and Sons (Mannheim, Germany).

Carnitine palmitoyltransferase was purified from calf-liver mitochondria according to FARSTAD *et al.*[2]. Palmitoyl-L-carnitine was synthesized according to BRENDEL AND BRESSLER[11] and was found to be chromatographically pure, as tested in the system described by BØHMER *et al.*[12].

Albumin was used, after dialysis and millipore filtration, as a clear neutral solution. Palmitate–albumin solutions were prepared by mixing potassium palmitate with albumin in a molar ratio of 7:1.

Animals

Male rats of the Wistar strain were used for the experiments (weight approx. 250 g). The animals had free access to food and water (except in the fasting experiments where only water was supplied *ad libitum*). The animals were bled after cervical fracture.

Homogenization and centrifugation procedures

Differential centrifugation. 4 g of liver were homogenized in 9 vol. of 0.25 M sucrose in a Potter-Elvehjem homogenizer with a Teflon pestle. The differential centrifugation was performed according to DE DUVE *et al.*[13], with slight modifications. The crude homogenate was centrifuged for 5 min at 900 $\times$ *g* in a Sorvall refrigerated centrifuge (Rotor SS 34). The pellet was rehomogenized in 20 ml of 0.25 M sucrose

and centrifuged again for 5 min at 900 × *g*. The last step was repeated once; and the final pellet, which consists of cellular debris, unbroken cells and nuclei, was suspended and designated nuclear fraction (N). The combined supernatants of fraction N were centrifuged for 10 min at 5100 × *g*. The pellet was resuspended in 30 ml of 0.25 M sucrose and centrifuged for the same integrated field. The last step was repeated once and the final pellet, after washing off the fluffy layer with some sucrose, was designated heavy mitochondrial fraction (M). The combined supernatants of the previous centrifugations were centrifuged for 10 min at 27000 × *g*. The pellet was resuspended in 30 ml of 0.25 M sucrose and centrifuged for the same integrated field. This was repeated once. The final pellet after washing off the fluffy layer was designated light mitochondrial fraction (L). The combined supernatants were centrifuged for 60 min at 131000 × *g* in the Beckman ultracentrifuge (Rotor SW 27); the resulting pellet was designated microsomal fraction (P) and the final supernatant soluble fraction (S).

Gradient centrifugation of 900 × g supernatant. 10 g of liver were homogenized in 4 vol. of 0.25 M sucrose. This homogenate was centrifuged for 5 min at 900 × *g*. 10 ml of the supernatant were layered on 28 ml of a continuous sucrose density gradient, with specific gravity varying from 1.025 to 1.275. The gradient was centrifuged for 2.5 h at 131000 × *g* in a Beckman ultracentrifuge (Rotor SW 27). After centrifugation, the content of the tube was collected in ten fractions. Beginning from the bottom of the tube there were nine fractions of 3 ml and one fraction (No. 10) of 11 ml, which included the soluble proteins from the layered homogenate. Fraction 10 was included to calculate the recoveries but was not considered in the data where percentage of total activity means percentage of activity in the Fractions 1–9. The activity in Fraction 10 was low for all the enzymes tested (see below).

Gradient centrifugation of mitochondria. The heavy mitochondrial fraction was isolated from 10 g of liver as described before, except that the mitochondria were washed twice by resuspending in 0.25 M sucrose and centrifugation at 12000 × *g* (10 min). The fluffy layer was carefully washed off. The final pellet was resuspended in 15 ml of 0.25 M sucrose of which 10 ml were layered on the continuous sucrose density gradient and centrifuged as described before. In the swelling experiments the final pellet was resuspended in 50 ml of 20 mM potassium phosphate buffer (pH 7.2) containing 0.02 % of bovine serum albumin and stirred for 20 min at 0–4°, as described by PARSONS *et al.*[14]. The swollen mitochondria were collected by centrifugation for 20 min at 48200 × *g* and resuspended in 15 ml of 0.25 M sucrose of which 10 ml were layered on the gradient and centrifuged as described before.

Enzyme assays

All the enzymes tested were assayed at 37°. Glucose-6-phosphatase (EC 3.1.3.9) was assayed according to BEAUFAY *et al.*[15]; monoamine oxidase (EC 1.4.3.4) according to WEISSBACH *et al.*[16] with kynuramine as substrate; cytochrome *c* oxidase (EC 1.9.3.1) was measured in an oxygraph, equipped with a "Clark" oxygen electrode; ascorbic acid was used as the electron donor as described by SOTTOCASA *et al.*[17]. Palmitoyl-CoA synthetase was assayed according to FARSTAD *et al.*[2] with several modifications. The incubation medium contained 20 mM KCl, 2 mM $MgCl_2$, 80 mM Tris-HCl, 0.5 mM EDTA, 0.5 mM potassium palmitate (complexed with 0.07 mM bovine serum albumin), 3 μg oligomycin, 5 mM L-[^{3}H]carnitine (specific activity, 0.032 μC/μmole), 0.2 ml of purified carnitine palmitoyltransferase (0.04 unit), 0.2 mM CoA, 2 mM ATP,

5 mM phosphoenolpyruvate and approx. 2 units of pyruvate kinase and adenylate kinase each. The reaction volume was 1 ml, and the pH was 7.4. The reaction was started with 0.1 mg of either mitochondrial or microsomal protein and stopped after 6 min of incubation with 0.1 ml of conc. HCl. After addition of 1.9 ml of water, the palmitoylcarnitine was extracted with 1.5 ml of *n*-butanol. After washing the butanol phase with 2 ml of butanol-saturated water, 0.5 ml of the butanol phase was transferred to a counting vial together with 10 ml of toluene (containing 5 g of 2,5-diphenyloxazole and 0.3 g of dimethyl-1,4-bis-(5-phenyloxazolyl-2)benzene per l) and sufficient Nuclear-Chicago solubilizer (Amersham Searle Corp.) or abs. ethanol to keep the solution clear. The counting efficiency was 29 % as determined by the channels ratio method. Carnitine palmitoyltransferase was assayed by two different methods: firstly, as described by NORUM[18] as the CoA-dependent incorporation of labeled carnitine into palmitoylcarnitine (the exchange reaction, Method I); secondly, as the formation of palmitoylcarnitine from palmitate, ATP, CoA and carnitine in the presence of an excess palmitoyl-CoA synthetase (forward reaction, Method II). The latter enzyme does not have to be added to the reaction medium, since it was found that both in mitochondria and in microsomes palmitoyl-CoA synthetase is present in excess of carnitine palmitoyltransferase. The incubation medium (Method II) is the same as that used for palmitoyl-CoA synthetase, except that the purified carnitine palmitoyltransferase from calf liver is omitted. The rate of the exchange reaction (Method I) was converted into the rate of the forward reaction (palmitoyl-CoA + carnitine → palmitoylcarnitine + CoA) by the use of the exchange rate expression as described in ref. 19.

The fractions prepared by differential centrifugation were treated with ultrasonic vibration before incubation (Branson S-75 sonifier; 1 min at 20 kHz). The temperature was maintained between 0 and 4° by cooling in an ice–salt bath.

Protein was determined with the biuret reaction after solubilization of particle-bound protein with deoxycholate according to JACOBS *et al.*[20].

RESULTS

Differential centrifugation studies on the localization of carnitine palmitoyltransferase and palmitoyl-CoA synthetase

Table I shows the distribution of carnitine palmitoyltransferase, palmitoyl-CoA synthetase and some marker enzymes in subcellular fractions from rat liver. The fractions were prepared by differential centrifugation, as described in MATERIALS AND METHODS. The results of this typical experiment are shown graphically in Fig. 1. The relative specific activities (for definition see legend to Fig. 1) are plotted against the percentage of the total protein as proposed by DE DUVE *et al.*[13]. The distribution of the marker enzymes, *i.e.* cytochrome *c* oxidase (mitochondrial) and glucose-6-phosphatase (microsomal), and protein correspond to those observed by other workers[13], except that our Fraction N is richer in protein and in enzyme content. This suggests a gentle homogenization of the liver, leaving a relatively large amount of unbroken cells in Fraction N. From the distribution of the marker enzymes it can be concluded that the mutual contamination of the different fractions is low. The distribution of monoamine oxidase resembles that of cytochrome *c* oxidase, except that a somewhat higher percentage is found in the microsomal fraction. As monoamine oxidase is

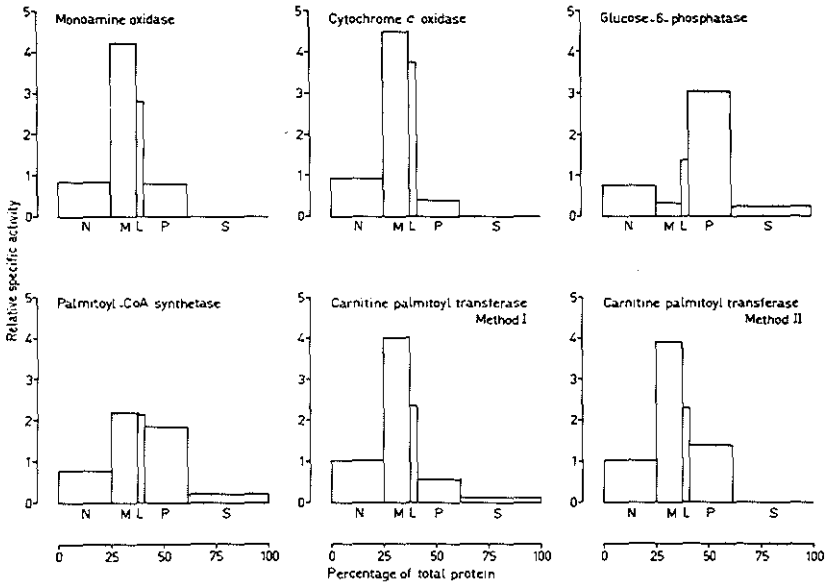

Fig. 1. The distribution patterns of carnitine palmitoyltransferase, palmitoyl-CoA synthetase and and some marker enzymes in fractions from rat liver. The relative specific activities (percentage of total activity divided by the percentage of total protein content) of the fractions are plotted against the percentage of total protein in each fraction. For recoveries and the meaning of N, M, L, P and S see Table I.

TABLE I

INTRACELLULAR DISTRIBUTION OF CARNITINE PALMITOYLTRANSFERASE, PALMITOYL-CoA SYNTHETASE AND SOME MARKER ENZYMES IN RAT LIVER

The enzymes and protein were assayed as described under MATERIALS AND METHODS. Protein is expressed in mg/g of liver (wet wt.). The enzyme activities in the total homogenate are given in μmoles of substrate metabolized per g of liver (wet wt.) per min. Fraction N is the nuclear fraction; M, the heavy mitochondrial fraction; L, the light mitochondrial fraction; P, the microsomal fraction; and S, the final supernatant.

Enzyme	*Total homogenate*	*N* (%)	*M* (%)	*L* (%)	*P* (%)	*S* (%)	*Recovery* (%)
Carnitine palmitoyltransferase (activity determined by Method I)	4.4	26.0	49.9	8.2	11.6	4.3	101
Carnitine palmitoyltransferase (activity determined by Method II)	2.2	26.1	48.5	8.1	28.4	0	111
Palmitoyl-CoA synthetase	12.4	19.1	27.1	7.5	37.7	8.5	91
Monoamine oxidase	0.17	21.7	52.6	9.8	16.0	0	92
Cytochrome *c* oxidase	88.0	23.4	55.5	13.2	7.9	0	92
Glucose-6-phosphatase	11.0	19.3	4.1	4.8	62.7	9.1	108
Protein	200.0	25.2	12.4	3.5	20.5	38.3	93

localized in the mitochondrial outer membrane[21,22], this suggests that a part of the mitochondrial outer membrane is separated from the mitochondria by the fractionation procedure. The distribution of palmitoyl-CoA synthetase supports the dual localization of the enzyme as reported by FARSTAD *et al.*[2]. In our hands a greater percentage is found in the mitochondria. From Table I it can be calculated that the

specific activities measured by us are higher than reported before, especially in the mitochondrial fraction[2,23].

The distribution of carnitine palmitoyltransferase is strongly dependent on the estimation used. When measured with the exchange reaction (Method I), the percentage of the total activity in the microsomal fraction is only slightly higher when compared to cytochrome *c* oxidase. However, when the forward reaction (Method II) is used, the distribution pattern suggests a dual localization of the enzyme with activity both in mitochondria and microsomes. It is suggested by several workers that the ratio of long-chain acylcarnitine to free carnitine is higher in livers from fasted rats than from fed rats (see for instance ref. 12). Microsomal enzymes are in general more sensitive to variations in the diet than mitochondrial enzymes. Therefore, we tested whether fasting activated the microsomal carnitine palmitoyltransferase more than the mitochondrial enzyme.

TABLE II

EFFECT OF FASTING (48 h) ON THE ACTIVITY OF CARNITINE PALMITOYLTRANSFERASE IN FRACTIONS FROM RAT LIVER

The enzyme activity was assayed using the CoA-dependent isotope-exchange reaction (Method I). The enzyme fractions were prepared by centrifugation of a 10% rat-liver homogenate for 5 min at 900 × *g*. The 900 × *g* supernatant is centrifuged for 10 min at 12000 × *g* to obtain the 12000 × *g* supernatant. Incubation was carried out with 2 mg (900 × *g* supernatant) or 6 mg (12000 × *g* supernatant) of protein. The enzyme activity is given as μmoles of substrate metabolized per amount of supernatant corresponding to 1 g of liver per min. The mean and the standard error of the mean of enzyme activity of 4 fasted and 4 normal rats are given. For further details see MATERIALS AND METHODS.

Treatment	*Number of rats*	*900 × g supernatant*	*12000 × g supernatant*
None	4	3.25 ± 0.56	0.83 ± 0.16
Fasted	4	4.00 ± 0.13*	1.19 ± 0.02**

* Statistically significant difference as compared with the values for untreated rats: $0.1 > P > 0.05$.

** $0.02 > P > 0.01$.

It can be seen from Table II, where the activities of carnitine palmitoyltransferase are compared in 900 × *g* and 12000 × *g* supernatants of livers from fed rats and rats fasted for 48 h, that the activity per g of liver is significantly increased by fasting only in the 12000 × *g* supernatant. This suggests that the increment induced by fasting is mainly due to activation of the extramitochondrial enzyme. Moreover, NORUM[9] found that after fasting, fat feeding and in diabetes the total activity increased and that the specific activity in the extramitochondrial compartment increased most. For these reasons and the smaller variation in enzyme activities in fasted rats (compare the standard errors in Table II), we decided to use fasted animals in further localization studies.

The distribution of carnitine palmitoyltransferase as studied with the use of continuous sucrose density gradients

It can be seen from Fig. 2, which shows a representative experiment of a series of 3 experiments, that also in continuous gradient centrifugation studies the enzyme

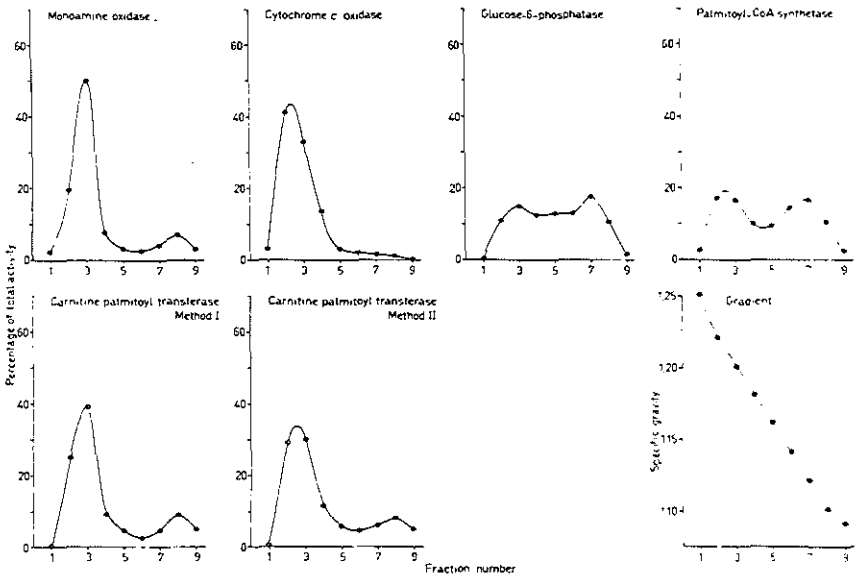

Fig. 2. The distribution of carnitine palmitoyltransferase, palmitoyl-CoA synthetase and some marker enzymes in fractions from rat liver, isolated by continuous sucrose density gradient centrifugation. A 48-h-fasted rat was used. For details see MATERIALS AND METHODS. The percentage of the total activity in Fractions 1–9 is plotted against the fraction number. The recovery of the activities of the different enzymes in the gradient fractions varied between 87 and 107%.

has a dual localization. Fractions 5–9 are very little contaminated with mitochondrial inner membranes (see the distribution of cytochrome *c* oxidase; compare ref. 13).

Fig. 2 shows that some monoamine oxidase is present in fractions not containing cytochrome *c* oxidase. Does the activity of carnitine palmitoyltransferase in gradient Fractions 5–9 correspond to particles containing monoamine oxidase (mitochondrial outer membranes) or particles containing glucose-6-phosphatase (microsomes)? In order to investigate this problem, we reinvestigated the intramitochondrial distribution of carnitine palmitoyltransferase. For these experiments, twice-washed mitochondria were used. The inner and outer membranes were separated by swelling in phosphate buffer as suggested by PARSONS *et al.*[14] (for details see MATERIALS AND METHODS) followed by centrifugation in a continuous sucrose density gradient, prepared exactly as that used in the fasting experiments. Monoamine oxidase (outer membranes) and cytochrome *c* oxidase (inner membranes) were used as marker enzymes for the mitochondrial membranes.

As can been seen from Fig. 3, this method yields a good separation of outer and inner membranes. Whereas after phosphate treatment the activity of cytochrome *c* oxidase is shifted to a region of higher density, about two thirds of the monoamine oxidase activity is shifted to a region of lower density. Carnitine palmitoyltransferase in these experiments behaves quantitatively as cytochrome *c* oxidase (95% of the activity shifts to regions of higher density). These experiments then strongly suggest that the mitochondrial carnitine palmitoyltransferase is localized in the inner membrane, confirming the observations of NORUM *et al.*[4]. Another enzyme also localized in the mitochondrial outer membrane, the rotenone-insensitive NADH–cytochrome

c reductase[17] behaves in these swelling experiments essentially like monoamine oxidase (not shown). These experiments then suggest that in the experiments of Fig. 2 the activity of carnitine palmitoyltransferase in Fractions 5–9 is not due to contamination with mitochondrial outer membranes (compare also the data of Table I) but is to be ascribed to the presence of microsomes. Rather unexpected is the distribution of glucose-6-phosphatase in the gradient experiment of Fig. 2. From differential centrifugation experiments (Table I) it can be seen that glucose-6-phosphatase is localized almost exclusively in the microsomes. However, when a 900 × *g* supernatant is centrifuged in a continuous sucrose density gradient apparently a considerable portion of the enzyme activity is found in the mitochondrial fractions (1–4). The same observation was made by FARSTAD *et al.*[2], when a mixture of isolated mitochondria and microsomes was centrifuged in about the same way. Probably part of the endoplasmic reticulum is rather tightly attached to the mitochondria (compare ref. 24). It is evident then that the percentage of the microsomal carnitine palmitoyltransferase is underestimated in these gradient experiments. This explains the apparent difference

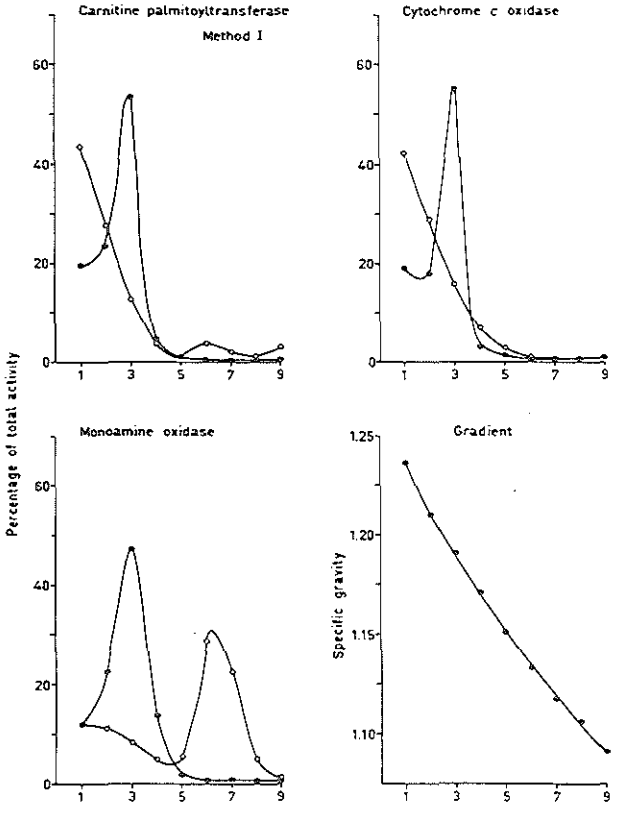

Fig. 3. Intramitochondrial localization of carnitine palmitoyltransferase. After swelling of the mitochondria in 20 mM phosphate buffer, the outer and inner membranes were separated in a continuous sucrose density gradient. For further details see MATERIALS AND METHODS. The percentage of the total activity in Fractions 1–9 is plotted against the fraction number. The recovery of the activities of the different enzymes in the gradient fractions varied between 89 and 103%. ●—●, intact mitochondria; ○—○, swollen mitochondria.

in percentage of microsomal carnitine palmitoyltransferase in the gradient studies (Fig. 2) and the differential centrifugation studies (Fig. 1). The gradient centrifugation studies thus confirm the differential centrifugation studies that carnitine palmitoyltransferase has a dual localization. The activity resides both in mitochondria and in microsomes. In mitochondria at least the bulk of the enzyme activity is localized in the inner membranes. Whether the outer membrane contains no carnitine palmitoyltransferase activity at all is difficult to decide. In the gradient centrifugation studies, the significant difference in transferase activity, measured by methods I and II as observed in the differential centrifugation studies, has disappeared. This requires further investigation.

Palmitoylcarnitine formation during palmitate oxidation in isolated mitochondria

The activities for the palmitoyl-CoA synthetase in sonicated mitochondria, as measured in the present studies, are much bigger than reported before[2,8,23]. From Table I a specific activity of 8.1 μmoles/mg protein per h can be calculated. From earlier experiments, carried out under different conditions, we concluded already that the capacity for long-chain fatty acid activation exceeds by far that for fatty acid oxidation[8]. If the rates of palmitate activation in sonicated and in intact mitochondria were the same, the oxidation of palmitate to acetyl-CoA (acetoacetate) would maximally correlate with an O_2 uptake of 1250 μl O_2 per mg protein per h (Q_{O_2}) at 37°. However, even when palmitoylcarnitine, a substrate which is oxidized more rapidly than palmitate by rat-liver mitochondria, is used, the Q_{O_2}, even for complete oxidation, never exceeds 250 (compare also ref. 25). The activity of mitochondrial carnitine palmitoyltransferase is also relatively high (2.6 μmoles/mg protein per h), so that it is also rather unlikely that this enzyme is rate limiting for long-chain fatty acid oxidation. In fact, as shown in Fig. 4, when palmitate is oxidized by rat-liver mitochondria in the presence of a physiological concentration of carnitine[26], the formation of palmitoylcarnitine can be demonstrated. That more palmitate (the spots marked with an asterisk) disappeared when the incubation was carried out with mitochondria (M) instead of mitochondrial supernatant (P + S) can be concluded from the autoradiogram. This extra amount was not recovered in the other spots, indicating a loss of material not extractable by chloroform–methanol, indicating oxidation of palmitate. Fig. 4 also shows that incubation with the mitochondrial supernatant results in the formation of palmitoylcarnitine (indicated by the arrow). This enzyme fraction is so little contaminated with mitochondria (compare the fractions from Table I), that the palmitoylcarnitine formation cannot be explained by mitochondrial contamination. The spot indicated by the arrow was further identified by two-dimensional thin-layer chromatography. The material was scraped from the thin-layer plate and, after elution from the silica gel with methanol, unlabeled palmitoylcarnitine was added. This mixture was chromatographed in two directions. The chromatogram was developed in chloroform–methanol–water (77:35:7, by vol.) and subsequently in chloroform–methanol–glacial acetic acid–water (50:15:6:2.5, by vol.). 80–96 % of the radioactivity was recovered in the palmitoylcarnitine spot. When in similar experiments [^{3}H]carnitine was used, ^{3}H was present in the palmitoylcarnitine spot.

These experiments then indicate that, even during palmitate oxidation in the presence of carnitine, palmitoylcarnitine accumulates.

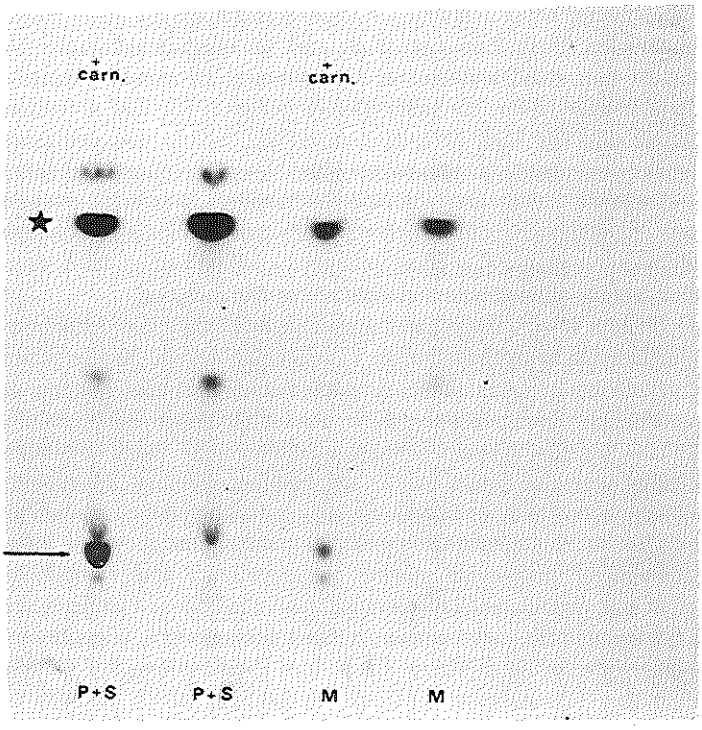

Fig. 4. Autoradiographic demonstration of palmitoylcarnitine synthesis in mitochondria (M) and in 27000 × *g* supernatant (P + S) from rat liver under aerobic condition. The reaction medium contained 80 mM Tris–HCl, 0.25 mM [1-^{14}C]palmitic acid (specific activity, 0.2 μC/μmole) complexed with 0.035 mM bovine serum albumin, 0.5 mM CoA, 2 mM $MgCl_2$, 20 mM KCl, 0.5 mM EDTA, 10 mM P_i, 2 mM ATP, 5 mM phosphoenolpyruvate, 2 units pyruvate kinase and 30 mM sucrose. Where indicated, 2 mM DL-carnitine (carn.) was added. Reaction volume, 1 ml; temp., 37°; pH 7.4. The enzyme fractions were prepared by centrifugation of a rat-liver homogenate in 0.25 M sucrose for 5 min at 900 × *g*. The pellet was discarded and the supernatant was centrifuged for 10 min at 27000 × *g*. The 27000 × *g* supernatant was carefully pipetted off. The 27000 × *g* pellet was washed twice by resuspending in 0.25 M sucrose and centrifugation for 10 min at 12000 × *g*. The fluffy layer was discarded and the mitochondrial pellet suspended in 0.25 M sucrose. After 20 min of incubation with 1.1 or 4.5 mg of protein (mitochondrial or 27000 × *g* supernatant fraction, respectively), the reactions were stopped by extraction with 3 ml chloroform–methanol (1:2, by vol.). The precipitate formed was reextracted with a mixture of 1.6 ml water, 4.0 ml methanol and 2.0 ml chloroform. After centrifugation the supernatants were combined and 7.7 ml of chloroform and 7.7 ml of water added. The resulting lower phase was quantitatively removed and evaporated to dryness. The residue was dissolved in 0.2 ml chloroform–methanol (1:2, by vol.) and applied to a thin-layer plate (silica gel). The chromatogram was developed with chloroform–methanol–water (77:35:7, by vol.). The references used (not shown) were palmitic acid, phosphatidylethanolamine, phosphatidylcholine, palmitoylcarnitine and a mixture of these compounds. Agfa–Gevaert Osray film was used (exposure time, 80 h).

DISCUSSION

The findings of other workers[2,23] that ATP-dependent palmitoyl-CoA synthetase has a dual localization is confirmed by us. The specific activities found by us are (at 37°) about 8 μmoles/mg protein per h in both mitochondria and microsomes. PANDE AND MEAD[23], who measured the activity of the enzyme based on the formation of hydroxamate in the presence of hydroxylamine, reported for microsomes a similar

figure. In mitochondria they found lower activities. FARSTAD *et al.*[2], on the other hand, measured much lower activities. Both FARSTAD *et al.* and we used in principle the same method: formation of palmitoylcarnitine in the presence of excess carnitine and carnitine palmitoyltransferase. Here, the discrepancy between the low activities described by FARSTAD *et al.* and the high activities described by us are probably to be ascribed to differences in incubation conditions. FARSTAD *et al.* report that fatty acid activation in isolated mitochondria and microsomes requires a soluble protein factor for optimal activity. In our experiments, however, such a soluble protein fraction was not required, as is demonstrated by the good recoveries of palmitoyl-CoA synthetase in the experiments on the subcellular distribution of the enzyme.

In agreement with NORUM *et al.*[4], the present paper reports that the mitochondrial localization of carnitine palmitoyltransferase is virtually 100% in the inner membrane *plus* matrix fraction. In fact, these authors found that the enzyme is localized in the inner membrane of the mitochondria. In earlier work of BREMER[10], in which no marker enzymes for the various cellular fractions were used, carnitine palmitoyltransferase activity was also found in the microsomal fraction. Moreover, NORUM[9] also found, especially in livers from fasted, diabetic and fat-fed rats, carnitine palmitoyltransferase activity in the extramitochondrial compartment. In a later publication, NORUM AND BREMER[3] now using marker enzymes state that "carnitine acyltransferases in rat liver are mitochondrial enzymes, and that these enzymes are not associated with the lysosomes, the microsomes or the cellular sap". The present paper, on the contrary, provides strong evidence for a dual localization of carnitine palmitoyltransferase in rat liver. As measured by our method II, which consists of the CoA- and ATP-dependent (endogenous) palmitoyl-CoA synthetase, followed by Reaction 1 from left to right, we find activities (at 37°) of 2.6 and 0.9 μmoles/mg protein per h in mitochondria and microsomes, respectively.

$$\text{Palmitoyl-CoA} + \text{carnitine} \rightleftharpoons \text{palmitoylcarnitine} + \text{CoA} \qquad (1)$$

With the very high activities reported here, it is unlikely that the palmitoyl-CoA synthetase or the carnitine palmitoyltransferase are rate-limiting enzymes in palmitate oxidation by rat-liver mitochondria. The mitochondrion, at least under certain conditions, has the apparatus to synthesize more activated fatty acids (acyl-CoA and acylcarnitine) than it can use for oxidation (compare also the experiment of Fig. 4). With the data presented in this paper we want to stress that acylcarnitine is a storage form of activated fatty acid, which can be formed and used both inside and outside the mitochondria.

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SHORT COMMUNICATIONS

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On the use of microsomal marker enzymes to distinguish the outer membrane of rat liver mitochondria from the microsomes

Several enzymes are generally accepted as marker enzymes for subcellular and submitochondrial fractions, such as glucose-6-phosphatase[1] (EC 3.1.3.9) and NADPH–cytochrome *c* reductase[2] for the microsomes, cytochrome *c* oxidase[2] (EC 1.9.3.1) for the mitochondrial inner membrane, and monoamine oxidase[3] (EC 1.4.3.4) and rotenone-insensitive NADH–cytochrome *c* reductase[2] for the mitochondrial outer membrane.

In a recent publication the use of some microsomal marker enzymes in distinguishing the outer membrane of rat liver mitochondria from the microsomes has been questioned[4]. In the present paper it is pointed out that specific activities alone are not sufficient to draw conclusions about the localization of a microsomal enzyme in the mitochondrial outer membrane, except if the specific activity in isolated mitochondrial outer membranes is significantly higher than in isolated microsomes. In the case of glucose-6-phosphatase and NADPH–cytochrome *c* reductase a several-fold increase in the specific activities has been reported in mitochondrial outer membranes, when compared to whole mitochondria[3,4]. However, the specific activities always remain far below the observed values for isolated microsomes. In this case we want to stress that the pattern of distribution of the total activity over the submitochondrial fractions, after separation of the mitochondria into outer and inner membranes, gives a more accurate picture of the localization of these enzymes than do the observed specific activities in the subfractions.

Fig. 1 shows a representative experiment from a series of 3 experiments. Glucose-6-phosphatase and NADPH–cytochrome *c* reductase are distributed in a different way over microsomal subfractions, isolated by centrifugation of a $27000 \times g_{max}$ (10 min) supernatant of a rat liver homogenate in a continuous sucrose density gradient.

Mitochondria are not present in the $27000 \times g$ supernatant used, so that the relatively high activity of glucose-6-phosphatase in Fractions 3–5 must be derived from microsomal membranes. These membranes have about the same density as mitochondria, when centrifuged in the same way (compare ref. 5). This explains the results shown in Table I, where the specific activities of glucose-6-phosphatase and NADPH–cytochrome *c* reductase in microsomes (P) and heavy mitochondria (M) are compared. Since the enzymes are not homogeneously distributed over the microsomal membranes, the ratio of the specific activities of microsomes to mitochondria (P/M) is not the same. We then tend to conclude that the microsomal contamination of washed mitochondria is due to the presence of "heavy" microsomes, contributing relatively much glucose-6-phosphatase activity.

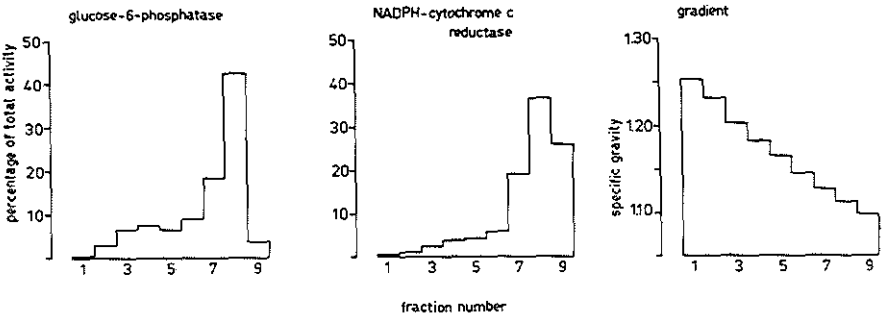

Fig. 1. The distribution of glucose-6-phosphatase and NADPH–cytochrome *c* reductase in fractions from rat liver microsomes, isolated by continuous sucrose density gradient centrifugation. A 20% rat liver homogenate, in 0.25 M sucrose–0.01 M Tris–HCl (pH 7.4), was centrifuged for 10 min at 27000 × g_{max}. 10 ml of the 27000 × *g* supernatant were brought on the gradient. For enzyme assays see the legend to Table I. The percentage of the total activity in Fractions 1–9 is plotted against the fraction No. For further details of gradient fractionation see ref. 5. The recovery of the enzyme activities in the gradient fractions was 81% for glucose-6-phosphatase and 101% for NADPH–cytochrome *c* reductase. The specific activities of the enzymes in isolated microsomes are given in Table I.

TABLE I

SPECIFIC ACTIVITIES OF GLUCOSE-6-PHOSPHATASE AND NADPH–CYTOCHROME *c* REDUCTASE IN MICROSOMES (P) AND HEAVY MITOCHONDRIA (M) ISOLATED FROM RAT LIVER

The subcellular fractions were prepared by differential centrifugation of a rat liver homogenate as described by DE JONG AND HÜLSMANN[7]. NADPH–cytochrome *c* reductase was measured spectrophotometrically according to SOTTOCASA *et al.*[2]. Rotenone (1.5 μM) was present and the reaction was started by the addition of cytochrome *c*. Glucose-6-phosphatase was measured as described by BEAUFAY *et al.*[1]. Enzyme activities were measured at 37°. The means and the standard error of the means of the enzyme activities are given as μmoles of substrate metabolized per mg of protein per h.

Enzyme	*Number of experiments*	*P*	*M*	*P/M*
Glucose-6-phosphatase	4	13.2 ± 3.3	1.40 ± 0.17	9.4
NADPH–cytochrome *c* reductase	4	11.2 ± 1.0	0.52 ± 0.10	21.6

BRUNNER AND BYGRAVE[4] conclude from their experiments with mitochondria washed several times that glucose-6-phosphatase and NADPH–cytochrome *c* reductase are localized not only in microsomes but also in the mitochondrial outer membrane. In order to investigate this further, we determined the distribution of these enzymes over inner and outer mitochondrial membranes. In Fig. 2 a representative experiment out of a series of 5 is shown. As marker enzymes for the inner and outer membranes, cytochrome *c* oxidase and rotenone-insensitive NADH–cytochrome *c* reductase are used, respectively. Glutamate dehydrogenase (EC 1.4.1.2) is used as a marker for the soluble mitochondrial matrix[6]. The mitochondria are subfractionated by a modification[5] of the method of PARSONS *et al.*[8]. The enzymes glucose-6-phosphatase and NADPH–cytochrome *c* reductase have a distribution totally different from the outer membrane marker and the inner membrane matrix markers. This

indicates very strongly that these enzymes (which are both firmly membrane-bound) are not localized in any mitochondrial membrane, but in the microsomes. The different distribution of these microsomal enzymes over the submitochondrial fractions from the gradient can be explained by the observed heterogeneous distribution over the microsomal subfractions as shown in Fig. 1. In Fig. 2 also, relatively more glucose-

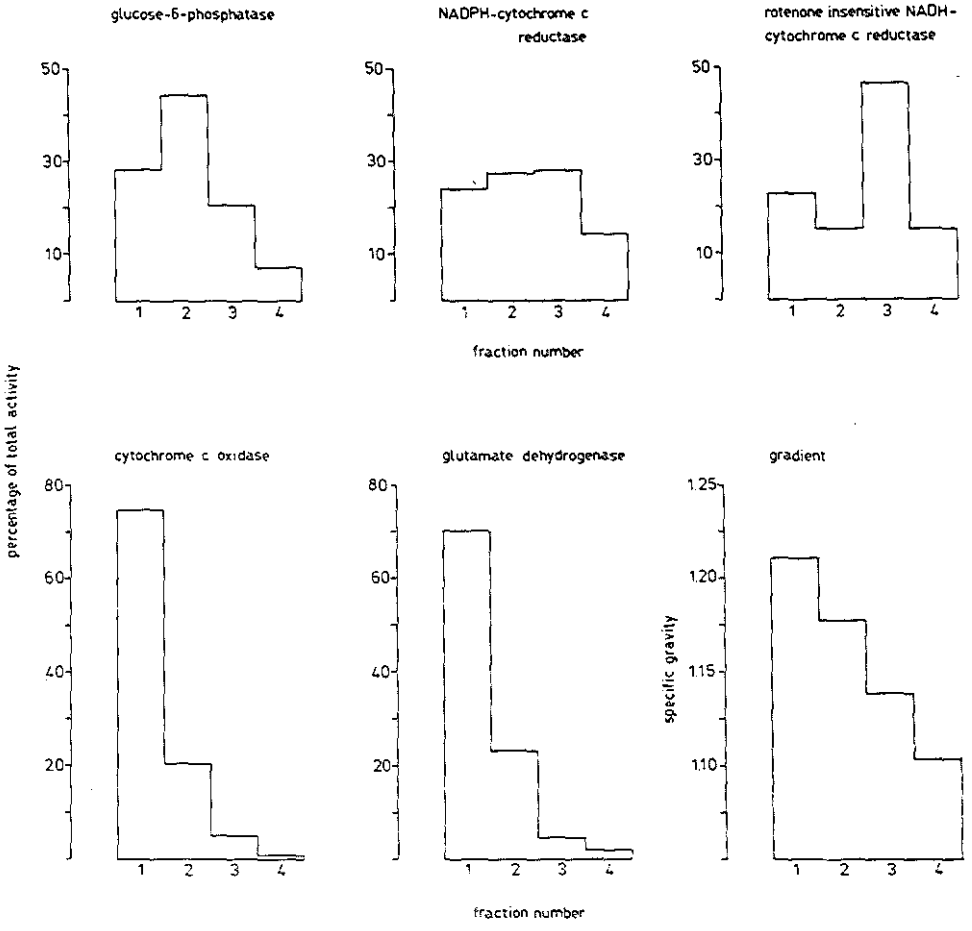

Fig. 2. The distribution of glucose-6-phosphatase, NADPH–cytochrome *c* reductase and some marker enzymes in submitochondrial fractions, isolated from twice-washed rat liver mitochondria. The mitochondria were subfractionated as described before[5], with a minor modification: instead of fractions of 3 ml, 4 fractions of 6 ml were collected. For the estimation of glucose-6-phosphatase and NADPH–cytochrome *c* reductase, see the references given in the legend to Table I. Rotenone-insensitive NADH–cytochrome *c* reductase and cytochrome *c* oxidase were measured according to Sottocasa *et al.*[2] and glutamate dehydrogenase according to Beaufay *et al.*, as modified by Hogeboom. Mitochondria and submitochondrial fractions were treated with ultrasonic vibration (Branson S-75 sonifier; 1 min at 20 kHz). The percentage of the total activity is plotted against the fraction No. The recovery of the activities of the different enzymes in the fractions varied between 85 and 110 %. The specific activities of glucose-6-phosphatase, NADPH–cytochrome *c* reductase in isolated mitochondria are given in Table I. The latter enzymes in sonicated mitochondria were 18.5, 112 and 13.3 μmoles/mg protein/min. Rotenone-insensitive NADH–cytochrome *c* reductase, cytochrome *c* oxidase and glutamate dehydrogenase respectively. All enzyme activities were measured at 37°.

6-phosphatase is found in the gradient at a site of higher density as compared with NADPH–cytochrome *c* reductase.

The heterogeneous distribution of microsomal marker enzymes over microsomal subfractions as presented here is in agreement with the data reported by DALLNER *et al.*[10,11] and by TATA[12].

It is concluded that glucose-6-phosphatase and NADPH–cytochrome *c* reductase are not endogenous constituents of mitochondrial outer membranes and can therefore be used as marker enzymes for microsomes.

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DUAL LOCALIZATION AND PROPERTIES OF ATP-DEPENDENT LONG-CHAIN FATTY ACID ACTIVATION IN RAT LIVER MITOCHONDRIA AND THE CONSEQUENCES FOR FATTY ACID OXIDATION

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SUMMARY

1. Direct evidence is given for the existence of two ATP-dependent palmitoyl-CoA synthesizing enzymes, localized in different compartments of the rat-liver mitochondrion.

2. About 90 % of the total activity of rat liver mitochondria is localized in the mitochondrial outer membrane and about 10 % in the inner membrane-matrix compartment.

3. The two enzyme systems show different apparent K_m's for fatty acid and ATP, and different apparent K_i's for AMP and adenosine.

4. The inner membrane-matrix enzyme, in contrast to the outer membrane and the microsomal enzyme, is strongly inhibited by octanoate.

5. Comparison of the kinetics of the two ATP-dependent long-chain fatty acid-activating enzymes with the kinetics of long-chain fatty acid oxidation shows, that during fatty acid oxidation at low concentrations of palmitate or oleate, in the presence of carnitine, the outer membrane acyl-CoA synthetase is operating. In the absence of carnitine and at high concentrations of long-chain fatty acids the activation reaction occurs in the inner membrane-matrix compartment of the mitochondrion.

6. The long-chain fatty acid concentration needed for half-maximal velocity of fatty acid oxidation by isolated rat liver mitochondria is about 1 μM in the presence of carnitine and 100–200 μM in the absence of carnitine.

INTRODUCTION

The subcellular distribution of the ATP-dependent long-chain acyl-CoA synthetase (acid: CoA ligase (AMP), EC 6.2.1.3) in the rat liver cell has been extensively studied[1–5]. It is generally agreed that the enzyme is localized both in mitochondria and microsomes. In fact we found that mitochondria and microsomes exhibit about equal activities[3,4]. The study of the intramitochondrial localization of long-chain fatty acid activation has obtained much less attention. NORUM *et al.*[6] reported "that the mitochondrial ATP-dependent enzyme most likely is confined to the outer membrane". Also from experiments of VAN DEN BERGH *et al.*[7], it can be concluded that the bulk of

the ATP-dependent long-chain fatty acid activation is localized in the mitochondrial outer membrane.

The data of the present paper support these findings. In addition we are able to demonstrate, by the use of Nagarse (subtilopeptidase A; EC 3.4.4.16), which destroys the greater part of ATP-dependent long-chain fatty acid activation in rat-liver mitochondria[4,8], that the activity remaining after Nagarse treatment is localized in the inner membrane-matrix compartment. Several properties of the two enzyme systems are described.

BREMER[9] and FRITZ[10] have suggested that activated long-chain fatty acids are transported through the mitochondrial inner membrane, which is impermeable for acyl-CoA[11], as acylcarnitines. This is very likely to be the case with heart sarcosomes, but for liver mitochondria an alternative mechanism may well exist. VAN DEN BERGH[12] and ROSSI *et al.*[13] reported carnitine-independent oxidation of long-chain fatty acids by rat liver mitochondria, an observation which is confirmed by the present study. DE JONG AND HÜLSMANN[14] showed that Nagarse treatment does not impair palmitate oxidation by rat liver mitochondria.

From the kinetics of the carnitine-independent long-chain fatty acid oxidation and the dual localization and properties of long-chain fatty acid activation, it is concluded that in the absence of carnitine the fatty acid activation needed for fatty acid oxidation occurs in the inner membrane-matrix compartment of the rat liver mitochondrion.

MATERIALS AND METHODS

Reagents

Nagarse was purchased from Serva Entwicklungslabor, Heidelberg. Bovine serum albumin, Fraction V, was supplied by Sigma Chemical Co., St. Louis, Mo. or by Pentex Inc., Kankakee, Ill. Fatty acids were removed from the albumin by charcoal treatment[15]. After dialysis and Millipore filtration, the albumin was used as a clear, neutral solution. Fatty acid–albumin solutions were prepared by mixing the potassium salt of the fatty acid with albumin in a molar ratio of 7:1. *p*-Dimethylaminobenzylamine (Lot-KP-1065) was purchased from Cyclo Chemical (Division Travenol Laboratories Inc., Los Angeles, Calif.), rotenone from Penick and Co., New York, N.Y. and antimycin A and oligomycin from Sigma.

DL-[*Me*-^{3}H]Carnitine with specific activity of 75 μC/μmole was kindly donated by Dr. J. Bremer from Oslo University, and L-carnitine was a gift from Otsuka Pharmaceutical Factory (Osaka, Japan). The radioactive carnitine was diluted with L-carnitine to a specific activity of 0.05 μC/μmole L-carnitine. Radioactive fatty acids were purchased from the Radiochemical Centre (Amersham, England). All other reagents were prepared exactly as described before[3].

Animals

Male rats of the Wistar strain were used for the experiments (weight 200–250 g). The animals had free access to food and water and were killed by cervical fracture and subsequent bleeding.

Preparation of subcellular fractions

Twice-washed heavy rat liver mitochondria were prepared exactly as described before[3]. Nagarse-treated mitochondria were prepared by stirring a 10% rat liver homogenate in 0.25 M sucrose–0.01 M Tris–HCl (pH 7.4) with 0.2 mg/ml Nagarse for 30 min at 0° before the mitochondria were isolated and washed twice. 12000 × *g* supernatant was isolated by carefully pipetting off the supernatant after centrifuging a 5100 × *g* supernatant of a 10% rat liver homogenate for 10 min at 12000 × g_{max}.

Separation of mitochondrial membranes

The method used to separate the mitochondrial outer membranes from the inner membranes is a modification of the method of PARSONS *et al.*[16] described before[3]. Instead of nine fractions of 3 ml, however, four fractions of 6 ml were collected (see also ref. 17). The remaining content of the gradient tube is not plotted in the figures, but is considered when calculating the recoveries.

Long-chain fatty acid oxidation by isolated rat liver mitochondria

Mitochondria, used for the oxidation experiments, were isolated from a 10% rat liver homogenate, as described before[3], and washed once. O_2 uptake was measured with a Clark "oxygen electrode". The reaction medium contained 32 mM KCl, 4.5 mM $MgCl_2$, 73 mM Tris–HCl, 1 mM EDTA, 1 mM DL-malate, 2.3 mM ATP, 2.3 mM ADP, 8 mM potassium phosphate buffer, 20 mM sucrose and about 3 mg of mitochondrial protein. Variable amounts of fatty acid, complexed to albumin in a molar ratio of 7:1, and 0.5 mM L-carnitine were added as indicated. The reaction volume was 2.2 ml, the temperature 37°, and the pH 7.4.

Enzyme assays

Long-chain acyl-CoA synthetase was measured in three ways. Firstly as a modification of the method of FARSTAD *et al.*[1], described before[3], in the presence of 5 mM KCN (Method A). Secondly by using the same incubation medium as in Method A except that [1-^{14}C]palmitate or [1-^{14}C]oleate was used together with nonradioactive carnitine. After incubation, the radioactive fatty acid, fatty acyl-CoA and acylcarnitine were extracted and separated as described before[18]. The chromatogram was developed with chloroform–methanol–water (77:35:7, by vol.). The radioactive acyl-CoA and acylcarnitine were scraped from the thin-layer plate and transferred to counting vials. After the addition of 10 ml of toluene (containing 5 g of 2,5-diphenyl-oxazole, 0.3 g of dimethyl-1,4-bis-[5-phenyl-oxazolyl-2]benzene and 30 g of thixotropic gel powder per l), the radioactivity was measured by liquid scintillation counting (Method B). In the third method (C), fatty acid activation was measured as hydroxamate formation, as described by PANDE AND MEAD[2]. The incubation medium was modified and contained: a hydroxylamine–Tris mixture of pH 7.4 (500 mM hydroxylamine and 100 mM Tris), 20 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5–1.0 mM potassium palmitate, 3 μg oligomycine, 5 mM GSH, 0.5 mM CoA, 5 mM ATP, 5 mM phosphoenolpyruvate, 2 units each of pyruvate kinase and adenylate kinase, and mitochondrial protein as indicated. After 1 min preincubation, the reaction was started with the addition of a warm solution of potassium palmitate in water. The reaction volume was 1 ml, the temperature, 37° and the reaction time, 30–60 min. Monoamine oxidase (EC 1.4.3.4) was assayed according to the method of DEITRICH AND ERWIN[19]

with *p*-dimethylaminobenzylamine as a substrate. Other enzymes and protein were measured as described elsewhere[3,17]. All enzyme assays were carried out at 37°.

Where indicated, mitochondria and submitochondrial fractions were treated with ultrasonic vibration before incubation (Branson S-75 sonifier; 3 min at 20 kHz). The temperature was maintained between 0 and 4° by cooling in an ice–salt bath.

RESULTS AND DISCUSSION

Intramitochondrial localization of ATP-dependent long-chain acyl-CoA synthetase in untreated mitochondria

In Fig. 1 the distribution of ATP-dependent long-chain acyl-CoA synthetase and the distribution of marker enzymes of submitochondrial fractions are shown. The figures represent one typical experiment out of a series of three. As marker enzymes for the inner and outer membranes, cytochrome *c* oxidase (EC 1.9.3.1) and rotenone-insensitive NADH–cytochrome *c* reductase are used, respectively[20,21] (*cf.* also refs. 3 and 17). After swelling in 20 mM phosphate buffer, the mitochondria are brought on a continuous sucrose density gradient and centrifuged for 2 h in the Beckman ultracen-

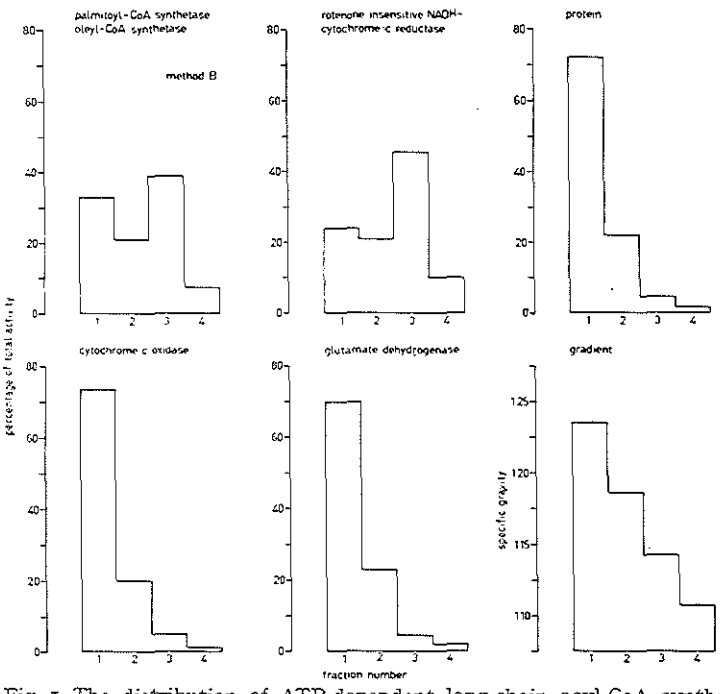

Fig. 1. The distribution of ATP-dependent long-chain acyl-CoA synthetase and some marker enzymes in submitochondrial fractions isolated from twice-washed rat liver mitochondria. After swelling in 20 mM phosphate buffer (pH 7.2) containing 0.02 % of bovine serum albumin, the outer and inner membranes were separated in a continuous sucrose density gradient. The gradient was fractionated and the enzymes were assayed as described in MATERIALS AND METHODS. Mitochondria and submitochondrial fractions were treated with ultrasonic vibration before incubation (see MATERIALS AND METHODS). The percentage of the total activity in Fractions 1–4 is plotted against the fraction number. The recovery of the activities of the different enzymes in the gradient fractions varied between 87 and 104 %. For specific activities in twice-washed rat liver mitochondria and further details of the enzyme assays, see Table I and refs. 3 and 17.

trifuge at 131000 × g_{max} (rotor SW-27). For details of the gradient fractionation, see MATERIALS AND METHODS. As can be seen from Fig. 1 this method yields a good separation of outer membranes and inner membranes. In these experiments the soluble matrix enzyme glutamate dehydrogenase (EC 1.4.1.2)[22] has the same distribution as the inner membrane enzyme cytochrome *c* oxidase. This indicates that the fractionation procedure is so mild that the inner membrane remains intact during the fractionation. More than half of the total activity of rotenone-insensitive NADH–cytochrome *c* reductase is found in Fractions 3 and 4. In these fractions the percentages of cytochrome *c* oxidase and glutamate dehydrogenase are very low, so that it can be concluded that the outer membrane fractions are very little contaminated with inner membrane-matrix enzymes. Palmitoyl-CoA synthetase and oleyl-CoA synthetase have exactly the same distribution and both very much like the outer membrane marker enzyme rotenone-insensitive NADH–cytochrome *c* reductase, except that a reproducible and significantly higher percentage of the total activity is recovered in the inner membrane-matrix fraction (Fraction 1). This indicates that the bulk of the ATP-dependent long-chain acyl-CoA synthetase is localized in the mitochondrial outer membrane, but it suggests also that some activity is present in the inner membrane-matrix fraction. From studies of DE JONG AND HÜLSMANN[4] and PANDE AND BLANCHAER[8] it is evident that Nagarse (a subtilopeptidase A) can very effectively destroy ATP-dependent long-chain acyl-CoA synthetase. The synthetase is destroyed for more than 90 %. In rat liver mitochondria a small percentage of the acyl-CoA synthetase is resistant to Nagarse (Table I, *cf.* ref. 14). This resistant part of the enzyme is probably not accessible to Nagarse. This, together with the information obtained from the gradient experiments (Fig. 1), led us to think of the possibility that the ATP-dependent long-chain acyl-CoA synthetase of rat liver mitochondria could have a double localization. We therefore decided to investigate this further and to perform the same experiments as illustrated in Fig. 1 with Nagarse-treated mitochondria.

Intramitochondrial localization of ATP-dependent long-chain acyl-CoA synthetase in Nagarse treated mitochondria

Table I shows the effect of Nagarse on the marker enzymes used and on the ATP-dependent long-chain acyl-CoA synthetase. Whereas cytochrome *c* oxidase is totally resistant, the outer membrane marker enzyme rotenone-insensitive NADH–cytochrome *c* reductase is about 35 % destroyed by the Nagarse treatment (see MATERIALS AND METHODS). Another outer membrane marker enzyme, monoamine oxidase, is totally resistant to Nagarse treatment[4,8]. Monoamine oxidase and rotenone-insensitive NADH–cytochrome *c* reductase have exactly the same distribution, whether mitochondria are treated with Nagarse prior to fractionation or not. Also Nagarse treatment neither changes cytochrome *c* oxidase nor glutamate dehydrogenase distribution patterns after fractionation (*cf.* Fig. 1 and Fig. 2). Therefore it can be concluded that Nagarse treatment has no effect on the localization of the marker enzymes used. However, Nagarse treatment has a very clear effect on the distribution pattern of the ATP-dependent long-chain acyl-CoA synthetase. Without Nagarse treatment of mitochondria, about 90 % of the total activity is localized in the outer membranes (see Fig. 1), but after Nagarse treatment there appears an opposite picture: about 90 % of the total activity is localized in the inner membrane-matrix fraction (see Fig. 2). We conclude from these experiments that the ATP-dependent long-chain fatty acid

TABLE I

EFFECT OF NAGARSE TREATMENT ON MITOCHONDRIAL ENZYME ACTIVITIES

Palmitoyl-CoA synthetase activity was proportional to protein in untreated as well as in Nagarse-treated mitochondria. In Method A about 0.1 mg (untreated mitochondria) or 1–2 mg (Nagarse-treated mitochondria) of protein was incubated for 5–10min with 0.5 mM potassium palmitate, complexed with 0.07 mM bovine serum albumin. In Method C about the same amounts of protein were incubated with 1 mM potassium palmitate for 30–60 min. For preparation of the mitochondria (untreated and Nagarse-treated) and for further details of enzyme assays, see MATERIALS AND METHODS. The means and the standard error of the means of the enzyme activities are given as nmoles of substrate metabolized per mg of protein per min.

Enzyme:	*Palmitoyl-CoA synthetase*				*Rotenone insensitive NADH–cytochrome c reductase*		*Cytochrome c oxidase*	
Number of experiments:	*7*		*5*		*4*		*3*	
	Method A	*Inhibition* (%)	*Method C*	*Inhibition* (%)		*Inhibition* (%)		*Inhibition* (%)
Untreated mitochondria	69.5 ± 14.5	—	77.7 ± 11.0	—	415 ± 98	—	2283 ± 517	—
Nagarse-treated mitochondria	3.5 ± 0.8	95	3.8 ± 0.7	95	268 ± 83	35	2400 ± 533	0

TABLE II

COMPARISON OF SOME KINETIC PROPERTIES OF THREE ATP-DEPENDENT LONG-CHAIN ACYL-CoA SYNTHETASES, LOCALIZED IN DIFFERENT PARTS OF THE RAT LIVER CELL

Palmitoyl-CoA synthetase is measured as described in MATERIALS AND METHODS (Method A). In untreated mitochondria, Nagarse-treated mitochondria and 12000 × *g* supernatant the properties of the fatty acid-activating enzymes, present, respectively, in the outer mitochondrial membrane, the inner mitochondrial membrane-matrix compartment and the microsomes, are estimated (for motivation, see the text). K_m and K_I values must be regarded as apparent and were determined by the Lineweaver–Burk method (see *e.g.* ref. 23 and Fig. 3). Inhibitions by AMP and adenosine were competitive with respect to ATP in all enzyme fractions tested. For preparation of the enzyme fractions and details of the enzyme assay, see MATERIALS AND METHODS. In the measurement of the K_I for AMP, the ATP-regenerating system (phosphoenolpyruvate, adenylate kinase and pyruvate kinase) was omitted from the incubation medium.

Enzyme fraction	*Untreated mitochondria*	*Nagarse-treated mitochondria*	*12000 × g supernatant*
K_m palmitate (mM)	0.05	0.18	0.11
K_m ATP (mM)	0.7	0.2	0.4
K_I AMP (mM)	0.2	0.8	0.2
K_I adenosine (mM)	0.1	0.2	0.1

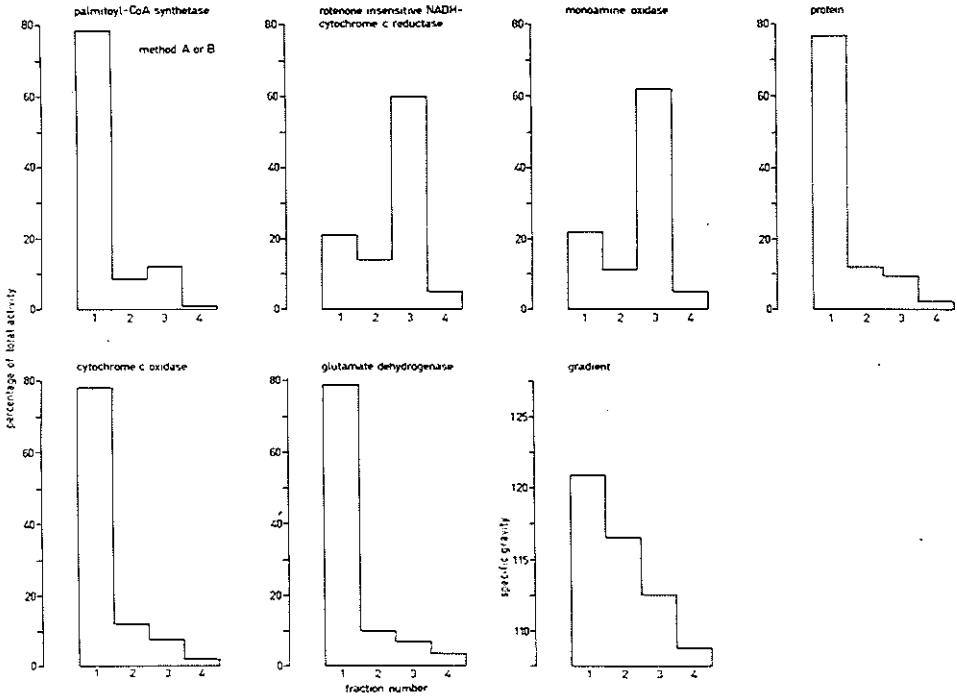

Fig. 2. The distribution of ATP-dependent long-chain acyl-CoA synthetase and some marker enzymes in submitochondrial fractions isolated from twice-washed Nagarse-treated rat liver mitochondria. For separation of mitochondrial membranes, fractionation of the sucrose gradient and enzyme assays, see MATERIALS AND METHODS and the legend to Fig. 1. The percentage of the total activity in Fractions 1–4 is plotted against the fraction number. The recoveries of the activities of the different enzymes in the gradient fractions varied between 91 and 110%. Specific activities in Nagarse-treated twice-washed rat liver mitochondria are given in Table I (see also refs. 3, 4 and 17).

activation in rat liver mitochondria has a dual localization. The enzyme is localized both in the outer membrane and in the inner membrane-matrix compartment.

Kinetics of the ATP-dependent long-chain fatty acid activating enzymes from rat liver

In Table II some properties of the differently localized, ATP-dependent long-chain fatty acid-activating enzymes of the rat liver cell are compared. For the microsomal enzyme we used 12000 × *g* supernatant (see MATERIALS AND METHODS). This supernatant does not contain a significant amount of mitochondria, and the soluble protein of the rat liver cell does not contain long-chain fatty acid activation[1–3]. For estimating the properties of the outer membrane-localized fatty acid activation we used twice-washed, intact, heavy rat liver mitochondria, which when prepared by differential centrifugation are little contaminated with microsomes[3]. Because about 90% of long-chain fatty acid activation resides in the outer membrane, it was not necessary to isolate the mitochondrial outer membranes separately. For the estimation of the properties of the inner membrane-matrix fatty acid-activating activity, Nagarse-treated mitochondria were used after sonication. Sonication enhances this activity under the experimental conditions used, whereas in mitochondria not treated with Nagarse, sonication has no detectable effect on the activity of long-chain fatty acid activation.

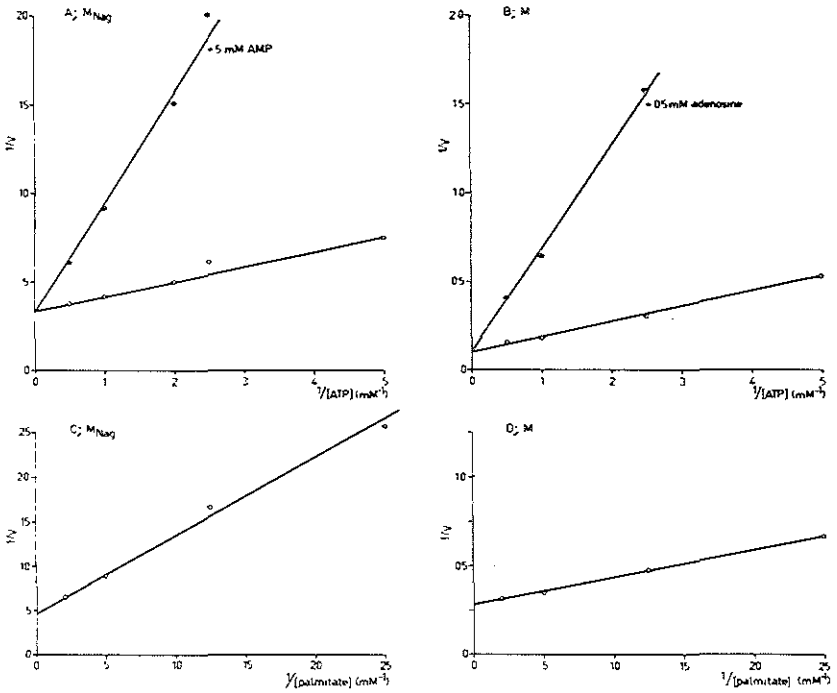

Fig. 3. Kinetic properties of two ATP-dependent long-chain acyl-CoA synthetases, present, respectively, in the outer membrane (B and D) and in the inner membrane-matrix compartment (A and C) of rat liver mitochondria. Palmitoyl-CoA synthetase is measured as described in MATERIALS AND METHODS (Method A). In untreated and Nagarse-treated mitochondria the properties of the fatty acid-activating enzymes, present, respectively, in the mitochondrial outer membrane and the mitochondrial inner membrane-matrix compartment, are estimated (for motivation see the text). K_m and K_i values derived from these figures (see Table II) must be regarded as apparent values. Lineweaver–Burk plots (see *e.g.* ref. 23) are given for the substrates palmitate (C and D) and ATP (A and B) in untreated and Nagarse-treated mitochondria. It can be seen that the inhibitions of AMP in Nagarse-treated mitochondria and of adenosine in untreated mitochondria are of the competitive type with respect to ATP. The enzyme activities (v) are given as μmoles of substrate metabolised per mg of protein per h.

As can be seen from Fig. 2, in Nagarse-treated mitochondria the bulk of the fatty acid activation is localized in the inner membrane-matrix compartment. The values given in Table II are derived from Lineweaver–Burk plots (*cf.* ref. 23) of which some are shown in Fig. 3. The inhibitions by AMP[2,14] and by adenosine[14] are both of the competitive type with respect to ATP in the microsomal enzyme (12 000 × g supernatant) as well as in the two mitochondrial enzyme systems (untreated and Nagarse-treated mitochondria). With respect to these inhibitions, the microsomal and the outer membrane enzyme respond in the same way, that is they have a low apparent K_i for AMP and adenosine when compared to the inner membrane-matrix enzyme system. In addition to this characteristically low K_i for AMP inhibition, there is another important characteristic: the apparent K_m for ATP is relatively high for the microsomal enzyme and even higher for the outer membrane fatty acid-activating enzyme. This means that a lowering of the phosphorylation state in the compartment outside the mitochondrial inner membrane could result in inhibition of fatty acid activation. The

TABLE III

INHIBITION OF THREE ATP-DEPENDENT LONG-CHAIN ACYL-CoA SYNTHETASES, LOCALIZED IN DIFFERENT PARTS OF THE RAT LIVER CELL BY SHORT- AND MEDIUM-CHAIN FATTY ACIDS

For preparation of the enzyme fractions and details of the palmitoyl-CoA synthetase assay (Method C), see MATERIALS AND METHODS and the legend to Table I. In the 12000 × *g* supernatant, untreated and Nagarse-treated mitochondria the properties of the palmitate activating enzymes, present respectively, in the microsomes, the mitochondrial outer membrane and the mitochondrial inner membrane-matrix compartment, are estimated (for motivation, see the text). The means and (if possible) the standard error of the means of the palmitoyl-CoA synthetase activities are given.

Additions	*Number of experiments*	*Palmitoylhydroxamate formation (nmoles/mg of protein per min)*					
		12000 × g supernatant	*Inhibition (%)*	*Untreated mitochondria*	*Inhibition (%)*	*Nagarse-treated mitochondria*	*Inhibition (%)*
None	4	43.3 ± 7.7	—	79.2 ± 12.5	—	4.0 ± 0.8	—
0.5 mM sodium octanoate	4	38.3 ± 6.0	12	67.2 ± 10.0	15	1.3 ± 0.2	68
5 mM sodium butyrate	2	44.5	0	80.0	0	2.0	50
10 mM sodium propionate	2	43.5	0	80.8	0	2.5	38

phosphorylation state in the inner membrane *plus* matrix fraction is much lower than that outside the inner membrane[24,25]. Moreover WALTER AND STUCKI[26] showed that the intramitochondrial AMP level during fatty acid oxidation is elevated about 5 times above the control level. Since the K_m for ATP of fatty acid activation in the inner membrane-matrix compartment is lower and the K_i for AMP is higher, when compared to fatty acid activation outside the mitochondrial inner membrane, fatty

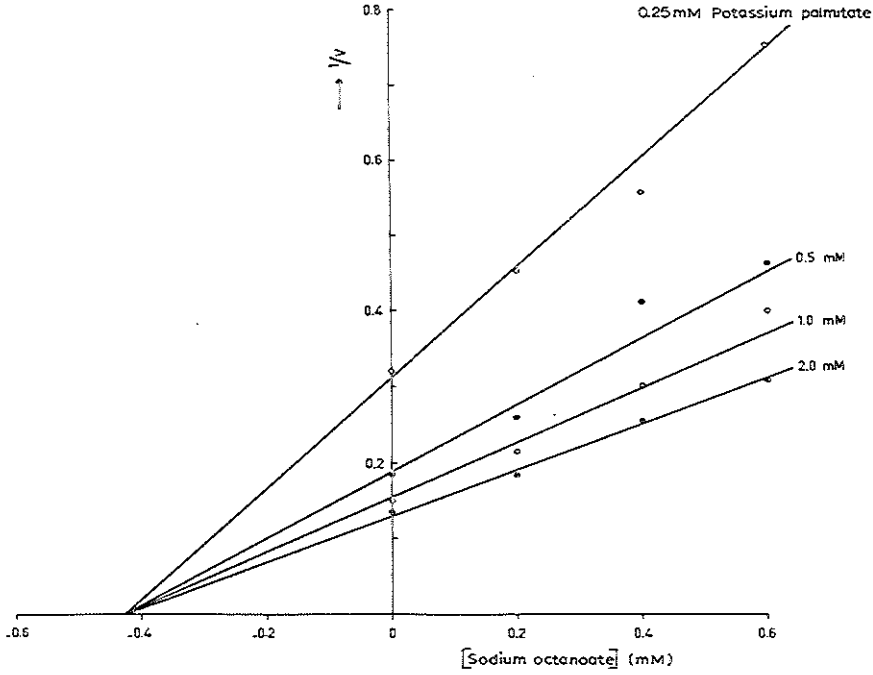

Fig. 4. Inhibition of ATP-dependent long-chain acyl-CoA synthetase by octanoate in Nagarse-treated twice-washed rat liver mitochondria. Palmitoyl-CoA synthetase was measured as described in MATERIALS AND METHODS (Method C). For preparation of Nagarse-treated mitochondria and further details of the enzyme assay see MATERIALS AND METHODS and the legend to Table I. The reciprocal value of the enzyme activity (v in nmoles of palmitate metabolised per mg of protein per min) is plotted against the octanoate concentration (Dixon plot, see *e.g.* ref. 23). As can be seen from the figure, the inhibition by octanoate is probably noncompetitive with respect to palmitate. The K_i for octanoate is 0.4 mM (compare Table III).

acid activation and oxidation can proceed under state 3 conditions (see Fig. 5). This is in agreement with the observation of DE JONG AND HÜLSMANN[14], who showed that in isolated rat liver mitochondria the rate of palmitate oxidation in state 3 is even higher than in state 4.

There is also an appreciable difference in the apparent K_m for palmitate (complexed to albumin) between the outer membrane enzyme and the inner membrane-matrix enzyme system. The inner membrane-matrix enzyme requires a 3–4 times higher palmitate concentration for half-maximal activity than the outer membrane enzyme.

From Table III and Fig. 4 it can be seen that the inner membrane-matrix ATP-dependent long-chain fatty acid activation is inhibited by 67 % with 0.5 mM octano-

ate. In these experiments fatty acid activation was measured as an ATP-dependent hydroxamate formation (see MATERIALS AND METHODS). 5 mM butyrate and 10 mM propionate, under the same conditions, inhibit 50 and 38 %, respectively. As can be seen from Fig. 4 the octanoate inhibition of the inner membrane-matrix enzyme is probably of a noncompetitive type with respect to palmitate. This, together with the relatively weak inhibitions by butyrate and propionate, suggests that the enzyme activating palmitate in the inner membrane-matrix compartment is different from the medium- and short-chain ATP-dependent fatty acid-activating enzymes reported by AAS AND BREMER[27] to be localized in the matrix of rat liver mitochondria. More experiments, however, are needed to differentiate definitely between different enzymes activating fatty acids of various chain lengths localized in the inner membrane and/or matrix of rat liver mitochondria. This study, mainly focussed on long-chain fatty acid activation, shows that there are two ATP-dependent long-chain activating enzyme systems in rat liver mitochondria, one in the outer membrane fraction and one in the inner membrane *plus* matrix fraction (*cf.* ref. 14).

Kinetics of carnitine-dependent and carnitine-independent long-chain fatty acid oxidation by isolated rat liver mitochondria

Fig. 5 shows the influence of the long-chain fatty acid concentration on the O_2 uptake by isolated rat liver mitochondria. The conditions used are optimal for O_2 uptake. It can be seen that in the presence of carnitine a normal Lineweaver–Burk plot is obtained. Oleate and palmitate give exactly the same oxidation rates. The apparent maximal Q_{O_2} in the presence of carnitine is 105 and the apparent K_m for long-chain fatty acid is 1 μM. Without added carnitine the apparent maximal Q_{O_2} is higher with palmitate than with oleate (133 and 100, respectively) and the apparent K_m for fatty acid is 100 μM for palmitate and 200 μM for oleate. The differences between the kinetics of palmitate and oleate oxidation are possibly due to a higher binding affinity of the bovine serum albumin used for oleate when compared to palmitate. As can be seen

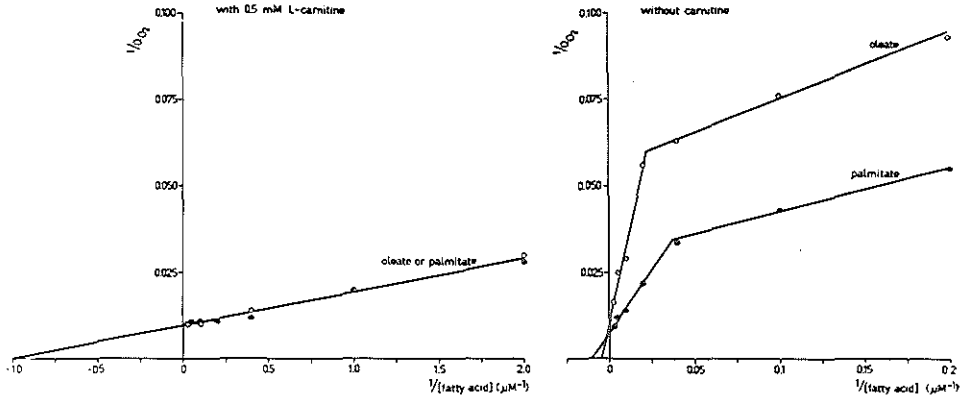

Fig. 5. Kinetics of carnitine-dependent and carnitine-independent long-chain fatty acid oxidation by isolated rat liver mitochondria. O_2 uptake was measured with a Clark "oxygen electrode". For details of the incubation conditions see MATERIALS AND METHODS. The reciprocal value of the Q_{O_2} (μl O_2 metabolized per mg protein per h) is plotted against the reciprocal value of the fatty acid concentration. The Q_{O_2} values shown in this Lineweaver–Burk plot are corrected for O_2 uptake in the absence of added fatty acid (about 40 μl O_2/mg protein per h). The fatty acids used were complexed to albumin in a molar ratio of 7:1.

from Fig. 5 the Lineweaver–Burk plot obtained in the absence of carnitine does not give one straight line in the range of fatty acid concentrations used (5–400 μM). If the fatty acid concentration is below about 40 μM (this is the range shown in the presence of carnitine), the Q_{O_2} is very low. Above about 40 μM there is a sharp cut off in the Lineweaver–Burk plot and the higher concentrations are oxidized rapidly. The sharp cut off indicates that a new enzyme reaction has become rate-limiting in the oxidation proces. Under the conditions used, the oxidative phosphorylation is not limited (ADP and P_i are present). Therefore the increased rate of O_2 consumption at higher concentrations of fatty acid cannot be due to uncoupling effects. In fact, under the conditions used, the mitochondria show respiratory control with palmitate at concentrations up to 0.4 mM (not shown; see also ref. 14). It is very likely then, that at higher fatty acid concentrations the fatty acid is activated by a different activating enzyme. The palmitoyl-CoA synthetase present in the mitochondrial outer membrane has an apparent K_m for fatty acid of 0.05 mM and the palmitoyl-CoA synthetase present in the inner membrane-matrix compartment requires 0.18 mM palmitate for half-maximal activity (see Table II). The latter K_m is of the same order of magnitude as the K_m for long-chain fatty acid in fatty acid oxidation in the absence of carnitine. Therefore we conclude that in the absence of carnitine if the fatty acid concentration is relatively high, the long-chain fatty acids are activated by the ATP-dependent long-chain acyl-CoA synthetase present in the inner membrane-matrix compartment of rat liver mitochondria. If palmitate is oxidized completely to CO_2 and H_2O, the measured activity of the palmitate activation in the inner membrane-matrix compartment, about 3.7 nmoles/mg protein per min (Table I), can theoretically result in a Q_{O_2} for palmitate oxidation at 37° of about 110. This agrees very well with the observed oxidation rates of 100–133 (see Fig. 5).

In the presence of carnitine, the fatty acids needed for fatty acid oxidation can be activated by acyl-CoA synthetase localized outside the barrier for acyl-CoA as well as by acyl-CoA synthetase localized inside this barrier. In the presence of low concentrations of fatty acid (below 40 μM) only the outer membrane enzyme can be operative because of its low K_m for fatty acid and its high activity. As can be seen from Fig. 5 the oxidation of long-chain fatty acid in the presence of carnitine shows an apparent K_m for fatty acid of 1 μM. With this fatty acid concentration the long-chain acyl-CoA synthetase localized in the outer membrane can only reach less than 5 % of its maximal activity (the apparent K_m for palmitate is 50 μM). Because of the very high activity of the ATP-dependent long-chain acyl-CoA synthetase localized in the outer membrane of rat liver mitochondria (Table I), enough acyl-CoA can still be synthesized for optimal fatty acid oxidation in the presence of carnitine at very low levels of long-chain fatty acid.

How much the ATP-dependent long-chain fatty acid activation localized in the inner membrane-matrix compartment *in vivo*, that is in the presence of carnitine[28], contributes to the total amount of activated fatty acid required for fatty acid oxidation and ketone body production has to await further experimentation. That carnitine plays a role in the interaction of fatty acid oxidation and gluconeogenesis is well established[29] and may be demonstrated by its beneficial effect in hypoglycin-induced vomiting sickness[30]. ENTMAN AND BRESSLER[30] showed that after hypoglycin intoxication, long-chain fatty acid oxidation was hampered and that carnitine administration restored palmitate oxidation to normal levels. Administration of carnitine to hypo-

glycin-treated mice prevented both the depression of palmitate oxidation and the hypoglycemia (see also refs. 31 and 32).

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SHORT COMMUNICATIONS

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The effect of carnitine and CoA on ketogenesis and citric acid cycle activity during long-chain fatty acid oxidation by isolated rat liver mitochondria

Recently it was found that in the presence of malate, ADP, ATP and relatively high concentrations of palmitate, maximal rates of oxygen uptake can be observed in the absence of carnitine[1]. In this case the palmitate oxidized is activated in the inner membrane–matrix compartment of the mitochondrion. In the presence of carnitine, the palmitoyl-CoA synthetase (acid:CoA ligase (AMP), EC 6.2.1.3) localized in the outer mitochondrial membrane contributes to the palmitate utilization in the inner membrane–matrix compartment[1]. In the present paper the hypothesis was tested as to whether carnitine addition influences the distribution between the end products of fatty acid oxidation in liver: CO_2 and ketone bodies. FRITZ[2] already observed a preferential stimulation of ketone body production by carnitine.

The hypothesis mentioned above was tested by comparison of oxygen uptake, CO_2 production and ketone body formation under several conditions. Table I shows the results of a representative experiment out of a series of 3 experiments. Carnitine or carnitine *plus* CoA stimulates ketogenesis 2-fold or more in the presence or absence of malate (compare also Fig. 1A). However, oxygen uptake is only stimulated by carnitine or carnitine *plus* CoA in the absence of malate (compare also ref. 1). An inhibition of $^{14}CO_2$ production from [1-^{14}C]palmitate is seen when carnitine or carnitine *plus* CoA is added. This indicates that carnitine and carnitine *plus* CoA stimulate ketogenesis and inhibit the complete oxidation of palmitate by inhibition of the citric acid cycle. During fatty acid oxidation the citric acid cycle may be inhibited at the level of the citrate synthase (EC 4.1.3.7) reaction[3–12]. It has been postulated that this inhibition is caused by a decrease of the intramitochondrial oxaloacetate concentration[7–12]. That carnitine, in the present investigation, also decreases the intramitochondrial concentration of oxaloacetate, is illustrated by the observation (Table I, Fig. 1) that carnitine addition increases the β-hydroxybutyrate/acetoacetate ratio. This correlates with an increase of the intramitochondrial malate/oxaloacetate ratio[13]. Indeed carnitine decreases the synthesis of products of oxaloacetate metabolism: citrate and phosphoenolpyruvate (Table I, Fig. 1B). In the presence of malate, the ATP concentration does not change under the influence of carnitine, and in the absence of malate, a significant hydrolysis of ATP is observed only in the absence of carnitine. It is known that a decrease of the phosphorylation state contributes to a lowering of NADH/NAD$^+$. The importance of intramitochondrial NADH/NAD$^+$ in the regulation of ketogenesis, citrate and phosphoenolpyruvate synthesis is again shown in Fig. 1 (a representative experiment out of a series of 3 is given). Here the time-course of the metabolic events is demonstrated. Ketone body production shows a lag time of about 5 min and is closely related to the β-hydroxybutyrate/acetoacetate ratio (Fig. 1A). The net synthesis of citrate levels off to zero after about 5 min, when the

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TABLE I

FACTORS AFFECTING KETOGENESIS AND THE FORMATION OF CITRATE AND PHOSPHOENOLPYRUVATE DURING PALMITATE OXIDATION

The incubation medium contained 65 mM Tris–HCl, 18.8 mM potassium phosphate buffer, 20 mM KCl, 1 mM EDTA, 25 mM sucrose, 10 mM $MgCl_2$, 7.5 mM ATP and 0.6 mM potassium [1-^{14}C]palmitate (specific activity, 0.4 mC/mmole) complexed with 0.086 mM bovine serum albumin. Where indicated, 0.5 mM L-carnitine, 0.05 mM CoA and/or 5 mM potassium DL-malate were added. Reaction volume, 2 ml; temp., 37°; pH 7.4. Heavy rat liver mitochondria were isolated as described before[16] and washed once. The reaction, carried out in Warburg vessels, was started by the addition of mitochondria (5.0 mg of protein) and incubation was carried out for 30 min in the Gilson differential respirometer. The total oxygen uptake was calculated by extrapolation. The centre well contained a KOH-soaked filter paper. The reaction was stopped by adding 0.1 ml 70% $HClO_4$ from the side arm and the vessels were allowed to shake for another 20 min. The radioactivity present on the filter paper was counted by liquid scintillation counting. The contents of the Warburg vessels was transferred to centrifuge tubes with 2 ml 4% $HClO_4$ and the protein was centrifuged off. The supernatant was neutralised in the cold with KOH, and after removing the $KClO_4$ by centrifugation, the supernatant was analysed for β-hydroxybutyrate[21], acetoacetate[22], citrate[23], phosphoenolpyruvate[24] and ATP[25].

Additions	*$\Delta^{14}CO_2$ (disint./min)*	*$-\Delta O_2$ (μmoles)*	*Δβ-Hydroxybutyrate + Δacetoacetate (μmoles)*	*ΔCitrate (μmoles)*	*ΔPhosphoenol-pyruvate (μmoles)*	*$-\Delta ATP$ (μmoles)*	*β-Hydroxybutyrate / Acetoacetate*
None	22 500	2.7	0.86	—	—	5.3	0.02
Carnitine	20 250	7.6	1.78	—	—	0.7	1.30
Carnitine + CoA	9 800	6.0	3.02	—	—	0.0	6.03
Malate	23 350	9.6	1.06	0.48	0.124	0.0	2.33
Malate + carnitine	10 250	9.5	2.20	0.36	0.062	0.0	9.22
Malate + carnitine + CoA	6 700	6.8	2.10	0.31	0.052	0.0	11.79

ratio increases (Fig. 1B). A less striking inversed relationship between the phosphoenolpyruvate synthesis and the β-hydroxybutyrate/acetoacetate ratio is also shown in Fig. 1B. It may be noted that the ATP concentration is constant under the conditions shown in Fig. 1 (see Table I).

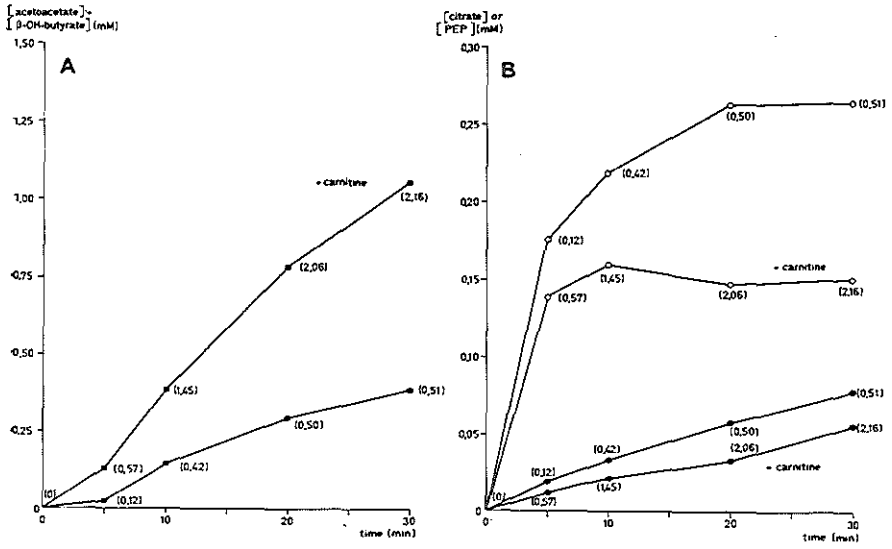

Fig. 1. Influence of carnitine on the time-course of ketogenesis (A), the β-hydroxybutyrate/acetoacetate ratio (given in parentheses) and the production of citrate and phosphoenolpyruvate (PEP) from malate (B), during palmitate oxidation. Incubation conditions were exactly the same as described in the legend to Table I (malate present). At the times indicated, samples of 2.0 ml were taken from the reaction mixture, and the metabolites were estimated in the deproteinized neutralized supernatant as described in the legend to Table I. The concentration of mitochondrial protein in the reaction medium was 2.4 mg/ml. ●—●, phosphoenolpyruvate formation; ○—○, citrate formation; ■—■, ketogenesis.

It may be of interest to note that although the bulk of the phosphoenolpyruvate carboxykinase (EC 4.1.1.32) activity in rat liver is localized in the extramitochondrial compartment[14], significant synthesis of phosphoenolpyruvate occurs in the mitochondria (Table I, Fig. 1B), confirming the observation of SCHOLTE AND TAGER[15].

In conclusion, carnitine addition influences the distribution between the end products of fatty acid oxidation in isolated rat liver mitochondria by making more activated fatty acid available to the β-oxidation system[16–18]. This results in an increased NADH/NAD^+ which decreases the citric acid cycle activity by lowering the intramitochondrial oxaloacetate concentration. This effect of carnitine is most pronounced in the presence of added CoA. Only in this case the full capacity of the palmitoyl-CoA synthetase present in the mitochondrial outer membrane can be used[1,19], since a suboptimal concentration of CoA exists in the sucrose space of isolated, washed rat liver mitochondria[17,19].

These present results are in agreement with the data obtained in perfusion experiments in which an inhibitor of the palmitoyl-CoA:carnitine palmitoyltransferase was employed[20].

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HEPATIC FATTY ACID OXIDATION

Activity, localization and function
of some enzymes involved