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Enzymological Studies of Mitochondrial Fatty Acid Beta-Oxidation

Kuo-Wei Yao

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1997

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This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

December 12, 1996
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ABSTRACT

Enzymological Studies of Mitochondrial Fatty Acid Beta-Oxidation

by

Kuo-Wei Yao

Adviser: Professor Horst Schulz

Mitochondrial enzymes of fatty acid β-oxidation have been studied with the aim of elucidating the regulation of this process in both liver and heart tissues and to develop specific assay methods for diagnostic purposes. Three projects are described. The first one describes a specific assay for the diagnosis of medium-chain acyl-CoA dehydrogenase (MCAD) which is absent in patients with MCAD deficiency. This assay measures spectrophotometrically at 308 nm the formation of cinnamoyl-CoA from 3-phenylpropionyl-CoA in the presence of phenazine methosulfate as an electron acceptor. Since absorbance changes at 308 nm caused by other reactions are less than 5% of the absorbance change due to cinnamoyl-CoA formation catalyzed by medium-chain acyl-CoA dehydrogenase, the assay can be used to measure the activity of this enzyme in crude tissue homogenates.

The second project addresses the relationship between mitochondrial activation and toxicity of some substituted carboxylic acids.

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The activation of 4-bromocrotonic acid, 4-bromo-2-octenoic acid, valproic acid, and 3-methylglycidic acid by conversion to their CoA thioesters and the effects of these carboxylic acids on palmitoylcarnitine-supported respiration were studied with rat liver and rat heart mitochondria. The results showed that the substituted-carboxylic acids that were activated also inhibited palmitoylcarnitine-supported respiration. This leads to the conclusion that substituted medium-chain carboxylic acids, which enter the mitochondria directly, may inhibit β-oxidation as long as they are activated. Liver is more susceptible to inhibition due to the broader substrate specificity of its mitochondrial medium-chain acyl-CoA synthetase.

The last project is a kinetic study of the recently discovered membrane-bound trifunctional β-oxidation complex enzyme having enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase in one complex. The degradation of 20 μM 2-*trans*-hexadecenoyl-CoA to myristoyl-CoA and acetyl-CoA catalyzed by the β-oxidation complex was kinetically analyzed. Quantification of intermediates by HPLC revealed that the steady-state concentrations of 3-hydroxyhexadecanoyl-CoAand 3-ketohexadecanoyl-CoA were lower than their calculated concentrations necessary to sustain the observed rate of the overall reaction. The observed lower-than-expected concentrations of intermediates are indicative of a channeling mechanism which would explain the virtual absence of β-oxidation intermediates from the mitochondrial matrix.

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ABBREVIATIONS

[1] AC, acetate, AD, acyl-CoA dehydrogenase; ADP/ATP, adenine diphosphate/triphosphate; AS, acyl-CoA synthetase; Δ^2C_{16}-CoA, 2-*trans*-hexadecenoyl-CoA; CoASH, Coenzyme A; CPT I, carnitine palmitoyltransferase I; CPT II, carnitine palmitoyltransferase II; DCPIP, 2, 6-dichlorophenolindophenol; DTNB, 5, 5'-dithiobis (2-nitrobenzoic acid); EGTA, ethylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid; EH, enoyl-CoA hydrase; ETF, eletron transfer flavoprotein; FA, fatty acid; FABP, fatty acid binding protein; $FADH_2$ (FAD^+), flavine adenine dinucleotide (oxidized); HAD, L-3-hydroxyacyl-CoA dehydrogenase; HPLC, high-performance liquid chromatography; I_1, 3-hydroxyhexadecanoyl-CoA; I_2, 3-ketohexadecanoyl-CoA; KT, 3-ketoacyl-CoA thiolase; LCAD, long-chain acyl-CoA dehydrogenase; LCEH, long-chain enoyl-CoA hydratase; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase; LCKT, long-chain 3-ketoacyl-CoA thiolase; MCAD, medium-chain acyl-CoA dehydrogenase; mcFA, medium-chain fatty acid; NADH (NAD^+), β-nicotinamide adenine dinuleotide (oxidized); NEM, N-ethylmaleimide; PMS, phenazine methosulfate; PP-CoA, 3-phenylpropionyl-CoA; SCAD, short-chain acyl- CoA dehydrogenase; T, carnitine:translocase; TCA, tricarboxylic acid; TOC, trifunctional β-oxidation complex; VLCAD, very long-chain acyl-CoA dehydrogenase;

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INTRODUCTION

During the last 15 years there has been a resurgence of interest in the mitochondrial β-oxidation of fatty acids. This development was fueled in part by the identification of membrane-bound enzymes of long-chain fatty acid β-oxidation (1, 2) and by the recognition of several inherited human diseases due to deficiencies of β-oxidation enzymes (3, 4). Other impulses have come from studies of the regulation of hepatic (5) and cardiac β-oxidation (6) and from the revision of the pathway for unsaturated fatty acid β-oxidation (7). It seems that mitochondrialβ-oxidation, which drives oxidative phosphorylation and supplies acetyl-CoA for ketogenesis, may not only be important for the oxidation of normal short-, medium-, and long-chain fatty acids, but also be crucial for the complete degradation of very long-chain fatty acid and methyl-substituted fatty acids, which had been thought to be substrates of the peroxisomal β-oxidation system.

Uptake of Fatty Acids by Animal Cells

Fatty acids, which are hardly soluble in water, are carried through animals by the circulatory system where they either bind to serum albumin or exist as triacylglycerol components of lipoproteins.Triacylglycerols are hydrolyzed by lipoprotein lipase at the endothelial surface of the vascular compartment to yield free fatty acids.

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The cellular uptake of fatty acids, although actively studied, has not been fully elucidated. Kinetic evidence has been obtained for a saturable and a non-saturable uptake of fatty acids (8). The saturable uptake, which predominates at nanomolar concentrations of free fatty acids, is assumed to be a carrier-mediated process. Detailed studies have led to the identification of a 40-kDa plasma membrane protein in rat liver which specifically binds fatty acids (9). Since antibodies raised against this membrane protein caused a partial inhibition of fatty acid uptake, it has been suggested that it mediates the cellular uptake of fatty acids (10). Studies of the hormonal regulation of cellular fatty acid uptake have yielded evidence for a 5 to 10-fold increase of fatty acids uptake by epinephrine treated adipocytes, whereas this stimulation was suppressed by insulin (11, 12). However, despite strong evidence for a carrier-mediated uptake and possibly for active transport of fatty acids by animal cells, it is still argued that fatty acid uptake takes place simply by nonspecific diffusion across the plasma membrane (13, 14). Cellular fatty acid uptake is obviously a complicated, probably multi-step process which is tightly coupled to the intracellular metabolism of fatty acids.

Once long-chain fatty acids have crossed the plasma membrane, they move to mitochondria, peroxisomes, and the endoplasmic reticulum where they are converted to their CoA thioesters. Whether the intracellular transport of fatty acids between membranes is a facilitated process or occurs by simple diffusion is an unresolved issue. The identification of fatty acid binding proteins (FABPs)[1] (Fig. 1) in the cytosol of animal cells has prompted speculations about their functions in intracellular fatty acid transport and the delivery of fatty acids to

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intracellular sites of fatty acid utilization including mitochondria (15). Six distinct, but homologous FABPs have been found. They are liver FABP, intestinal FABP, myelin P2, adipocyte and cutaneous FABP, and heart FABP. Liver FABP has been identified in liver, intestine, and kidney. Intestinal FABP is found in intestine and stomach. Myelin P2, adipocyte and cutaneous FABPs are present in peripheral nerve tissue, adipose tissue and skin cells. Heart FABP, existing abundantly in heart and red skeletal muscle cells, is also found in other tissues such as lung, kidney mammary gland and brain (16). The association of these proteins with tissues highly active in lipid synthesis or fatty acid oxidation indicates that FABPs play an important role in these processes.

Activation and Mitochondrial Uptake of Fatty Acids

The mitochondrial β-oxidation of fatty acids is preceded by their conversion to coenzyme A (CoA) thioesters (Fig. 1). This reaction is catalyzed by a group of acyl-CoA synthetases which differ in their chain length specificities and subcellular locations, but which catalyze the same type of reaction.

Acyl-CoA synthetases have customarily been grouped based upon their preference for different chain-length fatty acids . They are short-chain, medium-chain and long-chain acyl-CoA synthetases (17). A short-chain specific acetyl-CoA synthetase (EC. 6.2.1.1) has been isolated from beef heart mitochondria. This enzyme is most active with acetate as a substrate, although it exhibits some activity towards propionate. An acetyl-

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CoA synthetase has been detected in mitochondria of heart, skeletal muscle, kidney, adipose tissue and intestine, but not in liver mitochondria. A cytosolic acetyl-CoA synthetase was also identified in lipogenic tissues. It is possible that the cytosolic enzyme synthesizes acetyl-CoA for lipogenesis, whereas the mitochondrial acetyl-CoA synthetase activates acetate for oxidation. A distinct propionyl-CoA synthetase exists in liver mitochondria. This enzyme is active with acetate, propionate, and butyrate, but the Km value for propionate is much lower than the Km values for the other two substrates. Medium-chain acyl-CoA synthetases (EC 6.2.1.2) are present in mitochondrial matrix of various mammalian tissues. The partially purified enzyme from beef heart mitochondria acts on fatty acids with 3-7 carbon atoms, but is most active with butyrate. In contrast, a partially purified enzyme from bovine liver mitochondria activates fatty acids with 4-12 carbon atoms with octanoate being the best substrate. This enzyme can activate branched-chain, unsaturated, and hydroxy-substituted medium-chain carboxylic acids and, surprisingly, even act on aromatic carboxylic acids like benzoic acid and phenylacetic acid. Long -chain acyl-CoA synthetase (EC 6.2.1.3) is a membrane-bound enzyme that is associated with the mitochondrial outer membrane, the endoplasmic reticulum and peroxisomes. The rat liver enzyme, purified from mitochondria and microsomes (18), activates saturated fatty acids with 10 to 18 carbon atoms and unsaturated fatty acids with 16 to 20 carbon atoms. An immunological study showed that long-chain acyl-CoA synthetases associated with mitochondria, microsomes and peroxisomes are structurally similar (19). cDNA sequences of several clones for long-chain acyl-CoA synthetase from rat liver revealed a single sequence corresponding to a protein with a

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molecular mass of 78 kDa that is close to the subunit molecular mass of 76kDa determined with the purified enzyme (20). Evidence has also been obtained indicating the existence of a distinct very-long-chain acyl-CoA synthetase in peroxisomes, which, in contrast to long-chain acyl-CoA synthetase, is more active with lignoceric acid than with palmitic acid (21, 22, 23, 24, 25).

Since free CoASH and acyl-CoAs can not pass through the inner mitochondrial membrane, fatty acyl residues have to be converted to acyl-carnitine esters before being carried across the membrane. Carnitine palmitoyltransferase I (CPT I)[1], which is located at the outer mitochondrial membrane, transfers fatty acyl residues from CoA to L-carnitine (Fig. 1). The resultant fatty acyl carnitines can pass through the inner mitochondrial membrane via carnitine:acylcarnitine translocase (26, 27) by an 1:1 exchange for carnitine or other acylcarnitines including acetyl-carnitine. Once they are in the matrix, carnitine palmitoyltransferase II (CPT II)[1], which exists in the inner mitochondrial membrane, catalyzes the transfer of fatty acyl residues back from carnitine to CoASH (Fig. 1). CPT II has been purified from mitochondria of bovine heart and rat liver. The purified enzyme has a subunit molecular mass of approximately 70 kDa and catalyzes the reversible transfer of acyl residues with 10-16 carbon atoms between CoA and carnitine. The cDNAs of rat liver (28, 29) and human CPT II (30) have been cloned and sequenced. The predicted amino acid sequences of the corresponding 74 kDa proteins show a 82% homology with each other. CPT I has not been purified yet because of severe activity losses upon solubilization with detergents. CPT I, in contrast to CPT II, is reversibly inhibited by malonyl-CoA, its natural regulator, and is

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inactivated by CoA derivatives of certain alkyl-glycidic acids, such as 2-tetradecylglycidic acid. This property was used to label this protein for cloning and sequencing of the cDNAs coding for CPT I from rat and human liver (31). An isoform of liver CPT I is present in skeletal muscle. Both isoforms, which differ in size, kinetic properties, and sensitivity to malonyl-CoA, are expressed in heart mitochondria.

In addition to CPT I and CPT II, there exists a carnitine acetyltransferase (EC 2.3.1.7) in mitochondria. The enzyme from bovine heart has an estimated molecular mass of 60 kDa and consists of a single polypeptide chain. It catalyzes the reaction of transferring acyl groups with the chain length of 2 - 10 carbon atoms. The function of this enzyme has not been elucidated. It may function to regenerate free CoA in the mitochondrial matrix by transferring acetyl groups and other short-chain or medium-chain acyl residues from CoA to carnitine. The resulting acylcarnitines can leave mitochondria via the carnitine: acylcarnitine translocase. They can either be metabolized by the same or other tissues, or be excreted in urine.

Short-chain and medium-chain fatty acids with less than ten carbon atoms can enter mitochondria as free acids without the assistance of carnitine. They are activated to their CoA thioesters directly in the mitochondrial matrix where short-chain and medium-chain acyl-CoA synthetases are located.

The Mitochondrial β-Oxidation Spiral and Its Enzymes

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Acyl-CoAs, formed in the matrix from long-chain acylcarnitines or medium-chain fatty acids, are substrates of the β-oxidation spiral that yield acetyl-CoA, NADH, and $FADH_2$ (Fig. 2). NADH and $FADH_2$ are oxidized by the mitochondrial electron-transport chain, whereas acetyl-CoA is oxidized to CO_2 by the Krebs Cycle, which thereby regenerates CoASH. The enzymes catalyzing the reactions of the β-oxidation spiral are acyl-CoA dehydrogenase, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase. Since the discovery of a novel set of long-chain specific enzymes for mitochondrial β-oxidation (1, 2), the β-oxidation spiral has been reclassified into two systems, the long-chain fatty acid β-oxidation system, which consists of the membrane-bound very long-chain acyl-CoA dehydrogenase and trifunctional β-oxidation complex, working on the longer chain fatty acid acyl-CoAs, and the matrix β-oxidation enzyme system, which is working on the medium- or short-chain fatty acid acyl-CoAs (Fig. 2). These two systems may cooperate to complete the β-oxidation of long-chain fatty acids.

The observation that fatty acid β-oxidation in mitochondria proceeds without the accumulation of intermediates led to the suggestion that the soluble matrix enzymes catalyzing the individual reactions of this pathway may exist as a multienzyme complex. Subsequent studies with radioactively labeled fatty acids revealed the formation of low levels of chain shortened acyl-CoA intermediates in respiring mitochondria. In some studies only saturated acyl-CoA intermediates were identified, while in others very small amounts of 2-enoyl-CoA and 3-hydroxyacyl-CoA besides low levels of saturated acyl-CoA were detected. In a recent study of β-oxidation with human fibroblasts only saturated short-chain and

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medium-chain intermediates were detected in the incubation medium (32). However, with fibroblasts deficient with respect to certain β-oxidation enzymes different intermediate patterns were observed which are indicative of the enzyme defect. For example, a deficiency of a long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD)[1] resulted in the accumulation of long-chain saturated as well as 3-hydroxy intermediates. Thus, it seems that under normal conditions long-chain intermediates do not accumulate in fibroblasts and that of all possible short-chain and medium-chain intermediates only saturated ones escape from these cells presumably because of their intramitochondrial accumulation. One likely reason for the absence of free intermediates is their channeling between active sites of enzymes catalyzing consecutive reactions. Such channeling would most likely occur if the enzymes of β-oxidation were organized as multienzyme complexes. Although this has not been demonstrated for the soluble matrix enzymes, it has been established that long-chain enoyl-CoA hydratase (LCEH[1]), long-chain L-3-hydroxyacyl-CoA dehydrogenase (LCHAD[1]), and long-chain 3-ketoacyl-CoA thiolase (LCKT[1]) exist as a trifunctional β-oxidation complex in the inner mitochondrial membrane (Fig. 3). Since additionally very long-chain acyl-CoA dehydrogenase (VLCAD[1]) is located in the inner mitochondrial membrane, these four enzymes may act in concert with the result that long-chain intermediates are channeled between active sites and do not accumulate in the mitochondrial matrix (Fig. 3).

Regulation of Mitochondrial Fatty Acid Oxidation

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It has been reported that malonyl-CoA inhibits CPT I in liver and, therefore, controls the mitochondrial uptake and β-oxidation of fatty acids (33). This led to the proposal that malonyl-CoA is the regulatory signal that coordinates hepatic fatty acid synthesis and fatty acid oxidation. Whether this regulatory mechanism is also effective in nonhepatic tissues, such as heart, is still unresolved. Studies with isolated mitochondria revealed that CPT I of heart, skeletal muscle and other extrahepatic tissues is inhibited by malonyl-CoA and, in fact is more sensitive (10 to 100 times) toward malonyl-CoA than is hepatic CPT I (34, 35). Although malonyl-CoA was detected in heart and skeletal muscle (34), the origin of this compound in nonlipogenic tissues such as heart and skeletal muscle remained obscure. An isoform of acetyl-CoA carboxylase was identified and purified from heart (36, 37), but it has a very low affinity towards acetyl-CoA (38). Furthermore, in contrast to liver, the activity of CPT I in heart and its sensitivity toward malonyl-CoA do not change in response to fasting (39). Although fasting (34) and exercise (40) decrease the concentration of malonyl-CoA in heart and skeletal muscle, respectively, a clear relationship between malonyl-CoA concentration and the rate of fatty acid oxidation in heart and skeletal muscle has not been established. Hence, whether the control of myocardial fatty acid oxidation is via regulation of CPT I by malonyl-CoA remains an unresolved issue.

It has been proposed that the concentration of free fatty acids and the energy demand of the tissue are the main regulatory factors that control the oxidation of fatty acids in the heart (6, 41). Studies were done with coupled mitochondria allowing for the adjustment of the energy state by

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limiting ADP (state 4 respiration) or providing sufficient ADP (state 3 respiration) for oxidative phosphorylation. Because the ratios of both (acetyl-CoA)/(CoASH) and (NADH)/(NAD^+) change in response to shifts in the energy state of heart mitochondria (42), the effect of adjusting either of the two ratios on the rate of β-oxidation was investigated (41). When 10 mM L-carnitine was added to coupled rat heart mitochondria aimed at decreasing the ratio of (acetyl-CoA)/(CoASH), the rate of palmitoylcarnitine β-oxidation increased more than four-fold at state 4 respiration, whereas at state 3 respiration the rate of this process was enhanced slightly. Rates of β-oxidation at state 3 and state 4 respiration were the same in the presence of 10 mM L-carnitine even though rates of respiration remained unaffected. They were four to five times higher at state 3 than at state 4. The ratio of (acetyl-CoA)/(CoASH), which was four times higher at state 4 than that of state 3 in the absence of carnitine, was lowered by 10 mM L-carnitine 15 times and 53 times at state 3 and state 4, respectively (41). By contrast, the addition of 10 mM oxaloacetate or 5 mM acetoacetate to coupled rat heart mitochondria to lower the intramitochondrial ratio of NADH/NAD^+ did not stimulate palmitoylcarnitine-supported β-oxidation at state 4 respiration. It is likely that the rate of β-oxidation in heart mitochondria is regulated by (acetyl-CoA)/(CoASH) ratio in the mitochondrial matrix.

The identification of a novel set of long-chain specific enzymes of mitochondrial β-oxidation (1, 2, 43, 44) necessitates a study aimed at determining whether and, if so, how its activity is affected by changes in the (acetyl-CoA)/(CoASH) ratio. This evaluation is especially important because the long-chain specific thiolase of the trifunctional β-oxidation complex is assumed to function in the initial cycles of β-oxidation when

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long-chain fatty acids are chain shortened and where a regulatory mechanism might be effective.

Inhibitors of Mitochondrial Fatty Acid β-Oxidation

Studies of fatty acid oxidation, especially of its regulation, have been greatly aided by the availability of specific inhibitors which have also been investigated for their use as oral hypoglycemic agents. Three reactions of the mitochondrial β-oxidation pathway are inhibited by these inhibitors; those catalyzed by acyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolases, and carnitine palmitoyltransferase I (CPT I). These inhibitors are carboxylic acids and they must be converted to their coenzyme A thioesters and in most cases metabolized through the β-oxidation pathway to compounds that bind reversibly or irreversibly to the active site of the target enzyme, thereby inhibiting it. Generally these inhibitors have structural similarities to fatty acids. In addition, they carry a potentially reactive group which upon metabolic conversion will produce a highly reactive and active site-directed inhibitor.

The first inhibitor of mitochondrial β-oxidation reported was hypoglycin. It induces severe hypoglycemia probably due to the result of inhibiting β-oxidation upon ingestion (45). The amino acid hypoglycin, which is present in the arillus of the unripe ackee fruit, is metabolized in animals by deamination and oxidative decarboxylation to methylenecyclopropylacetyl-CoA. Methylenecyclopropylacetyl-CoA can inactivate butyryl-CoA dehydrogenase by forming a covalent adduct with the FAD cofactor of the enzyme (45).

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Of great interest for metabolic studies are effective inhibitors of CPT I, such as 2-tetradecylglycidic acid (46) and 2-bromopalmitic acid (47). In the cytosol these two compounds are converted to their CoA thioesters before they bind irreversibly to CPT I and inactivate it, thereby preventing the mitochondrial uptake of long-chain fatty acids for β-oxidation. The metabolism of medium-chain or short-chain fatty acids, which can pass through the mitochondrial membrane directly, are unaffected by these inhibitors.

Another useful group of β-oxidation inhibitors which can inactivate 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase are substituted carboxylic acids like 4-pentenoic acid (48), 4-bromocrotonic acid (49), 2-bromooctanoic acid (50) and 4-bromo-2-octenoic acid (51). These compounds are not inhibitory by themselves but become so after entering mitochondria where they are activated to their CoA thioesters and further metabolized via β-oxidation to their 3-keto derivatives. The resultant 3-keto derivatives bind to the active sites of thiolases where they undergo reactions leading to the formation of covalent bonds between the sulfhydryl groups of the enzyme and the inhibitor before or after their thiolytic cleavage (52).

Inherited Diseases of Fatty Acid β-Oxidation

Disorders of fatty acid oxidation were first reported in 1973 when patients with muscle weakness were shown to have either a carnitine deficiency (53) or a deficiency of carnitine palmitoyltransferase (54). Ten years later, the most common of these deficiencies, medium-chain acyl-

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CoA dehydrogenase (MCAD)[1] (EC 1.3.99.2) deficiency was reported (3). Human MCAD is synthesized in the cytosol with a leader peptide at the N-terminus (55, 56, 57). The precursor MCAD is imported into mitochondria and translocated into the matrix, where its leader peptide is proteolytically cleaved, producing the 396-amino acid (43.6 kDa) mature protein. The monomeric enzyme is then assembled into the native and biologically active homotetrameric form (55). Recent X-ray crystallographic studies indicate that the MCAD tetramer is actually a dimer of two dimers. Domains involved in the monomer-monomer interaction are different from those involved in the dimer-dimer interaction (58). Hereditary MCAD deficiency is mainly detected among Caucasian children who originate from northwestern Europe (59). It causes hypoketotic hypoglycemia and episodic vomiting and, if not treated, the patients may die. The incidence of MCAD deficiency is relatively high for a genetic metabolic disorder. The same mutation is found in 89% of all variant MCAD alleles. This mutation results in a glutamate being substituted for the normal lysine at position 304 (K304E) of the mature MCAD. This K304E-variant of MCAD is unstable and it is undetectable in patients' tissues (60). Since lysine 304 is located in the critical domain involved in the normal dimer-dimer interaction in the tetramer assembly (61), it has been suggested that this change of the charge at the interface of subunits impairs the normal assembly of variant subunits into tetramers. (62, 63, 64).

MCAD is generally assayed by coupling the substrate-dependent reduction of the flavine adenine dinucleotide (FAD) cofactor to the reduction of a dye which undergoes an absorbance change that can be

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measured spectrophotometrically. Several continuous assay procedures have been described in which a primary electron acceptor, e.g., electron transfer flavoprotein (ETF) (65), phenazine methosulfate (PMS)[1] (66), or meldolablau (67), facilitates the transfer of electrons from the enzyme to a secondary electron acceptor, eg., 2, 6-dichlorophenolindophenol (DCPIP)[1] (65, 66) or to iodonitrotetrazolium chloride (67), which undergoes a measurable absorbance change. Two additional assay procedures only require one electron acceptor: in one of them, the anaerobic reduction of ETF[1] (electron transfer flavoprotein) is measured fluorometrically (68), while in the other, the reduction of ferricenium hexafluorophosphate is followed spectrophotometrically (69, 70). A disadvantage of most of these assay procedures is interference by oxygen, thiol-containing compounds, and other redox carriers which react with the artificial electron carriers used in these assays. These interferences are most problematic when crude tissue extracts are assayed.

The discovery of the trifunctional β-oxidation complex has prompted detailed studies of long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency. A rapidly growing number of patients with a deficiency of long-chain 3-hydroxyacyl-CoA dehydrogenase of the trifunctional β-oxidation complex have been identified (71). Molecular cloning of the cDNA from patients with this deficiency revealed in many a G -> C mutation at nucleotide position 1528 in the cDNA sequence of the 3-hydroxyacyl-CoA dehydrogenase encoding region of the α-subunit of the enzyme complex. This single base change would result in the substitution of glutamine by glutamate at amino acid position 510. Although the enzyme impairment due to the mutation has not been elucidated, its identification would

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greatly aid in understanding this disease and in developing therapies for treating such patients.

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MATERIALS AND METHODS

Materials. 3-Phenylpropionic acid, cinnamic acid, valproic acid (2-(n-propyl)pentanoic acid) and 2-octynoic acid were purchased from Aldrich Chemical Company. Sigma was the source of phenazine methosulfate, 2,6-dichlorophenolindophenol, N-ethylmaleimide, CoASH, butyryl-CoA, crotonyl-CoA, octanoyl-CoA, dodecanoyl-CoA, L-3-hydroxyacyl-CoA dehydrogenase from pig heart, enoyl-CoA hydratase or crotonase from bovine liver, Histopaque-1077 and all other standard biochemicals. Palmitoyl-L-carnitine was a gift from Dr. K. Brendel, University of Arizona Medical School. 4-Bromocrotonic acid (72), 4-bromo-2-octenoic acid (51), and 3-methylglycidic acid (73) were synthesized as described. 2-*trans*-Hexadecenoic acid and 2-hexadecynoic acid were prepared by general methods developed for the synthesis of 2-trans-enoic acids (74) and 2-ynoic acid (75). Coenzyme A thioesters of 3-phenylpropionic acid, cinnamic acid, 2-octynoic acid, 4-bromocrotonic acid, 4-bromo-2-octenoic acid, 3-methylglycidic acid were prepared by the mixed anhydride method as detailed by Fong and Schulz (74). Acetoacetyl-CoA was synthesized as described (76) and 2-octynoyl-CoA was converted to 3-keto-octanoyl-CoA by crotonase as detailed by Thorpe (77). The purities of 3-phenylpropionyl-CoA (PP-CoA) and cinnamoyl-CoA were determined by HPLC[1]. Crude preparations of PP-CoA[1] did not contain any other acyl-CoA and therefore can be used without further purification. Cinnamoyl-CoA was purified by HPLC and found to have

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an A_{308}/A_{260} ratio of 1.16. Concentrations of acyl-CoAs were determined by measuring released CoASH by the method of Ellman (78) after quantitatively cleaving the thioester bond with 1 M hydroxylamine at pH 7. An extinction coefficient of 26,500 $M^{-1}cm^{-1}$ was determined for cinnamoyl-CoA by measuring the absorbance change at 308 nm upon cleavage of the thioester bond with 1 M hydroxylamine at pH 7. Valproyl-CoA was prepared from valproic acid and CoASH by the acid chloride method (79).

2-*Trans*-hexadecynoyl-CoA, after purification by hydrophobic chromatography on octyl-sepharose (75), was converted to 3-ketohexadecanoyl-CoA by incubating 1 mM 2-hexadecynoyl-CoA in 20 mM HEPES buffer (pH 7) with crotonase (40 units/ml) for 1 hr at 25°C as described by Thorpe (77). L-3-Hydroxyhexadecanoyl-CoA was prepared by incubating 1 mM 2-*trans*-hexadecenoyl-CoA in 0.1 M KPi (pH 7.6) with crotonase (10 units/ml) at 25°C for 30 min. All of the above substrates were purified by HPLC (see under *HPLC Analyses* for details). The concentration of 3-ketohexadecanoyl-CoA was determined by measuring its complete reduction by NADH at pH 7 in the presence of L-3-hydroxyacyl-CoA dehydrogenase. The concentration of purified L-3-hydroxyhexadecanoyl-CoA also was calculated based on its absorbance at 259 nm and an extinction coefficient of 15,400 $M^{-1}cm^{-1}$.

Bovine liver long-chain, medium-chain and short-chain acyl-CoA dehydrogenases were separated from each other and partially purified as described by Davidson and Schulz (80). Bovine liver enoyl-CoA hydratase (crotonase) (81) and pig heart 3-ketoacyl-CoA thiolase (82)

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were purified by established procedures. The trifunctional β-oxidation complex was purified from pig heart as described by Luo et al. (44).

Enzyme assays and protein determination. Most enzyme assays were performed on a Gilford recording spectrophotometer (Model 250) at 25°C. Absorbances were measured as a function of time or wavelength with a Gilford, Model 2600, microprocessor-controlled spectrophotometer interfaced with a recorder or Hewlett Packard 7225B graphic plotter. The conventional assay of acyl-CoA dehydrogenases is based on the substrate-dependent reduction of DCPIP in the presence of PMS. The assay mixture contained 0.1 M KP_i[1] (pH 8), 3.3 mM PMS, 35.7 μM DCPIP, 0.2 mM NEM, 0.45 mM KCN, 40 μM acyl-CoA, and enzyme to obtain an absorbance change of approximately 0.04 ΔA/min. The reaction was started by the addition of PMS and its progress was followed by measuring the decrease in absorbance at 600 nm at 25°C. An absorption coefficient for DCPIP at 600 nm of 21,300 M^{-1} cm^{-1} was used to calculate rates. The dehydrogenation of PP-CoA to cinnamoyl-CoA was measured spectrophotometrically at 308 nm. For measurements with partially purified enzyme, the assay mixture contained 0.1 M KP_i (pH 8), 4.5 mM PMS, 20 μM PP-CoA, and MCAD to give an absorbance change of approximately 0.04 ΔA/min. The reaction was initiated by the addition of substrate. An extinction coefficient of cinnamoyl-CoA at 308 nm of 26,500 M^{-1} cm^{-1} was used to calculate reaction rates. When tissue extracts were assayed for MCAD, the assay mixture contained 0.1 M KP_i (pH 8), 4.5 mM PMS, 0.2 mM NEM, 60 μM PP-CoA, and tissue extract containing either 5 μg of rat heart protein, or 10 μg of rat liver protein, or 150 μg of

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rat leukocyte protein. The reaction was initiated by the addition of PP-CoA and allowed to proceed for 10 min. The absorbance change at 308 nm, after correction for the absorbance change in the absence of substrate, was used to calculate the activity of MCAD. Thioesterase was assayed spectrophotometrically at 412 nm. The assay mixture contained 0.1 M Tris-HCl (pH 8), 50 mM KCl, 0.21 mM DTNB[1], 60 µM PP-CoA, bovine serum albumin (0.33 mg/ml), and the same amount of tissue homogenate used to assay MCAD. An extinction coefficient of 13,600 M^{-1} cm^{-1} (77) was used to calculate rates.

Enoyl-CoA hydratase (EC4.2.1.17) was assayed spectrophotometrically at 260 nm with crotonyl-CoA as substrate (74). The assay of L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35)was based on the NADH-dependent reduction of acetoacetyl-CoA measured at 340 nm (83). 3-Ketoacyl-CoA thiolase was assayed with 3-ketooctanoyl-CoA as a substrate as described (82). When the effect of 3-methylglycidoyl-CoA on the enzymes of β-oxidation was evaluated, rat liver mitochondria, sufficient for assaying the enzyme activities, were incubated in 0.1 ml of 50 mM KPi (pH 8) with 60 µM 3-methylglycidoyl-CoA in the presence of 0.05% Triton X-100 for 15 min. Thereafter, the assay components were added to a final volume of 1 ml and the assays were initiated by the addition of substrate.

The activation of carboxylic acid was measured by incubating 4 mM ATP, 0.5 mM CoASH, 10 mM $MgCl_2$, 0.5 mM carboxylic acid, 0.05% Triton X-100, and 4 mg of rat liver or rat heart mitochondrial protein in 1 ml of basal isotonic incubation buffer used for oxygen uptake measurements. After 15 min at 37°C the reaction was terminated by

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adjusting the pH to 1-2 with concentrated HCl and the formation of acyl-CoA was assessed by HPLC.

The three coupled reactions (overall reaction) catalyzed by TOC were assayed either by measuring spectrophotometrically the formation of NADH or by determining the concentration of acetyl-CoA or myristoyl-CoA by HPLC. A standard assay mixture contained in 1 ml of 0.1 KPi (pH 7.6) 1 mM NAD^+, 0.2 mM CoASH, 20 μM 2-*trans*-hexadecenoyl-CoA and 3 μg of TOC. Long-chain enoyl-CoA hydratase of TOC was assayed by either the direct or indirect method. The direct method is based on the decrease in absorbance at 280 nm due to the hydration of 2-enoyl-CoA. A standard assay mixture contained in 0.1 M KPi (pH 7.6) 20 μM 2-*trans*-hexadecenoyl-CoA and TOC to give an absorbance change of approximately 0.02 A/min. The molar extinction coefficient for calculating rates is 5,100 $M^{-1}cm^{-1}$ (84). The indirect method is based on a coupled assay in which L-3-hydroxyacyl-CoA formed by the hydration of 2-*trans*-enoyl-CoA is dehydrogenated and thiolytically cleaved by the combined actions of L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase in the presence of NAD^+ plus CoASH. A standard assay mixture contained 0.1 M KPi (pH 7.6) 1 mM NAD^+, 0.2 mM CoASH, 20 μM 2-*trans*-hexadecenoyl-CoA, pig heart L-3-hydroxyacyl-CoA dehydrogenase (2 units/ml), pig heart 3-ketoacyl-CoA thiolase (0.1 unit/ml), and TOC to give an absorbance change of approximately 0.02 A/min at 340 nm. Long-chain L-3-hydroxyacyl-CoA dehydrogenase of TOC was assayed by measuring the formation of NADH spectrophotometrically at 340 nm. A standard assay mixture contained 0.1 M KPi (pH 7.6) 1 mM NAD^+, 0.2 mM CoASH, 20 μM L-3-hydroxyhexadecanoyl-CoA, pig heart 3-ketoacyl-CoA thiolase (0.1 unit/ml)

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and TOC to give an absorbance change of approximately 0.02 A/min. Long-chain 3-ketoacyl-CoA thiolase of TOC was assayed by determining the concentration of acetyl-CoA or myristoyl-CoA by HPLC. A standard assay mixture of 1 ml contained 0.1 M KPi (pH 7.6) 0.2 mM CoASH, 20 μM 3-ketohexadecanoyl-CoA and 3 μg of TOC. After 45 sec of incubation, the reaction was terminated by adjusting the pH to 1-2 with concentrated HCl. An aliquot (250 μl) of the reaction was subjected to HPLC analysis to determine the concentration of acetyl-CoA or myristoyl-CoA. Kinetic parameters (Km, Vmax) were determined by nonlinear curve-fitting using the Sigma plot program. A milliunit (mU) of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 nmol of substrate to product per minute. Protein concentrations were determined by the method of Bradford (85). For trifunctional β-oxidational complex, protein concentrations were determined as described by Wang and Smith (86).

Preparation of tissue extracts from rat heart, liver and leukocytes. Rat heart or rat liver was minced and then homogenized at 0°C with 7 volumes of 0.1 M KP_i (pH 8) 6-times for 20 sec each with a Polytron homogenizer at top speed. Rat leukocytes were isolated by mixing 3 ml of rat blood containing 3.5% sodium citrate with 5 ml of 10 mM KP_i (pH 7.6) containing 120 mM NaCl and 2.7 mM KCl (buffer A). This suspension was layered on top of 3 ml of Histopaque-1077 in a 15 ml conical centrifuge tube and centrifuged at 200xg for 30 min. The clear upper layer was removed by aspiration and the opaque interface layer was collected and washed twice with buffer A by pelleting leukocytes by centrifugation at 200xg for 30 min and resuspending them with buffer A.

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Leukocytes suspended in 0.1 ml of buffer A were lysed by the addition of Triton X-100 to a final concentration of 0.2% (v/v). Rat liver and heart homogenates were also treated with 0.2% Triton X-100 which did not affect the dehydrogenase activity. After keeping these homogenates for 15 min at 0°C, they were clarified by centrifugation at 12,000xg for 10 min at 4°C. The resultant clear homogenates were assayed for MCAD as described above.

Isolation of rat liver and rat heart mitochondria. Rat heart mitochondria were isolated according to the procedure of Mela and Seitz (87). Rat liver mitochondria were obtained by the same procedure except that 0.25 M sucrose was used instead of 0.21 M mannitol plus 0.07 M sucrose and the treatment with Nagarse was omited. Protein concentrations were determined by the Biuret method (88).

Measurement of oxygen uptake by rat liver and rat heart mitochondria. Rat liver mitochondria (2 mg of protein) were suspended in 1.9 ml of a basal isotonic incubation buffer containing 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 4 mM KPi, 4 mM $MgCl_2$ and 0.1 mM EGTA. For measurements with rat heart mitochondria (1 mg of protein/ml) the basal isotonic medium contained 0.11 M KCl, 33 mM Tris-HCl (pH 7.4), 2 mM KPi, 2 mM $MgCl_2$ and 0.1 mM EGTA. To either suspension were added in the indicated sequence bovine serum albumin (0.5 mg/ml), 0.5 mM L-malate, 1 mM ADP, and 1 min later, varying amounts of the inhibitors. The mixtures were incubated for 3 min or the indicated periods of time. State 3 respiration was started by the addition of 30 μM of

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palmitoylcarnitine and oxygen uptake was measured polarographically with a Clark oxygen electrode attached to a Gilson oxygraph.

HPLC analyses - Prior to analysis by HPLC, reactions were terminated by adjusting the pH to 1-2 with concentrated HCl. Samples were filtered through 0.22 μm (pore size) membranes after which the pH was adjusted to 5.5 with KOH. The filtrates were applied to a Waters μBondapak C_{18} reverse-phase column (30cm x 3.9 mm) attached to a Waters gradient HPLC system (Waters 510 HPLC pump). The absorbance of the effluent was monitored at 254 nm. Separation of the substrate and the product of medium-chain acyl-CoA dehydrogenase was achieved by linearly increasing the acetonitrile/H_2O (9:1) content of the 25 mM ammonium phosphate elution buffer (pH 5.5) from 10% to 50% in 20 min at a flow rate of 2 ml/min. Separation for the studies of inhibitors and their activations was achieved by linearly increasing the acetonitrile/H_2O, 9:1 (V/V), content of the 10 mM ammonium phosphate elution buffer (pH 5.5) from 5% to 50% in 20 min at a flow rate of 2 ml/min. Separation for the channeling study was achieved by linearly increasing the acetonitrile/H_2O (9/1) content of the 50 mM ammonium phosphate elution buffer (pH 5.5) from 5% to 75% in 40 min at a flow rate of 2 ml/min. For the purification of different synthetic acyl-CoA substrates, the acetonitrile/H_2O (9/1) content of the buffer was linearly increased from 5% to 50% in 15 min. For the purification of acetyl-CoA, the acetonitrile/H_2O (9/1) content was increased from 5% to 25% in 15 min. Concentrations of intermediates and products

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were determined by use of standard curves that were established with HPLC-purified acyl-CoA thioesters.

Oxidation of (1-^{14}C) palmitoylcarnitine by rat heart mitochondria. The reaction mixture contained in a final volume of 0.7 ml, 0.11 M KCl, 3.3mM Tris-HCl (pH 7.4), 2 mM KPi, 2 mM $MgCl_2$, 0.1 mM EGTA, defatted bovine serum albumin (0.1 mg/ml), 0.5 mM L-malate, 20 μM (1-^{14}C) palmitoyl-L-carnitine (50,000 cpm), 60 μM ATP, 10 mM glucose, hexokinase (5U), and rat heart mitochondria (120 μg of protein). Glucose and hexokinase were added to the incubation medium to maintain mitochondria at state 3 respiration. State 4 respiration persisted in the absence of glucose and hexokinase. When the effect of pyruvate and L-carnitine on the rate of palmitoylcarnitine supported β-oxidation was studied, appropriate amounts of these compounds were added to the incubation medium before the reaction was started. Fatty acid oxidation was initiated by the addition of rat heart mitochondria to the incubation medium in a 25-ml Erlenmeyer flask. For quantitating acid-soluble β-oxidation products, the acidified incubation mixture was extracted three times with water-saturated butanol. An aliquot (0.2 ml) of the remaining aqueous phase was combined with 4 ml of ScintiVerse II and counted in a Parkard Tri-Carb 1500 liquid scintillation counter.

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RESULTS

A Specific Assay of Medium-Chain Acyl-CoA Dehydrogenase Based on the Spectrophotometric Measurement of Product Formation

Medium-chain acyl-CoA dehydrogenase is generally assayed by coupling the substrate-dependent reduction of their flavine adenine dinucleotide (FAD) cofactor to the reduction of a dye which undergoes an absorbance change which could be measured spectrophotometrically. Many other methods have also been described. But they are problematic especially when crude enzyme samples are used. The method described here is specific for MCAD and can be used with crude enzyme samples such as a leukocyte homogenate.

Spectrophotometric assay of MCAD based on the formation of cinnamoyl-CoA. The dehydrogenation of PP-CoA[1] to cinnamoyl-CoA is catalyzed by rat and human MCAD (89). This reaction, shown below, is accompanied by the emergence of a new chromophore in which the phenyl and thioester groups of the substrate are conjugated via the double bond introduced as a result of the dehydrogenation reaction.

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The time-dependent development of this chromophore centered around 308 nm was observed when PP-CoA was incubated with MCAD in the presence of PMS (see Fig. 4). An extinction coefficient of 26,500 M^{-1} cm^{-1} at 308 nm was determined for HPLC-purified cinnamoyl-CoA. The formation of cinnamoyl-CoA in the dehydrogenation reaction was confirmed by HPLC with chemically synthesized cinnamoyl-CoA as an authentic standard (data not shown). Quantitation of cinnamoyl-CoA formation by spectrophotometry and HPLC yielded similar results (13.7 vs. 15 nmol in one experiment and 26.2 vs. 26.4 in a second experiment) thereby establishing that the absorbance change at 308 nm is a measure of the rate of dehydrogenation.

The rate of PP-CoA dehydrogenation was determined as a function of the PMS concentration (Fig. 5). Although the rate increases with increasing PMS concentration up 7 mM, a PMS concentration of 4.5 mM was chosen for standard assays to keep the initial absorbance in the assay mixture below 1.2. It should be noted that the absorbance of PMS around 308 nm is at a minimum therefore permitting measurements at a relatively high PMS concentration of 4.5 mM. Since the quantitation of cinnamoyl-CoA by spectrophotometry and HPLC yielded similar results, it seems that the reduction of PMS does not contribute significantly to

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the absorbance change at 308 nm. This tentative conclusion was verified by a separate experiment in which the absorbance change at 308 nm due to the dehydrogenation of octanoyl-CoA by bovine MCAD in the presence of PMS was measured (see Fig. 6). The background rate due to enzyme and PMS remained unchanged when octanoyl-CoA was added. In contrast, the addition of PP-CoA caused a rapid and linear increase in the absorbance of 308 nm. Octanoyl-CoA is the preferred substrate of MCAD and is dehydrogenated to 2-*trans*-octenoyl-CoA which has an absorbance maximum at 263 nm. Thus, the increase in absorbance at 308 nm caused by the addition of PP-CoA only reflects cinnamoyl-CoA formation and hence can be used to calculate rates of dehydrogenation with the extinction coefficient determined for cinnamoyl-CoA.

Kinetic constants were determined for the dehydrogenation of octanoyl-CoA and PP-CoA by bovine MCAD (see Table 1). Kinetic parameters (apparent K_m and V_{max}) determined for PP-CoA by the conventional and new assay methods were identical. Interestingly, at low substrate concentrations PP-CoA is a slightly better substrate than is octanoyl-CoA. Since the apparent K_m for PP-CoA is 1.2 μM, a substrate concentration of 60 μM used in standard assays will yield maximal rates.

The specificity of the new assay procedure for measuring only the activity of MCAD was evaluated by comparing activities of bovine short-chain, medium-chain, and long-chain acyl-CoA dehydrogenases determined with their best substrates with activities measured with PP-CoA. As shown in Table 2, only MCAD is highly active with PP-CoA as a substrate. The short-chain dehydrogenase is inactive toward PP-CoA, while the long-chain enzyme shows with PP-CoA only 1% of the activity

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observed with its optimal substrate dodecanoyl-CoA. Thus, the contributions of the short-chain and long-chain enzymes to the rate of PP-CoA dehydrogenation are zero and negligible, respectively. This conclusion is in general agreement with data published by Rinaldo et al. (89) who also demonstrated that isovaleryl-CoA dehydrogenase and 2-methyl branched-chain acyl-CoA dehydrogenase exhibit very little activity toward PP-CoA.

Measurements of MCAD activity in tissue homogenates. The new assay of MCAD was evaluated by measuring the activity of this enzyme in homogenates of several rat tissues. The results obtained with a homogenate of rat leukocytes are most important because levels of MCAD in this tissue are very low compared to activities in heart and liver. Shown in Fig. 7A is the time-dependent increase in absorbance at 308 nm observed when the enzyme was assayed in a leukocyte homogenate. The rate was linear for at least 10 min. Acyl-CoA hydrolases or thioesterases may interfere with this assay by hydrolyzing substrate and/or product. Released CoASH reacts with PMS to form a product with an absorbance maximum at 288 nm and with a significant end absorbance at 308 nm (ε=3,500 M^{-1} cm^{-1}) (data not shown). When 0.2 mM NEM was included with CoASH and PMS, the increase in absorbance at 308 nm was reduced (ε=1,500 M^{-1} cm^{-1}) because part of the CoASH reacted with NEM instead of PMS (data not shown). The use of NEM at concentrations higher than 0.2 mM is not recommended because of a noticeable inhibition of MCAD at higher NEM concentrations. Hydrolysis of PP-CoA by a leukocyte homogenate was measured by use of

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a thioesterase assay (see MATERIALS AND METHODS) and the absorbance at 308 nm attributable to this side reaction was calculated assuming that 0.2 mM NEM was present. The result, shown in Fig. 7A, demonstrates that substrate hydrolysis does not interfere significantly with the assay of MCAD in a rat leukocyte homogenate. More specifically, 2% of the total absorbance change measured with PP-CoA (see Fig. 7A) is attributable to the reaction between PMS and CoASH formed by hydrolysis of PP-CoA. In rat liver and rat heart homogenates these non-specific absorbance changes were only 0.8% and 0.4%, respectively, of the total absorbance change at 308 nm.

Another reaction that might contribute to the absorbance change at 308 nm is the dehydrogenation of PP-CoA to cinnamoyl-CoA by peroxisomal acyl-CoA oxidase. Since oxygen serves as an electron acceptor in this dehydrogenation, the rate observed in the absence of PMS reflects the acyl-CoA oxidase reaction. In rat heart and leukocyte homogenates this activity was not detectable. In rat liver homogenate, the oxidase activity with PP-CoA was 1% of the dehydrogenase activity with the same substrate.

The stability of cinnamoyl-CoA in the reaction mixture was also evaluated. As shown in Fig. 7B, the absorbance at 308 nm due to cinnamoyl-CoA in the presence of rat leukocyte homogenate did not change significantly for 15 min. This result proves that cinnamoyl-CoA is not hydrolyzed or reacted upon by other enzymes, including enoyl-CoA hydratase (crotonase), to a degree that would interfere with the dehydrogenase assay.

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The activity of MCAD in homogenates of rat liver, heart, and leukocytes was determined as a function of the protein concentration. The results shown in Fig. 8 demonstrate that activities are linear over the concentration range of homogenate proteins appropriate for assaying MCAD. Specific activities of MCAD in homogenates of rat liver, heart, and leukocytes were found to be 29 ± 1, 68 ± 4, and 2.1 ± 0.1 nmol/min/mg, respectively.

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The Relationship between Mitochondrial Activation and Toxicity of Some Substituted Carboxylic Acids Which Inhibit β-Oxidation

Studies with inhibitors of mitochondrial β-oxidation have revealed a tissue specificity of some of these compounds. For example, 2-bromooctanoic acid, which is an inhibitor of β-oxidation in rat liver (50), is ineffective in rat heart mitochondria (49). Also, 4-bromo-2-octenoic acid, which specifically inactivates 3-ketoacyl-CoA thiolase and thereby inhibits β-oxidation in rat liver mitochondria, has no effect on the same process in rat heart mitochondria (51). Both 2-bromooctanoic acid and 4-bromo-2-octenoic acid must first be metabolized by mitochondrial β-oxidation before becoming inhibitory (50, 51). Since the mitochondrial β-oxidation systems present in liver and heart are not known to be different, either the mitochondrial uptake or activation of these two compounds must be different in heart and liver. In an attempt to elucidate the relationship between mitochondrial activation and toxicity, the conversion of 4-bromocrotonic acid, 4-bromo-2-octenoic acid, valproic acid, and 3-methylglycidic acid to their CoA thioesters and the effects of these compounds on mitochondrial β-oxidation were investigated.

The results obtained with 4-bromocrotonic acid and valproic acid are shown in Fig. 9. 4-Bromocrotonic acid, which was developed as an inhibitor of β-oxidation in rat heart mitochondria (49), is activated both by rat liver and rat heart mitochondria (see Fig. 9A & B). Since 4-bromocrotonyl-CoA is hydrated by enoyl-CoA hydratase (crotonase) (49)

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present in mitochondria, the main product of the activation of 4-bromocrotonic acid is 3-hydroxy-4-bromobutyryl-CoA (see Fig. 9A & B, peak 2). However, small amounts of 4-bromobutyryl-CoA (see Fig. 9A & B, peak 1) were detected. As reported previously (49), 4-bromocrotonic acid completely inhibited palmitoylcarnitine-supported respiration which is a direct measure of mitochondrial β-oxidation (see Fig. 9F). A near total inhibition of β-oxidation was also observed with rat liver mitochondria (see Fig. 9E). Valproic acid, an antiepileptic drug, is known to be activated in rat liver mitochondria (79, 90) and to inhibit hepatic fatty acid β-oxidation (90, 91, 92, 93). The conversion of valproic acid to its CoA thioester by rat liver mitochondria was confirmed (see Fig. 9C), whereas no activation was detected when rat heart mitochondria served as an enzyme source (see Fig. 9D). Inhibition of palmitoylcarnitine-supported respiration by valproic acid was observed with rat liver mitochondria but not with rat heart mitochondria (see Fig. 9G & H). The results obtained with 4-bromocrotonic acid and valproic acid support the conclusion that an inhibition of mitochondrial β-oxidation by these carboxylic acids correlates with their activation.

Summarized in Table 3 are the results obtained with 4-bromo-2-octenoic acid and 3-methylglycidic acid in addition to the above mentioned results observed with 4-bromocrotonic acid and valproic acid. 3-Methylglycidic acid was included in this study because it was evaluated as a potential mechanism-based inhibitor of β-oxidation enzymes. 4-Bromocrotonic acid was the only compound that was activated in both rat liver and rat heart mitochondria. 4-Bromo-2-octenoic acid and valproic acid were only activated by liver mitochondria, whereas 3-

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methylglycidic acid was activated neither in liver nor in heart mitochondria. A comparison of the activation of these four carboxylic acids with their effects on β-oxidation in liver and heart mitochondria supports the conclusion that these two properties are related to each other. Obviously, heart mitochondria have a much more limited capacity than liver mitochondria for activating substituted carboxylic acid which as a result of their activation may inhibit β-oxidation and perhaps exhibit other toxic effects. The different capacities of heart and liver mitochondria for activating short-chain and medium-chain carboxylic acids seem to be due to different specificities of their medium-chain acyl-CoA synthetases. Medium-chain acyl-CoA synthetase from bovine heart mitochondria was reported to only act on straight-chain carboxylic acids with 4 to 7 carbon atoms (94), whereas the corresponding synthetase from liver mitochondria is active with carboxylic acids having 4 to 12 carbon atoms (95). Moreover, substituted carboxylic acids and even aromatic carboxylic acids, like benzoic acid and phenylacetic acid, are activated by the liver synthetase (95)

Since 3-methylglycidic acid was not activated in mitochondria, this compound was not expected to inactivate β-oxidation enzymes. To confirm this assumption, coupled rat liver mitochondria were incubated for 3 min with and without 0.1 mM 3-methylglycidic acid and were assayed for enoyl-CoA hydratase (crotonase), L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase. The activities of all three enzymes were unaffected by incubation with 3-methylglycidic acid (data not shown). However, it remained to be determined whether or not 3-methylglycidoyl-CoA would inhibit any of these enzymes of β-oxidation.

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Only 3-ketoacyl-CoA thiolase was found to be inhibited by 70% when the aforementioned β-oxidation enzymes were incubated with 60 μM 3-methylglycidoyl-CoA for 15 min before being assayed (data not shown). A more detailed study of the inhibition of 3-ketoacyl-CoA thiolase by 3-methylglycidoyl-CoA revealed that the inhibitory compound affected the enzyme in a concentration-dependent and time-dependent manner (see Fig. 10). A 90% inhibition of this thiolase by 0.1 mM 3-methylglycidoyl-CoA was observed. When the concentration of 3-methylglycidoyl-CoA was kept constant at 30 μM, the enzyme lost 65% of its activity within 5 min (see Fig. 10). Since 3-ketooctanoyl-CoA, an effective substrate of this thiolase, did not prevent or even slow down the inactivation of the enzyme, 3-methyglycidoyl-CoA is most likely not an active site directed inhibitor. This conclusion is justified by the repeated observations that active site directed inhibitors of thiolases, as for example, 3-keto-4-bromobutyryl-CoA and 3-keto-4-bromooctanoyl-CoA formed from 4-bromocrotonic acid and 4-bromo-2-octenoic acid, respectively, inactivate 3-ketoacyl-CoA thiolase only slowly in the presence of substrate (49, 51). When 3-ketoacyl-CoA thiolase was first treated with 60 μM 3-methylglycidoyl-CoA for 15 min and then rapidly filtered through Sephadex G-50 by centrifugation, the lost activity (70%) was not restored (data not shown). This experiment is indicative of a mechanism of inactivation due to 3-methylglycidoyl-CoA reacting with 3-ketoacyl-CoA thiolase. Altogether, the experiments performed with 3-methylglycidoyl-CoA prompt the conclusion that this compound irreversibly inactivates 3-ketoacyl-CoA thiolase, most likely by forming a covalent adduct at a site other than the active site.

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Summarized in Figure 11 are that medium-chain substituted caroboxylic acids can enter into mitochondria directly and be activated to their CoA derivatives. These CoA derivatives can either become inhibitory to one or several enzymes involved in fatty acid β-oxidation or deplete free CoA in the mitochondria, thereby , shutting down the whole β-oxidation process.

Intermediate Channeling on the Trifunctional β-Oxidation Complex (TOC) from Pig Heart Mitochondria

Purified TOC[1] from pig heart was studied here. The results demonstrate that the two intermediates are either not formed or accumulate at levels insufficient to sustain the rate of the observed overall reaction. This finding agrees with the existence of a channeling mechanism which could explain the observed intermediate absence in whole cells.

Kinetic properties of TOC - The kinetic parameters (K_m,V_{max}) of TOC were determined with long-chain substrates at fixed coenzyme concentrations that were saturating (1 mM NAD^+) or close to the level known to exist in respiring mitochondria (0.2 mM CoASH) (42). The sequence of the three reactions, referred to as overall reaction (see Fig. 3) catalyzed by TOC, was measured either by recording spectrophotometrically the reduction of NAD^+ or by measuring the formation of acetyl-CoA by HPLC. Since both methods yielded the same results (data not shown), the

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more convenient spectrophotometric assay was used to determine the apparent K_m and V_{max} values for the overall reaction. In addition, the kinetic parameters of the individual reactions catalyzed by long-chain enoyl-CoA hydratase, long-chain 3-hydroxyacyl-CoA dehydrogenase, and long-chain 3-ketoacyl-CoA thiolase of TOC were determined. The results, shown in Table 4, indicate that the dehydrogenase catalyzes the slowest reaction in the sequence and that the apparent K_m values for 2-*trans*-hexadecenoyl-CoA in the first and overall reactions are similar but lower than the K_m values for the substrates of the second and third reactions. The K_m value of 3.9 µM for 3-hydroxyhexadecanoyl-CoA in the dehydrogenase reaction was obtained by assuming that the substrate was in equilibrium with its dehydration product, whereas the K_m value of 5.5 µM is based on the assumption that the substrate was not dehydrated.

Determination of intermediates and comparison of actual and calculated reaction rates - The substrate, intermediates, and products of the TOC-catalyzed overall reaction were separated by HPLC and quantified by use of standard curves established for each of the compounds with HPLC-purified acyl-CoA thioesters. As is apparent from Fig. 12A, only the first intermediate, 3-hydroxyhexadecanoyl-CoA (I_1) was detected besides the substrate (Δ^2C_{16}) and the two products myristoyl-CoA (C_{14}) and acetyl-CoA (C_2). The second intermediate, 3-ketohexadecanoyl-CoA (I_2), would have been detected, because it can be separated from all other compounds under the conditions used in this experiment (see Fig. 12B). The quantitative recovery of acyl-CoA thioesters was demonstrated by adding pentadecanoyl-CoA to the reaction mixture and determining its

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concentration by HPLC (data not shown). The concentration of intermediate I_1, 3-hydroxyhexadecanoyl-CoA, was determined as a function of the incubation time. As can be seen from Fig. 13A, the concentration of I_1 increased during the first minute of the reaction but declined thereafter. The measured concentration of I_1 and the kinetic parameters of the dehydrogenase (see Table 4) were used to calculate rates of NADH formation catalyzed by long-chain 3-hydroxyacyl-CoA dehydrogenase. The calculated formation of NADH , based on the concentration of free 3-hydroxyhexadecanoyl-CoA in the reaction mixture, is significantly lower than the observed formation of NADH (see Fig. 13B). Lines 1 and 2 in Fig. 13B represent the lower and upper limits, respectively, of the theoretical NADH formation based on K_m values of 3.9 μM and 5.5 μM. These K_m values reflect the lower and upper limits of the effective substrate concentrations in the assays of long-chain 3-hydroxyacyl-CoA dehydrogenase. Since the observed rate of NADH formation was higher than either of the calculated rates, the effective concentration of I_1 must be higher than the concentration of I_1 in the bulk phase. Such condition could be achieved if I_1 were channeled from the active site of long-chain enoyl-CoA hydratase to that of long-chain 3-hydroxyacyl-CoA dehydrogenase. The presence of I_1 in the bulk phase may be due to an excess capacity of the hydratase and a leaky channeling mechanism.

In contrast to the leaky channeling of I_1, 3-ketohexadecanoyl-CoA (I_2) was not detected at any time during the course of the overall reaction under the same conditions at which free I_1 was formed. Since 0.5 μM I_2 or less could have been detected, the observed formation of acetyl-CoA was compared with the calculated formation supported by 0.5 μM and 0.1 μM I_2.

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As shown in Fig. 14, the observed rate is much higher than the expected rate in the presence of 0.5 µM I_2 in the bulk phase. This discrepancy between observed and predicted rates supports the hypothesis of intermediate channeling between the active sites of the dehydrogenase and thiolase of TOC.

Effect of acetyl-CoA on the formation of intermediates and products of the TOC-catalyzed reaction sequence - The product inhibition of the TOC-catalyzed overall reaction by acetyl-CoA was studied with the aim of evaluating the proposed intermediate channeling. Acetyl-CoA inhibited the formation of myristoyl-CoA from 2-*trans*-hexadecenoyl-CoA by 50% when the acetyl-CoA concentration was raised from zero to 1 mM (see Fig. 15). However, the effect of this inhibitor on the concentration of intermediates was limited. Noteworthy is the accumulation of 0.5 µM I_2 in the presence of 1 mM acetyl-CoA, whereas this intermediate was not detected in the absence of acetyl-CoA. A small amount of I_2 also accumulated when the rate of the thiolase-catalyzed reaction was reduced by lowering the concentration of CoASH from 0.2 mM to 0.05 mM (data not shown). However, the concentration of I_1 changed insignificantly even though the hydration of 2-*trans*-hexadecenoyl-CoA had not reached an equilibrium. In fact, the observed concentration of I_1 was less than 50% of its equilibrium concentration. Acetyl-CoA, the product of the last of the three sequential reactions, was expected to inhibit thiolase. This assumption was proven to be correct by demonstrating that acetyl-CoA inhibited the thiolytic cleavage of 3-ketohexadecanoyl-CoA to myristoyl-CoA and acetyl-CoA (see Fig. 16A). Since 3-keto- acyl-CoAs, as for example

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acetoacetyl-CoA, are known inhibitors of 3-hydroxyacyl-CoA dehydrogenase (96), the effect of 3-ketohexadecanoyl-CoA on the reduction of NAD^+ in the overall reaction was determined. As shown in Fig. 16B, 3-ketohexadecanoyl-CoA at low micromolar concentrations inhibited the dehydrogenation of 3-hydroxyhexadecanoyl-CoA, the slowest reaction in the reaction sequence. This inhibition could explain the decreased formation of NADH by acetyl-CoA in the overall reaction because the latter compound inhibits thiolase with the result that I_2 accumulates which in turn inhibits the dehydrogenase-catalyzed formation of NADH. However if intermediate channeling occurs, the concentration of bound intermediate would most likely be higher than reflected by the concentration of free intermediate. Hence the degree of inhibition might be higher than could be accounted for by the concentration of the free intermediate. This seems to be the situation when the inhibition of the overall reaction by acetyl-CoA is analyzed. Although 1 mM acetyl-CoA caused an inhibition of the overall reaction by 50% (see Fig. 16A), the accumulation of 0.5 μM I_2 only explains a 30% decrease of the dehydrogenase-catalyzed reaction (see Fig. 16B) which is limiting the overall reaction. The difference between the observed and predicted degrees of inhibition are attributed to intermediate channeling.

Effects of pyruvate and L-carnitine on the β-oxidation of palmitoylcarnitine in coupled rat heart mitochondria A more direct method for assaying β-oxidation than the measurement of respiration is the determination of acid-soluble products and/or CO_2 formed from (1-

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^{14}C) palmitoylcarnitine. To maintain state 3 respiration, glucose and hexokinase were added to the reaction mixture. When rat heart mitochondria were incubated under these conditions for 10 min, mostly acid-soluble products were formed which remained in aqueous solution after the extraction of (1-^{14}C) palmitoylcarnitine with n-butanol. Since little $^{14}CO_2$ was formed from (1-^{14}C) palmitoylcarnitine under these conditions, only the formation of acid-soluble products (mostly acetyl-CoA and intermediates of the tricarboxylic acid cycle) was determined. As shown in Fig. 17, addition of pyruvate greatly inhibited β-oxidation at state 3. However, L-carnitine, which was added to reduce the (Acetyl-CoA)/(CoASH) ratio, could partially relieve the inhibition of β-oxidation caused by pyruvate at state 3 respiration.

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DISCUSSION

Activities of acyl-CoA dehydrogenases are usually measured spectrophotometrically by assays in which the reduction of electron acceptors is associated with measurable absorbance changes (65-67, 69, 70). Most of these assay methods give unsatisfactory results when they are used to measure acyl-CoA dehydrogenase activities in crude tissue homogenates. Problems are caused by the reoxidation of electron acceptors in the presence of oxygen and endogenous electron carriers and by the reaction of electron acceptors with endogenous sulfhydryl-containing compounds or with CoASH formed from acyl-CoA by thioesterases. These problems are avoided when a fluorometric assay based on the substrate-dependent reduction of ETF (68) is used. However, this assay method is cumbersome because it requires anaerobic conditions and substrate-level quantities of ETF which is not commercially available. Another disadvantage of all established assay methods is that they are not specific for any of the acyl-CoA dehydrogenases functioning in
β-oxidation due to overlapping chain length specificities of these enzymes.

The observation by Dommes and Kunau (97) that 4-decenoyl-CoA is a good substrate of MCAD but a poor one of long-chain acyl-CoA dehydrogenase provided a clue for the design of a specific assay of the MCAD. A recent report by Rinaldo et al. (89) showing that only MCAD effectively acts on PP-CoA suggested another possible substrate. Of

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several substrates considered here, PP-CoA was best suited, because the absorbance maximum of its dehydrogenation product cinnamoyl-CoA falls into a spectral region of low PMS absorptivity which is the preferred electron acceptor for the new assay method. Measurements with partially purified bovine liver acyl-CoA dehydrogenases confirmed the specificity of MCAD for PP-CoA.

When partially purified MCAD was used, the calculated values for cinnamoyl-CoA formation based on measured absorbance changes and an extinction coefficient of 26,500 M^{-1} cm^{-1} compared well with amounts of cinnamoyl-CoA determined by HPLC. However, in tissue homogenates, especially in those with low activity levels of MCAD, reactions other than the measured dehydrogenation may result in overestimated or underestimated activity values. An assessment of possible alternate reactions associated with absorbance changes at 308 nm revealed only minor problems. The absorbance increase observed at 308 nm when only PP-CoA was added to a rat liver homogenate was 1% of the absorbance change when additionally PMS was present. The slow reaction in the absence of PMS was attributed to peroxisomal acyl-CoA oxidase which dehydrogenates PP-CoA with oxygen as an electron acceptor. If necessary, the oxidase activity, which was even lower in extrahepatic tissues, can be corrected for by subtracting the absorbance increase measured in the absence of PMS from the change in absorbance determined with PMS. The observed stability of cinnamoyl-CoA in the assay mixture demonstrates the resistance of this compound toward thioesterases and enoyl-CoA hydratases. The latter enzymes act on cinnamoyl-CoA but the equilibrium of the hydration reaction is far to

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the side of the dehydrated reactant. However, thioesterases present in tissue homogenates hydrolyze PP-CoA thereby forming CoASH which reacts with PMS to yield a compound that contributes to the absorbance increase at 308 nm. When NEM was present in the assay mixture, the reaction between CoASH and PMS was reduced by more than 50%. With homogenates of rat leukocytes, which have low levels of MCAD, the thioesterase-dependent contribution to the absorbance change at 308 nm was 2% of the total ΔA_{308}. If necessary, an appropriate correction for the thioesterase-dependent contribution to the measured ΔA_{308} can be made by measuring the hydrolysis of substrate by thioesterase with DTNB (78) and calculating the resulting absorbance change at 308 nm by use of an extinction coefficient of 1,500 M^{-1} cm^{-1}.

Altogether, the assay of MCAD described here is specific, sensitive and relatively free of interferences by side reactions. If deviations up to 5% from the actual activity value are acceptable, the method can be used as described. If a greater accuracy is required some corrections may have to be made depending on the tissue that is being studied. Overall, the advantages of this first assay method based on the direct measurement of an enoyl-CoA product outweigh small interferences caused by side reactions.

The central role of free CoA (CoA) in the cellular metabolism of fatty acids (FA), medium-chain fatty acids (mcFA), and acetate (Ac) is summarized in Fig. 11. At least two separate pools of CoA, consisiting of free CoA and all of its acyl derivatives, exist in animal cells. One pool is only available for intramitochondrial reactions, whereas the other pool participates in extramitochondrial pathways. The distribution of total

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cellular CoA between the intra-and extramitochondrial pools is not the same in different tissues. In liver, the two pools are almost equal, whereas in heart 95% of the cellular CoA is estimated to be in mitochondria with the remaining 5% constituting the extramitochondrial pool (98). The extramitochondrial pool of CoA is most likely larger in liver than in heart because certain extramitochondrial CoA-requiring reactions are important in liver but are insignificant in heart. These reactions include the activation of fatty acids destined for lipid synthesis and peroxisomal degradation (see Fig. 11). Also the citrate cleavage reaction yielding acetyl-CoA for fatty acid biosynthesis is important in liver but not in heart. The activation of fatty acids, which are degraded by mitochondrial β-oxidation, is an important reaction in both liver and heart. In both hepatic and myocardial mitochondria, free CoA is required for generating fatty acyl-CoA from fatty acylcarnitine and for activating medium-chain fatty acids which directly enter mitochondria without the assistance of carnitine. The mitochondrial degradation of fatty acyl-CoA, pyruvate, and most amino acids requires free CoA which is regenerated by the metabolism of the resultant acetyl-CoA via the tricarboxylic acid (TCA)[1] cycle or by the utilization of acetyl-CoA for ketone body synthesis in liver.

The amount of free CoA both inside and outside of mitochondria is determined by the balance of CoA-requiring and CoA-regenerating reactions. Any xenobiotic compound disturbing this balance would affect the distribution of CoA between its different forms and thereby could affect all CoA-dependent pathways. Most serious would be either the inactivation of an enzyme catalyzing one of the major CoA-

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dependent reactions or the conversion of free CoA to inert acyl-CoA with resultant depletion of the free CoA pool.

Since long-chain fatty acids enter mitochondria via the carnitine-dependent uptake system (99), which is rather selective with respect to its substrates, xenobiotic carboxylic acids with the potential for causing damage intramitochondrially are more likely substituted medium-chain carboxylic acid that directly enter mitochondria. If such xenobiotic carboxylic acids are activated by medium-chain acyl-CoA synthetase in the mitochondrial matrix, they may tie up CoA and thereby reduce the concentration of free CoA and/or inhibit mitochondrial enzymes as for example 3-ketoacyl-CoA thiolase of β-oxidation (52, 100). The xenobiotic carboxylic acids illustrating the relationship between activation and toxicity are 4-bromocrotonic acid, 4-bromo-2-octenoic acid, valproic acid, and 3-methylglycidic acid. Of special interest for this discussion is 3-methylglycidic acid which has no effect on mitochondrial β-oxidation, but which strongly inhibits 3-ketoacyl-CoA thiolase once it is conjugated with CoA. Since it is reasonable to assume that this medium-chain carboxylic acid can penetrate the inner mitochondrial membrane, the absence of any effect of 3-methylglycidic acid on β-oxidation is most likely due to acyl-CoA synthetase not activating this acid. The observation that valproic acid and 4-bromo-2-octenoic acid are activated and toxic in rat liver, but not in rat heart mitochondria emphasizes tissue differences. Liver, which plays an key function in drug detoxification, is more likely damaged by reactive metabolites formed from xenobiotic compounds. In the case of 4-bromo-2-octenoic acid, the cause for the inhibition of β-oxidation is the inactivation of 3-ketoacyl-

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CoA thiolase (51). Although the mechanism of the valproate-induced inhibition of mitochondrial β-oxidation has not yet been elucidated, it is possible that the inhibition is a consequence of free CoA depletion due to intramitochondrial accumulation of relatively stable CoA-containing metabolites of valproic acid (79). Finally, 4-bromocrotonic acid illustrates that the mitochondrial metabolism in tissues other than liver can be poisoned as long as potentially toxic xenobiotic carboxylic acids are activated intramitochondrially. The question of whether and, if so, how rapidly normal mitochondrial function can be regained depends on the rate at which inhibited enzymes are reactivated or replaced and/or depends on the rate at which free CoA is regenerated from inert acyl-CoAs. Since free CoA can be regenerated from acyl-CoAs by acyl transfer to carnitine or by hydrolysis, the activities of mitochondrial carnitine acyltransfereses and acyl-CoA thioesterases toward xenobiotic acyl-CoAs are crucial for predicting the metabolic consequences of CoA sequestration.

In summary, substituted medium-chain carboxylic acids are potentially toxic compounds because they can directly enter mitochondria where they may be activated and further metabolized by β-oxidation. One or several of the resultant acyl-CoA metabolites may impair the mitochondrial metabolism of fatty acids and other compounds by reversibly or irreversibly inhibiting enzyme or by causing the depletion of free CoA. Such compounds will more likely be toxic to liver than in other tissues due to the broad substrate specificity of the hepatic medium-chain acyl-CoA synthetase which is essential for the activation and further metabolism of xenobiotic carboxylic acids.

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The recent characterization of several long-chain specific β-oxidation enzymes (1, 2, 43, 44) has made it necessary to modify the traditional view of how the enzymes of β-oxidation cooperate to completely degrade fatty acids. As schematically shown in Fig. 2, a set of four long-chain specific enzymes, located in the inner mitochondrial membrane and consisting of very long-chain acyl-CoA dehydrogenase, long-chain enoyl-CoA hydratase, long-chain 3-hydroxyacyl-CoA dehydrogenase, and long-chain 3-ketoacyl-CoA thiolase, are presumed to catalyze the chain shortening of long-chain fatty acyl-CoAs. After one or several rounds of β-oxidation, the soluble matrix enzymes, consisting of short-chain, medium-chain and perhaps long-chain acyl-CoA dehydrogenases in addition to enoyl-CoA hydratase (crotonase), 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase, take over to complete the degradation of acyl-CoAs. This revised hypothetical view of mitochondrial β-oxidation necessitates a reevaluation of the proposed control mechanism(s) of β-oxidation, especially of the energy-linked regulation in extra-hepatic tissues, e. g. in heart (6). Changes in the energy demand of a tissue oxidizing fatty acids were thought to cause changes in the concentrations of β-oxidation intermediates which in turn may control the activity of the pathway by regulating activities of key enzymes (6). However, the question of whether and if so, to which degree intermediates accumulate during β-oxidation *in vivo* has not been answered unambiguously.

The evidence presented here prompts the conclusion that long-chain intermediates of mitochondrial β-oxidation are channeled between the active sites of TOC. This conclusion does not exclude the possibility of

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intermediates dissociating from TOC under certain conditions and exiting from mitochondria and even cells. For example, 3-hydroxyacyl-CoAs have been reported to accumulate in isolated mitochondria and to exit from them as acylcarnitines, especially when the reoxidation of NADH is impaired (101-105) or when long-chain 3-hydroxyacyl-CoA dehydrogenase is deficient (106). In contrast, the accumulation of 3-ketoacyl-CoAs has not been observed. The observed accumulation of a relatively large quantity of 3-hydroxyhexadecanoyl-CoA (I_1) in this study may be a consequence of the specific experimental set-up with 2-hexadecenoyl-CoA serving as a substrate instead of hexadecanoyl-CoA and with the activity of long-chain 3-hydroxyacyl-CoA hydratase being high compared to the other activities of TOC.

This study prompts the conclusion that the channeling of long-chain β-oxidation intermediates is most likely the underlying cause for their absence from the mitochondrial matrix or for their presence at very low levels. This situation also would explain why long-chain intermediates of β-oxidation were not detected in the extracellular fluid of fibroblasts unless an enzyme defect like long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency impaired the further metabolism of long-chain intermediates (32). If experiments with isolated mitochondria and whole cells yield different results with respect to the formation of β-oxidation intermediates, the results obtained with whole cells may be more relevant to the *in vivo* situation because mitochondria present in whole cells, in contrast to isolated ones, are most likely undamaged. However, when extracellular or extra-mitochondrial levels of intermediates are measured, the question arises of whether they truly reflect the intramitochondrial concentrations

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of acyl-CoAs. In fact the available evidence indicates that this may not be the case (104).

In considering the metabolic consequences of the non-accumulation of β-oxidation intermediates, the availability of more free coenzyme A in the mitochondrial matrix is perhaps most important. If each intermediate of β-oxidation were present in the matrix only at a low micromolar concentration, a substantial amount of the available CoASH would be tied up by the 27 intermediates which are formed along the pathway from palmitoyl-CoA to acetyl-CoA. Moreover, if β-oxidation intermediates accumulate, they might inhibit various mitochondrial enzymes, especially those with binding sites for acyl-CoAs. In fact, 3-ketoacyl-CoA intermediates at nanomolar concentrations are effective inhibitors of acyl-CoA dehydrogenases (K_i= 80 nM) (80, 107). 3-Ketoacyl-CoAs also inhibit 3-hydroxyacyl-CoA dehydrogenase (108). Effective product inhibition has been observed with other enzymes of β-oxidation. For example, acyl-CoA dehydrogenases are strongly inhibited by 2-*trans*-enoyl-CoAs (79, 106) and enoyl-CoA hydratase (crotonase) is inhibited by L-3-hydroxyhexadecanoyl-CoA with a K_i of 0.35 μM (84). Altogether, the available evidence suggests that the accumulation of intermediates would strongly inhibit the flux through the β-oxidation spiral. Such inhibition may be avoided by the absence of intermediates due to channeling.

Although it has been argued that channeling of intermediates provides a kinetic advantage to the β-oxidation system (109), the importance of this property is less certain as many metabolic pathways do not seem to be designed for optimal kinetic or energetic efficiency. However, the control of β-oxidation, especially of the energy-dependent

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regulation in extra-hepatic tissues, may be greatly affected by intermediate channeling. In a pathway with intermediate channeling, the regulation of any reaction is expected to affect the whole system without any or significant changes in the concentration of intermediates. For example, the proposed regulation of 3-ketoacyl-CoA thiolase by the [acetyl-CoA]/[CoASH] ratio (6) could directly affect the activity of the first reaction of β-oxidation catalyzed by acyl-CoA dehydrogenase without 3-ketoacyl-CoA intermediates accumulating in the matrix and inhibiting acyl-CoA dehydrogenases (80).

β-Oxidation of long-chain fatty acid in mitochondria can be measured by determining the formation of acid-soluble products from (1-^{14}C) parmitoylcarnitine. My data shows that the addition of 5 mM pyruvate inhibits long-chain fatty acid β-oxidation by approximately 75% and that the addition of 10 mM L-carnitine partially relieves this inhibition of β-oxidation. This result leads to the conclusion that pyruvate inhibits fatty acid β-oxidation by affecting the ratio of (acetyl-CoA)/(CoASH). This observation supports the hypothesis that the rate of β-oxidation in extrahepatic mitochondria is regulated by the ratio of (acetyl-CoA)/(CoASH).

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TABLE 1

Kinetic Constants of MCAD with 3-Phenylpropionoyl-CoA (PP-CoA) and Octanoyl-CoA (C_8-CoA) as Substrates

Assay Method	Kinetic Constants[a]			
	PP-CoA		C_8-CoA	
	K_m µM	V_{max} mU/mg	K_m µM	V_{max} mU/mg
Reduction of DCPIP	1.2	320	2.8	350
Formation of Cinnamoyl-CoA	1.2	320		
	1.2	590*		

[a] For details of the assay procedure based on the reduction of DCPIP and the new assay procedure measuring the formation of cinnamoyl-CoA see under MATERIALS AND METHODS. All measurements were carried out at a fixed PMS concentration of 3.3 mM except for the value obtained with 4.5 mM PMS which is marked with an asterisk.

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TABLE 2

Activities of Acyl-CoA Dehydrogenases toward 3-Phenylpropionyl-CoA (PP-CoA) Relative to Activities Determined with Optimal Substrates

Acyl-CoA dehydrogenase	Specific Activity[a] Control[b]	PP-CoA	Activity with PP-CoA relative to control
	mU/mg		%
Long-chain	300	3.4	1.2
Medium-chain	350	320	92
Short-chain	120	0	0

[a] The conventional assay based on the substrate-dependent reduction of DCPIP was used. For details see MATERIALS AND METHODS.

[b] Control activities were determined with the following substrates: long-chain acyl-CoA dehydrogenase with dodecanoyl-CoA; MCAD with octanoyl-CoA; short-chain acyl-CoA dehydrogenase with butyryl-CoA.

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TABLE 3

Mitochondrial activation of 4-bromocrotonic acid, 4-bromo-2-octenoic acid, valproic acid, and 3-methylglycidic acid and effects of these carboxylic acids on palmitoylcarnitine-supported respiration in rat liver (RLM) and rat heart mitochondria (RHM)

Inhibitor	RLM			RHM		
	Act.[a]	Conc.	Inh.[a]	Act.[a]	Conc.	Inh.[a]
		mM	%		mM	%
4-Bromocrotonic acid	+	0.01	90	+	0.02	100[b]
4-Bromo-2-octenoic acid	+	0.02	100[c]	0	0.1	6
Valproic acid	+	0.5	43	0	0.5	0
3-Methylglycidic acid	0[d]	0.1	0	0[d]	0.1	0

a. Act., activation refers to the conversion of acids to their corresponding acyl-CoAs with "+" and "0" indicating formation and no formation of acyl-CoA, respectively. Inh., inhibition of palmitoylcarnitine-supported respiration at listed concentrations (Conc.) of inhibitors. For experimental details see Experimental Procedures.

b. From reference 17.

c. From reference 5.

d. Only trace amounts of 3-methylglycidoyl-CoA were detected.

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TABLE 4

Kinetic Parameters of the Trifunctional β-Oxidation Complex from Pig Heart

Enzyme [a]	Substrate	Km[b] µM	Vmax[b] U/mg
Overall Reaction	2-Hexadecenoyl-CoA	2.7	1.0
Hydratase	2-Hexadecenoyl-CoA	2.0	12.8
Dehydrogenase	3-Hydroxyhexadecanoyl-CoA	5.5 (3.9)[c]	1.1
Thiolase	3-Ketohexadecanoyl-CoA	8.4	2.0

[a] Overall reaction, the three coupled reactions catalyzed by TOC as measured by the formation of NADH; hydratase, long-chain enoyl-CoA hydratase; dehydrogenase, long-chain L-3-hydroxyacyl-CoA dehydrogenase; thiolase, long-chain 3-ketoacyl-CoA thiolase. For experimental details, see Experimental Procedures.

[b] Apparent K_m and V_{max} values are means of two determinations which differed by 12% or less.

[c] The Km value of 5.5 µM was obtained by assuming that the substrate was not dehydrated, whereas the Km value of 3.9 µM is based on the assumption that the equilibrium of the dehydration/hydration was reached instantaneously.

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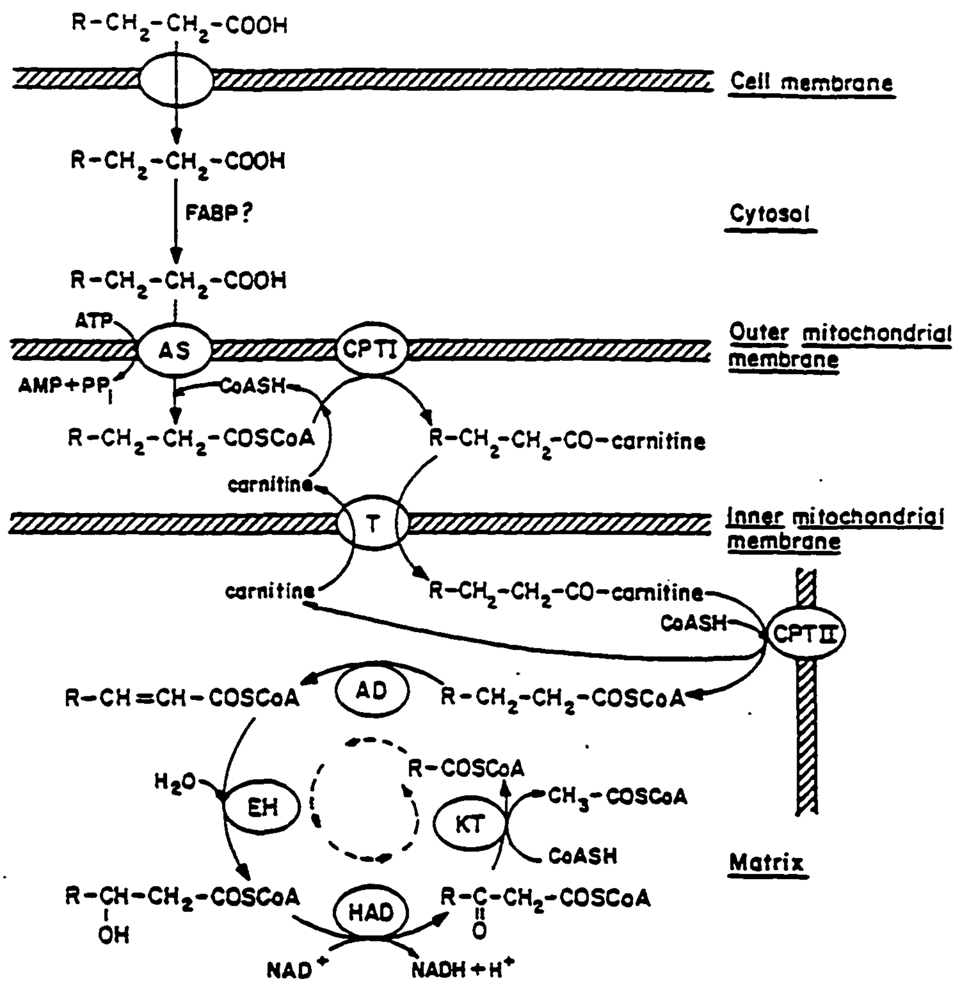

Fig. 1

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FIG. 1. Pathway of mitochondrial fatty acid oxidation. Enzymes of the pathway are : AS, acyl-CoA synthetase; CPT I, carnitine palmitoyltransferase I; T, carnitine: acylcarnitine translocase; CPT II, carnitine palmitoyltransferase II; AD, acyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; HAD, L-3-hydroxyacyl-CoA dehydrogenase; KT, 3-ketoacyl-CoA thiolase. Other abbrebiation; FABP, fatty acid binding protein.

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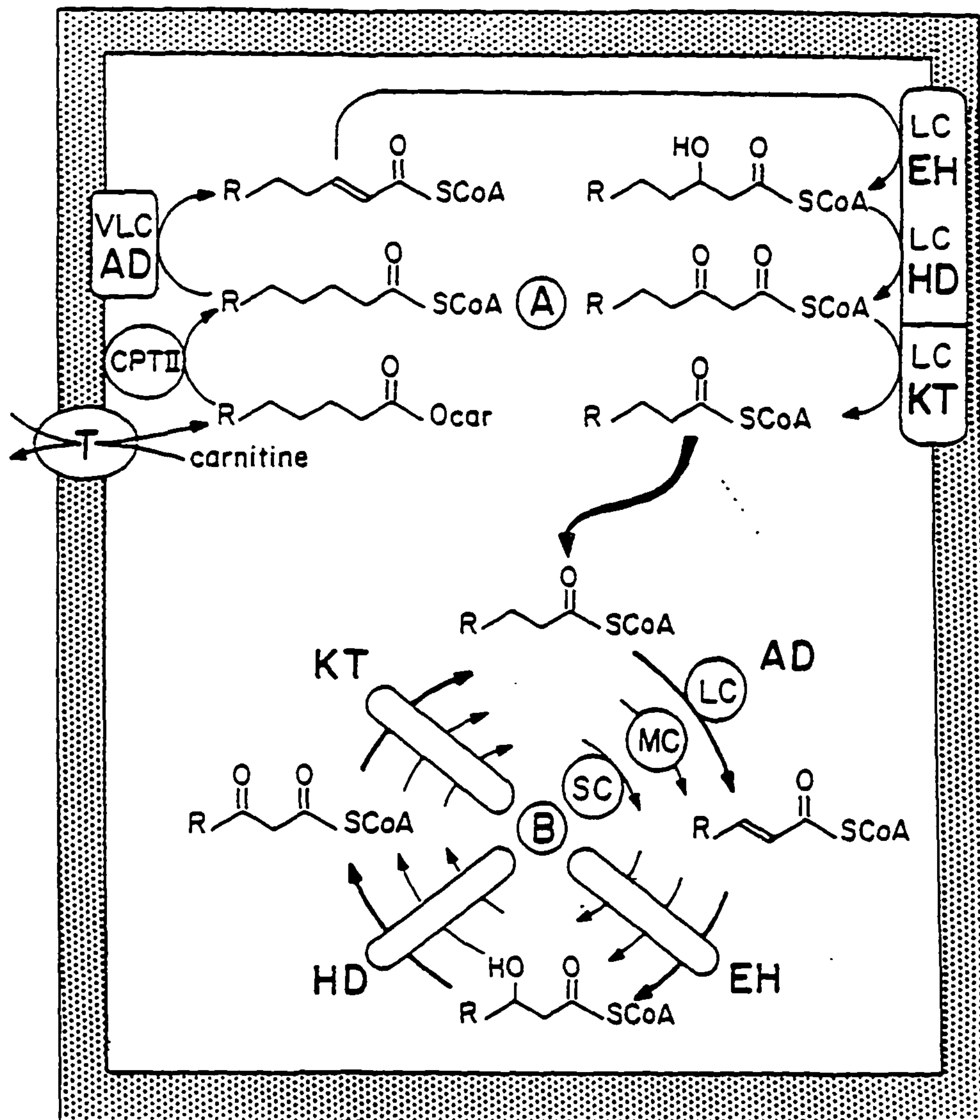

Fig. 2

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FIG. 2. Model of the functional and physical organization of β- oxidation enzymes in mitochondria. A. β-Oxidation system active with long-chain acyl-CoAs; B. β-Oxidation system active with long-chain, medium-chain, and short-chain acyl-CoAs. Abbreviations: T, carnitine:acylcarnitine translocase; CPT II, carnitine palmitoyltransferase II; AD, acyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; HD, L-3-hydroxyacyl-CoA dehydrogenase; KT, 3-ketoacyl-CoA thiolase; VLC, very-long-chain; LC, long-chain; MC, medium-chain; SC, short-chain.

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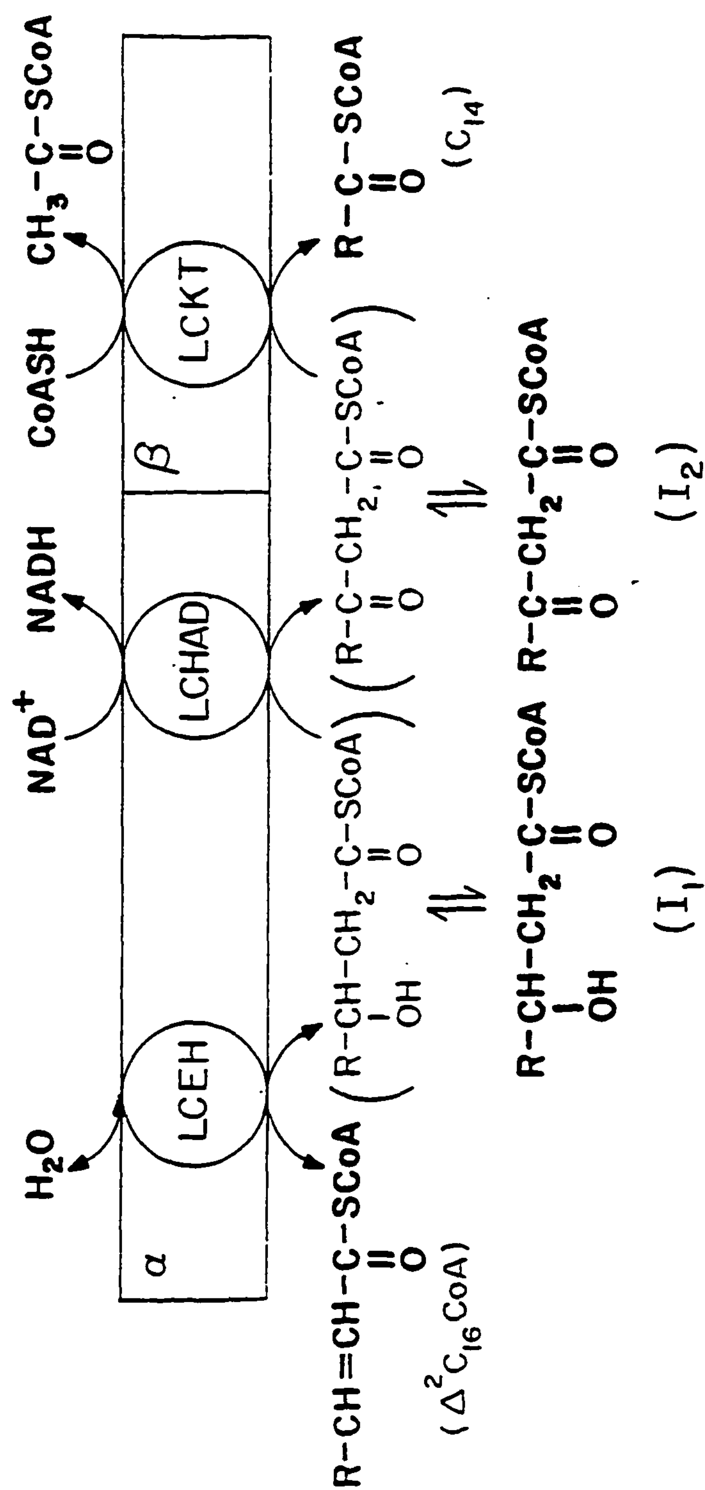

Fig. 3

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FIG. 3. Reaction catalyzed by the trifunctional β-oxidation complex. Abbriviations: LCEH, long-chain enoyl-CoA hydratase; LCHAD, long-chain L-3-hydroxyacyl-CoA dehydrogenase; LCKT, long-chain 3-ketoacyl-CoA thiolase; Δ^2-C_{16}-CoA, 2-*trans*-hexadecenoyl-CoA; C_{14}-CoA, myristoyl-CoA; I_1, 3-hydroxyhexadecanoyl-CoA; I_2, 3-ketohexadecanoyl-CoA.

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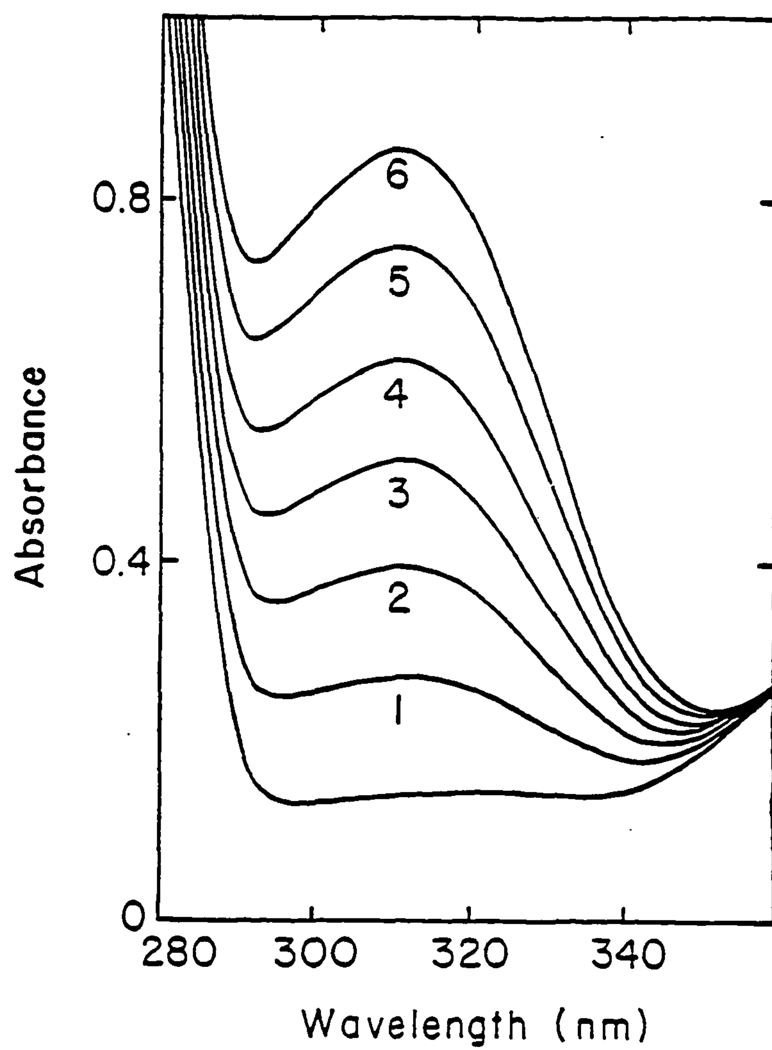

Fig. 4

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FIG. 4. Spectral change associated with the dehydrogenation of PP-CoA by bovine liver MCAD. The assay contained 60 µM PP-CoA, 0.1 mM PMS and partially purified enzyme. Spectra 1 through 6 were obtained by scanning the assay mixture 5, 10, 15, 20, 25 and 30 min, respectively, after initiating the reaction.

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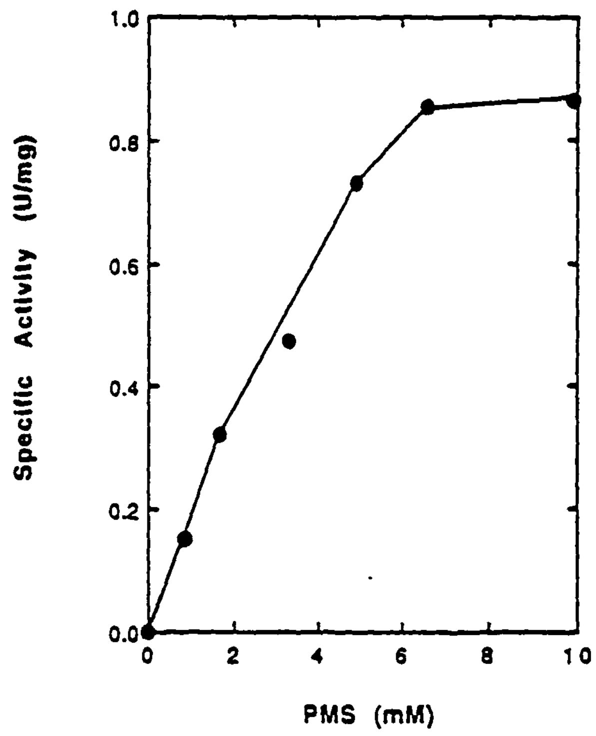

Fig. 5

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FIG. 5. Specific activity of bovine liver MCAD as a function of the PMS concentration. Substrate: 60 µM PP-CoA.

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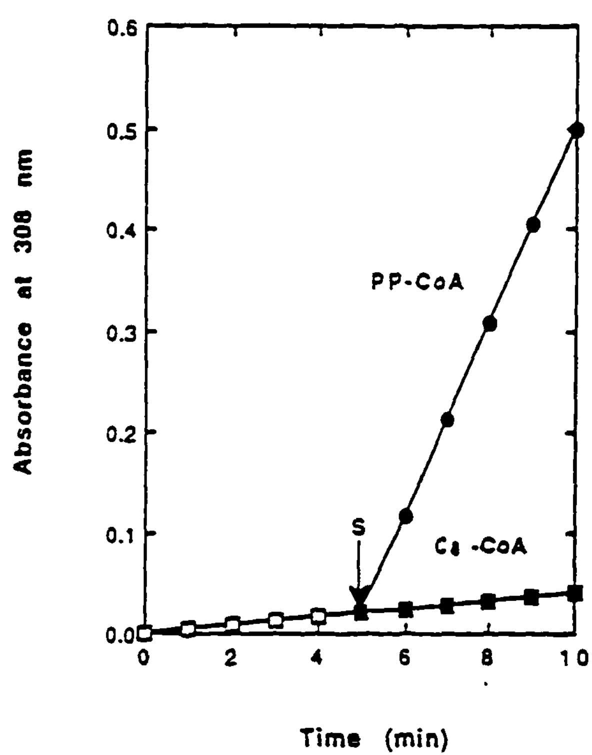

Fig. 6

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FIG. 6 Absorbance change at 308 nm as a function of time. Partially purified MCAD from bovine liver plus 4.5 mM PMS (□). The arrow marked S indicates the addition of substrates to the solution containing dehydrogenase plus PMS. The substrates were either 60 μM PP-CoA (●) or 60 μM octanoyl-CoA (C_8-CoA) (■).

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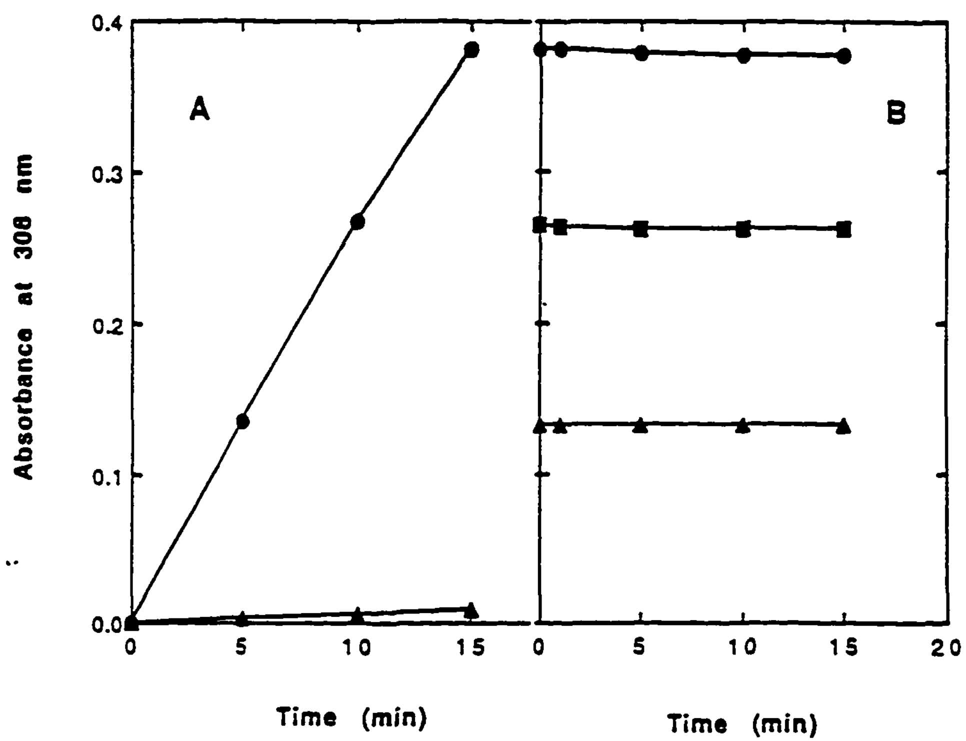

Fig. 7

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FIG. 7. Absorbance changes at 308 nm as a function of time. A. Measured absorbance change when MCAD was assayed with 60 μM PP-CoA, 4.5 mM PMS, and 0.2 mM NEM in a rat leukocyte homogenate (●). Calculated absorbance change due to the reaction between PMS and CoASH formed by hydrolysis of PP-CoA (▲). B. Absorbance of 5 μM (▲), 10 μM (■), and 15 μM (●) cinnamoyl-CoA in the presence of 4.5 mM PMS, 0.2 mM NEM, and rat leukocyte homogenate as a function of time.

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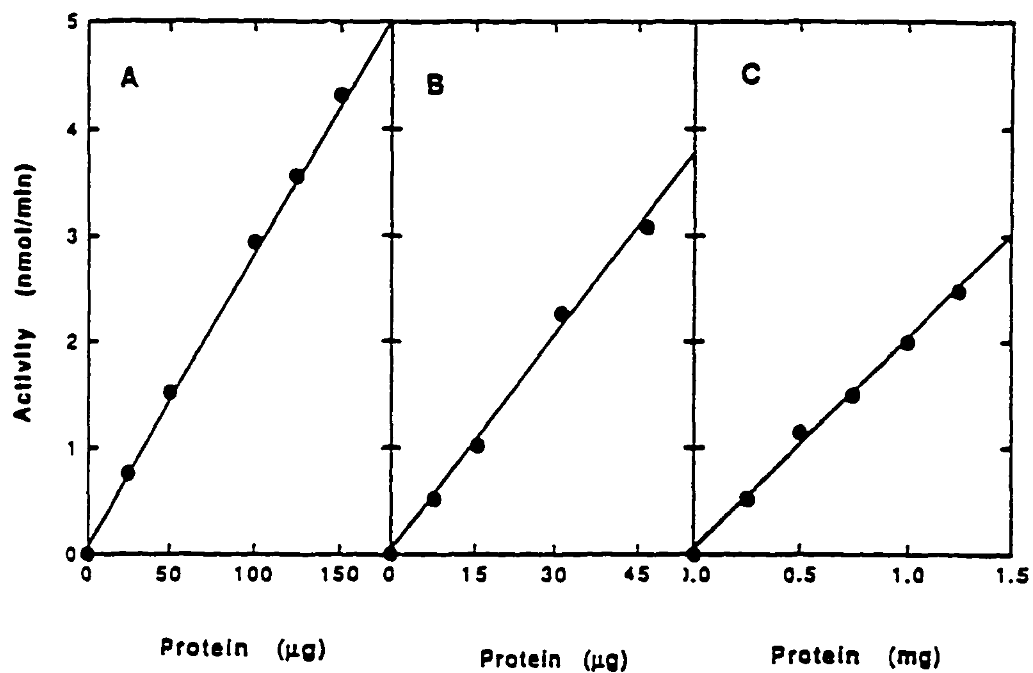

Fig. 8

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FIG. 8 Activity of MCAD as a function of the protein concentration. A, rat liver homogenate. B, rat heart homogenate. C, rat leukocyte homogenate.

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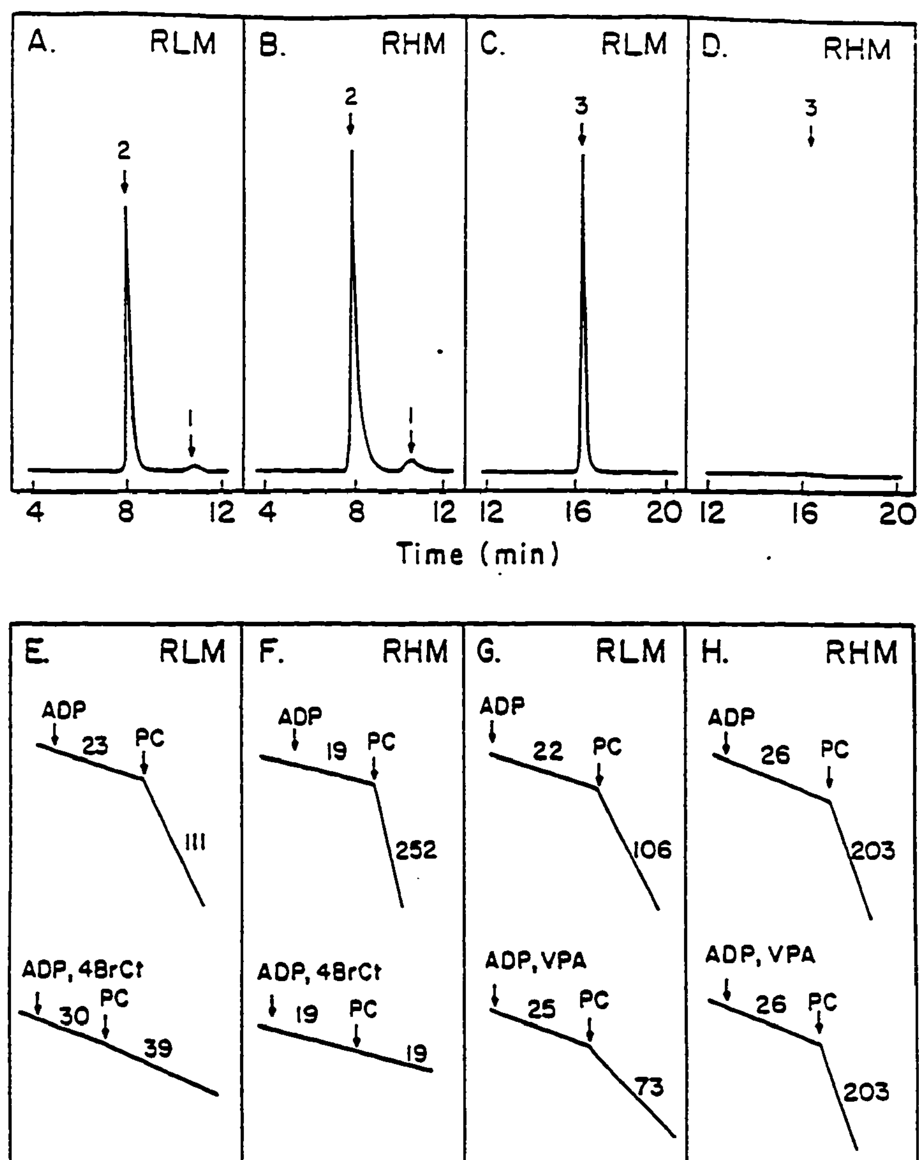

Fig. 9

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FIG. 9. Activation of 4-bromocrotonic acid and valproic acid by soluble extracts of rat liver and rat heart mitochondria and effects of these two compounds on palmitoylcarnitine-supported respiration in rat liver and rat heart mitochondria. HPLC analyses of activation products of 4-bromocrotonic acid (A, B) and valproic acid (C, D) formed in the presence of soluble extracts of rat liver mitochondria (A, C) and rat heart mitochondria (B, D). The following authentic compounds were eluted at times indicated by arrows marked 1, 4-bromocrotonyl-CoA; 2, 3-hydroxy-4-bromobutyryl-CoA, obtained by incubating 4-bromocrotonyl-CoA with crotonase; 3, valproyl-CoA. Rates of respiration of coupled rat liver mitochondria (E, G) and rat heart mitochondria (F, H) in the presence and absence of 4-bromocrotonic acid (E, F) and valproic acid (G, H). Numbers give rates of respiration in nanoatoms of oxygen per min and mg of protein. Abbreviations: RLM, rat liver mitochondria; RHM, rat heart mitochondria; PC, palmitoyl-L-carnitine; 4BrCt, 4-bromocrotonic acid; VPA, valproic acid. For experimental details see Experimental Procedures.

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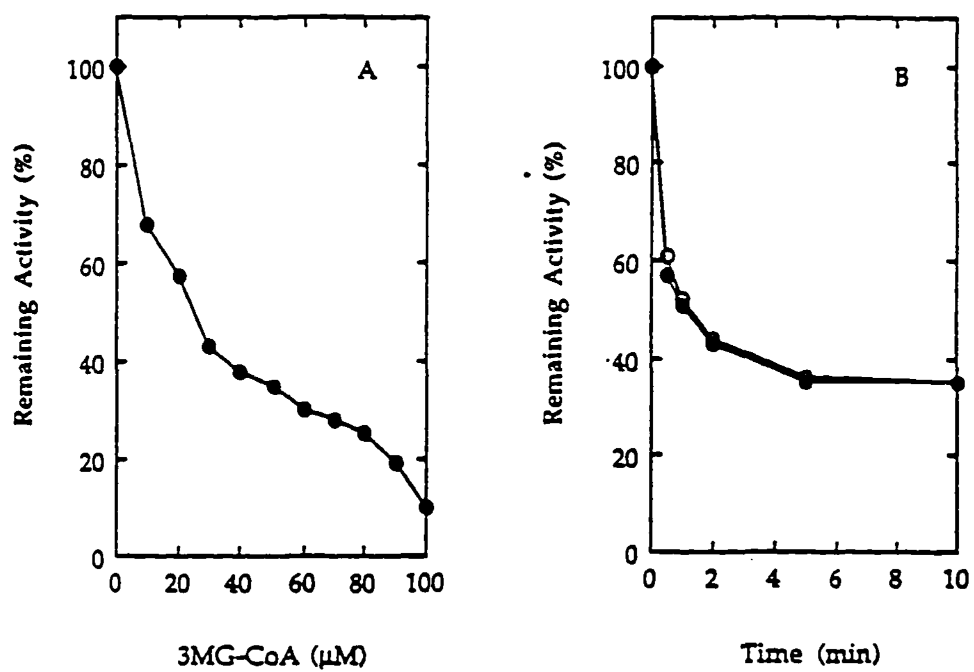

Fig. 10

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FIG. 10. Inhibition of 3-ketoacyl-CoA thiolase by 3-methylglycidoyl-CoA (3MG-CoA) as a function of its concentration and the incubation time. A, 3-Ketoacyl-CoA thiolase (0.1 μg/ml) was incubated with 3MG-CoA for 10 min at 25°C before being assayed with 60 μM 3-ketooctanoyl-CoA as substrate. The concentration of 3MG-CoA in the assay mixture was 10-time lower than in the incubation mixture. B, 3-Ketoacyl-CoA thiolase was pre incubated with 30 μM 3MG-CoA in the presence (O) or absence (●) of 400 μM 3-ketooctanoyl-CoA before being assayed with 40 μM 3-ketooctanoyl-CoA as substrate. The concentration of 3MG-CoA in the assay mixture was 3 μM.

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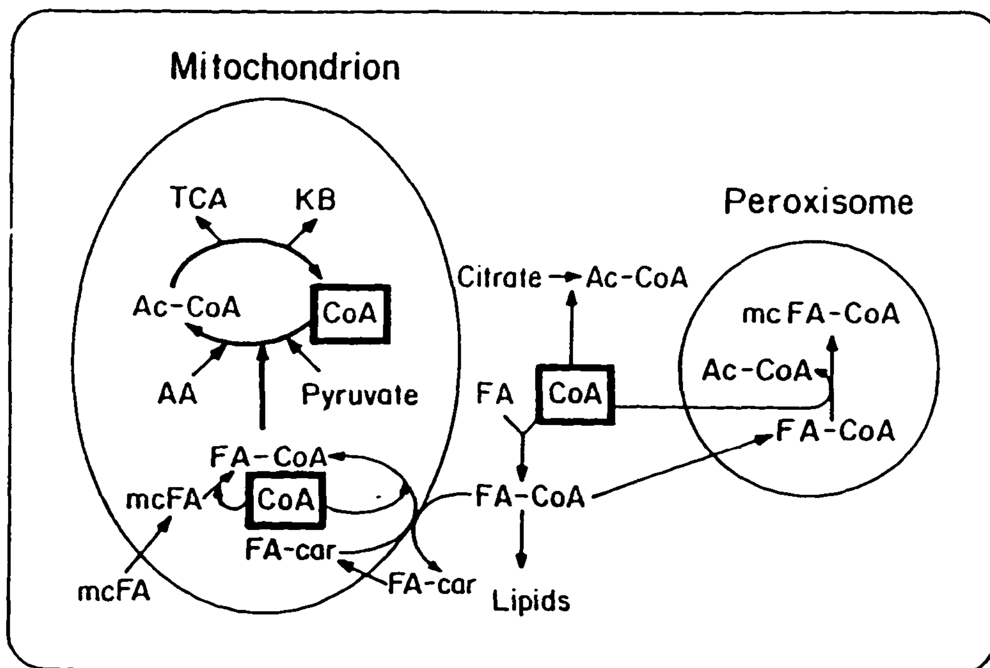

Fig. 11

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FIG. 11. Summary of important intramitochondrial and extramitochondrial reactions that either require or produce free coenzyme A. *Abbreviations:* CoA, free coenzyme A; FA-CoA, fatty acyl-CoA; mcFA-CoA, medium-chain fatty acyl-CoA; Ac-CoA, acetyl-CoA; FA-car, fatty acylcarnitine; FA, fatty acids; mcFA, medium-chain fatty acids; AA, amino acids; KB, ketone bodies; TCA, tricarboxylic acids.

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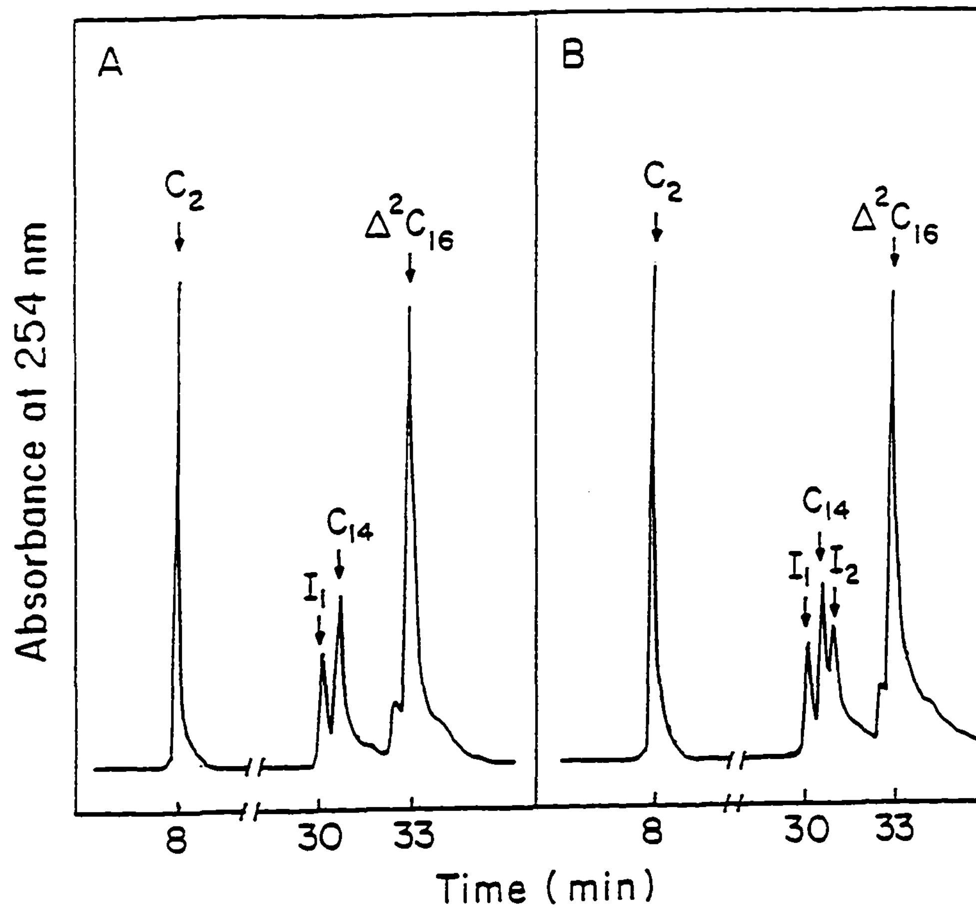

Fig. 12

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FIG. 12. HPLC analysis of intermediates and products formed in the overall reaction catalyzed by TOC. A. After incubating 20 μM 2-hexadecenoyl-CoA with 3 μg of TOC in the presence of 1 mM NAD^+ and 0.2 mM CoASH for 3 min. For experimental details see under Experimental Procedures. **B**. Same sample as in A except for the addition of 2.8 μM 3-ketohexadecanoyl-CoA. Peaks identified by use of authentic compounds were: C_2, acetyl-CoA; C_{14}, myristoyl-CoA; I_1, 3-hydroxyhexadecanoyl-CoA; I_2, 3-ketohexadecanoyl-CoA; Δ^2C_{16}, 2-hexadecenoyl-CoA.

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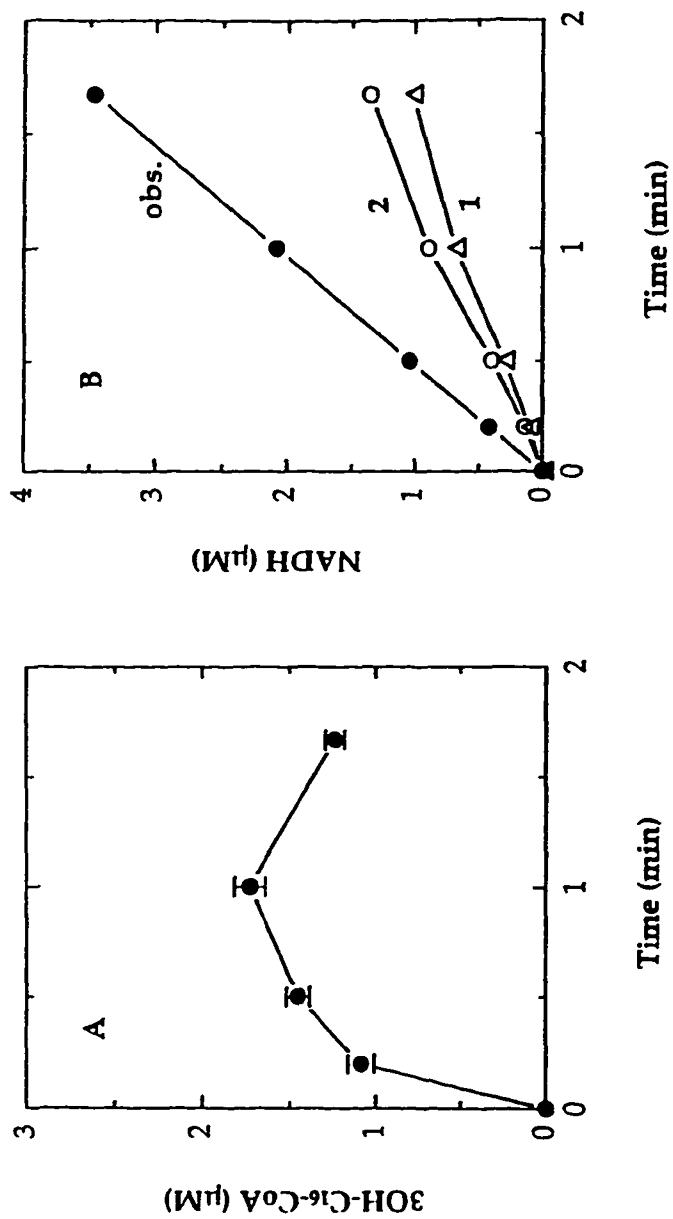

Fig. 13

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FIG. 13. Intermediate accumulation and NADH formation during the overall reaction catalyzed by TOC. A. Accumulation of 3-hydroxyhexadecanoyl-CoA (3-OH-C_{16}-CoA) when 10 µM 2-hexadecenoyl-CoA was incubated with 3 µg of TOC, 1 mM NAD^+, and 0.2 mM CoASH. The values are means based on six measurements. B. Observed (obs.) formation of NADH (o) at the incubation conditions indicated above. Calculated formation of NADH based on the measured concentrations of 3-hydroxyhexadecanoyl-CoA and the kinetic constants given in Table I. K_m values of 5.5 µM and 3.9 µM yielded lines 1 and 2, respectively.

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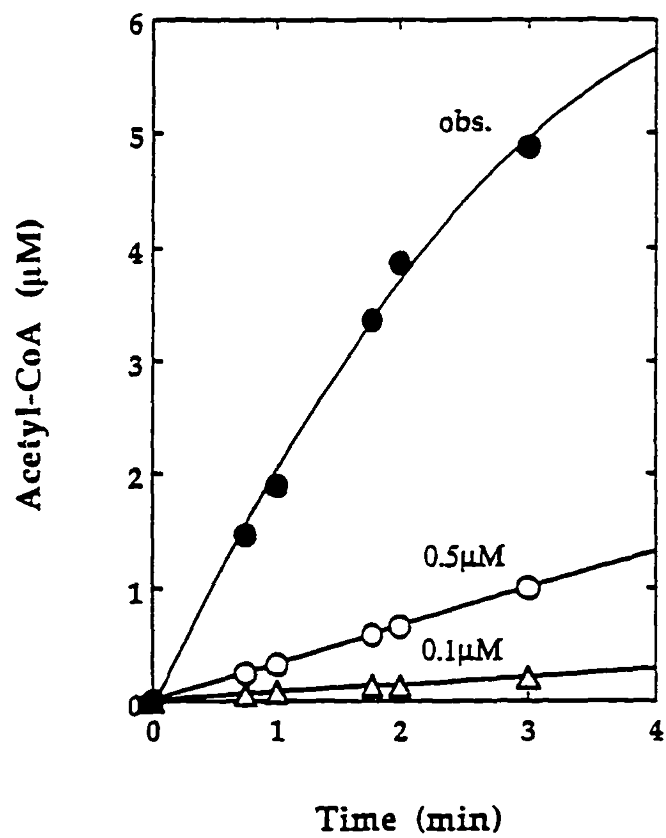

Fig. 14

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FIG. 14. Formation of acetyl-CoA during the overall reaction catalyzed by TOC. The concentrations of 2-hexadecenoyl-CoA, NAD^+, CoASH, and TOC were 20 µM, 1 mM, 0.2 mM, and 3µg/ml, respectively. Observed formation (o) and calculated formation (open symbols) of acetyl-CoA based on concentrations of free 3-ketohexadecanoyl-CoA (I_2) of 0.1 µM and 0.5 µM.

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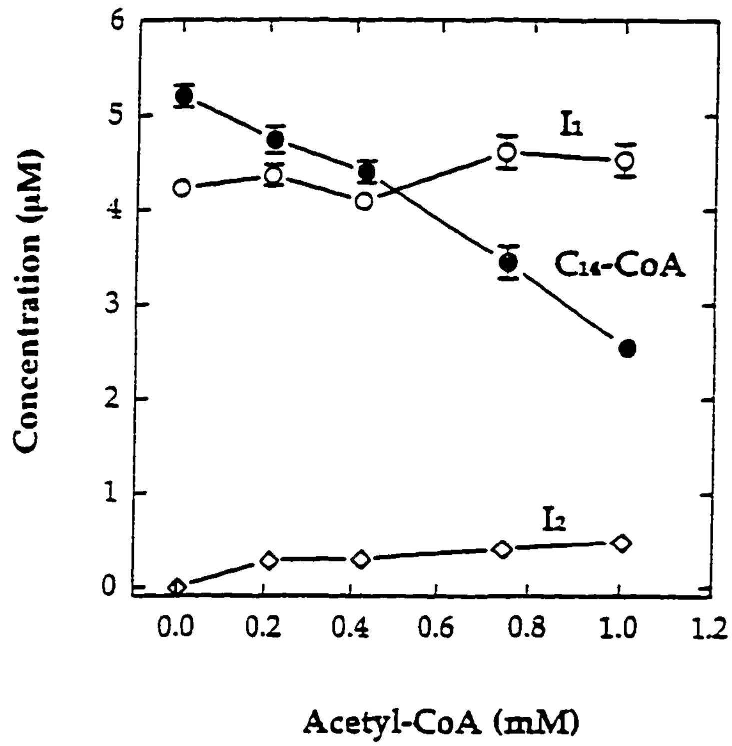

Fig. 15

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FIG.15. Effect of acetyl-CoA on the formation of product and intermediates of the TOC-catalyzed sequence of reactions. Reaction conditions were the same as g iven in the legend to Fig. 4. Reactions, run in triplicate, were terminated by acidification after 2 min and products were analyzed by HPLC as described under Experimental Procedures. Abbreviations: C_{14}, myristoyl-CoA; I_1, 3-hydroxyhexadecanoyl-CoA; I_2, 3-ketohexadecanoyl-CoA.

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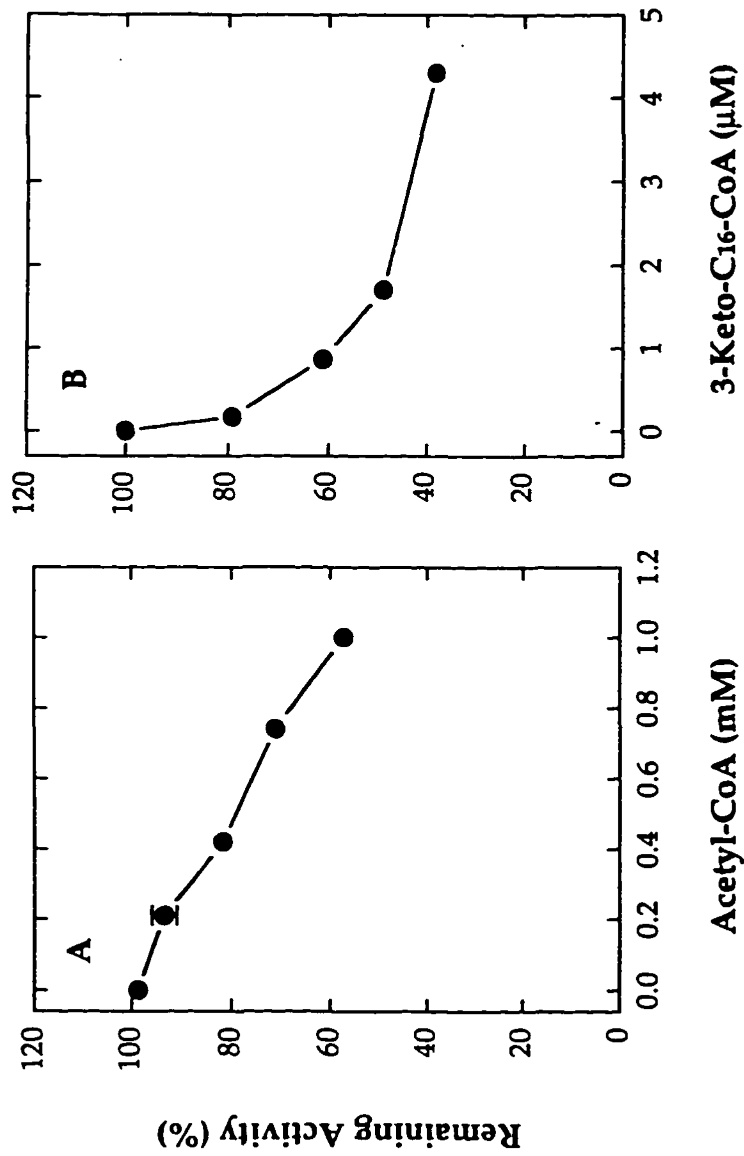

Fig. 16

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FIG.16. Product inhibition of long-chain 3-ketoacyl-CoA thiolase and long-chain 3-hydroxyacyl-CoA dehydrogenase. **A**. Inhibition of long-chain 3-ketoacyl-CoA thiolase by acetyl-CoA. For experimental conditions see under Experimental Procedures. The assay mixtures, except for 3-ketohexadecanoyl-CoA, were preincubated with acetyl-CoA at the indicated concentrations for 2 min. Reactions were started by the addition of substrate and terminated by acidification after 1 min. Reaction rates were determined by measuring the formation of myristoyl-CoA by HPLC. Data are means of three measurements. **B**. Inhibition of long-chain 3-hydroxyacyl-CoA dehydrogenase by 3-ketohexadecanoyl-CoA. The reaction mixtures contained in 0.1 M KP_i (pH 7.6) 20 μM 2-hexadecenoyl-CoA, 1 mM NAD^+, TOC (3 μg/ml), and the indicated concentrations of 3-ketohexadecanoyl-CoA but no CoASH.

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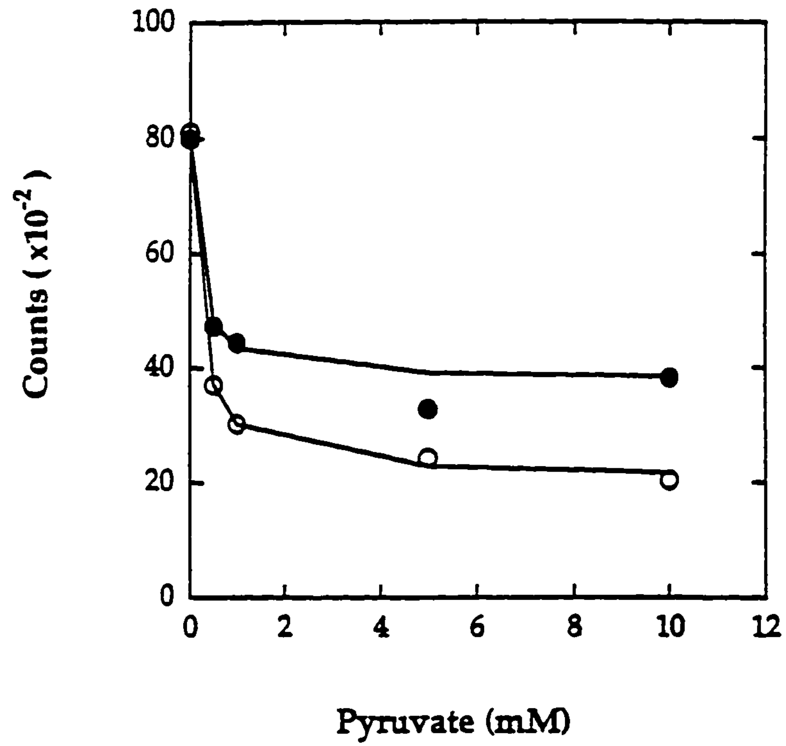

Fig. 17

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FIG. 17. Pyruvate inhibition of β-oxidation of (1-^{14}C) palmitoylcarnitine by coupled rat heart mitochondria at state 3 respiration and the partial removal of the inhibition by the addition of L-carnitine. Open circles, in the absence of L-carnitine; solid circles, in the presence of 5 mM L-carnitine. For experimental details see Materials and Methods.

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