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Alternate routes for the beta-oxidation of unsaturated fatty acids in peroxisomes, mitochondria, and *Escherichia coli*

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Alternate Routes for the Beta-Oxidation of Unsaturated Fatty Acids in Peroxisomes, Mitochondria, and *Escherichia coli*

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ABSTRACT

ALTERNATE ROUTES FOR THE BETA-OXIDATION OF UNSATURATED FATTY ACIDS IN PEROXISOMES, MITOCHONDRIA, AND ESCHERICHIA COLI

by

Tor Einar Smeland

Advisor: Professor Horst Schulz

Rat liver D-3-hydroxyacyl-CoA dehydratase was purified and found to act in combination with enoyl-CoA hydratase to catalyze the epimerization of 3-hydroxyacyl-CoA. The molecular weight of the dehydratase was estimated to be twice that of its 44 kDa subunit. It catalyzes the reversible dehydration of D-3-hydroxyacyl-CoA to 2-*trans*-enoyl-CoA, but does not act on 2-*cis*-enoyl-CoA. Virtually inactive toward crotonyl-CoA, it exhibits high activity with 2-*trans*-hexenoyl-CoA and acts with decreasing efficiency on all 2-enoyl-CoA's from 2-hexenoyl-CoA to 2-hexadecenoyl-CoA. It is suggested that 2-*cis*-enoyl-CoA intermediates formed during the beta-oxidation of polyunsaturated fatty acids in peroxisomes are hydrated by enoyl-CoA hydratase to D-3-hydroxyacyl-CoA's, which are epimerized to their L-isomers by the sequential actions of D-3-hydroxyacyl-CoA dehydratase and enoyl-CoA hydratase.

The 3-hydroxyacyl-CoA epimerase activity associated with the multienzyme complex of fatty acid oxidation from *E. coli*, was studied. The *E. coli* complex catalyzes the rapid, direct dehydration of D-3-hydroxy-4-*trans*-decenoyl-CoA to 2-*trans*,4-*trans*-decadienoyl-

CoA, which is slowly hydrated to L-3-hydroxy-4-*trans*-decenoyl-CoA. A kinetic analysis of the epimerase and its partial reactions established that epimerization of 3-hydroxyacyl-CoAs occurs via dehydration/hydration. A substrate competition study with D- and L-3-hydroxy-4-*trans*-decenoyl-CoA suggests that a single active site dehydrates the D- and L-isomers of 3-hydroxyacyl-CoAs.

The metabolism of 5-enoyl-CoAs, formed during the beta-oxidation of unsaturated fatty acids with odd-numbered double bonds, was studied. Metabolites were identified by high performance liquid chromatography. 5-*cis*-Octenoyl-CoA was dehydrogenated by medium-chain acyl-CoA dehydrogenase (EC 1.3.99.3) to 2-*trans*,5-*cis*-octadienoyl-CoA, which was isomerized to 3,5-octadienoyl-CoA either by mitochondrial 3-*cis*,2-*trans*-enoyl-CoA isomerase (EC-5.3.3.8) or by peroxisomal trifunctional enzyme. Further isomerization of 3,5-octadienoyl-CoA to 2-*trans*,4-*trans*-octadienoyl-CoA in the presence of soluble extracts of rat liver or heart mitochondria was attributed to a novel 3,5-2,4-dienoyl-CoA isomerase. A soluble extract of rat liver mitochondria catalyzed the isomerization of 2-*trans*,5-*cis*-octadienoyl-CoA to 2-*trans*,4-*trans*-octadienoyl-CoA, which upon addition of NADPH, NAD^+, and CoA was chain shortened. It is concluded that odd-numbered double bonds can be reductively removed during the beta-oxidation of polyunsaturated fatty acids.

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ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
BSA	Bovine serum albumin
CoA	Coenzyme A
DEAE	Diethylamino ethyl
EDTA	Ethylenediaminetetraacetate
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HPLC	High performance liquid chromatography
KIU	Kallikrein inhibitor units
KPi	Potassium phosphate
L fraction	A light mitochondrial fraction, enriched with respect to peroxisomes
NAD^+	Nicotinamide adenine dinucleotide, oxidized form
NADH	Nicotinamide adenine dinucleotide, reduced form
$NADP^+$	Nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
PMSF	Phenyl methane sulfonyl fluoride
TRIS-HCL	Tris(hydroxymethyl)aminomethane hydrochloride

INTRODUCTION

Historical Background

Serious inquiry into the metabolism of fatty acids began around the turn of the century with one of the earliest tracer experiments in the history of biochemistry. In 1904 in Germany, Franz Knoop fed fatty acids labeled with a non-metabolizable phenyl group at the ω carbon to dogs and examined the metabolites in their urine (1). Dogs fed phenyl-substituted fatty acids with an even number of carbon atoms excreted urinary phenyl aceturic acid, the glycine conjugate of phenylacetic acid. Those fed fatty acids containing an odd number of carbons excreted the glycine conjugate of benzoic acid. From these data, Knoop postulated what is essentially known today as β-oxidation. It was not until the 1940's that significant progress would again be made.

Following the demonstration of metabolism of fatty acids by subcellular extracts, it was found that degradation of fatty acids occurred in mitochondria. Acetyl-CoA was recognized as the activated form of acetate in biological systems, work for which Lynen (2) eventually received the Nobel Prize. These findings set the stage for the final effort for elucidating the mechanism whereby fatty acids are metabolized. This effort, to which several groups contributed and which provided a cohesive picture of β-oxidation, is described in an informative personal retrospective by Helmut

Beinert (3), a key player. An overview of the more salient aspects of this picture is presented in the following section. Several recent reviews are available which supply more detail than will be required here (4-6).

Uptake and Activation of Fatty Acids

The first step in the catabolism of fatty acids is their delivery into cells which contain the organelles and enzymes which are required for their degradation. Dietary triacylglycerols are cleaved by lipoprotein lipase located on the vascular endothelium, freeing fatty acids for their cellular uptake. Cellular uptake of fatty acids remains a controversial issue. Both saturable (7-9), and nonsaturable diffusion-dependent (10-12), uptake of fatty acids has been demonstrated.

Several low molecular weight (14-15 kD) cytosolic fatty acid binding proteins (FABPs) have been isolated and cloned from various tissues (13). Tissue-specific FABPs display high homology, about 80-95% across species, whereas in a single species, homology between FABPs in different tissues is generally on the order of 25-35%. Although these proteins have been shown to bind fatty acids, they have also been shown to bind other ligands, including heme, bilirubin, sterols, steroids, and fatty acyl-CoAs. The picture is further complicated by the existence of a 10 kD acyl-CoA binding protein, which has been shown to bind fatty acyl-CoAs, but is unable to bind free fatty acids, and shows no homology whatsoever with any FABP

(14). Apart from these disparate observations, little is known about the cytosolic transport of fatty acids. It is hoped that the coming years will see more progress on the events occurring between hydrolysis of serum triacylglycerols and the subsequent activation of the resultant fatty acids inside the cell.

Fatty acids are activated to their coenzyme A thioesters in an energy-dependent manner in a reaction requiring ATP by at least four acyl-CoA synthetases, presumably through an acyl-AMP intermediate. These enzymes, very long chain acyl-CoA synthetase, long chain acyl-CoA synthetase (EC 6.2.1.3), medium chain acyl-CoA synthetase (EC 6.2.1.2), and short chain acyl-CoA synthetase (EC 6.2.1.1) differ in their chain length specificities and subcellular locations (8). Short chain synthetases, which work only on acetate or propionate, are present in mitochondria and in the cytosol of lipogenic tissues. The mitochondrial enzyme presumably activates acetate for oxidation, whereas the cytosolic enzyme probably synthesizes acetyl-CoA for use in lipogenesis. Medium chain synthetases are present in the mitochondrial matrix. Long chain synthetases are found in three locations: the membranes of mitochondria, peroxisomes, and microsomes. The rat liver enzymes activate saturated fatty acids with 10 to 18 carbons, and activate unsaturated fatty acids with 16 to 20 carbons (15). The mitochondrial, peroxisomal, and microsomal enzymes are immunologically indistinguishable (16). A fourth, immunologically distinct, very long chain acyl-CoA synthetase was recently isolated

from brain microsomes (17). This enzyme, referred to as arachidonoyl-CoA synthetase, is relatively more active toward arachidonic acid, which is highly unsaturated, than palmitic acid. Thus the first step in the degradation of fatty acids, the energy-dependent conversion of the carboxylate moiety to a thioester of CoA, is handled by a range of enzymes differing in chain length specificity.

Transport Across the Mitochondrial Membrane

Short and medium chain fatty acids (those with less than ten carbons) can freely diffuse across the mitochondrial membrane system. Once inside the matrix, these fatty acids are activated and degraded by β-oxidation enzymes which are located in the mitochondrial matrix Neither CoA nor fatty acyl-CoAs are able to diffuse across the inner mitochondrial membrane. Therefore, following their activation at the outer mitochondrial membrane, long chain fatty acids must cross the inner membrane for further degradation by the enzymes of the mitochondrial β-oxidation system. The observation that mitochondrial degradation of long chain fatty acids is dependent upon L-carnitine sparked inquiry into the mechanism of fatty acid import into the mitochondrion. The existence of an activity on the outer mitochondrial membrane, which transfers fatty acyl residues from CoA to carnitine, was demonstrated (19, 20). This enzyme, carnitine palmitoyl transferase I (CPT I), is inhibited by malonyl-CoA, an early precursor in the

synthesis of fatty acids. The inhibition of CPT I by malonyl-CoA is believed to be a major physiological control mechanism for down-regulating mitochondrial β–oxidation by preventing entry of fatty acids into the mitochondrion for degradation under conditions of high intracellular malonyl-CoA concentration during fatty acid synthesis (21). Detailed structural and mechanistic studies of this enzyme have not been possible, due to the inability to obtain a stable purified form of this enzyme.

Acylcarnitines are unable to diffuse freely across the inner mitochondrial membrane. They have been shown to cross the inner membrane through the action of a translocase, which allows acylcarnitines to pass through the membrane in exchange for intramitochondrial carnitine or acylcarnitines (22-25). The exchange stoichiometry is one to one, but the translocase has also been found to allow a relatively slow unidirectional flux of carnitine (25). Translocase activity has been demonstrated in submitochondrial particles and in a reconstituted proteoliposomal system composed of submitochondrial particles and liposomes (26).

Once acylcarnitines have traversed the inner membrane of the mitochondrion they must be converted back to acyl-CoAs, the substrates of the β-oxidation system in the mitochondrial matrix. Another carnitine palmitoyltransferase activity, designated CPT II, catalyzes the intramitochondrial transfer of fatty acyl groups from carnitine to CoA at the inner mitochondrial membrane. The resulting acyl-CoAs are substrates of the β-oxidation spiral. The CPT

activity on the inner membrane, CPT II, was found to be insensitive to inhibition by malonyl-CoA (21). The rat liver and bovine heart enzymes have subunit molecular weights of close to 70,000 (27, 28). With the aid of specific CPT I inhibitors, rat liver CPT I was found to be larger than CPT II, which is inactivated by detergents used in solubilizing mitochondrial membrane (29). It is possible that CPT I and CPT II are identical, with CPT I harboring an extra subunit conferring malonyl-CoA sensitivity. Positive identification of the CPTs as distinct entities has been confounded by the inability to purify CPT I, by the existence of microsomal and peroxisomal carnitine acyltransferase activities (30), and by the difficulty of obtaining outer mitochondrial membrane fractions free of inner membrane and vice versa.

The Mitochondrial β-Oxidation Spiral

In the mitochondrial matrix, fatty acyl-CoAs are acted upon by acyl-CoA dehydrogenases containing tightly bound flavin adenine dinucleotides, which introduce a *trans* double bond between the second and third carbon atoms of the acyl chain. Both heart and liver contain identical short chain, medium chain, and long chain acyl-CoA dehydrogenases (31-34). The enzymes are most active toward fatty acyl-CoAs of four to six, six to 12, and eight or more carbons, respectively. All dehydrogenases studied thus far are tetramers of about 170-190 kD. Enzymes from different species display high homology, near 90%, whereas isoenzymes within a

species display approximately 30-35% homology. A crystal structure at 3 Å resolution has been obtained for the porcine liver medium chain enzyme (35). The mechanism involves proton abstraction at the α-carbon followed by β-hydride transfer to the FAD moiety of the dehydrogenase (36), resulting in the formation of enzyme-bound $FADH_2$. The FAD moiety is reoxidized by electron-transferring flavoprotein (ETF), also containing FAD, in a single electron transfer reaction (37). ETF then interacts with an iron-sulfur flavoprotein at the inner mitochondrial membrane, designated ETF:ubiquinone oxidoreductase (38), becoming reduced and therefore ready for reoxidation by another molecule of acyl-CoA dehydrogenase.

The 2-*trans*-enoyl-CoAs generated by the action of acyl-CoA dehydrogenase are hydrated to L-3-hydroxyacyl-CoAs (or S-3-hydroxyacyl-CoAs) by one of two enoyl-CoA hydratases present in mitochondria (39) with an equilibrium ratio of 3-hydroxy to 2-*trans* of about 2:1. The more well-characterized enzyme, porcine heart short chain enoyl-CoA hydratase (E.C. 4.2.1.17), more commonly referred to as crotonase, is a hexamer of 165 kD whose V_{max} is about 40 times higher for crotonyl-CoA (four carbons) than for 2-hexadecenoyl-CoA (16 carbons). The decline in V_{max}/K_M with chain length is primarily due to a decrease in V_{max} with chain length, since the K_M values for all substrates tested thus far fall between 10 and 30 μM (39). The long chain enzyme (E.C. 4.2.1.74), isolated from porcine mitochondria, has not been well characterized to date.

However, it has been observed to be inactive toward crotonyl-CoA, most active toward octenoyl-CoA, and active on all longer enoyl-CoAs thus far tested (39). Both enoyl-CoA hydratases found in mitochondria have been shown *in vitro* to catalyze the conversion of 2-*cis*-enoyl-CoAs to D- (or R-) 3-hydroxyacyl-CoAs. However, to date there is no evidence of formation of a 2-*cis*-enoyl-CoA from a saturated fatty acyl-CoA in mitochondria.

The L-3-hydroxyacyl-CoAs formed in the crotonase reaction undergo dehydrogenation to 3-ketoacyl-CoAs through the action of one of two NAD^+-dependent L-3-hydroxyacyl-CoA dehydrogenases, in a reaction whose equilibrium is pH-dependent. The porcine heart general chain L-3-hydroxyacyl-CoA dehydrogenase (HDH) (40) and rat liver HDH (41) have been purified to homogeneity. They are 64 kD homodimers whose amino acid sequence is known (42), and for which a crystal structure has been solved at a resolution of 2.8 Å (43). The x-ray structure reveals that the enzyme binds NAD^+ noncovalently at the amino terminus in a cleft at the interface of the two subunits. The K_Ms for all substrates examined fall below 10 μM, with the V_{max} values higher for medium chain fatty acids (44). The activity of 3-hydroxy-hexadecanoyl-CoA (16 carbons) is about 6% of the activity observed with medium chain substrates (44). Evidence has been presented for the association of this enzyme with the inner mitochondrial membrane through interaction with a specifically binding protein in an unconfirmed report (45), whose major appeal is that it would place the dehydrogenase in physical

proximity to the respiratory chain (46). A second L-3-hydroxyacyl-CoA dehydrogenase has been identified. This enzyme, termed long chain L-3-hydroxyacyl-CoA dehydrogenase, is more active toward long chain substrates (its activity toward four and six carbon substrates is about 2-5 % of the activity with 10 and 16 carbon substrates) and is believed to be an integral membrane protein of the inner mitochondrial membrane (47).

The final reaction in the β-oxidation spiral is the thiolytic cleavage of the 3-ketoacyl-CoA to yield acetyl-CoA and an acyl-CoA whose chain length is reduced by two carbons. The reaction is known to proceed through a covalently modified enzyme, where the acyl group is cleaved by nucleophilic attack at the β-carbon by an essential thiol residue at the active site, resulting in thiolysis and the release of acetyl-CoA. The thiol at the active site is regenerated as free CoA displaces the bound acyl group in the second thiolytic cleavage, freeing the chain-shortened acyl-CoA (48). The equilibrium of this reaction lies quite far to products, with the result that it draws the entire β-oxidation spiral forward at appropriate levels of free CoA. The two mitochondrial thiolases, thiolase I (E.C. 2.3.1.16), commonly referred to as 3-ketoacyl-CoA thiolase, and thiolase II (E.C. 2.3.1.9), referred to as acetoacetyl-CoA thiolase, differ in their chain length specificities. 3-Ketoacyl-CoA thiolase works on all 3-ketoacyl-CoAs from four carbons on up with K_Ms below 20 μM, whereas acetoacetyl-CoA thiolase is specific for acetoacetyl-CoA (49-51). It is likely that acetoacetyl-CoA thiolase does not participate in

β-oxidation, but rather functions solely in ketone body formation (50). Amino acid sequences have been attained for the two rat mitochondrial thiolases from their cDNA. Each thiolase is a tetramer with subunits of about 42 kD. Additionally, a long chain 3-ketoacyl-CoA thiolase activity associated with the mitochondrial membrane has been observed.

The β-oxidation spiral depicted in Figure 1 contains all the enzymes necessary for the mitochondrial β-oxidation of saturated fatty acids. However, for β-oxidation of unsaturated fatty acids at least two auxiliary enzymes are required which act on the double bond and allow the resultant product to enter the β-oxidation spiral. It should be kept in mind, however, that these products are only assumed to reenter the β-oxidation spiral; in many cases, no data is available to support the assumption that these intermediates are β-oxidized by the scheme outlined in Figure 1.

Auxiliary Enzymes for the β-Oxidation of Unsaturated Fatty Acids

Naturally occurring unsaturated fatty acids contain, for the most part, *cis* double bonds at odd and/or even numbered carbons. As a result of chain shortening by β-oxidation, these bonds would presumably appear as *cis* double bonds carbons two or three of an acyl-CoA. These two cases will be considered separately, since their further degradation involves two different auxiliary enzymes.

Whenever a *cis* double bond appears at an odd numbered carbon atom in an acyl-CoA, chain shortening by the β-oxidation spiral brings the *cis* bond to the three position as shown in Figure 2A. The resultant 3-*cis*-enoyl-CoA cannot be acted upon by any of the enzymes of the spiral. An activity which isomerizes 3-*cis*-enoyl-CoAs to their 2-*trans* isomers was discovered in liver extracts (52). The rat liver enzyme Δ^3,Δ^2-enoyl-CoA isomerase (E.C. 5.3.3.8) has been purified to homogeneity (53, 54) and found to have a molecular weight of 120,000 with a subunit molecular weight of 30,000. Recently, two chromatographically distinct isoenzymes have been isolated from rat liver mitochondria, and evidence has been presented that one of these isoenzymes is more active toward long chain 3-enoyl-CoAs (54). The enzyme is also active toward the 3-*trans* isomer; relative activities with the 3-*trans* isomers having six, ten, and 12 carbons are 9 : 2.5 : 1 (54). The isoenzyme preferentially active toward longer chain substrates has a native molecular weight of 200,000, and acts on substrates having six, ten, 12, and 16 carbon 3-*trans* isomers with an activity ratio of 1 : 2 : 3 : 1.7 (55). The resulting 2-*trans*-enoyl-CoAs are substrates of the β-oxidation spiral, and complete degradation presumably occurs following isomerization.

Fatty acids with *cis* double bonds at even numbered carbons were presumed to be chain shortened by the spiral until the *cis* double bonds appears at carbon two, as outlined in Figure 2B. The resulting 2-*cis*-enoyl-CoAs would then be acted upon by enoyl-CoA hydratase to yield the D- (or R-) 3-hydroxyacyl-CoA. D-3-

hydroxyacyl-CoAs are not substrates of the β-oxidation spiral due to the absolute stereospecificity of L-3-hydroxyacyl-CoA dehydrogenase for the L- (or S-) 3-hydroxy isomer. However, it was discovered that rat liver extracts are capable of catalyzing the epimerization of D-3-hydroxydecanoyl-CoA to its L-isomer (56). This activity, called 3-hydroxyacyl-CoA epimerase, had eluded purification prior to the work described in this thesis. Using linoleoyl-CoA as a model compound (useful because it contains double bonds at both odd and even numbered carbons) the roles of Δ^3,Δ^2-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase are illustrated in Figure 3.

The Δ^3,Δ^2-enoyl-CoA isomerase pathway, as presented above, is accepted as a major pathway by which fatty acids with double bonds at odd numbered carbons are degraded (5). However, the epimerase-dependent pathway for the degradation of fatty acids with double bonds at even numbered carbons has been disproven and evidence began to accumulate which led to the conclusion that these fatty acids, for the most part, are degraded by an entirely different pathway. In the late 1970's, an NADPH-dependent 2,4-dienoyl-CoA reductase, which uses NADPH to reductively remove one double bond of a 2,4-dienoyl-CoA to yield a 3-enoyl-CoA, was identified (57). In addition, it was demonstrated that 2-*trans*,4-*cis*-decadienoyl-CoA can not be efficiently β-oxidized by mitochondria (58), preliminary evidence suggested that 3-hydroxyacyl-CoA epimerase is absent from mitochondria (59), and NADPH is required for the β-oxidation of polyunsaturated fatty acids (60).

Accordingly, a new pathway was proposed in which 2,4-dienoyl-CoA reductase (E.C. 1.3.1.34) plays a key role in the β-oxidation of linoleic acid (61). This new scheme is outlined in Figure 4, alongside the old epimerase-dependent pathway. The flux of 2,4-dienoyl-CoA (formed during the β-oxidation of fatty acids with double bonds at even numbered carbons) through the epimerase-dependent pathway has been estimated to be absent in mitochondria, and, at most, to account for 2% of β-oxidation of unsaturated fatty acids in peroxisomes (61).

2,4-Dienoyl-CoA reductases have been purified from rat liver and bovine liver. The enzymes act on both 2-*trans*,4-*cis*-enoyl-CoAs and 2-*trans*,4-*trans*-enoyl-CoAs, are homotetramers with molecular weights of 124,000, and have K_M values of 3-10 μM for 2,4-dienoyl-CoAs and 100 μM for NADPH (62-64).

Peroxisomal β-Oxidation

Renewed interest in the β-oxidation of fatty acids was spurred by the discovery of a fatty acid oxidation system in peroxisomes which is inducible by clofibrate and other peroxisomal proliferators (65), and by the discovery of several inherited disorders of peroxisomal fatty acid metabolism (66, 67). This system is now well characterized and its component enzymes are described in detail in two recent and comprehensive reviews (6, 68); the system will therefore be discussed in brief.

Unlike mitochondria, whose uptake of long chain fatty acids is carnitine-dependent (19), long chain fatty acids are able to enter peroxisomes freely due to the existence of pores in the peroxisomal membrane allowing molecules with molecular weights of up to 10,000 to equilibrate with the cytosol (69). As mentioned above, peroxisomes (as well as microsomes) contain a long chain acyl-CoA synthetase which is identical to the mitochondrial enzyme (16). The resulting acyl-CoAs are dehydrogenated by an FAD-linked acyl-CoA oxidase, which oxidizes the acyl-CoA to yield a 2-*trans*-acyl-CoA, with the concomitant reduction of molecular oxygen to hydrogen peroxide. Peroxisomes, which contain a large amount of catalase, are capable of metabolizing H_2O_2 to water and oxygen. Rat liver acyl-CoA oxidase is a homodimer with a molecular weight of about 160,000, which may exist in two forms through proteolytic action on either one of its subunits (68), and is inactive toward both hexanoyl-CoA and butyryl-CoA. Two acyl-CoA oxidase mRNAs representing alternate splicing of two separate exons have been observed (70, 71), although the significance of this observation is unclear.

The second and third reactions of the β-oxidation spiral are catalyzed by a multifunctional protein harboring both activites on a single 79 kD polypeptide (72, 73). Recently, this enzyme was also shown to harbor Δ^3,Δ^2-enoyl-CoA isomerase as well (74), and will therefore be henceforth referred to as the peroxisomal trifunctional enzyme. The enoyl-CoA hydratase activity of the peroxisomal trifuctional enzyme acts with decreasing activity on crotonyl-CoA

(with a K_M of 83 μM) and all longer chain length acyl-CoAs so far tested (with K_M values of about 10 μM). The NAD^+-dependent activity of the enzyme displays decreasing K_M with increasing chain length for all L-3-hydroxyacyl-CoAs from four carbons to ten carbons (from 42 μM to 1 μM). The V_{max} values fall in the same series, with the effect that relative activities are similar.

The final reaction of the spiral in peroxisomes is catalyzed by a peroxisomal 3-ketoacyl-CoA thiolase, which is a homodimer of 41 kD subunit size whose substrate specificity is similar to that of the mitochondrial enzyme (51, 75).

As indicated above, peroxisomes contain a Δ^3,Δ^2-enoyl-CoA isomerase activity associated with the trifunctional enzyme. They also contain an NADPH-dependent 2,4-dienoyl-CoA reductase (76), and so are fully capable of degrading polyunsaturated fatty acids. Paradoxically, peroxisomes also contain a carnitine octanoyltransferase, presumably for conversion of acyl-CoAs to acylcarnitines for further β-oxidation in mitochondria, which would result in the regeneration of CoA in peroxisomes rather than in the cytosol (4). Since rat liver peroxisomes are incapable of degrading fatty acids completely to acetyl-CoA, further mitochondrial metabolism following fatty acid degradation in peroxisomes is essential.

The consensus in the literature indicates that the function of peroxisomal β-oxidation is in the chain shortening of very long

chain fatty acids, prostaglandins, dicarboxylic acids, xenobiotic compounds, and bile acid precursors (77-81).

Bacterial β-Oxidation: The *Escherichia coli* System

Bacteria, which contain neither mitochondria nor peroxisomes, are nevertheless capable of degrading fatty acids. In fact, *E. coli* can survive when supplied with long chain fatty acids as their sole carbon source, due to the existence of a long chain fatty acid-inducible β-oxidation system (82). The genes of the β-oxidation pathway, which constitute a regulon of five distinct loci on the *E. coli* chromosome, are under the coordinate control of a regulator protein and is sensitive to glucose and cAMP levels (83). Activation of fatty acids to their CoA thioesters in *E. coli* is presumably tightly coupled to uptake of fatty acids, since uptake mediated by a 43 kD membrane protein is dependent upon a functional acyl-CoA synthetase gene (83). As with mitochondria, short chain fatty acids can pass through the membrane by diffusion. Both ETF and acyl-CoA dehydrogenase remain unpurified and uncharacterized. *E. coli* also contain 2,4-dienoyl-CoA reductase as a monomeric 70 kD flavoprotein. *E. coli* reductase, in contrast to the mitochondrial and peroxisomal enzymes, converts 2,4-dienoyl-CoAs to 2-*trans*-enoyl-CoAs, rather than to 3-*trans*-enoyl-CoAs.

The remaining enzyme activities required for the β-oxidation of fatty acids reside on a multisubunit protein, the *E. coli* fatty acid oxidation complex (FAOC). FAOC is composed of a large

an $\alpha_2\beta_2$ arrangement with a native molecular weight of 260,000 (84). 3-Ketoacyl-CoA thiolase has been assigned to the small subunit, whereas enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyacyl-CoA epimerase, and Δ^3,Δ^2-enoyl-CoA isomerase activities are associated with the large subunit (84, 85). As with mitochondria, the degradation of unsaturated fatty acids in *E. coli* proceeds primarily by the reductase-dependent pathway. The β-oxidation of fatty acids as catalyzed by FAOC is shown in Figure 5.

Problematic Epimerization

If β-oxidation of unsaturated fatty acids indeed proceeds overwhelmingly by the reductase-dependent pathway in both mitochondria and in *E. coli*, a troubling question remains: what is the function of 3-hydroxyacyl-CoA epimerase? It seems reasonable to assume that it plays a role in β-oxidation, since it is physically linked with the evolutionarily precedent *E. coli* system. The direct β-oxidation of 2,4-dienoyl-CoA has been determined to be 2%, primarily due to channelling of 2-*trans*,4-*cis*-dienoyl-CoAs demonstrated on the peroxisomal trifunctional enzyme and on FAOC (86) from the enoyl-CoA hydratase site to the L-3-hydroxyacyl-CoA dehydrogenase site. This would result in an 3-keto-4-*cis*-enoyl-CoA, which would be cleaved by thiolase to yield a 2-*cis*-enoyl-CoA, whose further metabolism would begin with hydration to a D-3-hydroxyacyl-CoA by enoyl-CoA hydratase. This is essentially the epimerase-dependent pathway, as outlined in Figure

3. Therefore, wherever such multifunctional proteins are found — such as in peroxisomes and *E. coli* — epimerase activity would presumably be required to handle any metabolic flux due to channelling of such intermediates. However, a more serious problem remains to be addressed with respect to epimerization. Since the discovery of the ability of rat liver extracts to epimerize 3-hydroxyacyl-CoAs, purification of the activity has been elusive. This inability to identify an entity responsible for the activity has cast doubts on the existence of an epimerase *per se*, and raised questions as to the mechanism of epimerization. Experiments described in this thesis have resulted in the purification of the activity responsible for the observed epimerization activity and elucidation of the mechanism whereby epimerization is affected in both rat liver and on FAOC. The surprising findings from these experiments are described in this thesis.

NADPH-Dependent Removal of an Odd Numbered Double Bond in Mitochondria

According to the consensus in the literature on β-oxidation of unsaturated fatty acids, only the degradation of fatty acids with even numbered double bonds proceeds through an NADPH-dependent pathway (see above) via 2,4-dienoyl-CoA reductase. The β-oxidation of fatty acids with double bonds at odd numbered carbons presumably requires only one auxiliary enzyme — Δ^3,Δ^2-enoyl-CoA isomerase. Until recently, no evidence had been presented to

contradict this claim. However, a report recently appeared presenting data consistent with the NADPH-dependent β-oxidation of a 5-*cis*enoyl-CoA (87), along with a modified pathway in which a 5-*cis* double bond is directly reduced by a putative 5-*cis*-reductase. Since preliminary experiments failed to detect a 5-*cis*-reductase, an investigation as to the nature of the NADPH requirement was undertaken. The results of this investigation, a new metabolic route for the β-oxidation of unsaturated fatty acids with odd numbered double bonds, are presented in this thesis.

EXPERIMENTAL PROCEDURES

Materials. CoASH, NAD^+, NADPH, acetyl-CoA, butyryl-CoA, hexanoyl-CoA, octanoyl-CoA, bovine serum albumin, acyl-CoA oxidase from *Candida sp.*, pig heart L-3-hydroxyacyl-CoA dehydrogenase, sodium DL-3-hydroxybutyrate and all standard biochemicals were obtained from Sigma. Eastman Kodak Co. was the source of crotonic anhydride. Nycodenz was purchased from Accurate Chemical and Scientific Corp. (Westbury, NY). 3',5'ADP-Agarose was obtained from Pharmacia LKB, hydroxyapatite was purchased from Bio-Rad Laboratories, and phosphocellulose was purchased from Polysciences. 4-*trans*-Octadienal and 3-*trans*-octadecenoic acid were purchased from Bedoukian, Danbury, CT and Pfaltz and Bauer, respectively. 2-*trans*,4-*trans*-Octadienoic acid was prepared from 2-*trans*,4-*trans*-octadienal by oxidation with Ag_2O according to a general procedure for the oxidation of aldehydes to acids that are sensitive to strong oxidizing agents (58). 2-*trans*,4-*trans*-Decadienoic acid was prepared similarly from 2-*trans*,4-*trans*-decadienal. After crystallization from hexane, 2-*trans*,4-*trans*-octadienoic acid had a mp of 75-76°C (literature mp 76°C (88).

2-*trans*-Hexenoic acid, 2-*trans*-decenoic acid, methyl bromoacetate, hexanal, octanal, decanal, dodecanal, tetradodecanal, malonic acid, diketene, 2-*trans*,4-*trans*-decadienal, and 2-octynoic acid were purchased from Aldrich. The 2-*trans*-enoic acids with

even numbers of carbon atoms from 2-hexenoic acid to 2-hexadecenoic acid were synthesized by reacting malonic acid in the presence of pyridine with an aldehyde two carbons shorter than the desired acid as described by Linestead *et al.* (89). DL-3-Hydroxy-octanoic acid and DL-3-hydroxydecanoic acid were synthesized from methyl bromoacetate and hexanal or octanol, respectively, by a modified Reformatzky procedure described by Ruppert and White (90). DL-3-Hydroxyoctanoic acid was resolved into its L-isomer and D-isomer by neutralizing the acid with R-α-phenylethylamine and S-α-phenylethylamine, respectively, and by crystallizing the resultant salts to constant optical activity according to a procedure used by Stoffel *et al.* (56) for the resolution of DL-3-hydroxydodecanoic acid. DL-3-Hydroxybutyric acid was resolved similarly. DL-3-Hydroxy-4-*trans*-decenoic acid was synthesized and resolved as described previously (91). The CoA thioesters of all 2-*trans*-enoic acids, D-3-hydroxybutyric, DL-3-hydroxydecanoic acid, 2-octynoic acid and both enantiomers of 3-hydroxyoctanoic acid and D- and L-3-hydroxy-4-*trans*-decenoic acid were synthesized by the method of Goldman and Vagelos (92). Crotonyl-CoA was prepared as described by Weeks and Wakil (93). Acetoacetyl-CoA was prepared as previously described (94).

The methyl esters of 5-*cis*-octenoic acid and 5-*trans*-octenoic acid were generously provided by Dr. Howard Sprecher, Ohio State University. The purities of the *cis* and *trans*-isomers were 98% and 96%, respectively. The esters were saponified with a 3-fold molar

excess of aqueous 0.4 M KOH until the system became monophasic. The resultant acids were obtained after acidification and extraction with ether. 2-*trans*,5-*cis*-Octadienoyl-CoA and 2-*trans*,5-*trans*-octadienoyl-CoA were synthesized from the corresponding 5-octenoyl-CoAs by allowing them to react with oxygen in the presence of acyl-CoA oxidase from *Candida sp.* as described previously (86), except that catalase was omitted from the incubation mixture. The two isomeric 2,5-octadienoyl-CoAs were purified by HPLC. Concentrations of all CoA thioesters were determined by the method of Ellman (95) after cleaving the thioester bond with 1 M hydroxylamine (pH 7). The optical purities of D-3-hydroxyoctanoyl-CoA and L-3-hydroxyoctanoyl-CoA were determined by enzymatic dehydrogenation catalyzed by L-3-hydroxyacyl-CoA dehydrogenase at pH 8 in the presence of NAD^+, CoASH and pig heart 3-ketoacyl-CoA thiolase. The optical purities of the L-isomer and D-isomer were found to be 98% and 93%, respectively. 2-*cis*-Octenoyl-CoA was synthesized and purified as described elsewhere (96).

Mitochondrial Δ^3,Δ^2-enoyl-CoA isomerase (EC 5.3.3.8) was partially purified by chromatography of a soluble extract of rat liver mitochondria on hydroxyapatite as described by Kilponen *et al.* (55). Fractions corresponding to the first peak of isomerase activity were pooled, concentrated and stored at 70°C in the presence of 30% glycerol. 2,4-Dienoyl-CoA reductase (EC 1.3.1.34) was partially purified by chromatography of a soluble extract of rat liver

mitochondria on agarose-heptane-adenosine-2',5'-diphosphate following the procedure of Wang and Schulz (97). The peroxisomal bifunctional enzyme was purified from rat liver as described by Osumi and Hashimoto (72). Bovine liver enoyl-CoA hydratase (crotonase) (98) and pig heart 3-ketoacyl-CoA thiolase (50) were prepared as described.

Resolution of Rat Liver 3-Hydroxyacyl-CoA Epimerase Activity into Two Complementary Fractions from a DEAE-Cellulose Column. Rat liver light mitochondria were isolated as described in principle by Applemans *et al.* (99). A soluble extract of light mitochondria was prepared by subjecting them to treatment with a Polytron homogenizer for 6 x 20 s at 4° C followed by centrifugation at 105,000 x g for 60 min. The resultant supernatant, containing 17 mg of protein in 3 ml of 5 mM Tris-phosphate (pH 8), 10 mM mercaptoethanol and 10% glycerol, was applied to a DEAE-cellulose column (1 x 20 cm) equilibrated with the sample buffer. The column was developed with a gradient from 5 to 500 mM Tris-phosphate with 10 mM mercaptoethanol, 10% glycerol, 0.5 mM benzamidine, 0.1 mM PMSF, and 0.06 KIU aprotinin/ml. Early fractions, which were devoid of crotonase activity and showed epimerase activity only when supplemented with crotonase, were combined, concentrated and used in this study where they are referred to as early DEAE-cellulose fractions or early column fractions. 3-Hydroxyacyl-CoA epimerase was assayed spectrophotometrically at

340 nm and 25°C by coupling the epimerization of D-3-hydroxyoctanoyl-CoA to the dehydrogenation of the resultant L-isomer and finally to the thiolytic cleavage of the dehydrogenation product (100). Enoyl-CoA hydratase was assayed spectrophotometrically at 263 nm and 25°C. An assay mixture contained 60 μM L-or D-3-hydroxyoctanoyl-CoA in 0.22 M KP_i (pH 8) and enzyme to give an absorbance change of 0.07 per min or less.

Protein and Enzyme Assays of Rat Liver Activities Implicated in Epimerization. Protein concentrations were determined by the method of Bradford (101). The activities of rat liver crotonase, enoyl-CoA hydratase of the bifunctional enzyme and D-3-hydroxyacyl-CoA dehydratase were measured in the forward and reverse directions by a direct spectrophotometric assay at 263 nm, as described in principle by Fong and Schulz (39). A standard assay contained 0.22 M KP_i (pH 8), 60 μM substrate in a total volume of 0.6 ml and enzyme to give an absorbance change of 0.08 per min. D-3-Hydroxyacyl-CoA dehydratase was also assayed spectrophotometrically at 340 nm in the forward direction by coupling the dehydration of D-3-hydroxyacyl-CoA to its hydration by crotonase, followed by dehydrogenation and thiolytic cleavage in the presence of NAD^+, CoASH, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase. A standard assay contained 0.22 M KP_i (pH 8), 1 mM NAD^+, 0.3 mM CoASH, 100 μM D-3-hydroxyacyl-CoA, bovine liver crotonase (1 μg), L-3-

hydroxyacyl-CoA dehydrogenase (80 μg), and dehydratase in a total volume of 0.66 ml to give an absorbance change of 0.08 per min. Enzyme dilutions were made into 1 M KP_i (pH 8) containing 1 mg/ml BSA. The locations of mitochondria, peroxisomes, and microsomes on the Nycodenz gradient were detemined by assaying the marker enzymes glutamate dehydrogenase, catalase, and esterase, respectively. Glutamate dehydrogenase was assayed as described previously (102) except that the reaction was followed at 350 nm using an extinction coefficient of 5308 $M^{-1}cm^{-1}$ for NADH. Catalase (103) and esterase (104) were assayed as described previously. The dehydratase was measured in the forward direction by the coupled assay procedure. The kinetic parameters for the forward and reverse reactions of the D-specific dehydratase were determined by fittting intial rate data to the Michaelis Menten equation using a program written by Cleland (105) and transcribed from Fortran for use on an Apple II personal computer (106). A unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product per min.

Purification of D-3-Hydroxyacyl-CoA Dehydratase. Rat liver light mitochondria were isolated from the livers of 8 male Sprague-Dawley rats as described in principle by Appelmans *et al.* (99). A soluble extract was prepared by treating a suspension of rat liver light mitochondria (544 mg) with a Polytron homogenizer for 6 x 20 s at 4°C followed by centrifugation at 105,000 x g for 60 min in the

presence of 0.1 mM PMSF. The resultant supernatant, containing 102 mg of protein, was combined with 50 ml of 5 mM TRIS-phosphate (pH 8) in solution A, which contained 10 mM mercaptoethanol, 10% glycerol, 0.5 mM benzamidine and aprotinin (0.06 KIU/ml) and was applied to a DEAE-cellulose column (20 x 2.5 cm) equilibrated with the sample buffer. The column was developed with the same buffer using a linear gradient from 5 mM to 500 mM TRIS-phosphate. Fractions showing epimerase activity only when supplemented with crotonase were pooled and concentrated. These fractions were applied to a hydroxyapatite column (30 x 2.5 cm) equilibrated with 10 mM KP_i (pH 6.8) in solution A. The dehydratase was eluted with a gradient from 0 to 2 M KCl in the column buffer. Fractions containing dehydratase activity were combined, concentrated, and applied to a 3',5'ADP-Agarose column (13 x 0.75 cm) equilibrated with 10 mM KP_i (pH 8) in solution A following diafiltration through an Amicon PM 10 membrane. The enzyme was eluted with a linear KP_i (pH 8) gradient from 10 to 50 mM. Fractions containing the D-specific dehydratase were pooled, concentrated, and further purified on a Perkin-Elmer Isopure LC system with a TSK gel (type G3000SW) filtration column (30 x 0.75 cm) equilibrated with 100 mM KP_i (pH 8), containing 10% glycerol and 10 mM mercaptoethanol. Fractions containing dehydratase activity were concentrated and stored at 70°C after adjusting the glycerol concentration to 30%.

Subcellular Distribution of Rat Liver Epimerase Activity. Rat liver nuclei, mitochondria, light mitochondria, microsomes, and soluble proteins were isolated as described previously (107). For density gradient fractionation of rat liver light mitochondria, a 30% (w/v) solution of Nycodenz containing 1 mM EDTA, 5 mM HEPES (pH 7.3) and 0.1% ethanol was prepared at 4°C and 7.6 ml of this solution were placed in a 10 ml ultracentrifuge tube on top of 0.5 ml of a 60% sucrose cushion. The gradient was generated by centrifugation at 28,500 rpm (60,400 x g_{av}) in a T865.1 small angle rotor on a DuPont RC70 ultracentrifuge at 5°C for 24 hours. Light mitochondria (5.4 mg of protein in 0.5 ml), prepared according to Appelmans *et al.* (99), were layered on top of the gradient. A three-fold diluted isolation buffer (250 mM sucrose, 1 mM EDTA, 0.1% ethanol) was applied as a cover solution. The sample was centrifuged for 45 minutes at 32,000 rpm (76,200 x g_{av}) at 5°C. Ten fractions were collected from the bottom of the tube. Peroxisomes, microsomes, and mitochondria were localized by assaying the marker enzymes catalase, esterase, and glutamate dehydrogenase, respectively. D-3-Hydroxyacyl-CoA dehydratase was assayed in the forward direction as described above. Both glutamate dehydrogenase and the D-specific dehydratase were assayed at 350 nm. Dilutions of the Nycodenz fractions were made into 1 mM $NaHCO_3$ (pH 7.6) containing 1 mM EDTA, 0.01% Triton X-100, 0.1% ethanol, and 1 mg/ml BSA.

Determination of Equilibrium Constants. Extinction coefficients at 254 nm for 2-*cis*-octenoyl-CoA, 2-*trans*-octenoyl-CoA, D-3-hydroxyoctanoyl-CoA, and L-3-hydroxyoctanoyl-CoA were determined to be 21,122, 22,039, 15,941, and 15,421 M^{-1} cm^{-1}, respectively, as described by Li *et al.* (96).

Gel Electrophoresis. D-3-Hydroxyacyl-CoA dehydratase and the *E. coli* fatty acid oxidation complex were subjected to SDS-polyacrylamide gel electrophoresis on 10% gels according to the procedure of Laemmli (108).

Protein and Enzyme Assays of *E. coli* FAOC. Protein concentrations were measured by the method of Bradford with BSA as standard (101). Dehydration rates for D- and L-3-hydroxy-4-*trans*-decenoyl-CoA were determined by measuring the increase in absorbance at 300 nm due to the formation of 2,4-decadienoyl-CoA. A standard assay mixture contained 0.22 M KPi (pH 8), 30 μM 3-hydroxy-4-*trans*-decenoyl-CoA, and FAOC from *E. coli* to give an absorbance change of 0.08 min^{-1}. The molar extinction coefficient used for rate calculation was 25,400 $M^{-1}cm^{-1}$ (91). Hydration of 2-*cis*-octenoyl-CoA was assayed spectrophotometrically by measuring the decrease in absorbance at 263 nm. A standard assay contained, in a total volume of 0.61 ml, 0.22 M KPi (pH 8), 25 μM 2-*cis*-octenoyl-CoA, and FAOC or a soluble extract of *E. coli* to give an absorbance change of 0.02 min^{-1}. Hydration of 2-*trans*,4-*trans*-decadienoyl-CoA was

determined spectrophotometrically by measuring at 360 nm in a coupled assay the dehydrogenation of L-3-hydroxy-4-*trans*-decenoyl-CoA. A standard assay mixture contained, in a total volume of 0.63 ml, 0.22 M KPi (pH 8), 1 mM NAD^+, 0.3 mM CoASH, 30 μM 2-*trans*,4-*trans*-decadienoyl-CoA, pig heart 3-ketoacyl-CoA thiolase (38 mU), and *E. coli* FAOC to give an absorbance change of 0.04 min^{-1}. Epimerization of D-3-hydroxy-4-*trans*-decenoyl-CoA was determined spectrophotometrically at 360 nm by measuring, in a coupled assay, the dehydrogenation of L-3-hydroxy-4-*trans*-decenoyl-CoA. A standard assay mixture contained, in a total volume of 0.63 ml, 0.22 M KPi (pH8), 1 mM NAD^+, 0.3 mM CoASH, 30 μM D-3-hydroxy-4-*trans*-decenoyl-CoA, pig heart 3-ketoacyl-CoA thiolase (38 mU), and pig heart L-3-hydroxyacyl-CoA dehydrogenase (0.7 U), and *E. coli* FAOC to give an absorbance change of 0.04 min^{-1}. Dehydrogenation of L-3-hydroxy-4-*trans*-decenoyl-CoA was measured spectrophotometrically at 360 nm. A standard assay contained, in a total volume of 0.63 ml, 0.22 M KPi (pH8), 1 mM NAD^+, 0.3 mM CoASH, 30 μM L-3-hydroxy-4-*trans*-decenoyl-CoA, pig heart 3-ketoacyl-CoA thiolase (38 mU), and *E. coli* FAOC to give an absorbance change of 0.08 min^{-1}. Rates of NADH formation at 360 nm were calculated using an extinction coefficient of 4,087 $M^{-1}cm^{-1}$ (91). One unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product per minute.

HPLC Analysis of FAOC Metabolites of 3-Hydroxy-4-Enoyl-CoAs. Assays analyzed by HPLC were terminated by adjusting the pH to 1-2 with concentrated HCl. After filtration through 0.22 μm membranes, the pH was readjusted to 5 with KOH, and the samples were applied to a Waters HPLC μBondapak C18 reverse phase column (30 cm x 3.9 mm) attached to a Waters gradient HPLC system. Separation was achieved by linearly increasing the acetonitrile content of the 50 mM ammonium phosphate elution buffer (pH 5.5) from 10 to 40% in 45 minutes at a flow rate of 2 ml/min.

Purification of the *E. coli* Fatty Acid Oxidation Complex. An *E. coli* cellular supernatant was prepared as previously described (109) using recombinant *E. coli* strain LS6749pk52. The cell supernatant (432 mg protein in a total volume of 160 ml) was brought to pH 6.6 with phosphoric acid and applied to a phosphocellulose column (2.5 x 35 cm) equilibrated with 50 mM KPi (pH 6.6), 10 mM mercaptoethanol. Following application of the supernatant, the column was developed using a gradient of 50 to 500 mM KPi (pH 6.6), 10 mM mercaptoethanol, in a total volume of 900 ml. Fractions containing highest L-3-hydroxyacyl-CoA dehydrogenase activity as determined by measuring in 0.16 M KPi (pH 7), the oxidation of 150 μM NADH with 30 μM acetoacetyl-CoA as substrate, were pooled, concentrated by ultrafiltration with an Amicon PM10 membrane at 4°C and applied to a Sephacryl S-200 column (25 x 1.5 cm)

equilibrated with 50 mM KPi (pH 6.6), 10 mM mercaptoethanol. The fractions containing high L-3-hydroxyacyl-CoA dehydrogenase activity were pooled and concentrated as detailed above at 4°C. Both chromatographic steps were carried out at room temperature and in the absence of glycerol without deleterious effect on activity. For long term storage, glycerol was added to a final concentration of 30% and the preparation was kept at -70°C. Sodium dodecylsulfate polyacrylamide gel electrophoresis (118) followed by staining with Coomassie Blue R250 was carried out, and densitometric analysis of the resulting gel revealed the enzyme preparation to be 98% pure.

Preparation of a Soluble Mitochondrial Extract. Mitochondria, isolated from a single rat liver (110), were suspended in 0.1 M KP_i (pH 8) and sonicated for 10x20s at 0°C with an Ultrasonic sonifier (Model W-385) equipped with a micro-tip. The resultant suspension was centrifuged at 100,000 x g for 1 hr and the supernatant was passed through a Sephadex G-25 column equilibrated with 0.1 M KP_i (pH 8) to remove mitochondrial coenzymes. The soluble mitochondrial extract (5.9 mg/ml) was stored at -70°C. Protein concentrations were determined by the method of Bradford (101) with BSA as standard.

Enzyme Assays of 5-*cis*-Octenoyl-CoA and its Metabolites. Acyl-CoA dehydrogenase was assayed spectrophotometrically as described (33). When partially purified medium-chain acyl-CoA dehydrogenase

was assayed, the reaction was started by addition of the acyl-CoA substrate. With a soluble mitochondrial extract as an enzyme source, the reaction was initiated by the addition of phenazine methosulfate. Δ^3,Δ^2-Enoyl-CoA isomerase was assayed spectrophotometrically at 340 nm in a coupled assay (100) with 3-*trans*-octenoyl-CoA as substrate. 2,4-Dienoyl-CoA reductase was assayed spectrophotometrically at 340 nm with 2-*trans*,4-*trans*-octadienoyl-CoA as a substrate as previously described (57).

Metabolic Studies of the NADPH-Dependent Metabolism of 5-*cis* and 5-*trans*-Enoyl-CoAs. The hydration of 2,5-octadienoyl-CoA by crotonase and its isomerization to 3,5-octadienoyl-CoA were followed spectrophotometrically at 263 nm and 238 nm, respectively. Incubation mixtures contained 50 μM 2,5-octadienoyl-CoA in 0.1 M KP_i (pH 8) and either purified bovine liver crotonase, or purified trifunctional enzyme from rat liver peroxisomes, or partially purified Δ^3,Δ^2-enoyl-CoA isomerase from rat liver mitochondria was added to give an absorbance change of 0.08/min. The conversions of 2,5-octadienoyl-CoA or 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA were followed spectrophotometrically at 300 nm. Incubation conditions were the same as described above for the formation of 3,5-octadienoyl-CoA except that the enzyme source was a soluble mitochondrial extract (30 μg of protein/ml). When the chain shortening of 2,5-octadienoyl-CoA by β oxidation was studied, 50 μM substrate was incubated in 0.7 ml of 0.1 M KP_i (pH 8) with 60

μg of soluble mitochondrial extract until the absorbance at 300 nm ceased to increase. At that point, 170 μg of soluble mitochondrial extract and 0.3 ml of 0.1 M KP_i (pH 8) containing NAD^+, CoA, and NADPH were added to give final concentrations of the three coenzymes of 1 mM, 0.3 mM, and 0.1 mM, respectively. The progress of the reaction was monitored at 340 nm.

HPLC Analysis of 2-*trans*,5-*cis*-Octadienoyl-CoA Metabolites. Prior to analysis by HPLC, incubations were terminated by adjusting the pH to 1-2 with concentrated HCl. Samples were filtered through 0.22 μm membranes after which the pH was adjusted to 5 with KOH. The filtrates were applied to a Waters HPLC μBondapak C_{18} reverse phase column (30 cm x 3.9 mm) attached to a Waters gradient HPLC system. The absorbance of the effluent was monitored at 254 nm. Separation was achieved by linearly increasing the acetonitrile/H_2O (9:1) content of the 10 mM ammonium phosphate elution buffer (pH 5.5) from 10 to 50% in 30 min at a flow rate of 2 ml per min. When the chain shortening of 2,5-octadienoyl-CoA was studied, the acetonitrile/H_2O (9:1) content was increased from 0 to 10% in 10 min, followed by a linear increase from 10 to 50% in 20 min.

RESULTS

Resolution of Rat Liver 3-Hydroxyacyl-CoA Epimerase Activity into Two Complementary Fractions from a DEAE-Cellulose Column. Attempts to purify 3-hydroxyacyl-CoA epimerase from rat liver peroxisomes by a number of different chromatographic techniques invariably resulted in dramatic activity losses. These failures suggested that perhaps two components were being separated, both of which are necessary for the manifestation of epimerase activity. In pursuing this idea, it was observed that an apparent loss of epimerase activity upon chromatography on DEAE-cellulose is reversed if fractions are either recombined or supplemented with purified crotonase.

This initial observation led to application of a soluble extract of rat liver light mitochondria, which are enriched with respect to peroxisomes, to chromatography on DEAE-cellulose at low ionic strength to insure the complete adsorption of crotonase to the ion exchange material. Fractions unable to bind to DEAE-cellulose under these conditions were assayed for epimerase in the presence and absence of enoyl-CoA hydratase (crotonase). The elution profile is shown in Figure 6. Figure 7A illustrates the results of epimerase assays with the extract of rat liver light mitochondria and an early fraction from the DEAE-cellulose column as enzyme sources. The epimerase, which was present in the soluble extract of light mitochondria (Figure 7A, trace 1), apppeared to be absent from the

early column fraction (Figure 7A, trace 2a). However, when purified bovine liver crotonase, which was free of epimerase activity, was added to the assay of the early column fraction (Figure 6A, trace 2b), the epimerase activity was restored. When crotonase and the column fraction were added simultaneously (Figure 7A, trace 3), the progress curve was initially linear. However, when crotonase was added minutes after the column fraction (Figure 7A, trace 2b), the progress curve showed a burst followed by a linear rate similar to that seen when both enzymes were added simultaneously. This burst was thought to be caused by accumulated intermediate, which was rapidly acted upon when crotonase was added to the assay.

Since the enzymes present in the early column fractions, together with crotonase, facilitated the epimerization of a 3-hydroxyacyl-CoA thioester, it was speculated that it may be a 2-enoyl-CoA hydratase with a stereospecificity different from that of crotonase. Figure 7B represents the results of assays designed to test this hypothesis. The unknown enzyme dehydrates D-3-hydroxyoctanoyl-CoA, but not the L-isomer, whereas crotonase acts on L-3-hydroxyoctanoyl-CoA, but not on the D-isomer (see Figure 7B, traces 1-4). Since the equilibrium of the hydration of 2-*cis*-enoyl-CoA is almost completely to the side of the 3-hydroxyacyl-CoA, whereas the L-3-hydroxyacyl-CoA/2-*trans*-enoyl-CoA equilibrium is close to 3 (96), it is proposed that the new enoyl-CoA hydratase catalyzes the dehydration of D-3-hydroxyacyl-CoA to 2-*trans*-enoyl-

CoA. HPLC product analysis of the metabolites generated in Figure 7 have independently confirmed this proposal (111).

In conclusion, D-3-hydroxyacyl-CoAs are not directly converted to their L-isomers (indicated by the dashed line in Figure 8), but instead are dehydrated to 2-*trans*-enoyl-CoAs by a new enoyl-CoA hydratase (D-specific hydroxyacyl-CoA dehydratase) present in rat liver peroxisomes. The latter compounds can then be hydrated by mitochondrial crotonase or the peroxisomal bifunctional enzyme (L-specific hydratase) to L-3-hydroxyacyl-CoAs, which can be dehydrogenated and thiolytically cleaved to yield acetyl-CoA and chain-shortened acyl-CoAs. It follows that 3-hydroxyacyl-CoA epimerase is not a distinct enzyme; epimerization in rat liver is a reflection of the sequential actions of two enoyl-CoA hydratases with opposite stereospecificities.

Subcellular Localization of D-3-Hydroxyacyl-CoA Dehydratase. Although rat liver 3-hydroxyacyl-CoA epimerase is known to be associated with peroxisomes (59), it was important to also determine the subcellular location of D-3-hydroxyacyl-CoA dehydratase, which catalyzes one of the half reactions of 3-hydroxyacyl-CoA epimerization. For this purpose, analysis of the distribution of marker enzymes for peroxisomes, microsomes, and mitochondria were carried out on fractions containing nuclei and mitochondria (N + M), light mitochondria (L), microsomes (P), and soluble protein (S). The results of this analysis are shown in Figure 9. The

distribution pattern of D-3-hydroxyacyl-CoA dehydratase matches most closely with the pattern for catalase, a peroxisomal enzyme. Its highest specific activity appears in the L fraction, enriched with respect to peroxisomes. Further analysis was carried out using density gradient centrifugation of a rat liver L-fraction. The L fraction was prepared by differential centrifugation and further fractionated by centrifugation on a Nycodenz density gradient. Fractions were assayed for D-3-hydroxyacyl-CoA dehydratase in addition to catalase, esterase, and glutamate dehydrogenase as marker enzymes for peroxisomes, microsomes and mitochondria, respectively. The results of this experiment, shown in Figure10, reveal that catalase and dehydratase distribute nearly identically, whereas glutamate dehydrogenase (GDH) and esterase display two distinct and different distributions along the gradient. The inability to detect glutamate dehydrogenase activity at the bottom of the gradient, where catalase and the D-specific dehydratase activity is highest, indicates the absence of detectable levels of this activity in mitochondria. The pattern displayed by D-3-hydroxyacyl-CoA dehydratase conclusively establishes that this activity is peroxisomal.

Purification of Rat liver D-3-Hydroxyacyl-CoA Dehydratase. Since the light mitochondrial fraction (L-fraction) obtained from a rat liver homogenate by differential centrifugation is enriched with respect to peroxisomes, it was used as starting material for the

purification of D-3-hydroxyacyl-CoA dehydratase. Dehydratase present in the soluble extract, prepared from the L-fraction by treatment with a Polytron homogenizer followed by centrifugation, was purified by a four-step procedure involving column chromatographies on DEAE-cellulose, hydroxyapatite and 3',5'ADP-Agarose followed by gel filtration on an HPLC system. The results of this purification scheme are summarized in Table I.

The purified dehydratase, when subjected to polyacrylamide gel electrophoresis under non-denaturing conditions, gave rise to a single band of protein that exhibited dehydratase activity (96). When the dehydratase was subjected to polyacrylamide gel electrophoresis in the presence of SDS, one major band was observed corresponding to 90% of the protein (see Figure 11). Several faint bands could be detected by direct observation of the gel or on its scan, but are not visible on the photograph of the gel shown in Figure 11. The molecular weight of the dehydratase subunit was estimated to be 44,000 based on the relative mobilities of five standard proteins (see Figure 12). Molecular weight determination of the native enzyme by gel filtration on an HPLC system yielded a value of 106,000 (data not shown) whereas gel filtration of a soluble extract of the L-fraction on Sepharose CL-6B and of the purified enzyme on Sephadex G-200 gave values of 78,000 (data not shown) and 73,000 (see Figure 13), respectively. Thus, it seems that the dehydratase is composed of two, possibly identical, subunits.

Substrate Specificity and Kinetic Properties of D-3-Hydroxyacyl-CoA Dehydratase. The specificity of the dehydratase with respect to the acyl chain length of its substrates was investigated with three different substrates in the forward direction and with all even-numbered substrates from crotonyl-CoA to 2-*trans*-hexadecenoyl-CoA in the reverse direction. The results clearly demonstrate the virtual inactivity of this enzyme toward substrates with a four carbon acyl chain (see Table II). The residual activity shown in Table II represents the limit of the assay's sensitivity; the enzyme may be completely inactive toward D-3-hydroxybutyryl-CoA and crotonyl-CoA. The highest activity was observed with 2-*trans*-hexenoyl-CoA. The activity declined steadily with increasing chain length of the substrate from 2-*trans*-hexenoyl-CoA to 2-*trans*-hexadecenoyl-CoA so that the latter substrate was hydrated at only 4% of the rate observed with 2-*trans*-decenoyl-CoA.

Kinetic constants (K_M and V_{max}) were determined for the forward and reverse reactions with D-3-hydroxyoctanoyl-CoA and 2-*trans*-octenoyl-CoA as substrates, respectively. The V_{max} values for the forward and reverse reactions were estimated to be 67 U/mg and 57 U/mg, respectively, while the K_M values for D-3-hydroxyoctanoyl-CoA and 2-*trans*-octenoyl-CoA were 71 μM and 22.5 μM, respectively.

The effect of pH on the activity of 3-D-hydroxyacyl-CoA dehydratase was determined between pH 5 and 10. As shown in Figure 14, the enzyme is most active at a pH close to 8 but retains

more than 50% of its activity between pH 6 and 10. However, at pH 5 and below the enzyme is completely inactive.

Equilibrium Constants for the Hydration of 2-*trans*-Octenoyl-CoA and 2-*cis*-Octenoyl-CoA. A ratio of 2.7 was obtained for L-3-hydroxyoctanoyl-CoA/2-*trans*-octenoyl-CoA by calculating it according to the Haldane equation from the K_M and V_{max} values determined for the hydration of 2-*trans*-octenoyl-CoA and the dehydration of D-3-hydroxyacyl-CoA by the dehydratase. An independent estimate of the equilibrium of 3-hydroxyoctanoyl-CoA/2-*trans*-octenoyl-CoA was determined by HPLC and found to be 3.2; similarly, the equilibrium of D-3-hydroxyoctanoyl-CoA/2-*cis*-octenoyl-CoA was found to be 137 (96).

Dehydration of D-3-Hydroxy-4-*trans*-Decenoyl CoA and L-3-Hydroxy-4-*trans*-Decenoyl-CoA by the Fatty Acid Oxidation Complex from *E. coli*. Since the component enzymes of the *E. coli* fatty acid oxidation complex (FAOC) cannot be separated from each other, it would be difficult to establish with D-3-hydroxyacyl-CoA as a substrate whether or not epimerization proceeds by a dehydration/hydration mechanism. However, the use of 3-hydroxy-4-*trans*-decenoyl-CoA, which is rapidly dehydrated but only very slowly regenerated by hydration (91), provides a possible means for demonstrating the formation of a 2,4-dienoyl-CoA intermediate during the epimerization. With this approach in mind, the

dehydration of D-3-hydroxy-4-*trans*-decenoyl-CoA by *E. coli* FAOC was studied by use of HPLC. The dehydration of D-3-hydroxy-4-*trans*-decenoyl-CoA (see Figure 15A, peak 1) to 2,4-decadienoyl-CoA (see Figure 15B, peak 2) is catalyzed by FAOC and the equilibrium is, as previously demonstrated (91), almost completely to the side of the dehydration product. The stereochemistry of the dehydration product was established by comparing its retention time on HPLC with the retention times of 2-*cis*,4-*trans*-decadienoyl-CoA and 2-*trans*,4-*trans*-decadienoyl-CoA. The 2-*cis*,4-*trans*-decadienoyl-CoA isomer, which was generated from D-3-hydroxy-4-*trans*-decenoyl-CoA by reacting it with bovine liver crotonase (112), was clearly separated from the dehydration product formed by FAOC (see Figure 15C peaks 2 and 3). However, 2-*trans*,4-*trans*-decadienoyl-CoA, which was chemically synthesized and enzymatically formed from D-3-hydroxy-4-*trans*-decenoyl-CoA by D-3-hydroxyacyl-CoA dehydratase (4), coeluted with the dehydration product generated by FAOC (see Figure 15D, peak 2). Thus, FAOC-catalyzed dehydration of D-3-hydroxy-4-*trans*-decenoyl-CoA yields 2-*trans*,4-*trans*-decadienoyl-CoA. Dehydration of L-3-hydroxy-4-*trans*-decenoyl-CoA by FAOC produced, as expected, 2-*trans*,4-*trans*-decadienoyl-CoA (data not shown), which was identified as detailed above for the dehydration product of D-3-hydroxy-4-*trans*-decenoyl-CoA.

Analysis of Metabolites Formed During the β-Oxidation of D-3-Hydroxy-4-*trans*-Decenoyl-CoA by FAOC. The dehydration of both

the D- and L-isomers of 3-hydroxy-4-*trans*-decenoyl-CoA to 2-*trans*,4-*trans*-decadienoyl-CoA by FAOC agrees with, but does not prove, a dehydration/hydration mechanism of epimerization. Conversion of D-3-hydroxy-4-*trans*-decenoyl-CoA to 2-*trans*,4-*trans*-decadienoyl-CoA could also occur by epimerization of the D-3-hydroxy group followed by dehydration of the L-isomer due to the enoyl-CoA hydratase activity of FAOC. In order to exclude this possibility, D-3-hydroxy-4-*trans*-decenoyl-CoA was incubated with FAOC in addition to NAD^+, CoASH, pig heart thiolase, plus bovine liver L-3-hydroxyacyl-CoA dehydrogenase, and the β-oxidation intermediates formed were analyzed by HPLC. Under these conditions, any L-3-hydroxy-4-*trans*-decenoyl-CoA formed will be dehydrogenated and thiolytically cleaved to acetyl-CoA and 2-*trans*-octenoyl-CoA, which will be further degraded by β-oxidation to yield hexanoyl-CoA and acetyl-CoA. After 1 min of incubation, much of the D-3-hydroxy-4-*trans*-decenoyl-CoA was converted to 2-*trans*,4-*trans*-decadienoyl-CoA, with a small amount of hexanoyl-CoA having been formed (Figure 16A). As the D-3-hydroxy-4-*trans*-decenoyl-CoA was further depleted, 2-*trans*,4-*trans*-decadienoyl-CoA accumulated more rapidly than hexanoyl-CoA was formed (Figure 16B). Finally, all D-3-hydroxy-4-*trans*-decenoyl-CoA was converted to hexanoyl-CoA (Figure 16C). These observations suggest that D-3-hydroxy-4-*trans*-decenoyl-CoA is converted to 2-*trans*,4-*trans*-decadienoyl-CoA before it is chain shortened to hexanoyl-CoA.

Kinetic Analysis of the FAOC Enzymes Implicated in the Epimerization Reaction. Although the metabolite pattern observed during the β-oxidation of D-3-hydroxy-4-*trans*-decenoyl-CoA by FAOC is indicative of epimerization by a dehydration/hydration mechanism, the pattern is also compatible with a singe step epimerization if channeling of intermediates on the complex occurs as has been reported (91). To distinguish between these two possibilities, the rates of several component enzymes of the complex were determined and compared. The data presented in Table III show that the rate of epimerization at various concentrations of D-3-hydroxy-4-*trans*-decenoyl-CoA is 20 to 40 times slower than its dehydration but is equal to or slightly slower than the rate of hydration of 2-*trans*,4-*trans*-decadienoyl-CoA. In addition, this kinetic evaluation established that L-3-hydroxy-4-*trans*-decenoyl-CoA is much more slowly formed by epimerization than it is further metabolized by dehydrogenation or by dehydration to 2-*trans*,4-*trans*-decadienoyl-CoA. Altogether, the data presented in Table III fully support a mechanism of D-3-hydroxy-4-*trans*-decenoyl-CoA epimerization by rapid dehydration to 2-*trans*,4-*trans*-decadienoyl-CoA, followed by the much slower rehydration of 2-*trans*,4-*trans*-decadienoyl-CoA to L-3-hydroxy-4-*trans*-decenoyl-CoA, which in turn is rapidly dehydrogenated or dehydrated.

Effect of D-3-Hydroxy-4-*trans*-Decenoyl-CoA on the Rate of Dehydration of L-3-Hydroxy-4-*trans*-Decenoyl-CoA by FAOC. The

finding that FAOC from *E. coli* dehydrates both the L-isomer and D-isomer of 3-hydroxy-4-*trans*-decenoyl-CoA to 2-*trans*,4-*trans*-decadienoyl-CoA raises the question as to whether these two dehydration reactions occur at a single site or at two distinct active sites. With the aim of answering this question, the rates of dehydration of L-3-hydroxy-4-*trans*-decenoyl-CoA were determined as a function of the concentration of D-3-hydroxy-4-*trans*-decenoyl-CoA. Measured rates were compared with calculated rates assuming either the existence of separate and independent dehydration activities or the existence of a single active site for both substrates. Calculations by use of the Michaelis-Menten equation were based on the following apparent kinetic parameters determined as part of this study: L-3-hydroxy-4-*trans*-decenoyl-CoA K_M=8.7 μM, V_{max}=248 U/mg; D-3-hydroxy-4-*trans*-decenoyl-CoA K_M=38 μM, V_{max}=62 U/mg. The measured and calculated values shown in Figure 17 were obtained at a fixed concentration of L-3-hydroxy-4-*trans*-decenoyl-CoA of 3 K_M ([L']=3) whereas the concentration of D-3-hydroxy-4-*trans*-decenoyl-CoA was varied from three times its K_M ([D']=3) to six times its K_M ([D']=6). Substrate concentrations are normalized with respect to the K_M value to indicate the relative extent of saturation by each substrate. The observed and calculated rates in the absence of the D-isomer were virtually identical. With increasing concentration of D-3-hydroxy-4-*trans*-decenoyl-CoA, the total rate of dehydration decreased as predicted for competitive inhibition by alternative substrates. In contrast, the observed rate

was significantly lower than the rate expected if two separate and independent active sites for the two substrates were operative on FAOC.

Hydration of 2-*cis*-Octenoyl-CoA by *E. coli* Extracts and Purified FAOC. Since D-3-hydroxyacyl-CoAs are assumed to be the physiological substrates of the epimerase, the formation of these compounds by hydration of 2-*cis*-octenoyl-CoA in *E. coli* was investigated. In *E. coli* B cells grown on glucose no enoyl-CoA hydratase or crotonase activity was detected with 2-*cis*-octenoyl-CoA as a substrate. However, in an extract prepared from *E. coli* B cells grown on oleate as the sole carbon source, in which the enzymes of β-oxidation are induced, a low hydratase activity of 0.16 U/mg was observed with 2-*cis*-octenoyl-CoA. A similar 2-*cis*-octenoyl-CoA hydratase activity of 0.15 U/mg was also associated with purified FAOC.

Dehydrogenation of 5-Octenoyl-CoA. The mitochondrial β-oxidation of 5-enoyl-CoAs, which are presumed intermediates in the β-oxidation of unsaturated fatty acids with double bond extending from odd-numbered carbon atoms, was studied with 5-*cis*-octenoyl-CoA and 5-*trans*-octenoyl-CoA. The suggested reduction of 5-*cis*-enoyl-CoAs to acyl-CoAs by NADPH (87) was investigated. When 5-*cis*-octenoyl-CoA was incubated with NADPH in the presence of rat liver mitochondria, no oxidation of NADPH

was observed. Thus, it seems that 5-enoyl-CoAs are not directly converted to their saturated analogs by a hypothetical NADPH-dependent 5-enoyl-CoA reductase.

Purified 5-*cis*-octenoyl-CoA, which gave a single peak on HPLC (see Figure 18A), was reacted with acyl-CoA oxidase either in the presence or absence of catalase. The same major reaction product was obtained under both conditions and was purified by HPLC to remove unreacted starting material as well as a more polar reaction product. The product of this enzymatic reaction was assumed to be 2-*trans*,5-*cis*-octadienoyl-CoA (see Figure 18B) since acyl-CoA oxidases are known to dehydrogenate acyl-CoAs to 2-*trans*-enoyl-CoAs while reducing oxygen to H_2O_2 (113). The dehydrogenation product, 2-*trans*,5-*cis*-octadienoyl-CoA, was clearly separated from the starting material 5-*cis*-octenoyl-CoA by HPLC (see Figure 18C). The absorbance spectrum of 2-*trans*,5-*cis*-octadienoyl-CoA (not shown) is, as expected, characteristic of an acyl-CoA with a maximum close to 260 due to the adenine moiety of CoA. When 2-*trans*,5-*cis*-octadienoyl-CoA was incubated with crotonase, the absorbance around 260 nm decreased as expected for a 2-enoyl-CoA compound that is hydrated to 3-hydroxyacyl-CoA, reflecting hydration of the double bond with consequent disappearance of the chromophore (data not shown). The product of this reaction was also analyzed by HPLC and found to be eluted at a position expected for the more polar 3-hydroxy-5-*cis*-octenoyl-CoA (see Figure 18D). The elution time of 3-hydroxy-5-*cis*-octenoyl-CoA

formed by crotonase-catalyzed hydration of 2-*trans*,5-*cis*-octadienoyl-CoA was identical to the elution time of a minor and more polar reaction product formed during the dehydrogenation of 5-*cis*-octenoyl-CoA by acyl-CoA oxidase. When the acyl-CoA oxidase preparation was assayed for crotonase, this enzyme was detected. Separation of crotonase and acyl-CoA oxidase by chromatography on hydroxyapatite yielded an oxidase preparation which produced little of the more polar reaction product 3-hydroxy-5-*cis*-octenoyl-CoA during the dehydrogenation of 5-*cis*-octenoyl-CoA. Altogether, these experiments establish that the main product formed during the dehydrogenation of 5-*cis*-octenoyl-CoA by acyl-CoA oxidase is 2-*trans*,5-*cis*-octadienoyl-CoA. Virtually identical results were obtained when 5-*trans*-octenoyl-CoA was converted to 2-*trans*,5-*trans*-octadienoyl-CoA by acyl-CoA oxidase (data not shown).

The dehydrogenation of 5-*cis*-octenoyl-CoA and 5-*trans*-octenoyl-CoA by acyl-CoA dehydrogenase present in a soluble extract of a rat liver mitochondria were determined and compared with the dehydrogenation of octanoyl-CoA. Rates determined at saturating or near saturating concentrations (40-50 μM) of substrates were 21.1 mU/mg (100%) with octanoyl-CoA *vs* 17.4 mU/mg (82%) with either 5-*cis*-octenoyl-CoA or 5-*trans*-octenoyl-CoA. Kinetic measurements with partially purified medium-chain acyl-CoA dehydrogenase from bovine liver and 5-*cis*-octenoyl-CoA as well as 5-*trans*-octenoyl-CoA as substrates yielded relative maximal velocities which were almost identical but were 25% lower than the

maximal velocity obtained with octanoyl-CoA. Values of K_m for all three substrates were similar and in the low micromolar range (5-9 μM). The dehydrogenation product formed by bovine liver medium-chain acyl-CoA dehydrogenase with 5-*cis*-octenoyl-CoA as a substrate was indistinguishable from 2-*trans*,5-*cis*-octenoyl-CoA on HPLC (data not shown).

Isomerizations of 2,5-Octadienoyl-CoA. When 2-*trans*-5-*cis*-octadienoyl-CoA was incubated with a soluble extract of rat liver mitochondria, from which low molecular weight cofactors had been removed by filtration through Sephadex G-25, a single product was detected by HPLC (see Figure 18E). This compound, which eluted from a reverse phase HPLC column 1 min later than the starting material (see Figure 18F), was inseparable from authentic 2-*trans*,4-*trans*-octadienoyl-CoA. The same result was obtained when 2-*trans*,5-*trans*-octadienoyl-CoA was allowed to react with the soluble extract of rat liver mitochondria.

In an attempt to elucidate the isomerization of 2,5-octadienoyl-CoA, the 2-*trans*,5-*cis*-isomer was incubated with purified trifunctional enzyme from rat liver peroxisomes. Spectral analysis indicated that the absorbance at 260 nm decreased quickly, followed by a slower increase near 240 nm (114). The slower absorbance increase close to 240 nm is attributed to the formation of 3,5-octadienoyl-CoA catalyzed by the Δ^3,Δ^2-enoyl-CoA isomerase activity of the trifunctional enzyme (74). A partially purified

preparation of mitochondrial Δ^3,Δ^2-enoyl-CoA isomerase brought about the same absorbance changes (114). Product analysis by HPLC revealed a single peak, inseparable from the starting material 2-*trans*,5-*cis*-octadienoyl-CoA (data not shown). When an equimolar mixture of starting material and product was analyzed by HPLC, a slight separation was detectable (data not shown). However, the product, in contrast to the starting material, was neither hydrated by crotonase nor by the trifunctional enzyme. The addition of a soluble extract of rat liver mitochondria to 3,5-octadienoyl-CoA resulted in the disappearance of the absorbance around 240 nm and caused a corresponding absorbance increase centered around 300 nm (114). The spectrum, upon completion of the reaction, was characteristic of a 2,4-dienoyl-CoA compound. Product analysis by HPLC revealed the presence of a single UV-absorbing compound which coeluted with 2-*trans*,4-*trans*-octadienoyl-CoA (see Figure 18E) but which was clearly separated from the starting material (see Figure 18F).

Rates of isomerization from 2,5-octadienoyl-CoA to 2,4-octadienoyl-CoA and 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA were determined. With a soluble extract of rat liver mitochondria the 3,5→2,4 conversion proceeded twice as fast as the 2t,5t→2,4-isomerization and 15 times faster than the 2t,5c→2,4 conversion. With a fraction of the extract, which was obtained by chromatography on hydroxyapatite and which contained little Δ^3,Δ^2-enoyl-CoA isomerase activity, the 3,5→2,4 conversion was 6 times and 20 times faster than the conversions of 2t,5t→2,4 and

2t,5c→2,4 respectively. The isomerization of 2-*trans*,5-*cis*-octadienoyl-CoA to 2,4-octadienoyl-CoA was also catalyzed by a soluble extract of rat heart mitochondria.

Characterization of 2,4-Octadienoyl-CoA. The final isomerization product formed from either 2-*trans*,5-*cis*-octadienoyl-CoA, 2-*trans*,5-*trans*-octadienoyl-CoA, or 3,5-octadienoyl-CoA by a soluble extract of rat liver or rat heart mitochondria was tentatively identified as 2,4-octadienoyl-CoA based on its UV spectrum (114) and behavior on HPLC where it was indistinguishable from synthetic 2-*trans*,4-*trans*-octadienoyl-CoA. Further proof for its structure was obtained when NADPH was added to a mixture of 2,4-octadienoyl-CoA and a soluble extract of rat liver mitochondria, resulting in the absorbance at 300 nm disappearing and a decrease in absorbance at 340 nm occurred due to the NADPH-dependent reduction of 2,4-octadienoyl-CoA catalyzed by 2,4-dienoyl-CoA reductase present in the extract from rat liver mitochondria (114). When partially purified 2,4-dienoyl-CoA reductase was used, HPLC analysis revealed the formation of 3-octenoyl-CoA upon reduction of 2,4-octadienoyl-CoA by NADPH (see Figure 19A). Finally, when 2-*trans*,5-*cis*-octadienoyl-CoA was first completely converted to 2,4-octadienoyl-CoA by a soluble extract of rat liver mitochondria and then incubated for 5 min in the presence of NADPH, NAD^+, and CoA, the formation of hexanoyl-CoA, butyryl-CoA, and acetyl-CoA was detected by HPLC (see Figure 19B). Hexanoyl-CoA and acetyl-

CoA are the expected products if 2,4-octadienoyl-CoA, after reduction by NADPH-dependent 2,4-dienoyl-CoA reductase, completes one cycle of β-oxidation. Butyryl-CoA would be formed if 2,4-octadienoyl-CoA, without being reduced by 2,4-dienoyl-CoA reductase, passes twice through the β-oxidation cycle. This reaction proceeds at a significant rate when the 2,4-dienoyl-CoA intermediate has the all-*trans* configuration (21). Preliminary evidence also indicates that this mitochondrial extract has some acyl-CoA dehydrogenase activity which facilitates the complete degradation of hexanoyl-CoA and butyryl-CoA to acetyl-CoA (data not shown). Since it was observed that 2-*trans*,4-*cis*-decadienoyl-CoA and 2-*trans*,4-*trans*-decadienoyl-CoA can be separated by HPLC under conditions used to identify 2,4-octadienoyl-CoA, it seems that isomerizations of the two 2,5-octadienoyl-CoA isomers and of 3,5-octadienoyl-CoA yield 2-*trans*,4-*trans*-octadienoyl-CoA, because it coeluted with authentic 2-*trans*,4-*trans*-octadienoyl-CoA.

DISCUSSION

Epimerization in Rat Liver. It is by now well established that the β-oxidation of polyunsaturated fatty acids proceeds overwhelmingly, if not completely, via a pathway which requires Δ^3,Δ^2-enoyl-CoA isomerase (EC 5.3.3.8) and NADPH-dependent 2,4-dienoyl-CoA reductase (EC 1.3.1.34) as auxiliary enzymes in addition to the enzymes necessary for the β-oxidation of saturated fatty acids (61). However, in peroxisomes and *E. coli*, although not in mitochondria, the epimerase-dependent pathway, (for many years was thought to be the only pathway (56)), may make a minor contribution to the β-oxidation of polyunsaturated fatty acids (86). Attempts to purify this epimerase led to dramatic activity losses until it was recognized that recombining the chromatographic fractions resulted in increases of the epimerase activity. This observation provided the basis for demonstrating that the 3-hydroxyacyl-CoA epimerase activity present in rat liver peroxisomes (59) is due to the combined actions of two enoyl-CoA hydratases with opposite stereospecificities.

D-3-hydroxyacyl-CoA is not directly converted to its L-isomer (indicated by the dashed line in Figure 21), but instead is dehydrated to 2-*trans*-enoyl-CoA by a new enoyl-CoA hydratase (D-dehydratase) present in rat liver peroxisomes. The latter compound is then hydrated by mitochondrial crotonase or the peroxisomal bifunctional enzyme (L-hydratase) to L-3-hydroxyacyl-CoA which

can be dehydrogenated and thiolytically cleaved to yield acetyl-CoA and a chain-shortened acyl-CoA. 3-Hydroxyacyl-CoA epimerase, then, is not a distinct enzyme; epimerase activity, in reality, reflects the sequential actions of two enoyl-CoA hydratases with opposite stereospecificities. A D-specific hydratase (EC 4.2.1.55), which catalyzes the hydration of 2-*trans*-butenoyl-CoA (crotonyl-CoA) to D-3-hydroxybutyryl-CoA, has been identified in *Rhodospirillum rubrum* where it functions in the biosynthesis of poly-D-3-hydroxybutyrate, but it may also cooperate with an L-hydratase in the epimerization of D-3-hydroxybutyryl-CoA to the L-isomer (115).

The identification of D-3-hydroxyacyl-CoA dehydratase as a key enzyme in the epimerization of 3-hydroxyacyl-CoA adds another member to a group of hydratases that catalyze the hydration of 2-enoyl-CoA thioesters to 3-hydroxyacyl-CoA's. Among the best characterized in this group is mitochondrial enoyl-CoA hydratase, or crotonase, (EC 4.2.1.17) which acts on substrates from crotonyl-CoA to 2-hexadecenoyl-CoA with crotonyl-CoA being the best substrate (39, 98). Also located in mitochondria, long-chain-enoyl-CoA hydratase (EC 4.2.1.74), is inactive with crotonyl-CoA but active toward all longer chain substrates tested (39). A third enoyl-CoA hydratase is the one associated with the bifunctional β-oxidation enzyme of peroxisomes (72) which very recently was shown to be a trifunctional enzyme harboring Δ^3,Δ^2–enoyl-CoA isomerase in addition to enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase (74). This hydratase is active with all substrates

tested from crotonyl-CoA to 2-hexadecenoyl-CoA. The above three enoyl-CoA hydratases are presumed to function in β-oxidation where they catalyze the hydration of 2-*trans*-enoyl-CoA thioesters to L-3-hydroxyacyl-CoA's. In addition an enoyl-CoA hydratase, or rather a 3-hydroxyacyl-CoA dehydratase, is present in microsomes where it functions in the elongation of unsaturated fatty acids. An abstract reporting the existence of such a dehydratase in microsomes has been published (116), which prompted this study on the subcellular location of D-3-hydroxyacyl-CoA dehydratase. However, in the absence of detailed information, the identity of this dehydratase remains to be established. A known dehydratase that converts D-3-hydroxyacyl thioesters to 2-*trans*-enoyl thioesters is a component enzyme of the multifunctional fatty acid synthase that catalyzes the *de novo* synthesis of fatty acids in animals (117). In view of these many enoyl-CoA hydratases reported to exist in one cell, the uniqueness of the new 3-hydroxyacyl-CoA dehydratase needed to be established beyond doubt. The information provided here demonstrates that this dehydratase is a novel enzyme, since it hydrates 2-*trans*-enoyl-CoA to yield the D-isomer of 3-hydroxyacyl-CoA, in contrast to all enoyl-CoA hydratases involved in β-oxidation, which produce the L-isomer. It also differs from the dehydratase of the mammalian multienzyme complex of fatty acid oxidation, which has a molecular weight close to 500,000, and whose component enzymes act on intermediates covalently linked via thioester bonds to the acyl carrier protein of the synthase (117).

Although clearly a peroxisomal enzyme, the new D-3-hydroxyacyl-CoA dehydratase is distinct from the trifunctional β-oxidation enzyme since (a) it is inactive toward crotonyl-CoA and 2-*cis*-octenoyl-CoA, (b) it converts 2-*trans*-enoyl-CoA to D-3-hydroxyacyl-CoA, and (c) its subunit molecular weight is 44,000 vs. 78,000 for the trifunctional enzyme (74).

The purification of D-3-hydroxyacyl-CoA dehydratase by a five-step procedure yielded a highly purified preparation as judged by gel electrophoresis. Although the purification of this enzyme resulted only in a 25-fold increase in specific activity over an L-fraction (see Table I), it must be considered that an L-fraction is enriched with respect to peroxisomes. When the specific activity of the purified dehydratase (40 U/mg) is compared with its specific activity in a rat liver homogenate (0.19 U/mg), a more than 200-fold purification was achieved. In addition, it seems likely that substantial activity losses that occurred during the purification (see Table I) were not always due to losses of dehydratase protein. Thus, the final preparation may contain inactive dehydratase that would lower the specific activity of the pure enzyme and its fold purification.

D-3-Hydroxyacyl-CoA dehydratase was identified as the enzyme that catalyzes one of the half reactions during the epimerization of 3-hydroxyacyl-CoA thioesters (111, 118). This reaction is essential for the complete degradation of polyunsaturated fatty acids via the minor pathway, which may

account for 2% of the β-oxidation of linoleic acid in peroxisomes (91). The observations that 2-*trans*-enoyl-CoA's are intermediates in the epimerization reaction and that D-3-hydroxyacyl-CoA dehydratase does not act on 2-*cis*-enoyl-CoA thioesters necessitate a modification of the epimerase-dependent branch of the pathway (see Figure 20). 4-*cis*-Decenoyl-CoA (I), an intermediate in the β-oxidation of linoleic acid is dehydrogenated to 2-*trans*,4-*cis*-decadienoyl-CoA (II) which is overwhelmingly reduced to 3-*trans*-decenoyl-CoA (VII) and after isomerization is completely degraded via the β-oxidation spiral. Some non-mitochondrial β-oxidation systems, however, can directly, albeit slowly, β-oxidize 2-*trans*,4-*cis*-decadienoyl-CoA (II) to 2-*cis*-octenoyl-CoA (III) (91). In peroxisomes, this compound can only be hydrated by the enoyl-CoA hydratase of the trifunctional enzyme to yield D-3-hydroxyoctanoyl-CoA (IV), which when dehydrated by the novel D-hydroxyacyl-CoA dehydratase to 2-*trans*-octenoyl-CoA (V) can be rehydrated by the hydratase of the trifunctional enzyme to L-3-hydroxyoctanoyl-CoA (VI). The last compound is a substrate of the β-oxidation spiral.

If, as suggested, nonmitochondrial β-oxidation systems degrade polyunsaturated fatty acids to a limited degree via the epimerase-dependent pathway (1,3), D-3-hydroxyacyl-CoA dehydratase would be an essential enzyme in peroxisomal β-oxidation. It is possible, however, that the enzyme also functions in the β-oxidation of fatty acids substituted at odd-numbered carbon atoms with a D-hydroxy group. Examples of such compound are

5(S)-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), a product of the 5-lipoxygenase-catalyzed oxidation of arachidonic acid (117), and D-3-hydroxymyristic acid, a component of the lipid A portion of the *E. coli* lipopolysaccharide (119). The possible function of D-3-hydroxyacyl-CoA dehydratase in the degradation of such D-hydroxy fatty acids is unresolved, and thus requires further study.

Epimerization on the *E. coli* Fatty Acid Oxidation Complex. The demonstration that in rat liver peroxisomes, 3-hydroxyacyl-CoAs are epimerized by a two-step dehydration/hydration mechanism (111, 118) raised the question as to whether this mechanism is common to all 3-hydroxyacyl-CoA epimerases, including the epimerase activity associated with FAOC from *E. coli*. For studying the mechanism of the *E. coli* epimerase, D-3-hydroxyacyl-CoA could not be used as a substrate because 2-*trans*-enoyl-CoA, its presumed epimerization intermediate, can also be formed by dehydration of the epimerization product L-3-hydroxyacyl-CoA due to the association of enoyl-CoA hydratase with FAOC. In contrast, D-3-hydroxy-4-enoyl-CoA was a suitable substrate because the equilibrium of its dehydration is far to the side of the reaction product 2,4-dienoyl-CoA (91); D-3-hydroxy-4-enoyl-CoA is rapidly dehydrated to 2,4-dienoyl-CoA, which is very slowly hydrated and therefore should accumulate if epimerization proceeds by dehydration/hydration. When D-3-hydroxy-4-*trans*-decenoyl-CoA was incubated with FAOC, it was almost completely converted to a

product which was shown to be 2-*trans*,4-*trans*-decadienoyl-CoA. The identification of the product, including the elucidation of its geometric configuration, was made possible by the availability of the two geometric isomers 2-*trans*,4-*trans*-decadienoyl-CoA and 2-*cis*,4-*trans*-decadienoyl-CoA and by their separation on HPLC. This experiment established that *E. coli* FAOC can dehydrate D-3-hydroxyacyl-CoAs to the corresponding 2-*trans*-enoyl-CoAs. However, it remained to be demonstrated whether D-3-hydroxy-4-*trans*-decenoyl-CoA was directly dehydrated or was first epimerized to the L-hydroxy isomer and then dehydrated by the enoyl-CoA hydratase activity on FAOC. To distinguish between these two possibilities, the epimerization of D-3-hydroxy-4-*trans*-decenoyl-CoA was coupled to the dehydrogenation of the epimerization product. Under conditions at which the degradation of L-3-hydroxy-4-*trans*-decenoyl-CoA proceeded faster than its formation by epimerization, 2-*trans*,4-*trans*-decadienoyl-CoA was formed more rapidly than the final oxidation product hexanoyl-CoA. Hence, 2-*trans*,4-*trans*-decadienoyl-CoA was formed by direct dehydration of D-3-hydroxy-4-*trans*-decenoyl-CoA. Altogether, these observations lead to the conclusion that FAOC from *E. coli* is capable of epimerizing 3-hydroxyacyl-CoAs by a two-step dehydration/hydration mechanism.

To demonstrate that epimerization occurs exclusively by dehydration/hydration, the rates of epimerization were compared with the rates of the partial reactions. Since epimerization of D-3-

hydroxy-4-*trans*-decenoyl-CoA and hydration of 2-*trans*,4-*trans*-decadienoyl-CoA proceeded at almost equal rates, it follows that the kinetically relevant route of epimerization is via the two-step dehydration/rehydration sequence (I→II→III) outlined in Figure 21. The single-step epimerization marked by a dashed line in Figure 21 is either nonexistent or does not significantly contribute to the epimerization of 3-hydroxyacyl-CoAs. The dehydration of D-3-hydroxyacyl-CoA to 2-*cis*-enoyl-CoA, as illustrated in Figure 21 (see I→IV), is catalyzed by mammalian enoyl-CoA hydratase (112) but was not detected with FAOC. The extremely slow hydration of 2-*cis*-octenoyl-CoA by FAOC supports the conclusion that the enoyl-CoA hydratase of *E. coli* FAOC, in contrast to the mammalian enzyme, is almost inactive toward 2-*cis*-enoyl-CoA.

The involvement of two enoyl-CoA hydratase activities in the epimerization of 3-hydroxyacyl-CoAs by FAOC raises the question about their locations on the complex. Are the two hydratases associated with different regions of the complex or do they share the same active site? The observation that L-3-hydroxy-4-*trans*-decenoyl-CoA and its D-isomer behave as if they were competing substrates does not agree with the existence of separate active sites for the two substrates with each being unaffected by the other substrate. However, it was not possible to distinguish between two other arrangements: one where both substrates compete for a single site and another one with two separate active sites where each is inhibited by the substrate of the other site. The recent

sequencing of the *E. coli fadBA* operon (120-123) and the sequence analysis of the deduced amino acid sequence of FAOC (120, 123) are indicative of a single functional domain for enoyl-CoA hydratase which is associated with the amino terminal region of the large α subunit (123). When taken together, the conclusion of the sequence analyses and the results of the substrate competition study agree best with an arrangement where both hydratase activities are associated with a single active site.

Finally, this study of 3-hydroxyacyl-CoA epimerase again raises the question as to the metabolic function of this enzyme activity. Since the epimerase is associated with FAOC, which is induced in *E. coli* when the cells are grown on fatty acids as the sole carbon source, it is reasonable to assume that this enzyme functions in the β-oxidation of fatty acids. Specifically, it functions in the conversion of D-3-hydroxyacyl-CoAs to their L-isomers, which are substrates of the β-oxidation spiral. However, the origin of 3-hydroxy fatty acids that may be oxidized by *E. coli* has not been established. Formation of D-3-hydroxyacyl-CoAs by hydration of 2-*cis*-enoyl-CoAs seems to be insignificant in *E. coli* due to the extremely low hydratase activity of the complex toward 2-*cis*-enoyl-CoAs. Thus, the epimerase-dependent pathway of unsaturated fatty acid oxidation is even less significant than previously estimated (61). However, D-3-hydroxy myristic acid is a component of the lipid A portion of the *E. coli* lipopolysaccharide (119) and perhaps this acid, after epimerization, is degraded by β-oxidation if the

lipopolysaccharide is turned over or if the acid becomes available as a fuel due to cell death. It is interesting to speculate that mammals have retained epimerizing ability, probably essential for Gram negative bacteria, as an evolutionary advantage in degrading lipid A freed by macrophage metabolism during the immune response to Gram negative bacterial infections.

NADPH-Dependent Mitochondrial Metabolism of 5-*cis*-Enoyl-CoAs. Unsaturated fatty acids with odd-numbered double bonds, for example oleic acid with a double bond extending from carbon atom 9 and linolenic acid with two odd-numbered double bonds extending from carbon atoms 9 and 15, are believed to be chain shortened until the odd-numbered double bonds extend from carbon atom 3 (5). At this stage, Δ^3,Δ^2-enoyl-CoA isomerase converts 3-*cis* or 3-*trans* double bonds to a 2-*trans* double bond (58). The resultant 2-*trans*-enoyl-CoAs reenter the β-oxidation cycle beyond the first dehydrogenation step and are completely degraded. However, the observation of Tserng and Jin (87) that the effective β-oxidation of 5-*cis*-enoyl-CoAs requires NADPH raised doubts about the assumed chain shortening of 5-*cis*-enoyl-CoAs to 3-*cis*-enoyl-CoAs by a simple pass through the β-oxidation spiral. Since these authors observed the conversion of 5-*cis*-enoyl-CoAs to saturated acyl-CoAs with the same number of carbon atoms, they suggested that an NADPH-dependent 5-enoyl-CoA reductase may convert 5-enoyl-CoAs to the corresponding acyl-CoAs. Attempts to detect such

activity were unsuccessful, prompting this detailed study of the β-oxidation of 5-octenoyl-CoA, which is a metabolite of linolenic acid. Since the results obtained with 5-*cis*-octenoyl-CoA and 5-*trans*-octenoyl-CoA were qualitatively identical, only the β-oxidation of 5-*cis*-octenoyl-CoA will be discussed.

The proposed NADPH-dependent pathway by which 5-*cis*-octenoyl-CoA is chain shortened to hexanoyl-CoA is shown in Figure 22. All enzymes necessary for this pathway are present in a soluble extract of rat mitochondria. Mitochondrial medium-chain acyl-CoA dehydrogenase as well as peroxisomal acyl-CoA oxidase, which are known to introduce 2-*trans* double bonds into acyl-CoAs (5), catalyze the dehydrogenation of 5-*cis*-octenoyl-CoA (I) to 2-*trans*,5-*cis*-octadienoyl-CoA (II). The assigned structure of compound II is supported by the crotonase-catalyzed hydration of the 2-*trans* double bond observed spectrophotometrically and by HPLC. 2-*trans*,5-*cis*-Octadienoyl-CoA is acted upon by mitochondrial Δ^3,Δ^2-enoyl-CoA isomerase as well as by the trifunctional enzyme of rat liver peroxisomes and converted to 3,5-octadienoyl-CoA (III). The structure assigned to compound III is supported by several facts and observations. (1) Δ^3,Δ^2-Enoyl-CoA isomerases are known to catalyze the shift of double bonds from the 3,4- to 2,3-position and presumably catalyze the reverse reaction; (2) the inactivity of crotonase toward compound III agrees with the absence of a 2,3-double bond; (3) the observed decrease in absorbance around 260 nm and the increase in absorbance around 240 nm agree with the

disappearance of the 2,3-double bond and the formation of the 3,5-diene (114) for which an absorbance maximum at 228 nm has been observed with hexane as a solvent (124). The UV spectrum of the Δ^2,Δ^3-enoyl-CoA isomerase product was determined with water as solvent, the λ_{max} is expected to be shifted to the red by 10 to 20 nm (125). Even though the configuration of the diene of compound III has not been established, it is assumed that the 5-double remained unaffected by the isomerization, whereas the 3-double bond may have either the *trans* or *cis* configuration. Incubation of 3,5-octadienoyl-CoA (III) with a soluble extract of rat mitochondria produced an absorbance decrease centered around 240 nm and a corresponding increase centered around 300 nm in a time-dependent manner. These spectral changes are indicative of the formation of 2,4-octadienoyl-CoA (IV). Since the isomerization product IV and synthetic 2-*trans*,4-*trans*-octadienoyl-CoA could not be separated by HPLC, whereas 2-*trans*,4-*trans*-decadienoyl-CoA and 2-*trans*,4-*cis*-decadienoyl-CoA can be separated (unpublished observation), the 2,4-octadienoyl-CoA most likely has the all-*trans* configuration. The isomerization of 3,5-octadienoyl-CoA (III) to 2,4-octadienoyl-CoA (IV) could be the consequence of the two double bonds shifting either simultaneously or one at a time. If the two double bonds shift one-by-one, 2,5-octadienoyl-CoA would be an intermediate in the isomerization reaction. The observation that the 3,5→2,4 isomerization occurred much faster than the 2,5→2,4 isomerization argues against a mechanism involving separate shifts

of double bonds and favors the simultaneous shift of both double bonds. If so, a $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase is expected to be present in the mitochondrial extract. However, it remains to be established whether this enzymatic activity is due to a novel enzyme or is the unidentified activity of a known enzyme. The identity of 2,4-octadienoyl-CoA (IV) was established beyond doubt by the spectral changes observed when it was reduced by NADPH in the presence of 2,4-dienoyl-CoA reductase, by identification of the reduction product 3-octenoyl-CoA (V) on HPLC, and by its complete β-oxidation to hexanoyl-CoA (VII), butyryl-CoA and acetyl-CoA catalyzed by a mitochondrial extract in the presence of NADPH, NAD^+, and CoA. The reported conversion of 5-*cis*-enoyl-CoAs to saturated fatty acyl-CoAs in the presence of NADPH (87) could be the consequence of 2-*trans*-enoyl-CoAs (*e.g.* compound VI) being reduced to the saturated acyl-CoAs by NADPH-dependent 2-enoyl-CoA reductase which is present in mitochondria (126).

This study demonstrates that 5-octenoyl-CoA can be degraded via the pathway shown in Figure 22, which requires NADPH and results in the reductive removal of the pre-existing double bond. However, it is not yet clear if all 5-enoyl-CoA intermediates formed during the β-oxidation of polyunsaturated fatty acids are degraded via this pathway. It also remains to be established whether 5-enoyl-CoAs are exclusively degraded via the NADPH-dependent pathway or perhaps are metabolized by several routes, including the direct β-

oxidation of 5-*cis*-enoyl-CoAs to 3-*cis*-enoyl-CoAs, which until now was thought to be their only route of β-oxidation.

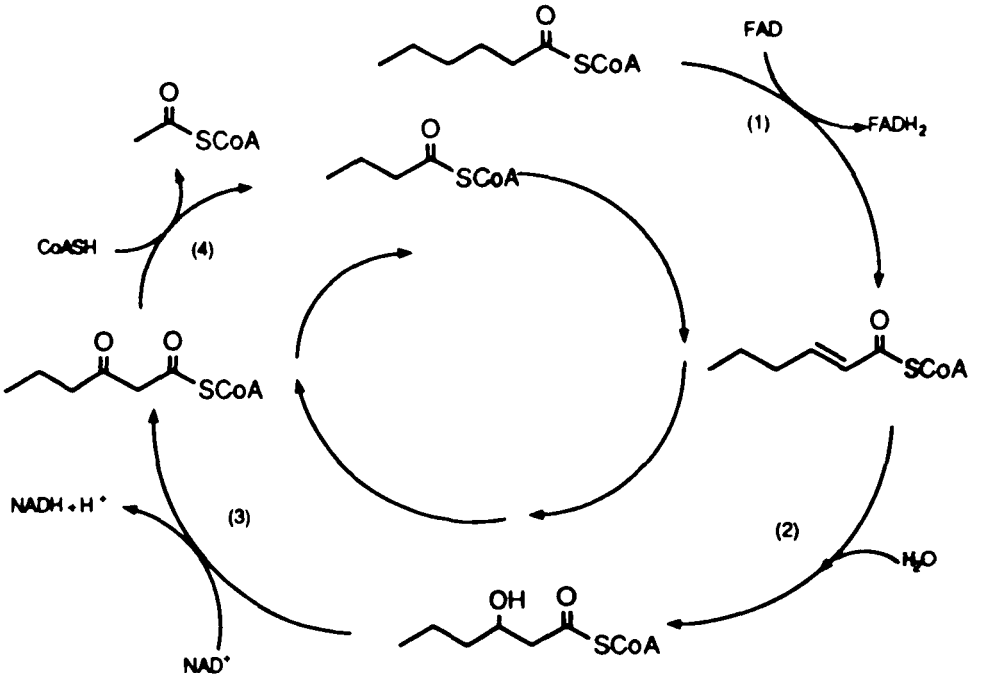

Figure 1. The β-Oxidation Spiral and its Enzymes. The reactions of the spiral are catalyzed by (1) acyl-CoA dehydrogenase; (2) enoyl-CoA hydratase; (3) L-3-hydroxyacyl-CoA dehydrogenase; (4) 3-ketoacyl-CoA thiolase.

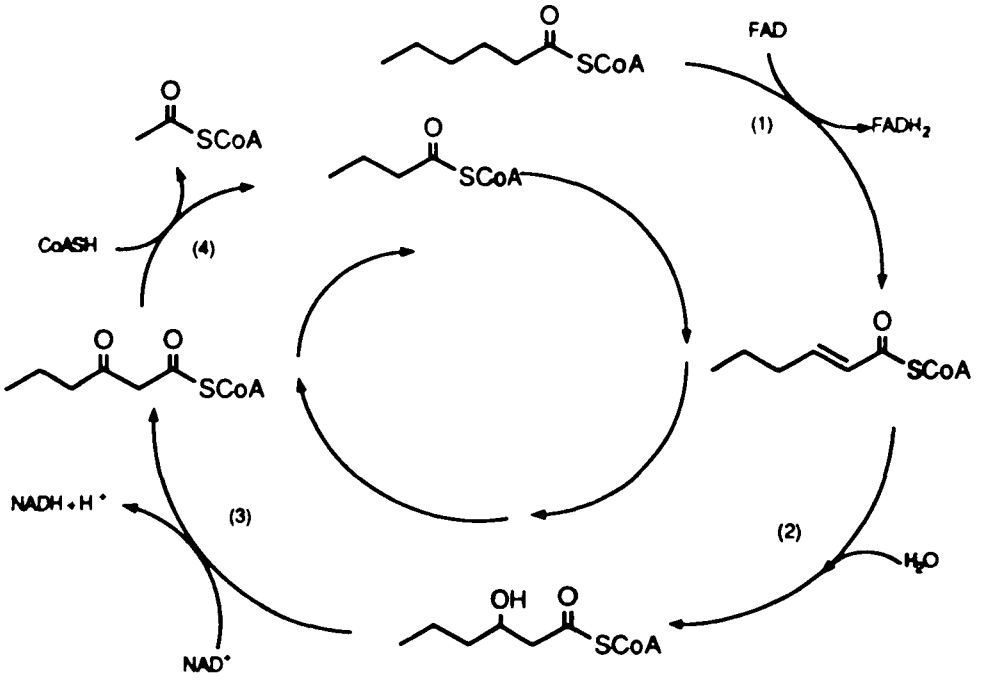

Figure 1. The β-Oxidation Spiral and its Enzymes. The reactions of the spiral are catalyzed by (1) acyl-CoA dehydrogenase; (2) enoyl-CoA hydratase; (3) L-3-hydroxyacyl-CoA dehydrogenase; (4) 3-ketoacyl-CoA thiolase.

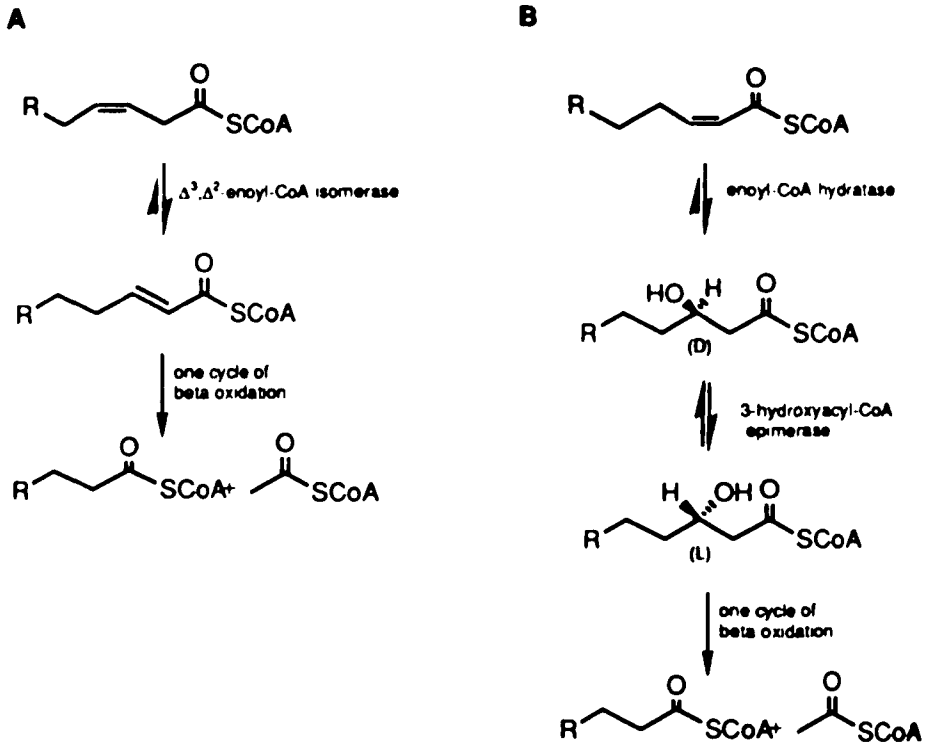

Figure 2. The Reactions Catalyzed by Δ^3,Δ^2-Enoyl-CoA Isomerase and 3-Hydroxyacyl-CoA Epimerase. (A) Chain shortening of fatty acids with *cis* double bonds at odd numbered carbons gives rise to 3-*cis*enoyl-CoAs, which enter the β-oxidation spiral only after being isomerized to the corresponding 2-*trans*-enoyl-CoA by Δ^2,Δ^3-enoyl-CoA isomerase. (B) Chain shortening of fatty acyl-CoAs with *cis* double bonds at even numbered carbons gives rise to 2-*cis*-enoyl-CoAs, which are acted upon by enoyl-CoA hydratase to give the D-3-hydroxyacyl-CoA. These may enter the spiral only after epimerization to the corresponding L-isomer by 3-hydroxyacyl-CoA epimerase.

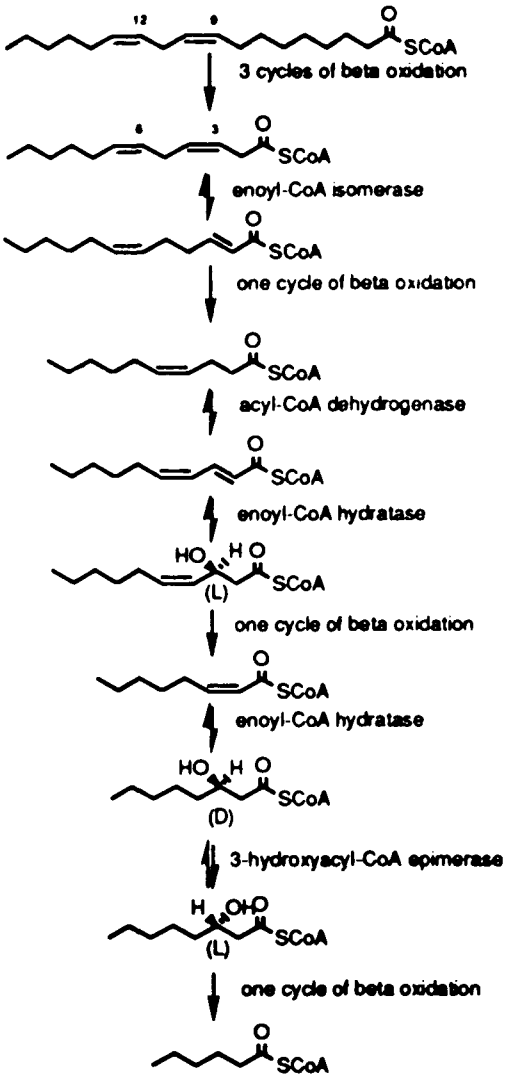

Figure 3. The Original Pathway for the β-Oxidation of Linoleic Acid. Both Δ^3,Δ^2-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase were once thought to be required for the β-oxidation of linoleoyl-CoA, as described in the scheme outlined above. Acetyl-CoAs generated during β-oxidation are omitted for clarity.

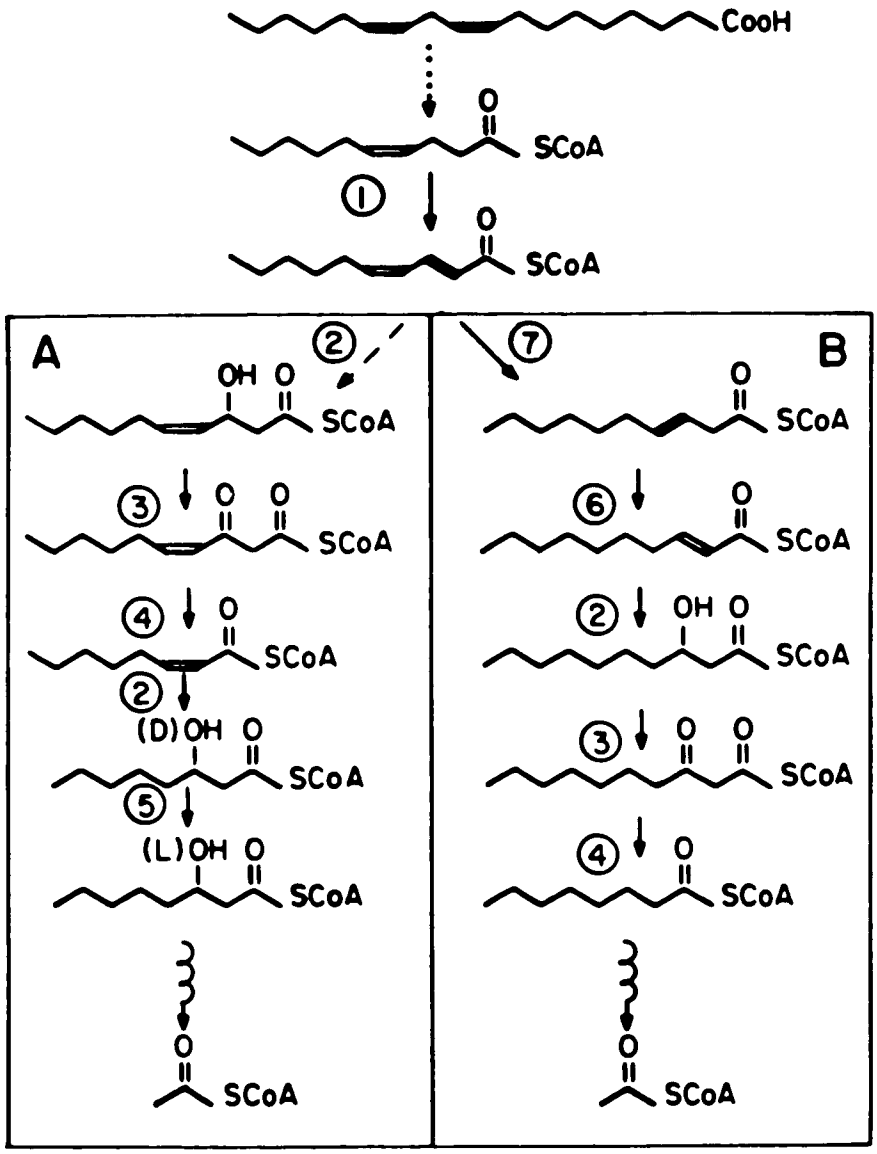

Figure 4. The Epimerase-Dependent (A) and Reductase-Dependent (B) Pathways of β-Oxidation. 1) acyl-CoA dehydrogenase, 2) enoyl-CoA hydratase, 3) L-3-hydroxyacyl-CoA dehydrogenase, 4) 3-ketoacyl-CoA thiolase, 5) 3-hydroxyacyl-CoA epimerase, 6) Δ^3,Δ^2-enoyl-CoA isomerase, 7) 2,4-dienoyl-CoA reductase.

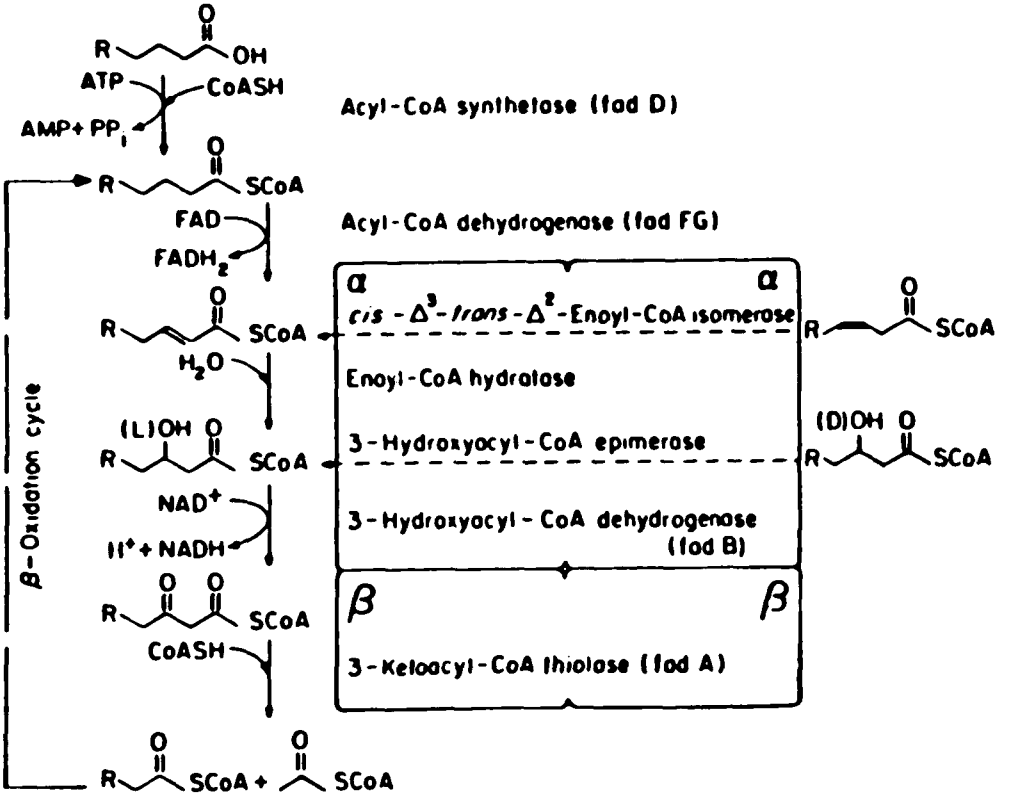

Figure 5. The *E. coli* Fatty Acid Oxidation Complex and its Component Activities.

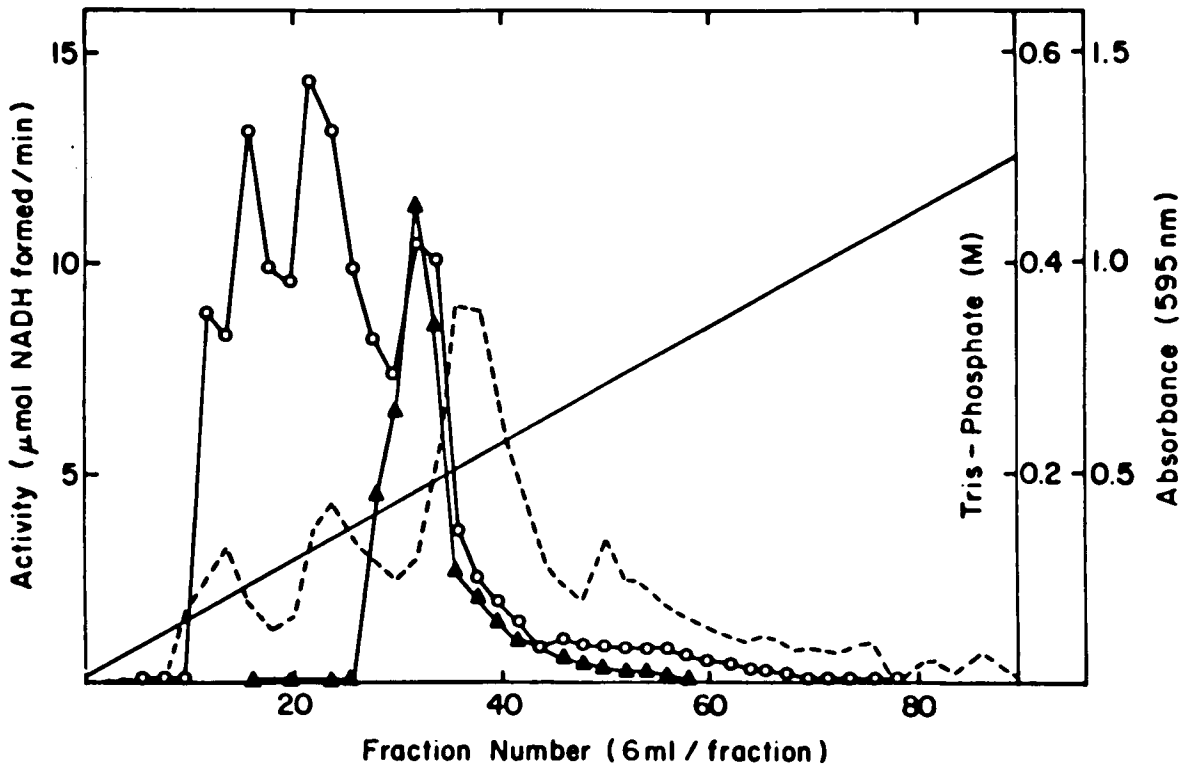

Figure 6. DEAE-Cellulose Chromatography of Rat Liver Light Mitochondria. The ability of each fraction to epimerize D-3-hydroxyacyl-CoA to the L-isomer was measured in the presence (open circles) and absence (closed circles) of externally added crotonase.

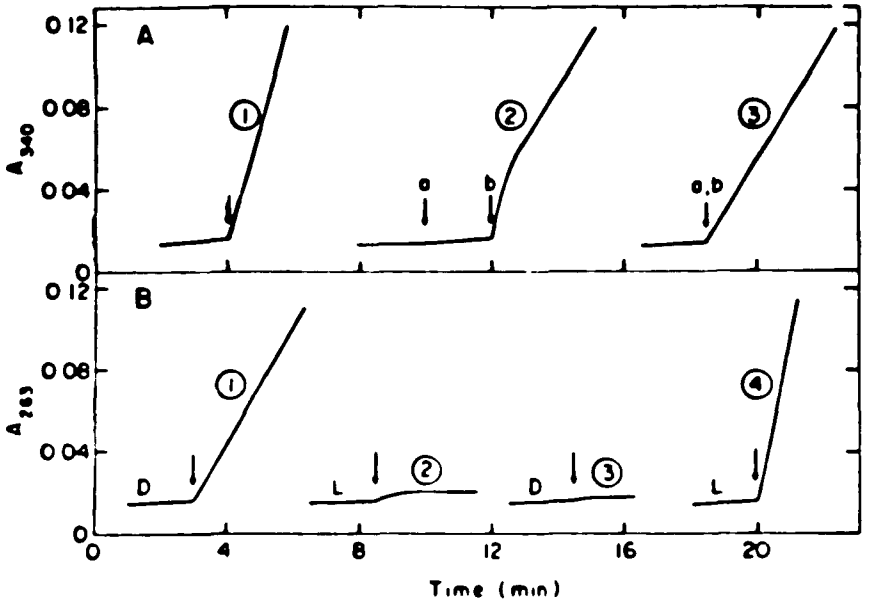

Figure 7. Assays of 3-Hydroxyacyl-CoA Epimerase and Enoyl-CoA Hydratase. A. 3-Hydroxyacyl-CoA epimerase assays: (1) with an extract of rat liver light mitochondria and (2) & (3) with (a) an early DEAE-cellulose fraction plus (b) crotonase. **B**. Enoyl-CoA hydratase assays: (1) & (2) with an early DEAE-cellulose fraction and (3) & (4) with crotonase. Substrates were either D-3-hydroxyoctanoyl-CoA or L-3-hydroxyoctanoyl-CoA referred to as D and L, respectively.

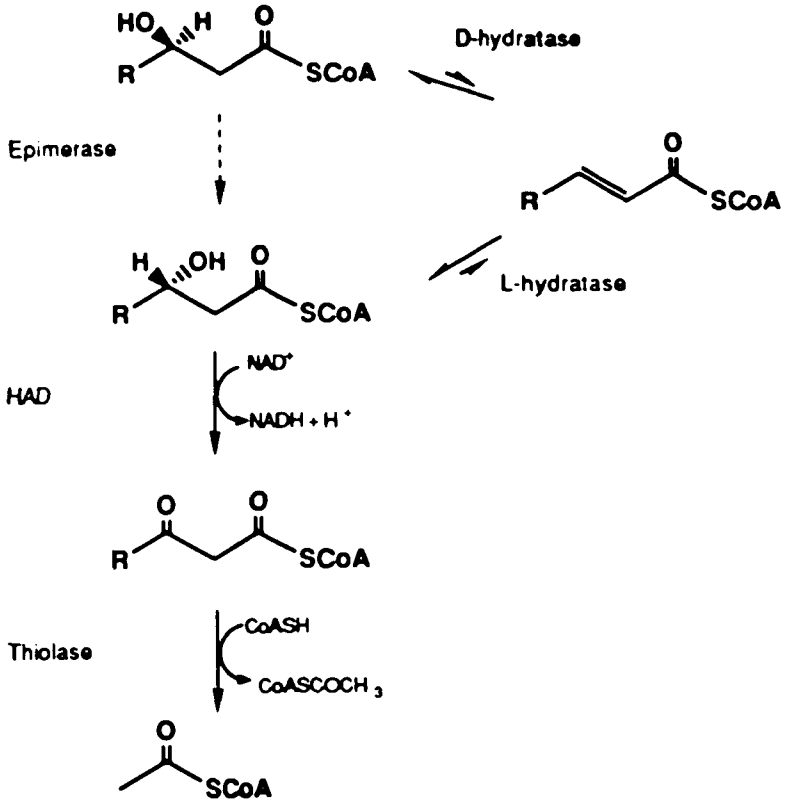

Figure 8. β-Oxidation of D-3-Hydroxyacyl-CoA. Epimerization of D-3-hydroxyacyl-CoA does not occur directly (dashed line) but instead proceeds via 2-*trans*-enoyl-CoA as an intermediate by the combined actions of a novel D-specific enoyl-CoA hydratase (D-hydratase) and an L-specific enoyl-CoA hydratase as for example the peroxisomal bifunctional enzyme or crotonase (L-hydratase).

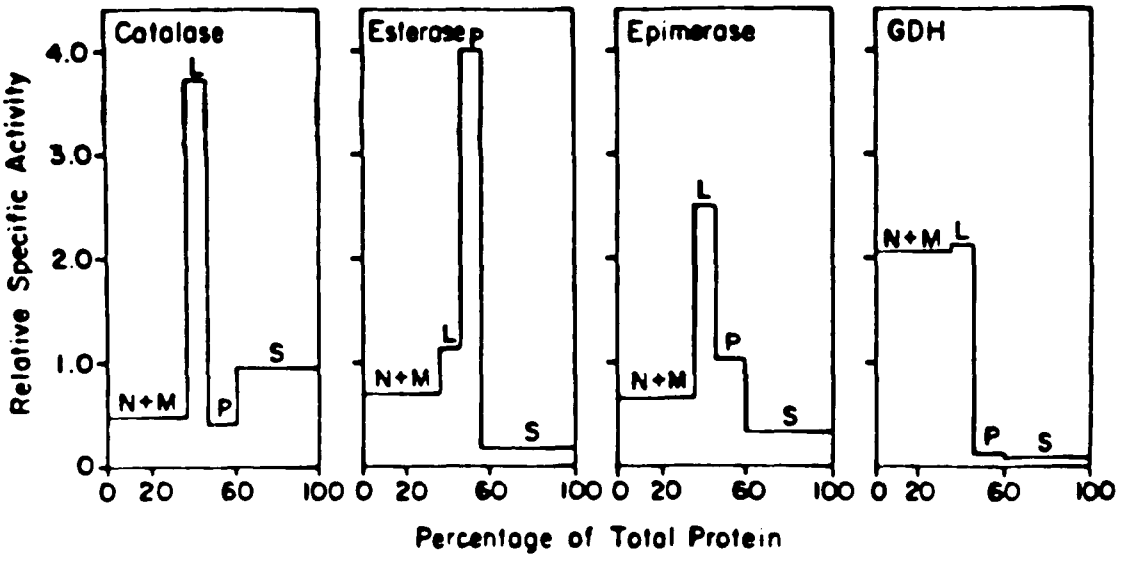

Figure 9. Subcellular Distribution of Epimerase Activity: NMLPS Analysis. Rat liver was fractionated by differential centrifugation into nuclei and mitochondria (N+M), light mitochondria (L), microsomes (P), and soluble protein (S) and each fraction was assayed for 3-hydroxyacyl-CoA epimerase in addition to catalase, esterase, and glutamate dehydrogenase (GDH) as marker enzymes for peroxisomes, microsomes, and mitochondria, respectively.

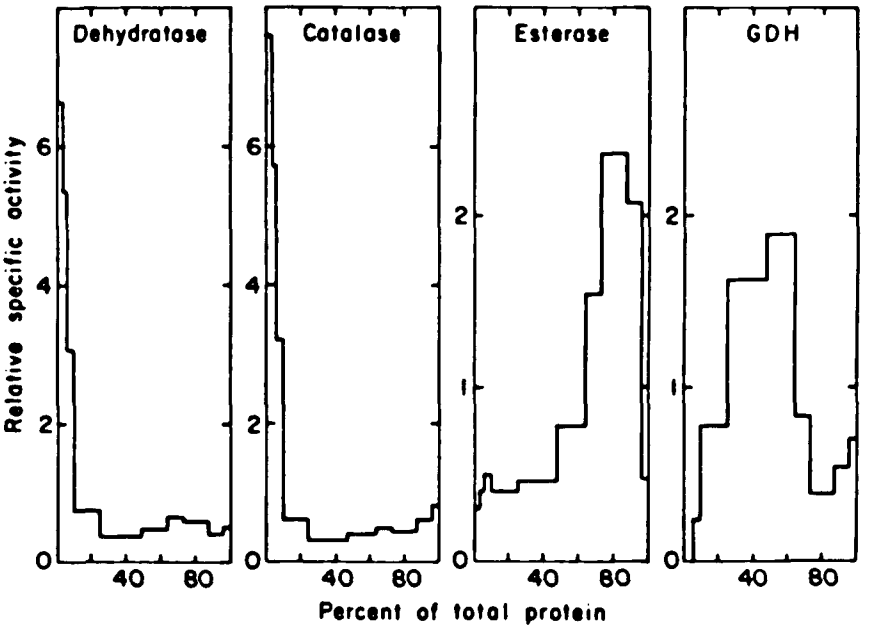

Figure 10. Separation of a Rat Liver L-fraction by Centrifugation on a Nycodenz Density Gradient. Fractions, collected from the bottom of the tube, were assayed for D-3-hydroxyacyl-CoA dehydratase (dehydratase) in addition to catalase, esterase, and glutamate dehydrogenase (GDH) as marker enzymes for peroxisomes, microsomes, and mitochondria, respectively.

Table I.
Purification of D-3-Hydroxyacyl-CoA Dehydratase

Purification steps	Total Activity (u*)	Total Protein (mg)	Specific Activity (u/mg)	Purification fold
L Fraction	886	544	1.63	1.00
Polytron treatment	833	544	1.63	0.99
L Fraction Extract	421	102	4.13	2.53
DEAE Cellulose	283	28.96	9.75	5.98
Hydroxyapatite	144	14.8	9.79	6.01
3',5'-ADP Agarose	90.4	4.34	20.8	12.8
HPLC Gel filtration	60.3	1.47	43.1	29.3

* 1 unit = 1 μmol/min

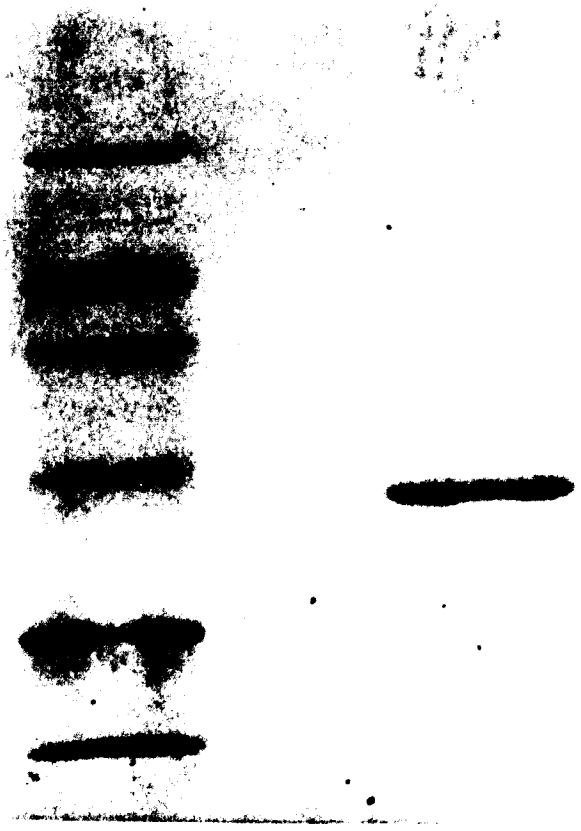

Figure 11. SDS Gel Electrophoresis of D-3-Hydroxyacyl-CoA Dehydratase. D-3-hydroxyacyl-CoA dehydratase (7.5 μg, right) was electrophoresced in the presence of SDS under reducing conditions on a 10% polyacrylamide gel followed by staining for protein with Coomassie blue. Standard proteins (left) were run on the same gel and their positions are, from the top, phosphorylase b (97.5 kD); bovine serum albumin (66 kD); catalase (57.5 kD); ovalbumin (45 kD); lactate dehydrogenase (35 kD); carbonic anhydrase b (29 kD).

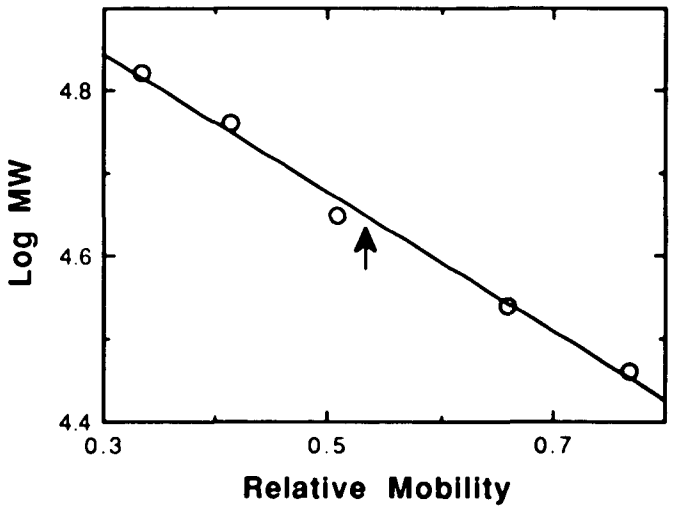

Figure 12. Subunit Molecular Weight of D-3-Hydroxyacyl-CoA Dehydratase. The positions of the standard proteins BSA, catalase, ovalbumin, lactate dehydrogenase, and carbonic anhydrase b from the SDS-PAGE gel shown in Figure 11 were determined and plotted as a function of the logarithm of their molecular weight. The molecular weight of D-3-hydroxyacyl-CoA dehydratase (see arrow) was determined to be 44,000.

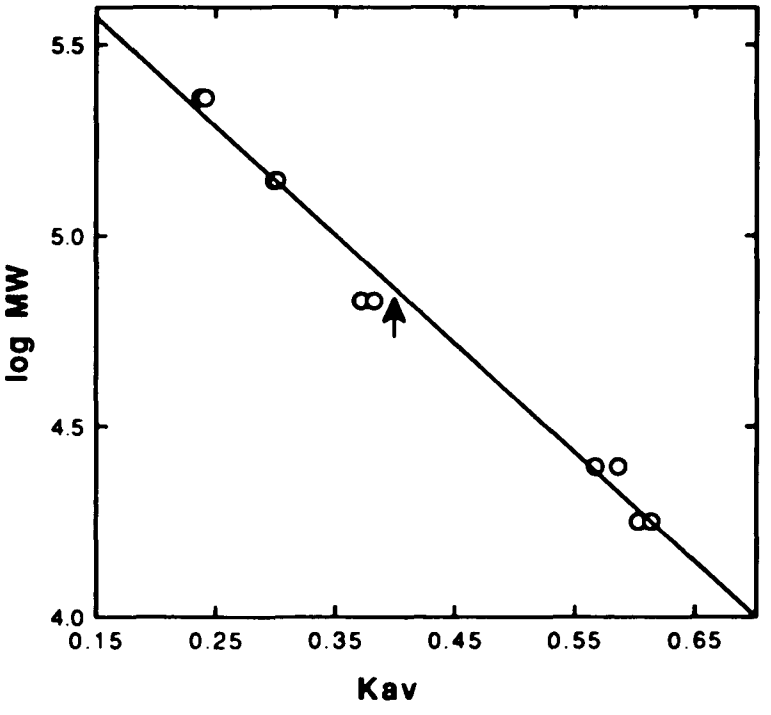

Figure 13. Gel filtration of D-3-Hydroxyacyl-CoA Dehydratase on Sephadex G-200. The native molecular weight of D-3-hydroxyacyl-CoA dehydratase was estimated to be 73,000 by comparison with the elution of standard proteins from a Sephadex G-200 column.

Table II

Chain length specificity of D-3-hydroxyacyl-CoA dehydratase. The enzyme was assayed in the forward and reverse directions by the direct spectrophotometric assay described under Experimental Procedures.

	Relative dehydratase activity	
Acyl chain length	2-*trans*-Enoyl-CoA	D-3-Hydroxyacyl-CoA
Number of carbon atoms	%	
4	1	0.4
6	114	
8	100	100
10	62	58
12	46	
14	21	
16	4	

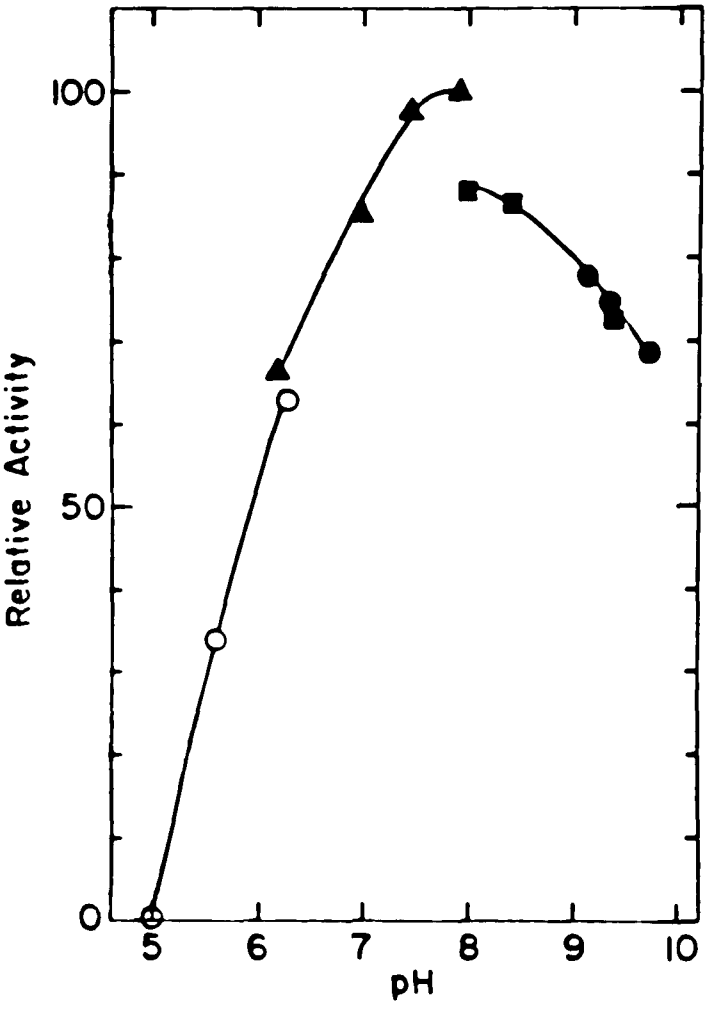

Figure 14. Activity of D-3-Hydroxyacyl-CoA Dehydratase as a Function of pH. The following buffers of the same ionic strength were used: (o) potassium acetate, (▲) potassium phosphate, (■) Tris-HCl, (●) potassium borate.

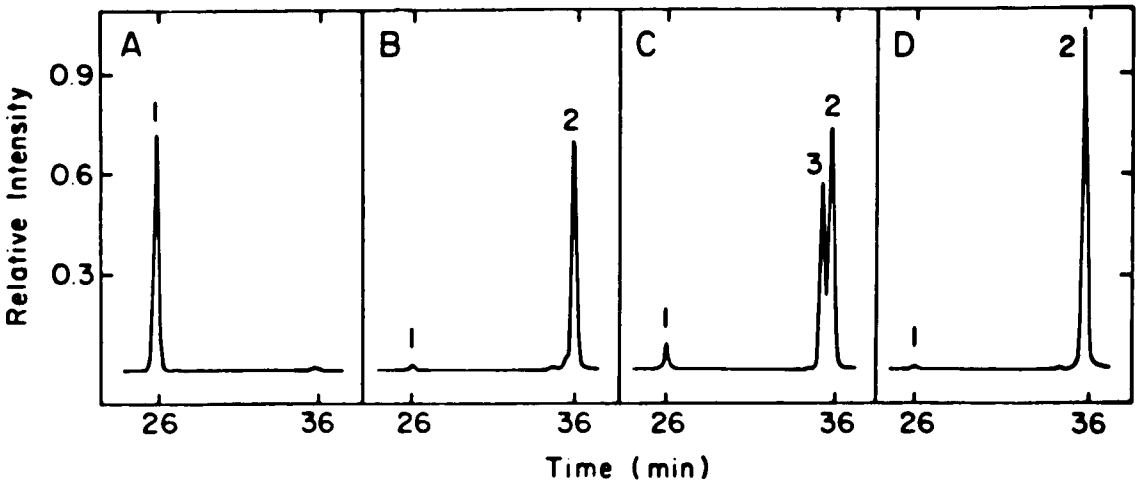

Figure 15. HPLC Analysis of the Product Formed by Dehydration of D-3-Hydroxy-4-*trans*-Decenoyl-CoA Catalyzed by FAOC from *E. coli*. A, D-3-Hydroxy-4-*trans*-decenoyl-CoA. B, Dehydration product of D-3-hydroxy-4-*trans*-decenoyl-CoA formed by FAOC. C, Same as B plus 2-*cis*,4-*trans*-decadienoyl-CoA. D, Same as B plus 2-*trans*,4-*trans*-decadienoyl-CoA. Peaks identified with authentic samples are (1) D-3-hydroxy-4-*trans*-decenoyl-CoA, (2) 2-*trans*,4-*trans*-decadienoyl-CoA, (3) 2-*cis*,4-*trans*-decadienoyl-CoA.

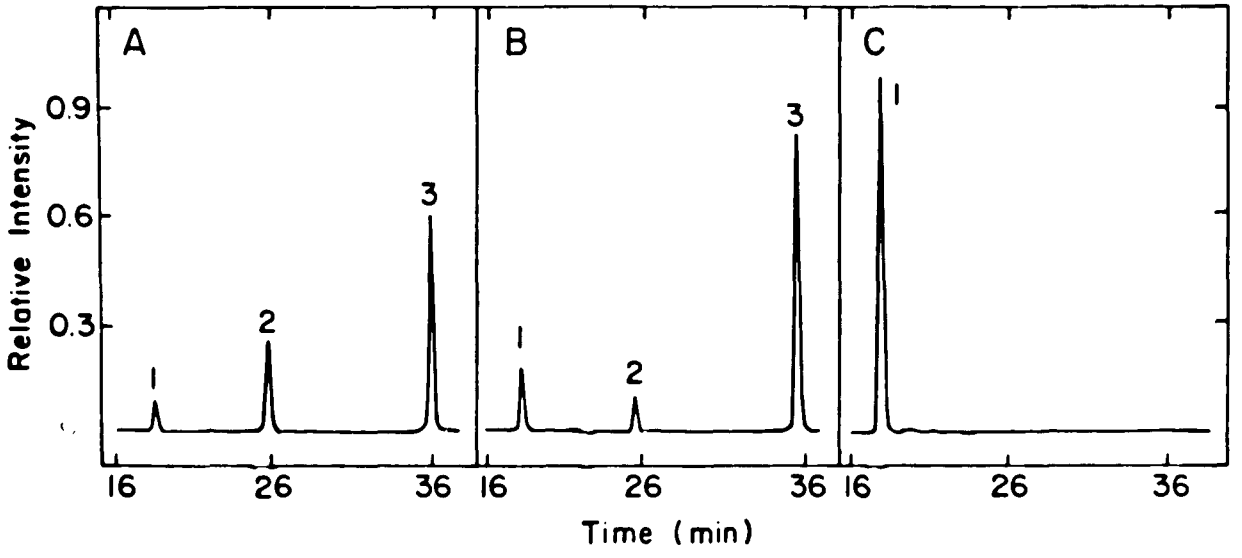

Figure 16. HPLC Analysis of Metabolites Formed During the β-Oxidation of D-3-Hydroxy-4-*trans*-Decenoyl-CoA in the Presence of *E. coli* FAOC, NAD^+, CoASH, Pig Heart L-3-Hydroxyacyl-CoA Dehydrogenase, and Pig Heart 3-Ketoacyl-CoA Thiolase. A, After 1 min; B, After 6 min; C, After 20 min. Peaks identified with authentic samples are (1) Hexanoyl-CoA. (2) D-3-Hydroxy-4-*trans*-decenoyl-CoA. (3) 2-*trans*,4-*trans*-Decadienoyl-CoA.

Table III

Rates of reactions catalyzed by the multienzyme complex of fatty acid oxidation. Rates of dehydration of L-3-hydroxy-4-*trans*-decenoyl-CoA (L-3-OH-4-*trans*) and D-3-hydroxy-4-*trans*-decenoyl-CoA (D-3-OH-4-*trans*), rates of epimerization of D-3-hydroxy-4-*trans*-decenoyl-CoA, rates of hydration of 2-*trans*,4-*trans*-decadienoyl-CoA (2-*trans*,4-*trans*), and rates of dehydrogenation of L-3-hydroxy-4-*trans*-decenoyl-CoA.

Conc. (μM)	Epimerization of D-3-OH-4-*trans* (U/mg)	Dehydration of D-3-OH-4-*trans* (U/mg)	Hydration of 2-*trans*,4-*trans* (U/mg)	Dehydration of L-3-OH-4-*trans* (U/mg)	Dehydrogenation of L-3-OH-4-*trans* (U/mg)
10	0.61 ± .01	13 ± 0.4	0.61 ± .01	133 ± 7	16 ± 1
30	0.68 ± .03	28 ± 1	0.76 ± .05	187 ± 4	78 ± 7
50	0.64 ± .01	35 ± 0.3	0.83 ± .02	214 ± 6	95 ± 4
70	0.67 ± .02	40 ± 1	0.87 ± .05	223 ± 3	96 ± 6

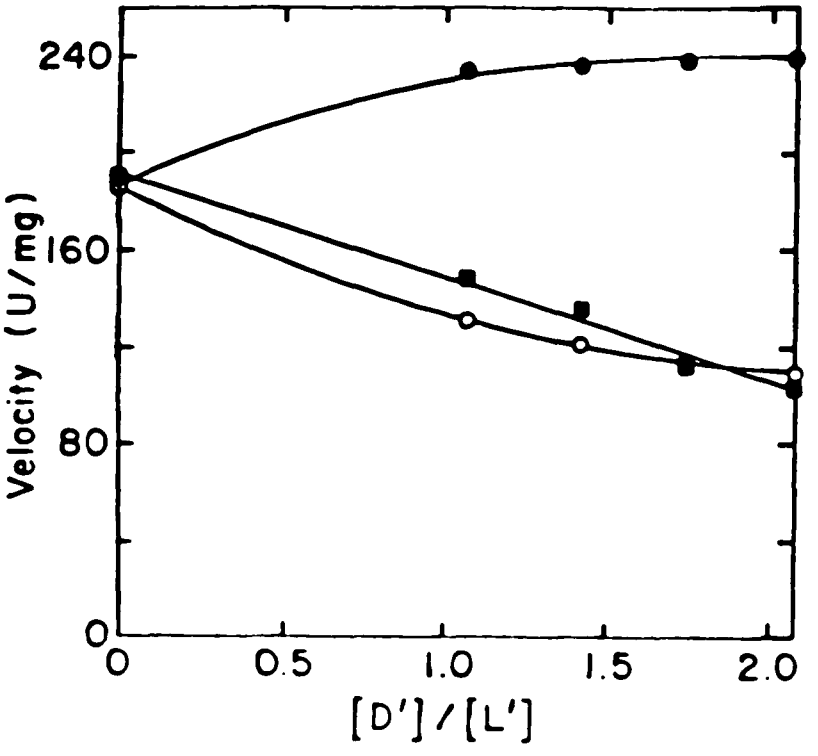

Figure 17. Comparison of Predicted and Observed Dehydration Rates of L-3-Hydroxy-4-*trans*-Decenoyl-CoA as a Function of the Concentration of D-3-Hydroxy-4-*trans*-Decenoyl-CoA. Closed circles: additive rates predicted by the Michaelis-Menten equation assuming separate and independent dehydration activities. Open circles: rates predicted by the initial velocity equation assuming competitive inhibition at a single active site by alternative substrates. Closed squares: observed rates of conversion of D- and L-3-hydroxy-4-*trans*-decenoyl-CoAs to 2-*trans*,4-*trans*-decadienoyl-CoA.

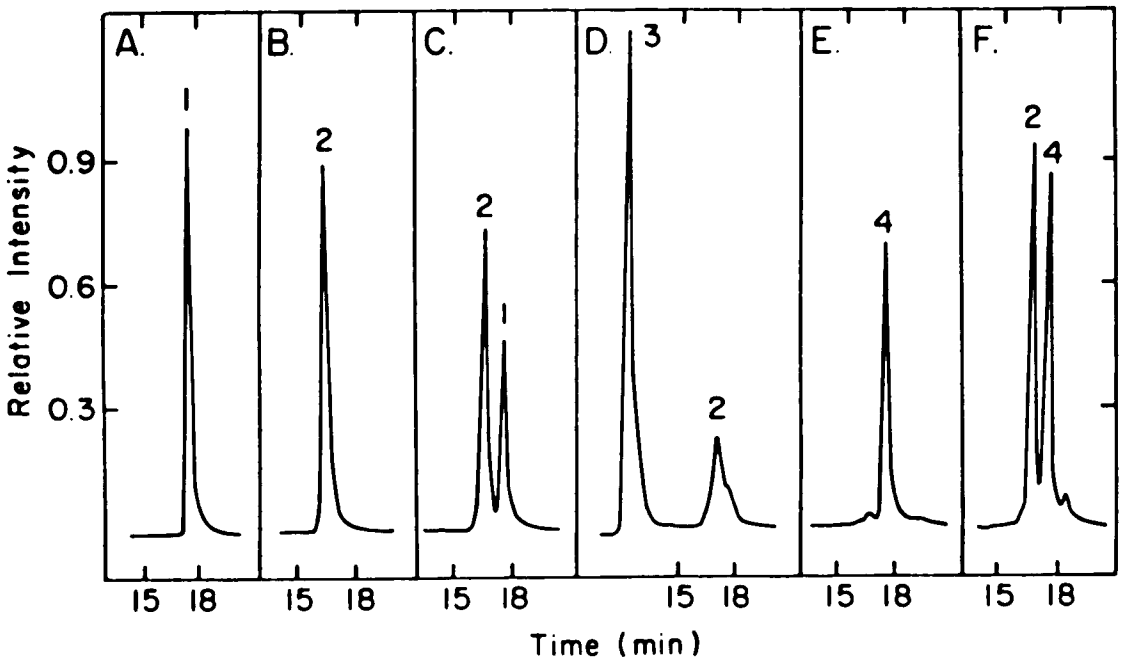

Figure 18. HPLC Analysis of Metabolites Formed by Enzymes of β-Oxidation from 5-*cis*-Octenoyl-CoA. A. 5-*cis*-Octenoyl-CoA (peak 1). B. 2-*trans*,5-*cis*-Octadienoyl-CoA (peak 2) formed from *cis*-5-octenoyl-CoA by acyl-CoA oxidase. C. 5-*cis*-Octenoyl-CoA (peak 1) and 2-*trans*,5-*cis*-octadienoyl-CoA (peak 2). D. Hydration of 2-*trans*,5-*cis*-octadienoyl-CoA (peak 2) by crotonase to 3-hydroxy-5-*cis*-octenoyl-CoA (peak 3). E. Isomerization of 2-*trans*,5-*cis*-octadienoyl-CoA to 2-*trans*,4-*trans*-octadienoyl-CoA (peak 4) by a soluble extract of rat liver mitochondria. F. 2-*trans*,5-*cis*-Octadienoyl-CoA (peak 2) and 2-*trans*,4-*trans*-octadienoyl-CoA (peak 4).

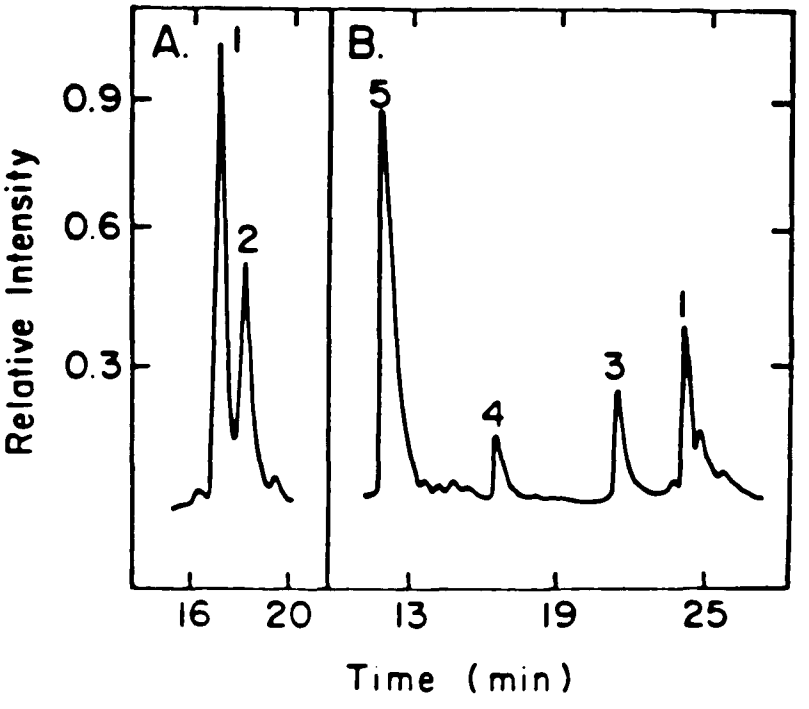

Figure 19. HPLC Analysis of Metabolites Formed by β-Oxidation from 2-*trans*,5-*cis*-Octadienoyl-CoA. 2-*trans*,5-*cis*-Octadienoyl-CoA was first converted to 2-*trans*,4-*trans*-octadienoyl-CoA by a soluble extract of rat liver mitochondria and (A) after removal of enzymes reduced by NADPH in the presence of partially purified 2,4-dienoyl-CoA reductase and (B) incubated for 5 min after the addition of NADPH, NAD^+, and CoA. For experimental details see *Experimental Procedures* Peaks identified by authentic materials: (1) 2-*trans*,4-*trans*-octadienoyl-CoA, (2) 3-*trans*-octenoyl-CoA, (3) n-hexanoyl-CoA, (4) n-butyryl-CoA, (5) acetyl-CoA.

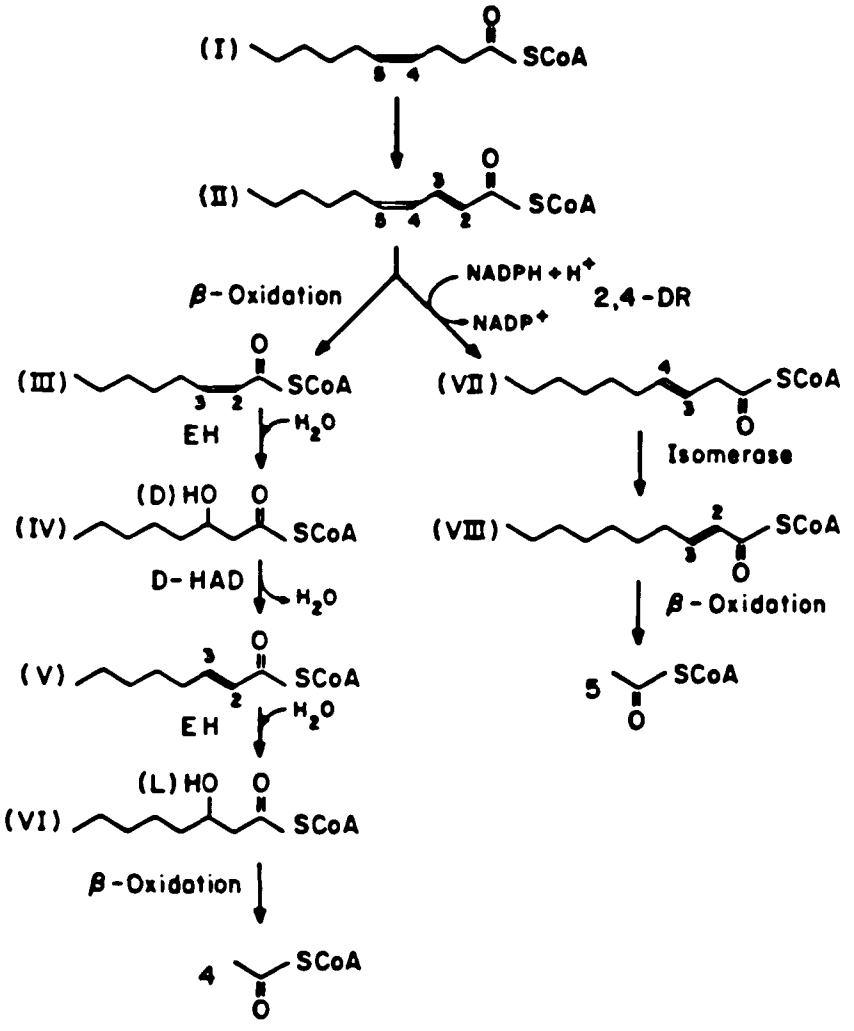

Figure 20. β-Oxidation of 4-*cis*-Decenoyl-CoA. 2,4-DR, 2,4-dienoyl-CoA reductase; EH, enoyl-CoA hydratase; D-HAD, D-3-hydroxyacyl-CoA dehydratase; isomerase, Δ^3,Δ^2-*trans*-enoyl-CoA isomerase.

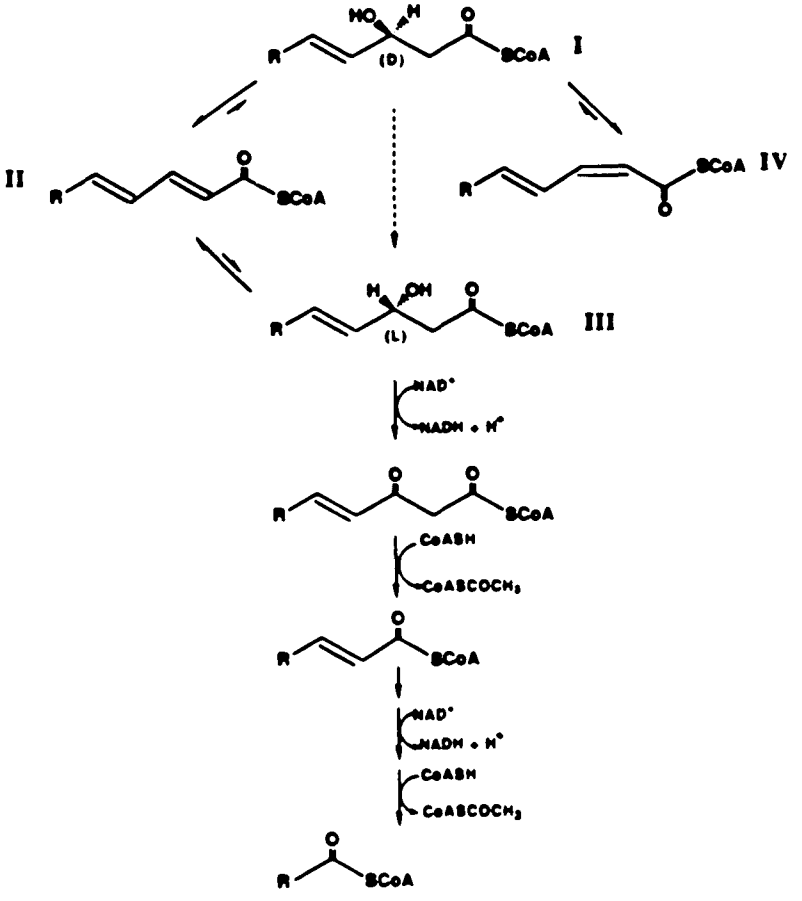

Figure 21. Metabolism of D-3-Hydroxy-4-*trans*-Decenoyl-CoA. D-3-hydroxy-4-*trans*-decenoyl-CoA (I) is not directly epimerized by FAOC from *E. coli* (dashed arrow). Instead, it is converted to 2-*trans*,4-*trans*-decadienoyl-CoA (II), which is slowly rehydrated to L-3-hydroxy-4-*trans*-decenoyl-CoA (III). The latter compound is degraded by FAOC to hexanoyl-CoA and two moles each of NADH and acetyl-CoA. In contrast, mammalian enoyl-CoA hydratase converts D-3-hydroxy-4-*trans*-decenoyl-CoA (I) to 2-*cis*,4-*trans*-decenoyl-CoA (IV). R represents CH_3-$(CH_2)_4$.

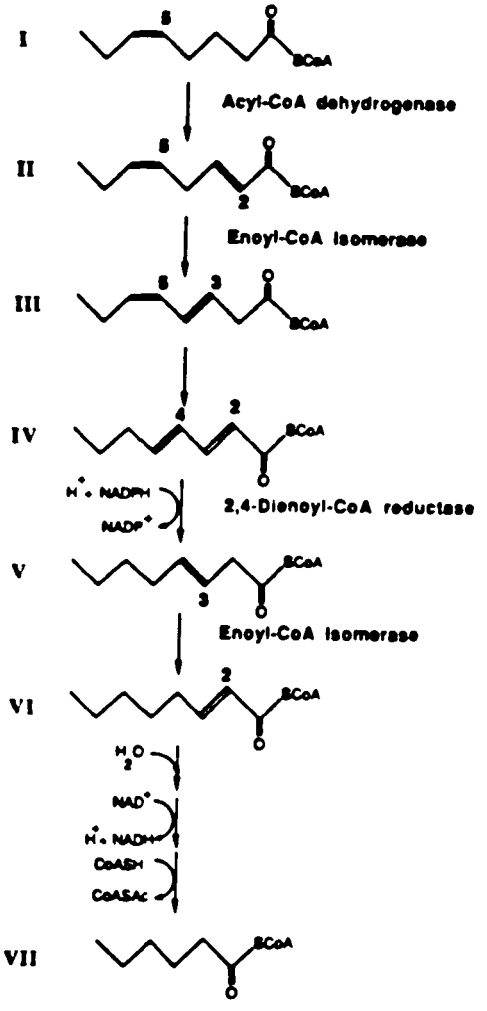

Figure 22. Proposed Pathway of the NADPH-Dependent β-Oxidation of 5-*cis*-Octenoyl-CoA. Enoyl-CoA isomerase is Δ^3,Δ^2-enoyl-CoA isomerase. The metabolites shown are: I, 5-*cis*-octenoyl-CoA; II, 2-*trans*,5-*cis*-octadienoyl-CoA; III, 3-*trans*,5-*cis*-octadienoyl-CoA; IV, 2-*trans*,4-*trans*-octadienoyl-CoA; V, 3-*trans*-octenoyl-CoA; VI, 2-*trans*-octenoyl-CoA; VII, n-hexanoyl-CoA.

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