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Delta 3,5, Delta 2,4-Dienoyl-CoA Isomerase in the Beta Oxidation of Unsaturated Fatty Acids: Characterization and Metabolic Significance

By

Khaled Shoukry

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

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

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Chair of Examining Committee

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Supervisory Committee

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Abstract

Delta 3,5, Delta 2,4-Dienoyl-CoA Isomerase in the Beta Oxidation of Unsaturated Fatty Acids: Characterization and Metabolic Significance

By

Khaled Shoukry

Adviser: Professor Horst Schulz

The degradation of unsaturated fatty acids by β-oxidation involves three auxiliary enzymes in addition to the enzymes required for the breakdown of saturated fatty acids. These auxiliary enzymes, 2,4-dienoyl-CoA reductase and Δ^3,Δ^2-dienoyl-CoA isomerase and $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase (dienoyl-CoA isomerase), are necessary for the metabolism of preexisting double bonds.

The recently detected mitochondrial dienoyl-CoA isomerase was purified 370-fold from rat liver at almost 30% yield by a six-step purification procedure. The molecular weights of the native enzyme and its subunit were estimated to be 126,000 and 32,000, respectively. The purification of dienoyl-CoA isomerase completes the characterization of the enzymes functioning in the reductase-dependent pathway for the β-oxidation of unsaturated fatty acids with odd-numbered double bonds. This novel pathway is operative in peroxisomes as

indicated by the subcellular localization of dienoyl-CoA isomerase in mitochondria and peroxisomes. The peroxisomal and the mitochondrial forms of the isomerase exhibit similar substrate specificities.

The metabolic significance of the reductase-dependent pathway was assessed with 2-trans-5-cis-octadienoyl-CoA (2,5-octadienoyl-CoA) and its products, all of which are metabolites of α-linolenic acid. A kinetic evaluation of β-oxidation enzymes revealed that the presence of a 5-*cis* double bond in the substrate most adversely affected the activity of 3-ketoacyl-CoA thiolase although not enough to become rate-limiting.

Concentration-dependent and time-dependent measurements indicated that most (80%) of 2,5-octadienoyl-CoA is metabolized via the isomerase-dependent pathway. The reason for the greater flux through the isomerase-dependent pathway is the higher activity of L-3-hydroxyacyl-CoA dehydrogenase as compared with Δ^3,Δ^2-enoyl-CoA isomerase. These two enzymes catalyze the rate-limiting steps in the isomerase-dependent and reductase-dependent pathways, respectively. Once 2,5-octadienoyl-CoA is converted to 3,5-octadienoyl-CoA (perhaps fortuitously because of the presence of Δ^3,Δ^2-enoyl-CoA isomerase), the only effective route for its degradation is via the reductase-dependent pathway. It is concluded that the reductase-dependent pathway assures the degradation of 3,5-dienoyl-CoA intermediates, thereby preventing the depletion of free coenzyme A and a likely impairment of mitochondrial oxidative function.

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Introduction

The interest in understanding fatty acid oxidation goes back to the first decade of our ending century when the term "β-oxidation" was first established by Franz Knoop. At that time, radioisotopes were not yet used to trace metabolic pathways and therefore Franz Knoop used chemical labels to trace fatty acid metabolism. In his pioneering experiment, in 1904, Knoop fed dogs fatty acids labeled at their ω (last)-carbon atom with a benzene ring. He isolated hippuric acid from urine of dogs fed odd-chain fatty acids, and phenylaceturic acid from those fed even-chain fatty acids.

These results led Knoop to postulate that the oxidation of the β-carbon atom is involved in fatty acid oxidation. Later on, he proposed, for the first time, a mechanism of fatty acid breakdown known as β-oxidation.

Almost 5 decades later, with the advent of modern biochemical techniques involving enzyme purification and the use of radioactive tracers, interest and progress in studying fatty acid oxidation resumed. The discovery of coenzymeA, carnitine and the isolation and characterization of fatty acid oxidation enzymes confirmed Knoop's hypothesis and founded a new era of research in fatty acid β-oxidation.

Undoubtedly, a major breakthrough that triggered more studies of fatty acid oxidation was the identification of the peroxisomal β-oxidation system in mammals (1). The recognition of several inherited human disorders due to deficiencies of β-oxidation enzymes (2,3) and studies of the regulation of hepatic β-oxidation (4) greatly added to the importance of such metabolic process.

Recently, with the beginning of the last decade of this century, a reassessment of the mitochondrial β-oxidation pathway for unsaturated fatty acids led to the identification of a novel enzyme involved in this pathway.

The characterization of this novel enzyme, $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase, opened a new chapter in the study of unsaturated fatty acids β-oxidation in mammals. This thesis dissertation presents novel findings in the area of unsaturated fatty acid β-oxidation.

Uptake and activation of fatty acids in animal cells:

Cellular uptake of fatty acids:

Fatty acids are present in the blood circulation as unesterified (free) fatty acids complexed to serum albumin or in the form of triacylglycerols associated with lipoproteins. Since cells take up only free fatty acids, the release of fatty acids from the triglycerides should occur outside the cells. Lipoprotein lipase hydrolyzes triacylglycerols outside the cells to form free fatty acids.

Several studies performed with isolated cells from heart, liver and adipose tissue did not yield a definite mechanism for the passage of fatty acid through the plasma membrane into cells. Until the present time, the mechanism of fatty acid entry into cells, even though extensively studied, remains to be elucidated. However, kinetic studies provided evidence for a saturable and a non-saturable uptake of fatty acids. The saturable uptake is assumed to occur by a carrier-mediated mechanism operating at nanomolar concentrations of free fatty acids. By contrast, the non-saturable uptake operates at higher concentrations of free fatty acids through their non-specific diffusion across the membrane.

Evidence for the involvement of transport proteins, identified by labeling with fatty acid analogs (7), has not produced a definite answer. However, a recent expression cloning study resulted in the identification of a 63 kDa adipocyte fatty acid transport protein which was found to promote, along with long-chain acyl-CoA synthetase, the cellular uptake of fatty acids (8).

Fatty acid binding proteins:

A class of cytosolic low molecular weight proteins named fatty acids binding proteins (FABP) has been identified (9,10). These proteins are believed to have an important function in fatty acid metabolism because they bind fatty acids and are most abundant in tissues that have high capacities for either fatty acid oxidation (heart, liver, skeletal muscle) or lipid metabolism (adipose tissue, intestine and liver). However, their specific function in fatty acid oxidation has not been demonstrated. Since these proteins are cytosolic, it is thought that they may facilitate the transfer of fatty acids from the cell membrane to intracellular sites of fatty acid metabolism (11). Moreover, the gradient-like distribution of FABP in heart cells and its tendency to self-aggregate was proposed to affect fatty acid oxidation by influencing the binding of acylcarnitines and their delivery to mitochondria for β-oxidation (12,13).

FABPs may also participate in the cellular uptake of fatty acids even though no experimental evidence for such function has been obtained. The primary structure of FABPs from several animals determined by protein or cDNA sequencing permitted their classification as distinct, but homologous, 14 - 15 kDa proteins. These groups are the heart FABP, adipocyte FABP, Myelin P2, liver FABP, intestinal FABP and cutaneous FABP (14). Structural studies of the intestine

FABP revealed it has a clam shell-like structure formed by 10 anti-parallel β-strands. The carboxyl group of the fatty acid molecule is believed to bind to the interior of the protein via a network of hydrogen-bonding in a ratio of 1 : 1 (14). In contrast to other FABPs, liver FABP has two binding sites for fatty acids.

Activation of fatty acids:

The term "fatty acid activation" implies the ligation of the fatty acid molecule with coenzyme A (CoA) to yield fatty acyl-CoA thioester, the substrate of the fatty acid for β-oxidation. This very first reaction of fatty acid degradation is catalyzed by a group of ATP-dependent enzymes named fatty acid : CoA ligases or acyl-CoA synthetases.

Acyl-CoA synthetases are classified based on their subcellular locations and their specificities for fatty acids of different chain lengths (15,16). They are classified as short chain, medium chain, long chain and very long chain acyl-CoA synthetases.

The short chain synthetase, such as acetyl-CoA synthetase and propionyl-CoA synthetase, are soluble enzymes located in the mitochondria matrix of most tissues. They act on acetate and propionate, respectively, but not on longer chain fatty acids. Medium chain acyl-CoA synthetase is also a soluble enzyme located in the mitochondrial matrix. The bovine liver medium chain acyl-CoA synthetase activates fatty acids having acyl chain with 4-12 carbon atoms. It also acts on branched-chain, unsaturated, hydroxy-substituted and even aromatic carboxylic acids. In contrast, the bovine heart enzyme acts on monocarboxylic acids with 3-7 carbon atoms, and its referred to as butyryl-CoA synthetase since it is most active towards butyrate. Long chain acyl-CoA synthetase is a membrane-bound enzyme

that is associated with the outer mitochondrial membrane, the membrane of the endoplasmic reticulum and peroxisomes. It acts on saturated and unsaturated fatty acids with 10-20 carbon atoms.

The mechanism of acyl-CoA formation studied with acetyl-CoA synthetase (17) revealed a two-step mechanism with acyl-AMP as an enzyme-bound intermediate as shown in the equation below:

$$E + R\text{-}COOH + ATP \longrightarrow Mg^{+}(E\cdot R\text{-}CO\text{-}AMP) + PPi$$

$$(E\cdot R\text{-}CO\text{-}AMP) + CoASH \longrightarrow R\text{-}CO\text{-}SCoA + AMP + E$$

However, a kinetic study of rat liver long chain acyl-CoA synthetase provided tentative evidence for a mechanism without an acyl adenylate as intermediate (18). Hence, the formation of acyl-AMPs intermediate remains to be demonstrated. It's believed that the active site or an essential domain of the long chain synthetase is exposed to the cytosolic compartment. This conclusion was derived from the results of topological studies for the mitochondrial (19), peroxisomal (20-22) and microsomal (23) enzymes performed by use of proteolytic inactivation.

Carnitine-dependent uptake of fatty acids by mitochondria.

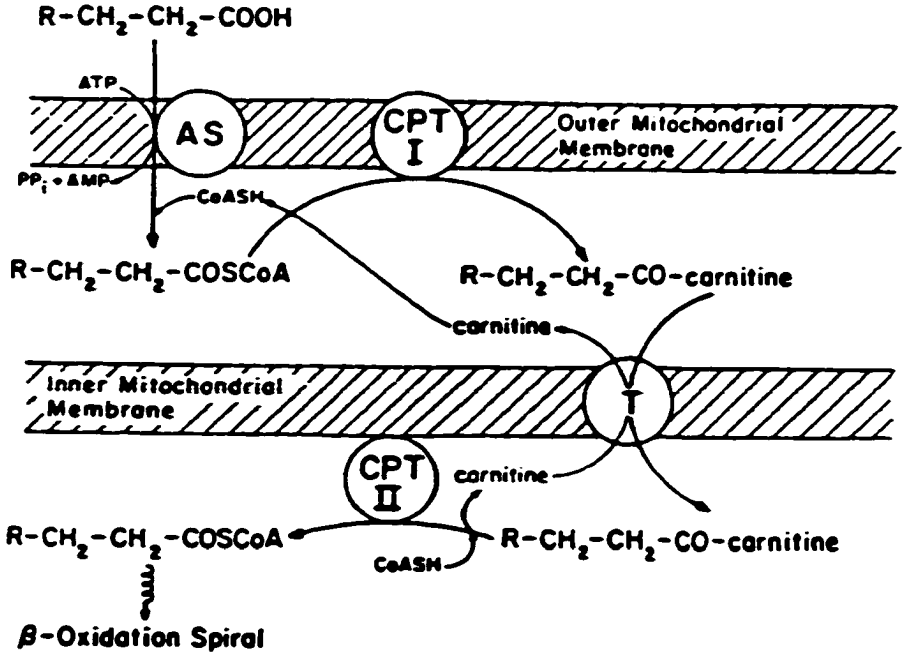

Fig. 1. Carnitine-dependent transfer of acyl groups across the inner mitochondrial membrane (7). Abbreviations: AS, acyl-CoA synthetase; CPTI and CPTII , carnitine palmitoyltransferase I and II, respectively; T. carnitine:acylcarnitine translocase.

The function of carnitine in the mitochondrial uptake of fatty acids was first observed when carnitine was shown to stimulate the β-oxidation of long chain fatty acids in mitochondria (24,25). The fatty acyl-residues are transferred from CoA to carnitine by carnitine palmitoyltransferase I (CPTI) to yield acyl-carnitine at the outer face of the inner mitochondrial membrane (26). After passing through the inner mitochondrial membrane, acylcarnitines are acted upon by CPTII which catalyzes the transfer of the acyl group from carnitine to CoA to form acyl-CoAs that are the substrates for β-oxidation.

Carnitine:acylcarnitine translocase (27,28), also named acylcarnitine: carnitine exchange protein (29,30), serves in the translocation of acylcarnitines into the mitochondrial matrix in exchange for free carnitine or acylcarnitines that exit from mitochondria. This translocase was identified in liver and heart mitochondria. It catalyzes the 1 : 1 exchange of L-carnitine or acylcarnitine for L-carnitine and shows unidirectional flux of carnitine.

The mechanism of acyl-CoA translocation across the inner mitochondrial membrane, as depicted in Fig.1, requires the action of two carnitine palmitoyltransferases (CPTI) and (CPTII) that are located to the outside and inside of the inner mitochondrial membrane, respectively. The specific inactivation of CPTI by certain inhibitors of fatty acid oxidation such as malonyl-CoA serves as a means of distinguishing between CPTI and CPTII (31,32,33). Carnitine acyltransferases were also identified and characterized in peroxisomes and microsomes. Both the peroxisomal and microsomal carnitine acyltransferases are active with medium chain and long-chain substrates (34), and are inhibited by malonyl-CoA (35,36).

Enzymes of the β-oxidation spiral:

The β-oxidation spiral is a sequence of 4 major reactions by which the fatty acyl chain is shortened by 2 carbon atoms that are released in the form of acetyl-CoA. All enzymes involved in the β-oxidation are located at the inner mitochondrial membrane or in the mitochondrial matrix. A schematic presentation of the reactions catalyzed by these enzymes is shown in Fig. 2 which also provides a hypothetical view of the physical and functional organization of these enzymes.

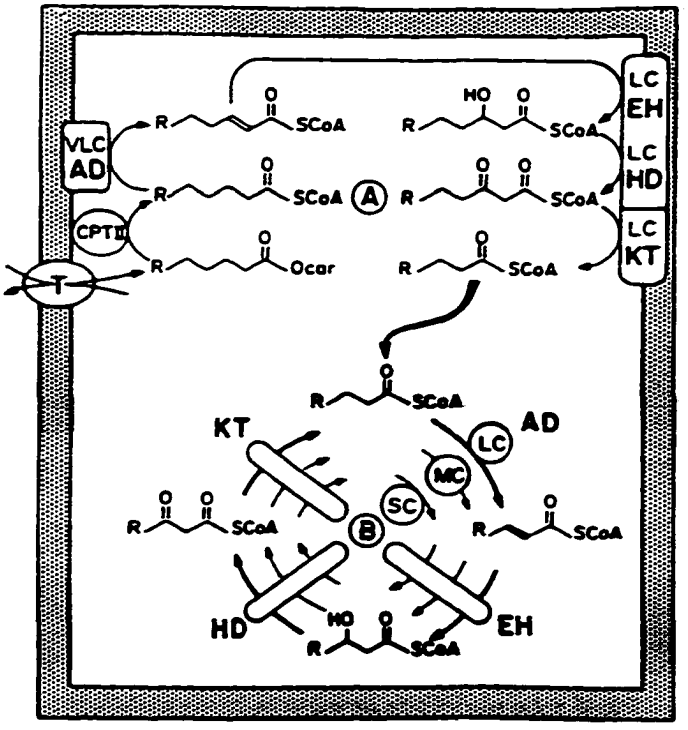

Fig. 2. Model of the functional and physiological organization of β-oxidation enzymes in mitochondria (7). (A) β-Oxidation system active with long-chain (LC) acyl-CoAs. (B) β-Oxidation system active with medium-chain (MC) and short-chain (SC) acyl-CoAs. Abbreviations: T, carnitine:acylcarnitine translocase; CPTII, carnitine palmitoyltransferase II; AD, acyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; HD, L-3-hydroxyacyl-CoA dehydrogenase; KT, 3-ketoacyl-CoA thiolase; VLC, very long-chain; car, carnitine.

Acyl-CoA dehydrogenases:

The first reaction of the β-oxidation spiral is the dehydrogenation of the fatty acyl-CoA to 2-*trans*-enoyl-CoA catalyzed by a group of acyl-CoA dehydrogenases which differ in their chain length specificities.

$$R\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}SCoA + FAD \longrightarrow R\text{-}CH{=}CH\text{-}CO\text{-}SCoA + FADH_2$$

Short chain, medium chain, and long-chain acyl-CoA dehydrogenases have been separated, purified and characterized from rat liver (37,38), bovine liver (39,40) and bovine heart (39). The short-chain dehydrogenase acts on butyryl-CoA and hexanoyl-CoA. Medium-chain dehydrogenase is highly active towards substrates from hexanoyl-CoA to dodecanoyl-CoA, whereas long-chain dehydrogenase acts on octanoyl-CoA and longer chain substrates.

The molecular weight of all dehydrogenases are between 170,000 and 190,000. They are composed of four identical subunits each of which carries a noncovalently-bound flavin adenine dinucleotide (FAD). A comparison of the same dehydrogenases from human and rat showed a high degree of homology (~ 90%). A significant homology (30-40%) has been observed for different dehydrogenases from the same animal. The three dimensional structure of the medium chain acyl-CoA dehydrogenase from pig liver (41) confirms that the enzyme is a homotetramer with one FAD bound per subunit in an extended conformation.

Another FAD-linked enzyme named electron-transferring flavoprotein (ETF) serves to reoxidize acyl-CoA dehydrogenases. One electron at a time is transferred from the reduced dehydrogenase to electron transferring flavoprotein (ETF) (42) which is reoxidized by an iron-sulfur flavoprotein of the inner mitochondrial membrane named ETF:ubiquinone oxidoreductase (43). This enzyme plays a central role in the flow of electrons from various mitochondrial dehydrogenases, including acyl-CoA dehydrogenases, via ETF into the mitochondrial respiratory chain (44). ETF:ubiquinone oxidoreductase was shown to be an iron-sulfur flavoprotein with one FAD and one [4Fe-4S] cluster per subunit.

Enoyl-CoA Hydratases:

The hydration of 2-trans-enoyl-CoA in the second step of the β-oxidation spiral is catalyzed by soluble matrix enzyme named enoyl-CoA hydratase. The products of this reaction are L-3-hydroxyacyl-CoAs.

$$R\text{-}CH{=}CH\text{-}CO\text{-}SCoA + H_2O \longrightarrow R\text{-}CH(OH)\text{-}CH_2\text{-}CO\text{-}SCoA$$

Enoyl-CoA hydratase was purified from pig heart (45) and rat liver (46). The enzyme is composed of 6 identical subunits with a total molecular weight of approximately 160,000. It acts on all substrates from crotonyl-CoA to 2-hexadecenoyl-CoA, but with a decreasing activity as the substrate chain length increases. Kinetic studies performed with the pig heart enzyme showed that the enzyme's Vmax with hexadecanoyl-CoA is only 1-2% of its Vmax with crotonyl-CoA (45). However, the Km values for all substrates are between 10 μM to 30 μM.

A second enoyl-CoA hydratase, named long-chain enoyl-CoA hydratase, was purified from pig heart. It exhibited no activity towards crotonyl-CoA and is most active with 2-octenoyl-CoA (45). The existence of this enzyme activity in mitochondria led to the suggestion that it complements crotonase to assure a high rate of hydration over the entire spectrum of enoyl-CoA intermediates.

A trifunctional β-oxidation enzyme complex purified from rat liver (47), human liver (48) and pig heart (49) was shown to contain long-chain specific activities of enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase. The characterization of the trifunctional β-oxidation complex from pig heart revealed that its enoyl-CoA hydratase activity is identical with the known long-chain hydratase. This enzyme complex is bound to the inner

mitochondrial membrane (47,49). It is composed of two types of subunits present in equimolar amounts. The molecular weight of the larger subunit is close to 80,000 whereas the smaller subunit has a molecular weight of 47,000 (47-49).

L-3-Hydroxyacyl-CoA dehydrogenase:

The dehydrogenation of L-3-hydroxyacyl-CoA to 3-ketoacyl-CoA is the third reaction of the β-oxidation process. This reaction is catalyzed by the enzyme L-3-hydroxyacyl-CoA dehydrogenase in the presence of the cofactor NAD^+.

$$R\text{-}CH(OH)\text{-}CH_2\text{-}CO\text{-}SCoA + NAD^+ \longrightarrow R\text{-}CO\text{-}CH_2\text{-}CO\text{-}SCoA + NADH + H^+$$

The enzyme was purified to apparent homogeneity from pig heart (50) and rat liver (51). It is composed of two identical subunits with a native molecular weight of 65,000. The enzyme is relatively specific for NAD^+, since the rate observed with $NADP^+$ is only 1-2% of the rate obtained with NAD^+. It only acts on the L(s)-isomer of 3-hydroxyacyl-CoAs.

Measurements of the dehydrogenation of substrates at pH 8.0 requires coupling to the thiolytic cleavage of the product and proved that the enzyme is more active towards medium-chain or short chain than with long chain substrates. However, Km values of less than 10 μM were determined for short chain, medium-chain and long chain substrates (52). The Vmax values were reported to be similar substrates of various acyl-chain lengths when the assays were performed at a pH close to 10. The purification and characterization of the membrane bound trifunctional β-oxidation complex revealed the presence of a second L-3-hydroxyacyl-CoA dehydrogenase in mammalian mitochondria. This enzyme is

active with medium-chain as well as long-chain, but not with short-chain (C_4) substrates.

Thiolases:

The last step of β-oxidation results in the thiolytic cleavage of 3-ketoacyl-CoA to yield acetyl-CoA and an acyl-CoA that is chain shortened by two carbon atoms.

$$R\text{-}CO\text{-}CH_2\text{-}CO\text{-}SCoA + CoASH \longrightarrow R\text{-}CO\text{-}SCoA + CH_3\text{-}CO\text{-}SCoA$$

Three types of thiolases have been reported to be present in mitochondria. Thiolase I or 3-ketoacyl-CoA thiolase is active with substrates of various chain lengths, whereas thiolase II or acetoacetyl-CoA thiolase acts on acetoacetyl-CoA. In addition, long-chain 3-ketoacyl-CoA thiolase is present as part of the trifunctional β-oxidation complex. This thiolase acts on a broad spectrum of substrates except for acetoacetyl-CoA toward which it is virtually inactive.

3-Ketoacyl-CoA thiolase has been purified from beef liver (53), pig heart (54) and rat liver (55) and acetoacetyl-CoA thiolase was purified from pig heart (56), beef liver (57-59) and rat liver (55,60). Evidence for the in vivo modification of both enzymes by a covalent but reversible binding to coenzyme A has been obtained (61-63) and reported to cause enzyme inactivation. Both enzymes are homotetramers with estimated subunit molecular weight between 40,000 and 46,000 (54,59,64-66).

Kinetic studies performed with 3-ketoacyl-CoA thiolase from pig heart (54), rat liver (66), and beef liver (53,67) indicated that its activity is similar towards

substrates of various chain lengths except for acetoacetyl-CoA which supports a Vmax that is only 25-30% of those observed with longer chain substrates. Apparent Km values for most 3-ketoacyl-CoAs are below 10 μM while Km values for CoASH vary from 2 to 40 μM depending on the chain length of the substrate. With beef liver acetoacetyl-CoA thiolase, the apparent Km values are 10 μM for acetoacetyl-CoA, 25-30 μM for CoASH and 80-90 μM for acetyl-CoA in the physiologically-important condensation reaction.

A characteristic feature of all thiolases is the presence of at least one essential and highly reactive sulfhydryl group at their active sites, at which alkylation causes irreversible inactivation of these enzymes.

β-oxidation of unsaturated fatty acids:

Unsaturated and polyunsaturated fatty acids are also degraded by β-oxidation. However, as the chain shortening of the unsaturated fatty acids proceeds by the β-oxidation system, the double bonds, either odd or even numbered, move closer to the β-carbon of the fatty acyl-CoA residue which is oxidized. Certain auxiliary enzymes, in addition to the enzymes of the β-oxidation spiral, are required to act on such double bonds. The best illustration of the function of these auxiliary enzymes in the breakdown of all unsaturated fatty acids is provided by a summary of the β-oxidation of linoleic acid which contains both an odd-numbered double bond and an even-numbered double bond between carbons 9 and 10 and 12 and 13, respectively (see Fig. 3).

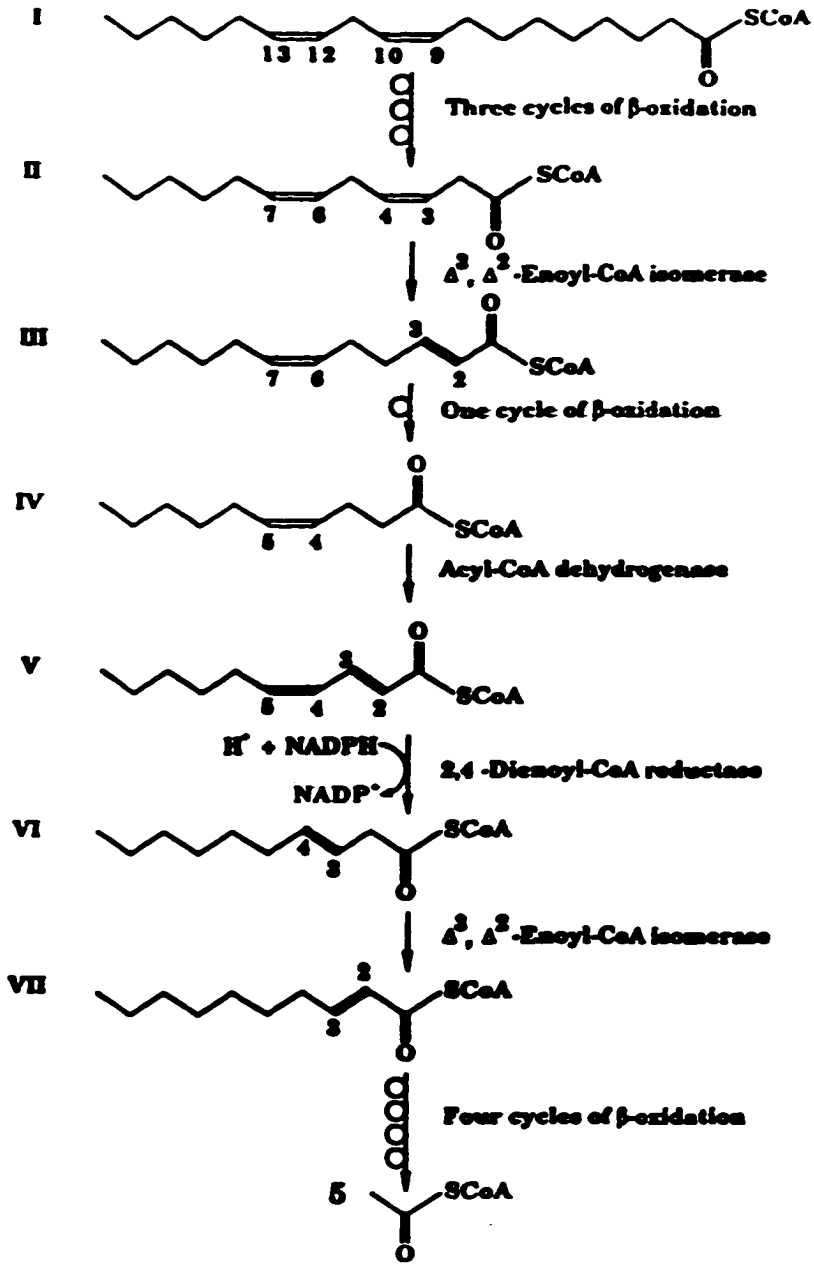

Fig. 3. β-Oxidation of linoleoyl-CoA (7)

As shown in Fig. 3, linoleoyl-CoA undergoes 3 cycles of β-oxidation to yield 3-*cis*, 6-*cis*-dodecadienoyl-CoA (III). Δ^3*cis*,Δ^2*trans*-Enoyl-CoA isomerase, the first auxiliary enzyme of β-oxidation, isomerizes the 3-*cis* double bond to yield 2-*trans*,-6-*cis*-dodecadienoyl-CoA (III), which is a substrate for another β-oxidation cycle that yields 4-*cis*-decenoyl-CoA (IV). 4-*cis*-Decenoyl-CoA is dehydrogenated to 2-*trans*-4-*cis*-decadienoyl-CoA by medium chain-acyl-CoA dehydrogenase. The later acyl-CoA cannot be further degraded by the β-oxidation spiral, but instead is reduced by NADPH in a reaction catalyzed by 2,4-dienoyl-CoA reductase, the second auxiliary enzyme of β-oxidation. The product of this reaction, 3-*trans*-decenoyl-CoA (VI), is isomerized by Δ^3,Δ^2-enoyl-CoA isomerase to 2-*trans*-decenoyl-CoA (VII), which can be completely degraded by 4 cycles of β-oxidation.

Altogether, the degradation of unsaturated fatty acids in mitochondria requires at least Δ^3, Δ^2-enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase as auxiliary enzymes in addition to the enzymes of the β-oxidation spiral.

2,4-Dienoyl-CoA reductase was purified from bovine liver (68), and later proved to be a mitochondrial matrix enzyme (69). The enzyme is a homotetramer with a native molecular weight of 124,000. Both, the 2-*trans*-4-*trans* and 2-*trans*-4-*cis* isomers are effectively reduced by 2,4-dienoyl-CoA reductase to yield 3-*trans*-enoyl-CoAs. The cDNA of the rat liver enzyme was cloned and sequenced and shown to code for a reductase precursor subunit with a molecular weight of 36,000 (70).

Two isomers of the mitochondrial Δ^3, Δ^2-enoyl-CoA isomerase have been reported to exist. A short chain Δ^3, Δ^2-enoyl-CoA isomerase, also referred to as 3-*cis*, 2-*trans*-enoyl-CoA isomerase, was purified from rat liver, (71-73), rat heart(72) and bovine liver (74). This enzyme is a homodimer with a subunit molecular

weight of close to 30,000 (71-74). The activity of this enzyme decreases with increasing chain length of the substrate (72-76), and it is 5-17 times more active with 3-*cis*-enoyl-CoAs than with the corresponding *trans* substrates (74,76). However, similar affinities of *cis* and *trans* substrates of various chain lengths to the enzyme have been observed as indicated by their Km values ranging from 10 to 30 μM (74,76). The second isoform is the long chain Δ^3, Δ^2-enoyl-CoA isomerase. The enzyme was partially purified from rat liver mitochondria but not further characterized. It has a preference for medium-chain substrates (77). Short chain isomerase has been crystallized (78) and its cDNA has been cloned and sequenced (74,79,80).

Site-directed mutagenesis of the cDNA coding for rat mitochondrial Δ^3, Δ^2-enoyl-CoA isomerase prompted the conclusion that Glu-165 is a residue involved in catalysis (81). Moreover, chemical modification of the enzyme suggests the involvement of a histidine residue in the enzyme's isomerization mechanism.

An interesting observation in the metabolism of unsaturated fatty acids with odd-numbered double bonds was the observed requirement for NADPH in the β-oxidation of 5-enoyl-CoAs by mitochondria (82). This observation received major attention since it suggested the existence of a novel NADPH-dependent pathway for the β-oxidation of unsaturated fatty acids with odd-numbered double bonds. This intriguing finding prompted a reassessment of the metabolism of unsaturated fatty acids at the stage of 5-enoyl-CoA intermediates.

The dissertation research described here is based on a metabolic study of the mitochondrial β-oxidation of 5-*cis*-octenoyl-CoA by a soluble extract of rat liver mitochondria and by purified β-oxidation enzymes. That study established the existence of a modified pathway according to which odd-numbered double bonds

in unsaturated enoyl-CoAs are removed by reduction with NADPH (5) (see Fig. 4). The novel, and key reaction, in the modified pathway is the isomerization of the odd-numbered double bonds of Δ^3, Δ^5-dienoyl-CoA to even-numbered positions in Δ^2, Δ^4-dienoyl-CoA. The resultant Δ^2, Δ^4-dienoyl-CoA, as reported earlier, undergoes β-oxidation via a pathway mediated by NADPH-dependent 2,4-dienoyl-CoA reductase.

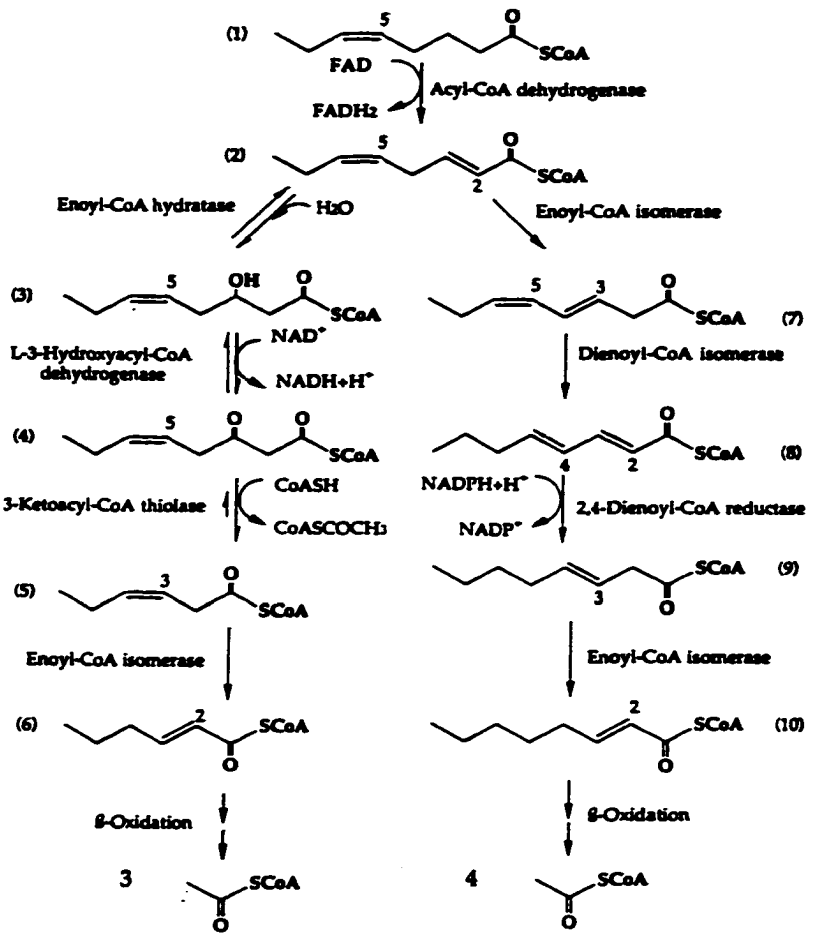

Fig. 4. Proposed pathway of the NADPH-dependent β-oxidation of 5-*cis*-octenoyl-CoA (5).

The key isomerization reaction by which the unsaturated 5-enoyl-CoAs become substrates for the NADPH-dependent pathway of β-oxidation is attributed to the mitochondrial matrix enzyme $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase (dienoyl-CoA isomerase) (5). A significant effort of this research project was dedicated to the (1) purification and characterization of dienoyl-CoA isomerase from rat-liver mitochondria (6) and (2) to the identification of the dienoyl-CoA isomerase in peroxisomes (83).

Dienoyl-CoA isomerase was purified from rat liver by a six-step purification procedure to apparent homogeneity (6). The molecular weight of the native enzyme and its subunit were estimated to be 126,000 and 32,000, respectively. The subcellular distribution of dienoyl-CoA isomerase was reinvestigated when a publication described the reductive removal of the odd-numbered double bond of arachidonic acid via β-oxidation in peroxisomes (84). This investigation resulted in the demonstration that dienoyl-CoA isomerase is present in peroxisomes (83). Moreover, the same study led to the conclusion that the peroxisomal isomerase is homologous to the mitochondrial enzyme as indicated by immunoblotting of the peroxisomal enzyme with the antiserum raised against the rat liver mitochondrial 32-kDa isomerase.

In 1998 it was demonstrated that dienoyl-CoA isomerase is actually the protein encoded by rECH1 (85). rECH1 is a peroxisomal rat liver cDNA that was believed to encode a novel member of the hydratase/isomerase protein superfamily with a type 1 peroxisomal targeting signal (SKL) at its C-terminal (86). This isolated gene was earlier named rat enoyl-CoA hydratase (rECH1), referring to its possible activity (86). In view of this finding the characterization of rECH1 was initiated. In this study the secondary structure prediction of dienoyl-CoA

isomerase was based on modeling the rECH1p structure using the coordinates of hydratase 1, the cloned and well characterized member of hydratase/isomerase superfamily, as a template (85). Moreover, the same study demonstrated the dual subcellular location of rECH1p in rat liver as judged by immunoblotting. 36,000 Da and 32,000 Da proteins were detected in purified peroxisomes and mitochondria, respectively, with anti-rECH1p. rECH1p was shown to contain a mitochondrial N-terminal signal sequence of 53 residues and a type 1 peroxisomal signal (SKL) at its C-terminal (85). The observed molecular mass of dienoyl-CoA isomerase protein (rECH1p) subunit is believed to be reduced from 36 kDa, for the precursor form to 32 kDa due to the cleavage of the mitochondrial signal sequence, on entering into the mitochondria (85).

The modeled subunits of rECH1p showed the presence of two domains, each composed of a trimer. This proposed modeling was confirmed in the same study by the results of the size exclusion chromatography which gave a molecular mass of 170,000 for the native enzyme.

The characterization of $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase at the molecular level took further progress when the crystal structure of the rat enzyme was determined at 1.5 A^0 resolution (87). The structural data confirmed that dienoyl-CoA isomerase is one of the few examples of proteins which are imported into both mitochondria and peroxisomes. It also revealed that the enzyme's active pocket is lined exclusively with hydrophobic residues, with the exception of the acidic residues Asp 176, Glu 196 and Asp 204. Moreover, the structural data supported a suggested mechanism according to which Glu 196 acts as a proton acceptor and Asp 204 acts as a proton donor. Asp 176 appeared to be important in optimizing the catalytic proton transfer properties of Glu 196.

The recognition of the modified pathway for the β-oxidation of unsaturated fatty acids with odd-numbered double bonds raised the question as to the contribution of this pathway to the β-oxidation of fatty acids with odd-numbered double bonds. All possible pathways for the degradation of 5-octenoyl-CoA and its intermediates are shown in Fig. 5. The name "isomerase-dependent pathway" was given to pathway A and "reductase-dependent pathway" was given to pathway B. Pathway C is referred to as a pathway dependent on enoyl-CoA isomerase and dienoyl-CoA isomerase but not on 2,4-dienoyl-CoA reductase.

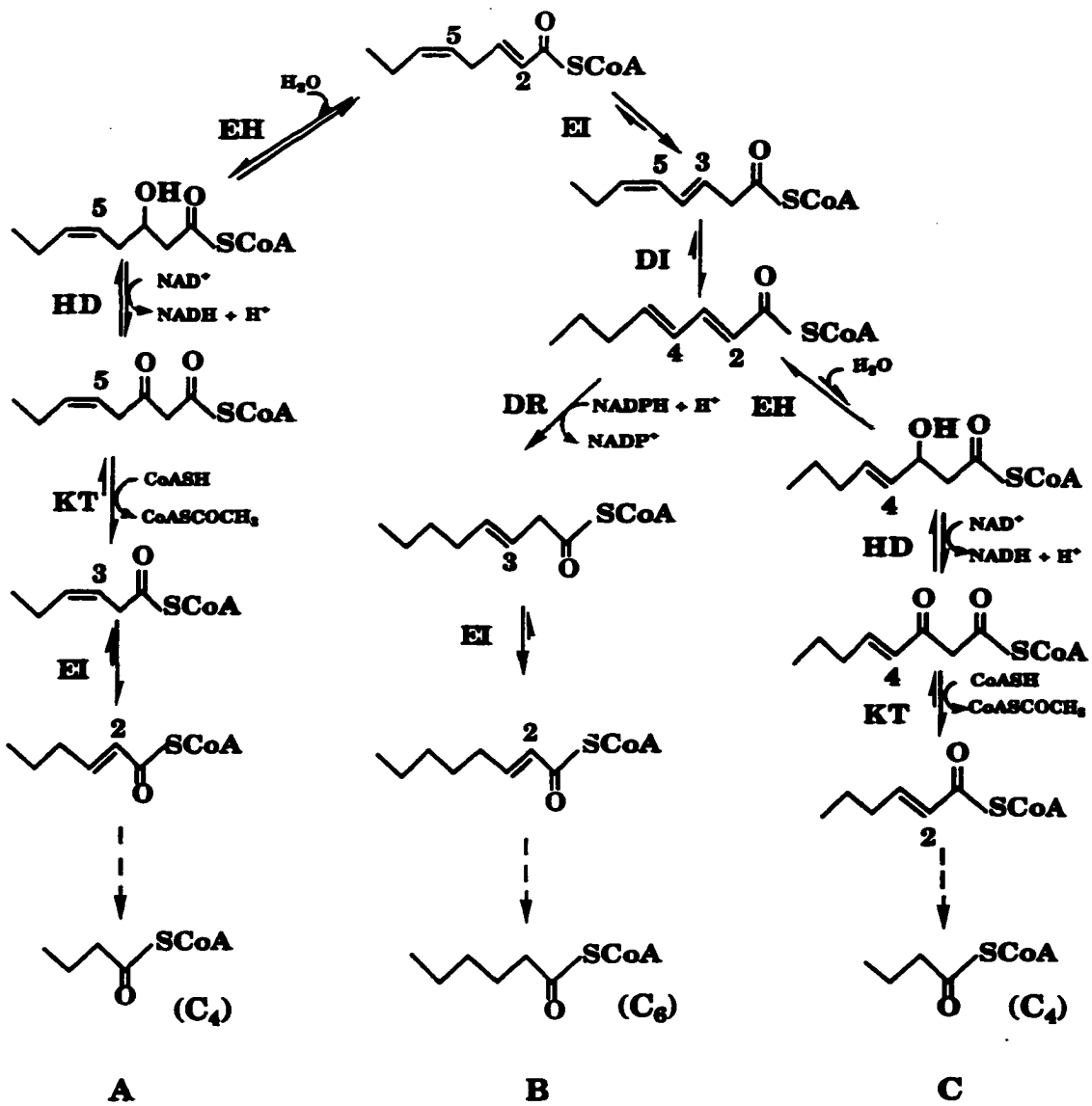

Fig. 5. Mitochondrial metabolism of 2-*trans*-5-*cis*-octadienoyl-CoA. *A*, isomerase-dependent pathway; *B*, reductase-dependent pathway; *C*, pathway dependent on enoyl-CoA isomerase and dienoyl-CoA isomerase but not on 2,4-dienoyl-CoA reductase. *EH*, enoyl-CoA hydratase; *HD*, 3-hydroxyacyl-CoA dehydrogenase; *KT*, 3-ketoacyl-CoA thiolase; *EI*, Δ^3,Δ^2-enoyl-CoA isomerase; *DI*, $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase; *DR*, 2,4-dienoyl-CoA reductase; *C4*, butyryl-CoA; *C6*, hexanoyl-CoA.

The evaluation of the metabolic significance of the reductase dependent pathway (Fig 5, pathway B) in rat liver mitochondria represents the major part of the study described in this dissertation. The study was conducted with 2-*trans*-5-*cis*-octadienoyl-CoA, the metabolite produced during the degradation of linolenic acid. The contribution of the reductase dependent pathway to the overall β-oxidation pathway of 2-*trans*-5-*cis*-octadienoyl-CoA was assessed through time-dependent and concentration-dependent HPLC measurements. The measurements indicated that most (80%) of 2,5-octadienoyl-CoA is metabolized via the isomerase dependent pathway. Kinetic evaluations of the effect of the Δ^5-*cis*-double bond in 5-octenoyl-CoA on the activities of the β-oxidation enzymes provided a significant, if not a complete, explanation for the greater flux through the isomerase-pathway as compared to the reductase pathway.

The other major objective of this study was to investigate whether the reductase-dependent pathway serves a special function in the metabolism of unsaturated fatty acids that cannot be assumed by the isomerase-dependent pathway.

Experimental Procedures

Materials - CoASH, butyryl-CoA, hexanoyl-CoA, arachidonic acid, NAD^+, NADH, NADPH, hexamethylphosphoramide, 2-mercaptoethanol, benzamidine hydrochloride, pepstatin-A, acyl-CoA oxidase from yeast, lactate dehydrogenase and other standard biochemicals were purchased from Sigma. Acyl-CoA oxidase from *Arthrobacter* species was purchased from Boehringer Mannheim. Bovine liver crotonase (85), pig heart L-3-hydroxyacyl-CoA dehydrogenase (50), pig heart 3-ketoacyl-CoA thiolase (54), rat liver ^{3}, ^{2}-enoyl-CoA isomerase (71 - 73), and rat liver ^{3,5}, ^{2,4} -dienoyl-CoA isomerase (6) were purified as described. 2-*trans*-4-*trans*-Octadienal was obtained from Bedoukian Research (Danburry, CT). 2-Octenoic acid, 2-octynoic acid, and 3-octenoic acid were purchased from Aldrich. Sep-Pak C18 cartridges used for concentrating acyl-CoAs were purchased from Waters. The methyl esters of 5-*cis*-octenoic acid and 5-*trans*-octenoic acid were generously provided by Dr. Howard Sprecher at Ohio State University and saponified as described (5).

Synthesis of Substrates - 5-*cis*-Octenoic acid, 5-*trans*-octenoic acid, and 2-*trans*, 4-*trans*-octadienoic acid were prepared from methyl-5-*cis*-octenoate (5), methyl-5-trans-octenoate (5), and 2-*trans*, 4-*trans*-octadienal (86), respectively, by established procedures. 5-*cis*-Octenoyl-CoA, 5-*trans*-octenoyl-CoA, 5-*cis*-tetradecenoyl-CoA, arachidonoyl-CoA, 3-octenoyl-CoA, 2-octynoyl-CoA, 2-*trans*-octenoyl-CoA, and 2-*trans*--4-*trans*-octadienoyl-CoA were synthesized from 5-*cis*-octenoic acid, 5-*trans*-octenoic acid, 5-*cis*-tetradecenoic acid, arachidonic acid, 3-octenoic acid, 2-octynoic acid, 2-*trans*-octenoic acid, and 2-*trans*-4-*trans*-octadienoic acid, respectively, by the

mixed anhydride method as described by Fong and Schulz (45). All acyl-CoAs were further purified by HPLC.

2-*trans*-5-*trans*-Octadienoyl-CoA was prepared by incubating 5-*trans*-octenoyl-CoA (3 µmol) with acyl-CoA oxidase (1U) in 3 ml of 50 mM KP_i (pH 8) at 37°C. Aliquots of 40 µl were withdrawn at various times and diluted to 1 ml with 0.1 M KP_i (pH 8) and analyzed for their absorbance at 263 nm. When the absorbance at 263 nm reached a maximum, which remained unchanged upon addition of more acyl-CoA oxidase, the reaction was judged to be complete. The conversion of 2-*trans*-5-*trans*-octadienoyl-CoA to 3,5-*trans*-octadienoyl-CoA was accomplished by the addition of peroxisomal trifunctional enzyme containing 0.1 U of ^{3,2}-enoyl-CoA isomerase activity. The isomerization reaction was monitored by measuring the absorbance at 238 nm until it reached a maximum when the reaction was judged to be complete. The reaction mixture was acidified with HCl to pH 2. After removal of precipitated protein by filtration through 0.22 µm (pore size) membranes, the pH was adjusted to 3.

For the synthesis of 2-*trans*-5-*cis*-octadienoyl-CoA, 10 µmoles of 5-*cis*-octenoyl-CoA were incubated with 5 units of acyl-CoA oxidase from *Arthrobacter* species in 60 ml of 0.1 M KP_i (pH 9.0) at room temperature. The conversion of 5-*cis*-octenoyl-CoA to 2-*trans*-5-*cis*-octadienoyl-CoA was monitored by analyzing aliquots of 50 µl, withdrawn from the reaction mixture at different time intervals, by HPLC. Under the condition described above, 3-*trans*-5-*cis*-octadienoyl-CoA was observed to be a minor product of the reaction. When 5-*cis*-octenoyl-CoA was completely converted to product, usually after one hour, the reaction was terminated by adjusting the pH to 1.0 with concentrated HCl to avoid further isomerization of 2-*trans*-5-*cis*-octadienoyl-CoA to 3-*trans*-5-*cis*-octadienoyl-CoA.

The protein was removed from the reaction mixture by filtration through a 0.22 μm, pore size, membrane and the pH was readjusted to 8.0 with 4.0 N KOH. To avoid the difficult separation of small amounts of 3,5-*cis*-octadienoyl-CoA from 2,5-*cis*-octadienoyl-CoA by HPLC, 0.5 units of purified dienoyl-CoA isomerase from rat liver were added to the reaction mixture to isomerize 3,5-*cis*-octadienoyl-CoA to 2-*trans*,4-*trans*-octadienoyl-CoA which could be separated more conveniently from 2,5-*cis*-octadienoyl-CoA by HPLC. After adjusting the pH to 1.0 with concentrated HCl, precipitated protein was removed from the reaction mixture by filtering the mixture through a 0.22 μm, pore size, membrane. The reaction mixture was concentrated, after adjusting its pH to 3.0, by passing it through a Sep-Pack mini column, and was eluted with methanol. Methanol was evaporated under a stream of N_2, the resultant acyl-CoA was dissolved in 1 ml H_2O, and the pH was adjusted to 3.0 with concentrated HCl. 2-*trans*-5-*cis*-Octadienoyl-CoA was further purified by HPLC.

3-*trans*-5-*cis*-Octadienoyl-CoA was synthesized by incubating 10 μmoles of 5-*cis*-octenoyl-CoA with 5 units of acyl-CoA oxidase from *Arthrobacter* species in 60 ml of 0.1 M KP_i (pH 8.0) at room temperature. The conversion of 5-*cis*-octenoyl-CoA to 3-*trans*-5-*cis*-octadienoyl-CoA was monitored by analyzing aliquots of 50 μl, taken at different time intervals from the reaction mixture, by HPLC. When 5-*cis*-octenoyl-CoA was completely converted to product the reaction was terminated by adjusting the pH to 1.0 with concentrated HCl and the precipitated protein was removed by filtration through a 0.22 μm, pore size, membrane after readjusting the pH to 5.0. The resultant acyl-CoA was concentrated by use of a Sep-Pack mini column as described above, and 3-*trans*-5-*cis*-octadienoyl-CoA was purified by HPLC.

3,5-*cis*-Tetradecenoyl-CoA and 3,5,8,11,14-eicosapentaenoyl-CoA were synthesized enzymatically from 5-*cis*-tetradecenoyl-CoA and arachidonoyl-CoA, respectively, by the procedure used to prepare 3-*trans*-5-*cis*-octadienoyl-CoA.

The synthesis of 3-hydroxy-5-*cis*-octenoyl-CoA was accomplished by incubating 10 μmoles of 5-*cis*-octenoyl-CoA with 15 units of acyl-CoA oxidase from yeast and 5 units of bovine liver crotonase in 60 ml of 0.1 M KP_i (pH 8.0) at room temperature. The conversion of 5-*cis*-octenoyl-CoA to 3-hydroxy-5-*cis*-octenoyl-CoA was monitored by analyzing aliquots of 50 μl, withdrawn from the reaction mixture at different time intervals, by HPLC. The reaction was terminated by adjusting the pH to 1.0 with concentrated HCl when no further increase in the formation of 3-hydroxy-5-*cis*-octenoyl-CoA was observed upon addition of more enzymes.

For the purpose of synthesizing 3-keto-5-*cis*-octenoyl-CoA, the above reaction was carried out in the presence of 10 units of pig heart L-3-hydroxyacyl-CoA dehydrogenase, 1.0 mM pyruvate, 1.0 mM NAD^+, and 15 units of lactate dehydrogenase. The conversion of 3-hydroxy-5-*cis*-octenoyl-CoA to 3-keto-5-*cis*-octenoyl-CoA was monitored by analyzing aliquots of 50 μl, withdrawn from the reaction mixture at different time intervals, by HPLC. The reaction was allowed to proceed for approximately 5 hours. The reaction was terminated by adjusting the pH to 1.0 with concentrated HCl and precipitated protein was removed by filtration through a 0.22 μm, pore size, membrane. The resultant 3-keto-5-*cis*-octenoyl-CoA was concentrated by use of a Sep-Pack mini column as described above, and purified by HPLC.

3-Ketooctanoyl-CoA was synthesized by incubating 5 μmoles of 2-octynoyl-CoA with 10 units of bovine liver crotonase in 5 ml of 50 mM MES (pH 6.0). The

reaction was allowed to proceed for 45 min. and was terminated by adjusting the pH to 1.0 with concentrated HCl. Precipitated protein was removed by filteration through a 0.22 μm, pore size, membrane and the pH was readjusted to 3.0. The product was purified by HPLC. Concentrations of all acyl-CoA thioesters were measured spectrophotometrically by quantifying CoASH with Ellman's reagent (90) after cleaving the thioesters bond with NH_2OH at pH 7.0 (45).

Preparation of a Soluble Extract of Rat Liver Mitochondria - Mitochondria were isolated as described (91) and stored at -70 °C. Mitochondria were precipitated by centrifugation and resuspended in an equal volume of 0.2M KP_i (pH 8.0) containing 20 mM 2-mercaptoethanol, 0.2% (v/v) hexamethylphosphoramide, 10 mM benzamidine, and pepstatin-A (2μg/ml). The resuspended mitochondria were sonicated 5 times for 5 sec. each under cooling at 4 °C and then were ultracentrifuged at 100,000 x g for 30 min. The supernatant represented the soluble extract of mitochondria.

Enzyme and Protein Assays - Dienoyl-CoA isomerase was assayed spectrophotometrically by measuring the increase in absorbance at 300 nm on a Gilford, Model 250, recording spectrophotometer at 25°C. A typical assay mixture contained 20 μM 3,5-octadienoyl-CoA in 0.2 M KP_i (pH 8) and enzyme to obtain an absorbance change of approximately 0.04 per min. The assay was started by the addition of enzyme. An extinction coefficient of 27,800 $M^{-1}cm^{-1}$ (92) was used to calculate rates. Kinetic parameters (Km, Vmax) were determined by nonlinear curve fitting using the Sigma plot program. Purified enoyl-CoA hydratase (crotonase) from bovine liver or crotonase present in a soluble extract of rat liver

mitochondria was assayed spectrophotometrically at 280 nm. A standard assay mixture contained 0.2 M KP_i, (pH 8.0), 20 μM 2-octenoyl-CoA or 2,5-octadienoyl-CoA and enzyme to give an absorbance change of approximately 0.04/min. A molar extinction coefficient of 5,100 $M^{-1}cm^{-1}$ was used to calculate rates. Purified 3-hydroxyacyl-CoA dehydrogenase from pig heart or 3-hydroxyacyl-CoA dehydrogenase present in a soluble extract from rat liver mitochondria was assayed spectrophotometrically by measuring the formation of NADH at 340 nm. A standard assay mixture contains 0.2 M KP_i, (pH 8.0), 20 μM 2-octenoyl-CoA or 2,5-octadienoyl-CoA, 1 mM NAD^+, 0.3 mM CoASH, crotonase (0.17 unit), 0.1 unit of purified pig heart 3-ketoacyl-CoA thiolase and enzyme to give an absorbance change of approximately 0.04/min. When the mitochondrial extract served as an enzyme source, no purified thiolase was added to the assay mixture. A molar extinction coefficient of 6,220 $M^{-1}cm^{-1}$ was used to calculate rates. Purified 3-ketoacyl-CoA thiolase from pig heart or 3-ketoacyl-CoA thiolase present in a soluble extract from rat liver mitochondria was assayed by measuring the formation of product by HPLC. A standard assay contained 0.2 M KP_i, (pH 8.0), 0.3 mM CoASH, 20 μM 3-ketooctanoyl-CoA or 20 μM 3-keto-5-*cis*-octadienoyl-CoA and enzyme to convert approximately 20% of the substrate to product during the one-minute incubation period. The reaction was terminated by adjusting the pH to 1.0 with concentrated HCl. After removal of precipitated protein by filtration through 0.22 μm, pore size, membranes, the pH was readjusted to 5 and the samples were analyzed by HPLC. The products were quantified by use of standard curves established with either hexanoyl-CoA or an equilibrium mixture of 2-*trans*-hexenoyl-CoA and 3-hydroxy-hexanoyl-CoA. With 3-keto-5-*cis*-octenoyl-CoA as the substrate, 3-*cis*-hexenoyl-CoA was formed when purified thiolase was used.

However, 3-hydroxyhexanoyl-CoA and 2-*trans*-hexenoyl-CoA were the products when the mitochondrial extract served as the enzyme source. The thiolytic product of 3-ketooctanoyl-CoA was hexanoyl-CoA. Enoyl-CoA isomerase was assayed spectrophotometrically with the purified enzyme and 2,5-octadienoyl-CoA as the substrate; the increase in absorbance at 240 nm, due to the formation of 3,5-*cis*-octadienoyl-CoA was recorded. An extinction coefficient of 18,843 $M^{-1}cm^{-1}$ was used to calculate rates. With the same substrate, but the mitochondrial extract as an enzyme source, the increase in absorbance due to the formation of 2,4-octadienoyl-CoA was recorded. Purified dienoyl-CoA isomerase, which was added as a coupling enzyme in some measurements, was found to produce maximally a 20% increase in the rate. An extinction coefficient of 27,000 $M^{-1}.cm^{-1}$ was used to calculate rates. With purified enoyl-CoA isomerase and 3-octenoyl-CoA as the substrate, an increase in absorbance at 263 nm, due to the formation of 2-enoyl-CoA, was recorded. An extinction coefficient of 6,700 $M^{-1}.cm^{-1}$ was used to calculate rates. A standard assay contained 0.2 M KP_i (pH 8.0), 20 µM 2,5-octadienoyl-CoA or 20 µM 3-enoyl-CoA and enzyme to produce an absorbance change of approximately 0.04 /min.

All spectrophotometric assays were performed an a Gilford, model 260, recording spectrophotometer at 25 °C. Kinetic parameters (apparent Vmax and Km) were obtained by non-linear curve fitting using the Sigma plot program. One unit of enzyme activity is defined as the amount of enzyme that converts 1 µmol of substrate to product per minute. Protein concentrations were determined as described by Bradford (93) with bovine serum albumin as the standard.

Characterization of the Purified $\Delta^{3,5}$-$\Delta^{2,4}$-Dienoyl-CoA Isomerase - Enzymatic conversions of 40 nmoles of 2,5-*trans*-octadienoyl-CoA or 30 nmoles of 3,5-*trans*-octadienoyl-CoA were monitored by recording the absorbance spectra of each substrate in 1 ml of 0.1 M KPi (pH 8.0) in the presence of either 0.14 U of dienoyl-CoA hydratase or 0.25 U of dienoyl-CoA isomerase. The enzymatic conversions of 30 nmoles of 3,5-*cis*-octadienoyl-CoA to 2,4-*trans*-octadienoyl-CoA by 0.1 U of purified dienoyl-CoA isomerase was monitored by recording the absorbance spectrum in 1 ml of 0.1 M KPi (pH 8.0).

HPLC Analyses and Purifications - Experimental conditions for the HPLC analyses of metabolites formed by the enzymatic conversions of 2,5-*trans*-octadienoyl-CoA and 3,5-*trans*.-octadienoyl-CoA were the same as given in the spectrophotometric analysis. However, the formed metabolites were identified by HPLC with and without the addition of 2,4-octadienoyl-CoA as an internal standard. Prior to analysis or purification by HPLC, reactions were terminated by adjusting the pH to 1-2 with concentrated HCl. Samples were filtered through 0.22-μm (pore size) membranes after which the pH was adjusted to 5 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 (v/v), content of 10 mM ammonium phosphate elution buffer (pH 5.5) from 10% to 50% in 30 min at a flow rate of 2 ml/min. For the purpose of purifying substrates, the desired fractions were collected and concentrated on a rotary evaporator under reduced pressure. When the kinetics of purified 3-ketoacyl-CoA thiolase with 3-keto-5-octenoyl-CoA as a substrate were studied, the isocratic elution was applied for 7 min. and the 5% to 50% gradient

was developed in 13 min. With 3-Ketooctanoyl-CoA as a substrate, the separation of substrate and product was achieved by linearly increasing the methanol content of 75 mM ammonium phosphate (pH 5.5) elution buffer from 30% to 60% in 30 min. at a flow rate of 2.5 ml/min. When the sample contained only acyl-CoAs, the isocratic part of the elution program was omitted and a linear gradient from 10% to 50% was used to achieve elution within 30 min. at a flow rate of 2 ml/min.

Metabolic Studies - For rate measurements, various amounts of 2,5-octadienoyl-CoA in 0.2 M KPi (pH 8.0) were incubated with a soluble extract of rat liver mitochondria. When the flux through the isomerase-dependent branch was evaluated, 1 mM NAD^+ and 0.3 mM CoASH were added and the formation of NADH was recorded at 360 nm. An extinction coefficient of 4,100 M^{-1}.cm-1 was used to calculate rates (94). When rates of metabolism via the reductase-dependent pathway were determined, either no cofactors or 1 mM NAD^+, 0.3 mM CoASH, 1 mM pyruvate and 1 U of lactate dehydrogenase were present. The absorbance increase at 300 nm was recorded which reflects the formation of 2, 4-octadienoyl-CoA. An extinction coefficient of 27,000 $M^{-1}.cm^{-1}$ was used to calculate rates. The rate of 2,4-octadienoyl-CoA degradation was measured spectrophotometrically by recording the decrease of the absorbance at 300 nm due to the disappearance of the substrate. The assay mixture contained in 0.2 M KP_i (pH 8.0), various amounts of 2,4-octadienoyl-CoA, 1 mM NAD^+, 0.3 mM CoASH, mitochondrial extract and either no or 0.5 mM NADPH. Observed absorbance changes were corrected for changes detected in the absence of cofactors which most likely were due to the hydrolysis of the substrate. An extinction coefficient of 27,000 $M^{-1}.cm^{-1}$ was used to calculate rates. When the time-dependent formation of metabolites from 2,5-

octadienoyl-CoA or 3,5-octadienoyl-CoA was studied, 20 μM of either substrate in 0.2 M KP_i (pH 8.0) was incubated with 1 mM NAD^+, 0.3 mM CoASH, 0.5 mM NADPH and mitochondrial extract (0.1 mg/ml). Reactions were terminated after various periods of incubation by adjusting the pH to 1 with concentrated HCl. After readjusting the pH to 5, samples were cleared by filtration through 0.22 μm, pore size, membranes and analyzed by HPLC. Standard curves established with purified acyl-CoAs were used to quantify the metabolites. In some experiments a reconstituted ß-oxidation system was used in place of the mitochondrial extract. Such incubation mixtures contained in 0.2 M KP_i (pH 8.0) either 20 μM 3,5-octadienoyl-CoA or 20 μM 2, 5-octadienoyl-CoA plus 1 mM NAD^+, 0.3 mM CoASH, 0.7 mU of enoyl-CoA isomerase, 0.5 U of enoyl-CoA hydratase, 18 mU of 3-hydroxyacyl-CoA dehydrogenase and 0.11 U of 3-ketoacyl-CoA thiolase. Samples were processed and analyzed by HPLC as described above.

Results

Characterization and subcellular localization of dienoyl-CoA isomerase from rat liver:

The purification of dienoyl-CoA isomerase completes the identification of the enzymes functioning in the NADPH-dependent pathway for the β-oxidation of unsaturated fatty acids with odd-numbered double bonds. The mitochondrial dienoyl-CoA isomerase, which catalyzes the conversion of 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA (see Fig. 4 & 5), was purified 370-fold from rat liver at almost 30% yield by a six-step purification procedure (6). The molecular weights of the native enzyme and its subunit(s) were estimated to be 126,000 and 32,000, respectively (6).

Mechanism of Dienoyl-CoA Isomerase -

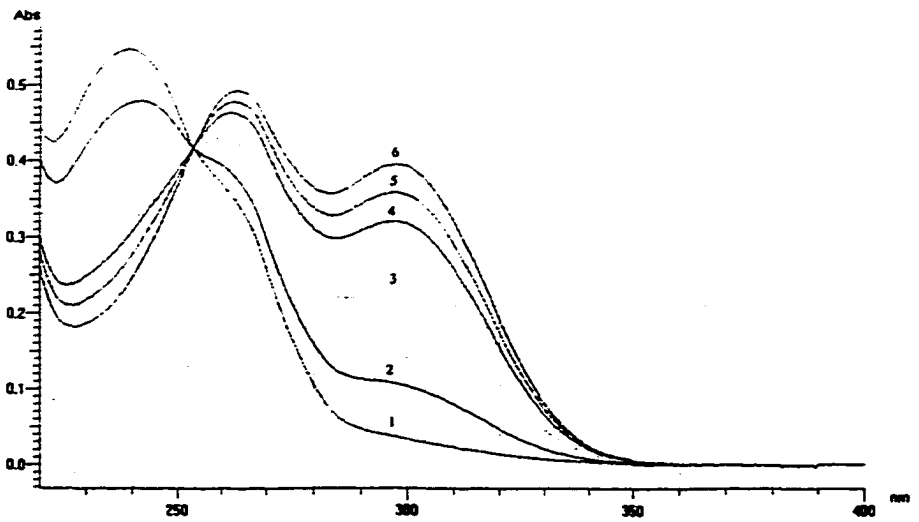

Fig. 6. Enzymatic conversions of 30 nmoles of 3,5-cis-octadienoyl-CoA to 2,4-trans-octadienoyl-CoA with 0.10 U of the purified dienoyl-CoA isomerase in 1.0 ml of 0.1 M KH_2PO4 (pH 8.0) as a function of time. 1, 0 min; 2, 1 min; 3, 4 min; 4, 6 min; 5, 8 min; 6, 10 min.

Although dienoyl-CoA isomerase catalyzed the conversion of 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA (see Fig. 6), it was not clear if this was the only reaction catalyzed by the enzyme and whether this reaction involved the simultaneous or consecutive shifts of the two double bonds.

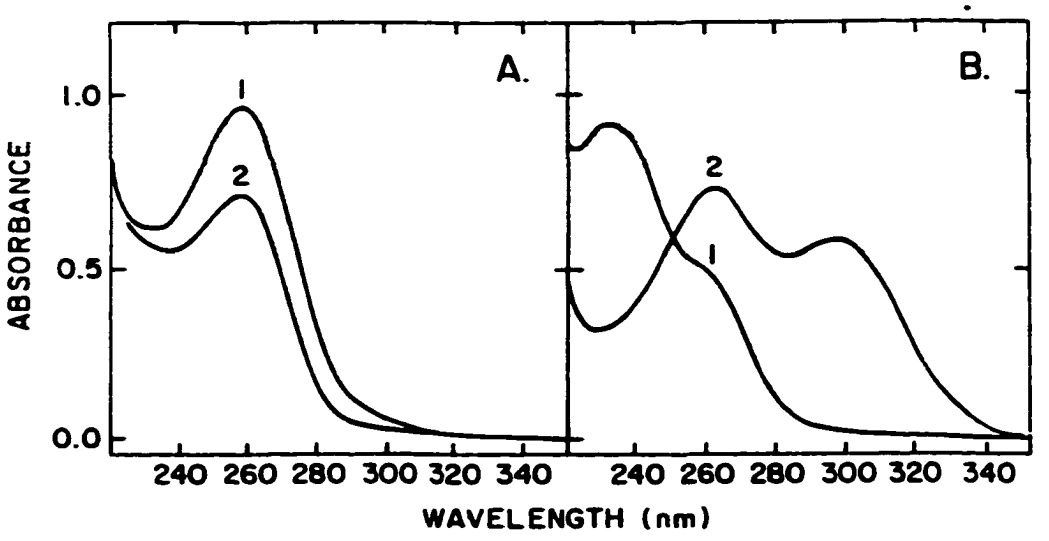

Fig. 7. Spectrophotometric analysis of the enzymatic conversions of 2-*trans*-5-*trans*-octadienoyl-CoA ($\Delta^{2,5}$), and 3,5-*trans*-octadienoyl-CoA ($\Delta^{3,5}$). **A**, to 40 nmol of $\Delta^{2,5}$ in 1 ml of 0.1 M KPi, pH 8.0 (curve 1) were added either 0.14 unit of crotonase (curve 2) or 0.25 unit of dienoyl-CoA isomerase (curve 1). **B**, to 30 nmol of $\Delta^{2,5}$ in 1 ml of 0.1 M KPi pH 8.0 (curve 1) were added either 0.14 unit of crotonase (curve 1) or 0.25 unit of dienoyl-CoA isomerase (curve 2).

The results shown in Fig. 7, panel A and Fig. 8, panels A-D clearly demonstrate that 2-*trans*-5-*trans*-octadienoyl-CoA is not acted upon by dienoyl-CoA isomerase, but is hydrated by crotonase to 3-hydroxy-5-*trans*-octenoyl-CoA. The separation of 2-*trans*-5-*trans*-octadienoyl-CoA from 2-*trans*-4-*trans*-octadienoyl-CoA by HPLC is documented in Fig. 8, panel D, whereas the 2,5- and 3,5-isomers could not be separated from each other under the conditions used in this study.

Thus, the metabolites marked ^{2,5} in Fig. 8, panel B and ^{3,5} in Fig. 8, panel G could also reflect the presence of the other isomer. In contrast to the hydration of 2-*trans*-5-*trans*-octadienoyl-CoA, 3,5-*trans*-octadienoyl-CoA was not acted upon by crotonase but was converted by dienoyl-CoA isomerase to 2-*trans*-4-*trans*-octadienoyl-CoA (see Fig. 7 spectrum B, and Fig. 8 panel E-H. The presence of only a small amount of 3,5-*trans*-octadienoyl-CoA after reacting it with dienoyl-CoA isomerase (see Fig. 8, panel G) suggests that the equilibrium of this reaction favors the formation of product. To confirm this conclusion, chemically synthesized and HPLC-purified 2-*trans*-4-*trans*-octadienoyl-CoA was incubated with dienoyl-CoA isomerase and analyzed by HPLC for the presence of the 3,5-and 2,4-isomers. Less than 5% of 2,4-isomer was converted to the 3,5-isomer (data not shown).

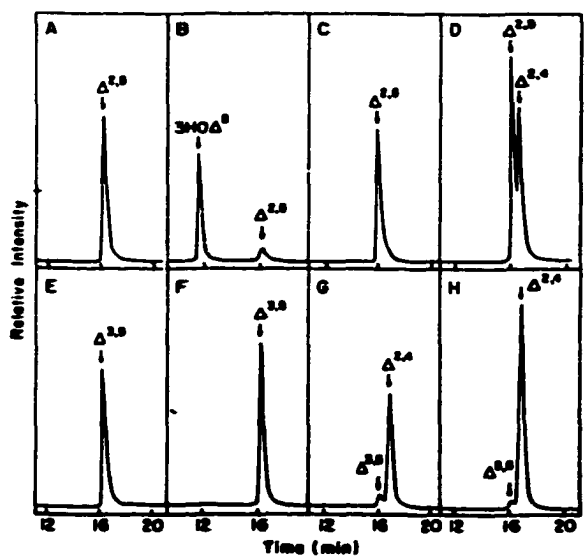

Fig. 8. HPLC analysis of the metabolites formed by enzymatic conversions of 2-*trans*-5-*trans*-octadienoyl-CoA ($\Delta^{2,5}$) and 3,5-*trans*-octadienoyl-CoA ($\Delta^{3,5}$). Experimental conditions were the same as given in the legend to Fig. 7. **A**, $\Delta^{2,5}$; **B**, $\Delta^{2,5}$ + crotonase; **C**, $\Delta^{2,5}$ + dienoyl-CoA isomerase; **D**, same as C plus $\Delta^{2,4}$; **E**, $\Delta^{3,5}$; **F**, $\Delta^{3,5}$+ crotonase; **G**, $\Delta^{3,5}$ + dienoyl-CoA isomerase; **H**, same as G plus $\Delta^{2,4}$. Other abbreviations are as follows: 3-*OH*Δ^{5}, 3-hydroxyl-5-*trans*-octenoyl-CoA; $\Delta^{2,4}$, 2-*trans*-4-*trans*-octadienoyl-CoA.

Kinetic Properties of Dienoyl-CoA Isomerase - The pH-activity profile of dienoyl-CoA isomerase has the appearance of a bell-shaped curve with an optimum close to pH 8 and half maximal activities at pH 5 and 10 (data not shown).

Kinetics parameters (Km, Vmax) were determined for dienoyl-CoA isomerase with 3,5-*cis*-octadienoyl-CoA and 3,5-*trans*-octadienoyl-CoA as substrates. 3,5-*cis*-Octadienoyl-CoA is the better of the two substrates with Km and Vmax values of 7.5 μM and 403 U/mg, respectively. The Km value for 3,5-*trans*-octadienoyl-CoA was 11.8 μM and the Vmax was 226 U/mg.

Subcellular Localization of Dienoyl-CoA Isomerase - Since mammalian cells contain two β-oxidation systems, one located in mitochondria and the other associated with peroxisomes, dienoyl-CoA isomerase may be present in either organelle or both. However, a recent observation that odd-numbered double bonds are reductively removed during the β-oxidation of arachidonic acid in peroxisomes is indicative of the presence of $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase in this organelle (84). Therefore the subcellular distribution of $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase was reinvestigated even though this activity had not been detected in peroxisomes during previous study (6). In the earlier study peroxisomes were isolated by centrifugation on a Nycodenz density gradient and assayed for dienoyl-CoA isomerase in the presence of Triton X-100. The effects of Nycodenz and Triton X-100 on the activity of purified dienoyl-CoA isomerase from rat liver were determined. Both compounds, at concentrations that were achieved in the assay mixture, strongly inhibited dienoyl-CoA isomerase. Given this situation, the subcellular distribution of dienoyl-CoA isomerase was redetermined except that fractions obtained by Nycodenz density gradient centrifugation were extensively dialyzed and sonicated before being assayed for dienoyl-CoA isomerase activity in

the absence of Triton X-100. Shown in Fig. 9 are the results of such an experiment with a light mitochondrial or L-fraction isolated from the liver of a rat fed rodent chow containing 2% di(ethylhexyl)phthalate to induce high levels of peroxisomal dienoyl-CoA isomerase.

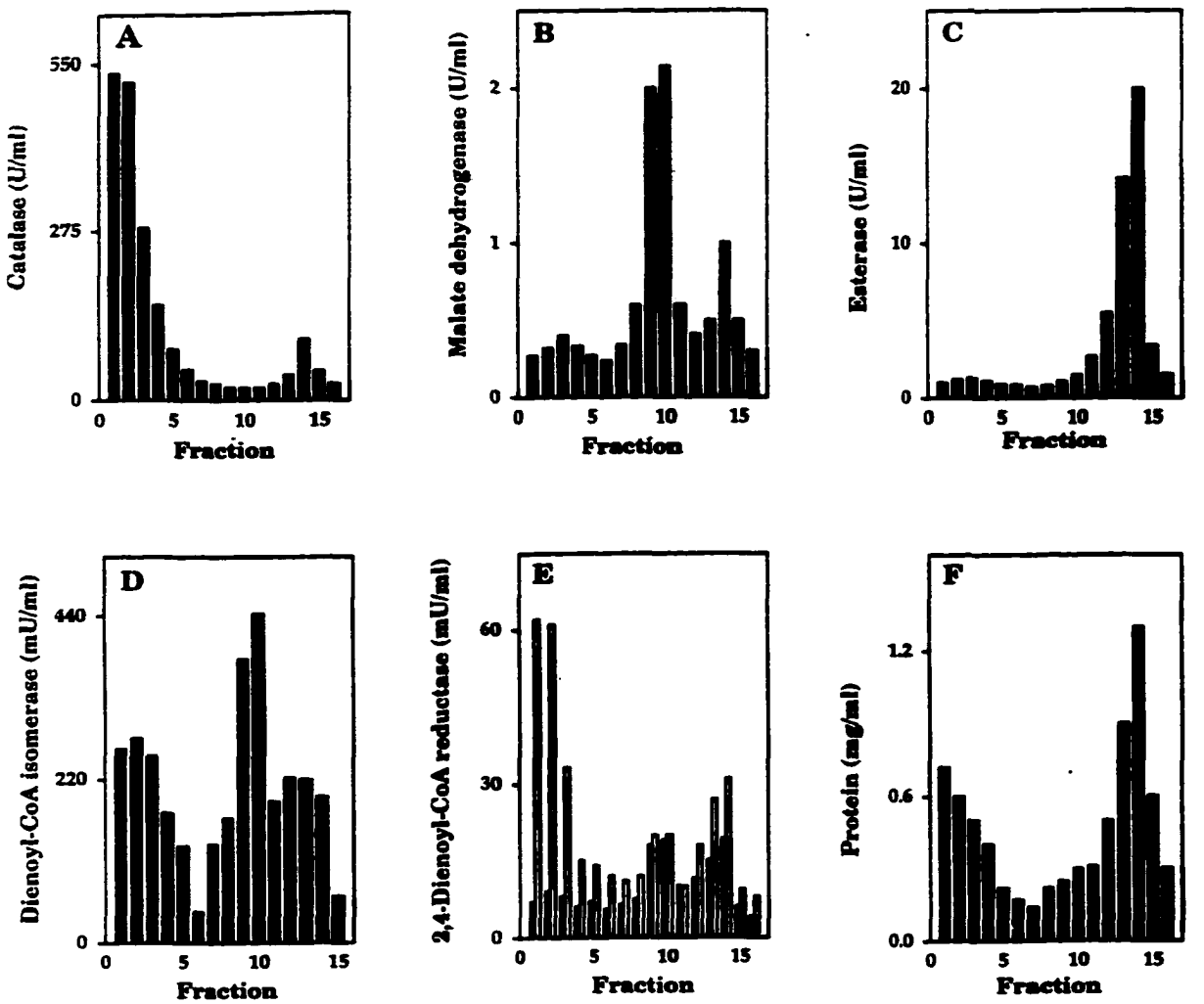

Fig. 9. Subcellular distribution of dienoyl-CoA isomerase and 2,4-dienoyl-CoA reductase. A light mitochondrial fraction from rat liver was fractionated by Nycodenz density gradient centrifugation and fractions were assayed for marker enzymes, dienoyl-CoA isomerase, and 2,4-dienoyl-CoA reductase with sorboyl-CoA (solid bars) and for 2,4-decadienoyl-CoA (open bars) as described under Experimental Procedures.

Panels A-C illustrate the separation and banding of peroxisomes, mitochondria, and microsomes, respectively. When the subcellular distribution of dienoyl-CoA isomerase, shown in Fig. 9 D, is compared with the locations of marker enzymes (see Fig. 9, A-C), it is apparent that dienoyl-CoA isomerase is present both in mitochondria and peroxisomes. The dienoyl-CoA isomerase activity detected in fractions 12-14, where microsomes are concentrated, is attributed to contaminating mitochondria and peroxisomes. The same subcellular fractions also were assayed for 2,4-dienoyl-CoA reductase with sorboyl-CoA and 2,4-decadienoyl-CoA as substrates. As expected, 2,4-dienoyl-CoA reductase activity was found to be present in peroxisomes and mitochondria (95), whereas the activity associated with the microsomal fractions most likely was due to contaminating peroxisomes and mitochondria. Interestingly, the ratio of activities measured with the two substrates in peroxisomes was different from the mitochondrial ratio. The relatively low sorboyl-CoA reductase activity in peroxisomes as compared to mitochondria agrees with the presumed function of the former organelle in the chain-shortening of fatty acids, in contrast to mitochondria which completely degrade fatty acids.

The further characterization of peroxisomal dienoyl-CoA isomerase required the use of purified peroxisomes, most importantly peroxisomes with only a minor mitochondrial contamination. Using the specific activity of catalase relative to that of malate dehydrogenase as a criterion, the best preparation was a peroxisomal fraction obtained by Nycodenz density gradient centrifugation of light mitochondria that had been isolated from the livers of rats fed a diet without the peroxisomal proliferator. The mitochondrial contamination of such peroxisomal fraction was well below 10%. The specific activities of dienoyl-CoA

isomerase with three different substrates were determined with purified rat liver peroxisomes and an extract from heavy mitochondria (see Table I).

Table I: $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase in peroxisomes and in an extract of heavy mitochondria from rat liver.

Enzyme	Substrate*	Specific Activity (nmol/min/mg)	
		Peroxisomes	Mitochondrial extract
$\Delta^{3,5},\Delta^{2,4}$-Dienoyl-CoA isomerase	C_8	180 (100%)	190 (100%)
	C_{14}	29 (16%)	36 (19%)
	C_{20}	22 (12%)	13 (7%)

*C_8, 3,5-octadienoyl-CoA; C_{14}, 3,5-tetradecadienoyl-CoA; C_{20}, 3,5,8,11,14-eicosapentaenoyl-CoA.

The chain length specificities of the peroxisomal and mitochondrial dienoyl-CoA isomerase were similar and not sufficiently different to distinguish between these two forms of the enzyme. When the specific activities measured with organelles from normal rats (see Table I) are compared with those from rats fed di(ethylhexyl)phthalate (see Fig. 9), it is apparent that the activity of dienoyl-CoA isomerase was induced less in peroxisomes (3-fold) than in mitochondria (<9-fold). A key experiment was the testing of the peroxisomal dienoyl-CoA isomerase by

immunoblotting with antiserum raised against the purified mitochondrial isomerase from rat liver. This experiment demonstrated that the peroxisomal form of the isomerase cross-reacts with the anti-mitochondrial antibody and hence may be highly homologous or identical with the mitochondrial isozyme (83).

The Metabolic Significance of the Novel Reductase-dependent Pathway:

The existence of dienoyl-CoA isomerase facilitates a reaction sequence by which the odd-numbered double bonds of unsaturated fatty acids are reduced by NADPH-dependent 2,4-dienoyl-CoA reductase. The starting metabolite in this reaction sequence is 2-*trans*-5-*cis*-dienoyl-CoA, which is formed from 5-*cis*-enoyl-CoA by acyl-CoA dehydrogenase. 2,5-Dienoyl-CoA can either complete the β-oxidation cycle via the isomerase-dependent pathway (Fig. 5, A) or can be metabolized via the reductase dependent pathway (Fig. 5, B) which requires dienoyl-CoA isomerase, 2,4-dienoyl-CoA reductase and enoyl-CoA isomerase.

Kinetic Study of the Mitochondrial Metabolism of 2,5-Octadienoyl-CoA -

The degradation of 2-*trans*-5-*cis*-octadienoyl-CoA, which is a metabolite of linolenic acid, only involves soluble enzymes that are located in the matrix of mitochondria. Hence, a soluble extract of mitochondria represents a suitable enzyme system to study the metabolism of this compound. The direct ß-oxidation of 2-*trans*-5-*cis*-octadienoyl-CoA requires the sequential actions of enoyl-CoA hydratase (crotonase), 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase (see Fig. 5, pathway A).

In an effort to determine if the 5-*cis* double bond might affect the rate at which 2-*trans*-5-*cis*-octadienoyl-CoA is metabolized via pathway A (see Fig. 5), the

apparent Km and Vmax values of these three ß-oxidation enzymes were determined using substrates with and without the 5-*cis* double bond. The results shown in Table II demonstrate that the 5-*cis* double bond affects the activities of the three enzymes differently.

Table II: The influence of a 5-*cis* double bond in the substrate on the kinetic parameters of several enzymes of β-oxidation.

Enzyme	Substrate	Vmax[1]	Km[1]
		Units/mg	µM
Enoyl-CoA hydratase	2-*trans*-octenoyl-CoA	320	9.4
	2-*trans*-5-*cis*-octadienoyl-CoA	930	12
3-Hydroxyacyl-CoA dehydrogenase	L-3-hydroxyoctanoyl-CoA	50	1.8
	L-3-hydroxy-5-*cis*-octenoyl-CoA	48	7.3
3-ketoacyl-CoA thiolase	3-Ketooctanoyl-CoA	139	2.1
	3-Keto-5-*cis*-octenoyl-CoA	24	11.3

[1]Apparent values of Vmax and Km were determined at fixed concentrations of 1 mM NAD^+ and 0.3 mM CoASH with 3-hydroxyacyl-CoA dehydrogenase and at 0.3 mM CoASH with 3-ketoacyl-CoA thiolase, respectively. For other experimental details see under Experimental Procedures.

With enoyl-CoA hydratase, a 3-fold higher Vmax was observed when the substrate contained the 5-*cis* double bond while the Km was virtually unaffected. The apposite observation was made with 3-hydroxyacyl-CoA dehydrogenase which yielded a 4-fold higher Km value for the unsaturated as compared to the saturated substrate. However, the maximal velocity of this enzyme was unaffected by the presence of the 5-*cis* double bond. The most severe effect was detected with 3-ketoacyl-CoA thiolase which exhibited with the unsaturated substrate only one-sixth of the maximal velocity observed with the saturated substrate. In addition, the Km value was 5-fold higher when the 5-*cis* double bond was present in the substrate. This data demonstrates that 5-*cis*-enoyl-CoA intermediates can be directly metabolized by β-oxidation, perhaps at somewhat slower rates compared to the corresponding saturated metabolites.

Metabolism of 2,5-Octadienoyl-CoA via the Isomerase-dependent and Reductase-dependent Pathways - Since 2-*trans*-5-*cis*-octadienoyl-CoA can be metabolized by both the isomerase-dependent (Fig. 5, A) and reductase-dependent (Fig. 5, B) pathways, it was important to determine the relative fluxes via these two routes. For estimating the flux through the isomerase-dependent pathway, rates of NADH formation in the presence of CoASH but absence of NADPH were measured spectrophotometrically (see Fig. 10, A). Since initial velocities were determined, the simultaneous entry of substrate into the reductase-dependent pathway was assumed to have little or no effect on the rates of degradation via the isomerase-dependent pathway. Flux through the reductase-dependent pathway was determined by measuring spectrophotometrically the formation of 2,4-dienoyl-CoA at 300 nm (see Fig. 10, B) in the absence of coenzymes.

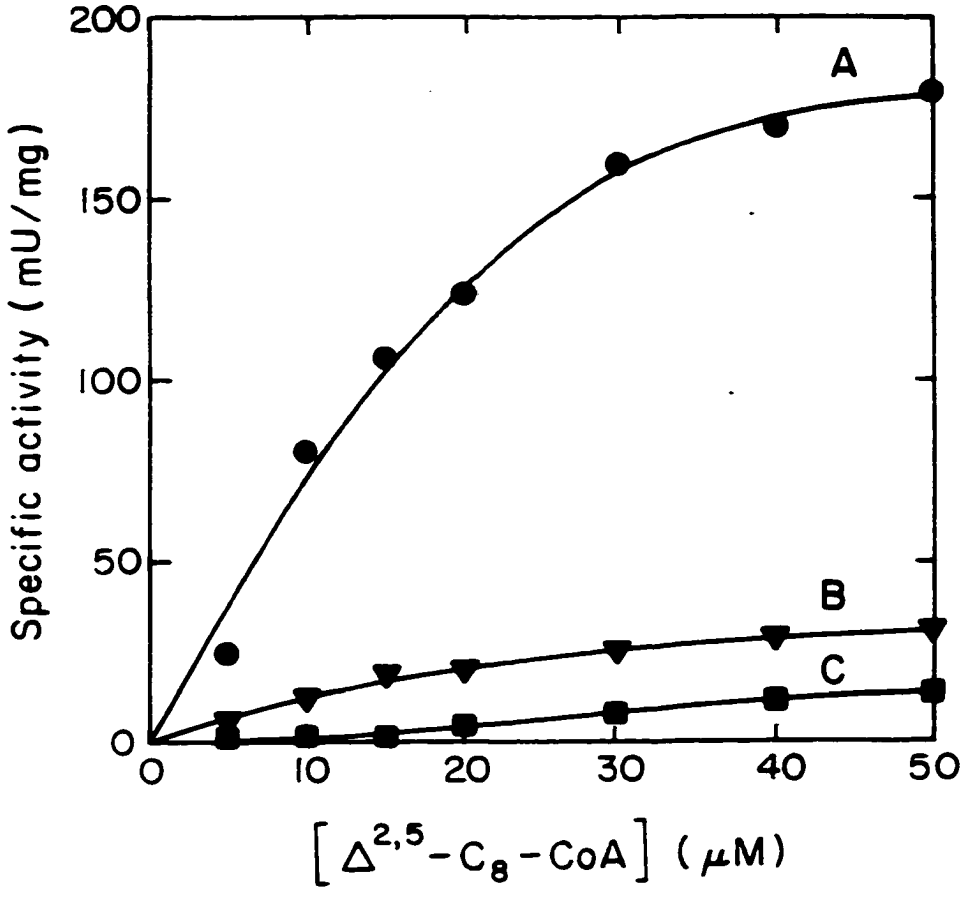

Fig. 10. Rates of 2,5-octadienoyl-CoA metabolism by a soluble extract of rat liver mitochondria as a function of the substrate concentration. A. Rates of NADH formation in the presence of 1 mM of NAD^+ plus 0.3 mM CoASH. B. Rates of 2,4-dienoyl-CoA formation in the absence of coenzymes. C. Rates of 2,4-dienoyl-CoA formation in the presence of 1 mM NAD^+, 0.3 mM CoASH, 1 mM pyruvate and 1 U of lactate dehydrogenase.

A comparison of the data shown in Fig 10 curves A and B, indicates that approximately 85% of 2,5-octadienoyl-CoA is metabolized by the isomerase-dependent pathway and only 15% via the reductase-dependent route. When the

rate of 2,4-dienoyl-CoA formation was measured in the presence of NAD^+, CoASH and pyruvate plus lactate dehydrogenase to re-oxidize NADH, rates were lower by approximately 50% than observed in the absence of coenzymes (compare curves B and C of Fig. 10). This decline in rates is attributed, at least in part to the ß-oxidation of 2,4-dienoyl-CoA via route C (see Fig 5) which requires the involvement of enoyl-CoA isomerase and dienoyl-CoA isomerase, but not the reductase. The direct β-oxidation of 2,4-dienoyl-CoA, as outlined in Fig. 5 C, is possible, albeit at a low rate, because this intermediate has the 2-*trans*-4-*trans* configuration which makes it a substrate of the mitochondrial β-oxidation system in contrast to the 2-*trans*-4-*cis*-isomer which is not directly oxidized (89). The β-oxidation of 2,5-octadienoyl-CoA by a mitochondrial extract from rat liver in the presence of NAD^+, CoASH, and NADPH was studied as the function of the incubation time. Metabolites were analyzed by HPLC (see Fig. 11)

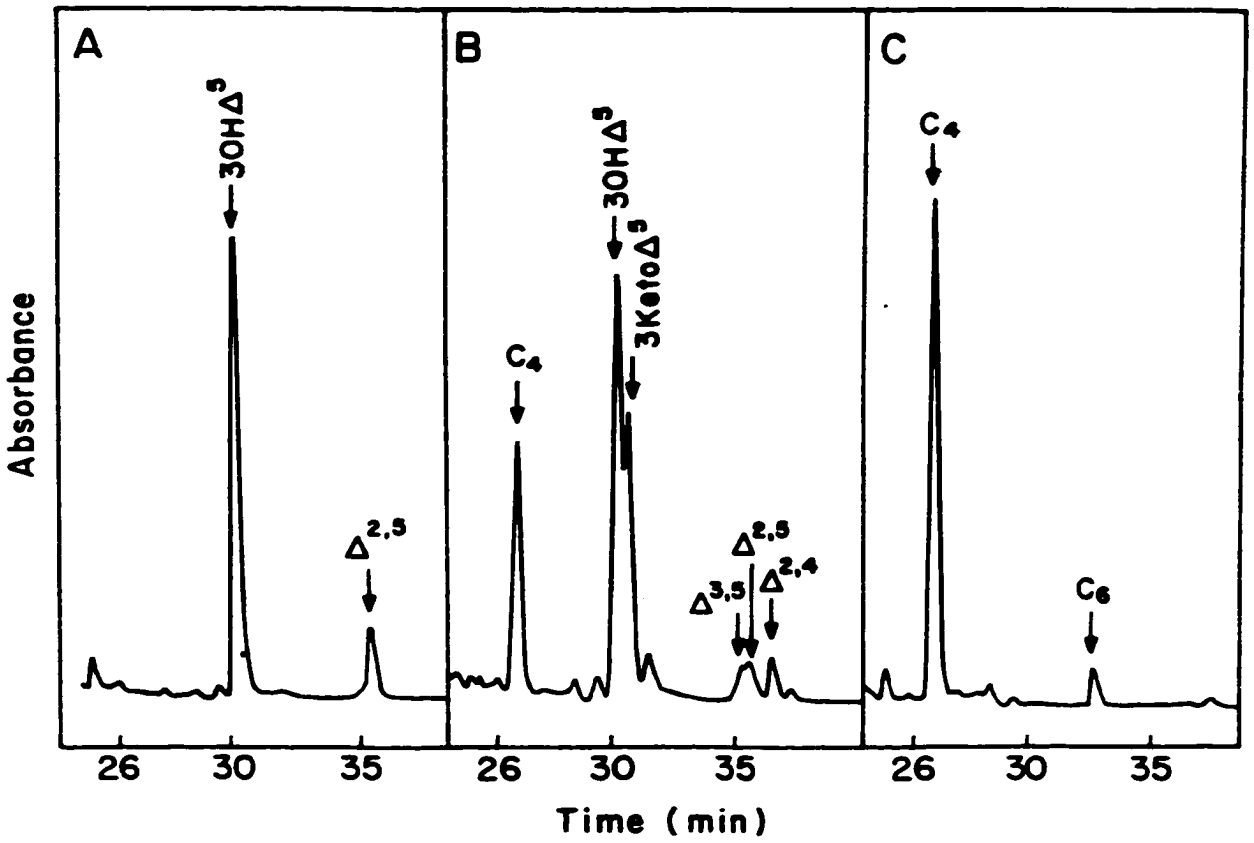

Fig. 11. HPLC analysis of metabolites formed from 2,5-octadienoyl-CoA by a soluble extract of rat liver mitochondria in the presence of NAD^+, CoASH, and NADPH. Products detected after: A, 5 sec; B, 1 min.; C, 5 min. Peaks identified by use of authentic compounds were: ^{2,5}, 2,5-octadienoyl-CoA; 3OH ^{5}, 3-hydroxy-5-octenoyl-CoA; C_4, butyryl-CoA; 3keto- ^{5}, 3-keto-5-octenoyl-CoA; ^{3,5}, 3,5-octadienoyl-CoA; ^{2,4}, 2,4-octadienoyl-CoA; C_6, hexanoyl-CoA. For details see under Experimental Procedures.

Striking was the almost instantaneous hydration of 2,5-octadienoyl-CoA to 3-hydroxy-5-octenoyl-CoA, the first metabolite of the isomerase-dependent pathway, (see Fig. 11, A). Purified enoyl-CoA hydratase was used to determine that the ratio of 3-hydroxy-5-octenoyl-CoA to 2,5-octadienoyl-CoA at equilibrium is 6.4:1. The reaction reached a steady-state within 1 min. when substantial amounts of 3-keto-octenoyl-CoA and butyryl-CoA, the final product of the isomerase-dependent pathway, had been formed (see Fig. 11, B). At the same time only small amounts of metabolites belonging to the reductase-dependent pathway had been formed (marked ^{3,5} and ^{2,4} in Fig. 10, B). Hexanoyl-CoA, the final product of the reductase-dependent pathway, was detected toward the end of the reaction when it constituted 18% of butyryl-CoA (see Fig. 11, C). The kinetic parameters (apparent Vmax and Km) of several β-oxidation enzymes, that are present in a soluble extract of rat liver mitochondria, were determined with substrates having 5-*cis* double bonds. As is apparent from the data shown in Table III, the activity of enoyl-CoA hydratase with 2,5-octadienoyl-CoA as a substrate is at least 50-fold higher the activity of the second most active enzyme in this group of ß-oxidation enzymes in rat liver mitochondria. The hydratase activity is sufficiently high to maintain an equilibrium or near equilibrium situation between 2,5-octadienoyl-CoA and its product of hydration. As a consequence, the actual concentration of 2,5-octadienoyl-CoA was only 14% of the expected concentration based on its formation.

Table III. [1]Apparent kinetic constants of several β-oxidation enzymes present in a soluble extract of rat liver mitochondria with substrates having 5-*cis* double bonds.

Enzyme	Substrate	Vmax[1]	Km[1]
		munits/mg	μM
Enoyl-CoA hydratase	2,5-Octadienoyl-CoA	48,600	4
3-Hydroxyacyl-CoA dehydrogenase	3-Hydroxy-5-octenoyl-CoA	28	10
3-Ketoacyl-CoA thiolase	3-Keto-5-octenoyl-CoA	840	15
Enoyl-CoA isomerase	2,5-Octadienoyl-CoA	48	23

[1]Apparent Vmax and Km values were determined at one fixed coenzyme concentration as detailed under Experimental Procedures.

Of the first three enzymes of pathway A (review Fig. 5) the least active one was 3-hydroxyacyl-CoA dehydrogenase. However, the specific activity of this enzyme was 6-times higher than the activity of enoyl-CoA isomerase which is the enzyme that determines the rate at which intermediates enter the reductase-

dependent pathway (see Fig. 5, B). The relative activities of the rate-determining enzymes of the isomerase-dependent and reductase-dependent pathway (Fig. 5, A-B) explain the observed greater flux through the former than through the latter pathway. If pathway A would be inhibited, then a larger percentage of 2,5-octadienoyl-CoA might enter pathway B. This hypothesis was tested by evaluating the effect of NADH on the fluxes through pathways A and B. Since NADH is a product inhibitor of 3-hydroxyacyl-CoA dehydrogenase, the flux through pathway A should be inhibited and more of the intermediate might enter pathway B.

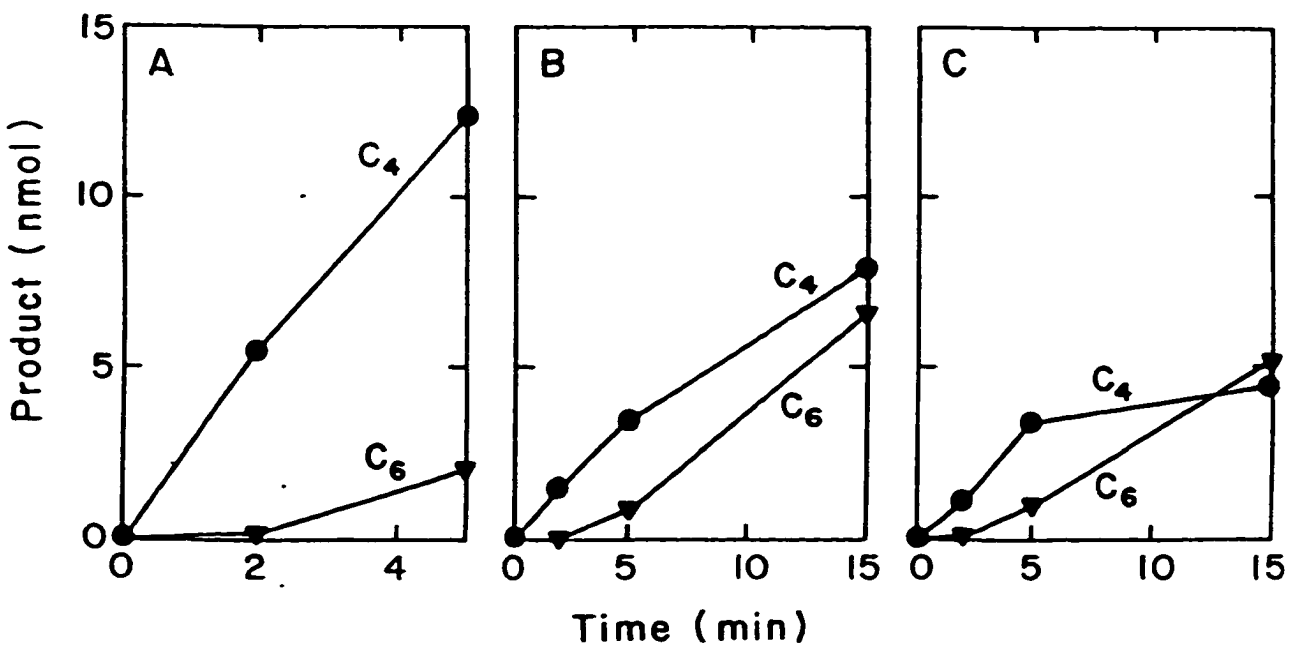

Fig. 12. Time-dependent degradation of 2,5-octadienoyl-CoA to butyryl-CoA and hexanoyl-CoA as a function of the incubation time. Incubations contained 1 mM NAD^+, 0.5 mM NADPH, 0.3 mM CoASH, and NADH at the following concentrations: A, 0 mM; B, 0.5 mM; C, 1 mM.

The results shown in Fig. 12, specifically the reduced formation of butyryl-CoA, prove the predicted inhibition of the flux through pathway A. However, the formation of hexanoyl-CoA via pathway B also was reduced but only slightly so that almost equal amounts of butyryl-CoA and hexanoyl-CoA were formed during the course of the experiments with either 0.5 mM or 1 mM NADH (see Fig. 12, B & C).

Metabolism of 3,5-octadienoyl-CoA -

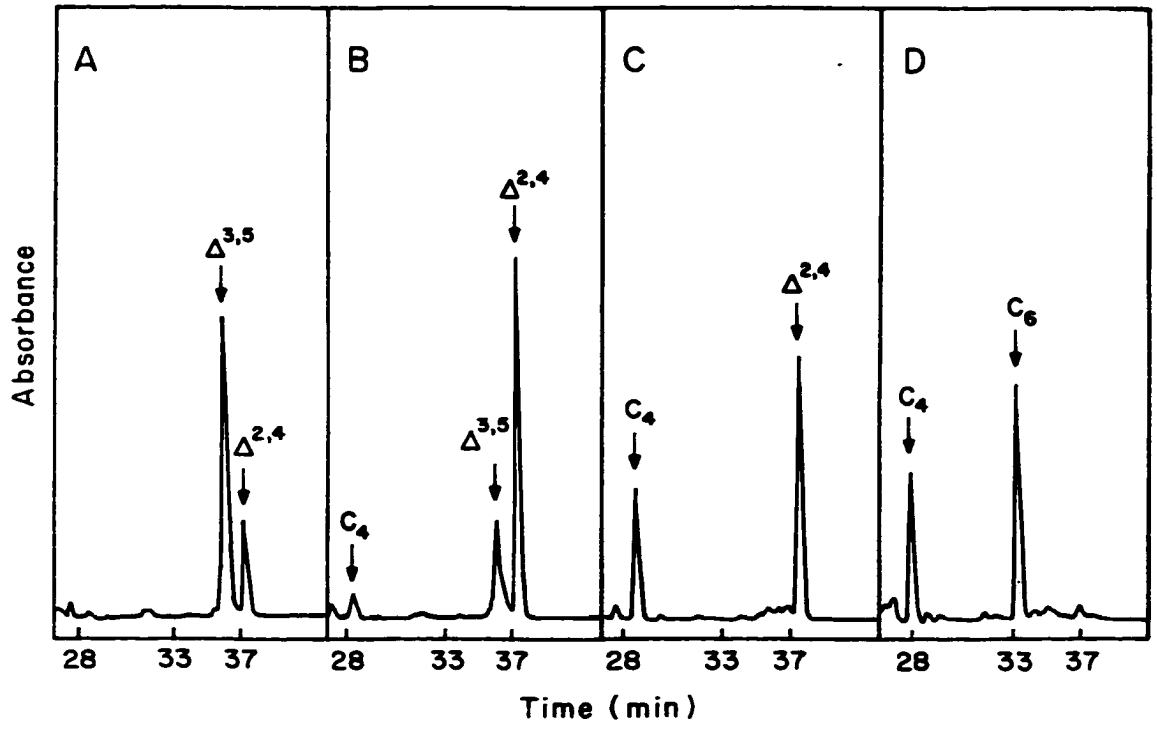

Fig. 13. HPLC analysis of metabolites formed from 3,5-octadienoyl-CoA by a soluble extract of rat liver mitochondria in the presence of NAD^+ and CoASH. Products detected after: A, 15 sec.; B, 1 min.; C, 5 min. D, product formed after 5 min. when NADPH was present in addition to NAD^+ and CoASH. Peaks identified by use of authentic compounds were: ^{3,5}, 3,5-octadienoyl-CoA; ^{2,4}, 2,4-octadienoyl-CoA; C_4, butyryl-CoA; C_6, hexanoyl-CoA. For details see under Experimental Procedures.

The isomerization of 2,5-octadienoyl-CoA to 3,5-octadienoyl-CoA could reduce the flux through the isomerase-dependent pathway (Fig. 5, pathway A) unless the isomerization is freely reversible. Since the equilibrium concentration of 3,5-octadienoyl-CoA was found to be at least 20-times higher than the concentration of the 2,5-isomer, the rate of the 3,5 --> 2,5 isomerization was expected to be slow. An analysis of metabolites formed from 3,5-octadienoyl-CoA by a soluble extract of rat liver mitochondria in the presence of NAD^+ and CoASH revealed the rapid conversion of 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA (see Fig. 13, A) and the slower appearance of butyryl-CoA (see Fig. 13, B & C).

This pattern of metabolite formation is indicative of the degradation of 3,5-octadienoyl-CoA via pathway C shown in Fig. 5 after its conversion to 2,4-octadienoyl-CoA. The absence of metabolites characteristic of pathway A suggests that butyryl-CoA was not formed via pathway A after the conversion of 3,5-octadienoyl-CoA to 2,5-octadienoyl-CoA. When NADPH was present besides NAD^+ and CoASH, hexanoyl-CoA and butyryl-CoA were formed in a ratio of 3:2 (see Fig. 13, D). A similar ratio was observed when the rate at which 2,4-octadienoyl-CoA was utilized by a soluble extract of rat liver mitochondria in the presence of either NAD^+, NADPH, and CoASH (B & C), or NAD^+ and CoASH (C) (see Fig. 14).

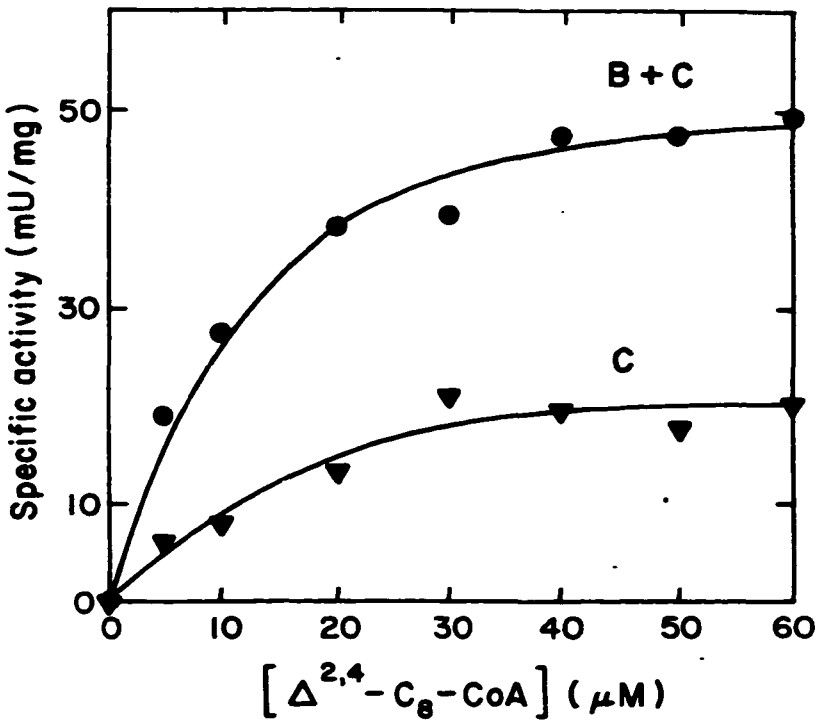

Fig. 14. Rates of 2,4-octadienoyl-CoA utilization by a soluble extract of rat liver mitochondria as a function of the substrate concentration. B+C, degradation in the presence of NAD^+, CoASH and NADPH via pathways B and C (see Fig.1). C, degradation in the presence of NAD^+ and CoASH via pathway C (see Fig.1). For details see Experimental Procedures.

This experiment revealed that 2,4-octadienoyl-CoA can be β-oxidized directly (see pathway C in Fig. 5) in addition to being degraded after the NADPH-dependent reduction of one double bond (see pathway B in Fig. 5). The fluxes

through the two pathways are of similar magnitude, with the reductive branch accommodating a slightly faster flux than the direct β-oxidation. Although the above mentioned results are indicative of the degradation of 3,5-octadienoyl-CoA via pathway B and C, a possible contribution of pathway A cannot be excluded. The possibility of a slow degradation of 3,5-octadienoyl-CoA via pathway A (see Fig. 5) was evaluated by use of a β-oxidation system reconstituted from purified enzymes.

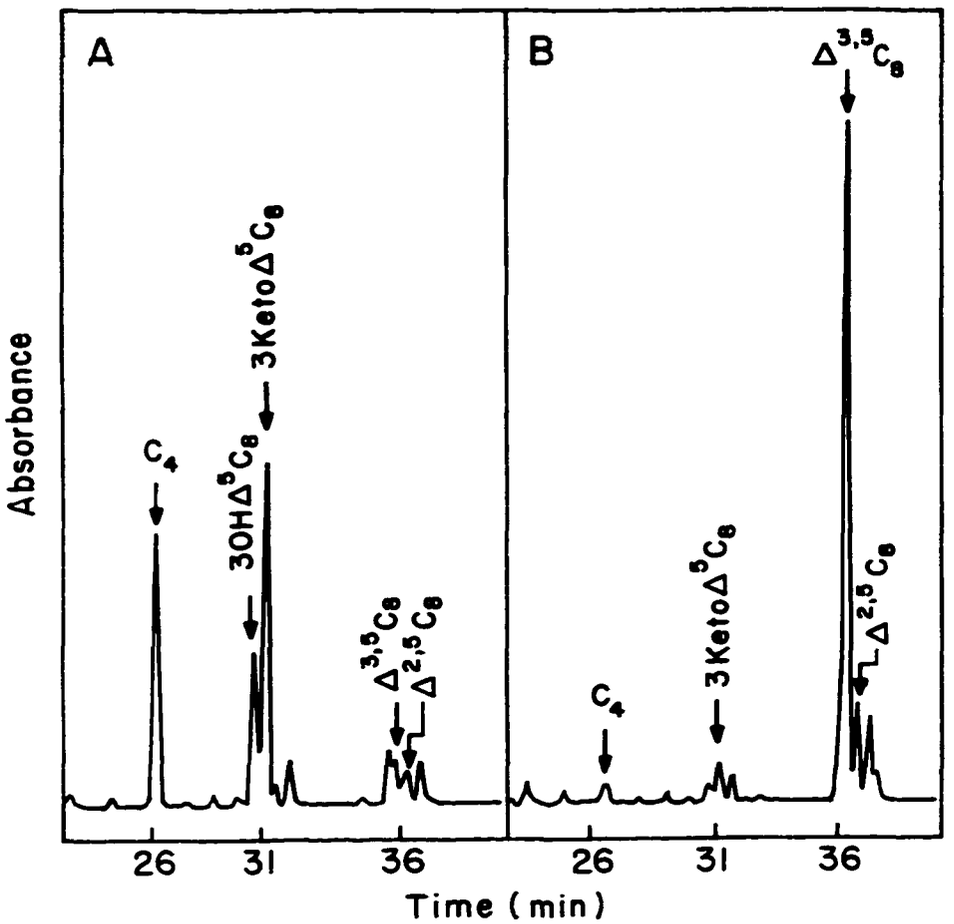

Fig. 15. HPLC analysis of metabolites formed from 2,5-octadienoyl-CoA (A) and 3,5-octadienoyl-CoA (B) by a reconstituted β-oxidation system which did not contain dienoyl-CoA isomerase or 2,4-dienoyl-CoA reductase. The incubation time was 1 min. For details see under Experimental Procedures.

All soluble β-oxidation enzymes necessary to degrade 3,5-octadienoyl-CoA via pathway A were combined at activity levels observed in a soluble extract of rat liver mitochondria. The absence of dienoyl-CoA isomerase prevented the degradation via pathways B and C. When 2,5-octadienoyl-CoA was incubated with the reconstituted β-oxidation system in the presence of NAD^+ and CoASH, its rapid degradation via pathway A was observed as indicated by the almost complete disappearance of the starting material within 1 min. (see Fig. 15, A). In contrast, when incubated under identical conditions, most of 3,5-octadienoyl-CoA was unchanged after the same incubation period (see Fig. 15, B). However, small amounts of butyryl-CoA and 3-keto-octenoyl-CoA were detected and are indicative of a slow degradation of 3,5-octadienoyl-CoA via pathway A.

Discussion

When the reductase-dependent pathway for the β-oxidation of 5-enoyl-CoAs was first identified (5), attention was immediately directed towards the purification of the novel enzyme dienoyl-CoA isomerase. The purification and characterization of dienoyl-CoA isomerase confirmed the proposed existence of an NADPH-dependent pathway for the β-oxidation of unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms. Characterization of the purified dienoyl-CoA isomerase demonstrated that the enzyme specifically catalyzes the isomerization of 3,5-enoyl-CoAs to 2,4-enoyl-CoAs. This demonstration disproved an earlier assumption that the isomerization of 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA may be catalyzed by Δ^3,Δ^2-enoyl-CoA isomerase. The absence of significant activities of other β-oxidation enzymes from the purified preparation established that the enzyme is an unique enzyme in the β-oxidation system that had remained unidentified. Overall, it is believed that the purification of dienoyl-CoA isomerase completed the characterization of the auxiliary enzymes essential in the metabolism of the unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms.

The demonstration that peroxisomes can metabolize arachidonic acid (84), a polyunsaturated long chain fatty acid with odd-numbered double bonds, prompted an investigation of the subcellular localization of dienoyl-CoA isomerase in peroxisomes. The subcellular localization of dienoyl-CoA isomerase in peroxisomes, as described in the results section, corrected a previous claim that dienoyl-CoA isomerase is present only in mitochondria but not in peroxisomes (6). Thus, the results of this study demonstrate that peroxisomes, similar to

mitochondria, contain all essential enzymes required for the β-oxidation of unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms.

Studies of the β-oxidation of 2,5-dienoyl-CoA were aimed at evaluating the contribution of the reductase-dependent pathway (Fig. 5, B) to the total degradation of unsaturated fatty acids. These studies relied on experiments with soluble extracts of rat liver mitochondria and with purified mitochondrial β-oxidation enzymes. Such an approach has the advantage that the concentrations of coenzyme can be controlled and that the concentrations of true intermediates can be measured. Since no detailed information about the operation of both pathways were available, results obtained with mitochondrial extracts and isolated enzymes should provide novel information.

The recognition that 2,5-dienoyl-CoAs, which are metabolites of unsaturated fatty acids with odd-numbered double bonds, can be degraded by the reductase-dependent pathway (see Fig. 5, B) (5), raised the question as to whether the isomerase-dependent pathway (see Fig. 5, A) is operative at all. If yes, the relative fluxes through both pathways needed to be determined. If the reductase-dependent pathway was found not to be essential for the rapid β-oxidation of unsaturated fatty acids, then its metabolic function needed to be elucidated.

The metabolic studies demonstrated that 2,5-dienoyl-CoAs can be degraded via the isomerase-dependent pathway. Kinetic evaluations of enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase revealed that the presence of a 5-*cis* double bond in the substrate had a positive effect on the hydration reaction, but negatively affected the dehydrogenation reaction and the thiolytic cleavage. Most severely affected by the 5-*cis* double bond was 3-ketoacyl-

CoA thiolase. The Vmax value determined with 3-keto-5-octenoyl-CoA as the substrate was almost 6-times lower than the Vmax obtained with 3-ketooctanoyl-CoA. In addition the Km for the substrate was 5-times higher when a 5-*cis* double bond was present in the acyl chain. In contrast, the Vmax of 3-hydroxyacyl-CoA dehydrogenase is unaffected by the presence of a 5-*cis* double bond, whereas the Km for the unsaturated substrate is 4-times higher than the value for the saturated substrate. Since the activity of the dehydrogenase was found to be lower than that of the thiolase in the soluble extract of rat liver mitochondria, it seems that the 5-*cis* double bond may have only a small, if any, impact on the flux of 5-*cis* enoyl-CoAs through this part of the β-oxidation spiral. Moreover, it remains to be established which part of the β-oxidation spiral limits the rate of fatty acid oxidation. If the degradation of medium-chain intermediates does not restrict flux through the overall pathway of β-oxidation, the lower activity of 3-hydroxyacyl-CoA dehydrogenase toward substrates with 5-*cis* double bonds would not affect the rate of unsaturated fatty acid oxidation.

Since 2,5-dienoyl-CoAs can be metabolized via the isomerase-dependent (see Fig. 5, A) and the reductase-dependent (see Fig. 5, B) pathways, it was important to determine the contributions of both pathways to the metabolism of 2,5-octadienoyl-CoA. The results of concentration-dependent and time-dependent measurements are indicative of a much slower flux through the reductase-dependent branch (15-20%) than through the isomerase-dependent branch (80-85%). The main reason for the slow degradation of 2,5-octadienoyl-CoA via the reductase-dependent pathway is the low activity of enoyl-CoA isomerase toward 2,5-octadienoyl-CoA relative to the activity of 3-hydroxyacyl-CoA dehydrogenase with 3-hydroxy-5-octenoyl-CoA in rat liver mitochondria. Moreover, the steady-

state concentration of 2,5-octadienoyl-CoA in an extract of rat liver mitochondria is low due to a high ratio of [3-hydroxy-5-octenoyl-CoA] to [2,5-octadienoyl-CoA] at equilibrium which is maintained because of the high activity of enoyl-CoA hydratase in rat liver mitochondria. However, a restriction in the flux through the isomerase-dependent pathway could result in an increased entry of 2,5-octadienoyl-CoA into the reductase-dependent pathway. This possibility was explored by inhibiting 3-hydroxyacyl-CoA dehydrogenase with NADH and studying the effect of this measure on product formation via both pathways. As expected, the flux through the isomerase-dependent pathway was reduced, but so was the degradation via the reductase-dependent pathway. However, the fluxes through both pathways, although reduced, were almost equal at [NADH]/[NAD^+] ratios of 0.5 and 1. Thus, the relative contribution of the reductase-dependent pathway to the degradation of unsaturated fatty acids with odd-numbered double bonds appears to be more significant when the intramitochondrial [NADH]/[NAD^+] ratio is high, e.g. under conditions of restricted energy utilization.

If the reductase-dependent pathway is not essential for the efficient β-oxidation of 5-enoyl-CoAs, it may serve another metabolic function. Experiments with 3,5-octadienoyl-CoA as a substrate indicated that this intermediate can be metabolized at a significant rate only by its conversion to 2,4-octadienoyl-CoA. Hence, 3,5-octadienoyl-CoA seems to be an obligatory metabolite of the reductase-dependent pathway. The major reason for this situation is the greater thermodynamic stability of 3,5-octadienoyl-CoA as compared to the 2,5-isomer. This difference in stabilities is reflected by a 20:1 ratio of [3,5-isomer] to [2,5-isomer] at equilibrium. Consequently, the re-entry of the 3,5-isomer into the

isomerase-dependent pathway is an unfavorable reaction. The experiment with a reconstituted system of β-oxidation enzymes, which was devoid of dienoyl-CoA isomerase, demonstrated that the metabolism of 3,5-octadienoyl-CoA via the isomerase-dependent pathway is extremely slow even when this compound is present at a high concentration. Because enoyl-CoA isomerase is essential for the degradation of unsaturated fatty acids, it is present in mitochondria and will catalyze the conversion of 2,5-dienoyl-CoAs to their 3,5-isomers. Once formed, 3,5-dienoyl-CoAs cannot be metabolized efficiently via the isomerase-dependent pathway. Hence they must be metabolized via the reductase-dependent pathway or they would accumulate and compromise the metabolic function of mitochondria. If CoA is tied up as 3,5-dienoyl-CoA, the concentration of free mitochondrial CoA is lower with the result that all oxidative pathways in mitochondria will be inhibited. Obviously, such deleterious consequence of the unintended 2,5 -> 3,5 isomerization must be prevented. Since dienoyl-CoA isomerase assures the removal of 3,5-dienoyl-CoAs, it may be present in mitochondria (5, 6) and peroxisomes (83) as a detoxification enzyme.

A surprising finding of this study was the observation that 2,4-octadienoyl-CoA can be degraded by β-oxidation without being first reduced by 2,4-dienoyl-CoA reductase (see Fig. 5, C). This result is not completely surprising as it had been reported that 2-trans, 4-trans-decadienoyl-CoA is a substrate for both direct β-oxidation and NADPH-dependent reduction, whereas the 2-*trans*-4-*cis*-isomer can only be degraded after reduction by 2,4-dienoyl-CoA reductase (89). Although the reduction of 2,4-octadienoyl-CoA is more rapid than its direct β-oxidation, both pathways contribute significantly to the degradation of this metabolite.

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