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Study of 2,4-dienoyl-CoA reductase in unsaturated fatty acid beta-oxidation

Nada, Mohamed Abou El-Yazeid, Ph.D.

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U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

STUDY OF 2,4-DIENOYL-CoA REDUCTASE IN UNSATURATED FATTY ACID BETA-OXIDATION

by

MOHAMED ABOU EL-YAZEID NADA

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The City University of New York

ABSTRACT

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IN
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MOHAMED A. NADA

Advisor: Professor Horst Schulz

A disorder in the β-oxidation of polyunsaturated fatty acids is described that appears to be caused by a deficiency of 2,4-dienoyl-CoA reductase (EC 1.3.1.34). Further studies of this disorder, with small samples of human tissues or human fibroblasts, require a more sensitive assay of 2,4-dienoyl-CoA reductase than is currently used. A radioactive method for assaying 2,4-dienoyl-CoA reductase is described. The assay measures the incorporation of tritium from [4B-^{3}H]NADPH into 2-*trans*,4-*cis*-decadienoyl-CoA or 2-*trans*,4-*trans*-decadienoyl-CoA which, after cleavage of the thioester bond with hydroxylamine, can be separated from the radioactive coenzyme by extraction with toluene. This assay is at least 30-times more sensitive than the spectrophotometric assay, even though rates determined by the radioactive method are 10 times lower than rates obtained spectrophotometrically due to a primary kinetic isotope effect. The linearity of this assay with respect to time and protein concentration is sufficient for determining 2,4-dienoyl-CoA reductase activities in extracts from small samples of human fibroblasts.

The spectrophotometric assay of 2,4-dienoyl-CoA reductase was modified to improve the sensitivity and linearity of this method. A new substrate, 5-phenyl-2,4-pentadienoyl-CoA, was introduced which has an absorbance maximum at 340 nm with an extinction coefficient of 44,338 M^{-1} cm^{-1}. This assay, which measures the decrease in absorbance at 340 nm due to the reduction of 5-phenyl-2,4-pentadienoyl-CoA and the oxidation of NADPH, is more linear and two times more sensitive than the currently used spectrophotometric assay.

Activities of carnitine palmitoyl transferase I (EC. 2.3.1.21) and mitochondrial respiration rates were measured to determine whether the reaction catalyzed by CPT I is a rate-limiting step in the mitochondrial oxidation of long chain unsaturated fatty acids in heart. Although activities of CPT I were higher than rates of fatty acyl-CoA oxidation measured by oxygen consumption, incubation of intact rat heart mitochondria with varying concentrations of 2-tetradecyglycidyl-CoA (TDG-CoA), which is known to irreversibly inhibit CPT I, caused parallel inhibitions of CPT I activities and respiration. Initial rates of respiration were higher with docosahexaenoylcarnitine than with docosahexaenoyl-CoA. However, several minutes into the reaction, the rate of respiration supported by docosahexaenoyl-CoA approached the rate observed with docosahexaenoylcarnitine. Thus it seems that the CPT I catalyzed reaction may not be the rate-limiting step in β-oxidation in heart mitochondria unless the intracellular concentration of malonyl-CoA is sufficiently high to cause a significant inhibition of CPT I activity.

The mitochondrial metabolism of 5-enoyl-CoAs, which are formed during the β-oxidation of unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms, was studied with mitochondrial ex-

tracts and purified enzymes of β-oxidation. Metabolites were identified spectrophotometrically and by high performance liquid chromatography. 5-*cis*-Octenoyl-CoA, a putative metabolite of linolenic acid, was efficiently 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 Δ^3,Δ^2-enoyl-CoA isomerase (EC 5.3.3.8) or by the peroxisomal trifunctional enzyme. Further isomerization of 3,5-octadienoyl-CoA to 2-*trans*-4-*trans*-octadienoyl-CoA in the presence of soluble extracts of either rat liver or rat heart mitochondria was observed and attributed to a novel $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase. Qualitatively similar results were obtained with 2-*trans*,5-*trans*-octadienoyl-CoA formed by dehydrogenation of 5-*trans*-octenoyl-CoA. 2-*trans*,4-*trans*-Octadienoyl-CoA was a substrate for NADPH-dependent 2,4-dienoyl-CoA reductase. 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 to hexanoyl-CoA, butyryl-CoA, and acetyl-CoA. It is concluded that odd-numbered double bonds, like even-numbered double bonds, can be reductively removed during the β-oxidation of polyunsaturated fatty acids.

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ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CoA	coenzyme A
CoASH	coenzyme A
DEAE	diethylaminoethyl
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol bis-(aminoethyl ether) N',N-tetraacetic acid
FAD	flavine adenine dinucleotide (oxidized)
$FADH_2$	flavine adenine dinucleotide (reduced)
HPLC	high performance liquid chromatography
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
Tris-HCl	tris(hydroxymethyl) aminomethane hydrochloride
UV	ultraviolet

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INTRODUCTION

β-Oxidation of fatty acids provides a major portion of the energy in some tissues of the fed animal and is essential for supplying energy in fasting animals. Tissues like heart normally gain most of their energy from β-oxidation of fatty acids. A sufficient level of fatty acid oxidation is therefore essential for these tissues to provide a steady supply of ATP for muscle contraction. In prokaryotes, as in the gram-negative bacterium *E. coli*, fatty acids can serve as the sole carbon and energy source (1,2) so that all cellular constituents are derived from the oxidation products of fatty acids. Recently several inherited human diseases due to deficiencies of β-oxidation enzymes have been identified (3). Depending on the enzyme deficiency, these inherited defects may only cause mild muscle weakness or may result in life-threatening coma or death (4,5). The oxidation of fatty acids, therefore, is an important cellular process that is common to many living organisms, and enormous efforts have been undertaken to investigate the details of this metabolic process, including the identification of metabolic intermediates, clarification of its enzymology, and the elucidation of its regulation.

The mechanism by which free fatty acids cross the plasma membrane when they enter the cell is not totally understood even though significant progress has been made in the characterization of a 40-kDa fatty acid binding protein present in the plasma membrane of liver and other tissues (6). The metabolism of fatty acids requires their prior conversion to fatty acyl-coenzyme A thioesters. This reaction is catalyzed by a group of acyl-CoA synthetases which differ in their chain length specificities and

subcellular locations, but which catalyze the same kind of reaction (7). Fatty acyl residues are transferred by carnitine acyltransferase I (CPT I) from CoA to carnitine at the outer mitochondrial membrane (8) to yield acylcarnitines, which pass through the inner mitochondrial membrane, catalyzed by carnitine-acylcarnitine translocase, into the matrix space where acyl groups are transferred back from carnitine to coenzyme A by carnitine acyltransferase II (CPT II) before entering the β-oxidation spiral (9). The outer carnitine acyltransferase (CPT I), which is facing the inter-membrane space, is subject to regulation by malonyl-CoA, whereas CPT II, which is facing the matrix space, is not affected by malonyl-CoA (10).

Enzymes required for β-oxidation of saturated fatty acids

Long-chain acyl-CoAs generated in the mitochondrial matrix by the reaction catalyzed by CPT II and medium-chain acyl-CoAs formed by activation of free acids in the matrix are substrates of β-oxidation. Acyl-CoA residues are degraded by a sequence of four reactions that yield acetyl-CoA and an acyl-CoA shortened by two carbon atoms (11). Each cycle of this sequence of four reactions results in the removal of two carbon atoms from the fatty acyl residues in the form of acetyl-CoA (see Fig. 1).

The first reaction is the dehydrogenation of acyl-CoA to 2-*trans*-enoyl-CoA which is catalyzed by a group of four acyl-CoA dehydrogenases which differ in their chain length specificities (EC 1.3.99.2) and EC 1.3.99.3).

The second reaction in the β-oxidation pathway is the hydration of 2-*trans*-enoyl-CoA to L-3-hydroxyacyl-CoA catalyzed by the soluble ma-

trix enzyme enoyl-CoA hydratase or crotonase (EC 4.2.1.17), and a long chain enoyl-CoA hydratase (EC 4.2.1.74).

The third reaction is the dehydrogenation of L-3-hydroxyacyl-CoA dehydrogenase to 3-ketoacyl-CoA catalyzed by L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) and long chain L-3-hydroxyacyl-CoA dehydrogenase.

The fourth and final reaction of the β-oxidation spiral is the thiolytic cleavage of 3-ketoacyl-CoA thioesters by 3-ketoacyl-CoA thiolase (EC 2.3.1.16), and long chain 3-ketoacyl-CoA thiolase. Products of this reaction are acyl-CoA chain-shortened by two carbon atoms and acetyl-CoA.

Peroxisomal β-oxidation

Peroxisomes in animals and other organisms contain a β-oxidation system which is distinct from the mitochondrial system. Peroxisomal β-oxidation is inducible and the enzymes are coded for by genes which differ from the genes of the mitochondrial β-oxidation enzymes (12).The first step of the peroxisomal fatty acid β-oxidation pathway differs from the first step of mitochondrial β-oxidation in that the peroxisomal dehydrogenation is catalyzed by acyl-CoA oxidase, which transfers electrons from acyl-CoA via its cofactor flavine adenine dinucleotide to O_2 to form H_2O_2 (13). The hydration of 2-*trans*-enoyl-CoA to L-3-hydroxyacyl-CoA and the NAD^+-dependent dehydrogenation are catalyzed by a trifunctional enzyme, which harbors enoyl-CoA hydratase, Δ^3,Δ^2-enoyl-CoA isomerase and L-3-hydroxyacyl-CoA dehydrogenase activities (11). The final step of β-oxidation in peroxisomes is the thiolytic cleavage of 3-ketoacyl-CoA catalyzed by 3-ketoacyl-CoA thiolase. Acyl-CoA oxidase from rat liver

peroxisomes, which is thought to catalyze the rate-limiting step in peroxisomal β-oxidation, is almost inactive toward octanoyl-CoA and shorter chain acyl-CoAs (13). Consequently, the peroxisomal β-oxidation system of rat liver does not catalyze the complete degradation of fatty acids.

β-Oxidation of unsaturated fatty acids

All double bonds found in unsaturated and polyunsaturated fatty acids can be classified either as double bonds extending from odd-numbered carbon atoms, like the 9-*cis* double bond present in oleic acid, linoleic acid and many other polyunsaturated fatty acids, or as double bonds extending from an even-numbered carbon atoms like the 12-*cis* double bond of linoleic acid. Unsaturated fatty acids are also degraded via the β-oxidation spiral except that the degradation of unsaturated fatty acids by β-oxidation involves at least two auxiliary enzymes in addition to the enzymes required for the breakdown of saturated fatty acids (11). The auxiliary enzymes acting on double bonds are 2,4-dienoyl-CoA reductase or 4-enoyl-CoA reductase (EC 1.3.1.34) and Δ^3,Δ^2-enoyl-CoA isomerase (EC 5.3.3.8) (14).

Chain shortening of unsaturated fatty acids with double bonds extending from even-numbered carbon atoms leads to the formation of 4-enoyl-CoAs, which are dehydrogenated by acyl-CoA dehydrogenase to 2,4-dienoyl-CoAs. An NADPH-dependent 2,4-dienoyl-CoA reductase, originally described by Kunau and Dommes (15), catalyzes the reduction of 2,4-dienoyl-CoAs to 3-enoyl-CoAs, which after isomerization by Δ^3,Δ^2-enoyl-CoA isomerase to 2-enoyl-CoAs, can be completely degraded via the β-oxidation spiral.

Unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms are, according to Stoffel and Caesar (16), chain shortened to 3-enoyl-CoAs, which, after isomerization to 2-enoyl-CoAs by Δ^3,Δ^2-enoyl-CoA isomerase, reenter the β-oxidation spiral. 5-Enoyl-CoAs are intermediates which would pass once more through the β-oxidation spiral before being acted upon by Δ^3,Δ^2-enoyl-CoA isomerase. This prediction, however, is contradicted by a recent observation of Tserng and Jin (17) who reported that the mitochondrial β-oxidation of 5-enoyl-CoAs is dependent on NADPH. Their analysis of metabolites by gas chromatography/mass spectrometry led them to propose that the double bonds of 5-enoyl-CoAs are reduced by NADPH to yield the corresponding saturated fatty acyl-CoAs, which are then further degraded by β-oxidation.

2,4-Dienoyl-CoA reductases from bovine liver (18), rat liver (19), and *E. coli.* (18,20) have been purified to homogeneity and their properties have been studied extensively (19,21,22). Although they have similar substrate specificities, enzymes from different organisms differ in their molecular weight and catalytic properties. Eukaryotic reductases (21,23,24) catalyze the reduction of 2,4-dienoyl-CoA thioesters to 3-*trans*-enoyl-CoAs (20.21) whereas the reductase from *E. coli* yields 2-*trans*-enoyl-CoAs (21). The eukaryotic enzyme is a homotetramer with a native molecular weight of 124,000 daltons (21), whereas the *E. coli* enzyme is a monomer with a molecular weight of 70,000 daltons (20). NADPH is absolutely required for all three reductases all of which act on 2-*trans*,4-*cis*-dienoyl-CoA and 2-*trans*-4-*trans*-dienoyl-CoA,

Peroxisomal β-oxidation of unsaturated and polyunsaturated fatty acids proceeds overwhelmingly by the reductase-dependent pathway (23). A small percentage of polyunsaturated fatty acids may be degraded via the original epimerase dependent-pathway which differs from the reductase-dependent pathway shown in Fig. 2 only in the metabolism of 2-*trans*,4-*cis*-dienoyl-CoA. A small percentage, estimated to be 2% (25), may pass through an additional cycle of β-oxidation to yield 2-*cis*-enoyl-CoA as a result of intermediate channeling on the trifunctional enzyme. 2-*cis*-Enoyl-CoA is hydrated by enoyl-CoA hydratase to D-3-hydroxyacyl-CoA and epimerized by two enzymes acting in sequence; a novel peroxisomal D-3-hydroxyacyl-CoA dehydratase converts D-3-hydroxyacyl-CoA to 2-*trans*-enoyl-CoA which is rehydrated by enoyl-CoA hydratase to L-3-hydroxyacyl-CoA (26,27). Although rat liver peroxisomes are capable of chain-shortening regular fatty acids, their main function seems to be the partial β-oxidation of very long chain fatty acids, prostaglandins, dicarboxylic acids, and hydroxylated 5-β-cholestanoic acid. (28).

Regulation of mitochondrial fatty acids β-oxidation

Regulation of hepatic β-oxidation is more complex than is the regulation in other tissues, since liver has high capacities for oxidizing and synthesizing fatty acids in addition to ketogenesis. Livers of fed animals convert carbohydrates to fatty acids, but in the fasting or diabetic animal fatty acids are mostly oxidized to produce ketone bodies. Thus an inverse relation exists between fatty acid oxidation and fatty acid synthesis. The rate-limiting and regulated step in fatty acid oxidation is thought to be the reaction catalyzed by carnitine palmitoyltransferase I (CPT I) (29). The

proposed regulator, malonyl-CoA, is formed by acetyl-CoA carboxylase in the rate-limiting reaction of fatty acid synthesis and it effectively inhibits CPT I at low micromolar concentrations (29). When fatty acid synthesis and thus the concentration of malonyl-CoA are high, CPT I and consequently β-oxidation are inhibited. However, during fasting, when the glucagon concentration increases, acetyl-CoA carboxylase is inactivated by phosphorylation and the malonyl-CoA concentration decreases with the results that CPT I is deinhibited and the rate of fatty acid oxidation increases. The regulation of carnitine palmitoyltransferase (CPT I) by malonyl-CoA determines the flux of fatty acids into mitochondria and thereby controls rates of β-oxidation and ketogenesis (29) . This regulatory mechanism assures that during fasting fatty acids are directed toward oxidation in mitochondria, whereas in the fed animal they are mostly incorporated into lipids at the endoplasmic reticulum. Whether or not this regulatory mechanism is important in the control of fatty acid oxidation in extra-hepatic tissues remains to be established. However, in heart, which obtains most of its energy from the oxidation of fatty acids (30), the rate of β-oxidation also changes in response to changes in the energy demand of the tissue. Studies with perfused hearts led Neely and coworkers (31) to conclude that at low plasma concentrations of palmitate (≤0.6 mM) the cellular uptake of fatty acids seems to limit β-oxidation, whereas at higher palmitate levels β-oxidation seems to be restricted by the oxidation of acetyl-CoA. Increasing the ventricular pressure to simulate the imposition of a higher workload on the heart resulted in higher O_2 consumption, higher CO_2 formation, and a decrease in acetyl-CoA concentration. Experiments with coupled rat heart mitochondria (32), showed that the higher energy demand of state 3 respiration as compared to state 4 respi-

ration is associated with lower ratios of [acetyl-CoA]/[CoASH] and [NADH]/[NAD^+] in the mitochondrial matrix. The thiolytic cleavage catalyzed by 3-ketoacyl-CoA thiolase may be the site at which β-oxidation is controlled by the [acetyl-CoA]/[CoASH] ratio (33). Since long-chain acyl-CoAs, which are the substrates of β-oxidation, are present in the matrix at concentrations sufficient to support high rates of β-oxidation in isolated mitochondria at state 4 respiration or in perfused hearts with low workloads imposed, the removal of β-oxidation products, specifically the oxidations of NADH and $FADH_2$ as well as the conversion of acetyl-CoA to CoASH, seems to restrict the rate of β-oxidation. Evidence has been presented showing that the NAD^+-linked oxidation of 3-hydroxyacyl-CoAs is more easily suppressed than is the FAD-linked dehydrogenation of acyl-CoAs (34).

Regulation of unsaturated fatty acids β-oxidation

According to the original proposal, Δ^3,Δ^2-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase (EC 5.1.2.3) function as auxiliary enzymes (16). However, when an NADPH-dependent 2,4-dienoyl-CoA reductase, originally referred to as 4-enoyl-CoA reductase, was identified and shown to effectively reduce 2-*trans*,4-*cis*-decadienoyl-CoA, an intermediate of linoleate β-oxidation, to 3-*trans*-decenoyl-CoA (15), a modified pathway was proposed (14). Demonstration that (a) 2-*trans*,4-*cis*-decadienoyl-CoA cannot be directly chain-shortened by the mitochondrial β-oxidation enzymes (35), (b) 3-hydroxyacyl-CoA epimerase is not present in mitochondria (36), and (c) NADPH is required for the degradation of polyunsaturated fatty acids (37), led to the acceptance of the reductase-dependent pathway. The NADPH required to sustain 2,4-

dienoyl-CoA reductase activity in mitochondria is furnished by the activity of isocitrate dehydrogenase (EC 1.1.1.42) or NAD(P)H transhydrogenase (EC 1.6.1.1) or by glutamate dehydrogenase (EC 1.4.1.3) (28).

Isolated mitochondria can oxidize polyunsaturated fatty acids. Most of them are oxidized at rates comparable to those of the corresponding saturated and mono-unsaturated fatty acids. The position of a single double bond, however, affects rates of mitochondrial β-oxidation: those isomers with their double bonds near the carboxyl group are oxidized at slower rates (38) than fatty acids with double bonds removed from the carboxyl end. Some polyunsaturated fatty acids, e.g. γ-linolenate, arachidonate, and docosahexaenoate, give low rates of β-oxidation under optimal incubation conditions. In contrast to other fatty acids, β-oxidation of these fatty acids is stimulated by substrates generating intra-mitochondrial NADPH (e.g. glutamate), stimulated by a high intra-mitochondrial concentration of NADH, and consequently inhibited by conditions facilitating re-oxidation of intra-mitochondrial NADH, and inhibited by uncouplers of oxidative phosphorylation. This inhibition can be overcomed by stimulation of NAD(P)H transhydrogenase activity, or by facilitating other reactions which generate NADPH. High intramitochondrial concentrations of NADPH stimulate β-oxidation of polyunsaturated fatty acids, while low concentrations impair their oxidation. (39)

Long chain unsaturated fatty acids have an important function as components of biomembrane phospholipids which regulate membrane-associated cell functions, such as enzyme activity, cell fusion, endocytosis, permeability, and transmembrane transport (40). In addition polyunsaturated fatty acids are precursors for prostanoids which are involved in

various physiological and pathological processes such as hemostasis, thrombosis, and lipoprotein metabolism. It has been shown that stimulation of mitochondrial protein synthesis (41) and changes in the composition of mitochondrial phospholipids are caused by growth hormones (42). Growth hormone treatment of hypophysectomized rats results in an increased incorporation of polyunsaturated fatty acids, such as docosahexaenoic acid, into mitochondrial phospholipids (42). These alterations of mitochondrial phospholipids correlate with changes in membrane properties which may in turn result in activity changes of some membrane-bound enzymes. Specific examples include activity increases of NADH dehydrogenase and of the energy-dependent NAD(P)H transhydrogenase as a result of growth hormone treatment of hypophysectomized rats.

The slower oxidation rate of docosahexaenoic acid (C_{22}:6) and other long chain polyunsaturated fatty acids as compared to that of palmitic acid or oleic acid could be a result of their higher degree of unsaturation or their longer chain lengths. Recently, it has been shown that liver mitochondrial 2,4-dienoyl-CoA reductase activity is subject to regulation by growth hormone (43). Growth hormone treatment of hypophysectomized rats (deficient in growth hormone) caused a three fold increase in the activity of 2,4-dienoyl-CoA reductase in mitochondria. The activities of other β-oxidation enzymes remained unchanged. The same study also showed that rates of respiration supported by polyunsaturated fatty acids and rates of β-oxidation of polyunsaturated fatty acids were stimulated in rats treated with growth hormone. These results suggest that the rates at which polyunsaturated fatty acids are oxidized in rat liver mitochondria are limited by the availability of NADPH

or the activity of 2,4-dienoyl-CoA reductase. However, it is generally assumed that carnitine palmitoyl transferase (CPT I) catalyzes the rate-limiting reaction in the β-oxidation of long chain fatty acids. It is known that the activity of this enzyme in rat liver mitochondria declines as the acyl chain length of substrates exceeds sixteen carbon atoms (44).

In an attempt to identify the rate-limiting step in β-oxidation of polyunsaturated fatty acids in heart, rates of 2,4-dienoyl-CoA reductase and CPT I relative to rates of fatty acid oxidation were determined and analyzed. In addition I measured activities of 2,4-dienoyl-CoA reductase in tissue samples of a patient with a suspected deficiency of 2,4-dienoyl-CoA reductase. This collaborative work prompted the design of more sensitive assay methods of 2,4-dienoyl-CoA reductase. Finally I participated in an effort to elucidate the NADPH-dependent β-oxidation of 5-enoyl-CoAs.

MATERIALS AND METHODES

D-[1-^{3}H]Glucose, and [1-^{14}C]docosahexaenoic acid were purchased from DuPont/New England Nuclear. [Methyl-1-^{3}H]L-carnitine and [1-^{14}C]palmitic acid were obtained from Amersham. Fisher Scientific was the source of Scinti Verse II. Defatted bovine serum albumin, pig heart L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), acyl-CoA oxidase from *Arthrobacter sp.*, hexokinase, D-glucose 6-phosphate dehydrogenase, catalase, bovine growth hormone, acetyl-CoA, n-butyryl-CoA, n-hexanoyl-CoA, n-octanoyl-CoA, behenoyl-CoA, malonyl-CoA, malonic acid, NADPH, NAD^{+}, $NADP^{+}$, ATP, CoASH, L-carnitine, and other biochemicals were purchased from Sigma. Palmitoyl-L-carnitine was a gift from Dr. R. Bressler, University of Arizona Medical School. 2-Tetradecylglycidic acid was provided by Dr. John Lowenstein, Brandeis University. 4-*cis*-Decenal was obtained from K & K Laboratories, Inc. Aldrich Chemical Co. was the source of 2-*trans*,4-*trans*-decadienal, 2-*trans*-decenoic acid and *trans*-cinnemaldehyde. 2-*trans*,4-*trans*-Octadienal and 3-*trans*-octadecenoic acid were purchased from Bedoukian, Danbury, CT and Pfaltz and Bauer, respectively. Docosahexaenoyl-L-carnitine was prepared by the method of Cervenka and Odmundsen (45), and was purified by high performance liquid chromatography on a reverse phase Sep-Pak cartridge, and stored as a methanolic solution in the presence of an antioxidant, butylated hydroxy toluene, at -20^{o}C. 5-Phenyl-2,4-pentadienoic acid was synthesized by reacting malonic acid in the presence of pyridine with cinnemaldehyde, an aldehyde two carbons shorter than the desired acid, as described in principle by Linestead et al. (46). The acid was crystalized at -20 ^{o}C from

petroleum ether/ ether in the form of white needles with a mp of 166-168 ^{o}C (literature mp 166-167 ^{o}C[47]).

4-*cis*-Decenoic acid, 2-*trans*,4-*trans*-decadienoic acid, and 2-*trans*-4-*trans*-octadienoic acid were prepared from 4-*cis*-decenal, 2-*trans*, 4-*trans*-decadienal, and 2-*trans*-4-*trans*-octadienal respectively, by oxidation with Ag_2O according to a general procedure for the oxidation of aldehydes to acids that are sensitive to strong oxidizing agents (48). 2-*trans*,4-*trans*-Octadienoic acid, after crystallization from hexane, had a mp of 75-76°C (literature mp 76°C (49). 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 methyl 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. The CoA derivatives of 5-*cis*-octenoic acid, 5-*trans*-octenoic acid, 3-*trans*-octenoic acid, 4-*cis*-decenoic acid, 2-*trans*,4-*trans*-decadienoic acid, 5-phenyl-2,4-pentadienoic acid, and 2-*trans*,4-*trans*-octadienoic acid were synthesized according to the procedure of Goldman and Vagelos (50). All synthetic acyl-CoAs used in HPLC or spectrophotometric experiments were purified by HPLC. 2-*trans*,5-*cis*-Octadienoyl-CoA and 2-*trans*,5-*trans*-octadienoyl-CoA , and 2-*trans*,4-*cis*-decadienoyl-CoA were synthesized from the corresponding 5-octenoyl-CoAs, and 4-*cis*-decadienoyl-CoA, respectively, by allowing them to react with oxygen in the presence of acyl-CoA oxidase from *Arthrobacter sp.* as described previously (25) except that catalase was omitted from the incubation mixture. The concentrations of

acyl-CoA thioester solutions were determined by the method of Ellman (51) after cleaving the thioester bond with 1 M hydroxylamine at pH 7. The two isomeric 2,5-octadienoyl-CoAs were purified by HPLC. Bovine liver enoyl-CoA hydratase (EC 4.2.1.17) or crotonase (52), the trifunctional enzyme from rat liver peroxisomes (53,54), and pig heart 3-ketoacyl-CoA thiolase (EC 2.3.1.16) (55) were purified as previously described. 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 hydroxylapatite as described by Kilponen *et al.* (56). 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 (57). Medium chain acyl-CoA dehydrogenase was partially purified from bovine liver mitochondria as described (58).

Synthesis of [4B-^{3}H]NADPH.

[4B-^{3}H]NADPH was synthesized essentially as described (59). In brief, 17 nmol of D-[1-^{3}H]glucose (250 μCi) were combined with 100 μl of a solution containing 0.1 μmol of ATP, 5 μmol of Tris-HCl (pH 8), 1 μmol of $MgCl_2$, and hexokinase (0.1 U). The solution was kept at room temperature for 15 min after which time 50 nmol of $NADP^+$ and glucose 6-phosphate dehydrogenase (0.15 U) were added. After continuing the incubation for 30 min the solution was diluted with water to give a final volume of 1 ml and applied to a DEAE-cellulose mini column (0.2 ml bed volume) which had been equilibrated with ammonium formate and

thereafter washed with water. The column was developed with 1 ml of 75 mM ammonium formate, followed by 4 ml of 150 mM ammonium formate and 1.5 ml of 500 mM ammonium formate. All components of the reaction mixture, except for NADPH, were removed from the column after the initial two gradient steps. [4B-^{3}H]NADPH was eluted with 500 mM ammonium formate and diluted more than 1000-fold with NADPH to a specific radioactivity of 30,000 dpm/nmol. Aliquots containing 2 μCi were lyophilized in ampoules which were sealed under N_2 and stored at -78°C.

Preparation of tissue extracts.

Human fibroblasts (approximately $5x10^6$ cells obtained from one 75 cm^2 tissue culture flask), provided by Dr. J.-H. Ding, Duke University Medical Center, were suspended in 1 ml of a solution containing 0.25 M sucrose, 5 mM Tris-HCl (pH 7.4), 1 mM [ethylenebis-(oxyethylenenitrilo]tetraacetic acid (STE buffer) and sonicated ten times for eight seconds each with a Branson sonifier (Model W-385) equipped with a microtip. The sonicated homogenate was assayed for 2,4-dienoyl-CoA reductase. For the preparation of bovine liver homogenates, and the human soluble tissue extracts, tissue pieces were finely cut with chilled sharp scissors and washed several times with ice-cold STE buffer to remove extracellular proteins. The minced livers were homogenized in a Potter-Elvehjem tissue homogenizer and sonicated as described above, whereas psoas muscle was homogenized with a polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY). The resultant homogenates were centrifuged for 1 hr at 100,000 x g and the resultant supernatant was used to assay the enzyme activities. Isolated bovine liver mitochondria or rat liver mitochondria were sonicated and centrifuged as

described above to yield mitochondrial soluble extracts. Protein concentrations of the homogenates were determined by the Bradford method (60).

Isolation of mitochondria and respiration measurements.

Rat heart mitochondria were isolated as described by Chappell and Hansford (61). For respiration measurements, rat heart mitochondria (1 mg of protein) were suspended in 1.9 ml of a basal iso-osmotic medium containing 0.11 M KCl, 3.3 mM Tris-HCl (pH 7.4), 2 mM KP_i, 2 mM $MgCl_2$, and 0.1 mM EGTA. To this suspension were added, in the indicated sequence, defatted bovine serum albumin (1mg/ml), 0.5 mM L-malate, and 0.25 mM ADP . Respiration was started by the addition of 40 µM palmitoyl-L-carnitine, or 40 µM palmitoyl-CoA, or 20 µM docosahexaenoyl-CoA. When acyl-CoAs were used, 0.4 mM L-carnitine was added to the reaction mixture prior to the addition of the acyl-CoA. Respiration rates were measured polarographically at 30°C with a Clark oxygen electrode attached to a Gilson oxygraph. When malonyl-CoA or tetradecylglycidyl-CoA were included in this standard incubation mixture, their concentrations are indicated in the Figures. Rat liver mitochondria were isolated as described above except that nagarse was omitted.

Radiolabelled assay of CPT I

For measuring CPT I activity in mitochondria isolated from control rats, hypophysectomized rats, or hypophysectomized rats treated with growth hormones, a modification of "assay II", described by McGarry et al. (62) which measures the formation of palmitoylcarnitine from palmitoyl-

CoA plus carnitine, was employed. Reactions were carried out at 30 °C in glass culture tubes (13 mm x 100 mm). The standard incubation mixture initially contained, in a volume of 0.9 ml: Tris/HCl (pH 7.4), 105 μmol; reduced glutathione, 0.25μmol; ATP, 4μmol; $MgCl_2$, 4μmol; KCl, 15μmol; KCN, 2μmol; rotenone. 40 μg; defatted albumin, 10 mg; palmitoyl-CoA, 40 nmol (or 30nmol docosahexaenoyl-CoA); L-carnitine, 400nmol; and DL-[methyl-^{3}H]carnitine, 1μCi. Reactions were initiated by the addition of 0.1ml of the mitochondria suspension (0.2-0.5 mg of protein) and were terminated with 1 ml of ice cold 1.2 M HCl. The formed acylcarnitine was extracted and counted as described (62).

When both rates of respiration and CPT I activities were determined with rat heart mitochondria, the incubation mixture contained, in addition to the substrates, 0.11 M KCl, 3.3 mM Tris-HCl (pH 7.4), 2 mM KP_i, 2 mM $MgCl_2$, and 0.1 mM EGTA. Defatted bovine serum albumin was added at a concentration of 1 mg/ml (63). Malonyl-CoA or tetradecylglycidyl-CoA were included in control reactions to completely inhibit CPT I. All reactions were terminated after 2-5 min.

Assays of 2,4-dienoyl-CoA reductase.

(a) Spectrophotometric assay.

2,4-Dienoyl-CoA reductase was assayed spectrophotometrically by measuring the oxidation of NADPH at 340 nm with a Gilford (Model 240) recording spectrophotometer. The assay mixture (1 ml) contained 0.2 M potassium phosphate (pH 8), 0.1 mM NADPH, 25 μM 2-*trans*,4-*cis*-

decadienoyl-CoA or 25 μM 2-*trans*,4-*trans*-decadienoyl-CoA or 25 mM 5-phenyl-2,4-pentadienoyl-CoA and tissue extract to give an absorbance change of 0.01 to 0.025 A/min. The reaction was started by the addition of 2,4-dienoyl-CoA.

(b) High performance liquid chromatography assay.

The assay mixture was the same as described above. The reaction was terminated by the addition of 0.1 ml of concentrated HCl to the assay mixture of 1 ml. After keeping the mixture for 30 min at 4°C it was clarified by centrifugation in a table top centrifuge. The pH of the supernatant was adjusted to 5.5 with 1 N KOH. Final clarification of the solution was achieved by centrifugation followed by filtration through 0.22 μm filters. Aliquots were analyzed for $NADP^+$ by high performance liquid chromatography (HPLC) using a μBondapak C_{18} reverse-phase column (30 cm x 3.9 mm) attached to a Waters gradient HPLC system. Separation of $NADP^+$ from other components was achieved by linearly increasing the methanol content of the 50 mM ammonium phosphate elution buffer (pH 5.5) from 0 to 20% in 25 min. $NADP^+$ was quantitated by use of a standard curve.

(c) Radioactive assay.

The assay mixture of 0.2 ml in a glass culture tube (16 x 125 mm) contained 0.2 M potassium phosphate (pH 8), 0.1 mM [4B-^{3}H] NADPH (0.272 μCi), 25 μM 2-*trans*,4-*cis*-decadienoyl-CoA and tissue extract with approximately 15 μg of protein. The reaction was initiated by the addition of 2,4-decadienoyl-CoA and terminated after 2 min or at the indicated times by the addition of 0.5 ml of 2 M hydroxylamine

(pH 7). After keeping the mixture for 20 min at 25°C, it was acidified by the addition of 2 ml of 1.2 M HCl and extracted with 2 ml of toluene by vortexing and centrifuging the tube. One ml of the toluene phase was withdrawn, mixed with 4 ml of Scinti Verse II and analyzed for radioactivity in a liquid scintillation counter. 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.

Metabolic studies with 2,5-octadienoyl-CoA.

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. 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.

Spectrophotometric measurements.

All scans were recorded on a microprocessor-controlled Gilford 2600 UV-VIS spectrophotometer interfaced with a Hewlett Packard 7225B graphics plotter. The incubation conditions were the same as those detailed under 2,5-octadienoyl-CoA metabolic studies.

HPLC Analysis.

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. The product of the radioactive 2,4-dienoyl-CoA assay, and the product of the reductase with 5-phenyl-2,4-pentadienoyl-CoA as a substrate, were purified by HPLC by linearly increasing the acetonitrile content of the 25 mM ammonium phosphate elution buffer (pH 5.5) from 20 to 80% in 20 minutes at a flow rate of 2 ml/min. For quantitating the radioactivity of the eluted peaks, eluted fractions were collected every 30 sec using a Pharmacia LKB 200 fractionator. Aliquots of 0.2 ml were mixed with 4 ml of Scinti Verse II and analyzed for radioactivity in a liquid scintillation counter.

Animals and growth hormone treatment:

Male Sprague-Dawely rats were obtained from Taconic Farms, Inc., Germantown, N.Y. and maintained on Purina laboratory chow. Control rats weighed 200-250 g. while hypophysectomized rats weighed 117-141 g. Hypophysectomized rats were provided with 5% dextrose solution instead of water. Hypophysectomized rats were kept for two weeks , then bovine growth hormone (0.1 IU/100g body weight) was injected subcutaneously daily for seven days. Hypophysectomized rats injected with growth hormone gained approximately 23 g over seven days of hormone injection while untreated hypophysectomized rats did not gain any weight during this period.

RESULTS

I. The rate limiting step in the β-oxidation of long chain polyunsaturated fatty acids in heart

In heart, docosahexaenoic acid (C_{22}:6) and other long chain polyunsaturated fatty acids are degraded by β-oxidation at slower rates than palmitic acid or oleic acid (64). These rate differences could be due to the longer chain lengths or higher degrees of unsaturation of the polyunsaturated fatty acids. Evidence presented by Osmundsen et. al. (65) suggests that the removal of double bonds extending from even-numbered carbon atoms may be rate limiting in the degradation of long polyunsaturated fatty acids because either the activity of 2,4-dienoyl-CoA reductase or the concentration of NADPH are suboptimal in rat liver mitochondria. Clejan and Schulz (43) showed that the mitochondrial 2,4-dienoyl-CoA reductase activity in rat liver is regulated by growth hormone. Growth hormone treatment of hypophysectomized rats caused a three fold increase in the activity of 2,4-dienoyl-CoA reductase in mitochondria. The same study demonstrated that growth hormone treatment also resulted in increased rates of respiration supported by polyunsaturated fatty acids. These results suggest that the rates at which polyunsaturated fatty acids are oxidized in rat liver mitochondria may be limited by the activity of 2,4-dienoyl-CoA reductase and / or the availability of NADPH.

Abd El-Aleem (64) extended this study to heart and assessed how growth hormones affects the rates of β-oxidation of oleate and docosahexaenoate in rat heart mitochondria and myocytes isolated from control rats, hypophysectomized rats, and hypophysectomized rats

treated with growth hormones (64). Although growth hormone treatment increased the activity of 2,4-dienoyl-CoA reductase 2-fold, the rate of docosahexaenoate oxidation was unaffected by growth hormone treatment. Since the slower oxidation rates of long chain polyunsaturated fatty acids could be due to their slower uptake by mitochondria, activities of carnitine palmitoyltransferase I (CPT I) in control rats, hypophysectomized rats, and hypophysectomized rats treated with growth hormones were measured. Both hypophysectomy, and hypophysectomy followed by growth hormone treatment did not significantly affect the CPT I activity in either rat liver or rat heart (Tables 1 and 2). The results also show that the activities measured with behenoyl-CoA ($C_{22:0}$) or docosahexaenoyl-CoA ($C_{22:6}$) as substrates are similar and about 15% of the activity measured with palmitoyl-CoA ($C_{16:0}$). The rates of heart CPT I activity in this study were lower than rates reported in the literature (63), most likely due to the higher concentration of serum albumin in the assay mixture used in this study (10 mg/ml). Serum albumin is known to bind long chain fatty acyl-CoAs thereby lowering their free concentrations (66) and possibly rates of acyl transfer. The activities of CPT I in intact rat heart mitochondria were also measured with docosahexaenoyl-CoA and with palmitoyl-CoA in the presence of lower concentration of bovine serum albumin (1 mg/ml). The results are listed in Table 3. Although the activities measured with docosahexaenoyl-CoA and palmitoyl-CoA are higher at the lower concentration of bovine serum albumin, the ratios of docosahexaenoyl transfer to palmitoyl transfer were the same at both concentrations of bovine serum albumin (compare Tables 2 and 3). The lower activity obtained with docosahexaenoyl-CoA as compared to the activity with palmitoyl-CoA is not surprising since it is known that the

activity of CPT I in rat liver decreases as the acyl chain length of the substrate exceeds sixteen carbon atoms (44). Rates of mitochondrial respiration were determined with $C_{22:6}$-CoA, $C_{22:6}$-carnitine , $C_{16:0}$-CoA , $C_{16:0}$-carnitine as substrates. With palmitoyl-CoA and palmitoylcarnitine, higher rates were observed than with the derivatives of docosahexaenoic acid. The initial rate of respiration with $C_{22:6}$-CoA as a substrate was lower than the rate obtained with $C_{22:6}$-carnitine but increased with time until both rates were the same (see Fig. 3). This finding suggests that CPT I is not catalyzing the rate-limiting step in the β-oxidation of $C_{22:6}$-CoA. A comparison of the CPT I activities (see Table 3) and rates of respiration (see Table 4) also supports this conclusion, whereas a study in which CPT I inhibitors were used, seems to contradict it (see Figs. 4, and 5). In this study, the inhibition of CPT I by 2-tetradecyglycidyl-CoA (TDG-CoA), which is known to inhibit CPT I activity irreversibly (67), and by malonyl-CoA, which inhibits the enzyme reversibly and is believed to regulate CPT I *in vivo* (29), was compared with the effect of the two inhibitors on the rates of respiration. Intact rat heart mitochondria were incubated with varying concentrations of the inhibitor prior to measuring CPT I activities and respiration rates. Both the CPT I activities and respiration rates showed similar responses to TDG-CoA (Fig. 4 and Fig. 5) and malonyl-CoA inhibition (data not shown). Both TDG-CoA and malonyl-CoA when added to rat heart mitochondria, caused parallel inhibitions of CPT I activity and of respiration rates supported by either palmitoyl-CoA or docosahexaenoyl-CoA. As expected the inhibitors did not affect rates of respirations supported by either palmitoylcarnitine or docosahexaenoylcarnitine.

II. 2,4-Dienoyl-CoA reductase deficiency: A possible new disorder of fatty acid oxidation

Recently an unusual acylcarnitine was observed by fast atom bombardment tandem mass spectrometry in the urine of a hypotonic patient (68). Analysis of the patient's urine by this technique indicated that the intermediate was an acylcarnitine with an acyl group having 10-carbon atoms and two double bonds ($C_{10:2}$). Its origin from dietary long chain unsaturated fatty acids was strongly supported when a change of the diet to a new formula enriched with medium chain triglycerides but with a very low unsaturated fat content, resulted in a marked reduction of the putative $C_{10:2}$ acyl-carnitine in favor of acetylcarnitine, the normal metabolite. Further structural information was obtained when the acyl-carnitine recovered from the patient's urine was subjected to mild alkaline hydrolysis and the liberated acids were analyzed as methyl esters by capillary GC/MS. The mass spectrum of the main peak was identical with authentic methyl 2-*trans*,4-*cis*-decadienoate. Since 2-*trans*,4-*cis*-decadienoyl-CoA and 2-*trans*,4-*trans*-decadienoyl-CoA are known substrates of NADPH-dependent 2,4-dienoyl-CoA reductase, the activity of the reductase in the patient's liver and muscle was assayed with 2-*trans*,4-*trans*-decadienoyl-CoA which is an easily available substrate. Although the reductase activities in the patient's liver and muscle were lower than in control tissues, the residual activity in the patient was relatively high with this substrate (see Table 5). Since the fatty acid metabolite identified in the urine of the patient was 2-*trans*,4-*cis*-

decadienoylcarnitine, 2-*trans*,4-*cis*-decadienoyl-CoA was used for assaying the reductase activities. The residual reductase activity in the patient's muscle with 2-*trans*,4-*cis*-decadienoyl-CoA was only 17% of the activity found in control muscle. In liver reductase activity was 40% of the control (Table 5).

Since the patient was hyperlysinemic, the effect of lysine on the activity of 2,4-dienoyl-CoA reductase was evaluated. The presence of lysine in the reductase assay resulted only in a slight inhibition (≤20%) which remained virtually unchanged when the lysine concentration was changed from 1 to 10 mM (Table 6). To determine wether this deficiency is a result of an inherited genetic disorder, reductase activities of the patient's sibling and parent were measured in lymphocytes. Although the reductase activity was normal in the patient's sibling, the enzyme activity in the father's lymphocytes was about 50% of the control (see Table 7). Unfortunately, lymphocytes from the mother have not yet become available.

III. Design of sensitive assays for 2,4-dienoyl-coenzyme A reductase

1. Radioactive Assay

2,4-Dienoyl-CoA reductase is routinely assayed by measuring the oxidation of NADPH spectrophotometrically at 340 nm. Since the enzyme activity required for each spectrophotometric assay is close to one mU and since the specific activity of 2,4-dienoyl-CoA reductase in human tissues is only 10 mU per mg of protein or less (68), the spectrophotometric assay is not sufficiently sensitive for measuring levels of this enzyme in small tissue samples available from patients suspected of having a reductase deficiency. However, a radioactive assay would be several orders of magnitude more sensitive than the spectrophotometric method.

An outline of a radioactive assay for 2,4-dienoyl-CoA reductase is shown in Fig. 6. The assay is based on the reductase-catalyzed transfer of tritium from NADP(^{3}H) to substrate which after cleavage of the thioester bond can be easily separated from the radioactive coenzyme. Since Dommes and Kunau (20) have demonstrated that the bovine liver reductase catalyzes a 1,4-addition of hydrogen across the 2,4-diene system, the hydride ion from NADPH is assumed to be transferred to carbon atom 5 and hence will be resistant to exchange with protons of water. Dommes and Kunau (21) also established that bovine liver 2,4-dienoyl-CoA reductase catalyzes the transfer of hydrogen from the B side of NADPH (pro-S hydrogen) to the substrate. Thus, [4B-^{3}H]NADPH is required for this ra-

dioactive assay of 2,4-dienoyl-CoA reductase. It was prepared by the enzymatic reduction of $NADP^+$ with D-[1-^{3}H]glucose 6-phosphate in the presence of glucose 6-phosphate dehydrogenase as described by Moran et al. (59). When the activity of 2,4-dienoyl-CoA reductase was determined spectrophotometrically either with commercial NADPH or with synthesized [4B-^{3}H]NADPH the rate with the latter coenzyme preparation was found to be 85% of the former.

Termination of the reductase assay and the simultaneous conversion of substrate and product (s) to compounds which can be extracted with organic solvents was achieved by treating the assay with hydroxylamine at pH 7. The resultant radioactive hydroxamic acids were completely removed from the aqueous phase by a single extraction with toluene. This extraction procedure was unsatisfactory when sorboyl-CoA, a commonly used substrate of 2,4-dienoyl-CoA reductase, was utilized in this assay. The use of toluene resulted in low background values of 200 dpm in the blank which contained all assay components except for the substrate. The radioactivity associated with the reductase assays ranged from twice the blank value to 30 times this value. However, the sensitivity and range of the radioactive assay can be increased by using [4B-^{3}H]NADPH with a higher specific radioactivity.

Shown in Table 8 are the results which were obtained when the 2,4-dienoyl-CoA reductase activity of a bovine liver homogenate was determined as a function of time by three different methods. Although the formation of products determined by the radioactive assay was reasonably linear with time, the values were much lower than those obtained by the spectrophotometric method which is based on the disappearance of NADPH. To solve this discrepancy, the amount of $NADP^+$ formed under

identical conditions was quantitated by HPLC. The data shown in Table 8 clearly indicate that the values for NADPH disappearance and $NADP^+$ formation are similar although the latter values are slightly lower than the former values. In contrast, the ratio of radioactive product(s) formed *vs* NADPH oxidized was only close to 10% irrespective of the incubation time. Although the reductase rates obtained spectrophotometrically with [4B-^{3}H]NADPH were slightly lower (15%) than rates observed with commercial NADPH, this difference does not explain the lower rates obtained with the radioactive assay method. However, since either tritium or hydrogen is transferred during the reduction and since the transfer step may be rate-limiting, a primary kinetic isotope effect in the order of ten is not surprising (69). If absolute reductase activities are required, rates obtained by use of the radioactive assay must be multiplied with a correction factor, which is close to 10, but should be determined every time the assay is set up.

The products of the reductase reaction were separated by HPLC and analyzed for radioactivity. When a soluble extract of rat liver mitochondria was used as a source of 2,4-dienoyl-CoA reductase, most of the radioactivity was associated with 3-hydroxydecanoyl-CoA (see peak 1 in Fig. 7A) while little radioactivity was detected in the fractions containing 3-decenoyl-CoA and 2-decenoyl-CoA (see peak 2 in Fig. 7A). This product pattern was expected because Δ^3,Δ^2-enoyl-CoA isomerase (EC 5.3.3.8) and enoyl-CoA hydratase (EC 4.2.1.17) are present in a soluble mitochondrial extract and will convert 3-*trans*-decenoyl-CoA, the reductase reaction product, to 2-*trans*-decenoyl-CoA and further on to L-3-hydroxydecanoyl-CoA. In contrast, a partially purified preparation of 2,4-dienoyl-CoA reductase yielded mostly 3-decenoyl-CoA/2-decenoyl-CoA

and little 3-hydroxydecanoyl-CoA (see Fig. 7B). The radioactivity corresponding to peaks 1 and 2 accounted for 70 to 80% of the radioactivity extracted with toluene after cleavage of the thioester bond. This result suggests that the reductase products are completely extracted with toluene. The specific radioactivity of 3-hydroxydecanoyl-CoA corresponding to peak 1 in Fig. 7A was estimated and found to be approximately seven times lower than the specific radioactivity of [^{3}H]NADPH. Thus, a kinetic isotope effect is most likely responsible for the 10-fold lower incorporation of tritium into reductase products than would be expected from the spectrophotometrically observed oxidation of NADPH.

The activity of 2,4-dienoyl-CoA reductase present in an extract of bovine liver mitochondria was almost linearly dependent on the protein concentration irrespective of whether the activity was determined spectrophotometrically (see Fig. 8A) or by use of the radioactive assay (see Fig. 8B). Since a spectrophotometric assay required at least 130 µg of extracted mitochondrial protein, whereas 4 µg of protein was sufficient for the radioactive determination, the latter assay is at least 30 times more sensitive than the former one. This increased sensitivity of the radioactive method permitted 2,4-dienoyl- CoA reductase to be assayed in extracts of human fibroblasts which were only available in small quantities and which have low reductase levels. Shown in Fig. 9 are the results obtained with a homogenate of human fibroblasts. The formation of product was reasonably linear during the first two minutes of the reaction (see Fig. 9A) and the activity was linearly dependent on the protein concentration up to 20 µg per assay (see Fig. 9B). Based on these observations, a standard radioactive assay for measuring 2,4-dienoyl-CoA reductase in a fibroblast homogenate was established which contained 15 µg of protein

and was allowed to proceed for 2 minutes. Specific activities of 2,4-dienoyl-CoA reductase in human fibroblasts determined with this assay procedure varied between 1.8 mU/mg and 5.8 mU/mg of protein with a mean of 3.7 ± 1.55 U/mg based on data obtained with six fibroblast lines.

2. Assays of 2,4-dienoyl-CoA reductase with 5-phenyl-2,4-pentadienoyl-CoA substrate

5-Phenyl-2,4-pentadienoyl-CoA was synthesized and its U.V. spectrum was determined (see Fig. 10). The absorbance maximum of the 5-phenyl-2,4-dienoyl thioester chromophore is at 340 nm with an extinction coefficient of 44,338 M^{-1} cm^{-1}.

When 5-phenyl-2,4-pentadienoyl-CoA was incubated with NADPH and 2,4-dienoyl-CoA reductase, its characteristic spectrum with a maximum at 340nm disappeared in a time-dependent manner (Fig. 11). The observed absorbance change was dependent on the presence of NADPH and reductase. Similar results were observed when 5-phenyl-2,4-pentadienoyl-CoA was incubated with NADPH and a soluble mitochondrial extract (data not shown). The apparent kinetic constants (V_{max} and K_m) of 2,4-dienoyl-CoA reductase were determined with both 2-*trans*,4-*cis*-decadienoyl-CoA and 5-phenyl-2,4-pentadienoyl-CoA at a fixed concentration of 0.1mM NADPH. The apparent V_{max} of the reduction of 5-phenyl-2,4-pentadienoyl-CoA was 83 nmol/min per mg protein while that obtained for 2,4-decadienoyl-CoA was 579 nmol/min per mg protein The apparent K_m value for 5-phenyl-2,4-pentadienoyl-CoA was 8.4 μM and the value for 2,4-decadienoyl-CoA was 5.9 μM.

More evidence for the reduction of 5-phenyl-2,4-pentadienoyl-CoA and its subsequent β-oxidation was obtained by HPLC analysis. Incubation of 5-phenyl-2,4-pentadienoyl-CoA, NADPH, and 2,4-dienoyl-

CoA reductase resulted in the formation of a single product (see Fig. 12 b, peak 2) which was eluted from a reverse-phase HPLC column 1 min ahead of the starting material (Fig. 12B, peak 1). When a soluble extract of rat liver mitochondria, from which low molecular weight cofactors had been removed by filtration through Sephadex G-25, was used as a source of 2,4-dienoyl-CoA reductase, most of the product was eluted earlier (see peak 3 in Fig. 12C) while little product was associated with peak 2 This product pattern was expected because Δ^3,Δ^2-enoyl-CoA isomerase and enoyl-CoA hydratase are present in a soluble mitochondrial extract and might convert 3-*trans*-enoyl-CoA, the reductase reaction product, to 2-*trans*-enoyl-CoA and further on to L-3-hydroxyacyl-CoA. In contrast, a partially purified preparation of 2,4-dienoyl-CoA reductase yielded mostly the product eluted in peak 2 (3-decenoyl-CoA/2-decenoyl-CoA) and little product eluted in peak 3 (3-hydroxydecanoyl-CoA) (see Fig. 12B).

The reduction of 5-phenyl-2,4-pentadienoyl-CoA by the partialy purified rat liver reductase should result in the removal of one double bond thus the product should be 5-phenyl-pentenoyl-CoA, most likely 5-phenyl-3-pentenoyl-CoA because eukaryotic reductases catalyse the reduction of 2,4-dienoyl-CoA thioesters to the 3-*trans*-enoyl-CoA derivatives (18,19). If so , this product should not be hydrated by enoyl-CoA hydratase (crotonase) since crotonase only acts on 2-enoyl-CoA thioesters. This prediction was shown to be correct when little of the reduction product of the reductase (Fig.13A peak 2) was hydrated with a purified crotonase (see Fig. 13B). In contrast, incubation of the reductase reaction product with either purified trifunctional enzyme or mitochondrial soluble extract resulted in the conversion of 5-phenyl-pentenoyl-CoA into a more polar compound (see Fig. 13C, peak 3) which was eluted from a

reverse-phase HPLC column approximately 5 min ahead of the starting material. This product pattern was expected because Δ^3,Δ^2-enoyl-CoA isomerase and enoyl-CoA hydratase are present both in the soluble mitochondrial extract as well as in the trifunctional enzyme. Further evidence for the structure of the hydration product, presumed to be 5-phenyl-3-hydroxypentanoyl-CoA (peak 3), was obtained when it was converted by purified crotonase to a compound which emerged from HPLC at a position where 5-phenyl-pentenoyl-CoA was eluted (see Fig. 13E).

With 5-phenyl-2,4-pentadienoyl-CoA as a substrate, the activity of 2,4-dienoyl-CoA reductase present in an extract of rat liver mitochondria was linearly dependent on the protein concentration (see Fig. 14A) Shown also are the activities measured in the presence of 2-*trans*,4-*cis*-decadienoyl-CoA. Similar results were obtained when a rat heart mitochondrial extract was used as the source of 2,4-dienoyl-CoA reductase (data not shown). While the absorbance changes observed with either 2-*trans*,4-*cis*-decadienoyl-CoA or 2-*trans*,4-*trans*-decadienoyl-CoA as substrates showed less linearity with time, the formation of the product of 5-phenyl-2,4-pentadienoyl-CoA, using rat liver soluble mitochondrial extract as source of enzyme, was at least linear for five minutes (see Fig. 14B). Under the same conditions, the absorbance changes per min observed with 5-phenyl-2,4-pentadienoyl-CoA were higher than the absorbance changes measured with any of the other substrates.

IV. NADPH-dependent β-oxidation of unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms.

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 (17) 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 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 since acyl-CoA oxidases are known to dehydrogenate acyl-CoAs to 2-*trans*-enoyl-CoAs while reducing oxygen to H_2O_2 (53). The dehydrogenation product, 2-*trans*,5-*cis*-octadienoyl-CoA, was clearly separated from the starting material 5-*cis*-octenoyl-CoA by HPLC. The absorbance spectrum of 2-*trans*,5-*cis*-octadienoyl-CoA (see Fig. 15) is 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 (see Fig. 15). 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 (data not shown). The elution time of 3-hydroxy-5-*cis*-octenoyl-CoA formed by the 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 hydroxylapatite 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).

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 . This compound, which was eluted from a reverse phase HPLC column 1 min later than the starting material (data not shown), 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. As is apparent from Fig. 15B, the absorbance around 260 nm decreased immediately whereas an absorbance maximum close to 240 nm developed more slowly. The decrease in absorbance close to 260 nm was most likely due to the instantaneous hydration of the 2,3 double bond catalyzed by the high enoyl-CoA hydratase activity of the trifunctional enzyme. 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 (52). A partially purified preparation of mitochondrial Δ^3,Δ^2-enoyl-CoA isomerase brought about the same absorbance changes (data not shown). 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 (see Fig. 15C). 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 was coeluted with 2-*trans*,4-*trans*-octadienoyl-CoA but which was clearly separated from the starting material.

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 hydroxylapatite 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 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. As shown in Fig. 16, the absorbance at 300 nm disappeared 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. 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 Fig. 17A). 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 Fig. 17B). 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 (35). 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 , 5-octadienoyl-CoA yield 2-*trans*,4-*trans*-octadienoyl-CoA, because it was coeluted with authentic 2-*trans*,4-*trans*-octadienoyl-CoA.

DISCUSSION

The first *in vivo* evidence for the essential function of 2,4-dienoyl-CoA reductase in β-oxidation of unsaturated fatty acids was obtained with a prokaryotic organism. A mutation of 2,4-dienoyl-CoA reductase in *Eshcerichia coli* rendered the organism unable to grow on petroselinic acid (6-*cis*-octadecenoic acid) without affecting growth on oleic acid (70). Thus, in *E. coli* 2,4-dienoyl-CoA reductase is required for the *in vivo* degradation of unsaturated fatty acids with double bonds extending from even-numbered carbon atoms. A similar conclusion was reached with respect to eukaryotic organisms based on the evaluation and study of a patient with a defect in fatty acid oxidation. Accumulation of 2-*trans*,4-*cis*-decadienoylcarnitine in the patient's blood, and the low activity of the reductase activity in the patient's muscle lead to the conclusion that degradation of linoleic acid in humans also requires the involvement of 2,4-dienoyl-CoA reductase. This case study represents the first *in vivo* evidence that polyunsaturated fatty acid oxidation in humans proceeds by the reductase-dependent pathway (14). The low activity levels of 2,4-dienoyl-CoA reductase in animal tissues (43) and the dramatic effect that a reduced activity level of this enzyme has on the β-oxidation of polyunsaturated fatty acids, prompted the question of whether the reaction catalyzed by 2,4-dienoyl-CoA reductase is limiting polyunsaturated fatty acid oxidation.

An increase in the activity of 2,4-diencyl-CoA reductase in liver can be induced by growth hormone treatment of hypophysectomized rats (43). Although the specific activity of 2,4-dienoyl-CoA reductase in heart was

increased 2-fold by growth hormone treatment of hypophysectomized rats, the rate of β-oxidation of either oleate or docosahexaenoic acid in myocytes remained unaffected by this treatment (64). Also, in contrast to liver mitochondria, the rate of oxidation of docosahexaenoylcarnitine remained unchanged upon growth hormone trearment. A possible explanation is that, in rat heart, 2,4-dienoyl-CoA reductase does not catalyze the rate-limiting reaction in the oxidation of long chain polyunsaturated fatty acids such as docosahexaenoic acid.

During the last 15 years it has become increasingly evident that CPT I plays an important role in the regulation of fatty acid oxidation in liver due to its marked inhibition by malonyl-CoA (29). This situation has prompted efforts to determine whether the CPT I catalyzed reaction is a limiting step in the mitochondrial oxidation of long chain unsaturated fatty acids in heart. The activities of CPT I with CoA derivatives of long chain saturated fatty acids ($C_{16:0}$), very long chain saturated fatty acids ($C_{22:0}$), and very long unsaturated fatty acids ($C_{22:6}$) were studied and compared to study the effect of both chain length and unsaturation on the rate of this reaction. Growth hormone treatment of hypophysectomized rats had no significant effect on CPT I activities measured with either palmitoyl-CoA, behenoyl-CoA, or docosahexaenoyl-CoA both in rat liver and rat heart mitochondria. Rates with behenoyl-CoA, or docosahexaenoyl-CoA, as substrates, were similar but lower than rates observed with palmitoyl-CoA (Tables 1, 2, and 3). Intact rat heart mitochondria incubated with varying concentrations of 2-tetradecyglycidyl-CoA (TDG-CoA), which is known to inhibit CPT I activity irreversibly, resulted in parallel inhibitions of CPT I and β-oxidation (Fig. 4 and Fig. 5). Additionally it was observed that the

rates of mitochondrial respiration were higher with saturating concentrations of acylcarnitine than with acyl-CoA. Taken together these *in vitro* observations suggest that the CPT I reaction might be the rate-limiting step in β-oxidation of long chain unsaturated fatty acids, since rates of respiration seemed to be reduced to the same extent that rates of CPT I were inhibited. On the other hand, the ratio of CPT I activities with docosahexaenoyl-CoA and palmitoyl-CoA, as substrates, was similar to the ratio of respiration rates supported by docosahexaenoylcarnitine and palmitoylcarnitine. Also, activities of CPT I were higher than rates of fatty acyl-CoA oxidation measured by oxygen consumption. However, it was observed that the initial rate of respiration was higher with acylcarnitine than with acyl-CoA, especially in the case of docosahexaenoyl derivatives. Only after several minutes, when the concentration of docosahexanoylcarnitine had increased, did the rate of respiration supported by $C_{22:6}$-CoA approched the rate observed with $C_{22:6}$-carnitine.These observartions suggest that CPT I may not be catalyzing the rate-limiting step in β-oxidation in heart mitochondria. However, since the CPT I activity is close to the rate of β-oxidation, as illustrated by the parallel inhibitions of CPT I and respiration by TDG-CoA, this enzyme may be catalyzing the rate-limiting reaction in β-oxidation *in vivo*, if the concentration of malonyl-CoA is sufficiently high to cause a significant inhibition of the CPT I activity.

The radioactive assay of 2,4-dienoyl-CoA reductase described here is sensitive enough to permit measurements of the enzyme in small samples of biological material. With this assay it was possible to determine reductase activities in a homogenate of human fibroblasts

which were obtained from a single culture flask. Enough assays could be performed with such homogenates to obtain reliable values of the activity even though the mean specific reductase activity in fibroblasts is only 3.7 mU. If necessary, the sensitivity of the assay can be increased up to 1000-fold by preparing [4B-^{3}H]NADPH with a higher specific radioactivity. If the reductase activity is measured to compare the levels of this enzyme in different samples, the data obtained by use of the radioactive method can be used without any correction. However, when the true activity of 2,4-dienoyl-CoA reductase is required, the values obtained by use of the radioactive assay must be corrected. The correction factor determined in this study was close to 10 and is attributed to a primary kinetic isotope effect associated with the hydrogen transfer which occurs during the reduction. Whenever a new preparation of [4B-^{3}H]NADPH is being used, the correction factor should be determined by measuring under identical conditions. It is anticipated that this radioactive assay will permit reductase measurements of sufficient accuracy to establish the inheritance pattern of the recently described 2,4-dienoyl-CoA reductase deficiency (68).

2,4-Dienoyl-CoA reductase is routinely assayed by measuring the oxidation of NADPH spectrophotometrically at 340 nm .The most common substrates used for this assay are 2-*trans*,4-*cis*-decadienoyl-CoA and 2-*trans*,4-*trans*-decadienoyl-CoA. Even though 2-*trans*,4-*cis*-decadienoyl-CoA is known to be the physyological substrate, the all-*trans* substrate is more often used because it is easier to prepare. This assay suffers from lack of linearity seconds after its initiation possibly due to the competitive inhibition of the enzyme by the product of this reaction which is known to

have a K_i at low micromolar concentrations (71). This problem complicates the measurement of reductase activities by the standard spectrophotometric assay method.

The problem of nonlinearity in 2,4-dienoyl-CoA reductase assay was overcome by use of a new substrate, 5-phenyl-2,4-pentadienoyl-CoA. The structure of 5-phenyl-2,4-pentadienoyl-CoA (I) and its reduction by NADPH catalysed by 2,4-dienoyl-CoA reductase is shown in Fig. 18. If the mechanism of this reductive step is identical with the reduction of other 2,4-dienoyl-CoA compounds, 5-phenyl-3-pentenoyl-CoA (II) would be formed. The later compound may then be converted to 5-phenyl-2-pentenoyl-CoA (III) by Δ^3,Δ^2-enoyl-CoA isomerase. The subsequent β-oxidation reaction is the hydration of this compound by crotonase to yield 5-phenyl-3-hydroxypentanoyl-CoA (IV). The formation of product was at least linear over the first five minutes of the reaction (see Fig. 14B) and the activity was linearly dependent on the protein concentration (see Fig. 14A). The U.V. spectra of the products of the reductase reaction (Fig.13; peak 2 due to 5-phenyl-3-pentenoyl-CoA and/or 5-phenyl-2-pentenoyl-CoA and peak 3 due to 5-phenyl-3-hydroxy-pentanoyl-CoA) were obtained and showed no absorbance at 340 nm, the wavelength at which the assay is performed. The reductase assay with 5-phenyl-2,4-pentadienoyl-CoA as a substrate is twice as sensitive as the assay with the commonly used substrate 2,4-decadienoyl-CoA. Overall, the use of 5-phenyl-2,4-pentadienoyl-CoA in the assay will permit measurements of 2,4-dienoyl-CoA reductase in small samples of biological material, including homogenates of human tissues with higher sensitivity, better linearity, and more accuracy.

Unsaturated fatty acids with odd-numbered double bonds, as 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 thought to be chain shortened until the odd-numbered double bonds extend from carbon atom 3 (11). At this stage, Δ^3,Δ^2-enoyl-CoA isomerase converts 3-*cis* or 3-*trans* double bonds to a 2-*trans* double bond (16). 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 (17) 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. An attempt to detect such enzyme activity was unsuccessful and prompted 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 Fig. 19. 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 (11), 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 for which an absorbance maximum at 228 nm has been observed with hexane as a solvent (72). Since the U.V. spectrum shown in Fig. 15 was determined with water as solvent, the λ_{max} is expected to be shifted to the red by 10 to 20 nm (73). Even though the configuration of the diene of compound III has not been established, it is assumed that the 5-double bond 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 around 240 nm and a corresponding increase 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, 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 (43) 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 (24).

This study demonstrates that 5-octenoyl-CoA can be degraded via the pathway shown in Fig. 19, which requires NADPH and results in the

reductive removal of the preexisting 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.

Table 1

Effect of hypophysectomy and growth hormone treatment on carnitine palmitoyltransferase I (CPT I) activity of rat liver mitochondria

Substrate	Rate of CPT I activity (nmol/min/mg protein)		
	normal	hypophysect.	hypophysect.+ GH
P-CoA	5.03 ± 0.7 (3)	4.90 ± 0.40 (3)	5.44 ± 1.50(3)
D-CoA	0.65 ± 0.2 (3)	0.58 ± 0.26 (3)	0.57 ± 0.03(3)
B-CoA	0.74 ± 0.2 (3)	0.34 ± 0.13 (3)	0.78 ± 0.14(3)

Activities were measured in the presence of 10mg/ml bovine serum albumin.
GH: growth hormone, P-CoA: palmitoyl-CoA, D-CoA: docosahexaenoyl-CoA, B-CoA: behenoyl-CoA

Table 2

Effect of hypophysectomy and growth hormone treatment on carnitine palmitoyltransferase I (CPT I) activity of rat heart mitochondria

Substrate	Rate of CPT I activity (nmol/min/mg protein)		
	normal	hypophysect.	hypophysect.+ GH
P-CoA	2.29 ± 0.3 (3)	3.60 ± 0.20 (3)	3.56 ± 0.40(3)
D-CoA	0.37 ± 0.1 (3)	0.20 ± 0.06 (3)	0.45 ± 0.03(3)
B-CoA	0.33 ± 0.1 (3)	0.17 ± 0.09 (3)	0.57 ± 0.10(3)

Activities were measured in the presence of 10mg/ml bovine serum albumin.
GH: growth hormone, P-CoA: palmitoyl-CoA, D-CoA: docosahexaenoyl-CoA, B-CoA: behenoyl-CoA

Table 3

Activities of rat heart mitochondrial carnitine palmitoyltransferase I

Substrate	Specific activity nmol/min/mg protein	Relative activity %
P-CoA	23.0 ± 2.1 (3)	100
D-CoA	04.4 ± 1.4 (3)	19.7

Activities are measured in the presence of 1mg /ml of bovine serum albumin. P-CoA: palmitoyl-CoA, and D-CoA: docosahexaenoyl-CoA.

Table 4

Respiration rates of rat heart mitochondria

Substrate	Rate of respiration		Relative rate
	ngO2 /min/mg	nmol/min/mg	%
PC	200 ± 9 (4)	5.0	100
DC	74 ± 10 (4)	1.2	24
P-CoA	224 ± 10 (4)	4.5	100
D-CoA	48 ± 8 (4)	0.77	17

Respiration supported by PC, palmitoylcarnitine; DC, docosahexaenoylcarnitine; P-CoA, palmitoyl-CoA; and D-CoA, docosahexaenoyl-CoA. Bovine serum albumin concentration is 1mg/ml.

Table 5

2,4-Dienoyl-CoA reductase activity in liver and psoas muscle of a patient with a suspected deficiency in the oxidation of polyunsaturated fatty acids

Tissue and source	Activity (nmol/min/mg protein)			
	2t,4c	%	2t,4t	%
Liver, control	13 ± 2.6 (5)	100	8.4 ± 2.2 (5)	100
Liver, patient	5.1	40	5.5	65
Muscle, control	4.6 ± 0.8 (5)	100	3 ± 0.28 (5)	100
Muscle, patient	0.8	17	1.24	43

2t,4c: reductase activity with 2-*trans*,4-*cis*-decadienoyl-CoA as substrate
2t,4t: reductase activity with 2-*trans*,4-*trans*-decadienoyl-CoA as substrate

Table 6

Effect of lysine on the activity of human 2,4-dienoyl-CoA reductase

Lysine concentration (mM)	Specific activity (nmol/min/mg protein)	Inhibition (%)
00.0	5.75	0.0
01.0	4.90	15
05.0	4.60	20
10.0	4.70	18

Rates were measured with 2-*trans*,4-*cis*-decadienoyl-CoA as a substrate

Table 7

2,4-Dienoyl-Coenzyme A Reductase activity in human lymphocytes*

Lymphocytes	Activity* (nmol/min/mg)	Activity in tested cells vs control(%)
Control	0.429 ± 0.004 (4)	100
Patient's sibling	0.429 ± 0.050	100
Patient's father	0.224 ± 0.004	52

* Activities were measured by using the radioactive assay.

Table 8

2,4-Dienoyl-CoA Reductase Activity in Bovine Liver Homogenate

Time (min)	Product formed (nmol/mg protein)			B/A(%)
	Spectrophotometric assay (A)	Radioactive assay (B)	HPLC assay	
0.5	1.73 ± 0.20 (8)			
1.0	3.42 ± 0.26 (8)	0.35 ± 0.01 (6)	2.43 ± 0.2 (2)	10
1.5	5.12 ± 0.32 (6)			
2.0	6.36 ± 0.21 (6)	0.58 ± 0.02 (6)	6.09 ± 0.1 (2)	9.1
2.5	8.10 ± 0.19 (4)	0.74 ± 0.02 (3)		9.1
3.0	9.94 ± 0.30 (4)	0.82 ± 0.04 (5)	8.18 ± 0.1 (2)	8.3

Incubation mixtures contained 25 μM 2-*trans*,4-*cis*-decadienoyl-CoA and other components as described under Materials and Methods. B/A; radioactive product as a percentage of NADPH oxidized.

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

Figure 1

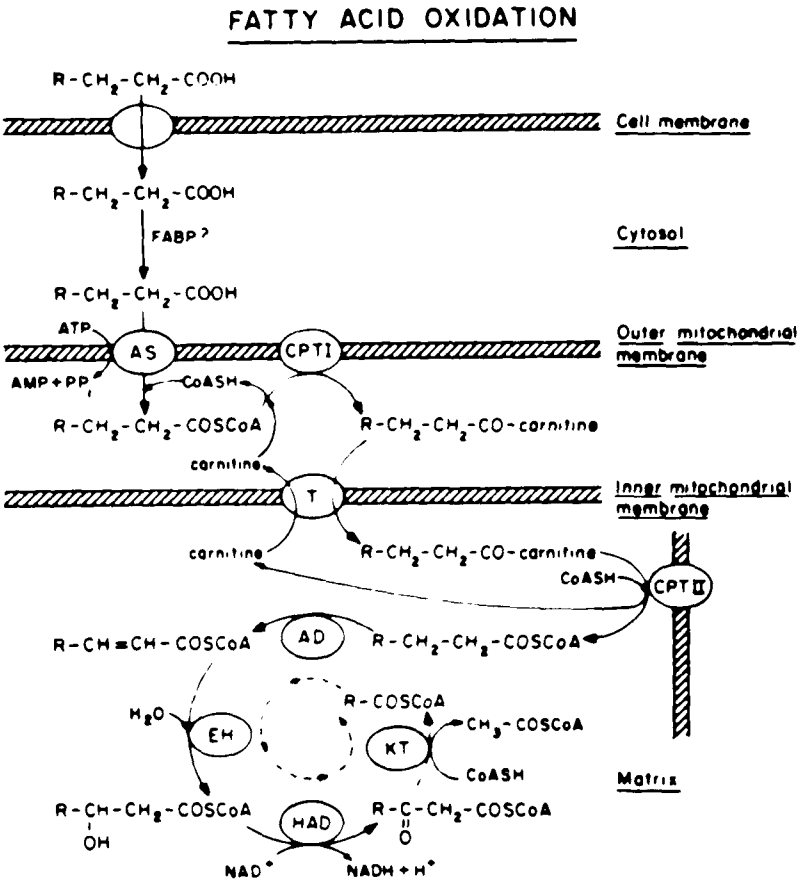

FATTY ACID OXIDATION
R-CH2-CH2-COOH
Cell membrane
R-CH2-CH2-COOH
FABP?
Cytosol
R-CH2-CH2-COOH
ATP
AS
CPTI
Outer mitochondrial membrane
AMP+PPi
CoASH
R-CH2-CH2-COSCoA
R-CH2-CH2-CO-carnitine
carnitine
T
Inner mitochondrial membrane
carnitine
R-CH2-CH2-CO-carnitine
CoASH
CPT II
R-CH=CH-COSCoA
AD
R-CH2-CH2-COSCoA
R-COSCoA
H2O
EH
KT
CH3-COSCoA
CoASH
Matrix
R-CH-CH2-COSCoA
OH
HAD
R-C-CH2-COSCoA
O
NAD+
NADH+H+

Figure 2. **β-Oxidation of linoleoyl-CoA**
A. Epimerase-dependent pathway.
B. Reductase-dependent pathway.

Figure 2

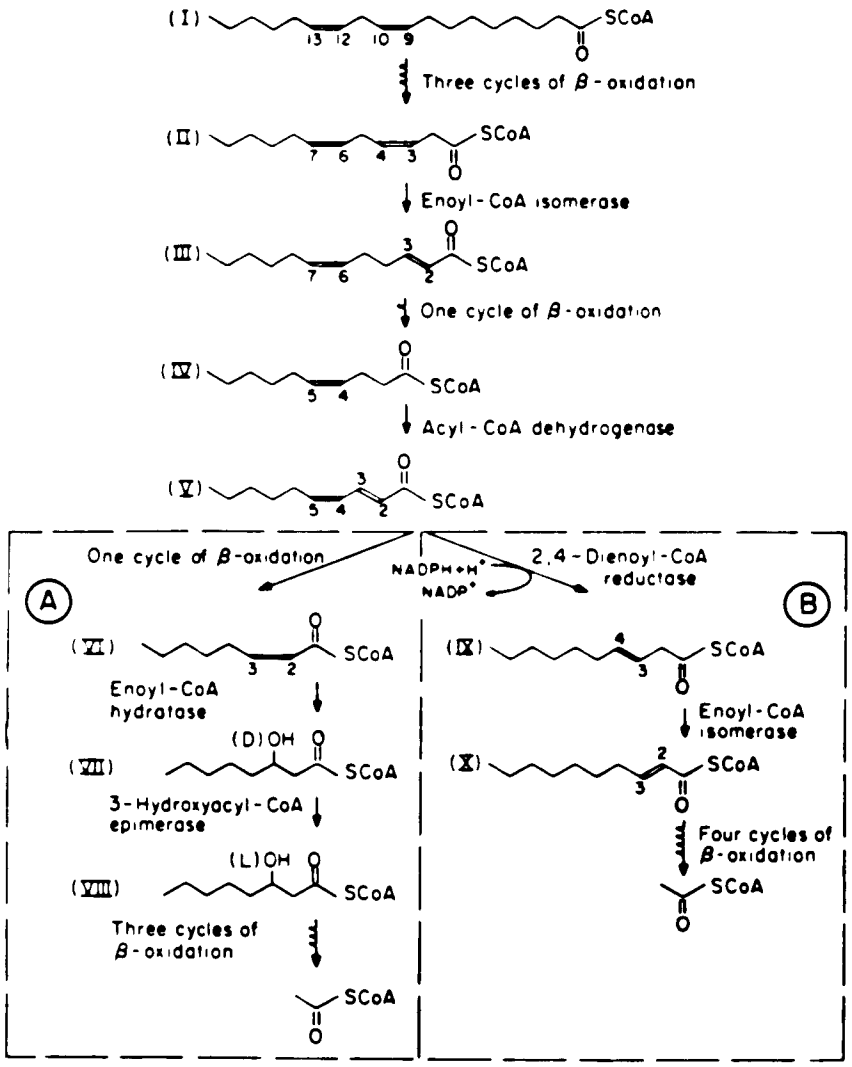

(I)
SCoA
13 12 10 9
Three cycles of β-oxidation
(II)
SCoA
7 6 4 3
Enoyl-CoA isomerase
(III)
SCoA
7 6
One cycle of β-oxidation
(IV)
SCoA
5 4
Acyl-CoA dehydrogenase
(V)
SCoA
5 4 2
One cycle of β-oxidation
NADPH+H+
NADP+
2,4-Dienoyl-CoA reductase
A
B
(VI)
SCoA
3 2
Enoyl-CoA hydratase
(D)OH
(VII)
SCoA
3-Hydroxyacyl-CoA epimerase
(L)OH
(VIII)
SCoA
Three cycles of β-oxidation
SCoA
(IX)
SCoA
4 3
Enoyl-CoA isomerase
(X)
SCoA
2 3
Four cycles of β-oxidation
SCoA

Figure 3 **Respiration rates of rate heart mitochondria.** Abbreviations: P-CoA, palmitoyl-CoA; PC, palmitoyl-carntine; D-CoA, docosahexaenoyl-CoA; DC, docosahexaenoyl-carnitine. Numbers give the respiration rates in ng-atoms of O_2/ min per mg of protein.

Figure 3

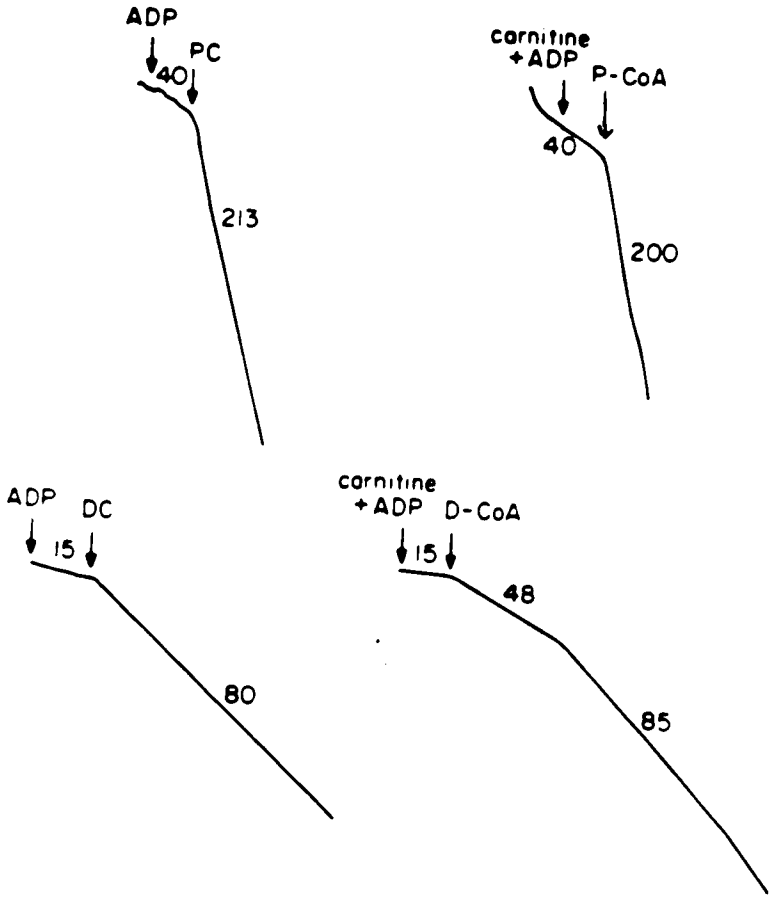

ADP
PC
40
213
carnitine
+ADP
P-CoA
40
200
ADP
DC
15
80
carnitine
+ADP
D-CoA
15
48
85

Figure 4. **Effect of TDG-CoA on respiration and the rate of carnitine palmitoyltransferase I in rat heart mitochondria.** Respiration was supported by docosahexaenoyl-CoA(•) or docosahexaenoyl-carnitine (Δ). CPTI was measured with docosahexaenoyl-CoA as a substrate (o).

Figure 4

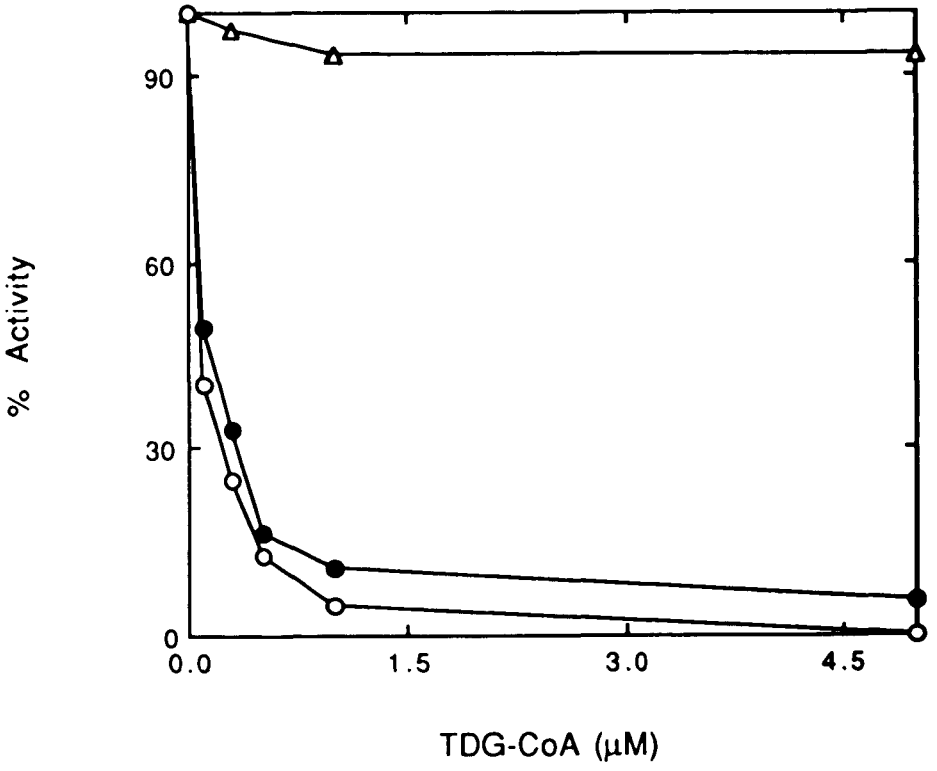


Figure 5. **Effect of 2-tetradecylglycidyl-CoA on respiration and the rate of carnitine palmitoyltransferase I in rat heart mitochondria.** Respiration was supported by palmitoyl-CoA (•) or palmitoylcarnitine (Δ). CPTI was measured with palmitoyl-CoA as a substrate (o).

Figure 5

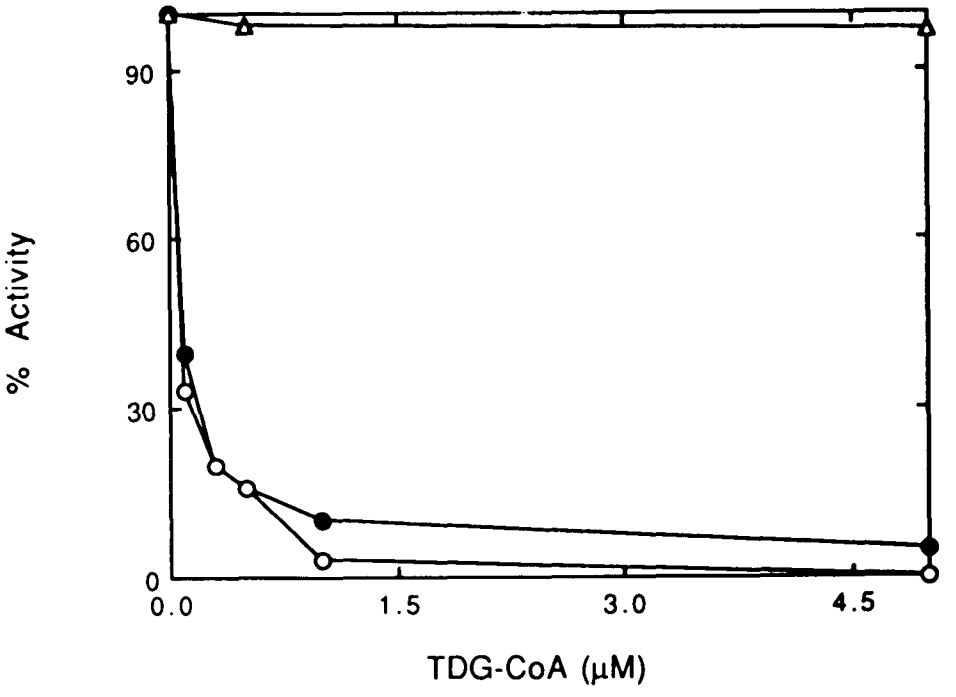


Figure 6. Outline of the radioactive method for assaying 2,4-dienoyl-CoA reductase with [4B-^{3}H]NADPH synthesized enzymatically from D-[1-^{3}H]glucose, ATP, and $NADP^{+}$.

Figure 6

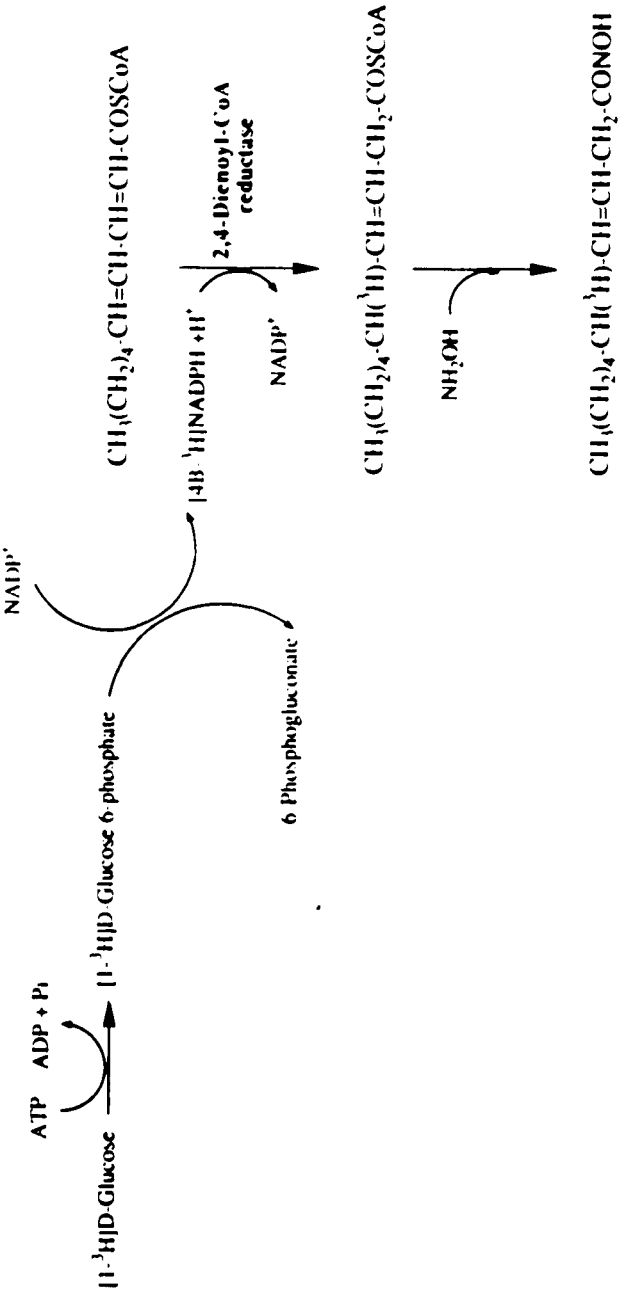

ATP
ADP + Pi
[1-³H]D-Glucose
[1-³H]D-Glucose 6-phosphate
NADP⁺
6 Phosphogluconate
[4B-³H]NADPH +H⁺
NADP⁺
2,4-Dienoyl-CoA reductase
CH₃(CH₂)₄-CH=CH-CH=CH-COSCoA
CH₃(CH₂)₄-CH([³H])-CH=CH-CH₂-COSCoA
NH₄OH
CH₃(CH₂)₄-CH([³H])-CH=CH-CH₂-CONOH

Figure 7. **Analysis of products formed in the radioactive assay. Source of 2,4-dienoyl-CoA reductase:** A,C. Soluble extract from rat liver mitochondria. B, D Partially purified rat liver reductase. Reaction mixtures were separated by HPLC and analyzed for radioactivity as described under Materials and Methods. An equilibrium mixture of L-3-hydroxy-decanoyl-CoA and 2-*trans*-decenoyl-CoA, obtained by incubating 2-*trans*-decenoyl-CoA with enoyl-CoA hydratase (crotonase), was used to identify peak 1 as 3-hydroxydecanoyl-CoA and peak 2 as 2-decenoyl-CoA and/or 3-decenoyl-CoA.

Figure 7

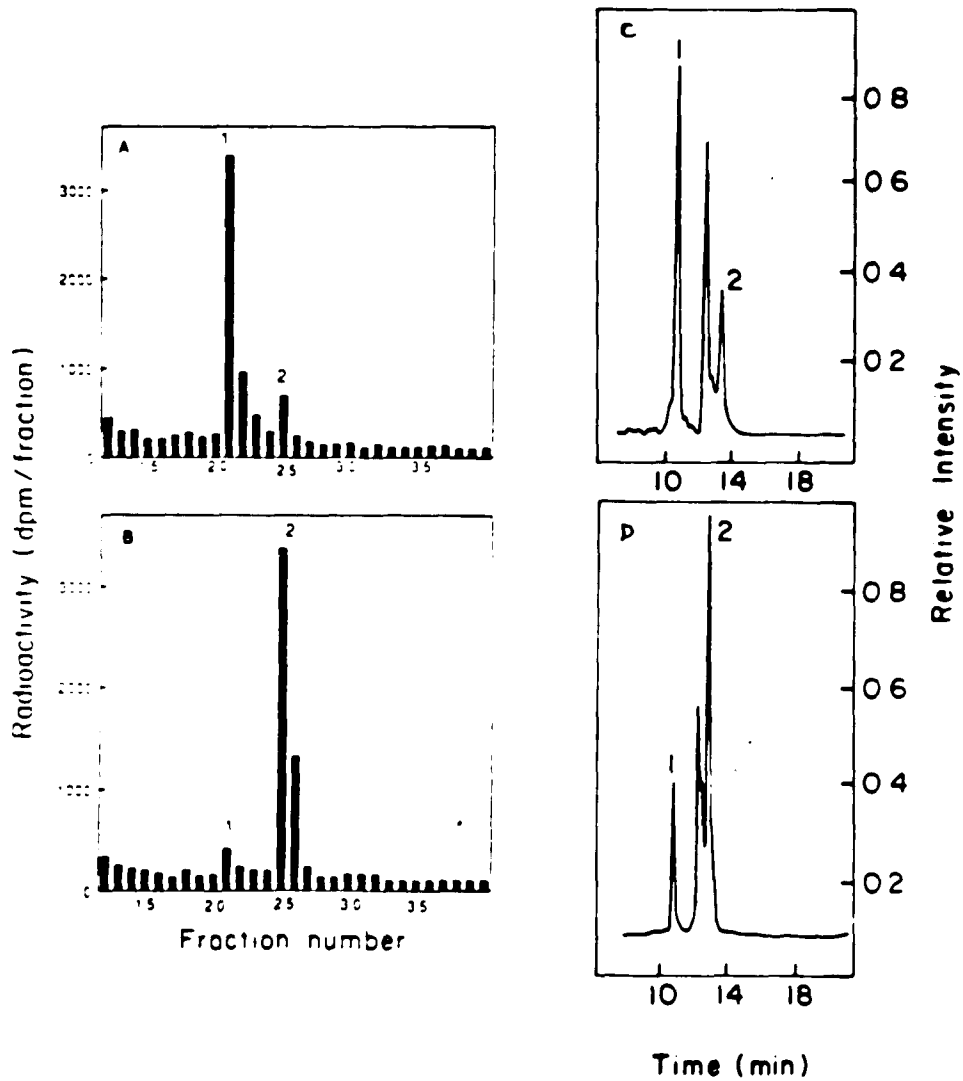

A
B
C
D
1
2
Radioactivity (dpm / fraction)
Fraction number
Relative Intensity
Time (min)
10
14
18
0 2
0 4
0 6
0 8

Figure 8. **Activity of 2,4-dienoyl-CoA reductase in a soluble extract of bovine liver mitochondria as a function of the protein concentration.** (A) Spectrophotometric assay. (B) Radioactive assay.

Figure 8

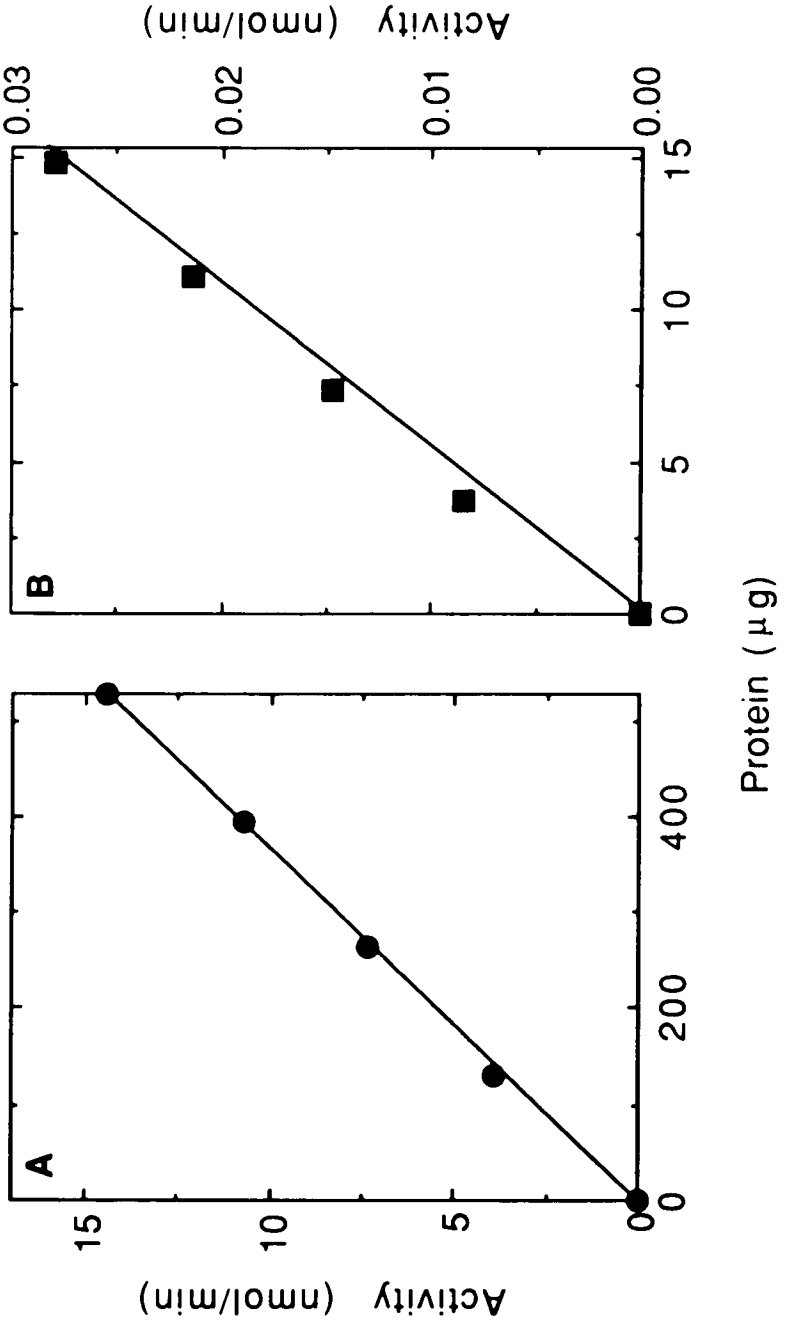

Activity (nmol/min)
0.03
0.02
0.01
0.00
B
0
5
10
15
Protein (μg)
Activity (nmol/min)
A
0
200
400
0
5
10
15

Figure 9. **Activity of 2,4-dienoyl-CoA reductase in a homogenate of human fibroblasts.** (A) Product formation as a function of time with 15 μg protein. (B) Activity as a function of protein concentration. Incubation time was 90 sec.

Figure 9

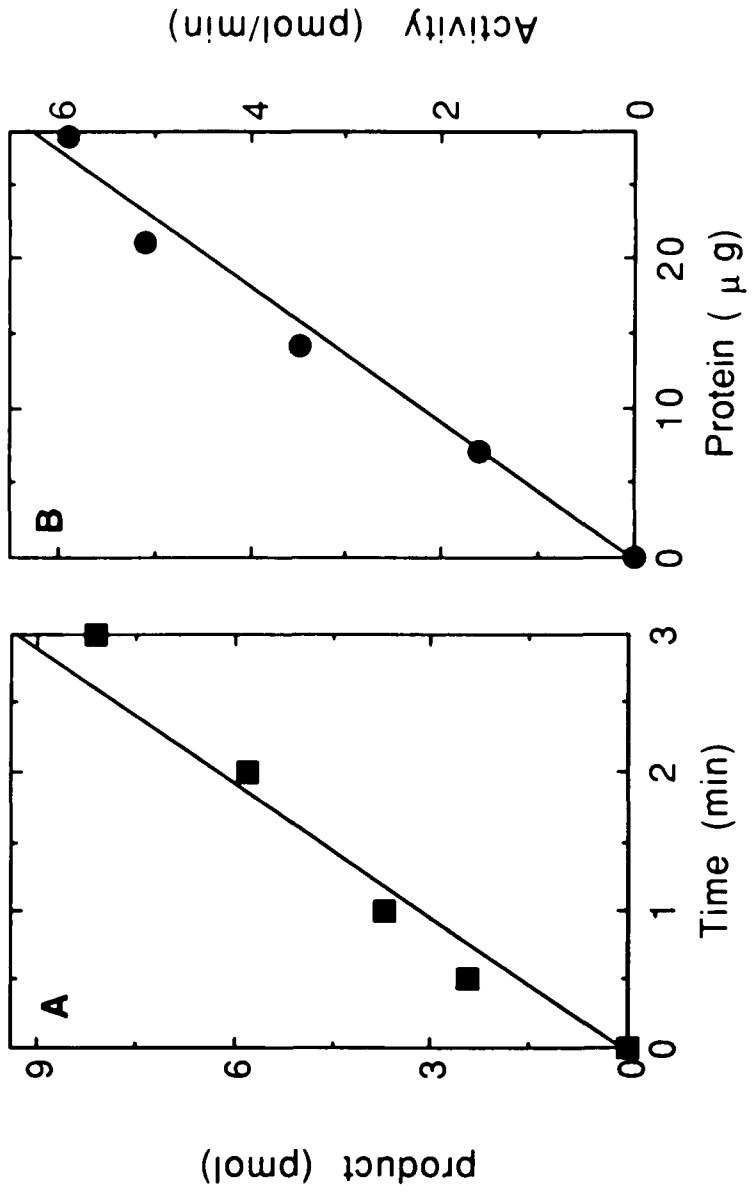

product (pmol)
9
6
3
0
A
0
1
2
3
Time (min)
Activity (pmol/min)
6
4
2
0
B
0
10
20
Protein (μ g)

Figure 10. U.V. spectrum of 5-phenyl-2,4-pentadienoyl-CoA

Figure 10

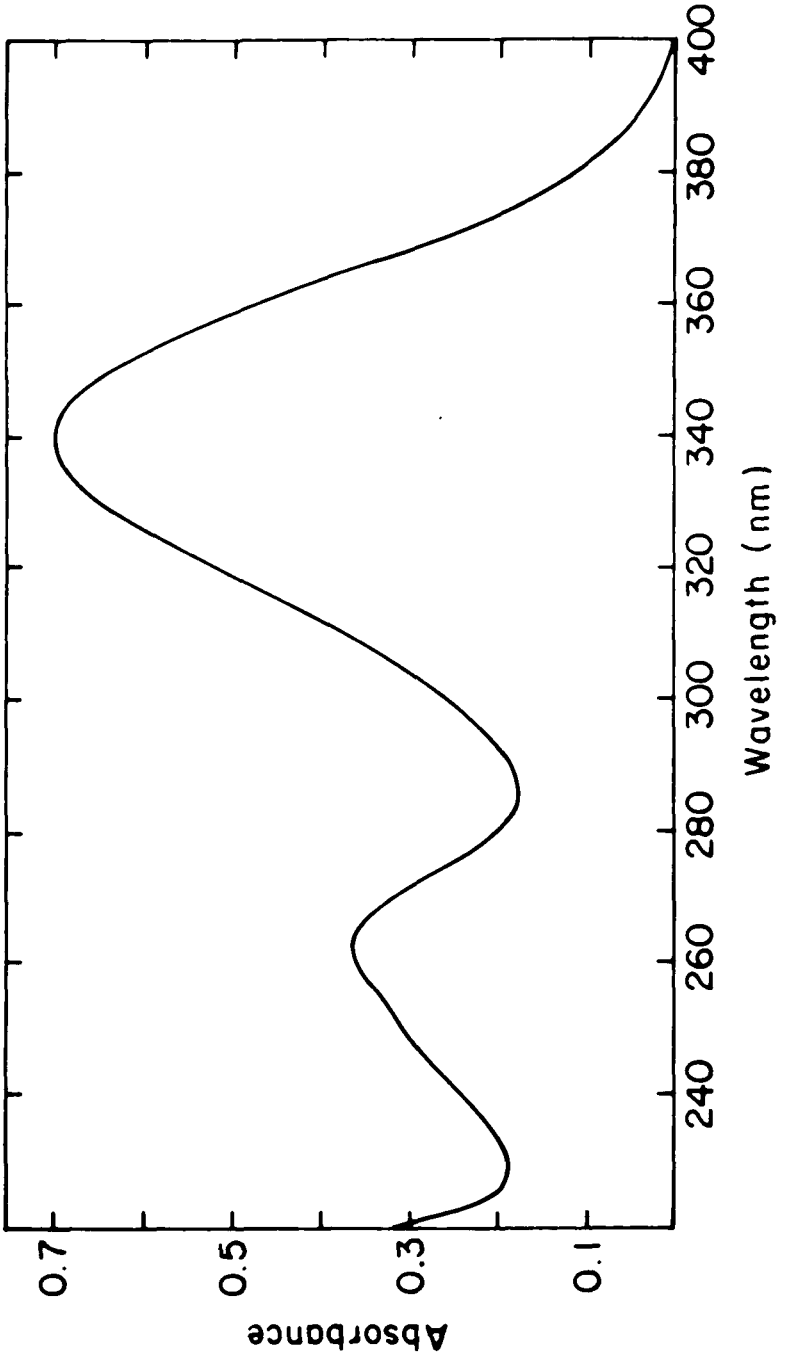

Absorbance
0.7
0.5
0.3
0.1
240
260
280
300
320
340
360
380
400
Wavelength (nm)

Figure 11. **Spectral changes associated with the reduction of 5-phenyl-2,4-pentadienoyl-CoA in the presence of 2,4-dienoyl-CoA reductase and NADPH.** The reaction time was (1) 0 min, (2) 3 min, (3) 8 min, (4) 30 min.

Figure 11

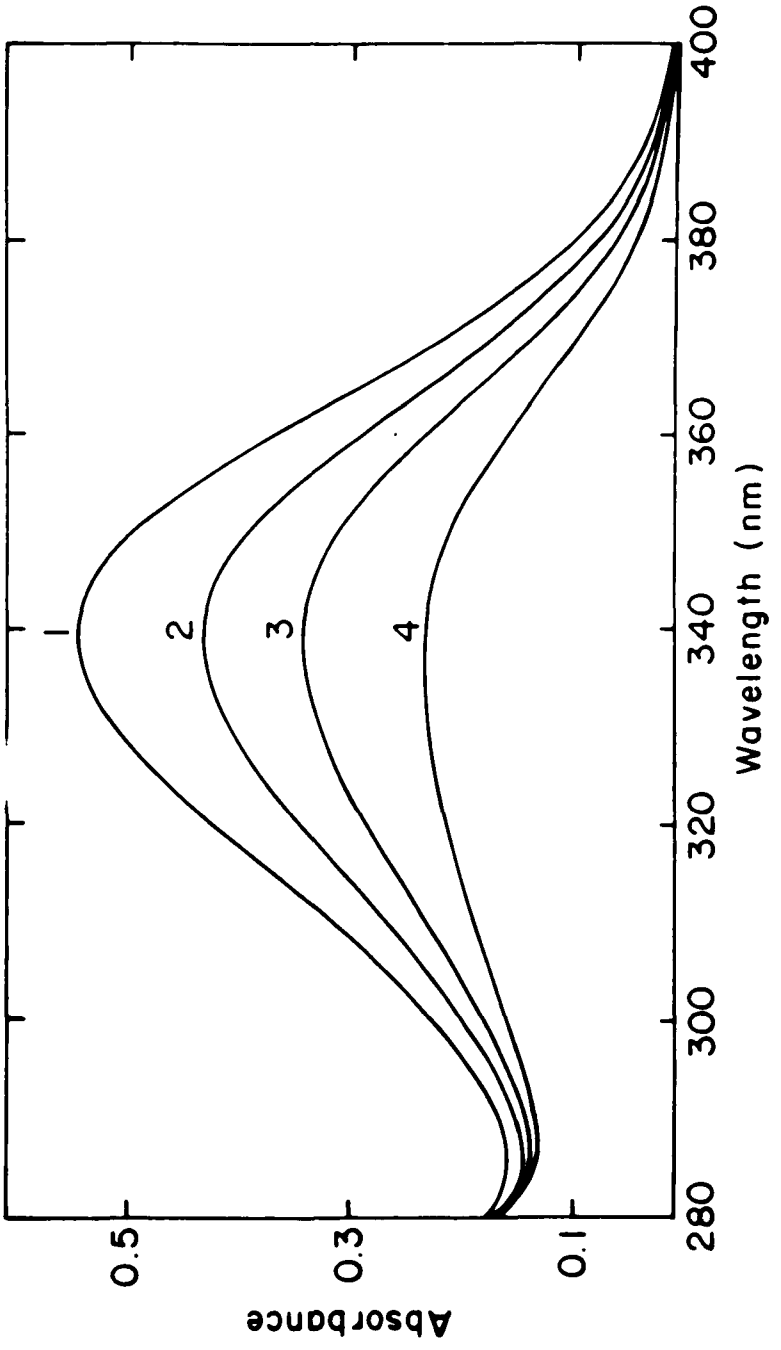

Absorbance
0.5
0.3
0.1
280
300
320
340
360
380
400
Wavelength (nm)
1
2
3
4

Figure 12. **HPLC analysis of metabolites formed from 5-phenyl-2,4-pentadienoyl-CoA by 2,4-dienoyl-CoA reductase or by a soluble extract of rat mitochondria.** A. 5-Phenyl-2,4-pentadienoyl-CoA (peak 1). B. 5-Phenyl-3-pentenoyl-CoA (peak 2) formed from 5-phenyl-2,4-pentadienoyl-CoA and NADPH by partially purified 2,4-dienoyl-CoA reductase. C. NADPH-dependent reduction of 5-phenyl-2,4-pentadienoyl-CoA (peak 1) by the trifunctional enzyme to yield 5-phenyl-3-pentenoyl-CoA / 5-phenyl-2-pentenoyl-CoA (peak 2), and 5-phenyl-3-hydroxy pentanoyl-CoA (peak 3).

Figure 12

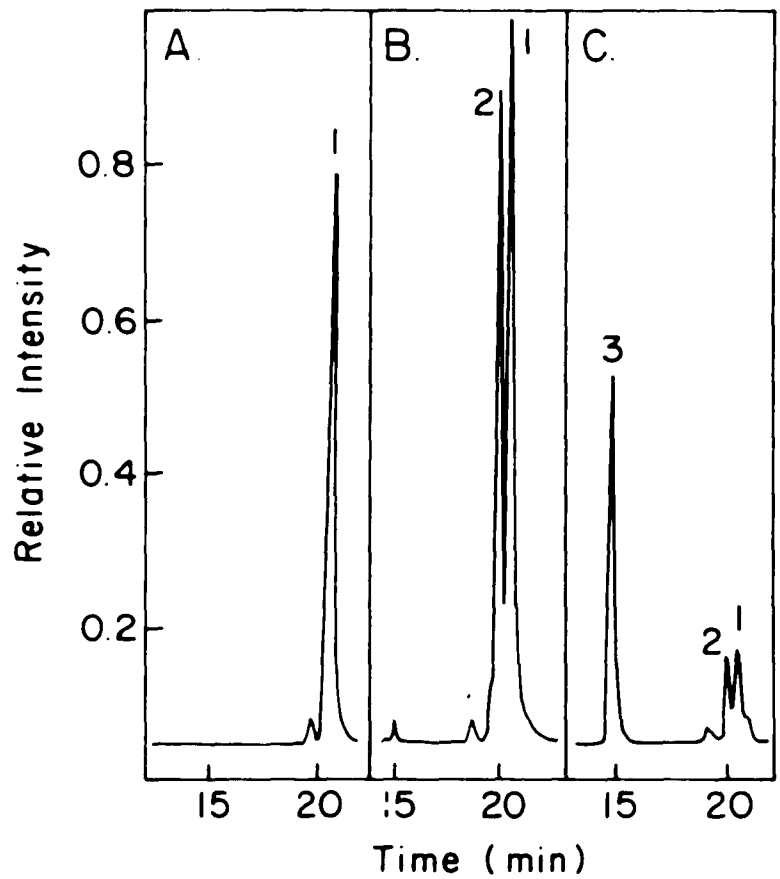


Figure 13. **HPLC identification of metabolites formed from 5-phenyl-2,4-pentadienoyl-CoA by β-oxidation enzymes.** A. Purified 5-phenyl-3-pentenoyl-CoA formed from 5-phenyl-2,4-pentadienoyl-CoA by NADPH and partially purified reductase. B. Incubation of 5-phenyl-3-pentenoyl-CoA with purified crotonase. C. Isomerization and hydration of 5-phenyl-3-pentenoyl-CoA (peak 2) by purified trifunctional enzyme from rat liver peroxisomes. D. Purified 5-phenyl-3-hydroxy-pentanoyl-CoA (peak 3). E. Dehydration of 5-phenyl-3-hydroxypentanoyl-CoA (peak 3) by purified crotonase.

Figure 13

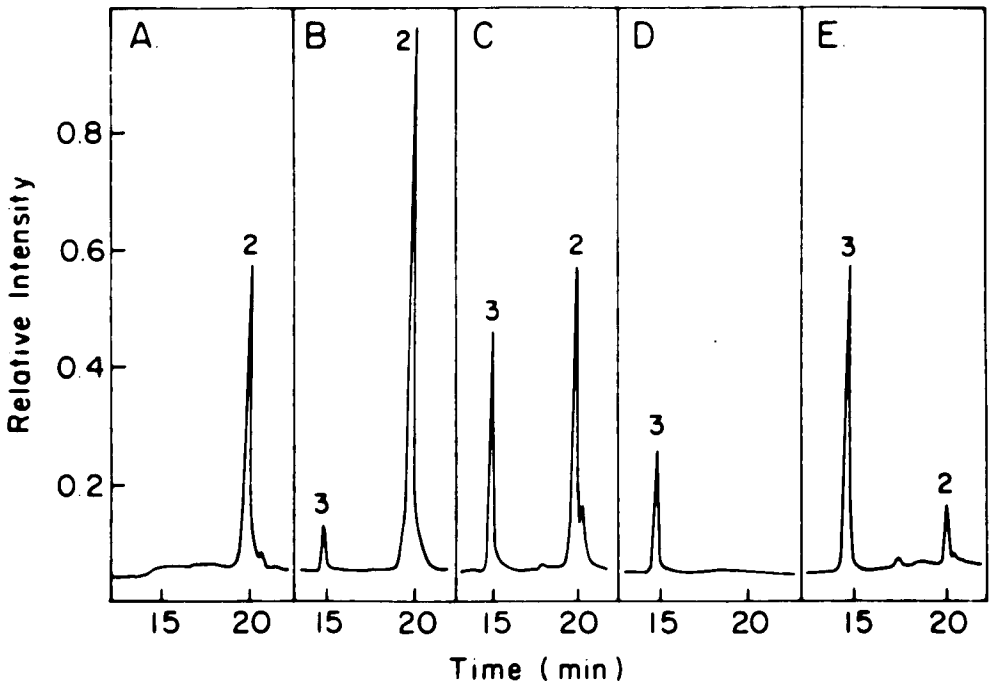


Figure 14. **NADPH-dependent reduction of 2-*trans*,4-*cis* (*trans*)-decadienoyl-CoA or 5-phenyl-2,4-pentadienoyl-CoA by2,4-dienoyl-CoA reductase present in a soluble extract of rat liver mitochondria .** (A) Activity as a function of protein concentration with 2-*trans*-4-*cis*-decadienoyl-CoA (•) or 5-phenyl-2,4-pentadienoyl-CoA (Δ) as substrates. (B) Absorbance change as a function of time of a reaction mixture that contained 0.12 mg of mitochondrial protein, 0.1mM NADPH, and 40 μM 2-*trans*-4-*cis*-decadienoyl-CoA (•) ,or 40 μM 2-*trans*-4-*trans*-decadienoyl-CoA (O), or 40 μM 5-phenyl-2,4-pentadienoyl-CoA (Δ).

Figure 14

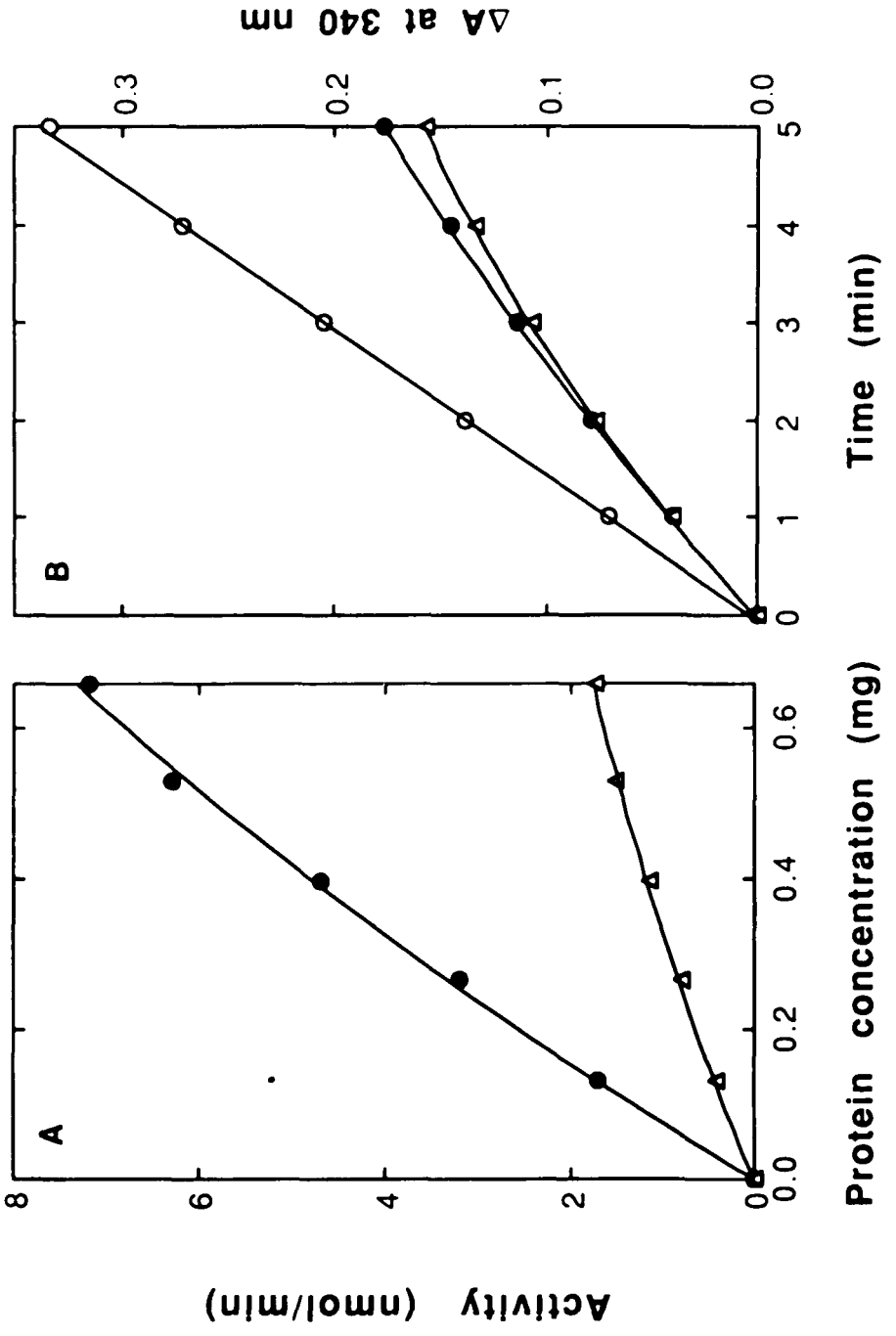

ΔA at 340 nm
0.0
0.1
0.2
0.3
B
0
1
2
3
4
5
Time (min)
Activity (nmol/min)
0
2
4
6
8
A
0.0
0.2
0.4
0.6
Protein concentration (mg)

Figure 15. **Spectral changes associated with the hydration and isomerizations of 2-*trans*,5-*cis*-octadienoyl-CoA.** A. Hydration of 2-*trans*,5-*cis*-octadienoyl-CoA by crotonase. (1) No addition, (2) 90 sec and (3) 5 min after addition of crotonase. B. Isomerization of 2-*trans*-5-*cis*-octadienoyl-CoA to 3,5-octadienoyl-CoA catalyzed by the peroxisomal trifunctional enzyme. (1) No addition, (2) 90 sec, (3) 3 min, (4) 6 min after the addition of enzyme. C. Isomerization of 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA catalyzed by a soluble extract of rat liver mitochondria. (1) No addition, (2) 90 sec, (3) 3 min, (4) 6 min after the addition of enzyme.

Figure 15

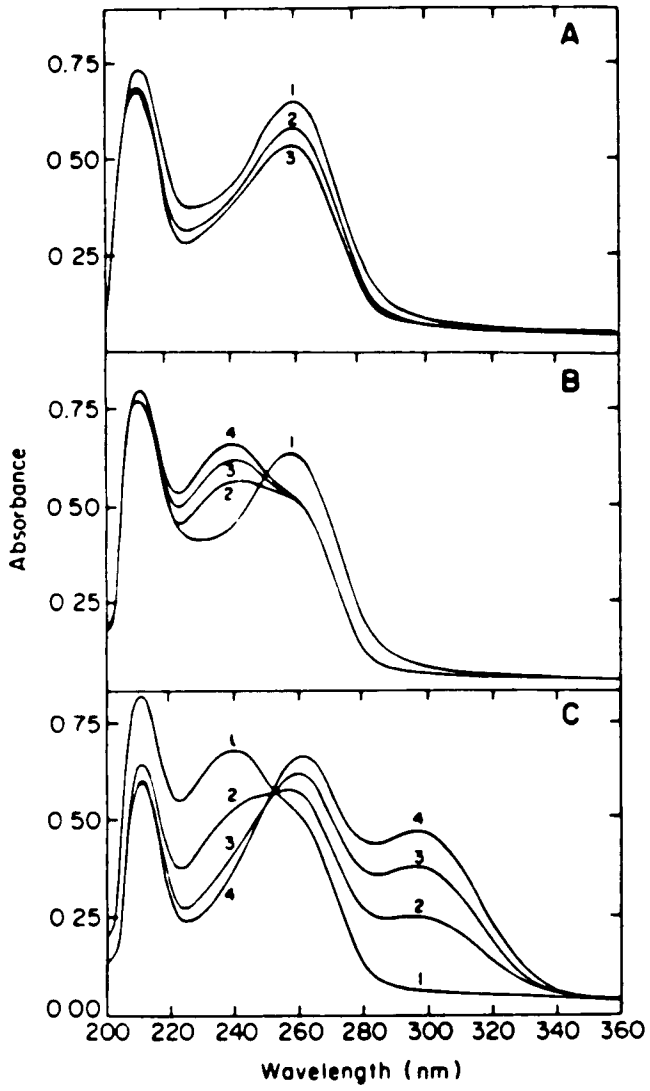

A
B
C
0.75
0 50
0 25
0 00
Absorbance
200 220 240 260 280 300 320 340 360
Wavelength (nm)

Figure 16. **Spectral changes associated with the reduction of 2,4-octadienoyl-CoA by NADPH in the presence of a soluble extract of rat liver mitochondria.** (1) Before addition of NADPH, (2) 3 min, (3) 9 min, (4) 20 min after the addition of NADPH to both the measuring and reference cuvettes.

Figure 16

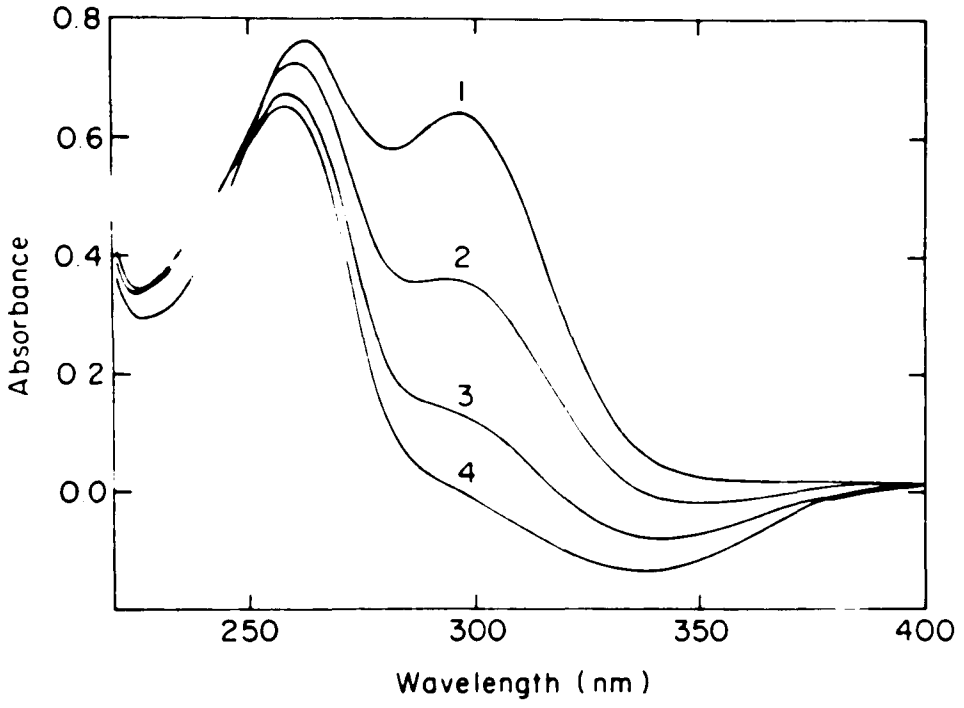


Figure 17. **HPLC analysis of metabolites formed by β-oxidation from 2-*trans*-5-*cis*-octadienoyl-CoA.** 2-*trans*-5-*cis*-Octadienoyl-CoA was first converted to 2-*trans*-4-*trans*-octadienoyl-CoA by a soluble extract of rat liver mitochondria and (A) after removal of enzymes reduced by NADPH in the 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 *Materials and Methods*. 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 17

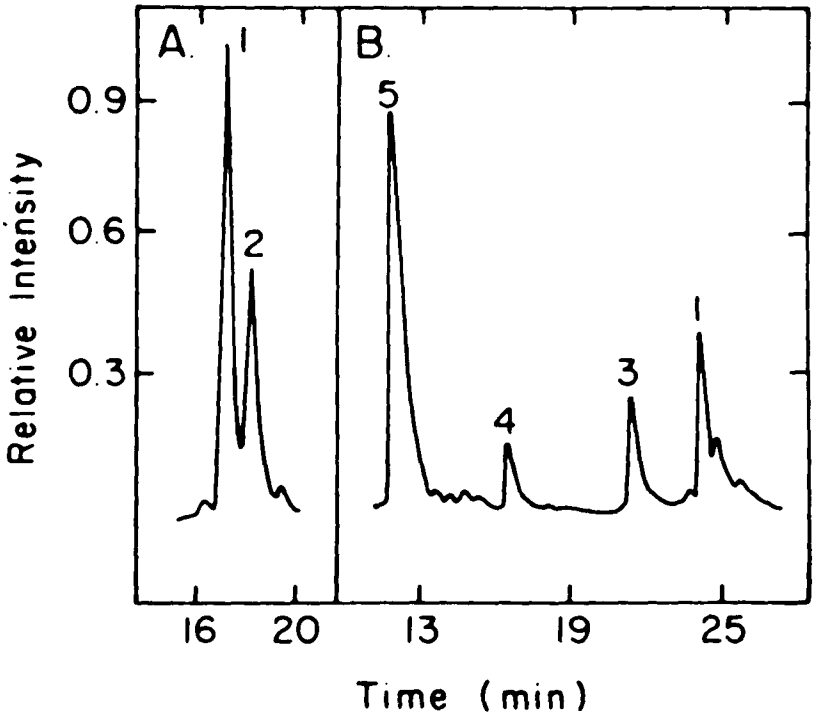


Figure 18. **Proposed degradation of 5-phenyl-2,4-pentadienoyl-CoA by β-oxidation enzymes.** The metabolites shown are: I, 5-phenyl-2,4-pentadienoyl-CoA; II, 5-phenyl-3-pentenoyl-CoA; III, 5-phenyl-2-pentenoyl-CoA; 5-phenyl-3-hydroxy-pentanoyl-CoA.

Figure 18

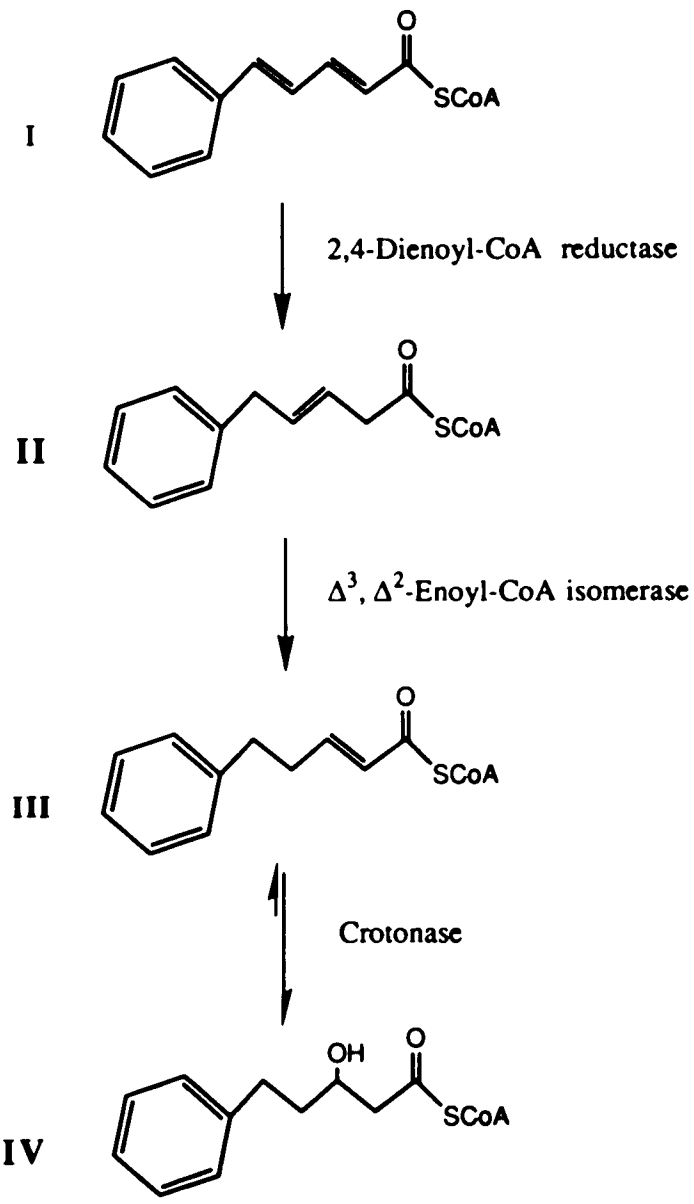
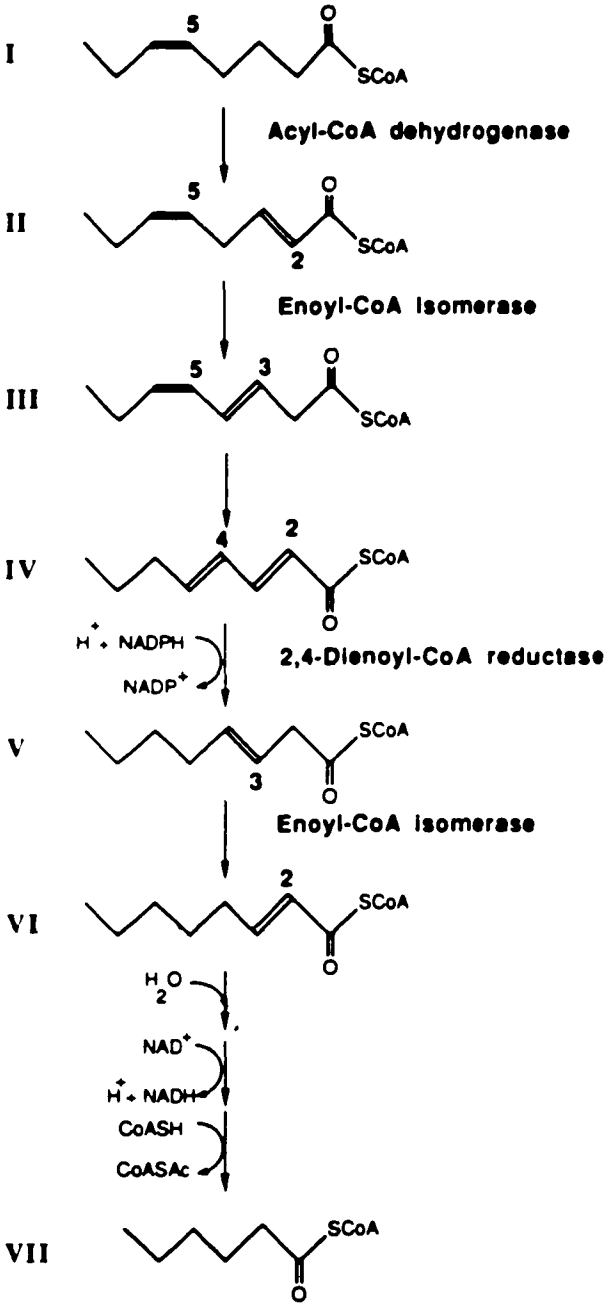

I
SCoA
2,4-Dienoyl-CoA reductase
II
SCoA
Δ^3, Δ^2-Enoyl-CoA isomerase
III
SCoA
Crotonase
IV
OH
SCoA

Figure 19. **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.

Figure 19


I
5
O
SCoA
Acyl-CoA dehydrogenase
II
5
2
Enoyl-CoA Isomerase
III
5
3
IV
4
2
H+ + NADPH
NADP+
2,4-Dienoyl-CoA reductase
V
3
Enoyl-CoA Isomerase
VI
H2O
NAD+
H+ + NADH
CoASH
CoASAc
VII

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