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Inhibitors of mitochondrial beta-oxidation and the mechanism of 3-hydroxyacyl-CoA epimerization

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INHIBITORS OF MITOCHONDRIAL BETA-OXIDATION AND THE MECHANISM OF 3-HYDROXYACYL-CoA EPIMERIZATION

by

Jianxun Li

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

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ABSTRACT

INHIBITORS OF MITOCHONDRIAL BETA-OXIDATION AND THE MECHANISM OF 3-HYDROXYACYL-CoA EPIMERIZATION

by

Jianxun Li

Adviser: Professor Horst Schulz

In an attempt to develop a compound which would inhibit ß-oxidation by specifically inactivating 3-ketoacyl-CoA thiolase (EC 2.3.1.16) in whole mitochondria, 4-bromo-2-octenoic acid was synthesized and studied. After rat liver mitochondria were preincubated with 4-bromo-2-octenoic acid for 3 min, respiration supported by either palmitoylcarnitine or pyruvate was completely abolished, whereas no inhibition was observed with rat heart mitochondria. Addition of carnitine stimulated respiration supported by pyruvate without relieving inhibition of palmitoylcarnitine-dependent respiration. Hence, this compound seems to be a specific inhibitor of ß-oxidation. When the enzymes of ß-oxidation were assayed in a soluble extract prepared from mitochondria preincubated with 4-bromo-2-octenoic acid, only 3-ketoacyl-CoA thiolase was found to be inactivated. 4-Bromo-2-octenoic acid is metabolized by mitochondrial ß-oxidation enzymes to 3-keto-4-bromooctanoyl-CoA which effectively and irreversibly inhibits 3-ketoacyl-CoA thiolase but not acetoacetyl-CoA thiolase (EC 2.3.1.9). Even though 3-keto-4-bromooctanoyl-CoA inhibits the latter enzyme reversibly, 4-bromo-2-octenoic acid does not inhibit ketogenesis in rat liver mitochondria with acetylcarnitine as a substrate. It is concluded that 4-bromo-2-octenoic acid specifically inhibits mitochondrial fatty acid oxidation by inactivating 3-ketoacyl-CoA thiolase in rat liver mitochondria.

The metabolism of 2-propylpentanoic acid (valproic acid), which is an antiepileptic drug but also causes inhibition of ß-oxidation, was studied in rat liver mitochondria by spectrophotometric assays and high performance liquid chromatography. 2-Propylpentanoic acid was converted to 2-propylpentanoyl-CoA by Triton X-100 solubilized mitochondria at a rate of 0.25 nmol/min/mg of mitochondrial protein. 2-Propylpentanoyl-CoA is dehydrogenated by 2-methyl-branched chain acyl-CoA dehydrogenase to 2-propyl-2-pentenoyl-CoA (Ito *et al*, 1988) which is hydrated by enoyl-CoA hydratase to 3-hydroxy-2-propylpentanoyl-CoA. The later compound is dehydrogenated by an unknown NAD^+-dependent 3-hydroxyacyl-CoA dehydrogenase to 3-keto-2-propylpentanoyl-CoA, the structure of which was confirmed by mass spectrometry. The further metabolism of 3-keto-2-propylpentanoyl-CoA by either thiolytic cleavage, hydrolysis or transfer to carnitine was not detectable by our assay methods. These results agree with the observed accumulation of 3-keto-2-propylpentanoyl-CoA when coupled rat liver mitochondria were incubated with 2-propylpentanoic acid. The rate at which 3-keto-2-propylpentanoyl-CoA and the CoA thioesters of 2-propylpentanoic acid plus its metabolites were formed in coupled rat liver mitochondria are 0.24 and 0.44 nmol/min/mg of mitochondrial protein, respectively. It is concluded that 2-propylpentanoic acid is converted intramitochondrially to 3-keto-2-propylpentanoyl-CoA which possibly inhibits ß-oxidation by tying up CoA and moreover may directly inhibit ß-oxidation enzyme. 3-Keto-2-propylpentanoic acid observed in the plasma of patients treated with valproic acid may be derived from mitochondrial 3-keto-2-propylpentanoyl-CoA by a slow and so far undetected hydrolytic process.

The mechanism of 3-hydroxyacyl-CoA epimerase, which functions in the minor pathway of unsaturated fatty acid ß-oxidation, was investigated.

Chromatography of a soluble extract from a rat liver L fraction on DEAE-cellulose led to the separation of a novel D-3-hydroxyacyl-CoA dehydratase and enoyl-CoA hydratase which only when combined catalyze the epimerization of 3-hydroxyacyl-CoA's. The novel dehydratase was purified by a five step procedure and found to be a soluble peroxisomal protein with a native molecular weight of 106,800 and a subunit molecular weight of 44,000. It catalyzes the reversible dehydration of D-3-hydroxyacyl-CoA to 2-*trans*-enoyl-CoA but, in contrast to enoyl-CoA hydratase, does not act on 2-*cis*-enoyl-CoA's. It is suggested that 2-*cis*-enoyl-CoA intermediates formed during the ß-oxidation of polyunsaturated fatty acids in peroxisomes are hydrated by enoyl-CoA hydratase to D-3-hydroxyacyl-CoA's, which are epimerized to their L-isomers by the sequential actions of D-3-hydroxyacyl-CoA dehydratase and enoyl-CoA hydratase.

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TABLE OF CONTENTS

Abstract iii

Acknowledgements vi

List of Tables xii

List of Figures xiii

Abbreviations xvi

INTRODUCTION 1

EXPERIMENTAL PRECEDURES 10

Materials 10

Synthesis of 4-Bromo-2-octenoic Acid 11

Isolation of Rat Liver Mitochondria and Preparation
of a Soluble of Extract from Rat Liver Mitochondria 12

Measurement of Oxygen Uptake by Rat Liver Mitochondria 12

Enzyme Assays 13

Enzymatic Conversion of 4-Bromo-2-octenoyl-CoA to
3-Keto-4-bromooctanoyl-CoA and
Its Effect on Purified Thiolases 14

Measurement of Acetoacetate
Formation in Rat Liver Mitochondria 15

HPLC Analysis of Reaction Products 15

Synthesis of 2-Propyl-2E-pentenoyl-CoA
and 2-Propylpentanoyl-CoA 15

Degradation of 2-Propyl-2E-pentenoyl-CoA by
Triton X-100 solubilized Rat Liver Mitochondria 16

Metabolism of 2-Propylpentanoic Acid
by Coupled Rat Liver Mitochondria 17

Synthesis of 2-*cis*-Octenoyl-CoA 17

Purification of D-3-Hydroxyacyl-CoA Dehydratase 28

Determination of Equilibrium Constants for the
Hydration of 2-*cis*-Octenoyl-CoA and 2-*trans*-Octenoyl-CoA
by D-3-Hydroxyacyl-CoA Dehydratase 19

Electrophoresis 20

STUDY OF ß-OXIDATION INHIBITOR

Part I: 4-Bromo-2-octenoic Acid 21

RESULTS 21

Synthesis of 4-Bromo-2-octenoic Acid 21

Effect of 4-Bromo-2-octenoic Acid on
Fatty Acid Oxidation in Mitochondria 21

Metabolism of 4-Bromo-2-octenoic Acid by
ß-Oxidation Enzymes and the Effect of Its
Metabolites on the Activity of 3-Ketoacyl-CoA Thiolase 24

Effect of 4-Bromo-2-octenoic Acid on
Ketogenesis in Rat Liver Mitochondria 26

DISCUSSION 37

Part II: 2-Propylpentanoic Acid 30

RESULTS 30

Activation of 2-Propylpentanoic Acid 30

Hydration of 2-Propyl-2E-pentenoyl-CoA 30

Dehydrogenation of 3-Hydroxy-2-propylpentanoyl-CoA 32

Metabolism of 2-Propylpentanoic Acid by
Coupled Rat Liver Mitochondria 34

DISCUSSION 36

THE MECHANISM OF 3-HYDROXYACYL-CoA EPIMERIZATION 44

RESULTS 40

Purification of D-3-Hydroxyacyl-CoA Dehydratase 40

Mechanism of Epimerization 41

Synthesis of 2-*cis*-Octenoyl-CoA 42

Substrate Specificity of D-3-hydroxyacyl-CoA Dehydratase
and Determination of Equilibrium Constants 44

DISCUSSION 46

TABLES 49

FIGURES 53

REFERENCES 90

LIST OF TABLES

Table I: Effect of 4-bromo-2-octenoic acid on the activities of β-oxidation enzymes 49

Table II: Equilibrium constants for the hydrations of enoyl-CoA's to 3-hydroxyacyl-CoA's 51

Table III: Purification of D-3-hydroxyacyl-CoA dehydratase 52

LIST OF FIGURES

FIGURE 1: The fatty acid ß-oxidation cycle 53

FIGURE 2: Pathway of linoleic acid degradation 55

FIGURE 3: Effect of 4-bromo-2-octenoic acid on respiration in rat liver mitochondria 57

FIGURE 4: Effect of 4-bromo-2-octenoic acid on palmitoylcarnitine-supported respiration and thiolase activities in rat liver mitochondria 59

FIGURE 5: Spectrophotometric evidence for the metabolism of 4-bromo-2-octenoyl-CoA by ß-oxidation enzymes 61

FIGURE 6: Spectrum of the Mg^{2+}-enolate complex of 3-keto-4-bromooctanoyl-CoA 63

FIGURE 7: Effect of 4-bromo-2-octenoyl-CoA and its ß-oxidation metabolites on the 3-ketoacyl-CoA thiolase activity present in a soluble extract of rat liver mitochondria 65

FIGURE 8: Proposed pathway for the metabolism of 4-bromo-2-octenoic acid..... 67

FIGURE 9: HPLC analysis of products formed during the degradation of 2-propylpentanoic acid 68

FIGURE 10: Formation of NADH during the dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA 70

FIGURE 11: Mass spectrum of 3-keto-2-propylpentanoyl-CoA 72

FIGURE 12: Degradation of 2-propylpentanoic acid by coupled mitochondria 74

FIGURE 13: Proposed pathway of the mitochondrial metabolism of 2-propylpentanoic acid 76

FIGURE 14: Polyacrylamide gel electrophoresis of D-3-hydroxyacyl-CoA dehydratase under nondenaturing conditions 78

FIGURE 15: Native and subunit molecular weight of D-3-hydroxyacyl-CoA dehydratase 80

FIGURE 16: HPLC analysis of products formed during the ß-oxidation of D-3-hydroxyoctanoyl-CoA 82

FIGURE 17: HPLC analysis of the products formed during ß-oxidation of 2-*cis*-octenoyl-CoA 84

FIGURE 18: HPLC analysis of 2-*cis*-octenoyl-CoA and 2-*trans*-octenoyl-CoA incubated with crotonase and D-3-hydroxyacyl-CoA dehydratase 86

FIGURE 19: ß-Oxidation of 4-*cis*-decenoyl-CoA by the epimerase-dependent pathway 88

Abbreviations

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
DEAE	diethylamino ethyl
DTNB	5,5'-dithio-*bis*-(2-nitrobenzoic acid)
EDTA	ethylenediamineteraacetate
EGTA	[ethylenebis(oxyethylenenitrilo)]tetraacetic acid
HSCoA	coenzyme A
HPLC	high performance liquid chromatography
NAD^+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
$NADP^+$	nicotinamide adenine diuncleotide phosphate, oxidized form
NADPH	nicotinamide adenine diuncleotide phosphate, reduced form
PMSF	phenyl methane sulfonyl fluoride
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride

INTRODUCTION

ß-Oxidation of fatty acids plays a major role in energy metabolism in mammals, especially in heart and skeletal muscle. The pathway of saturated fatty acid oxidation in mitochondria (see Figure 1, for a review see Schulz, 1985) and in peroxisomes is well established (Lazarow and De Duve, 1976). But unlike many other metabolic pathways, for example glycolysis, gluconeogenesis, or glycogenolysis, the regulation of fatty acid ß-oxidation is still poorly understood.

Three of the ß-oxidation enzymes may play a role in regulation of this pathway. The first enzyme is carnitine acyl-CoA transferase (CPT I) (EC.2.3.1.21), which facilitates the entrance of long-chain acyl-CoA's into mitochondria, while short-chain and medium-chain fatty acids can directly diffuse into mitochondria where they are activated. The second enzyme of interest is acyl-CoA dehydrogenase (EC 1.3.99.3) which catalyzes the formation of 2-*trans*-enoyl-CoA from acyl-CoA. The third enzyme is 3-ketoacyl-CoA thiolase (EC 2.3.1.16) which catalyzes the last reaction of ß-oxidation by cleaving 3-ketoacyl-CoA's to acetyl-CoA and acyl-CoA's shortened by two carbon atoms.

Compounds which specifically inhibit key regulatory enzymes are important tools in studying the control of metabolic pathways. Fatty acid oxidation is one of the pathways whose regulation has been probed by the use of several inhibitors (Olowe & Schulz, 1982; Declercq *et al.*, 1987). The known inhibitors of this pathway affect one of three reactions (for a recent review, see Schulz, 1987): those catalyzed by carnitine palmitoyltransferase I, a key regulatory enzyme in liver (McGarry & Foster, 1980); 3-ketoacyl-CoA thiolase, a suggested regulatory enzyme in heart (Olowe & Schulz, 1980); and acyl-CoA dehydrogenase, which catalyzes the first step of ß-

oxidation.

An example for the use of inhibitors in studying the regulation of ß-oxidation was reported by Olowe and Schulz (1982). Their earlier work (Olowe and Schulz, 1980) showed that 3-ketoacyl-CoA thiolase activity was significantly inhibited by acetyl-CoA at concentrations of HSCoA and acetyl-CoA which are assumed to exist intramitochondrially at state-4 respiration. This result suggested that fatty acid oxidation may be controlled via the regulation of 3-ketoacyl-CoA thiolase by the acetyl-CoA/HSCoA ratio which is determined by the rate of the citric acid cycle and consequently by the energy demand of the tissue. To determine whether 3-ketoacyl-CoA thiolase catalyze the rate-limiting step in ß-oxidation, an inhibitor of this enzyme was required to study effect of inhibition thiolase on the rate of ß-oxidation. For this purpose, 4-bromocrotonic acid was synthesized and tested in rat heart mitochondria. This compound is first activated to its HSCoA derivative and then metabolized by two ß-oxidation steps to yield 4-bromo-3-keto-butyryl-CoA which inactivates 3-ketoacyl-CoA thiolase as well as acetoacetyl-CoA thiolase. It was observed that a decrease in thiolase activity was paralleled by a decreasing rate of mitochondrial respiration supported by palmitoylcarnitine. Based on this finding it was suggested that 3-ketoacyl-CoA thiolase catalyzes the rate-limiting reaction or perhaps one of several equally slow reactions of ß-oxidation in rat heart mitochondria. Thus ß-oxidation in rat heart mitochondria can be regulated by altering 3-ketoacyl-CoA thiolase activity. It is obvious that the identification or design of synthetic enzyme inhibitors can be very useful in studying the regulation of metabolic pathways.

It is noteworthy that all ß-oxidation inhibitors known so far, except for acylaminocarnitine, are fatty acids with a reactive or potentially reactive functional

group. They must first be converted to their HSCoA derivatives and some of them have to be further metabolized by the ß-oxidation pathway before they become inhibitory and bind to the active sites of enzymes. For example, 4-pentenoic acid was found to be a thiolase inhibitor in rat heart mitochondria as well as in rat liver mitochondria. This compound is metabolized via two competing pathways (Schulz, 1983). The common part of these two pathway are the activation of 4-pentenoic acid to its HSCoA derivative followed by dehydrogenation to 2,4-pentadienoyl-CoA. Metabolism of 2,4-pentadienoyl-CoA in the major pathway involves 2,4-dienoyl-CoA reductase which catalyzes the reduction of 2,4-pentadienoyl-CoA to 3-*trans*-pentenoyl-CoA. This compound is then isomerized to 2-pentenoyl-CoA which can be degraded by ß-oxidation to generate acetyl-CoA and propionyl-CoA (Schulz, 1983). In the minor pathway, 2,4-pentadienoyl-CoA is directly ß-oxidized to acryloyl-CoA and acetyl-CoA. Only an intermediate in the minor pathway, 3-keto-4-pentenoyl-CoA, is the inhibitor of thiolase (Schulz, 1983). In general, inhibitors of ß-oxidation enzymes should be structurally similar to a fatty acid and should be metabolized in mitochondria to a compound which resembles the substrates and in addition carries a reactive groups.

Although several inhibitors of 3-ketoacyl-CoA thiolase are known, none inactivates only this enzyme. For example, 4-bromocrotonic acid is metabolized intramitochondrially to 3-keto-4-bromobutyryl-CoA which effectively and irreversibly inactivates 3-ketoacyl-CoA thiolase as well as acetoacetyl-CoA thiolase, thereby inhibiting both fatty acid oxidation and ketone body degradation (Olowe & Schulz, 1982). 2-Bromooctanoic acid (Raaka & Lowenstein, 1978) and 4-pentenoic acid (Schulz,1983) also inactivate both mitochondrial thiolases.

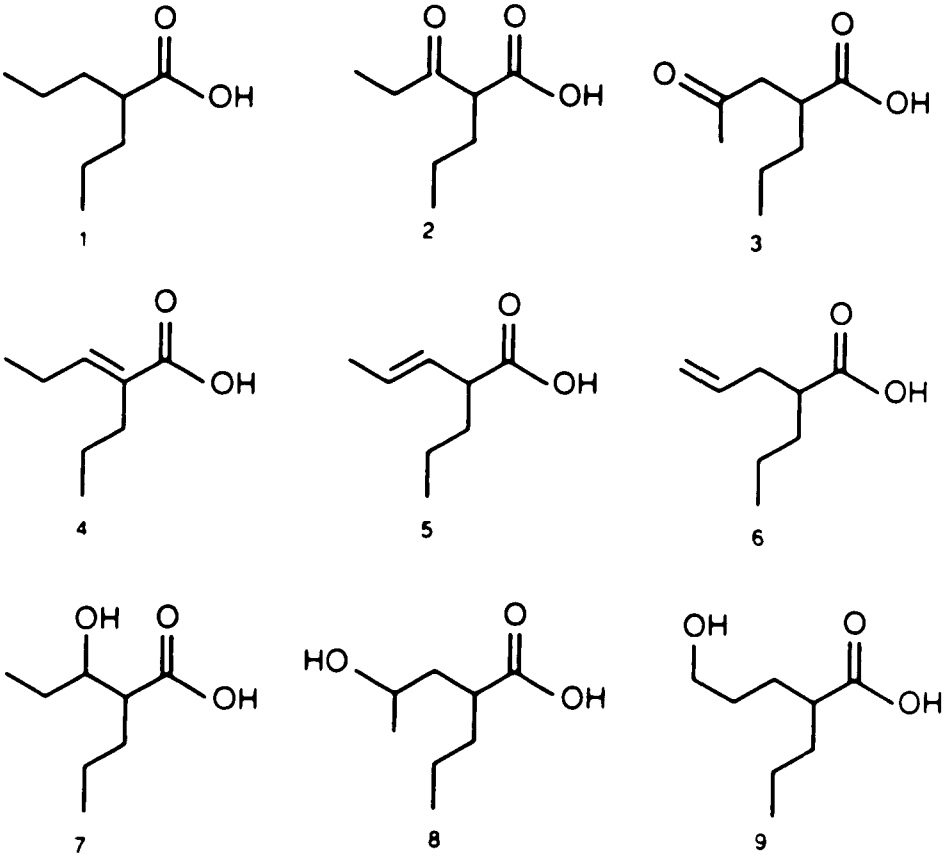
Since all known inhibitors of thiolase inactivate 3-ketoacyl-CoA thiolase as

well as acetoacetyl-CoA thiolase, an attempt was made to design a compound which would specifically inactivate 3-ketoacyl-CoA thiolase. In 4-bromo-2-octenoic acid, a longer chain homolog of 4-bromocrotonic acid, I found such compound.

Another fatty acid β-oxidation inhibitor of interest is 2-propylpentanoic acid (valproic acid), a widely used antiepileptic drug for the treatment of several types of epilepsy. It has been in use in Europe since the late 1960's and was introduced into the United States in 1978. Interestingly, it was first used as a solvent in testing potential anticonvulsant drugs. Later, it was discovered that the solvent itself is a strong anticonvulsant drug. Unfortunately it is toxic in some pateints and may cause fatal hepatic injuries. More then 100 fatal cases of hepatotoxicity have been reported worldwide (Scheffner *et al.* 1988). The cause for the toxicity of 2-propylpentanoic acid remains unknown though it has been studied for many years.

After supplying 2-propylpentanoic acid to living animals, a number of metabolites have been identified in urine and plasma by gas chromatography and mass spectroscopy. The major metabolites and their structures are listed below:

1. 2-Propylpentanoic acid
2. 3-Keto-2-propylpentanoic acid
3. 4-Keto-2-propylpentanoic acid
4. 2-Propyl-2-pentenoic acid
5. 2-Propyl-3-pentenoic acid
6. 2-Propyl-4-pentenoic acid
7. 3-Hydroxy-2-propylpentanoic acid
8. 4-Hydroxy-2-propylpentanoic acid
9. 5-Hydroxy-2-propylpentanoic acid



It has been reported by Prickett and Baillie (1984) that the 3-hydroxy, 4-hydroxy and 5-hydroxy derivatives of 2-propylpentanoic acid are formed in rat liver by cytochrome P-450-dependent ω oxidation, ω-1 oxidation and ω-2 oxidation that occur in the endoplasmic reticulum. When 2-propylpentanoic acid was incubated with a rat liver microsomal fraction, the formation of these three compounds was observed. Inhibitors of cytochrome P-450 inhibited ω-oxidation and phenobarbital treatment of rats resulted in a stimulation. A recent report from the same laboratory (Rettie *et al.*, 1988) indicated that cytochrome P-450 also catalyzes the formation of 2-propyl-4-pentenoic acid at a rate about 30-times slower than the formation of hydroxylated metabolites.

Dienes derived from 2-propylpentanoic acid have also been observed in the urine and plasma of patients on valproic acid therapy (Kochen and Sprunck, 1984). The location and source of the enzymes involved have not been reported.

2-Propyl-2-pentenoic acid, 3-hydroxy-2-propylpentanoic acid and 3-keto-2-propylpentanoic acid are thought to be formed by mitochondrial ß-oxidation of valproic acid. Although the conversion of 2-propylpentanoic acid to 2-propylpentanoyl-CoA in hepatocytes and mitochondria has been reported (Becker and Harris, 1983, Turnbull *et al.*, 1983), little is known about the metabolism of 2-propylpentanoic acid by mitochondria, or by the ß-oxidation enzymes. A preliminary report by Ito *et al.* (1987) provided evidence for the conversion of 2-propylpentanoyl-CoA to 2-propyl-2-pentenoyl-CoA in mitochondria by 2-methyl-branched chain acyl-CoA dehydrogenase, one of the enzymes of isoleucine degradation. This reaction may account for the formation of 2-propyl-2-pentenoic acid observed in urine and plasma, although this compound may also be formed by dehydration of 3-hydroxy-2-propylpentanoyl-CoA formed by ω-2 oxidation in the endoplasmic reticulum. The

further metabolism of 2-propyl-2-pentenoyl-CoA in mitochondria is largely unknown.

Several reports have provided evidence for the inhibition of fatty acid degradation by 2-propylpentanoic acid. It has been observed that 2-propylpentanoic acid not only inhibits the degradation of long-chain fatty acids (Turnbull *et al.*, 1983, Coude, *et al.*, 1983) and medium-chain fatty acids (Bjorge and Baillie, 1985) in rat liver homogenate, but also inhibits pyruvate-supported mitochondrial respiration (Turnbull *et al.*, 1983, Rumbach *et al.*, 1983). 2-Propyl-4-pentenoic acid, one of the metabolites of 2-propylpentanoic acid, has been reported to have a strong inhibitory effect on fatty acid oxidation (Bjorge and Baillie, 1985). Its inhibitory effect may be similar to the well-known inhibition of ß-oxidation by 4-pentenoic acid, because of their structural similarity (Schulz, 1983).

Unresolved questions about the ß-oxidation of 2-propylpentanoic acid have prompted this investigation of the metabolism of 2-propylpentanoic acid by isolated rat liver mitochondria and the purified enzymes of ß-oxidation.

While the pathway of saturated fatty acid oxidation has long been established, most efforts are now aimed at elucidating its regulation, some questions remain regarding the pathway by which in the polyunsaturated fatty acids are degraded, however. The double bonds of polyunsaturated fatty acids can extend from odd-number or even-number carbon atoms. As shown in Figure 2, linoleic acid which has one *cis* double bond each extending from an odd-numbered and an even-numbered carbon atom is converted to 3-*cis*,6-*cis*-dodecadienoyl-CoA after three cycles of ß-oxidation. The further metabolism of this compound requires the involvement of an auxiliary enzyme named Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase

(EC 5.3.3.8), which converts the 3-*cis* double bond to a 2-*trans* double bond (Stoffel *et al.*, 1964). This enzyme has been purified and characterized. It was found to isomerize 3-*cis* as well as 3-*trans* double bonds to 2-*trans* double bonds (Stoffel, 1978, Miesowicz, 1979).

After two more ß-oxidation cycles, 2-*trans*, 6-*cis*-dodecadienoyl-CoA yields 2-*cis*-enoyl-CoA. According to Wakil (1956) 2-*cis*-enoyl-CoA is hydrated by crotonase to D-3-hydroxyacyl-CoA. Since L-3-hydroxyacyl-CoA dehydrogenase only acts on the L-isomer, another auxiliary enzyme, 3-hydroxyacyl-CoA epimerase (Stoffel *et al.*, 1965), is required to convert D-3-hydroxyacyl-CoA to L-3-hydroxyacyl-CoA (Figure 2A). However, 2-*cis*-octenoyl-CoA, a proposed intermediate of linoleic acid degradation, has never been observed.

Kunau and Dommes reported the existence of another auxiliary enzyme, 2,4-dienoyl-CoA reductase (Figure 2B). This enzyme, in the presence of NADPH, catalyzes the reduction of 2-*trans*-4-*cis*-decadienoy-CoA to 3-*trans*-decenoyl-CoA which is converted by the isomerase to 2-*trans*-decenoyl-CoA before being completely degraded by ß-oxidation. A number of experiments support this revised pathway. For example, the oxidation of docosahexenoylcarnitine ($\Delta^{4,7,10,13,16,19}$-$C_{22:6}$) in contrast to that of palmitoylcarnitine, is inhibited in uncoupled mitochondria suggesting that energy is required for its degradation. This inhibition is partly or completely overcome by the addition of either ATP or glutamate. These results have been interpreted in terms of a requirement for NADPH which can be generated by glutamate dehydrogenase (EC 1.4.1.4) or by $NAD(P)^+$ transhydrogenase in uncoupled mitochondria driven by ATP. Cuebas and Schulz reported (1982) that 2-*trans*-4-*cis*-decadienoyl-CoA cannot be degraded to 2-*cis*-octenoyl-CoA in a reconstituted ß-oxidation system. Upon addition of 2,4-dienoyl-

CoA the reductase and NADPH a rapid disappearance of substrate and NADPH was observed. This result indicated that linoleic acid is most likely degraded via the reductase pathway rather than by the epimerase-dependent pathway.

However, the existence of 3-hydroxyacyl-CoA epimerase prompted further studies aimed at characterizing it and elucidating its biological function. When epimerase activity was assayed in rat heart mitochondria, the observed activity was found to be lower than the rate of linoleic acid degradation (Chu, 1984). An analysis of the subcellular distribution of epimerase revealed that it is located in peroxisomes but not in mitochondria (Chu and Schulz, 1985). Most likely the reductase-dependent pathway is the only pathway of linoleic acid degradation in mitochondria. Since both reductase (Dommes *et al.*, 1981) and epimerase are present in peroxisomes, the contribution of the epimerase-dependent pathway in this organelle was studied. A careful study led Yang *et al.* (1986) to conclude that in peroxisomes, unlike in mitochondria, the epimerase-dependent pathway may contribute a small percentage to the degradation of polyunsaturated fatty acids. In spite of great efforts, the purification of the epimerase was not achieved, mostly due to dramatic activity losses during various chromatographies. In order to study the significance of this minor pathway, attempts were made to purify and characterize this enzyme.

EXPERIMENTAL PROCEDURES

Materials

Sigma was the source of NAD^+, HSCoA, $NADP^+$, EGTA, bovine serum albumin, pigeon breast muscle carnitine:acetyl-CoA transferase, pig heart 3-hydroxyacyl CoA dehydrogenase and all standard biochemicals. n-Butyryl-CoA, n-decanoyl-CoA, n-palmitoyl-CoA, ATP and ADP were purchased from Pharmacia Biotechnology, Inc. 2-Decenoic acid, 2-octynoic acid, 2-propylpentanoic acid and acetonitrile (HPLC grade) were obtained from Aldrich. 2-Octenoic acid was synthesized from hexanal and malonic acid in the presence of pyridine as described in principle by Box and Linstead (1931). D-3-Hydroxy and L-3-hydroxyoctanoyl-CoA was synthesized as described (Smeland, *et al.*, 1989). Coenzyme A thioesters of 2-decenoic acid, 4-bromo-2-octenoic acid, D-hydroxyoctanoic acid, L-3-hydroxyoctanoic acid, 2-octenoic acid and 2-octynoic acid were synthesized by the mixed-anhydride method as described in principle by Goldman and Vagelos (1961). 3-Ketooctanoyl-CoA was prepared from 2-octynoyl-CoA as described by Thorpe (1986). The concentrations of all coenzyme A derivatives were determined by the method of Ellman (1959) after cleavage of the thioester bond with hydroxylamine at pH 7. Pig heart 3-ketoacyl-CoA thiolase (Staack et al., 1978), pig heart acetoacetyl-CoA thiolase (Yang *et al.*, 1987) and bovine liver crotonase (Steinmmam & Hill, 1979) were prepared as described. Palmitoyl-L-carnitine, acetyl-L-carnitine and L-carnitine were gifts from Dr. K. Brendel, University of Arizona, College of Medicine. 2-Propyl-2E-pentenoic acid was generously provide by Dr. H. Nau, Freie University Berlin, Berlin, F.R.G. A rat liver microsomal fraction was prepared as described in principle by Applemans *et al.*(1955).

Synthesis of 4-Bromo-2-octenoic Acid

The procedure of Bellassoued et al. (1983) developed for the synthesis of 4-bromocrotonic acid was used to prepare 4-bromo-2-octenoic acid. To 8 g of 2-octenoic acid (56 mmol) and 9 ml of chlorotrimethylsilane (71 mmol) in 200 ml of anhydrous ether was added 6.2 ml of anhydrous pyridine dropwise under vigorous stirring. The resulting mixture was heated under reflux for 3 hr after which time the formed precipitate was removed by filtration and the ether was distilled off under reduced pressure. The resultant 11.5 g of trimethylsilyl 2-octenoate (54 mmol) was used without further purification. Ten grams (47 mmol) of this compound dissolved in 70 ml of CCl_4 was combined with 8.9 g of N-bromosuccinimide (50 mmol). After the mixture was heated to the boiling point, 0.5 g of dibenzoyl peroxide was added to start the reaction, and the refluxing was continued for 3 hr. The formed precipitate was removed by filtration, and CCl_4 was evaporated under reduced pressure. The remaining oil was distilled and the fraction boiling between 130 and 135 ^{o}C at 3.5 mmHg was collected. In order to hydrolyze trimethylsilyl ester, this fraction was combined with an equal volume of water, shaken for 10 min, and 4-bromo-2-octenoic acid was extracted 3 times each with 5 ml of ether. The etherel extracts were combined dried over Na_2SO_4. After evaporation of ether, 6.1 g (27.6 mmol) of 4-bromo-2-octenoic acid was obtained (49% yield). Its ^{1}H NMR spectrum in $CDCl_3$ gave the following resonances (TMS = 0 ppm): 4.17 ppm (d of t, J_d=8 Hz, J_t=6.2 Hz, 1 H, -CHBr-), 5.54 ppm (d, J=15 Hz, 1 H, =CH-COO-), and 6.6 ppm (d of d, J=15 Hz and 8 Hz, 1 H, R-CH=). The result of an elemental analysis is shown below:

$C_8H_{14}O_2Br$

Calculated: C, 43.44; H, 5.88; Br, 36.17

Found: C, 43.44; H, 5.86; Br, 35.46

Isolation of Rat Liver Mitochondria and Preparation of a Soluble Extract from Rat Liver Mitochondria

Rat heart mitochondria were isolated according to the procedure of Chappel and Hansford (1969). Rat liver mitochondria were isolated in a similar manner except that 0.25 M sucrose was used instead of 0.21 M mannitol plus 0.07 M sucrose and the treatment with Nagarse was omitted. Mitochondria suspended in isolation buffer were sonicated at 0^{o}C 5 times for 5 s each with a Branson sonifier (Model W-185) equipped with a microtip. The resulting mixture was centrifuged at a 105,000 g for 1 hr. Protein concentrations of the mitochondrial suspension and the soluble mitochondrial extract were determined by the biuret method (Gornall et., 1949).

Measurement of Oxygen Uptake by Rat Liver Mitochondria

Rat liver mitochondria (2 mg) were suspended in 1.9 ml of a basal isotonic incubation buffer containing 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 4 mM KP_i, 4 mM $MgCl_2$, and 0.1 mM EGTA. To this suspension were added in the indicated sequence: bovine serum albumin (0.5 mg/ml), 0.5 mM L-malate, 1 mM ADP, and 1 min later, varying amounts of 4-bromo-2-octenoic acid. The mixture was incubated for 3 min or the indicated periods of time. State 3 respiration was started by the addition of 30 µM of palmitoylcarnitine or 5 mM pyruvate. Oxygen uptake was measured polarographically with a Clark oxygen electrode attached to a Gilson oxygraph.

Rat liver mitochondria were incubated with varying concentrations of 4-bromo-2-octenoic acid for 3 min as described in the preceding paragraph. No respiratory substrate had been added when samples were quickly frozen in a dry ice/acetone bath and stored at -76^{O}C until enzyme assays were performed.

Enzyme Assays

Acyl-CoA dehydrogenase (EC 1.3.99.3), butyryl-CoA dehydrogenase (EC 1.3.99.2) and 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) were assayed as described by Olowe and Schulz (1982). The activities of acetoacetyl-CoA thiolase (EC 2.3.1.9) and 3-ketoacyl-CoA thiolase (EC 2.3.1.16) were determined as described by Binstock and Schulz (1981). Extinction coefficients of 5,500 cm^{-1} M^{-1} and 13,300 cm^{-1} M^{-1} were used when 3-ketooctanoyl-CoA and acetoacetyl-CoA were used as substrates, respectively. The activity of acetoacetyl-CoA thiolase was obtained by subtracting from the combined activities of both thiolases measured with acetoacetyl-CoA the part due to 3-ketoacyl-CoA thiolase. The activity of the latter enzyme with acetoacetyl-CoA as a substrate was calculated from its 3-ketooctanoyl-CoA thiolase activity, which is between 3.7 and 3.9 times greater than its activity with acetoacetyl-CoA (Middleton, 1975). Enoyl-CoA hydratase (EC 4.2.1.1) and D-3-hydroxyacyl-CoA dehydratase were assayed in both the forward and the reverse direction as described by Smeland *et al.* (1989). All enzyme assays were performed at 25 ^{O}C on a Gilford recording spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product per min.

Enzymatic Conversion of 4-Bromo-2-octenoyl-CoA to 3-Keto-4-bromooctanoyl-CoA and its Effect on Purified Thiolases.

To 1 ml of 20 μM 4-bromo-2-octenoyl-CoA in 0.1 M HEPES buffer (pH 8) were added 15 mU of enoyl-CoA hydratase. When the hydration reaction had reached equilibrium as judged by observing no further decrease in the absorbance at 263 nm, 1mM NAD^+ and 20 mM $MgCl_2$ were added. The dehydrogenation reaction was started by the addition of 2 U of 3-hydroxyacyl-CoA dehydrogenase, and the progress of the reaction was monitored spectrophotometrically at 340 nm. After the reaction had reached equilibrium, 10 mU of 3-ketoacyl-CoA thiolase or 4 mU of acetoacetyl-CoA thiolase were added and incubated for 3 min before the addition of 60 μM CoASH and either 12 μM 3-ketooctanoyl-CoA or 16 μM acetoacetyl-CoA. The absorbance of the solution was recorded at 303 nm.

Samples containing in 1 ml of 0.1 M HEPES buffer (pH 8), 20 μM 4-bromo-2-octenoyl-CoA, 15 mU of enoyl-CoA hydratase, 1 mM NAD^+, 20 mM $MgCl_2$, 2 U of 3-hydroxyacyl-CoA dehydrogenase, 10 mU of 3-ketoacyl-CoA thiolase, or 4 mU of acetoacetyl-CoA thiolase were incubated for 3 min and dialyzed for 36 h against 2 L of 0.75 M Tris-HCl (pH 8), containing 25% glycerol, 10 mM mercaptoethanol, and bovine serum albumin (1 mg/ml). The dialysis buffer was changed once after 20 h. After the dialysis was completed, 200 μl of the sample was transferred into 700 μl of H_2O containing 60 μM CoASH and 20 mM $MgCl_2$. Either 12 μM 3-ketooctanoyl-CoA or 16 μM acetoacetyl-CoA was added to start the reaction. Extinction coefficients of 14,400 cm^{-1} M^{-1} and 21,300 cm^{-1} M^{-1} were used to calculate rates for the thiolysis of 3-ketooctanoyl-CoA and acetoacetyl-CoA, respectively. The changes in volume that occurred during dialysis were corrected for by weighing of samples before and after dialysis.

Measurement of Acetoacetate Formation in Rat Liver Mitochondria.

Liver mitochondria from rats fasted for 24 h were suspended in the incubation system used for respiration measurements. In addition 3.3 mM malonate was present. Reactions were started by the addition of 30 μM palmitoylcarnitine or 3 mM acetylcarnitine and allowed to proceed for 5 min at which time the reaction was stopped by bringing the pH to 1.8 with $HClO_4$ followed immediately by adjusting the pH to 6 with 1 M KOH. The concentration of acetoacetate formed was determined as described by Krebs *et al.* (1969).

HPLC Analysis of Reaction Products

The HPLC analysis was performed on a μBondapak C_{18} reverse phase column (30 cm x 3.9 mm) attached to a Waters gradient HPLC system. Separation was achieved by linearly increasing the acetonitrile content of the 50 mM ammonium phosphate (pH 5.5) elution buffer from 10% to 45% in 30 min at a flow rate of 2 ml/min.

Synthesis of 2-Propyl-2E-pentenoyl-CoA and 2-Propylpentanoyl-CoA

To 62 mg of dried 2-propyl-2E-pentenoic acid (or 60 mg of 2-propylpentanoic acid) were added 2 ml of $SOCl_2$. This mixture was incubated at 60°C for 20 hr. At the end of the reaction, $SOCl_2$ was evaporated under reduced pressure. The residue was dissolved in 4 ml freshly distilled anhydrous THF and added dropwise to a flask containing 15 mg HSCoA, 30 mg $KHCO_3$, 2 ml H_2O and 3

ml THF under a N_2 atmosphere. During the reaction, additional 20 mg of $KHCO_3$ were added to maintain the pH at 8. The free HSCoA content was tested with Ellman's reagent. When all HSCoA had reacted after about 25 minutes, the reaction was stopped by adjusting the pH to 4 with concentrated HCl. THF was evaporated under reduced pressure. The resultant solution was extracted 4 times with ether and ether remaining in the solution was removed by bubbling N_2 through it. The sample was then further purified by HPLC. The concentration of 2-propylpentanoyl-CoA was determined spectrophotometrically using the extinction coefficient of HSCoA at 260 nm of 14,600 $cm^{-1}M^{-1}$. The concentration of 2-propyl-2E-pentenoyl-CoA was determined in the same way using the extinction coefficient for 2-*trans*-octenoyl-CoA at 260 nm of 21,300 $cm^{-1}M^{-1}$.

Degradation of 2-Propyl-2E-pentenoyl-CoA by Rat Liver Mitochondria

Hydration of 2-propyl-2E-pentenoyl-CoA was carried out in a mixture containing 0.2 M Tris-HCl (pH 10) and 30 μM of 2-propyl-2E-pentenoyl-CoA. The reaction was started by the addition of either crotonase or rat liver mitochondria solubilized with 0.07% Triton X-100 and was monitored spectrophotometrically at 263 nm. Dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA was assayed in the presence of 0.2 M Tris-HCl (pH 10), 0.07% Triton X-100, 1.6 mM of NAD^+ or 1.6 mM of $NADP^+$, 30 μM of 2-propyl-2E-pentenoyl-CoA and 29 U of crotonase. The reaction was started by the addition of either rat liver mitochondria or L-3-hydroxyacyl-CoA dehydrogenase and monitored spectrophotometrically at 340 nm. The rate of synthesis of 2-propylpentanoyl-CoA from 2-propylpentanoic acid was assayed according the procedure described by Cuebas (1985). To 0.2 M Tris-HCl (pH 8) buffer were added 0.5 mM 2-propylpentanoic acid, 10 mM $MgCl_2$, 4 mM ATP and 0.5 mM HSCoA. The reaction was started by the addition of rat liver

mitochondria. At indicated times, the reaction was stopped by acidifying the mixture to pH 1 with HCl. After 1 min, the samples were filtered and adjusted to pH 5 with 4 M KOH. They were then centrifuged at 9220 xg for 5 min at 4^{O}C and filtered through a 0.22 μm filter before being subjected to HPLC analysis under the condition described before.

Metabolism of 2-Propylpentanoic Acid by Intact Rat Liver Mitochondria

Rat liver mitochondria (4 mg) were suspended in 1 ml of a basal isotonic incubation buffer containing 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 4 mM KP_i,4 mM $MgCl_2$, and 0.1 mM EGTA. To this suspension were also added bovine serum albumin (0.5 mg/ml), 0.5 mM L-malate, 1 mM ADP, 4 mM ATP and 2 mM carnitine. The reaction was started by the addition of 0.5 mM 2-propylpentanoic acid. The reaction was then stopped at indicated times by the addition of 1 drop of concentrated HCl to bring the pH to 1 and filtered through an Millipore membrane (0.22 μm). The sample was adjusted to pH 5 with 4 M KOH and the precipitate was removed by centrifugation at 9220 xg for 5 min. The supernatant was filtered again through 0.22 μm filter before being subjected to HPLC analysis under the condition described before.

Synthesis of 2-*cis*-octenoyl CoA

Two ml of 6.4 mM octynoyl-CoA in water (pH 2) were added to 5 ml of methanol containing 69 mg of Pd/C (5%) and 60 mg of quinoline. This mixture was shaken vigorously under 1 atm H_2 for 1 hr at which time one drop of acetic acid was added to stop the reaction. Particles were removed by filtration. After evaporating methanol under reduced pressure at room temperature in the dark, 3 ml of crude

product was obtained. It was further purified by HPLC on a μBondapak C_{18} column (30 cm x 3.9 mm) at the flow rate of 1 ml/min by linearly increasing the methanol content of the 50 mM ammonium phosphate (pH 5.5) elution buffer from 10% to 50% in 30 min and then continuing the elution at the final condition for an additional 15 min. At each run 250 μl of the crude 2-*cis*-octenoyl-CoA were injected. The main peak, emerging between minor peaks due to unreacted 2-octynoyl-CoA and fully hydrogenated octanoyl-CoA, was assumed to be 2-*cis*-octenoyl-CoA. Fractions corresponding to the major peak were collected from a number of separate runs and concentrated under reduced pressure at 25 ^{O}C in the dark to remove methanol. The resultant solution was further concentrated by ultrafiltration through an Amicon YC05 membrane to yield 1.5 ml of 1.53 mM 2-*cis*-octenoyl-CoA. Final yield 18%. Rechromatography of the sample on HPLC showed the remaining impurity to be less then 4%.

Purification of D-3-Hydroxyacyl-CoA Dehydratase

Rat liver light mitochondria were isolated from 8 male Sprague-Dawley rats as described in principle by Appelmans *et al.* (1955). Rat liver light mitochondria (543 mg of protein) were suspended in 55 ml of 5 mM Tris-phosphate buffer (pH 8) in solution A which contained 10% glycerol, 10 mM mercaptoethanol, 0.5 mM benzamidine and aprotinin (0.06 KIU/ml). A soluble extract was prepared by treating this suspension with a Polytron homogenizer for 6x20 s at 4^{O}C in the presence of 0.1 mM PMSF followed by centrifugation at 105,000xg for 60 min. The resultant supernatant containing 102 mg of protein in 50 ml was applied to a DEAE-cellulose column (20 x 2.5 cm) equilibrated in 55 ml of 5 mM Tris-phosphate buffer (pH 8) in solution A. The column was developed with the with a linear gradient of Tris-phosphate increasing from 5 mM to 500 mM in solution A. Fractions showing

epimerase activity only when supplemental with crotonase were pooled and concentrated by ultrafiltration using an Amicon PM 10 membrane. By repeating the dilution and concentration 4 times, the sample buffer was changed to 18 ml of 10 mM KP_i (pH 6.8) in solution A. This sample was applied to a hydroxylapatite column (30 x 2.5 cm) equilibrated with the sample buffer. The dehydratase was eluted with a gradient from 0 to 2 M KCl in the same buffer. Fractions containing hydratase activity were combined and concentrated by ultrafiltration. By repeately diluting and concentrating the buffer was changed to 7.7 ml of 10 mM KP_i (pH 8) in solution A. This sample was applied to a 3',5'-ADP Agarose column (13 x 0.75 cm) equilibrated with the sample buffer. The sample was eluted with a linear gradient of 10 mM to 50 mM KP_i (pH 8) in solution A. Fractions containing the dehydratase were pooled and concentrated by ultrafiltration to 1.6 ml. Further purification was achieved on a Perkin-Elmer Isopure LC system having on line a TSK gel filtration column (type G 3000 sw, 30 x 0.75 cm). The column was equilibrated with 100 mM KP_i (pH 8) containing 10% glycerol and 10 mM mercaptoethanol. Fractions containing dehydratase activity were pooled and concentrated. After adjusting the glycerol concentration to 30%, the enzyme was stored at -76^{O}C.

Determination of Equilibrium Constants

To 50 μM 2-enoyl-CoA or 3-hydroxyoctanoyl-CoA in 0.22 M KP_i buffer (pH 8) was added either 0.4 μg of crotonase or 4 μg of D-3-hydroxyacyl-CoA dehydratase. Where indicated, the reaction was coupled with excess amount of 3-ketoacyl-CoA thiolase and L-3-hydroxyacyl-CoA dehydrogenase in the presence of 1.6 mM NAD^+ and 0.3 mM HSCoA to remove contaminating 2-*trans*-enoyl-CoA and L-3-hydroxyacyl-CoA. The reaction was monitored spectrophotometrically until no further absorbance change was detected. The reaction was then terminated by

adjusting the solution to pH 1 with one drop concentrated HCl followed by filtering it through an Millipore membrane (0.22 μm) to remove precipitated protein. After adjusting the pH to 5, the sample was subjected to HPLC analysis under the condition described before. The areas under the peaks were calculated according to peak height x half height width/2 and corrected for different molar absorbances at 254 nm of 2-trans-octenoyl-CoA ($22x10^3$ $M^{-1}cm^{-1}$), 2-*cis*-octenoyl-CoA ($21.1x10^3$ $M^{-1}cm^{-1}$), L-3-hydroxyoctanoyl-CoA ($15.4x10^3$ $M^{-1}cm^{-1}$) and D-3-hydroxyoctanoyl-CoA ($15.9x10^3$ $M^{-1}cm^{-1}$).

Electrophoresis

Polyacrylamide gel electrophoresis of D-3-hydroxyacyl-CoA dehydratase (5 μg protein) under nondenaturing conditions was performed according to the procedure described by Ausubel (1987) with modification. Samples were run on 7% polyacrylamide gels with 90 mM Tris/80 mM H_3BO_3 adjusted to pH 9.6 with KOH at 10 ^{o}C at a fixed current of 8 mA. Upon completion, half of the gel was stained with Coomassie blue R and scanned with an LKB Ultrascan XL laser densitometer while the other half was cut into segments which were extracted for 24 hr with the electrophoresis buffer adjusted to pH 8 and containing 1 mg/ml BSA. Dehydratase activity was assayed in the forward direction as described before.

INHIBITORS OF MITOCHONDRIAL β-OXIDATION

Part I

4-Bromo-2-octenoic Acid

RESULTS

Synthesis of 4-Bromo-2-octenoic Acid

Although 2-enoic acids can be directly, albeit slowly, brominated with N-bromosuccinimide at carbon atom 4, the resulting 4-bromo-2-enoic acid are obtained in poor yield, thus complicating their purification. In contrast, bromination of the esters of 2-enoic acids with N-bromosuccinimide precedes rapidly and yields mostly the desired product. The procedure developed by Bellassoued *et al.* (1983) for the synthesis of 4-crotonic acid and some other short-chain 4-bromo-2-enoic acids was used to prepare 4-bromo-2-octenic acid. According to their approach, 2-octenoic acid was converted to its trimethylsilyl ester by reacting the acid with chlorotrimethylsilane in the presence of pyridine. The resulting trimethylsilyl 2-octenoate was brominated with N-bromosuccinimide to yield trimethylsilyl 4-bromo-2-octenoate which was hydrolyzed upon addition of water in the absence of base thereby preventing hydrolysis of the allylic bromide.

Effect of 4-Bromo-2-octenoic Acid on Fatty Acid Oxidation in Mitochondria

Since 4-bromocrotonic acid causes the inactivation of 3-ketoacyl-CoA thiolase as well as acetoacetyl-CoA, it inhibits both fatty acid oxidation and ketone

body degradation (Olowe & Schulz, 1982). It was reasoned that a longer chain homologue of 4-bromocrotonic acid, like 4-bromo-2-octenoic acid, may inactivate only 3-ketoacyl-CoA thiolase and thereby inhibit fatty acid oxidation without affecting ketone body metabolism. When coupled rat heart mitochondria were preincubated with 4-bromo-2-octenoic acid, respiration supported by palmitoylcarnitine remained unaffected (data not shown). This finding was not completely surprising because 2-bromooctanoic acid, an effective inhibitor of fatty acid oxidation in rat liver mitochondria (Raaka & Lowenstein, 1981), also had no significant effect on the same pathway in rat heart mitochondria (Olowe & Schulz, 1982). However, when coupled rat liver mitochondria were preincubated for 3 min with 20 μM 4-bromo-2-octenoic acid, respiration supported by either palmitoylcanitine (see Figure 3A, B) or pyruvate (see Figure 3C, D) was completely inhibited. Addition of carnitine to these inhibited mitochondrial suspensions restored pyruvate-supported respiration to 60% of the control level without relieving the inhibition of respiration sustained by palmitoylcarnitine (see Figure 3B, D). Thus, 4-bromo-2-octenoic acid seems to specifically inhibit fatty acid oxidation. When mitochondria were uncoupled with 2,4-dinitrophenol before being incubated with 4-bromo-2-octenoic acid, palmitoylcarnitine-supported respiration was not inhibited (see Figure 3E, F). Thus, it appears that the inhibitor is only effective when ATP or some other form of energy is available, possibly in order to meet the energy requirement for conversion of the inhibitor to its coenzyme A thioester.

In order to determine which reaction of the β-oxidation cycle may be inhibited by 4-bromo-2-octenoic acid, rat liver mitochondria were incubated in the presence 0.1 mM inhibitor for 3 min and rapidly frozen. After the mitochondrial suspension was quickly thawed, the enzymes of β-oxidation were assayed immediately. As is apparent from the data presented in Table I, 3-ketoacyl-CoA

thiolase is the only enzyme that is significantly inhibited. The activity of acetoacetyl-CoA thiolase was obtained by assaying both types of mitochondrial thiolases with acetoacetyl-CoA and deducting from the measured value that part of the activity which was due to 3-ketoacyl-CoA thiolase (for details, see Experimental Procedures). The ß-oxidation enzymes were released from the matrix space by solubilizing the mitochondrial membrane with Triton X-100. Since this treatment results in the extensive dilution of the matrix content, a reversible inhibition of 3-ketoacyl-CoA thiolase should not have persisted. Thus, it appears that the inhibition of 3-ketoacyl-CoA thiolase is at least in part due to an irreversible inactivation of the enzyme.

The effect of 4-bromo-2-octenoic acid on the activities of the two thiolases and palmitoylcarnitine-supported respiration as a function of the inhibitor concentration was studied in rat liver mitochondria. As shown in Figure 4A, 10 µM 4-bromo-2-octenoic acid completely inhibited respiration under the conditions used in this experiment. Inhibition by 50% was obtained with approximately 2 µM inhibitor. In contrast, 3-ketoacyl-CoA thiolase was only partially inhibited while the activity of acetoacetyl-CoA thiolase remained unaffected by the inhibitor up to a concentration of 0.1 mM. It should be noted that the thiolase activities were assayed after the mitochondrial membrane was dissolved and the contents of the matrix space were highly diluted so that a reversible inhibition would not have been detected. When carnitine was present during the preincubation of mitochondria with 4-bromo-2-octenoic acid, the inhibition was less severe than that observed in the absence of carnitine (data not shown). The inhibition of respiration caused by 20 µM 4-bromo-2-octenoic acid increased with time up to 2 min when respiration was completely inhibited (see Figure 4B).

Metabolism of 4-Bromo-2-octenoic Acid by ß-Oxidation Enzymes and the Effect of Its Metabolites on the Activity of 3-Ketoacyl-CoA Thiolase

Since 4-bromo-2-octenoic acid does not cause the inhibition of fatty acid oxidation in rat heart mitochondria or uncoupled rat liver mitochondria, it is likely that this compound must first be activated to its coenzyme A thioester before becoming inhibitory. However, it remained unclear whether 4-bromo-2-octenoyl-CoA itself or a metabolite derived from it causes the inactivation of 3-ketoacyl-CoA thiolase. With the aim of clarifying this point, 4-bromo-2-octenoyl-CoA was synthesized, and its metabolism via ß-oxidation was studied. Purified enzymes of ß-oxidation were used in a spectrophotometric evaluation of the ß-oxidation of 4-bromo-2-octenoyl-CoA. In the presence of enoyl-CoA hydratase, 4-bromo-2-octenoyl-CoA was hydrated as evidenced by the decrease in absorbance at 263 nm shown in Figure 5. The reaction product must have been L-3-hydroxy-4-bromo-octanoyl-CoA because the addition of L-3-hydroxyacyl-CoA dehydrogenase together with NAD^+ gave rise to an absorbance change at 340 nm due to the formation of NADH (see Figure 5). If so, 3-keto-4-bromooctanoyl-CoA should have been formed which in the presence of $MgCl_2$ gives rise to a Mg^{2+}-enolate complex with an absorbance maximum around 300nm. Since the addition of 3-ketoacyl-CoA thiolase and CoASH did not cause an absorbance decrease at 303 nm (see Figure 5), 3-keto-4-bromooctanoyl-CoA is not a substrate of thiolase. In fact, this compound caused the inhibition of 3-ketoacyl-CoA thiolase since the final addition of the thiolase substrate 3-ketooctanoyl-CoA did not result in its thiolytic cleavage detectable at 303 nm. Spectroscopic evidence for the formation of 3-keto-4-bromooctanoyl-CoA was obtained by incubating 4-bromo-2-octenoyl-CoA with enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase in the presence of NAD^+ and $MgCl_2$. An absorbance, which increased with time, was detected around 320 nm with a shoulder

at 340 nm (see Figure 6A). Addition of EDTA to the absorbing material corresponding to curve 3 in Figure 6A resulted in the disappearance of the absorbance close to 320 nm without affecting the absorbance centered at 340 nm. The absorbance at 340 nm was due to NADH since it disappeared upon addition of pyruvate and lactate dehydrogenase. Thus, the material giving rise to the absorbance around 320 nm must have been the Mg^{2+}-enolate complex of 3-keto-4-bromooctanoyl-CoA. The spectrum of this compound without interference by the overlapping spectrum of NADH is shown in Figure 6B; its absorbance maximum is at 317 nm. The above findings lead me to propose that mitochondrial metabolism of 4-bromo-2-octenoic acid proceeds as shown in Figure 8.

In an attempt to determine which of the metabolites of 4-bromo-2-octenoic acid causes the inactivation of 3-ketoacyl-CoA thiolase, a soluble extract of rat liver mitochondria was incubated with either 4-bromo-2-octenoyl-CoA or 4-bromo-2-octenoyl-CoA plus NAD^+. Since only in the latter experiment 3-ketoacyl-CoA thiolase was rapidly and completely inactivated, 3-keto-4-bromooctanoyl-CoA, which can only be formed in the presence of NAD^+, must be the metabolite causing the inactivation of this thiolase (see Figure 7). Protection against inactivation is provided by the thiolase substrate 3-ketooctanoyl-CoA (see Figure 8). This observation suggests that the inhibitor 3-keto-4-bromooctanoyl-CoA is directed against the active site of the enzyme. The limited inactivation of 3-ketoacyl-CoA thiolase in the presence of 4-bromo-2-octenoyl-CoA (see Figure 8) may reflect the formation of a small amount of 3-keto-4-bromooctanoyl-CoA due to the presence of some NAD^+ in the soluble mitochondrial extract.

The question as to whether 3-keto-4-bromooctanoyl-CoA causes the reversible inhibition of 3-ketoacyl-CoA thiolase or its irreversible inactivation was

attempted to be answered by treating the enzyme with 20 μM inhibitor which was subsequently removed by dialysis. The results of this experiment demonstrate an insignificant reactivation of 3-ketoacyl-CoA thiolase from 8.8% of the control activity before dialysis to 12.4% after dialysis. Thus, 3-ketoacyl-CoA thiolase seems to be inactivated by 3-keto-4-bromooctanoyl-CoA in an irreversible fashion. Surprisingly, acetoacetyl-CoA thiolase was inhibited by 50% in the presence of 20 μM 3-keto-4-bromooctanoyl-CoA, but this inhibition was completely reversed upon dialysis.

Effect of 4-Bromo-2-octenoic Acid on Ketogenesis in Rat Liver Mitochondria

Since 4-bromo-2-octenoic acid causes the irreversible inhibition of the β-oxidation enzyme 3-ketoacyl-CoA thiolase, but does not inactivate acetoacetyl-CoA thiolase which is assumed to function in ketone body formation, the inhibitor was expected to inhibit ketogenesis from fatty acids but not from acetate. To test this hypothesis I determined the effect of 0.1 mM 4-bromo-2-octenoic acid on acetoacetate formation in rat liver mitochondria. As expected, acetoacetate formation from palmitoylcarnitine declined by 85% from 30.2 nmol $(5\ min)^{-1}$ $(mg\ of\ protein)^{-1}$ in control mitochondria to 4.7 nmol $(5\ min)^{-1}$ $(mg\ of\ protein)^{-1}$ in mitochondria preincubated with 0.1 mM 4-bromo-2-octenoic acid. In contrast, synthesis of acetoacetate from acetylcarnitine remained unchanged at 9.4 nmol $(5\ min)^{-1}$ $(mg\ of\ protein)^{-1}$ in the presence of the inhibitor. Hence, 4-bromo-2-octenoic acid seems to be a specific inhibitor of fatty acid oxidation.

DISCUSSION

With 4-bromo-2-octenoic acid I have identified a compound which inhibits fatty acid oxidation without affecting ketone body synthesis from acetate and possibly amino acids in rat liver mitochondria. This selectivity can be attributed to the chain length of the inhibitor which after conversion to 3-keto-4-bromooctanoyl-CoA inactivates the β-oxidation enzyme 3-ketoacyl-CoA thiolase but not acetoacetyl-CoA thiolase which is assumed to function in ketogenesis only. The inhibitor also exhibits tissue specificity as reflected by its inertness in heart mitochondria in contrast to being a good inhibitor in liver mitochondria. The different effects of the inhibitor in heart and liver may be a consequence of 4-bromo-2-octenoic acid being converted to its coenzyme A thioester in liver but not in heart mitochondria. This interpretation agrees with the reported specificities of medium-chain acyl-CoA synthetase in liver and heart mitochondria; the latter of the two has a much narrower specificity than the liver enzyme (Webster *et al.*, 1965; Mahler *et al.*, 1953). The foregoing interpretation is identical with the explanation presented for the observed inhibition of fatty acid oxidation in rat liver mitochondria by 2-bromooctanoic acid which is almost ineffective in rat heart mitochondria and myocytes (Schulz, 1987). Underlying the preceding discussion is the assumption that 4-bromo-2-octenoic acid must be converted to its coenzyme A thioester in order to become inhibitory. Evidence in support of this assumption is provided by the finding that the inhibitor is ineffective in uncoupled rat liver mitochondria which are deprived of ATP necessary for coenzyme A thioester formation.

Although respiration supported by either pyruvate or palmitoylcarnitine is inhibited by 4-bromo-2-octenoic acid, inhibition of the former pathway is partially

relieved by the addition of carnitine. It is likely that the inhibition of pyruvate oxidation is a consequence of the inhibitor tying up HSCoA, thereby causing the inhibition of metabolic pathways dependent on free coenzyme A. If, however, carnitine is present during the preincubation of mitochondria with the inhibitor, fatty acid oxidation is less severely inhibited than it is in the absence of carnitine. Although I have not further studied this effect of carnitine, it seems possible that in the presence of carnitine one or several metabolites of 4-bromo-2-octenoic acid may be transferred by carnitine acetyltransferase from HSCoA to carnitine thereby lowering the intramitochondrial concentration of the inhibitory metabolite 3-keto-4-bromooctanoyl-CoA.

When the activities of the ß-oxidation enzymes were assayed in solubilized mitochondria preincubated with the inhibitor, only 3-ketoacyl-CoA thiolase was found to be significantly inhibited. Since solubilization of mitochondria resulted in the dilution of the matrix content by several hundred fold, only an irreversible inhibition should have persisted. This suggestive conclusion was confirmed by the demonstration that the inhibition of 3-ketoacyl-CoA thiolase by 3-keto-4-bromooctanoyl-CoA persisted even after prolonged dialysis. Surprisingly, acetoacetyl-CoA thiolase was also inhibited, but its inhibition was reversed by dialysis and thus seems to be reversible. It is possible that 3-keto-4-bromooctanoyl-CoA binds to the active site of the enzyme. If 3-keto-4-bromooctanoyl-CoA is not a substrate of the enzyme or is a poor one, the inhibition will be reversible until a reaction between inhibitor and enzyme leads to the covalent modification and thus inactivation of the enzyme. The most likely reaction between inhibitor and enzyme would be a displacement of the 2-keto bromide by a nucleophilic group on the enzyme. This covalent modification of the enzyme could occur either before or after the initial catalytic event in which an 2-bromohexanoyl-S-enzyme intermediate would

be formed. If inactivation of the enzyme is the consequence of an intramolecular reaction of the 2-bromohexanoyl-S-enzyme intermediate, it is obvious that acetoacetyl-CoA thiolase will not be irreversibly inactivated because this enzyme is inactive toward substrates with acyl chains having more than four carbons. A reversible inhibition of 3-ketoacyl-CoA thiolase, which would not have been detected in experiments with dissolved mitochondria, may account for the apparently less severe inhibition of 3-ketoacyl-CoA thiolase as compared to the inhibition of palmitoylcarnitine-supported respiration. Thus it is possible that the combined reversible and irreversible inhibitions of the enzyme in whole mitochondria may be equal to the inhibition of respiration.

The observed reversible inhibition of acetoacetyl-CoA thiolase by 3-keto-4-bromooctanoyl-CoA prompted the question as to whether ketogenesis from substrates other than fatty acids may be affected by the inhibitor. The answer is apparently "no" since acetoacetate formation in rat liver mitochondria with acetylcarnitine as a substrate was not inhibited by 4-bromo-2-octenoic acid. The absence of an effect of 4-bromo-2-octenoic acid on ketogenesis from acetate may be the consequence of an insufficient accumulation of 3-keto-4-bromo-octanoyl-CoA in mitochondria so that acetoacetyl-CoA thiolase is not inhibited significantly. In addition, this enzyme does not catalyze the rate-limiting step in acetoacetate formation under the conditions of this study. Thus 4-bromo-2-octenoic acid is the desired specific inhibitor of fatty acid oxidation in rat liver mitochondria.

INHIBITORS OF MITOCHONDRIAL ß-OXIDATION

Part II

2-Propylpentanoic Acid

RESULTS

Activation of 2-Propylpentanoic Acid

2-Propylpentanoyl-CoA has been found in hepatocytes and liver mitochondria isolated from rat treated with 2-propylpentanoic acid (Becker and Harris, 1983). Since 2-propylpentanoic acid is a fatty acid with eight carbon atoms, it can pass freely through the mitochondrial membrane in an L-carnitine independent manner and then be converted to its CoA thioester in the mitochondria matrix. The activation of 2-propylpentanoic acid to its coenzyme A derivative was investigated with rat liver mitochondria solubilized with Triton X-100. In the presence of ATP, HSCoA and Mg^{2+}, and 2-propylpentanoic acid, the formation of 2-propylpentanoyl-CoA was confirmed by analyzing the reaction mixture on HPLC. The rate of formation of 2-propylpentanoyl-CoA plus its metabolites was found to be 0.247 nmol/min/mg protein. Thus at least one enzyme exists in the mitochondria which can convert 2-propylpentanoic acid to its coenzyme A derivative.

Hydration of 2-Propyl-2E-pentenoyl-CoA

It has previously been observed by Ito *et al.*, (1987) that 2-propylpentanoyl-

CoA can be dehydrogenated to 2-propyl-2-pentenoyl-CoA. However, the configuration of the dehydrogenation product has not been determined. Since a compound with the 2E configuration was predominant among the unsaturated metabolites of 2-propylpentanoic acid in urine and plasma, I chose 2-propyl-2E-pentenoic acid as the substrate for this study. The successful synthesis of 2-propyl-2E-pentenoyl-CoA enabled me to study the hydration of 2-propyl-2E-pentenoyl-CoA. The results prove that purified crotonase from bovine liver causes a decrease in the absorbance at 263 nm (data not shown), thereby prompting the suggestion that 2-propyl-2E-pentenoyl-CoA underwent hydration. When the reaction mixture was analyzed by HPLC, a product peak 3 (see Figure 9A) was observed which confirms that 2-propyl-2E-pentenoyl-CoA is a substrate of crotonase. It has been well established that the hydration product of 2-*trans*-enoyl-CoA catalyzed by crotonase is 3-hydroxyacyl-CoA with a 3S-hydroxy group and a 2-pro-R hydrogen atom (Willadsen and Eggerer, 1975). Thus the hydration product of 2-propyl-2E-pentenoyl-CoA by crotonase must be 3S-hydroxy-2S-propylpentanoyl-CoA. The ratio of 2-propyl-2E-pentenoyl-CoA to 3-hydroxy-2-propylpentanoyl-CoA at equilibrium is 3.1 to 1 determined by HPLC. When Triton X-100 solubilized rat liver mitochondria were used as an enzyme source, I observed the same reaction as seen in Figure 9D. Peak 4 in Figure 9D is a further metabolite which is most likely formed because of the presence of NAD^+ in mitochondria and will be discussed in the next section. The rate of hydration of 2-propyl-2E-pentenoyl-CoA catalyzed by mitochondria solubilized with Triton X-100 was determined to be 25.6 nmol/min/mg mitochondrial protein. This result proves that 2-propyl-2E-pentenoyl-CoA can be hydrated in rat liver mitochondria most likely by crotonase.

Dehydrogenation of 3-Hydroxy-2-propylpentanoyl-CoA

When 2-propyl-2E-pentenoyl-CoA was incubated with solubilized rat liver mitochondria supplemented with purified crotonase in the presence of NAD^+, an increase in absorbance at 340 nm was observed (Figure 10, curve 1-3). Upon addition of pyruvate, which is the substrate for lactate dehydrogenase present in the reaction mixture, the peak at 340 nm disappeared (Figure 10, curve 4), proving that NADH is formed during the reaction. However, upon addition of $MgCl_2$, no absorbance change at 303 nm could be detected which is characteristic of the Mg^{2+}-enolate complex of 3-ketoacyl-CoA compounds. For this reason the reaction mixture was further analyzed by HPLC. The result is shown in Figure 9B. All of the 2-propyl-2E-pentenoyl-CoA was converted to product (peak 4), which emerged in a region where normally 3-ketoacyl-CoA's are eluted. To further analyze the structure of this product, the material corresponding to this peak was collected and analyzed by mass spectrometry as seen in Figure 11 which shows the MH^+ peak as 908.4 confirming that this compound is keto-2-propylpentanoyl-CoA. A requirement of this reaction for NAD^+ is shown in Figure 9D. In the absence of NAD^+, only trace amount of keto-2-propylpentanoyl-CoA formed, while the hydration product was observed. This suggested that keto-2-propylpentanoyl-CoA was generated from 2-propyl-2E-pentenoyl-CoA with 3-hydroxy-2-propylpentanoyl-CoA as an intermediate. Since this keto-2-propylpentanoyl-CoA was generated by dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA, it must be 3-keto-2-propylpentanoyl-CoA. The rate of dehydrogenation was determined and found to be 30 nmol/min/mg mitochondrial protein.

I examined whether or not 3-keto-2-propylpentanoyl-CoA can be thiolytically cleaved to generate n-pentanoyl-CoA and n-propanoyl-CoA. When pig

heart 3-ketoacyl-CoA thiolase plus HSCoA and additional mitochondrial extract were added to the reaction mixture containing 3-keto-2-propylpentanoyl-CoA, the rate of thiolysis of 3-keto-2-propylpentanoyl-CoA was less than 0.01 nmole/min/mg protein (shown in Figure 9C).

Interestingly, no dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA was observed with purified pig heart 3-hydroxyacyl-CoA dehydrogenase at a concentration $3x10^5$ higher than that required to observe a rate with 3-hydroxyoctanoyl-CoA as a substrate. When using mitochondria solubilized with Triton X-100, the ratio of 3-hydroxy-2-propylpentanoyl-CoA dehydrogenation to 3-ketooctanoyl-CoA dehydrogenation was 0.35. When a mitochondrial membrane fraction was used as the enzyme source, the ratio increased to 0.59, while with a soluble mitochondrial extract no dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA was observed. These results suggested that a mitochondrial membrane-associated dehydrogenase was responsible for the dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA rather than 3-hydroxyacyl-CoA dehydrogenase of the ß-oxidation pathway.

When $NADP^+$ was substituted for NAD^+, the rate was less than 0.2 nmol/min/mg mitochondrial protein as detected by monitoring the reaction spectrophotometrically at 340 nm. A rat liver microsomal fraction was also testd as an enzyme source instead of mitochondria and an activity of only 0.4 nmole/min/mg microsomal protein was observed. This low rate maybe due to a mitochondrial contamination in the microsomal fraction. The rat liver peroxisomal bifunctional enzyme was also tested and found to be inactive toward 3-hydroxy-2-propylpentanoyl-CoA at a concentration $3.4x10^3$ higher than that required to observe a rate with 3-hydroxyoctanoyl-CoA as a substrate. The rate of

dehydrogenation catalyzed by rat heart mitochondria, which contain few peroxisomes, was found to be 2.3 nmole/min/mg protein. Thus the possible role of peroxisomes in catalyzing this reaction was excluded. Taken together, these results indicate that a mitochondrial membrane associated enzyme is involved in the dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA.

Metabolism of 2-Propylpentanoic Acid by Intact Mitochondria

Whether 2-propylpentanoic acid can be metabolized by coupled mitochondria as suggested by the above experiments which were performed with broken mitochondria remained to be established. Also, the possible cause of the inhibitory effects of 2-propylpentanoic acid on fatty acid oxidation remained to be examined. In order to answer these questions, the metabolism of 2-propylpentanoic acid by coupled rat liver mitochondria was studied in the presence of ATP, Mg^{2+}, malate and L-carnitine. I found that two major CoA containing metabolites accumulated in mitochondria when 2-propylpentanoic acid was incubated with mitochondria (see Figure 9E). One is 2-propylpentanoyl-CoA and the other is 3-keto-2-propylpentanoyl-CoA. No difference was observed in the presence and absence of L-carnitine (data not shown). Trace amounts of 2-propyl-2E-pentenoyl-CoA were also observed. The time dependency of this metabolism was studied and the results are shown in Figure 12. The amounts of 2-propylpentanoyl-CoA and 3-keto-2-propylpentanoyl-CoA increased with time. However, the ratio of 3-keto-2-propylpentanoyl-CoA to 2-propylpentanoyl-CoA increased with time because the formation of 2-propylpentanoyl-CoA reached a maximum after 10 min and then began to decline, whereas 3-keto-2-propylpentanoyl-CoA continued to accumulate. After 20 min, 3-keto-2-propylpentanoyl-CoA was the predominant metabolite,

perhaps as a result of being metabolically stable and tying up all coenzyme A, thereby inhibiting the further generation of 2-propylpentanoyl-CoA.

DISCUSSION

For a long time 2-propylpentanoic acid was thought to be degraded in part by ß-oxidation because a number of its metabolites detected in plasma and urine are identical with the hydrolytic products of ß-oxidation intermediates. However, few studies were published over the past years aimed at examining the mitochondrial ß-oxidation of this compound. Although it has been reported that 2-propylpentanoyl-CoA is present in hepatocytes and mitochondria isolated from rats treated with 2-propylpentanoic acid (Becker and Harris, 1983), the site of its activation remained unknown.

Since 2-propylpentanoic acid is an eight carbon fatty acid, it most likely behaves as a medium-chain fatty acid and can enter mitochondria freely. This work indicated that this compound can be activated in coupled mitochondria in the absence and presence of L-carnitine, suggesting that the entrance of this compound into mitochondria is not via the carnitine-dependent transport system. Since acyl-CoA cannot enter mitochondria by itself, the activation of this compound observed in this study must occur inside mitochondria. It has been reported that a medium-chain acyl-CoA synthetase which may catalyze this reaction exists in mitochondria (EC 6.2.1.2, Mahler, 1953).

Ito *et al.* (1987) reported the formation of 2-propyl-2-pentenoyl-CoA catalyzed by the mitochondrial 2-methyl branched-chain acyl-CoA dehydrogenase. Unfortunately the configuration of the double bond was not determined. In this work, the further metabolism of 2-propyl-2E-pentenoyl-CoA was studied. 2-Propyl-2E-pentenoyl-CoA was hydrated by crotonase to 3-hydroxy-2-propylpentanoyl-CoA. The same product was also observed when mitochondria solubilized with Triton X-

100 were used as an enzyme source. Clearly, mitochondrial crotonase can function in the ß-oxidation of 2-propylpentanoic acid even though another hydratase exists in mitochondria, the long-chain enoyl-CoA hydratase described by Schulz (1984), which was not included in this study.

In the absence of NAD^+, mitochondria solubilized with Triton X-100 catalyzed the formation of 3-hydroxy-2-propylpentanoyl-CoA and in addition a small amount of 3-keto-2-propylpentanoyl-CoA was formed, which was most likely due to the presence of a small amount of NAD^+ in mitochondria. When NAD^+ was supplied, all 2-propyl-2E-pentenoyl-CoA and 3-hydroxy-2-propylpentanoyl-CoA were completely converted to 3-keto-2-propylpentanoyl-CoA, indicating that the formation of 3-hydroxy-2-propylpentanoyl-CoA is an intermediate step. Interestingly, the result indicated that 3-hydroxy-2-propylpentanoyl-CoA is not a substrate for 3-hydroxyacyl-CoA dehydrogenase, the normal ß-oxidation enzyme. Neither microsomes nor peroxisomes had the capacity to catalyze this dehydrogenation reaction. Rather, it appeared that a mitochondrial membrane-associated dehydrogenase was involved in this reaction. It has been reported that a long-chain 3-hydroxyacyl-CoA dehydrogenase is associated with the inner mitochondrial membrane (EL-Fakhri and Middleton, 1982). Whether or not this enzyme was involved remains to be established.

In order to verify these reactions in whole mitochondria, 2-propylpentanoic acid was incubated with coupled mitochondria. Two metabolites accumulated, 2-propylpentanoyl-CoA and 3-keto-2-propylpentanoyl-CoA. Trace amounts of 2-propyl-2-pentenoyl-CoA were also observed but 3-hydroxy-2-propylpentanoyl-CoA was not detected. Prickett *et al.* (1984) reported the formation of 3-hydroxy-2-propylpentanoic acid is catalyzed by microsomal cytochrome P-450 whereas 3-

hydroxy-2-propylpentanoyl-CoA did not accumulate in mitochondria, suggesting that the 3-hydroxylated metabolites observed in urine and plasma are not formed in mitochondria. Presumably, all or most of 2-propylpentanoic acid is converted intramitochondrially to 3-keto-2-propylpentanoyl-CoA.

When the thiolytic cleavage of 3-keto-2-propylpentanoyl-CoA was investigated by adding 3-ketoacyl-CoA thiolase and HSCoA to a solution of 3-keto-2-propylpentanoyl-CoA, no reaction was observed. The presence of the carnitine derivative of 2-propylpentanoic acid in the plasma of patients treated with 2-propylpentanoic acid has been reported (Millington *et al.*, 1985). This compound may be formed from 2-propylpentanoyl-CoA by carnitine acyltransferase and leave mitochondria as do short-chain, medium chain and branched-chain acylcarnitines (Lysiak *et al.*, 1986). The hydrolysis of 2-propylpentanoyl-CoA by rat liver homogenate has also been reported by Moore *et al.* (1988) who suggested a possible escape route for this metabolite from mitochondria.

Together, these observation lead me to propose the pathway for the mitochondrial ß-oxidation of 2-propylpentanoic acid shown in Figure 13. Once 2-propylpentanoic acid enters mitochondria, it is activated to its coenzyme A thioester. Dehydrogenation yields 2-propyl-2-pentenoyl-CoA which is hydrated to 3-hydroxy-2-propylpentanoyl-CoA. Last, dehydrogenation gives rise to 3-keto-2-propylpentanoyl-CoA. This metabolite accumulates in mitochondria where it might be slowly hydrolyzed or transferred to carnitine and thus leave mitochondria either as a free acid or its carnitine derivative.

The inhibitory effects of 2-propylpentanoic acid on fatty acid oxidation have been studied. Since fatty acid ß-oxidation requires free CoA for activation and

thiolysis and since pyruvate metabolism also requires free CoA for the formation of acetyl-CoA, the accumulation of 3-keto-2-propylpentanoyl-CoA in mitochondria may tie up CoA and thereby inhibit oxidative metabolism in mitochondria as suggested by Becker & Harris (1983) and Turnbull *et al.* (1983). Although 2-propyl-4-pentenoic acid is a strong inhibitor of fatty acid oxidation *in vitro* (Bjorge et al., 1985), the rate of its formation is only 10.5 pmol/min/mg microsomal protein (Rettie *et al.*, 1988), which is 40 times slower than the rate at which metabolites of 2-propylpentanoic acid accumulate in mitochondria. Thus fatty acid oxidation should be inhibited by CoA depletion long before enough 2-propyl-4-pentenoic acid has been generated. Also 2-propyl-4-pentenoic acid must first be converted to its CoA thioester before it becomes inhibitory to ß-oxidation. Hence, depletion of free CoA would prevent its activation and becoming inhibitory to ß-oxidation.

THE MECHANISM OF EPIMERIZATION OF D-3-HYDROXYACYL-CoA

RESULTS

Purification of D-3-Hydroxyacyl-CoA Dehydratase

Attempt to pu[illegible]3-hv[illegible]oxyacyl-CoA epimerase by ion exchange chromatography invariably resulted in large activity losses. Since part of the activity was regained when all fractions were combined, it appeared that at least two components are required for the expression of epimerase activity. When the subcellular location of epimerase was determined, Smeland (unpublished results) confirmed its presence in peroxisomes as previously suggested by Chu and Schulz (1985).

This initial observation led me to pursue the purification and characterization of the enzyme(s) responsible for the epimerization of 3-hydroxyacyl-CoA thioesters in cooperation with Tor Smeland.

By subjecting a soluble extract of rat liver light mitochondria (L fraction), which is enriched with respect to peroxisomes, to chromatography on DEAE-celllulose at low ionic strength (5 mM Tris-phosphate, pH 8), total loss of epimerase activity was observed. Only when crotonase was added to the assay mixture, was epimerase activity observed in the early fractions emerging from the column. It was also observed that these fractions catalyzed the dehydration of D-3-hydroxyoctanoyl-CoA but did not act on L-3-hydroxyoctanoyl-CoA (Smeland et al., 1989). Hence the

enzyme present in the early fractions was named D-3-hydroxyacyl-CoA dehydratase.

The further purification of D-3-hydroxyacyl-CoA dehydratase was achieved by chromatography on hydroxylapatite and 3',5'-ADP-Agarose followed by HPLC gel filtration as described under "Experimental Procedures". The results are summarized in Table III. Electrophoresis of native D-3-hydroxyacyl-CoA dehydratase on a 7% polyacrylamide gel at pH 9.6 demonstrated the presence of only one intensive protein band which coincided with enzyme activity thereby proving the association of the dehydratase activity with the major protein (Figure 14). When the same preparation was subjected to polyacrylamide gel electrophoresis in the presence of SDS, one strong band was observed and in addition some faint bands were only seen when a large quantity of protein was applied. The purity of this preparation was estimated to be approximately 90% by gel scanning (Smeland, unpublished result). The native molecular weight was found to be 106,800 by HPLC gel filtration and the subunit molecular weight was estimated to be 44,000 as shown in Figure 15.

Mechanism of Epimerization of D-3-Hydroxyacyl-CoA

Since D-3-hydroxyacyl-CoA dehydratase present in the early DEAE-cellulose fractions in combination with crotonase catalyzed the epimerization of 3-hydroxyacyl-CoA, it was speculated that the epimerization of 3-hydroxyacyl-CoA is probably due to the combined actions of two hydratases which catalyze the same type of reaction but exhibit opposite stereospecificities. The following experiments were carried out to examine this hypothesis. D-3-Hydroxyloctanoyl-CoA was incubated with the crotonase in the presence of excess L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, NAD^+ and HSCoA. Products formed were analyzed by HPLC and the result is shown in Figure 16A. Since L-3-hydroxyacyl-CoA

dehydrogenase only acts on L-3-hydroxyacyl-CoA (Wakil, 1960), no hexanoyl-CoA should be formed. As expected, only a trace amount of hexanoyl-CoA was observed reflecting the presence of a small amount of L-3-hydroxyoctanoyl-CoA in the preparation of the D-isomer. After removal of the enzymes from the mixture by ultrafiltration, an aliquot of the early DEAE-cellulose fraction devoid of crotonase activity plus L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase were added to this solution and the sample was analyzed by HPLC. Figure 16B shows the appearance of a new peak at the position of the *cis* and *trans* isomers of 2-octenoyl-CoA. To determine the configuration of the 2-octenoyl-CoA formed, the enzymes were removed from the reaction mixture by ultrafiltration and crotonase together with coupling enzymes were added to the solution. The result shown in Figure 16C demonstrates that 2-octenoyl-CoA (Peak No.3 in Figure 16B) formed by the early DEAE-cellulose fraction was converted into hexanoyl-CoA, thereby proving that 2-octenoyl-CoA identified in Figure 16B had a *trans* configuration. Based on these findings it was concluded that this dehydratase catalyzes the conversion of D-3-hydroxyacyl-CoA to 2-*trans*-enoyl-CoA. When L-3-hydroxyoctanoyl-CoA was used as a substrate no reaction was observed (Smeland *et al.*, 1989). Therefore, this enzyme was named D-3-hydroxyacyl-CoA dehydratase. The apparent epimerase activity observed in peroxisomes was due to the combined actions of the D-3-hydroxyacyl-CoA dehydratase and enoyl-CoA hydratase.

Synthesis of 2-*cis*-Octenoyl-CoA

I attempted to synthesize 2-*cis*-octenoyl-CoA from the corresponding free acid by the mixed-anhydride method (Goldman and Vagelos, 1961). However, more than 90% of the product was isomerized to 2-*trans*-octenoyl-CoA during thioesterification. Thus I attempted to directly hydrogenate 2-octynoyl-CoA in the

presence of Pd/C even at the risk of cleaving the thioester bond. After 1 hr of hydrogenation, 70% of 2-octynoyl-CoA was converted to 2-*cis*-octenoyl-CoA without significant loss of thioester (data not shown). The product was purified by HPLC and its structure was confirmed enzymatically. The following assay was performed based on the known fact that crotonase catalyzes the formation of D-3-hydroxyacyl-CoA from 2-*cis*-enoyl-CoA (Wakil, 1956) and that L-3-hydroxyacyl-CoA dehydrogenase does not act on D-3-hydroxyacyl-CoA (Wakil, 1960). Figure 17A shows the HPLC chromatography of 2-*cis*-octenoyl-CoA. When this compound was incubated with crotonase, a decrease in absorbance at 263 nm was observed due to the disappearance of double bond conjugated with the thioester. HPLC analysis of this reaction mixture demonstrated the formation of 3-hydroxyoctanoyl-CoA with either D or L configuration (see Figure 17B). When 2-*cis*-octenoyl-CoA was incubated with crotonase in the presence of 3-ketoacyl-CoA thiolase, L-3-hydroxyacyl-CoA dehydrogenase, NAD^+ and coenzyme A, only a small absorbance increase was observed at 340 nm, which was due to the degradation of a small amount of 2-*trans*-octenoyl-CoA contaminating the preparation of 2-*cis*-octenoyl-CoA. HPLC analysis showed that 3-hydroxyoctanoyl-CoA remained unchanged and only a small amount of hexanoyl-CoA was formed (see Figure 17C). This observation proved that the 3-hydroxyoctanoyl-CoA formed by crotonase had the D-configuration. The amount of hexanoyl-CoA observed by HPLC greater than the quantity expected based on the spectrophotometric assay. This was due to a large quantity of coupling enzymes used which contained trace amounts of epimerase and hence some of the D-3-hydroxyoctanoyl-CoA was converted to its L-isomer and then to hexanoyl-CoA. When partially purified D-hydroxyacyl-CoA dehydratase was added, a dramatic increase in absorbance at 340 nm was observed. HPLC analysis showed that all of the 3-hydroxyoctanoyl-CoA was converted to hexanoyl-CoA (see Figure 17D). These results proved the synthesized compound to be 2-*cis*-octenoyl-CoA. Based on the

spectrophotometric assay, the contaminating amount of 2-*trans*-octenoyl-CoA was calculated to be less than 3% of the total.

Substrate Specificity of D-3-Hydroxyacyl-CoA Dehydratase and Determination of Equilibrium Constants

Since the possibility existed that D-3-hydroxyacyl-CoA dehydratase, which has a stereospecificity opposite to that of crotonase, could catalyze the formation of L-3-hydroxyacyl-CoA from 2-*cis*-enoyl-CoA, the effect of the D-3-hydroxyacyl-CoA dehydratase on this compound was investigated. When 2-*cis*-octenoyl-CoA was incubated with purified D-3-hydroxyacyl-CoA dehydratase, no absorbance change was observed at 263 nm suggesting that no hydration of the *cis* double bond had occurred. Analysis of the reaction mixture by HPLC showed only trace amounts of 3-hydroxyoctanoyl-CoA (Figure 18A), which stemmed from contaminating *trans* isomer in this preparation of 2-*cis*-octenoyl-CoA. In contrast, crotonase catalyzed the almost complete hydration of 2-cis-octenoyl-CoA to D-3-hydroxyacyl-CoA, as seen in Figure 18B. This observation explains why no dehydration of L-3-hydroxyoctanoyl-CoA catalyzed by crotonase was observed. When 2-*trans*-octenoyl-CoA was used as a substrate, both crotonase (Figure 18C) and D-3-hydroxyacyl-CoA dehydratase (Figure 18D) catalyzed its hydration. When the purified bifunctional enzyme from rat liver peroxisomes was studied, results identical to those obtained with crotonase were obtained (data not shown). The equilibrium ratio of 3-hydroxyoctanoyl-CoA to 2-octenoyl-CoA in the presence of either crotonase or D-3-hydroxyacyl-CoA dehydratase were determined for both the hydration and dehydration reactions (see Table II). When the hydration of 2-*cis*-octenoyl-CoA and the dehydration of D-3-

hydroxyoctanoyl-CoA by crotonase were investigated, L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, 1.6 mM NAD^+ and 0.3 mM HSCoA were present to assure the removal of trace amounts of contaminating 2-*trans*-octenoyl-CoA and L-3-hydroxyoctanoyl-CoA respectively.

DISCUSSION

Since the epimerase-dependent pathway accounts for only a small percentage of polyunsaturated fatty acid degradation, its function in peroxisomal ß-oxidation still remains a mystery. The key enzyme in this minor pathway had not been purified even though substantial efforts had been made. In the work reported here the existence of an epimerase was excluded and instead proof was provided for the existence of a distinct D-3-hydroxyacyl-CoA dehydratase. The results show that the epimerization of 3-hydroxyacyl-CoA's was due to the combined actions of two hydratases with opposite stereospecificities. One is D-3-hydroxyacyl-CoA dehydratase which catalyzes the dehydration of D-3-hydroxyacyl-CoA to 2-*trans*-enoyl-CoA, and differs from crotonase in that it does not act on 2-*cis*-enoyl-CoA.

The discovery of a novel D-3-hydroxyacyl-CoA dehydratase followed by its purification and characterization necessitates a modification of the pathway for polyunsaturated fatty acid degradation as shown in Figure 19. The difference between the original and modified pathways is the initial conversion of 2-*cis*-octenoyl-CoA to D-3-hydroxyoctanoyl-CoA by enoyl-CoA hydratase, since D-3-hydroxyacyl-CoA dehydratase does not act on this 2-*cis*-enoyl-CoA's (Figure 18A). Thereafter D-3-hydroxyoctanoyl-CoA is dehydrated by D-3-hydroxyacyl-CoA dehydratase to 2-*trans*-octenoyl-CoA, whereupon enoyl-CoA hydratase acts again to generate L-3-hydroxyacyl-CoA, which can be completely degraded by the regular ß-oxidation pathway.

It has been reported that crotonase catalyzes the conversion of 2-*trans*-enoyl-CoA to L-3-hydroxyacyl-CoA and 2-*cis*-enoyl-CoA to D-3-hydroxyacyl-CoA (Wakil, 1956). The peroxisomal bifunctional enzyme exhibits the same stereospecificity as crotonase toward 2-*trans*-enoyl-CoA (Hashimoto, 1980) and 2-*cis*-enoyl-CoA (this study). The dehydratase described here is clearly distinct from crotonase and peroxisomal bifunctional enzyme. I originally expected that this enzyme may catalyze 2-*cis*-enoyl-CoA directly to L-3-hydroxyacyl-CoA which is a ß-oxidation intermediate. However the data shown in Figure 18A show that 2-*cis*-enoyl-CoA is not a substrate of D-3-hydroxyacyl-CoA dehydratase. Some other D-specific dehydratase exists in animal cells, one of them is ß-hydroxyacyl-ACP dehydratase associated with the fatty acid synthetase. Since this enzyme is located in the cytosol and uses ß-hydroxyacyl-ACP as substrates, whereas the D-hydroxyacyl-CoA dehydratase described here is located in peroxisomes (Smeland, unpublished results) and acts on CoA derivatives, they obviously are different enzymes. Another D-specific dehydratase may be present in microsomes where it would function in fatty acid elongation. However, no significant amounts of D-3-hydroxyacyl-CoA dehydratase was observed to be associated with microsomes (Smeland, unpublished results). Another reported hydratase is long-chain enoyl-CoA hydratase (Schulz, 1984) which catalyzes the conversion of 2-*trans*-enoyl-CoA to L-3-hydroxylacyl-CoA and is located in mitochondria. The activity of long-chain enoyl-CoA hydratase towards 2-*cis*-enoyl-CoA's has not been studied.

Since only a very small portion of unsaturated fatty acid may be degraded via the epimerase-dependent pathway, the possiblity exists that D-3-hydroxyacyl-CoA dehydratase has another function *in vivo*. Since the enzyme exists in all ß-oxidation systems tested except for mitochondria, it may be probably essential for the

degradation of fatty acids carrying D-hydroxy group at odd number carbon atoms.

Table I: Effect of 4-bromo-2-octenoic acid on the activities of β-oxidation enzymes. Rat liver mitochondria (2 mg) were suspended in 1.9 ml of a basal isotonic incubation buffer containing 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 4 mM KP_i,4 mM $MgCl_2$, and 0.1 mM EGTA. To this suspension were added bovine serum albumin (0.5 mg/ml) 0.5 mM L-malate, 1 mM ADP, and 1 min later, varying amounts of 4-bromo-2-octenoic acid. The mixture was incubated for 3 min before being frozen in dry ice/acetone and the β-oxidation enzymes were assayed as described under "Experimental Procedures".

enzyme	substrate	specific activity µmol/min/mg protein		remaining activity %
		no inhibitor	+ inhibitor[b]	
acyl-CoA dehydrogenase	n-butyryl-CoA	0.024	0.0235	98
	n-decanoyl-CoA	0.011	0.095	86
	palmitoyl-CoA	0.0095	0.0087	92
enoyl-CoA hydratase	crotonyl-CoA	5.6	6	107
	2-decenoyl-CoA	2.35	2.35	100
3-hydroxyacyl-CoA dehydrogenase	acetoacetyl-CoA	0.92	0.93	101
3-ketoacyl-CoA thiolase	3-ketooctanoyl-CoA	0.872	0.154	18
acetoacetyl-CoA thiolase	acetoacetyl-CoA	0.19	0.2	105

[a] For experimental details see EXPERIMENTAL PROCEDURES.

[b] Inhibitor: 0.1 mM 4-bromo-2-octenoic acid.

Table II: Equilibrium ratio of 2-enoyl-CoA/3-hydroxyacyl-CoA. The equilibrium ratios were determined for the forward and backward reactions. 2-*cis*-Octenoyl-CoA, 2-*trans*-octenoyl-CoA, D-3-hydroxyacyl-CoA and L-3-hydroxyacyl-CoA were incubated with excess amount of crotonase or D-3-hydroxyacyl-CoA dehydratase. The reaction products were analyzed by HPLC. Ratios were calculated as described under "Experimental Procedures".

equilibrium ratio	crotonase	D-dehydratase
L-3-hydroxyoctanoyl-CoA 2-*trans*-octenoyl-CoA	3.22	
D-3-hydroxyoctanoyl-CoA 2-*trans*-octenoyl-CoA		3.20
D-3-hydroxyoctanoyl-CoA 2-*cis*-octenoyl-CoA	137	

Table III: Purification of D-3-hydroxyacyl-CoA dehydratase.

purification step	total activity (U)	total protein (mg)	specific activity (U/mg)	Purification fold
L fraction	886	544	1.63	1.00
polytron treatment	833	544	1.63	0.99
L fraction extract	421	102	4.13	2.53
DEAE-cellulose	283	28.9	9.75	5.98
hydroxyapatite	144	14.8	9.79	6.01
3,5-ADP-agarose	90.4	4.34	20.8	12.8
HPLC gel filtration	60.3	1.47	43.1	29.3

FIGURE 1: The fatty acid ß-oxidation cycle. Reactions catalyzed by (1) Acyl-CoA dehydrogenase; (2) enoyl-CoA hydratase; (3) L-3-hydroxyacyl-CoA dehydrogenase; (4) 3-ketoacyl-CoA thiolase.

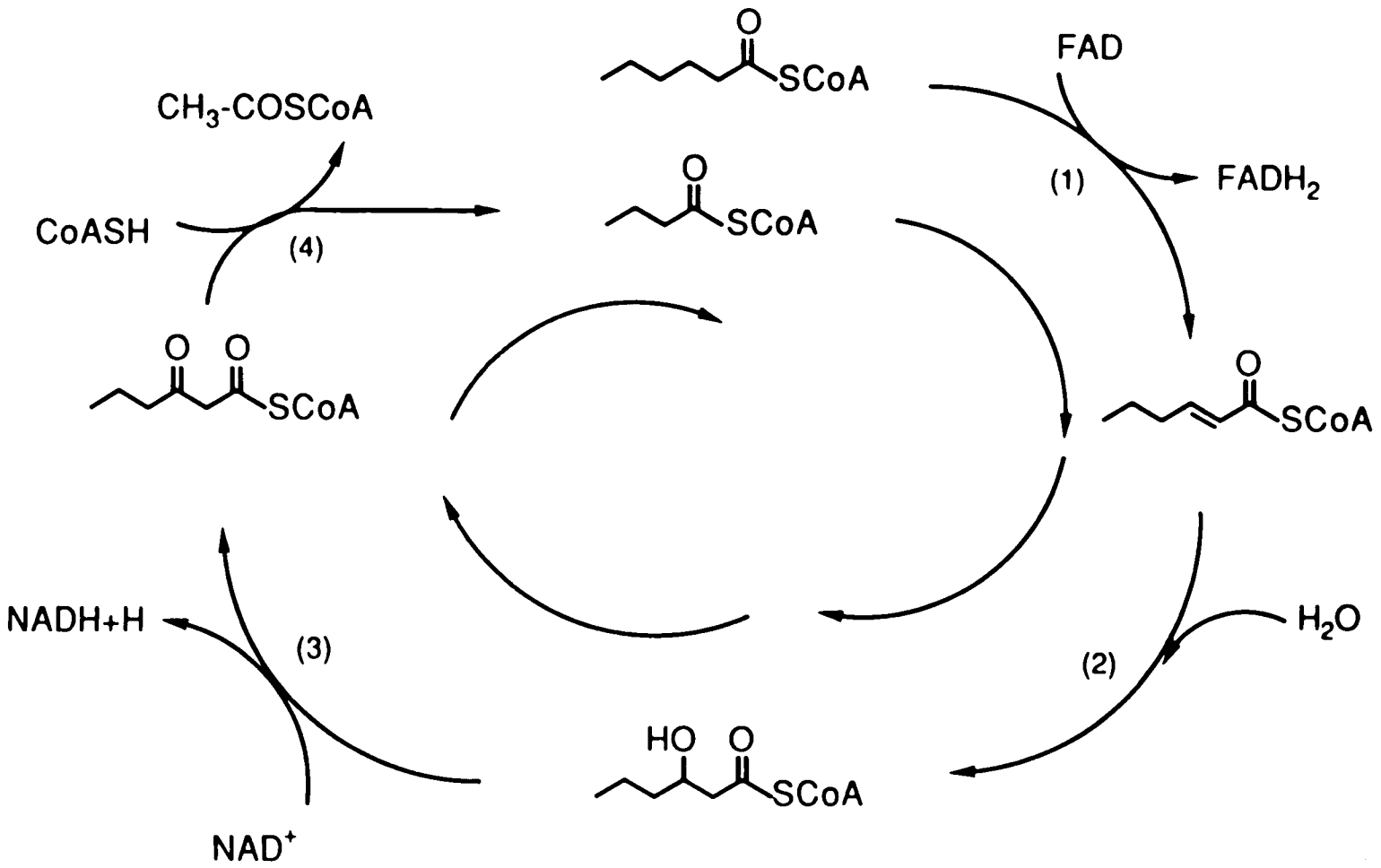

CH3-COSCoA
CoASH
(4)
SCoA
FAD
(1)
FADH2
SCoA
SCoA
NADH+H
(3)
NAD+
HO
SCoA
(2)
H2O

FIGURE 2: Pathway of linoleic acid degradation. A. Epimerase-dependent pathway; B. Reductase-dependent pathway. Reaction catalyzed by (1) acyl-CoA dehydrogenase; (2) enoyl-CoA hydratase; (3) L-3-hydroxacyl-CoA dehydrogenase; (4) 3-ketoacyl-CoA thiolase; (5) 3-hydroxyacyl-CoA epimerase; (6) 3-*cis*-2-*trans*-enoyl-CoA isomerase; (7) 2,4-dienoyl-CoA reductase;

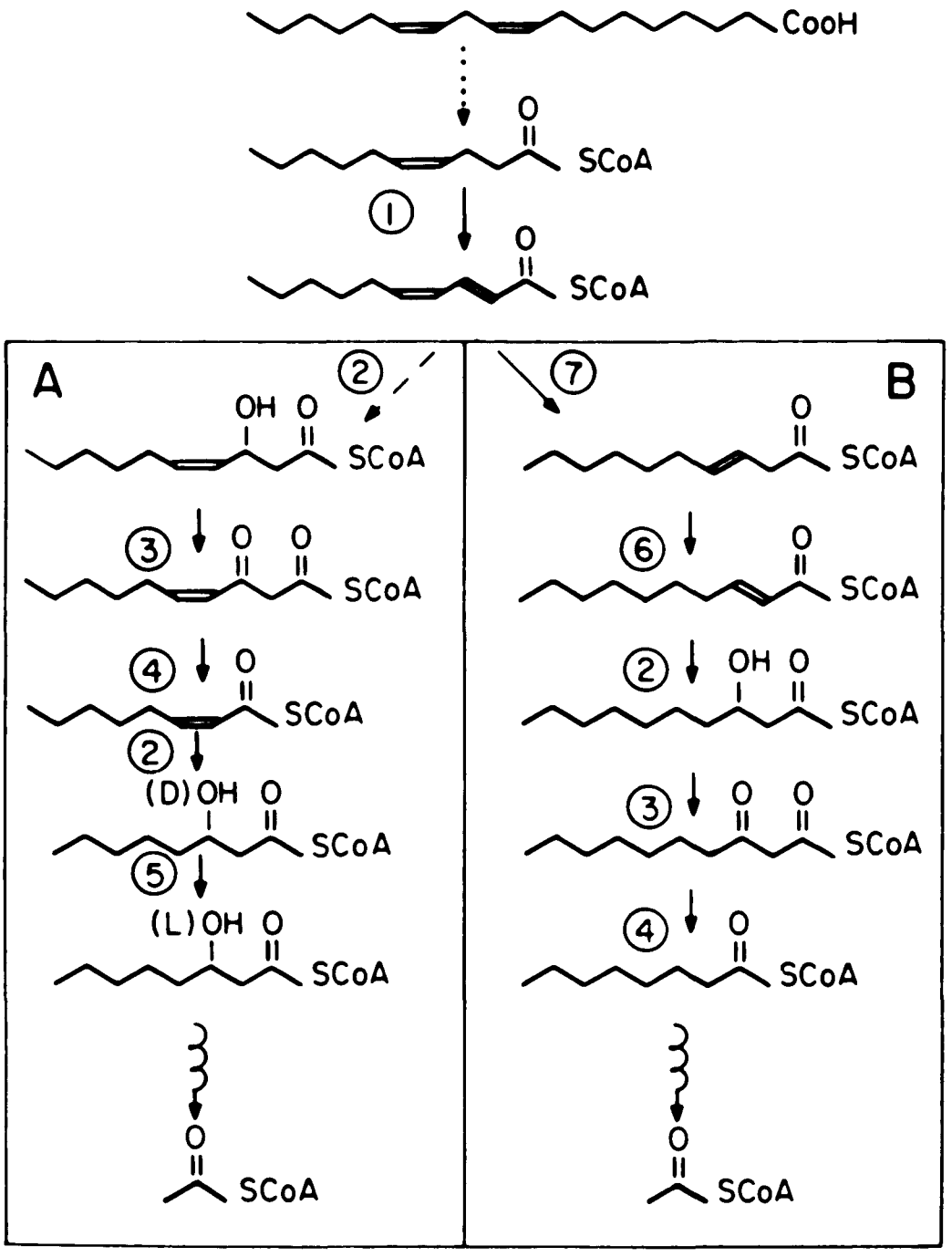

CooH
SCoA
1
A
2
OH O
3
4
(D) OH
5
(L) OH
B
7
6

FIGURE 3: Effect of 4-bromo-2-octenoic acid on respiration in rat liver mitochondria. Rat liver mitochondria (2 mg) were suspended in 1.9 ml of a basal isotonic incubation buffer containing 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 4 mM KP_i,4 mM $MgCl_2$, and 0.1 mM EGTA. To this suspension were added in the indicated sequence bovine serum albumin (0.5 mg/ml), 0.5 mM L-malate, 1 mM ADP, and 1 min later, varying amounts of 4-bromo-2-octenoic acid. The mixture was incubated for 3 min or the indicated periods of time. State 3 respiration was started by the addition of 30 μM of palmitoylcarnitine or 5 mM pyruvate. Oxygen uptake was measured polarographically with a Clark oxygen electrode attached to a Gilson oxygraph. PC, 30 μM palmitoylcarnitine; C, solvent used to dissolve the inhibitor; Br $_2C_8$, 20 μM 4-bromo-2-octenoic acid dissolved in ethanol/incubation buffer (1:1); Car, 2.2 mM L-carnitine; Pyr, 6.25 mM pyruvate; DNP, 0.1 mM 2,4-dinitrophenol. The numbers give the rates of respiration in nanoatoms of oxygen min^{-1} (mg of protein)$^{-1}$.

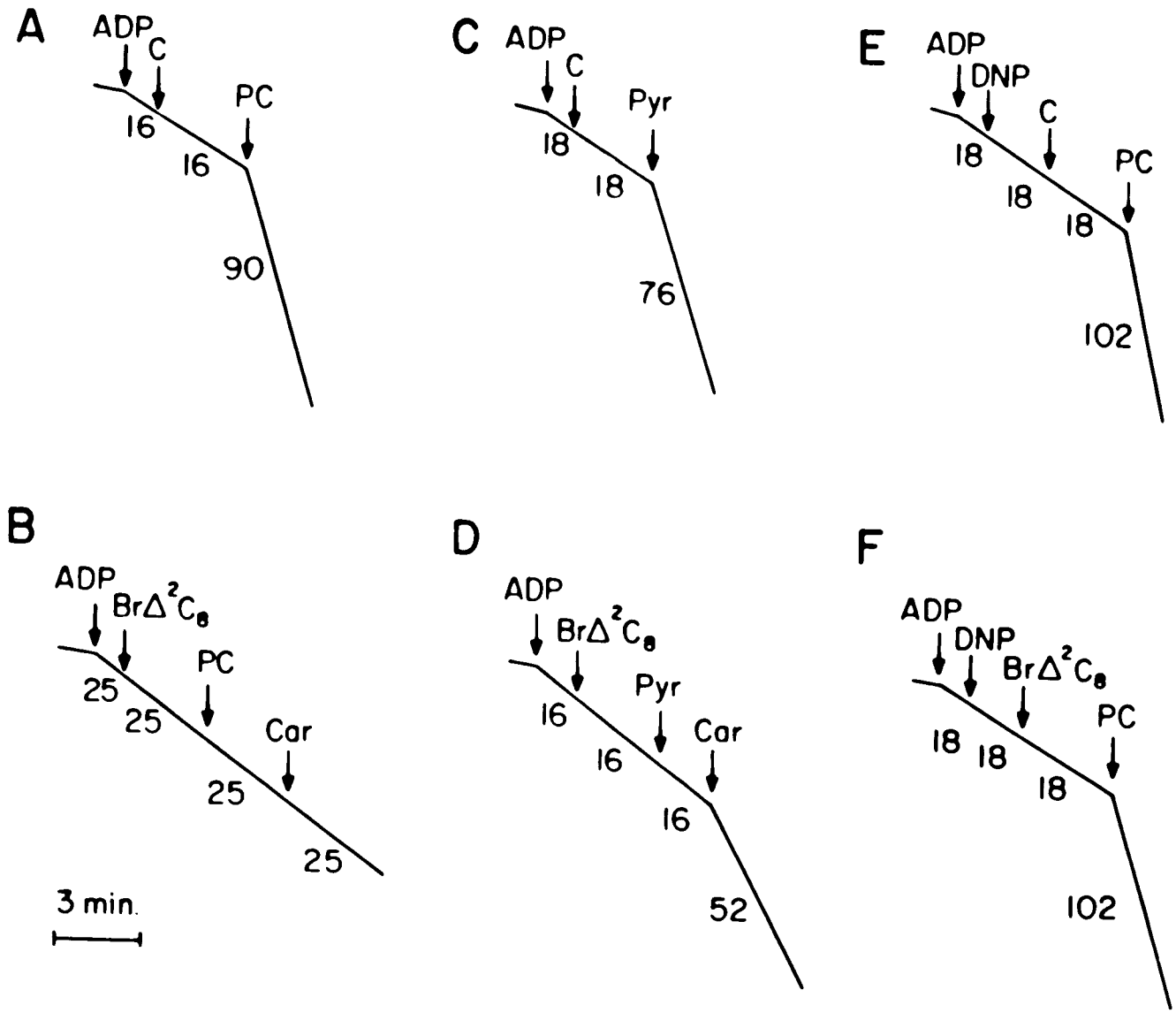

A
ADP
C
PC
16
16
90
B
ADP
BrΔ²C₈
PC
Car
25
25
25
25
3 min.
C
ADP
C
Pyr
18
18
76
D
ADP
BrΔ²C₈
Pyr
Car
16
16
16
52
E
ADP
DNP
C
PC
18
18
18
102
F
ADP
DNP
BrΔ²C₈
PC
18
18
18
102

FIGURE 4: Effect of 4-bromo-2-octenoic acid on palmitoylcarnitine-supported respiration and thiolase activities in rat liver mitochondria. (A) Inhibition of respiration supported by palmitoylcarnitine and of the activity of 3-ketoacyl-CoA thiolase as a function of the 4-bromo-2-octenoic acid concentration. Coupled rat liver mitochondria were preincubated with the inhibitor for 3 min before respiration measurements and thiolase assays were performed as described under "Experimental Procedures". (B) Inhibition of palmitoylcarnitine-supported respiration by 4-bromo-2-octenoic acid as a function of time. Coupled rat liver mitochondria were preincubated with the inhibitor. (●) Respiration supported by palmitoylcarnitine; (▲) activity of 3-ketoacyl-CoA thiolase measured with 3-ketooctanoyl-CoA; (■) activity of acetoacetyl-CoA thiolase determined as described under Experimental Procedures.

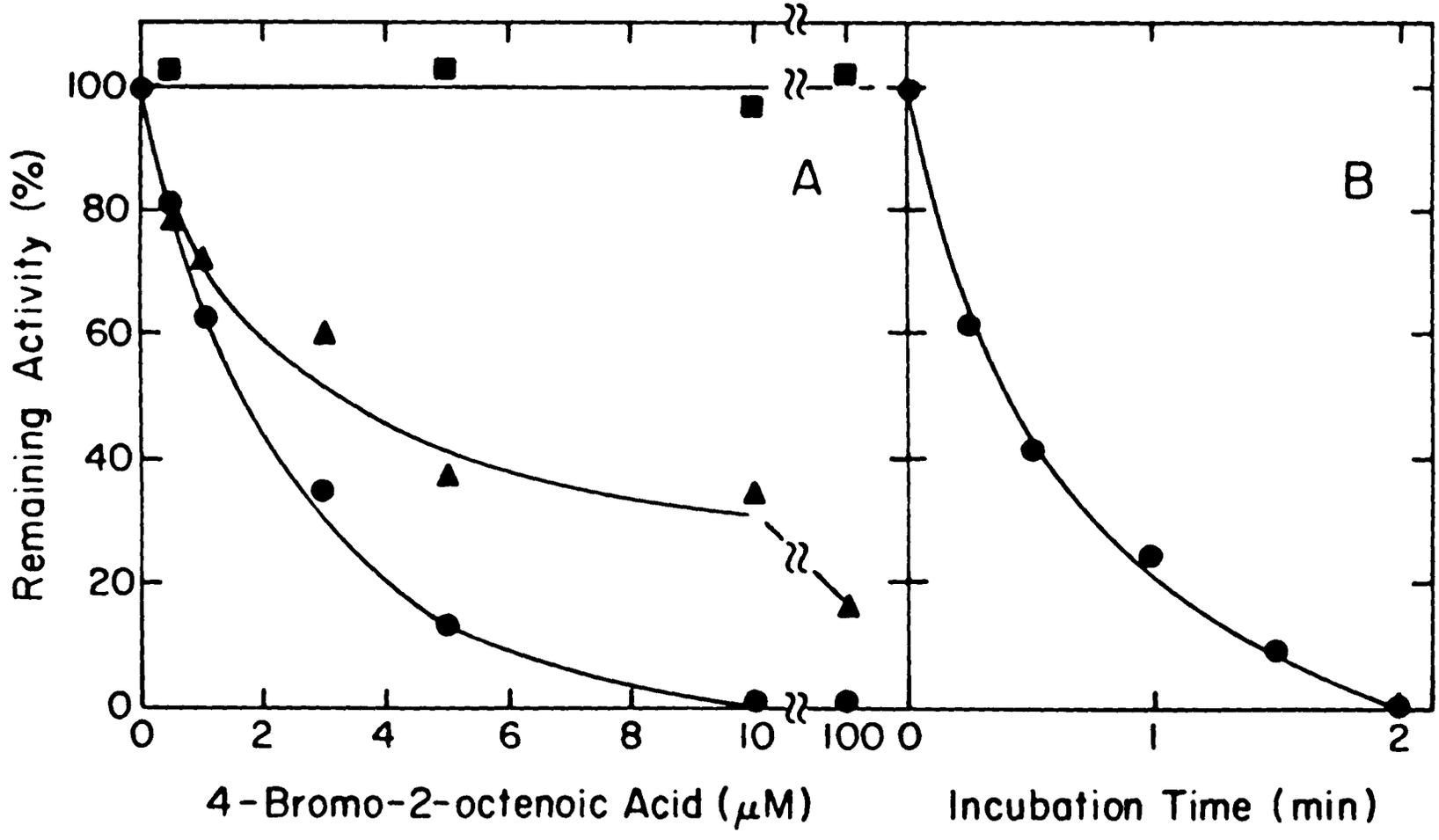

Remaining Activity (%)
100
80
60
40
20
0
A
B
0
2
4
6
8
10
100
0
1
2
4-Bromo-2-octenoic Acid (μM)
Incubation Time (min)

FIGURE 5: Spectrophotometric evidence for the metabolism of 4-bromo-2-octenoyl-CoA by β-oxidation enzymes. To 20 nmol of 4-bromo-2-octenoyl-CoA in 1 ml of 0.1 M HEPES (pH 8.1) was added 15 mU of enoyl-CoA hydratase. After no further change in absorbance at 263 nm could be observed, the wavelength was changed to 340 nm, and 20 mM $MgCl_2$ and 1 mM NAD^+ were added followed by 2 U of L-3-hydroxyacyl-CoA dehydrogenase. After completion of the reaction, the wavelength was changed to 303 nm, and 10 mU of 3-ketoacyl-CoA thiolase was added followed by 60 μM CoASH and finally 12 μM 3-ketooctanoyl-CoA.

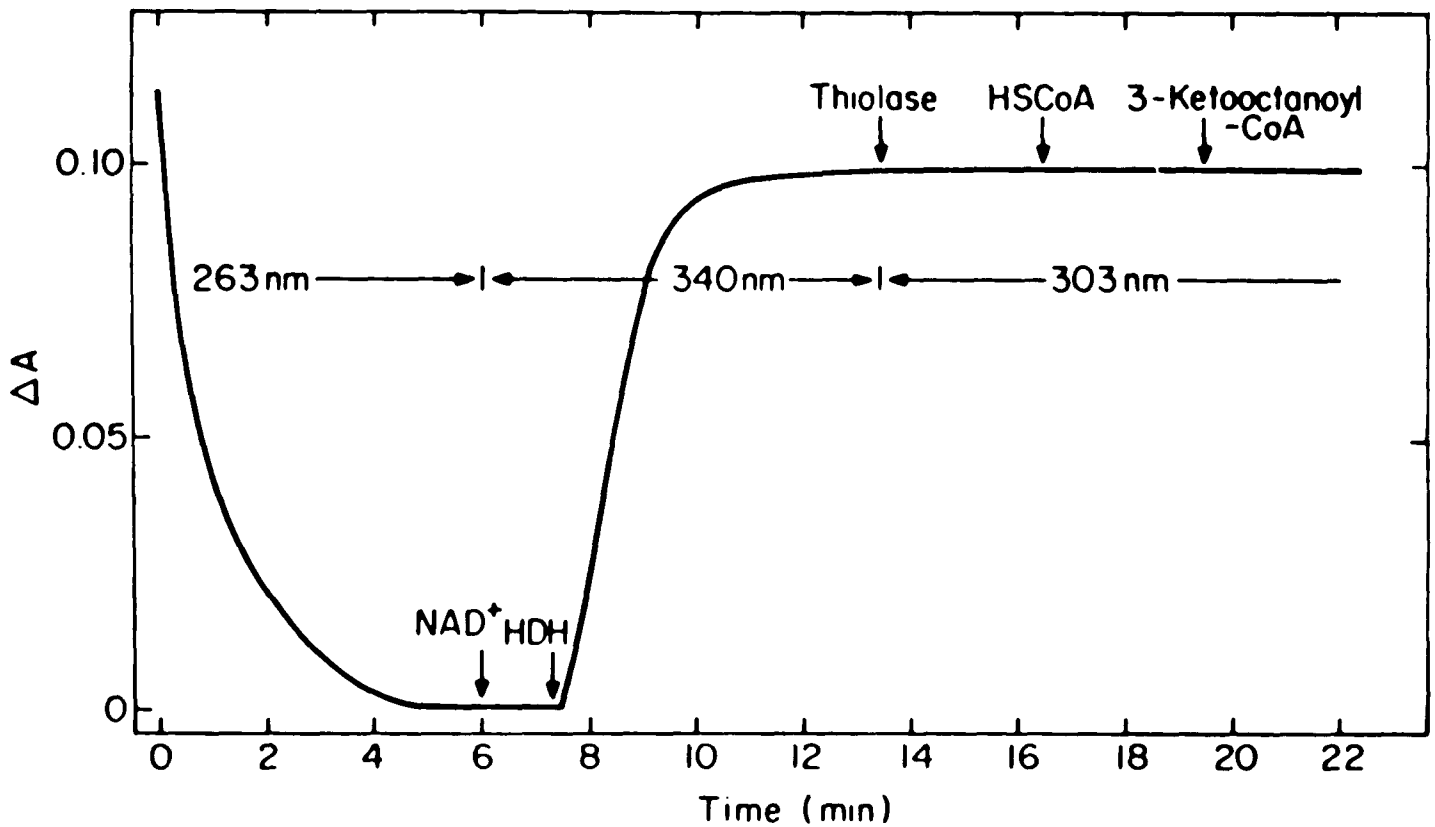

Thiolase
HSCoA
3-Ketooctanoyl -CoA
0.10
0.05
0
ΔA
263nm
340nm
303nm
NAD+ HDH
0
2
4
6
8
10
12
14
16
18
20
22
Time (min)

FIGURE 6: Spectrum of the Mg^{2+}-enolate complex of 3-keto-4-bromooctanoyl-CoA. The reaction mixture contained in 1 ml of 0.1 M HEPES (ph 8.1) 20 nmol of 4-bromo-2-octenoyl-CoA, 15 mU of enoyl-CoA hydratase, 20 μmol of $MgCl_2$, and 1 μmol of NAD^+. The reaction was started by the addition of 2 U of L-3-hydroxyacyl-CoA dehydrogenase. (A) Spectra (1) after 20 s, (2) after 1.5 min, (3) after 8 min, (4) after addition of 30 μmol of EDTA to the solution corresponding to spectrum 3, and (5) after addition of pyruvate and lactate dehydrogenade to the solution corresponding to spectrum 4. (B) Spectrum of the Mg^{2+}-enolate complex of 3-keto-4-bromo-octanoyl-CoA obtained by subtracting spectrum 4 of panel A from spectrum 3 of panel A.

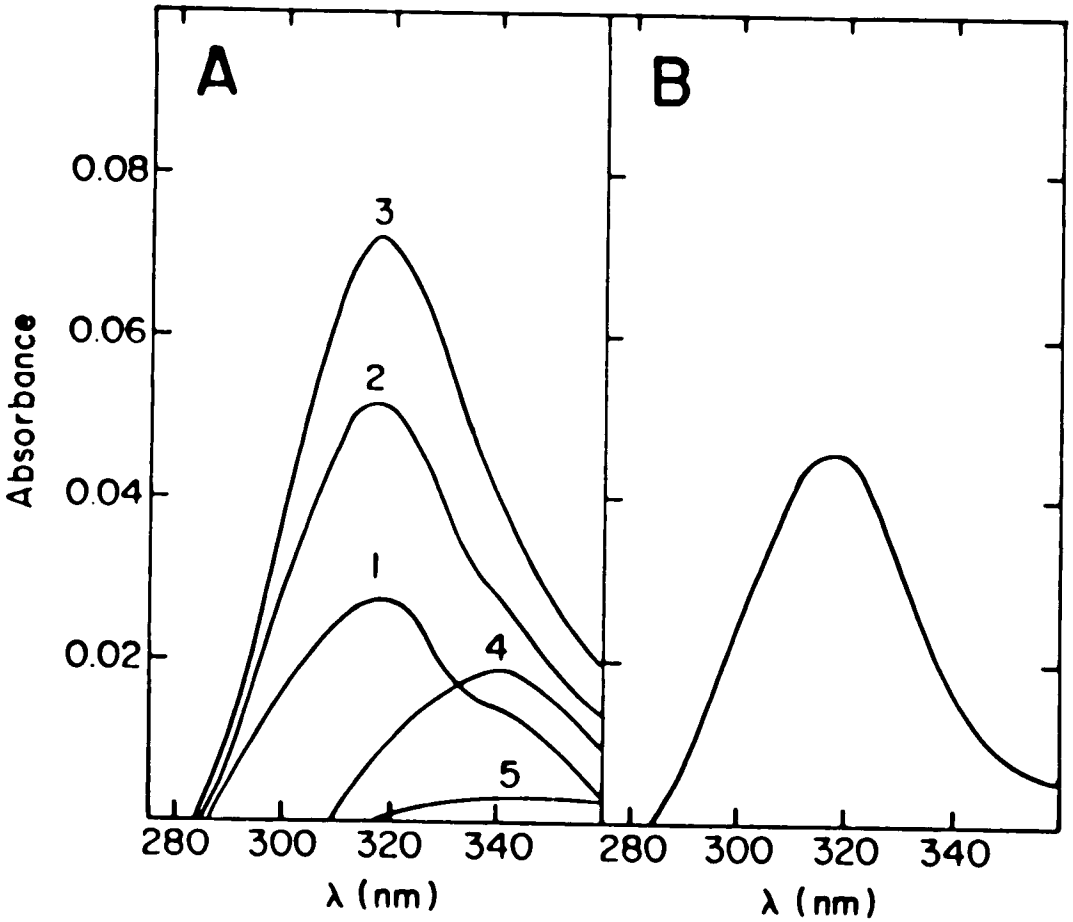

A
B
0.08
0.06
0.04
0.02
Absorbance
3
2
1
4
5
280
300
320
340
λ (nm)
280
300
320
340
λ (nm)

FIGURE 7: Effect of 4-bromo-2-octenoyl-CoA and its ß-oxidation metabolites on the 3-ketoacyl-CoA thiolase activity present in a soluble extract of rat liver mitochondria. A soluble extract of rat liver mitochondria (0.278 mg of protein in 1 ml of buffer used in respiration measurements) was incubated with 4 mM NAD^+ (●), 20 µM 4-bromo-2-octenoyl-CoA (▲), 4 mM NAD^+ plus 20 µM 4-bromo-2-octenoyl-CoA (■), or 4 mM NAD^+ plus 20 µM 4-bromo-2-octenoyl-CoA plus 1 mM 3-ketooctanoyl-CoA (◆). Samples of 10 µl were taken at the indicated times and assayed for 3-ketoacyl-CoA thiolase with 3-ketooctanoyl-CoA as a substrate.

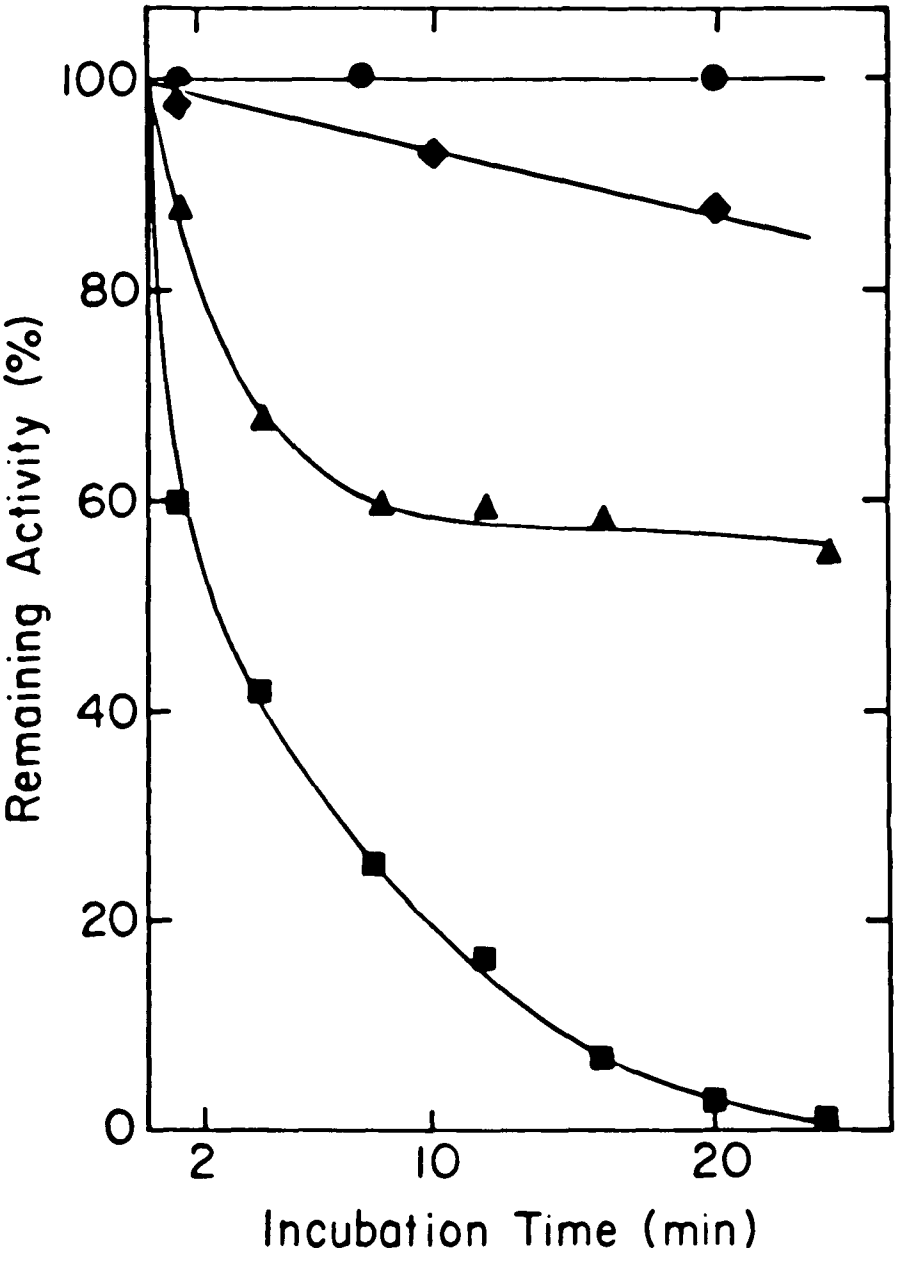

100
80
60
40
20
0
Remaining Activity (%)
2
10
20
Incubation Time (min)

FIGURE 8: Proposed pathway for the metabolism of 4-bromo-2-octenoic acid.

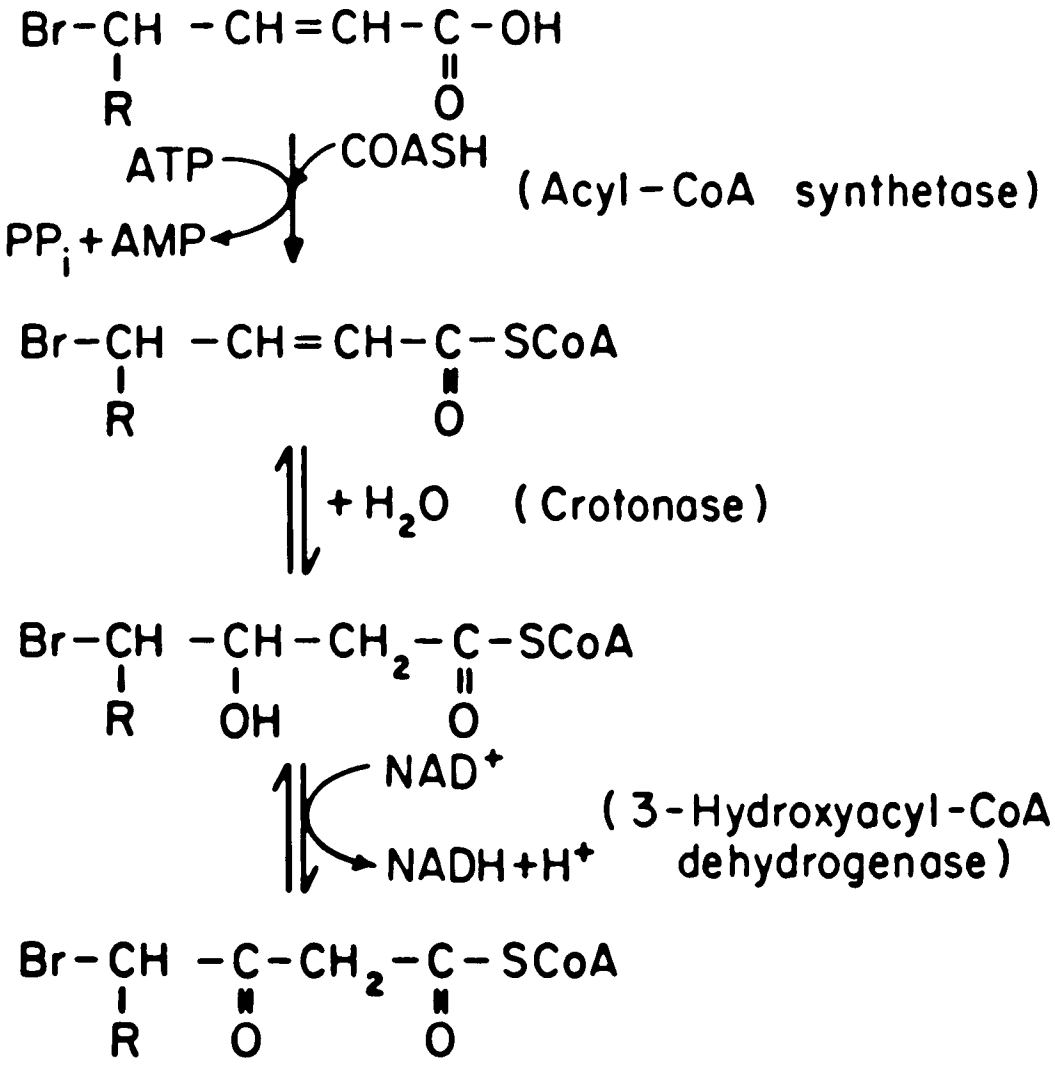


FIGURE 9: HPLC analysis of products formed during the degradation of 2-propylpentanoic acid. Reactions were performed as described under "Experimental Procedures". A. Incubation of 2-propyl-2E-pentenoyl-CoA with purified bovine liver crotonase; B. Incubation of 2-propyl-2E-pentenoyl-CoA with broken rat liver mitochondria in the presence of NAD^+ and excess crotonase. C. Same as B with additional 3-ketoacyl-CoA thiolase and 0.3 mM coenzyme A. D. Same as B in the absence of NAD^+. E. Incubation of 2-propylpentanoic acid with intact rat liver mitochondria for 2.5 min. Peaks identified by use of authentic samples were: 1. 2-propylpentanoyl-CoA; 2. 2-propyl-2E-pentenoyl-CoA; 3. 3-hydroxy-2-propylpentanoyl-CoA; 4. 3-keto-2-propylpentanoyl-CoA.

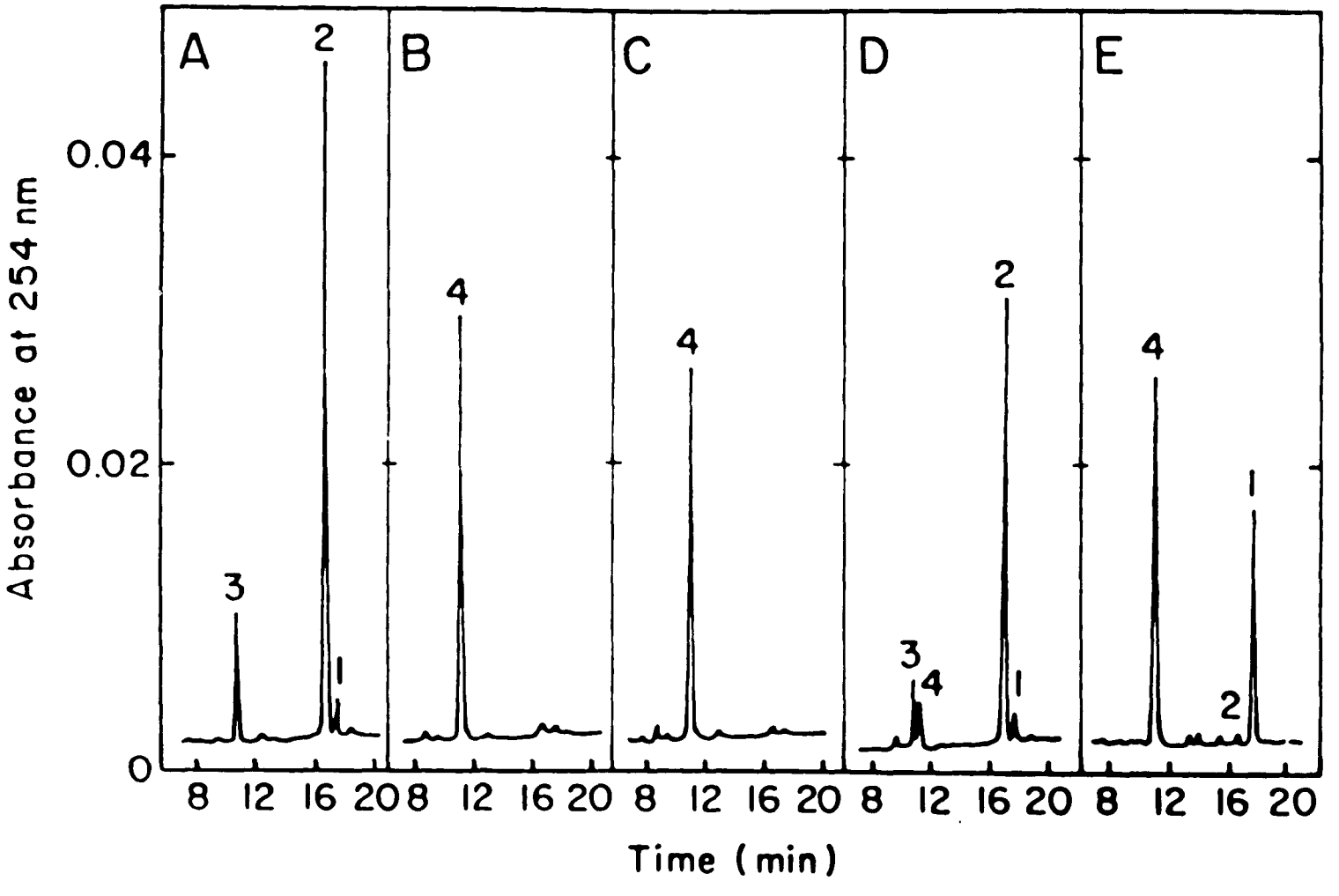

A
B
C
D
E
2
4
3
1
0.04
0.02
0
Absorbance at 254 nm
8 12 16 20
Time (min)

FIGURE 10: Formation of NADH during the dehydrogenation of 3-hydroxy-2-propylpentanoyl-CoA. Details are given under "Experimental Procedures". Incubation of 2-propyl-2E-pentenoyl-CoA with broken rat liver mitochondria in the presence of 1.6 mM NAD^+ and excess crotonase. Spectra taken were 1. after 1 min, 2. after 4 min, 3. after 20 min, 4. after the addition of pyruvate to the sample corresponding to spectrum 3.

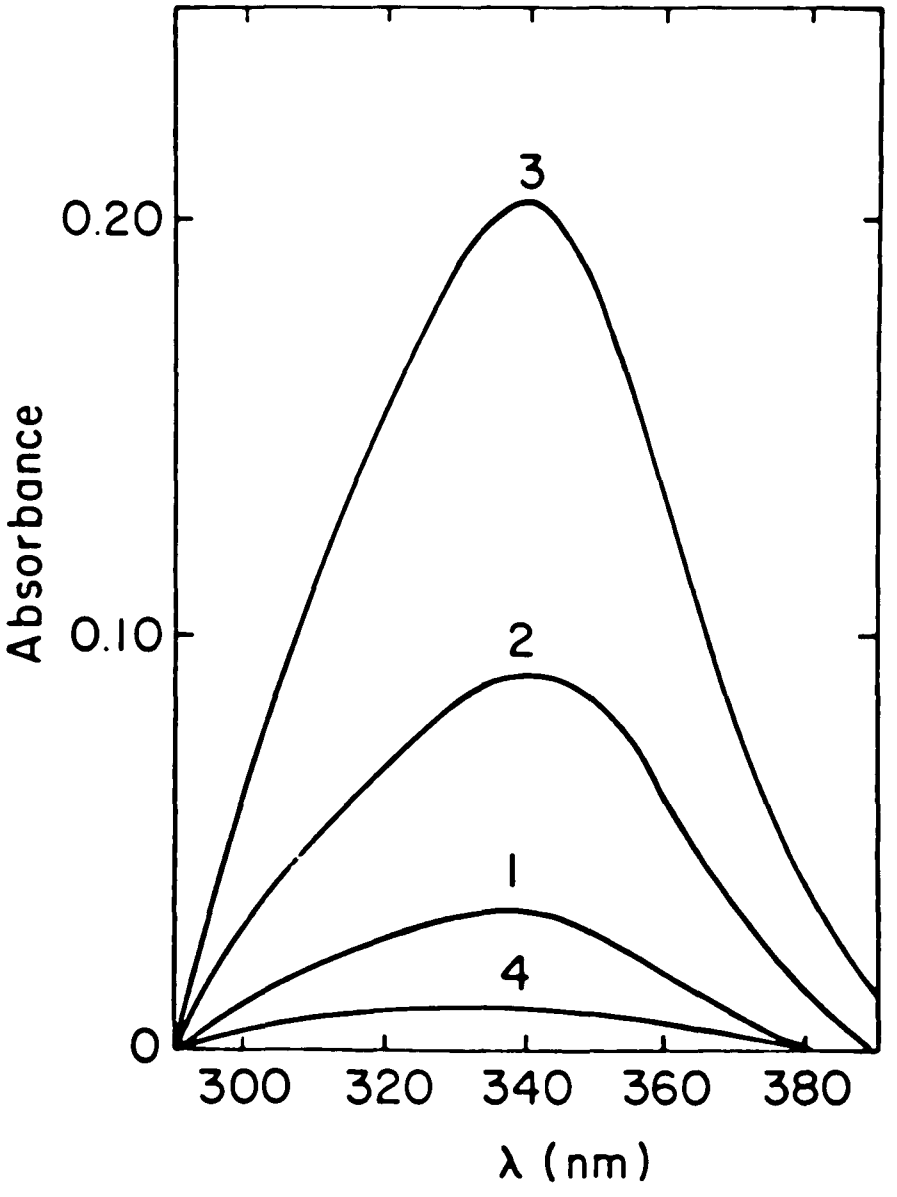

Absorbance
0.20
0.10
0
3
2
1
4
300
320
340
360
380
λ (nm)

Figure 11: Mass spectrum of 3-keto-2-propylpentanoyl-CoA. MH^+ peak is shown at m/z 908.4.

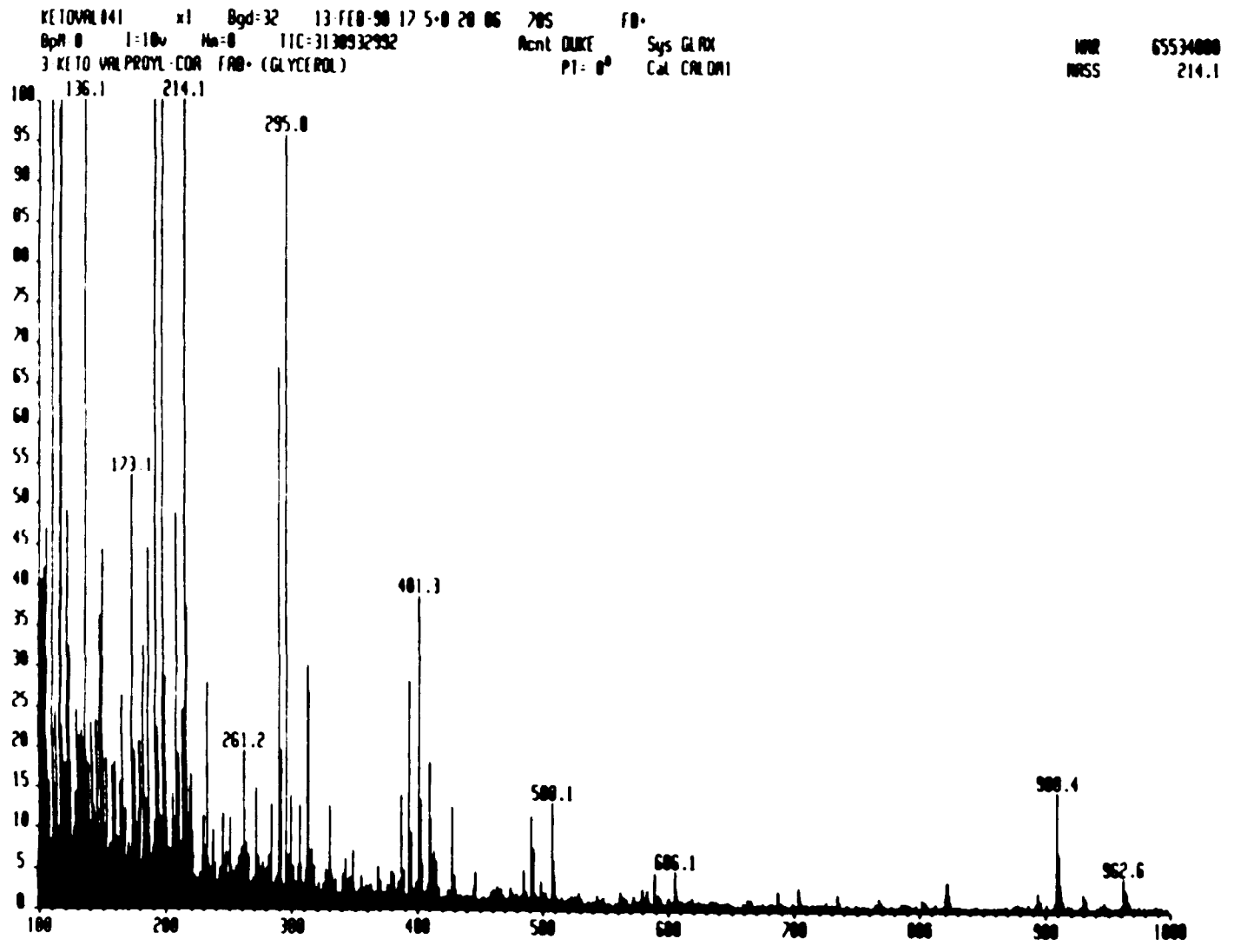

KETOVAL041 x1 Bgd=32 13-FEB-90 17 5-0 20 0G 70S FB-
BpM 0 I=10v Na=0 TIC=3138932992 Acnt DUKE Sys GLRX NMR 65534000
3 KETO VALPROYL-COR FAB- (GLYCEROL) P1= 0° Cal CALDA1 MASS 214.1
136.1
214.1
295.0
173.1
401.3
261.2
500.1
606.1
900.4
962.6
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
100
200
300
400
500
600
700
800
900
1000

FIGURE 12: Degradation of 2-propylpentanoic acid by intact mitochondria. 2-Propylpentanoic acid was incubated with intact rat liver mitochondria in the presence of 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 4 mM KP_i,4 mM $MgCl_2$, and 0.1 mM EGTA. To this suspension were added bovine serum albumin (0.5 mg/ml), 0.5 mM L-malate, 1 mM ADP, 4 mM ATP and 2 mM carnitine. The reaction was started by the addition of 0.5 mM 2-propylpentanoic acid. (▲), Total coenzyme A derivatives; (■), 3-keto-2-propylpentanoyl-CoA; (●), 2-propylpentanoyl-CoA; (○), 2-propyl-2E-pentenoyl-CoA.

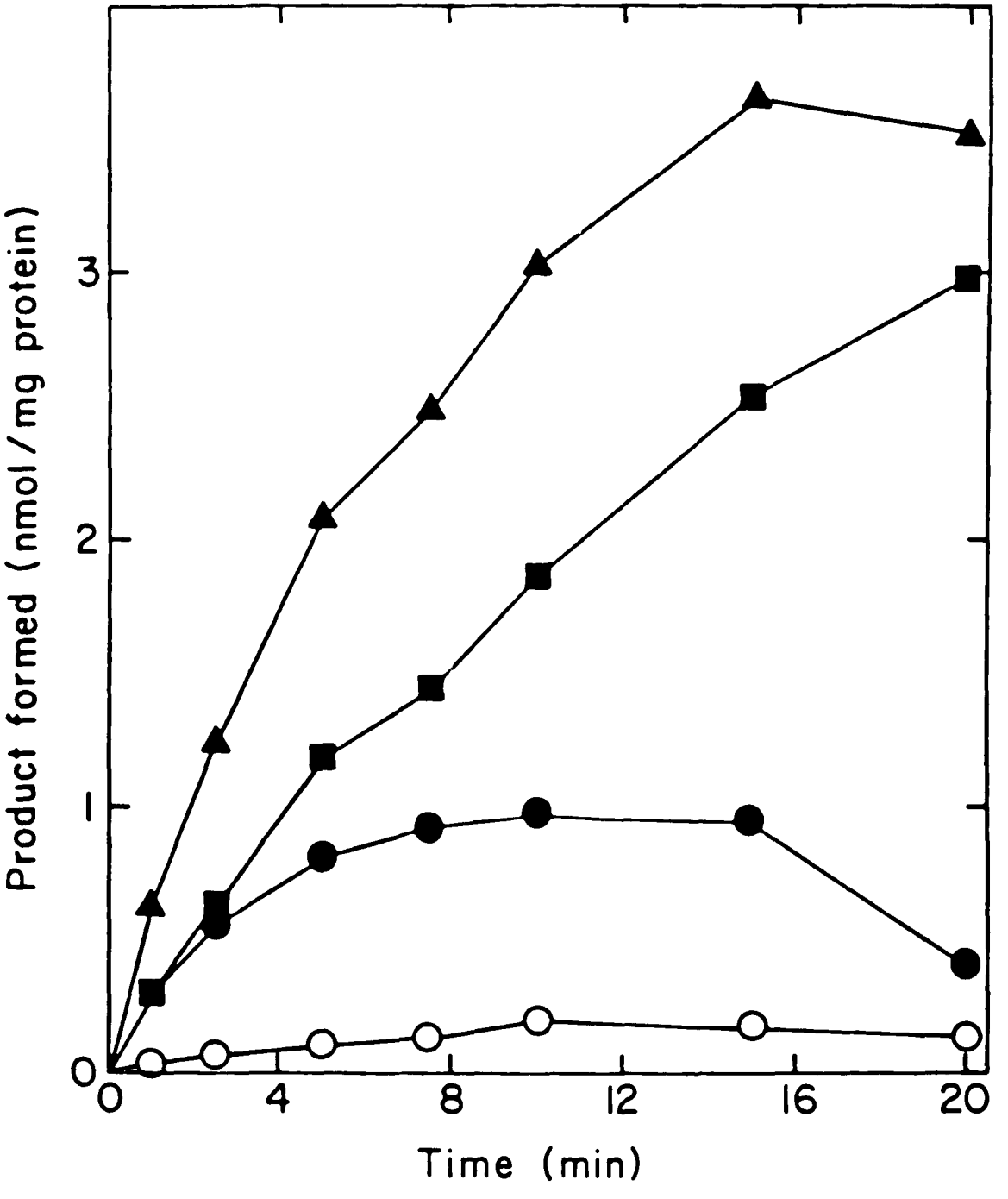

Product formed (nmol / mg protein)
0
1
2
3
0
4
8
12
16
20
Time (min)

FIGURE 13: Proposed pathway of the mitochondrial metabolism of 2-propylpentanoic acid. 1. 2-propyl-pentanoic acid; 2. 2-propylpentanoyl-CoA; 3. 2-propyl-2E-pentenoyl-CoA; 4. 3-hydroxy-2-propylpentanoyl-CoA; 5. 3-keto-2-propylpentanoyl-CoA.

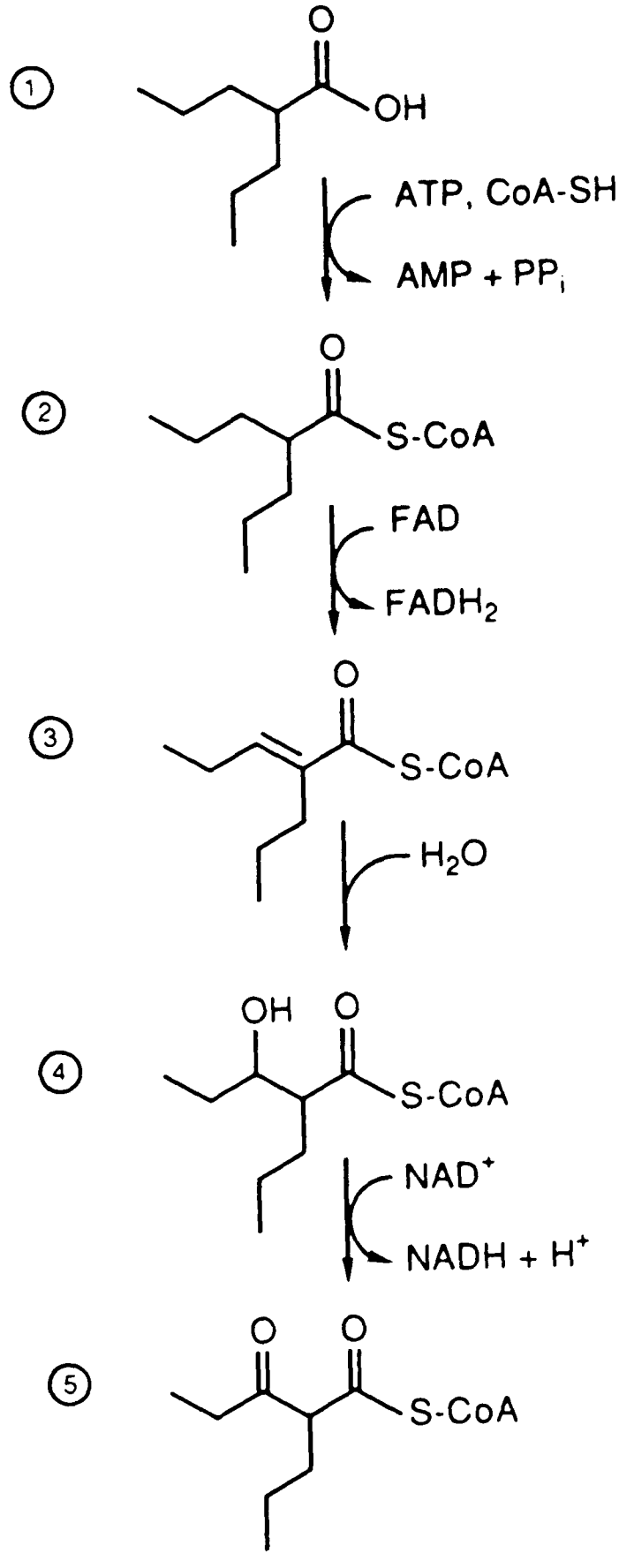

1
O
OH
ATP, CoA-SH
AMP + PPi
2
O
S-CoA
FAD
FADH2
3
O
S-CoA
H2O
4
OH
O
S-CoA
NAD+
NADH + H+
5
O
O
S-CoA

FIGURE 14: Polyacrylamide gel electrophoresis of D-3-hydroxyacyl-CoA dehydratase under nondenaturing conditions. D-dehydratase (5ug) was subjected to electrophoresis on a 7% polyacrylamide gel with 90 mM Tris/80 mM H_3BO_3 adjusted to pH 9.6 with KOH at 10 ^{o}C at fixed current of 8 mA. Upon completion, half of the gel was stained with Coomassie blue R and scanned with an LKB Ultrascan XL laser densitometer while the other half was cut into 2 mm segments which were extracted for 24 hr with the electrophoresis buffer adjusted to pH 8 containing 1 mg/ml BSA. D-hydratase activity was assayed as described in "Experimental Procedure". Dashed line, activity of D-3-hydroxyacyl-CoA dehydratase; solid line, absorbance of protein.

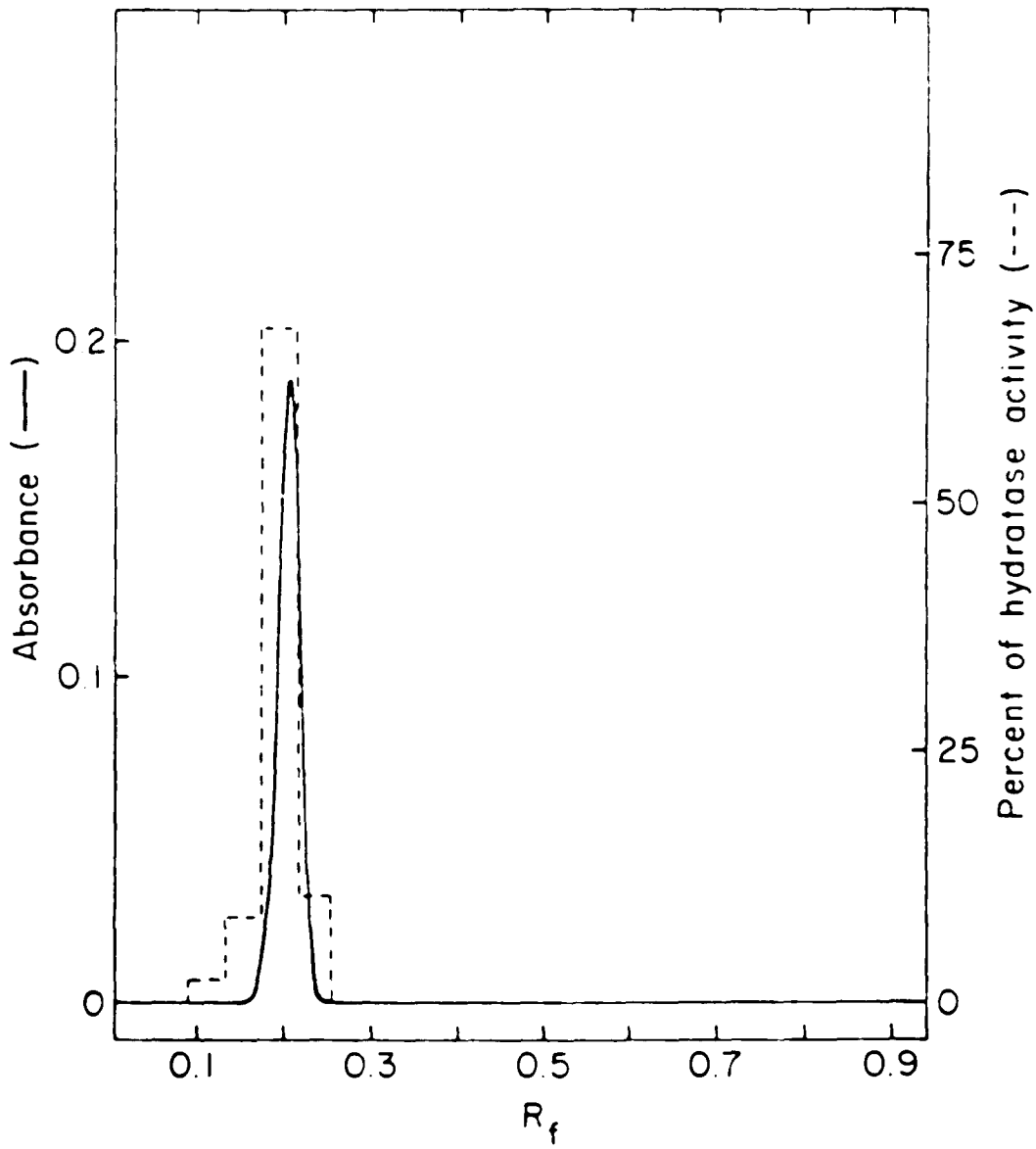

Absorbance (——)
0.2
0.1
0
75
50
25
0
Percent of hydratase activity (- - -)
0.1
0.3
0.5
0.7
0.9
R_f

FIGURE 15: Native and subunit molecular weights of D-3-hydroxyacyl-CoA dehydratase. The subunit molecular weight was determined by polyacrylamide gel electrophoresis in the presence of SDS (◆). The native molecular weight was determined by HPLC gel fitration (■)(see experimental precedures for details). (1) Ferritin, 440,000 Da; (2) catalase, 232,000 Da, subunit 57,500 Da; (3) lactate dehydrogenase, 150,000 Da, subunit 35,000 Da; (4) hexokinase, 102,000 Da; (5) L-3-hydroxyacyl-CoA dehydrogenase, 76,000 Da; (6) chymotrypsinogen A, 25,000 Da; (8) bovine serum albumin, 66,000 Da; (9) ovalbumin, 45,000 Da; (10) carboxylase, 29,000 Da; ***Arrow*** D-3-hydroxyacyl-CoA dehydrogenase. The native molecular weight is 106,800 Da.; the subunit molecular weight is 44,000 Da.

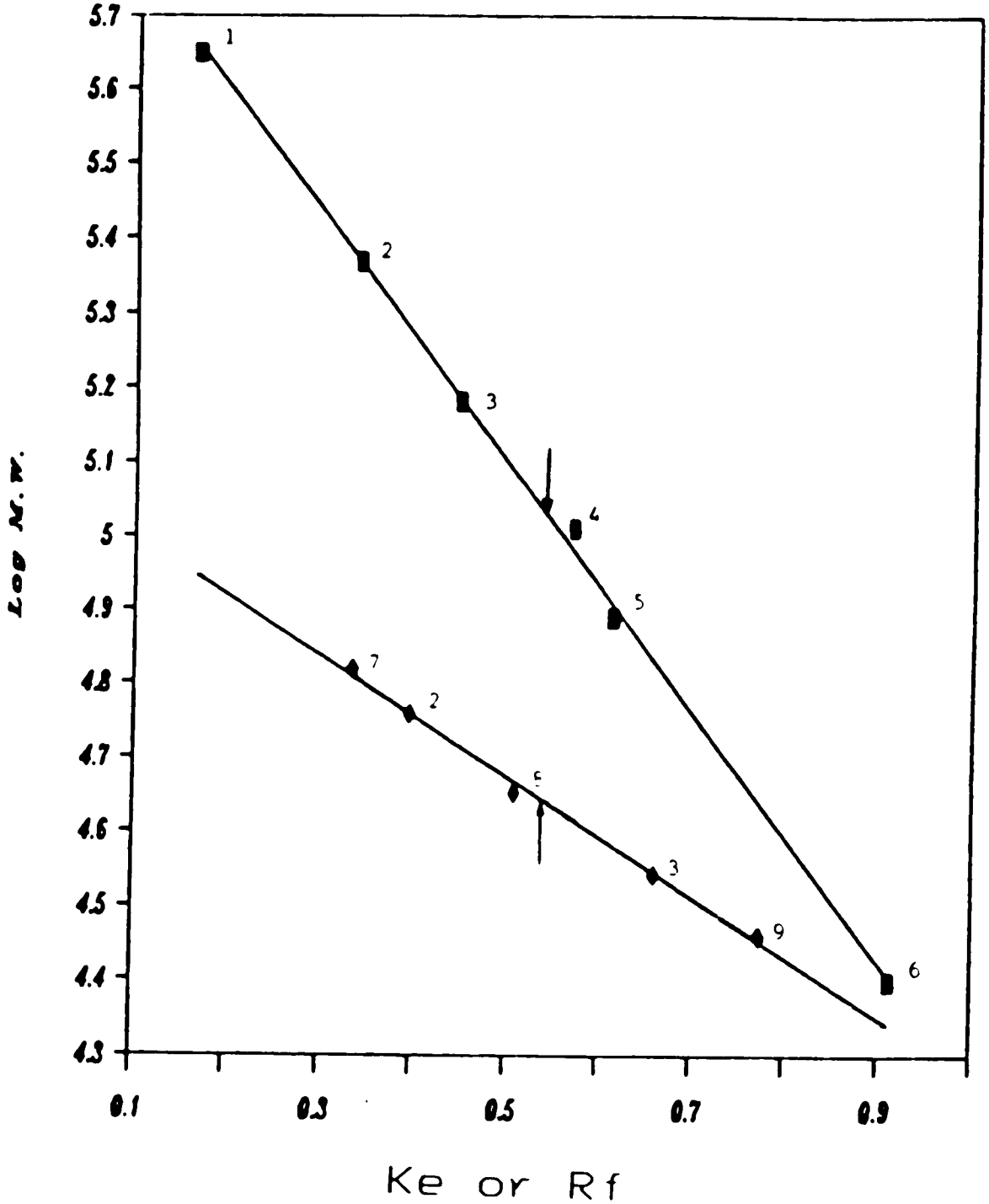

5.7
5.6
5.5
5.4
5.3
5.2
5.1
5
4.9
4.8
4.7
4.6
4.5
4.4
4.3
Log M.W.
0.1
0.3
0.5
0.7
0.9
Ke or Rf
1
2
3
4
5
6
7
8
9

FIGURE 16: HPLC analysis of products formed during the β-oxidation of D-3-hydroxyoctanoyl-CoA. Product formed when D-3-hydroxyoctanoyl-CoA was incubated: A. with L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, crotonase, 1.6 mM NAD^+, and 0.3 mM coenzyme A; B. with the early DEAE-cellulose fraction in addition to the components listed under A except crotonase; C with the components listed under B then ultrafiltered and incubated with the componets listed under A. Peaks identified by use of authentic samples were: 1. D-3-Hydroxyoctanoyl-CoA; 2. n-hexanoyl-CoA; 3. 2-*trans*-octenoyl-CoA.

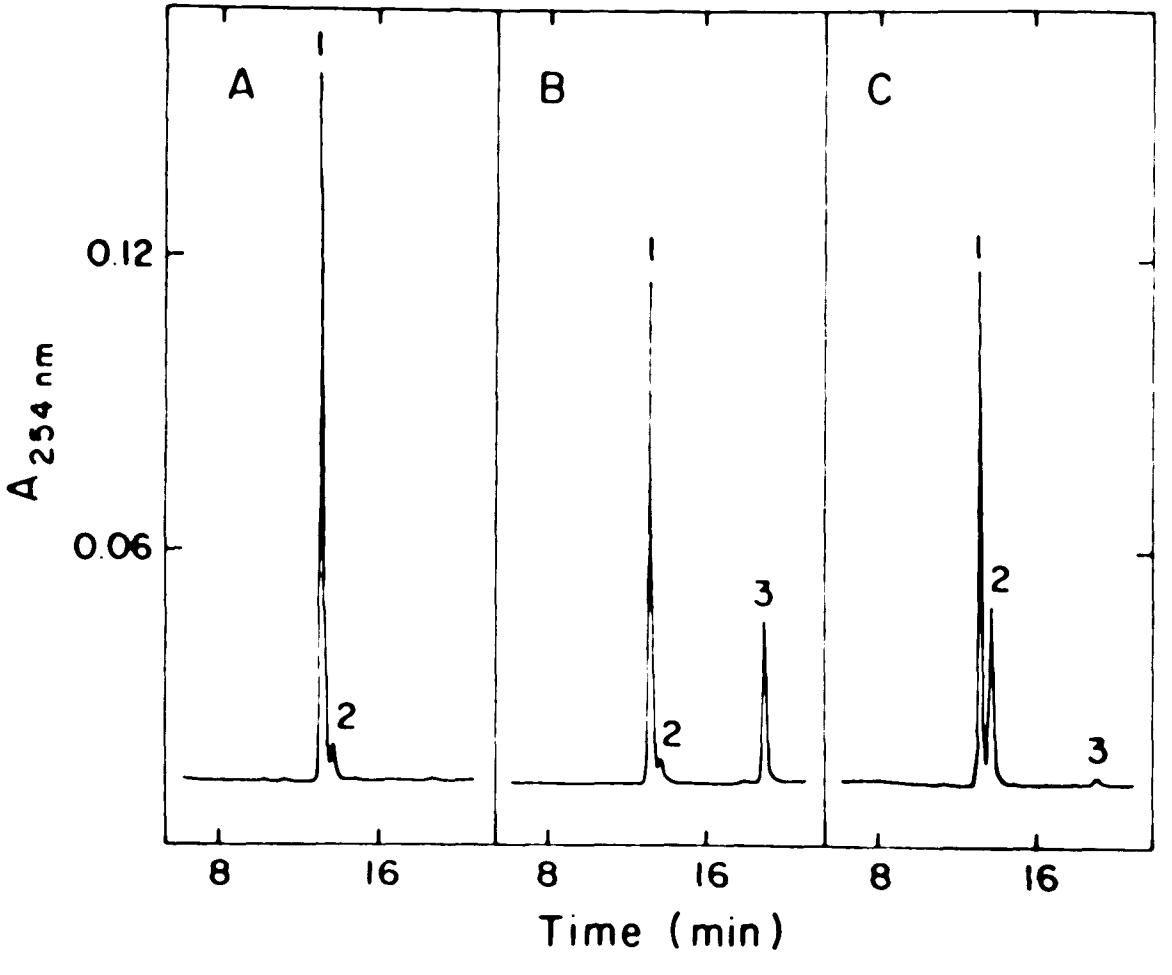

A
B
C
1
2
3
0.12
0.06
$A_{254\ nm}$
8
16
Time (min)

FIGURE 17: HPLC analysis of the product formed during β-oxidation products of 2-*cis*-octenoyl-CoA. Product formed when 2-*cis*-octenoyl-CoA was incubated: A. with buffer only; B. with crotonase; C. with crotonase, L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, 1.6 mM NAD^+ and 0.3 mM coenzyme A; D. with partially purified D-3-hydroxyacyl-CoA dehydratase in addition to the components listed under C; Peaks identified by use of authentic samples were: 1. 2-*cis*-octenoyl-CoA; 2. D-3-hydroxyoctanoyl-CoA; 3. n-hexanoyl-CoA.

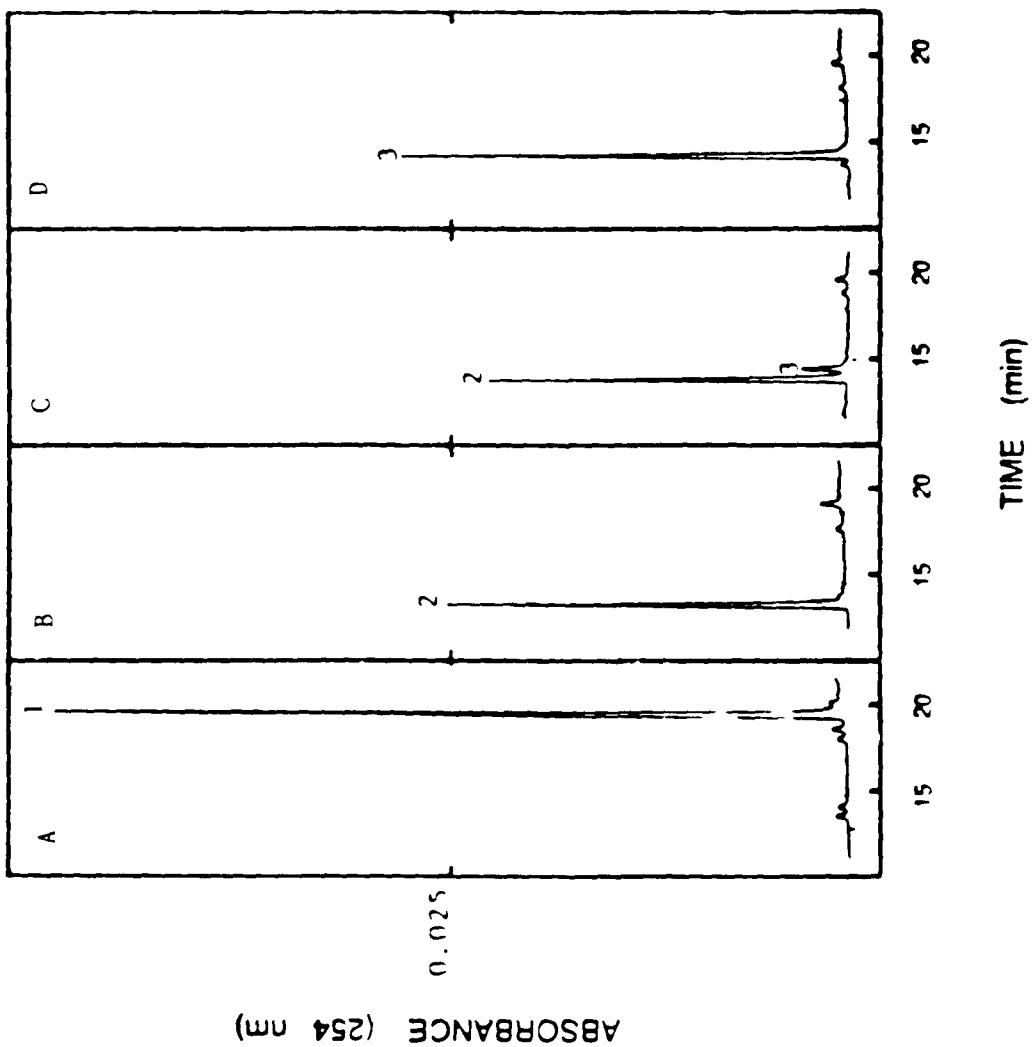

A
B
C
D
1
2
3
2
3
3
15
20
15
20
15
20
15
20
TIME (min)
0.025
ABSORBANCE (254 nm)

FIGURE 18: HPLC analysis of products formed when 2-*cis*-octenoyl-CoA or 2-*trans*-octenoyl-CoA were incubated with crotonase or D-3-hydroxyacyl-CoA dehydratase. 2-*cis*-Octenoyl-CoA was incubated in 0.2 M KP_i with: A. D-3-hydroxyacyl-CoA dehydratase. B. crotonase. 2-*trans*-Octenoyl-CoA was incubated in 0.2 M KP_i with: C. D-3-hydroxyacyl-CoA dehydratase. D. crotonase. Peaks identified by use of authentic samples are 1. 3-Hydroxyoctanoyl-CoA; 2. 2-octenoyl-CoA

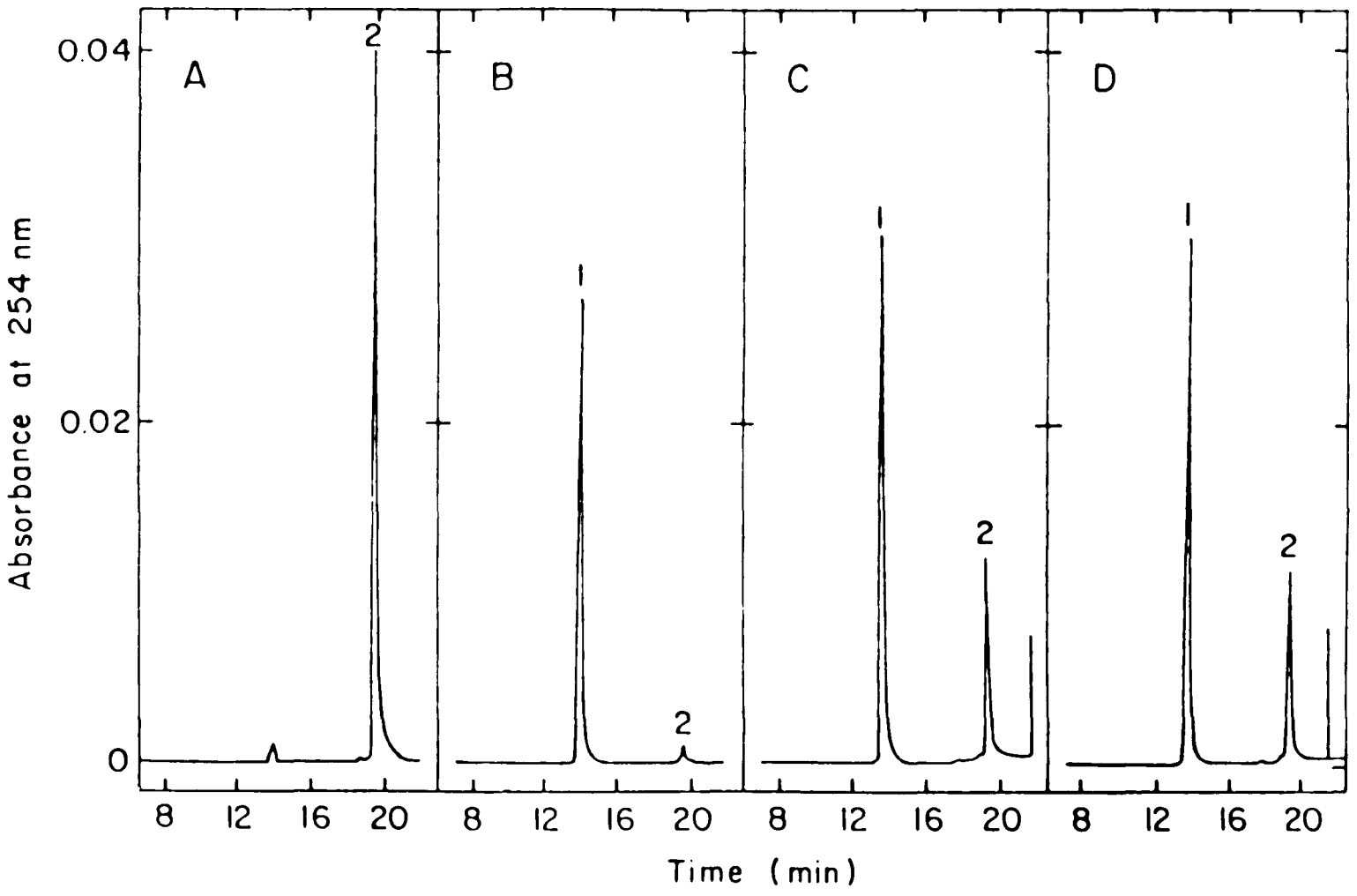
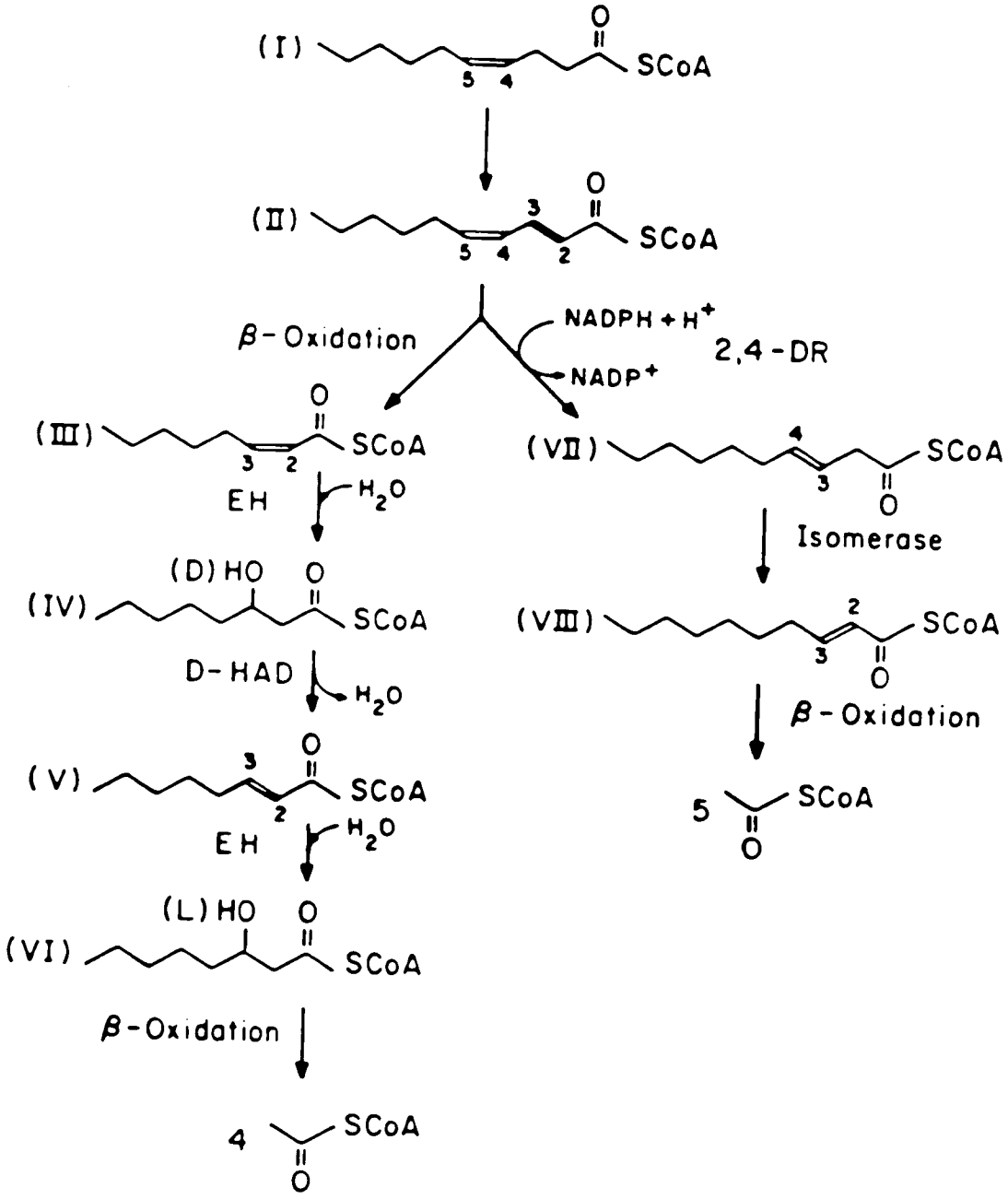

A
B
C
D
1
2
0.04
0.02
0
Absorbance at 254 nm
8
12
16
20
Time (min)

FIGURE 19: β-Oxidation of 4-*cis*-decenoyl-CoA by the epimerase-dependent pathway. 2,4-DR: 2,4-dienoyl-CoA reductase; EH: enoyl-CoA hydratase; D-HAD: D-3-hydroxyacyl-CoA dehydratase.


(I)
SCoA
(II)
SCoA
β-Oxidation
NADPH + H+
NADP+
2,4-DR
(III)
SCoA
EH
H2O
(VII)
SCoA
Isomerase
(D) HO
(IV)
SCoA
D-HAD
H2O
(VIII)
SCoA
β-Oxidation
(V)
SCoA
EH
H2O
5
SCoA
(L) HO
(VI)
SCoA
β-Oxidation
4
SCoA

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