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# Metabolic and enzymological studies of mitochondrial fatty acid beta-oxidation

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# METABOLIC AND ENZYMOLOGICAL STUDIES OF MITOCHONDRIAL FATTY ACID BETA-OXIDATION

by

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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

## METABOLIC AND ENZYMOLOGICAL STUDIES OF MITOCHONDRIAL FATTY ACID BETA-OXIDATION

by

Hang-Yong Wang

Advisor: Professor Horst Schulz

The possible control of fatty acid oxidation in heart by the intramitochondrial ratio of acetyl-CoA/CoASH or/and by the ratio of $NADH/NAD^+$ was investigated with coupled rat heart mitochondria at respiration states 3 and 4. When 10 mM L-carnitine was added to coupled rat heart mitochondria at state 4 respiration, the rate of palmitoylcarnitine β-oxidation increased more than 4-fold, while the same addition had little effect on β-oxidation at state 3 respiration. Rates of respiration were unaffected by the addition of carnitine. Neither oxaloacetate nor acetoacetate, added to mitochondria to lower the intramitochondrial $NADH/NAD^+$ ratio, stimulated β-oxidation. Determination of the intramitochondrial ratio of acetyl-CoA/CoASH by high performance liquid chromatography yielded a value close to 10 for state 4 respiration as compared to 2.5 at state 3 respiration. Addition of 10 mM carnitine caused a dramatic decrease of this ratio to less than 0.2 at both respiration states. All data agree with the hypothesis that in heart mitochondria the rate of β-oxidation is controlled by the acetyl-CoA/CoASH ratio via the regulation of 3-ketoacyl-CoA thiolase (EC 2.3.1.16).

A long-chain L-3-hydroxyacyl-CoA dehydrogenase (HDH) was solubilized from pig heart mitochondrial membranes with Triton X-100 and separated from the soluble general HDH (EC 1.1.1.35) by gel filtration in the presence of 0.1 M EDTA and 2.5 M KCl. Gradient gel electrophoresis of native proteins followed by activity staining with substrates showed that the detergent-solubilized long-chain HDH activity behaved like a high molecular protein similar to its behavior on a gel filtration column. In contrast to the almost complete immunoprecipitation of general HDH, the long-chain HDH activity could not be precipitated with antibodies raised against general HDH. Furthermore, the long-chain activity in mitochondrial extracts solubilized with 1% octyl glucoside could be separated from general HDH activity by antibody affinity chromatography. On the other hand, preliminary results using Western blotting suggested that the long-chain HDH activity could be recognized by anti-general HDH antibodies. The long-chain HDH activity has been partially purified by several steps which include the removal of soluble proteins by centrifugation, gel filtration and mono Q HPLC. The low efficiency of the long-chain HDH purification by conventional column chromatography may be due to the poor interaction of the long-chain enzyme with the chromatography media in the presence of detergents.

The mitochondrial β-oxidation of all *trans*-octa-2,4,6-trienoic acid was studied with the aim of elucidating the degradation of unsaturated fatty acids with conjugated double bonds. Octa-2,4,6-trienoic acid was found to be a respiratory substrate of coupled rat liver mitochondria, but not of rat heart mitochondria. Octa-2,4,6-trienoyl-CoA, the product of the inner-mitochondrial activation of the acid, was chemically synthesized and its degradation by purified enzymes of β-oxidation was studied

spectrophotometrically and by use of HPLC. This compound is a substrate of NADPH-dependent 2,4-dienoyl-CoA reductase or 4-enoyl-CoA reductase (EC 1.3.1.34), which facilitates its further β-oxidation. The product obtained after the NADPH-dependent reduction of octa-2,4,6-trienoyl-CoA and one round of β-oxidation was 4-hexenoyl-CoA, which can be completely degraded via β-oxidation. It is concluded that polyunsaturated fatty acids with two conjugated double bonds extending from even-numbered carbon atoms can be completely degraded via β-oxidation because their presumed 2,4,6-trienoyl-CoA intermediates are substrates of 2,4-dienoyl-CoA reductase.

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## ABBREVIATIONS

| | |
|---|---|
| ADP | adenosine 5'-diphosphate |
| ATP | adenosine 5'-triphosphate |
| BSA | bovine serum albumin |
| CAPS | (3-[Cyclohexylamino]-1-propanesulfonic acid) |
| CoA | coenzyme A |
| CoASH | coenzyme A |
| DEAE | ethylene diamine tetraacetic acid |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylene diamine tetraacetic acid |
| EGTA | ethylene glycol bis-(aminoethyl ether)N,N'-tetraacetic acid |
| HDH | 3-hydroxyacyl-CoA dehydrogenase |
| HPLC | high performance liquid chromatography |
| $NAD^+$ | nicotinamide adenine dinucleotide |
| NADH | reduced nicotinamide adenine dinucleotide |
| $NADP^+$ | nicotinamide adenine dinucleotide phosphate |
| NADPH | reduced nicotinamide adenine dinucleotide phosphate |
| SDS | sodium dodecyl sulfate |
| Tris | tris(hydroxymethyl)amino methane |
| UV | ultraviolet |

# INTRODUCTION

β-Oxidation of fatty acids is a major catabolic pathway in animals and in other living organisms. Some tissues, like heart and muscle, normally gain the bulk of their energy from the thermodynamically favored β-oxidation of fatty acids; in animal heart, in fact, fatty acids are the preferred fuel (1). A sufficient level of fatty acid oxidation is therefore essential for these tissues to provide a steady supply of ATP for muscle contraction. Tissues, like brain (2), that do not derive these energy from fatty acid oxidation still contain β-oxidation enzymes. Ketogenesis in animal liver also requires an active β-oxidation enzyme system to generate acetyl-CoA (3). In prokaryotes as in the gram-negative bacterium *E. coli.* fatty acids can serve as the sole carbon and energy source (4,5) so that all cellular constituents that contain carbon atoms are derived from the oxidation products of fatty acids. Recent research has identified several inherited human diseases due to deficiencies of β-oxidation enzymes (6). These inherited defects can result in a mild muscle weakness to death (7,8). The oxidation of fatty acids, therefore, is an important cellular process that is common to many living organisms and enormous efforts have been undertaken to investigate the details of the pathway, including the identification of metabolic intermediates, its enzymology and regulation.

Fatty acid β-oxidation has been studied since the beginning of this century by Knoop and Dakin (9, 10) who proposed the basic scheme for the degradation of the carbon skeleton of fatty acids. The enzymology of this pathway and other details were elucidated by various groups in the fifties (11). It was found that fatty acid oxidation occurs in the mitochondrial fraction

obtained by differential centrifugation of rat liver homogenates (12). The discovery of coenzyme A (13) and its function as a carrier of activated fatty acids (14) in the early 1950s led to the isolation and characterization of the majority of mitochondrial fatty acid β-oxidation enzymes (15). Finally, the discoveries in 1955 of the acetylation of carnitine (16) and of carnitine as a cofactor in fatty acid oxidation (17) have led to the elucidation of carnitine as a carrier of fatty acyl residues across the mitochondrial membrane. Fig. 1. shows the overall scheme for fatty acid activation, translocation and the four reactions involved in the β-oxidation cycle.

Several recent reviews (18,19,20) have summarized the current understanding of the molecular and enzymological details of fatty acid β-oxidation. The most important new findings during the past decade are: 1) the identification of an inducible fatty acid β-oxidation system in peroxisomes (21, 22); 2) the regulation of long chain fatty acid uptake by liver mitochondria via the control of carnitine palmitoyltransferase I by malonyl-CoA (23); 3) the degradation of polyunsaturated fatty acids via a pathway which is dependent on an NADPH-dependent 2,4-dienoyl-CoA reductase (24); 4) the existence of the enzymes of β-oxidation in *E. coli* as a multifunctional complex which can be purified in an intact form (25) and 5) the recognition of inborn defects of β-oxidation enzymes in mitochondria and peroxisomes as the cause of human diseases(26, 27). All these findings have raised further questions and have prompted a number of laboratories to seek answers to these questions.

An important aspect of the study of fatty acid oxidation is the elucidation of its regulation. The regulatory mechanisms of glycolysis, gluconegenesis and

many other important metabolic pathways are reasonably well understood. In contrast, evidence for the regulation of β-oxidation by hormones or its second messengers, by covalent modification or allosteric control is very limited, and therefore a major effort of recent research of fatty acid oxidation has been focused on elucidating factors that control the rate of β-oxidation.

The rate of fatty acid oxidation is dependent upon the fatty acid concentration in plasma and, in many tissues, on the energy demand of the tissue. The plasma fatty acid concentration is related to the nutritional and hormonal state of the tissue. When an animal is in the fasting state, the plasma fatty acids are derived from triacylglycerols in adipose tissue. Hydrolysis of triacylglycerols (lipolysis) in adipose cells is catalyzed by hormone-sensitive lipase. The lipase activity, in turn, is covalently regulated by phosphorylation and dephosphorylation (28). It has been demonstrated that, in the fasting state, an increased level of lipolytic hormones such as glucagon can activate receptor-coupled adenylate cyclase to produce the second messenger cAMP from ATP (29). The cAMP-activated protein kinase then phosphorylates the lipase thereby activating it. On the other hand, insulin can inhibit this process and thereby inactivates the lipase (30). Therefore, metabolic states associated with a rapid rate of fatty acid oxidation (starvation, diabetes) always correlate with low insulin/glugagon ratios.

The regulation of hepatic fatty acid oxidation is more complex and differs from the regulation of β-oxidation in cadiac and skeletal muscle. In liver, lipid metabolism, carbohydrate metabolism, and ketogenesis are closely related. In the transition from the fed to the fasted state, the liver switches from carbohydrate utilization and fatty acid synthesis to fatty acid oxidation

and ketone body production (31). Clearly, a reciprocal relationship exists between fatty acid synthesis and fatty acid degradation (32). Evidence for this proposal was supplied recently by McGarry and Foster (23). The regulation of carnitine palmitoyltransferase (CPT I) by malonyl-CoA determines the rates of β-oxidation and ketogenesis. Fatty acid oxidation is shut down at high tissue levels of malonyl-CoA, which acts as a potent inhibitor of CPT I (33). Conversely, the suppression of malonyl-CoA synthesis and a simultaneous increase in hepatic carnitine by an unknown mechanism (34) leads to the activation of fatty acid oxidation and ketogenesis. Furthermore, malonyl-CoA is formed from acetyl-CoA carboxylase in the rate-limiting reaction of fatty acid synthesis (35). Acetyl-CoA carboxylase activity is controlled through phosphorylation and dephosphorylation in response to hormones like glucagon and epinephrine (36). When fatty acids are synthesized, acetyl-CoA carboxylase activity is high and malonyl-CoA levels are elevated. In contrast, when the glucagon concentration increases during fasting, acetyl-CoA carboxylase is inactivated and the malonyl-CoA concentration decreases (37). The coordination of fatty acid synthesis and degradation in liver, therefore, is achieved by hormonal regulation. This regulatory mechanism at a step prior to the mitochondrial uptake of fatty acids seems to be most appropriate for liver, where fatty acids can either be degraded in mitochondria or can be utilized for lipid synthesis at the endoplasmic reticulum. However, several groups have reported that starvation also causes an increase in total CPT I activity and a decrease in the sensitivity of the enzyme toward malonyl-CoA (see 38 and refs. therein). This observation may be explained by covalent modification of CPT I in the fasting state; a recent unconfirmed report has shown that glucagon could stimulate the phosphorylation of CPT I with resultant activation (39). The increased activity of CPT I and β-oxidation

during starvation when the glucagon/insulin ratio is high may due a consequence of a lower concentration of malonyl-CoA, a higher total CPT I activity and a lower sensitivity of CPT I to malonyl-CoA (20).

Since Neely and coworkers (40) have experimentally studied fatty acid utilization in isolated perfused rat hearts at varying substrate concentrations and energy consuming states, attention has been recently focused on the regulation of fatty acid oxidation in animal heart. In heart, where little or no fatty acid synthesis and ketogenesis takes place, fatty acids are mainly degraded to drive oxidative phosphorylation and most of the tissue's energy is produced by fatty acid oxidation (1). In heart, then, the rate of fatty acid oxidation should be tuned to the energy demand of the tissue. However, the rate of β-oxidation in rat heart cells does not seem to be affected by hormones (41), and despite an extensive search for allosteric modulators of β-oxidation enzymes, none have been found. Studies with perfused hearts at various imposed workloads have led Neely and coworkers (40) to conclude that the rate of fatty acid oxidation in rat heart is a function of both the plasma free fatty acid concentration and the energy demand of the tissue. At low extracellular concentrations of palmitate (<0.6 mM), fatty acid uptake and/or activation seems to limit β-oxidation, whereas at sufficiently high palmitate levels, β-oxidation seems to be restricted by the oxidation of acetyl-CoA. Increasing the ventricular pressure to simulate a higher workload resulted in higher $O_2$ consumption and $CO_2$ formation but lower levels of acetyl-CoA. Thus, the rate of fatty acid oxidation may be tuned to the energy demand of the tissue via changes of the intramitochondrial acetyl-CoA/CoASH ratio. It has been suggested that intramitochondrial changes of the acetyl-CoA/CoASH ratio are transmitted to the cytosol via parallel changes of the

acetylcarnitine/carnitine ratio, which in turn may modulate the cytosolic CoASH concentration and thereby the activation of fatty acids (42). With regard to the β-oxidation cycle itself, the possibility of product inhibition of β-oxidation enzymes by the various intermediates formed during this process has been investigated. Olowe and Schulz have proposed that changes in the mitochondrial acetyl-CoA/CoASH ratio may determine the activity of 3-ketoacyl-CoA thiolase (EC 2.3.1.16), which is strongly inhibited by acetyl-CoA ($K_i$ = 4 μM), especially at low concentrations of CoASH, and thereby determine the rate of β-oxidation (43). The same authors demonstrated by use of the thiolase inhibitor 4-bromocrotonic acid that the rate of β-oxidation is linearly dependent on the activity of 3-ketoacyl-CoA thiolase (44). The ratio of $NADH/NAD^+$ may also change in response to variations in energy demand and this change may affect β-oxidation via a modulation of L-3-hydroxyacyl-CoA dehydrogenase activity (EC 1.1.1.35). For a complete understanding of the regulation of cardiac β-oxidation, more effort must be invested to study how the concentrations of mitochondrial and cytosolic pools of substrates, coenzymes, and intermediates would be affected by changes in the tissue's energy demand and how these concentration changes would modulate enzyme activities in this pathway.

Recent results have indicated that the enzymes of β-oxidation in mitochondria may also exist as organized complexes (45) which may be associated with the inner mitochondrial membrane (46). If this is true, it may explain why intermediates of fatty acid oxidation ordinarily do not accumulate in mitochondria (47). Obviously, further investigations of how β-oxidation enzymes are organized in mitochondria are pivotal in the elucidation of the regulation of fatty acid β-oxidation.

A prerequisite to understand the regulation of a metabolic pathway is the characterization of the enzymes that function in that pathway. Unresolved questions about the number of enzymes functioning in mitochondrial β-oxidation have curtailed attempts to elucidate the regulation of fatty acid oxidation and impeded clarification of the biochemical basis of inherited diseases related to β-oxidation. Although the existence of more than one isozyme for each of the four reactions of the β-oxidation cycle has been reported (18), only the essential function of three acyl-CoA dehydrogenases in β-oxidation has been demonstrated by the observed impairment of fatty acid oxidation in patients who have a deficiency of any of these three enzymes (6). On the other hand, the function of other recently identified β-oxidation enzymes, such as the long-chain L-3-hydroxyacyl-CoA dehydrogenase (48) and the long-chain enoyl-CoA hydratase (49), in β-oxidation remains to be established. More information is especially needed about the long-chain L-3-hydroxyacyl-CoA dehydrogenase since a long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (50) and another inherited disease, namely 3-hydroxyoctanoic aciduria (51), appear to be due to deficiencies of 3-hydroxyacyl-CoA dehydrogenases.

L-3-Hydroxyacyl-CoA dehydrogenase [HDH, (EC 1.1.1.35)], the third enzyme of the fatty acid β-oxidation pathway, reversibly catalyzes the $NAD^+$-dependent dehydrogenation of 3-hydroxyacyl-CoA to 3-ketoacyl-CoA. HDH has been isolated from several tissues. Both, the rat liver (52) and pig heart (53) enzymes have been studied extensively. It has a molecular weight of approximately 67,000 and is composed of two identical subunits which contain 307 amino acids of known sequence (55). In eukaryotic cells, L-3-

hydroxyacyl-CoA dehydrogenase is encoded by a nuclear gene and is synthesized in the cytoplasm as a precursor that appears to contain an additional 30-35 amino acids (55). A study of its conformation at 2.8-A resolution has revealed that it is a bilobal protein with an $NAD^+$-binding site in its amino-terminal domain (56). Kinetic measurements in the forward direction with the pig heart enzyme revealed the $K_m$ values for all substrates to be below 10 μM and $V_{max}$ values to be highest with medium-chain substrates (57). 3-Hydroxyacyl-CoA dehydrogenase is also present in the peroxisomes of mammalian cells (58). The peroxisomal enzyme differs in many properties from the mitochondrial dehydrogenase. Both, however, show a broad acyl-chain length specificity. Peroxisomal HDH activity, in fact, exists as a component of a trifunctional polypeptide along with enoyl-CoA hydratase and 3-*cis*,2-*trans*-enoyl-CoA isomerase activities (59). Recently, a second mitochondrial 3-hydroxyacyl-CoA dehydrogenase activity was identified (60). This dehydrogenase activity is associated with the inner mitochondrial membrane and exhibits a high preference for long-chain substrates (48). Since the general HDH exhibits little activity with 3-hydroxyhexadecanoyl-CoA as a substrate (57), a long-chain 3-hydroxyacyl-CoA dehydrogenase may be required to act in the first cycles of β-oxidation. However, whether or not the long-chain specific activity is a novel enzyme requires further investigation. Although the apparent native molecular weight of the long chain enzyme determined by gel filtration is much higher (180,000) than the soluble HDH (70,000) (48), it cannot be concluded that this is a completely different enzyme, because protein aggregation (protein-protein interaction) can result in an increase of the apparent molecular weight. Also, Triton X-100, always present in the isolation buffer, forms micelles into which long-chain HDH may be incorporated. The enzyme within micelles may

exhibit different acyl-chain length specificity and an increased apparent molecular weight. In this regard, the most important finding was the observation made by Sumegi and Srere, that soluble HDH can bind to the mitochondrial inner membrane through an integral membrane protein (46), suggesting the possible association of this enzyme with the respiratory chain, where NADH is reoxidized to $NAD^+$. Although the membrane protein has been isolated and partially characterized (61), the unique property of interaction between the membrane protein with the general HDH and the substrate chain-length specificities of the enzyme when associated with the membrane protein have not yet been studied. If long-chain specific HDH were immunologically related to the very well characterized general HDH, it is possible that the long-chain form of the enzyme is a complex of general 3-hydroxyacyl-CoA dehydrogenase with the dehydrogenase-binding protein of the inner mitochondrial membrane (61). On the other hand, the long-chain specific HDH activity may be a novel enzyme that normally functions in fatty acid β-oxidation.

Another area of recent interest is the β-oxidation of unsaturated fatty acids. Most naturally occurring unsaturated fatty acids contain *cis* double bonds and these can extend from either odd-numbered or even-numbered carbon atoms of the fatty acid. Degradation of unsaturated fatty aid containing double bonds requires auxiliary enzyme in addition to the enzymes necessary for the β-oxidation of saturated fatty acids (62). When unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms are β-oxidized to the point where a 3-enoyl-CoA derivative is formed, an auxiliary enzyme, 3-*cis*,2-*trans*-enoyl-CoA isomerase (EC 5.3.3.8), converts this intermediate to a 2-*trans*-enoyl-CoA, which can further be degraded through the β-oxidation

cycle. This isomerase has been purified and characterized (63, 64, 65). In the case of double bonds extending from even-numbered carbons, degradation can proceed via β-oxidation until a 4-enoyl-CoA derivative is formed. This intermediate is a substrate of medium-chain acyl-CoA dehydrogenase [EC 1.3.99.3] that can introduce a 2-*trans* double bound to form a 2,4-dienoyl-CoA derivative. Originally, it was proposed that this intermediate is degraded by an epimerase dependent pathway (66) which is referred to as the original pathway (See Fig. 2). However, most of the hypothesized intermediates in this pathway have not been identified, and reactions involved in the the pathway have not been experimentally verified. Furthermore, recent results have shown that an epimerization activity is only present in peroxisomes (67) and the observed 'epimerase' activity is actually due to the combined action of a novel peroxisomal D-3-hydroxyacyl-CoA dehydratase, and enoyl-CoA hydratase (68,69). Rat liver peroxisomal D-specific dehydratase has been purified to near homogeneity and has been partially characterized (70).

Recently, a novel reductase that is capable of reducing 2,4-dienoyl-CoA intermediates to 3-*trans*-enoyl-CoA's was identified (71,72). Kunau and others (24) have provided convincing evidence for the essential function of this enzyme in the degradation of 2,4-dienoyl-CoA intermediates generated during the β-oxidation of polyunsaturated fatty acids. These finding are: a) NADPH is required for the degradation of polyunsaturated fatty acids (73); b) 2-*trans*, 4-*cis*-decadienoyl-CoA, an intermediate of linoleic acid degradation, cannot be directly chain-shortened by the reconstituted β-oxidation system or by rat heart mitochondria (74); and c) 3-hydroxyacyl-CoA epimerase activity is not present in mitochondria (67). As a result, a revised pathway (the reductase-dependent pathway) by which polyunsaturated fatty acids are β-

oxidized was proposed (24) (see Fig. 2). The key reaction of this revised pathway is the reduction of 2-*trans*,4-*cis*-dienoyl-CoA by 2,4-dienoyl-CoA reductase, which has been identified in all tissues and organelles capable of β-oxidation (24). Also, the identification of a human genetic disorder, 2,4-dienoyl-CoA reductase deficiency (75), and the isolation of an *E. coli* 2,4-dienoyl-CoA reductase mutant (76) have proven the essential function of 2,4-dienoyl-CoA reductase in the β-oxidation of polyunsaturated fatty acids.

2,4-Dienoyl-CoA reductases from bovine liver (77), rat liver (78), and *E. coli* (77, 79) have been purified to homogeneity and their properties have been studied extensively (78, 80, 81). Although they have similar substrate specificities, enzymes from different organisms differ in their molecular and catalytic properties. Eukaryotic reductases (80,82, 83) catalyze the reduction of 2,4-dienoyl-CoA thioesters to the 3-*trans*-enoyl-CoA derivatives, whereas the *E. coli* reductase yields 2-*trans*-enoyl-CoA derivatives (79, 80). The eukaryotic enzyme is a homotetramer with a native molecular weight of 124,000 (80), whereas the *E. coli* enzyme is a monomer with a molecular weight of 70,000 (79). NADPH is absolutely required for all three reductases and all act on 2-*trans*,4-*trans*-decadienoyl-CoA and 2-*trans*, 4-*cis*-decadienoyl-CoA, which are metabolites of unsaturated fatty acids with *trans* and *cis* double bonds, respectively. $K_m$ values for substrates are 3-10 μM and for NADPH it is close to 0.1 mM (for detail see 24). Recently, it has been reported that 2,4-dienoyl-CoA reductase is also present in rat liver peroxisomes (82) and that the peroxisomal 2,4-dienoyl-CoA reductase is involved in the β-oxidation of unsaturated fatty acids in that organelle (84).

An important aspect of unsaturated fatty acid oxidation is the elucidation of the metabolism of polyunsaturated fatty acids with conjugated double bonds. Fatty acids with conjugated double bonds constitute an interesting class of natural substances (85). They occur mainly as components of the glycerides of plant seed oils, but some are found in other parts of higher plants, not necessarily as glycerides (85). Also, fatty acids with conjugated double bonds can be formed during the autoxidation of lipids (86) and during the partial hydrogenation of vegetable oils (87). Those fatty acids having conjugated double bonds at even-numbered carbon atoms might yield 2,4,6-trienoyl-CoA thioesters as a result of chain shortening and the introduction of a double bond at the 2-position. The question would be whether a compound like octa-2,4,6-trienoic acid or its CoA derivative can be further degraded by β-oxidation, and if so, by which sequence of reactions?

Questions about the regulation of fatty acid oxidation in heart mitochondria, the existence of a long-chain HDH and the metabolism of polyunsaturated fatty acids prompted further investigation. This thesis presents the results of these investigations. The determination of intramitochondrial ratios of acetyl-CoA/CoASH by high performance liquid chromatography have demonstrated that the ratio of acetyl-CoA/CoASH is high (close to 10) at state 4 and low (about 2.5) at state 3 respiration. The addition of 10 mM L-carnitine dramatically decreased this ratio to less than 0.2, and stimulated fatty acid oxidation especially at state 4 respiration. These results strongly support the hypothesis that in heart mitochondria the rate of β-oxidation is controlled by the acetyl-CoA/CoASH ratio via the regulation of 3-ketoacyl-CoA thiolase. Also, gel filtration, enzyme activity staining and immunological analysis all suggest that the long-chain HDH activity is a

novel enzyme. The purification of this membrane-bound enzyme is still in progress. Finally, the degradation pathway of octa-2,4,6-trienoic acid, an analogue of the metabolic intermediates of polyunsaturated fatty acids with conjugated double bounds, was demonstrated (88).

## EXPERIMENTAL PROCEDURES

### Materials

Acetyl-CoA, succinyl-CoA, $NAD^+$, NADH, NADPH, $NADP^+$, octyl-Sepharose CL-4B, Sephacryl S-200, Mono Q column, blue Sepharose, and agarose-hexane-adenosine 3',5'-bisphosphate were purchased from Pharmacia. DuPont was the source of [1-$^{14}$C]palmitoyl-L-carnitine (45 mCi/mmol). ScintiVerse II was obtained from Fisher. Palmitoyl-L-carnitine was a gift from Dr. R. Bressler, University of Arizona Medical School. Sigma was the source of CoASH, ADP, n-octyl-β-D-glucoside, nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), 5-bromo-4-chloro-3-indolyl phosphate, *Staphylococcus aureus* cells (Cowan Strain), glycine, CAPS, Triton X-100, hexokinase, L-carnitine, pig heart L-3-hydroxyacyl-CoA dehydrogenase, citrate synthase, lactate dehydrogenase, acyl-CoA oxidase, catalase, DL-3-hydroxybutyric acid, and all standard biochemicals. Alkaline phosphatase, nitrocellulose membranes and prestained standard proteins were bought from Bio-Rad. Octa-all-*trans*-2,4,6-trienoic acid, 2-octynoic acid, deca-2-*trans*,4-*trans*-dienal, diketene, ethyl chloroformate, 2,4-dinitrophenol and triethylamine were bought from Aldrich Chemical Co. AcA 34 Ultrogel was purchased from IBF biotechnics. The fatty acid oxidation complex from *Escherichia coli* was purified as previously described (89). Rabbit anti-3-hydroxyacyl-CoA dehydrogenase and anti-crotonase antisera were prepared by Pocono Rabbit Farm & Laboratory, Canadensis, PA. Crotonase (90), L-3-hydroxyacyl-CoA dehydrogenase (53) and 3-ketoacyl-CoA (91) thiolase were purified by Mr. Chin-Hung Chu, City College of New York, according to published procedures.

Synthesis of Substrates

Synthesis of fatty acids:

DL-3-hydroxyoctanoic acid was synthesized from methyl bromoacetate and hexanal by a modified Reformatzky procedure (92). DL-3-Hydroxyoctanoic acid was resolved into its L-isomer and D-isomer according to a procedure used by Stoffel *et al.* (93). Commercially available all-*trans*-2,4,6-octatrienoic acid was purified immediately before use by extraction with hot hexane followed by crystallization at -20 $^0$C. After recrystallization from toluene/methanol, the compound was obtained in the form of white needles with a melting point of 190-193 $^0$C (literature m.p.189 $^0$C) (94). Deca-2-*trans*,4-*trans*-dienoic acid was prepared from deca-2-*trans*,4-*trans*-dienal by oxidation with $Ag_2O$ (95), and dec-3-*cis*-enoic acid was synthesized from oct-3-yn-l-ol as described (96). 2-Hexadecynoic acid was synthesized according to a procedure used for the synthesis of 2-pentadecynoic acid and purified by recrystallization (97). Structures of the acids were confirmed by $C^{13}$ and $H^1$ NMR (data not shown).

Synthesis of CoA derivatives and determination of their concentrations:

Acetoacetyl-CoA was prepared as described by White and Jencks (98). The CoA derivatives of DL-3-hydroxybutyric acid, DL-3-hydroxyoctanoic acid, deca-2,4-dienoic acid, octa-2,4,6-trienoic acid, dec-3-*cis*-enoic acid, 2-hexadecynoic acid, and 2-octynoic acid were synthesized from the free acids and CoASH by the mixed anhydride method of Goldman and Vagelos (99). The concentrations of CoA thioesters, except for 3-ketoacyl-CoA derivatives, were determined by the method of Ellman (100) after cleaving the thioester bond with 1 M hydroxylamine at pH 7. The concentration of 3-ketoacyl-CoA

derivatives were measured by following the oxidation of NADH at 340 nm in the presence of 3-hydroxyacyl-CoA dehydrogenase at pH 7. The UV spectrum of octa-2,4,6-trienoyl-CoA after purification by HPLC exhibited an absorbance maximum at 337 nm with an absorption coefficient of 49300 $M^{-1} \cdot CM^{-1}$. The absorption coefficient at 340 nm was 48780 $M^{-1} \cdot CM^{-1}$(see Fig. 3).

Preparation of 3-ketooctanoyl-CoA and 3-ketohexadecanoyl-CoA:

2-Octynoyl-CoA (2 mM in 20 mM HEPES buffer pH 7.0) was directly converted to 3-ketooctanoyl-CoA by purified crotonase (2 U/ml). However, the 2-hexadecynoyl-CoA had to be purified by hydrophobic column chromatography before it could be converted to 3-ketoacyl-CoA derivative by crotonase. The cloudy colloidal CoA derivative suspension was suspended in 1 M LiCl and applied to an octyl-Sepharose column equilibrated with 0.5 M LiCl. The column was eluted with a decreasing salt gradient (LiCl-water). CoASH and dimer CoA were eluted with 0.5 M LiCl, whereas the long-chain CoA derivatives were eluted at very low ionic strength (see Fig. 4). The purified 2-hexadecynoyl-CoA was concentrated and adjusted to an appropriate concentration (1 mM ) with 20 mM HEPES buffer (pH 7.0). The crotonase (40 U/ml) was then added to convert 2-hexadeceynoyl-CoA to 3-ketohexadecanoyl-CoA. The reaction mixture was incubated for 1 hour at room temperature (25 $^0$C) and reaction was terminated by adjusting the pH to 2-3. The HPLC elution profiles were used to monitor product formation (see Fig. 5).

Isolation of Mitochondria and Respiration Measurements

Rat and pig heart mitochondria were isolated as described by Chappell and Hansford (101). Rat liver mitochondria were prepared by a similar procedure, except that 0.25 M sucrose was used instead of 0.21 M-mannitol plus 0.07 M sucrose, and Nagarse treatment was omitted. Protein concentrations of mitochondrial suspensions were determined by the biuret method of Gornall *et al.* (102). For respiration measurements, rat liver mitochondria (2 mg of protein) were suspended in 1.9 ml of a basal iso-osmotic medium containing 0.1 M KCl, 20 mM-Tris/HCl (pH 7.4), 4 mM potassium phosphate, 4 mM-$MgCl_2$ and 0.1 mM-EGTA; rat heart mitochondria (1 mg of protein) were suspended in 1.9 ml of a basal iso-osmotic medium containing 0.11 M KCl, 3.3 mM Tris-HCl (pH 7.4), 2 mM $KP_i$, 2 mM $MgCl_2$, and 0.1 mM EGTA. To these suspensions were added, in the indicated sequence, bovine serum albumin (0.1 mg/ml), 0.5 mM L-malate, 20 μM palmitoyl-L-carnitine or another substrate and 1 min later 0.25 mM ADP. State 3 respiration was maintained by the addition of 10 mM glucose and hexokinase (7 U/ml) to the incubation mixture. Respiration rates were measured polarographically at 25°C with a Clark oxygen electrode attached to a Gilson oxygraph.

Preparation of Soluble Extracts from Rat Liver and Heart Mitochondria

Rat liver mitochondria (50 mg) suspended in 7 ml of 25 mM-potassium phosphate (pH 7.2) containing 0.25 mM-dithiothreitol and 0.5 mM-EDTA were sonicated for 6 x 6s with a Branson sonifier (Model W-185) equipped with a micro-tip, and centrifuged at 105000 g for 1 h. For the purpose of partially purifying 2,4-dienoyl-CoA reductase, mitochondria were first precipitated by centrifugation at 5900g for 8 min and resuspended in 50 mM-potassium phosphate (pH 7.2) containing 5 mM-dithiothreitol and 1 mM-

EDTA. The resulting mitochondrial suspension was sonicated as described above and centrifuged at 105000 g for 1 h. For preparing an extract of rat heart mitochondria, a mitochondrial suspension was sonicated for 7 x 7s and centrifuged at 105,000xg for 1h. Protein concentrations of the mitochondrial extracts were determined either by the biuret procedure (102) or by the method of Lowry *et al.* (103).

Enzyme Assays

All assays were performed on a Gilford recording spectrophotometer (Model 250) at 25 $^0$C. One unit of activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product per min.

L-3-Hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase assays:

The assay of L-3-hydroxyacyl-CoA dehydrogenase is based on the oxidation of NADH which was followed spectrophotometrically at 340 nm and 25°C. The assay mixture contained 0.1 M $KP_i$ (pH 8), 60 μM acetoacetyl-CoA or 30 μM 3-ketooctanoyl-CoA or 20 μM 3-ketohexadecanoyl-CoA, 0.15 mM NADH and pig heart 3-hydroxyacyl-CoA dehydrogenase to give an absorbance change between 0.02 and 0.07 A/min. For measuring L-3-hydroxyacyl-CoA dehydrogenase in the forward direction, a standard mixture contained 0.2 M KPi (pH 8), fatty acid-free bovine serum albumin (0.2 mg/ml), 0.5 mM $NAD^+$, 0.25 mM CoASH, 30 μM 3-hydroxyacyl-CoA, pig heart 3-ketoacyl-CoA thiolase (80 mU) and pig heart 3-hydroxyacyl-CoA dehydrogenase to give an absorbance change of 0.03 A/min at 340nm. The 3-ketoacyl-CoA thiolase activity was measured with acetoacetyl-CoA as substrate (104). Protein concentrations were determined by the method of Lowry *et al,* (103).

2,4-Dienoyl-CoA reductase, 3-*cis*,2-*trans*-enoyl-CoA isomerase and thioesterase assays:

2,4-Dienoyl-CoA reductase was assayed as described in principle by Kunau & Dommes (72). The spectrophotometric assay is based on measuring the decrease of NADPH absorbance at 340 nm. A standard assay mixture (1 ml) contained 0.2 M-potassium phosphate (pH 8), 0.1 mM-NADPH, 30 μM-octa-2,4,6-trienoyl-CoA or 30 μM-deca-2,4-dienoyl-CoA and an amount of partially purified rat liver 2,4-dienoyl-CoA reductase to give an $A_{340}$ change of approx. 0.08/min. The thioesterase assay was based on measuring the $A_{340}$ decrease owing to the hydrolysis of octa-2,4,6-trienoyl-CoA at pH 8. A standard assay mixture (1 ml) contained 0.2 M-potassium phosphate (pH 8), 30 μM-octa-2,4,6-tri-enoyl-CoA and enzyme to give an $A_{340}$ change of approx. 0.06/min. The 3-*cis*,2-*trans*-enoyl-CoA isomerase (EC 5.3.3.8) was assayed spectrophotometrically at 340 nm with dec-3-*cis*-enoyl-CoA as a substrate at pH 8 and 25 $^0$C (89). The activity of the *E. coli* fatty acid β-oxidation complex is defined by the activity of its component enzyme 3-*cis*, 2-*trans*-enoyl-CoA isomerase.

Enzymatic Determination of the Product Formed from Octa-2,4,6-trienoyl-CoA by 2,4-Dienoyl-CoA Reductase

After completion of the NADPH-dependent reduction of octa-2,4,6-trienoyl-CoA catalyzed by 2,4-dienoyl-CoA reductase, the assay mixture was passed through an Amicon membrane (PM 5) to remove proteins. $NAD^+$ and CoASH (final concns. 1.2 mM and 0.25 mM respectively) were added to the resultant filtrate, followed by *E. coli* fatty acid oxidation complex (12 munits/ml). Difference spectra of this mixture versus a control, containing

all components except the enzyme, were recorded between 280 and 400 nm. When the absorbance ceased to change, L-malate and malate dehydrogenase were added to final concentrations of 3.2 mM and 50 m units/ml respectively. The conversion of acetyl-CoA into citrate was initiated by the addition of citrate synthase (2 munit/ml) after the malate dehydrogenase reaction had reached an equilibrium. When the $A_{340}$ did not increase any further, pyruvate (6 mM) and lactate dehydrogenase (6 munits/ml) were added to demonstrate that the absorbance peak centered at 340 nm was due to NADH.

The product formed from octa-2,4,6-trienoyl-CoA after one cycle of β-oxidation was generated by incubating 30 μM of the substrate in 0.2-potassium phosphate (pH 8) with 120 mM-NADPH, 0.5 mM-$NAD^+$ and 0.1 mM-CoASH in the presence of 2,4-dienoyl-CoA reductase (0.78 munit/ml) and *E. coli* fatty acid oxidation complex (12 munits/ml). After reaching equilibrium, the enzymes were removed by ultrafiltration (Amicon membrane YM 5). To the filtrate were added catalase (2 μg/ml) and acyl-CoA oxidase (0.1 unit/ml) and the $A_{300}$ was recorded. When it reached a maximum, 60 μM NADPH and 2,4-dienoyl-CoA reductase (0.78 muit/ml) were added and the $A_{300}$ was recorded.

Partial Purification of 2,4-Dienoyl-CoA Reductase

The soluble rat liver mitochondrial extract (30 ml, containing 180 mg of protein) obtained from mitochondria (420 mg of protein) was applied to an agarose-hexane-adenosine 3',5'-bisphosphate column (1 cm x 5 cm) equilibrated with 50 mM-potassium phosphate (pH 7.2) containing 5 mM-dithiothreitol and 1 mM-EDTA. The column was washed with 10 column volumes of the same buffer and was developed with a linear $NADP^+$ gradient made up of 200 ml each of the starting buffer with and without 1.2 mM

$NADP^+$. Fractions were assayed for 2,4-dienoyl-CoA reductase with 2-*trans*, 4-*trans*-decadienoyl-CoA as a substrate, and for thioesterase with octa-2,4,6-trienoyl-CoA as substrate. The elution profile of rat liver mitochondrial 2,4-dienoyl-CoA reductase is shown in Fig 6. Fractions 38 to 47 containing high reductase but negligible thioesterase activities were pooled and concentrated in an Amicon concentrator (PM 10 membrane). The purification procedure was carried out three times with virtually the same result. All operation, except for enzyme assays, were performed at 4 $^0$C.

Partial Purification of Long-chain L-3-Hydroxyacyl-CoA Dehydrogenase

Frozen and thawed pig heart mitochondria were centrifuged (100,000xg for 30 min) and the pellet was solubilized with 1% of Triton X-100 containing 0.1 mM phenylmethanesulfonyl fluoride and 1 mM pepstatin A. The soluble fraction of this homogenate obtained by centrifugation at 100,000xg for 30 min was applied to an FPLC Sephacryl S-200 column (2 x 50 cm) and developed with 50 mM KPi, pH 7.0, containing 0.1 M EDTA, 20% glycerol and 0.01% of Triton X-100. The fractions corresponding to the long-chain 3-hydroxyacyl-CoA dehydrogenase activity peak were combined and dialyzed against Mono Q column buffer (20 mM KPi, pH 8.0, 0.05% Triton X-100 and 20 mM mercaptoethanol). The resultant enzyme source was centrifuged at 100,000xg for 30 min and the soluble fraction was concentrated (5 mg/ml). An 0.2 ml aliquot of this fraction was applied to an HPLC Mono Q column (0.5 x 5 cm). The long-chain 3-hydroxyacyl-CoA dehydrogenase activity was eluted with KCl gradients (1.5 ml/min) from 0-0.4 M for 5 min, 0.4-0.6 M for 10 min and 0.6-1.0 M for 5 min. Fractions (1 ml) were collected and assayed with

acetoacetyl-CoA and 3-ketohexadecanoyl-CoA as substrates. The purification profile is shown in Fig. 19.

Gel Filtration, Native Gradient Gel Electrophoresis and HDH Activity Staining with Substrates

Gel filtration:

Pig heart mitochondria were dissolved with varying amounts of Triton X-100. The whole homogenate was chromatographed in 0.05 M potassium phosphate buffer (pH 7.0) containing 20% (w/v) glycerol, 0.01% (w/v) Triton X-100, 2.5 M KCl, 0.1 M EDTA on a 1.6x70 cm column of Ultrogel AcA 34. Fractions were collected and assayed with 3-ketohexadecanoyl-CoA, 3-ketooctanoyl-CoA and acetoacetyl-CoA.

Native gradient gel electrophoresis and HDH activity staining:

Linear gradient (4-20%) polyacrymide gels were cast in cathode buffer (0.1 M CAPS-$NH_3$ , pH 10.2, 0.1% of Triton X-100) by a simple gradient mixing apparatus. Electrophoresis was carried out on Hoefer Scientific apparatus in 100 mM CAPS-$NH_3$ buffer, pH 10.2. In the cathode buffer, 0.1% of Triton X-100 was included. High salt samples were dialyzed before applying them to the gel and samples were applied about 1.5 cm from the cathode. All samples were diluted 2 x with sample buffer (100 mM CAPS-$NH_3$, pH 10.2, 0.2% of Triton X-100, 20% glycerol and 20 mM mercaptoethanol). The gel was prerun at 100 V for 30 min at 10 $^0$C, and separation was achieved at 200 V overnight (12-15 hour) at 10 $^0$C. The gel was subsequently stained with staining mixture which was composed of 2.5 volumes of $NAD^+$ (2 mg/ml), 5 volumes of nitroblue tetrozolium (NBT, 2 mM), 1.5 volumes of phenazine methosulfate

(PMS 2 mM) and 250 μM substrates (final concentration) in 0.1 M glycine-NaOH buffer (pH 10.2) for 1 hour at room temperature. The control gel staining mixture contains all components except the substrate.

Immunotitration, Immunoaffinity Chromatography and Western Blotting Analysis

Anti-general HDH antibodies and anti-crotonase antibodies were partially purified (105). The partially purified antibodies were used for immunotitration, immunoaffinity chromatography and Western blotting analysis.

Immunotitration:

The pig heart mitochondrial homogenates (in 50 mM $KP_i$, pH 7.0, 1 M KCl, 10% of glycerol, 50 mM EDTA and 0.5% of Triton X-100) were incubated with varying amounts of antibodies for 15 min at 25 $^0$C, after which time 120 μl of assay buffer containing 0.1 M HEPES (pH 7.0), 25 mM $MgCl_2$, bovine serum albumin (0.2 mg/ml), 20 mM mercaptoethanol, and 5% (v/v) glycerol was added. This mixture was incubated together with 45 μl of a 10% cell suspension of *S. aureus* (Cowan strain) for 2 min at 25 $^0$C and then 0.83 ml of $H_2O$ and 0.12 ml of the assay buffer were added and well mixed. The resulting mixture was centrifuged on a Beckman Microcentrifuge B for 3 min, and 0.6 ml of the supernatant was assayed for 3-hydroxyacyl-CoA dehydrogenase activities with acetoacetyl-CoA, 3-ketooctanoyl-CoA and 3-ketohexadecanoyl-CoA, respectively.

Immunoaffinity chromatography:

Immunoaffinity support was synthesized by Mr. Luo according to a procedure of Kerner and Bieber (45). Frozen and thawed pig heart mitochondria (20 mg/ml) in the isolation buffer were dissolved with 1% (w/v) of octyl glucoside. After standing for 30 min on ice with occasional mixing, the 100,000 g soluble fraction was prepared. An aliquot (3 ml) of the supernatant was loaded onto the immunoaffinity column at room temperature. The column had been previously equilibrated with 150 mM NaCl and 25 mM potassium phosphate, pH 7.25. Unbound proteins were removed by extensive washing with mitochondrial isolation buffer until proteins elution from the column ceased. Bound HDH and other proteins were eluted with ice-cold 20% glycerol and 50 mM $NH_3$, pH 10.6. Fractions were assayed for HDH activity with acetoacetyl-CoA ($C_4$), 3-ketooctanoyl-CoA ($C_8$) and 3-ketohexadecanoyl-CoA ($C_{16}$) and protein concentrations were determined by the procedure of Bradford (106).

Western blotting:

Protein samples were diluted with 2 x SDS sample buffer and denatured by heating for 5 min in a boiling water bath. The samples were then subjected to electrophoresis on 8% SDS polyacrylamide gels. Proteins were electrophoretically transferred to a nitrocellulose membrane and probed with partially purified antibody to general HDH. The primary antibody was detected by allowing it to react with goat anti-rabbit IgG alkaline phosphatase conjugate which was visualized by incubating the nitrocellulose membrane in the presence of alkaline phosphatase (107).

Oxidation of [1-$^{14}$C]Palmitoylcarnitine by Rat Heat Mitochondria

The reaction mixture contained in a final volume of 0.7 ml, 0.11 M KCl, 3.3 mM Tris-HCl (pH 7.4), 2 mM $KP_i$, 2 mM $MgCl_2$, 0.1 mM EGTA, fatty acid-free bovine serum albumin (0.1 mg/ml), 0.5 mM L-malate, 20 μM [1-$^{14}$C]palmitoyl-L-carnitine (50,000 cpm), 60 μM ATP, 10 mM glucose, hexokinase (5 U) and rat heart mitochondria (120 μg of protein). Glucose and hexokinase were added to the incubation medium to maintain mitochondria at state 3 respiration. State 4 respiration persisted in the absence of glucose and hexokinase. When the effect of L-carnitine, oxaloacetate or acetoacetate on the rate of palmitoylcarnitine β-oxidation was studied, appropriate amounts of these compounds were added to the incubation medium before the reaction was started. Fatty acid oxidation was initiated by the addition of rat heart mitochondria to the incubation medium in a 25 ml Erlenmeyer flask covered with a rubber septum to which a plastic center well was attached. After incubating the mixture under shaking for the indicated periods of time at 25°C, 0.3 ml of 1 M hyamine hydroxide was injected into the center well followed by the injection of 0.1 ml of 70% perchloric acid into the incubation medium to stop the reaction. The flask was shaken overnight in a water bath kept at 37°C to allow the complete absorption of $^{14}CO_2$ by hyamine hydroxide. The center well was then transferred to a scintillation vial, mixed with 4 ml of ScintiVerse II and counted in a liquid scintillation counter. For quantitating acid-soluble β-oxidation products, the acidified incubation mixture was extracted three times with water-saturated butanol. An aliquot (0.2 ml) of the remaining aqueous phase was combined with 4 ml of ScintiVerse II and counted in a liquid scintillation counter.

Determination of Mitochondrial Concentrations of CoASH and Acetyl-CoA

Reaction mixtures of 30 ml with or without 10 mM L-carnitine contained exactly the same components and at the same concentrations as were used to measure mitochondrial respiration. The only change was that 60 μM ATP was added instead of ADP to maintain state 4 respiration, whereas state 3 respiration was sustained by the additional addition of 10 mM glucose and hexokinase (7 U/ml). Reactions were started by the addition of rat heart mitochondria (15 mg) and incubations were continued for 3 min at 25°C under thorough stirring. Reactions were terminated by adjusting the pH to 2.5 followed by the addition of 0.6 ml of 5% Tween-20 to disrupt mitochondria. After concentrating the resultant solution by filtration through an Amicon YC05 membrane, aliquots of the concentrate corresponding to 2.5 mg of mitochondrial protein were analyzed by HPLC. Peaks corresponding to CoASH and acetyl-CoA were identified by the addition of authentic materials and quantitated by use of standard curves obtained by determining peak areas for various concentrations of CoASH and acetyl-CoA determined under identical conditions. Intramitochondrial concentrations of CoASH and acetyl-CoA were estimated by assuming that the density of the mitochondrial matrix is 1 μl/mg in mitochondrial protein (108).

Product Analysis by HPLC

A Waters HPLC system was used, consisting of two model 6000A pumps, a model 660 solvent programmer, a model U6K sample injector and a model 441 absorbance detector attached to a Houston Omniscribe chart recorder. A

Waters μBondapak $C_{18}$ column (30 cm x 3.9 mm) was placed on-line and equilibrated with 50 mM ammonium phosphate (pH 5.5). The following program was used for the identification and quantitation of acetyl-CoA and CoASH: After injection of the sample the column was developed at a flow rate of 1 ml/min for 20 min with a linear (0-8%) methanol gradient, then kept for 2 min at 8% methanol, followed by a second linear (8-20%) methanol gradient for 15 min after which time the elution was continued for 10 min with 20% methanol. The aqueous phase contained 50 mM ammonium phosphate (pH 5.5). The column was finally washed with 70% methanol containing 50 mM ammonium phosphate (pH 5.5). Acetyl-CoA and CoASH were eluted with the second methanol gradient and were detected spectrophotometrically at 254 nm with a full-scale recorder setting of 0.05 A unit. For identification of metabolites of octa-2,4,6-trienoyl-CoA, samples were injected and eluted at a flow rate of 1 ml/min with a methanol gradients (detailed in Fig. 23) containing 50 mM ammonium phosphate (pH 5.5). The eluted substrates, products and coenzymes were detected at 254 nm with a full-scale recorder setting of 0.2 A unit. All solvents and samples were clarified by filtration through 0.45 μm-pore-size HA Millipore filters.

# Regulation of Fatty Acid β-Oxidation In Rat Heart Mitochondria

## RESULTS

Preliminary results have been obtained showing that partially purified 3-ketoacyl-CoA thiolase is strongly inhibited in a non-linear fashion by acetyl-CoA at low CoASH concentrations, while L-3-hydroxyacyl-CoA dehydrogenase was inhibited by NADH with an $K_i$ of 25 μM (109). These observation suggested that the β-oxidation products acetyl-CoA and/or NADH may play a regulatory role in β-oxidation by modulating the activities of 3-ketoacyl-CoA thiolase and L-3-hydroxyacyl-CoA dehydrogenase, respectively. The possible control of fatty acid oxidation in heart by the intramitochondrial ratio of acetyl-CoA/CoASH or/and by the ratio of NADH/$NAD^+$ was therefore investigated.

### Mitochondrial Fatty Acid Oxidation at State 3 and State 4 Respiration

An *in vitro* assay system was established to study the regulation of mitochondrial fatty acid oxidation in response to the energy demand of the

tissue. Rat heart mitochondria were isolated and incubated under condition that sustain either state 3 or state 4 respiration to mimic situations of high and low energy demands, respectively. ATP was added to the incubation medium to maintain state 4 respiration, while state 3 respiration persisted when additionally glucose and hexokinase were added to continuously convert ATP to ADP (108). However, optimal rates of state 3 respiration were only obtained when the ATP concentration was 1 mM or slightly lower. At higher ATP concentrations rates of respiration were not only suboptimal but declined further during the course of the measurements (see Fig. 7). The reason for lower than expected rates of respiration at high concentrations of ATP may be the built-up of a sufficiently high concentration of glucose 6-phosphate which might inhibit hexokinase and thereby the efficient conversion of ATP to ADP.

Also, a procedure for direct measurements of fatty acid β-oxidation in rat hart mitochondria was established with [1-$^{14}$C]palmitoyl-L-carnitine as a substrate. When 120 μg of mitochondria were incubated in the standard assay mixture (for details, see Experimental Procedures) with [1-$^{14}$C]palmitoyl-L-carnitine, the acid-soluble products accumulated linearly during the first 15 min at both state 3 and state 4 respiration (see Fig. 8). Therefore, subsequent β-oxidation measurements were run for 15 min.

Effects of L-Carnitine and Oxaloacetate on Palmitoylcarnitine-supported Respiration in Coupled Rat Heart Mitochondria

β-Oxidation is frequently assayed in an indirect manner by measuring rates of mitochondrial respiration with palmitoylcarnitine as a substrate. In coupled mitochondria in the presence of malate, ADP, and phosphate,

respiration will change from state 3 to state 4 when all ADP is converted to ATP. However, state 3 respiration can be maintained by the addition of glucose and hexokinase to facilitate the conversion of ATP to ADP (see Fig. 9A). When the respiration state changes from 3 to 4, the matrix concentrations of acetyl-CoA and/or NADH will increase, whereas those of CoASH and $NAD^+$ will decrease (108). Since L-carnitine is assumed to lower the ratio of acetyl-CoA/CoASH in the matrix and since either oxaloacetate or acetoacetate should decrease the NADH/$NAD^+$ ratio, the effect of these compounds on the rate of palmitoylcarnitine-supported respiration was investigated. As shown in Fig. 9B-D, neither L-carnitine, nor oxaloacetate, nor the addition of both compounds together caused stimulation of state 4 respiration. Acetoacetate did not have any effect either (data not shown). Since the addition of glucose plus hexokinase fully restored state 3 respiration (see Fig. 9B-D), neither L-carnitine nor oxaloacetate had a deleterious effect on palmitoylcarnitine-supported respiration.

Effects of L-Carnitine, Oxaloacetate, and Acetoacetate on the β-Oxidation of Palmitoylcarnitine in Coupled Rat Heart Mitochondria

A more direct method for assaying β-oxidation than measurement of respiration is the determination of acid-soluble products and/or $CO_2$ formed from [1-$^{14}$C]palmitoylcarnitine. To maintain state 4 respiration, ATP was added instead of ADP. If state 3 respiration was to be sustained, glucose and hexokinase were also added. When rat heart mitochondria were incubated under these conditions for 15 min, mostly acid-soluble products were formed which remained in aqueous solution after the extraction of [1-$^{14}$C]palmitoyl-carnitine with n-butanol. Little $^{14}CO_2$ was formed from [1-$^{14}$C]palmitoyl-

carnitine under these conditions. Shown in Table I are the results obtained when the effects of L-carnitine, oxaloacetate, or acetoacetate on the rate of [1-$^{14}$C]palmitoylcarnitine β-oxidation in coupled rat heart mitochondria was investigated. Since carnitine can be translocated into mitochondria in exchange for acetylcarnitine by acetylcarnitine:carnitine translocase, the externally added carnitine can be converted to acetylcarnitine by intramitochondrial acetyl-CoA:carnitine transferase and regenerate free CoASH from acetyl-CoA thereby decreasing the intramitochondrial ratio of acetyl-CoA/CoASH. Also, either oxaloacetate or acetoacetate can enter the mitochondrial matrix and oxidize NADH catalyzed by either malate dehydrogenase or 3-hydroxybutyrate dehydrogenase thereby reducing the intramitochondrial NADH/NAD$^+$ ratio. At state 3 respiration, the addition of 10 mM L-carnitine, which is assumed to decrease the intramitochondrial ratio of acetyl-CoA/CoASH, stimulated β-oxidation 34%, whereas at state 4 respiration the stimulation was 334%. In contrast, oxaloacetate or acetoacetate, which are expected to lower the intramitochondrial ratio of NAD/NAD$^+$, did not cause a significant stimulation of β-oxidation at either state 3 or state 4 respiration. In fact, at state 3 respiration 10 mM oxaloacetate inhibited β-oxidation by 50% possibly due to the oxidation of pyruvate formed from oxaloacetate by decarboxylation. The inhibition of β-oxidation by acetoacetate may be caused by its own oxidation and resultant repression of β-oxidation. The formation of acid-soluble products via the β-oxidation of [1-$^{14}$C]palmitoylcarnitine was determined as a function of time. As shown in Fig. 10, the rate of β-oxidation at state 3 respiration was linear during a 20-min incubation period and several fold higher than β-oxidation at state 4 respiration (see Fig. 10). Addition of 10 mM L-carnitine to the incubation mixture at state 4 respiration stimulated β-oxidation so that the rate was

virtually identical with the rate observed at state 3 respiration (see Fig. 10). However, at state 3 respiration, the addition of 10 mM L-carnitine had only a slight stimulatory effect on the formation of acid-soluble products. The rates of $CO_2$ formation from palmitoylcarnitine in the absence or presence of 10 mM L-carnitine were very similar and much lower than the formation of acid-soluble products (data not shown). Since the amount of $^{14}CO_2$ released when β-oxidation was immediately terminated by acidification was higher than the amount of $CO_2$ due to β-oxidation, $CO_2$ formation was not considered when rates of β-oxidation were determined.

Determination of Intramitochondrial Concentrations of Acetyl-CoA and CoASH

To test our hypothesis that the intramitochondrial ratio of acetyl-CoA/CoASH may determine the activity of 3-ketoacyl-CoA thiolase and thereby regulate the rate of β-oxidation, we determined the intramitochondrial concentrations of acetyl-CoA and CoASH during palmitoylcarnitine β-oxidation at respiration states 3 and 4 in the absence or presence of 10 mM L-carnitine. For these determinations, β-oxidation was terminated after 3 min by acidification and mitochondria were dispersed by the addition of Tween-20. After concentrating the resultant solutions by ultrafiltration, acetyl-CoA and CoASH were separated by reverse phase HPLC. The peaks corresponding to acetyl-CoA and CoASH were identified by spiking samples with authentic materials and the amounts of the two compounds were estimated from peak areas using standard curves. Representative chromatograms are shown in Fig. 11 and estimated intramitochondrial concentrations of acetyl-CoA and CoASH as well as acetyl-CoA/CoASH ratios

are presented in Table II. Even though the estimated concentrations of acetyl-CoA and CoASH may be too low due to losses that might have occurred during the work-up of samples, the acetyl-CoA/CoASH ratios are believed to be close to the ratios that prevailed in whole mitochondria. Most noteworthy are the following observations: (1) CoASH was present even at state 4 respiration; (2) the acetyl-CoA/CoASH ratio at state 4 respiration was almost four times higher than at state 3; (3) the addition of 10 mM L-carnitine to β-oxidizing mitochondria caused the acetyl-CoA/CoASH ratios to decline 15-fold at state 3 respiration and more than 50-fold at state 4 respiration.

## DISCUSSION

In contrast to the regulation of hepatic fatty acid oxidation, which has recently received much attention (1), control of β-oxidation in heart and muscle remains little explored and poorly understood. A study by Neely and coworkers in the seventies with perfused working hearts demonstrated that at sufficiently high levels of free fatty acids (0.6-1.2 mM) in the perfusate, increased ventricular pressure resulted in an increased oxidation of fatty acids to $CO_2$ and a concomitant decrease of the cellular acetyl-CoA/CoASH ratio (40). In a subsequent study, Oram *et al.* (42) proposed that β-oxidation may be regulated at the fatty acid activation step in the cytosol where the availability of CoASH may limit acyl-CoA formation and where the concentration of CoASH may fluctuate in concert with mitochondrial changes of CoASH. This proposal is based on the assumption that 1) mitochondrial and cytosolic ratios of acetyl-CoA/CoASH are in equilibrium with the corresponding

acetylcarnitine/carnitine ratios due to the actions of carnitine acetyltransferases and that 2) the mitochondrial and cytosolic concentrations of acetylcarnitine and carnitine are equal or similar because of their ability to freely pass through the innermitochondrial membrane.

Since Olowe and Schulz observed a severe inhibition of purified pig heart 3-ketoacyl-CoA thiolase by acetyl-CoA at low concentrations of CoASH (43), they suggested that β-oxidation may be turned to the energy demand of the tissue via the regulation of 3-ketoacyl-CoA thiolase activity by the intramitochondrial acetyl-CoA/CoASH ratio. In this study an attempt was made to test this hypothesis and to evaluate the possible control of β-oxidation via the regulation of L-3-hydroxyacyl-CoA dehydrogenase by the $NADH/NAD^+$ ratio.

The severe inhibition of 3-ketoacyl-CoA thiolase by acetyl-CoA at low concentrations of CoASH, originally observed with the purified pig heart enzyme (43), was confirmed with partially purified rat heart 3-ketoacyl-CoA thiolase (110). Since plots of 1/v *vs.* the acetyl-CoA concentration were non-linear, a $K_i$ value for acetyl-CoA could not be determined. However 3-ketoacyl-CoA thiolase, in contrast to acetoacetyl-CoA thiolase, was completely inhibited by 1 mM acetyl-CoA at 20 μM CoASH (110). As expected NADH inhibited L-3-hydroxyacyl-CoA dehydrogenase, most pronouncedly at low concentrations of $NAD^+$. Hence, the control of β-oxidation via the regulation of either thiolase or the dehydrogenase as outlined in Fig. 12 seemed to be a possibility and needed to be investigated.

For an evaluation of these control mechanisms with isolated mitochondria it was necessary to modulate the intramitochondrial ratios of acetyl-CoA/CoASH and $NADH/NAD^+$. The acetyl-CoA/CoASH ratio in heart mitochondria can be lowered by the addition of L-carnitine, which passes

through the inner mitochondrial membrane into the matrix space where it accepts acetyl groups from acetyl-CoA in a reaction catalyzed by carnitine acetyltransferase. The intramitochondrial NADH/$NAD^+$ ratio can be decreased by the addition of either oxaloacetate or acetoacetate which enter the mitochondrial matrix and oxidize NADH in a reaction catalyzed by either malate dehydrogenase or 3-hydroxybutyrate dehydrogenase. Rates of β-oxidation were measured with coupled rat heart mitochondria at state 3 respiration and state 4 respiration which may resemble conditions in the heart when the energy demand is extremely high or very low, respectively. When rates of palmitoylcarnitine-supported respiration were measured, neither the addition of L-carnitine nor the addition of oxaloacetate or acetoacetate to the incubation mixture had any effect on the rate of oxygen consumption. This observation is not surprising because rates of respiration in coupled mitochondria are controlled by rates of oxidative phosphorylation which were not expected to be influenced by the above additions. A more direct measure of β-oxidation is the formation of acid-soluble products and/or $CO_2$ from palmitoylcarnitine. Since malate is present in the incubation mixture, most radioactivity released during the β-oxidation of [1-$^{14}$C]palmitoylcarnitine is initially associated with the acid-soluble fraction which includes intermediates of the tricarboxylic acid cycle in addition to acetyl-CoA. The formation of $^{14}CO_2$ is initially insignificant and in addition was difficult to measure accurately because acidification of [1-$^{14}$C]palmitoyl-carnitine resulted in the release of a large amount of $^{14}CO_2$. Since the addition of 10 mM L-carnitine strongly stimulated palmitoylcarnitine β-oxidation at state 4 respiration, but had only a slight effect on this process at state 3 respiration, and since the addition of either oxaloacetate or acetoacetate did not cause a significant stimulation, it is likely that β-oxidation at state 4

respiration is restricted by a high intramitochondrial ratio of acetyl-CoA/CoASH.

Measurements of the acetyl-CoA/CoASH ratios by HPLC confirmed a high value of 10 at state 4 respiration in contrast to a ratio of 2.5 at state 3 respiration. As expected, the addition of 10 mM L-carnitine resulted in a dramatic drop of this ratio to less than 0.2 at both respiration states. These data agree with the hypothesis that β-oxidation is turned to the rate of oxidative phosphorylation via the intramitochondrial acetyl-CoA/CoASH ratio. Given a situation in coupled heart mitochondria in which the rate of oxidative phosphorylation is low or where in heart the imposed workload is light, acetyl-CoA oxidation via the tricarboxylic acid cycle slows down leading to a build-up of acetyl-CoA and a concomitant decrease of CoASH in the mitochondrial matrix. This increase in the acetyl-CoA/CoASH ratio may cause inhibition of 3-ketoacyl-CoA thiolase and thereby a slowdown of β-oxidation (see Fig. 12). The concentrations of acetyl-CoA and CoASH given in Table II were used to estimate the effective activity of 3-ketoacyl-CoA thiolase in the mitochondrial matrix; at state 3 respiration it is 55% of the maximal activity determined with a soluble mitochondrial extract under optimal conditions in contrast to 15% at state 4 respiration (109). Since the maximal activity of 3-ketoacyl-CoA thiolase in whole mitochondria is not known, the specific activity of this enzyme at either state 3 or 4 respiration could not be calculated. However, the concentration differences observed for acetyl-CoA and CoASH between respiration states 3 and 4 and their estimated effects on the activity of 3-ketoacyl-CoA thiolase are consistent with, although do not prove, the suggested control of β-oxidation via the regulation of thiolase by the acetyl-CoA/CoASH ratio (see Fig. 12). In contrast, the possible control of β-oxidation via the regulation of L-3-hydroxyacyl-CoA

dehydrogenase by the $NADH/NAD^+$ ratio (see Fig. 6) does not seem to be an effective mechanism during the β-oxidation of palmitoylcarnitine, but may be important when other substrates support the intramitochondrial reduction of $NAD^+$.

Since intermediates of β-oxidation do not accumulate in mitochondria (47), acceptance of the proposed regulatory mechanism raises the question as to how the entry of fatty acids into the β-oxidation spiral is controlled. Further studies are therefore required to search for possible feedback connections between the last reaction of β-oxidation catalyzed by 3-ketoacyl-CoA thiolase and the initial step of the spiral or preceding events such as fatty acid uptake or activation. One possibility is the regulation of acyl-CoA dehydrogenases by 3-ketoacyl-CoA thioesters, which may accumulate when 3-ketoacyl-CoA thiolase is inhibited. This hypothesis is based on the strong inhibition of acyl-CoA dehydrogenases by 3-ketoacyl-CoA compounds which was previously observed and reported (110).

# Identification and Partial Purification of a Membrane-associated Long-chain L-3-Hydroxyacyl-CoA Dehydrogenase in Pig Heart Mitochondria

## RESULTS

### Separation of Mitochondrial 3-Hydroxyacyl-CoA Dehydrogenases by Gel Filtration and Gradient Gel Electrophoresis

Although preliminary evidence was published showing that a long-chain 3-hydroxyacyl-CoA dehydrogenase activity is associated with the inner mitochondrial membrane (41), a further study of this novel activity was initiated. Since Triton X-100 (0.2% w/v) can solubilize up to 90% of the mitochondrial long-chain activity (48), the mitochondrial homogenate was prepared for further enzyme separation. Approximately 300 mg of pig heart mitochondria were suspended in 15 ml of buffer containing 2.5 M KCl, 0.1 M EDTA, 50 mM KPi (pH 7.0), 20% of glycerol (w/v) and 0.2% (w/v) Triton X-100 and homogenized with a Polytron homogenizer (3 x 2 min) to solubilize the long-chain HDH. The whole homogenate was subjected to gel filtration on Ultrogel AcA 34 (exclusion limit 350,000). The resulting chromatogram

showed two peaks of activity measured with 3-ketohexadecanoyl-CoA ($C_{16}$) (see Fig. 13), which is in accord with previous observations (48). The recovery of HDH activity from the gel filtration column chromatography was 2.5% for acetoacetyl-CoA ($C_4$), 12% for 3-ketooctanoyl-CoA ($C_8$) and 17.5% for 3-ketohexadecanoyl-CoA ($C_{16}$) indicating that the long-chain activity is more stable than the short-chain activity. The loss of HDH activity may be due to denaturation or digestion by proteases since no protease inhibitor was present in the homogenization and isolation buffers. The higher stability of long-chain activity may be due to protection by detergent against protease attack or to a generally lower sensitivity of long-chain HDH to proteolytic degradation (48).

More detailed information about the physical nature of the mitochondrial HDHs was obtained by gradient gel electrophoresis followed by activity staining (see Fig. 14). The long-chain HDH activity from the gel filtration column gave rise to a single slower moving band (C, lane b), whereas the commercial general HDH showed a single faster moving band (C, lane c) with 3-hydroxyoctanoyl-CoA as substrate. When the gel was stained with 3-hydroxybutyryl-CoA as substrate, however, the band due to the long-chain specific HDH was very faint (B, b) in agreement with a low activity of long-chain HDH toward short chain substrates. An important finding was that 1% Triton X-100 can solubilize the long-chain activity as judged by activity staining with 3-hydroxyoctanoyl-CoA (14 C, lane a). However, no long-chain activity band was observed when a soluble mitochondrial extract was used. This finding agrees with the strong association of long-chain HDH with the mitochondrial membrane. Even sonication did not solubilize the long-chain HDH (data not shown). Thus by simple centrifugation most of the soluble mitochondrial protein can be removed from the long-chain HDH. This is not

only additional evidence for the existence of a separate long-chain HDH, but also provides a way to localize HDH on a native gradient gel if electrophoresis is used to purify the enzyme.

Relationship between General HDH and Long-chain HDH

Since gel filtration and electrophoresis resolved two HDH activities, the immediate question was the structural and functional relationships of these two HDHs. Long-chain HDH activity and general HDH share some properties as for example a specificity for the L-3-hydroxy isomer of the substrate and a requirement for $NAD^+$ as a cofactor.

The immunological relationship between general HDH and long-chain HDH was evaluated with antibodies raised against general HDH. A bacterial suspension of *S. aureus*, which carries protein A on its surface, was used to facilitate the precipitation of HDH-antibody complexes by centrifugation (111). After centrifugation, the HDH activity remaining in the supernatant was measured. A pig heart mitochondrial homogenate (10 mg/ml, 0.5% Triton X-100 w/v) was used as a source of general and long-chain HDH. The immunotitration curves (see Fig. 15) obtained showed that short chain ($C_4$) HDH activity present in the mitochondrial homogenate was removed by immunoprecipitation with antibodies to general HDH. Part of the medium-chain ($C_8$) activity (25%) was also precipitated whereas only 10% of the long-chain activity ($C_{16}$) was removed. This observation suggested that the long-chain HDH in its native state is immunologically unrelated to general HDH or that the native structure of long-chain HDH is not similar to that of general HDH. Alternatively, the long-chain HDH may be trapped in Triton X-

100 micelles, so that it is not accessible to antibodies. The HDH activity remaining in the solution may be due to long-chain HDH.

Another, more direct way of differentiating between the two types of HDH was the separation of long-chain HDH and general HDH by immunoaffinity chromatography. Freeze-thawed pig heart mitochondria (20 mg/ml) were dissolved by adding 1% (w/v) octyl glucoside and centrifuged. The resulting 100,000 g supernatant was applied to an anti-general HDH column and washed with mitochondria isolation buffer. After protein ceased be eluted from the column, a high pH-elution (ice-cold $NH_4OH$, pH 10.6) was initiated (see Fig. 16). The elution profile shows two peaks of HDH activity. The flow through fractions contain an HDH activity that has a marked preference toward the long-chain substrate ($C_{16}$), whereas the fraction eluted at high pH contained HDH activity with a preferance for the short-chain substrate (see, Fig. 16). This finding further strengthens the conclusion that there are two HDHs present in a pig heart mitochondrial homogenate; one most active with short-chain substrate ($C_4$) which is immunologically related to general HDH and another specific toward long-chain substrate ($C_{16}$) which is not recognized by anti-general HDH antibodies. These two enzymes can be effectively separated by immunoaffinity chromatography.

Partial Purification of Long-Chain HDH

Various lines of evidence suggested that long-chain HDH is associated with the inner mitochondrial membrane. The purification and partial characterization of the long-chain enzyme was initiated. To increase the flow rate in gel filtration, the column was run at room temperature (25 $^0$C). Surprisingly, the short-chain activity was almost completely lost after three

days of chromatography at room temperature, in contrast to the long-chain activity which did not decline significantly (see Fig. 17). General HDH is either more temperature-sensitive or less resistant to proteases. An interesting observation was that the total long-chain activity recovered from the column was 50% higher than the activity present after mitochondria were homogenized with 1% (w/v) Triton X-100 (see Fig. 18), suggesting that some of the short-chain HDH activity might be converted to long-chain HDH activity. The peak fractions of long-chain activity were combined, concentrated, and dialyzed against 20 mM Mops buffer pH 6.6 containing 1mM EDTA, 0.125 dithiothreitol and 0.2% of octyl glucoside. Although the major part of the long-chain activity was retained in the 100,000xg supernatant, the subsequent partial purification of the enzyme by classical ion exchange techniques ( *e.g* CM-cellulose) was unsuccessful since all proteins appeared in the flow through fraction (data not shown). This may be due to the difficulty of removing detergent from the protein by dialysis and preventing the enzymes embedded in detergent micelles from interacting with the ion exchange media. Among the various chromatographic techniques so far tried, only Sepharose Q (an anion exchanger) effectively adsorbed the long-chain enzyme dissolved in detergent-containing buffer.

Since the long-chain enzyme is associated with the mitochondrial inner membrane, the general HDH can be separated from the long-chain enzyme by centrifugation. For this purpose, the freeze-thawed pig heart mitochondria were centrifuged for 30 min at 100,000 g. The soluble fraction was discarded and the pellet was homogenized with 1% Triton X-100. The resulting suspension was centrifuged (100,000g x1 hour) and the soluble fraction was applied to an FPLC Sephacryl S-200 gel filtration column. Although the

resolution was not as good as by conventional gel filtration due to the high flow rate, the FPLC procedure resolved long-chain and a general HDH activities (data not shown). The fractions enriched in long-chain HDH activity were pooled, dialyzed, and concentrated. The resultant sample was applied to an HPLC Mono Q column (a strong anionic exchange column). Fig. 19 shows the elution profile from the Mono Q column. Although the procedure requires further improvement, the process can achieve up to 5-fold of purification (see Table III).

# DISCUSSION

Experimental results presented here provide further evidence that a novel long-chain specific L-3-hydroxyacyl-CoA dehydrogenase activity is associated with the inner mitochondrial membrane. The long-chain HDH was reported to be an enzyme of the inner mitochondrial membrane (48), but its absence from other subcellular components such as the outer mitochondrial membrane, endoplasmic reticulum and peroxisome has not been established. Although, native long-chain HDH and general HDH can be separated by immunoprecipitation and chromatography on an anti-general HDH affinity column, the cross-reactivity of the long-chain HDH with antibodies raised against the short-chain enzyme by Western blotting (see Fig. 20) indicated that at least part of the long-chain enzyme may be structurally related to the general HDH. The idiotypic conserved parts of the long-chain enzyme in the native state are not recognized by anti-general HDH antibodies, perhaps as a result of long-chain HDH being entrapped by detergent or due to both protein aggregation and detergent protection.

Another interesting observation made during the course of these studies was the presence of several HDH bands upon substrate staining after native gradient gel electrophoresis. Thus, HDH may associate with other protein(s) or is present in different states of aggregation. Although preliminary results indicate that the long-chain HDH is structurally related to general HDH in the denatured state, the relationship of long-chain HDH to general HDH is unclear because the commercially available general HDH is not homogenous as judged by SDS gel electrophoresis, and thus the antibodies raised against this preparation may not be mono-specific. In considering all evidence it seems most likely that long-chain specific HDH is a novel enzyme or that the

long-chain HDH activity is the result of short-chain HDH interacting with the HDH-binding protein on the inner mitochondrial membrane. To solve this problem, either the long-chain HDH must be purified or the complex of HDH binding protein and general HDH must be studied. The resultant complex should be assayed with substrates of various chain lengths to test whether the general HDH interacting with the HDH-binding protein results in a different chain length specificity with a preferance for long-chain substrates. If the activity of this complex does not show long-chain specific activity, long-chain HDH must be purified. Unfortunately, the preliminary results showed that various chromatographic procedures, including CM-cellulose, Sepharose Q, hydrophobic interaction, blue Sepharose and ADP-agarose, were not very effective for the purification of long-chain HDH due to the poor interaction of protein with the chromatographic materials in buffers containing detergent. Although the strong anionic exchange column (mono Q) can bind the long-chain enzyme dissolved in 0.1% Triton X-100 buffer, the majority of the proteins coeluted with long-chain HDH activity (see Fig. 19). When mitochondria were treated with10% of $N_aHCO_4$ pH 10.7 to dissolve peripheral mitochondrial membrane proteins (112) the long-chain HDH activity was solubilized. However, the long-chain HDH activity solubilized by this method was easily lost, so that this procedure cannot be used for the purification of long-chain HDH. Currently, various detergents with different properties are tested to find a suitable one that can solubilize the long-chain HDH activity, and in addition does not prevent the enzyme from interacting with support materials such as affinity and ion exchange materials.

# β-Oxidation of Polyunsaturated Fatty Acids with Conjugated Double Bonds

## Mitochondrial metabolism of octa-2,4,6-trienoic acid

## RESULTS

### Octa-2,4,6-trienoic Acid as a Respiratory Substrate of Mitochondria

Coupled rat liver mitochondria, which respire in the presence of exogenously added octanoic acid, ADP and phosphate, also utilize octa-2,4,6-trienoic acid as a respiratory substrate (see Figs. 21a and 21b). However, the rate of respiration observed with the all-trans isomer of octa-2,4,6-trienoic acid was only 30 ng-atoms of O/min per mg of protein. This value represents 40% of the rate of 72 ng-atoms/min per mg observed with octanoic acid as a substrate. The rate of respiration when determined as a function of the concentration of octa-2,4,6-trienoic acid approached a maximal value of 35 ng-atoms of O/min per mg of protein at a substrate concentration of 30 μM (results not shown). Neither octanoic acid nor octa-2,4,6-trienoic acid supports respiration in uncoupled rat liver mitochondria (see Figs. 21c and 21d), presumably owing to a lack of intramitochondrial ATP required for the conversion of these medium-chain fatty acids into their CoA thioesters, which are the substrate of β-oxidation and thus of respiration.

In contrast with its ready metabolism in rat liver mitochondria, octa-2,4,6-trienoic acid did not support respiration in coupled rat heart mitochondria, even though octanoic acid served as a respiratory substrate (see Figs. 21e and 21f). This inability of rat heart mitochondria to utilize octa-2,4,6-trienoic acid is most likely a consequence of the relatively narrow substrate specificity of the heart mitochondrial medium-chain acyl-CoA synthetase (113), which must activate the substrate before it can be oxidized.

Reduction of Octa-2,4,6-trienoyl-CoA by 2,4-Dienoyl-CoA Reductase and Its Subsequent β-Oxidation

When octa-2,4,6-trienoyl-CoA was incubated with NADPH and 2,4-dienoyl-CoA reductase, its characteristic spectrum with a maximum at 337 nm disappeared in a time-dependent manner (see Fig. 22a). The observed absorbance change was dependent on the presence of both NADPH and the reductase. The apparent kinetic constants ($V_{max}$ and $K_m$) of 2,4-dienoyl-CoA reductase were determined with both deca-2,4-dienoyl-CoA and octa-2,4,6-trienoyl-CoA as substrates at a fixed concentration of 60 μM-NADPH. The apparent $V_{max}$ of the reduction of octa-2,4,6-trienoyl-CoA (90 nmol/min per mg of protein) was less than one-tenth of the rate of 980 nmol/min per mg obtained with deca-2,4-dienoyl-CoA. The apparent $K_m$ value for octa-2,4,6-trienoyl-CoA was 50 μM, and thus 5 times the $K_m$ value of 10 μM determined for deca-2,4-dienoyl-CoA.

After establishing that octa-2,4,6-trienoyl-CoA can be reduced by NADPH-dependent 2,4-dienoyl-CoA reductase, the further β-oxidation of the reduction product was studied. For this purpose a reaction mixture obtained after the complete reduction of octa-2,4,6-trienoyl-CoA was freed of protein by

ultrafiltration and incubated with purified *E. coli* fatty acid-oxidation complex in the presence of $NAD^+$ and CoASH. Since the complex contains all β-oxidation enzymes except for acyl-CoA dehydrogenase and 2,4-dienoyl-CoA reductase, it should β-oxidize the product of the reduction as long as the latter compound contains an isolated double bond in the 2,3-position or 3,4-position. As documented in Fig. 22(b), an increase in the $A_{340}$ was observed, and was attributed to the formation of NADH formed during β-oxidation. Since completion of a cycle of β-oxidation would also yield acetyl-CoA, its presence was tested for and confirmed by a further increase in $A_{340}$ when L-malate, malate dehydrogenase and citrate synthase were added to the reaction mixture (see Fig. 22b). The molar proportions of octa-2,4,6-trienoyl-CoA reduced:NADH formed during β-oxidation:NADH formed during the conversion of acetyl-CoA and malate into citrate were close to 1:1:1. The absorbance increases centered around 340 nm were shown to be due to the formation of NADH, because the absorbance disappeared when pyruvate and lactate dehydrogenase were added to the reaction mixture (see Fig. 22b).

More direct evidence for the reduction of octa-2,4,6-trienoyl-CoA and its subsequent β-oxidation was obtained by HPLC analysis. Incubation of octa-2,4,6-trienoyl-CoA, NADPH and 2,4-dienoyl-CoA reductase resulted in the formation of $NADP^+$ and compound X, which was eluted from a reverse-phase HPLC column ahead of octa-2,4,6-trienoyl-CoA (see Fig. 23a). When the *E. coli* fatty acid oxidation complex, $NAD^+$ and CoASH were added to the above reaction mixture, β-oxidation occurred, as evidenced by the appearance of NADH and acetyl-CoA (see Fig. 23b). The peak for octa-2,4,6-trienoyl-CoA disappeared almost completely, whereas peak X increased in size (see Fig. 23b). It remains unclear, however, why the product of octa-2,4,6-trienoyl-CoA

reduction and the product of its reduction plus β-oxidation seem to emerge at the same position marked 'X'.

Structure of the Product Obtained After Reduction and β-Oxidation of Octa-2,4,6-trienoyl-CoA

Since the reduction of octa-2,4,6-trienoyl-CoA followed by one cycle of β-oxidation should result in the removal of two double bonds and one acetate unit, the product was expected to be hexenoyl-CoA, most likely with a double bond in the 4,5-position. If so, introduction of a 2,3-double bond should yield hexa-2,4-dienoyl-CoA (sorboyl-CoA), which is a substrate of 2,4-dienoyl-CoA reductase (114). This prediction was shown to be correct when the product obtained after reduction and β-oxidation of octa-2,4,6-trienoyl-CoA was converted by acyl-CoA oxidase and catalase into a compound with an absorbance at 300 nm characteristic of a 2,4-dienoyl-CoA chromophore (see Fig. 24). Since addition of NADPH and 2,4-dienoyl-CoA reductase to this presumed 2,4-dienoyl-CoA-containing reaction mixture led to the disappearance of the absorbance at 300 nm (see Fig. 24), the absorbing compound was most likely sorboyl-CoA. It follows that the product formed as a result of the reduction and β-oxidation of octa-2,4,6-trienoyl-CoA must have been hex-4-enoyl-CoA.

# DISCUSSION

The question addressed in this study was whether fatty acids with conjugated double bonds extending from even-numbered carbon atoms can be degraded via the reductase-dependent pathway of unsaturated fatty acid oxidation. Chain-shortening of such fatty acids should lead to the formation of 4,6-dienoyl-CoA intermediates, which upon dehydrogenation by acyl-CoA dehydrogenase would yield 2,4,6-trienoyl-CoAs. If 2,4-dienoyl-CoA reductase catalyses the NADPH-dependent reduction of 2,4,6-trienoyl-CoA thioesters, this conjugated system of four double bonds would be completely disrupted at a substantial cost in free energy. If the reductase does not act on 2,4,6-trienoyl-CoA thioesters, fatty acids with conjugated double bonds are either degraded by another pathway, or are not at all, or not completely, degraded, with possible deleterious consequences to the animal.

The initial observation that coupled rat liver mitochondria respire with octa-2,4,6-trienoic acid as a substrate proved this compound to be metabolized in liver mitochondria. Presented in Fig. 25 is the sequence of steps by which octa-2,4,6-trienoyl-CoA is most likely degraded. The key question is the reduction of octa-2,4,6-trienoyl-CoA (I) by NADPH, catalyzed by 2,4-dienoyl-CoA reductase. If the mechanism of this reductive step is identical with the reduction of 2,4-dienoyl-CoA compounds, octa-3,6-dienoyl-CoA (II) would be formed. The latter compound is then converted into octa-2,6-dienoyl-CoA (III) by 3-*cis*-2-*trans*-enoyl-CoA isomerase, which also acts on compounds with 3-*trans* double bonds, although at a slower rate (96). The subsequent β-oxidation involving the hydration of octa-2,6-dienoyl-CoA (III), followed by the dehydrogenation of L-3-hydroxyoct-6-enoyl-CoA (IV) and the thiolytic cleavage of 3-oxo-oct-6-enoyl-CoA (V), can occur, as evidenced by the

formation of NADH and acetyl-CoA, which were detected spectrophotometrically and by HPLC. If this sequence of reactions did occur, then hex-6-enoyl-CoA (VI) should have been formed. The following evidence supports the predicted formation of hex-6-enoyl-CoA. The reaction of presumed hex-6-enoyl-CoA with oxygen in the presence of acyl-CoA oxidase and catalase gave rise to a compound with an absorbance maximum at 300 nm, as expected if hexa-2,4-dienoyl-CoA (sorboyl-CoA, VII) were formed. Since this chromophore disappeared upon addition of NADPH and 2,4-dienoyl-CoA reductase, the compound with the chromophore at 300 nm was sorboyl-CoA and its precursor must have been hex-6-enoyl-CoA.

The degradation of octa-2,4,6-trienoic acid in mitochondria require its initial conversion into octa-2,4,6-trienoyl-CoA, most likely catalyzed by medium-chain acyl-CoA synthetase. The subsequent sequence of reactions is outlined in Fig. 25. The key reaction is the NADPH-dependent reduction of octa-2,4,6-trienoyl-CoA by 2,4-dienoyl-CoA reductase. The subsequent reactions are catalyzed by the mitochondrial β-oxidation enzymes, all of which are associated with different proteins. The conversion of hex-4-enoyl-CoA (VI) into hexa-2,4-dienoyl-CoA (VII) is presumed to be catalyzed by medium-chain acyl-CoA dehydrogenase, which is the only one of the mitochondrial acyl-CoA dehydrogenases capable of introducing a double bond between the thioester group and an existing double bond in the 4,5-position (115). The resultant hexa-2,4-dienoyl-CoA (sorboyl-CoA) is reduced by NADPH-dependent 2,4-dienoyl-CoA reductase to yield hex-3-enoyl-CoA (VIII). The complete conversion of hex-3-enoyl-CoA (VIII) into 3 mole of acetyl-CoA requires a shift of the 3,4-double bond to the 2,3-position catalyzed by enoyl-CoA isomerase, followed by two cycles of β-oxidation.

Table I

Effects of L-Carnitine, Oxaloacetate (OAA), and Acetoacetate (AcAc) on the Rates of Palmitoylcarnitine β-Oxidation in Coupled Rat Heart Mitochondria.

| Addition | Relative rates of β-oxidation* State 3 | State 4 |
|---|---|---|
| None | 1 | 1 |
| 2 mM carnitine | 1.24 ±0.41 | 2.88 ±0.85 |
| 10 mM carnitine | 1.34 ±0.24 | 4.34 ±0.56 |
| 1 mM OAA | 0.8 ±0.22 | 1.01 ±0.56 |
| 10 mM OAA | 0.5 ±0.19 | 1.18 ±0.08 |
| 1 mM AcAc | 0.96 ±0.3 | 0.85 ±0.22 |
| 5 mM AcAc | 0.83 ±0.11 | 0.71 ±0.5 |
| 10 mM carnitine + 10 mM OAA | 1.56 ±0.5 | 3.58 ±0.67 |

*Rates of palmitoylcarnitine β-oxidation in the absence of any additions were 0.82 nmoles of acid-soluble compounds formed per min and mg of mitochondrial protein at state 3 respiration and 0.29 nmol at state 4 respiration. Values are means ± S.D. from at least three sets of experiments.

Table II

Concentrations of Acetyl-CoA (AcCoA) and CoASH in Rat Heart Mitochondria

| Respiration state | Addition | AcCoA* | CoASH* | AcCoA / CoASH |
|---|---|---|---|---|
| | | mM | mM | |
| 3 | None | 0.12 ±0.02 | 0.049 ±0.004 | 2.5 |
| | 10 mM carnitine | 0.04 ±0.006 | 0.24 ±0.01 | 0.17 |
| 4 | None | 0.23 ±0.05 | 0.024 ±0.003 | 9.6 |
| | 10 mM carnitine | 0.029 ±0.006 | 0.16 ±0.006 | 0.18 |

*Values are means ± SD from three different sets of experiments

Table III

Purification of Pig heart Long-chain Specific L-3-Hydroxyacyl-CoA Dehydrogenase. Dehydrogenase activities were assayed with 3-ketohexadecanoyl-CoA as substrate

| Step | Total activity | Total protein | Specific activity | Purification | Recovery |
|---|---|---|---|---|---|
| | units | mg | units/mg | -fold | % |
| Mitochondria | 40.5 | 130.7 | 0.31 | 1 | 100 |
| Mitochondria pellet | 28.6 | 66.5 | 0.43 | 1.39 | 70.6 |
| FPLC gel filtration | 7.2 | 31.1 | 0.23 | 0.74 | 17.6 |
| Dialysis | 5.6 | 20.8 | 0.27 | 0.87 | 10.2 |
| HPLC Mono Q | 3.1 | 2.3 | 1.34 | 4.32 | 7.7 |

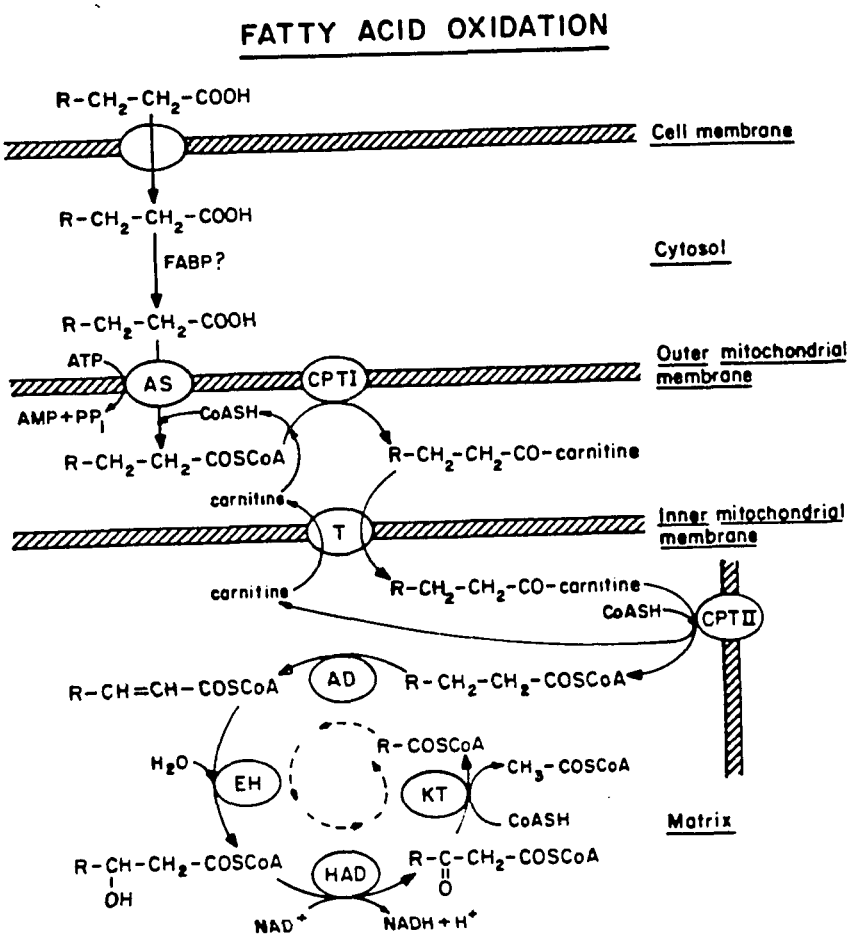


**Fig. 1. Pathway of mitochondrial fatty acid oxidation.** Enzymes of the pathway are: AS, acyl-CoA synthetase; CPT I, carnitine palmitoyltransferase I; T, carnitine:acylcarnitine translocase; CPT II, canitine palmitoyl-transferase II; AD, acyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; HAD, L-3-hydroxyacyl-CoA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

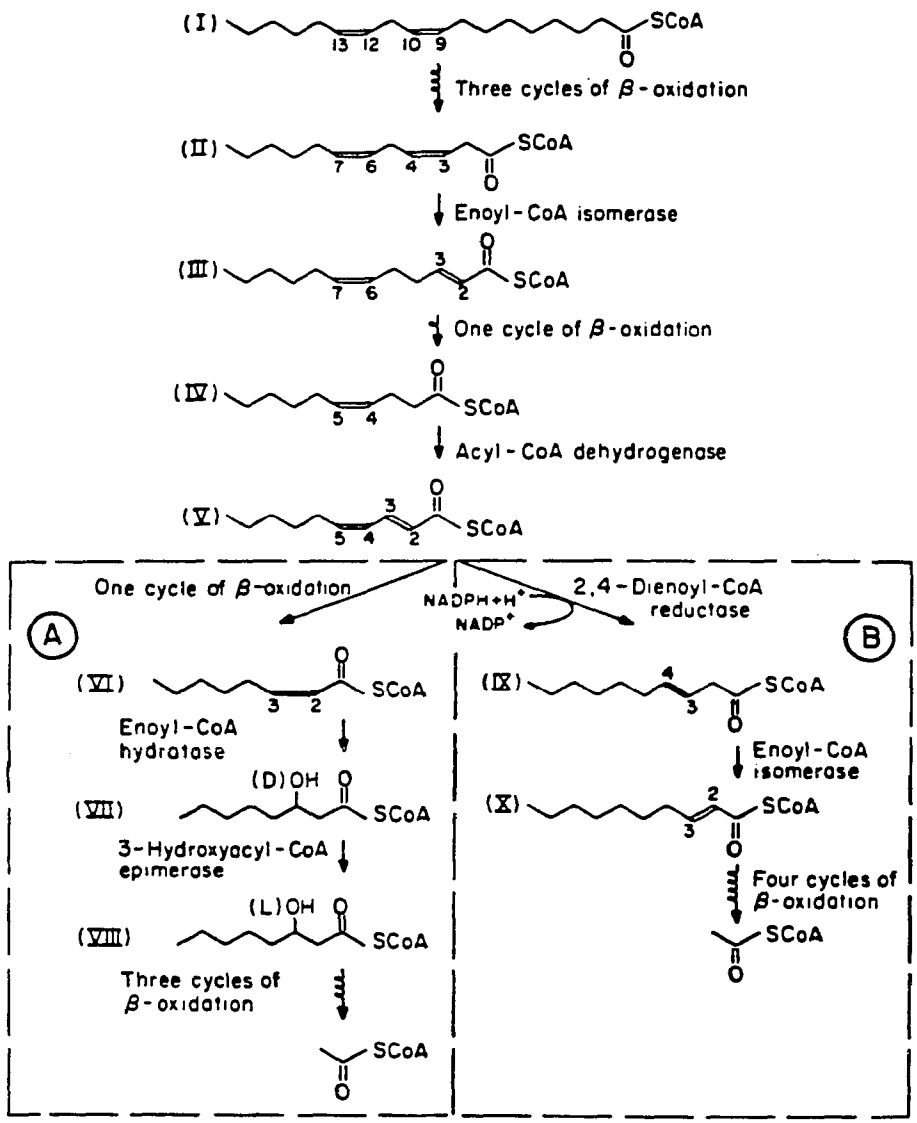


**Fig. 2. β-Oxidation of linoleoyl-CoA.** (a) Original pathway dependent on 3-hydroxyacyl-CoA epimerase. (b) Revised pathway dependent on 2,4-dienoyl-CoA reductase.

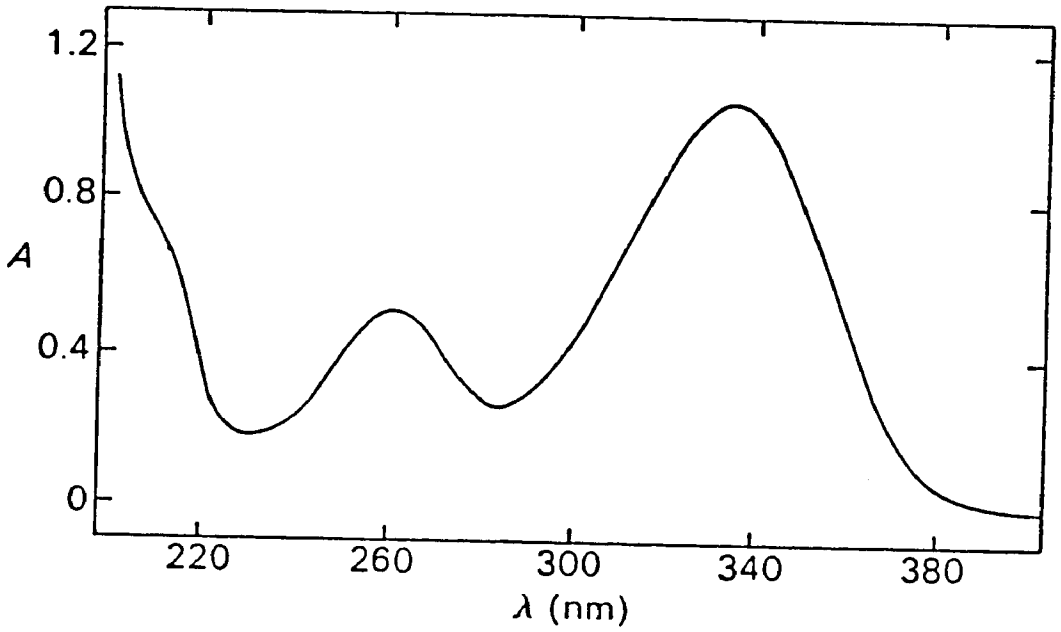


**Fig. 3. UV spectrum of octa-2,4,6-trienoyl-CoA**

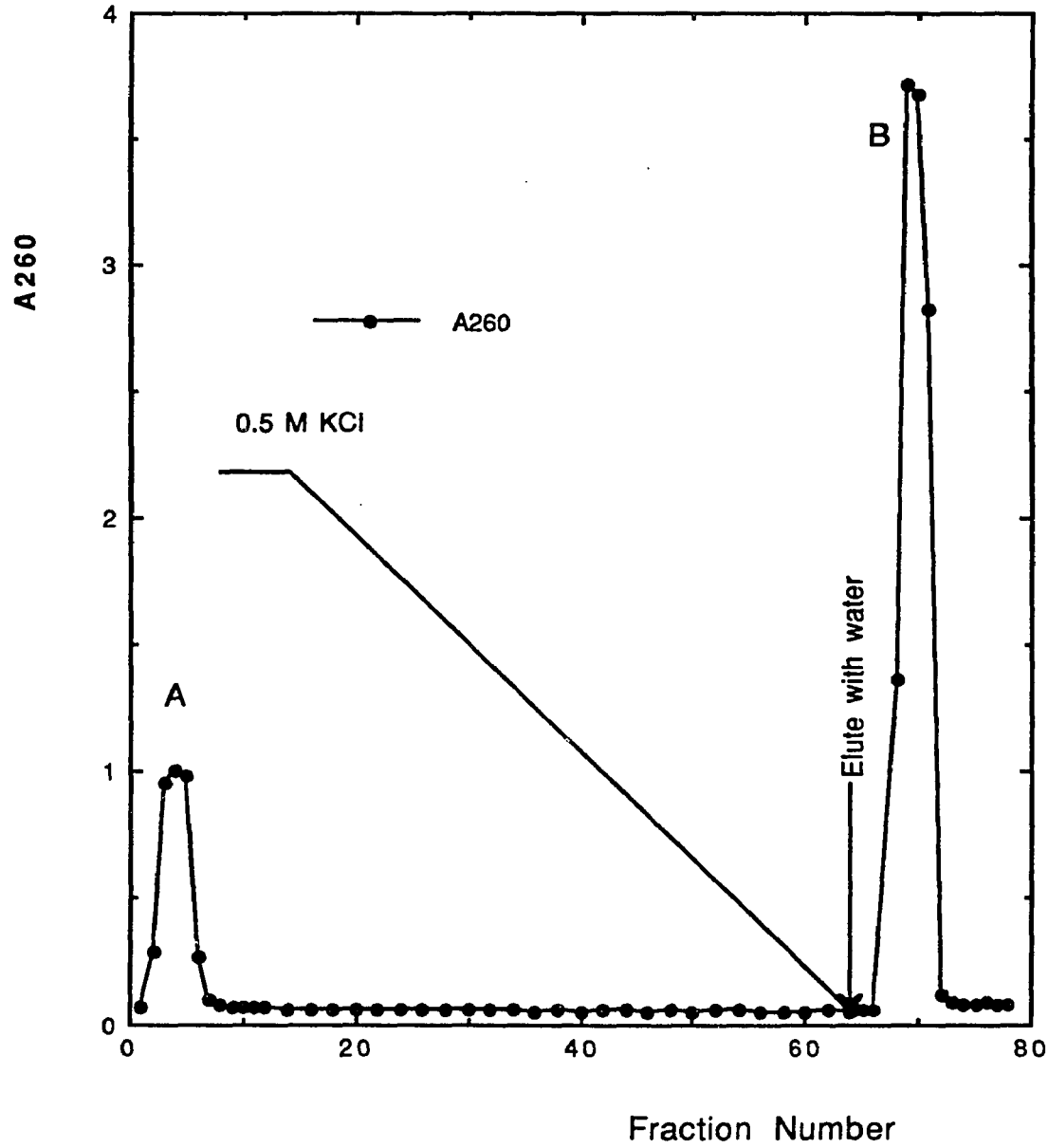


**Fig. 4. Purification of 2-hexadecynoyl-CoA by octyl-Sepharose column chromatography.** A, CoASH, and dimer CoA; B, 2-hexadecynoyl-CoA and other long-chain CoA derivatives.

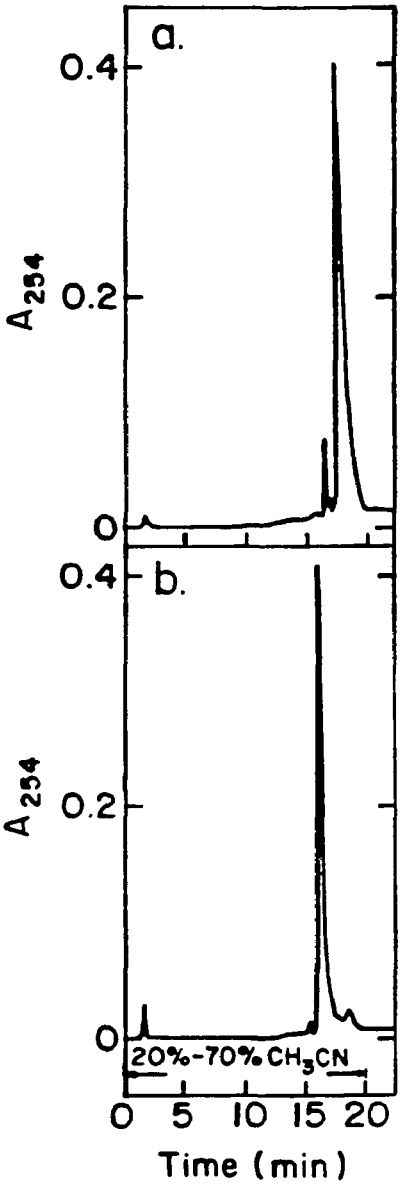


**Fig. 5. Conversion of 2-hexadecynoyl-CoA to 3-keto-hexadecanoyl-CoA detected by HPLC.** A. Elution profile of hexadecynoyl-CoA; B. Elution profile of 3-ketohexadecanoyl-CoA.

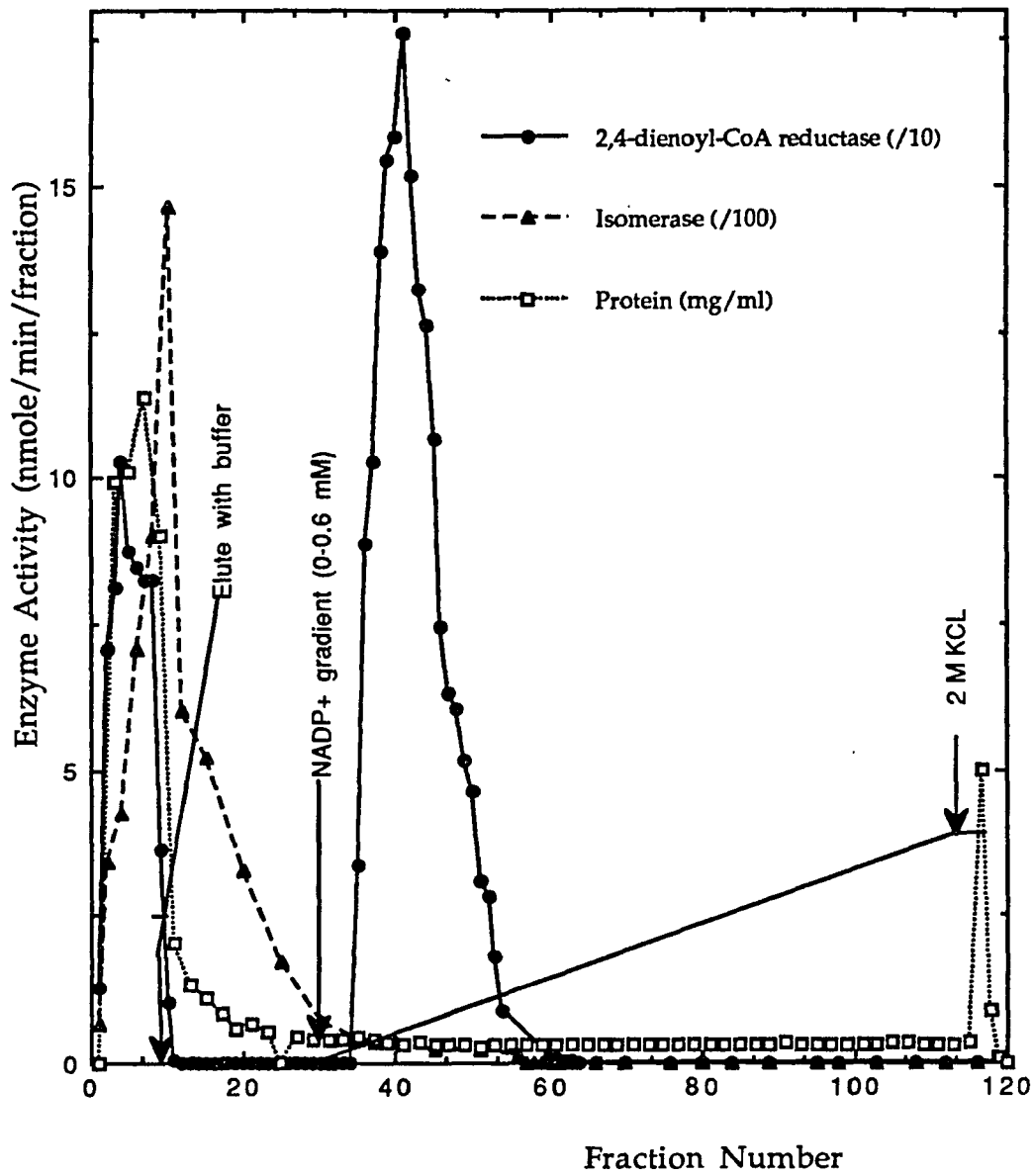


**Fig. 6. Partial purification of 2,4-dienoyl-CoA reductase by ADP affinity chromatography.** 2,4-Dienoyl-CoA reductase activities were assayed with 2-*trans*,4-*trans*-decadienoyl-CoA as a substrate and 3-*cis*-,2-*trans*-enoyl-CoA isomerase was assayed with dec-3-*cis*-enoyl-CoA as a substrate. Protein concentrations were determined by the method of Lowry *et al.* (103).

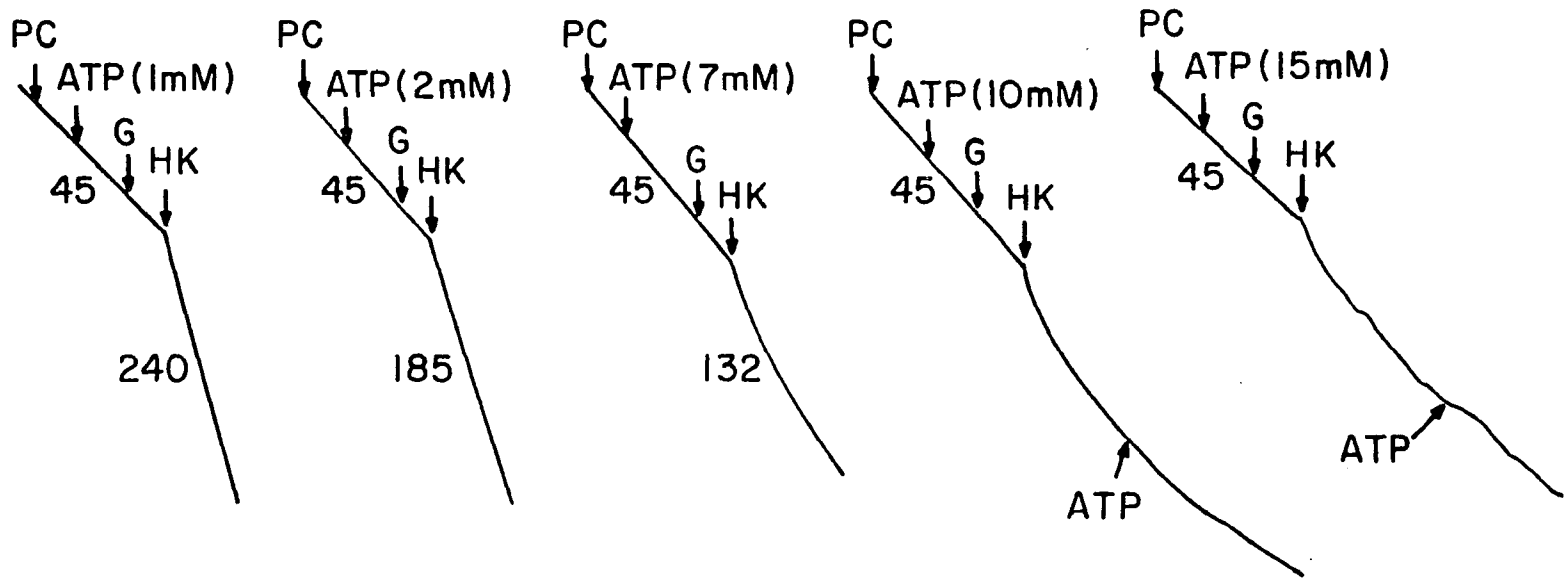


**Fig. 7. Effect of ATP concentration on the respiration of rat heart mitochondria.** Abbreviations: PC, 20 μM palmitoyl-L-carnitine; G, 10 mM glucose; HK, hexokinase (7 U/ml). Numbers give rates of respiration in nanoatoms of oxygen $min^{-1}$ (mg of protein)$^{-1}$.

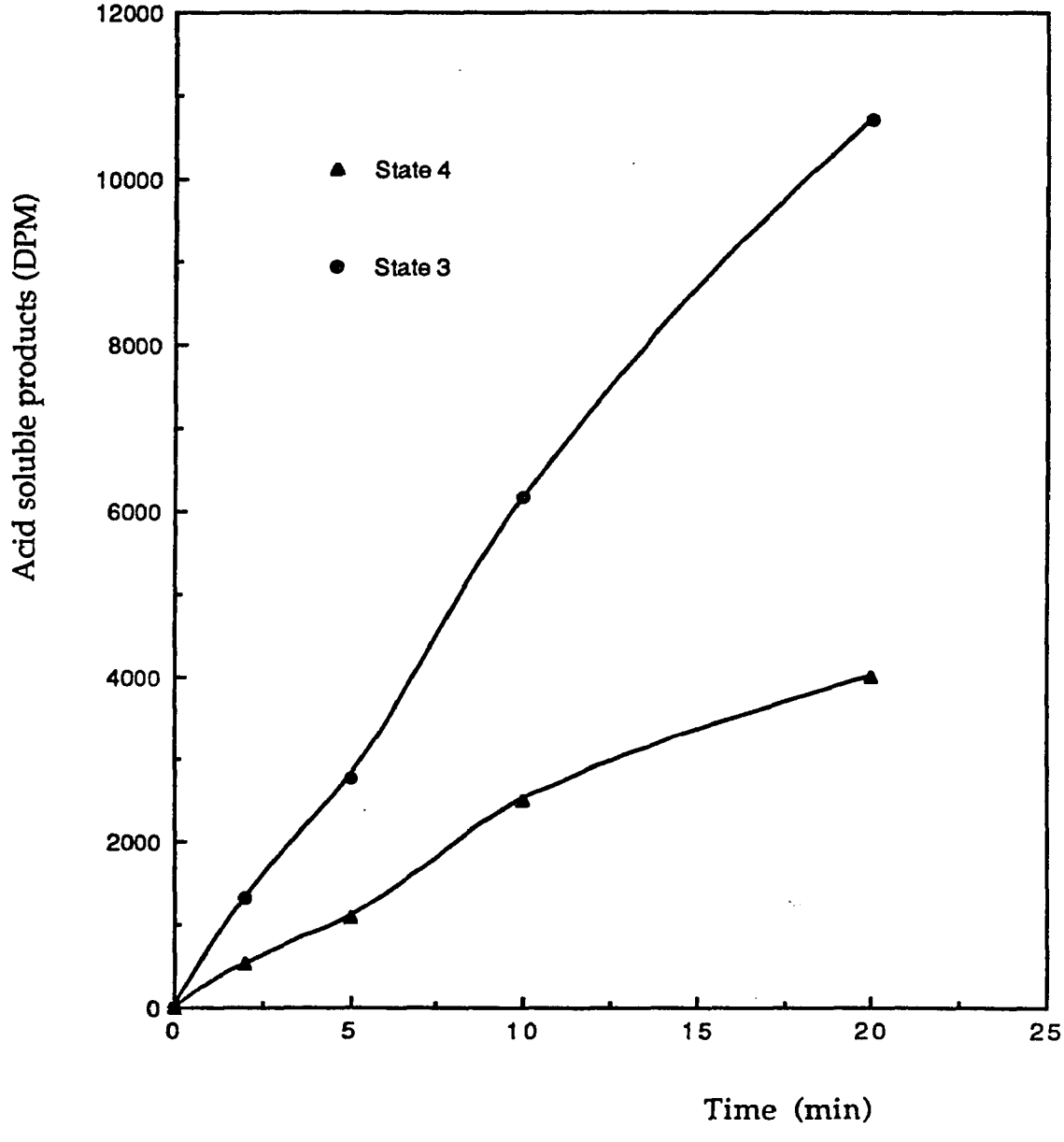


**Fig. 8. Oxidation of [1-$^{14}$C]palmitoyl-L-carnitine by rat heart mitochondria as a function of time.** Triangles, state 4 respiration; circles, state 3 respiration.

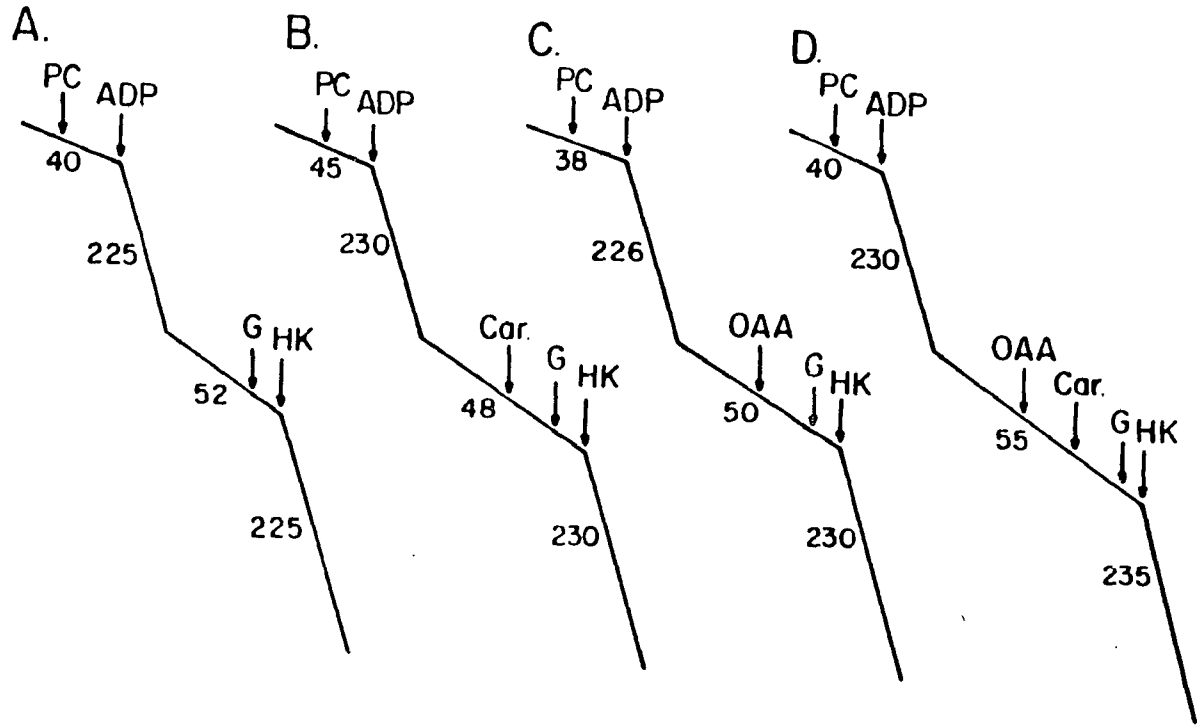


**Fig. 9. Effect of L-carnitine and oxaloacetate on palmitoylcarnitine-supported respiration of coupled rat heart mitochondria.** PC, 20 μM palmitoylcarnitine; G, 10 mM glucose, HK, hexokinase (12 U); Car, 10 mM L-carnitine; OAA, 10 mM oxaloacetate. Numbers give the rates of respiration in nanoatoms of oxygen $(min)^{-1}$ $(mg\ of\ protein)^{-1}$.

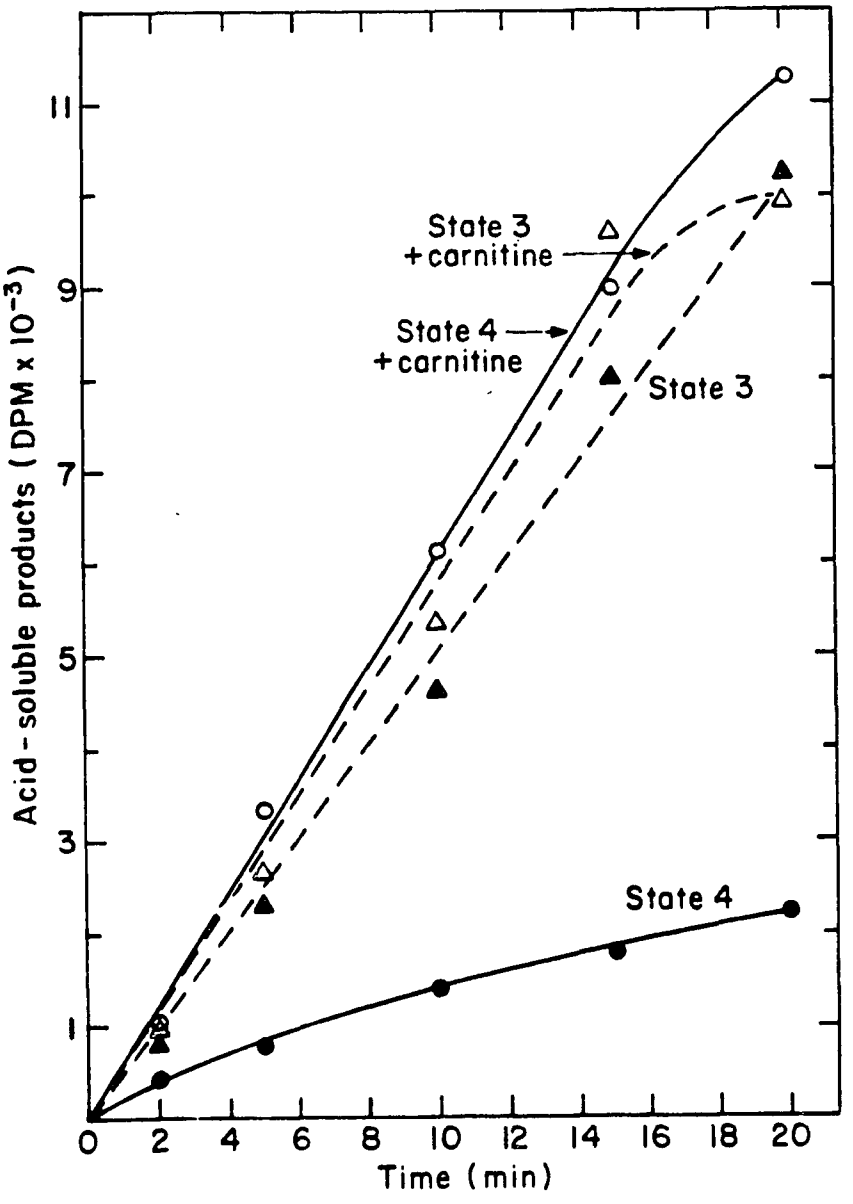


**Fig. 10. β-Oxidation of [1-$^{14}$C]palmitoylcarnitine by coupled rat heart mitochondria.** Triangles, state 3 respiration; circles, state 4 respiration. Solid symbols, in the absence of L-carnitine; open symbols, in the presence of 10 mM L-carnitine.

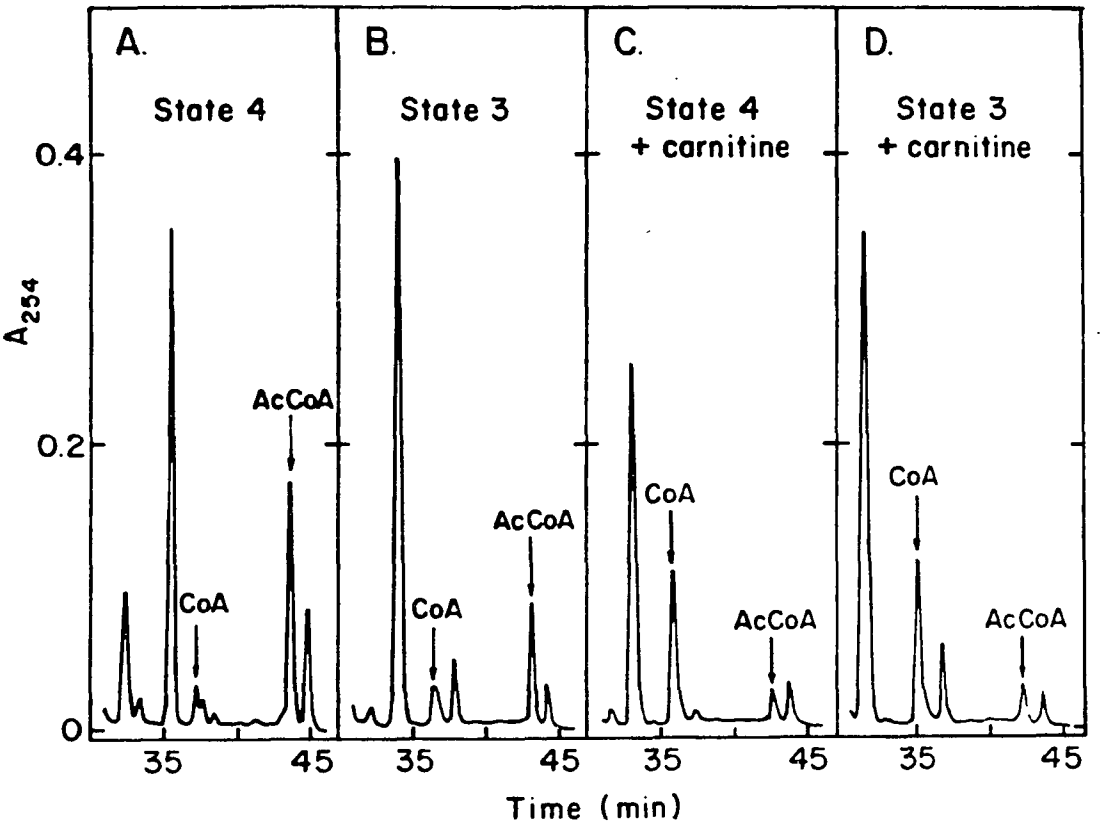


**Fig. 11. Separation by HPLC of acetyl-CoA and CoASH present in a solution of Tween-20-solubilized rat heart mitochondria.** CoA, coenzyme A, AcCoA, acetyl-CoA. State 3 and state 4 refer to state 3 respiration and state 4 respiration, respectively.

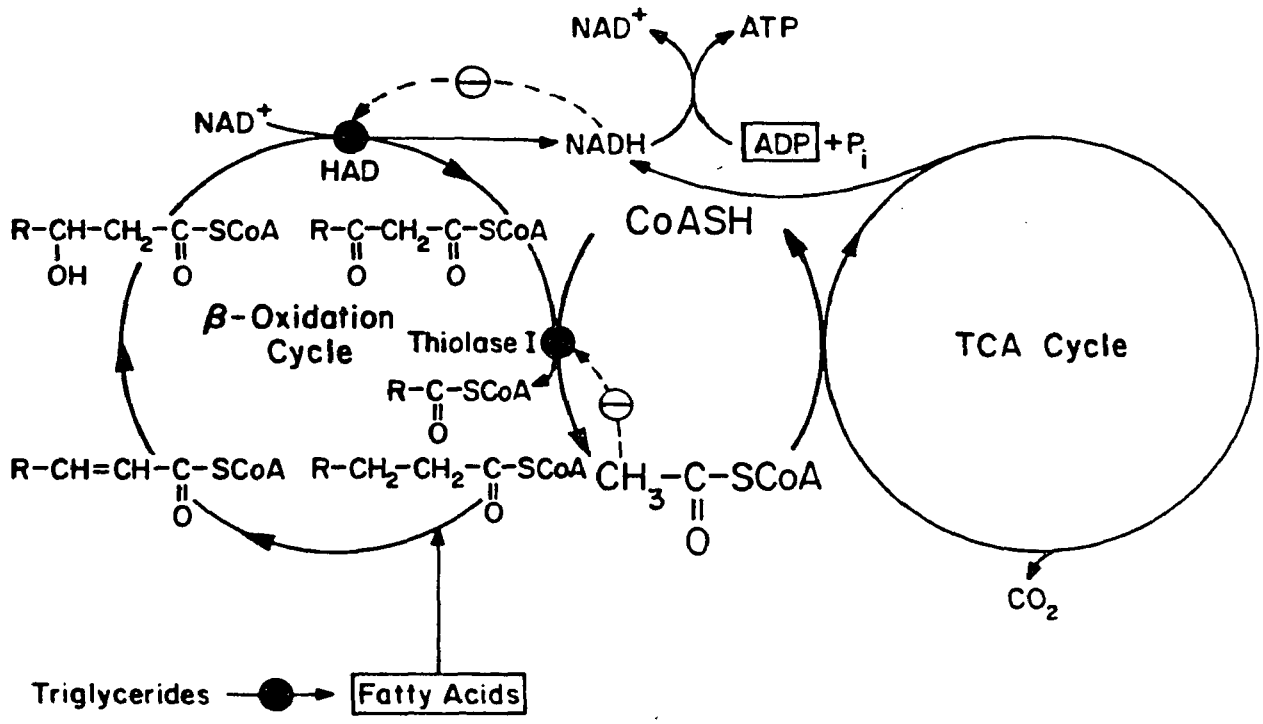


**Fig. 12. Regulation of β-oxidation in heart.** The arrow heads on the solid lines designate the usual directions of flux through the pathways marked by standard abbreviations. Solid circles designate known or possible points of control. Dotted lines marked by circles with negative signs indicate possible feedback inhibitions.

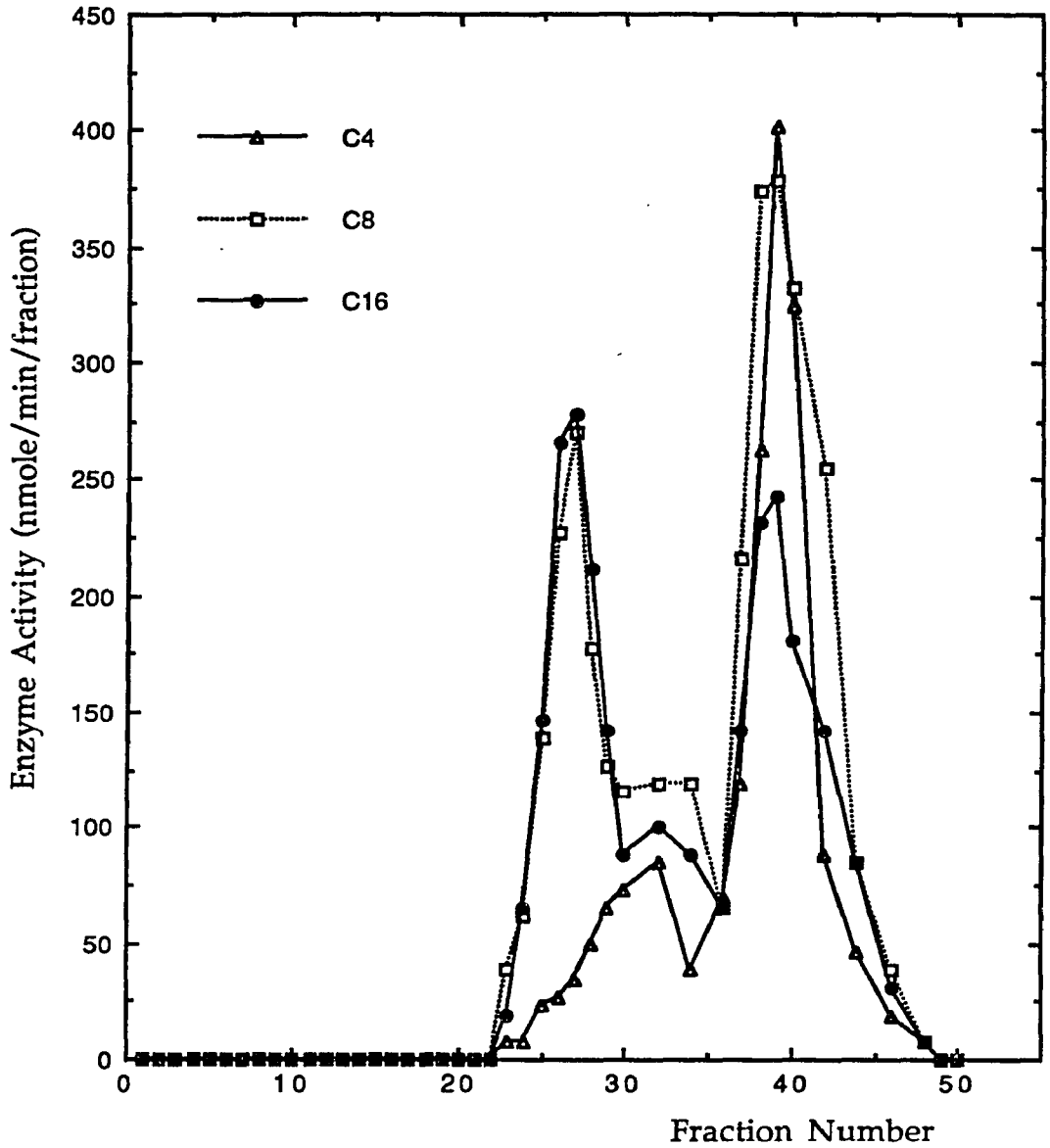


**Fig. 13. Separation of pig heart mitochondrial HDH activities by gel filtration.** Pig heart mitochondria were dissolved with Triton X-100. The whole homogenate in 0.05 M potassium phosphate buffer (pH 7.0) containing 20% (w/v) glycerol, 0.01% (w/v) Triton X-100, 2.5 M KCl, 0.1 M EDTA was chromatographed on a Ultrogel AcA 34 column (1.6X70 cm). Fractions (2.0 ml) were collected and assayed with acetoacetyl-CoA ($C_4$), 3-ketooctanoyl-CoA ($C_8$), and 3-ketohexadecanoyl-CoA ($C_{16}$) as substrates.

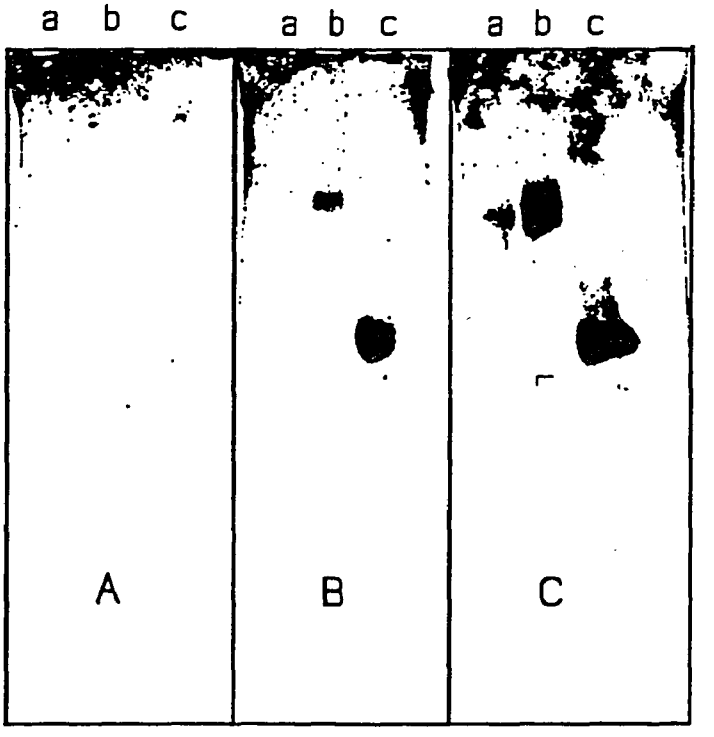


**Fig. 14. Separation and localization of HDH activities by native gradient gel electrophoresis.** A, Enzyme activities were stained with A, no substrate; B, with 3-hydroxybutyryl-CoA; C, 3-hydroxyoctanoyl-CoA. Lane a, mitochondrial homogenate solubilized with 1% of Triton X-100 (25 mU with 3-ketohexadecanoyl-CoA as substrate); lane b, long-chain HDH from Ultrogel AcA (25 mU with 3-ketohexadecanoyl-CoA as substrate); lane c, 25 mg of general HDH.

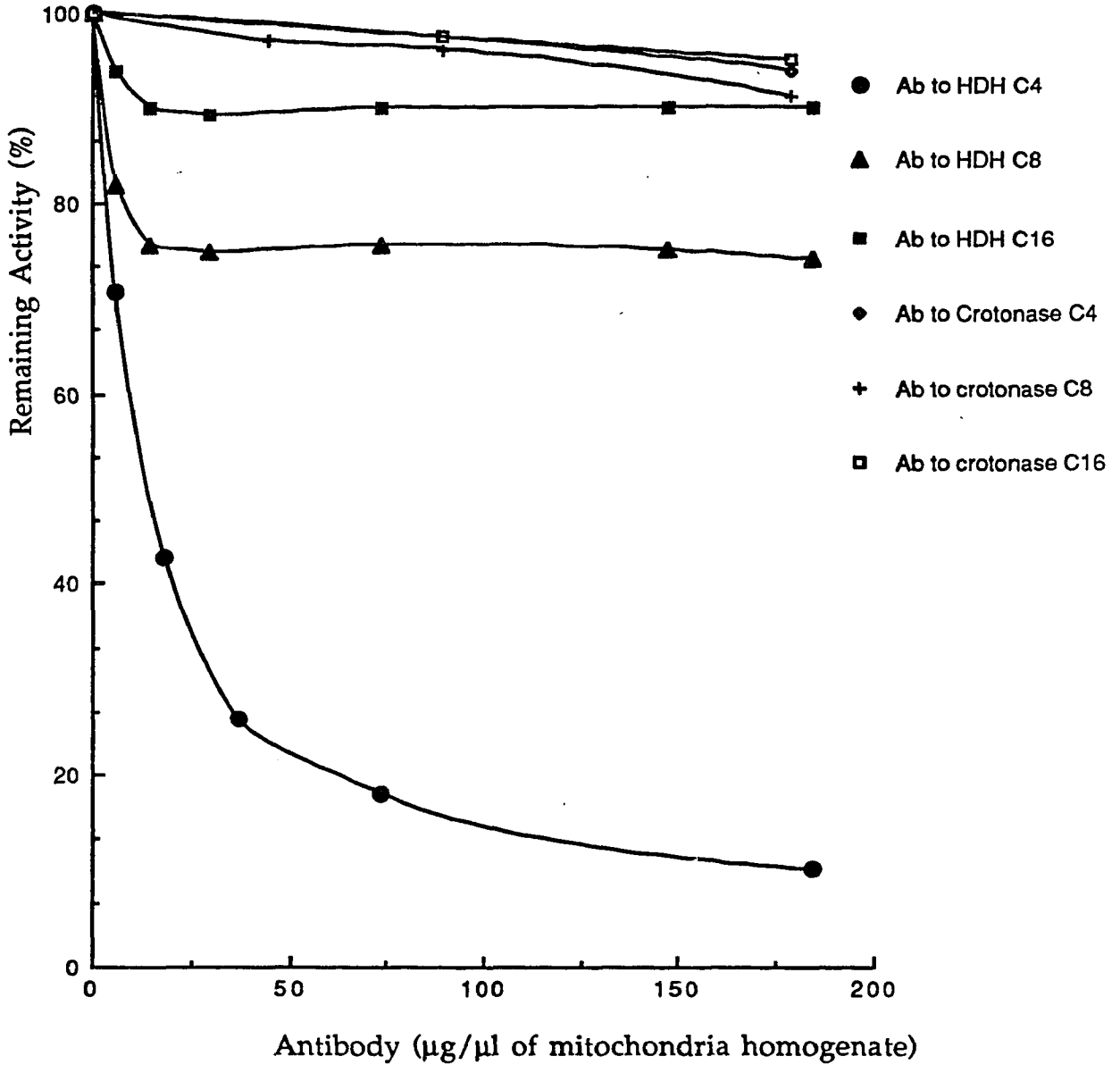


**Fig. 15. Immunotitration of 3-hydroxyacyl-CoA dehydrogenase of a pig heart mitochondrial homogenate.** The percent of remaining HDH activity in the supernatant after precipitation of immune complexes was plotted as a function of the amount of added antibody (Ab) per ml of mitochondrial homogenate. Anti-crotonase antibody was used as a control. HDHs activities were assayed with acetoacetyl-CoA ($C_4$), 3-ketooctanoyl-CoA ($C_8$) and 3-ketohexadecanoyl-CoA ($C_{16}$) as substrates.

**Fig. 16. Separation of HDH activities by anti-general HDH affinity chromatography.** Frozen pig heart mitochondria were treated with 1% of octyl glucoside as described under 'Experimental Procedures'. Three ml of a 100,500xg supernatant containing 27.7 U of HDH assayed with acetoacetyl-CoA and 9.6 U of HDH assayed with 3-ketohexadecanoyl-CoA as substrates was applied to a 5 ml anti-general HDH antibodies immunoaffinity column that was equilibrated with 150 mM NaCl and 25 mM potassium phosphate, pH 7.25, at room temperature. The flow rate was approximately 0.2ml/min. Unbound protein was removed by washing with several column volumes of mitochondrial isolation buffer at room temperature; bound proteins were eluted (arrow) with 50 mM ammonium (pH 10.6) containing 20% glycerol at 4 $^0$C. Fractions of 3 ml were assayed for HDH activities with acetoacetyl-CoA ($C_4$), 3-ketooctanoyl-CoA ($C_8$), and 3-ketohexadecanoyl-CoA ($C_{16}$) as substrates. The protein concentrations were determined by the Bradford method (106).

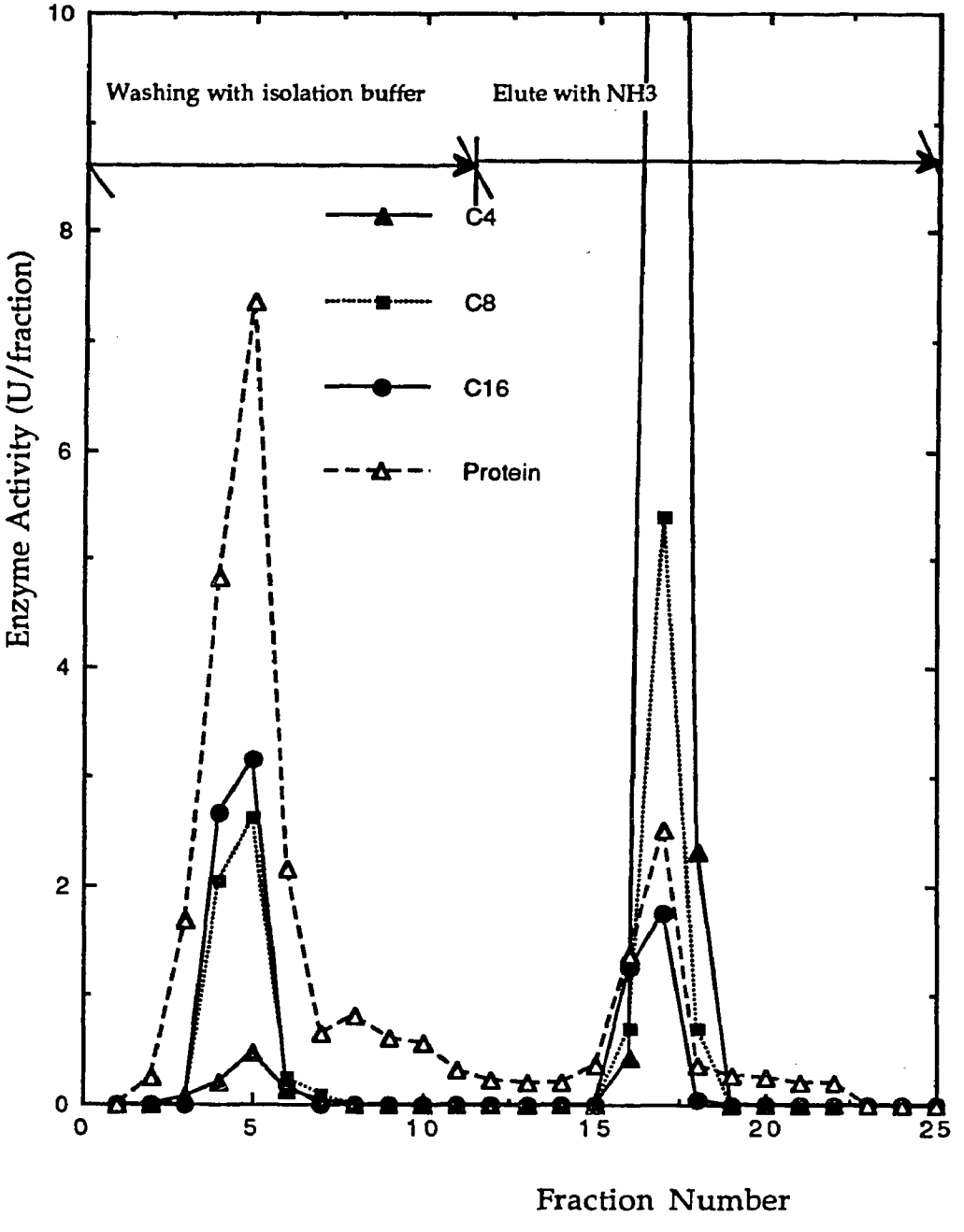
Washing with isolation buffer
Elute with NH3
C4
C8
C16
Protein
Enzyme Activity (U/fraction)
Fraction Number
0
2
4
6
8
10
0
5
10
15
20
25

Fig. 16.

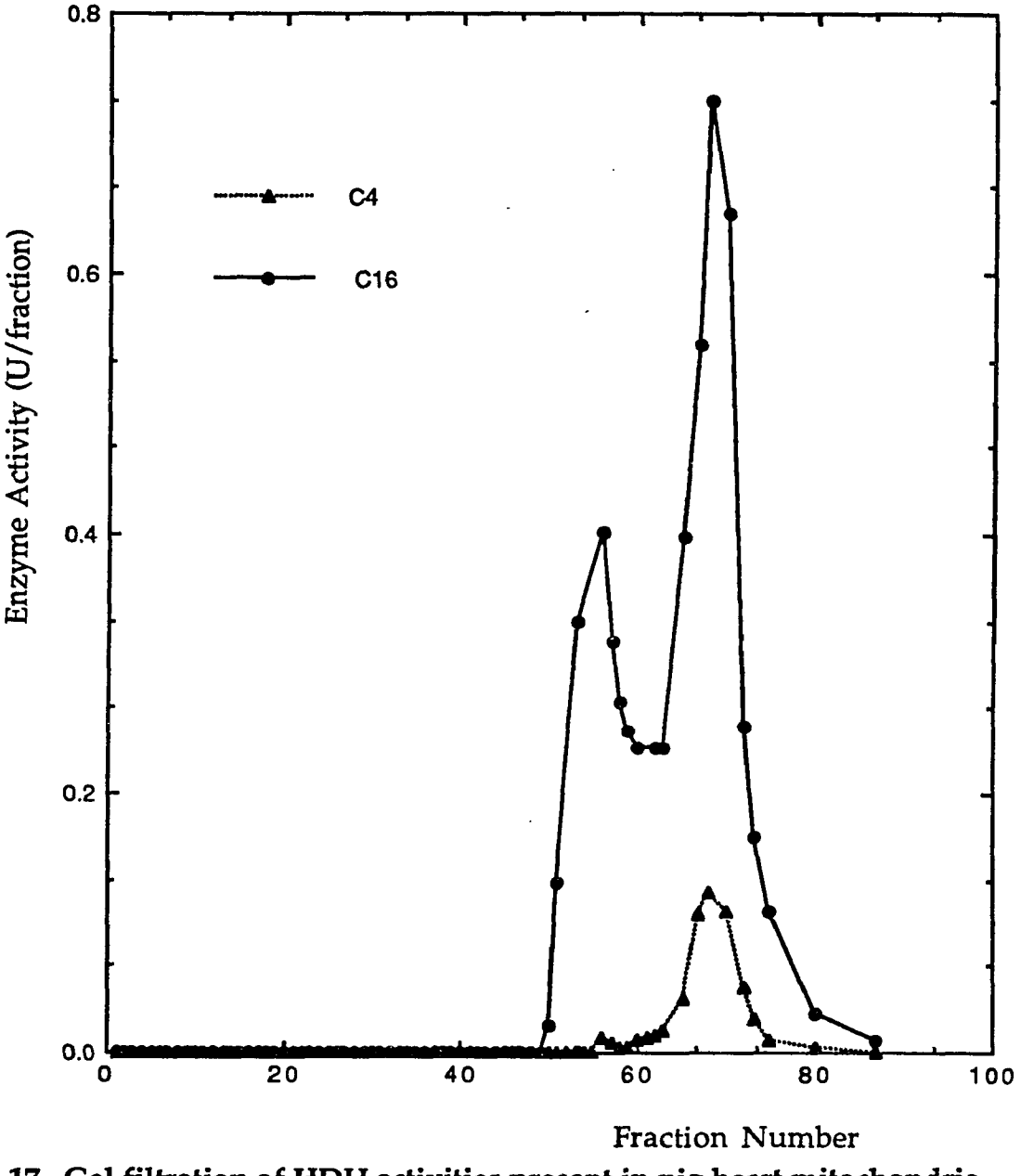


**Fig. 17. Gel filtration of HDH activities present in pig heart mitochondria homogenate.** Mitochondria were suspended in the buffer of Fig. 13, except that 0.5% (w/v) Triton X-100 was included. The mitochondrial homogenate was chromatographed on a 2.5x100 cm Ultrogel AcA 34 column at room temperature with the same buffer as mentioned in the legend of Fig. 13. Fractions (4 ml) were collected and assayed with 3-ketohexadecanoyl-CoA ($C_{16}$) and acetoacetyl-CoA ($C_4$) as substrates.

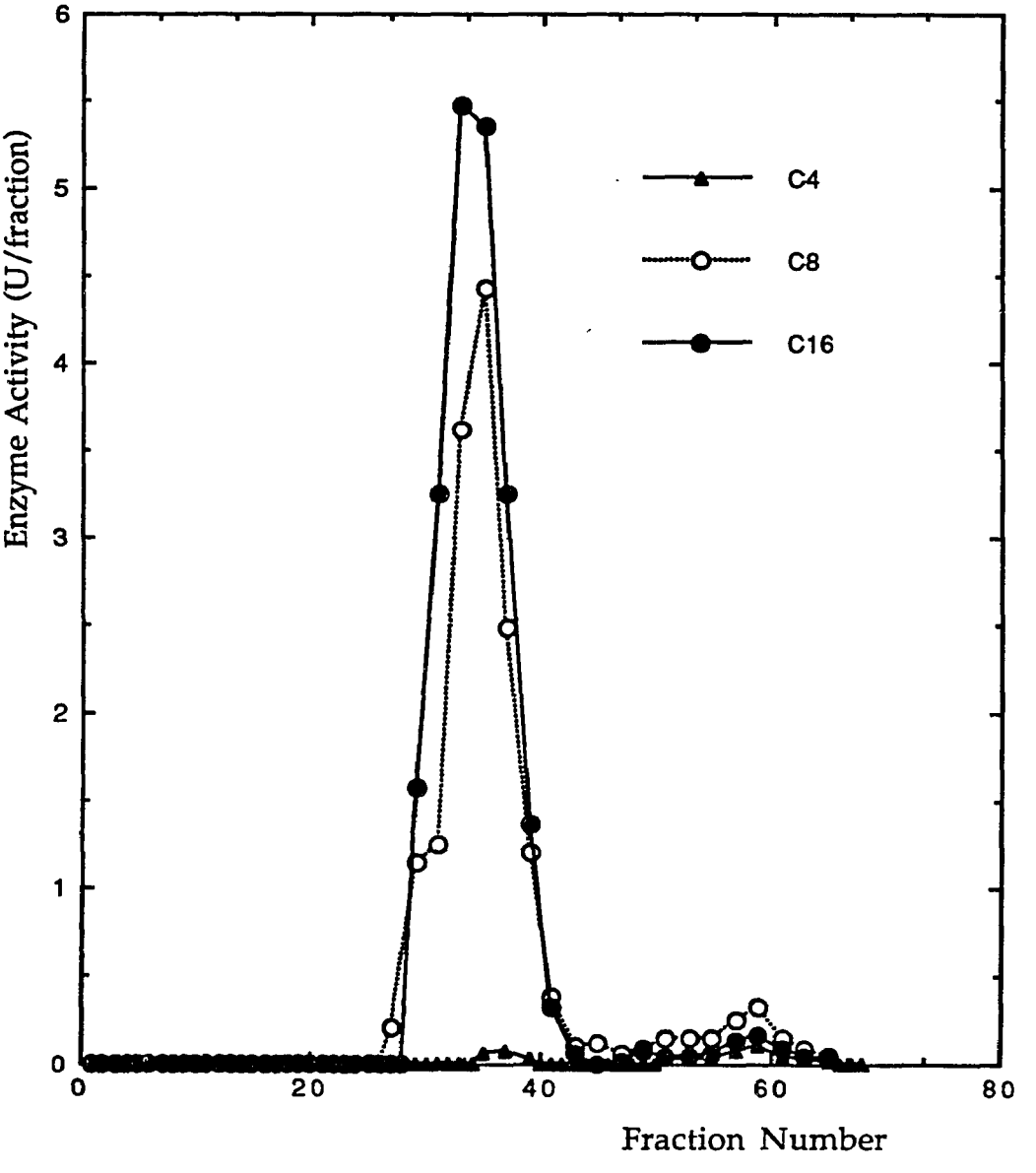


**Fig. 18. Effect of high detergent concentration on the gel filtration of mitochondrial HDHs.** All procedures were the same as described in Fig. 17, except that 1% (w/v) of Triton X-100 was used instead with 0.5% to dissolve pig heart mitochondria.

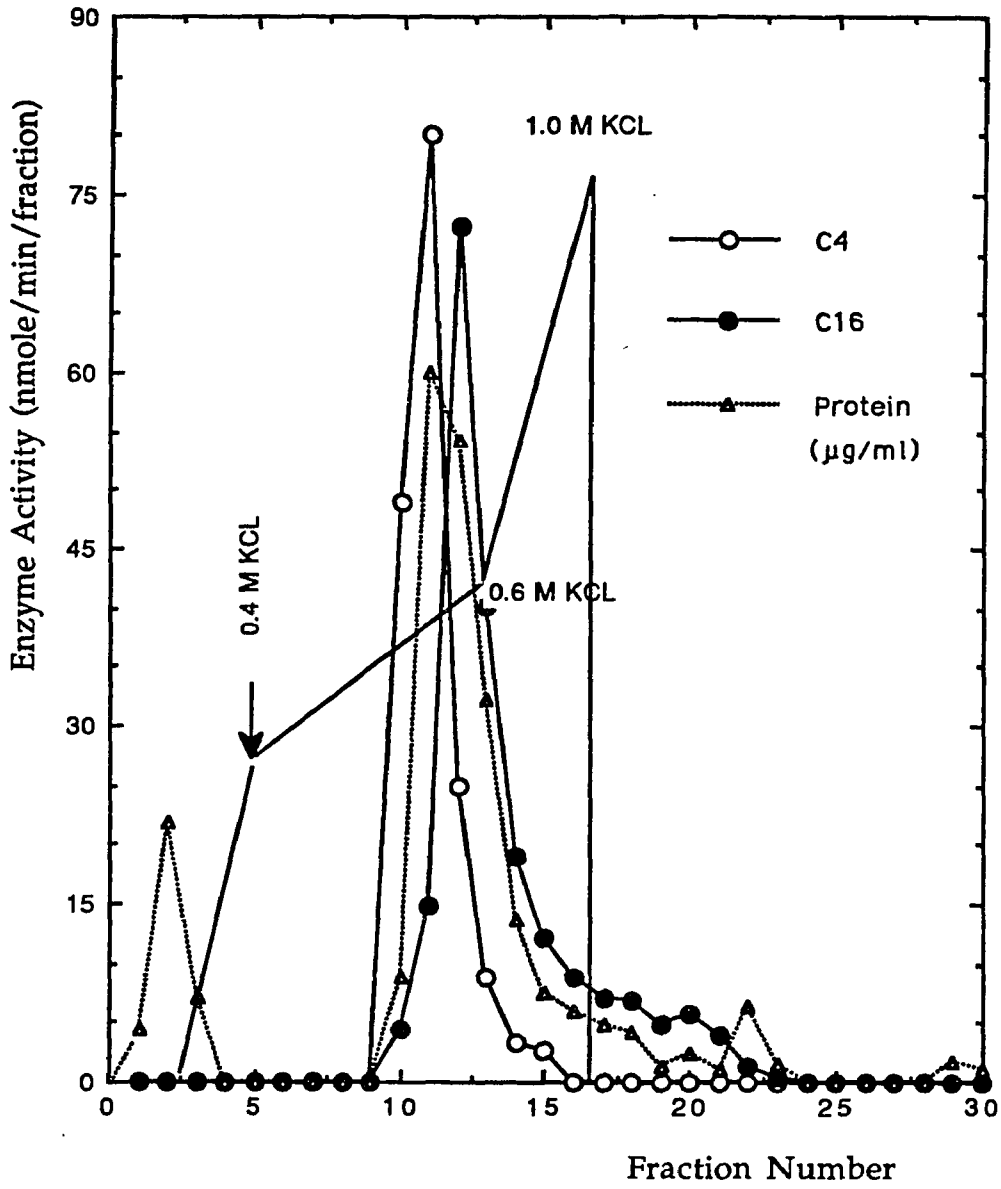


**Fig. 19. Purification of long-chain HDH on a HPLC Mono Q column.** The enriched long-chain HDH activity fractions from an FPLC Sephacryl S-200 gel filtration column were pooled, dialyzed, and concentrated. 0.2 ml of the enzyme solution (0.1 U) was applied to an HPLC Mono Q column (0.5 x 5 cm). KCl gradients were applied as shown to elute HDHs. Fractions (1.0 ml) were collected and HDH activities were assayed with acetoacetyl-CoA ($C_4$), 3-ketooctanoyl-CoA ($C_8$), and 3-ketohexadecanoyl-CoA ($C_{16}$). Protein concentrations were determined by the Bradford method (106).

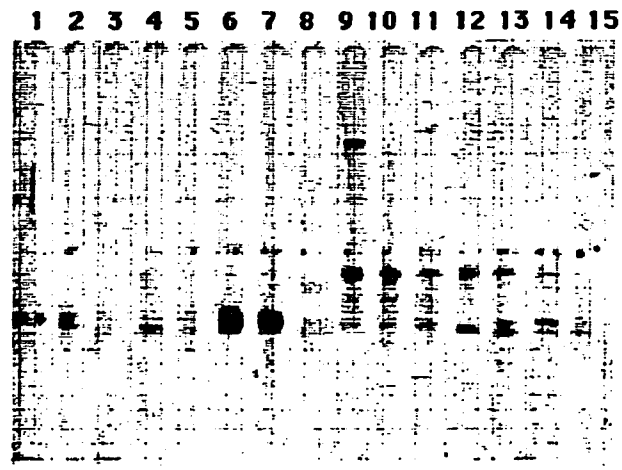


**Fig. 20. Western blotting analysis of HDHs.** Lane 2, 50 mg and lane 3, 20 mg of pig heart mitochondrial extract containing 1% of Triton X-100; lane 4, 50 mg and lane 5, 20 mg of pig heart mitochondrial extract; lane 6, 5 mg and lane 7, 2 mg of purified general HDH; lane 9, 50 mg, lane 10, 25 mg and lane 11, 12.5 mg of partially purified long-chain HDH (free of short-chain HDH activity); lane 12, 10 mg, lane 13, 5 mg and lane 14, 2 mg of general HDH prepared by immunoaffinity chromatography.

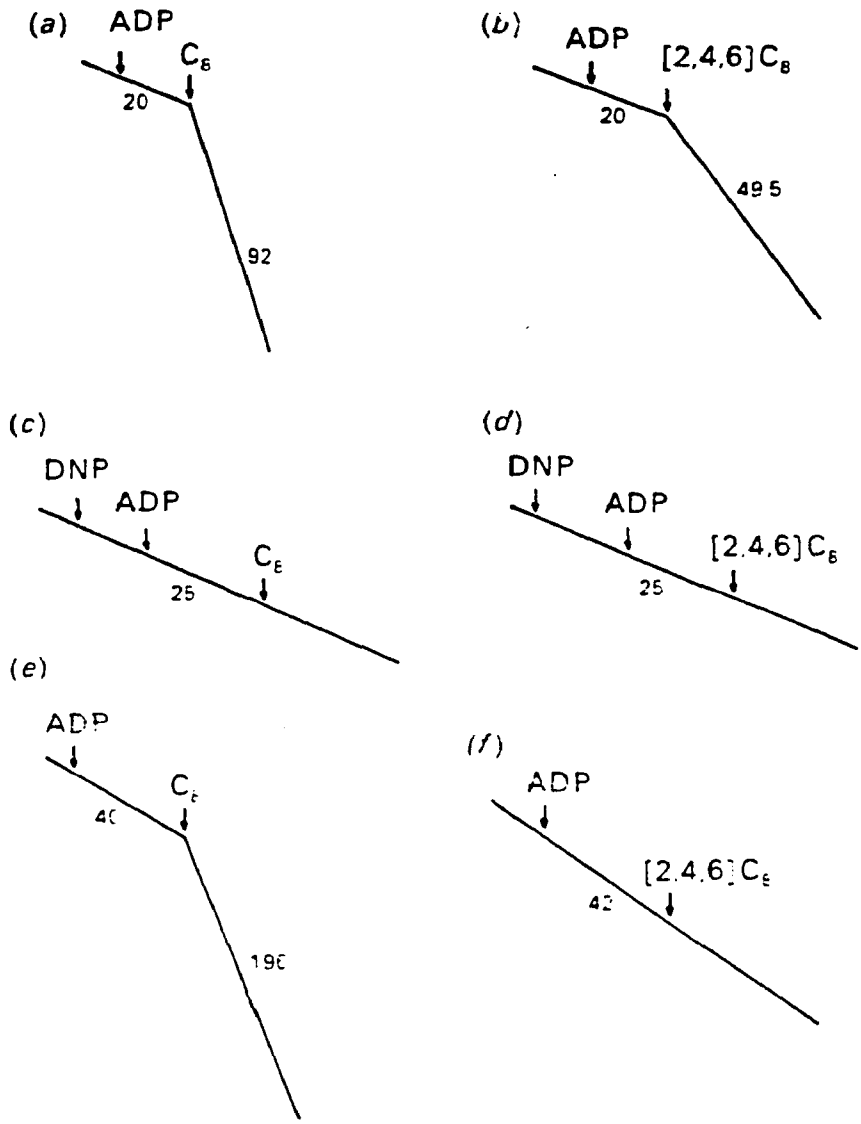


**Fig. 21. Rates of mitochondrial respiration: a-d, with rat liver mitochondria; e and f, with rat heart mitochondria**

Abbreviations: C8, octanoic acid; [2,4,6]C8, octa-2,4,6-trienoic acid; DNP, 2,4-dinitrophenol. Numbers give the respiration rates in ng-atoms of O/min per mg of protein.

**Fig. 22. Spectral changes associated with the reduction of octa-2,4,6-trienoyl-CoA and its subsequent β-oxidation**

(a) Absorbance changes recorded when octa-2,4,6-trienoyl-CoA was incubated with NADPH and 2,4-dienoyl-CoA reductase for (1) 0 min, (2) 2 min, (3) 12 min, (4) 70 min. (b) Absorbance changes recorded when the reduction product formed as described in (a) was incubated with the *E. coli* fatty acid oxidation complex plus CoA and NAD+ for (0) min, (1) 6 min, and (2) 10 min. The reference cuvette contained all components except for the fatty acid oxidation complex. Further absorbance changes were recorded at (3) 5 min and (4) 8 min after the addition of L- malate, malate dehydrogenase, and citrate synthase to the mixture that gave rise to spectrum 2. When lactate dehydrogenase and pyruvate were added to the sample that yielded spectrum 4, recording 5 was obtained.

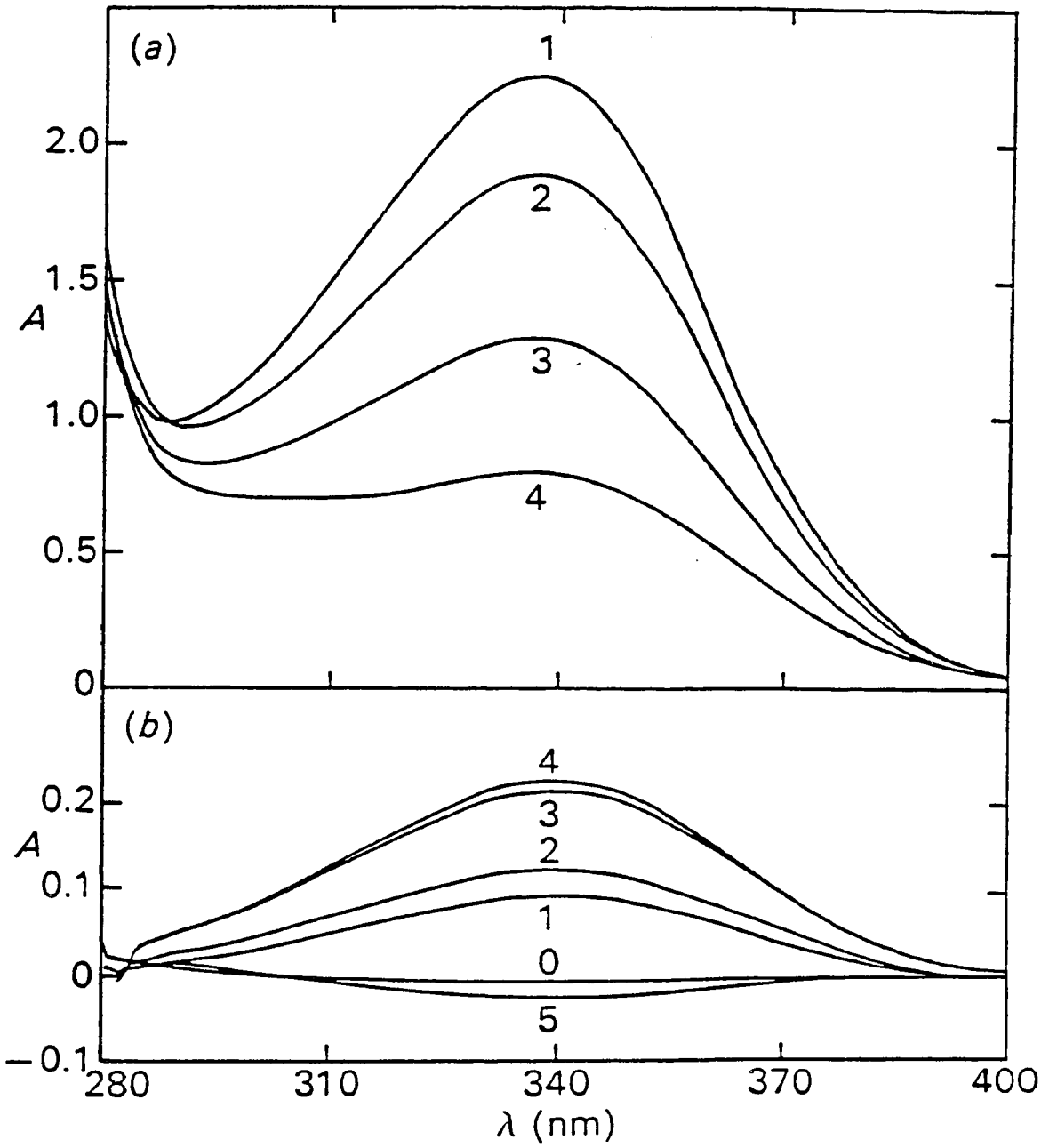


Fig. 22.

**Fig. 23. HPLC analysis of the products formed during the reduction of octa-2,4,6-trienoyl-CoA and its subsequent β-oxidation**

(a) Products identified when octa-2,4,6-trienoyl-CoA was incubated for 86 min with NADPH and 2,4-dienoyl-CoA reductase. (b) Products identified 12 min after the *E. coli* fatty acid oxidation complex, CoA and $NAD^+$ were added to the reaction mixture containing the products shown in panel (a). Peaks labeled $NADP^+$, NADPH, $NAD^+$, NADH, CoA, AcCoA (acetyl-CoA) and 2,4,6C8CoA (octa-2,4,6-trienoyl-CoA) were identified by use of authentic compounds. Samples were eluted with a methanol gradient detailed below the absorbance tracings on both chromatograms.

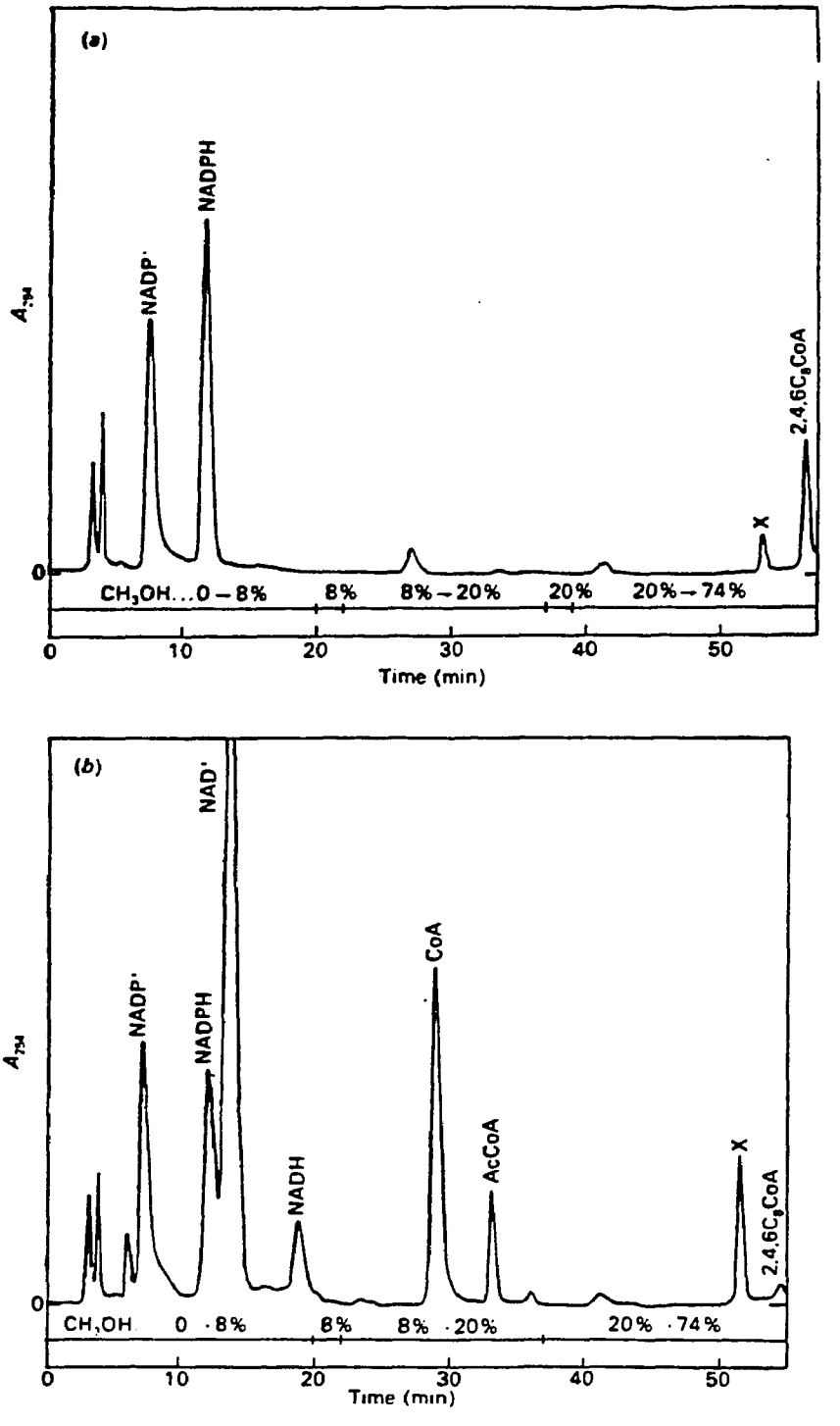
(a)
NADP·
NADPH
X
2,4,6C8CoA
0
CH3OH...0–8%
8%
8%–20%
20%
20%–74%
0
10
20
30
40
50
Time (min)
(b)
NAD·
NADP·
NADPH
CoA
NADH
AcCoA
X
2,4,6C8CoA
0
CH3OH 0 ·8%
8%
8% ·20%
20% ·74%
0
10
20
30
40
50
Time (min)

Fig. 23.

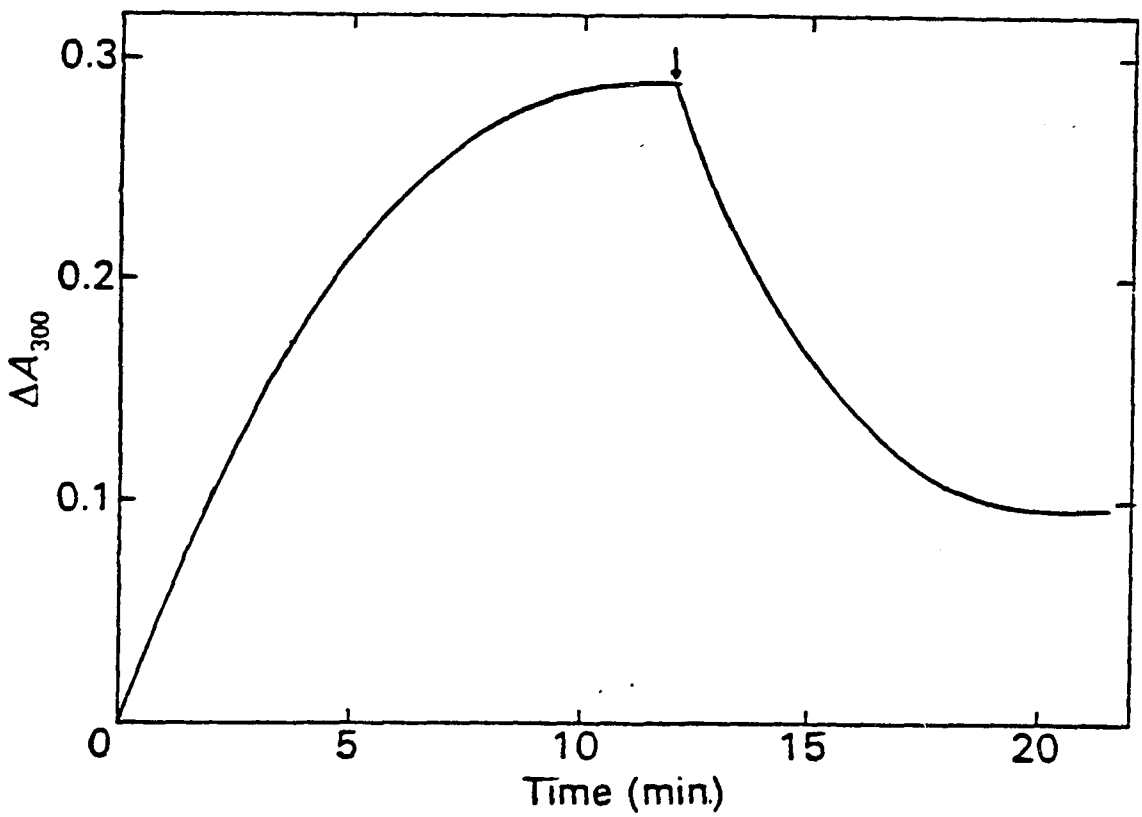


**Fig. 24. Absorbance changes observed when the product of the reduction and β-oxidation of octa-2,4,6-trienoyl-CoA was first reacted with acyl-CoA oxidase and catalase and then with 2,4-dienoyl-CoA reductase and NADPH**

Acyl-CoA oxidase and catalase were added at zero time. 2,4-Dienoyl-CoA reductase and NADPH were added at at the time marked by the arrow.

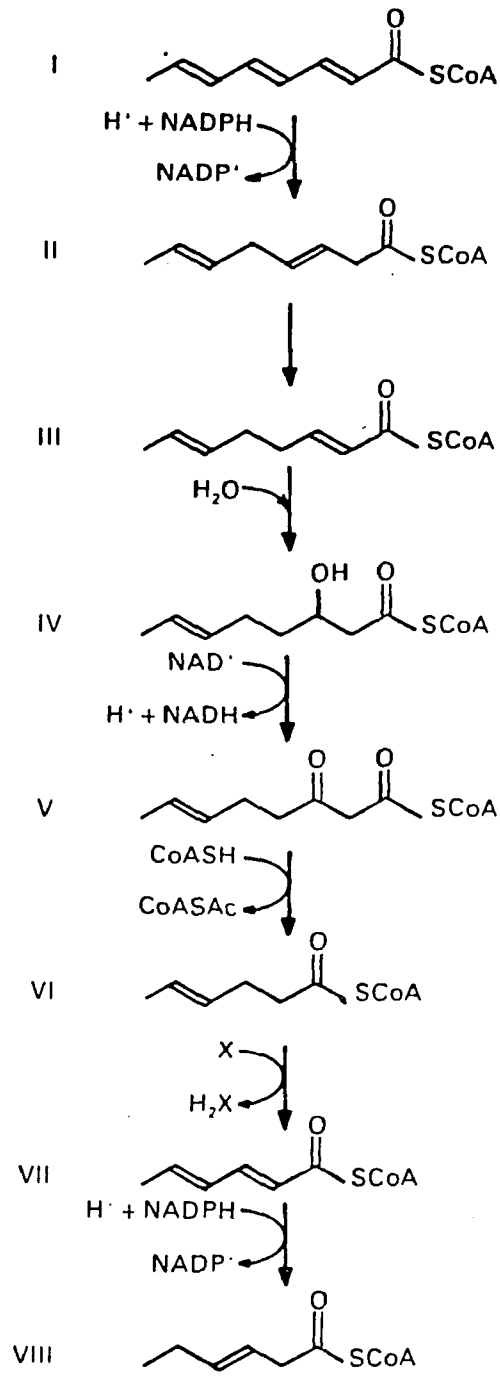


**Fig. 25. Proposed degradation of octa-2,4,6-trienoyl-CoA by β-oxidation**

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