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City University of New York PH.D. 1985

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REGULATION OF ACYL-CoA DEHYDROGENASES AND FATTY ACID OXIDATION IN HEART

by

BRUCE PAUL DAVIDSON

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.

1985

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

2-15-85
Date

Chairman of Examining Committee

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Abstract

REGULATION OF ACYL-CoA DEHYDROGENASES
AND FATTY ACID OXIDATION IN HEART

by

Bruce Paul Davidson

Adviser: Professor Horst Schulz

Bovine liver butyryl-CoA dehydrogenase, general acyl-CoA dehydrogenase and long-Chain acyl-CoA dehydrogenase, all of which are believed to function in fatty acid oxidation, have been separated and partially purified by a simple two-step procedure. the same procedure was used to separate the bovine heart acyl-CoA dehydrogenases. Butyryl-CoA dehydrogenase, general acyl-CoA dehydrogenase and long-chain acyl-CoA dehydrogenase were thus identified and found to be identical with the bovine liver enzymes.

The control of ß-oxidation via the regulation of acyl-CoA dehydrogenase has been investigated. All three acyl-CoA dehydrogenases are strongly inhibited by 3-ketoacyl-CoA's and less so by 2-enoyl-CoA compounds. However, most severely

inhibited is long-chain acyl-CoA dehydrogenase, which may catalyze the first step in the oxidation of long-chain fatty acids. The following inhibition constants were determined with long-chain acyl-CoA dehydrogenase: K_I = 1.3 µM and K_I = 0.075 µM for 3-ketodecanoyl-CoA with palmitoyl-CoA and decanoyl-CoA as substrates, respectively; K_I = 0.2 µM for 3-ketopalmitoyl-CoA with palmitoyl-CoA as a substrate.

It is proposed that a decrease in the energy demand of heart muscle leads to an increased concentration of acetyl-CoA which causes the inhibition of 3-ketoacyl-CoA thiolase (Y. Olowe and H. Schulz (1980) Eur. J. Biochem. 109, 425-429). The consequence of an inhibition of 3-ketoacyl-CoA thiolase may be an accumulation of 3-ketoacyl-CoA compounds which would inhibit most effectively long-chain acyl-CoA dehydrogenase, the enzyme that presumably catalyzes the first step of ß-oxidation.

In order to determine the presence of fatty acid oxidation intermediates in mitochondria, coupled rat heart mitochondria were incubated for 2 min with [16-^{14}C]palmitoyl-CoA at state 3 and state 4 respiration. At state 4, in contrast to state 3 respiration large amounts of hydroxy acids, presumably in the form of their CoA derivatives, accumulated. Identification and quantitation of methyl ketones derived from 3-ketoacyl-CoA's provided evidence for a 3-fold increase in their concentration to 3mM when the respiration state was changed from 3 to 4. These observations support the hypothesis that ß-oxidation may be

controlled via the regulation of long-chain acyl-CoA dehydrogenase by 3-ketoacyl-CoA and possibly 2-enoyl-CoA compounds.

To the memory of my father Jesse and uncle Milton.

Acknowledgment

I wish to express my sincere appreciation to Professor Horst Schulz, who for over ten years has acted as both friend and mentor. His steady guidance, patience and concern for his students will always serve as an example of the finest among educators and men. Special thanks to my mother Ruth for her endless patience in the preparation of this manuscript. I also want to thank Emily Sabbagh whose excellent technical skills in isolation and polarographic measurement of mitochondria helped bring this thesis to a fruitful conclusion.

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INTRODUCTION

In 1904 Knoop proposed that fatty acids are degraded by ß-oxidation. More than forty years later Kennedy and Lehninger proved ß-oxidation to take place in the mitochondrion and to yield two-carbon units which are further metabolized by the citric acid cycle. The individual steps of the ß-oxidation pathway were elucidated primarily by the groups of Green (1), Lynen (2), and Ochoa (3) and are summarized below. Whereas the uptake of long-chain fatty acids by mitochondria is dependent on carnitine, short-chain and medium-chain fatty acids freely diffuse into the matrix where they are activated by short-chain (EC 6.2.1.1) and medium-chain acyl-CoA synthetase (EC 6.2.1.2). The entry of long-chain fatty acids from the cytosol into the mitochondria requires three reactions:

1. The ATP-dependent thioester bond formation between free fatty acid and extra mitochondrial CoASH to yield fatty acyl-CoA. This step, referred to as activation, is catalyzed by long-chain acyl-CoA synthetase (EC 6.2.1.3). Long-chain acyl-CoA synthetase studied in heart is most active with long-chain fatty acids and is located mainly in the outer mitochondrial membrane (4,5,6). In liver, the same enzyme is present in the outer mitochondrial membrane as well as in the endoplasmic reticulum.

2. Transesterification of the acyl groups from CoA to

carnitine to yield acylcarnitine catalyzed by carnitine palmitoyl-transferase I (EC 2.3.1.21).

3. Translocation of acyl carnitine through the inner mitochondrial membrane in exchange for carnitine catalyzed by carnitine acylcarnitine translocase. (7,8). After crossing the inner mitochondrial membrane, the acyl unit is transferred back to CoA by carnitine palmitoyl transferase II (EC 2.3.1.21) (9). The carnitine palmitoyl transferase I is located on the outer surface of the inner mitochondrial membrane and carnitine palmitoyl transferase II is on the inner surface of the inner mitochondrial membrane.

Once present in the mitochondrial matrix fatty acyl-CoA's can enter the ß-oxidation system (10) as outlined below:

1) Acyl-CoA + FAD^+ $\rightleftharpoons$ 2-enoyl-CoA + $FADH_2$

2) 2-Enoyl-CoA + H_2O $\rightleftharpoons$ 3-hydroxyacyl-CoA

3) 3-Hydroxyacyl-CoA + NAD^+ $\rightleftharpoons$ 3-ketoacyl-CoA + NADH + H^+

4) 3-Ketoacyl-CoA + CoASH $\rightleftharpoons$ acyl-CoA(-2C) + acetyl-CoA

The first reaction of ß-oxidation is catalyzed by three acyl-CoA dehydrogenases which differ in their chain length specificities and which are named butyryl-CoA dehydrogenase (EC 1.3.99.2), medium-chain acyl-CoA dehydrogenase (EC 1.3.99.3) and long-chain acyl-CoA dehydrogenase (EC 1.3.99.3). Their combined actions result in the dehydrogenation of fatty acyl-CoA's with chain lengths from four through twenty-two carbons. The products of the reactions catalyzed by these flavin

nucleotide enzymes are the trans-$\Delta^{2,3}$- enoyl-CoA thioesters.

Steriospecific hydration of $\Delta^{2,3}$ - enoyl-CoA to L-3-hydroxyacyl-CoA is catalyzed by enoyl-CoA hydratase (EC 4.2.1.17). Two enoyl-CoA hydratases are present in pig heart mitochondria (11). Crotonase is specific for short-chain enoyl-CoA's, whereas the other hydratase is active with medium-chain and long-chain substrates.

The resulting L-3-hydroxyacyl-CoA's are oxidized to the 3-ketoacyl-CoA thioesters by L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35). Only one dehydrogenase which exhibits a broad chain length specificity was known until recently when an additional long-chain specific L-3-hydroxyacyl-CoA dehydrogenase was identified (95). Both dehydrogenases act only on L-hydroxy-substrates.

The final reaction of the ß-oxidation cycle is the thiolytic cleavage of 3-ketoacyl-CoA's to yield acetyl-CoA and acyl-CoA's which are shortened by two carbons. Two thiolases are known to be present in pig heart mitochondria (12). One is 3-ketoacyl-CoA thiolase or thiolase I (EC 2.3.1.16), which is active with 3-ketoacyl-CoA's of all chain lengths and functions in ß-oxidation. In contrast acetoacetyl-CoA thiolase or thiolase II (EC 2.3.1.9) which acts only on acetoacetyl-CoA is believed to function in the metabolism of ketone bodies, which are formed in liver and utilized by peripheral tissues.

Also present in mitochondria are enzymes which function in

ß-oxidation of unsaturated fatty acids. Very recently Cuebas and Schulz (13) have confirmed the modified pathway presented by Kunau and Dommes (14) for the degradation of linoleate by rat heart mitochondria. Cuebas and Schulz show that 2-trans 4-cis-decadienoyl-CoA, a metabolite of linoleate oxidation, is not directly degraded via the ß-oxidation cycle as was previously proposed by Stoffel and Caesar (15). In contrast, this metabolite is reduced by NADPH-dependent 2,4-dienoyl-CoA reductase to 3-decenoyl-CoA The latter is isomerized by cis-$\triangle^3$-trans-$\triangle^2$-enoyl-CoA isomerase (EC 5.3.3.3) to 2-trans-decenoyl-CoA which can be further degraded via ß-oxidation.

Acyl-CoA Dehydrogenases

Acyl-CoA dehydrogenases and butyryl-CoA dehydrogenase are ubiquitous enzymes catalyzing the initial reaction of ß-oxidation. Several investigators, many of whom were coworkers of Beinert, were the first to demonstrate that three different flavin nucleotide enzymes are involved in the first dehydrogenation step of ß-oxidation in pig liver mitochondria (16,17), and at least two in bovine liver (17,18), bovine heart (19,20) and sheep liver (21). Butyryl-CoA dehydrogenase from mycobacteria was first purified by Gelbard and Goldman (22) and at least two acyl-CoA dehydrogenases are present in E. Coli (23). The original work of Beinert and coworkers (17) and even the more recent separation of these enzymes carried out by Hall

and Kamin (19,24) have only demonstrated the presence of butyryl-CoA dehydrogenase and long-chain acyl-CoA dehydrogenase in bovine heart. It is possible that these procedures did not result in the complete separation of all three enzymes or that the lengthy purification procedures resulted in the loss of one of the enzymes, or that only two dehydrogenases are present in heart. The nomenclature used by Beinert (25) to describe these three dehydrogenases was based on the distinct visual properties and substrate specificities of the individual enzymes. These flavin nucleotide enzymes were classified according to their distinctive color and their chain length specificities G (C4-C8) is green in color and acts on substrates with four to eight carbon chain lengths, Y(C4-C16) is yellow in color and acts on substrates with four to sixteen carbon chain lengths, and Y'(C6-C16) is deep yellow in color and acts on substrates with six to sixteen carbon chain lengths. The three acyl-CoA dehydrogenases are now commonly referred to as butyryl-CoA dehydrogenase, medium-chain or general acyl-CoA dehydrogenase, and long-chain or palmitoyl-CoA dehydrogenase, respectively. The acyl-CoA dehydrogenases have similar physical and catalytic properties. All acyl-CoA dehydrogenases so far studied are of approximately equal molecular weight between 180,000 and 200,000 daltons (19,24,25,26). The enzymes from several sources display pH optima between 7.6 and 8.4. All acyl-CoA dehydrogenases are composed of four, possibly identical subunits which bind one

FAD each (19,24,26). The flavin nucleotides are non-covalently, but tightly bound to the apoenzyme. A K_D of 0.44 μM has been determined for FAD of butyryl-CoA dehydrogenase from bovine liver (27). However, in three instances (21,22,27) separation of the flavoprotein into flavin nucleotides and protein and recombination of the components to yield active enzymes was achieved. FAD accepts electrons from the substrate and transfers them to the mitochondrial electron transport chain via electron transfer flavoprotein (ETF) (28). The transfer of electrons from ETF to the mitochondrial electron transport chain has been suggested by Ruzicka and Beinert (29) to be catalyzed by ETF-ubiquinone oxidoreductase, an iron-sulfur flavoprotein highly purified from beef heart mitochondria. McKean et al., (30) have recently found ETF to be composed of two nonidentical subunits only one of which contains a FAD. The reported molecular weights of the subunits are 31,000 and 27,000 daltons. ETF is significantly more fluorescent than are the acyl-CoA dehydrogenases. The fluorescence of ETF at its emission peak at 485 to 490 nm is 3.5 times greater than that of an equivalent amount of FAD. Fluorescence of general acyl-CoA dehydrogenase, on the other hand, is less than 1% of that of the free flavin dinucleotide (24). As is the case with most flavoproteins, a bleaching of the yellow color of acyl-CoA dehydrogenase can be observed when substrate is added. This constitutes reasonable proof that the flavin nucleotide which is present in these

enzymes is in fact participating in the catalytic turnover (25). More than thirty years ago Mahler (27) attributed the deep green color of butyryl-CoA dehydrogenase to the presence of copper. However, there exists no convincing evidence that copper or any other metal ion is contributing to the spectrum or is involved in the catalytic activity of these enzymes. Williamson, Massey and Coworkers (31) have recently found that the greening ligand in native butyryl-CoA dehydrogenase from M. elsdenii, and general acyl-CoA dehydrogenase from pig kidney is a CoA persulfide. They suggest that in vivo formation of such a ligand most likely involves some form of sulfur donor. They suggest the sulfur is probably donated by hydrogen sulfide released by M. elsdenii in culture. However since mammalian tissues are sensitive to low levels of hydrogen sulfide, a more specific sulfur donor must be involved. A well documented sulfur donating enzyme rhodanese is mainly associated with the mitochondria in vivo, and may function in mammals as a sulfur donor.

The spectral characteristics of acyl-CoA dehydrogenase-substrate complexes have been extensively studied in attempts to elucidate the catalytic mechanism and kinetics of the dehydrogenase reaction (25,32,33,34). Recent studies by Hall and Lambeth (35) on the nature of the electron transfer from highly purified pig liver general acyl-CoA dehydrogenase to ETF has provided evidence for a rapid reduction of the ETF to a

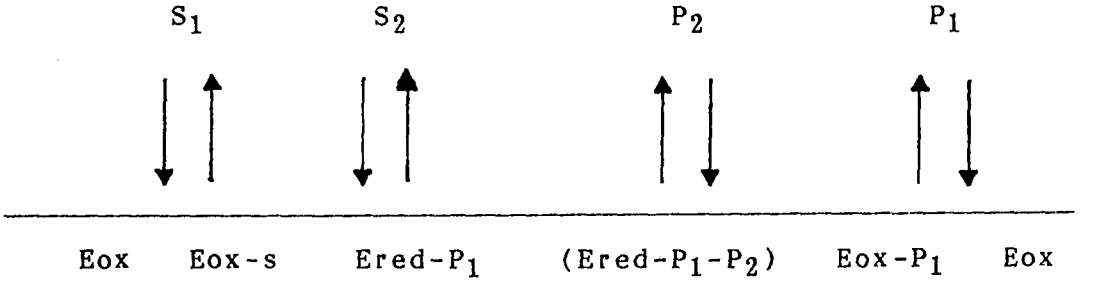
one-electron anionic semiquinone followed by the slow transfer of a second electron to form the fully reduced ETF. Hall's results show that the acyl-CoA dehydrogenase-ETF system utilizes the semiquinone catalytically. The recent work of Frerman et al. (36) on the chemical modification of the carboxyl groups of crystalized pig liver general acyl-CoA dehydrogenase has demonstrated that carboxyl residues are essential for the transfer of electrons from the enzyme to ETF. In another publication (26) he also reported the amino acid composition, isoelectric point, and a procedure for the crystalization of general acyl-CoA dehydrogenase which has facilitated mechanistic studies in greater detail.

A kinetic investigation of highly purified pig liver general acyl-CoA dehydrogenase, using the 2,6-dichlorophenolindophenol (DCPIP) - ETF coupled dye assay, has recently been reported by McKean et al. (37). The results are consistent with an ordered Bi-Bi mechanism for the oxidation of butyryl-CoA. The proposed scheme (McKean et al.)[1] is illustrated below. The final step in the dehydrogenation, which appears to be rate-limiting, is the release of tightly bound $\triangle^{2,3}$ -trans- enoyl-CoA product. The authors' results are consistent with the original spectroscopic data of Beinert and Page (33), which indicate that the product remains tightly bound to the enzyme following

---

1. McKean, M.C., Frerman, F.E., and Mielke, D. M. (1979) J. Biol. Chem. 254, Pg. 2732.

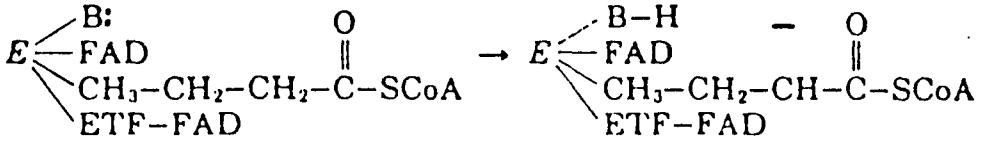
reoxidation of the dehydrogenase flavin nucleotide by the transfer of electrons
to oxidized ETF. It has been suggested that this strong product binding may be involved in the regulation of ß-oxidation in heart, under metabolic conditions at which ß-oxidation intermediates accumulate.

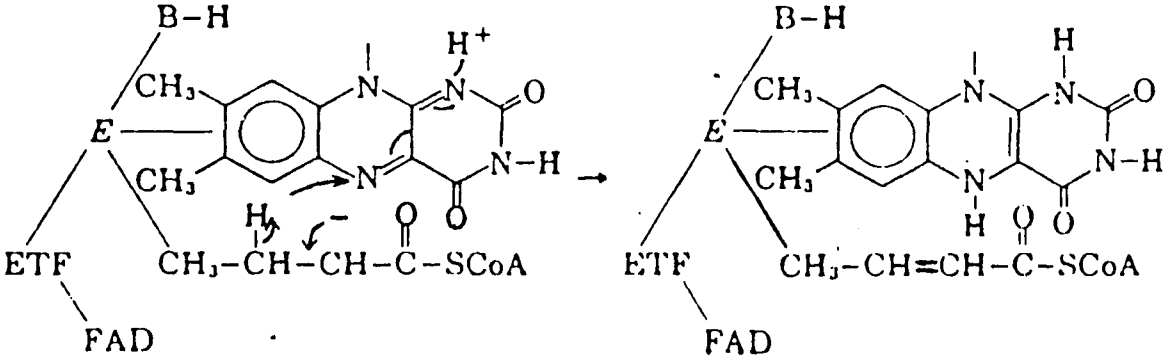


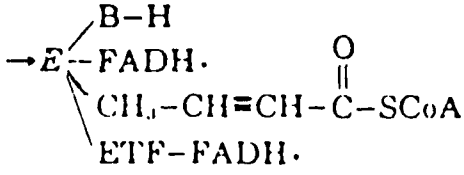
Key: S_1 - oxdizable acyl-CoA
S_2 - ETF oxidized
P_2 - ETF reduced
P_1 - enoyl -CoA

The kinetic mechanism of highly purified pig liver general acyl-CoA dehydrogenase has recently been studied by Schmidt et al. (38). Results of a Resonance Raman investigation of electron transfer between acyl-CoA substrate and oxidized flavin at the enzyme active site are consistent with base catalyzed formation of a charge transfer complex between the C-2 anion of the substrate and oxidized flavin through which electrons are trans-

ferred from substrate to N-5, C-4a of the flavin isoalloxazine ring, as illustrated below, (Schmidt et al.)[2]. It has recently been suggested that electron transfer between acyl-CoA and oxidized flavin occurs by steriospecific hydride transfer.







There is a paucity of information on in vivo and in vitro inhibitors specific for acyl-CoA dehydrogenase. Mahler (27) reported reversible inhibition of butyryl-CoA dehydrogenase

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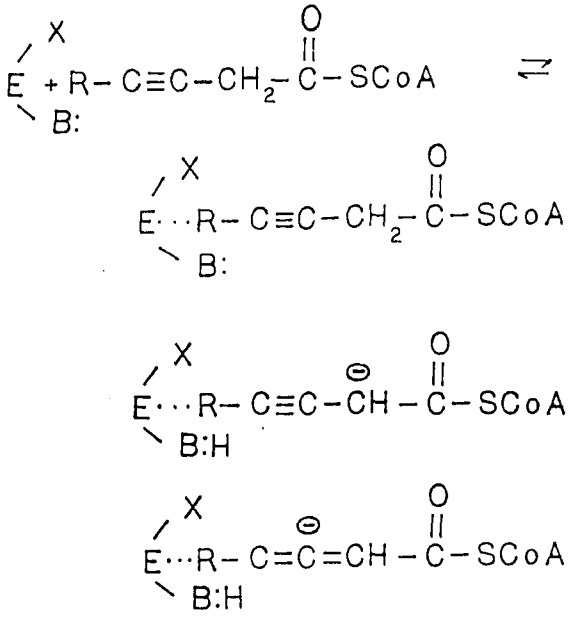
2. Schmidt, J., Reinsch, J., and McFarland, J. T. (1981) J. Biol. Chem. 256, Pg. 11667.

from bovine liver by mercurials. However, 50% inhibition of the enzyme required an inhibitor concentration of 0.3 mM. Acetoacetyl-CoA and crotonyl-CoA have been shown to inhibit reversibly acyl-CoA dehydrogenase (37), and butyryl-CoA dehydrogenase (39). Hypoglycin, an unusual amino acid, is the active and toxic component of the unripe ackee fruit. While the ripe fruit serves as a dietary staple in Jamaica, ingestion of unripe fruit causes severe hypoglycemia and often death in man (40,41). Hypoglycin was found to be metabolized to methylenecyclopropylacetate (MCPA) in mammals (42). The latter has been implicated in some of the toxic effects (40), and its CoA-ester is believed to inhibit oxidation of fatty acids both *in vivo* (43) and *in vitro* (44,45). Ghisla et al. (46) have recently demonstrated the irreversible inhibition of highly purified general acyl-CoA dehydrogenase from pig kidney by MCPA-CoA. The authors present spectral evidence for formation of a covalent adduct between the inhibitor and enzyme-bound FAD and a corresponding irreversible inactivation of the enzyme. Frerman et al. (47) have demonstrated that 3-octynoyl-CoA is a potent irreversible inhibitor of highly purified pig liver general acyl-CoA dehydrogenase. The presence of a triple bond between carbons 3 and 4 of this acyl-CoA is believed to inactivate the dehydrogenase via the following mechanism as illustrated

below. (Frerman et al.)[3].

Step No. 1

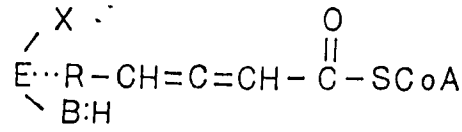
The base-catalyzed abstraction of a proton at the number 2 carbon of the 3-ynoyl-CoA, followed by isomerization to the Δ^2, Δ^3 - allene carbanion.



3. Frerman, F. E. Miziorko, H.M., and Beckmann, J.D. (1980) J. Biol. Chem. 255, Pg. 11197

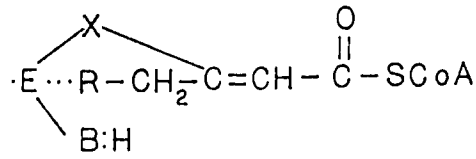
Step No. 2

Protonation of the carbanion.



Step No. 3

Reaction of a nucleophile at the enzyme active site with the number 3 carbon of the $\Delta^{2,3}$-dienoyl-CoA. A covalent bond is formed between the inhibitor and the protein and not with the flavin nucleotide as was previously suggested (48).



During our study of the regulation of fatty acid oxidation in heart several 3-ynoic compounds were tested in vitro for their ability to inhibit the bovine heart and rat heart acyl-CoA dehydrogenases.

Most recently, while writing this thesis, Tanaka's group (49) reported the separation and properties of five distinct

acyl-CoA dehydrogenases from rat liver mitochondria. Two hitherto unknown dehydrogenases which are specific for branched chain acyl-CoA's were separated from the three previously known straight chain acyl-CoA dehydrogenases. At the present time there has been no demonstration of the presence of these branch chain acyl-CoA dehydrogenases in rat heart or in other mammalian tissues.

The Regulation of Fatty Acid Oxidation in Heart

Under normal physiological conditions the heart preferentially derives energy from the mitochondrial oxidation of fatty acids (50). Glycogenolysis and specifically the oxidation of pyruvate derived from glycogen provide most of the energy during the onset of heavy work. Under conditions where the blood contains high concentrations of ketone bodies, these metabolites can be oxidized and contribute significantly to the energy metabolism in heart muscle (51).

The regulation of fatty acid oxidation in heart has been extensively studied by many scientists. However, a unifying picture of how the rate of fatty acid oxidation is tuned to the energy demand of the cell has not yet emerged. More specifically the regulatory sites, mechanisms, and feedback metabolites have not been identified.

Although there is no consensus as to the regulation of

fatty acid oxidation in heart muscle, it is generally accepted that the rate of fatty acid oxidation is directly related to the plasma free fatty acid concentration and to the energy demand of the tissue. The plasma concentration of free fatty acids is hormonally regulated via the hormone sensitive lipase of adipose tissue (52). Hormone sensitive lipase is regulated by phosphorylation -dephosphorylation in response to several hormones. The phosphorylated enzyme is active and predominates when the intracellular glucagon and epinephrine is high (53). Insulin causes dephosphorylation of the enzyme and thus inhibits lipolysis (54). Circulating albumin-bound non-esterified fatty acids reach tissues where their uptake by cells and their cytosolic transport remain incompletely characterized. However, several workers have recently suggested that a fatty acid binding protein is involved in the transfer of fatty acids across the cytosol of heart cells. Z protein may function in this same capacity in liver (55,56,57).

It has been suggested that the activation of fatty acids by acyl-CoA synthetase and/or their subsequent transfer to carnitine via carnitine palmitoyl transferase I may be regulatory steps in fatty acid oxidation. Acyl-CoA synthetase may be regulated under physiological conditions by palmitoyl-CoA. Pande (58) reports that palmitoyl-CoA inhibits the synthetase by increasing the K_m for CoASH. Oram et al. (59) propose that the intramitochondrial concentration of acetyl-

CoA may determine the cytosolic CoASH concentration and thereby the activity of acyl-CoA synthetase. The author suggests that the rate of fatty acid activation could be restricted by low levels of cytosolic CoASH when the rate of acetyl-CoA production from ß-oxidation exceeds the rate of its oxidation through the TCA cycle as it occurs in hearts perfused with high concentrations of exogenous palmitate (60).

Recently Mc Garry and Foster (61) have suggested that in liver malonyl-CoA may regulate the activity of carnitine palmitoyl transferase I. However, this type of regulation is unlikely to function in heart where malonyl-CoA is not synthesized. In contrast to these results, in vitro measurements performed by Pande (62) led him to conclude that the optimal activities of acyl-CoA synthetase and carnitine palmitoyl transferase I are higher than the capacity of heart mitochondria to oxidize fatty acids. It has also been proposed that acyl-CoA dehydrogenase may catalyze a slow step in the pathway. Based on in vitro assays acyl-CoA dehydrogenase appears to be 10 times less active than subsequent ß-oxidation enzymes (63). However, results of in vitro assays may be poor indicators of in vivo enzyme activities, and in the case of the acyl-CoA dehydrogenases may yield lower than optimum activity values because the assays are performed with artificial electron acceptors.

Present in the matrix space of mitochondria are compounds

which theoretically may function in the regulation of one or more of the enzymes of the ß-oxidation pathway. Lumeng, Davis and Coworkers (64) suggest that the activity of 3-hydroxyacyl-CoA dehydrogenase may be regulated by the $NADH/NAD^+$ ratio. Olowe and Schulz (65) suggest that 3-ketoacyl-CoA thiolase is regulated by intramitochondrial acetyl-CoA which tunes the rate of fatty acid oxidation to the rate of the citric acid cycle. Fong and Schulz (66) have shown by the use of the inhibitor 4-pentenoic acid that in coupled rat heart mitochondria, fatty acid oxidation and 3-ketoacyl-CoA thiolase are inhibited in a parallel fashion. Similarly Olowe and Schulz (67) in a recent kinetic evaluation of the inhibition caused by 4-bromocrotonic acid in coupled rat heart mitochondria, demonstrated that 3-ketoacyl-CoA thiolase and respiration supported by palmitoylcarnitine are inactivated at equal rates. These findings point to the thiolase I catalyzed reaction as a (the) rate-limiting step in fatty acid oxidation in heart.

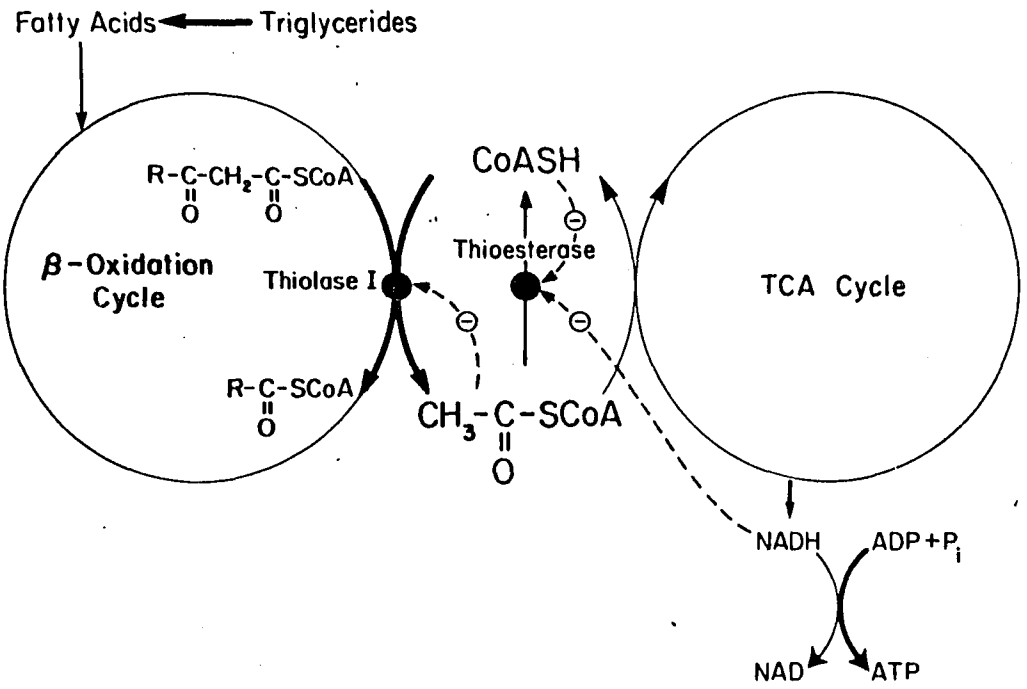
Schulz's group propose that the rate of fatty acid oxidation in heart is adjusted to the rate of the citric acid cycle and ultimately to the energy demand of the cell via feedback regulation of 3-ketoacyl-CoA thiolase by acetyl-CoA. The acyl-CoA thioesterase (EC 3.1.2.1) to (EC 3.1.2.11) in turn is regulated by NADH, CoASH and ATP (68). This enzyme provides free CoASH for oxidative metabolism when the energy requirement of the cell is high, but the citric acid cycle operates at an

insufficient rate. Under such conditions the thioesterase will uncouple fatty acid oxidation from the citric acid cycle, thereby allowing ß-oxidation to operate at an optimal rate, a diagramatic representation is presented in Fig. 1. However, this proposal raises but does not answer the question whether a feedback connection exists between the last and first steps of ß-oxidation. A major goal of this thesis is to answer this question.

Fig. 1. Diagrammatic representation of the integrated pathways of β-oxidation, TCA cycle and oxidative phosphorylation.

Fig. 1

REGULATION OF FATTY ACID OXIDATION IN HEART



Little is known about a possible physical association of the enzymes of ß-oxidation in mammals. In contrast, the enzymes of ß-oxidation in E. Coli have been shown by Schulz and coworkers to be part of a 260,000 dalton multi enzyme complex (69,70). Early reports (71) about the absence of intermediates of fatty acid oxidation in mitochondria led to the suggestion that the ß-oxidation enzymes are organized intramitochondrially as a loosely associated complex to which the ß-oxidation intermediates remain bound (72,73). However, more recent attempts to detect intermediates of ß-oxidation have demonstrated that various long-chain 3-hydroxy intermediates including 3-hydroxy fatty acids accumulate in rat heart mitochondria poisoned with rotenone (74). Very recently Moore et al. have demonstrated that medium and long-chain 3-hydroxy fatty acids accumulate in coupled rabbit heart mitochondria incubated with rotenone (75). However, terminal respiratory chain inhibition by cyanide sufficient to inhibit palmitoyl-CoA oxidation by 85% did not cause 3-hydroxy intermediates to accumulate, even though $NADH/NAD^+$ ratios were comparable to those attained with rotenone.

Whether other regulatory metabolites or sites exist must be established before the entire picture of the regulation of fatty acid oxidation in heart is complete. To this end my thesis addresses the following questions:

1. How many acyl-CoA dehydrogenases are present in heart?

2. What are the kinetic properties of these dehydrogenases?

3. Do mitochondrial coenzymes and metabolites inhibit and thus regulate these dehydrogenases?

4. Do regulatory metabolites change in concentration in response to a changing energy demand of the heart?

5. Does acyl-CoA dehydrogenase catalyze the rate limiting, or one of the slow reactions in ß-oxidation?

EXPERIMENTAL PROCEDURES

Materials - CoASH and CoA derivatives of saturated fatty acids were purchased from P-L Biochemicals, Inc. Sigma Chemical Company was the source of DL-3-hydroxybutyric acid, phenazine methosulfate, 2,6-dichlorophenolindophenol, 9-octadecynoic acid, and L-3-hydroxyacyl-CoA dehydrogenase. 2-Undecanone, trans-2-decenoic acid, and trans-2-hexadecenoic acid were obtained from Aldrich Chemical Company. 3-octyne-1-ol was purchased from CDS Laboratories. Methyl 3-ketopalmitate was provided by Professor Horst Schulz, Department of Chemistry, City College of New York. [16-^{14}C] Palmitic acid was purchased from New England Nuclear. L-Carnitine and palmitoyl-(L)-carnitine were generously provided by Dr. K. Brendel, University of Arizona College of Medicine. Fresh bovine heart and liver were bought from Max Insel Cohen Company, Livingston, N. J. DL-3-Hydroxydecanoic acid was synthesized by reduction with $NaBH_4$

of ethyl 3-ketodecanate, prepared according to an established procedure (76), followed by hydrolysis. The CoA derivatives of DL-hydroxybutyric acid, 2-decenoic acid, 2-hexadecenoic acid, 3-octynoic acid, and DL-3-hydroxydecanoic acid were synthesized from the corresponding free acids and CoA by the method of Goldman and Vagelos (77). 3-Ketodecanoyl-CoA, and 3-ketopalmitoyl-CoA were prepared enzymatically from the respective trans-2-enoyl-CoA according to the procedure of Seubert et al. (78). Acetoacetyl-CoA (79) and crotonyl-CoA (80) were prepared by established procedures. 3-Octynoic acid was prepared by oxidation of 3-octyn-1-ol as described by Stoffel et al. (81). [16-^{14}C] Palmitoyl-CoA was synthesized from the corresponding free acid (57 mCi/mmol) and CoA by a modification of the method of Goldman and Vagelos (77). The product had a specific activity of 55 x 10^3 dpm/nmol. The concentrations of 3-ketodecanoyl-CoA, and 3-ketopalmitoyl-CoA were estimated by recording at 340 nm the oxidation of NADH in the presence of 3-hydroxyacyl-CoA dehydrogenase. The concentrations of all other acyl-CoA compounds were determined by the method of Ellman (82) after cleaving the thioester bond with hydroxylamine at pH 7.0.

Acyl-CoA Dehydrogenase and Protein Assays - All three acyl-CoA dehydrogenases were assayed spectrophotometrically by following at 600 nm the acyl-CoA dependent reduction of 2,6-dichlorophenolindophenol in the presence of phenazine

methosulfate as outlined by Hoskins (83). The assay mixture contained 0.1 M potassium phosphate (pH 7.6) 28 µM 2,6-dichlorophenolindophenol, 0.65 mM phenazine methosulfate, 20 µM acyl-CoA, 0.2 mM N-ethylmaleimide, and enzyme to obtain a ΔA/min of approximately 0.06. The reaction was initiated by the addition of enzyme when partially purified acyl-CoA dehydrogenase was assayed. Phenazine methosulfate was added last when mitochondria or a mitochondrial extract was used as an enzyme source. Additional components were 0.09% Triton X-100 to disrupt the mitochondria and 0.45 mM KCN to prevent the nonspecific reoxidation of phenazine methosulfate. When general acyl-CoA dehydrogenase was assayed, the concentration of phenazine methosulfate (PMS) was increased to 6.5 mM. N-Ethylmaleimide was present in the assay mixture to reduce the background rate due to the reaction of 2,6-dichlorophenolindophenol with sulfhydryl groups. N-Ethylmaleimide at the concentration used had no effect on the activities of the acyl-CoA dehydrogenases. Assays were performed at 25°C and an extinction coefficient of 21,300 M^{-1} was used to calculate rates which were corrected for nonspecific reactions. A unit of enzyme activity is defined as the amount of enzyme that catalyzes the dehydrogenation of one micromole of substrate per minute. K_I values were determined graphically from Dixon plots. Lines were drawn based on least-squares treatment of the data. Protein concentrations were determined

according to Lowry et al. (84).

Separation and Partial Purification of Acyl-CoA Dehydrogenases from Bovine Liver and Heart - Mitochondria from fresh bovine liver were prepared essentially by the method of Fleischer et al. (85). Mitochondria from fresh bovine heart were prepared by the method of Crane et al. (86). Frozen mitochondria (5-10 gm) were quickly thawed and diluted with ten volumes of 10 mM potassium phosphate (pH 7.6) and subjected to sonic oscillation in an ice-cooled Rosette cell for 90 s at 50 W with a Branson sonifier, (Model W185D) equipped with a micro tip. Soluble mitochondrial proteins were isolated by centrifugation at 100,000 x g for 60 min at 4°C. The sonic supernatant containing 100-500 mg of protein was applied to a DEAE-cellulose column (2.5 x 30 cm) which had previously been equilibrated with 50 mM potassium phosphate (pH 7.6). The column was washed with three column volumes of the same buffer until material absorbing light at 280 nm ceased to be eluted. The resulting column was washed with six column volumes of 100 mM potassium phosphate (pH 7.6) and fractions of 19 ml were collected until all butyryl-CoA dehydrogenase activity had been eluted. The most active fractions were combined and concentrated to approximately 1 ml in an Amicon concentrator (PM-10 membrane) and stored at -76°C. The column was then washed with three column volumes of 0.5 M potassium phosphate (pH 7.6). Fractions of 18 ml were collected and assayed with decanoyl-CoA and palmitoyl-CoA as substrates.

The most active fractions were combined and concentrated to approximately 10 ml and dialyzed against 6 liters of 10 mM potassium phosphate (pH 7.6) for 6 hours with two changes of buffer. The dialyzate was centrifuged at 20,000 x g for 15 min to remove small amounts of precipitated material. The resulting supernatant was diluted with 2.5 volumes of dialysis buffer and applied to a hydroxylapatite column (2.5 x 7 cm) which had previously been equilibrated with the dialysis buffer. The column was washed with 1.5 column volumes of dialysis buffer and 2 column volumes of 50 mM potassium phosphate (pH 7.6) until material absorbing light at 280 nm ceased to be eluted. The resulting column was developed with a gradient made up of 5 column volumes each of 50 mM potassium phosphate (pH 7.6) and 300 mM potassium phosphate (pH 7.6) at a flow rate of 1.5-2 ml per min. Fractions of 5 ml were collected and assayed with butyryl-CoA, decanoyl-CoA, and palmitoyl-CoA substrates. Fractions of highest activity representing either medium-chain or long-chain acyl-CoA dehydrogenase were separately combined, concentrated to approximately 1ml and stored at -76°C.

Disc Gel Electrophoresis Disc gel electrophoresis was performed on 7.5% polyacrylamide gels (pH 8.9) at 16°C as described by Davis. (87) Gels were stained for protein with Coomassie blue R, destained with 7% acetic acid and scanned at 550 nm. Duplicate unstained gels were sliced into 2 mm sections and extracted for 12 hrs. at 4°C with 1 ml of 0.3 M potassium phosphate (pH 7.6)

containing 1 mg/ml of bovine serum albumin. Assays of extracted gels were performed as described above at optimal concentrations of substrate and PMS. The assay reaction was started by the addition of acyl-CoA after recording background changes in absorbance for one min.

Isolation of Mitochondria Coupled rat heart mitochondria were isolated by the procedure of Chappell and Hansford (88). The isolation buffer contained 0.21 M mannitol, 0.07 M sucrose, 5 mM Tris-HCl (pH 7.4) and 1 mM EGTA[4]. Protein concentrations were determined by the biuret method (89). All experimental data presented was obtained using mitochondria with RCR values between 4.0 and 6.0.

Measurement of Oxygen Uptake by Mitochondria - Mitochondria (0.5 to 1 mg/ml) were incubated in 1.9 ml of a basal isotonic medium containing 0.11 M KCl, 33 mM Tris-HCl (pH 7.4), 2 mM KPi, 2 mM $MgCl_2$ and 0.1 mM EGTA. To this suspension were added bovine serum albumim (0.5 mg/ml), 0.5 mM L-malate, and 60 µM L-carnitine when palmitoyl-CoA served as substrate. Respiration states were adjusted to 3 or 4 by addition of 1 mM ADP, or 50 µM ADP respectively. Respiration rates were measured polarographically by use of a Clark oxygen electrode with either palmitoyl-(L)-carnitine (30 µM) or palmitoyl-CoA (3.75 µM-7.5 µM) substrates. Values observed with the above mentioned

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4. EGTA; ethylene glyco-bis (ß-aminoethylether) -N,N' -tetra acetic acid.

substrates at optimal and limiting concentration of ADP are listed in table 1. (see also Fig. 2).

Table I

Oxygen uptake by coupled rat heart mitochondria at an optimal concentration of ADP (1 mM), and a limiting concentration of ADP (50 μM). Oxygen uptake measurements are described under "Experimental Procedures".

Substrates	Concentration	Oxygen uptake	ADP Concentration
		(ng atoms O_2/Min/mg protein)	
Palmitoylcarnitine	30 μM	206 ± 18	1 mM
Palmitoylcarnitine	30 μM	43 ± 8	50 μM
Palmitoyl-CoA	3.75 μM	218 ± 10	1 mM
Palmitoyl-CoA	3.75 μM	48 ± 5	50 μM
Palmitoyl-CoA	7.5 μM	340 ± 30	1 mM
Palmitoyl-CoA	7.5 μM	75 ± 10	50 μM

Fig. 2. State 3 and state 4 oxidation rates of palmitoyl-CoA as a function of ADP concentration in coupled rat heart mitochondria. For experimental details see under "Experimental Procedures". The numbers represent the rates of respiration in ng atoms O_2/min/mg protein.

A. C_{16}-CoA, palmitoyl-CoA (3.5 µM),

B. C_{16}-CoA, palmitoyl-CoA (3.5 µM). The concentration of ADP appears next to the corresponding slope.

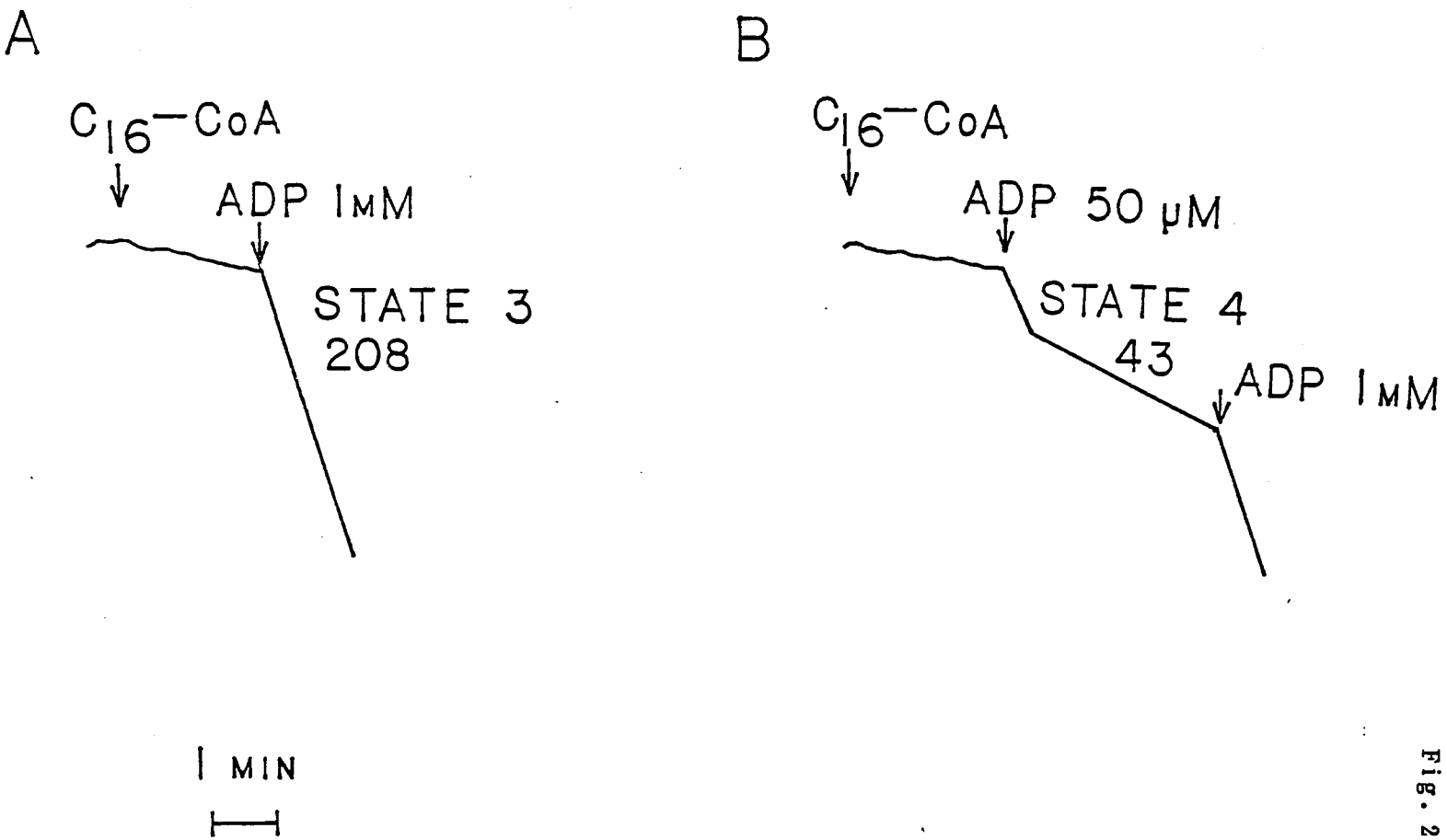

A
C_{16}—CoA
ADP 1mM
STATE 3
208
1 MIN
B
C_{16}—CoA
ADP 50 μM
STATE 4
43
ADP 1mM

Fig. 2

Determination of Enzyme Activities in Mitochondria Incubated with Inhibitors of Acyl-CoA Dehydrogenase - Potential inhibitors of acyl-CoA dehydrogenase were tested for their effectiveness by preincubating them with mitochondria for up to 5 min. Aliquots of the incubation mixture (50 µl-200 µl) were removed and rapidly frozen in dry ice - methanol and stored at -76°C until assayed. The remaining acyl-CoA dehydrogenase activity was determined by assaying each sample at an optimal concentration of PMS with butyryl-CoA, decanoyl-CoA or palmitoyl-CoA as described previously.

Mitochondrial Metabolites of [16 - ^{14}C] Palmitoyl-CoA Formed During Respiration States 3 and 4 - Incubations were carried out in a shaker bath at 30°C in 25 ml stoppered flasks with center wells which contained 0.25 ml of 1 M hyamine hydroxide. Incubation mixtures contained between 3.75 and 7.5 µM [16 - ^{14}C] palmitoyl-CoA (2×10^6 - 4×10^6 dpm) in 5-10 ml of basal medium. Respiration states were adjusted to 3 or 4 by addition of the amounts of ADP to give final concentrations of 1 mM and 50 µM, respectively. The incubation periods were predetermined from polarographic measurements of oxygen consumption for equivalent concentrations of unlabelled palmitoyl-CoA during state 3 and state 4 respiration periods (e.g. Fig. 2). Reactions were initiated by the rapid addition of mitochondrial protein (5-10 mg) to a final concentration of 1 mg per ml. Incubations were carried out for the predetermined period (1-2 min) and were

then terminated by addition of 8 M NaOH to a final concentration of 1.25 M. Reaction products, including labelled acyl-CoA, were saponified by heating the incubation mixture, at 90°C for 2 hrs., or at 25°C for 12 hrs. The hydrolyzate was adjusted with 6 M HCl to pH 2.0 to which was added a mixture of 1 mg each of 3-hydroxy decanoic acid, decanoic acid and palmitic acid and extracted 5-times into equal volumes of diethyl ether. The products were concentrated under a stream of dry nitrogen to a volume of approximately 0.25 ml, diluted with 2 ml of diethyl ether and applied equally to two silica G plates (20 x 20 cm).

Each of these duplicate TLC plates was placed into a separate chromatography tank containing 100 to 200 ml of either solvent system 1 or 2. A separate TLC plate containing standards of 3-hydroxy and saturated fatty acid was developed separately.

Solvent System 1:

Hexane: ether: acetic acid (70:30:2)

Solvent System 2:

Hexane: ether: methanol: acetic acid (50:50:5:1)

All TLC plates were developed until the solvent front had moved 16 cm from the origin. The plates were dried at room temperature in a fume hood. TLC plates with the mixture of standard fatty acids were exposed to iodine vapors or were sprayed with a solution of bromocresol green. TLC plates containing the mitochondrial extract were divided into 2 cm-wide sections. The Silica of each section was carefully scraped from the plate and

placed in a scintillation vial. Each fraction was counted in 10 ml of a toluene-based cocktail for 2- 20 min periods using the wide ^{14}C - channel of a Beckman scintillation counter (Model LS 150). The data was corrected for sample quenching and plotted as radioactivity vs. distance of migration. The identity of a radioactive peak with a fatty acid was assumed to be proven when the migration distance of the unknown matched that of the standard fatty acid in both solvent systems. Concentrations of mitochondrial metabolites were calculated on the basis of the known specific activity of 55 x 10^3 dpm per nanomole [16 -^{14}C] palmitoyl-CoA and assuming a matrix volume of 1 µl per mg of rat heart mitochondria. [16-^{14}C] 3-Ketoacyl-CoA thioesters were converted to [16 -^{14}C] methyl ketones. For this purpose the incubation mixtures were saponified as described and were then refluxed for 6 hrs. in 100 ml boiling flasks containing 2 mg of 2-undecanone and methyl 3-ketopalmitate as carriers. This treatment resulted in decarboxylation of 3-keto acids to their 1-carbon shorter methyl ketones. Products were extracted from the alkaline reaction solution with 5 volumes of petroleum ether. The ether phase was washed 3-times with 10% $NaHCO_3$ thereby reducing the carryover of labelled palmitate. TLC plates were prepared as described and developed in the following solvent systems:

Solvent System 1:

Toluene: diethyl ether (95:5)

Solvent System 2:

Chloroform: petroleum ether: diethyl ether (70:30:2)

2-Undecanone and 2-pentadecanone, which served as standards, were visualized by spraying the plates with a saturated solution of diaminophenylhydrazine in 2 N HCl. The ^{14}C - containing products were quantitated as described above.

RESULTS

Separation and Identification of Acyl-CoA Dehydrogenases of Bovine Liver and Heart - When a soluble extract of either bovine liver or bovine heart mitochondria was subjected to chromatography on DEAE-cellulose, a complete separation of butyryl-CoA dehydrogenase from medium-chain and long-chain acyl-CoA dehydrogenase activities was achieved by a simple step-wise elution procedure (see Figs. 3, 4) The separation of the two acyl-CoA dehydrogenases co-chromatographing on DEAE-cellulose was accomplished by chromatography on hydroxylapatite (see Figs. 5, 6). Development of the hydroxylapatite column with a linear potassium phosphate gradient resulted in the elution of first an acyl-CoA dehydrogenase that was most active with decanoyl-CoA as a substrate, less active with palmitoyl-CoA and almost inactive with butyryl-CoA (see Figs. 5, 6). Following the first acyl-CoA dehydrogenase, but well separated from it, appeared a second dehydrogenase that was highly active with both decanoyl-CoA and palmitoyl-CoA as substrates but was significantly less active towards butyryl-CoA. Based on their relative activities towards these three substrates it was not possible to match the two bovine liver acyl-CoA dehydrogenases with the corresponding pig liver enzymes described by Beinert (25). Of greatest importance was the finding that extracts from bovine liver and heart mitochondria yielded identical purification patterns for the acyl-CoA dehydrogenases. Thus

Fig. 3. Separation of bovine liver butyryl-CoA dehydrogenase from medium-chain and long-chain acyl-CoA dehydrogenases by chromatography on DEAE-cellulose. For experimental details see under "Experimental Procedures". Substrates used for assaying acyl-CoA dehydrogenases were:

(▲), butyryl-CoA; (●), decanoyl-CoA;

(■), palmitoyl-CoA.

Fig. 3

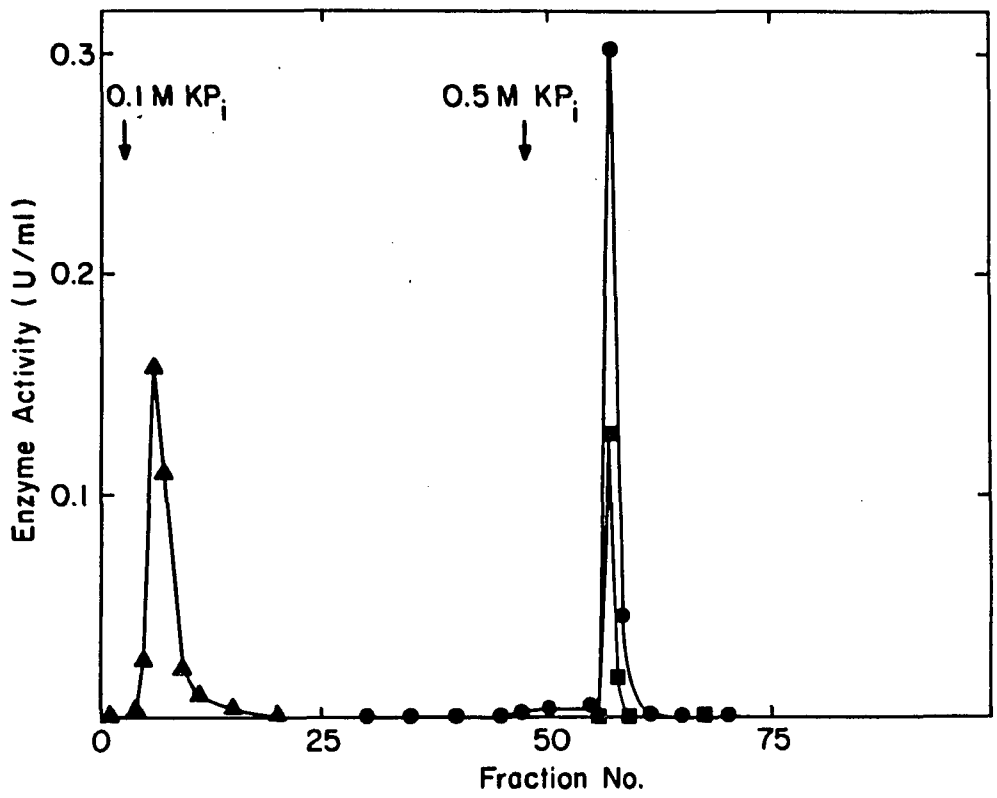

0.1 M KP$_i$
0.5 M KP$_i$
Enzyme Activity (U/ml)
0.3
0.2
0.1
0
25
50
75
Fraction No.

Fig. 4. Separation of bovine heart butyryl-CoA dehydrogenase from medium-chain and long-chain acyl-CoA dehydrogenases by chromatography on DEAE-cellulose. For experimental details see under "Experimental Procedures". Substrates used for assaying acyl-CoA dehydrogenases were:

(▲), butyryl-CoA; (●), decanoyl-CoA;

(■), palmitoyl-CoA.

Fig. 4

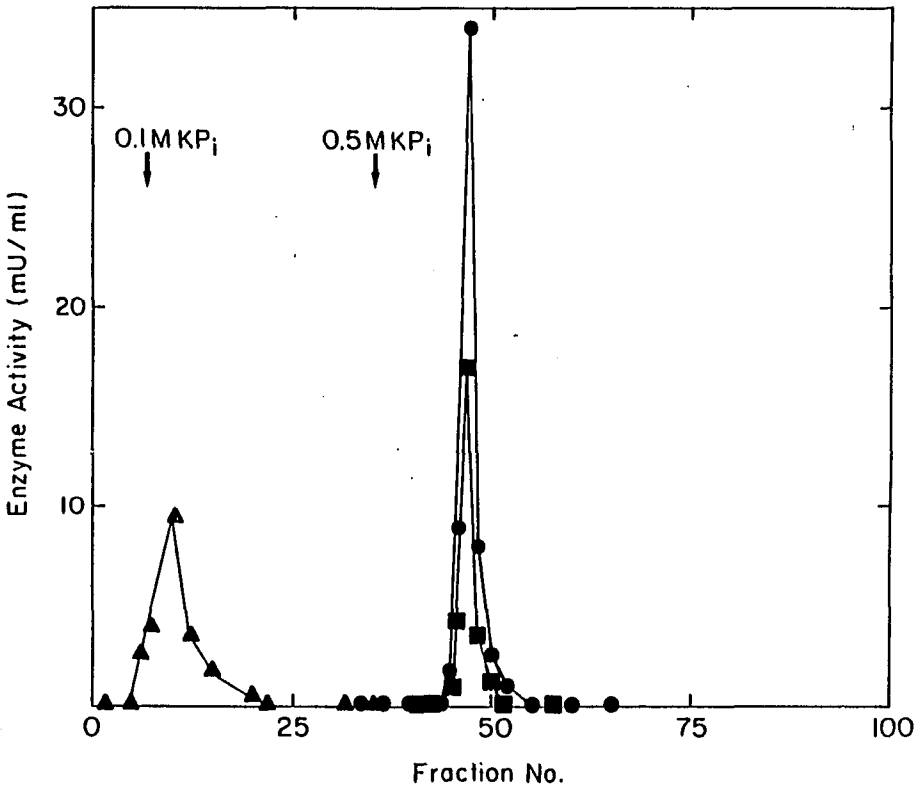

0.1M KPi
0.5M KPi
Enzyme Activity (mU/ml)
30
20
10
0
25
50
75
100
Fraction No.

Fig. 5. Separation of bovine liver medium-chain and long-chain acyl-CoA dehydrogenases on hydroxylapatite. For experimental details see under "Experimental Procedures". Substrates used for assaying acyl-CoA dehydrogenase were:

(▲), butyryl-CoA; (●), decanoyl-CoA;

(■), palmitoyl-CoA.

Fig. 5.

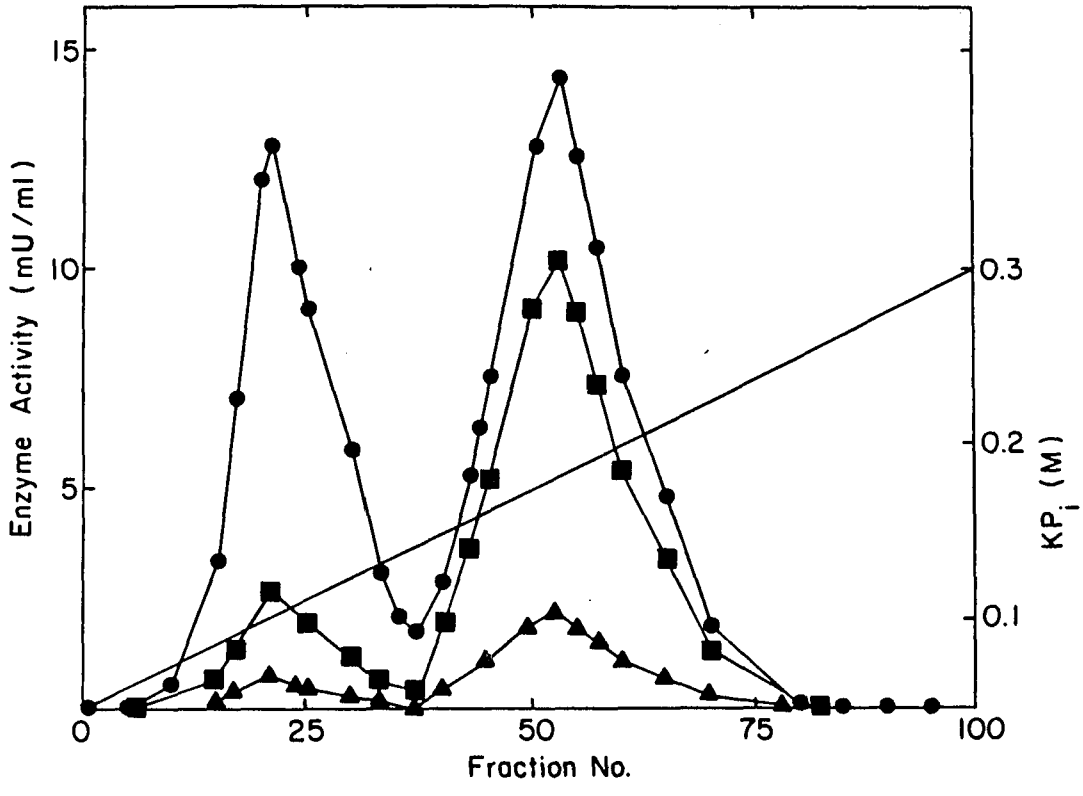
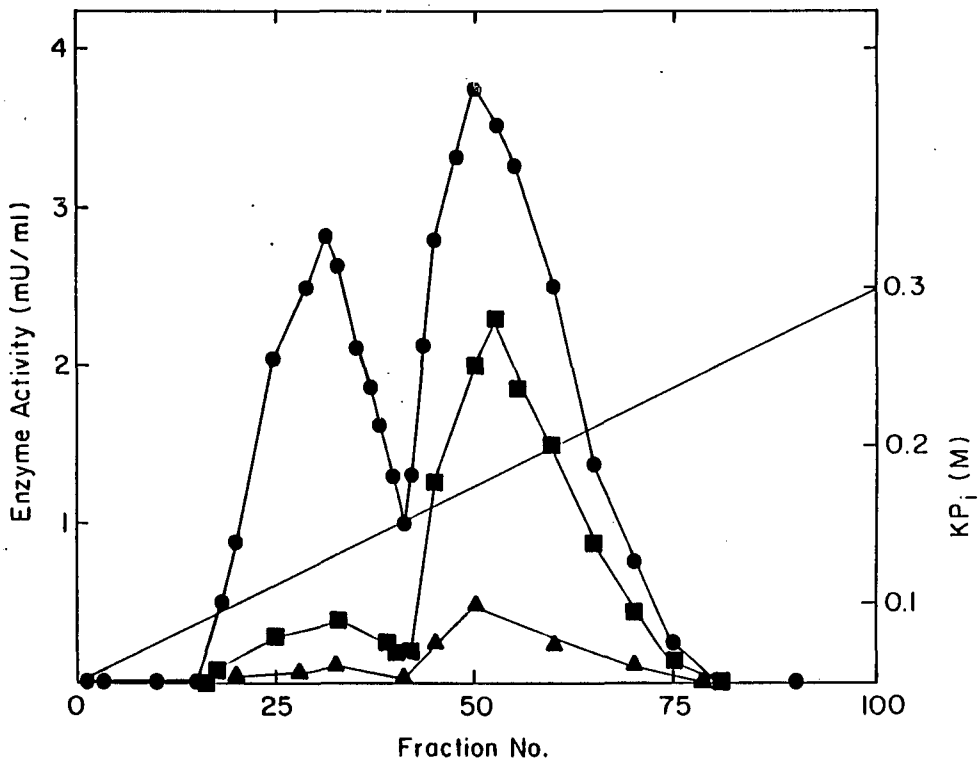

15
10
5
0
25
50
75
100
Fraction No.
Enzyme Activity (mU/ml)
0.3
0.2
0.1
KP_i (M)

Fig. 6. Separation of bovine heart medium-chain and long-chain acyl-CoA dehydrogenases on hydroxylapatite. For experimental details see under "Experimental Procedures". Substrates used for assaying acyl-CoA dehydrogenase were:

(▲), butyryl-CoA; (●), decanoyl-CoA;

(■), palmitoyl-CoA.

Fig. 6.


Enzyme Activity (mU/ml)
4
3
2
1
0
25
50
75
100
Fraction No.
0.3
0.2
0.1
KPi (M)

both organs contain possibly identical sets of three acyl-CoA dehydrogenases. The three acyl-CoA dehydrogenases isolated from bovine liver and heart were subjected to polyacrylamide disc gel electrophoresis and were found to contain several proteins (see Figs. 7, 8, 9). The most abundant of these proteins, which accounted for approximately 30% of the material, was identified as acyl-CoA dehydrogenase by assaying extracts of gel segments. Furthermore, these experiments proved that the corresponding acyl-CoA dehydrogenases from bovine liver and bovine heart have the same relative mobilities on polyacrylamide gels, (Fig. 10).

Chain Length Specificities and Some Kinetic Properties of Acyl-CoA Dehydrogenases - The chain length specificities of the three acyl-CoA dehydrogenases isolated from bovine liver and bovine heart were determined at 20 μM substrate concentrations (see Fig. 11). The patterns of activity versus substrate chain length for corresponding dehydrogenases from heart and liver were found to be identical. Butyryl-CoA dehydrogenase is most active with butyryl-CoA and only slightly less active with hexanoyl-CoA. However, it was found to be virtually inactive with longer chain substrates (see Fig. 11). The acyl-CoA dehydrogenase which was eluted last from the hydroxylapatite column, was found to be most active with octanoyl-CoA as a substrate. When this dehydrogenase was assayed at the standard concentration of phenazine methosulfate of 0.65 mM, its activity

Fig. 7. Disc gel electrophoresis of partially purified bovine liver butyryl-CoA dehydrogenase. The trace shows a spectrophotometric scan of gel (III)-L, (see Fig. 10) which had been stained for protein. The total protein applied to duplicate unstained gels was increased to 450 µg to compensate for a low percentage of recoverable enzyme activity in the extracts of frozen gels. Extracts of 2 mm gel slices were assayed with 50 µM butyryl-CoA. For experimental details see under "Experimental Procedures".

Fig. 7

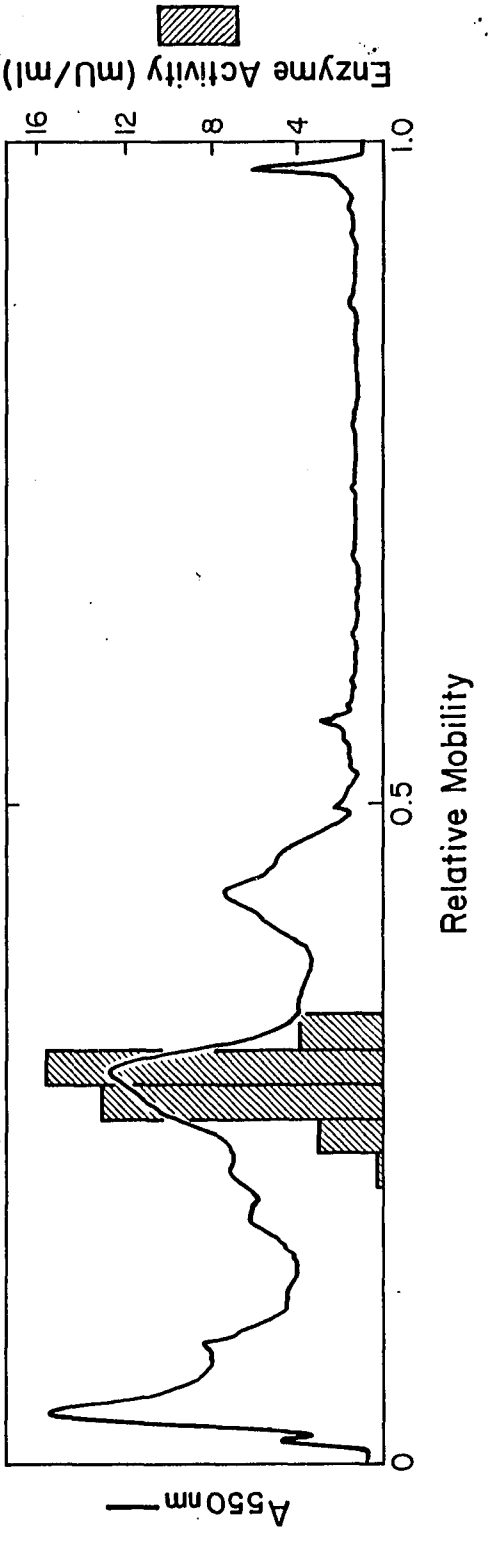

Enzyme Activity (mU/ml)
16
12
8
4
1.0
0.5
0
Relative Mobility
A550nm

Fig. 8. Disc gel electrophoresis of partially purified bovine liver medium-chain acyl-CoA dehydrogenase. The trace shows a spectrophotometric scan of gel (I)-L, (see Fig. 10) which had been stained for protein. The total protein applied to duplicate unstained gels was increased to 180 µg to compensate for a low percentage of recoverable enzyme activity in the extracts of frozen gels. Extracts of 2 mm gel slices were assayed with 50 µM octanoyl-CoA. For experimental details see under "Experimental Procedures".

Fig. 8

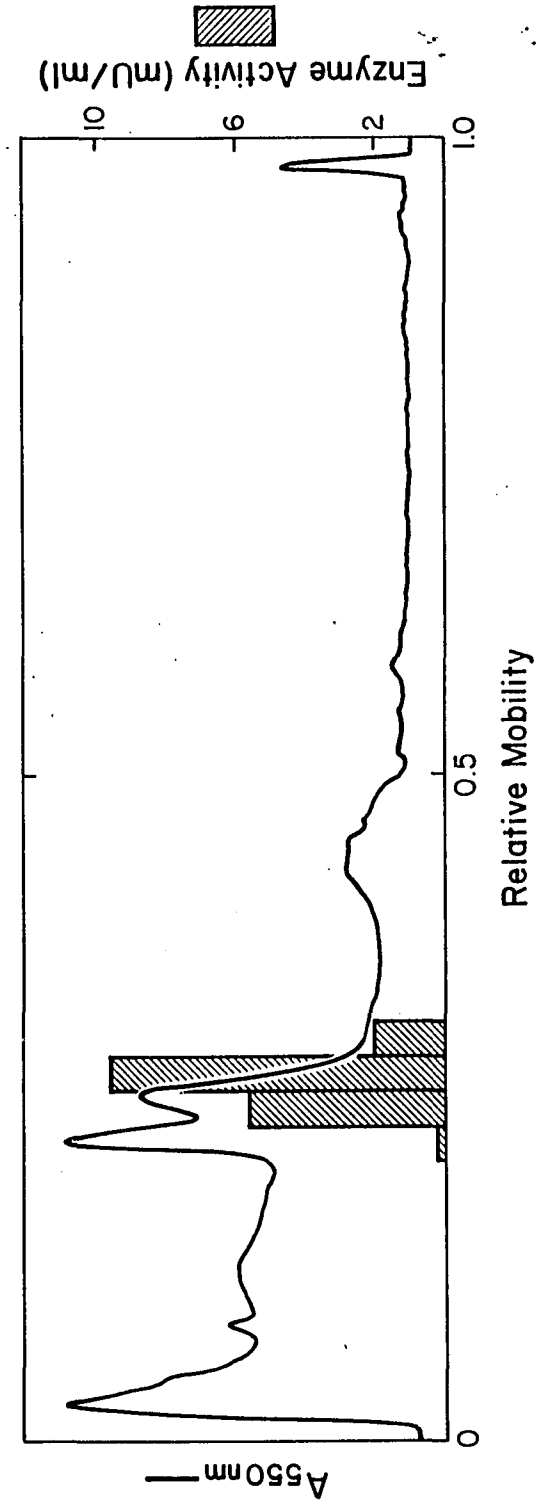


Fig. 9. Disc gel electrophoresis of partially purified bovine liver long-chain acyl-CoA dehydrogenase. The trace shows a spectrophotometric scan of gel (II)-L, (see Fig. 10) which had been stained for protein. The total protein applied to duplicate unstained gels was increased to 560 µg to compensate for a low percentage of recoverable enzyme activity in the extracts of frozen gels. Extracts of 2 mm gel slices were assayed with 50 µM dodecanoyl-CoA. For experimental details see under "Experimental Procedures".

Fig. 9

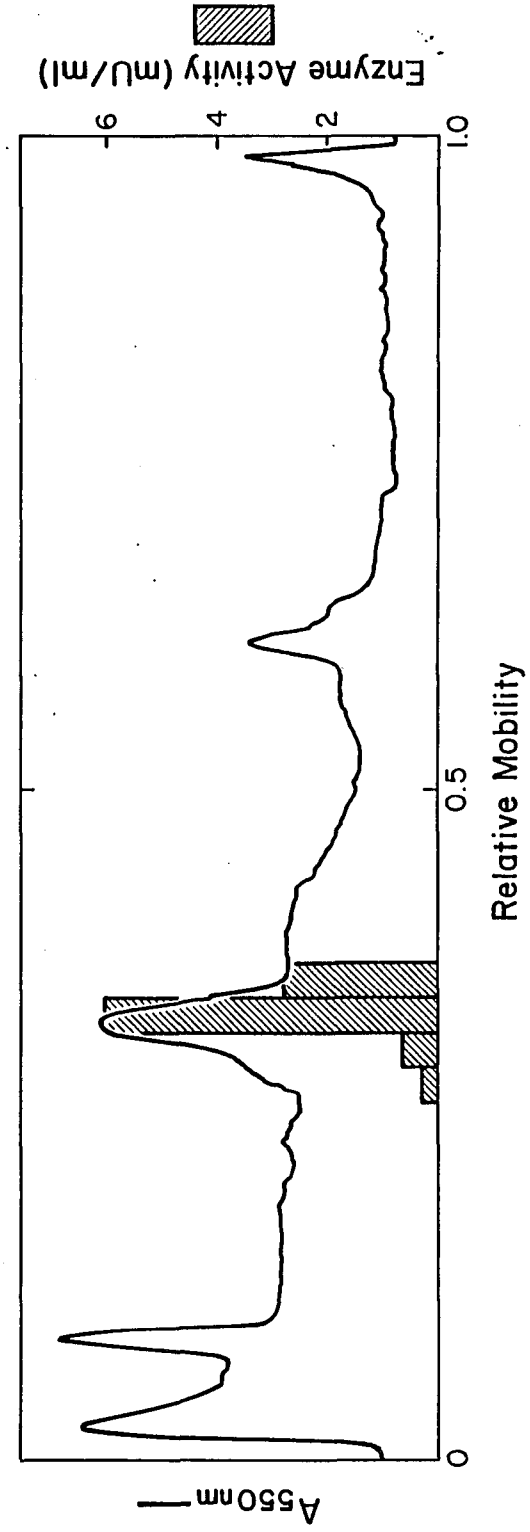


Fig. 10. Disc gel electrophoresis of the partially purified acyl-CoA dehydrogenases on 7.5% potyacrylamide gels. The gels each contained 25 µg protein and were stained with coomassie blue R. For experimental details see under "Experimental Procedures". (I), (II), (III). Refer to medium-chain acyl-CoA dehydrogenase, long-chain acyl-CoA dehydrogenase and butyryl-CoA dehydrogenase respectively. (L), Liver enzyme, (H), Heart enzyme. The arrows indicate the position of the acyl-CoA dehydrogenase activities.

Fig. 10

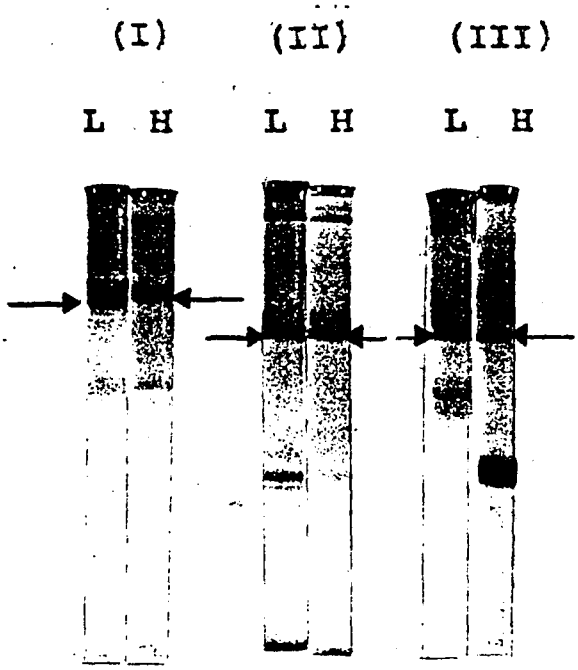
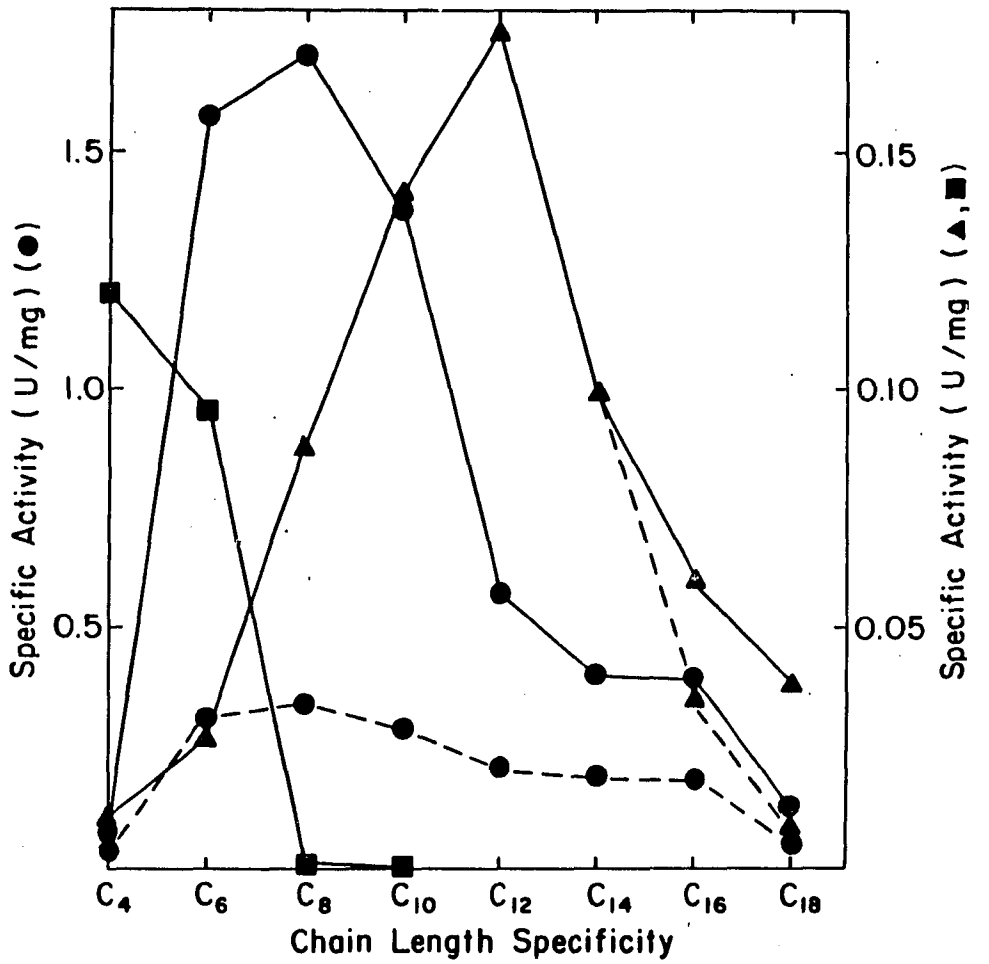


Fig. 11. Chain length specificities of bovine acyl-CoA dehydrogenases. (■), butyryl-CoA dehydrogenase; (●), medium-chain acyl-CoA dehydrogenase; (▲), long-chain acyl-CoA dehydrogenase. Solid lines represent activities obtained at optimal concentrations of phenazine methosulfate; broken lines represent activities observed at a suboptimal concentration of 0.65 mM phenazine methosulfate. The activities of long-chain acyl-CoA dehydrogenase with myristoyl-CoA or shorter-chain substrates were unaffected by a change in the concentration of phenazine methosulfate from 0.65 to 6.5 mM.

Fig. 11


Specific Activity (U/mg) (●)
Specific Activity (U/mg) (▲,■)
1.5
1.0
0.5
0.15
0.10
0.05
C4
C6
C8
C10
C12
C14
C16
C18
Chain Length Specificity

varied little with the substrate chain lengths as long as the acyl chain was between six and sixteen carbons long, (see Fig. 11). However, at an optimal concentration of phenazine methosulfate of 6.5 mM the enzyme is highly active with medium-chain substrates, moderately active with long-chain substrates (C_{12} -C_{16}) and virtually inactive with butyryl-CoA (see Fig. 11). Consequently, this enzyme is best characterized as a medium-chain acyl-CoA dehydrogenase. The acyl-CoA dehydrogenase, that was eluted first from the hydroxylapatite column, was found to be most active with dodecanoyl-CoA as a substrate, but it acts effectively on medium-chain and long-chain substrates including stearoyl-CoA. It is virtually inactive with butyryl-CoA and marginally active with hexanoyl-CoA (see Fig. 11). Consequently, it is best described as a long-chain acyl-CoA dehydrogenase. Its activity with palmitoyl-CoA or longer chain substrates was significantly increased when the concentration of phenazine methosulfate was raised from the standard 0.65 mM to 6.5 mM (see Fig. 11).

Some kinetic constants (K_m and relative V_{max} values) were determined for all three bovine acyl-CoA dehydrogenases. All K_m values were found to be in the low micromolar range except for values determined with medium-chain acyl-CoA dehydrogenase for butyryl-CoA and with long-chain acyl-CoA dehydrogenase for hexanoyl-CoA (see Table 2 and Figs. 12, 13, 14). These two high K_m values explain why medium-chain and long-chain acyl-CoA

Table II

Kinetic properties of bovine acyl-CoA dehydrogenases

Substrate	butyryl-CoA dehydrogenase		medium-chain acyl-CoA dehydrogenase		long-chain acyl-CoA dehydrogenase	
	K_m (μM)	rel. V_{max} (%)	K_m (μM)	rel. V_{max} (%)	K_m (μM)	rel. V_{max} (%)
Butyryl-CoA	3	100	>100	31	--	--
Hexanoyl-CoA	12	100	3.5	100	80	50
Octanoyl-CoA			2	100	15	46
Decanoyl-CoA			0.7	67	10	100
Dodecanoyl-CoA					4	100
Palmitoyl-CoA					2	16

Fig. 12. Activity of bovine liver butyryl-CoA dehydrogenase as a function of the concentration of butyryl-CoA (C_4, ○); and hexanoyl-CoA (C_6, ●). Data are plotted according to Lineweaver-Burk.

Fig. 12

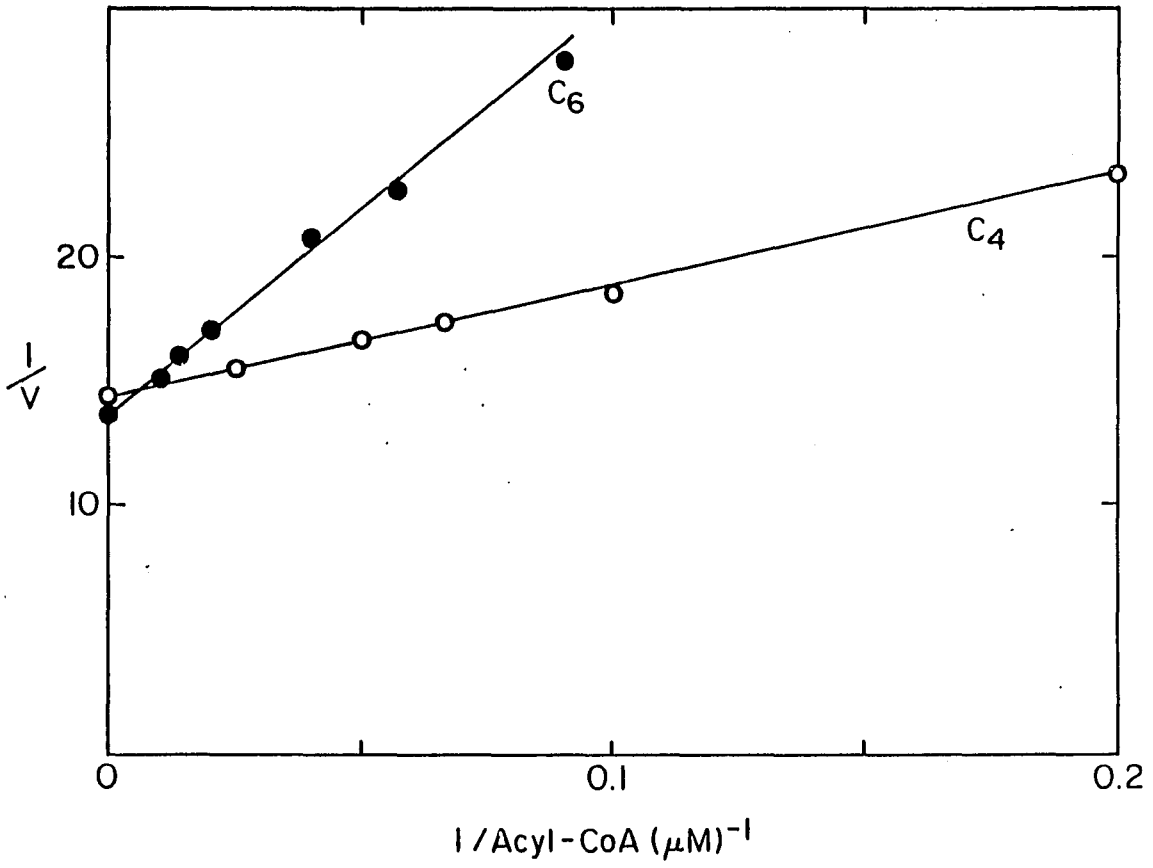


Fig. 13. Activity of bovine liver medium-chain acyl-CoA dehydrogenase as a function of the concentration of hexanoyl-CoA, (C_6, ●); octanoyl-CoA (C_8, ●); and decanoyl-CoA (C_{10}, ●). Data are plotted according to Lineweaver-Burk.

Fig. 13

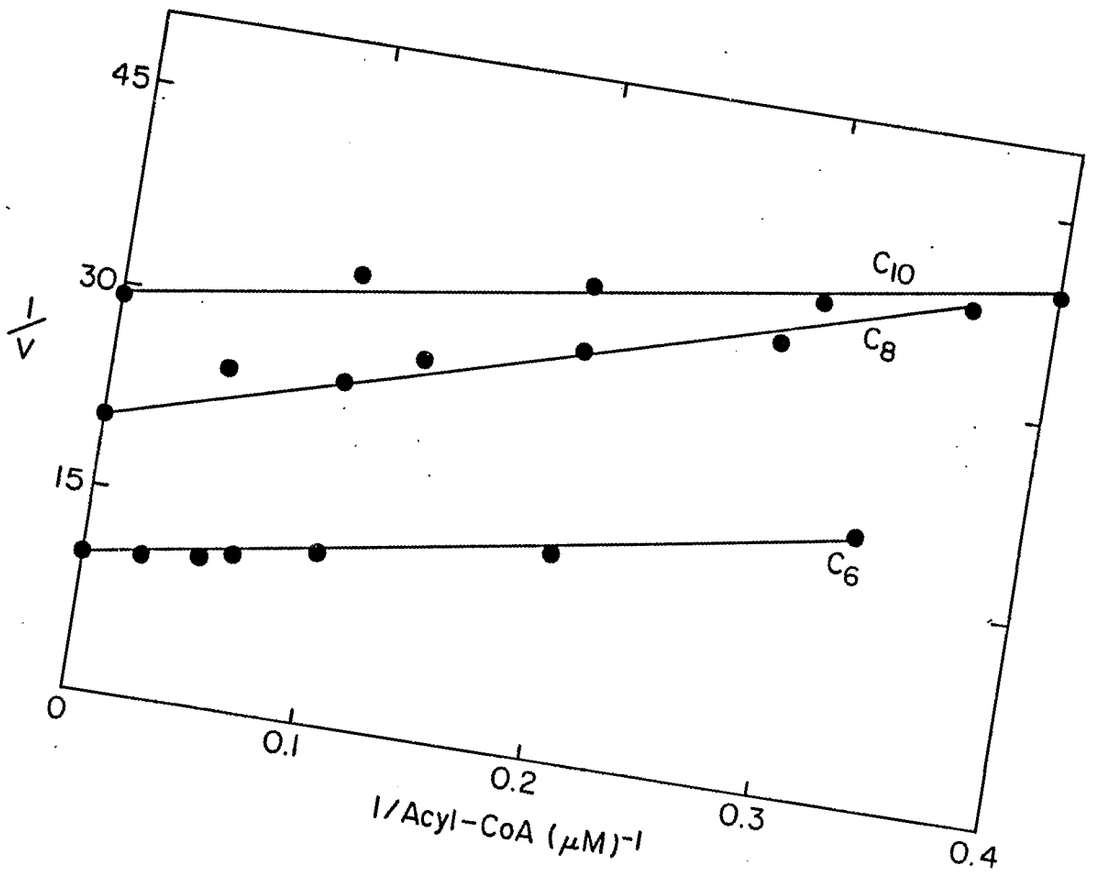
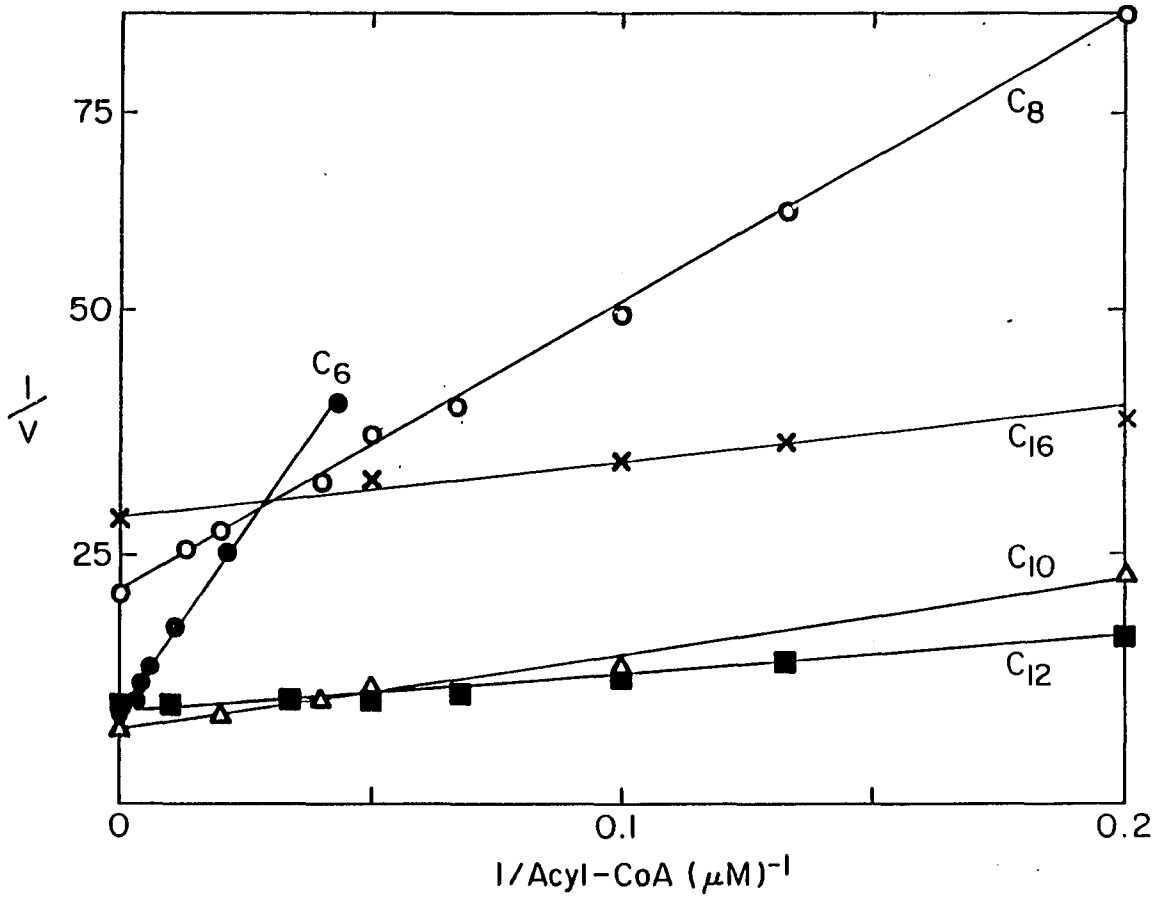


Fig. 14. Activity of bovine liver long-chain acyl-CoA dehydrogenase as a function of the concentration of hexanoyl-CoA, (C_6, ●); octanoyl-CoA (C_8, ○); decanoyl-CoA (C_{10}, △); dodecanoyl-CoA (C_{12}, ■) and palmitoyl-CoA (C_{16}, X). Data are plotted according to Lineweaver-Burk.

Fig. 14



dehydrogenases are nearly inactive towards butyryl-CoA and hexanoyl-CoA, respectively when assayed at 20 μM substrate concentrations used to determine the chain length specificities of these enzymes (see Fig. 11). The K_m values obtained with both medium-chain and long-chain acyl-CoA dehydrogenase were found to decrease with increasing chain lengths of the substrates. An opposite trend was observed for butyryl-CoA dehydrogenase. In all cases tested the K_m values for substrates of medium-chain acyl-CoA dehydrogenase were lower by approximately a factor of ten than the values obtained for the same substrates with long-chain acyl-CoA dehydrogenase. The decrease of V_{max} values with increasing substrate chain length observed both with medium-chain and long-chain acyl-CoA dehydrogenase may be partially due to the detergent properties of the substrates.

The Effects of Various Mitochondrial Coenzymes and Metabolites on the Activities of Bovine Acyl-CoA Dehydrogenase - Several mitochondrial coenzymes and metabolites were tested for their ability to either inhibit or stimulate the three bovine acyl-CoA dehydrogenases. None of the following coenzymes tested at the indicated concentrations affected the activities of the dehydrogenases: ATP (5 mM), ADP (5 mM), GTP (5 mM), GDP (5 mM), FAD (10 mM), NAD (1 mM), NADP (1 mM), carnitine (5 mM), acetyl-CoA (1 mM) and succinyl-CoA (0.1 mM). Also the tricarboxylic acid cycle intermediates citrate, isocitrate,

succinate, 2-ketoglutarate, malate and fumarate at 10 mM concentrations were without effect. However, several intermediates of fatty acid oxidation were found to be effective inhibitors of these dehydrogenases. Tested for their inhibitory potential were the short-chain fatty acid oxidation intermediates crotonyl-CoA, DL-3-hydroxybutyryl-CoA and acetoacetyl-CoA as well as the medium-chain intermediates 2-decenoyl-CoA, DL-3-hydroxydecanoyl-CoA and 3-ketodecanoyl-CoA, and long-chain intermediate 3-ketopalmitoyl-CoA. The results obtained with butyryl-CoA dehydrogenase are presented in Fig. 15A. The most effective inhibitor is acetoacetyl-CoA. This finding agrees with a previous report in which the inhibition of the same enzyme from Peptostreptococcus elsdenii by acetoacetyl-CoA was demonstrated (90). In contrast, 3-ketodecanoyl-CoA is a weak inhibiter possibly because its long alkyl chain does not fit into the substrate binding site. Crotonyl-CoA, the product of the butyryl-CoA-catalyzed reaction, is less inhibitory than is acetoacetyl-CoA for which a K_I of 10^{-6} M was determined (see Fig. 16). Both 3-hydroxyacyl-CoA compounds had no effect on the activity of butyryl-CoA dehydrogenase. Medium-chain acyl-CoA dehydrogenase is inhibited only by 3-decenoyl-CoA. and more severely by 3-ketodecanoyl-CoA (see Fig. 15B) for which a K_I of 8×10^{-7} M was obtained (see Fig. 17). Acetoacetyl-CoA, which has been

Fig. 15. Effects of ß-oxidation intermediates on the activities of partially purified acyl-CoA dehydrogenases. A: Butyryl-CoA dehydrogenase. B: Medium-chain acyl-CoA dehydrogenase. C: Long-Chain acyl-CoA dehydrogenase. Inhibitors (I): (○), Acetoacetyl-CoA; (●), 3-ketodecanoyl-CoA; (□), crotonyl-CoA; (■), 2-decenoyl-CoA; (△), DL-3-hydroxybutyryl-CoA; and (▲), DL-3-hydroxydecanoyl-CoA.

Fig. 15

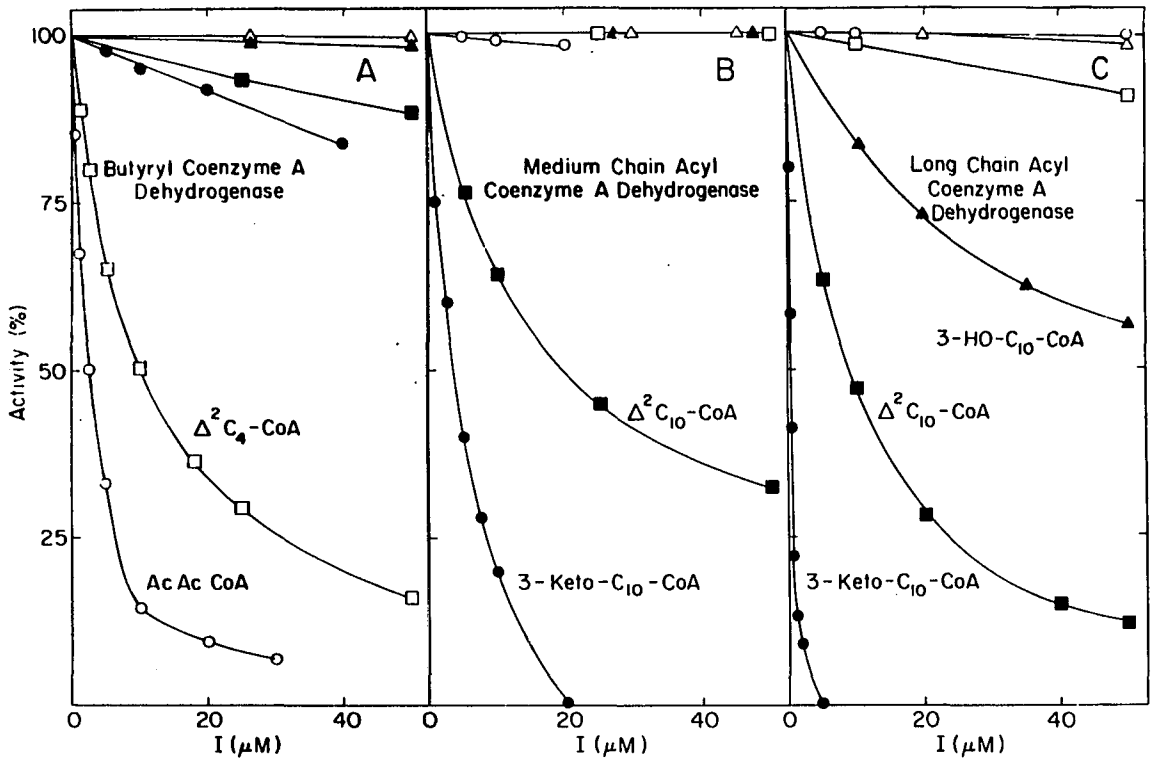

100
75
50
25
Activity (%)
A
B
C
Butyryl Coenzyme A Dehydrogenase
Medium Chain Acyl Coenzyme A Dehydrogenase
Long Chain Acyl Coenzyme A Dehydrogenase
Δ²C₄-CoA
Ac Ac CoA
Δ²C₁₀-CoA
3-Keto-C₁₀-CoA
3-HO-C₁₀-CoA
Δ²C₁₀-CoA
3-Keto-C₁₀-CoA
0
20
40
I (μM)

Fig. 16. Inhibition of bovine liver butyryl-CoA dehydrogenase by acetoacetyl-CoA (AcAcCoA) at various fixed concentrations of butyryl-CoA. The concentration of butyryl-CoA is indicated next to the corresponding line. Data are plotted according to Dixon.

Fig. 16

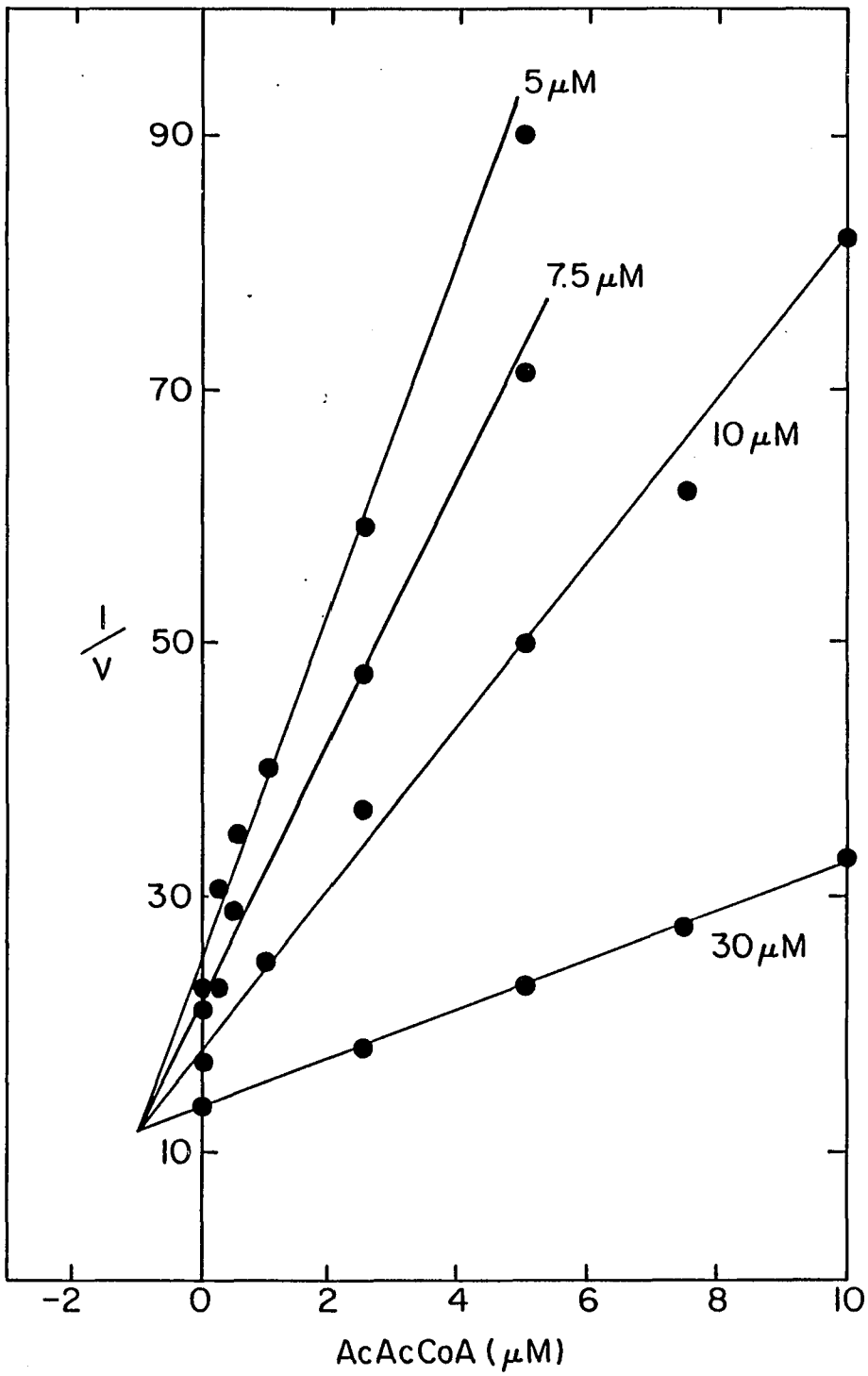
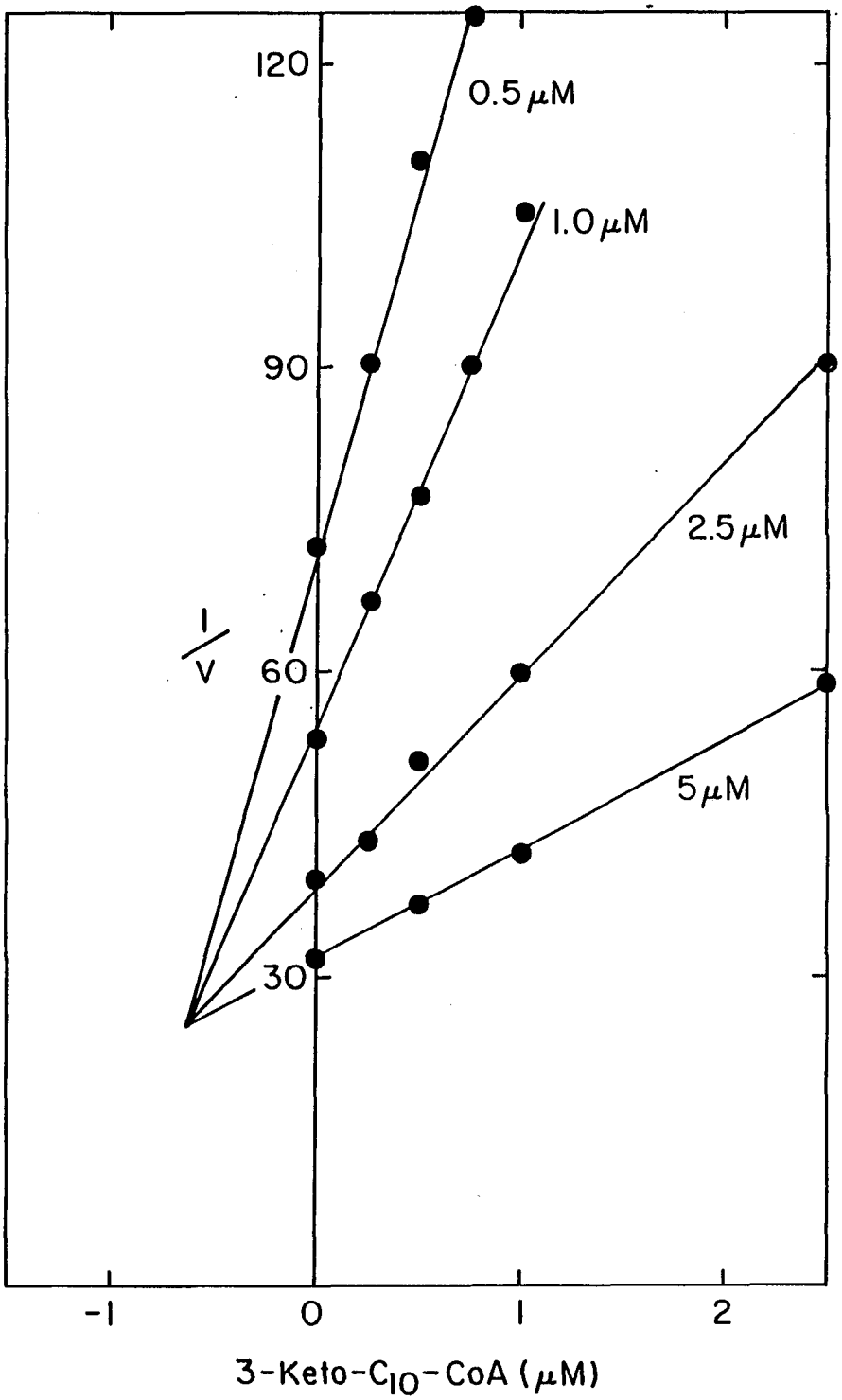


Fig. 17. Inhibition of bovine liver medium-chain acyl-CoA dehydrogenase by 3-ketodecanoyl-CoA (3-keto-C_{10}-CoA) at various fixed concentrations of decanoyl-CoA. The concentration of decanoyl-CoA is indicated next to the corresponding line. Data are plotted according to Dixon.

Fig. 17



reported to be an inhibitor of pig liver general acyl-CoA dehydrogenase (37), has virtually no effect on the bovine liver enzyme over the concentration range tested. The most interesting result was obtained when the inhibition of long-chain acyl-CoA dehydrogenase was studied (see Fig. 15C). 3-Ketodecanoyl-CoA was found to be an extremely effective inhibitor of this enzyme with a K_I value of 7.5×10^{-8} M (see Fig. 18). As expected, 2-decenoyl-CoA is also inhibitory, although to a lesser degree. Surprisingly, even DL-3-hydroxydecanoyl-CoA causes a significant inhibition. Crotonyl-CoA is a very weak inhibitor whereas both acetoacetyl-CoA and DL-3-hydroxybutyryl-CoA are virtually without effect. Also tested with long-chain acyl-CoA dehydrogenase was the effect of long-chain 3-ketoacyl-CoA. As expected 3-ketopalmitoyl-CoA was found to be a more effective inhibitor of palmitoyl-CoA dehydrogenation than was 3-ketodecanoyl-CoA. The K_I values were 0.2 µM (see Fig. 19) and 1.3 µM (see Fig. 20), respectively. Fig. 21 illustrates the inhibition of long-chain acyl-CoA dehydrogenase as a function of 3-ketoacyl-CoA chain length and substrate chain length. Summarized in table 3 are the K_I values obtained for the acyl-CoA dehydrogenases with their respective 3-ketoacyl-CoA inhibitor and substrate.

Presence of Fatty Acid Oxidation Intermediates in Mitochondria

If acyl-CoA dehydrogenase is regulated *in vivo* by intermediates of ß-oxidation, the presence of these metabolites should be

Fig. 18. Inhibition of bovine liver long-chain acyl-CoA dehydrogenase by 3-ketodecanoyl-CoA (3-keto-C_{10}-CoA) at various fixed concentrations of decanoyl-CoA. The concentration of decanoyl-CoA is indicated next to the corresponding line. Data are plotted according to Dixon.

Fig. 18

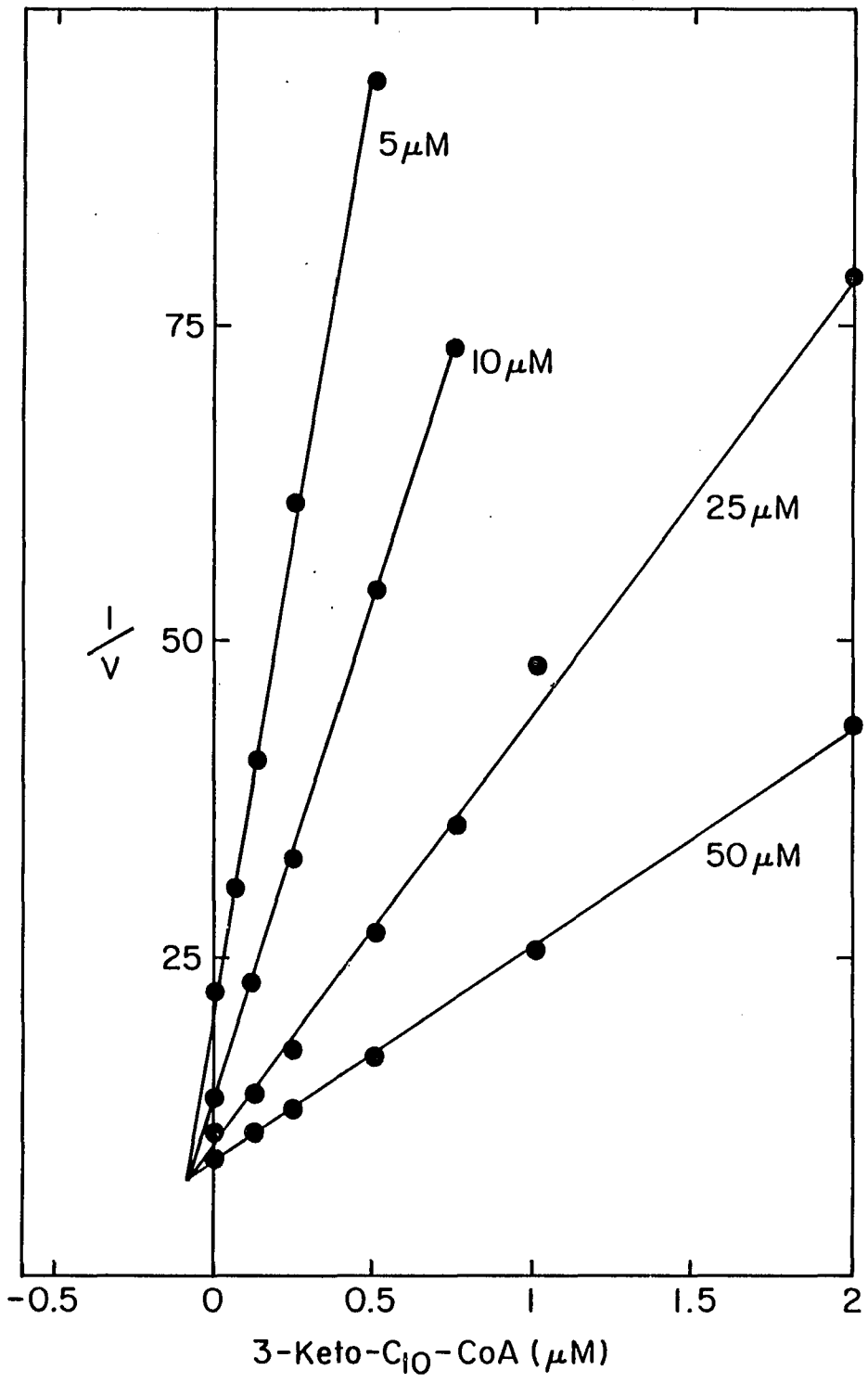


Fig. 19. Inhibition of bovine liver long-chain acyl-CoA dehydrogenase by 3-ketodecanoyl-CoA (3-keto-C_{10}-CoA) at various fixed concentrations of palmitoyl-CoA. The concentration of palmitoyl-CoA is indicated next to the corresponding line. Data are plotted according to Dixon.

Fig. 19

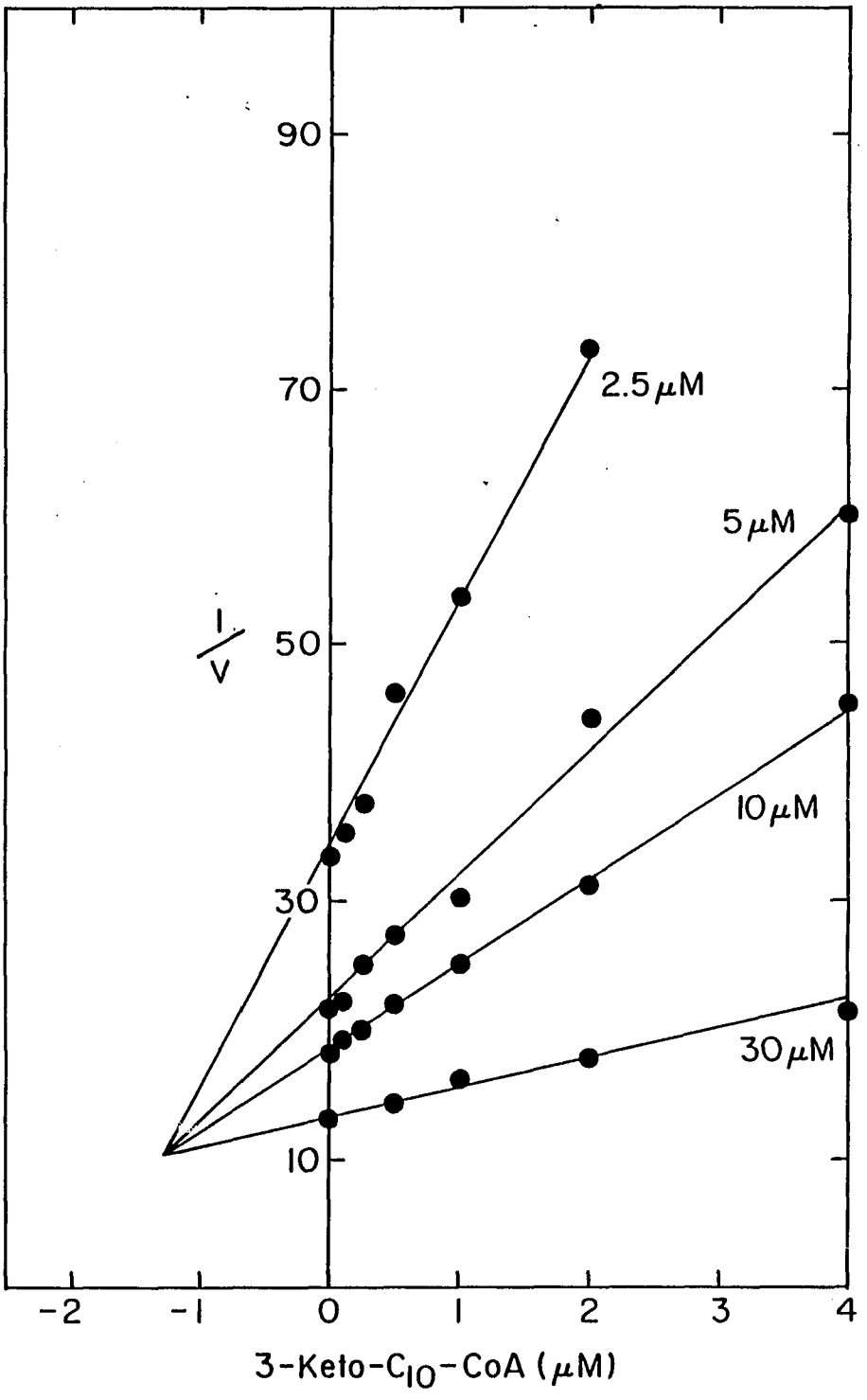


Fig. 20. Inhibition of bovine liver long-chain acyl-CoA dehydrogenase by 3-ketopalmitoyl-CoA (3-keto-C_{16}-CoA) at various fixed concentrations of palmitoyl-CoA. The concentration of palmitoyl-CoA is indicated next to the corresponding line. Data are plotted according to Dixon.

Fig. 20

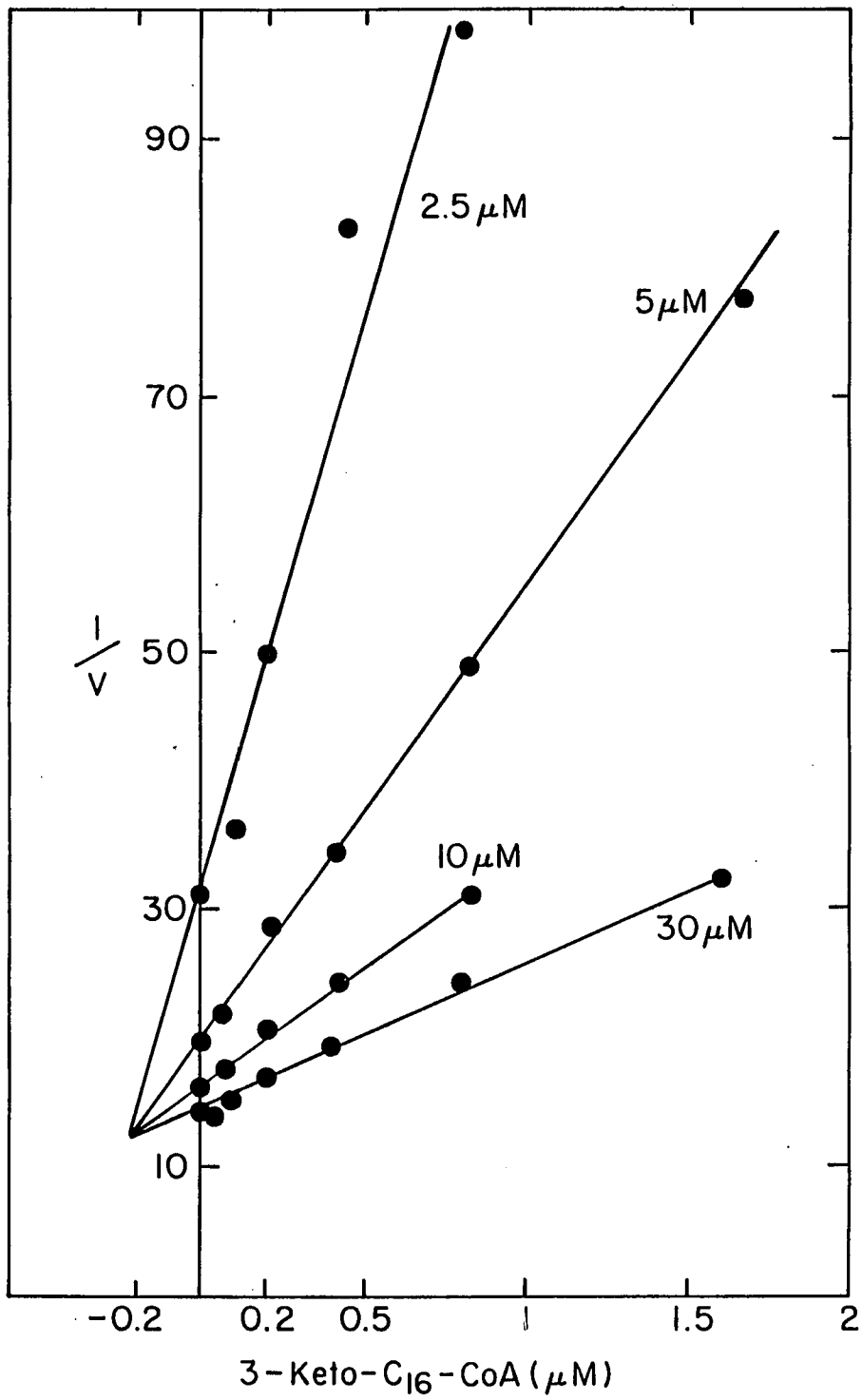

90
70
50
30
10
1/V
2.5 μM
5 μM
10 μM
30 μM
-0.2
0.2
0.5
1
1.5
2
3-Keto-C16-CoA (μM)

Fig. 21. Inhibition of bovine liver long-chain acyl-CoA dehydrogenase as a function of 3-ketoacyl-CoA chain length and substrate chain length.

(●), 3-ketodecanoyl-CoA and 30 μM palmitoyl-CoA.

(■), 3-ketopalmitoyl-CoA and 30 μM palmitoyl-CoA.

(▲), 3-ketodecanoyl-CoA and 50 μM decanoyl-CoA.

Data are plotted according to Dixon.

Fig. 21

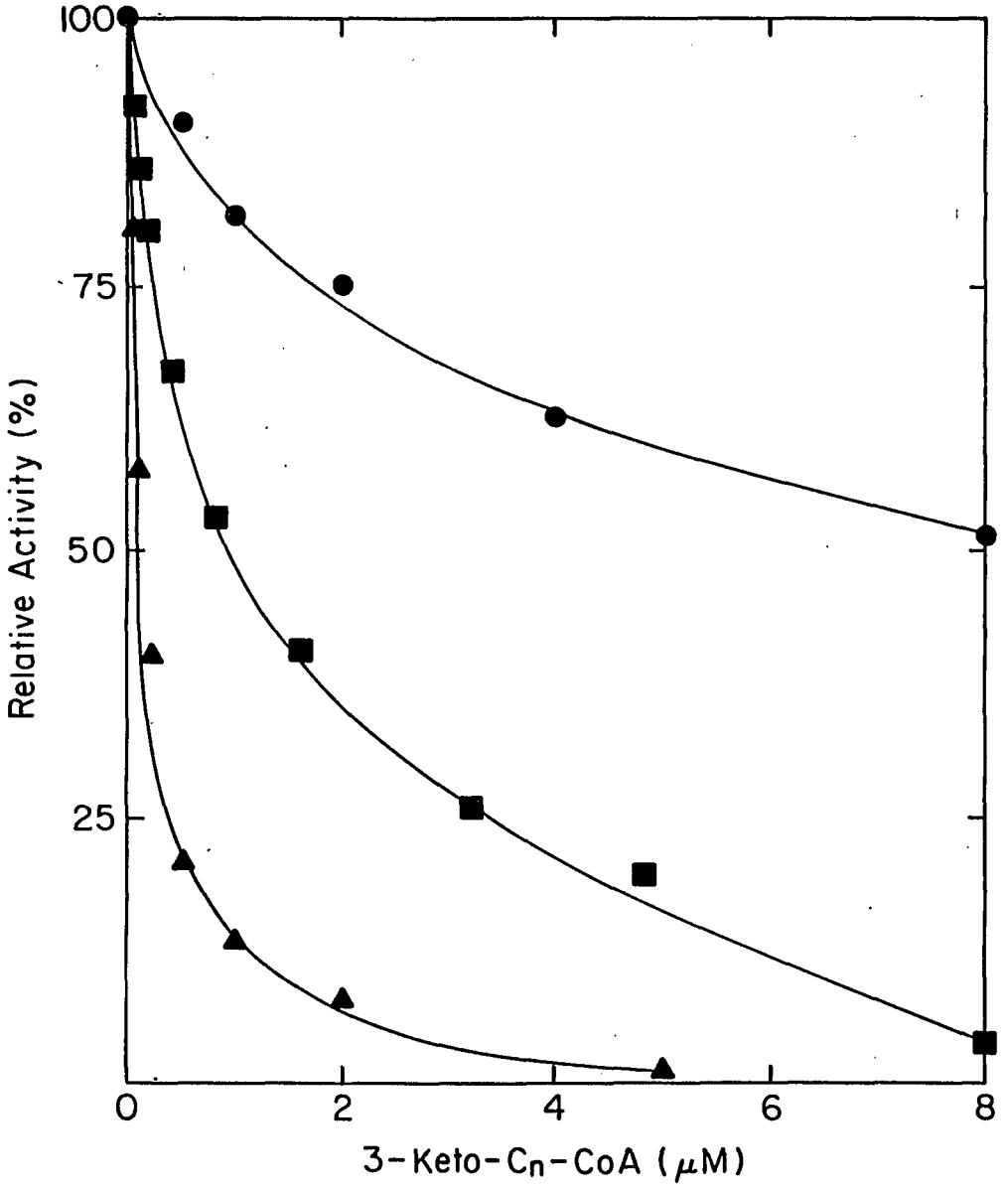


Table III

Summary of K_I values for the bovine acyl-CoA dehydrogenses with their respective 3-ketoacyl-CoA inhibitors.

Enzyme	Substrate	Inhibitor	K_I
			μM
Butyryl-CoA dehydrogenase	Butyryl-CoA	Acetoacetyl-CoA	1
Medium-chain acyl-CoA dehydrogenase	Decanoyl-CoA	3-Ketodecanoyl-CoA	0.8
Long-chain acyl-CoA dehydrogenase	Decanoyl-CoA	3-Ketodecanoyl-CoA	0.075
	Palmitoyl-CoA	3-Ketodecanoyl-CoA	1.3
	Palmitoyl-CoA	3-Ketopalmitoyl-CoA	0.2

observable when the rate of this pathway decreases. This hypothesis was tested by incubating mitochondria with [16-^{14}C] palmitoyl-CoA of high specific radioactivity (for details see under "Experimental Procedures") and by identifying labelled metabolites as illustrated in Fig. 22 and described under "Experimental Procedures". A 10-fold increase in the concentration of 3-hydroxy acids was observed when the respiration state was changed from 3 to 4 (see Fig. 23, and Table 4). Because of the rapid equilibrium between 3-hydroxyacyl-CoA and 2-enoyl-CoA catalyzed by crotonase, the concentrations of $\triangle^{2,3}$- enoyl-CoA should also increase dramatically when the respiration is shifted from state 3 to 4. Unfortunately, separation on TLC of long-chain $\triangle^{2,3}$ enoic acids from saturated fatty acids was not possible. The most interesting finding was the presence of increased concentration of 3-ketoacyl-CoA at state 4 respiration (see Fig. 24). Their estimated concentration of 3 μM (see Table 4) in the mitochondrial matrix could account for the inhibition of long-chain acyl-CoA dehydrogenase because K_I values in the nM range have been determined with long-chain acyl-CoA dehydrogenase for 3-ketoacyl-CoA thioesters.

Inhibition of Acyl-CoA Dehydrogenase by Acetylynic Acyl-CoA Compounds - In an attempt to determine if acyl-CoA dehydrogenases catalyze a slow or rate-limiting step in ß-oxidation several in vitro inhibitors of the dehydrogenases were tested for their

Fig. 22. Proposed metabolism of [16-^{14}C] palmitoyl-CoA in rat heart mitochondria. Coupled rat heart mitochondria were incubated with [16-^{14}C] labelled palmitoyl-CoA at state 3 and state 4 respirations. After terminating the incubations, the fatty acyl-CoA metabolites were identified by TLC and quantitated by scintillation counting. For experimental details see under "Experimental Procedures".

Fig. 22

Mitochondrial Metabolites of Fatty Acids

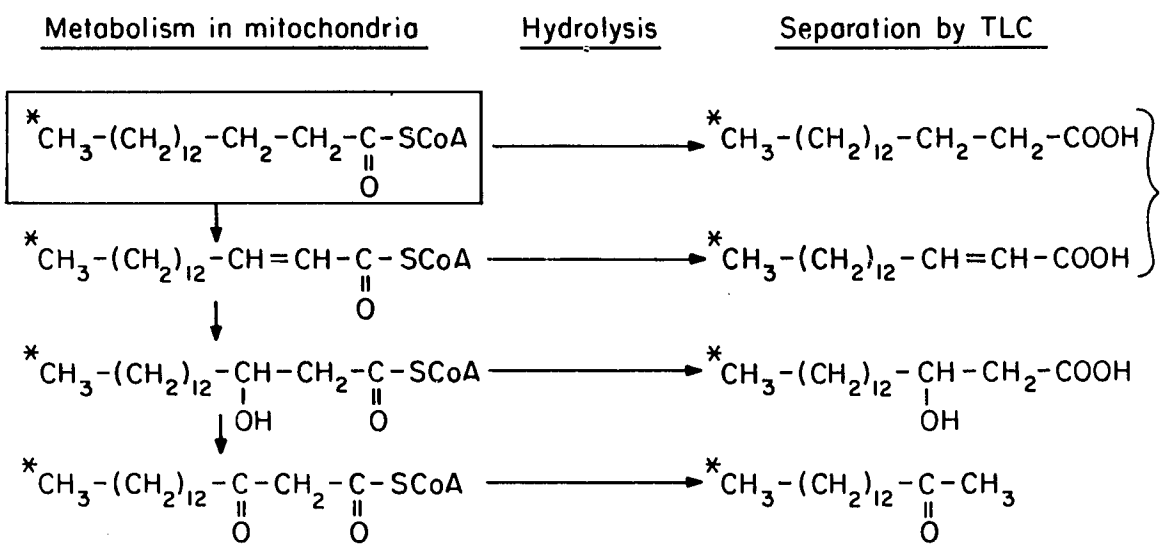


Fig. 23. Separation by TLC of (▨) 3-hydroxy fatty acids and (■) $\triangle^{2,3}$- enoic plus saturated fatty acids accumulating during state 3 and state 4 respirations. For experimental details see under "Experimental Procedures".

Fig. 23

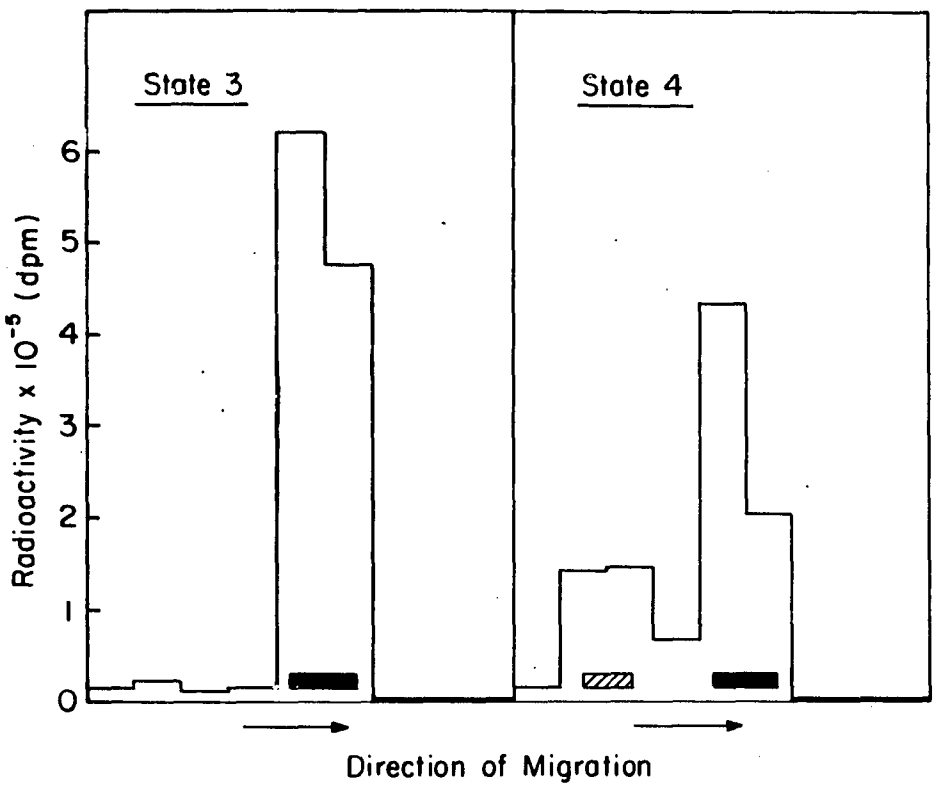

State 3
State 4
Radioactivity x 10^{-5} (dpm)
0
1
2
3
4
5
6
Direction of Migration

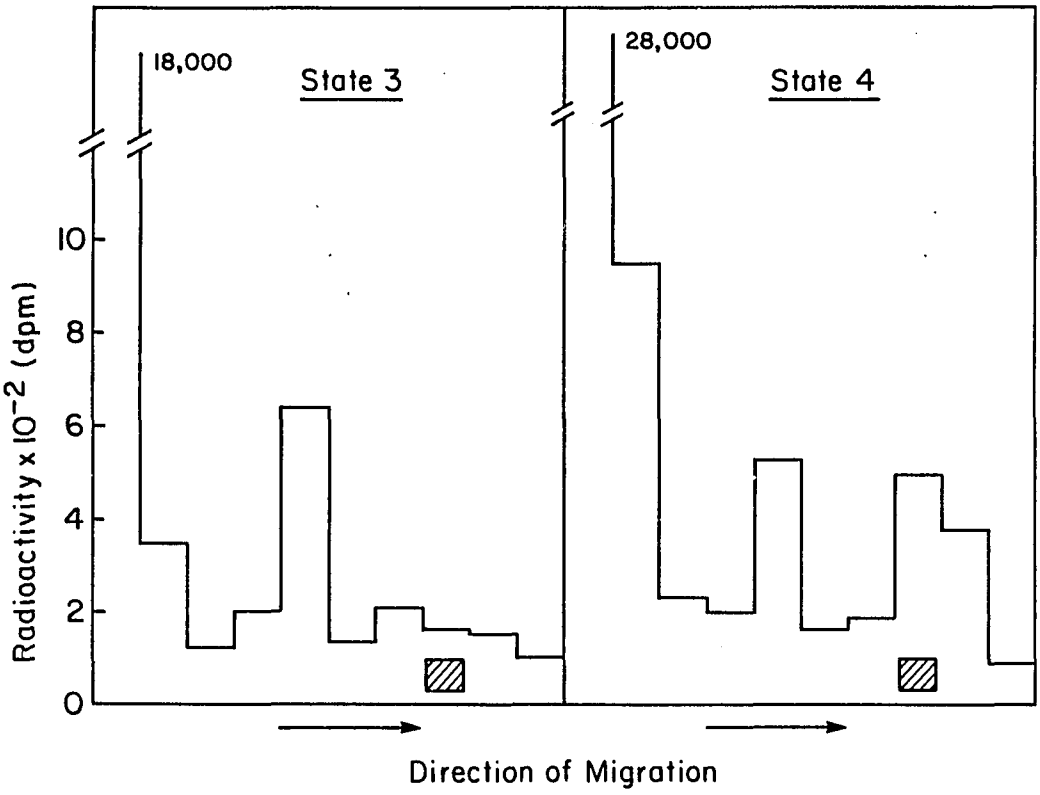
Table IV

Concentrations of [16-^{14}C] labelled 3-hydroxy acids and 3-keto acids accumulating during state 3 and state 4 respirations. Concentrations were calculated by assuming a matrix volume of 1 µl per mg of rat heart mitochondria. For experimental details see under "Experimental Procedures".

Metabolite	Mitochondrial Concentration		
	State 3	State 4	Change
	μM	μM	μM
3-Hydroxy acid	90±10	1240±130	1150
3-Keto acid	0.9±0.5	3±1	2.1

Fig. 24.- Separation by TLC of (). 3-keto fatty acids (as their methyl ketones) accumulating during state 3 and 4 respirations. For experimental details see under "Experimental Procedures".

Fig. 24

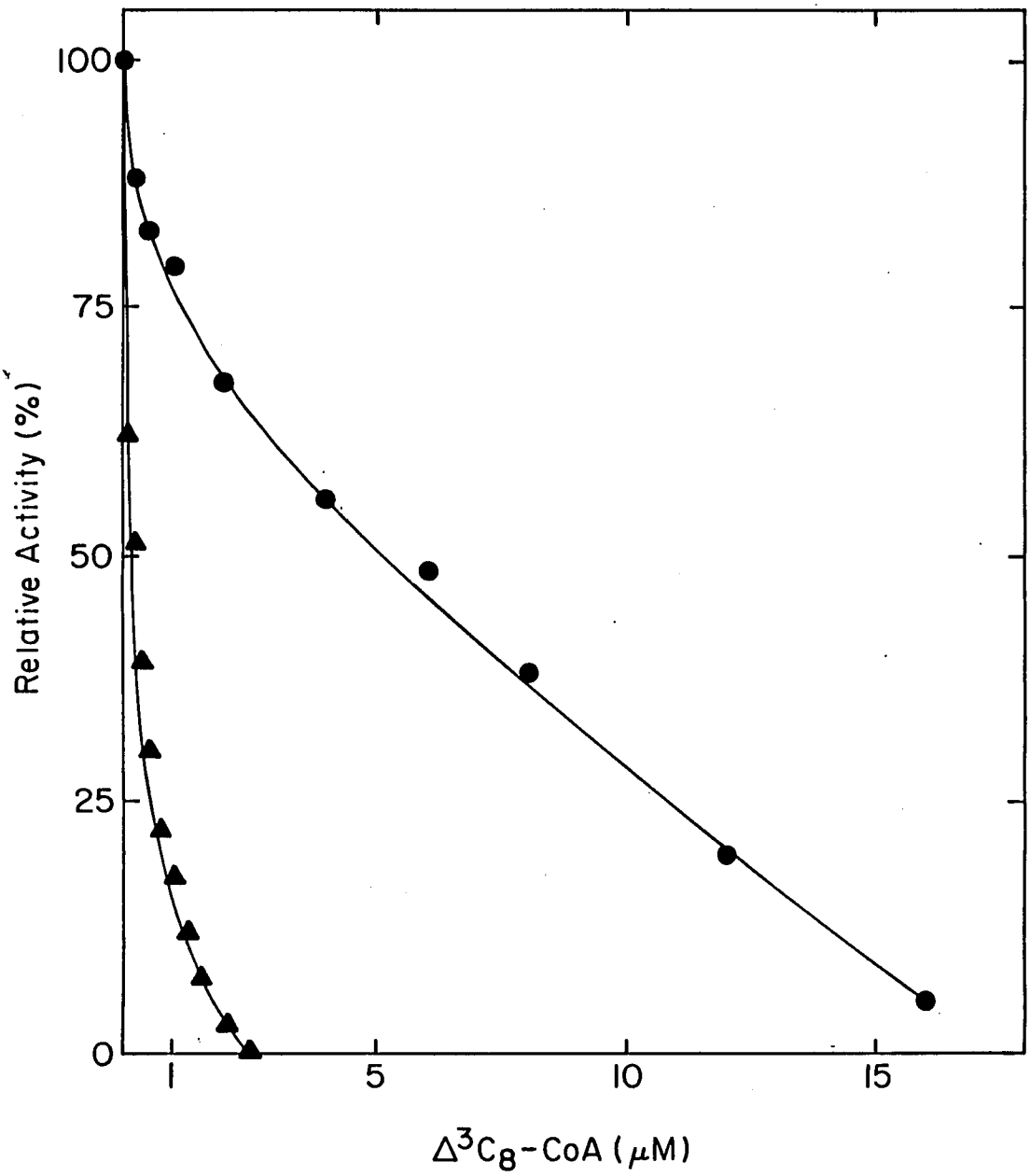

18,000
State 3
28,000
State 4
Radioactivity x 10^{-2} (dpm)
0
2
4
6
8
10
Direction of Migration

effect on the rate of palmitoyl-(L)-carnitine supported respiration in coupled rat heart mitochondria. Similar rates of inhibition of the dehydrogenases and ß-oxidation would suggest that dehydrogenation is a slow or rate-limiting reaction. If ß-oxidation would be inhibited at a slower rate than the dehydrogenases, the reaction catalyzed by them would most likely not be limiting.

In vitro assay of the partially purified bovine heart dehydrogenases with 2.5 µM, and 16 µM 3-octynoyl-CoA resulted in 100% inhibition of medium-chain acyl-CoA dehydrogenase, and 95% inhibition of long-chain acyl-CoA dehydrogenase respectively (see Fig. 25). However, 3 minute preincubation of coupled rat heart mitochondria in the presence of 140 µM 3-octynoyl-CoA resulted in only 56% depression of palmitoyl-(L)-carnitine supported respiration (data not shown). Since assaying the dehydrogenases in mitochondria preincubated with high concentrations of 3-octynoyl-CoA proved impracticable, a second approach which eliminated this problem was chosen. In this approach I used 9-octadecynoyl-CoA, an analogue of stearoyl-CoA, which after passing three-times through the ß-oxidation cycle would theoretically yield 3-dodecynoyl-CoA. This metabolite is expected to act as a potent and specific inhibitor of medium-chain and long-chain acyl-CoA dehydrogenase. Since this metabolite can be formed only in the mitochondrial matrix, an accurate in vitro determination of dehydrogenase inhibition

Fig. 25. Inhibition of (▲), medium-chain acyl-CoA dehydrogenase and (●), long-chain acyl-CoA dehydrogenase as a function of the concentration of 3-octynoyl-CoA. The partially purified bovine liver dehydrogenases were assayed at optimal concentrations of PMS and (▲), 20 μM octanoyl-CoA or (●), 20 μM dodecanoyl-CoA as substrates. Assays are described under "Experimental Procedures". Data are plotted according to Dixon.

Fig. 25



from incubated samples was possible. Preincubation of coupled rat heart mitochondria for 3 minutes with 25 µM 9-octadecynoyl-CoA resulted in 86% inhibition of palmitoyl-(L)-carnitine - supported respiration. However, contrary to my expectations the total dehydrogenase activity in the preincubated samples remained equal to that of the controls. This finding suggests that inhibition had occurred at a different site in the fatty acid oxidation pathway.

These results may be explained in light of a very recent finding by Wood and Lee (91). The author describes a pathway in rat liver mitochondria by which acetylynic acyl-CoA's are metabolized. Through the sequential activities of isomerase and crotonase the inhibitory $\triangle^{2,3}$ allene intermediate is rapidly metabolized to 3-ketoacyl-CoA which is further degraded by ß-oxidation. Such a pathway could explain why acyl-CoA dehydrogenases are not inhibited in mitochondria by 3-octynoic acid. However, the severe depression of palmitoyl-(L)-carnitine - supported respiration remains unexplained. Inhibition of palmitoyl-(L)-carnitine - supported respiration without any apparent inhibition of the first reaction of ß-oxidation is possibly due to a block at sites required for translocation of acetylynic acyl-CoA and/or substrate into the mitochondrial matrix or due to an inhibition of product removal. I suggest that fatty acids containing a triple bond may either inhibit the rat heart carnitine acylcarnitine translocase and/or

carnitine dependent transferases thus causing a decrease in metabolism of substrates dependent on these activities e.g. palmitoyl-(L)-carnitine.

DISCUSSION

By use of a simple two-step procedure described in this thesis I have been able to separate the three acyl-CoA dehydrogenases expected to be present in bovine liver. More importantly, I have separated the acyl-CoA dehydrogenases of bovine heart by use of the same procedure and have thus demonstrated the presence of three acyl-CoA dehydrogenases in heart muscle. A comparison of the chromatographic behaviors, the chain length specificities and kinetic properties of the acyl-CoA dehydrogenases from bovine liver and bovine heart has led to the conclusion that the two sets of enzymes present in liver and heart are identical.

The two-step purification procedure described above has the advantage of resulting in the complete separation of the two acyl-CoA dehydrogenases that are active with medium-chain and long-chain substrates. Consequently, the chain length specificities determined for these two enzymes are truly those of the uncontaminated dehydrogenases. It is not clear whether previous purification procedures have achieved the same degree of separation of these two acyl-CoA dehydrogenases (17,19,24,25). Thus, some doubt has remained about the reported chain length specificities of these dehydrogenases and consequently about their physiological functions. A comparison of the chain length specificities reported herein for the bovine acyl-CoA dehydrogenases with those determined by Beinert (25)

for the pig liver enzymes demonstrates an obvious similarity only in the case of the butyryl-CoA dehydrogenases. The two sets of longer chain acyl-CoA dehydrogenases from bovine and pig tissues differ significantly. They only resemble each other in that one enzyme from each animal is most active with octanoyl-CoA as a substrate, whereas the other acts preferentially on dodecanoyl-CoA. According to their preferred substrates the C_4 -C_{16} enzyme from pig liver (25) corresponds to the bovine medium-chain acyl-CoA dehydrogenase and the C_6 -C_{16} pig enzyme resembles the long-chain acyl-CoA dehydrogenase isolated as described in this thesis. However, it is possible that differences in the assay procedure, especially the use of electron-transferring flavoprotein as a primary electron acceptor by Beinert and coworkers and phenazine methosulfate herein may account for some of the observed differences in chain length specificities.

Although butyryl-CoA dehydrogenase and the two acyl-CoA dehydrogenases identified in pig and bovine tissues are assumed to function in fatty acid oxidation, their specific roles under physiological conditions have not been established. An evaluation of the chain length specificities of the dehydrogenases from pig liver suggests that these enzymes may complement each other to assure a high rate of dehydrogenation of all common fatty acyl compounds. However, the cooperation of butyryl-CoA dehydrogenase and acyl-CoA dehydrogenase (C_6

-C_{16}) alone should result in the efficient dehydrogenation of all fatty acids. The chain length specificities of the bovine enzymes determined via the present investigation also suggests that butyryl-CoA dehydrogenase together with long-chain acyl-CoA dehydrogenase would catalyze effectively the dehydrogenation of all fatty acids with four to eighteen carbons. However, the relatively high K_m values for medium-chain substrates observed with long-chain acyl-CoA dehydrogenase may result in a low capacity to metabolize medium-chain fatty acids if they are present at low concentrations unless medium-chain acyl-CoA dehydrogenase participates in this process. Since the effective concentrations of ß-oxidation intermediates under physiological conditions are not known, the question concerning the necessary cooperation of two or three dehydrogenases in the degradation of saturated fatty acids cannot yet be answered. Another function for medium-chain acyl-CoA dehydrogenase has recently been proposed by Kunau and Dommes (14) who showed that cis-4-decenoyl-CoA, an intermediate in the degradation of linoleic acid, can only be dehydrogenated by medium-chain acyl-CoA dehydrogenase. Thus, it is possible that the degradation of saturated fatty acids requires the presence of butyryl-CoA dehydrogenase and long-chain acyl-CoA dehydrogenase, whereas the medium-chain dehydrogenase is essential for the breakdown of polyunsaturated fatty acids.

The strong inhibitory effect of 3-ketodecanoyl-CoA and,

3-ketopalmitoyl-CoA on long-chain acyl-CoA dehydrogenase, which presumably catalyzes the initial step in ß-oxidation, leads to the suggestion that 3-ketoacyl-CoA compounds may function in vivo as feedback inhibitors of fatty acid oxidation. Although some intermediates of ß-oxidation have been found to accumulate in mitochondria under normal conditions as well as in the presence of rotenone (74,75), previous to this study no attempts had been made to identify and quantitate medium-chain and long-chain 3-ketoacyl-CoA compounds, which may be effective regulators of ß-oxidation at nanomolar concentrations. I have demonstrated by using coupled rat heart mitochondria that a significant increase in the concentration of ß-oxidation intermediates, most notably, long-chain 3-ketoacyl-CoA's occurred when the respiration state was changed from 3 to 4. The estimated concentration of 3-ketoacyl-CoA's at state 4 respiration of 3 µM was found to agree closely with the estimated value of 2.5 µM which was calculated based upon the following three points:

No. 1. The equilibrium constant (K_{deh}) reported by Wakil et al. (92) for the following reaction.

L-3-hydroxyhexanoyl-CoA +NAD^+ 3-ketohexanoyl-CoA +NADH + H^+ is = 2.5 x 10^{-11} M, at 25°C, (pH 8.0).

No. 2. The K_{deh} for the above reaction can be defined by the following equation (Wakil et al.)[1]

$$K_{deh} = \frac{[\text{3-ketoacyl-CoA}]\ [\text{NADH}]\ [H^+]}{[\text{L-3-hydroxyacyl-CoA}]\ [NAD^+]}$$

No. 3. The following intramitochondrial concentrations of dinucleotides and 3-hydroxyacyl-CoA metabolites are assumed to be present in the mitochondrial matrix during state 4 respiration.

A. L-3-hydroxyacyl-CoA, 1 mM

B. NADH, 3 mM; NAD^+, 3 mM

The state 4 concentration of 3-ketoacyl-CoA metabolites can then be approximated by solving the resultant equation.

$$\frac{(2.5 \times 10^{-11}\ \text{M})}{(\text{3-Ketoacyl-CoA M})} = \frac{(3 \times 10^{-3}\ \text{M})\ (1 \times 10^{-8}\ \text{M})}{(1 \times 10^{-3}\ \text{M})\ (3 \times 10^{-3}\ \text{M})}$$

3-ketoacyl-CoA = 2.5×10^{-6} M

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1. Wakil, S.J., Green, D.E., Mii, S., and Mahler, H.R. (1954) J. Biol. Chem. 207, Pg. 636.

3-Ketoacyl-CoA's were in all cases tested the most effective inhibitors of the three acyl-CoA dehydrogenases. However, the estimated I_{50} of 8 µM for $\triangle^{2,3}$ decenoyl-CoA (see Fig. 15C) could account for the inhibition of long-chain acyl-CoA dehydrogenase which additionally may be inhibited by 3-hydroxyacyl-CoA compounds. It is possible that fine tuning of the rate of ß-oxidation to the energy demands of the cell is more complex than expected in that the activity at multiple sites by several intermediates and/or coenzymes collectively exert a regulatory effect.

I have shown that both medium-chain and long-chain acyl CoA dehydrogenase from bovine heart and liver are inhibited in vitro by 3-octynoyl-CoA. These results are in agreement with the findings of Frerman et al. (47) on the inhibition of purified pig liver medium-chain acyl-CoA dehydrogenase by 3-octynoyl-CoA suggesting that this group of dehydrogenases are inhibited by a similar mechanism.

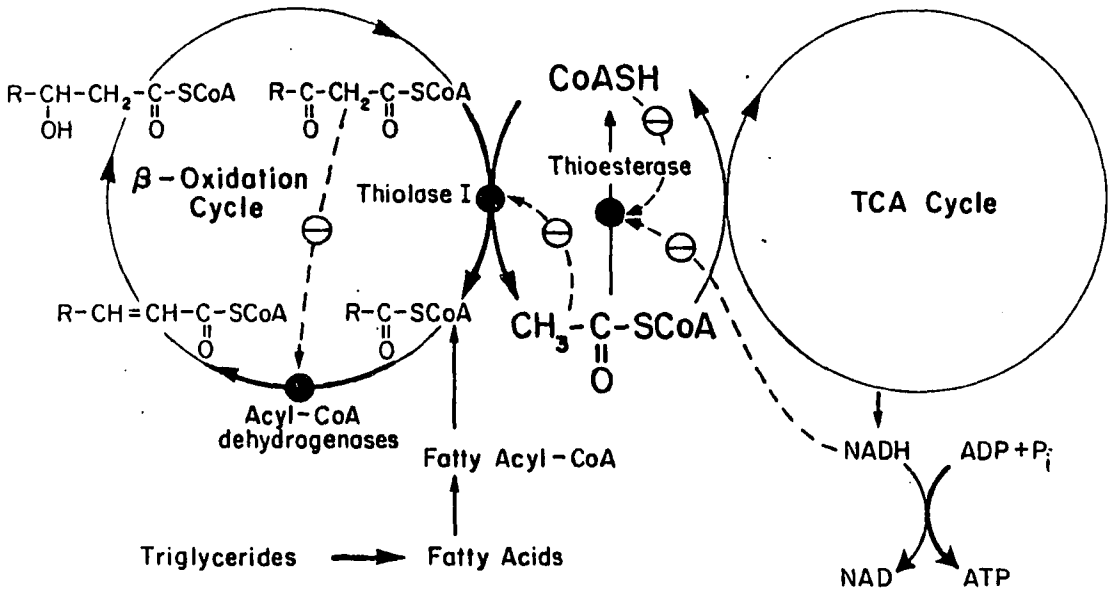
It was, unfortunately, not possible to rule out the nonspecific inhibition of fatty acid oxidation by acetylynic compounds, or the detoxification of these inhibitory compounds by the pathway described by Wood and Lee (91). Despite numerous attempts I was unable to demonstrate parallel inhibition of fatty acid oxidation and acyl-CoA dehydrogenase activity.

However, the present studies of the inhibition of the acyl-CoA dehydrogenases by 3-ketoacyl-CoA thioesters and the evidence of an increase in the concentration of these metabolites when respiration is shifted from state 3 to 4 provides strong support for the existence of a feedback connection between the last and first steps of ß-oxdidation. In conclusion, on the basis of findings reported in this thesis and elsewhere (65) it is proposed that the rate of fatty acid oxidation is tuned to the energy demand of heart via several connecting feedback controls as outlined in Fig. 26. It has been demonstrated both with isolated mitochondria (75,93) and in the perfused heart (60,94) that a decrease in the energy demand leads to an increase in the concentration of acetyl-CoA and to a corresponding decrease of CoASH. Increases in the acetyl-CoA/CoASH ratio of the magnitude observed in mitochondria and the perfused heart result in the inhibition of 3-ketoacyl-CoA thiolase (65) which catalyzes one of the slow steps of ß-oxidation (66). A slow-down of ß-oxidation as a result of the inhibition of the thiolase-catalyzed last step of the pathway, leads to the accumulation of fatty acid intermediates. These intermediates possibly inhibit the first step of the pathway catalyzed by long-chain acyl-CoA dehydrogenase thereby preventing the further entry of fatty acyl-CoA's into the cycle.

Fig. 26. Diagramatic representation of the integrated pathways of ß-oxidation, TCA cycle and oxidative phosphorylation.

Fig. 26

REGULATION OF FATTY ACID OXIDATION IN HEART



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