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Purification, Properties and Regulation of Mitochondrial Acyl Coenzyme A Thioesterase from Pig Heart

by

Kwan Yong Lee

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

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Abstract

PURIFICATION, PROPERTIES AND REGULATION OF MITOCHONDRIAL ACYL COENZYME A THIOESTERASE FROM PIG HEART

by

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A thioesterase which is located in mitochondria has been isolated from pig heart muscle and has been partially purified. This thioesterase appears to be specific for CoA esters since it is inactive towards derivatives of pantetheine, glutathione and N-acetylcysteamine. In contrast, it shows less specificity with regard to the acyl portion of the substrate as evidenced by its ability to act on CoA derivatives of fatty acids containing 2 to 16 carbons as well as on acetoacetyl-CoA, 3-ketodecanoyl-CoA and succinyl-CoA. Crotonyl-CoA, 3-hydroxybutyryl-CoA, and 3-hydroxydecanoyl-CoA are poor substrates and malonyl-CoA is not hydrolyzed at all. The same K_m-value of 48 μM was determined for acetoacetyl-CoA, decanoyl-CoA, butyryl-CoA, succinyl-CoA, and acetyl-CoA whereas the corresponding relative V_{max} values were found to be 1, 0.93, 0.88, 0.34, 0.29 respectively. The thioesterase has an estimated molecular weight of 300,000, a pH_I of 4.5 and a pH optimum of 8-8.5. The activity of the enzyme is stimulated by Ca^{2+}

but not by Mg^{2+}. Various experimental results such as the co-chromatographies of the various thioesterase activities on DEAE-cellulose and hydroxylapatite, their co-banding during isoelectric focusing and polyacrylamide gel electrophoresis, their parallel thermal inactivation and the non-additivity of thioesterase activities measured with different substrates suggest that the hydrolyses of the different substrates are catalyzed by a single enzyme.

The effects of various metabolites and coenzymes on the activity of the enzyme have been investigated in an effort to establish its physiological function of this enzyme. Among the compounds tested CoASH, NADH and ATP inhibited the enzyme significantly at concentrations considered to be physiological whereas the inhibitory effects of ADP, AMP and citrate were less pronounced. Inhibitions by oxidized CoA (K_I = 53 μM) and ATP (K_I = 0.52 mM) were competitive with respect to acetyl-CoA while the inhibition by NADH (K_{IS} = 195 μM, K_{II} = 115 μM) was noncompetitive. The K_I value for CoASH was estimated to be 8 μM. With palmitoyl-CoA as a substrate the K_I values for oxidized CoA and NADH were 85 μM and 0.5 mM respectively. The inhibitions caused by oxidized CoA and NADH were found to be additive. Calculations lead to the conclusion that the thioesterase is almost completly inhibited at state 3 respiration or state 4 respiration when either the concentrations of CoASH plus ATP or NADH plus ATP are high.

However, the enzyme would be active under conditions at which CoASH and NADH concentrations are low. It is suggested that the function of this thioesterase in heart, similarly to that of the pathway of ketone body synthesis in liver, is to provide free CoASH for the oxidative metabolism in mitochondria when ATP is required but the citric acid cycle operates at an insufficient rate.

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I am also thankful to Dr. Charlotte Russell for her helpful questions and suggestions regarding the introduction of this dissertation.

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INTRODUCTION

Thioester hydrolases (EC 3.1.2.1 to EC 3.1.2.11) also referred to as thioesterases, which catalyze the hydrolysis of the thioester bond of acyl derivatives of coenzyme A (CoA), glutathione and acyl carrier protein (ACP), have been detected in many organisms including mammals, bacteria and plants (1-20). But important questions about this class of enzymes, such as their subcellular locations, substrate specificities and physiological roles have generally not been answered. An exception represents the well-studied long chain thioesterase which is an integral part of the fatty acid synthetase of higher organisms and which is responsible for the release of palmitic acid and stearic acid from the multi-enzyme complex (12, 18, 19). It catalyzes the hydrolysis of the thioester bond through which fatty acids are covalently linked to the acyl carrier protein of the synthetase. Control of the chain length of the growing fatty acids synthesized by this multi-enzyme was suggested by Barnes (12) and Porter (21) on the ground that the specificity of the pigeon liver thioesterase permits fatty acid synthesis to continue until palmitoyl-ACP is formed. But this suggestion is disputed by Kurooka (11), because in mammalian tissues the content of myristic acid is generally only one tenth of that of palmitic acid and stearic acid despite the fact that myristoyl-CoA is hydrolyzed by the long chain thioesterase of rat tissues

at almost the same rate as is palmitoyl-CoA. Long chain fatty acyl-ACP or acyl-CoA thioesterases which are not part of the multi-enzyme complex of fatty acid synthesis have been found in mammals, bacteria and plants (11, 12, 15, 17). A study of the tissue distribution of long chain acyl-CoA thioesterase in rat (11) showed that the activity of this enzyme in brain and testis (30 and 15 μmoles/min/g tissue respectively) is about 10-times higher than in kidney, liver, spleen and heart tissue. The reason for this unequal distribution is unknown. Two long chain thioesterases have been purified from *E. coli* (12-14). Their molecular weights were found to be 22,000 and 122,000. In addition to their difference in molecular weights, the two enzymes differ in their susceptibilities to inhibitors and in their substrate specificities (12, 13, 14). The smaller in contrast to the larger enzyme is inhibited by diisopropyl fluorophosphate and does not hydrolyze β-hydroxyacyl-CoA esters. The Michaelis constants (K_m) of both enzymes for enzymatically synthesized palmitoyl-ACP are 100 to 200 μM, values which are about 10-times higher than those observed with chemically-synthesized palmitoyl-ACP or palmitoyl-CoA (22). This difference appears to be due to some chemical modification of the ACP moiety which may have occurred during the chemical synthesis of the substrates (23, 24, 25). Although the two long-chain acyl-ACP thioesterases in *E. coli* may possibly function in

regulating the chain length of newly synthesized fatty acids, evidence presented by Greenspan et al. (26) suggests that β-ketoacyl-ACP synthetase serves this function by only elongating ACP derivatives of fatty acids which contains 14 or less carbons. The role of long chain fatty acyl thioesterases in _E. coli_ and mammalian tissue may be to regenerate ACP needed for fatty acid synthesis and to prevent accumulation of palmitoyl-ACP or palmitoyl-CoA which are known to inhibit many enzymes generally due to their detergent effects (12, 22). The thioesterases may prevent the accumulation of intracellular acyl-CoA in _E. coli_ even when the cells are involved in β-oxidation (27, 28). A small protein of M.W. 10,500 from _Mycobacterium_ which participates in the process of long chain fatty acid elongation has been studied by Vance et al. (15). This protein was required for the high molecular weight synthetase from _Mycobacterium_ to elongate palmitoyl-CoA to lignoceroyl-CoA. It was found to be a potent thioesterase for palmitoyl-CoA and other long chain acyl-CoA derivatives and appears to stimulate elongation by regulating the levels of both palmitoyl-CoA and free CoA. A plant thioesterase which is several times more active with oleoyl-ACP than with stearoyl-ACP was detected in safflower, avocado and spinach (17). The isolation of oleic acid, as the desaturation product of stearoyl-ACP (29) and the desaturation of oleoyl-CoA but not of oleoyl-ACP by safflower microsomal preparations (30) led to the suggestion by Shine et al. (17)

that this long chain thioesterase from plants is involved in conversion of oleoyl-ACP to oleoyl-CoA by acting in concert with an acyl thiokinase. The switching from ACP ester to CoA ester is necessary for the further desaturation of oleic acid and its incorporation into polar lipids (30) because both processes require the CoA derivative of this fatty acid.

Medium chain fatty acyl-thioester hydrolases from lactating rabbit (8) and rat (9) mammary glands, both of which have a molecular weight of about 30,000 and are not part of the fatty acid synthetase complex, apparently function in terminating fatty acid synthesis at the level of medium chain fatty acids which are characteristic components of milk fats. The major role of this medium chain thioesterase is therefore that of shifting the product specificity of mammary gland fatty acid synthetase from predominantly long chain fatty acids to mainly medium chain fatty acids. These thioesterases have negligible hydrolase activity with butyryl and hexanoyl thioesters and seem to be unique to the mammary gland.

In contrast to the possible roles of long chain and medium chain fatty acyl thioester hydrolases in the termination and elongation of fatty acid synthesis described above, the function of short chain fatty acyl thioesterases, which have been observed in different tissues of various organisms, remain poorly understood. Acetyl-CoA hydrolase,

although known for many years, has only recently been studied in detail. This enzyme is widely distributed in animal tissues. The tissue with the highest activity is liver and most of the activity is localized in the mitochondrial fraction (6, 7, 31, 32). Two distinct short chain thioesterases have been identified in rat liver mitochondria by DEAE-cellulose chromatography (31). Acetyl-CoA was the best substrate for one enzyme whereas propionyl-CoA was preferred by the other. Both enzymes also hydrolyzed succinyl-CoA and butyryl-CoA, but had minimal activities towards long chain acyl-CoA compounds. Rat brain acyl-CoA thioesterase, partially purified by Robinson (7), was found to be most active with acetyl-CoA but it hydrolyzed also malonyl-CoA, acetoacetyl-CoA, succinyl-CoA, butyryl-CoA and even decanoyl-CoA. This enzyme was inactive towards palmitoyl-CoA. It is however possible that both investigators, Robinson (7) and Knowles (6), might have missed palmitoyl-CoA thioesterase activity in their enzyme preparation because they used very high concentrations of palmitoyl-CoA (0.24 mM (7) and 0.5-4 mM (6)) which could have caused the inactivation of the enzyme due to its detergent effect. When acetyl-CoA hydrolase activity in various rat tissues was determined (6, 7), liver (4 μmoles/min/g of wet tissue) was found to have the highest activity whereas the activity in kidney, heart and brain was about 5 to 10 times lower. Sheep tissues have about

one third the activity of the corresponding tissues of rat (6). Robinson (7) observed about 10 to 20 times more acetyl-CoA thioesterase activity in rat tissues of brain, kidney and heart than did Knowles (6). However both investigators reported for liver the same activity of 4 μmoles/min/g of wet tissue. The reason for this discrepancy is not known, but it may be partly due to differences in their assay methods. Bernson (20) demonstrated that the brown adipose tissue mitochondria prepared from cold adapted rats and guinea pigs require malate for significant palmitoyl-L-carnitine oxidation, but the mitochondria prepared from cold-adapted hamster (a hibernator) rapidly oxidized palmitoyl-L-carnitine in the absence of added malate. The major product of malate-independent fatty acid oxidation in mitochondria of hamster adipose brown tissue was identified as acetate. The rate of acetate formation was found to be temperature independent in the range of 37°-10°C whereas the capacity of the citric acid cycle was substantially reduced at low temperatures. Acetate was also produced from pyruvate and palmitoyl-L-carnitine when rat heart mitochondria were incubated with fluorocitrate or fluorocitrate plus rotenone (6, 33, 34, 35). These findings indicate that an inhibition of the citric acid cycle results at least in some tissues in the formation of acetate which is produced from accumulated acetyl-CoA by the action of acetyl-CoA hydrolase instead

of being utilized for the synthesis of citrate. Obviously this bypass impairs vital reactions such as substrate-level phosphorylation and reactions involving the citric acid cycle intermediates and also results in a lower energy yield within the tissue. Since an uncontrolled hydrolysis of acetyl-CoA represents a waste of energy, acetyl-CoA thioesterase is expected to be carefully regulated, especially in those tissues which have high levels of thioesterase with a high affinity for acetyl-CoA as in hamster brown adipose tissue (20). K_m values for various substrates including acetyl-CoA were reported to be less than 100 μM for the thioesterase from rat brain (7) and 51 μM for the enzyme from hamster brown adipose tissue (20). But rat and sheep liver mitochondria contained acetyl-CoA hydrolases which have more than 10-times lower affinities for acetyl-CoA (6, 31). Free CoA was found to be a strong inhibitor of the enzyme from hamster brown adipose tissue (K_I = 0.5 μM (20)), rat brain (K_I < 100 μM (7)), rat and sheep liver (31). NADH was a non-competitive inhibitor (K_I = 15 μM) for the enzyme from hamster (20). In contrast ADP activated the enzyme from hamster and acted as an inhibitor of the enzyme from rat brain (7). It is possible that these metabolically-important coenzymes may participate in regulating these enzymes in vivo. Thus, when the citric acid cycle is operative and CoASH is rapidly regenerated, acetyl-CoA thioesterase will be

inhibited and acetate formation suppressed. Furthermore, under normal physiological conditions, acetyl-CoA will be preferentially utilized for citrate formation because the K_m of citrate synthetase for acetyl-CoA is only half of that of acetyl-CoA thioesterase and because the specific activity of citrate synthetase in hamster brown adipose tissue is 5 to 6-times higher than that of the hydrolase. When the flow through the citric acid cycle is restricted, the acetyl-CoA/CoA ratio would increase, but in contrast to liver mitochondria in which a high acetyl-CoA/CoA leads to the production of ketone bodies (36), mitochondria from hamster brown adipose tissue (32) and rat heart (16) produce acetate. Another enzyme competing in the utilization of acetyl-CoA is carnitine acetyl-transferase, which has an apparent K_m similar to that of the hydrolase but twice the V_{max}/mg soluble mitochondrial protein. In summary, when the citric acid cycle is inhibited in non-hepatic tissues, acetyl-CoA formed from either pyruvate or fatty acids may be hydrolyzed by acetyl-CoA hydrolase. The acetate thus formed is redistributed throughout the body and further metabolized. Therefore, acetate metabolism in non-hepatic tissues seems to have a function similar to that of ketone body metabolism in liver.

In this work, I report the partial purification of a pig heart thioesterase, present evidence for its location in mitochondria and demonstrate its uniqueness among the

many thioesterases mentioned in this introduction by showing that it is apparently a single enzyme which acts on a wide range of substrates including acetyl-CoA, palmitoyl-CoA, acetoacetyl-CoA and succinyl-CoA. Furthermore, the effect of various mitochondrial coenzymes and metabolites on the activity of the enzyme were investigated and the results are discussed in the light of this enzyme's metabolic function.

EXPERIMENTAL PROCEDURES

Materials - Coenzyme A, acetyl-CoA, n-butyryl-CoA, n-decanoyl-CoA, palmitoyl-CoA, oleoyl-CoA, malonyl-CoA, agarose-hexane-CoA, NADH, NAD^+, NADPH and $NADP^+$ were purchased from P-L Biochemicals, Inc. Oxidized-CoA, ATP, GTP, ADP, AMP, bovine serum albumin (BSA), N-methylmaleimide (NMM), cytochrome c were purchased from Sigma Chemical Co. [1-^{14}C]palmitoyl-CoA was a product of New England Nuclear. Crotonic anhydride was bought from Eastman Kodak Co. Ethylchloroformate, tri-ethanolamine, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Aldrich Chemical Co. Phenylmethylsulfonyl fluoride and pantethine were purchased from Calbiochem. Hydroxylapatite and Selectacel DEAE-cellulose were products of Bio-Rad Laboratories and Brown Company respectively. Glutathione was from E. M. Laboratories. All other chemicals used were of reagent grade. Thioesterase used for the study of its properties was isolated from fresh pig heart purchased from Max Insel Cohen Co., New Jersey.

Preparation of Substrates - The following substrates were prepared according to published procedures: crotonyl-CoA (37), acetoacetyl-CoA (38), succinyl-CoA (39) and 3-keto-decanoyl-CoA (40). The CoA derivatives of DL-3-hydroxy-butyric acid and DL-3-hydroxydecanoic acid were synthesized by the method of Goldman and Vagelos (41). The S-aceto-acetyl derivatives of glutathione, pantetheine and N-acetyl-

cysteamine were prepared by reacting the thiol compounds with diketene analogously to the synthesis of acetoacetyl-CoA (38). N-Acetylcysteamine and pantetheine were obtained by reducing N-Diacetylcystamine and pantethine respectively with $NaBH_4$. N,N'-Diacetylcystamine was synthesized by reacting an alkaline solution of cystamine with acetic anhydride. The concentrations of all CoA derivatives were determined by the method of Ellman (42) after cleaving the thioester bond with hydroxylamine at pH 7.

Enzyme Assays and Protein Determination - Thioesterase was routinely measured by following spectrophotometrically the increase in absorbance at 412 nm due to the reaction of liberated CoASH with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) (standard assay). A molar extinction coefficient of 13,600 $M^{1}cm^{1}$ (42) was used for calculating units of activity which are defined as μmoles of substrate hydrolyzed per min. Bovine serum albumin (BSA) was added routinely to the assay mixture to alleviate the detergent effect of long chain fatty acyl-CoAs. The thioesterase was found to have negligible activity with palmitoyl-CoA as a substrate in the absence of BSA but exhibited optimal activity when 200 μg of BSA were added to 0.6 ml of assay mixture containing 10 nmoles of palmitoyl-CoA. The same amount of BSA increased thioesterase activity with decanoyl-CoA as a substrate by 30% and had no effect on the hydrolysis of the short chain substrates, acetyl-CoA,

butyryl-CoA and acetoacetyl-CoA. A typical assay contained in a final volume of 0.6 ml : 60 μmoles of Tris-HCl (pH 8), 30 μmoles of KCl, 200 μg of bovine serum albumin, 128 nmoles of DTNB, 10 nmoles of acyl-CoA and 1 μg of thioesterase. All assays were performed at 28°. Occasionally thioesterase was assayed by following spectrophotometrically at 232 nm the decrease in absorbance due to the hydrolysis of the thioester bond (direct assay). The assay conditions were the same as those given above except that DTNB was omitted from the mixture. A molar extinction coefficient of 4,250 $M^{1}cm^{-1}$ was determined and used for calculating activities. For studying the inhibition of the thioesterase-catalyzed hydrolysis of palmitoyl-CoA by acetyl-coA, the release of [1-^{14}C]palmitic acid from palmitoyl-CoA was followed (radioactive assay). The assay mixture contained in addition to the components presented under "standard assay", 6 nmoles [1-^{14}C] palmitoyl-CoA (containing 8.8 x 10^4 dpm) and various amounts of acetyl-CoA. The reactions were terminated after 2 min by acidification with 10% trichloroacetic acid. After addition of unlabeled palmitic acid to a final concentration of 1 mM, the reaction mixtures were extracted 3 times with 3 ml each of pentane and the combined extracts were evaporated to dryness under a stream of N_2. The residues were extracted with a mixture of 2.5 ml of heptane and 1 ml of H_2O. Portions of the heptane phases (2 ml)

were transferred to counting vials and mixed with 10 ml of toluene based scintillation fluid for radioactivity measurements in a Beckman liquid scintillation counter. All results were corrected for counts extracted in the absence of enzyme. To prevent the loss of palmitic acid due to the adsorption to the glass, quartz cuvettes were used as reaction vessels and plastic tubes were used for the extraction. The recovery of [1-^{14}C]palmitic acid produced in the thioesterase reaction was better than 90%. All reactions were started by the addition of thioesterase. Acid phosphatase was assayed by the method of Fiske-Subbarow (43) except that ascorbic acid and 0.1 M sodium acetate-acetic acid buffer (pH 4) were used instead of the Fiske-Subbarow reagent and 5N H_2SO_4 respectively (44). Fumarase (45) and succinate cytochrome c reductase (46) were assayed according to published procedures. Protein was determined by the biuret method of Gornall et al. (47) or by the method of Lowry et al. (48).

Purification of Pig Heart Thioesterase - 1069 g of fresh pig heart were trimmed of fat, cut into small pieces and forced through a meat grinder. Batches of half of the material were blended together with 600 ml of precooled acetone (-15°) in a Waring blender for 2 min. at low speed and 2 min. at high speed. The resulting acetone suspensions were rapidly filtered through cheese cloth. The solid retentate was washed with 600 ml of precooled

acetone and then with the same amount of precooled ether. The residual ether was removed under vacuum. A total of 283 g of dried acetone powder was thus obtained. The acetone powder was extracted overnight by stirring it with 1600 ml of 0.02 M potassium phosphate (pH 6.6). All purification steps were performed at 4°C unless otherwise indicated. Insoluble material was removed by centrifugation at 16,600 x g for 30 min. The supernatant was filtered through cheese cloth yielding 1120 ml of filtrate. The filtrate was then applied to a phosphocellulose column (5 x 41 cm) which had been previously equilibrated with 3 column volumes of 0.02 M potassium phosphate (pH 6.6). The column was washed with the same buffer until no further ultraviolet-absorbing material was eluted and then developed with 0.5 M potassium phosphate (pH 6.6). A total of 1150 ml eluant was concentrated in an Amicon concentrator (PM-10 membranes) to 91 ml. A 30 ml portion of it was extensively dialyzed against 0.02 M potassium phosphate (pH 6.6) and applied to a hydroxylapatite column (4 x 30 cm) which had been equilibrated with 0.02 M potassium phosphate (pH 6.6). The column, after having been extensively washed with the same buffer, was developed with a gradient made up of 1 liter of each of 0.02 M potassium phosphate (pH 6.6) and 0.5 M potassium phosphate (pH 6.6). Fractions of 20 ml were collected at a flow rate of 80 ml per hour. The fractions with high thio-

esterase were pooled and concentrated to 20 ml. The concentrate was extensively dialyzed against 0.02 M Tris-HCl (pH 8) and adsorbed on a DEAE-cellulose column (2.5 x 45 cm) which had been previously equilibrated with 0.02 M Tris-HCl (pH 8) containing 0.05 M NaCl. After washing the column with the same buffer, it was developed with a gradient made up of 0.6 liter each of 0.02 M Tris-HCl (pH 8) containing 0.05 M NaCl and 0.02 M Tris-HCl (pH 8) containing 0.35 M NaCl. The fractions with high thioesterase activity were pooled and concentrated. The concentrated and dialyzed preparation after chromatography on DEAE-cellulose was applied to a DEAE-Sephadex A-50 column previously equilibrated with 0.02 M Tris-HCl (pH 8) containing 0.1 M NaCl. After washing the column with the same buffer, it was developed with a gradient made up of 500 ml each of 0.02 M Tris-HCl (pH 8) containing 0.1 M NaCl and 0.02 M Tris-HCl (pH 8) containing 0.3 M NaCl. The fractions with high thioesterase activity were pooled, concentrated and stored at -76°.

Subcellular Fractionation of Pig Heart Homogenate - For subcellular fractionation, the fresh pig heart was trimmed of fat and connective tissue and cut into small pieces, then passed through a meat grinder and washed thoroughly with 0.01 M Tris-HCl (pH 7.8) containing 0.25 M sucrose. The minced material was then suspended in the same buffer and homogenized in a loose fitting teflon-pestled

Potter-Elvehjem homogenizer. The glass tube containing the minced suspension was kept in ice and was given a single upward stroke against the rapidly rotating pestle (1400 rpm). The homogenate thus obtained was filtered through a double layer of cheese cloth. The residual material was rehomogenized and the two filtrates were combined. The combined filtrates were fractionated as described by DeDuve et al. (49). The following five fractions were obtained under the indicated conditions: 1, 700 x g for 10 min; 2, 24,000 x g for 10 min; 3, 106,00 x g for 60 min; 4, 105,000 x g for 100 min and 5, soluble fraction. All fractions were assayed as described above for thioesterase, fumarase, which is located in the mitochondrial matrix (50), acid phosphatase, a marker enzyme for lysosomes (51), and succinate cytochrome c reductase which is associated with the inner mitochondrial membrane (52). All assays were performed after sonicating the fractions, except for the soluble fraction, to assure the complete release of enzymes from the organelles.

RESULTS

Intracellular Localization of Pig Heart Thioesterase - During studies of fatty acid metabolism in heart muscle, an acyl-CoA thioesterase, which interfered with acyl-CoA dehydrogenase assays, was observed in pig heart homogenates. Since the thioesterase, if present in mitochondria, may have a function in mitochondrial fatty acid metabolism and its regulation, the subcellular location of this enzyme was investigated. For this purpose a pig heart homogenate was separated into five subcellular fractions by differential centrifugation as described by deDuve et al. for liver homogenate (49). Each of the five fractions was assayed for fumarase, acid phosphatase, succinate cytochrome c reductase and butyryl-CoA thioesterase activities. The data, when plotted as specific activities vs. the percentage of total protein present in each fraction, yielded the graph shown in Fig. 1. The presence of fumarase in the supernatant indicated that a fraction of the mitochondria has been damaged, a fact which is not surprising in view of the toughness of pig heart muscle. Since the distribution of thioesterase between the five subcellular fractions was virtually identical to that of fumarase but different from that of acid phosphatase, it is concluded that this thioesterase like fumarase is a soluble mitochondrial enzyme. This conclusion is strengthened by a closer examination of the above results which show that in every subcellular

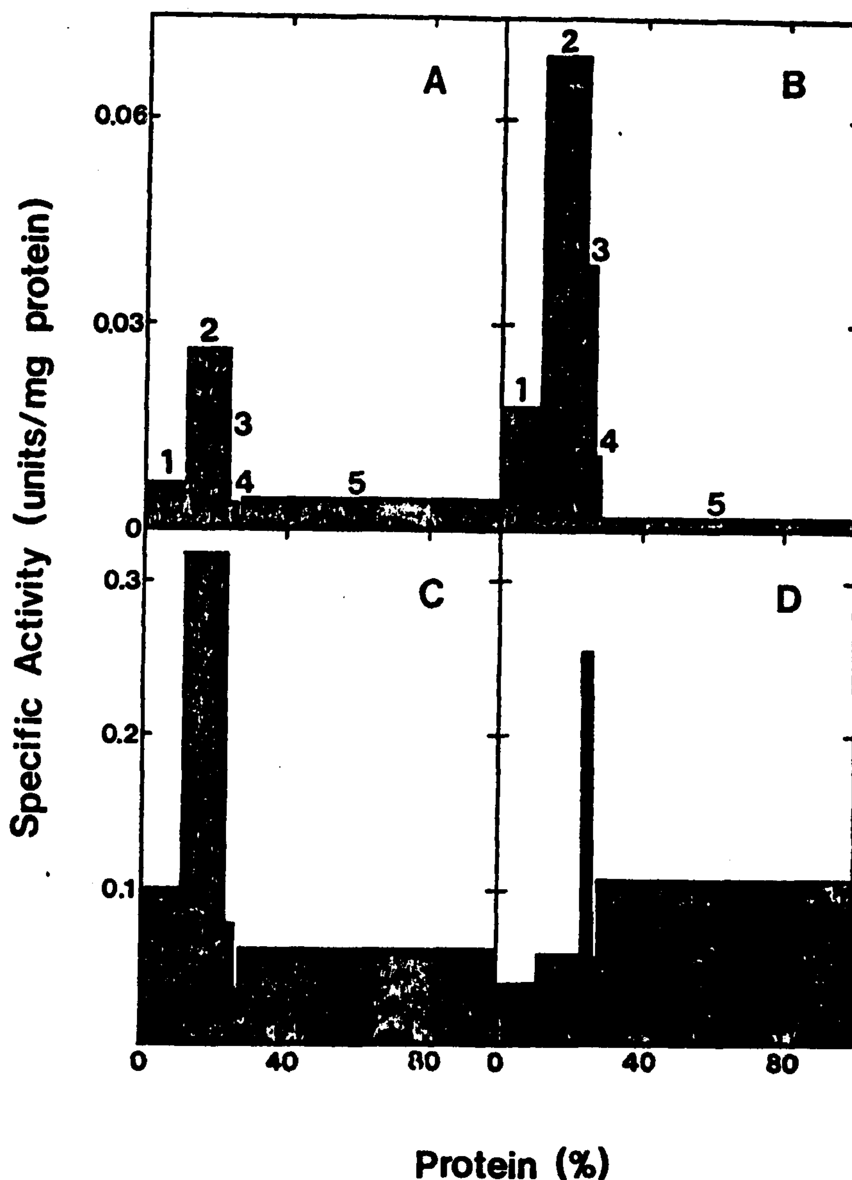


Fig. 1—

Subcellular distribution of pig heart thioesterase and sevral marker enzymes. Ordinate: specific enzyme activities. Abscissa: percent of total protein present in five subcellular fractions. Subcellular fractionation and standard assays were performed as described under "Experimental Procedures". A, butyryl-CoA thioesterase; B, succinate cytochrome C reductase; C, fumarase; D, acid phosphatase. Subcellular fractions were obtained under the following conditions: 1, 700 x g for 10 min.; 2, 24,000 x g for 10 min.; 3, 106,000 x g for 60 min.; 4, 105,000 x g for 100 min. and 5, soluble fraction.

fraction the relative amounts of thioesterase and fumarase are virtually equal. This observation is illustrated in Fig. 2. When the subcellular fractions were assayed for thioesterase activities with acetyl-CoA, decanoyl-CoA and palmitoyl-CoA as substrates, the results shown in Fig. 3 were obtained. The thioesterase was found to be concentrated in the mitochondrial fraction. If additionally the damage to mitochondria and the concomittant release of thioesterase from these organelles is considered (see above), most of the thioesterase activities observed in pig heart appears to be located in mitochondria. The finding that the relative thioesterase activities of the mitochondrial fraction were identical to those of the partially purified enzyme preparation (vide infra) provides evidence for the mitochondrial location of the partially purified thioesterase extracted from a pig heart acetone powder.

Partial Purification of Thioesterase from Pig Heart - Fresh pig heart packed in ice and received directly from the slaughter-house were used for the isolation of the enzyme. The purification was started by preparing an acetone powder from the whole tissue rather than from mitochondria since the isolation of intact mitochondria in large quantities from pig heart poses difficult technical problems. Chromatography on phosphocellulose was used as the first step in the purification because by this step, one

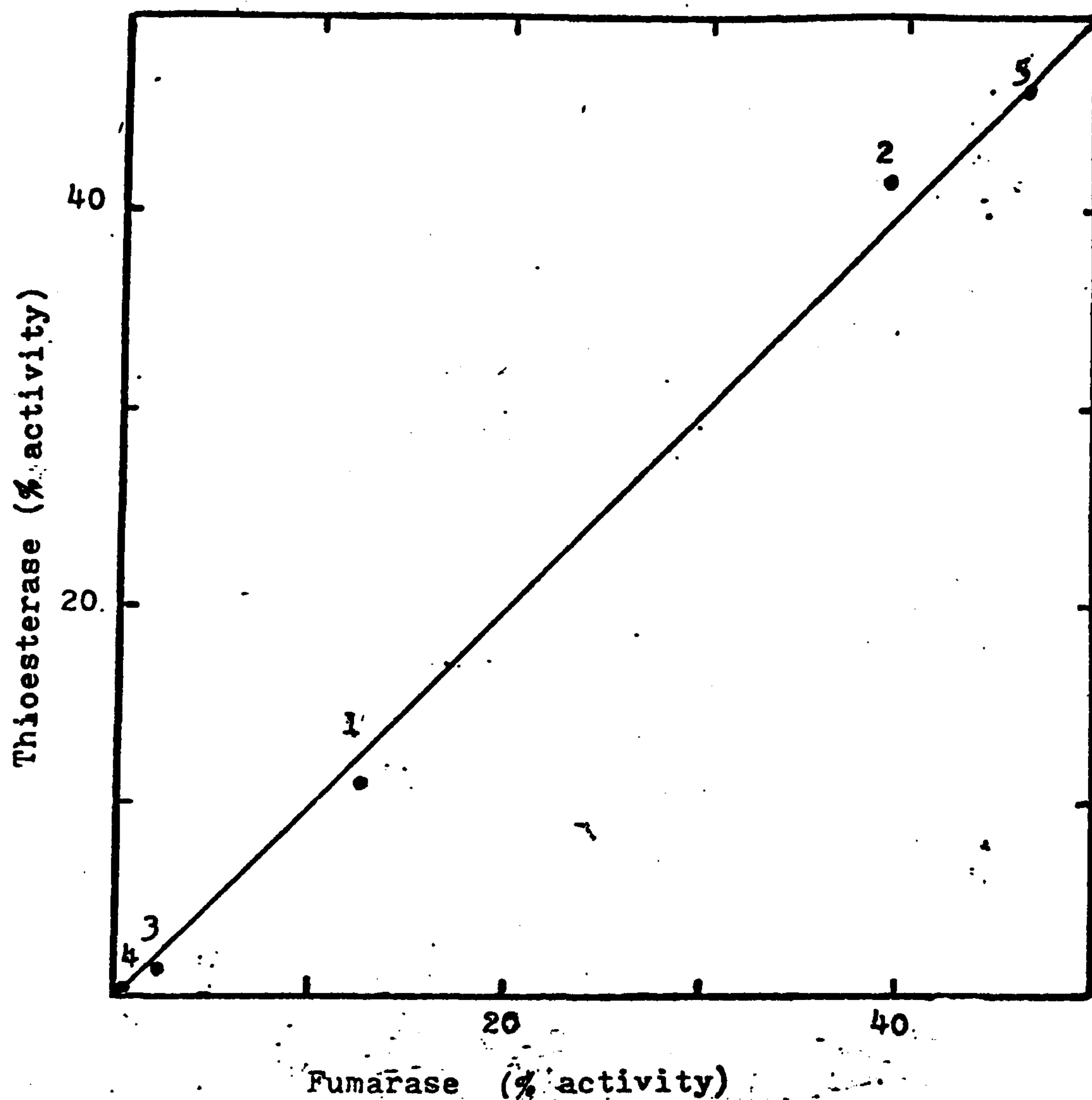


Fig. 2.
Distribution of fumarase and butyryl-CoA thioesterase activities in subcellular fractions of pig heart: Subcellular fractionation and standard enzyme assays were performed as described under "Experimental Procedures". Subcellular fractions were obtained under the following conditions: 1, 700 x g for 10 min; 2, 24,000 x g for 10 min; 3, 106,000 x g for 60 min; 4, 105,000 x g for 100 min and 5, soluble fraction.

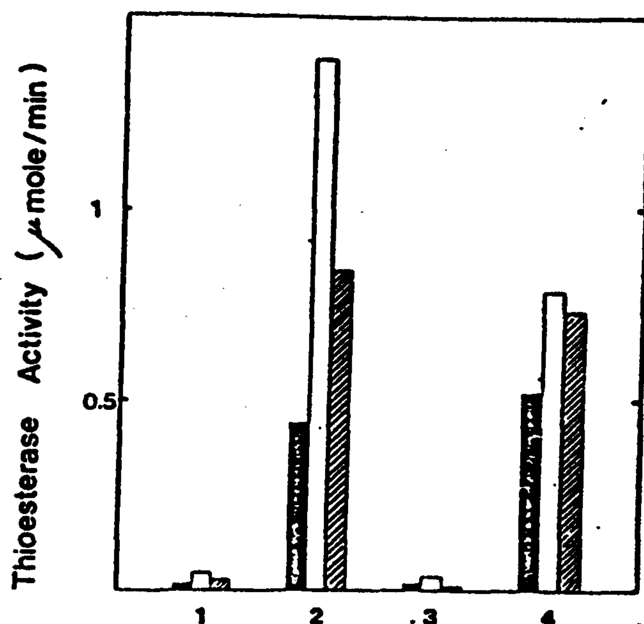


Fig. 3.
Thioesterase activities in subcellular fractions of pig heart muscle. Solid bars, acetyl-CoA thioesterase; open bars, decanoyl-CoA thioesterase; hatched bars, palmitoyl-CoA thioesterase. The subcellular fractionation and standard enzyme assays were performed as described under "Experimental Procedures". Subcellular fractions were obtained under the following conditions: 1, 700xg for 10 min; 2, 24,000xg for 10 min; 3, 106,000xg for 60 min and 4, soluble fraction.

third of the protein was removed without significant loss of thioesterase activity as shown in Table I. The results of further purification steps by column chromatographies on hydroxylapatite, DEAE-cellulose and DEAE-Sephadex A-50 are summarized in Table I. Thioesterase which adhered to these three column materials was eluted with the linear gradients as described under "Experimental Procedures". Unfortunately the thioesterase emerged from all three columns in form of a broad peak close to or as part of one of the main protein fractions. As a result of this situation, the enzyme after the four-step purification procedure was purified only 44-fold over the acetone powder extract. Because of the large activity losses which occurred during the purification of this thioesterase, further attempts to purify the enzyme by standard procedures seemed unpromising. This conclusion was confirmed by the finding that neither chromatography on Sephadex G-200 nor electrofocusing resulted in significant increases in the specific activity of the partially purified enzyme. The ultimate purification of this thioesterase was therefore attempted by affinity chromatography on agarose-hexane-coenzyme A to which the enzyme adhered in the presence of 0.1 M Tris-HCl (pH 8) and from which it was desorbed with 0.1 M Tris-HCl (pH 8) containing 0.5 M NaCl. However, again due to large activity losses (over 50%), the specific activity of the enzyme

Table I

Purification of pig heart thioesterase[a]

	Step	Protein	Total activity	Specific activity	Yield	Purification
		mg	µmoles/min	µmoles/min/ mg of protein	%	fold
1.	Acetone powder extract	5525	337	0.061	100	1
2.	Phospho-cellulose	3822	321	0.084	95.3	1.4
3.	Hydroxylapatite	650	197.1	0.3	58.5	4.9
4.	DEAE-cellulose	63.8	47.9	0.75	14.2	12.3
5.	DEAE-Sephadex A-50	7.2	19.4	2.7	5.8	44.3

[a] The thioesterase activity was measured by the standard assay method with butyryl-CoA as substrate as described under "Experimental Procedures". The acetone powder was prepared from 356 g of pig heart.

did not increase significantly. It has also been found during this study that neither ammonium sulfate, nor glycerin, nor mercaptoethanol stabilize this thioesterase. At -13°C, the enzyme lost half of its activity gradually over a week. But the enzyme was remarkably stable when it was kept frozen at -76°C and virtually no loss of its activity was observed when the partially purified thioesterase was kept for over one year at this temperature. For further studies the preparation which was obtained after the four-step purification procedure summarized in Table I and which was only partially purified as judged by polyacrylamide gel electrophoresis (Fig. 4) was used. The molecular weight of this thioesterase was estimated by chromatography on Sephadex G-200 (53) to be 300,000 (Fig. 5).

Properties of Pig Heart Thioesterase - The substrate specificity of the partially purified thioesterase was established by measuring the rates of the thioesterase-catalyzed hydrolysis of a wide range of thioesters listed in Table II. Since acetoacetyl-CoA was the preferred substrate while the acetoacetyl derivatives of glutathione, N-acetylcysteamine and even pantetheine were not hydrolyzed, the thioesterase seems to be specific for CoA esters. In contrast, the enzyme is less specific with regard to the acyl portion of the substrate as evidenced by the near equal rates of hydrolysis observed with

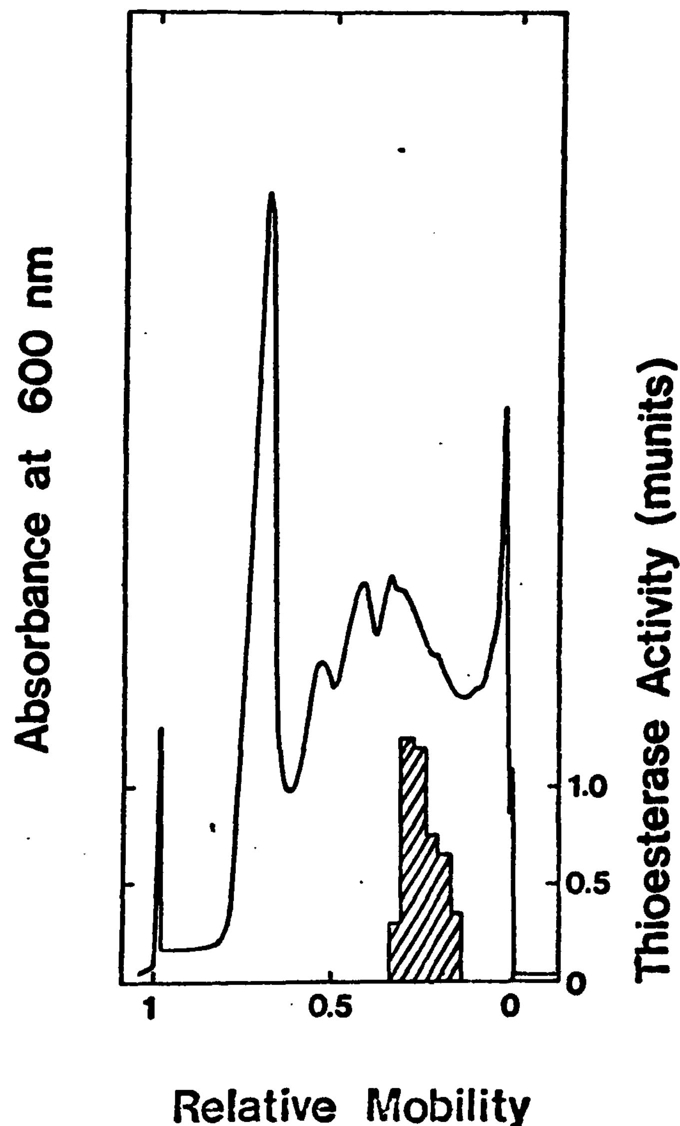


Fig. 4.
Polyacrylamide disc gel electrophoresis of partially purified pig heart thioesterase. Disc gel electrophoresis was performed with two identical 7.5% polyacrylamide gels at pH 8.5 with 27.7 μg of protein per gel. After the electrophoresis, one gel was stained with Commassie blue R and scanned at 600 nm while the other was cut into 1.5 mm thin slices. Each slice was extracted overnight with 0.2ml of 0.2 M potassium phosphate (pH 8) at 4° C and assayed (standard method) for thioesterase activity with decanoyl-CoA as substrate. The hatched region represents the area of the gel where the thioesterase activity was located.

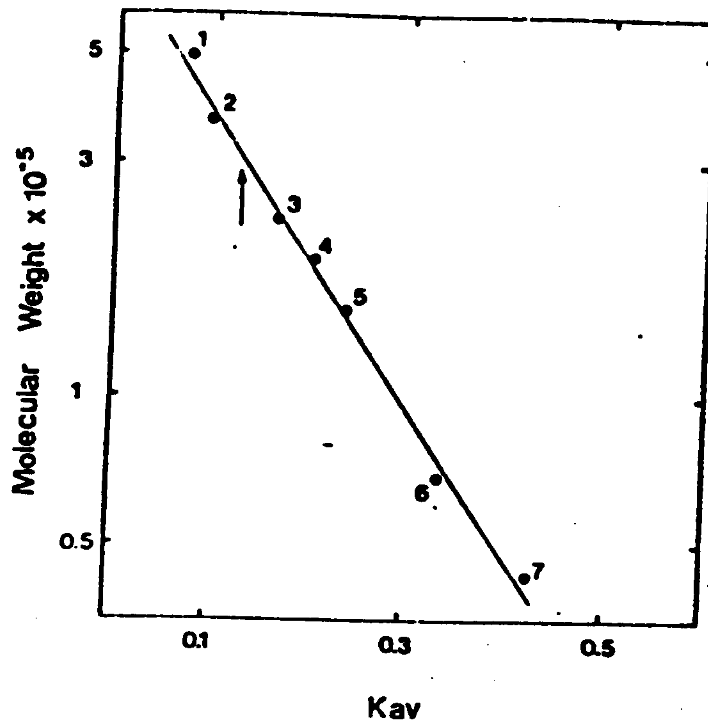


Fig. 5.

Molecular weight determination of thioesterase by chromatography on Sephadex G-200. A Sephadex G-200 column (2.6 x 46 cm) was equilibrated with 0.05 M potassium phosphate PH 7.8. The following standards were used : 1, L-glutamate dehydrogenase; 2, phosphorylase a; 3, catalase; 4, phosphorylase b; 5, lactate dehydrogenase; 6, ovalbumin; 7, bovine serum albumin. All standards (10 mg/ml) were applied to the column in 1 ml samples and their elution volumes were determined by measuring absorbance at 280 nm. A 1 ml sample of thioesterase (10 mg protein) was applied separately to the column and its elution volume was determined by assaying thioesterase activities with acetyl-CoA, decanoyl-CoA and palmitoyl-CoA as substrates (standard assay method). The arrow indicates the position of thioesterase.

Table II

Substrate specificity

Substrate	Rel. Activity[a]	V_{max}
	%	units/mg
Acetoacetyl-CoA	100	9.5
n-Decanoyl-CoA	89	8.8
n-Butyryl-CoA	81	8.1
Palmitoyl-CoA	60	
Oleoyl-CoA	40	
Acetyl-CoA	33.4	2.7
Succinyl-CoA	33.2	3.2
3-Ketodecanoyl-CoA	32	
Crotonyl-CoA	9.4	
3-Hydroxybutyryl-CoA	4.3	
3-Hydroxydecanoyl-CoA	1.4	
Malonyl-CoA	0	
Acetoacetyl glutathione	0	
Acetoacetyl N-acetylcysteamine	0	
Acetoacetyl pantetheine	0	

[a] Standard assays were performed as described under "Experimental Procedures" with 17 μM substrate at several enzyme concentrations.

decanoyl-CoA and butyryl-CoA. Acetyl-CoA is a poorer substrate than its longer chain homologs whereas the lower rates of hydrolysis seen with palmitoyl-CoA and oleoyl-CoA were perhaps due to their detergent effects. This explanation is based on the observed stimulation of the hydrolysis of long chain but not of medium or short chain substrates by bovine serum albumin. Since this thioesterase may possibly function in the regulation of fatty acid oxidation, its activity towards various fatty acid oxidation intermediates was investigated. As mentioned above, acetoacetyl-CoA, the preferred substrate was hydrolyzed at a slightly higher rate than was butyryl-CoA. In contrast, 3-ketodecanoyl-CoA was hydrolyzed at only one third of the rate observed with decanoyl-CoA as the substrate. However, even 3-ketodecanoyl-CoA was a good substrate when compared to crotonyl-CoA, 3-hydroxybutyryl-CoA and 3-hydroxydecanoyl-CoA. This thioesterase acted also on succinyl-CoA which was hydrolyzed as efficiently as acetyl-CoA was, whereas the enzyme was inactive towards malonyl-CoA. The K_m values for all good substrates (the top eight on Table II) with the exception of palmitoyl-CoA, oleoyl-CoA and 3-ketodecanoyl-CoA which caused substrate inhibition even in the presence of bovine serum albumin, were determined and found to be 48 μM (Fig. 6). Hence, the relative activities listed in Table II are independent of the substrate

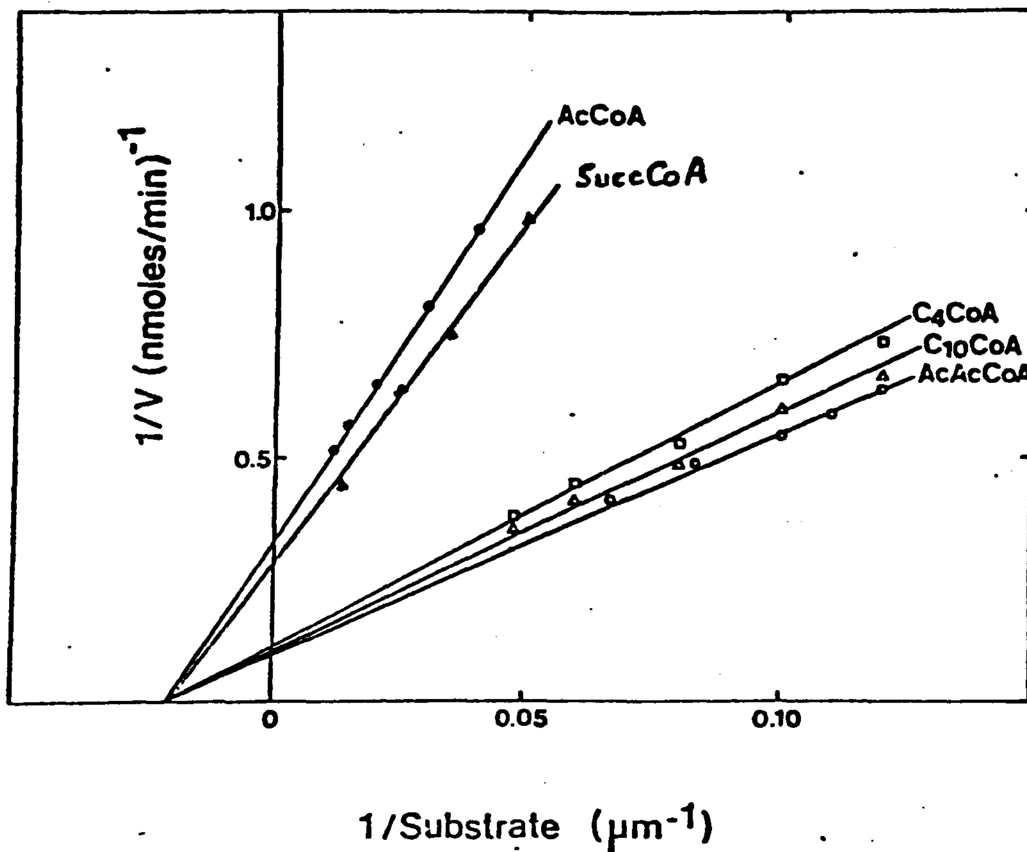


Fig. 6.
Rates of thioesterase-catalyzed hydrolysis of five substrates as a function of their concentrations. Data are plotted on reciprocal coordinates. Standard assays were performed as described under "Experimental Pricedures". AcCoA. acetyl-CoA; succCoA, succinyl-CoA; C_4CoA, butyryl-CoA; C_{10}CoA, decanoyl-CoA; AcAcCoA, acetoacetyl-CoA.

concentration at which the activity measurements were performed.

The pH dependence of the thioesterase activity was determined with butyryl-CoA as a substrate by measuring rates of hydrolysis spectrophotometrically at 232 nm (Fig. 7). The enzyme exhibited optimal activity between pH 8 and pH 8.5. However, the activity was found to be strongly pH-dependent as evidenced by a 50% decrease in activity upon decreasing the pH from 8 to 7. Although several thioesterases have been found to be serine hydrolases as judged by their inhibition in the presence of phenylmethylsulfonyl fluoride (18, 54), this thioesterase was not inactivated by treatment with 5 mM phenylmethylsulfonyl fluoride. Since the pig heart thioesterase was fully active in the presence of 8 mM N-methylmaleimide, the enzyme does not seem to have a reactive essential sulfhydryl group either. The presence of an essential multivalent cation was assessed by measuring the activity after exhaustive dialysis of the enzyme against EDTA. Since this treatment did not affect the activity of the enzyme, the effect of added cations on the thioesterase activity was investigated. As shown in Table III, Ca^{2+} was the only cation which stimulated the thioestersase activity. While Mg^{2+} and Fe^{2+} did not affect the reaction, all other divalent cations, but most pronouncedly Ni^{2+}, inhibited the

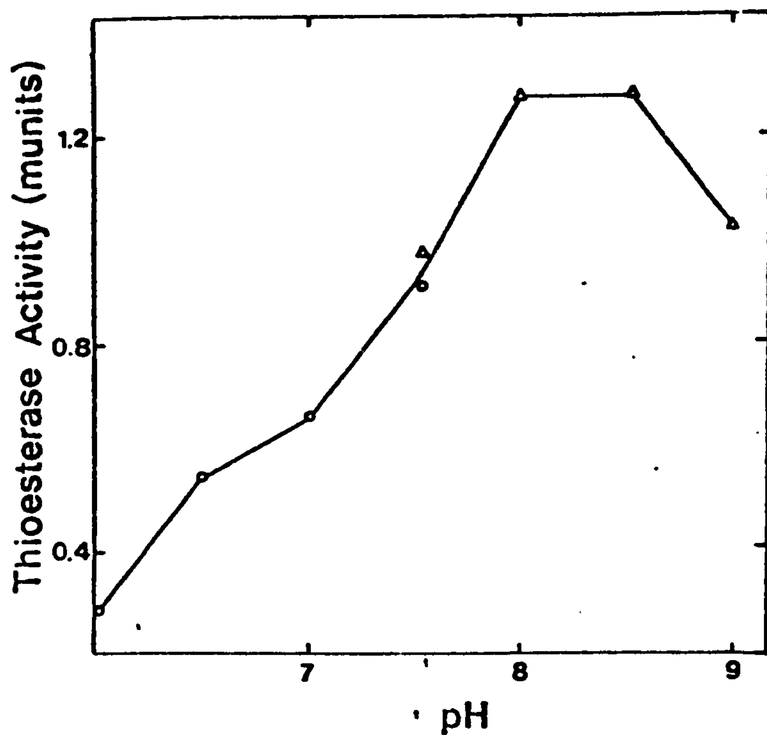


Fig. 7.
Thioesterase activity as a function of pH.
The direct assay method was used as described under "Experimental Procedures" with butyryl-CoA as a substrate. 0.1 M Bis-Tris propane (o—o) and 0.1 M Tris (Δ—Δ) were used as buffers.

Table III

Effect of divalent cations

Divalent cations added	Relative activity[a]			
	0.1 mM	0.5 mM	1 mM	2 mM
		%		
Ca^{2+}	125	137	144.5	164
Fe^{2+}			100	
Mg^{2+}			97.5	
Mn^{2+}			73.3	
Zn^{2+}			55.8	
Co^{2+}			45.4	
Cu^{2+}			23.2	
Ni^{2+}	63	25	13.3	

[a] Thioesterase activities were measured by the standard assay method with butyryl-CoA as substrate as described under "Experimental Procedures". Activity measured in the absence of divalent cations was taken as 100%.

enzyme significantly.

Are the Various Substrates Hydrolyzed by a Single Thioesterase? - Since the thioesterase was not obtained in a homogeneous form, the possibility existed that the partially purified enzyme preparation contained two or more thioesterases whose combined action accounted for the established substrate specificity. However, several lines of evidence do not support this possibility. Most important was the finding that the various thioesterase activities co-migrated or co-banded when partially purified enzyme preparations were subjected to chromatographies on DEAE-cellulose (Fig. 8), and hydroxylapatite (Fig. 9), polyacrylamide gel electrophoresis (Table IV) or to iso-electric focusing (Fig. 10). In the latter experiment the isoelectric pH of thioesterase was determined to be 4.5 (see Fig. 10). Another argument in favor of the presence of a single thioesterase in the partially purified enzyme is based on the observation that the activity ratios were the same in various preparations purified to different degrees (see Table V). Also the parallel thermal inactivations of acetyl-CoA, acetoacetyl-CoA, and palmitoyl-CoA thioesterase activities associated with the partially purified enzyme (see Table VI) attest to the presence of a single thioesterase in this preparation. Finally, I have determined whether or not the activities with different substrates are additive. Specifically

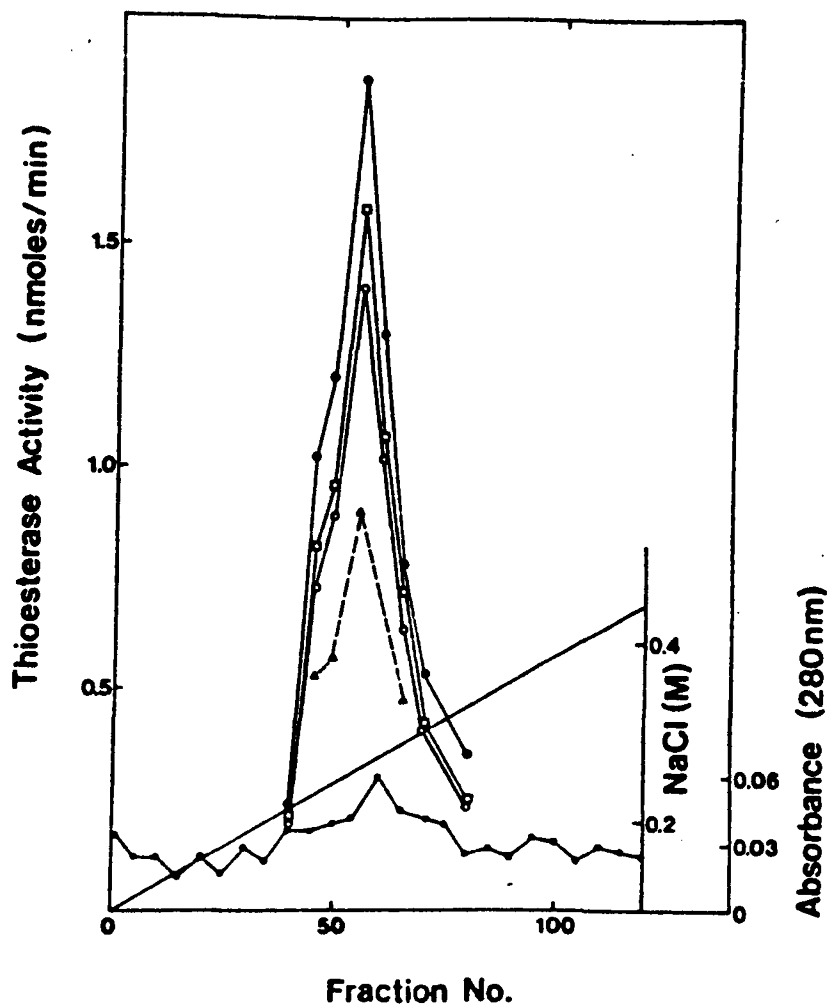


Fig. 8.
Chromatography of partially purified thioesterase on DEAE-cellulose. Thioesterase (2.5 mg) obtained after purification as summarized in Table 1 was applied to a DEAE-cellulose column (1.2 x 42.5 cm) and eluted with a gradient made up of 150 ml of 0.02 M Tris-HCl (pH 8), 0.1 M NaCl and 150 ml of 0.02 M Tris-HCl (pH 8), 0.5 M NaCl. Fractions of 2.4 ml were collected and assayed (standard method) for thioesterase activities with acetoacetyl-CoA (-●-), decanoyl-CoA(-□-), butyryl-CoA (-○-) and palmitoyl-CoA(-▲-) as substrates.

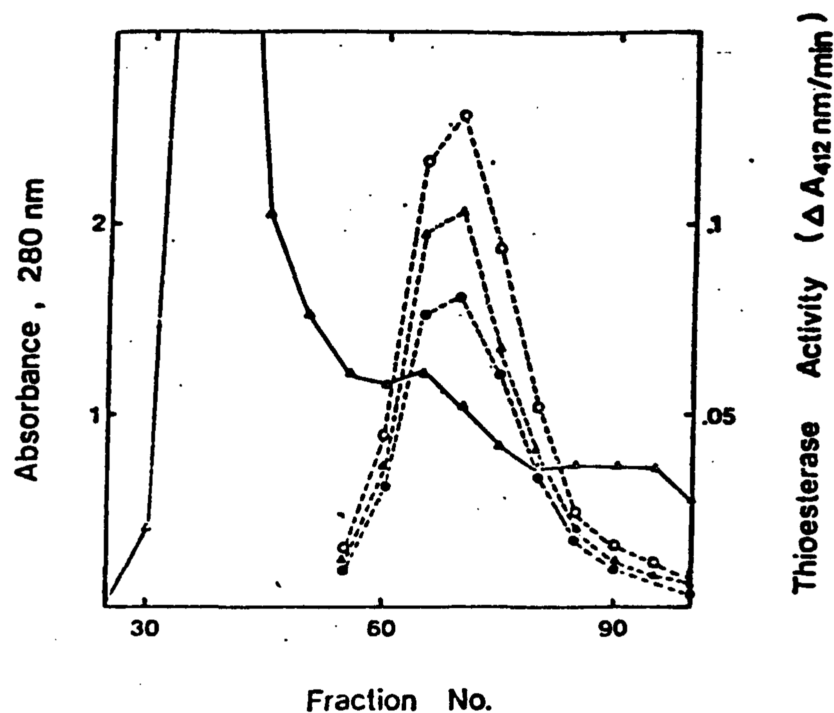


Fig. 9.
Chromatography of thioesterase on hydroxyapatite. Thioesterase (1.7 g of protein), which had been partially purified by chromatography on DEAE-cellulose, was applied to a hydroxylapatite column (1.25 x 43 cm) and eluted with a gradient made up of 600 ml each of 0.02 M and 0.5 M potassium phosphate, pH 6.6. Fractions of 10 ml were collected and assayed (standard method) for thioesterase with butyryl-CoA (▲), decanoyl-CoA(o), and palmitoyl-CoA(•) as substrate. Absorbance at 280 nm (Δ).

Table IV

Comigration of various thioesterase activities on polyacrylamide gels.

Substrate[a]	Relative activities (%)[b]			
	Gel 1	Gel 2	Gel 3	Mean
$C_{10}CoA$	100	100	100	100
AcAcCoA	113	123		118
C_4CoA	89	100	77	89
$C_{16}CoA$		51		51
AcCoA	39			39

[a] $C_{10}CoA$, decanoyl-CoA; AcAcCoA, acetoacetyl-CoA; C_4CoA, butyryl-CoA; $C_{16}CoA$, palmitoyl-CoA; AcCoA, acetyl-CoA.

[b] Determined with extracts of 1.5 mm-wide gel slices which showed the highest thioesterase activities. Procedural details were identical to those given in the legend of Fig. 4.

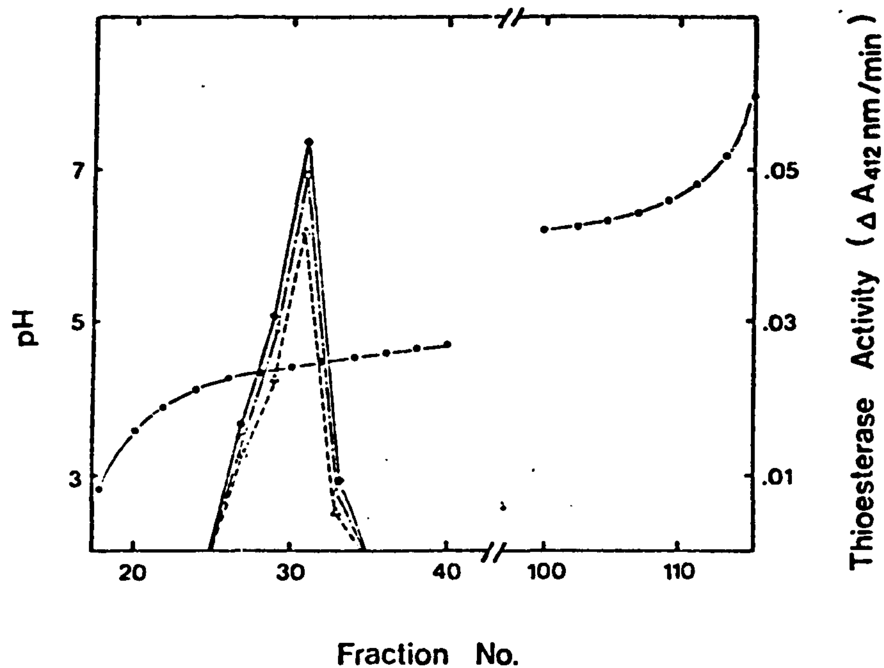


Fig. 10.

Isoelectric focusing partially purified thioesterase (16.7 mg) on a sucrose gradient column (volume 110 ml). The thioesterase was electrofocused for 45 hours at 4° C and 600 V. Fractions of 1 ml were collected and assayed (standard method) for thioesterase activities with butyryl-CoA (o), decanoyl-CoA (◆), and palmitoyl-CoA (△) as substrates. pH (•).

Table V

Relative activities of various thioesterase preparations

Substrate[a]	Relative activities (%)								
	Homog.[b]	Fig.3[c]	Fig.8[c]	Fig.9[c]	Fig.10[c]	Table II[c]	Table IV[c]	V_{max}	Mean
C_{10}CoA	100	100	100	100	100	100	100	100	100
AcAcCoA	125		110			112	118	110	117±5
C_4CoA	99		89	80	95	91	89	92	91±4
C_{16}CoA	67	60	57	63	(84)	67	(51)		62±4
AcCoA		32				37.5	39	31	35±3
Succ-CoA						37		36	36.5±1

[a] C_{10}CoA, decanoyl-CoA; AcAcCoA, acetoacetyl-CoA; C_4CoA, butyryl-CoA; C_{16}CoA, palmitoyl-CoA; AcCoA, acetyl-CoA; Succ-CoA, succinyl-CoA.

[b] Based on activities measured with a crude pig heart homogenate.

[c] Based on activities presented in the indicated Figures or Tables.

Table VI

Effect of temperature on thioesterase activities

		Remaining activity		
Temp	Time	AcCoA	AcAcCoA	C_{16}CoA
^{o}C	min		%	
45	5	82	81	85
45	10	62	62	65
47.5	5	61	65	64
50	5	18	17	17

[a] The thioesterase (0.55 mg/ml) in 0.02 M Tris (pH 8) containing 0.3 M NaCl was incubated at the indicated temperatures for 5 min and 10 min respectively. Its activities were then determined by the standard assay method with acetyl-CoA (AcCoA), acetoacetyl-CoA (AcAcCoA) and palmitoyl-CoA (C_{16}CoA) as described under "Experimental Procedures".

the additivity of acetyl-CoA and palmitoyl-CoA thioesterase activities was evaluated because none of the thioesterase characterized so far has been found to be active with both of these substrates. As shown in Table VII the activities obtained with acetyl-CoA and palmitoyl-CoA were definitely not additive. Similar results were obtained with butyryl-CoA and acetoacetyl-CoA in the presence of acetyl-CoA. This conclusion is also supported by the observed inhibition of the hydrolysis of palmitoyl-CoA in the presence of acetyl-CoA (Fig. 11). Since no evidence for the presence of more than one thioesterase in the partially purified preparation was obtained, it appears that a single enzyme is responsible for the hydrolysis of the various substrates listed in Table II.

Effect of Mitochondrial Coenzymes and Metabolites on Pig Heart Thioesterase Activities - Although the mitochondrial thioesterase of pig heart muscle acts on a wide range of acyl-CoA compounds (Table II), only the hydrolyses of acetyl-CoA and palmitoyl-CoA were studied in detail. These two compounds were chosen because they represent extremes in the range of substrates of this enzyme and because both are present at significant concentrations in mitochondria (54). Since the continuous action of thioesterase would represent a waste of energy, the activity of this enzyme was expected to be tightly regulated. In order to elucidate possible regulatory mechanisms, the effects of various

Table VII

Rates of hydrolysis in the presence of palmitoyl-CoA plus acetyl-CoA

Substrates		Activity[a]	
$C_{16}CoA$[b]	AcCoA[b]	Observed	Calc.[c]
μM	μM	mU	mU
33	-	0.66	
33	33	0.66	1.0
33	66	0.62	1.2
33	133	0.64	1.3

[a] Activities were measured by the standard assay method as described under "Experimental Procedures".

[b] $C_{16}CoA$, palmitoyl-CoA; AcCoA, acetyl-CoA.

[c] Calculated by assuming that activities are additive.

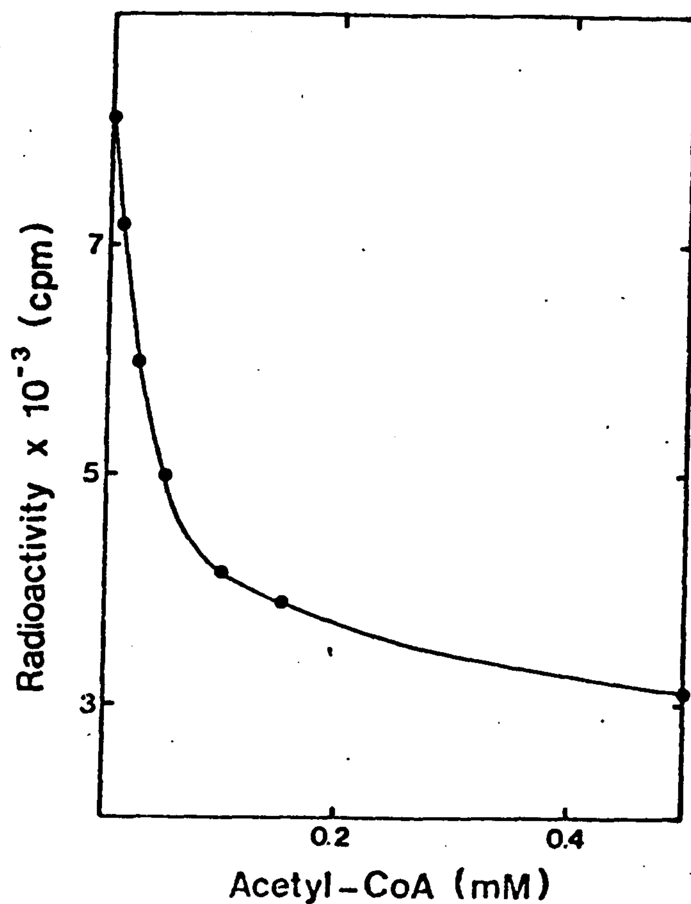


Fig. 11.
Inhibition of the thioesterase-catalyzed hydrolysis of palmitoyl-CoA by acetyl-CoA. The radioactive assay method described under "Experimental Procedures" was used..

mitochondrial coenzymes and metabolites on the activity of this enzyme were investigated. As shown in Table VIII several of the listed compounds inhibited the thioesterase-catalyzed hydrolysis of both acetyl-CoA and palmitoyl-CoA. Most pronounced was the inhibition by CoASH which at a concentration of 0.1 mM almost completely inhibited the acetyl-CoA hydrolase activity of this enzyme. In view of the phsyiological concentrations of NADH and ATP, which under certain conditions can be as high as 3 mM and 8 mM respectively (54-56), the inhibitions caused by these two coenzymes seem also to be physiologically important. In contrast, the inhibitory effects of ADP, AMP and citrate, even at high concentrations, are not very likely to affect the activity of this enzyme significantly. All inhibitors of the acetyl-CoA hydrolase activity also inhibited the thioesterase-catalyzed hydrolysis of palmitoyl-CoA although to a lesser extent.

For a kinetic study of the inhibition of acetyl-CoA hydrolysis by CoASH, the direct assay which does not require DTNB had to be used. However, since CoASH is formed during the course of the reaction, the direct assay yields satisfactory results only at high substrate concentrations. In order to avoid this complication, I used oxidized CoA (CoA-S-S-CoA) instead of CoASH for a kinetic evaluation of the inhibition caused by this coenzyme. As shown in Fig. 12, both forms of the coenzyme inhibited the reaction

Table VIII

Effects of mitochondrial coenzymes and metabolites on the activity of thioesterase.

Inhibitor	Conc.	Remaining Activity[a]	
		AcCoA	C_{16}CoA
	mM	%	
CoASH	0.1	13[b]	n.d.[c]
Oxidized CoA	0.4		39
Oxidized CoA	0.67	20	
NADH	1	19	46
NAD	10	100	100
NADPH	1	100	100
NADP	1	100	100
ATP	10	12	48
ADP	10	31	76
AMP	5	73	n.d.[c]
GTP	5	18	n.d.[c]
Citric Acid	10	36	67
Malic Acid	10	87	89
Glutamic Acid	10	96	102
α-Ketoglutaric Acid	10	104	n.d.[c]
Pyruvic Acid	10	105	107
Alanine	10	100	107
Aspartic Acid	10	104	102
Succinic Acid	10	92	94

[a] Activities were measured by the standard assay method described "Experimental Procedures" with either 16.7 μM acetyl-CoA (AcCoA) or 16.7 μM palmitoyl-CoA (C_{16}CoA) as substrates.

[b] The direct assay method was used (see under "Experimental Procedures") with 66.7 μM acetyl-CoA.

[c] Not determined.

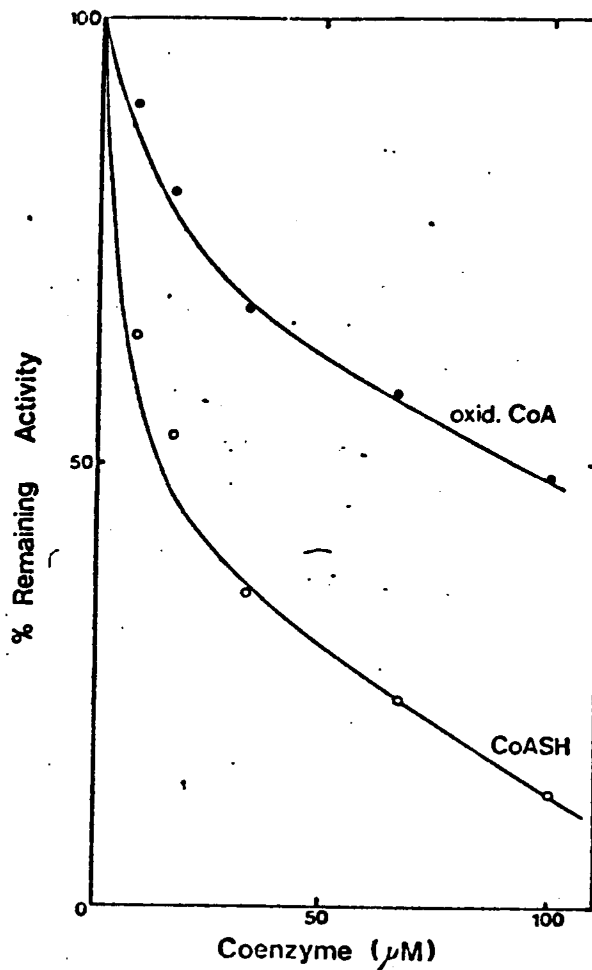


Fig. 12.
Effects of CoASH and oxidized CoA on the hydrolysis of acetyl-CoA catalyzed by the mitochondrial thioesterase from pig heart. Rates were measured by the direct assay method described under "Experimental Procedures" with 66.7 uM acetyl-CoA as substrate, (o) CoASH; (●) oxidized CoA.

but an inhibition of 50% required 7-times more oxidized CoA than free CoASH. Since both forms of the coenzyme were assumed to inhibit the reaction in the same manner and since oxidized CoA but not free CoASH could be used in the standard assay which involved DTNB, I have studied the nature of the CoA inhibition with oxidized CoA. As shown in Fig. 13, oxidized CoA inhibited the reaction competitively with respect to acetyl-CoA. The K_I for oxidized CoA was found to be 53 μM. Since free CoASH was 7-times more effective in inhibiting this reaction than was oxidized CoA, the K_I for CoASH is estimated to be 8 μM. The competitive nature of the inhibition by CoA was expected because the absolute requirement of this thioesterase for CoA-containing substrates suggests that the binding of substrates to the enzyme is dependent on the interaction between the CoA moiety and the enzyme. Consequently, CoASH or oxidized CoASH would be expected to compete with substrates for the CoA-binding region at the active site. In contrast, the inhibition caused by NADH was found to be noncompetitive with respect to acetyl-CoA (see Fig. 14). The inhibition constants obtained from replots of slopes and intercepts were 195 μM and 115 μM respectively. Since NADH but not NAD^+ inhibited the reaction, the dihydropyridine ring appears to be essential for the manifestation of an inhibition. In view of the noncompetitive nature of the inhibition

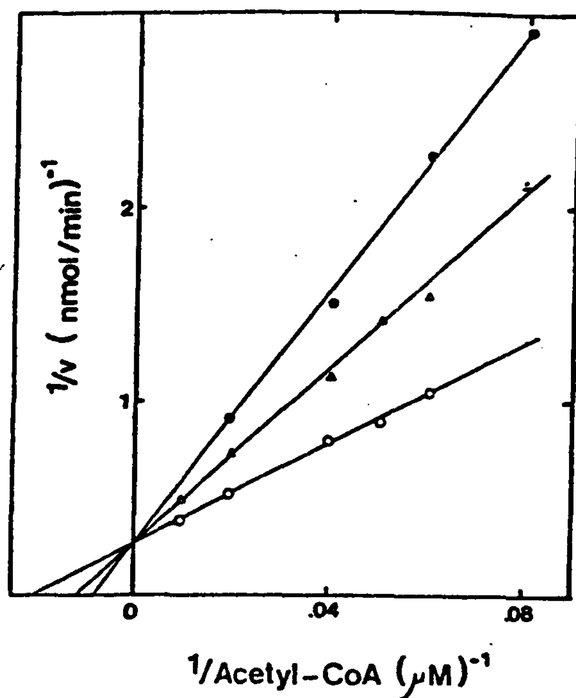


Fig. 13.
Inhibition of acetyl-CoA hydrolysis by oxidized CoA. Rates were measured by the standard assay method described under "Experimental Procedures". Data are plotted according to Lineweaver and Burk. (o) No oxidized CoA; (▲) 40 μM oxidized CoA; (●) 80 μM oxidized CoA.

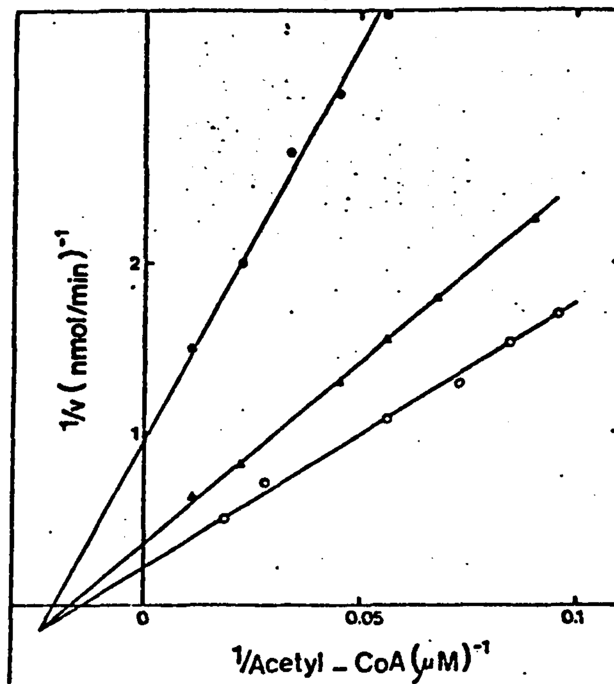


Fig. 14. Inhibition of acetyl-CoA hydrolysis by NADH. Rates were measured by the standard assay method described under"Experimental Procedures". Data are plotted according to Lineweaver and Burk. (o) No NADH; (▲) 100 mM NADH; and (●) 400 mM NADH.

and because of the absence of a structural element in the substrate resembling the dihydropyridine ring of NADH, it seems likely that NADH inhibits the enzyme by binding to a site different from the active site. The inhibition of acetyl-CoA hydrolysis by ATP was found to be competitive with a K_I of 0.52 mM (see Fig. 15). This surprising finding leads us to suggest that ATP competes with the substrate for the region of the active site where the 3'-phosphoadenosine 5'-diphosphate moiety of the substrate interacts with the enzyme (see Fig. 16 for structures of CoASH, ATP and ADP). The decreasing effectiveness of the inhibition when ATP, ADP and AMP are compared (see Fig. 16) leads to the conclusion that every phosphate group of ATP contributes to the binding of the nucleotide to the enzyme. Based on the K_I value of 0.52 mM for ATP and based on the relative inhibitions by ATP and ADP (see Fig. 17), the K_I for ADP was estimated to be 1.5 mM. In view of this high K_I value and the usually low ADP concentration in mitochondria (54), the regulation of thioesterase by ADP, and more so by AMP, appears to be unlikely. Since the inhibition of acetyl-CoA hydrolysis by citrate was found to be comparable to that caused by ADP (see Fig. 17), citrate is not believed to be an important regulator of this enzyme either.

A kinetic study of this thioesterase with palmitoyl-CoA as a substrate was complicated by the severe substrate

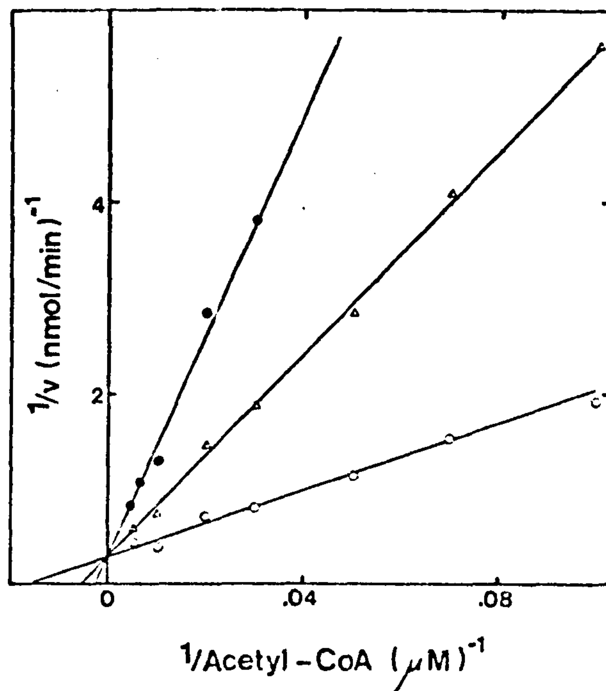
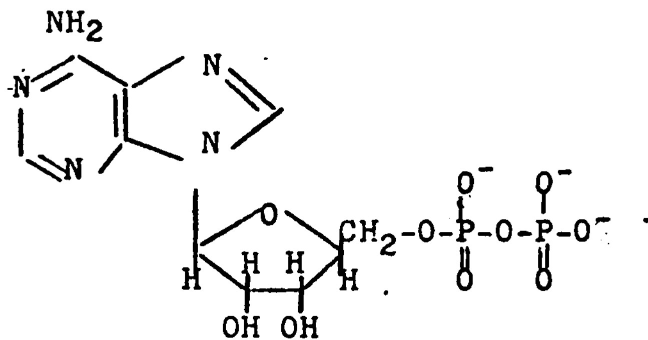
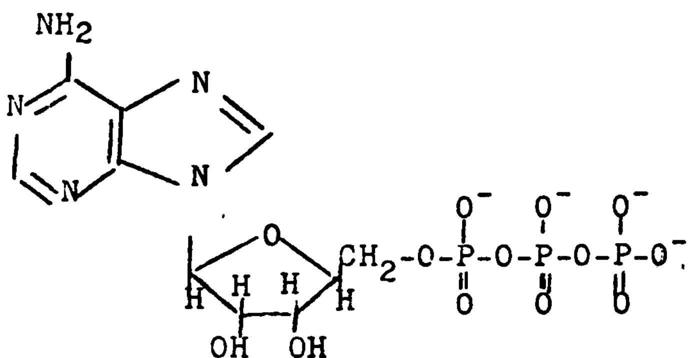


Fig. 15.
Inhibition of acetyl-CoA hydrolysis by ATP. Rates were measured by the standard assay method described under "Experimental Procedures". Data are plotted according to Lineweaver and Burk. (o) No ATP; (△) 1 mM ATP; and (●) 3 mM ATP.





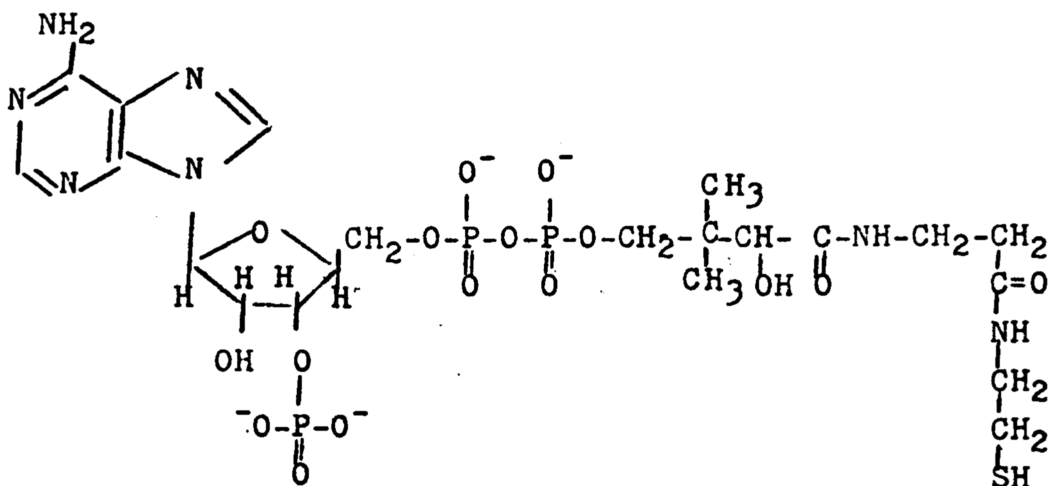


Fig. 16.
Structures of adenosine diphosphate (ADP), adenosine triphosphate (ATP) and coenzyme A (CoA).

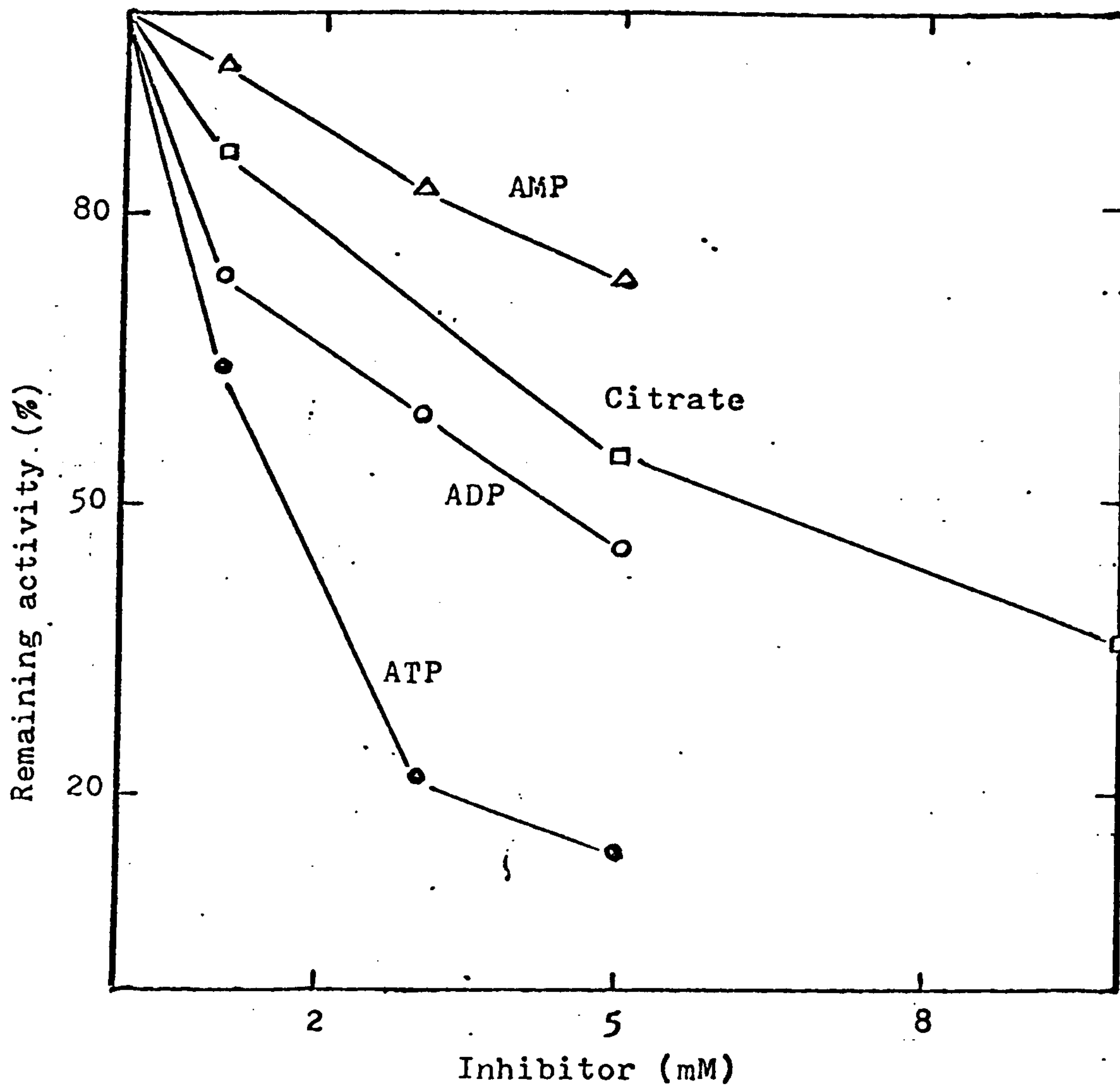


Fig. 17.
Inhibition of thioesterase by ATP, ADP, AMP and citrate. Activities were determined by the standard assay method described under "Experimental Procedures " with 16.7 uM acetyl-CoA as substrate.

inhibition which only at lower concentrations of palmitoyl-CoA could be relieved by the presence of bovine serum albumin. I therefore limited the kinetic investigation of palmitoyl-CoA hydrolysis to determining the K_I values for oxidized CoA (85 μM, see Fig. 18) and NADH (0.5 mM, see Fig 19) by use of Dixon plots. The inhibition patterns obtained were compatible with a competitive inhibition for oxidized CoA (Fig. 18) and noncompetitive inhibition for NADH (Fig. 19). Most important was the finding that both, oxidized CoA and NADH, inhibited the hydrolysis of palmitoyl-CoA less severely than that of acetyl-CoA.

In order to assess the contributions of the various inhibitors to the total inhibition under physiological conditions, it was important to determine whether or not the effects caused by the noncompetitive inhibitor NADH and by the competitive inhibitors CoASH and ATP are additive. As shown in Fig. 20, the inhibitions due to oxidized CoA and NADH were found to be nearly additive. It is therefore concluded that CoASH, NADH and ATP contribute to the inhibition of mitochondrial thioesterase which is present in pig heart muscle at levels 5 to 10-times higher than in pig liver, rat heart or rat liver. Typical activities observed in pig heart extracts, when measured at substrate concentrations of 17 μM, were in units per g of wet tissue: 1.5 with acetyl-CoA, 3.3 with butyryl-CoA, 3.4 with n-decanoyl-CoA, 2.3 with palmitoyl-CoA and 4.2 with acetoacetyl-CoA.

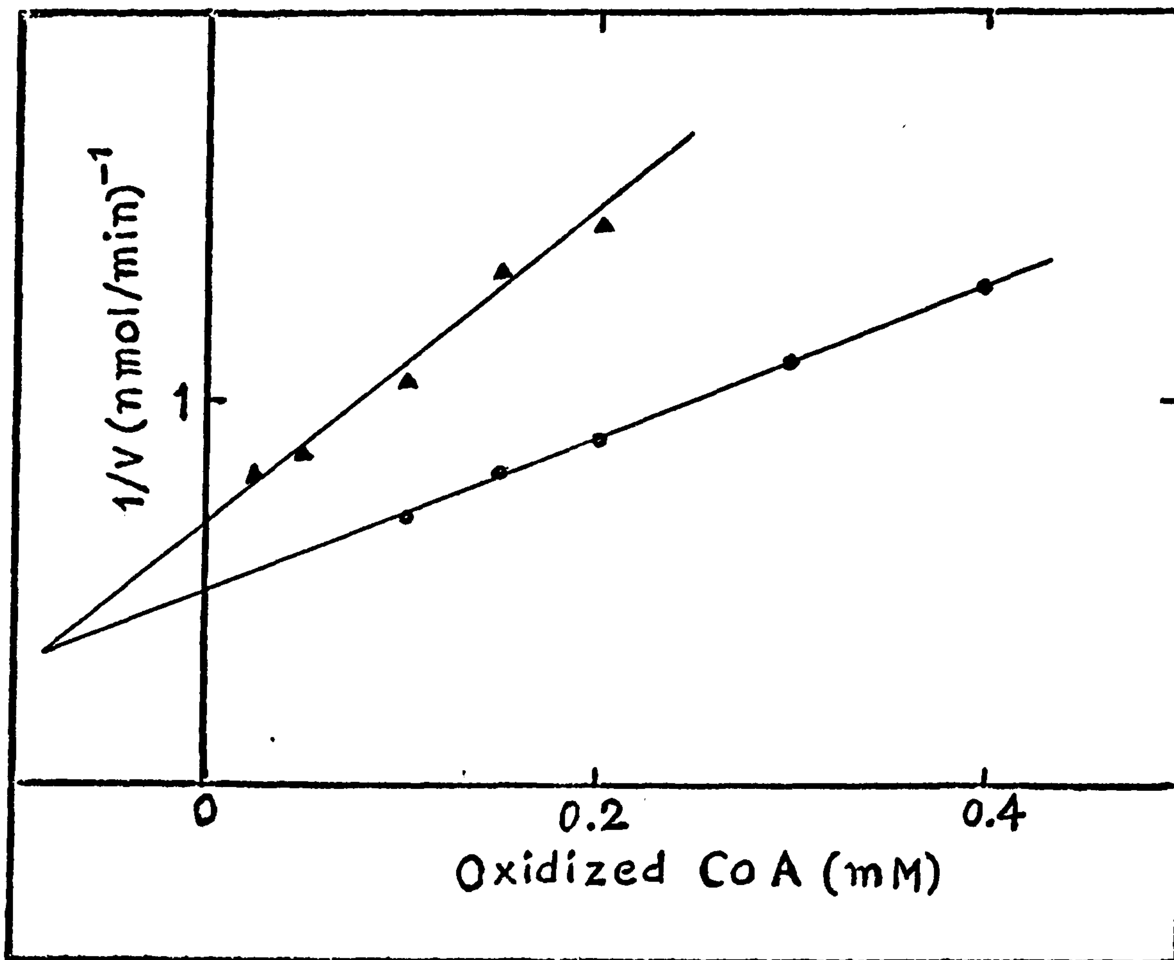


Fig. 18.

Inhibition of palmitoyl-CoA hydrolysis by oxidized CoA Rates were measured by the standard assay method described under "Experimental Procedures". Data are plotted according to Dixon.

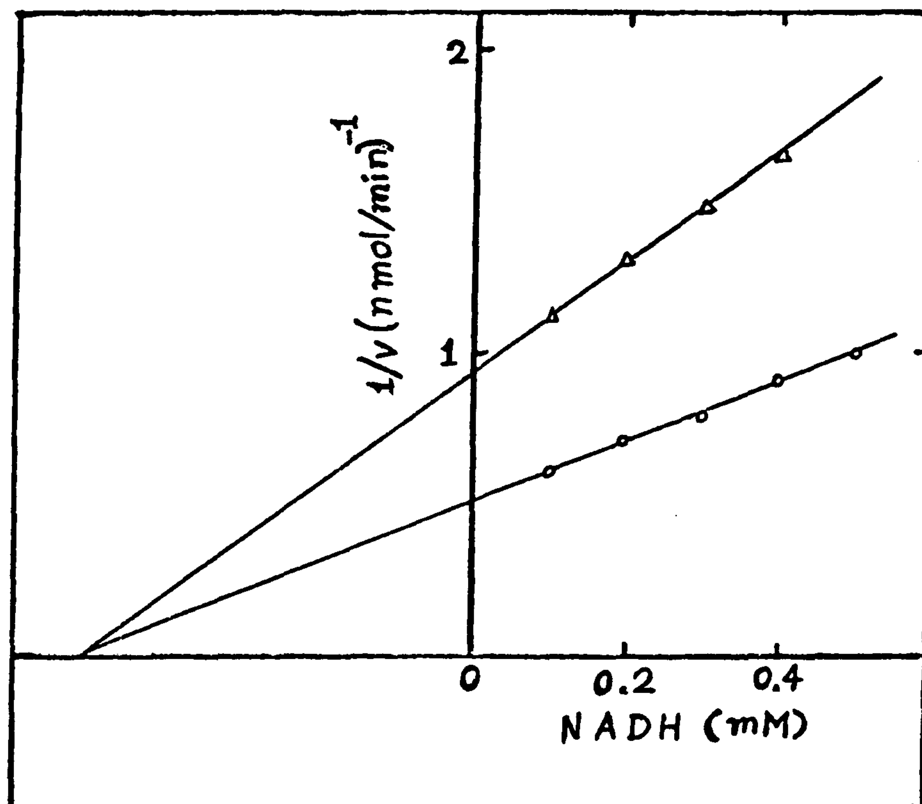


Fig. 19.

Inhibition of palmitoyl-CoA hydrolysis by NADH. Rates were measured by the standard assay method described under "Experimental Procedures". Data are plotted according to Dixon.

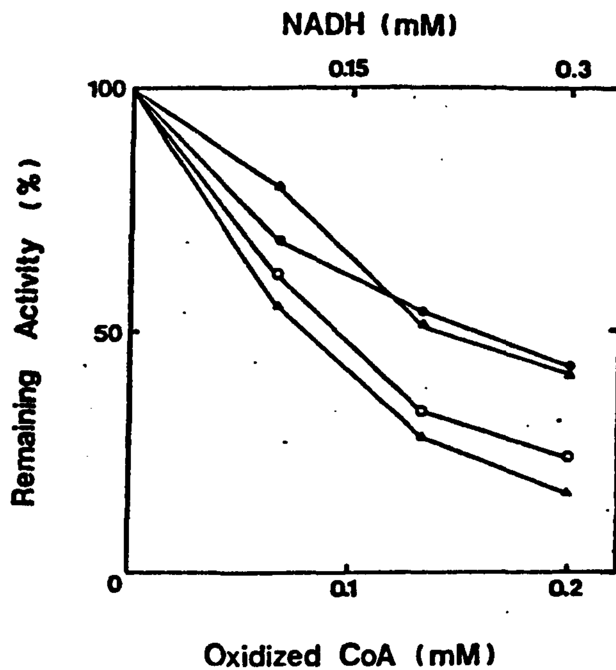


Fig. 20.
Additivity of oxidized-CoA-dependent and NADH-dependent inhibition of acetyl-CoA hydrolysis catalyzed by pig heart thioesterase. Activities were determined by the standard assay method described under "Experimental Procedures" in the presence of oxidized CoA (●) alone, NADH (△) alone and oxidized CoA plus NADH (o). Calculated activities, which are based on the assumption that the experimentally determined inhibitions by oxidized CoA and NADH are additive, are also presented (▲).

DISCUSSION

The results of this study lead to the conclusion that pig heart mitochondria contain a thioesterase which is specific for CoA esters but which hydrolyzes a wide variety of acyl-CoA compounds. Several lines of evidence suggest that the various thioesterase activities observed in a partially purified preparation are due to the action of a single enzyme for which the name "general thioesterase" or "general acyl-CoA hydrolase" is suggested. Since this enzyme is active with acetyl-CoA, butyryl-CoA, decanoyl-CoA, palmitoyl-CoA and oleoyl-CoA as substrates it has the properties of both a long chain and a short chain thioesterase and since it acts also on acetoacetyl-CoA and succinyl-CoA, it fits the classification of the four following, separately listed, thioester hydrolases: acetyl-CoA hydrolase (EC 3.1.2.1), palmitoyl-CoA hydrolase (EC 3.1.2.2), succinyl-CoA hydrolase (EC 3.1.2.3), and acetoacetyl-CoA hydrolase (EC 3.1.2.11). It thus seems possible that a number of reports regarding different thioesterase activities are in fact based on observations of the same enzyme acting on various substrates.

A literature review of acyl-CoA hydrolases reveals the existence of long chain thioesterases or palmitoyl-CoA hydrolases, which are virtually inactive towards short chain substrates, in a variety of organisms as for example *E. coli* (12, 13, 14), *Mycobacterium phlei* (15), avocado (17)

and higher animals (10, 11, 12). This group of enzymes appears to be involved in the termination of fatty acid synthesis or elongation as is the medium chain thioesterase recently isolated from lactating mammary gland (8,9). Less clear is the situation with regard to short chain thioesterases and thioesterases which act on acetoacetyl-CoA, succinyl-CoA and other acyl-CoA derivatives. This study leads to the conclusion that a variety of short chain acyl-CoA derivatives can be hydrolyzed by the general thioesterase of mitochondria which additionally can act on long chain substrates. It remains, however, to be established whether this general thioesterase is present in mitochondria of different animals and tissues. A preliminary report of a thioesterase from rat brain mitochondria indicates that this enzyme has also a broad substrate specificity (7). The main difference between the rat brain and pig heart thioesterase is the inability of the former to hydrolyze palmitoyl-CoA (7). Also, two short chain acyl-CoA hydrolases have been isolated from sheep liver. Both enzymes act on acetyl-CoA, succinyl-CoA and other short chain acyl-CoA compounds but are virtually inactive towards decanoyl-CoA (31). However, the reported inactivities of short chain acyl-CoA thioesterases towards long chain substrates may have been due to substrate inhibition which was observed with the mitochondrial pig heart thioesterase at low concentrations of palmitoyl-CoA. This

inhibition was prevented by bovine serum albumin at low but not at high substrate concentrations.

Although the physiological function of the pig heart thioesterase has not been definitely established, it is assumed that this enzyme functions in generating free CoASH from acyl-CoA compounds in mitochondria. If this assumption is correct, the broad specificity of this thioesterase is not surprising because it could hydrolyze long chain fatty acyl-CoA, acetyl-CoA and succinyl-CoA which are the main acyl-CoA compounds present in mitochondria.

Although numerous reports concerning thioesterases have appeared in the literature, an acetyl-CoA hydrolase from hamster brown adipose tissue (20) is the only thioesterase which has been specifically studied with regard to its regulatory properties. Some of the properties of this enzyme are similar to those of the pig heart thioesterase. Both enzymes are located in mitochondria and have almost identical K_m values of about 50 μM for acetyl-CoA. Both enzymes were extensively inactivated during their purification, but were found to be stable for several months to a year at low temperatures (at -18°C for the hamster brown adipose tissue acetyl-CoA hydrolase and at -76°C for the pig heart thioesterase). They are stimulated by $CaCl_2$ and are inhibited by palmitoyl-CoA. In contrast to these similarities, the two enzymes exhibit major differences in their responses towards important

mitochondrial coenzymes which may regulate both thioesterases *in vivo*. The mitochondrial acetyl-CoA hydrolase of brown adipose tissue from hamster is noncompetitively inhibited by CoASH and NADH and noncompetitively activated by ADP. ATP, GDP and AMP at concentrations at which ADP caused significant stimulation did not affect the activity of the enzyme. The K_I values reported by Bernson (20) for NADH (K_{IS} = 13 μM, K_{II} = 17 μM) and CoASH (K_{IS} = 0.5 μM) are one order of magnitude smaller than the inhibition constant for the same coenzymes obtained with the pig heart thioesterase. In contrast to the hamster brown adipose thioesterase the pig heart enzyme is competitively inhibited by CoASH and ATP and is not stimulated, but instead weakly inhibited, by ADP. It thus appears that the two thioesterases differ significantly although the activities of both enzymes are affected by several important mitochondrial coenzymes.

In order to assess the physiological function of the pig heart thioesterase, the residual acetyl-CoA hydrolase activity of this enzyme at state 3 and state 4 respiration was calculated. The concentrations of acetyl-CoA, CoASH, NADH and ATP listed in Table IX are based on values obtained by Hansford and Johnson (54) with rabbit heart mitochondria. The total amounts of NADH plus NAD^+ and of ATP plus ADP plus AMP in beef heart mitochondria were reported to be 6 nmoles/mg and 8 nmoles/mg of mitochondrial

Table IX

Calculated activities of thioesterase towards acetyl-CoA at state 3 and state 4 respirations.

Inhibitor	State 3 Conc.	State 3 Act.[a]	State 3 Rel.Act.	State 4 Conc.	State 4 Act.[a]	State 4 Rel.Act.
	mM	U/g tissue	%	mM	u/g tissue	%
None		3.9	100		5.1	100
CoASH	0.2	0.43	11	0.01	4.4	86
NADH	0.33	1.1	28	3	0.2	4
ATP	7.6	0.7	18	8	1.8	35
CoASH + NADH + ATP	as above	0.02	0.6	as above	0.06	1.2

[a] The listed activities are based on an acetyl-CoA thioesterase activity of 1.5 units per g wet tissue determined with 16.7 μM acetyl-CoA. The K_m for acetyl-CoA is 48μM. The concentrations of acetyl-CoA were assumed to be 100 μM and 350 μM for state 3 and state 4 respirations respectively (54). The inhibition constants for CoASH, NADH and ATP are listed under "Results".

protein respectively (54, 55). The concentrations were calculated by assuming that the matrix space of mitochondria is 1 μl/mg of mitochondrial protein (54) and that the mitochondrial coenzymes are located in their free forms in the matrix. The values presented in Table IX lead to the conclusion that the acetyl-CoA hydrolase activity of pig heart mitochondrial thioesterase is almost completely inhibited at state 3 and state 4 respirations. At state 3 the inhibition is caused mainly by CoASH and ATP, whereas at state 4 the inhibitory effect of NADH is most important. It is obvious that a metabolic situation which is characterized by low concentrations of CoASH and NADH and by high levels of acetyl-CoA would leave the thioesterase active. Such situation could arise when at state 3 respiration the rate of fatty acid oxidation exceeds that of the citric acid cycle perhaps due to limiting amounts of oxaloacetate and its precursors. In agreement with this prediction is the observation that acetate is the main product of fatty acid and pyruvate oxidations in heart mitochondria when the citric acid cycle is inhibited by either malonate or fluoroacetate (33-35). With malonate as an inhibitor acetoacetate in addition to acetate is formed (35). The formation of acetate and acetoacetate is believed to involve the mitochondrial thioesterase which will be deinhibited when the acetyl-CoA/CoASH ratio increases and the concentration of NADH decreases in mitochondria which

rapidly oxidize fatty acids in the presence of malonate. Acetate formation under physiological conditions has been observed in mitochondria from brown adipose tissue of cold-adapted hamster in which apparently acetyl-CoA hydrolysis represents a way to bypass the citric acid cycle which operates poorly at low temperatures (32). Acetate formation seems to be also important in tumor cells (57), in housefly flight muscle mitochondria (58) and in human skeletal muscle during exercise (59).

Important reactions by which acetyl-CoA can be metabolized in heart mitochondria are summarized in Fig. 21. Under normal conditions acetyl-CoA is known to be metabolized preferentially by the citric acid cycle. When the rate of acetyl-CoA formation increases, significant amounts of acetate units can be temporarily stored in the form of acetylcarnitine. However, the buffering capacity provided by carnitine and carnitine acetyltransferase is limited by the amount of carnitine. Hence, when the rate of formation of acetyl-CoA continues to exceed its removal in form of citrate, the acetyl-CoA/CoASH ratio will increase. If at the same oxidative phosphorylation occurs at a high rate, the mitochondrial thioesterase should be de-inhibited and would catalyze the hydrolysis of acetoacetyl-CoA, formed by condensation of two molecules of acetyl-CoA, of succinyl-CoA and of long chain acyl-CoA derivatives. Especially important may be the hydrolysis of CoA

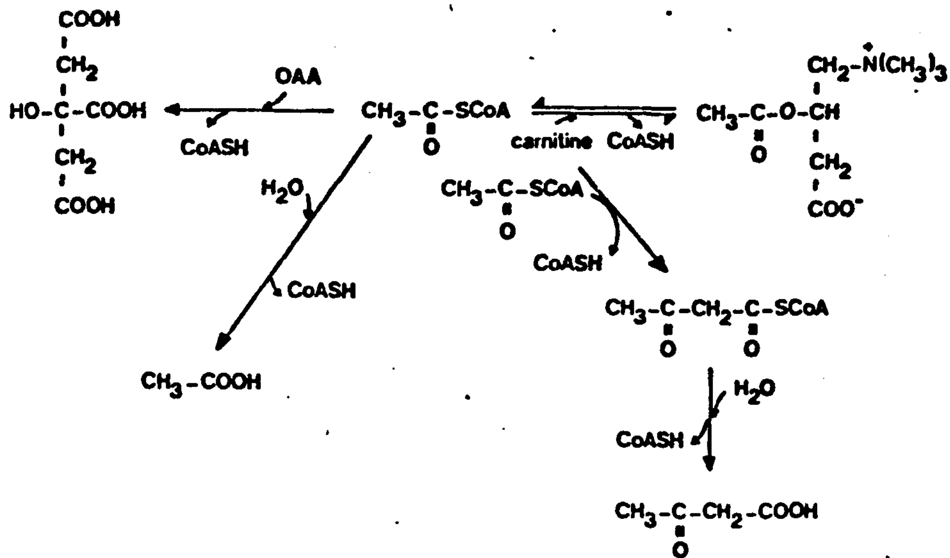


Fig. 21.
Established and suggested major pathways of acetyl-CoA metabolism in heart muscle.

derivatives of long chain fatty acids because their concentration in mitochondria can exceed that of acetyl-CoA when the influx of long chain acylcarnitine is high (54). Since the hydrolysis of palmitoyl-CoA is less sensitive to inhibition by CoASH and NADH than is that of acetyl-CoA, this thioesterase may act on long chain acyl-CoA compounds at concentrations of CoASH and NADH at which it is still inhibited towards acetyl-CoA. Additionally, the long chain acyl-CoA hydrolase activity of this enzyme may function in the termination of fatty acid elongation in heart mitochondria because part of the elongation products have been found to exist in the form of free fatty acids (60).

Based on the activities and regulatory properties of the mitochondrial thioesterase from pig heart, it is concluded that this enzyme functions to provide free CoASH required for the oxidative metabolism in mitochondria at situations where the capacity of the citric acid cycle is limited while the energy demand is high. Thus acetate formation catalyzed by this thioesterase in extra-hepatic tissue may have a physiological function similar to that of ketone body formation in liver.

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