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THIOLASES OF PIG HEART MUSCLE: PURIFICATION/CHARACTERIZATION AND PHYSIOLOGICAL FUNCTIONS.

CITY UNIVERSITY OF NEW YORK, PH.D., 1978

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THIOLASES OF PIG HEART MUSCLE: PURIFICATION/CHARACTERIZATION AND PHYSIOLOGICAL FUNCTIONS

by

HAROLD MICHAEL STAACK

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.

1978

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.

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Abstract

THIOLASES OF PIG HEART MUSCLE: PURIFICATION/CHARACTERIZATION AND PHYSIOLOGICAL FUNCTIONS

by

HAROLD MICHAEL STAACK

Adviser: Professor Horst Schulz

A thiolase (EC 2.3.1.16) which acts on substrates of various chain lengths (thiolase I) has been purified 366-fold to near homogeneity from pig heart muscle. Its molecular weight was estimated to be 200,000 in the absence and 48,000 in the presence of sodium dodecyl sulfate. Kinetic measurements with acetoacetyl-CoA, 3-ketohexanoyl-CoA, 3-ketooctanoyl-CoA and 3-ketodecanoyl-CoA yielded apparent K_m-values of 16 μM, 8.3 μM, 2.4 μM and 1.8 μM, respectively, whereas apparent V_{max} values of 65-69 μmoles/min/mg were obtained with all substrates except for acetoacetyl-CoA with which a value of 26.5 μmoles/min/mg was observed. Antibodies prepared against this thiolase were used to demonstrate that thiolase I and acetoacetyl-CoA thiolase (thiolase II) from pig heart mitochondria are immunologically unrelated. The antibodies cross-reacted, however, with thiolase I from beef heart mitochondria. Thiolases I and II were found to have different pH optima when both were assayed with acetoacetyl-CoA. Thiolase I was found to be more stable to dilution than was thiolase II but both thiolases were inhibited by thiol reagents.

The two thiolases of pig heart are distinct from each other and on the basis of a comparison with E. coli thiolases the two

pig heart thiolases are thought to have distinct physiological functions. It is proposed that thiolase I from pig heart is the only one involved in fatty acid metabolism whereas thiolase II functions solely in ketone body degradation.

Antibodies to pig heart thiolase I inhibited almost completely and in a parallel fashion thiolase I and the acetyl-CoA-dependent fatty acid elongation system present in an acetone powder extract of pig heart mitochondria. This finding leads to the conclusion that mitochondrial fatty acid elongation occurs by reversal of fatty acid oxidation. Several lines of evidence point to the thiolase catalyzed condensation reaction as the rate limiting step in the formation of elongated products. However, the accumulation of hydroxy acids suggests that the enoyl-CoA reductase activity is limiting in the synthesis of saturated fatty acids.

ACKNOWLEDGMENTS

This thesis is dedicated to my loving wife who gave me the support to see this project through to the end.

I also wish to express my sincerest appreciation to Dr. Horst Schulz who, by his fine example and endless patience, taught me the pleasure and sometimes the frustration of doing research and for this I will always be in his debt.

I am most grateful to Dr. Judith Feigenbaum Binstock for supplying me with antibodies to E. coli thiolase II and for her many helpful suggestions. Also my thanks to Bruce Davidson who helped with the analysis of the products of fatty acid elongation and to Lawrence Coll who helped with the native molecular weight determination of thiolase I. I am also grateful to the many people at City College who, by their ideas and suggestions, helped to bring this project to a fruitful conclusion.

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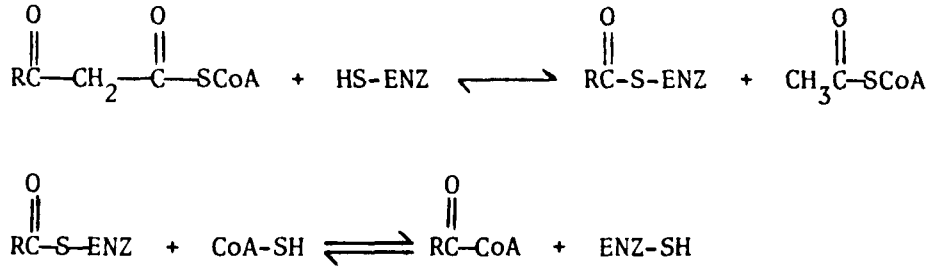
INTRODUCTION I

Fatty acids are one of the principal fuels of oxidative metabolism in higher organisms. They originate in adipose tissue and are transported as free fatty acids in the plasma to points in the body where they are needed for energy production (1, 2, 3, 4). Their metabolic conversions take place at different functional sites within the cell. The esterification and deesterification of fatty acids are primarily microsomal functions, whereas their oxidation occurs in the mitochondria (5, 6, 7). The universal pathway by which fatty acids are successively oxidized to acetate units in the mitochondria is the β oxidation cycle, which was first formulated by Knoop in 1904. The steps of the pathway were confirmed by Green (8), Lynen (9), Ochoa (10) and others and are outlined as follows (see Fig. 1 part A): The fatty acid is first activated by fatty acyl-CoA synthetase to yield the fatty acyl-CoA thioester; this compound is dehydrogenated to the trans-$\Delta^{2,3}$-enoyl-CoA compound by acyl-CoA dehydrogenase, an FAD containing enzyme which transfers electrons from acyl-CoA to an electron transferring protein which also contains FAD and can pass the electrons to cytochrome b of the electron transport chain; the resulting trans-$\Delta^{2,3}$-enoyl-CoA intermediate is hydrated by enoyl-CoA hydratase to yield the L-3-hydroxyacyl-CoA intermediate which, in turn, is oxidized by the NAD^+-specific 3-hydroxyacyl-CoA dehydrogenase to the 3-ketoacyl-CoA compound; the 3-ketoacyl-CoA compound is cleaved in the last step by thiolase to the two-carbon shorter acyl-CoA compound and acetyl-CoA.

The thiolase reaction proceeds as follows:



Free reduced CoA is required for the thiolytic cleavage of 3-ketoacyl-CoA in which an acyl-S-enzyme intermediate (11) is formed between the active thiol group of the enzyme and the β carbon of the 3-ketoacyl-CoA substrate. The thiolase reaction is reversible (11, 12) but a large negative free energy change (ΔG^{o} = -6.65) favors the cleavage of 3-ketoacyl-CoA (K_{eq} = 6 X 10^{4}) and also supplies the energy to drive β oxidation. Thus, the thiolase catalyzed reaction may be a possible regulated step in β oxidation.

In addition to the above enzymes, enzymes are found in the mitochondria which are necessary for the oxidation of unsaturated and odd chain length fatty acids. An isomerase which catalyzes the conversion of the cis-Δ^{3} isomer to the trans-Δ^{2}-enoyl-CoA derivative (13) and an epimerase which converts the D-3-hydroxyacyl-CoA derivative to the L enantiomer (13, 14) are required for the oxidation of unsaturated fatty acids. Also, enzymes have been isolated from pig heart which catalyze the conversion of propionyl-CoA, produced by the oxidation of odd chain fatty acids in a three-step process to succinyl-CoA (15). All of these additional reactions occur in mitochondria (13, 14, 15).

Although β oxidation in mitochondria is the principal means of fatty acid oxidation in eukaryotic cells, very little is known about the control of the enzymes of β oxidation or their organization within mitochondria. Attempts to demonstrate the accumulation of acyl-CoA intermediates of β oxidation have proved unsuccessful and it has therefore been concluded that intermediates of β oxidation remain enzyme bound, in a way analogous to the situation in fatty acid synthesis (16, 17). But since the enzymes of β oxidation can be separated from each other, the low concentrations of intermediates may be due to their movement from one enzyme to another without being released into the medium. Thus, the enzymes of β oxidation are thought to exist in a highly ordered form, possibly maintained by their association with the inner mitochondrial membrane (16, 17).

In addition to fatty acids, extrahepatic tissues oxidize ketone bodies (3-hydroxybuterate and acetoacetate), which are produced in liver and are transported in blood to such tissues as skeletal muscle and heart. The physiological significance of ketone body generation in liver is the prompt regeneration of coenzyme A during periods of high fatty acid oxidation such as during fasting or in the diabetic state. Liver under these conditions produces, by reversal of the thiolase catalyzed reaction, acetoacetyl-CoA, which is converted to acetoacetate via 3-hydroxy-3-methylglutaryl-CoA (18). In the fasted rat the amount of energy provided from the blood in the form of ketone bodies is approximately the same as that provided as free fatty acids. The combined utilization of free fatty acids and ketone bodies in the fasted animal provides as much oxidizable

substrate as does glucose in the fed animal (19). Even under normal conditions ketone bodies serve as a quantitatively important source of energy. Heart muscle preferentially uses acetoacetate even in the presence of glucose and insulin or in the presence of lactate (20). The pathway by which heart utilizes ketone bodies is shown in Fig. 1 part B.

It can be seen in Fig. 1 that both β oxidation and ketone body metabolism share, as a common intermediate, acetoacetyl-CoA and that both these mitochondrial pathways involve thiolases. In addition acetoacetyl-CoA is synthesized from acetyl-CoA in the cytoplasm by cytoplasmic thiolase, which is involved in cholesterol biosynthesis. Middleton (21) has identified three classes of thiolases in animal tissues: A mitochondrial thiolase active with 3-ketoacyl-CoA compounds of various chain lengths, a mitochondrial thiolase active only with acetoacetyl-CoA and a cytoplasmic acetoacetyl-CoA-specific thiolase. Clearly the mitochondrial thiolase active on various 3-ketoacyl-CoA compounds is involved in β oxidation but Middleton found that it has less activity with acetoacetyl-CoA (21, 22). Thus, the mitochondrial acetoacetyl-CoA-specific thiolase may be a necessary component enzyme in β oxidation if all 3-ketoacyl-CoA intermediates are to be cleaved at equal rates and therefore this thiolase may function in both ketone body metabolism and β oxidation.

In order to obtain evidence regarding the physiological functions of the mitochondrial thiolases, the work presented in this thesis was undertaken. To do this it was necessary to purify and characterize the mitochondrial thiolase active on 3-ketoacyl-CoA

substrates of various chain lengths. This thiolase (EC 2.3.1.16) is referred to as thiolase I in order to distinguish it from the other mitochondrial thiolase (EC 2.3.1.9) named thiolase II. Before this work was begun thiolase II from pig heart had been purified to homogeneity by Lynen and coworkers (23). It was found to have a molecular weight of 170,000, to be composed of four subunits of 42,000 molecular weight (24) and to contain an active site cysteine (25). Most thiolases that have been studied have native and subunit molecular weights similar to those above and all contain an active site cysteine (21, 26, 27, 28, 29). Thiolase I from beef liver mitochondria was the first and only 3-ketoacyl-CoA thiolase to be purified to homogeneity (30) but it was poorly characterized. Middleton (22) had partially purified thiolases I and II from beef liver mitochondria and determined their app. K_m values for coenzyme A and acetoacetyl-CoA but not for longer chain substrates.

Pig heart was chosen for this work because heart lacks cytoplasmic thiolase, a situation which made the purification of the mitochondrial thiolases easier. In addition many of the other β oxidation enzymes of pig heart have already been purified and characterized. Additionally pig heart offered the advantage that the acetoacetyl-CoA-specific thiolase (thiolase II) had been previously purified and studied (23, 24, 25) and thus pig heart offered the opportunity to complete the in-depth investigation of the mitochondrial thiolases present in one organ. As a result of the work presented in this thesis, it was possible to compare the properties of the two pig heart thiolases with those of the E. coli thiolases

which had been purified to homogeneity and studied in detail (26, 31).

In E. coli the enzymes of fatty acid oxidation, including thiolase I, are co-inducible (32) and form a "fad" (fatty acid degradation) operon (33). Genetic studies (32) have shown that the activities of thiolase II and acetate CoA transferase in E. coli are stimulated by a factor of 3000 in the presence of acetoacetate. The structural genes for these enzymes are linked with a regulatory gene forming an "ato" operon (acetoacetate degradation), which proved to be located on the E. coli chromosome map at a point different from the location of the "fad" operon. Thus, a comparative study of the pig heart thiolases with the thiolases of E. coli, which have known physiological roles (32, 33, 34), leads to the suggestion that only thiolase I is involved in the mitochondrial metabolism of fatty acids, whereas thiolase II functions only in ketone body degradation.

EXPERIMENTAL PROCEDURES I

Materials

Coenzyme A, NAD^+ and NADH were obtained from P-L Biochemicals, Inc. N-Methylmaleimide, bovine serum albumin, catalase, cytochrome c, lactate dehydrogenase, phosphorylases a and b were purchased from Sigma Chemical Co. α-Iodoacetamide was bought from Calbiochem. Ethyl chloroformate, triethylamine, diketene, 2-decenoic acid and 2-octenoic acid were obtained from Aldrich Chemical Co. 2-Hexadecenoic acid and 3-hydroxyacyl-CoA dehydrogenase were purchased from Miles Laboratories and Boehringer Mannheim, respectively. trans-$\Delta^{2,3}$-Hexenoic acid, trans-$\Delta^{2,3}$-dodecenoic acid and trans-$\Delta^{2,3}$-tetradecenoic acid were synthesized by reacting malonic acid in the presence of pyridine with n-butyraldehyde, n-decanal and n-dodecanal, respectively, according to a procedure by Linestead et al. (35). Crotonase (EC 4.2.1.17) was prepared from beef liver by the method of Steinman and Hill (36). Fresh pig hearts were purchased from Max Insel Cohen Co. Pig heart mitochondria were isolated as described by Crane et al. (37) for beef heart mitochondria. An acetone powder of pig heart mitochondria was prepared according to the procedure of Dahlen and Porter (38). New Zealand white rabbits were purchased from Marland Breeding Farms, N. J., and immunodiffusion plates as well as Freund's adjuvant were bought from Cappel Laboratories.

Methods

Preparation of Substrates

The CoA derivatives of $\Delta^{2,3}$-hexenoic acid and its longer chain homologs were prepared by reacting their mixed anhydrides with CoA, as described by Schulz (39). The concentrations of the resulting $\Delta^{2,3}$-enoyl-CoA substrates were determined by the method of Ellman (40) after cleaving the thioester bond with hydroxylamine at pH 7. The 3-ketoacyl-CoA substrates, other than acetoacetyl-CoA, were synthesized enzymatically from their respective $\Delta^{2,3}$-enoyl-CoA derivatives, in the presence of crotonase and 3-hydroxyacyl-CoA dehydrogenase as described by Feigenbaum and Schulz (26). Acetoacetyl-CoA was prepared from CoA and diketene according to the method of Seubert (41).

Protein and Enzyme Determinations

Protein concentrations were determined by the method of Lowry *et al.* (42). Thiolase activities were routinely measured as previously described (26) except that the pig heart enzymes were assayed at pH 8.3 and in the absence of mercaptoethanol. Since the extinction coefficients of the Mg^{2+}-3-ketoacyl-CoA complexes are dependent on pH, the 3-ketoacyl-CoA and Mg^{2+} concentrations, all routine assays were performed at pH 8.3 in the presence of 25 mM $MgCl_2$ and 10 μM 3-ketoacyl-CoA (33 μM in the case of acetoacetyl-CoA). The extinction coefficients under these conditions were determined by measuring the total change of absorbance at 303 nm in the presence of 70 μM CoASH and thiolase (5 mU/0.6 ml) and by measuring the concentration of the 3-ketoacyl-CoA substrates with 3-hydroxyacyl-CoA dehydrogenase

(10 mU/0.6 ml) as previously described (26). The molar extinction coefficients (ε) thus obtained were: acetoacetyl-CoA, 21,400 $M^{-1}cm^{-1}$; 3-ketohexanoyl-CoA, 16,600 $M^{-1}cm^{-1}$; 3-ketooctanoyl-CoA, 14,400 $M^{-1}cm^{-1}$; 3-ketodecanoyl-CoA, 13,900 $M^{-1}cm^{-1}$; 3-ketododecanoyl-CoA, 11,000 $M^{-1}cm^{-1}$; 3-ketotetradecanoyl-CoA, 11,600 $M^{-1}cm^{-1}$; 3-ketohexadecanoyl-CoA, 9,900 $M^{-1}cm^{-1}$. Enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase were assayed as described (26, 39). Units of enzyme activity are expressed as μmoles of substrates utilized or products formed per min.

Purification of the Thiolase with Broad Chain Length Specificity (Thiolase I) from Pig Heart

All operations were performed at 4^{o} C. Fresh pig heart (579 g) were minced with a meat grinder and homogenized together with 2.2 l of 0.05 M potassium phosphate (pH 6.6) containing 5% glycerol and 10 mM mercaptoethanol for 2 min in a Waring Blender operated at top speed. The homogenate was centrifuged for 1 hr at 11,900 x g and the resulting supernatant was applied to a phosphocellulose column (5 x 50 cm) which had been previously equilibrated with 0.05 M potassium phosphate (pH 6.6) containing 5% glycerol and 10 mM mercaptoethanol. The column was washed with the same buffer until UV-absorbing material ceased to be eluted. Another extract was prepared from 272 g of pig heart by the above procedure and applied to the same column. However, only half of the additional thiolase I adhered to the column, which was then eluted with 0.15 M potassium phosphate (pH 6.6) containing 5% glycerol and 10 mM mercaptoethanol. The eluate was collected in batches of 200 ml and most of the

thiolase I activity was found in two of these batches which were concentrated in an Amicon concentrator (PM-10 membrane) to approximately 30 ml. The resulting column was kept for the purification of thiolase II which was eluted with a higher ionic strength buffer. The potassium phosphate concentration of the thiolase I concentrate was adjusted to 0.05 M by the addition of water containing 5% glycerol and 10 mM mercaptoethanol. The resulting solution was applied to a phosphocellulose column (2.5 x 36 cm) equilibrated with 0.05 M potassium phosphate (pH 6.6) containing 25% glycerol and mercaptoethanol. The column was washed with three volumes of the same buffer and developed with a gradient made up of 1 l each of 0.05 M potassium phosphate (pH 6.6) 10% glycerol, 10 mM mercaptoethanol and 0.2 M potassium phosphate (pH 6.6) 10% glycerol, 10 mM mercaptoethanol. Fractions of 18 ml were collected and assayed for thiolase activity with acetoacetyl-CoA and 3-ketodecanoyl-CoA as substrates. Both activities peaked in the same fractions and the fractions with high thiolase activity were pooled and concentrated on an Amicon concentrator (PM10 membrane). The sample was dialyzed against 4 l of 0.01 M potassium phosphate (pH 6.6) containing 20% glycerol and 10 mM mercaptoethanol and applied to a CM-cellulose column (1.2 x 46 cm) which had been equilibrated with the dialysis buffer. After washing with 5 column volumes of the same buffer, the column was developed with a gradient made up of 200 ml each of 0.01 M potassium phosphate (pH 6.6) 20% glycerol, 10 mM mercaptoethanol and 0.1 M potassium phosphate (pH 6.6) 20% glycerol, 10 mM mercaptoethanol. Fractions of 3.3 ml were collected and assayed for thiolase activity. The

most active fractions were combined, concentrated and stored at -76° C after the addition of glycerol and dithiothreitol. No loss of activity was observed when this preparation was stored at -76° C for more than a year.

Purification of Acetoacetyl-CoA Thiolase (Thiolase II) from Pig Heart

The phosphocellulose column (5 x 50 cm) from which thiolase I had been removed with 0.15 M potassium phosphate was washed with 1 l of 0.4 M potassium phosphate (pH 6.6) containing 5% glycerol and 10 mM mercaptoethanol. The eluate was concentrated in an Amicon concentrator (PM10 membrane). The resulting solution was extensively dialyzed against 0.1 M potassium phosphate (pH 6.6) containing 5% glycerol and 10 mM mercaptoethanol and applied to a phosphocellulose column (2.5 x 26 cm) which had been equilibrated with the same buffer except that it contained 10% glycerol. The column was washed with this buffer until all UV-absorbing material ceased to be eluted and was then developed with a gradient made up of 500 ml each of 0.1 M potassium phosphate (pH 6.6) 10% glycerol, 10 mM mercaptoethanol and 0.35 M potassium phosphate (pH 6.6) 10% glycerol, 10 mM mercaptoethanol. Fractions of 10 ml were collected and assayed for thiolase activity. The most active fractions were pooled and concentrated in an Amicon concentrator. The concentrate was extensively dialyzed against 0.01 M potassium phosphate (pH 6.6) containing 20% glycerol and 10 mM mercaptoethanol. After removing an inactive precipitate which had formed during dialysis, the solution was adsorbed to a CM-cellulose column (1.2 x 25 cm) which had been equilibrated with the dialysis buffer. After washing the column with several column

volumes of the same buffer, thiolase II was eluted with 0.1 M potassium phosphate (pH 6.6) containing 25% glycerol and 10 mM mercaptoethanol. The resulting material was rechromatographed on a CM-cellulose column (1.2 x 25 cm) as described above, except that the column was developed with a gradient made up of 100 ml of 0.01 M potassium phosphate (pH 6.6) 25% glycerol, 10 mM mercaptoethanol and 100 ml of 0.1 M potassium phosphate (pH 6.6) 25% glycerol, 10 mM mercaptoethanol. Fractions of 2 ml were collected and assayed for thiolase activity. The most active fractions were pooled and concentrated to 1 ml. After the addition of 0.1 ml of 1 M KCl, 0.5 ml of glycerol and 3.2 mg of dithiothreitol to the concentrate, the enzyme preparation was stored at -76^{o} C for more than a year without loss of activity.

Disc Gel Electrophoresis

Disc gel electrophoresis of thiolases I and II from pig heart were performed on standard 7.5% polyacrylamide gels at pH 8.5 and 15^{o} C according to the procedure of Davis (43) except that 10 mM mercaptoethanol was included both in the separating gel and gel buffer. Gels were stained for protein with either Coomassie Brilliant Blue or Fast Green and destained with 7% acetic acid. Gels containing thiolase I were assayed for enzymatic activity by slicing duplicate unstained gels and incubating each slice overnight at 5^{o} C in Tris-HCl buffer (pH 8.2) containing 5% glycerol, bovine serum albumin (1 mg/ml) and 10 mM mercaptoethanol. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out as described by Weber and Osborn (44).

Molecular Weight Determination

Molecular weight estimations were carried out on a standardized Sephadex G-200 column following the procedure of Andrews (45). The following protein standards were used: phosphorylases a and b, catalase, lactate dehydrogenase and bovine serum albumin.

Inhibition of Thiolase I by Sulfhydryl Binding Agents, N-Methylmaleimide and Iodoacetamide

Thiolase I was diluted to a final concentration of 5 µg/ml with 0.1 M Tris-HCl (pH 8.1) and allowed to react with 10-100 µM sulfhydryl reagent for 0, 15 or 30 min. The control did not contain the sulfhydryl reagent. The reactions were terminated by the addition of 1.0 ml of 0.1 M Tris-HCl (pH 8.1) containing 15 mM mercaptoethanol to 0.5 ml aliquots of the reaction mixtures. The enzyme was then assayed as described above with acetoacetyl-CoA as the substrate. In order to assess the protection of thiolase I by its substrate, 13 µg of thiolase I were preincubated with 125 nmoles of acetoacetyl-CoA in 0.1 ml of 0.1 M Tris-HCl (pH 8.1) for 10 min. The enzyme was then diluted by the addition of 2.5 ml 0.1 Tris-HCl (pH 8.1) and immediately a 0.5 ml aliquot was mixed with 1.0 ml Tris-HCl (pH 8.1), containing 15 mM mercaptoethanol and assayed for acetoacetyl-CoA thiolase activity. To the remaining 2.1 ml of the thiolase I solution, 0.21 µmoles of iodoacetamide were added. After 30 min at room temperature, mercaptoethanol was added to remove free iodoacetamide, prior to assaying thiolase.

Preparation of Antibodies

A medium sized (3.4 kg) New Zealand white rabbit was injected subcutaneously into each of two footpads with 1 mg of pig heart thiolase I which had been extensively dialyzed against saline solution containing 20 mM sodium phosphate (pH 7.2) and had then been emulsified with an equal volume of Freund's complete adjuvant. One week later a booster, containing 1.5 mg of pig heart thiolase I, prepared as above, was injected subcutaneously into two footpads. Blood was taken from the vein of the ear 10 and 13 days after giving the booster injection. Ouchterlony plates showed a strong immunoprecipitation of pig heart thiolase I by serum taken on these days. The rabbit was terminally bled at the carotid artery 14 days after giving the booster injection. Whole blood (85 ml) was collected and stored overnight at 4^{o} C. The clotted cells formed during storage were loosened from the walls of the tube with a wooden spatula and collected by centrifugation at 1,000 x g for 15 min. Purification of the antibodies to thiolase I on DEAE-cellulose followed an established procedure (46). Purified antibodies raised against thiolase II from E. coli were kindly supplied by Dr. Judy Feigenbaum Binstock. Additionally, unfractionated serum of a rabbit immunized with lactate dehydrogenase from beef heart was prepared following the above procedure.

Immunotitration of Thiolases

Since the formation of the antibody-thiolase I (pig heart) complex led to only 60% inhibition of the enzymatic activity, the

antibody-antigen complexes were removed by either ultrafiltration or centrifugation. According to the first method antibodies and thiolase I solutions were combined and kept for 1 min at 25° C. After adding 0.33 ml of 0.3 M Tris-HCl (pH 8.3) containing 75 mM $MgCl_2$ and 0.67 ml of 0.1 M KCl, the solution was filtered through a Millipore filter (0.45 μ) into a cuvette. Acetoacetyl-CoA (25 nmoles) or 10 nmoles of 3-ketodecanoyl-CoA were added and the assay was started by the addition of 60 nmoles of CoA. According to the second method antibodies and antigen were allowed to react for 1 min at 25° C. After the addition of 1 ml of 50 mM glycylglycine (pH 7.8) containing 10 mM mercaptoethanol and 0.18 mg of bovine serum albumin, the solution was centrifuged for 30 min at 127,000 x g and the supernatant was assayed for thiolase activity as described above.

RESULTS I

Purification and Stabilities of Thiolases I and II from Pig Heart Muscle

Chromatography of a pig heart homogenate on phosphocellulose led to the separation of two thiolases, one of which acted on both acetoacetyl-CoA and 3-ketodecanoyl-CoA, whereas the second one was specific for acetoacetyl-CoA (see Fig. 2). No evidence for the existence of yet another thiolase was obtained. This finding is in general agreement with a previous report in which it was additionally shown that thiolases of heart muscle, including those of pig heart, are located in mitochondria. Since only one acetoacetyl-CoA-specific thiolase (thiolase II) was found in pig heart, this thiolase must be the one which had previously been purified to homogeneity and extensively studied by Gehring et al. (23, 24, 25). The thiolase, which was active with acetoacetyl-CoA as well as with longer chain substrates (thiolase I), was purified by chromatography on phosphocellulose and CM-cellulose as summarized in Table I and as described in detail under "Experimental Procedures." Chromatography on the first phosphocellulose column served as a means of reducing the amount of extracted protein to a manageable level and resulted in the separation of thiolases I and II. After the three-step purification procedure, summarized in Table I, thiolase I was purified 366-fold with a recovery of 32% of the original activity. The final chromatography of thiolase I on CM-cellulose is shown in Fig. 3. Polyacrylamide gel electrophoresis demonstrated the presence of one intensive protein band which coincided with both the acetoacetyl-CoA-thiolytic and

3-ketodecanoyl-CoA-thiolytic activities detected on an identical gel (see Fig. 4). When the same preparation was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (see Fig. 5), approximately 90% of the protein banded in one position, a finding which is indicative of the presence of small amounts of impurities. Assays for other enzymes of β oxidation showed that this preparation contained 0.65 U/mg of 3-hydroxyacyl-CoA dehydrogenase (assayed with acetoacetyl-CoA) and 5.8 U/mg of crotonase (assayed with crotonyl-CoA) which, because of their high specific activities (47, 48), can account for only 1% of the protein of the purified thiolase I preparation.

Thiolase II, which had been previously purified to homogeneity (23), was purified 308-fold by a modified procedure (see under "Experimental Procedures") which allowed for the simultaneous purification of both thiolases. The final specific activity of thiolase II was 43 units/mg. The purification of thiolase II is summarized in Table II and the final chromatography of thiolase II on CM-cellulose is shown in Fig. 6. This preparation of thiolase II showed two major protein bands when subjected to polyacrylamide gel electrophoresis (Fig. 7), but no activity was detected on an identical gel, which indicates that thiolase II is less stable than thiolase I under the conditions of polyacrylamide gel electrophoresis. This preparation showed also two major protein bands when subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. One of the bands observed on polyacrylamide gel in the presence of sodium dodecyl sulfate corresponded to a polypeptide with

a molecular weight of approximately 43,000 similar to that reported for the pig heart acetoacetyl-CoA thiolase studied by Gehring and Riepertinger (24). No attempts were made to purify thiolase II to homogeneity because a partially purified preparation was sufficient for the purpose of this study.

Although both thiolases were found to be stable for more than a year when stored at -76° C in the presence of glycerol and dithiothreitol (see under "Experimental Procedures"), their stabilities in dilute solution and at higher temperatures had to be determined before kinetic measurements could be performed. Thiolase II was found to be more labile than thiolase I (see Fig. 9A). This was especially pronounced at 25° C at which temperature thiolase II, when diluted to 1 μg/ml, lost its activity rapidly (data not shown) whereas thiolase I at the same concentration was stable for several days as long as mercaptoethanol was present and the ionic strength of the buffer was sufficiently high (see Fig. 9B). Bovine serum albumin and glycerol which were routinely added to stabilize both thiolases were not necessary for the stabilization of thiolase I (see Fig. 9B). In contrast, thiolase II in dilute form was stable for several days only when kept at 5° C in the presence of 50 mM potassium phosphate containing glycerol, mercaptoethanol and bovine serum albumin (see Fig. 9A). The deletion of any of these components as well as a decrease in the buffer concentration resulted in activity losses (see Fig. 9A). The fact that both thiolases were found to be stable only in the presence of thiol compounds points to the existence of an essential sulfhydryl group on these enzymes. The

presence of such a sulfhydryl at the active site of thiolase II has been previously demonstrated (23). Since, as shown in Table III, thiolase I lost 84% of its activity when reacted for 15 min with 10^{-4} M iodoacetamide but only 14% when in addition 1 mM acetoacetyl-CoA was present, it is concluded that this thiolase too has an essential sulfhydryl group at its active site.

pH Optima of Thiolases I and II from Pig Heart

The two thiolases of pig heart mitochondria differ with respect to their pH optima (see Fig. 10). Thiolase I showed greatest activity when assayed at pH 7.6 whereas thiolase II was most active at pH 8.6. As shown in Fig. 10, the molar extinction of acetoacetyl-CoA is a function of pH. Therefore, values for the extinction coefficient were determined for each pH. The fact that these two thiolases have different pH optima, when assayed with the same substrate, indicates that they may differ in their active sites, a suggestion also supported by their differences in substrate specificity. This finding contrasts somewhat with pH optima of thiolases I and II from E. coli, which were found to be 7.8 and 8.2, respectively (26). Both in pig heart and E. coli, thiolase I had a lower pH optimum than did thiolase II.

Physical and Kinetic Properties of Thiolase I and II from Pig Heart

The native molecular weight of thiolase I from pig heart (see Fig. 11) was estimated by gel filtration on a standardized Sephadex G-200 column, according to the procedure of Andrews (45). A value of 200,000 was obtained for pig heart thiolase I. Polyacrylamide gel

electrophoresis in the presence of sodium dodecyl sulfate yielded a subunit molecular weight of 48,000 (see Fig. 12). Hence, it is concluded that this thiolase is composed of four, possibly identical, subunits, as has been established for pig heart thiolase II (24), for cytoplasmic thiolases (29, 49) and recently for E. coli thiolase II (26, 50).

The chain length specificity of pig heart thiolase I was determined by measuring the rates of the thiolytic cleavage of a number of CoA derivatives of even-numbered 3-keto acids having 4 to 14 carbons. As shown in Fig. 13, the rate observed with acetoacetyl-CoA at 10 μM concentration was approximately one-fifth of those measured with longer chain substrates for all of which nearly equal rates were determined. The decrease in rate seen with 3-ketotetradecanoyl-CoA is most likely due to its non-specific inhibition of thiolase I because this inhibition was found to be dependent on the bovine serum albumin concentration in the assay mixture. In order to further analyze the rate difference between the cleavage of acetoacetyl-CoA and those of longer chain substrates, the apparent K_m and V_{max} values for several substrates were determined. Since the thiolase assay is based on the disappearance of the Mg^{2+}-3-ketoacyl-CoA complex, whose extinction coefficient is a function of the 3-ketoacyl-CoA concentration, values of the extinction coefficients were determined at various substrate concentrations as described under "Experimental Procedures." The results obtained with various 3-ketoacyl-CoA compounds are shown in Fig. 14. Only in the case of the Mg^{2+}-acetoacetyl-CoA complex did the extinction coefficient not change dramatically. When rates, measured in the presence of various concentrations of

3-ketohexanoyl-CoA, were corrected for the concentration dependence of the extinction coefficient and were plotted according to Lineweaver and Burk, they fell on a straight line (see Fig. 16). However, when the values were calculated using a constant extinction coefficient, a complex result was obtained which could be taken as evidence for substrate inhibition (see Fig. 16). Thus, the K_m and V_{max} values for the various chain lengths of 3-ketoacyl-CoA substrates as presented in Table IV were determined by the above outlined approach, using a saturating level of CoA (70 μM) as shown in Fig. 15. Kinetic parameters for longer chain substrates than listed were not determined because these compounds severely inhibited the enzyme. Although the listed apparent Michaelis-Menten constants for 3-ketooctanoyl-CoA and 3-ketodecanoyl-CoA are only approximate values, the results clearly demonstrate that the higher rates of thiolase I with 3-ketohexanoyl-CoA and longer chain substrates as compared to that measured with acetoacetyl-CoA are due to increases in maximal velocities as well as to decreases in K_m values. Especially the low K_m values for 3-ketooctanoyl-CoA and longer chain substrates explain the high rates observed with these substrates at low substrate concentrations (see Fig. 13). It is also obvious that the ratios of activities determined with substrates of various chain lengths depend on the substrate concentration at which the measurements were made.

The apparent K_m for CoASH was determined with thiolase I but only with acetoacetyl-CoA as substrate and was found to be 8.7 μM (see Fig. 17). A much higher value of 51.3 μM was determined for

CoASH with thiolase II (see Fig. 18). While this value is similar to that reported by Gehring et al. (24), their K_m value for acetoacetyl-CoA of 30 μM is higher than the value of 13.5 μM determined in this study (see Fig. 19).

Immunological Studies

Antibodies prepared against purified pig heart thiolase I were used to study the structural relationships between the two thiolases of pig heart and between E. coli and pig heart thiolases. As shown in Fig. 20, antibodies to pig heart thiolase I inhibited this enzyme, but maximally only 60%. It was therefore necessary to remove the thiolase-antibody complex by either ultrafiltration or centrifugation before determining the remaining activity. The results of such an experiment with purified thiolase I are shown in Figs. 21 and 22. The smooth immunoprecipitation curve observed in response to increasing amounts of antibodies as well as the virtually total loss of activity at higher antibody concentrations are indicative of the specific and complete precipitation of thiolase I. This experiment also demonstrates that the antibodies to thiolase I did not cross-react with pig heart thiolase II even at 2500-times higher concentration than required to achieve 50% inhibition of thiolase I (see Fig. 21). This finding leads to the conclusion that the two mitochondrial thiolases differ in their structures. Not surprising is the observation that antibodies prepared against E. coli thiolase II did not react with either of the two pig heart thiolases. The possible reactions of antibodies to pig heart thiolase I and to E. coli thiolase II with thiolase I and II from both pig heart and E. coli were also evaluated

by double diffusion according to Ouchterlony (51). In agreement with the results obtained from the immunotitration experiment, antibodies to pig heart thiolase I reacted only with pig heart thiolase I and antibodies to E. coli thiolase II gave a precipitation line only with E. coli thiolase II (data not shown). This result not only supports the above conclusion concerning the distinct antigenic properties of thiolase I and II from pig heart but also demonstrates that functionally related thiolases from pig and E. coli are structurally dissimilar. On the other hand, antibodies to pig heart thiolase I cross-reacted with thiolase I from beef heart (see Fig. 23A), a finding which conforms to the rule that functionally identical enzymes of closely related species have similar structures and antigenic sites. The availability of antibodies to pig heart thiolase I also permitted the determination of the contributions of thiolases I and II to the total acetoacetyl-CoA thiolytic activity present in an acetone powder extract of pig heart mitochondria. As shown in Fig. 23B, immunoprecipitation resulted in the complete removal of long chain thiolase activity whereas the acetoacetyl-CoA thiolase activity was only reduced by 35%. Since only two thiolases have been identified in pig heart, the remaining 65% of the acetoacetyl-CoA thiolase activity must have been due to thiolase II. The contribution of thiolases I and II to the total acetoacetyl-CoA thiolase activity of pig heart determined by immunoprecipitation are in good agreement with values previously obtained by chromatographic separation of the two enzymes (21) (see Fig. 2).

DISCUSSION I

The pig heart thiolase which acts on substrates of various chain lengths (thiolase I) is the second mammalian thiolase of its type to be purified to near homogeneity and it is the first of its type to be studied with respect to its native and subunit molecular weights and with respect to its kinetic parameters with longer chain substrates. The observed molecular weights of 200,000 and 48,000 for the native enzyme and its subunits, respectively, are only slightly larger than those of the mitochondrial and cytoplasmic acetoacetyl-CoA thiolases for which values of approximately 170,000 and 42,000, respectively, have been reported (24, 29, 49). Hence, it is concluded that all three types of thiolases found in animals have the same quaternary structure. They all are tetrameric proteins which are composed of four, possibly identical, polypeptide chains. However, despite the similarities in gross quarternary structure, the two mitochondrial thiolases from pig heart differ significantly in their tertiary structure, so that antibodies prepared against one of them do not cross-react with the other. If the two types of mitochondrial thiolases have emerged from the same ancestral gene, this event most likely occurred early during evolution for otherwise a greater structural similarity would be expected to exist. The fact that a type I and a type II thiolase occurs in E. coli supports the conclusion concerning the emergence early during evolution of these two types of thiolases. An interesting question remains concerning the similarities of the mitochondrial and cytoplasmic acetoacetyl-CoA thiolases. Since the cytoplasmic enzyme is believed to be involved

in cholesterol biosynthesis, it may have emerged later during evolution and may have possibly evolved from the gene of the mitochondrial acetoacetyl-CoA thiolase. If so, these two thiolases may be structurally more similar to each other than to thiolase I.

The availability of purified pig heart thiolase I also afforded the opportunity to determine the kinetic parameters (V_{max}, K_m) for the thiolytic cleavage of substrates of various chain lengths. Pig heart thiolase I, similarly to the corresponding thiolase from beef liver (30) and E. coli (31), is several fold more active with medium and long chain substrates than it is with acetoacetyl-CoA. The results of the kinetic measurements (see Table IV) clearly indicate that the increase in activity with increasing chain lengths of the substrates is due to a decreasing apparent K_m for the substrates. Additionally this increase reflects a 3-fold increase in apparent V_{max} for the reactions with 3-ketohexanoyl-CoA or longer chain substrates as compared to that with acetoacetyl-CoA. The apparent K_m values of approximately 2 μM for medium and long chain substrates point to the effective cleavage of these substrates even at low concentrations. In view of this finding, it is not surprising that the accumulation of 3-ketoacyl-CoA intermediates, which are formed during fatty acid oxidation, has not been observed. Should any 3-ketoacyl-CoA compound accumulate, it would be acetoacetyl-CoA on the basis of the kinetic parameters. However, because of the presence of thiolase II in mitochondria, the accumulation of unbound acetoacetyl-CoA is not very likely except when the concentration of free CoASH approaches zero.

Such a situation may occur when fatty acids are oxidized but the resulting acetyl-CoA is not further metabolized.

Of great importance to a full understanding of the regulation of β oxidation is the possible cooperation of thiolases I and II which, if effective, would assure a high rate of degradation of all 3-ketoacyl-CoA compounds formed during fatty acid oxidation. Although no definite evidence of this cooperation is available, a comparison of the E. coli fatty acid oxidation enzymes with those found in animals might provide further insight into this problem. Most important in this context is the recent demonstration that the E. coli fatty acid oxidation system exists as a multi-enzyme complex which includes thiolase, 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase (31). Although it has been suggested that the enzymes of fatty acid oxidation exist in mitochondria in a highly organized arrangement (17), E. coli is the first organism in which this was shown to be the case. This finding adds weight to the contention that a similar situation may exist in mitochondria except that the enzymes would be more loosely bound to one another. Since thiolase II of E. coli was shown to be a separate enzyme (26), it is suggested that also in mitochondria thiolase II is not part of the putative multi-enzyme arrangement of β oxidation. Further suggestive evidence concerning the physiological functions of thiolases I and II of heart is derived from a comparison of E. coli and pig heart thiolases. A compilation of properties of both thiolases from E. coli and pig heart is presented in Table V. Clearly, the two thiolases I are strikingly similar in that they both act on substrates of various chain lengths

and both act more efficiently on longer chain substrates than on acetoacetyl-CoA. Additionally, their kinetic parameters are very similar, specifically the low K_m values for the longer chain substrates. Since the E. coli thiolase I is part of a multi-enzyme complex, its true molecular weight and subunit structure are still unknown. Also the two thiolases II are nearly identical with respect to the listed properties except for their K_m values for CoASH, which differ by a factor of 7. It is thus concluded that the two types of thiolases present in heart mitochondria and E. coli which are strikingly similar may have identical functions. Since thiolases I and II of E. coli are induced by, and thus function in, the degradation of fatty acids and acetoacetate, respectively, it is suggested that thiolase I of pig heart mitochondria is the sole thiolase required for β oxidation of fatty acids, whereas thiolase II functions only in ketone body degradation.

INTRODUCTION II

Three systems of fatty acid synthesis are found in mammalian cells. De novo synthesis is accomplished by the cytoplasmic multi-enzyme complex which produces fatty acids for triglyceride and phospholipid synthesis in liver, adipose tissue and mammary gland. Elongation of preexisting saturated or unsaturated fatty acids is accomplished by either a mitochondrial or a membrane bound microsomal system. The most important system of fatty acid synthesis in heart is in the mitochondria which represents 70-90% of the total fatty acid synthesizing activity of heart (52).

Fatty acid synthesis in mitochondria is unique in that it occurs by an acetyl-CoA-dependent elongation of fatty acids. It has been observed that heart mitochondria incorporates acetyl-CoA into fatty acids more rapidly than malonyl-CoA (38, 53, 54). Additionally, the absence of acetyl-CoA carboxylase in rabbit heart mitochondria (55) and the failure of avidin to inhibit the fatty acid synthesizing activity in a mitochondrial membrane preparation (56) support the conclusion concerning non-involvement of malonyl-CoA in mitochondrial fatty acid synthesis. Evidence presented by Dahlen and Porter (38) indicates that malonyl-CoA is first decarboxylated to acetyl-CoA before it is incorporated into fatty acids in mitochondria.

The location and nature of the mitochondrial fatty acid synthesizing system(s) has been the subject of a continued controversy for many years. In 1968, Dahlen and Porter (38) reported the existence

of an enzyme system located on the outer membrane of beef heart mitochondria which was capable of fatty acid elongation. Whereat et al. (56), working with rabbit heart mitochondria, reported the separation of two synthetic systems, one located on the outer mitochondrial membrane and another associated with the inner membrane. The inner membrane system required ATP and was thought to be capable of de novo fatty acid synthesis. However, Dahlen and Porter (38) were unable to find any evidence for de novo synthesis with beef heart mitochondrial preparations. Additionally, the ATP requirement could be eliminated by adding acyl-CoA to mitochondrial preparations (30, 38, 59). It therefore seems unlikely that de novo synthesis occurs in heart mitochondria.

The enzymes involved in mitochondrial fatty acid elongation have not been completely elucidated, although it has been suggested that elongation of fatty acids occurs by a reversal of the last three steps of β oxidation (30, 58). As shown in Fig. 24, the steps of β oxidation reversed during elongation are those catalyzed by thiolase I (EC 2.3.1.16), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) and enoyl-CoA hydratase (EC 4.2.1.17). The last step of the elongation process is not catalyzed by acyl-CoA dehydrogenase (EC 1.3.99.3) but instead by a pyridine-nucleotide dependent, membrane bound enoyl-CoA reductase (EC 1.3.1.8), which transfers hydrogen from NADH or NADPH to enoyl-CoA. The reaction catalyzed by the pyridine-nucleotide dependent enoyl-CoA reductase is important to the equilibrium of the elongation pathway because, as Seubert et al. (30) have pointed out, the reversal of all four steps of β oxidation is

thermodynamically unfavorable whereas the pathway outlined in Fig. 24 favors elongation when the concentrations of NADH or NADPH are high as during cellular anoxia.

The mitochondrial system of fatty acid elongation is widely distributed among mammalian tissues but the systems differ from tissue to tissue with respect to their pyridine nucleotide requirements. Liver, kidney cortex and the mitochondria of brown adipose tissue require both NADH and NADPH, whereas heart muscle, skeletal muscle and aortic intimal mitochondria require only NADH (59). It would appear that enoyl-CoA reductase of liver is different from that of heart muscle. Consequently, a distinction is made in the literature between two types of mitochondrial fatty acid elongation systems: the "liver type" which requires both NADH and NADPH and the "heart type" which requires NADH only. Hinsch and Seubert (59, 60) have proposed different physiological roles for each type. The "heart type" they propose may be of importance in the conservation of reducing equivalents or acetate units in the anaerobic state. The "liver type" may play a role in the transfer of hydrogens from NADPH to the respiratory chain via enoyl-CoA reductase coupled to acyl-CoA dehydrogenase, which can transfer electrons to cytochrome b through an electron transferring flavoprotein (37, 61). This action of acyl-CoA dehydrogenase, coupled to enoyl-CoA reductase, allows an ADP-independent bypass of the first part of the respiratory chain.

A final clarification of the physiological role of the mitochondrial fatty acid elongation system requires the elucidation of the enzymes involved in this process. As mentioned above, the

elongation of fatty acids has been suggested to occur by reversal of β oxidation. This suggestion is supported by the observed elongation of acyl-CoA primers in the presence of a mixture of purified enzymes consisting of thiolase I, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase (30). However, there are reports which represent evidence that the fatty acid elongation activity is associated with a single protein having a molecular weight of maximally 135,000 (38, 62) and which therefore does not agree with the proposed involvement of β oxidation enzymes in this process.

In order to determine whether or not mitochondrial fatty acid elongation occurs by a reversal of fatty acid oxidation, the dependence of the elongation system on thiolase I was investigated. For this purpose, thiolase I was removed by immunoprecipitation from a mitochondrial extract which was then assayed for fatty acid elongation activity. In addition, an attempt was made to identify the rate limiting step in mitochondrial fatty acid elongation.

EXPERIMENTAL PROCEDURES II

Materials

Acetyl-CoA, octanoyl-CoA and malonyl-CoA were obtained from P-L Biochemicals, Inc. (1-^{14}C) acetyl-CoA and (1,3-^{14}C) malonyl-CoA were purchased from New England Nuclear, Inc. Rotenone was purchased from Sigma Biochemicals, Inc.

Methods

Preparation of Substrates

Crotonyl-CoA was prepared according to a published procedure (39). Other substrates were prepared as described in "Experimental Procedures I." D,L-3-hydroxydecanoyl-CoA was synthesized from D,L-3-hydroxydecanoic acid by the method used to prepare $\Delta^{2,3}$-decenoyl-CoA (see under "Experimental Procedures I").

Enzyme Assays

Enoyl-CoA hydratase was assayed as described by Schulz (39) except that each assay contained 30 μmoles of glycylglycine (pH = 7.8), 12 nmoles of $\Delta^{2,3}$-decenoyl-CoA or 16 nmoles crotonyl-CoA and 30 ng of acetone powder extract or pig heart homogenate in an assay volume of 0.6 ml. When enoyl-CoA hydratase was assayed in the direction of dehydration, the assay contained 21 nmoles of D,L-3-hydroxydecanoyl-CoA and 50-90 μg of acetone powder extract or pig heart homogenate.

Thiolase was assayed in the cleavage direction as described in "Experimental Procedures I" but in the condensation direction the assay

solution contained: 0.06 mmoles of Tris-HCl (pH 8.8), 0.03 mmoles of acetyl-CoA, to which 5.2 μg of purified thiolase I were added. The increase in absorbance at 303 nm was measured and an approximate molar extinction coefficient of 19,800 was determined and used to calculate activity. A ratio of activities in the cleavage vs. the condensation direction of 210 was thus determined with pure thiolase and used to calculate thiolase catalyzed condensation activities in acetone powder extracts and pig heart homogenate.

L-3-Hydroxyacyl-CoA dehydrogenase activity in acetone powder extracts and whole pig heart homogenates was determined with acetoacetyl-CoA, as described previously (26). Both acetone powder extracts and whole homogenates were found to contain high levels of lactic acid dehydrogenase. Consequently, 3-ketodecanoyl-CoA produced enzymatically as described in "Experimental Procedures I" was not suitable as a substrate for these measurements because this substrate contained pyruvate which in the presence of lactate dehydrogenase led to the rapid oxidation of NADH. Therefore, the activity ratio of 2 determined for the reduction of acetoacetyl-CoA vs. that of 3-ketodecanoyl-CoA with pure L-3-hydroxyacyl-CoA dehydrogenase from pig heart was used to calculate the values listed in Table VII from reductase activities determined with acetoacetyl-CoA as substrate.

The acetyl-CoA-dependent fatty acid elongation was determined by measuring the incorporation of (1-^{14}C) acetyl-CoA into ether-extractable fatty acids at 38^{o} C. The reaction mixture contained in a total volume of 1 ml:40 μmoles of glycylglycine (pH 7.8), 130 nmoles of octanoyl-CoA, 65 nmoles of (1-^{14}C) acetyl-CoA

(6000 dpm/nmole), 0.56 μmole of NADH and 25 μmoles of rotenone. The reaction was started by the addition of an acetone powder extract (0.2 mg of protein) prepared from pig heart mitochondria and was terminated after 15 min by the addition of either KOH or HCl. Termination with HCl permitted the separate extraction of free acids, followed by the extraction of CoA-bound fatty acids after alkaline hydrolysis. After washing the ether extracts with water, the solvent was removed in vacuum and the residues were counted in a liquid scintillation counter. In experiments in which thiolase I was removed from the acetone powder extracts by immunoprecipitation, the acetone powder extract was first centrifuged at 31,000 x g for 15 min and was then reacted with 0-400 μg of antibodies per 140 μg of extracted protein. After 1 min at room temperature, 0.05 M glycylglycine (pH 7.8), containing 10 mM 2-mercaptoethanol and bovine serum albumin (0.18 mg/ml), was added to a final volume of 1 ml. The solution was centrifuged at 31,000 x g for 30 min and the resulting supernatant was immediately assayed for thiolase and fatty acid elongation activities. Units of enzyme activity are expressed as μmoles of substrates utilized or product formed per min. Protein concentrations were determined by the method of Lowry et al. (42).

Thin Layer Chromatography of Products

The radioactive fatty acids formed during the acetyl-CoA-dependent fatty acid elongation were dissolved in a minimal volume of diethyl ether and were spotted on activated silica gel plates. Standards of 3-hydroxydecanoic acid, $\Delta^{2,3}$-decenoic acid and n-decanoic acid as well as a mixture of the 3 acids were spotted on the same

plate which was then developed with a hexane-diethyl ether-acetic acid (70:30:2) solvent system. After development in a closed container, the plate was cut vertically and the resulting strips were cut into equal segments and counted in a liquid scintillation counter. The standards were visualized with iodine vapors in a closed container. Under conditions used in this separation, polar 3-hydroxy fatty acids remained close to the origin whereas the saturated and unsaturated standards moved nearly to the solvent front. Unfortunately, the saturated and unsaturated free acids nor their methyl esters could be resolved by this method or by chromatography on silica plates impregnated with $AgNO_3$.

Preparation of Pig Heart Homogenate

Pig heart (6.05 g) was cut into small pieces and homogenized in 20 ml of buffer containing 1 mmole of glycylglycine (pH 7.8) and 56 μmoles of 2-mercaptoethanol. The homogenate was filtered through one layer of cheesecloth. The volume after filtration was 16 ml and it contained 151 mg of protein.

Preparation of Pig Heart Mitochondria

Small pieces of fresh pig heart were forced through a meat grinder and suspended in 0.25 M sucrose, 0.01 M 2-mercaptoethanol, 0.02 M Tris-HCl (pH 7.5). The suspension was homogenized in a Waring Blender for 30 sec and filtered through two layers of cheesecloth. The filtrate was centrifuged at 390 x g for 10 min. The pellet was resuspended in the same buffer and centrifuged a second time. The combined supernatants were centrifuged at 11,700 x g for

20 min. The pellet was resuspended and centrifuged again. The mitochondrial pellet was stored frozen at -76° C.

Preparation of Mitochondrial Acetone Powder

The procedure of Dahlen and Porter (38) was modified to prepare acetone powder from the frozen mitochondrial pellet. Mitochondria (8.9 g) were slowly added with stirring to 150 ml of an acetone-water-ammonia mixture (95:5:0.3) at -20° C. After one hour of stirring in the cold, the powder was filtered off with suction, washed once with cold acetone and dried in vacuo at -20° C. The yield of dry powder was 1.14 g, which was stored in a dark bottle at -76° C. For the preparation of acetone powder extracts, 25 mg of powder were suspended in 3 ml of 0.05 M glycylglycine buffer (pH 7.8) containing 2.8 mM 2-mercaptoethanol and were sonicated for 30 sec. In experiments with antibodies, the sonicated suspension of acetone powder was centrifuged at 127,000 x g for 30 min or 31,000 x g for 15 min before use.

RESULTS II

Acetyl-CoA-dependent Fatty Acid Elongation

As shown in Fig. 25, the incorporation of (1-^{14}C) acetyl-CoA into fatty acids was a linear function of the amount of pig heart acetone powder extract. Results presented in Table VI demonstrate the requirement for an acyl-CoA primer, such as octanoyl-CoA, and thereby proof for the absence of a de novo synthesizing system in this preparation. These results are consistent with those of Dahlen and Porter (38) with beef heart and of Hinsch et al. (59) with pig heart acetone powder extracts. The observed slight incorporation of malonyl-CoA into fatty acids was most likely due to the presence of small amounts of acetyl-CoA in the malonyl-CoA preparation.

Immunotitration of Acetyl-CoA-dependent Chain Elongation

The removal of thiolase I from acetone powder extracts by immunoprecipitation resulted in a nearly complete inhibition of the fatty acid elongation system. The results of a detailed inhibition study of both thiolase I and the fatty acid elongation system by antibodies to thiolase I are presented in Fig. 26. This data shows that antibodies to thiolase I, in contrast to unspecific antibodies, inhibited thiolase I and the fatty acid elongation system in a parallel fashion, a finding which suggests that thiolase I is a component enzyme of the mitochondrial fatty acid elongation and possibly catalyzes the rate-limiting step in this pathway. The small thiolase activity not precipitated at optimal concentrations was most likely the cause for the residual fatty acid elongation activity.

Since this experiment suggests that the thiolase-catalyzed condensation of octanoyl-CoA and acetyl-CoA is the rate limiting step of the elongation reactions, the effects of additional purified thiolase I on the rate of fatty acid elongation was investigated. As shown in Table VI, the elongation activity was stimulated by the addition of thiolase I, a finding which supports the conclusion concerning the rate limiting step of the pathway. To further substantiate this conclusion, the individual reactions of the fatty acid elongation system, except for the NADH-dependent enoyl-CoA reductase, were measured. The latter enzyme could not be measured because L-3-hydroxyacyl-CoA dehydrogenase, which is present in the acetone powder in high levels, interferes with both the spectrophotometric and radioactive assay of the NADH-dependent enoyl-CoA reductase. The activities of thiolase, L-3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase in the direction of fatty acid elongation are listed in Table VII. These approximate values indicate again that the rate of fatty acid elongation is determined by the rate of the thiolase catalyzed condensation reaction. The lower values of the condensation reaction as compared to the elongation activities are most likely due to difference in conditions at which the two types of assays were performed.

Products of Fatty Acid Elongation

Analyses of the elongation products, as shown in Table VIII, revealed that approximately 50% of the fatty acids were present as free acids; the free and the CoA-bound fatty acids were found to be mostly hydroxy acids. The ratios of hydroxy acids to saturated and

unsaturated acids were approximately 3:1 and thus were similar to those observed by Hinsch et al. (59). The high percentage of hydroxy acids which is typically observed with mitochondrial extracts of heart, skeletal muscle and other extrahepatic tissues, but not with liver mitochondria (59, 60), points to enoyl-CoA reductase as the rate limiting activity in the formation of saturated fatty acids. It is thus concluded that the condensation reaction catalyzed by thiolase I is the rate limiting step in heart mitochondria, when the incorporation of acetyl-CoA into all ether-extractable fatty acids and fatty acid intermediates is measured, whereas the reduction of the enoyl-CoA intermediates is possibly the rate limiting step in the formation of saturated fatty acids.

DISCUSSION II

Mitochondrial fatty acid elongation was studied with an acetone powder extract of pig heart mitochondria because this elongation system is soluble and has recently been investigated by Hinsch et al. (50) who demonstrated that it catalyzes most efficiently the acetyl-CoA-dependent elongation of medium chain fatty acyl-CoA's and requires as an electron donor only NADH. Similar results have been obtained by Dahlen and Porter with an acetone powder extract of beef heart mitochondria (38). As shown in Fig. 25, the elongation activity is a linear function of the amount of protein added to the assay.

In agreement with other reports concerning the absence of a de novo fatty acid synthesizing system in heart mitochondria is the finding that acetyl-CoA is not incorporated into fatty acid in the absence of octanoyl-CoA primer (38, 54, 63). In contrast to the cytoplasmic and microsomal systems, malonyl-CoA does not participate in the elongation reaction. This fact together with the nearly complete inhibition of the fatty acid elongation system by antibodies to thiolase I indicates that the first step in the elongation process in mitochondria is the thiolase catalyzed condensation of acyl-CoA and acetyl-CoA. The parallel and virtually complete inhibitions of thiolase I and of the fatty acid elongation activity by antibodies to thiolase I provide proof that fatty acid elongation in heart mitochondria occurs by reversal of fatty acid oxidation. Since enoyl-CoA reductase is membrane bound (12) and since a significant fraction of the β oxidation enzymes remain membrane associated even when

mitochondria are broken, the fatty acid elongation is best characterized as membrane associated. Because of the well-known location of the enzymes of fatty acid oxidation within the space surrounded by the inner mitochondrial membrane (64), the fatty acid elongation system is assumed to be associated with the inner mitochondrial membrane. In view of the conclusions reached concerning the enzymes of the mitochondrial fatty acid elongation system in heart, it is suggested that the observed behavior of the elongation activity as a single protein with a molecular weight of 135,000, as reported by Dahlen and Porter (38), may be a reflection of the molecular weight range of maximal overlap between the four component enzymes of the system.

The observed accumulation of 3-hydroxy fatty acids suggests that enoyl-CoA reductase activity in heart mitochondria is low compared to the levels of the other enzymes of β oxidation. However, Hinsch et al. (59) have reported that in a pig heart mitochondrial acetone powder extract the rate of enoyl-CoA reduction was 6.4-times higher than the rate of formation of saturated fatty acid and yet they found hydroxy acids to be the main products. Thus, it seems that hydroxy acids are the preferred products of mitochondrial fatty acid elongation in heart.

Little is known about the physiological significance of mitochondrial fatty acid elongation in heart. Whereat et al. (53, 65) demonstrated that fatty acid synthesis in heart mitochondria increases with increasing NADH/NAD ratio. An increase in fatty acid synthesis during hypoxia can also be demonstrated in the arterial wall (66).

These findings suggest a physiological function for fatty acid elongation in the storage of reducing equivalents and acetate units under hypoxia. This capacity would also provide a mechanism for mitochondria to conserve energy during metabolic states characterized by high phosphate potentials. However, to date there is no solid evidence to indicate what role, if any, fatty acid elongation plays in the metabolic processes of mitochondria. For heart, though, fatty acids can only be modified by this process and an understanding of the nature of the enzymes involved in fatty acid elongation is important. A "heart type" mitochondrial fatty acid elongation system, which is characterized by a requirement for NADH and not for NADPH in the reduction of enoyl-CoA intermediates, is also found in beef aortic intima (59). The fatty acids produced in this tissue may affect the incorporation of cholesterol into plaques on the vessel wall. Elevated plasma cholesterol concentrations were found to stimulate fatty acid synthesis in the walls of the vessel (67) and perhaps these fatty acids become esterified to cholesterol, as suggested by Lofland et al. (68, 69). If this is correct, the control mechanism of fatty acid synthesis in vascular tissue may be important for an understanding of the fundamental defect leading to the development of atherosclerosis.

The data presented here indicates that mitochondrial fatty acid elongation occurs by reversal of β oxidation reactions and that the thiolase I activity may determine the rate of synthesis of the pool of 3-hydroxy and other fatty acids.

Table I

Purification of Thiolase I from Pig Heart

For details of the purification, see "Experimental Procedures I." Assays were performed as described under "Experimental Procedures I" with 3-ketodecanoyl-CoA as the substrate.

Step	Total Protein	Total Activity	Specific Activity	Purification	Yield
	g	units	units/mg	-fold	%
Homogenate	20.24	3,300	0.163	1	100
Phosphocellulose (batch)	1.468	2,935	2.0	12	89
Phosphocellulose (gradient)	0.116	1,484	12.8	79	45
CM-cellulose	0.018	1,056	59.7	366	32

Table II

Purification of Thiolase II from Pig Heart

For details of the purification, see "Experimental Procedures I." Assays were performed as described under "Experimental Procedures I" with acetoacetyl-CoA as the substrate.

Step	Total Protein	Total Activity	Specific Activity	Purification	Yield
	g	units	units/mg	-fold	%
Homogenate[a]	20,240	2794	0.138	1	100
Phosphocellulose (batch)	279	614	2.2	16	22
Phosphocellulose (gradient)	57	339	6.0	43	12
CM-cellulose (batch)	9.2	163	18	129	5.8
CM-cellulose (gradient)	1.1	48	43	308	1.7

[a] Pig heart homogenate contains thiolases I and II, both of which are active with acetoacetyl-CoA. The total thiolase units assayed with acetoacetyl-CoA in the homogenate was 3,894 units. This homogenate contained 3,300 thiolase units using 3-ketodecanyl-CoA as a substrate (see Table I); since the activity ratio for thiolase I is 1:3, it was possible to estimate that 1,100 units and 2,794 units were due to thiolases I and II, respectively.

Table III

Inhibition of Thiolase I by Sulfhydryl Reagents

Assays were performed as described under "Experimental Procedures I."

Inhibitor	Concentration	Percent Activity Remaining After 15 min	After 30 min
	mM		
None		96	87
Iodoacetamide	0.1	16	14
Iodoacetamide	0.05	46	25
Iodoacetamide	0.01	84	64
Iodoacetamide + 12.5 μM acetoacetyl-CoA	0.1	100	86
N-Methylmaleimide	0.1	70	41
N-Methylmaleimide	0.5	40	23

Table IV

Apparent Kinetic Constants of Thiolase I with Substrates of Various Chain Lengths

Thiolase was assayed as described under "Experimental Procedures I", except that the molar extinction coefficients were determined for each Mg^{+2}-3-ketoacyl-CoA complex as a function of 3-ketoacyl-CoA concentration in the range of 5 to 40 μM. Activity as a function of 3-ketoacyl-CoA concentration was plotted according to the method of Lineweaver and Burk and apparent kinetic constants were determined. The coenzyme A concentration in each assay was 70 μM.

Substrate	Apparent K_m	Apparent V_{max}	Relative V_{max}
	μM	units/mg	
Acetoacetyl-CoA	17	29	1.0
3-Ketohexanoyl-CoA	8.3	69	2.4
3-Ketooctanoyl-CoA	2.4	65	2.2
3-Ketodecanoyl-CoA	1.8	67	2.3

Table V

Comparison of the Thiolases from Pig Heart and *E. coli*

	Chain Length Specificity	Activity Ratio C_4:C_{10}	K_m Values		Molecular Weight	Function
				μM		
Heart mitochondria						
Thiolase I	C_4-C_{16}	1:6	C_4-CoA	17.3	200,000	Fatty acid
			C_{10}-CoA	1.8	(4 X 46,000)	oxidation
			CoA-SH	8.7		
Thiolase II	C_4		C_4-CoA	13.5	170,000[a]	Ketone body
			CoA-SH	51.3	(4 X 42,000[a])	degradation
E. coli						
Thiolase I	C_4-C_{16}[b]	1:20[c]	C_4-CoA	31	320,000[c]	Fatty acid
			C_{10}-CoA	2	(complex)	oxidation
			CoA-SH	20		
Thiolase II	C_4[b]		C_4-CoA	7.5	175,000[d]	Acetoacetate
			CoA-SH	7.6	(4 X 41,000[d])	degradation

[a]See reference 24.

[b]See reference 26.

[c]See reference 31.

[d]See reference 70.

Table VI

Fatty Acid Elongation with an Acetone Powder Extract of Pig Heart Mitochondria

Experiments were performed as described under "Experimental Procedures II."

Experiment	Additions	Deletions	Specific Activity	Relative Activity
			munits/mg	%
#1	None	None	1.00	100
	None	Octanoyl-CoA	0	0
	Thiolase (9.7 mU)[a]	None	1.34	134
	Thiolase (19.5 mU)	None	1.62	162
	Thiolase (39.2 mU)	None	1.92	191
	Thiolase (404 mU)	None	2.66	266
#2	None	None	0.587	100
	Ab to thiolase (94 µg)	None	0.016	3
	Malonyl-CoA	Acetyl-CoA	0.02	3
	Malonyl-CoA + 94 µg of Ab to thiolase	Acetyl-CoA	0.015	3

[a]Thiolase assays were performed in the cleavage direction with 3-ketodecanoyl-CoA as described under "Experimental Procedures I."

Table VII

Activities of the Fatty Acid Elongation System and of Several of its Component Enzymes in a Homogenate and Acetone Powder Extract of Pig Heart Mitochondria

Each enzyme was assayed as described under "Experimental Procedures II." Thiolase was assayed in the direction of condensation; enoyl-CoA hydratase was assayed in the dehydration direction.

Enzyme	Specific Activity	
	Homogenate	Acetone Powder
	mUnits/mg	mUnits/mg
Fatty acid elongation system	0.88	0.37
Thiolase	0.5	0.25
3-Hydroxyacyl-CoA dehydrogenase	586	173
Enoyl-CoA hydratase	24	18

Table VIII

Products of Mitochondrial Fatty Acid Elongation in Pig Heart

The products of acetyl-CoA-dependent fatty acid elongation were determined as described in "Experimental Procedures II." The acetone powder extract was used without centrifugation; 50 µl containing 0.21 mg of protein were added to the assay mixture at 38° C. When the pig heart homogenate was used as the enzyme source, 20 µl containing 0.188 mg of protein were used in each assay mixture. The reaction was terminated after either 15 or 30 min by the addition of 0.1 ml of 4 M HCl and the products were saponified, extracted and dried as described in "Experimental Procedures II." In each experiment one half of the extracted material was counted in a liquid scintillation counter and the second half was analyzed by thin layer chromatography as described under Experimental Procedures II."

Enzyme Source	Proportion of the Product		Reaction Time
	Free Acid	3-Hydroxy	
	%	%	min
Acetone powder	43	76	15
	33	79	30
Homogenate	59	69	15
	50	67	30

Fig. 1 Fatty acid degradation in heart muscle. The β oxidation pathway is shown at the top (A) of the figure, while the reactions of ketone body degradation in heart are shown below (B). The individual reactions of β oxidation are catalyzed by the following enzymes: (1) acyl-CoA synthetase; (2) acyl-CoA dehydrogenase; (3) enoyl-CoA hydratase; (4) L-3-hydroxyacyl-CoA dehydrogenase; (5) thiolase I. The enzymes of ketone body degradation are: (6) thiolase II; (7) succinic-acetoacetyl thiophorase; and (8) D-3-hydroxybutyric acid dehydrogenase.

Figure 1

Fatty Acid Oxidation and Ketone Body Degradation

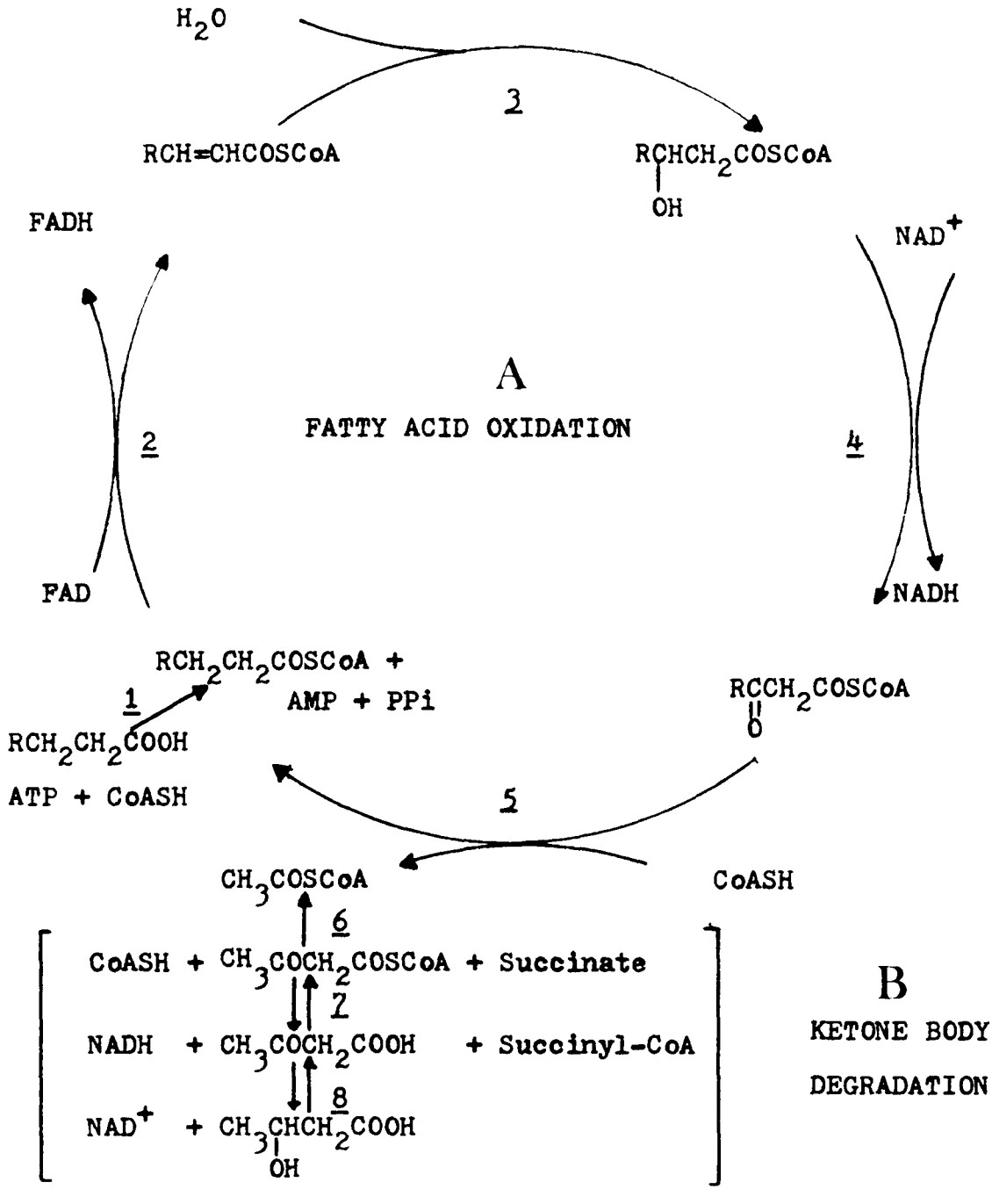


Fig. 2 Separation of pig heart thiolases I and II on phosphocellulose. A homogenate of 10 g of pig heart in 200 ml of 0.02 M potassium phosphate (pH 6.6) containing 10 mM mercaptoethanol and 25% glycerol was applied to a phosphocellulose column (1.2 x 37 cm) equilibrated with the same buffer. After washing the column with the starting buffer, the column was developed with a linear gradient made up of 200 ml of 0.02 M potassium phosphate (pH 6.6) containing 10 mM mercaptoethanol and 25% glycerol and 200 ml of 0.5 M potassium phosphate containing the same amount of mercaptoethanol and glycerol. Sixty fractions of 6.4 ml each were collected and assayed for both acetoacetyl-CoA (▲) and 3-ketodecanoyl-CoA (●) thiolase activities as described under "Experimental Procedures I."

Figure 2

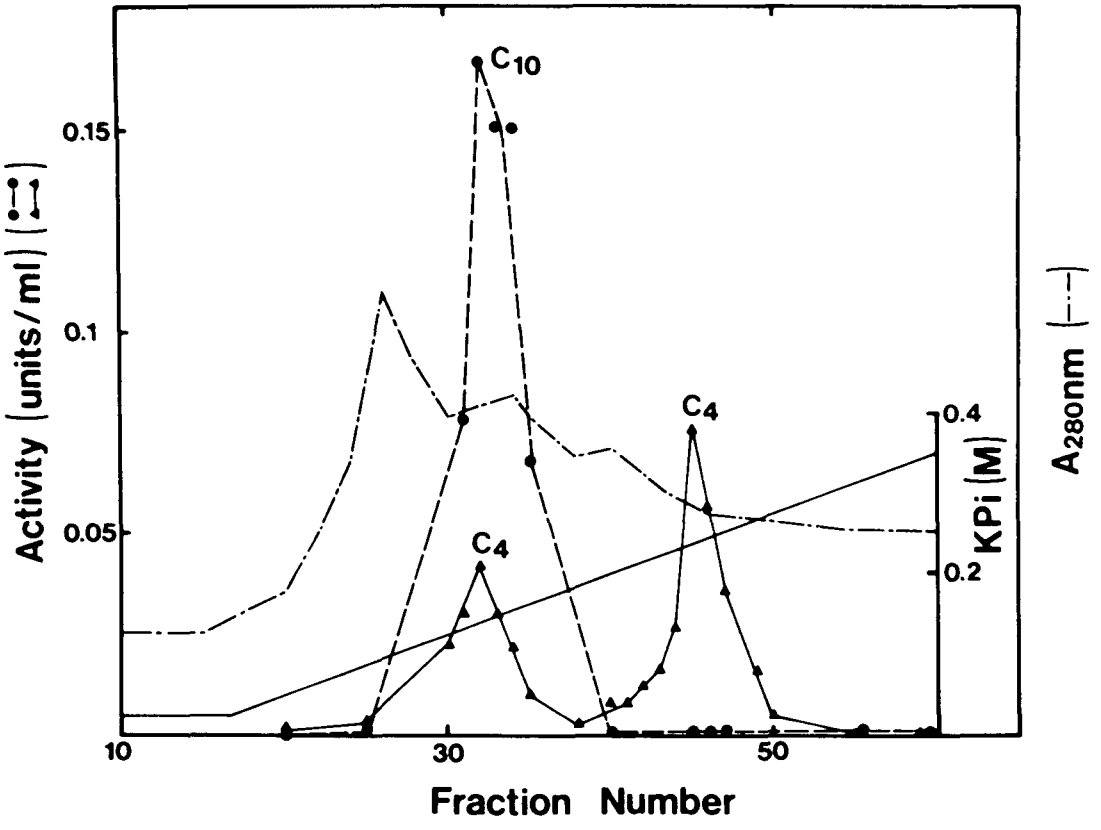

C10
C4
C4
0.15
0.1
0.05
0.4
0.2
10
30
50
Activity (units/ml) (•—•)
KPi (M)
A280nm (—)
Fraction Number

Fig. 3 Purification of thiolase I by CM-cellulose chromatography. Thiolase I (116 mg), which had been partially purified by phosphocellulose chromatography, was applied to a CM-cellulose column (1.2 x 46 cm) and the column was developed with a linear gradient of potassium phosphate between 0.01 M and 0.1 M (for details see "Experimental Procedures I"). Each fraction contained 3.3 ml. Fractions 105 to 110 and fractions 111 to 115 were pooled separately and stored as described under "Experimental Procedures I."

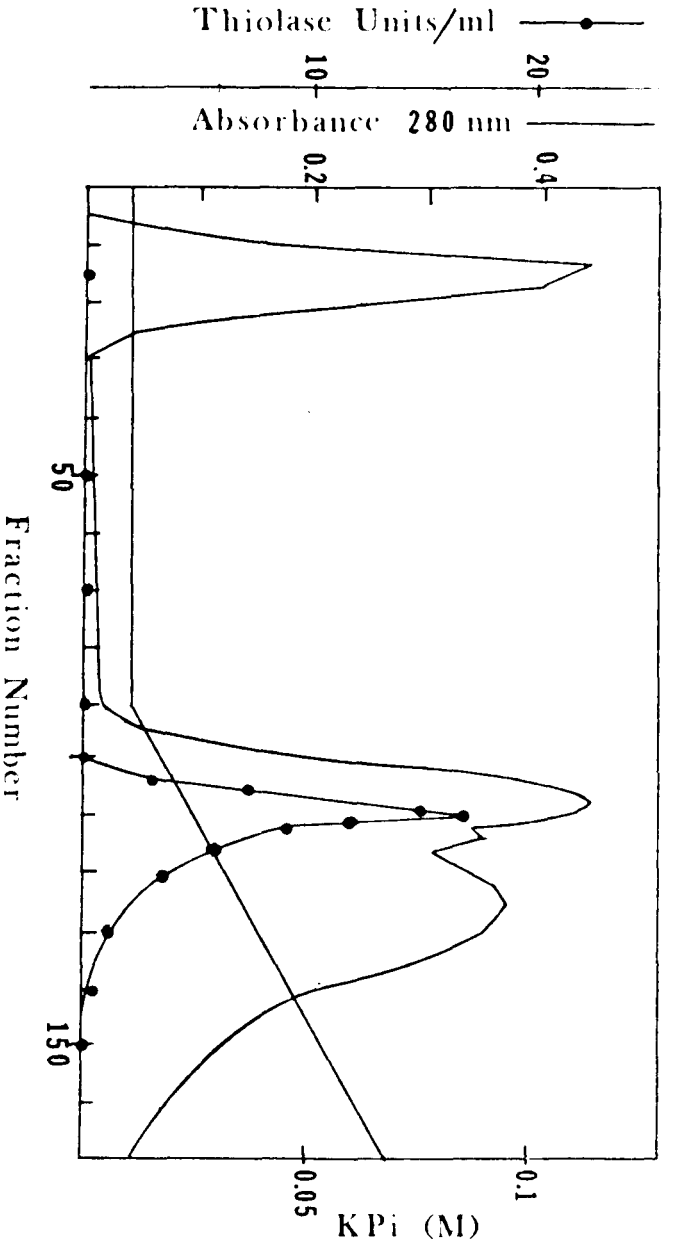

Thiolase Units/ml
10
20
Absorbance 280 nm
0.2
0.4
50
150
Fraction Number
0.05
0.1
KPi (M)

Figure 3

Fig. 4 Disc gel electrophoresis of purified thiolase I (20 μg) on a 7.5% polyacrylamide gel. Gels were run in the presence of 10 mM mercaptoethanol and stained for protein with Fast Green. Duplicate unstained gels were sliced and each slice was extracted for 12 hr with 0.2 ml of 0.05 M Tris-HCl buffer (pH 8.1) containing 5% glycerol, 1 mg/ml bovine serum albumin and 10 mM mercaptoethanol. The extracts were assayed for thiolase activities with acetoacetyl-CoA and 3-ketodecanoyl-CoA as described under "Experimental Procedures I." The open and solid bars indicate 3-ketodecanoyl-CoA and acetoacetyl-CoA thiolase activities, respectively.

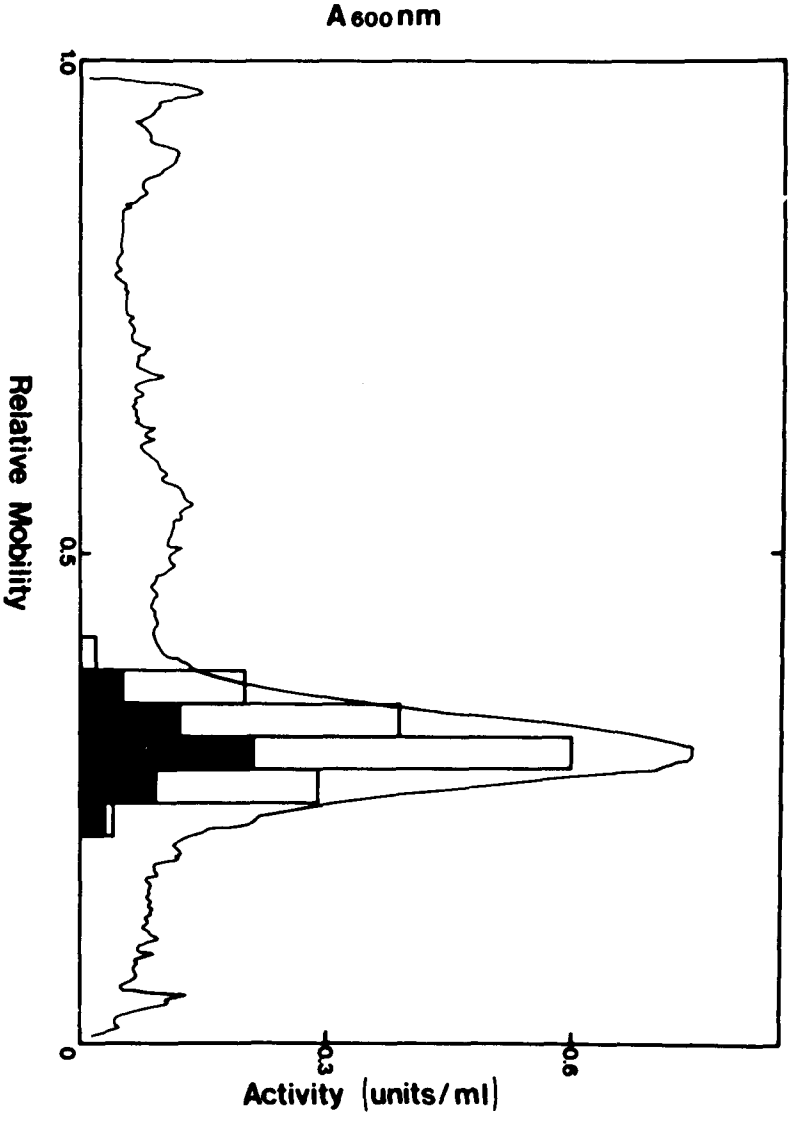


Figure 4

Fig. 5 Gel electrophoresis of purified thiolase I in the presence of sodium dodecyl sulfate on a 10% polyacrylamide gel. Thiolase I (5 μg from CM-cellulose fractions 111-115) was incubated at 37° C for 2 hr in 0.01 M sodium phosphate containing 10% sodium dodecyl sulfate and 1% mercaptoethanol. The gel was stained with Fast Green and destained in 7% acetic acid.

Figure 5

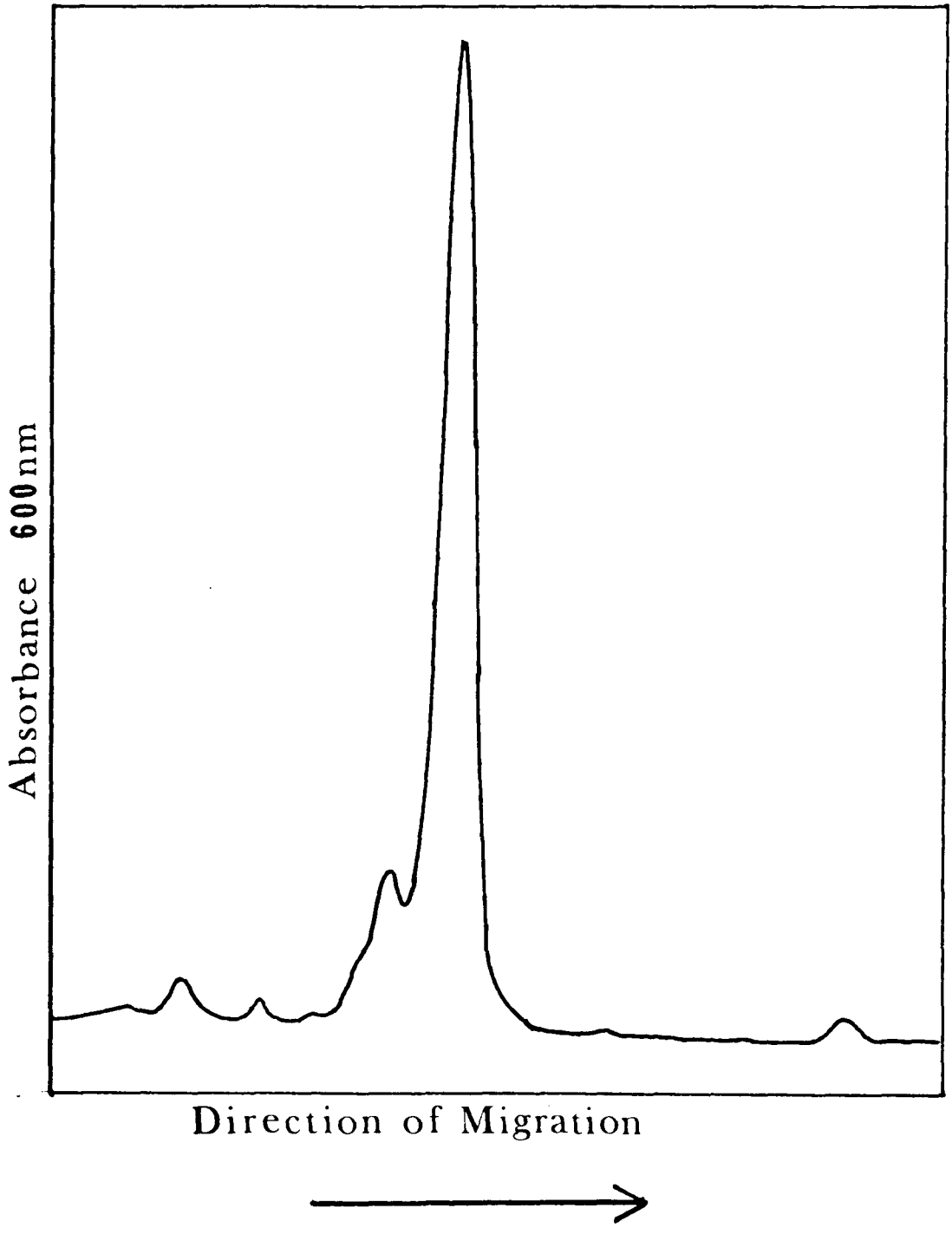


Fig. 6 Purification of thiolase II by CM-cellulose chromatography. Thiolase II (9.2 mg), which had been partially purified by chromatography on CM-cellulose (batch procedure), was applied to a second CM-cellulose column (1.2 x 25 cm) and this was developed with a linear gradient of potassium phosphate between 0.01 M and 0.1 M (for details see "Experimental Procedures I"). Symbols: (●), thiolase activity assayed with acetoacetyl-CoA. Fractions 62 to 76, containing 2.0 ml each, were pooled and stored as described in "Experimental Procedures I."

Figure 5

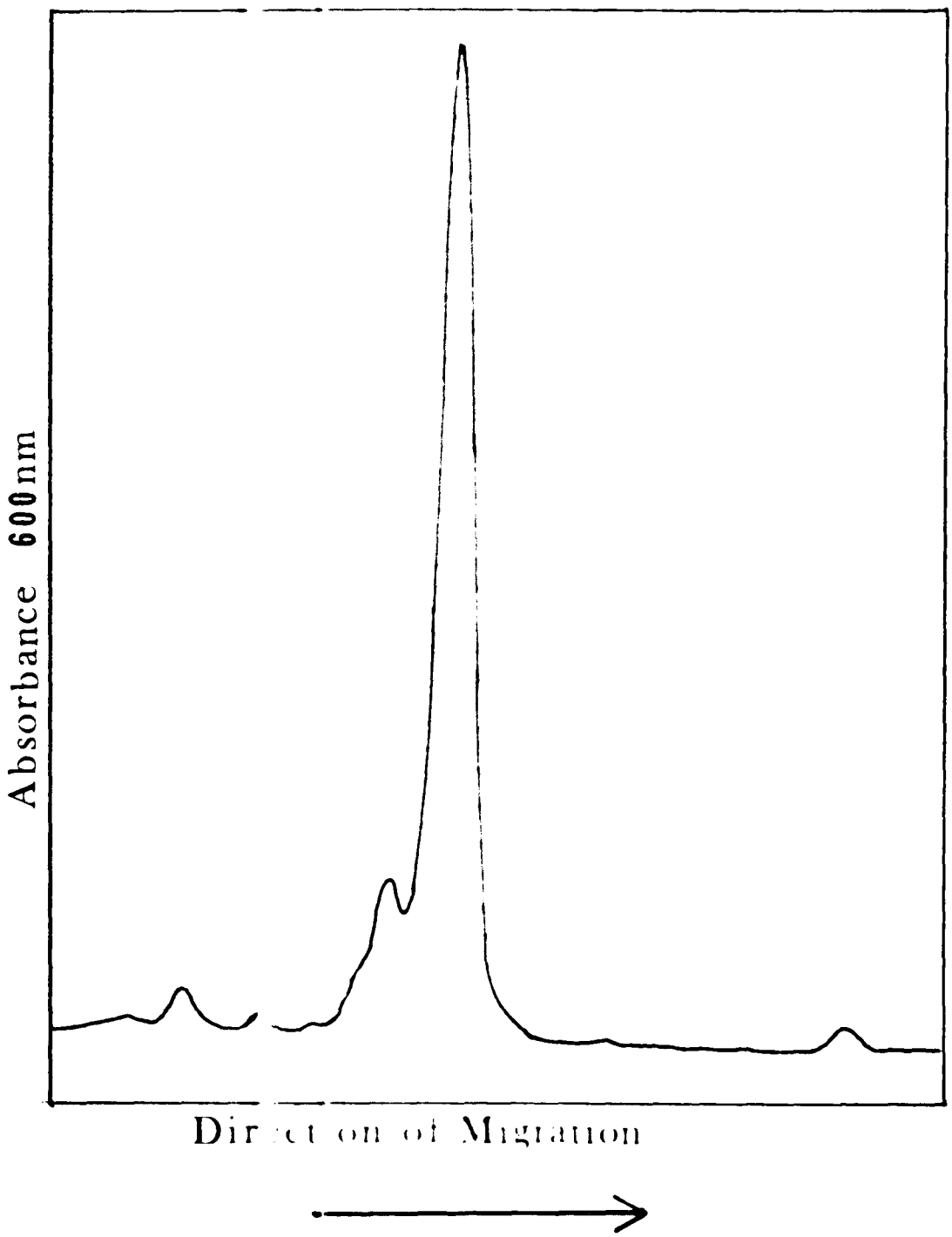


Figure 6

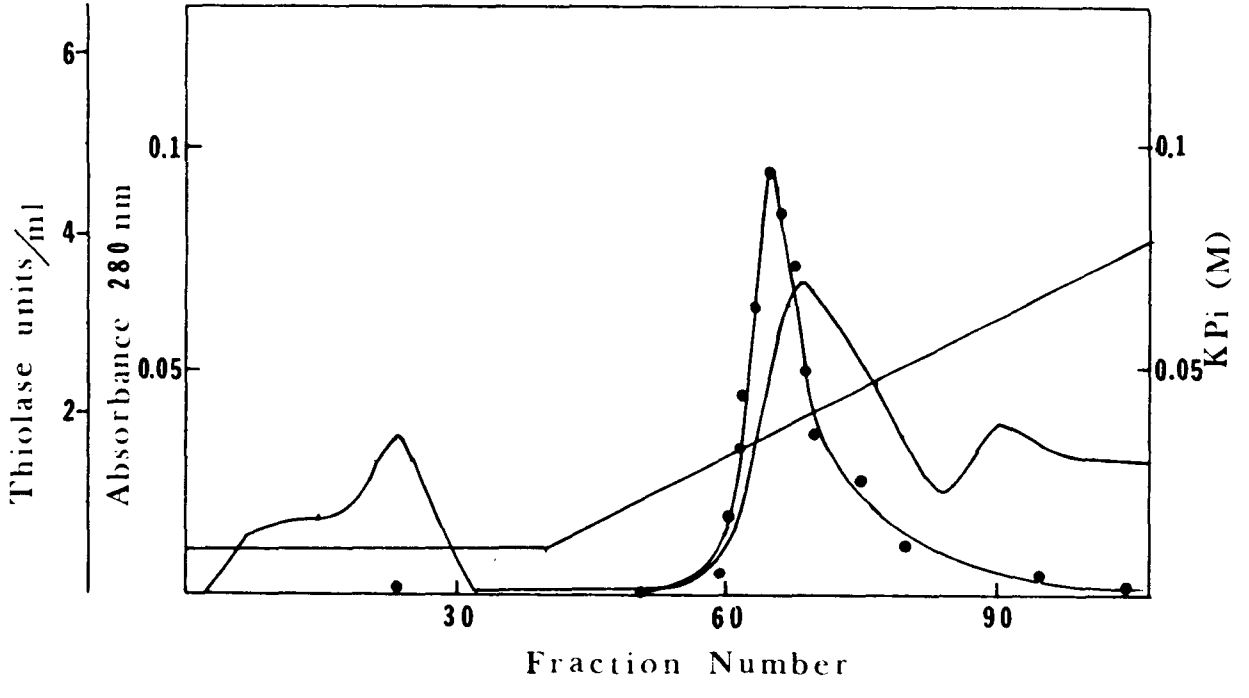

Thiolase units/ml
6
4
2
Absorbance 280 nm
0.1
0.05
0.1
0.05
KPi (M)
30
60
90
Fraction Number

Fig. 7 Disc gel electrophoresis of partially purified thiolase II on a 7.5% polyacrylamide gel. Thiolase II (20 μg from CM-cellulose fractions 62 to 76) was applied to the gel. The gel was stained in Fast Green and destained in 7% acetic acid.

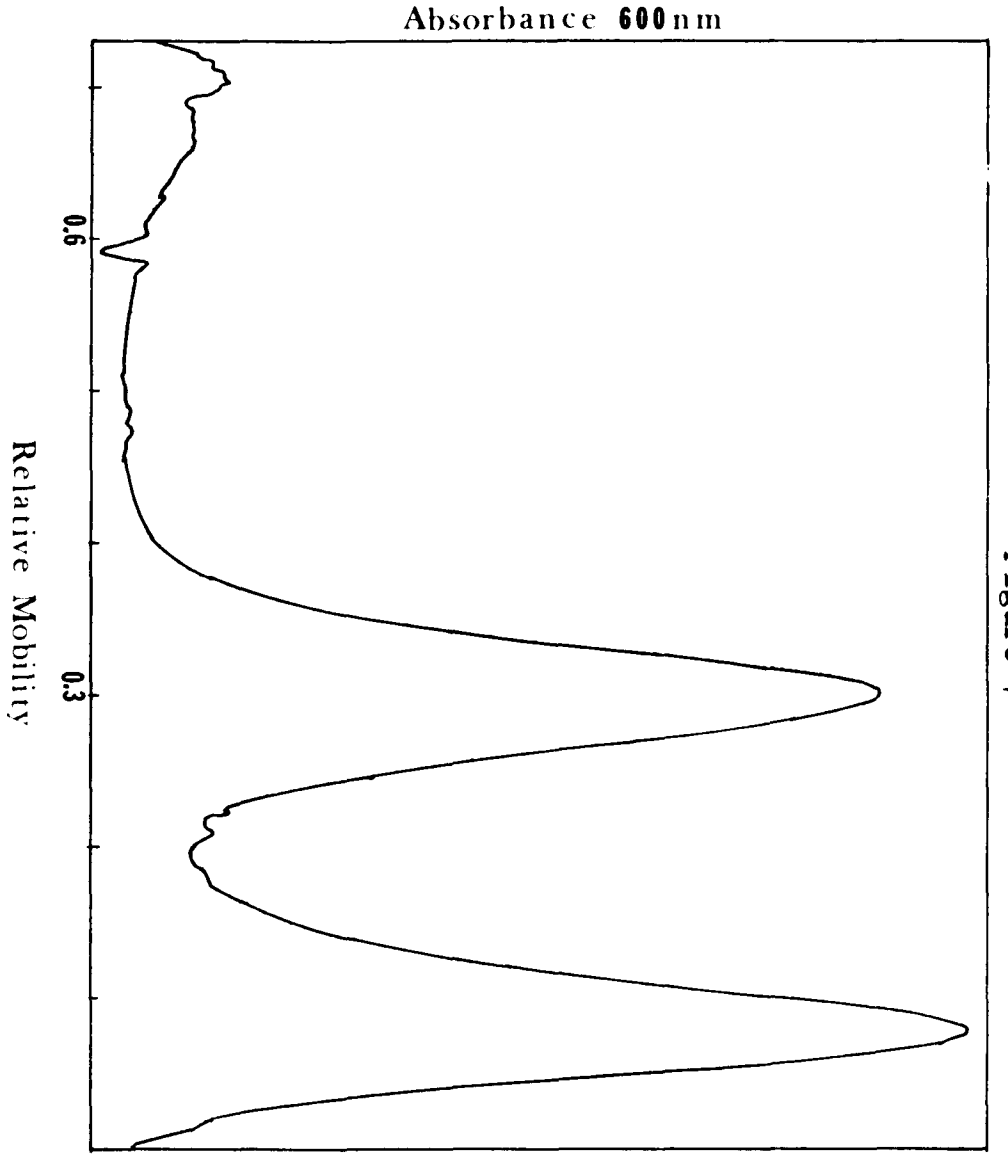


Figure 7

Fig. 8 Gel electrophoresis of partially purified thiolase II in the presence of sodium dodecyl sulfate on a 10% polyacrylamide gel. Thiolase II (10 μg from CM-cellulose fractions 62 to 76) was incubated at 37° C for 2 hr in 0.01 M sodium phosphate containing 10% sodium dodecyl sulfate and 1% mercaptoethanol. The gel was stained with Fast Green and destained in 7% acetic acid.

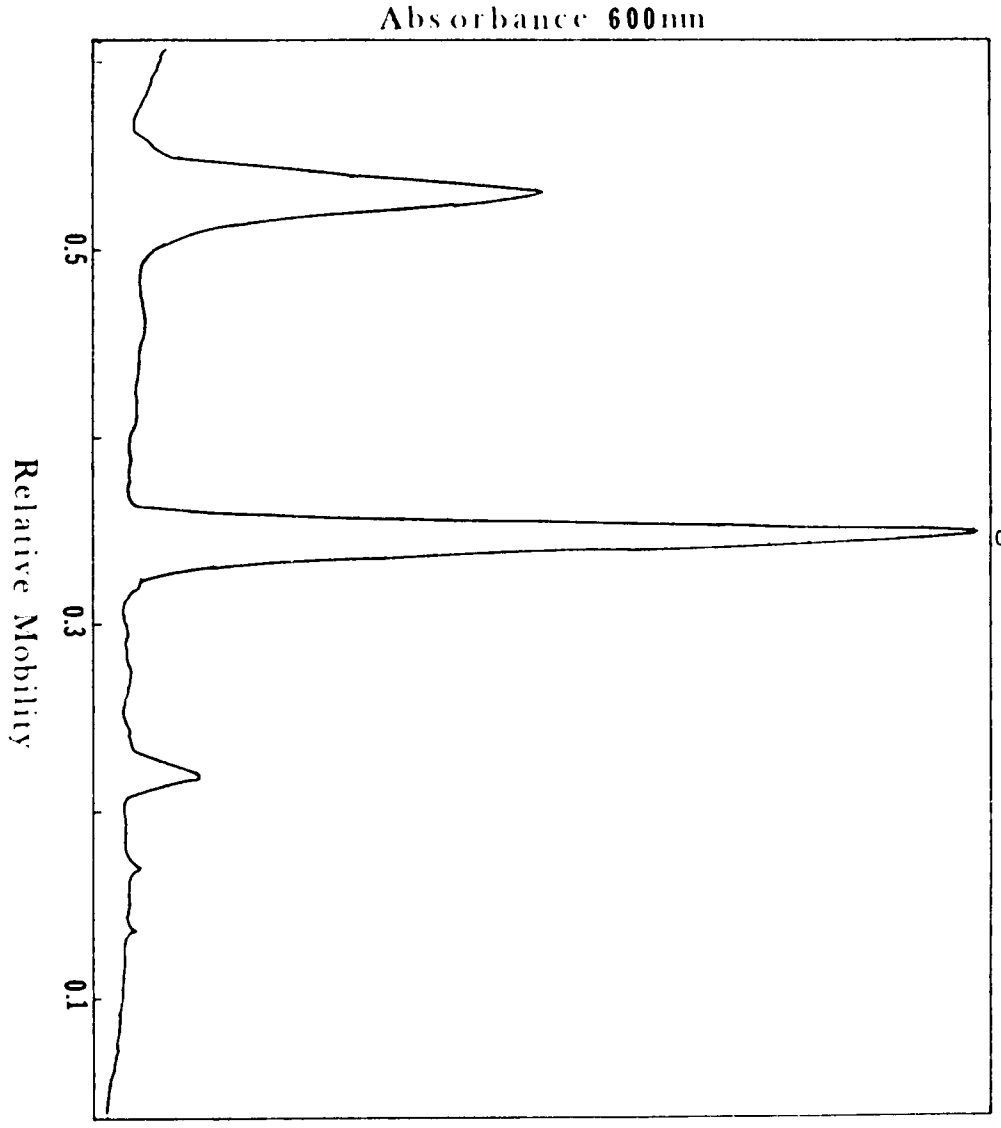


Figure 8

Fig. 9 Stabilities of pig heart thiolases I and II. All assays were performed with acetoacetyl-CoA as described under "Experimental Procedures I." A: Thiolase II was diluted to a final concentration of 1 µg/ml with the following buffers: (○), 50 mM potassium phosphate (pH 6.6) containing 25% glycerol, bovine serum albumin (1 mg/ml) and 10 mM mercaptoethanol; (■), same without glycerol; (□), same without bovine serum albumin; (▲), same without mercaptoethanol; (△), 50 mM potassium phosphate (pH 6.6); (●), 20 mM potassium phosphate (pH 6.6) containing 25% glycerol, bovine serum albumin (1 mg/ml) and 10 mM mercaptoethanol. The diluted enzyme solutions were kept at 5^{o} C.
B: Thiolase I was diluted to a final concentration of 1 µg/ml with the following buffers: (○), 0.75 M Tris-HCl (pH 8.1) containing 25% glycerol, bovine serum albumin (1 mg/ml) and 10 mM mercaptoethanol; (■), same without glycerol; (▲), same without mercaptoethanol; (□), same without bovine serum albumin; (△), 0.75 M Tris-HCl (pH 8.1); (●), 20 mM Tris-HCl (pH 8.1) containing 25% glycerol, bovine serum albumin (1 mg/ml) and 10 mM mercaptoethanol.

Figure 9

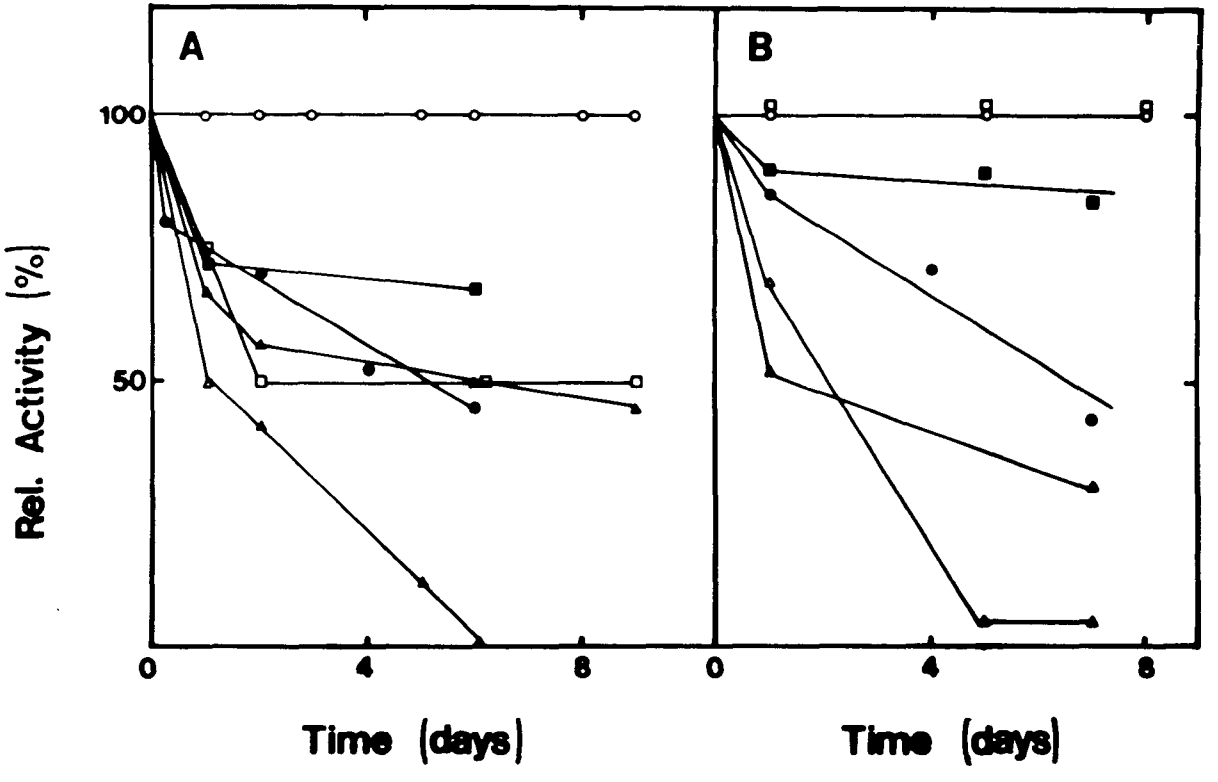


Fig. 10 The pH optima of thiolase I (A) and thiolase II (B). The buffers used were 0.1 M Tris-propane (between pH 6.71 and 7.00) and Tris-HCl (between pH 7.29 and 8.98). In addition to the buffer, the assay mixture contained 27 mM $MgCl_2$, 37 µM acetoacetyl-CoA, 70 µM CoA-SH and 0.052 µg thiolase I or 0.015 µg thiolase II. The molar extinction coefficient (ε) of the Mg^{+2}-acetoacetyl-CoA complex was determined as a function of pH as shown in part C, the values of which were used to determine the thiolase activities presented in parts A and B.

Figure 10

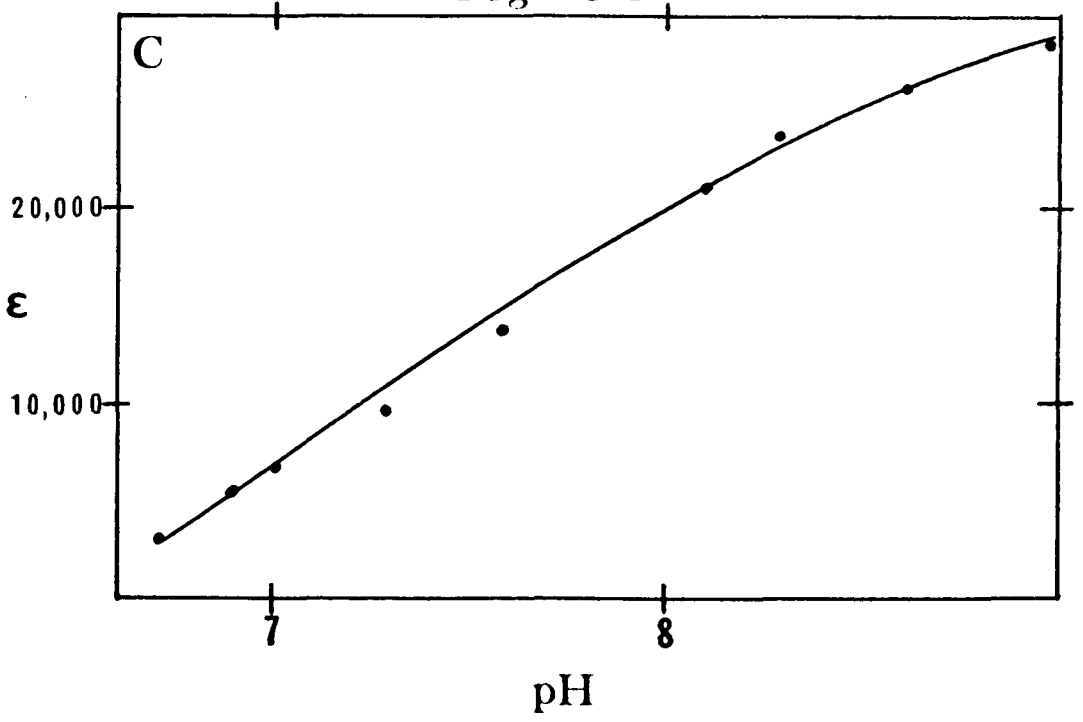


Figure 10

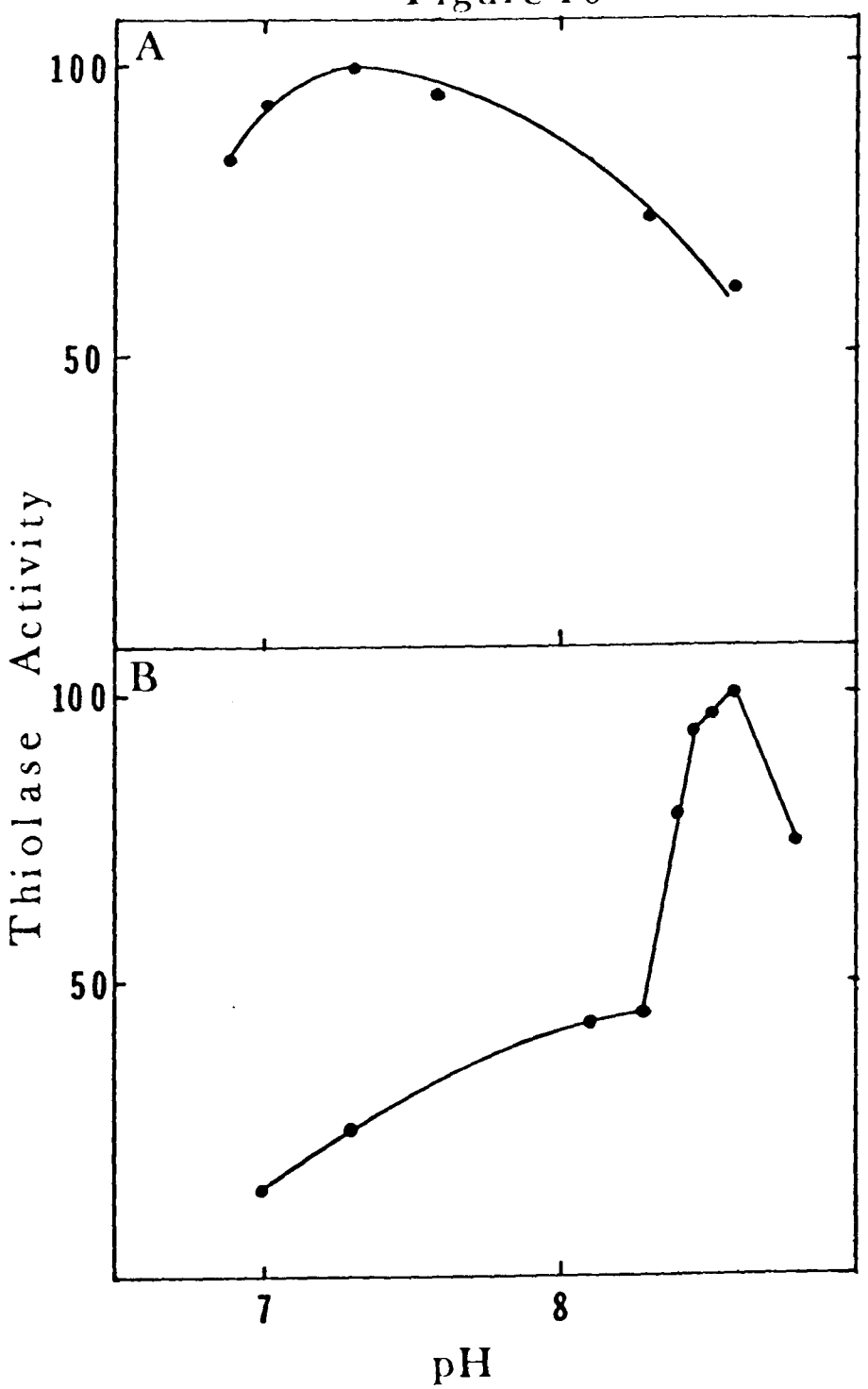


Fig. 11 Molecular weight determination of thiolase I on Sephadex G-200. A Sephadex G-200 column (2.6 x 58 cm) was equilibrated with 0.5 M potassium phosphate (pH 7) containing 0.1 M NaCl and 10 mM 2-mercaptoethanol. The void volume was determined with blue dextran. The following standards were used: phosphorylase a (400,000), catalase (232,000), phosphorylase b (200,000), lactate dehydrogenase (150,000) and bovine serum albumin (68,000). All standards (10 mg/ml) were applied to the column in 2 ml samples and their elution volumes were determined by measuring absorbance at 280 nm. A 1.1 ml sample of thiolase I (2.5 mg) was applied to the column and the elution volume determined by measuring the thiolase activity in 1.25 ml fractions. The position of thiolase I is indicated (+).

Figure 11

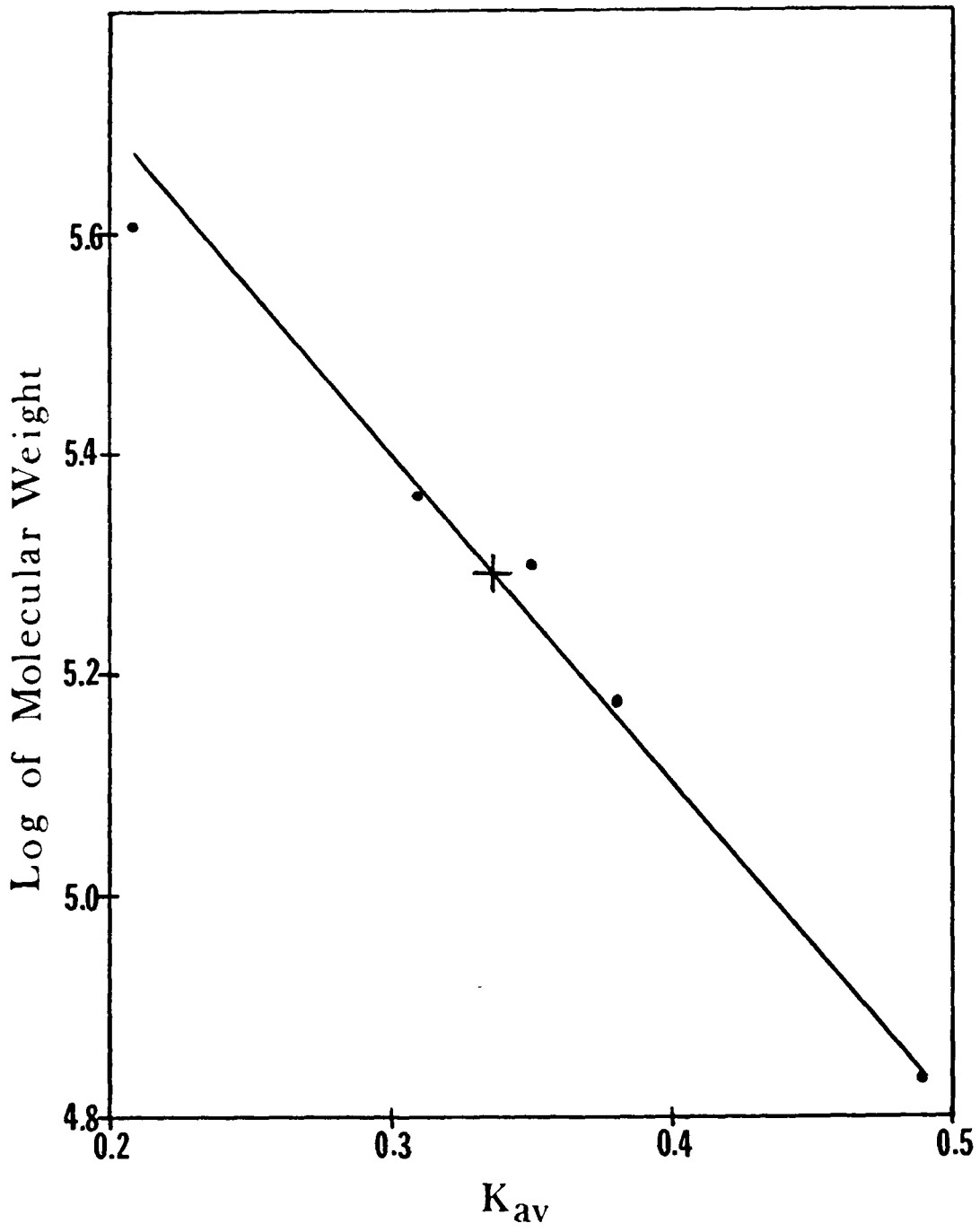


Fig. 12 Subunit molecular weight of thiolase I as determined by gel electrophoresis in the presence of sodium dodecyl sulfate. Five μg each of thiolase I, bovine serum albumin (molecular weight 68,000), ovalbumin (molecular weight 43,000), pepsin (molecular weight 35,000), chymotrypsinogen (molecular weight 25,700), as well as 3 μg each of myoglobin (molecular weight 17,200) and cytochrome c (molecular weight 11,700) were incubated at 37° C for 2 hr in 0.01 M sodium phosphate (pH 7.0) containing 10% sodium dodecyl sulfate and 1% mercaptoethanol before being applied to a 10% polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue and the relative mobilities of each band were plotted against the log of the molecular weights of the standards. The relative mobility of thiolase I is marked on the line (+).

Figure 12

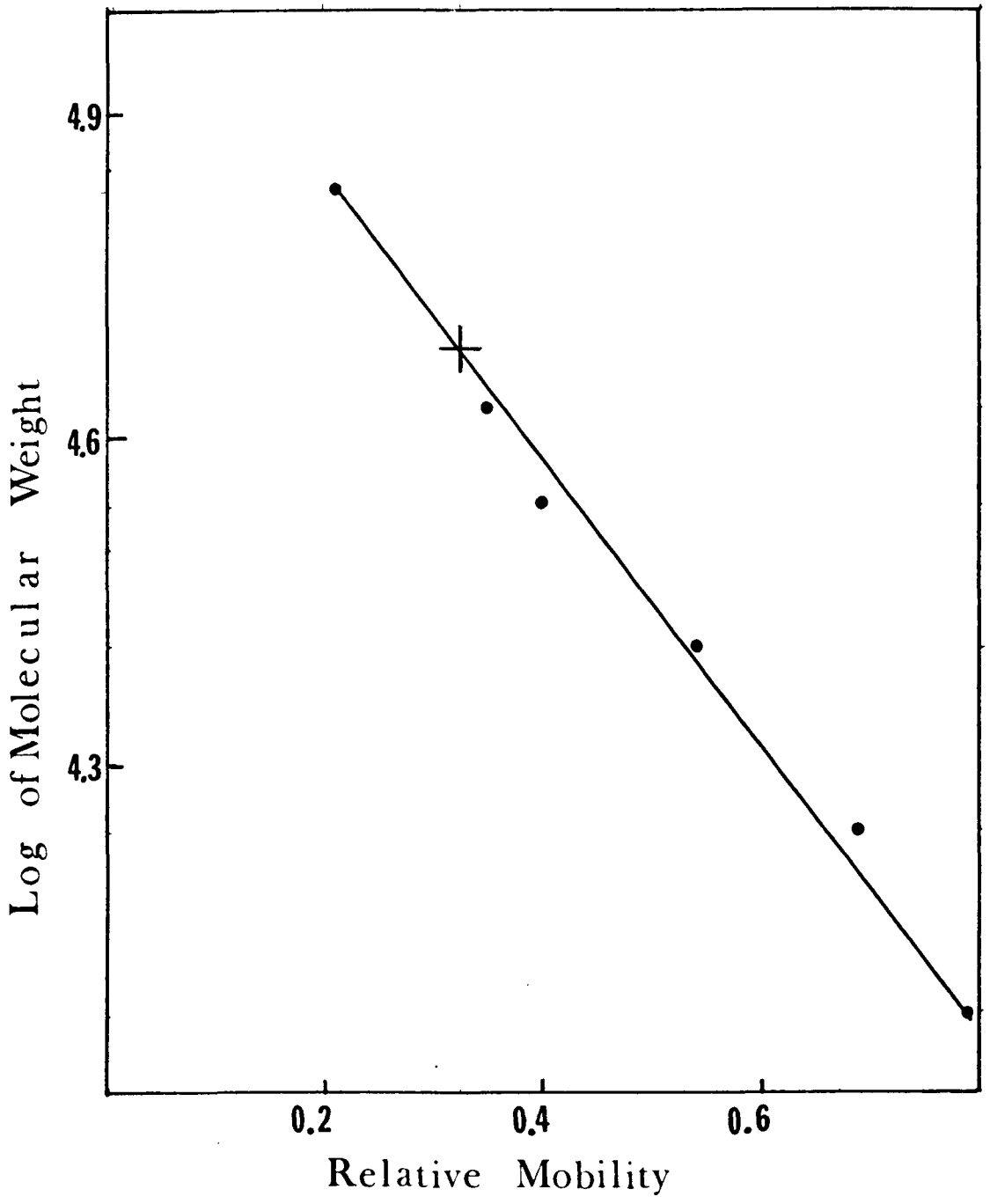


Fig. 13 The chain length specificity of thiolase I. Each assay mixture contained 10 μM 3-ketoacyl-CoA and 70 μM CoA-SH. Assays were performed as outlined in "Experimental Procedures I." The following molar extinction coefficients were used: acetoacetyl-CoA, 22,200; 3-ketohexanoyl-CoA, 16,600; 3-ketooctanoyl-CoA, 14,400; 3-ketodecanoyl-CoA, 13,900; 3-ketododecanoyl-CoA, 11,000; 3-ketotetradecanoyl-CoA, 11,600 $M^{-1}cm^{-1}$.

Figure 13

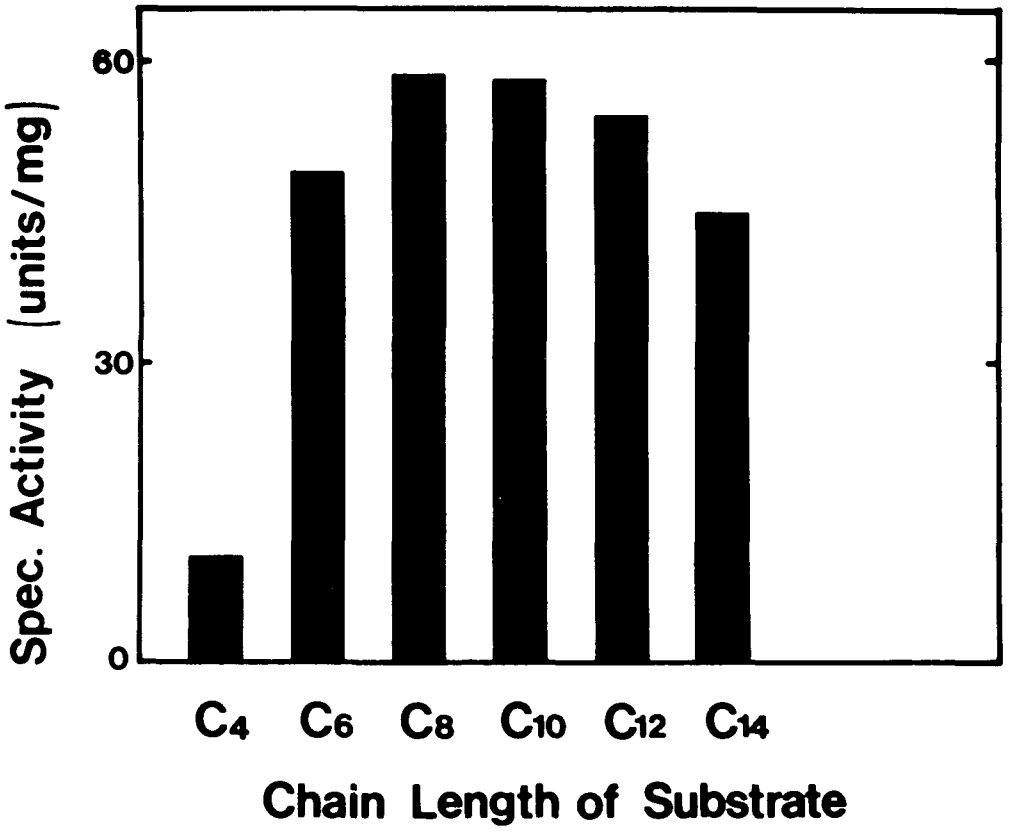

60
30
0
Spec. Activity (units/mg)
C4
C6
C8
C10
C12
C14
Chain Length of Substrate

Fig. 14 The molar extinction coefficients of Mg^{+2}-3-ketoacyl-CoA complexes as a function of the 3-ketoacyl-CoA concentrations. Molar extinction coefficients were determined as described in "Experimental Procedures I." Symbols: (●), acetoacetyl-CoA; (○), 3-ketohexanoyl-CoA; (▲), 3-ketooctanoyl-CoA; (△) 3-ketodecanoyl-CoA.

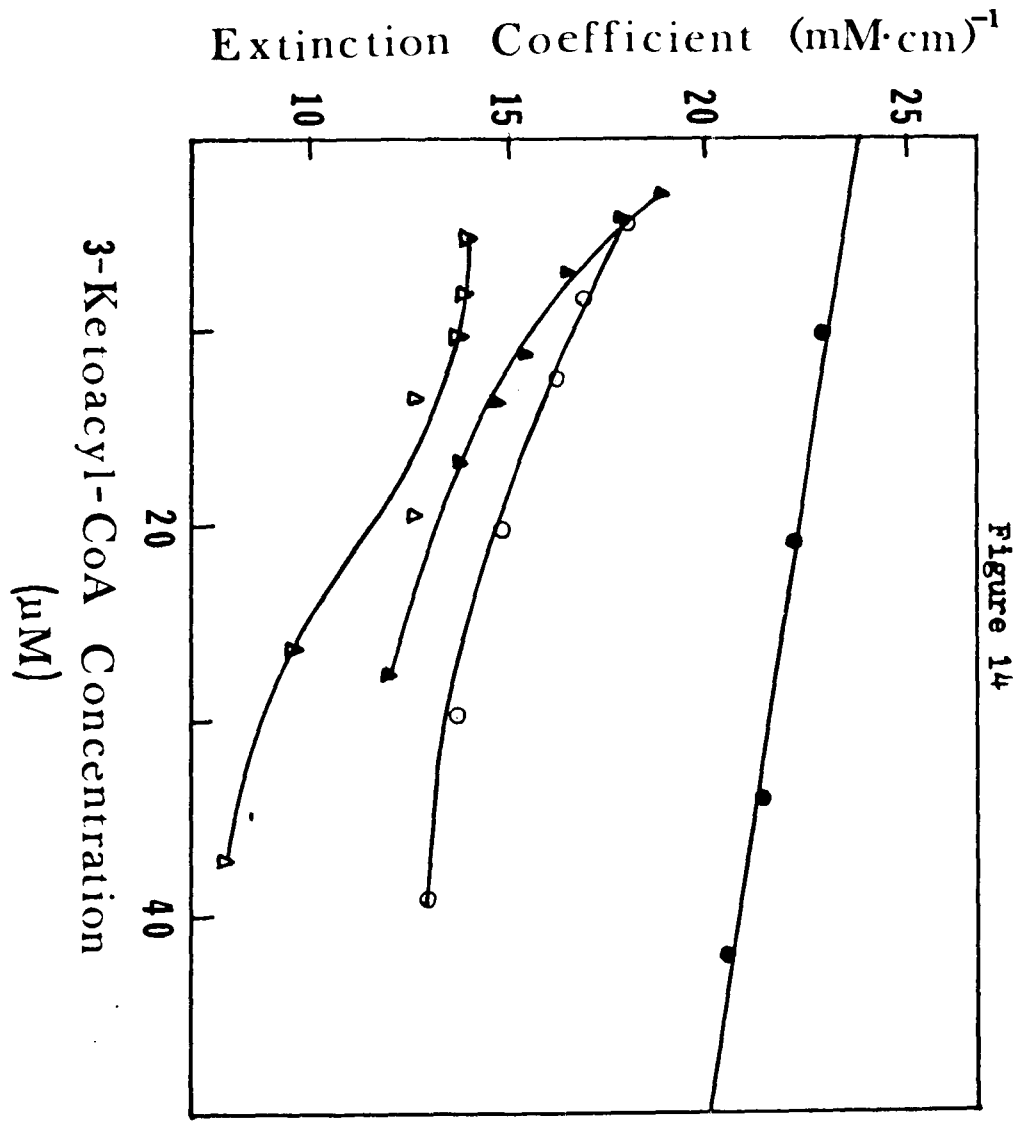

Extinction Coefficient (mM·cm)$^{-1}$
10
15
20
25
20
40
3-Ketoacyl-CoA Concentration
(μM)

Figure 14

Fig. 15 Double reciprocal plot of thiolase I activity versus 3-ketoacyl-CoA concentration. All assays were performed as described in "Experimental Procedures I" at a constant CoA-SH concentration of 70 μM with 0.026 μg of thiolase I per assay. The extinction coefficients from Fig. 14 were used to calculate thiolase activities. Symbols: (●), acetoacetyl-CoA; (○), 3-ketohexanoyl-CoA; (▲), 3-ketooctanoyl-CoA; (△), 3-ketodecanoyl-CoA.

Figure 15

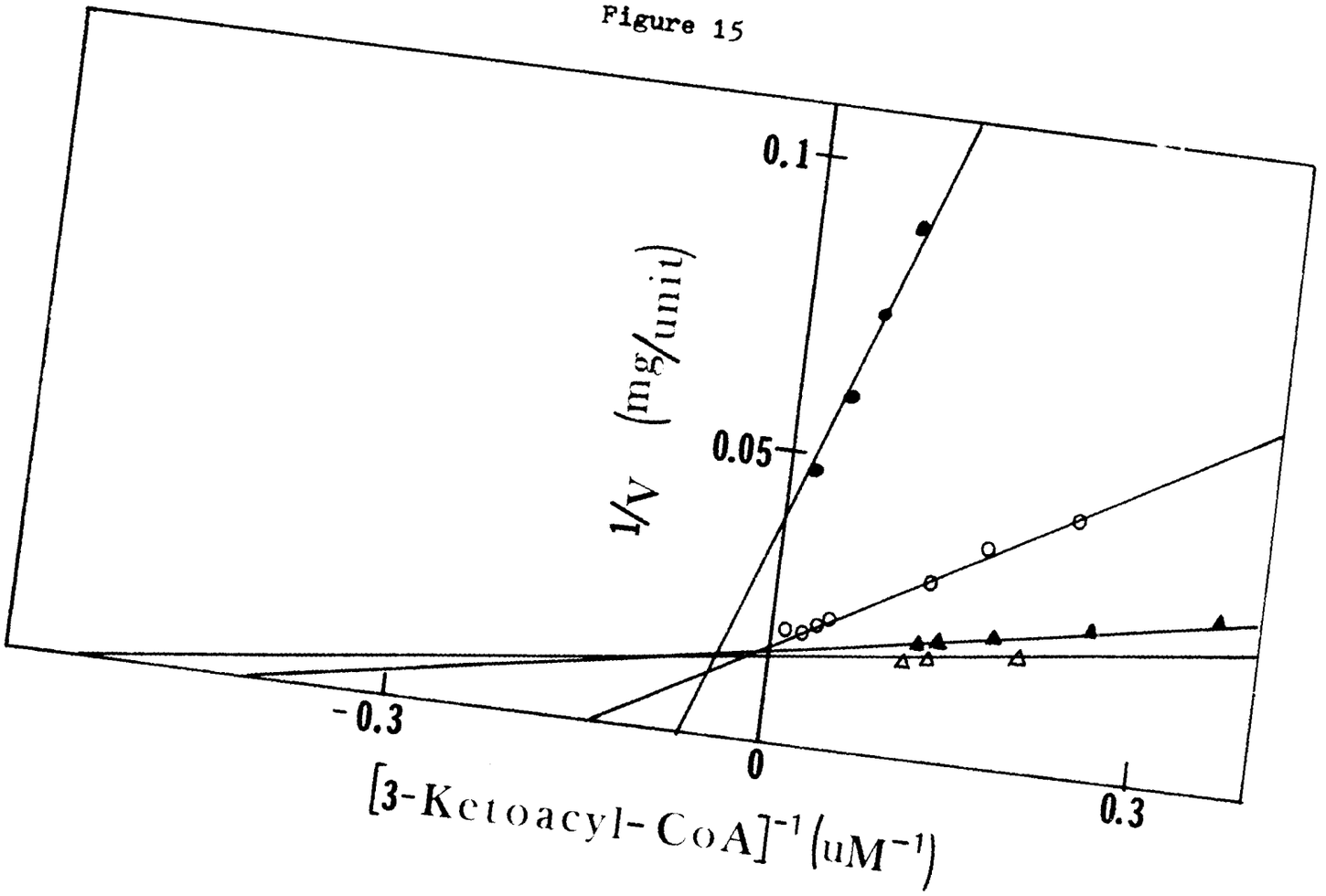


Fig. 16 Double reciprocal plot of thiolase I activity versus 3-ketohexanoyl-CoA concentration. Symbols: (Δ), activities are calculated using a fixed molar extinction coefficient of 13,900 $M^{-1}cm^{-1}$; (▲), activities are calculated as in Fig. 15 using the molar extinction coefficients determined in Fig. 14.

Figure 16

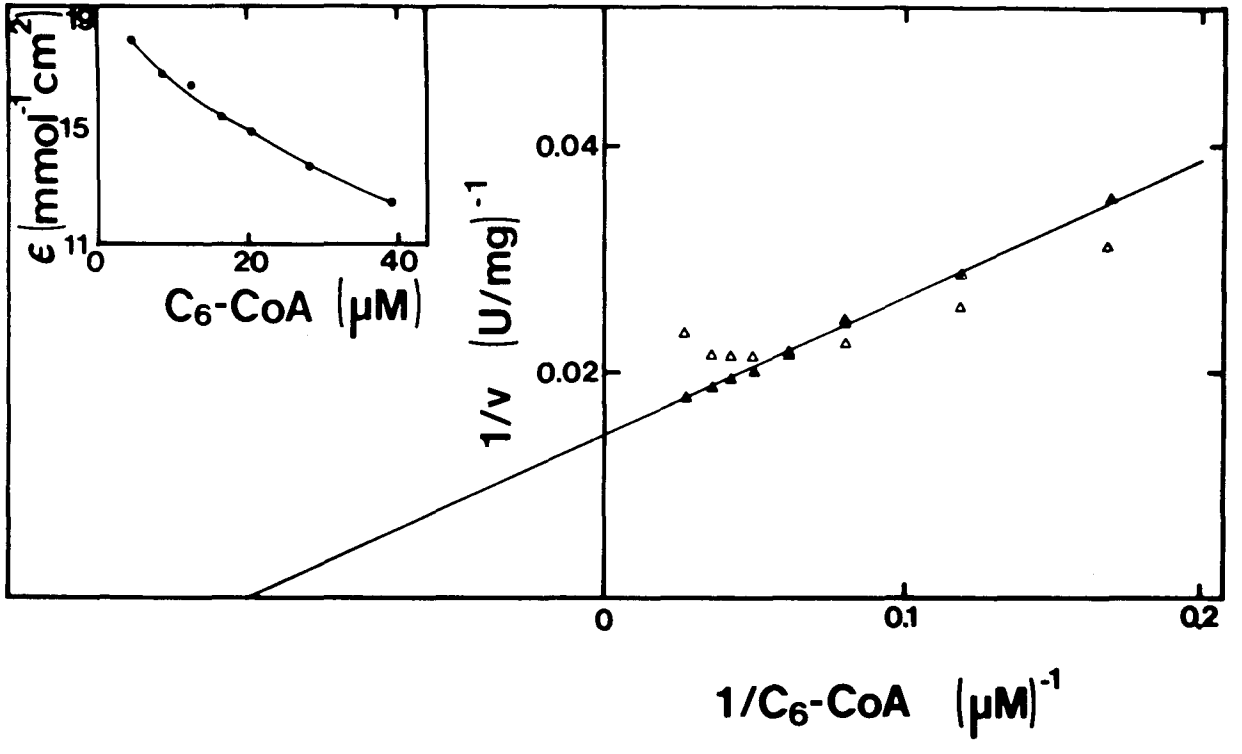

ε (mmol⁻¹ cm²)
15
11
0
20
40
C6-CoA (μM)
0.04
0.02
1/v (U/mg)⁻¹
0
0.1
0.2
1/C6-CoA (μM)⁻¹

Fig. 17 Double reciprocal plot of thiolase I activity versus the concentration of CoA-SH. All assays were performed as described in "Experimental Procedures I." Acetoacetyl-CoA concentration was maintained at 33 μM and 0.026 μg of thiolase I was added to each assay.

Figure 17

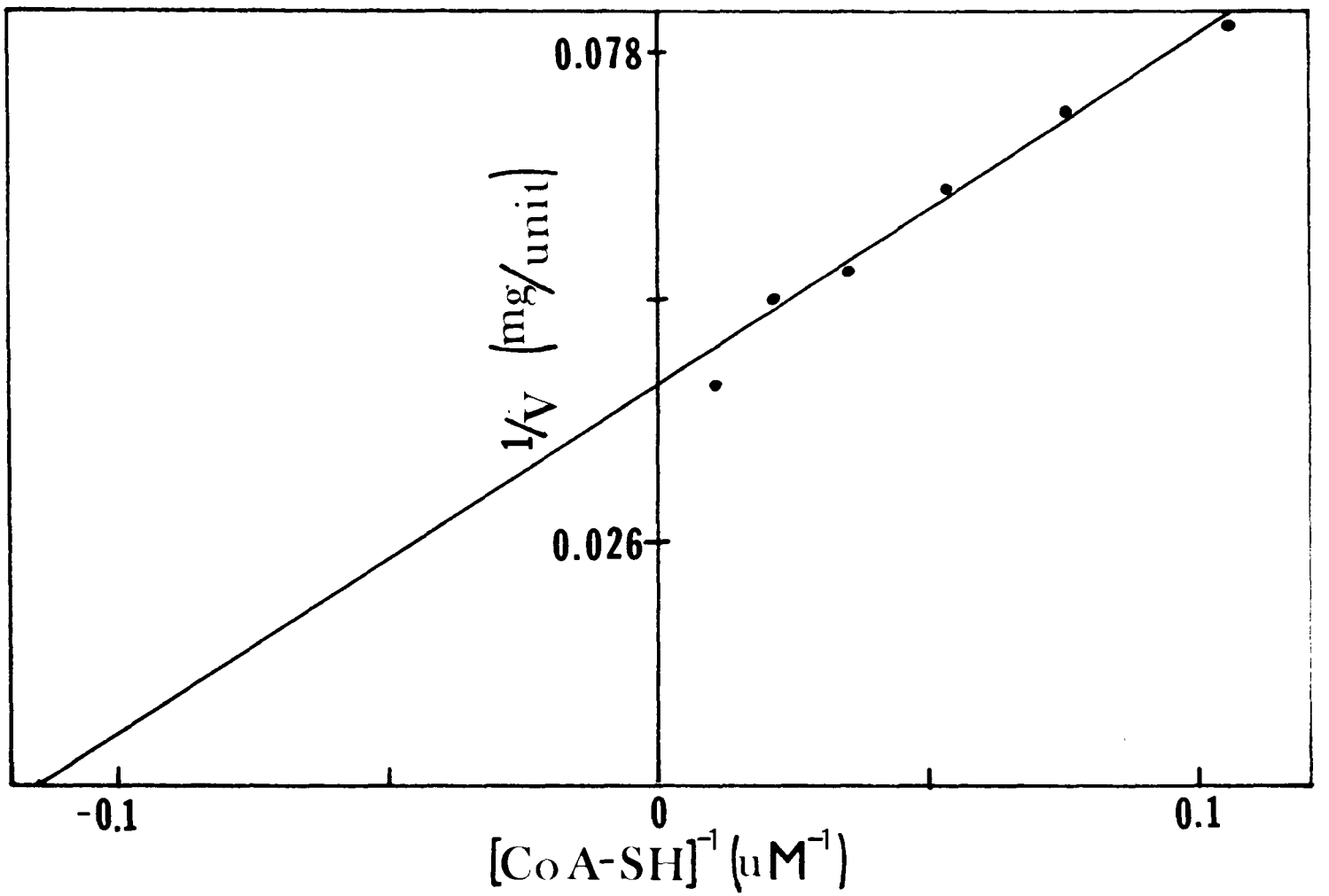


Fig. 18 Double reciprocal plot of thiolase II activity versus the concentration of CoA-SH. All assays were performed as described in "Experimental Procedures I." Acetoacetyl-CoA concentration was maintained at 33 μM. Thiolase II (0.038 μg) was added to each assay.

Figure 18

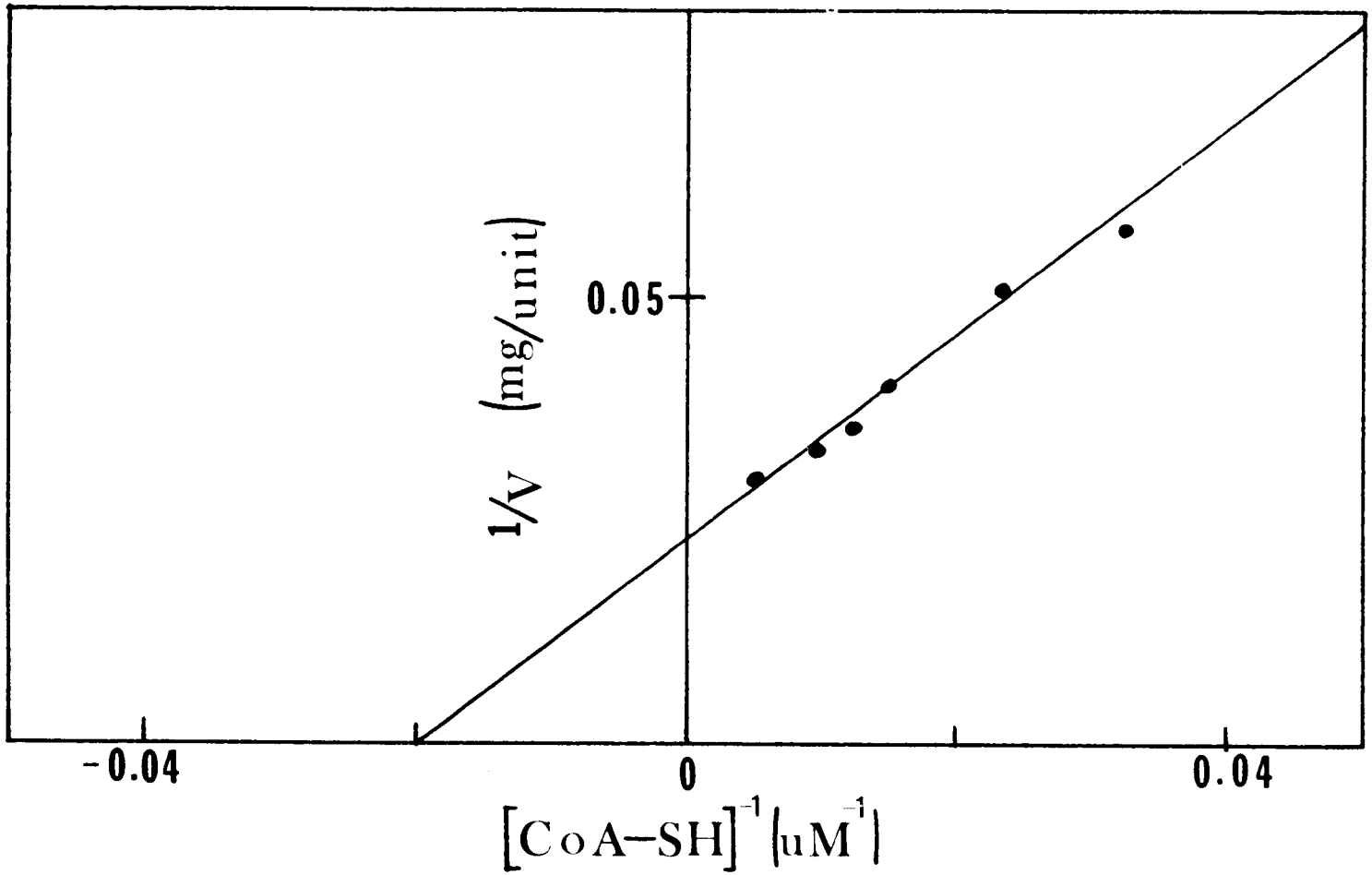


Fig. 19 Double reciprocal plot of thiolase II activity versus acetoacetyl-CoA concentration. Assays were performed as described in "Experimental Procedures I", except that a fixed molar extinction coefficient of 23,500 $M^{-1}cm^{-1}$ was used to calculate activities and a CoA-SH concentration of 85 μM was maintained. Thiolase II (0.038 μg) was used in each assay.

Figure 19

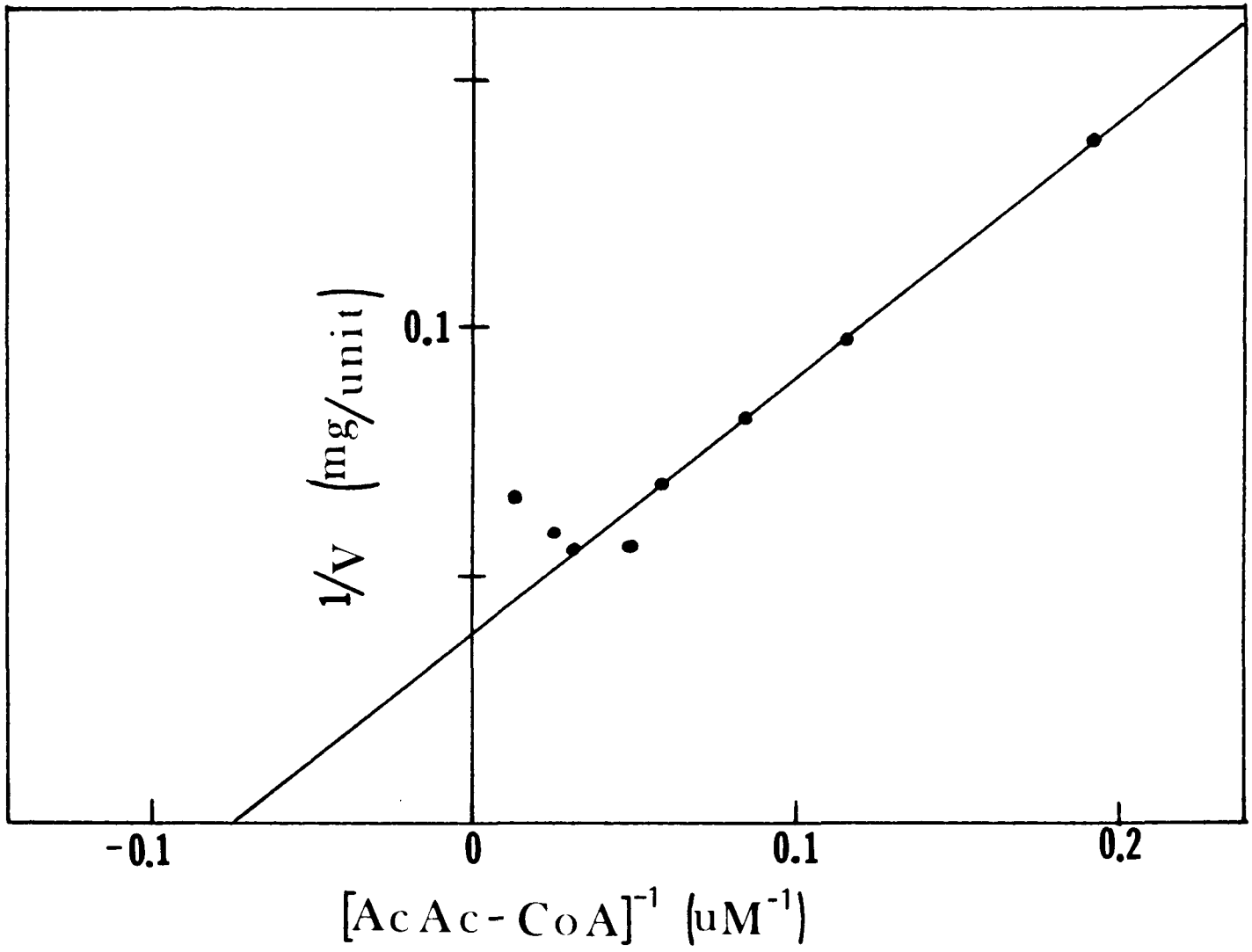


Fig. 20 The inhibition of purified thiolase I by antibodies to thiolase I. Assays were performed as described under "Experimental Procedures I" with acetoacetyl-CoA as substrate. In each experiment, the indicated amount of antibodies was added to 26 μg of purified thiolase I (0.63 units). The addition of 170 μg of antibodies to E. coli thiolase II failed to inhibit pig heart thiolase I (data not shown).

Figure 20

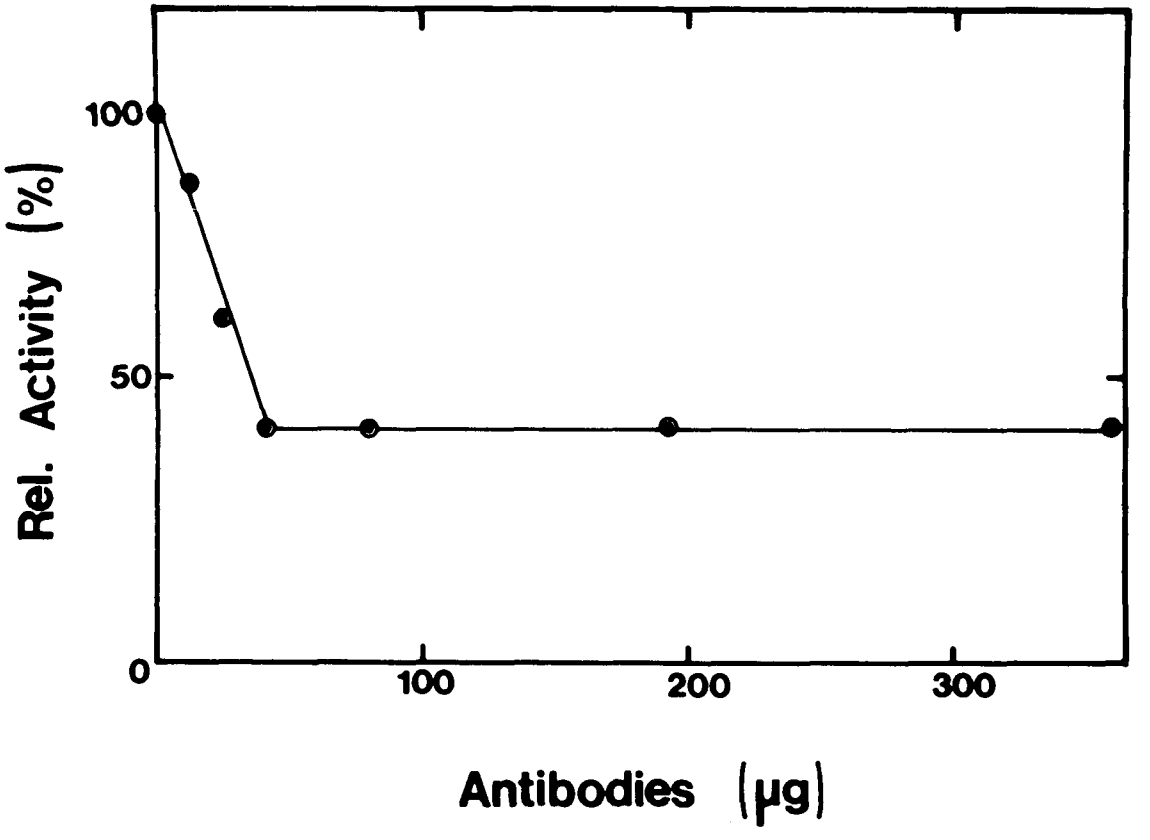


Fig. 21 Immunotitrations of thiolases I and II from pig heart. The experiments were performed as described under "Experimental Procedures I." The antigen-antibody complexes were removed by filtration through Millipore filters (0.45 μ). The percent of acetoacetyl-CoA thiolase activity remaining in the filtrate is plotted as a function of the antibody concentrations. The immunotitration curves shown are (▲), pig heart thiolase I (0.26 μg) with antibodies to pig heart thiolase I; (△), pig heart thiolase I (0.26 μg) with antibodies to E. coli thiolase II; no inhibition was observed in the presence of up to 685 μg of antibodies (data not shown); (○), pig heart thiolase II (75 ng) with antibodies to pig heart thiolase I; no inhibition was observed in the presence of up to 376 μg of antibodies (data not shown); (□), pig heart thiolase II (75 ng) with antibodies to E. coli thiolase II; no inhibition was observed in the presence of up to 1.37 mg of antibodies (data not shown).

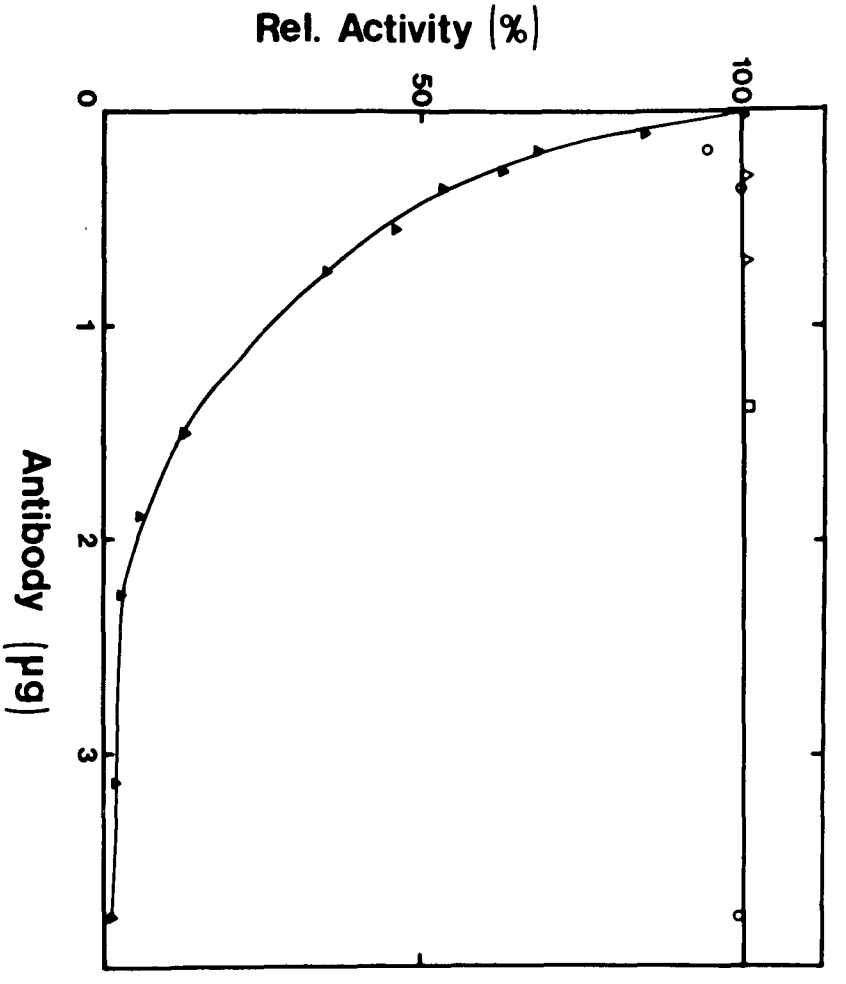


Figure 21

Fig. 22 Immunotitration of antibodies to thiolase I with thiolase I. The antibody-antigen complex was removed by the filtration technique explained under "Experimental Procedures I." A fixed amount of antibody was added to each assay. Two immunotitration curves are shown: (●), contained 3.76 μg of antibodies per assay; (▲), contained 1.88 mg of antibodies to lactic acid dehydrogenase per assay. Increasing amounts of pure thiolase I were added to each assay mixture and the thiolase activity remaining after filtration was measured. The dashed line represents the expected theoretical linear increase in activity if no thiolase were lost upon filtration. All assays were performed as described under "Experimental Procedures I" with 10 μM 3-ketodecanoyl-CoA and 70 μM CoA-SH.

Figure 22

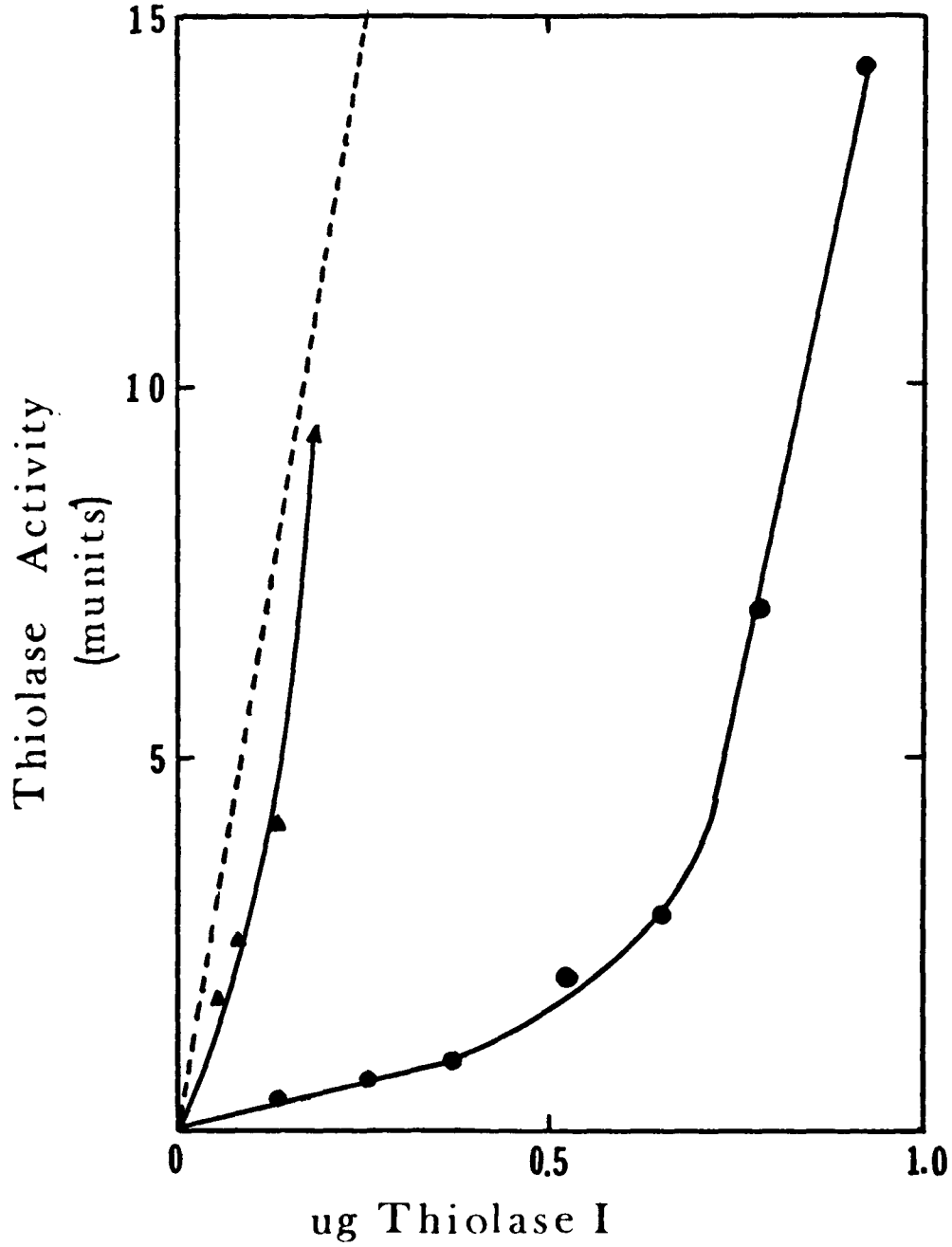


Fig. 23 Inhibition of beef and pig heart thiolase I present in mitochondrial acetone powder extracts by antibodies to pig heart thiolase I. Acetone powders were suspended in 0.05 M glycylglycine buffer (pH 7.8) containing 3 mM mercaptoethanol, sonicated for 30 sec and centrifuged at 127,000 x g for 30 min. The antigen-antibody complexes were removed by centrifugation as described under "Experimental Procedures I." A: Extracts from 76 µg of beef heart (Δ), 55 µg (●) and 88 µg of pig heart (○) mitochondrial acetone powders containing 0.096 units, 0.116 units and 0.186 units of 3-ketodecanoyl-CoA thiolase activities, respectively, were immunotitrated with antibodies to pig heart thiolase I. B: An extract of 148 µg of pig heart mitochondrial acetone powder containing 0.204 units of 3-ketodecanoyl-CoA thiolase activity and 0.283 units of acetoacetyl-CoA thiolase activity was immunotitrated with antibodies to pig heart thiolase I. (○), acetoacetyl-CoA thiolase activity; (●), 3-ketodecanoyl-CoA thiolase activity.

Figure 23

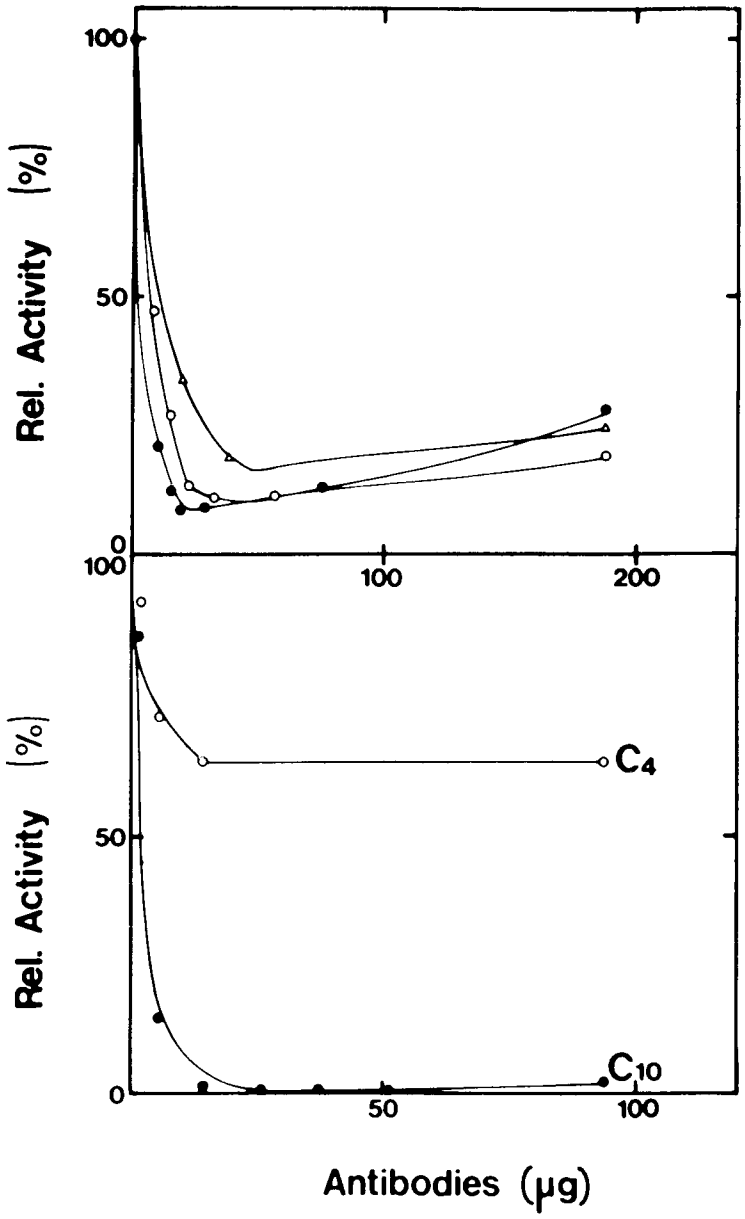

100
50
0
Rel. Activity (%)
100
200
100
50
0
Rel. Activity (%)
C4
C10
50
100
Antibodies (µg)

Figure 24

Mitochondrial Fatty Acid Elongation in Heart

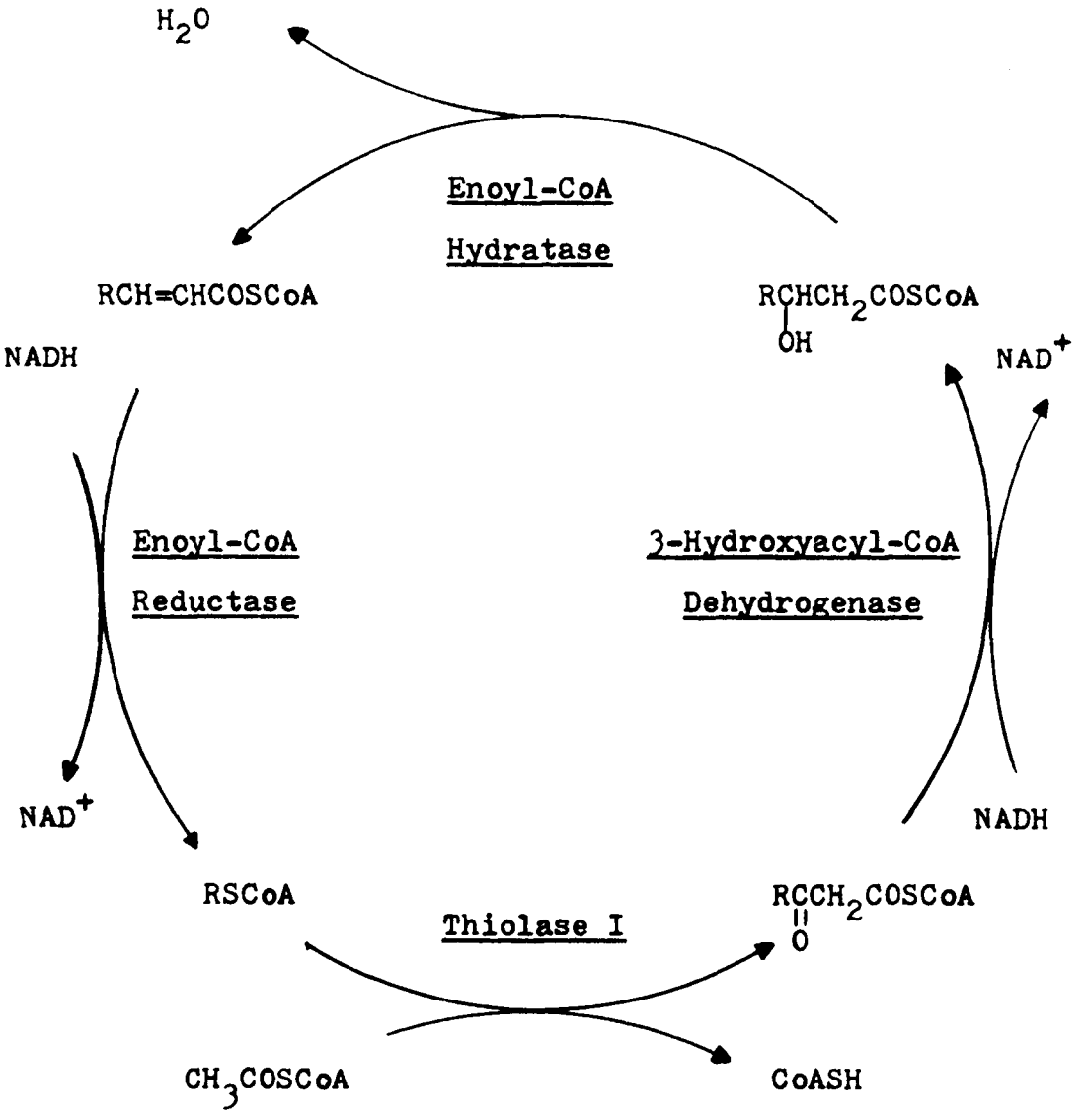


Fig. 25 The incorporation of (1-C^{14}) acetyl-CoA into ether-extractable fatty acids as a function of the amount of pig heart mitochondrial acetone powder extract added to the assay. The acetone powder extract was prepared by centrifugation at 31,000 x g for 15 min before use as described under "Experimental Procedures II." Fatty acid elongation was assayed as described in "Experimental Procedures II", and the activity is expressed as the amount of acetyl-CoA incorporated into fatty acid (in nmoles) after 15 min of reaction time at 38^{o} C.

Figure 25

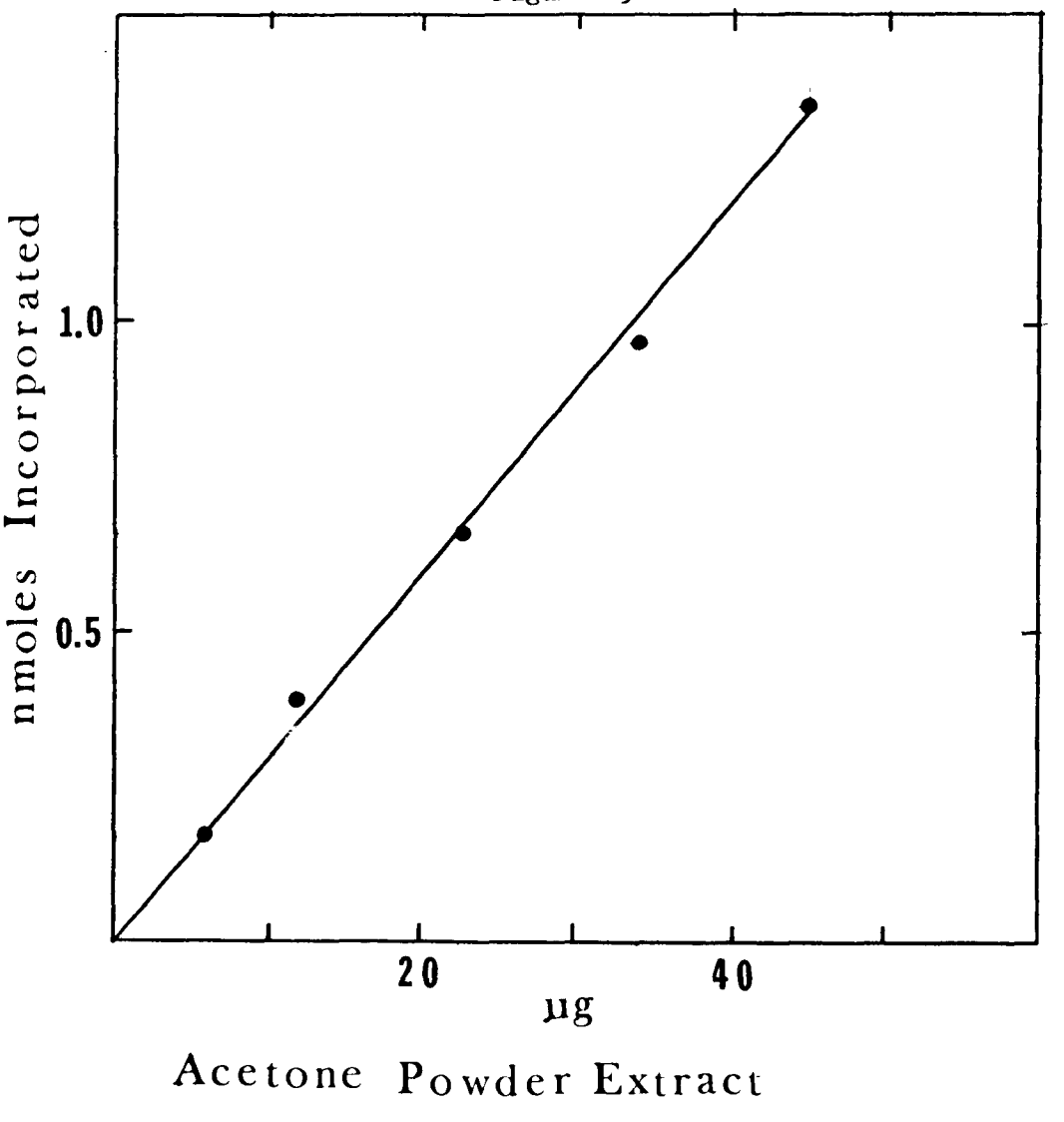
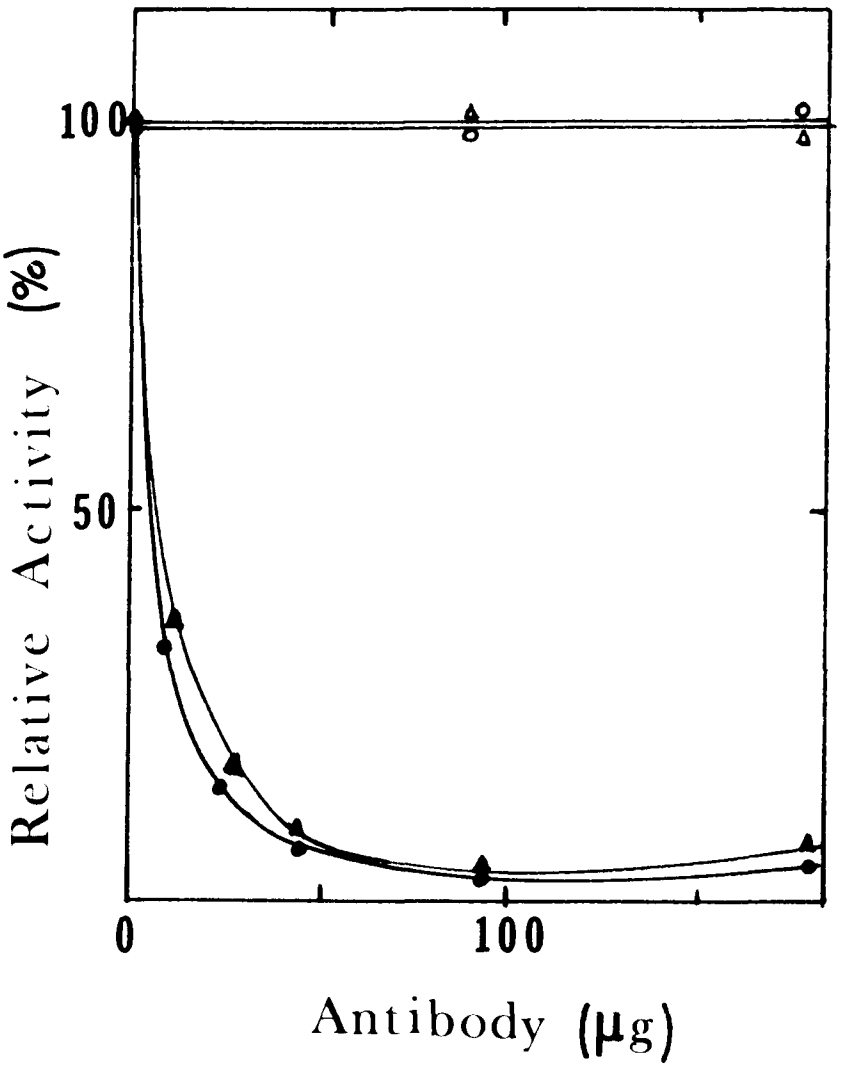


Fig. 26 Effects of antibodies to thiolase on the activities of thiolase and of the mitochondrial fatty acid elongation system. Experiments were performed as described under "Experimental Procedures II." Thiolase I activity (▲) and fatty acid elongation activity (●) after addition of antibodies prepared against thiolase I. Thiolase I activity (△) and fatty acid elongation activity (○) after addition of antibodies prepared against E. coli thiolase II.

Figure 26



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