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THE STRUCTURE OF THE MULTIENZYME COMPLEX OF FATTY ACID OXIDATION FROM ESCHERICHIA COLI

by

SHASHI PAWAR

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.

1981

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.

May 8, 1981
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ABSTRACT

The Structure of the Multienzyme Complex of Fatty Acid Oxidation from Escherichia Coli

by

Shashi Pawar

Adviser: Professor Horst Schulz

The purified multienzyme complex of fatty acid oxidation from E. coli B was found to possess 3-hydroxyacyl-CoA epimerase (EC 5.1.2.3) and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase (EC 5.3.3.3) activities in addition to the previously identified enoyl-CoA hydratase (EC 4.2.1.17), L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) and 3-ketoacyl-CoA thiolase (EC 2.3.1.16). Co-chromatography of these enzymes on phosphocellulose, their co-migration on polyacrylamide gel electrophoresis and their parallel inactivation by Tris-hydrochloride represent sufficient proof for the association of the five enzymes in a multienzyme complex. The structure of the purified multienzyme complex of fatty acid oxidation from E. coli B has been studied. The molecular weight of the native complex was estimated by two methods to be close

to 260,000. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate led to the identification of two types of subunits whose molecular weights were estimated to be 78,000 and 42,000 respectively. The two subunits are present in the complex in equimolar amounts. When the complex was rapidly isolated by immunoprecipitation in the presence of several protease inhibitors, its subunit structure was found to be identical to that of a preparation purified by the standard procedure. It is therefore concluded that the complex is composed of two copies each of the 78,000 dalton and 42,000 dalton subunit. The complex contains additionally phospholipids (63 nmoles of lipid phosphate per mg of protein) which were identified as phosphatidylethanolamine, phosphatidylglycerol and cardiolipin. Immunotitration of a soluble E. coli extract with antibodies raised against the pure complex provided evidence for the association of all 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase and crotonase activities with the complex. However, a long chain enoyl-CoA hydratase exists as a separate protein. By specifically labeling 3-ketoacyl-CoA thiolase and cis-Δ^3-trans-Δ^2-enoyl-CoA hydratase with N-[2-^{14}C]ethylmaleimide, it was shown that the former component enzyme resides on the 42,000 dalton subunit, whereas the latter one is associated with the 78,000 polypeptide. Various attempts to prove the location of 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase on the 78,000 dalton subunit have failed so far.

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to Professor Horst Schulz for his advice, encouragement and guidance throughout the course of this work.

I am thankful to Mr. Ajay Pramanik who provided valuable help specially in the beginning of this project. I am also thankful to Professor Thomas Haines and Professor Charlotte Russell for many helpful discussions.

Finally, I am thankful to Mrs. Edna Hughes for carefully typing the thesis.

Shashi Pawar

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INTRODUCTION

The pathway by which fatty acids are degraded in biological systems began to be unraveled as early as 1904 by the work of Knoop. Subsequent studies done by the groups of Lynen, Green, Ochoa and other investigators have led to the formulation of a universal pathway by which fatty acids are successively oxidized to acetate units. The various steps involved in this sequence of reactions are: (1) The formation of fatty acyl-coenzyme A (CoA) thioester, catalyzed by acyl-CoA synthetase (EC 6.2.1.3); (2) the α-β-dehydrogenation of acyl-CoA by the action of acyl-CoA dehydrogenase (EC 1.3.99.2 and 99.3); (3) the hydration of α-β-unsaturated fatty acyl-CoA by enoyl-CoA hydratase (EC 4.2.1.17); (4) the oxidation of the β-hydroxyacyl-CoA, catalyzed by L-β-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) and (5) the thiolytic cleavage of β-ketoacyl-CoA by β-ketoacyl-CoA thiolase (EC 2.3.1.16) (see Fig. 1A). Unsaturated fatty acids are oxidized by the same general pathway. However, they require two additional enzymes to convert (1) cis-Δ^3-double bonds to trans-Δ^2 ones and (2) D-3-hydroxy intermediates to L-3-hydroxyacyl-CoA's. The first reaction is catalyzed by cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase (EC 5.3.3.3) and the second one involves 3-hydroxyacyl-CoA epimerase (EC 5.2.1.17) (see Fig. 1B).

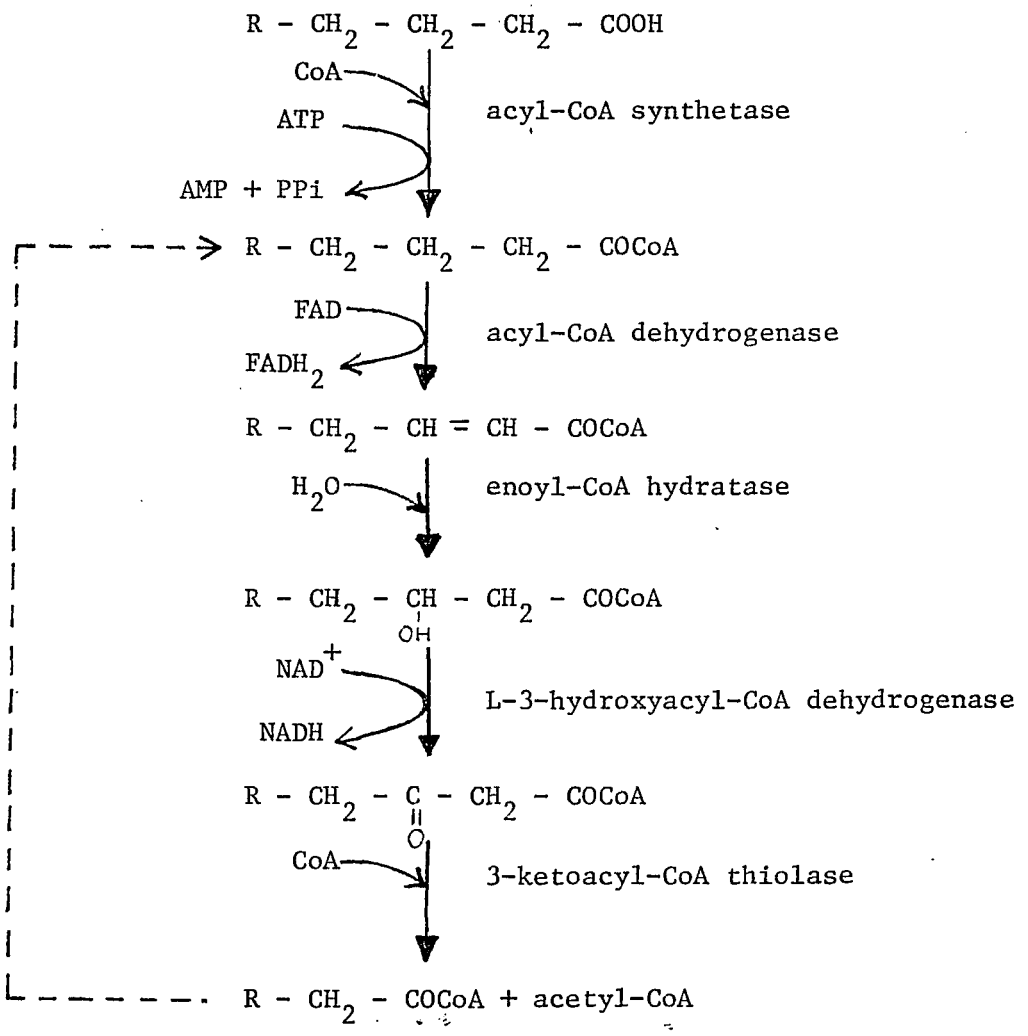

Figure 1A: Pathway of fatty acid oxidation.

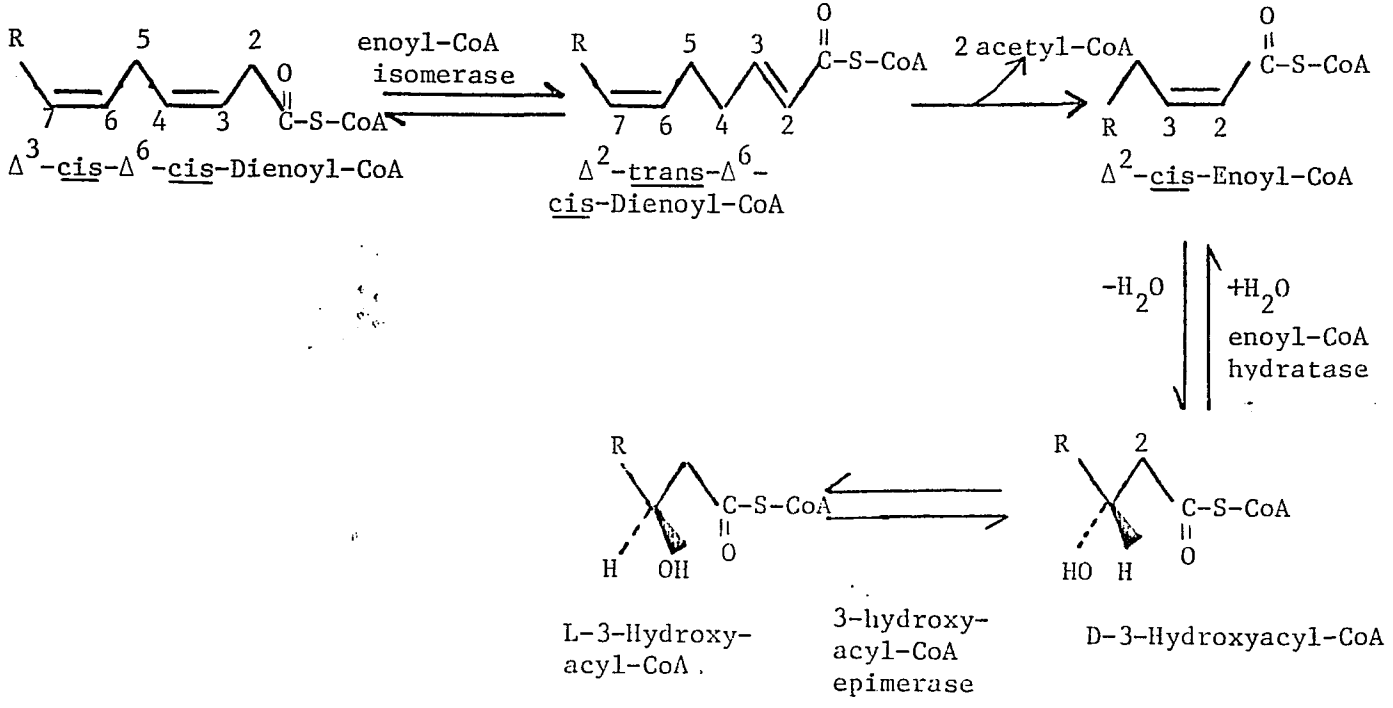

Figure 1B: Pathway of oxidation of unsaturated fatty acids showing the action of auxiliary enzymes.

The various enzymes catalyzing the successive steps in fatty acid oxidation have since been isolated in highly purified form and studied in significant details (1). However, the regulation of this important metabolic pathway and the in vivo organization of the enzymes of β-oxidation remain poorly understood.

Since the complete degradation of long chain fatty acid requires a large number of reactions one would expect to find a number of intermediates. However Garland, Shephard and Yates, who studied the β-oxidation of palmitoyl-CoA in intact rat mitochondria were unable to demonstrate the accumulation of appreciable amounts of fatty acid oxidation intermediates (2). This early observation led to the suggestion that the β-oxidation enzyme function in a coordinate manner and perhaps exist in vivo in a highly ordered arrangement. However, no such multienzyme complex has yet been isolated from eukaryotic cells, although it is believed that the enzymes of β-oxidation in mitochondria exist as a well organized complex which is disrupted when mitochondria are broken.

Over the years studies of bacterial metabolism and genetics have contributed considerably to the understanding of many metabolic control mechanisms. In the hope of gaining further insight into the organization of the enzymes of β-oxidation and the regulation of this pathway, we have studied the oxidation of fatty acid in E. coli.

During the earlier studies of β-oxidation in microorganisms it was observed that E. coli and other bacteria can grow on a

variety of fatty acids as the sole carbon source (3). Other investigators on the other hand could not demonstrate the presence of the enzymes of β-oxidation in the E. coli extracts (4,5). The presence of the enzymes of β-oxidation in extracts of E. coli was not established until 1967 when Overath and coworkers showed that fatty acid oxidation enzymes in E. coli are highly induced when the bacteria is grown on long chain fatty acids as the sole carbon source (6). The synthesis of the enzymes of fatty acid degradation in E. coli is strongly repressed by the presence of glucose in the growth medium (6). Addition of inducers, e.g. oleate could not relieve this repression but addition of cAMP did cause derepression (7) These observations may explain why in the earlier investigations the presence of the β-oxidation enzymes in E. coli extracts could not be demonstrated. It is likely that the synthesis of the enzymes of fatty acid degradation (fad) is regulated by a mechanism similar to that of the lac operon, first proposed by Jacob and Monod (8).

Overath et al. (6, 9), Weeks et al. (10) and Klein et al (11) have demonstrated that acyl-CoA synthetase, at least two acyl-CoA dehydrogenases, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA epimerase and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase are induced when E. coli cells are grown on long chain fatty acids as the sole carbon source. Extensive genetic work involving the isolation and mapping of mutants

of fatty acid oxidation has led to the conclusion that the structural genes for the β-oxidation enzymes are located on at least 5 separate regions of the E. coli chromosome (see Fig. 2).

Mutants that harbor a defect in one of these structural genes, the fad D gene lack acyl-CoA synthetase and cannot be induced to synthesize the fad enzymes (9). These results led Overath and co-workers (9) to suggest that the fatty acyl-CoA derivatives rather than fatty acids serve as inducers.

Although all enzymes of β-oxidation in E. coli were coordinately induced when the cells were grown on long chain fatty acid as a sole carbon source, ideal coordinate induction was observed only for thiolase, crotonase and 3-hydroxyacyl-CoA dehydrogenase, as compared to the slightly lower rates of induction for acyl-CoA synthetase and acyl-CoA dehydrogenase (9,10). These results led Overath and coworkers to hypothesize that genes for thiolase, crotonase and 3-hydroxyacyl-CoA dehydrogenase form a unit expression, 'an operon', with a common operator, whereas the structural genes for acyl-CoA synthetase and acyl-CoA dehydrogenase are not linked to this operon and presumably have their own operators. However, the expression of all the fad genes is believed to be regulated by the same regulator gene (9).

Further genetic work has led to the isolation and characterization of several other mutants. The fad 5 mutant lacks thiolase, crotonase, 3-hydroxyacyl-CoA dehydrogenase, cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase, but has inducible wild

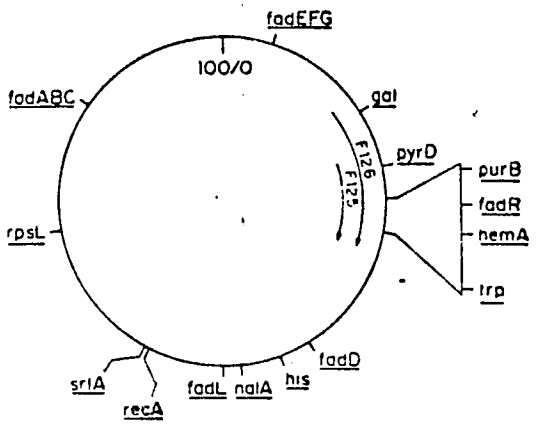

Figure 2: Location of fad mutations on the genetic map of E. coli.

Strain	Characteristics
Fad A	Deficient in 3-ketoacyl-CoA thiolase
Fad B	Deficient in L-3-hydroxyacyl-CoA dehydrogenase
Fad 5*	Deficient in thiolase, crotonase, L-3-hydroxyacyl-CoA dehydrogenase, cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase, and 3-hydroxyacyl-CoA epimerase
Fad E	Deficient in electron transfer flavoprotein
Fad F	Deficient in butyryl-CoA dehydrogenase
Fad G	Deficient in acyl-CoA dehydrogenase
Fad L	Deficient in fatty acid permease
Fad R	Deficient in a regulatory protein: a repressor

*Location of the fad 5 mutation not shown in Fig. 2. However, it maps close to the fad A and the fad B genes.

type levels of acyl-CoA synthetase and acyl-CoA dehydrogenase (6). The fad A and the fad B mutants were found to be devoid of 3-ketoacyl-CoA thiolase and 3-hydroxyacyl-CoA dehydrogenase respectively (6,9). Mapping data showed that the fad 5, the fad A, and the fad B genes were closely linked together on the E. coli chromosome (9) (also see Fig. 2). However, the fad D gene which codes for acyl-CoA synthetase was located on a separate region of the E. coli chromosome unlinked to the fad A, the fad B and the fad 5 gene (9) (also see Fig. 2). Thus the ideal coordinate induction observed only for thiolase, crotonase and 3-hydroxyacyl-CoA dehydrogenase, and the close location of the fad A, the fad B and the fad 5 on the E. coli chromosome support the hypothesis formulated by Overath et al, that genes for these enzymes apparently form an operon.

The location of other fad genes have since been identified on the E. coli chromosome. The fad E, the fad F and the fad G genes map closely together on the E. coli chromosome at a locus distinct from all other fad loci (12) (see Fig. 2). The fad F and the fad G gene were found to code for butyryl-CoA dehydrogenase and acyl-CoA dehydrogenase respectively (12). Mutants in the fad E gene were found to be deficient in electron transfer flavo-protein, a protein that couples the acyl-CoA dehydrogenase to the respiratory chain (12). Electron transfer flavoprotein was found to be constitutive in E. coli (12).

Recent studies by Nunn and coworkers (13,14) have led to the identification of a new gene locus (fad L) required for the utilization of the long chain fatty acids (see Fig. 2). Although the

exact nature of the fad L mutation is unknown, preliminary findings suggest that fad L gene may be the structural gene for a protein that facilitates the entry of long chain fatty acids through the cytoplasmic membrane (13,14).

Medium chain fatty acids cannot themselves serve as inducers, however they can serve as substrate for the fad enzymes (9,10,11). Overath and coworkers first showed that mutants which were able to utilize medium chain fatty acids as the sole carbon source were constitutive for the fad enzymes (9). These mutants have been termed as fad R, and Overath et al. (9), have suggested that the fad R gene codes for a regulatory protein, possibly a repressor. Further studies by Simons et al., have shown that fad R gene maps at a locus distinct from all other fad loci (15) (see Fig. 2) and it codes for a polypeptide which is a repressor molecule (16).

Thus the structural genes for the enzymes of fatty acid oxidation in E. coli form a complex regulon , referred to as fatty acid degradation (fad) regulon.

Little is known about the structural or the kinetic properties of the fad regulon enzymes. However, some of the enzymes of β-oxidation have been studied. Overath et al., (9) and Samuel et al., (17) have reported the partial purification of acyl-CoA synthetase from E. coli. Acyl-CoA synthetase from E. coli like the mammalian synthetase (18) was found to be partially membrane bound. However, the other β-oxidation enzymes were found in the soluble fraction of a E. coli homogenate (9). Beadle et al., (19)

have recently reported evidence for two enoyl-CoA hydratase activities in E. coli K12. A membrane associated enoyl-CoA hydratase displayed preference for long chain substrates whereas a cytoplasmic hydratase displayed activity towards substrates of varying chain lengths (19). Additionally, a study of β-oxidation enzymes in E. coli has led to the isolation and purification of a multienzyme complex which was reported to exhibit enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase activities (20,21). However, acyl-CoA-synthatase and more interestingly acyl-CoA dehydrogenase, which catalyze the first reaction of the β-oxidation cycle are not associated with the complex (20,22).

In the present thesis I report that the purified multienzyme complex from E. coli exhibits additionally 3-hydroxyacyl-CoA epimerase and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase activities, both of which are required for the degradation of unsaturated fatty acids. The fatty acid oxidation complex from E. coli is composed of two 78,000 dalton and two 42,000 dalton subunits each. The small subunit carries the thiolase activity and the large subunit probably carries cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase activity. Evidence is also presented for the association of all soluble thiolase, 3-hydroxyacyl-CoA dehydrogenase and crotonase activities with the complex, whereas a long chain enoyl-CoA hydratase exists as a separate protein.

The last part of my thesis describes attempts to elucidate the intramolecular location of crotonase and 3-hydroxyacyl-CoA dehydrogenase.

EXPERIMENTAL PROCEDURES

Materials - CoA, NAD^+ and NADH were purchased from P-L Biochemicals, Inc. 2-Decenoic acid was obtained from Aldrich Chemical Co. 3-Octyn-1-ol was bought from Pfaltz and Bauer. N-[2-^{14}C]Ethyl maleimide was supplied by New England Nuclear. [1-^{14}C]Oleic acid and NCS tissue solubilizer were purchased from Amersham Co. Phosphatidylethanolamine, phosphatidylglycerol and cardiolipin were bought from Supelco Inc. Polyacrylamide gradient gels (4-30%) and Sepharose CL-6B were supplied by Pharmacia Inc. N-Ethylmaleimide, L-3-hydroxyacyl-CoA dehydrogenase, and all proteins used for molecular weight determinations as well as most biochemical reagents were obtained from Sigma Chemical Co.

cis-3-Octenoic acid was synthesized from 3-octyn-1-ol by the procedure of Stoffel and Ecker (23). DL-3-Hydroxydodecanoic acid was prepared by reduction of ethyl 3-ketododecanoate with $NaBH_4$, followed by alkaline hydrolysis. Ethyl 3-ketododecanoate was synthesized by an established procedure (24). Pig heart 3-ketoacyl-CoA thiolase (25) and bovine liver enoyl-CoA hydratase (26) were purified according to published procedures.

Growth of Cells - E. coli B (ATCC 11775) cells were grown in M9 mineral salts medium with oleate (0.1%; v/v) as the sole carbon source, as described in detail by Overath et al. (9). Triton X-100 (0.4%; v/v) was used instead of Brij 35 to disperse oleate. For the preparation of ^{14}C-labeled fatty acid oxidation complex, E. coli cells were grown in the above mentioned medium which contained [1-^{14}C]oleate (1 μCi/ml). After harvesting and washing the cells with M9 mineral salts medium they were mixed with four parts of unlabeled cells. The ^{14}C-labeled fatty acid oxidation complex was isolated from these cells as described by Binstock et al. (20,27,28).

Preparation of Substrates - Crotonyl-CoA (29) and acetoacetyl-CoA (30) were synthesized according to published procedures. The CoA derivatives of cis-3-octenoic acid, DL-3-hydroxydodecanoic acid and 2-decenoic acid were prepared by the mixed anhydride method as detailed by Goldman and Vagelos (31). The concentrations of CoA derivatives were determined by the method of Ellman (32) after cleaving the thioester bond with hydroxylamine at pH 7. 3-Ketodecanoyl-CoA was synthesized enzymatically from 2-decenoyl-CoA as described (27). The concentration of 3-ketodecanoyl-CoA was determined by measuring the oxidation of NADH at pH 7 in the presence of 3-hydroxyacyl-CoA dehydrogenase.

Protein and Enzyme Assays - Protein concentrations were determined by the method of Lowry (33). Thiolase, L-3-hydroxyacyl-CoA

dehydrogenase and enoyl-CoA hydratase were assayed spectrophotometrically at 303 nm, 340 nm and 263 nm respectively as described in principal by Lynen and Ochoa (34) and as detailed by Binstock and Schulz (28). Acyl-CoA synthetase was assayed by the method of Kornberg and Pricer (35) as described by Overath et al. (4). Acyl-CoA dehydrogenase assays were performed as described by Beinert (36). 3-Hydroxyacyl-CoA epimerase activities were measured spectrophotometrically at 340 nm and at 30°C by an assay in which the epimerase-dependent formation of L-hydroxydodecanoyl-CoA was coupled to its dehydrogenation and thiolytic cleavage, which were catalyzed by L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, respectively (22). cis-Δ^3-trans-Δ^2-Enoyl-CoA isomerase activity was measured spectrophotometrically at 340 nm and at 30°C by an assay in which the isomerase-dependent formation of trans-Δ^2-enoyl-CoA was coupled to its hydration, dehydrogenation and finally thiolytic cleavage, which were catalyzed by crotonase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase respectively (22). One unit of enzyme activity is defined as 1 μmole of substrate converted to product per min.

Purification of the Fatty Acid Oxidation Complex - E. coli B cells (18 g) induced for the enzymes of fatty acid oxidation were homogenized by sonication for 4 min at 0° in 36 ml of 10 mM KP_i (pH 7.0) containing 10 mM 2-mercaptoethanol, 25% (v/v) glycerol and 2 mM phenyl methane sulfonyl flouride. Purification of the complex to

homogeneity from the crude homogenate was achieved by chromatography on a phosphocellulose column (5 × 44 cm) as described in detail by Binstock and Schulz (20,28).

Molecular Weight Determination - The molecular weight of the native complex was estimated by gel filtration on Sepharose CL-6B and by electrophoresis on polyacrylamide gradient gels. Gel filtration was performed on a Sepharose CL-6B column (2.7 × 74 cm) equilibrated with 20 mM KP_i (pH 7.0) containing 0.1 M NaCl and 10 mM 2-mercaptoethanol. The molecular weight was determined as described by Andrews (37). Electrophoresis on 4 to 30% polyacrylamide gradient gels was performed as previously described (22). The buffer used was 0.09 M Tris-boric acid (pH 8.35) containing 2.5 mM EDTA. The relative mobility of the complex was unaffected by the electrophoresis time between 6 and 18 hours.

Gel Electrophoresis - Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate was performed at pH 8.3 according to the method of Laemmli (38) and at pH 7.2 as outlined by Nielson and Reynolds (39). Electrophoresis in the presence of sodium dodecyl sulfate plus urea (3%; w/v) was carried out as described by Bassford et al. (40). Polyacrylamide gradient gel electrophoresis in the presence of sodium dodecyl sulfate was performed on 4 to 30% polyacrylamide gradient gels (7.5 × 7.5 cm, Pharmacia). The gradient gels were pre-electrophoresed at 70 V for

1 hour. The electrophoresis buffer was 0.04 M Tris, 0.02 M sodium acetate (pH 7.4) containing 2 mM EDTA and 0.2% sodium dodecyl sulfate. Protein samples (1-3 mg/ml) were dissolved in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 1% sodium dodecyl sulfate, 5% 2-mercaptoethanol and boiled for 5 minutes. Aliquots containing 5-10 µg of protein were mixed with 0.5 µl of bromphenol blue (1%) and an equal volume of glycerol and were applied to the gel. Electrophoresis was performed at a constant voltage of 300 V until samples had entered the gel. Thereafter electrophoresis was continued for 2 hours at 150 V. The gel was then stained for 40 minutes with Coomassie Blue (1%) and destained with acetic acid (7%) for 1 hour after which protein bands were visible. Destaining for several hours in acetic acid (7%) in the presence of wool resulted in the complete removal of background stain.

Preparation of Antibodies - Purified fatty acid oxidation complex from E. coli B (1.9 mg) was extensively dialyzed against a saline solution containing 10 mM NaP_i (pH 7.5), emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into two footpads of a New Zealand white rabbit (3.5 kg). One week later an equal amount of fatty acid oxidation complex prepared in an identical manner, except that Freund's incomplete adjuvant was used, was injected into the two other footpads of the same rabbit. Blood was taken from a vein of the ear two weeks after giving the booster injection. Serum was obtained by allowing the blood to

clot overnight at 4° and by removing the carefully resuspended precipitate by centrifugation at 1000 × g for 30 minutes. The serum thus obtained gave rise to a strong precipitin line against purified fatty acid oxidation complex on an Ouchterlony plate. The rabbit was then terminally bled at the carotid artery and the blood (45 ml) was processed to serum as described above. Partial purification of the immunoglobulin fraction was achieved by chromatography on DEAE-cellulose as described (41). The preparation of rabbit antibodies against pig heart 3-ketoacyl-CoA thiolase has been described by Staack et al. (25).

Immunotitration of the Purified Multienzyme Complex - Fatty acid oxidation complex (5 μg) in 0.2 ml of 0.2 M potassium phosphate, pH 8.0, was incubated with antibodies raised against the fatty acid oxidation complex. The weight ratio of complex to antibody was varied from 2 to 0.1. After incubation for 5 minutes enoyl-CoA hydratase was assayed in the incubation mixture or in the supernatant obtained after centrifugation of the incubation mixture at 120,000×g for 45 minutes.

Immunoprecipitation of the Fatty Acid Oxidation Complex from E. coli B Homogenates - E. coli B cells (0.2 g) induced for the enzymes of fatty acid oxidation were suspended in 1 ml of 0.2 M KP_i (pH 8.0) containing 5 mM phenyl methane sulfonyl flouride, 5 mM o-phenanthroline, 2 mM p-chloromercuribenzoate and 10 mM EDTA. The cell suspension

was sonicated for 1 minute at 0° and the resulting homogenate was centrifuged at 120,000 × g for 2 hours at 0°. Antibodies raised against the fatty acid oxidation complex (125 μg/mg of soluble protein) were added to the high-speed supernatant and kept for 5 minutes at 25°. The antibody-antigen complex was isolated by centrifugation at 120,000 × g for 45 minutes at 4°C. The resulting precipitate was washed with 0.2 M KP_i (pH 8.0) containing 0.1% deoxycholate and was finally subjected to gel electrophoresis in the presence of sodium dodecyl sulfate.

The immunotitration experiments were performed as described above except that only 2 mM phenyl methane sulfonyl fluoride was used to prevent proteolysis. The final supernatants were assayed for thiolase, 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase as previously described (28).

Quantitation and Identification of the Phospholipids Associated with the Fatty Acid Oxidation Complex from E. coli B - Purified fatty acid oxidation complex (1-2 mg) was extensively dialyzed against water and lyophilized to dryness. Lipids were extracted with a 2:1 mixture of chloroform and methanol. The phosphate content of the lipid extract was determined by the procedure of King (42) after ashing the sample by the method of Ames and Dubin (43). For identification, the phospholipids were radioactively labeled by growing E. coli cells on [1-^{14}C]oleate as the sole carbon source.

The complex was isolated from these cells and the lipids were extracted as described above. The lipid extract was combined with a mixture of phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol and cardiolipin. The phospholipids were separated by thin layer chromatography on silica-coated plates. The solvent system used was a mixture of chloroform, acetone, methanol, acetic acid and water in a ratio of 50:20:10:10:5. The phospholipid spots were visualized by exposing the plate to I_2 vapor. The radioactivity associated with the phospholipids was determined by removing regions of the silica layer from the plate and counting their radioactivity in a liquid scintillation counter.

Labeling of the Fatty Acid Oxidation Complex with N-[2-^{14}C]-Ethylmaleimide - Fatty acid oxidation complex (0.3 mg) in 0.3 ml of 0.2 M KP_i (pH 8.0) containing 10 mM N-ethylmaleimide, 2 mM phenylmethane sulfonyl fluoride and 2 mM acetoacetyl-CoA was preincubated for 40 minutes at 0°. 2-Mercaptoethanol was then added to a final concentration of 50 mM. The complex was separated from the incubation mixture by centrifugation-filtration through Sephadex G-50 as described by Penefsky (44). The filtrate was reacted with 5 mM or 10 mM [^{14}C]N-ethylmaleimide (4-5 μCi/μmol) at 0° for 10 minutes or 40 minutes respectively. The reaction was terminated by the addition of 2-mercaptoethanol to a final concentration of 50 mM. The protein was separated from the incubation mixture by the centrifugation-gel filtration procedure of Penefsky (44).

The fatty acid oxidation complex (10-20 μg) labeled with [^{14}C]N-ethylmaleimide was subjected to polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate at pH 8.3 (38). Gels were stained with Coomassie Blue and destained in 7% acetic acid. Records of the gels were obtained by scanning their absorbance at 500 nm. The gels were then sliced into 2 mm wide segments, each of which was incubated overnight with a mixture (1 ml) of NCS tissue solubilizer and water (9:1) at 50° and thereafter combined with 10 ml of a toluene based counting solution.

RESULTS

Association of cis-Δ^3-trans-Δ^2-Enoyl-CoA Isomerase and 3-Hydroxyacyl-CoA Epimerase Activities with the Purified Fatty Acid Oxidation Complex - Isolation and purification of a fatty acid oxidation complex has been previously reported by Binstock, Pramanik and Schulz (20). Enoyl-CoA hydratase (EC 4.2.1.17), L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), and 3-ketoacyl-CoA thiolase (EC 2.3.1.16), three of the seven known enzyme activities required for the degradation of fatty acid to acetyl-CoA, were found to be associated with this multienzyme complex (20,28). The purified complex was devoid of acyl-CoA synthetase and acyl-CoA dehydrogenase activities (20).

Further studies by us with the purified complex led to the identification of two additional enzyme activities which apparently were associated with the multienzyme complex. These enzyme activities were found to be cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase (EC 5.3.3.3) and 3-hydroxyacyl-CoA-epimerase (EC 5.1.2.3), both of which are required for the degradation of unsaturated fatty acids. To prove their association with the complex, the co-purification of enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA epimerase, and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase was studied. When an E. coli homogenate was subjected to chromatography on a phosphocellulose column it was observed that all five enzymes

were eluted coincidentally (see Fig. 3). The results of a typical purification experiment are summarized in Table I. The five enzymes associated with the complex, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA epimerase and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase, were purified approximately to the same extent. Slight differences in the fold purification shown in Table I are due to difficulties in accurately assaying some of the enzymes in the crude homogenate. The specific activities of the component enzymes of the complex are comparable to those reported in our earlier publications (22) except for the activity of enoyl-CoA hydratase which is significantly higher when the complex is isolated in the presence of phenyl methane sulfonyl fluoride. When the purified complex was subjected to polyacrylamide disc electrophoresis (20) or chromatography on Sephacryl SS 300 (data not shown) a single band or peak was observed. These findings lead to the conclusion that all five enzymes listed in Table I are associated with one protein which is homogeneous.

Inactivation of the Fatty Acid Oxidation Complex by Tris-HCl - During our studies on the fatty acid oxidation complex it was observed that 3-ketoacyl-CoA thiolase (thiolase I) which is a component enzyme of the fatty acid oxidation complex, was easily inactivated in dilute solution, although it was surprisingly heat stable (20,22). Further studies proved that this inactivation was caused by Tris-hydrochloride buffer, which was routinely used for

Figure 3

Purification of the enzymes of fatty acid oxidation from E. coli by chromatography on phosphocellulose. A crude extract prepared from 18 g of E. coli B cells as described under "Experimental Procedure" was applied to a phosphocellulose column (5 × 44 cm) equilibrated with 50 mM potassium phosphate (pH 6.6) containing 10 mM β-mercaptoethanol, 25% glycerol (v/v). The column was developed with linear gradient of 0.05 M to 0.5 M potassium phosphate (pH 6.6) containing 10 mM β-mercaptoethanol, and 25% glycerol (v/v). (x) 3-Ketoacyl-CoA thiolase, (●) cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase, (▲) L-3-Hydroxyacyl-CoA dehydrogenase, (○) Enoyl-CoA hydratase, (△) 3-Hydroxyacyl-CoA epimerase. The absorbance at 280 is not shown because a very small amount of protein was eluted with the potassium phosphate gradient.

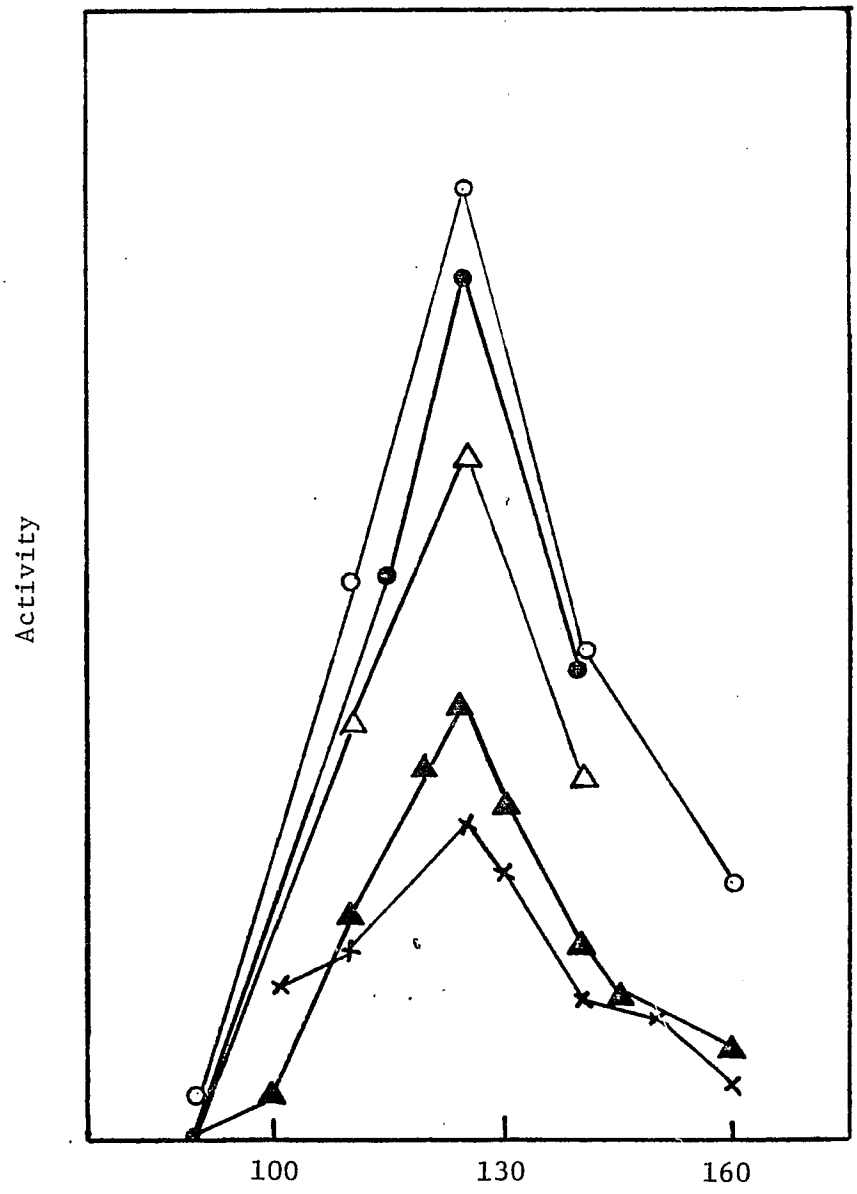

Figure 3

TABLE I

Purification of the Multienzyme Complex of Fatty Acid Oxidation from E. Coli[a]

Enzyme	Substrate	Specific Activity: Homogenate[b] (U/mg)	Specific Activity: Purified Complex[c] (U/mg)	Purification (-fold)
Enoyl-CoA hydratase	Crotonyl-CoA	4.1	115	28
L-3-Hydroxyacyl-CoA dehydrogenase	Acetoacetyl-CoA	0.71	20	28
3-Ketoacyl-CoA thiolase[d]	Acetoacetyl-CoA	0.22	5.5	25
3-Hydroxyacyl-CoA epimerase	D-Hydroxydodecanoyl-CoA	0.07	2.4	34
cis-Δ^3-trans-Δ^2-Enoyl-CoA isomerase	cis-Δ^3-Octenoyl-CoA	0.24	8	33

[a]Data for 20 g of E. coli B cell paste.

[b]Contained 1.7 g of protein.

[c]After chromatography on phosphocellulose. Contained 11.2 mg of protein. Since all assays are performed with 30μM substrates, these specific activities are significantly lower than the corresponding V_{max} values. The complex is usually obtained in 20% yield.

[d]The assay buffer was HEPES.

diluting and assaying this enzyme. A systematic study of this phenomenon revealed that all component enzymes of the complex were inactivated by Tris-hydrochloride. The half-times for the inactivation were 2 minutes for thiolase and 4 to 4.5 minutes for the other four enzymes when the complex (at a concentration of 27 µg/ml) was kept in 1M Tris-hydrochloride, pH 8.1 (see Fig. 4). Under the same conditions but in the presence of 0.2 M potassium phosphate, pH 8, or in 1 M potassium N-2-hydroxyethylpiperazine-N'-2 ethane sulfonate, pH 8.1, instead of Tris-HCl none of the enzymes was significantly inactivated. Since the complex is rapidly inactivated in Tris-HCl buffer, potassium-N-2-hydroxyethylpiperazine-N-2-ethane sulfonate was routinely used instead of Tris-HCl for the dilution and the assay of the thiolase.

In order to elucidate the effect of Tris-hydrochloride on the complex, it was incubated with several different buffers and the activity of 3-ketoacyl-CoA thiolase activity, a component enzyme of the complex, as a function of time was determined. As can be seen from Fig. 5, 1 M imidazole, pH 8.1, caused the complete loss of thiolase activity with a half time of 4 minutes. In contrast, in 1 M glycylglycine, pH 8.1, or 1 M $KHCO_3$, pH 8.1, or 1 M $NaHCO_3$, pH 8.1, 60% of the enzyme's activity remained, even after 30 minutes of incubation (see Fig. 5). However, the results presented in Fig. 5 and Table II do not provide a conclusion regarding the mechanism by which the complex is inactivated in Tris-hydrochloride. Attempts to reactivate the complex by transferring it from 1 M

Figure 4

Effects of Tris-hydrochloride and potassium phosphate on the activities of the multienzyme complex of fatty acid oxidation. Purified complex (27 μg/ml) at 0°C in the presence of 1 M Tris-hydrochloride, pH 8.1 (—) or 0.2 M potassium phosphate, pH 8 (---). The activities of the five component enzymes were determined as a function of time. Symbols: (●) thiolase activity with acetoacetyl-CoA; (o), thiolase activity with 3-ketodecanoyl-CoA; (▲), L-3-hydroxyacyl-CoA dehydrogenase with acetoacetyl-CoA; (Δ), L-3-hydroxyacyl-CoA dehydrogenase with 3-ketodecanoyl-CoA; (x), cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase with cis-3-octenoyl-CoA; (◪), 3-hydroxyacyl-CoA epimerase with D -3-hydroxydodecanoyl-CoA. The inactivation curve for enoyl-CoA hydratase with crotonyl-CoA was virtually identical to that of L-3-hydroxyacyl-CoA dehydrogenase.

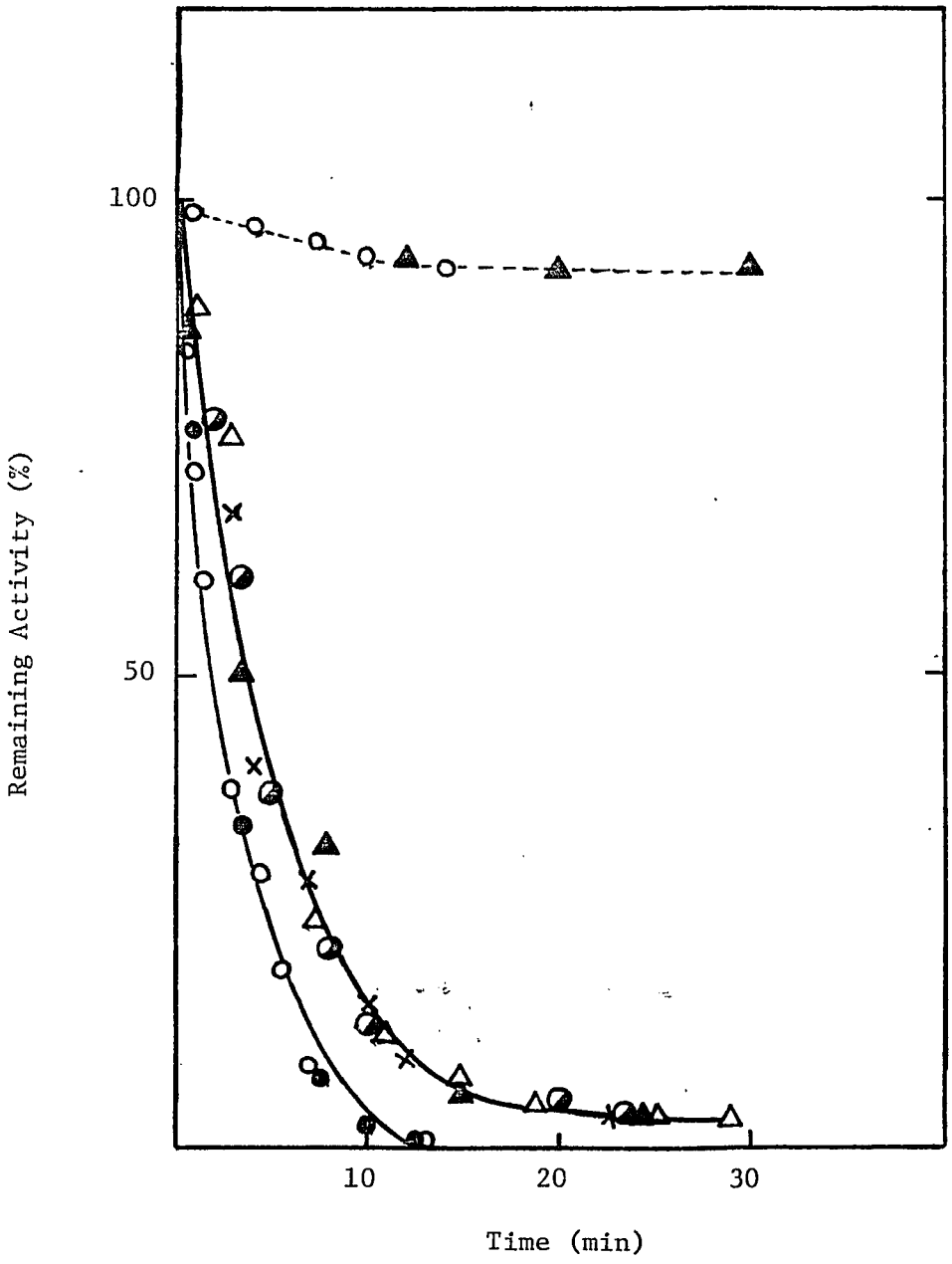

Figure 4

Figure 5

Effect of several buffers on the stability of the thiolase activity of the complex. Fatty acid oxidation complex (27 μg/ml) was kept at 0°C, (Δ) in 1 M imidazole (pH 8.1); (o) in 1 M potassium bicarbonate (pH 8.1); (●) in 1 M sodium bicarbonate (pH 8.1); (◐) in 1 M glycylglycine (pH 8.1). Thiolase activity was determined as a function of time with acetoacetyl-CoA as a substrate.

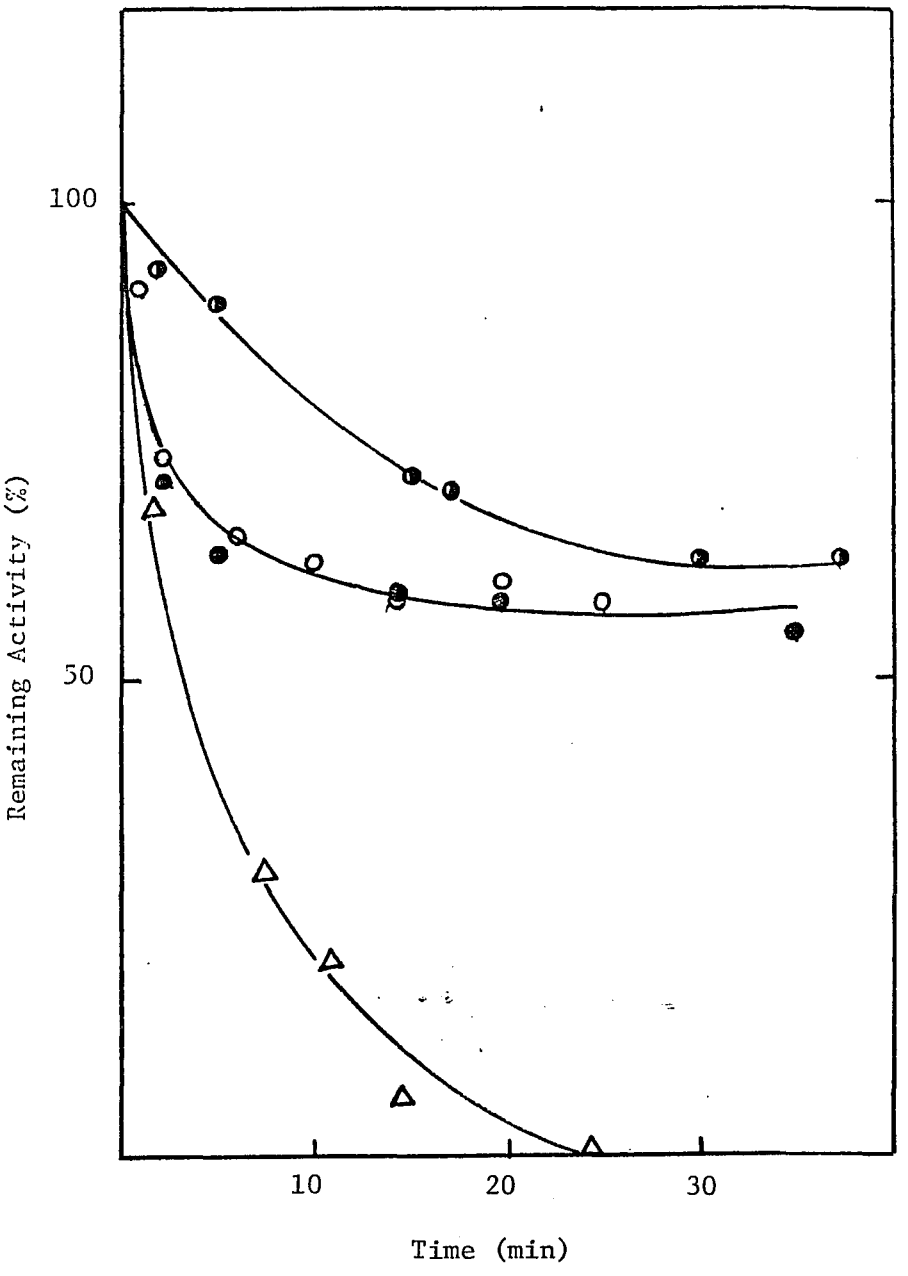

Figure 5

TABLE II

Effect of different buffers on the stability of the thiolase activity of the multienzyme complex of fatty acid oxidation from E. coli

Buffers	Substrate	Conc of multi-enzyme complex µg/ml	Half Time for inactivation (min)
0.02M Tris-HCl (pH 8.2)	Acetoacetyl-CoA	27	20.5
0.05M Tris-HCl (pH 8.2)+ 25% glycerol	"	"	38
1M Tris-HCl (pH 8.1)	"	"	2.0
1M Tris-HCl (pH 8.1) + 1 mM NAD^+	"	"	20
1M Tris-HCl (pH 8.1) + 1 mM Acetoacetyl-CoA	"	"	2.4
1M Tris-HCl (pH 8.1) + 1 mM Crotonyl-CoA	"	"	8.5
0.5M glycylglycine (pH 8.0)	"	"	28
1M glycylglycine (pH 8.0)	"	"	No inactivation
1M imidazole (pH 8.0)	"	"	3.4
0.2M $NaHCO_3$ (pH 8.0)	"	"	18.5
0.2M $KHCO_3$ (pH 8.0)	"	"	18.5
0.02M Tris-HCl (pH 8.2)	3-Ketodecanoyl-CoA	27	18.0
1M Tris-HCl (pH 8.1)	"	"	2.0

Tris-HCl, pH 8.1 buffer into 0.2 M potassium phosphate, pH 8, containing 10 mM β-mercaptoethanol, by the centrifugation-gel filteration procedure of Penefsky (44) were unsuccessful.

In addition, the protection against inactivation of the complex by Tris-hydrochloride was studied. It was observed that the presence of NAD^+ or crotonyl-CoA in contrast to acetoacetyl-CoA, protected thiolase significantly against inactivation by Tris-hydrochloride (see Fig. 6).

The Quaternary Structure of the Fatty Acid Oxidation Complex - The molecular weight of the native complex was estimated by electrophoresis on 4-30% polyacrylamide gradient gels and by gel filtration on Sepharose CL-6B. As shown in Fig. 7A and Fig. 7B, the linear standard curves yielded an estimated molecular weight close to 260,000 for the complex.

The subunit structure of the complex was determined by polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate at pH 8.3. An absorbance scan of a resulting gel stained for protein with Coomassie Blue (see Fig. 8A) demonstrates the presence of two protein bands. The molecular weights of the two corresponding polypeptides were estimated to be 78,000 (I) and 42,000 (II). When the complex was isolated in the absence of phenyl methane sulfonyl fluoride, the subunit pattern showed the presence of a third band corresponding to a protein with a molecular weight of 35,000 (see Fig. 8B). Since this

Figure 6

Protection of the thiolase activity of the fatty acid oxidation complex against inactivation by Tris-hydrochloride. The multienzyme complex at a concentration of 27 µg/ml was kept at 0^{o} in (▲), 1 M Tris-HCL (pH 8); (o) 1 M Tris-HCl (pH 8) plus 1 mM acetoacetyl-CoA; (●), 1 M Tris-HCl (pH 8) plus 1 mM crotonyl-CoA; (Δ), 1 M Tris-HCl (pH 8) plus 1 mM NAD^{+}. Thiolase activity was determined as a funtion of time with acetoacetyl-CoA as a substrate.

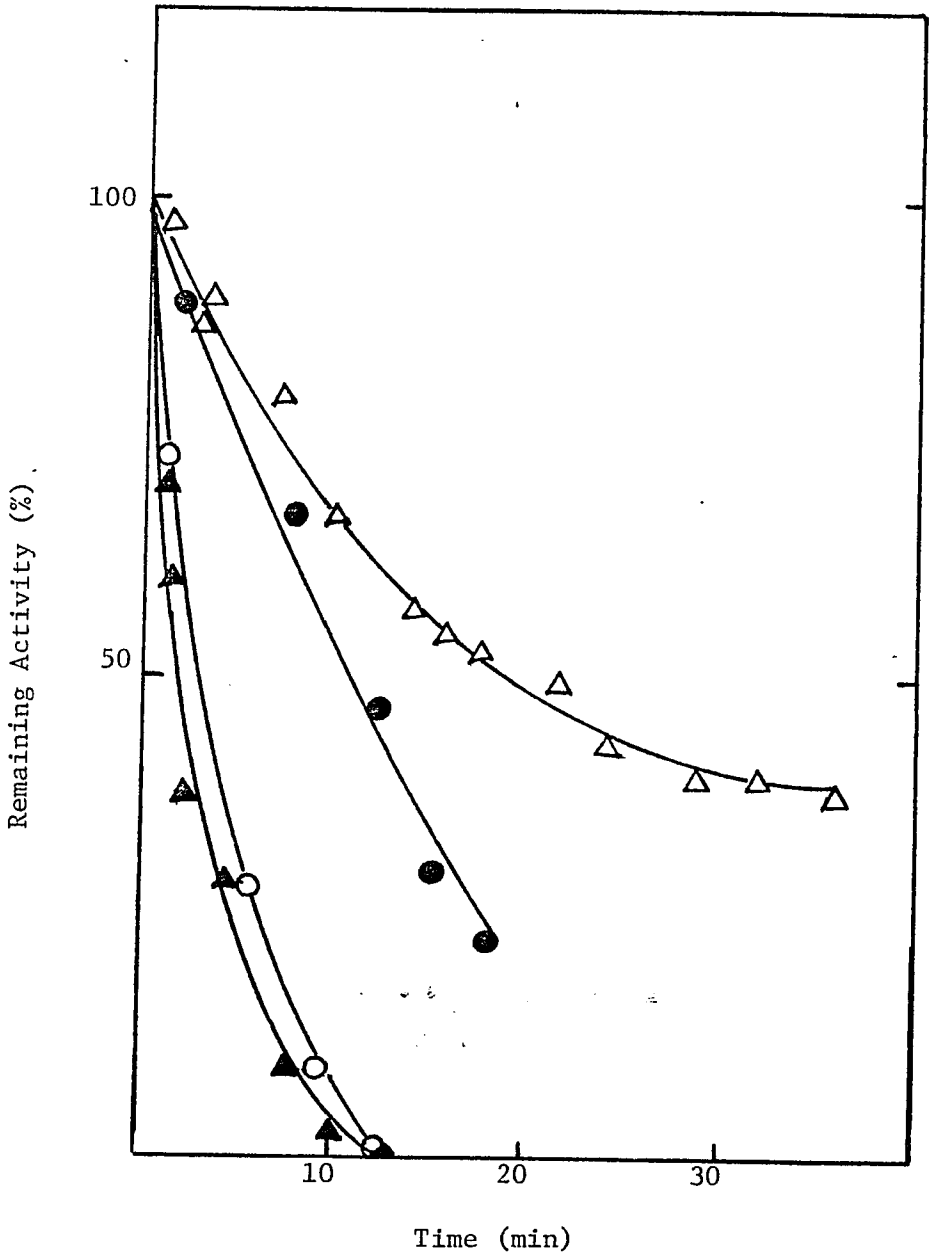

Figure 6

Figure 7

Native molecular weight of the fatty acid oxidation complex. (A) Polyacrylamide gradient gel electrophoresis, (B) Gel filtration on Sepharose CL-6B. For experimental details see "Experimental Procedures." Standards are: (1) Apoferritin, (2) catalase (3) lactate dehydrogenase, (4) bovine serum albumin, (5) ovalbumin, (6) β-galactosidase, (7) phosphorylase a, (8) aldolase.

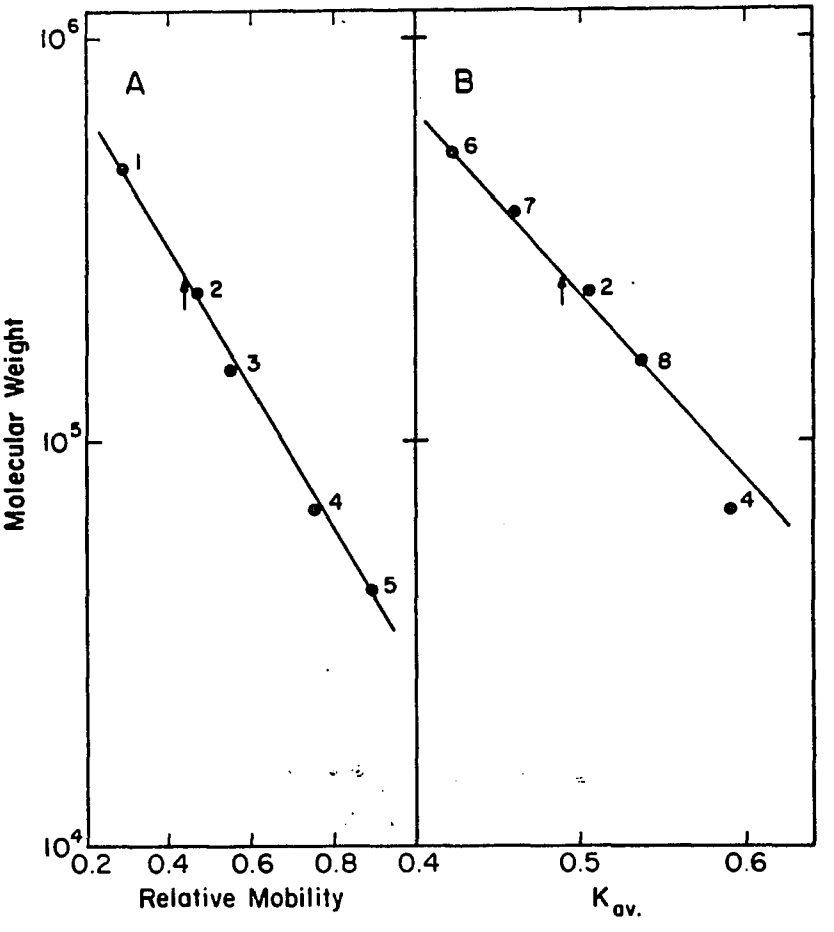
A
B
Molecular Weight
10^6
10^5
10^4
1
2
3
4
5
6
7
8
0.2
0.4
0.6
0.8
0.4
0.5
0.6
Relative Mobility
$K_{av.}$

Figure 7

Figure 8

Disc gel electrophoresis of the fatty acid oxidation complex in the presence of sodium dodecyl sulfate on 10% polyacrylamide gels, as described under "Experimental Procedures". (A) Enzyme preparation isolated in the presence of phenyl methane sulfonyl fluoride. (B) Enzyme preparation isolated in the absence of phenyl methane sulfonyl fluoride.

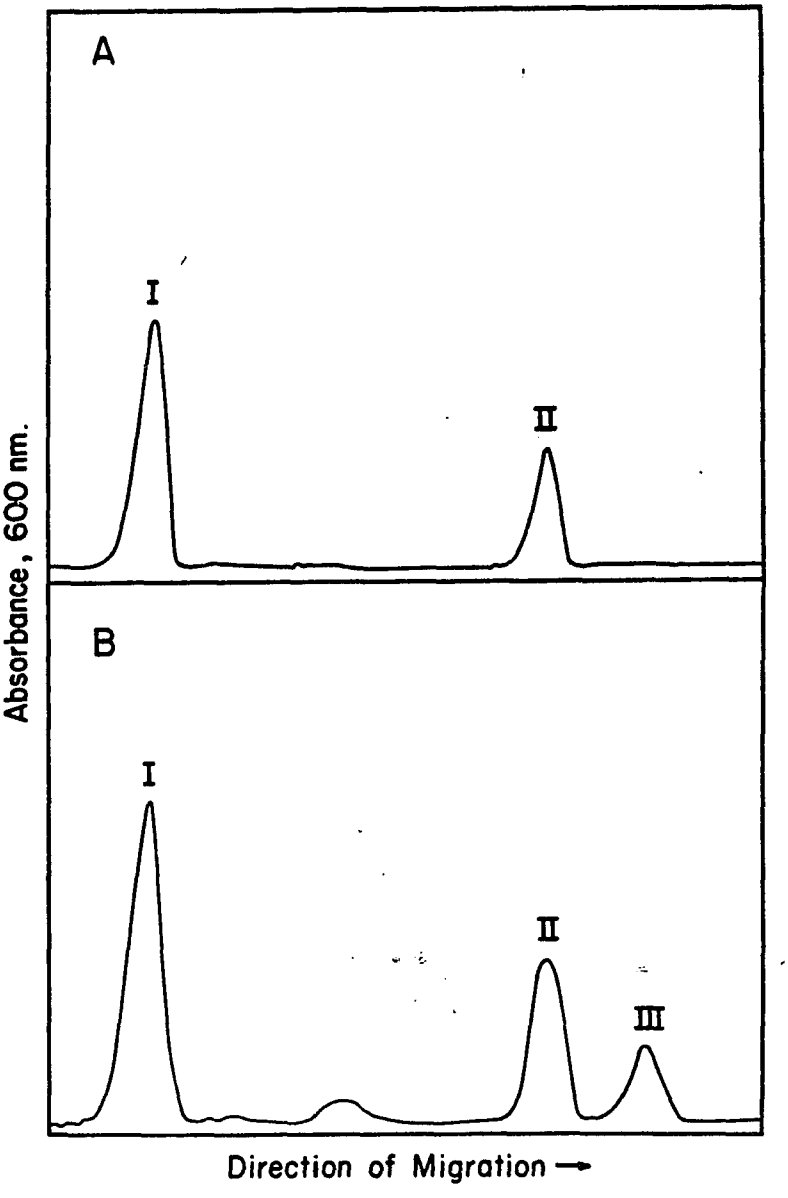

Figure 8

smaller polypeptide was only present when the complex was isolated in the absence of the protease inhibitor, we conclude it was a proteolytic degradation product of one of the two larger polypeptides. The subunit structure of the complex was additionally determined by polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate at pH 7.2 and in the presence of sodium dodecyl sulfate plus urea at pH 8.3. Under both conditions patterns identical to that shown in Fig. 8A were obtained. Since electrophoresis on a 4 to 30% polyacrylamide gradient gel in the presence of sodium dodecyl sulfate yielded the same two-band pattern, it is concluded that the fatty acid oxidation complex from E. coli B is composed of two types of subunits whose molecular weights are estimated to be 78,000 and 42,000. The molar ratio of the two subunits was estimated by determining the staining intensities of the bands observed on polyacrylamide gels in the presence of sodium dodecyl sulfate. For this purpose gels were loaded with various amounts of complex (2.5 to 25 μg), electrophoresed, and stained with either Coomassie Blue or Fast Green. Absorbance ratios for the two bands were determined and used to calculate molar ratios (see Table III). Average values of 1.2 and 1.05 were obtained for the molar ratio of the 42,000 dalton to 78,000 dalton subunit when the gels were stained with Coomassie Blue and Fast Green respectively (see Table III). Since staining with Fast Green has been reported to yield patterns more reflective of the relative amounts of protein than staining with Coomassie Blue

TABLE III

Subunit Stoichiometry of the Fatty Acid Oxidation Complex

(For details see under "Results")

Amt. of Protein µg	Molar Ratio of 42,000/78,000 Subunit		
	Coommassie Blue	Fast Green	^{14}C-label
7.5	1.23	0.98	
10	1.13	1.05	1.16
12.5	1.18	1.06	1.10
15	1.22	1.11	
20	1.3		
25	1.21		
Average	1.2	1.05	1.13

does (45), the molar ratio of 1.05 should be more reliable. In order to definitely establish the molar ratio of the two subunits, we have subjected ^{14}C-labeled complex to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and have determined the radioactivity associated with the two bands (see Fig. 9). An average molar ratio of 1.1 for the 42,000 dalton to 78,000 dalton subunit was thus obtained (see Table III). In view of these values for the molar ratio of the two polypeptides, it is concluded that the two subunits are present in the complex in equimolar amounts.

A report presenting evidence for the presence of lipid phosphorous in the partially purified complex (21), prompted us to determine the amount and types of phospholipids associated with the purified protein. An average value of 63 nmoles of lipid-bound phosphate per mg of complex was obtained. For their identification radioactive phospholipids were extracted from ^{14}C-labeled fatty acid oxidation complex and separated by thin layer chromatography. By comparison with standard phospholipids phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and cardiolipin (CL) were identified (see Fig. 10). The approximate molar ratios for PG:PE:CL in the complex were calculated to be 1:3:2.

The presence of phospholipids in the complex raises the question of whether phospholipids are required for the enzymatic activities of the complex or for maintaining its structural integrity. To answer this question, the complex was treated separately with phospholipases A_2,C (snake venom) and drugs like tetracaine and/or

Figure 9

Polyacryamide disc gel electrophoresis of the ^{14}C-labeled fatty acid oxidation complex in the presence of sodium dodecyl sulfate. For experimental details see under "Experimental Procedures".

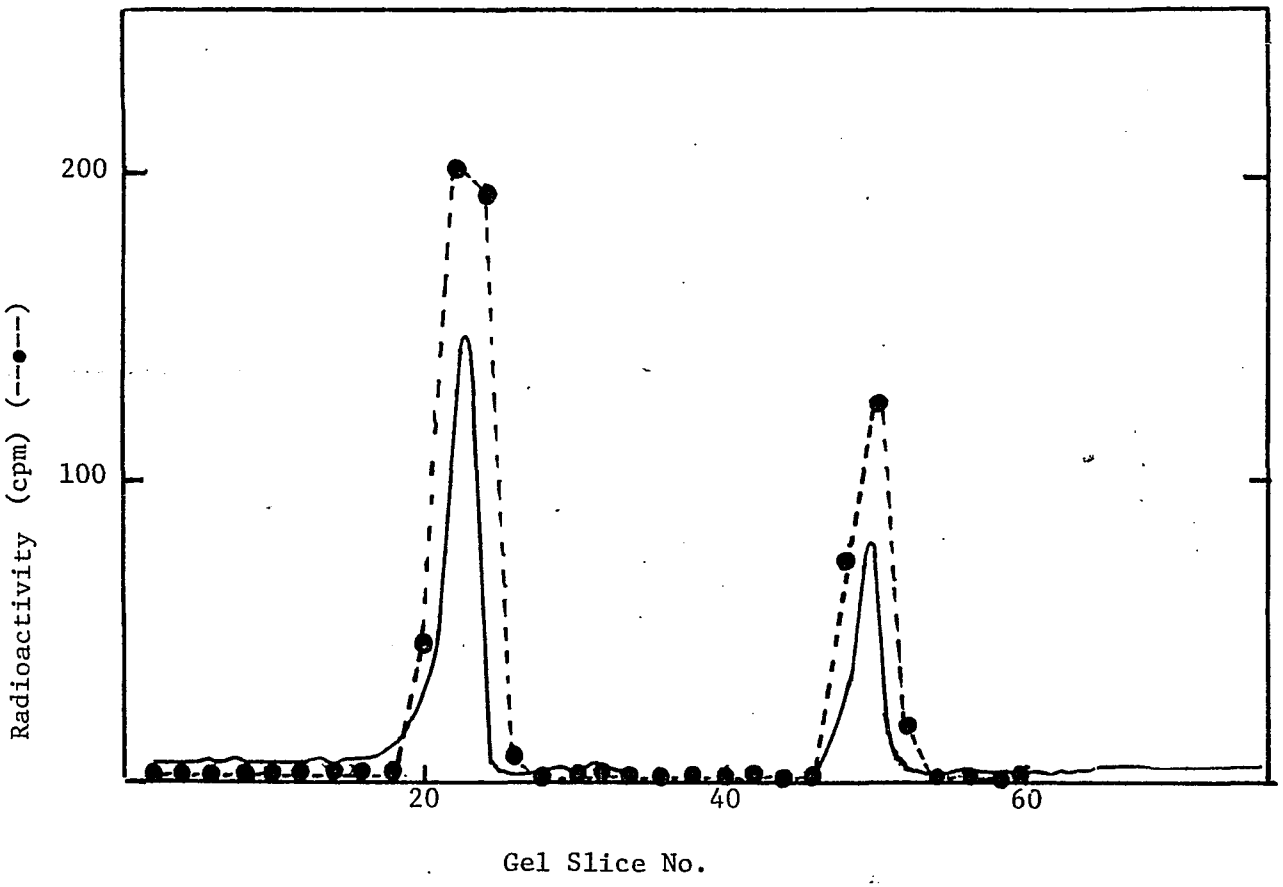

Figure 9

Figure 10

Thin layer chromatography of ^{14}C-labeled lipids extracted from ^{14}C-labeled fatty acid oxidation complex. The solvent system used for developing the chromatogram was a mixture of chloroform, acetone, methanol, acetic acid and water in a ratio 50:20:10:10:5. The phospholipids spots were visualized by exposing the plate to I_2 vapor The radioactivity associated with the phospholipids was determined by removing the silica layer in 1 cm^2 regions from the plate and counting the radioactivity in a liquid scintillation counter. PC, PG, PE and CL represent spots of phosphatidylcholine, phosphatidylglycerol,phosphotidylethanolamine and cardiolipin used as standards.

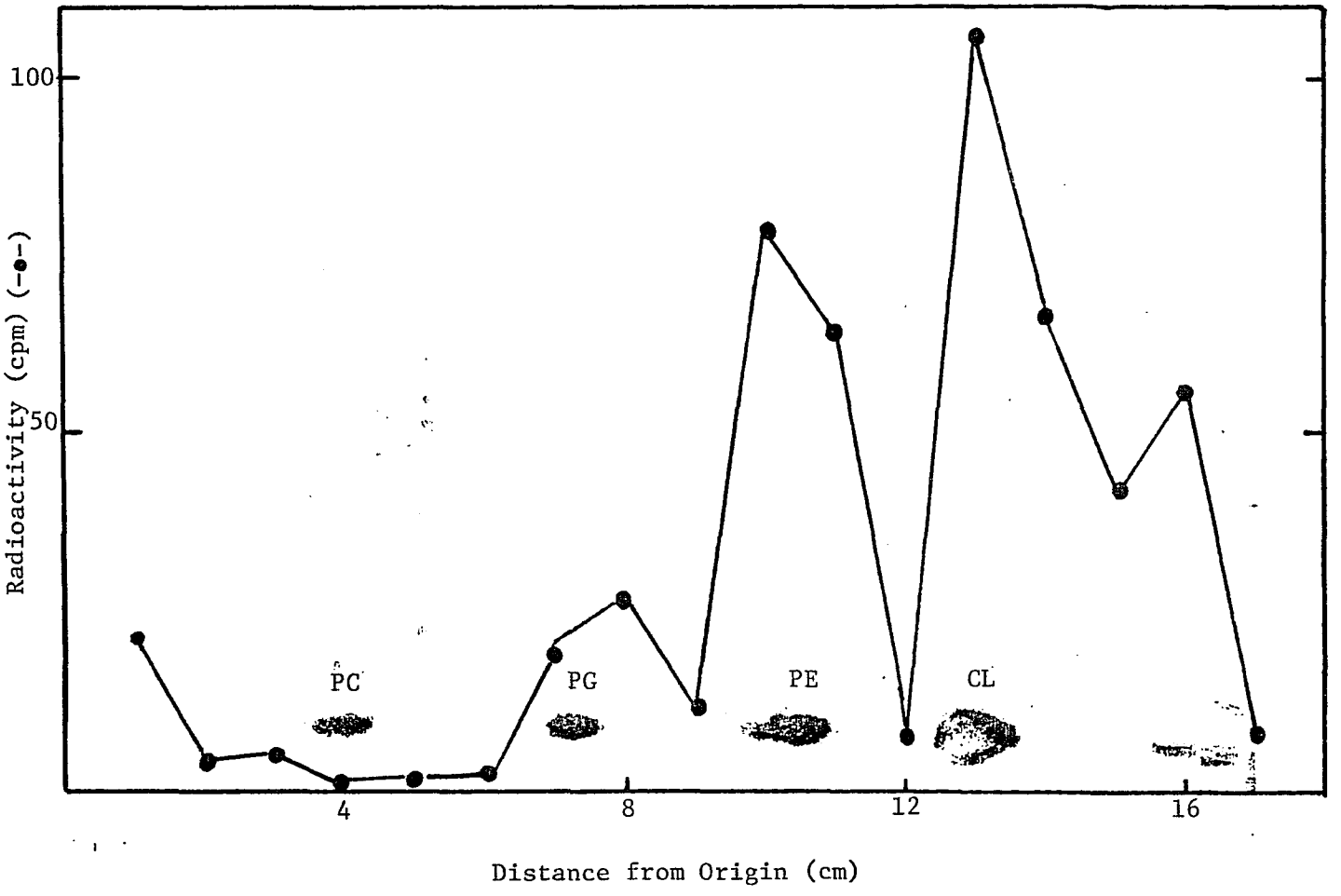
100
50
Radioactivity (cpm) (-●-)
PC
PG
PE
CL
4
8
12
16
Distance from Origin (cm)

Figure 10

dibucaine which are known to form complexes with phospholipids (46). Additionally a mixture of phospholipids containing phosphatidylethanolamine, phosphatidylglycerol and cardiolipin was added to the complex in the form of either liposomes (47) or as mixed micelles with Triton X-100. All treatments described above did not affect 3-ketoacyl-CoA thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities of the complex significantly. Thus phospholipids in the complex may not be required for the component enzymes to be active. Pretreated complex as described above was also subjected to polyacrylamide gradient gel electrophoresis and was found to behave identically to the untreated complex. This finding suggests that phospholipids present in the complex are either not removed by the described treatment or are not required for the structural integrity of the complex. Much harsher treatments like extraction of phospholipids from the complex with $CHCl_3$: CH_3OH (2:1) (Folch's extraction (48)) led to the precipitation of the complex which could not be solubilized with a buffer containing 1% Triton X-100.

Immunological Studies - An immunotritation study of the pure complex showed that even in the presence of an optimal amount of antibodies, the complex retained 40% of its enoyl-CoA hydratase activity (see Fig. 11). This finding suggests that antibodies do not completely block the active site of enoyl-CoA hydratase. However, at

Figure 11

Immunotitration of the pure fatty acid oxidation complex with antibodies raised against the purified complex. For experimental details see under "Experimental Procedures". (o) Enoyl-CoA hydratase assayed with crotonyl-CoA after addition of antibody. (•) Enoyl-CoA hydratase assayed with crotonyl-CoA in the supernatant obtained after removing the antigen-antibody complex by centrifugation at 120,000 × g for 45 minutes.

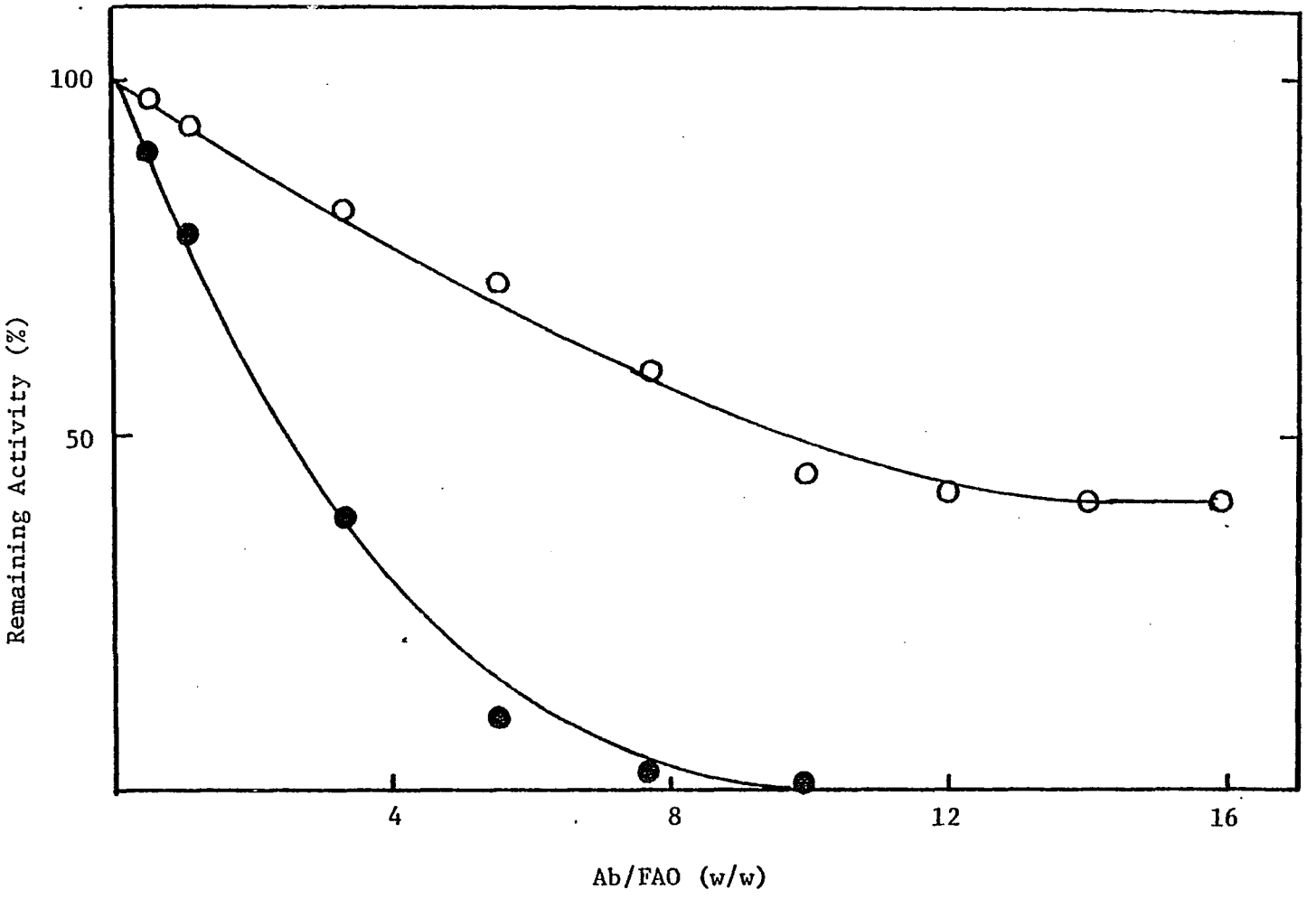

Figure 11

optimal antibody concentrations all enoyl-CoA hydratase activity was removable from the incubation mixture by immunoprecipitation.

Since the fatty acid oxidation complex is susceptible to proteolysis, it is possible that the two types of subunits which are present in the complex in equimolar amounts, may have been derived from a large common precursor by proteolysis. In order to rule out that this processing of the complex has occurred during its purification, we have rapidly isolated the complex from a homogenate by immunoprecipitation in the presence of the protease inhibitors phenyl methane sulfonyl fluoride, o-phenanthroline, p-chloromercuribenzoate and EDTA. When the immunoprecipitate was analyzed by polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate, the pattern shown in Fig. 12 was obtained. Present in the antigen-antibody precipitate were only the 78,000 (I) and 42,000 (II) subunits of the fatty acid oxidation complex as well as the heavy (H) and light (L) chains of the immunoglobulins. This data suggests that the subunit structure of the complex was not altered during its isolation and purification.

The identification of thiolase, 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase as component enzymes of the fatty acid oxidation complex raises the question of whether the total cellular activities of these three enzymes are associated with the complex. To answer this question an extract of soluble proteins from E. coli was immunotitrated with antibodies to the complex and the enzyme activities remaining in solution after centrifugation

Figure 12

Polyacrylamide disc gel electrophoresis of the antibody: fatty acid oxidation complex precipitate in the presence of sodium dodecyl sulfate. For experimental details see under "Experimental Procedures".

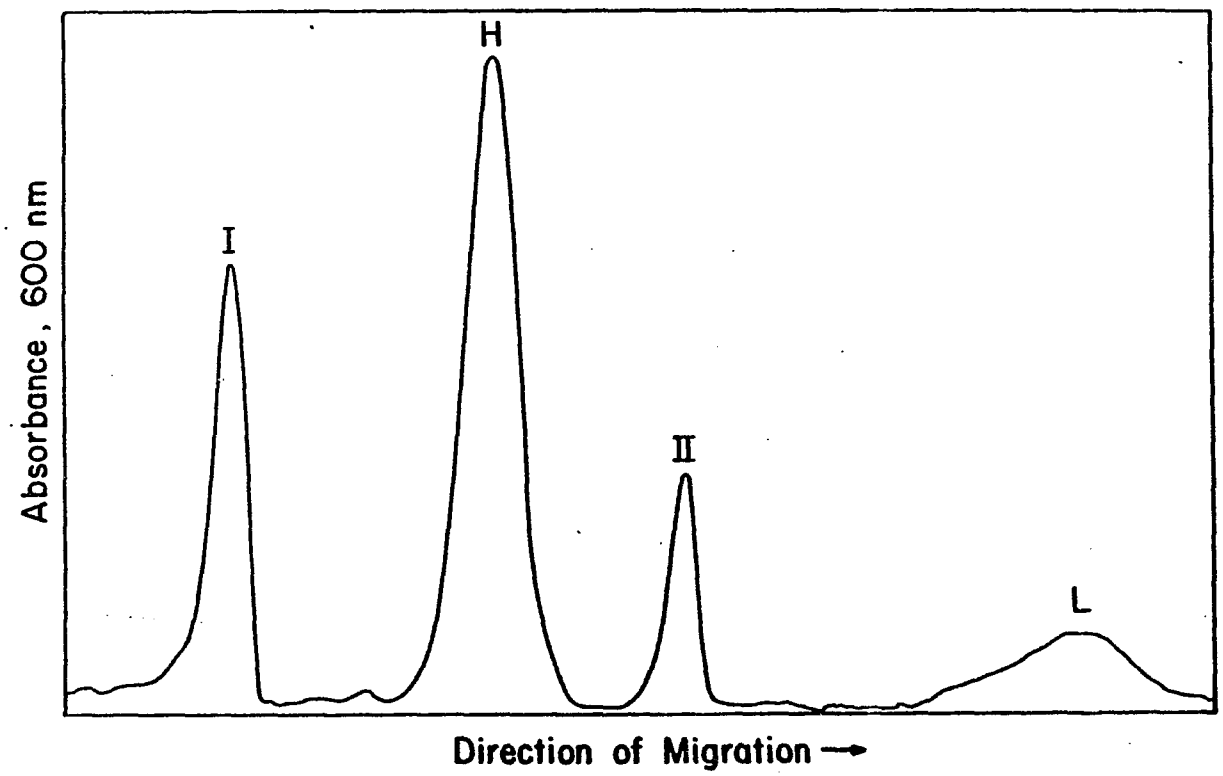

Figure 12

were determined. As shown in Fig. 13A, both thiolase and 3-hydroxyacyl-CoA dehydrogenase activities detected with acetoacetyl-CoA as well as with 3-ketoacyl-CoA as substrates were completely precipitated by antibodies to the complex. The immunotitration curves for these two enzymes are identical (see Fig. 13A). However, a more complex result was obtained for enoyl-CoA hydratase. Immunoprecipitation of short chain enoyl-CoA hydratase (crotonase) was complete and paralleled those of thiolase and 3-hydroxyacyl-CoA dehydrogenase whereas only one-third of the long chain enoyl-CoA hydratase activity was removed with antibodies to the complex (see Fig. 13B). When the soluble extract was first heated for 3 minutes to 60°, two-thirds of the long chain enoyl-CoA hydratase activity, but none of the crotonase activity, was destroyed. The remaining one-third of the long chain enoyl-CoA hydratase activity was immunoprecipitated with antibodies to the complex completely and in parallel with crotonase (see Fig. 13B). The conclusion of this experiment is that all thiolase, 3-hydroxyacyl-CoA dehydrogenase and crotonase activities present in a soluble extract of E. coli B are associated with the complex whereas a long chain enoyl-CoA hydratase activity, which is more thermo-labile than the complex, exists as a separate entity. We have observed that a significant fraction of the long chain enoyl-CoA hydratase activity precipitates during centrifugation. This finding suggests that the long chain enoyl-CoA hydratase is at least partially membrane-bound.

Figure 13

Immunotitration of the fatty acid oxidation complex present in a soluble E. coli B extract with antibodies raised against the purified complex. For experimental details see under "Experimental Procedures". (A) 3-Ketoacyl-CoA thiolase assayed with acetoacetyl-CoA (▲) and 3-ketodecanoyl-CoA (Δ) as substrates. 3-Hydroxyacyl-CoA dehydrogenase assayed with acetoacetyl-CoA (●) and 3-ketodecanoyl-CoA (o) as substrates. (B) Enoyl-CoA hydratase assayed with crotonyl-CoA (▲) and decenoyl-CoA (Δ) as substrates before heat-treatment. Enoyl-CoA hydratase assayed with crotonyl-CoA (●) and decenoyl-CoA (o) as substrates after heat-treatment.

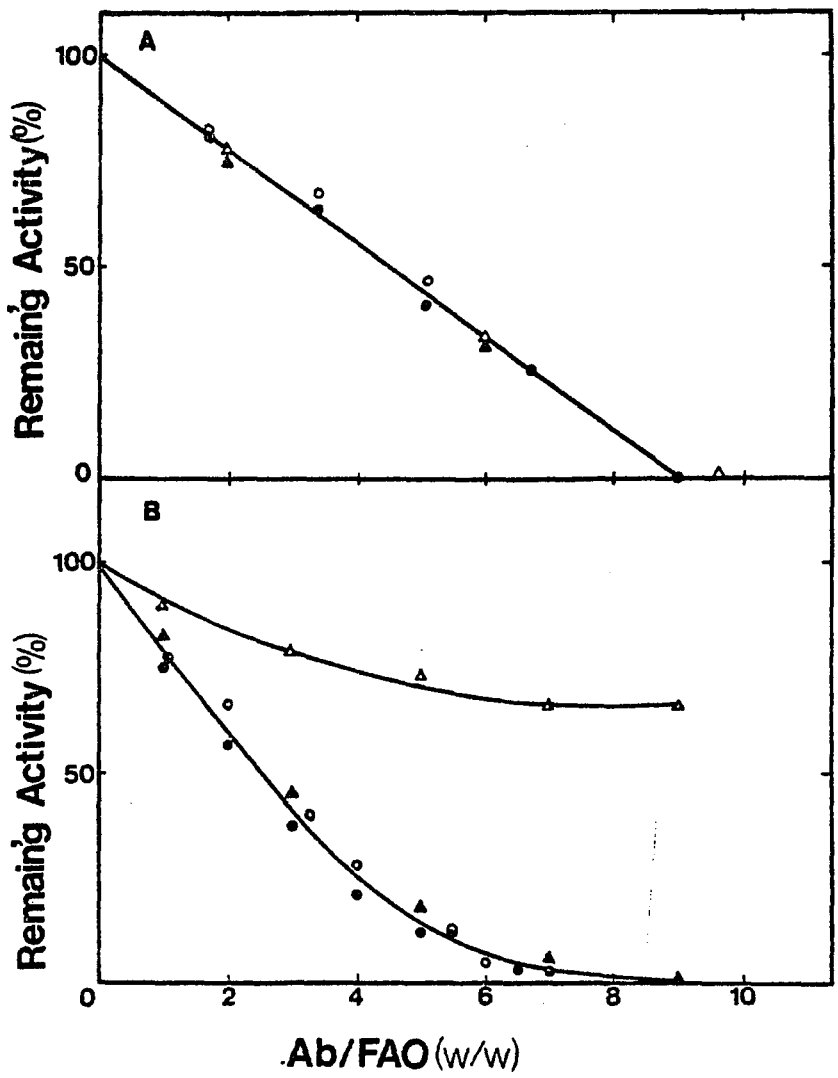
A
B
100
50
0
Remaing Activity(%)
Remaing Activity (%)
0
2
4
6
8
10
Ab/FAO (w/w)

Figure 13

Intramolecular Location of 3-Ketoacyl-CoA Thiolase and cis-Δ^3-trans-Δ^2-Enoyl-CoA Isomerase - The reaction of N-ethylmaleimide with the complex resulted in the rapid inactivation of thiolase and in the much slower inhibition of isomerase (see Table IV). The other three enzymes, i.e. enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-hydroxyacyl-CoA epimerase were not affected by the treatment with N-ethylmaleimide. Both thiolase and isomerase are significantly protected against inactivation by N-ethylmaleimide in the presence of acetoacetyl-CoA (see Table IV). The inactivation of only thiolase and isomerase by N-ethylmaleimide and the protection against this inhibition provided the means of identifying the location of these two enzymes with respect to the two subunits. For this purpose the fatty acid complex was preincubated for 40 minutes with N-ethylmaleimide in the presence of acetoacetyl-CoA. The complex was then rapidly separated from the incubation medium by rapid gel filtration (44). The pretreated complex was allowed to react for 10 minutes and 40 minutes respectively with N-[2-^{14}C] ethylmaleimide. The amount of radioactive label associated with each of the two subunits was determined after their separation by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. As shown in Fig. 14A, incubation for 10 minutes, which led to a significant inactivation of thiolase, resulted in the labeling of only the 42,000 dalton subunit. Incubation for 40 minutes caused the complete inactivation of thiolase and the partial inhibition of isomerase. Both the 78,000 and 42,000 subunits were radioactively labeled

TABLE IV

Effects of N-Ethylmaleimide on the Fatty Acid Oxidation Complex from *E. coli*

For experimental details see under "Experimental Procedures".

Enzyme	Remaining Activity (%)		
	+Acetoacetyl-CoA	-Acetoacetyl-CoA	
	40 min	10 min	50 min
3-Ketoacyl-CoA Thiolase	80	35	7
cis-Δ^3-*trans*-Δ^3-Enoyl-CoA Isomerase	85	70	35
3-Hydroxyacyl-CoA Dehydrogenase	102	100	95
Enoyl-CoA Hydratase	96	104	95
3-Hydroxyacyl-CoA Epimerase	105	95	90

Figure 14

Disc gel electrophoresis of the [^{14}C]N-ethylmaleimide-labeled fatty acid oxidation complex on 10% polyacrylamide gels in the presence of sodium dodecyl sulfate. For experimental details see under "Experimental Procedures". The complex was reacted with [^{14}C]N-ethylmaleimide for 10 minutes (A) and 40 minutes (B).

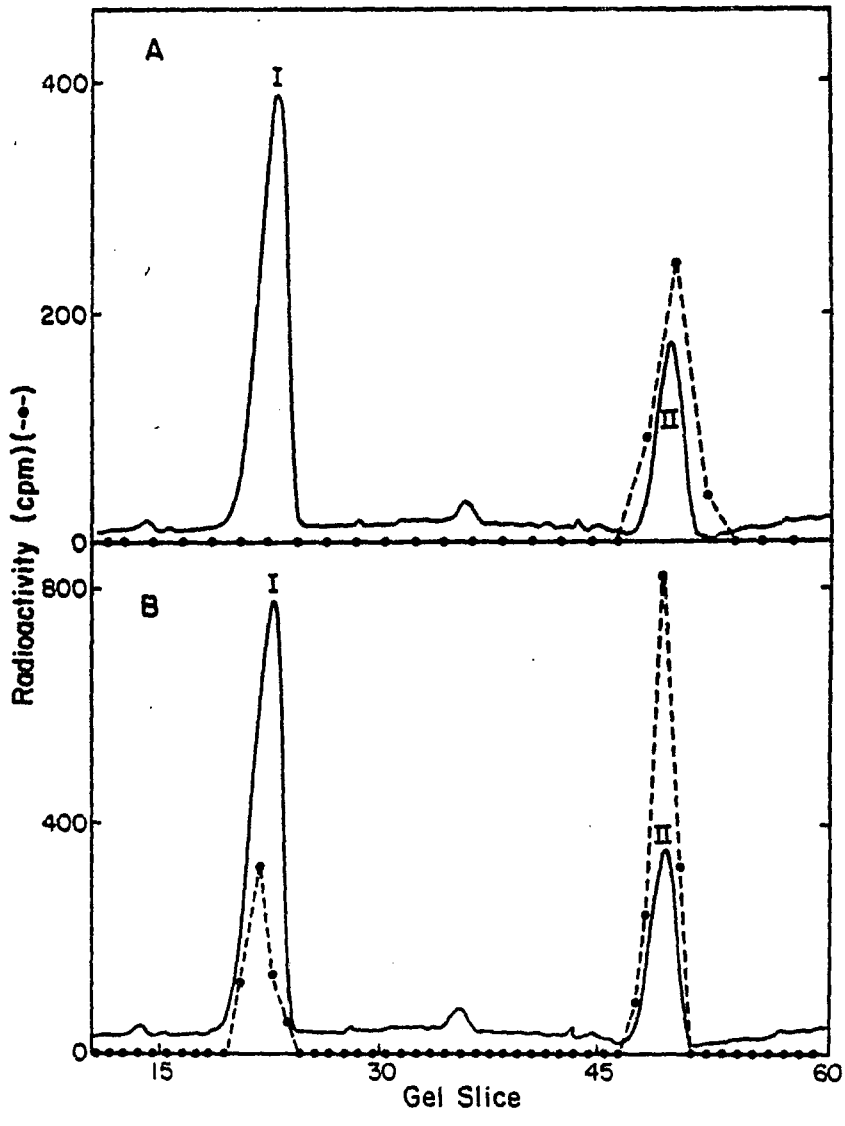
A
B
I
II
400
200
0
800
400
0
Radioactivity (cpm)(-•-)
15
30
45
60
Gel Slice

Figure 14

(see Fig. 14B). Based on these observations it is concluded that thiolase is associated with the 42,000 dalton subunit whereas cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase is most likely a component enzyme of the 78,000 dalton subunit.

Studies on the Intramolecular Localization of Enoyl-CoA Hydratase and 3-Hydroxyacyl-CoA Dehydrogenase - After successfully localizing 3-ketoacyl-CoA thiolase and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase on the 42,000 dalton and 78,000 dalton subunit respectively, we attempted to determine the subunit location of enoyl-CoA-hydratase and L-3-hydroxyacyl-CoA dehydrogenase. Our approach was based on the observation that the complex is susceptible to proteolysis. This proteolysis leads to the formation of a 35,000 dalton subunit and simultaneously to a loss of enoyl-CoA hydratase activity as mentioned above. We believe that this 35,000 dalton subunit may be a proteolytic product of the 78,000 dalton polypeptide. Thus the larger subunit may also carry the enoyl-CoA hydratase activity.

To further substantiate this hypothesis we treated the complex (1 mg/ml) at 30° with either trypsin, α-chymotrypsin, or pronase (protease concentration 1% to 5% of the complex concentration) in the presence of 1 mM NADH. The activities of 3-ketoacyl-CoA thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA were measured as a function of time. All three activities were found to decrease only slowly and in a nearly identical fashion. When the complex after treatment with different proteases was subjected

to polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate, the ratio of smaller to larger subunit was unchanged. It thus appears that proteolytic cleavage of complex to yield the 35,000 dalton subunit requires a protease with a specificity different from those used by us.

Since the 35,000 subunit is possibly produced by an endogeneous protease of E. coli, we incubated pure multienzyme complex with a homogenate of E. coli fad 5 (fad 5 mutant lacks all the enzymes of fatty acid oxidation complex). However, this incubation did not cause the inactivation of any of the component enzymes of the complex.

In another approach towards determining the subunit localization of L-3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase, the complex was to be dissociated under mild conditions and the two types of polypeptides were to be separated and analyzed for their enzymatic activities. For this purpose the multienzyme complex was treated with either 2% Triton X-100, 2% Brij, 2 M urea, 30 mM octanyl-pyranoglucoside, phospholipase A_2 or phospholipase C, 4 M KCL, 50 mM EDTA or 4 M guanidinium-hydrochloride. The resulting complex was then subjected to polyacrylamide gradient gel electrophoresis. No dissociation of the multienzyme complex was observed. It is interesting to note that in the presence of 4 M KCl the complex was insoluble.

It has recently been reported that some enzymes will renature after treatment with sodium dodecyl sulfate when the detergent is

being removed (49). However, this technique has been successful only with enzymes which are composed of a single subunit or several identical subunits. In the hope of identifying the subunit location of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase we have treated the fatty acid complex (1 mg/ml) with 2% sodium dodecyl sulfate. The sodium dodecyl sulfate treated complex was then diluted a hundred-fold with 1 M Hepes pH 8.1 containing 1 mM NADH and assayed for its different component enzymes. No crotonase, thiolase or 3-hydroxyacyl-CoA activity was detected. Hence, no further experiments were performed, aimed to separate the sodium dodecyl sulfate treated complex on polyacrylamide gradient gel and to assay the subunits in order to identify the locations of various enzymatic activities on the two subunits.

During our studies of the subunit structure of the complex an interesting observation was made. When the complex, treated with 2% sodium dodecyl sulfate + 0.02 M calcium phosphate in Tris-hydrochloride, pH 8.1, was subjected to polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate, only one polypeptide band with a molecular weight of 42,000 was observed. This observation would be explained by the possible tight association of the larger subunit, but not of the smaller subunit with insoluble calcium phosphate. The large subunit would then be immobilized and not enter the polyacrylamide gel. An attempt was made to dissociate the subunits by treatment of the complex with 1% Triton X-100. After addition of $Ca_3(PO_4)_2$, the complex was subjected to

polyacrylamide gradient gel electrophoresis. However, no separation of the smaller and the larger subunit was observed. We believe that only the sodium dodecyl sulfate treated multienzyme complex and not the Triton X-100 treated complex adsorbs to $Ca_3(PO_4)_2$. Since sodium dodecyl sulfate treatment renders the complex completely inactive, further studies based on the binding of sodium dodecyl sulfate treated subunits of the complex to $Ca_3(PO_4)_2$ were not carried out.

Recently the preparation and properties of arylazido-β-NAD^+, an NAD^+ photoaffinity analogue, have been reported (50). This analogue is composed of NAD^+ which acts as an active site directed group and of the arylazido group which is a photoreactive group. Since 3-hydroxyacyl-CoA dehydrogenase, a component enzyme of the complex, requires NAD^+ as a cofactor, we attempted to specifically label 3-hydroxyacyl-CoA dehydrogenase of the complex with this reagent. However, our preliminary experiments showed that arylazido-β-alanine NAD^+ in the presence of light did not inhibit 3-hydroxyacyl-CoA dehydrogenase significantly and that NADH did not protect this against inhibition. Therefore this technique could not be used.

In a last attempt to label the active site of 3-hydroxyacyl-CoA dehydrogenase we attempted to specifically modify an essential arginine residue suspected of functioning in the binding of NAD^+. A specific modifying agent of arginine is phenylglyoxal. Phenylglyoxal was found to inhibit 3-hydroxyacyl-CoA dehydrogenase activity (see

Fig. 15). However neither NADH nor acetoacetyl-CoA did protect against this inactivation (see Fig. 15). Additionally, phenylglyoxal was found to inhibit also crotonase and thiolase. (Figure 15 shows the inhibition of only crotonase, data for thiolase are not shown). Hence, 3-hydroxyacyl-CoA dehydrogenase could not be specifically labeled with phenylglyoxal. This experiment, however, tells us that one or more arginine groups of the complex are important for the activities of several component enzymes.

Figure 15

Effect of phenylglyoxal on the fatty acid oxidation complex from E. coli B. Purified complex (25 μg/ml) was reacted at 0°C with 10 mM phenylglyoxal in 0.3 M potassium bicarbonate (pH 8.2). 3-Hydroxyacyl-CoA dehydrogenase and crotonase activities were determined as a function of time. Crotonase activity in the absence (●) and the presence (o) of 2 mM NADH respectively. 3-Hydroxyacyl-CoA dehydrogenase in the absence (▲) and the presence (Δ) of 2 mM NADH respectively. The inactivation curve for crotonase and 3-hydroxyacyl-CoA dehydrogenase in the presence of 1 mM acetoacetyl-CoA was virtually identical to that of 3-hydroxyacyl-CoA dehydrogenase in the absence of 2 mM NADH.

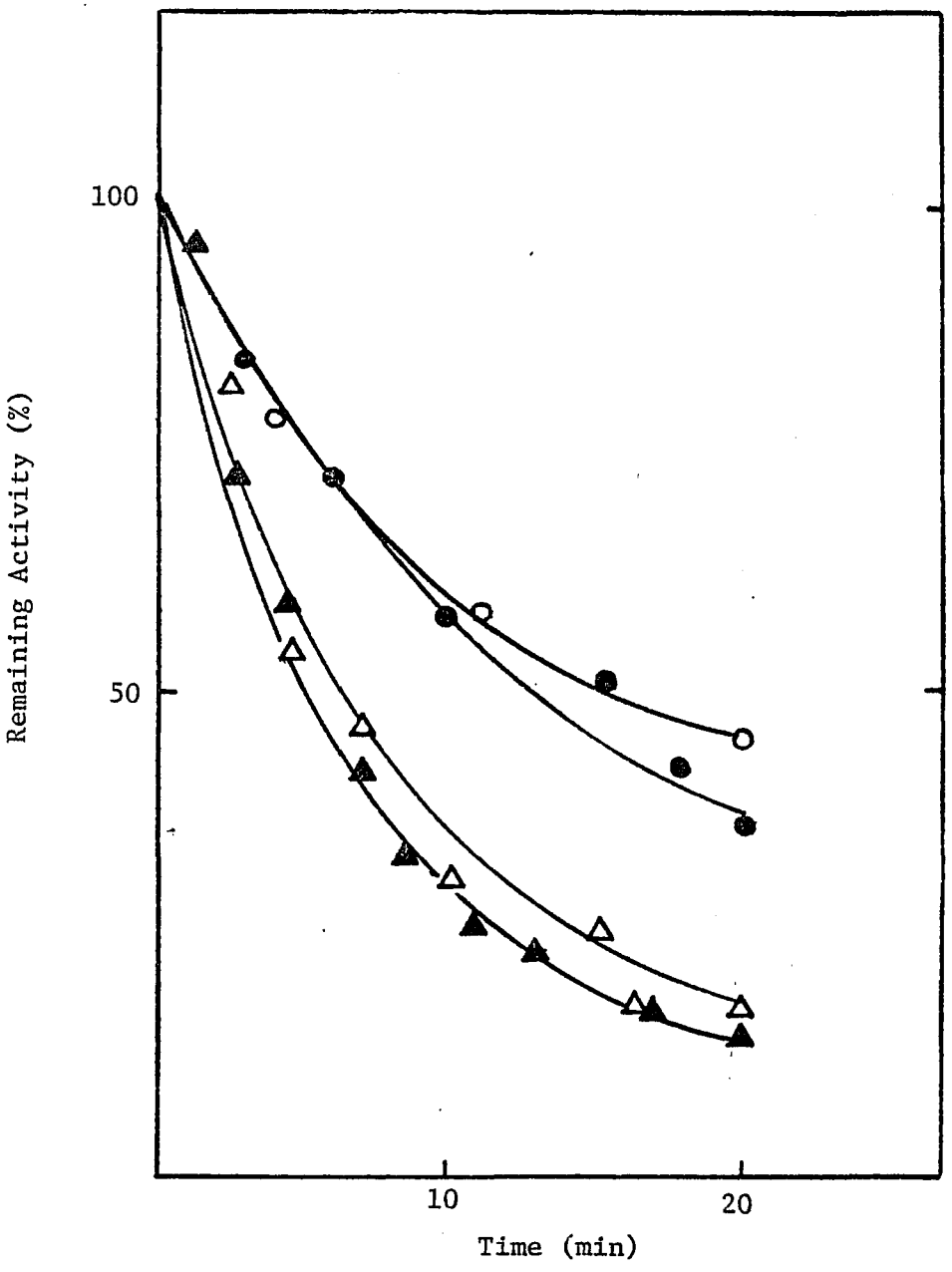

Figure 15

DISCUSSION

The data presented above demonstrates that the purified multienzyme complex of fatty acid oxidation from Escherichia coli possesses 3-hydroxyacyl-CoA epimerase and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase activities in addition to the previously identified enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase activities (20,22). Acyl-CoA synthetase and acyl-CoA dehydrogenase activities are apparently not part of the complex (20,22). The Co-purification of all five enzymes and the fact that they remain associated under a variety of conditions, support the conclusion that they are component enzymes of a multienzyme complex.

The molecular weight of the native fatty acid oxidation complex was estimated by two methods to be close to 260,000. This value agrees fairly well with previous estimates of 245,000 (21), 270,000 (20) and 320,000 (20). Two types of polypeptides with estimated molecular weights of 78,000 and 42,000 were identified as subunits of the complex. A third polypeptide with a molecular weight of 35,000, which was observed when the complex was isolated in the absence of phenyl methane sulfonyl fluoride, was most likely a proteolytic degradation product of one of the two larger subunits. Since the two subunits are present in the complex in equimolar amounts, the fatty acid oxidation complex is suggested to have an $\alpha_2\beta_2$ structure, where α and β denote the 78,000 dalton and 42,000

dalton subunit respectively. The calculated molecular weight for this complex is 240,000, a value which agrees reasonably well with the estimated value of 260,000. If additionally the phospholipid content is considered, the calculated molecular weight would be 250,000. This good agreement between the estimated and calculated molecular weight of the native complex lends further support to its proposed quaternary structure. Since limited proteolysis of the complex has been observed during the course of this study, the possibility existed that the 78,000 and 42,000 dalton subunits had been derived from a 120,000 dalton or even larger precursor. However, when the complex was isolated rapidly by immunoprecipitation in the presence of several protease inhibitors, its structure was found to be unaltered. Thus we tentatively conclude that the observed quaternary structure of the complex is not an artifact of the purification procedure. It also remains to be established whether or not post-translational processing occurs intracellularly and is in fact a necessary step in the formation of the active complex.

During our studies, rapid and parallel inactivation of all the component enzymes of the complex by Tris-HCl was observed. At first we thought that the primary amino group of Tris-HCl might be reacting with a carbonyl group on the protein to form a Schiff's base, thereby causing inactivation of the complex. If this were true, the same kind of inactivation would be expected with glycylglycine, a compound having a primary amino group with a pK value similar to that of Tris. On the other hand imidazole, which does not have a

primary amino group should not affect the complex. However the observed inactivation of the complex by imidazole and its stability in the presence of glycylglycine rule out the Schiff's base formation theory as the cause for inactivation of the complex.

Our study of how different buffers affect the complex shows that only cationic buffers like Tris-HCl and imidazole inactivate the thiolase activity of the complex, whereas anionic buffers like bicarbonate and phosphate and zwitterionic buffer like glycylglycine seem to have virtually no effect. Components of the complex of unknown importance are phospholipids which were identified as anionic cardiolipin and phosphotidylglycerol, and neutral phosphotidylethanolamine. It is possible that the inactivation of thiolase and the other component enzymes is a consequence of an ionic interaction of the cationic buffers with the anionic phospholipids of the complex. Since Tris-HCl inactivates all component enzymes of the complex, the ionic interaction may result in the disruption of the structural integrity of the complex. In addition, a study of how the complex can be protected against inactivation by Tris-HCl revealed that NAD^+ but not crotonyl-CoA or acetoacetyl-CoA protected thiolase significantly against inactivation. The binding of NAD^+ to the complex may result in a tighter complex where the anionic phospholipids of the complex become less susceptible to the ionic interaction with cationic buffers. However, binding of acetoacetyl-CoA and crotonyl-CoA apparently do not exert a similar protective effect. The observed inactivation of the complex by some of the

buffers at low concentration but not at high concentrations may be non-specific and could be due to the low ionic strength of the solution. Apart from all the speculation, however, the true mechanism of inactivation of the complex by Tris-HCl remains to be elucidated.

Preliminary tests aimed at determining the function of the phospholipids associated with the complex have been inconclusive. It is interesting to note that the phospholipids identified as components of the complex are also the major phospholipids found in the membrane of E. coli (51). Thus it could be argued that the phospholipids associated with the complex are part of the bacterial membrane which remains associated with the complex during its isolation. However, this proposal seems unlikely because the ratio of phosphotidylethanolamine to phosphotidylglycerol to cardiolipin in the bacterial membrane is 15:4:1 respectively in contrast to a ratio of 3:1:2 determined for the same phospholipids of the complex.

Since the fatty acid oxidation complex is composed of only two types of subunits, the question arises of how the five component enzymes of the complex are distributed between the two subunits. So far the association of thiolase with the 42,000 dalton subunit has been established and suggestive evidence for the location of cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase on the 78,000 dalton polypeptide has been obtained. Since three other component enzymes are associated with the complex, at least one of the two types of subunits of the complex must be a multifunctional enzyme. The

observation that prevention of proteolysis during the purification of the complex yielded a preparation with higher crotonase activity and the apparently higher susceptibility of the 78,000 dalton subunit to proteolysis suggests that crotonase may be associated with the larger subunit. Attempts to prove this hypothesis by subjecting the purified complex to proteolytic digestion with proteases failed. We believe that the complex is very resistant to most proteolytic enzymes and that proteolysis, which leads to the formation of the 35,000 dalton subunit is probably dependent on a specific protease present in E. coli.

Several attempts to dissociate the complex and locate the different enzymatic activities on the types of subunits have failed. The negative outcome of these experiments points to the tight association of the subunits in the complex and possibly reflects the instability of the subunits after their dissociation. Consequently, the dissociation of the complex without loss of activity may not be feasible or may only be achieved after conditions for stabilizing the separated subunits have been worked out.

O'Brien et al have reported the separation of thiolase, crotonase and 3-hydroxyacyl-CoA dehydrogenase activities of the complex (21). However, our study of the quaternary structure of the complex clearly demonstrates that the complex is composed of two types of subunits. Consequently the three component enzymes, thiolase, crotonase and 3-hydroxyacyl-CoA dehydrogenase cannot be associated with three different polypeptide chains.

Attempts to specifically label the active site of 3-hydroxyacyl-CoA dehydrogenase of the complex with arylazido-β-alanine NAD^+ failed. We speculate that the analogue did not function as a photoaffinity label because the Km for NAD^+ is very high and most likely arylazido-β-alanine NAD^+ did not saturate the complex sufficiently to label it substantially. Although two experiments aimed at specifically labeling the active sites of a component enzyme of the complex failed, we are confident that other successful approaches to achieve this aim can be found. Although the subunit location of the other three enzymes remains to be elucidated, we can conclude that at least one of the two types of subunits must be a multifunctional enzyme. It is tempting to speculate that the 78,000 dalton subunit may harbor both the 3-hydroxyacyl-CoA dehydrogenase and crotonase activity as does the 80,000 dalton and 75,000 dalton protein of peroxisomes (53) and glyoxysomes (54) respectively. The observation that the thiolase activity of the complex is more rapidly inactivated by Tris-HCl ($t_{1/2}$ = 2 minutes) than are the other four component enzymes, all of which yielded $t_{1/2}$ values of 4.5 minutes, agrees best with the location of thiolase on one, the 42,000 dalton subunit and the presence of all other enzymes on the 78,000 dalton polypeptide.

Overath et al. (6,9) have suggested that the genes for 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase as well as crotonase and possibly those for 3-hydroxyacyl-CoA epimerase and

cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase form an operon. This suggestion is based on the highly coordinate induction observed for the first three enzymes (6,10) and on the mapping and properties of mutants deficient in (a) all five enzymes (fad 5), (b) thiolase (fad A) and (c) 3-hydroxyacyl-CoA dehydrogenase (fad B). In view of Overath's and our findings it seems justified to hypothesize that the structural genes for the five enzymes of the complex are transcribed into one continuous mRNA which may then be translated into a large polypeptide. Dimerization of the polypeptide followed by proteolysis or vice versa would yield the complex with its $\alpha_2\beta_2$ structure.

By immunotitrating an E. coli extract with antibodies to the complex we were able to demonstrate that all soluble thiolase, 3-hydroxyacyl-CoA dehydrogenase and crotonase activities are associated with the fatty acid oxidation complex. However, an enoyl-CoA hydratase, which can be detected with 3-decenoyl-CoA as a substrate but not with crotonyl-CoA, is not part of the complex. This observation agrees with a report by Beadle et al. (19) who have identified separate short-chain and long-chain enoyl-CoA hydratases in an E. coli homogenate. Our immunological evaluation suggests that the long chain enoyl-CoA hydratase which is not precipitated by antibodies to the complex, is most likely not present in the complex. If so, the enoyl-CoA hydratase activity of the complex detected with medium and long chain substrates (20), must be due to crotonase. This situation would be similar to the one

observed in higher organisms where a separate crotonase (short chain enoyl-CoA hydratase) and long chain enoyl-CoA hydratase have been identified (55,56). These two hydratases are thought to complement each other so that the cellular enoyl-CoA hydratase activity is high with fatty acid intermediates of all chain lengths. Crotonase, which is very active with crotonyl-CoA as a substrate, exhibits little activity towards hexadecenoyl-CoA, whereas the long chain enoyl-CoA hydratase, which is virtually inactive towards crotonyl-CoA, is highly active with medium and long chain substrates (19,55). Similarly the two E. coli hydratases may complement each other, although it is not obvious why the long chain enoyl-CoA hydratase is not part of the complex. In further comparing the β-oxidation enzymes from mammalian mitochondria and from E. coli it is interesting to recognize their similarities. Both systems contain two or three acyl-CoA dehydrogenases, two enoyl-CoA hydratases and one 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase each. Even the chain length specificities of the mitochondrial and E. coli β-oxidation enzymes are very similar. The only apparent difference is the organization of the E. coli enzymes, in contrast to the mitochondrial enzymes, in a multienzyme complex. However, we believe that in mitochondria the β-oxidation enzymes are also organized in a complex, which in contrast to the one in E. coli, dissociates when the mitochondria are disrupted. The advantage of having the enzymes of β-oxidation organized in a multienzyme complex is the possible greater kinetic efficiency due to the

channeling of fatty acid oxidation intermediates from one active site to the next without equilibrating with the medium. Additionally, such a situation would prevent the accumulation of intermediates which would tie up equimolar amounts of CoASH.

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