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MULTIENZYME COMPLEXES OF FATTY ACID OXIDATION FROM WILD TYPE ESCHERICHIA COLI AND FROM FATTY ACID OXIDATION MUTANTS

*City University of New York* PH.D. 1983

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# MULTIENZYME COMPLEXES OF FATTY ACID OXIDATION FROM WILD TYPE *Escherichia coli* AND FROM FATTY ACID OXIDATION MUTANTS

by

Ajay Pramanik

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.

1983

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.

7/26/83
Date

Chairman of Examining Committee

August 4, 1983
Date

Executive Officer

Supervisory Committee

ABSTRACT

MULTIENZYME COMPLEXES OF FATTY ACID OXIDATION FROM WILD TYPE Escherichia coli AND FROM FATTY ACID OXIDATION MUTANTS

by

AJAY PRAMANIK

Adviser: Professor Horst Schulz

An E. coli mutant (fadB64) with a defective L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) which is unable to grow on long chain fatty acids as the sole carbon source, was shown to possess a fatty acid oxidation complex that contains five β-oxidation enzymes including L-3-hydroxyacyl-CoA dehydrogenase. The fatty acid oxidation complex from this mutant was purified to near homogeneity and the complex from its parental strain was highly purified to near homogeneity. A comparative study of these two complexes and that from E. coli B demonstrated the immunological and gross structural identity of all three fatty acid oxidation complexes despite significant differences between their specific activities. A kinetic evaluation of the complexes led to the conclusion that the mutation has affected the active site of L-3-hydroxyacyl-CoA dehyrogenase so that it is inactive with acetoacetyl-CoA as a substrate, but exhibits an increasing percentage of the parental dehydrogenase activity with increasing chain length of the substrate. However, even with 3-ketodecanoyl-CoA as a substrate the mutant complex is 20-times less active than the wild-type complex. This behavior is apparently due to a strong dependence

of the maximal velocity on the chain length of the substrate whereas the $K_m$ value for the substrate seems to be little or not at all affected by the mutation.

An E. coli strain (RS 3084) with a mutation in the fadABC region was found to contain the parental level of long-chain enoyl-CoA hydratase while being devoid of any of the enzyme activities associated with the fatty acid oxidation complex. However, other fadABC mutants including mutant fad5 did neither contain long-chain enoyl-CoA hydratase nor the fatty acid oxidation complex. These observations together suggest that long-chain enoyl-CoA is a gene product of the fadABC operon but is not associated with the fatty acid oxidation complex.

The rapid inactivations of all component enzymes of the fatty acid oxidation complex by Tris-hydrochloride has been observed. The half time of the inactivation of thiolase was faster than those of the four other component enzymes which were inactivated at equal rates. Crotonyl-CoA was found to protect both enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase, but not thiolase against this inactivation. These observations suggest the locations of enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase to be in close proximity, possibly on one subunit which does not seem to harbor thiolase.

Treatment of the complex with acetic anhydride results in the inactivation of all of its component enzymes. Crotonyl-CoA protects only enoyl-CoA hydratase against this inactivation. Attempts to specifically label the active site of enoyl-CoA hydrates with [1-$^{14}$C]acetic anhydride failed because of the extensive non-specific

labeling of the complex.

Evidence is also presented for the absence of cis-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase activities in the peroxisomal bifunctional enzyme which exhibits enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase activities.

ACKNOWLEDGEMENTS

I am grateful to Professor Horst Schulz for his advise, encouragement and guidance throughout the course of this work.

My sincere thanks are to my loving sister, Dr. Sujata Roy and my brother-in-law, Dr. Asit Roy for their valuable suggestions. I am grateful to my mother and late father. I would highly appreciate the sacrifices, encouragement and inspiration of my beloved wife Bani.

Finally, I am thankful to Mrs. Barbara Alexander for carefully typing the thesis.

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# INTRODUCTION

It was first suggested by Knoop in 1904 that fatty acids are degraded in biological system through β-oxidation. The mechanism involves a sequence of four reactions in which two carbon atoms are removed from a fatty acid as acetyl-CoA. Subsequent investigations were done by Lynen, Green, Ochoa and others in mammalian tissues. These studies led to the formulation of a universal pathway by which fatty acids are successively oxidized to acetate units (for a review see Ref. 1). The steps in this pathway are:

1) The formation of fatty acyl-CoA from free fatty acids and CoA catalyzed by acyl-CoA synthetase (EC 6.2.1.2);
2) dehydrogenation of acyl-CoA to 2-trans-enoyl-CoA catalyzed by acyl-CoA dehydrogenase (EC 1.3.99.2);
3) hydration of 2-trans-enoyl-CoA to L-3-hydroxyacyl-CoA catalyzed by enoyl-CoA hydratase (crontonase) (EC 4.2.1.17);
4) oxidation of L-3-hydroxyacyl-CoA to 3-ketoacyl-CoA catalyzed by L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35); and
5) cleavage of 3-ketoacyl-CoA by 3-ketoacyl-CoA thiolase (EC 2.3.1.16) to acetyl-CoA and an acyl-CoA two carbon shorter in length.

The resulting acyl-CoA compounds pass through the β-oxidation cycle again as shown in Fig 1, until they are completely degraded to acetyl-CoA.

The oxidation of unsaturated fatty acids containing cis double bonds was believed to require, additionally cis-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA

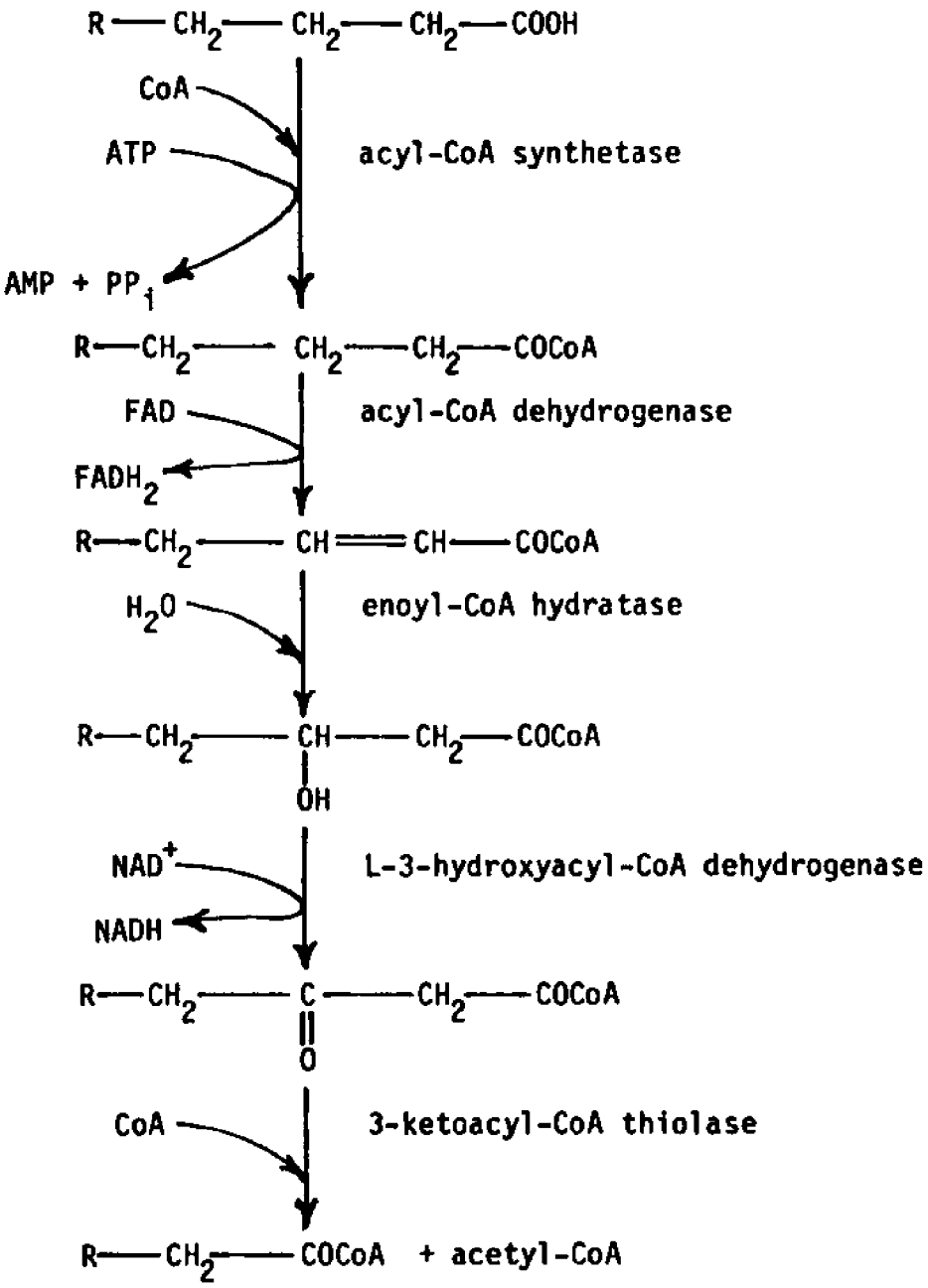


Figure 1: Pathway of Fatty Acid Oxidation.

isomerase (EC 5.3.3.1) and 3-hydroxyacyl-CoA epimerase (EC 5.1.2.3) (2). The former enzyme acts on 3-cis-enoyl-CoA intermediates derived from unsaturated fatty acids contaning cis double bonds extending from odd-numbered carbons. The products of the isomerization reaction are 2-trans-enoyl-CoA compounds (3) that can be further degraded by β-oxidation. Unsaturated fatty acids with cis double bonds extending from even-numbered carbons were assumed to be chain-shortened by β-oxidation to 2-cis-enoyl-CoA's which can be hydrated by enoyl-CoA hydratase to D-3-hydroxyaycl-CoA derivatives (4). The latter compounds are isomerized by 3-hydroxyacyl-CoA epimerase to the L-isomers (2) which can be completely degraded by β-oxidation (see Fig. 2A). Although cis-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase are present in mitochondria, the degradation of unsaturated fatty acids via the proposed pathway has neither been proven nor disproven.

In 1979 Kunau and Dommes (5) proposed a modified pathway for the metabolism of 4-cis-decenoyl-CoA by acyl-CoA dehydrogenase II and a newly detected NADPH dependent 2,4-dienoyl-CoA reductase (Fig. 2B). However, the modified pathway has not been generally accepted until recently, when Cuebas and Schulz (6) convincingly demonstrated by use of a reconstituted β-oxidation system and rat heart mitochondria that 2-trans,4-trans-decadienoyl-CoA, but not a mixture of 2-trans, 4-cis-decadienoyl-CoA and 2-cis,4-cis-decadienoyl-CoA can be directly degraded via β-oxidation. However, all three isomers of 2,4-decadienoyl-CoA can be oxidized by the proposed pathway of Kunau and

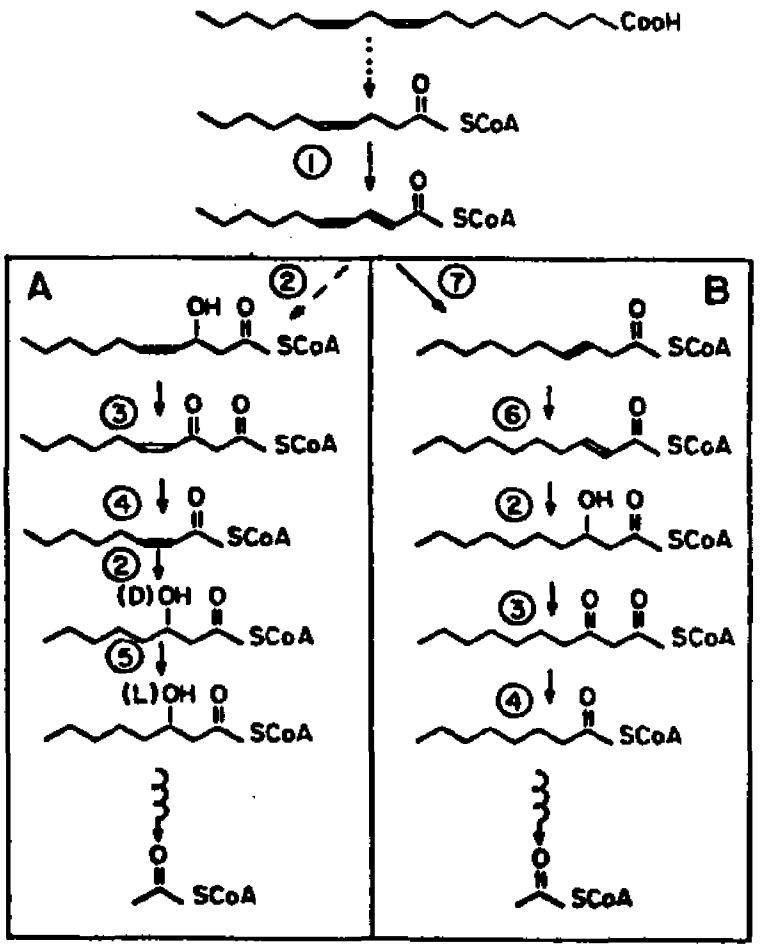


Figure 2. Linoleic Acid Degradation. A: Pathway proposed by Stoffel and Caesar (3) B: Modified pathway proposed by Kunau and Dommes (5) and by Cuebas and Schulz (6). Reactions catalyzed by: 1, acyl-CoA dehydrogenase; 2, enoyl CoA hydratase; 3, L-3-hydroxyacyl-CoA dehydrogenase; 4, 3-ketoacyl-CoA thiolase; 5, 3 hydroxyacyl-CoA epimerase; 6, cis-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA isomerase; 7, 2,4-dienoyl-CoA reductase.

Dommes (5). Linoleate degradation by the modified pathway which involves the NADPH-linked reduction of 2-trans,4-cis-decadienoyl-CoA catalyzed by 2,4-dienoyl-CoA reductase is shown in Fig. 2B. Interestingly, this pathway raises a new question about the physiological function of 3-hydroxyacyl-CoA epimerase.

The association of β-oxidation with mitochondria has been known for a long time (1). However, more recently peroxisomes and glyoxisomes were shown to also degrade long-chain fatty acids (7,8). Hepatic peroxisomal and mitochondrial fatty acid β-oxidation differ in many ways. For example, peroxisomal -oxidation is carnitine independent, whereas mitochondrial β-oxidation of long-chain fatty acid is carnitine dependent (9,10). Instead of the FAD-linked acyl-CoA dehydrogenase of mitochondria, peroxisomes in protozoa (11), liver (12,13,) and yeast (14,15,) as well as seed glyoxisomes (16) contain a FAD-linked acyl-CoA oxidase that transfers electrons to $O_2$ to form $H_2O_2$, which in turn may be decomposed by catalase. It appears that hepatic peroxisomal β-oxidation is specific for long-chain fatty acyl-CoAs, whereas mitochondria can degrade both long-chain and short-chain substrates. Interestingly, the end products of peroxisomal β-oxidation appear to be acetylcarnitine and octanoylcarnitine. These carnitine derivatives can passively diffuse out of the peroxisomes into the cytoplasm (9,10,17,18). Thus peroxisomal β-oxidation may provide acetyl units for other cellular synthetic purposes and chain-shortened acylcarnitine for futher oxidation in mitochondria.

β-Oxidation in glyoxysomes is not carnitine dependent (19).

Acetyl-CoA from β-oxidation is utilized in the same glyoxysomes by the glyoxylate cycle to form succinate (20).

In 1951 it was first suggested by Silker and Rittenberg (21) that the enzymes of fatty acids in E. coli can be induced. But subsequent studies (22,23) failed to demonstrate the presence of β-oxidation enzymes in other microorganisms. The presence of the enzymes of β-oxidation in an extract of E. coli was not established until 1967 when Overath and coworkers (24,25,26) demonstrated that the enzymes of fatty acid oxidation are highly induced in E. coli when cells are grown on long-chain fatty acids as the sole carbon source. The synthesis of the enzymes of fatty acid degradation in E. coli. is strongly repressed by the presence of glucose in the growth medium (24). Inducers like oleate had no benefical effect on the repression by glucose but addition of cAMP did relieve this repression (27). This observation might explain why the presence of the β-oxidation enzymes in E. coli extract could not be demonstrated in previous studies (22,23). The synthesis of the enzymes of fatty acid degradation is most probably regulated by a mechanism similar to that of the lac operon, first postulated by Jacob and Monod (28). Also, like many other inducible enzymes systems, expression of the enzymes of the fad system are subject to catabolite repression (27,9).

Overath et al. (24,25), Weeks et al. (26) and Klein et al. (29) 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-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA isomerase are induced when E. coli cell are grown on long chain fatty acids as the sole carbon source. Extensive genetic work (29,30,31) has led to the conclusion that the structural genes for the β-oxidation enzymes are located on at least five separate region of the E. coli chromosome (see Fig. 3). The fadD gene lacks acyl-CoA synthetase and cannot be induced to synthesize the fad enzymes. This observation led Overath and coworkers (24) to suggest that the fatty acyl-CoA derivatives rather than fatty acids serve as inducers of the enzymes of fatty acid oxidation.

When E. coli cells were grown on long-chain fatty acids as a sole carbon source, ideal coordinate induction was observed only for thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, while acyl-CoA synthetase and acyl-CoA dehydrogenase (24,26) showed slightly lower rates of induction. On the basis of the above results, Overath and coworkers hypothesized that genes for thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase form a unit expression, "an operon", with a common operator, while the genes of acyl-CoA synthetase and acyl-CoA dehydrogenase are not controlled by this operon and possibly have their own operators. However, the expression of all fad genes is possibly regulated by the same regulator gene (24).

Several fatty acid degradation mutants (fad5, fadB64, fadA30) have been isolated by Overath et al., (24,25) which carry mutations in the 85-minute region (ABC region of Fig. 3) on the E. coli.

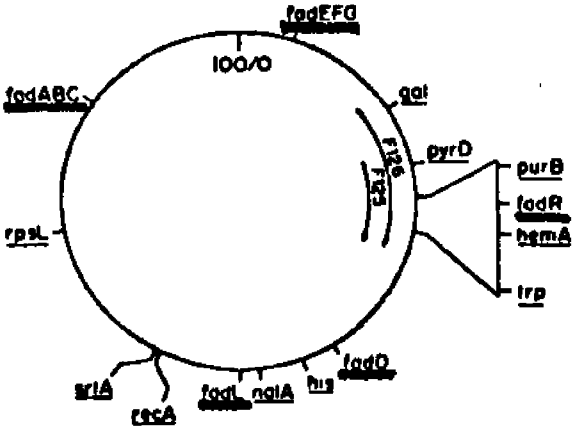


Figure 3: Location of fad Mutations on the Genetic Map of E. coli.

| Mutant | Characteristics |
|---|---|
| fadA | Deficient in 3-ketoacyl-CoA thiolase |
| fadB | Deficient in L-3-hydroxyacyl-CoA dehydrogenase |
| fadC** | Deficient in enoyl-CoA hydratase |
| fadD | Deficient in acyl-CoA synthetase |
| fadE | Deficient in electron transfer flavoprotein |
| fadF | Deficient in butyryl-CoA dehydrogenase |
| fadG | Deficinet in acyl-CoA dehydrogenase |
| fadL | Deficient in fatty acid permease |
| fadR | Deficient in a regulatory protein: a repressor |
| fad5* | Deficient in thiolase, enoyl-CoA hydratase L-3-hydroxyacyl-CoA dehydrogenase, cis-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA isomerase, and 3-hydroxyacyl-CoA epimerase |

* Location of fad5 mutation not shown in Fig. 2. However, it maps close to the fadA and the fadB genes.

** No fadC mutant has yet been isolated.

chromosome. The mutants were isolated after mutagenesis with N-methyl-N'-nitro-N-nitroso-guanidine (32) by plating first on minimal glucose plates and replica plating on minimal oleate. Mutant fadB64 has no detectable L-3-hydroxyacyl-CoA dehydrogenase activity when assayed with acetoacetyl-CoA as a substrate and fadA30 is devoid of acetoacetyl-CoA thiolase activity. However, O'Brien et al., (33) reported that 3-ketoacyl-CoA thiolase and L-3-hydroxyacyl-CoA dehydrogenase can be detected in fadA30 and fadB64 mutants respectively if substrates with sixteen carbon chains are used in the enzyme assays. Mutant fad5 is devoid of enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, thiolase, cis-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase, but has inducible wild type levels of acyl-CoA synthetase and acy-CoA dehydrogenase (24,25). Mapping data suggest that the fad5, fadA and fadB genes are closely linked and possibly form an operon (25) (see Fig. 3).

The location of other fad genes on the E. coli. chromosome is interesting. The fadD gene is located on a region well separated from the fadA, fadB and fad5 regions. The fadE, fadF and fadG genes map close together at a locus distinct from all other fad loci (Fig. 3). The fadF and fadG genes code for butyryl-CoA dehydrogenase and acyl-CoA dehydrogenase respectively. Nunn and coworkers (30,34) identified a new gene locus (fadL) which may be the structural gene for a protein that facilitates the entry of long-chain fatty acids through the cytoplasmic membrane (30,34). The fadR gene codes for a regulatory protein, possibly a repressor. It

has been demonstrated by Simons et al., (35,36) that *fadR* gene maps at a fifth *fad* locus and that it codes for a polypeptide acting like a repressor.

The fatty acid degradation (*fad*) regulon in *E. coli* is a complex entity composed of a regulatory gene and the structural genes for the enzymes of fatty acid oxidation. Our knowledge about the structural and kinetic properties of enzymes of the *fad* regulon are limited. Overath et al., (24) and Samuel et al., (37) have partially purified acyl-CoA synthetase from *E. coli*. The latter enzyme is partially membrane bound like the mammalian synthetase (38).

In a previous publication the isolation of a multienzyme complex of fatty acid oxidation from *E. coli* B cells was reported and it was shown to contain enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase activities (39). In a subsequent publication it was additionally shown to posess 3-hydroxyacyl-CoA epimerase and *cis*-$_{\Delta}^{3}$-*trans*-$_{\Delta}^{2}$-enoyl-CoA isomerase activities (40). The complex has a native molecular weight of 260,000 and contains two types of subunits with molecular weights of 78,000 and 42,000, respectively. The complex appears to be composed of two copies each of the two subunits (41). The complex contains additionally the phospholipids phosphatidylethanolamine, phosphotidylglycerol, and cardiolipin (41). By specific labeling with N-[1-$^{14}$C]ethylmaleimide, it was shown that 3-ketoacyl-CoA thiolase resides on the 42,000-dalton subunit, whereas the *cis*-$_{\Delta}^{3}$-*trans*-$_{\Delta}^{2}$-enoyl-CoA hydratase is most likely associated with the 78,000-dalton polypeptide (41).

Beadle et al.,(42) have recently presented evidence for the existence of two enoyl-CoA hydratase activities in E. coli K12. A partially membrane-associated enoyl-CoA hydratase has a preference for long chain substrates, whereas a cytoplasmic hydratase shows activity towards substrates of varying chain length (42). Immunotitration of the fatty acid oxidation complex present in a soluble E. coli B extract with antibodies raised against the purified complex showed that a long-chain enoyl-CoA, possibly identical with that described by Beadle et al.,(42), is not part of the multienzyme complex (41).

In the present thesis the properties of the fatty acid oxidation complex from of E. coli have been studied. A comparative study of the complexes from wild-type E. coli B and K12 as well as from fatty acid oxidation mutants demonstrated their immunological and gross structural identity. A kinetic evaluation of the complexes from mutant fadB64 with a defective L-3-hydroxyacyl-CoA dehydrogenase and from its parental strain led to the suggestion that the mutation may have affected the active site of L-3-hydroxyacyl-CoA dehydrogenase.

## MATERIALS AND METHODS

Chemicals. Ethyl chloroformate, triethylamine, diketene, pyruvic acid and trans-$\Delta^2$-decenoic acid were obtained from Aldrich Chemical Company. CoA, $NAD^+$, NADH were purchased from PL-Biochemicals. L-3-Hydroxyacyl-CoA dehydrogenase (EC1.1.1.35) was bought from Boehringer Mannheim Corp. 3-Octyn-1-ol was obtained from Pfaltz and Bauer. Lactate dehydrogenase, bovine serum albumin, catalase, ovalbumin were purchased from Sigma. Apoferritin was bought from Miles Laboratories, Inc. Sephadex G-50, polyacrylamide gradient gels (4-30%) were supplied by Pharmacia, Inc. [1-$^{14}$C]Acetic anhydride was bought from New England Nuclear. NCS tissue solubilizer was supplied by Amersham Co. All other chemicals were of reagent grade. Tetrahydrofuran, ethyl chloroformate and diketene were distilled before use. 3-Ketoacyl-CoA thiolase was isolated from pig heart according to the procedure of Staack et al., (43). Bovine liver enoyl-CoA hydratase was purified according to Steinman and Hill (44). The dye for protein assays was obtained from Bio Rad.

Synthesis of Substrates. cis-3-Octenoic acid was prepared from 3-octyn-1-ol, by the procedure of Stoffel and Ecker (45). trans-2-Hexenoic acid was synthesized from n-butanal and malonic acid according to the procedure of Linestead et al., (46). DL-3-Hydroxydodecanoic acid was obtained by reduction of ethyl 3-ketododecanoate with $NaBH_4$ followed by alkaline hydrolysis. Ethyl 3-ketododecanoate was prepared by an established procedure (47). Acetoacetyl-CoA (48)

and crotonyl-CoA (49) were synthesized as described. The CoA derivatives of 2-hexenoic acid, 2-decenoic acid, and DL-3-hydryoxy-dodecanoic acid were prepared by the mixed anhydride procedure of Goldman and Vagelos (50). 3-Ketohexanoyl-CoA and 3-ketodecanoyl-CoA were formed enzymatically from the corresponding enoyl-CoA compounds (51). The concentrations of all CoA derivatives, except for 3-ketohexanoyl-CoA and 3-ketodecanoyl-CoA, were determined by the method of Ellman (52) after cleaving the thioester bond with hydroxylamine at pH 7. The concentrations of 3-ketohexanoyl-CoA and 3-ketodecanoyl-CoA were determined by measuring the oxidation of NADH at pH 7 in the presence of L-3-hydroxyacyl-CoA dehydrogenase. The preparation of antibodies against the multienzyme complex of fatty acid oxidation has recently been described (41).

Growth Conditions of Bacterial Strains. E. coli K-12 YMel (CGSC strain #5032) was obtained from Yale University School of Medicine, Department of Human Genetics. Strains fadB64, fad5, and fadA30 derived from E. coli K-12 YMel, were provided by Dr. P. Overath, Max Planck Institut fur Biologie, Tubingen, West-Germany. Strain fadB64 and E. coli K-12-YMel were grown on a minimal medium consisting of M 9 mineral salts, oleate (0.1%; v/v), Triton X-100 (0.5%, w/v) and tryptone (0.5%, w/v). Cells taken from a slant were grown overnight in nutrient broth (10 ml). An aliquot (1 ml) of this cell suspension was added to 10 ml of minimal medium and grown to stationary phase. The resulting culture was diluted 25-fold into minimal

medium and grown again to stationary phase. Finally the cell suspension was diluted 8-fold with minimal medium to a total volume of 2-liters. The cells were grown to an absorbance of 1.4 at 420 nm (protein concentration 0.4 mg/ml) and stored at 4°C overnight. During this period of time the protein concentration increased 5 to 30-fold depending on the E. coli strain. More importantly the specific activity of enoyl-CoA hydratase increased approximately 2-fold. The cells were then harvested by centrifugation, washed twice with M-9 mineral salts medium and stored at -70°C.

All RS strains, defective in the fatty acid degradation enzymes, were supplied by Dr. W. Nunn of the University of California at Irvine. These strains were first grown overnight in nutrient broth (N.B.) medium in the presence of 0.003% (w/v) kanamycin and 0.002% (w/v) tetracycline. The resulting RS cultures were then used to pick single colonies from plates of solid nutrient broth medium containing the above mentioned antibioties. E. coli B (ATCC 11775) and all other strains were routinely grown in M-9 mineral media with oleate and acetate as carbon sources as described by Overath et al., (24,25).

Purification of the Fatty Acid Oxidation Complex. Fatty acid oxidation complexes were isolated from E. coli strains B, K12 and fadB64. Cells (18 g) were suspended in 36 ml of cell suspension buffer and homogenized by sonication for 5 x 1 minute at 0°C. Immediately before sonication phenyl methane sulfonyl fluoride (PMSF)

was added to the suspension to a final concentration of 1 mM. Thereafter the same amount of PMSF was added after each one-minute sonication so that the final concentration of PMSF reached 10 mM. Purification of the complex to homogeneity from the crude homogenate was achieved by chromatography on a phosphocellulose column (5 x 44 cm) as described in detail by Binstock and Schulz (53). In the case of strain fadB64 cells (9 g) were used and the homogenate after centrifugation was directly applied to the phosphocellulose column (2.5 x 44 cm) without being dialyzed first.

Protein and Enzyme Assays. Protein concentrations were determined by the method of Lowry (54) or by the Bio Rad Protein Assay, a dye-binding assay based on the method of Bradford (55). Enoyl-CoA hydratase was assayed by following the decrease in absorbance at 263 nm due to the hydration of the $\Delta^{2,3}$-double bond of the substrate, as described in principal by Lynen and Ochoa (56) and as detailed by Binstock and Schulz (53). L-3-Hydroxyacyl-CoA dehydrogenase was routinely assayed by measuring the decrease in absorbance at 340 nm due to the dehydrogenation of NADH, as detailed by Binstock and Schulz (53). 3-Ketoacyl-CoA thiolase was assayed by measuring the decrease in absorbance at 303 nm due to the disappearance of the $Mg^{2+}$-enolate complex of the substrate, as described by Binstock and Schulz (53). L-3-Hydroxyacyl-CoA dehydrogenase was also assayed in the forward direction. First the L-3-hydroxyacyl-CoA substrates were generated enzymatically by reacting 47 μM $\Delta^{2}$-enoyl-CoA in 0.2 M potassium phosphate (pH 8) with

beef liver enoyl-CoA hydratase (0.25 U). After the reaction had reached equilibrium it contained 24 μM L-3-hydroxyacyl-CoA to which were added 0.22 mM CoASH, 1 mM $NAD^+$ and pig heart 3-ketoacyl-CoA thiolase (3 mU). The assay was started by the addition of fatty acid oxidation complex and was followed by measuring the increase in absorbance at 340 nm. 3-Hydroxyacyl-CoA epimerase was assayed by following at pH 8 the increase in absorbance at 340 nm due to the formation of NADH (53). The assay is based on the epimerase-dependent formation of L-3-hydroxydodecanoyl-CoA coupled to its dehydrogenation and thiolytic cleavage in the presence of L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, $NAD^+$ and CoASH. cis-$\Delta^3$-trans-$\Delta^2$-Enoyl-CoA isomerase was assayed at pH 8 by following the increase in absorbance at 340 nm due to the formation of NADH (53). The assay is based on isomerase-dependent formation of trans-$\Delta^2$-octenoyl-CoA coupled to its hydration, dehydrogenation and finally thiolytic cleavage in the presence of crotonase, L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, $NAD^+$ and CoASH. A unit of enzyme activity was defined as that which catalyzes the conversion of one micromole of substrate to product per minute. All lines in kinetic plots were drawn based on least-squares treatment of the data.

Immunoprecipitation of the Purified Multienzyme Complex. Double immunodiffusion experiments, were performed according to Ouchterlony (57). In these experiments E. coli B complex (65 μg), K-12-Y-Mel complex (68 μg) and E. coli fadB64 complex (40 μg) were

placed in the outer wells of a plate whereas the antibodies (210 μg) raised against the pure complex from E. coli B were placed in the inner well. The plate was kept overnight at room temperature in a desiccator containing distilled water. The plate was then washed overnight with 1 liter of saline solution containing 0.85% NaCl and 20 mM potassium phosphate (pH 7.4). The Ouchterlony plates were stained with the Amido Black (0.1% w/v) in 7% acetic acid for 2-24 hrs., destained for several days with 7% acetic acid and stored in a desiccator.

Gel Electrophoresis . Electrophoresis of native fatty acid oxidation complex was performed on 4 to 30% polyacrylamide gradient gels (7.6 x 7.5 cm). After application of the protein samples, the gels were subjected to electrophoresis for 5 hrs at 100V (constant voltage) in a Pharmacia GE-4 electrophoresis apparatus. The electrophoresis buffer was 0.09M Tris boric acid (pH 8.35) containing 2.5 mM EDTA. After completion of electrophoresis narrow slices cut vertically from each side of the slab were stained for 40 min with 1% Coomassie Brilliant blue and destained for 1 hr in 7% acetic acid, after which time the protein bands were visible. The remainder of the gel was cut horizontally into several segments so that each of the main protein bands was associated with a separate slice. All slices were extracted for 18 hrs in a minimal volume of 0.02 M potassium phosphate (pH 8) containing 10% glycerol, bovine serum albumin (1 mg/ml), and 5 mM mercaptoethanol. Polyacrylamide disc gel electrophoresis in the

presence of sodium dodecyl sulfate was performed at pH 8.3 and at a constant current of 2.5 mA/gel as outline in principle by Laemmli (58).

Immunoprecipitation of the Fatty Acid Oxidation Complex from Cell Homogenates. Either E. coli K-12 YMel (0.5 g) induced for the enzymes of fatty acid oxidation or strain fad5 (0.5 g) grown on oleate plus acetate were suspended in 1 ml of 0.2 M $KP_i$ (pH 8.0) containing 5 mM phenylmethane sulfonyl fluoride. The cell suspensions were sonicated for 2 min at 0°C and centrifuged at 120,000 x g for 2 hours at 0°C. Antibodies raised against the multienzyme complex of fatty acid oxidation from E. coli B were added to the supernatant (125 μg Ab per mg of supernatant protein). After 5 min at room temperature the resulting antibody-antigen complex was isolated by centrifugation at 120,000 x g for 45 min at 4°C. The antibody-antigen precipitate was washed with 0.2 M $KP_i$ (pH 8.0) containing 0.1% deoxylcholate. The remaining precipitate was subjected to gel electrophoresis in the presence of sodium dodecyl sulfate.

Labelling of the Fatty Acid Oxidation Complex with [1-$^{14}$C]Acetic Anhydride. The multienzyme complex of fatty acid oxidation was first filtered as described by Penefsky (59) through a small Sephadex G-50 column (1 ml) equilibriated with 0.1 M $KP_i$ (pH 7.0). To the filtrate (0.4 mg in 0.3 ml) 0.1 ml of 0.1 M $KP_i$ (7.0), crotonyl-CoA to a final concentration of 1 mM followed by acetic anhydride in tetrahydrofuran

to a final concentration of 5 mM was added. After 3 min at 0°C the protein was separated from the incubation mixture by the centrifugation-gel filtration procedure of Penefsky (59). A small aliquot of this filtrate was used for protein determination and for enzyme assays. The remainder was divided into two equal volumes. One part was allowed to react with [1-$^{14}$C]acetic anhydride at a final concentration of 5 mM. The other part was reacted identically except that additionally 1 mM of crotonyl-CoA was present. Both mixture were kept for 2 min at 0°C. The protein was separated from the incubation mixture by centifugation-gel filtration as described (59). The fatty acid oxidation complex (20-30 µg) labeled with [1$^{14}$C]acetic anhydride was subjected to polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate at pH 8.3 (58). Gels were stained with Coomassie blue and destained in 7% acetic acid. The gels were first scanned at 500 nm and then sliced into 2 mm wide segments. Each segment was incubated with 1 ml of a mixture of NCS tissue solubilizer and water (9:1) at 50 °C overnight. The resulting fractions were counted in a liquid scintillation counter after addition of 10 ml each of a toluene-based counting solution.

## RESULTS

Study of the Fatty Acid Oxidation Complex With Cell-Free Extracts of Fatty Acid Oxidation Mutants. The fadABC region of the E. coli chromosome is known to code for the fatty acid oxidation complex (24,29). Several fatty acid oxidation mutants known to have mutations in the fadABC region have been studied in the hope of finding a complex devoid of one or several of its enzyme activities. Cell-free extracts of these mutants were assayed for enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase activities with both four-carbon chain substrates ($C_4$) and ten-carbon chain substrates ($C_{10}$). Strain K-12YMel is the parental strain of mutants fadA30, fadB64 and fad5. Strain RS 3065 is the parental strain of mutants RS 3081 and RS 3110, whereas strains RS 3084 and RS 3112 were derived from strain RS 3072.

Mutant fadB64 has been found to be devoid of L-3-hydroxyacyl dehydrogenase when assayed with acetoacetyl-CoA as a subsrate (24,25). However in a later study, preliminary evidence for the presence of an activity with long-chain substrates was obtained (33). The data shown in Table I clearly demonstrate this mutant to be inactive toward acetoacetyl-CoA, but to exhibit 5% of the wild-type activity with 3-ketodecanoyl-CoA as a substrate. The 3-ketoacyl-CoA thiolase activity of this mutant is only half of that of the parent strain, whereas its enoyl-CoA hydratase activity is slightly elevated. Mutant fadA30 which had been reported to be devoid of thiolase activity exhibited low activities with short-chain and medium chain

TABLE I

Enoyl-CoA Hydratase, 3-Hydroxyacyl-CoA Dehydrogenase and 3-Ketoacyl-CoA Thiolase Activities in Homogenates of Different Fatty Acid Oxidation Mutants.

| | Enoyl-CoA Hydratase | | | | L-3-Hydroxyacyl-CoA dehydrogenase | | | | 3-Ketoacyl-CoA Thiolase | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | $C_4$[a] | | $C_{10}$[b] | | $C_4$[c] | | $C_{10}$[d] | | $C_4$[c] | | $C_{10}$[d] | |
| | U/mg | Relative Activities (%) | U/mg | Relative Activities (%) | U/mg | Relative Activities (%) | U/mg | Relative Activities (%) | U/mg | Relative Activities (%) | U/mg | Relative Activities (%) |
| K-12-YMel | 1 | 100 | 0.42 | 100 | 0.82 | 100 | 1.38 | 100 | 0.15 | 100 | 0.99 | 100 |
| fadB64 | 1.24 | 124 | 0.3 | 71 | 0 | 0 | 0.065 | 5 | 0.06 | 40 | 0.66 | 67 |
| fadA30 | 2.6 | 260 | 1.16 | 276 | 0.77 | 94 | 1.21 | 88 | 0.004 | 2.7 | 0.02 | 2.1 |
| fad5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Table I Continued

| | Enoyl-CoA Hydratase | | | | L-3-Hydroxyacyl-CoA dehydrogenase | | | | 3-Ketoacyl-CoA Thiolase | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $C_4$[a] | | $C_{10}$[b] | | $C_4$[c] | | $C_{10}$[d] | | $C_4$[c] | | $C_{10}$[d] | |
| Strain | U/mg | Relative Activities (%) | U/mg | Relative Activities (%) | U/mg | Relative Activities (%) | U/mg | Relative Activities (%) | U/mg | Relative Activities (%) | U/mg | Relative Activities (%) |
| RS 3065 | 3.94 | 100 | 0.92 | 100 | 1.6 | 100 | 2.84 | 100 | 0.17 | 100 | 2.5 | 100 |
| RS 3081 | 0.12 | 0.3 | 0.06 | 6 | 0 | 0 | 0.01 | 0.4 | 0 | 0 | 0.02 | 0.9 |
| RS 3110 | 0 | 0 | 0.07 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0.02 | 0.7 |

Table I Continued

| | Enoyl-CoA Hydratase | | | | L-3-Hydroxyacyl-CoA dehydrogenase | | | | 3-Ketoacyl-CoA Thiolase | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $C_4$[a] | | $C_{10}$[b] | | $C_4$[c] | | $C_{10}$[d] | | $C_4$[c] | | $C_{10}$[d] | |
| Strain | U/mg | Relative Activities (%) | U/mg | Relative Activities (%) | U/mg | Relative Activities (%) | U/mg | Relative Activities (%) | U/mg | Relative Activities (%) | U/mg | Relative Activities (%) |
| RS 3072 | 0.53 | 100 | 0.18 | 100 | 0.155 | 100 | 0.6 | 100 | 20.3 | 100 | 0.325 | 100 |
| RS 3084 | 0.06 | 11 | 0.2 | 111 | 0 | 0 | 0 | 0 | 29 | 143 | 0 | 0 |
| RS 3112 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 42 | 206 | 0 | 0 |

[a] Crotonyl-CoA; [b] Decenoyl-CoA; [c] Acetoacetyl-CoA [d] 3-Ketodecanoyl-CoA

substrates. Its enoyl-CoA hydratase activity was elevated. Mutant fad5 was found to be devoid of all enzyme activities as has previously been reported (24,25). All RS mutants were obtained by insertion of a transposon into the fadABC region. Not surprisingly these mutants are devoid of all enzymes associated with the fatty acid oxidation complex. Of special interest is mutant RS 3084 derived from strain RS 3072, which is constitutive for short-chain thiolase. The gene of short-chain thiolase is located at a region of the E. coli chromosome different from the fadABC region. As a consequence of the mutation strain RS 3084 does not exhibit the enzyme activities associated with the fatty acid oxidation complex i.e. it is devoid of the fatty acid oxidation complex. However, this mutant exhibits long-chain enoyl-CoA hydratase activity. This long-chain enoyl-CoA hydratase first described by Beadle et al., (42) was shown by Pawar and Schulz (41) not to be part of the fatty acid oxidation complex. The location of the gene for this long chain enoyl-CoA hydratase is not known. The fact that the gene for long-chain enoyl-CoA hydratase is expressed in one but not in three other ABC mutants suggests that it is part of the fadABC operon.

Condition for Growing E. coli K-12-YMel and fadB64 with High Contents of Fatty Acid Oxidation Complex. Over the years we have observed that E. coli extracts with high specific activities of fatty acid oxidation enzymes yield the purest enzyme preparation. This observation had aided me in determining the optimal growth conditions for different

TABLE II

Condition for Growing *E. coli* K-12-YMel and *fadB64* with high contents of fatty acid oxidation complex. Cells were grown for 8 hrs at 37°C and then kept overnight at 4°C. Samples of 5 ml were taken from a 2 liter *fadB64* cell suspension at various time points and assayed for crotonase activity and protein concentration as described under "Materials and Methods".

| Time (hr) | Enoyl-CoA Hydratase (U/ml) | Protein (mg/ml) | Specific Activities |
|---|---|---|---|
| 4½ | 0.436 | 0.306 | 1.42 |
| 5 | 0.820 | 0.436 | 1.88 |
| 6 | 1.130 | 0.406 | 2.78 |
| 8 | 1.040 | 0.419 | 2.48 |
| 25 | 7.480 | 1.800 | 4.16 |

E. coli strains. For this study samples of 5 ml were removed from cell suspensions at various growth times. They were homogenized by sonication and assayed for enoyl-CoA hydratase activity and protein concentration. As shown in Table II the specific activity of enoyl-CoA hydratase increased during the first six hours of growth at 37°C, but declined slightly thereafter. When the cell suspension was cooled from 37°C to 4°C and stored overnight at 4°C, the protein concentration increased 5-fold, but more importantly the specific activity of enoyl-CoA hydratase increased approximately 2-fold. The cells were harvested at this point by centrifugation, washed twice with M9 mineral salts media and stored at -70°C. E. coli K-12-YMel and fadB64 were grown by following the above outlined protocol.

Purification of Fatty Acid Oxidation Complexes from Several E. coli Strains. The isolation and purification of the multienzyme complex of fatty acid oxidation has been previously described (39,40,53). This multienzyme complex exhibits enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, cis-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase acitivities. When a homogenate of wild type E. coli B cells was subjected to chromatography on a phosphocellulose column and the column eluate was assayed for all five enzyme activities, a typical elution profile as shown in Fig. 4 was obtained. The result of a purification experiment are summarized in Table III. All five enzymes were co-eluted from the phosphocellulose column and were purified approximately to the same

Figure 4

Purification of the enyzmes 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 "Materials and Methods" was applied to a phosphocellulose column (5 x 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 a 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*-$\Delta^3$-*trans*-$\Delta^2$-enoyl-CoA isomerase, (▲) L-3-hydroxyacyl-CoA dehydrogenase, (o) enoyl-CoA hydratase, ($\Delta$) 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.

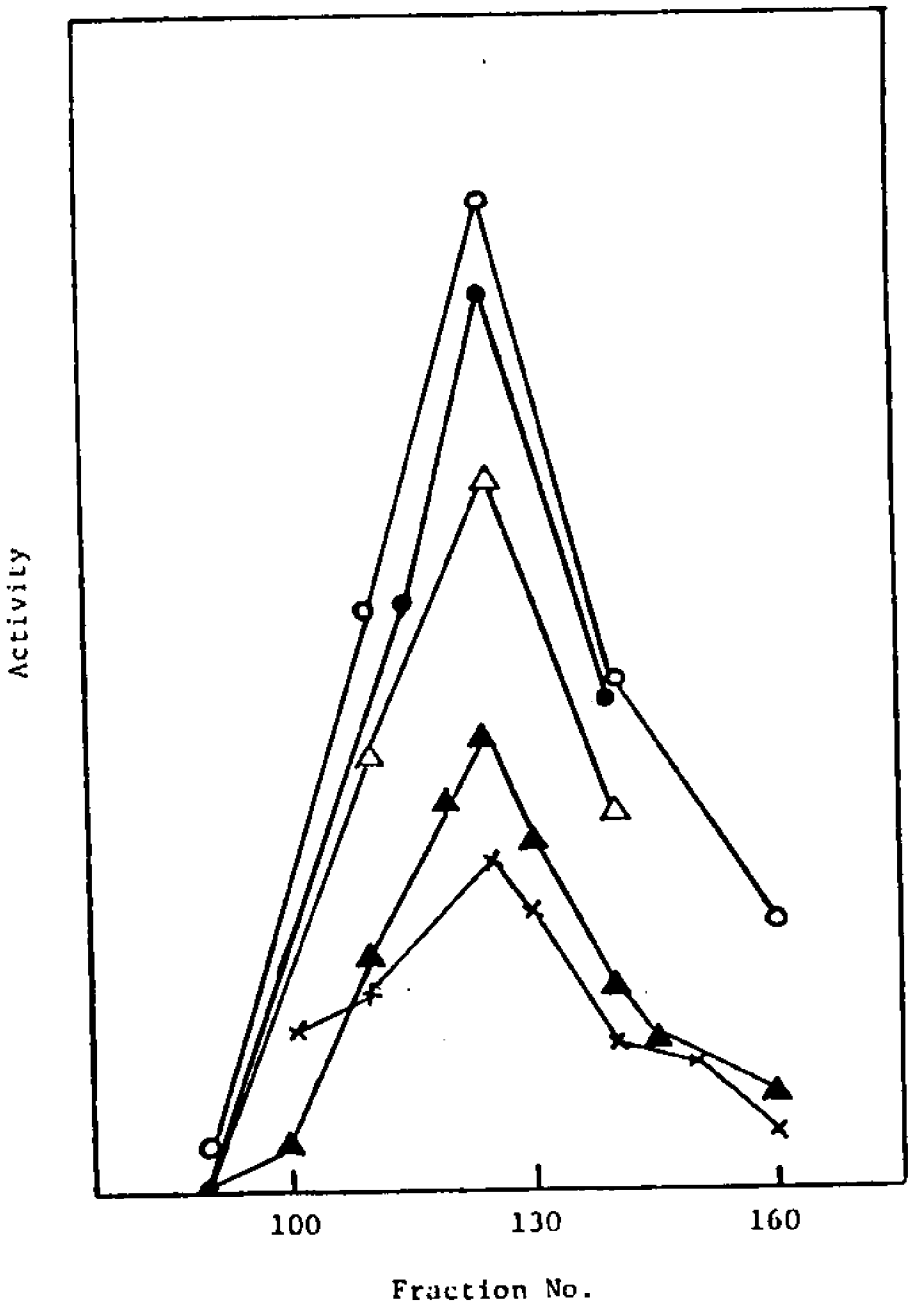


Figure 4

TABLE III

Purification of the Multienzyme Complex of Fatty Acid Oxidation from *E. coli* B[a]

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

Table III Continued

[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 30 μM substrates, these specific activities are significantly lower than the corresponding $V_{max}$ values. The complex is usually obtained in 30-40% yield.

extent.

Study of the Polymeric Forms of the Fatty Acid Oxidation Complex.

When the isolated complex from wild-type E. coli B was subjected to electrophoresis on a 4-30% polyacrylamide gradient gel (Fig. 5a) two intense protein bands were observed. As shown in Fig. 5b, the linear standard curves yielded estimated molecular weights of 265,000 (peak A) and of 580,000 (peak B) respectively (Fig. 5b). Peak A represents the monomeric complex (39) whereas peak B was possibly due to a dimeric form of the complex. Occasionally a small amount of a trimeric form of the complex was observed when the material was subjected to polyacrylamide gradient gel electrophoresis. Definite proof for the close relationship of proteins A and B (Fig. 5a) was obtained by the identification of all five enzymes listed in Table III in gel regions A and B. The specific and relative activities of all five enzymes associated with regions A and B of the polyacrylamide gradient gel are given in Table IV. In view of the low activities present in the gel extracts, the relative activities of the enzymes located in regions A and B of the gel agree reasonably well with those of the starting material (Table III) except for thiolase, which under a variety of conditions was found to be inactivated more easily than the other enzymes. The activities of all five enzymes in the regions adjacent and between the peaks were either very low or undetectable. We therefore conclude that protein B is an enzymatically active dimer of the multienzyme complex of fatty acid oxidation.

Figure 5a. Electrophoresis of the purified complex of fatty acid oxidation complex (0.36 mg) on a 4 to 30% polyacrylamide gradient gel. The recorder tracing was obtained by scanning the absorbance at 500 nm of a gel slice stained for protein. The bars represent enoyl-CoA hydratase activity measured in the extracts of various gel segments. For details see "Materials and Methods".

Figure 5b. Relative mobilities of bands A and B with respect to standards (1) apoferritin (2) catalase, (3) lactate dehydrogenase, (4) bovine serum albumin (5) ovalbumin.

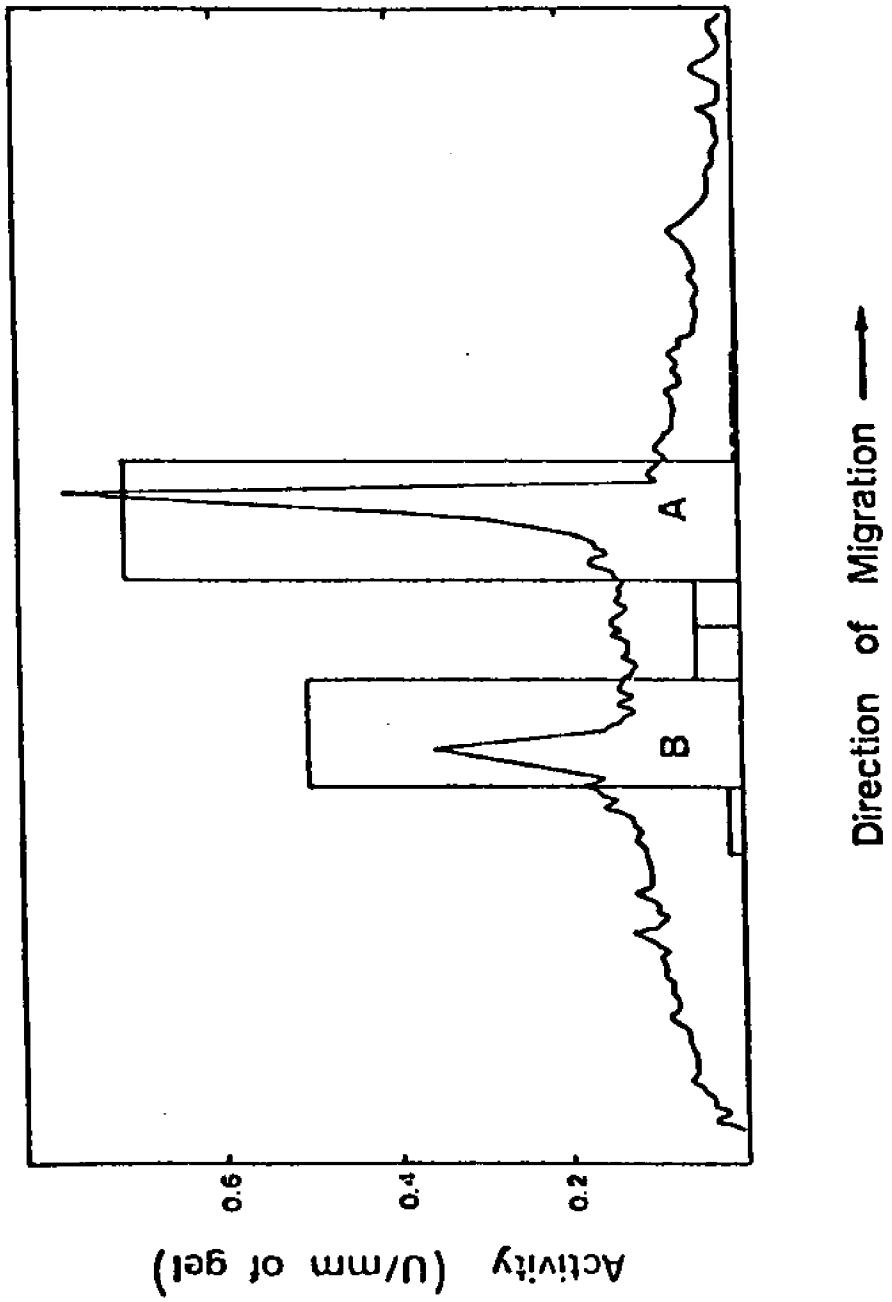


Figure 5a

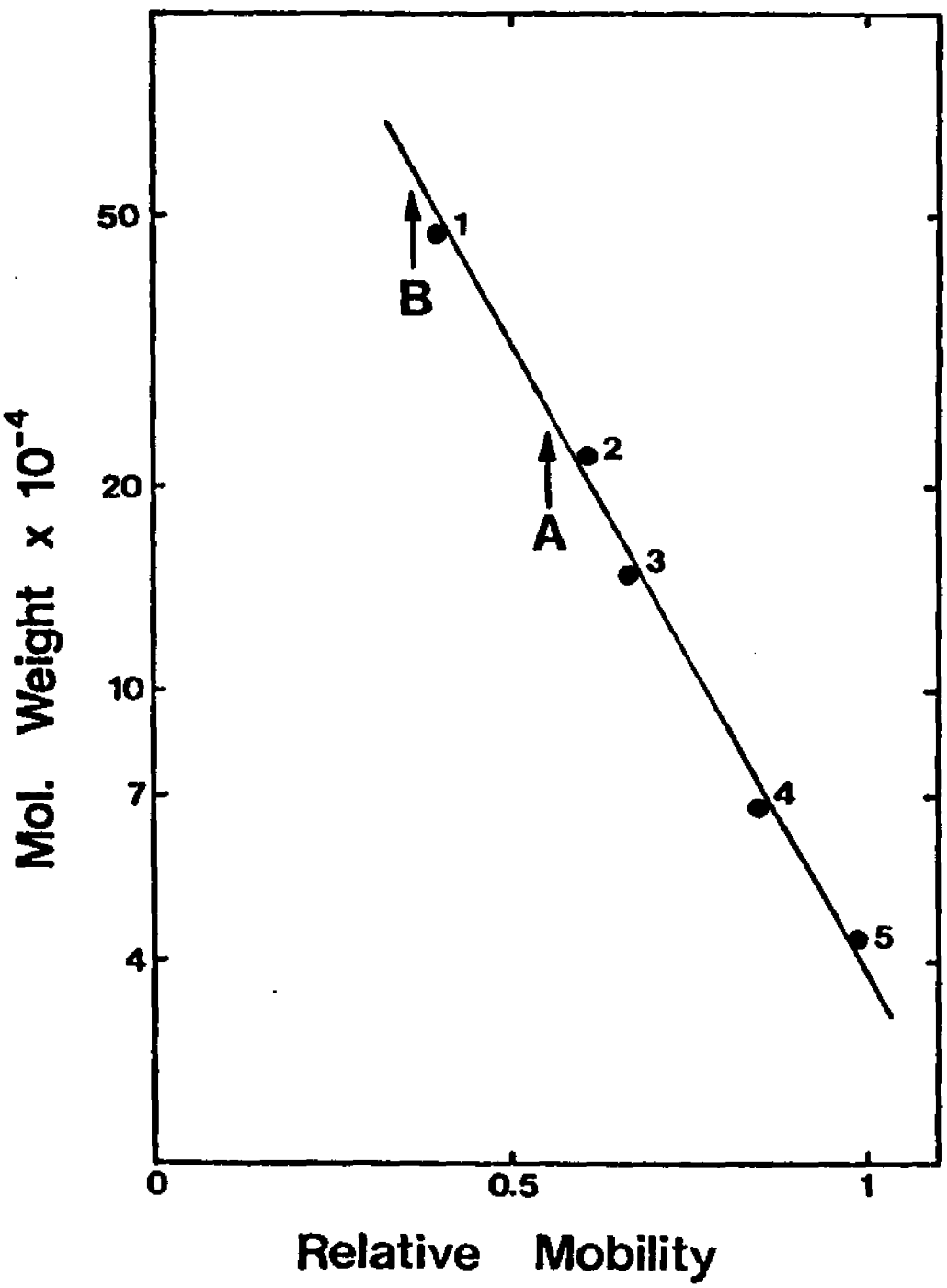


Figure 5b

TABLE IV

Activities of the Monomer and Dimer of the Multienzyme Complex of Fatty Acid Oxidation from *E. coli*[a]

| Enzyme | Substrate | Sp act (μmol/min per mm of gel)[d] | | Relative Activities | | |
|---|---|---|---|---|---|---|
| | | A[b] | B[b] | PC[c] | A[b] | B[b] |
| Enoyl-CoA hydratase | Crontonyl-CoA | 0.71 | 0.5 | 100 | 100 | 100 |
| L-3-Hydroxyacyl-CoA dehydrogenase | Acetoacetyl-CoA | 0.24 | 0.21 | 29 | 34 | 42 |
| 3-Ketoacyl-CoA thiolase | 3-Ketodecanoyl-CoA | 0.2 | 0.15 | 69 | 28 | 30 |
| 3-Hydroxyacyl-CoA epimerase | D-3-Hydroxydodecanoyl-CoA | 0.085 | 0.046 | 10 | 12 | 9.1 |
| *cis*-$\Delta^3$-*trans*-$\Delta^2$-Enoyl-CoA isomerase | *cis*-$\Delta^3$-Octenoyl-CoA | 0.021 | 0.014 | 3.2 | 2.9 | 2.7 |

[a]Separation of the monomer and dimer of the complex and enzyme assays were performed as

Table IV. Continued

described in the text and in the legend to Fig. 5a.

[b]A and B, Extracts from regions A and B of polyacrylamide gradient gel (Fig. 5a).

[c]PC, Purified complex of fatty acid oxidation. Specific activities of this preparation are given in Table III, except for thiolase activity with 3-ketodecanoyl-CoA, which was 39.3 U/mg.

[d]Because the gel was divided into unequal slices, the enzyme activities determined in the gel extracts were normalized with respect to the width of the gel slices.

Isolation and Characterization of Mutant Complexes. When extracts of E. coli K-12-YMel, fadB64, and fadA30 cells were subjected to chromatography on phosphocellulose, the same elution patterns for the fatty acid oxidation complexes were obtained. The result obtained with E. coli fadB64 are shown in Fig. 6. Most important is the finding that all five enzyme activities were co-eluted including the remaining L-3-hydroxyacyl-CoA dehydrogenase activity detected with 3-ketodecanoyl-CoA as a substrate. Hence all five enzyme activities seem to be associated with the fatty acid oxidation complex. Additionally the fatty acid oxidation complex was isolated from E. coli K-12-YMel cells which is the parental strain of the fadB64 mutant. The subunit compositions of the purified complexes from E. coli strains B, K-12-YMel, and fadB64 were determined by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. Scans of the resulting gels after staining with Coomassie Brilliant blue are shown in Fig. 7. The best preparations of the purified complex from E. coli B only show two bands (band I and II) corresponding to proteins with molecular weights of 78,000 and 42,000 (see Fig. 7A). As shown in Fig. 7B the major proteins of the complex from E. coli K-12-YMel are the 78,000 dalton (band I) and 42,000 dalton (band II) polypeptides identified as the subunits of the complex from E. coli B (41). The additional band III has previously been observed with some preparations from E. coli B and is possibly due to the proteolytic degradation of one of the two subunits of the complex (41). The protein giving rise to band IV is present in small

Figure 6. Chromatography on phosphocellulose of the enzymes of β-oxidation from E. coli fadB64. The column was developed with a 0.05 M to 0.5 M potassium phosphate (pH 6.6) gradient as previously described (39). 2-Enoyl-CoA hydratase (○), L-3-hydroxyacyl-CoA dehydrogenase (▲), 3-ketoacyl-CoA thiolase (□), cis-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA isomerase (△), and 3-hydroxyacyl-CoA epimerase (●).

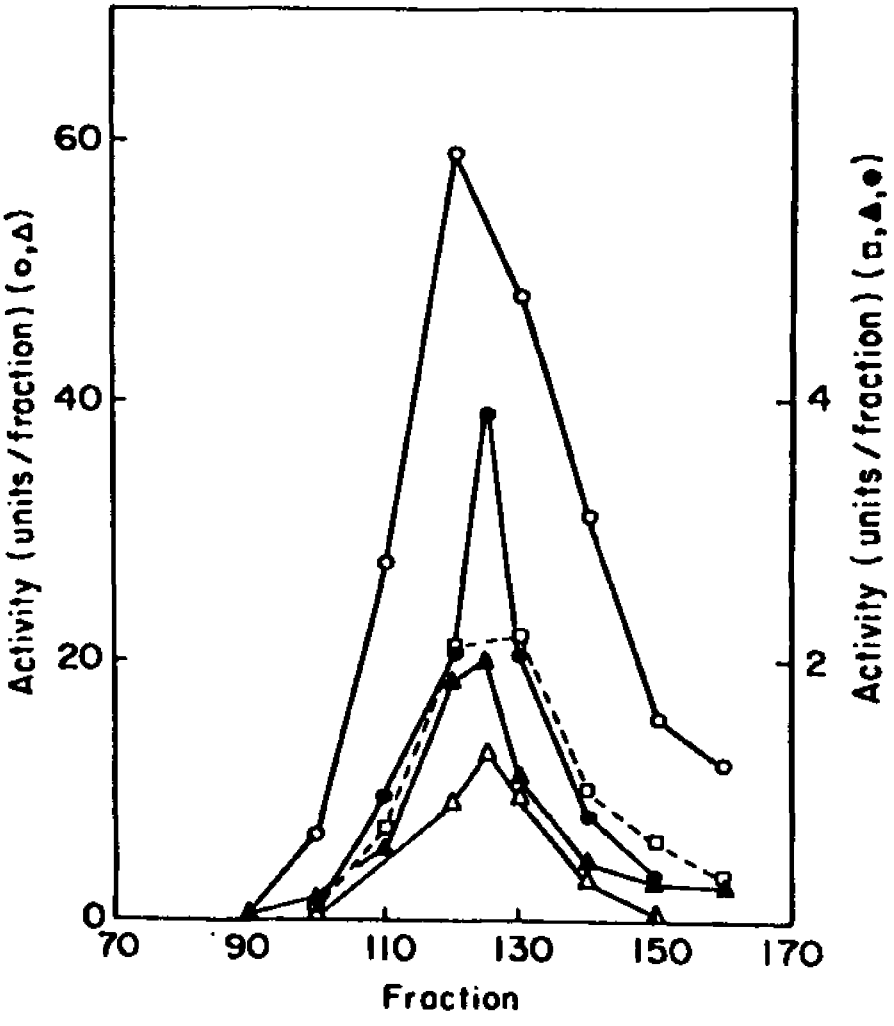
60
40
20
0
4
2
70
90
110
130
150
170
Fraction
Activity (units / fraction) (o,Δ)
Activity (units / fraction) (□,▲,●)

Figure 6

Figure 7. Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate of the fatty acid oxidation complexes from: (A), *E. coli* B; (B), *E. coli* K-12 YMel; and (C), *E. coli fadB64*.

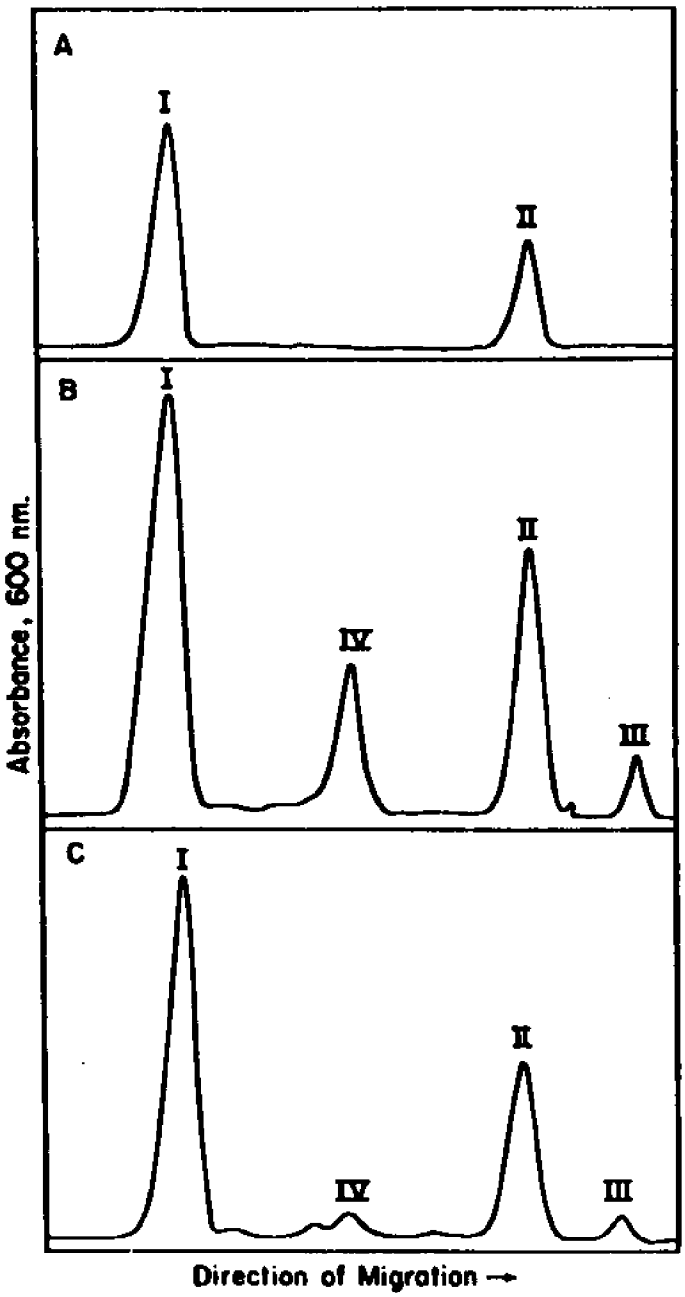
A
I
II
B
I
II
IV
III
C
I
II
IV
III
Absorbance, 600 nm.
Direction of Migration →

Figure 7

amounts in other preparations and is possibly a contaminant that is unrelated to the fatty acid oxidation complex. This and other impurities are the cause for the lower than expected specific activity of this preparation from E. coli K-12-YMel as compared to that isolated from E. coli B (see Table V). Most interesting was the observation that the subunit compositions of the complexes from E. coliB and fadB64 are virtually identical (compare Figs. 7A and 7C). Since complexes isolated from E. coli B cells in the absence of phenylmethanesulfonyl fluoride contained more of the 35,000 dalton polypeptide (39,41), it is possible that this subunit was formed by the degradation of one of the two larger subunits (41). Some of the multienzyme complex preparations also contained a small amount of a protein with a molecular weight close to 55,000. The fractions of the fadB64 complex corresponding to the first and second half of the material eluted from phosphocellulose were separately analyzed by SDS gel electrophoresis. For this purpose fractions 75-85 and 86-100 of the phosphocellulose eluate (see Fig. 8) were separately pooled and concentrated, and subjected to SDS gel electrophoresis. The part of the complex that was eluted first from the column gave rise (see Fig. 9A) the two main bands I (M.W. 78,000) and II (M.W. 42,000). In addition bands III (M.W. 39,000), IV (M.W. 52,000), and V (M.W. 56,000) were detected. However, the second part of peak was devoid of these additional bands. This data shows that the fatty acid oxidation complex from fadB64 is only composed of two types of subunits whose molecular weights are estimated to be 78,000 and 42,000.

TABLE V. Specific Activities of the Component Enzymes of Fatty Acid Oxidation Complexes from *E. coli*.[a]

| Enzyme | Substrate | Sp act (μmol/min/mg) | | | Relative Activities (%) | | |
|---|---|---|---|---|---|---|---|
| | | K-12-YMel | *fadB64* | B | K-12-YMel | *fadB64* | B |
| Enoyl-CoA hydratase | 27 μM Crotonoyl-CoA | 21 | 51 | 115 | 100 | 100 | 100 |
| | 20 μM 2-Decenoyl-CoA | 3.9 | 9.5 | 19.6 | 18.6 | 18.6 | 17 |
| L-3-Hydroxyacyl-CoA | 27 μM Acetoacetyl-CoA | 9.9 | 0 | 20 | 47 | - | 17.4 |
| dehydrogenase | 27 μM 3-Ketohexanoyl-CoA | 24.7 | 0.04 | - | 118 | 0.1 | - |
| | 20 μM 3-Ketodecanoyl-CoA | 16.6 | 1.5 | 37 | 79 | 3 | 32 |
| | 23 μM L-3-Hydroxybutyryl-CoA | 2 | 0 | - | 9.5 | - | - |
| | 24 μM L-3-Hydroxydecanoyl-CoA | 5 | 0.5 | - | 24 | 1 | - |
| 3-Ketoacyl-CoA thiolase | 27 μM Acetoacetyl-CoA | 2.2 | 5.4 | 5.5 | 10.5 | 10.6 | 4.8 |
| | 20 μM 3-Ketodecanoyl-CoA | 13.4 | 40 | 60 | 64 | 78 | 52 |
| 3-Hydroxyacyl-CoA epimerase | 30 μM D-3-Hydroxydodecanoyl-CoA | 0.4 | 0.6 | 2.4 | 2 | 1.2 | 2 |
| *cis*-$\Delta^3$-*trans*-$\Delta^2$-Enoyl-CoA | 27 μM *cis*-3-Octenoyl-CoA | 2.6 | 7.5 | 8 | 12.4 | 14.7 | 7 |
| isomerase | | | | | | | |

[a]The enzymes were assayed as described under "Experimental Procedures."

Figure 8. Purification of the enzymes of fatty acid oxidation from E. coli fadB64 by chromatography on phosphocellulose. The purification procedure has been described under "Materials and Methods". (O), 2-Enoyl-CoA hydratase activity with crotonyl-CoA as a substrate and (▲) absorbance of 595 nm due to protein. Fractions 75 to 85 and fractions 86-100 were separately pooled and concentrated.

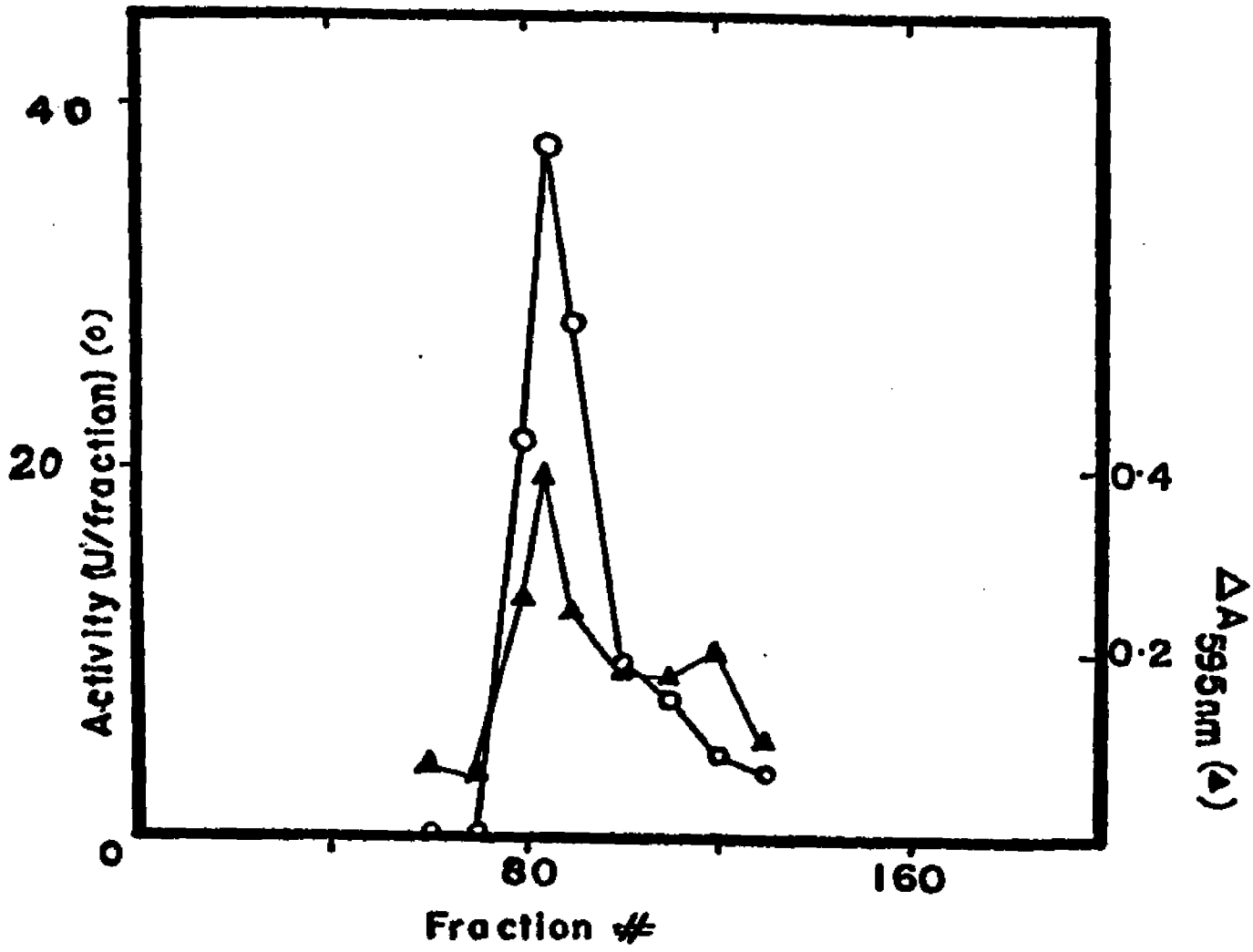


Figure 8

Figure 9. Disc gel electrophoresis of the fatty acid oxidation complex in the presence of sodium dodecyl sulfate on 10% polyacrylamide gel, as described under "Materials and Methods".

A. Fractions 75 to 85 from the phosphocellulose column shown in Fig. 8.

B. Fractions 86 to 100 from the phosphocellulose column shown in Fig. 8.

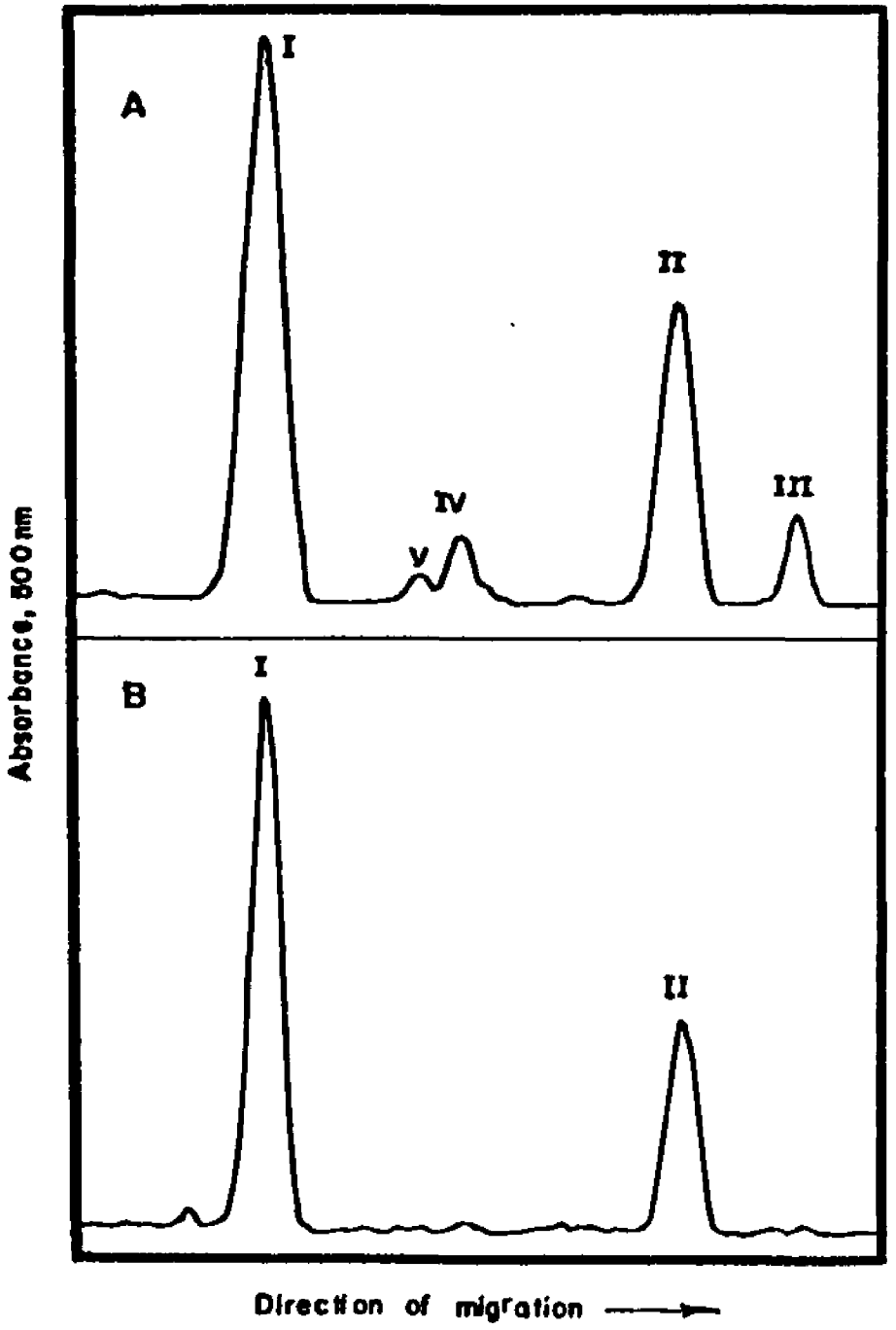


Figure 9

The specific activities of the five component enzymes associated with the purified fatty acid oxidation complexes are listed in Table V. The specific activities of the complex from K-12-YMel are lower than those of the other two complexes partly because the former was obtained in a less pure form (see Fig. 7). However, when the activities of the wild-type complex from K-12-YMel and of the mutant complex from fadB64 are presented as relative activities with enoyl-CoA hydratase set as 100% (see Table V), 3-hydroxyacyl-CoA dehydrogenase appears to be the only enzyme that is significantly affected by the mutation. The relative enzyme activities of the complex from E. coli B are for the most part lower than those of the other two complexes because the specific activity of its enoyl-CoA hydratase was high. However, the specific activity of this component enzyme was found to vary from one purified preparation to the next between 60 to 120 U/mg. If one uses the lower value of 60 U/mg for enoyl-CoA hydratase, most of the relative activities of the complex from E. coli B are similar to those from the other two complexes. The most obvious defect of the complex isolated from mutant fadB64 is the inactivity of its L-3-hydroxyacyl-CoA dehydrogenase with either acetoacetyl-CoA or L-3-hydroxybutyryl-CoA as substrates. With 3-ketohexanoyl-CoA as a substrate a specific activity of 40 mU/mg was observed which represents less than 0.2% of the activity observed with the wild-type complex (see Table V). With 3-ketodecanoyl-CoA or L-3-hydroxydecanoyl-CoA as substrates the activity ratio of the wild-type complex to the mutant complex are approximately 10:1. If one considers the lower degree of purification

achieved for the complex from the parental strain, the dehydrogenase activity of the mutant complex with either 3-ketodecanoyl-CoA or L-3-hydroxydecanyl-CoA as substrates is approximately 5% of the wild-type activity. This value is close to that determined with crude homogenates. Other enzymatic activities of the mutant complex, especially those of 3-ketoacyl-CoA thiolase and cis-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA isomerase are apparently not affected by the mutation. Even the enoyl-CoA hydratase activity of 51 U/mg is very similar to activities observed with earlier preparations from E. coli B (39,40). Only 3-hydroxyacyl-CoA epimerase of the fadB64 complex is 3- to 4-fold less active than the epimerase of E. coli B and this may be a consequence of the mutation.

Kinetic Study of Fatty Acid Oxidation Complexes. In order to elucidate the basis for the low L-3-hydroxyacyl-CoA dehydrogenase activity of the mutant complex, we have performed some kinetic measurements. Lineweaver-Burk plots for L-3-hydroxyacyl-CoA dehydrogenase (Fig. 10, 11), enoyl-CoA hydratase (Fig. 12), and 3-ketoacyl-CoA thiolase (Fig. 13) activities are presented. The kinetic constants ($V_{max}$ and $K_M$) for these three enzymes are listed in Table VI. The higher $V_{max}$ values observed for 3-ketoacyl-CoA thiolase and enoyl-CoA hydratase with the mutant complex as compared to those of the wild-type complex are due to the greater purity of the former preparation. Most interesting is the virtual identity of the $K_m$ values for 3-ketodecanoyl-CoA determined with L-3-hydroxyacyl-CoA dehydrogenase of the

Figure 10. Lineweaver-Burk plot of L-3-hydroxyacyl-CoA dehydrogenase activity *versus* NADH concentration with 3-ketodecanoyl-CoA (57 μM) as a substrate. Assays were performed as described under "Materials and Methods". Activities with the complexes from K-12-YMel (●), and from *fadB64* (▲).

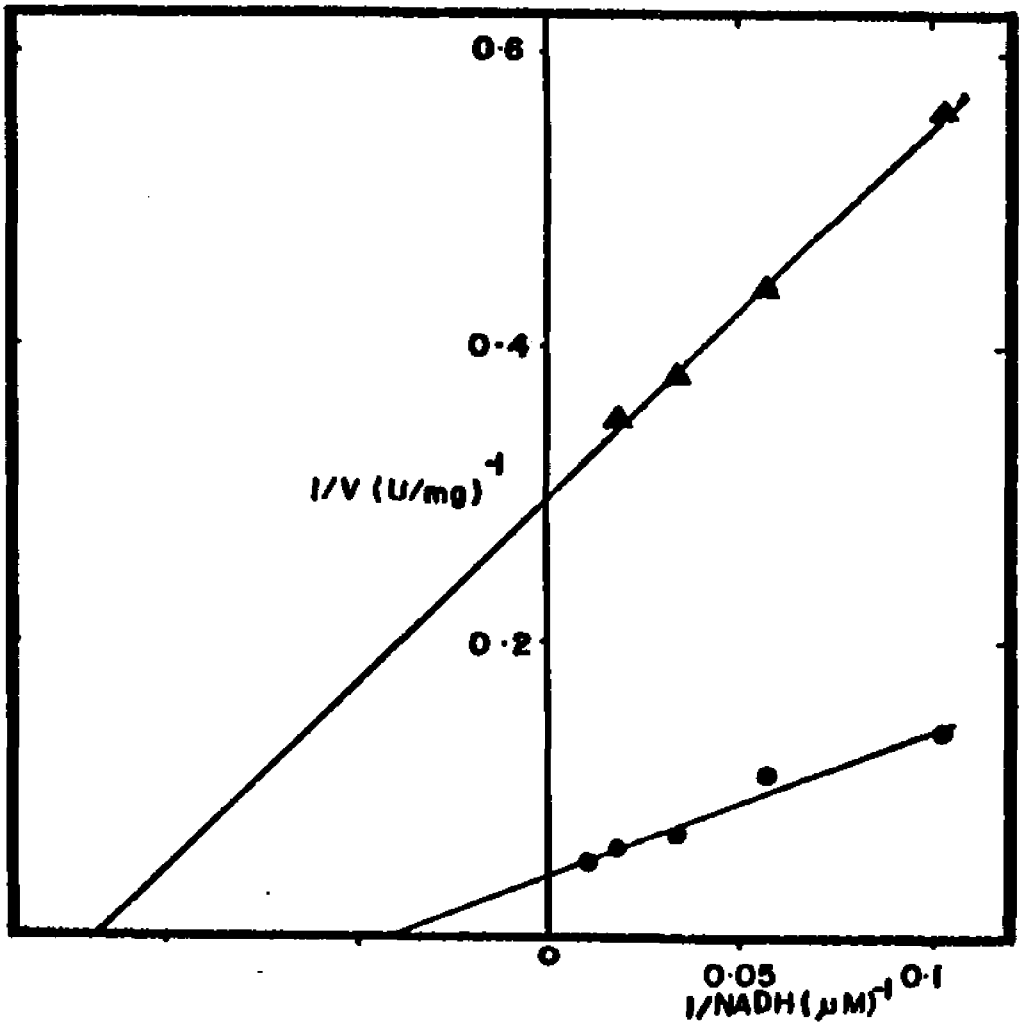


Figure 10

Figure 11. Lineweaver-Burk plot of L-3-hydroxyacyl-CoA dehydrogenase activity versus 3-ketodecanoyl-CoA (β-keto $C_{10}$) concentration at a fixed NADH concentration of 116 μM. Assays were performed as described under "Materials and Methods". "Complexes from E. coli K-12-YMel (●), and from E. coli fadB64 (▲).

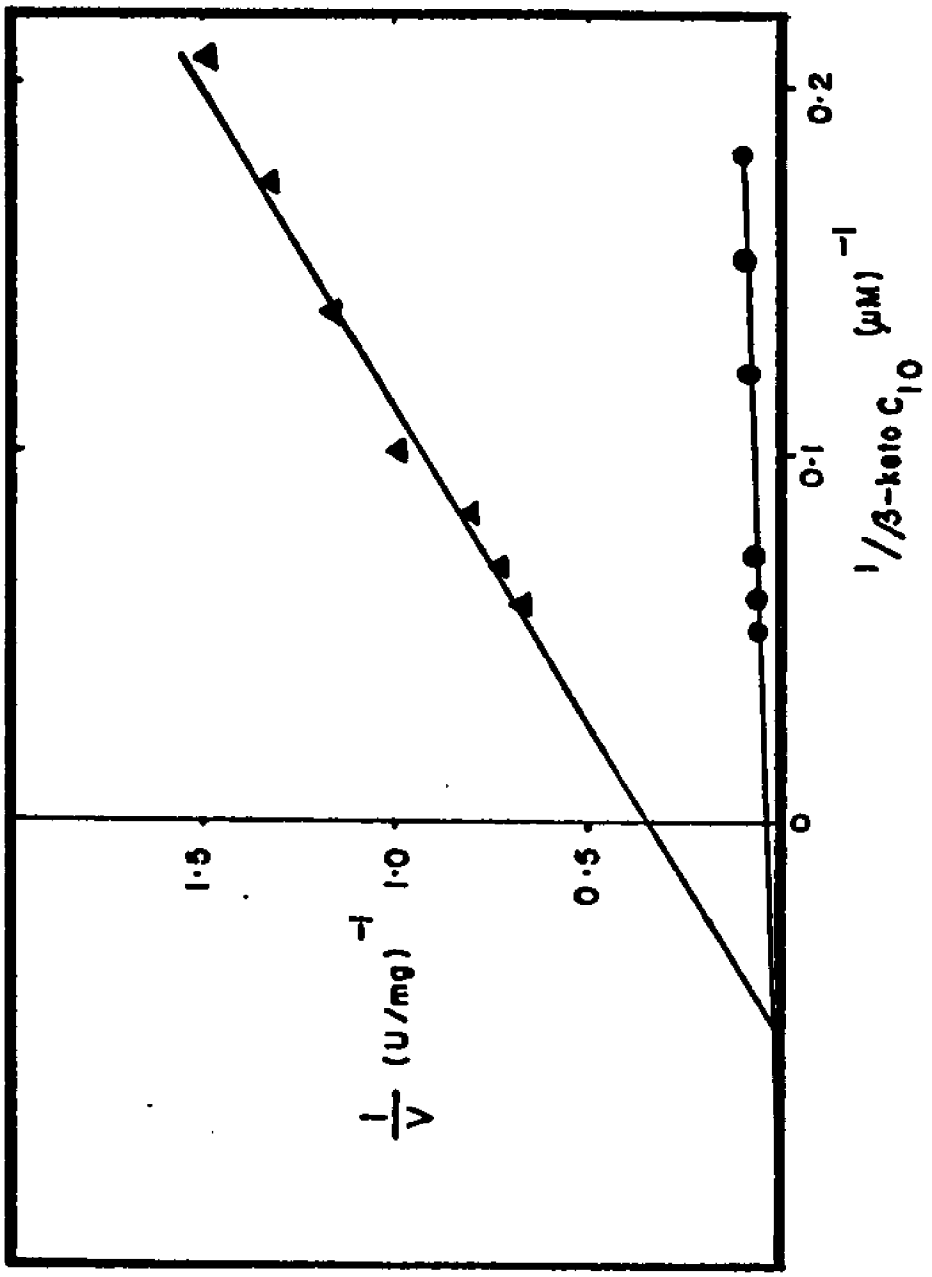


Figure 11

Figure 12. Lineweaver-Burk plot of enoyl-CoA hydratase activity *versus* crotonyl-CoA ($\Delta^2C_4$CoA) concentration. Assays were performed as described under "Materials and Methods". Complexes from *E. coli* K-12-YMel (●), and *E. coli fadB64* (▲).

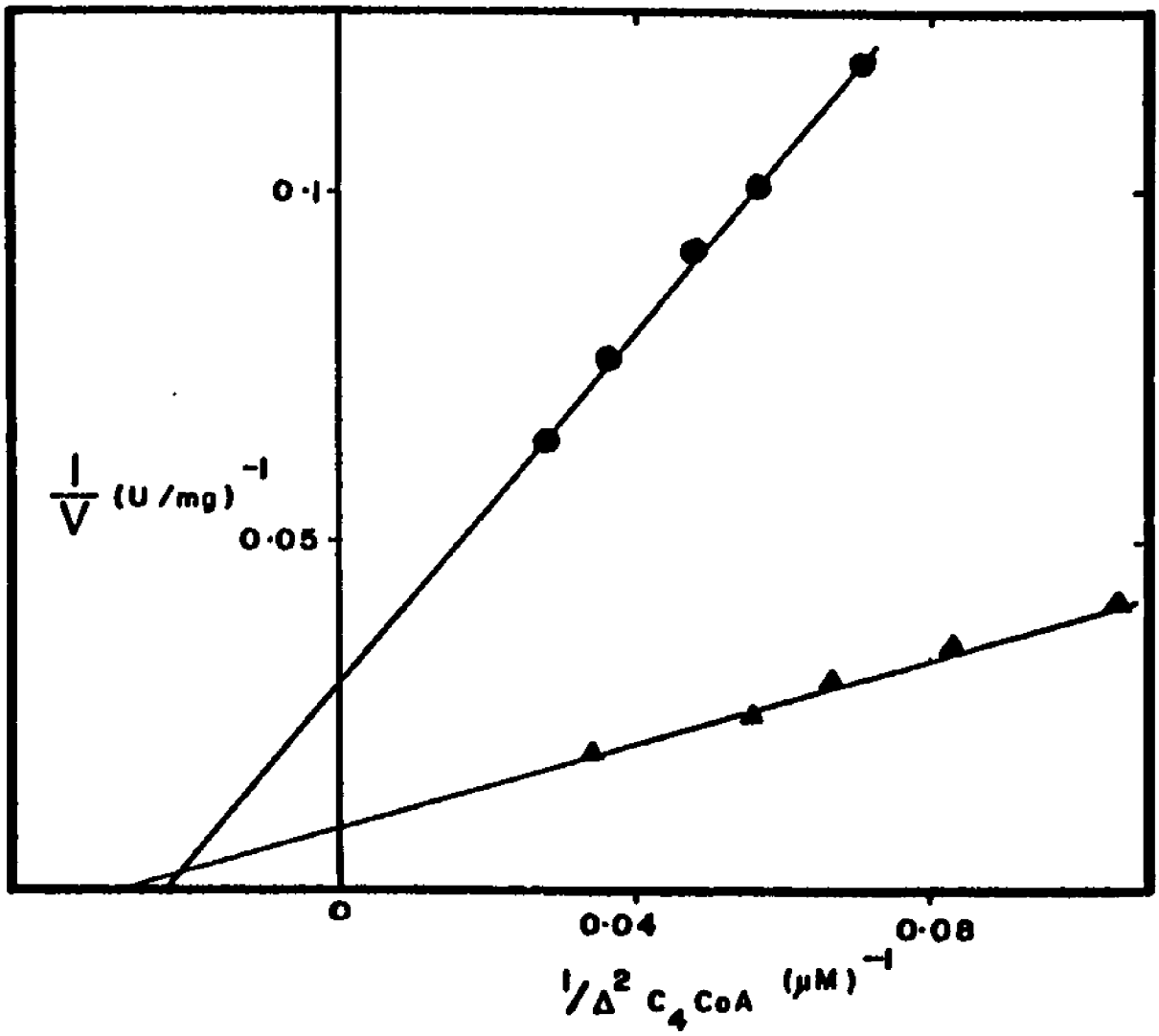


Figure 12

Figure 13. Lineweaver-Burk plot of thiolase activity versus acetoacetyl-CoA (AcAcCoA) concentration at a fixed CoASH concentration of 137 μM. Assays were performed as described under "Materials and Methods". Complexes from E. coli K-12 YMel (●), and from E. coli fadB64 (▲).

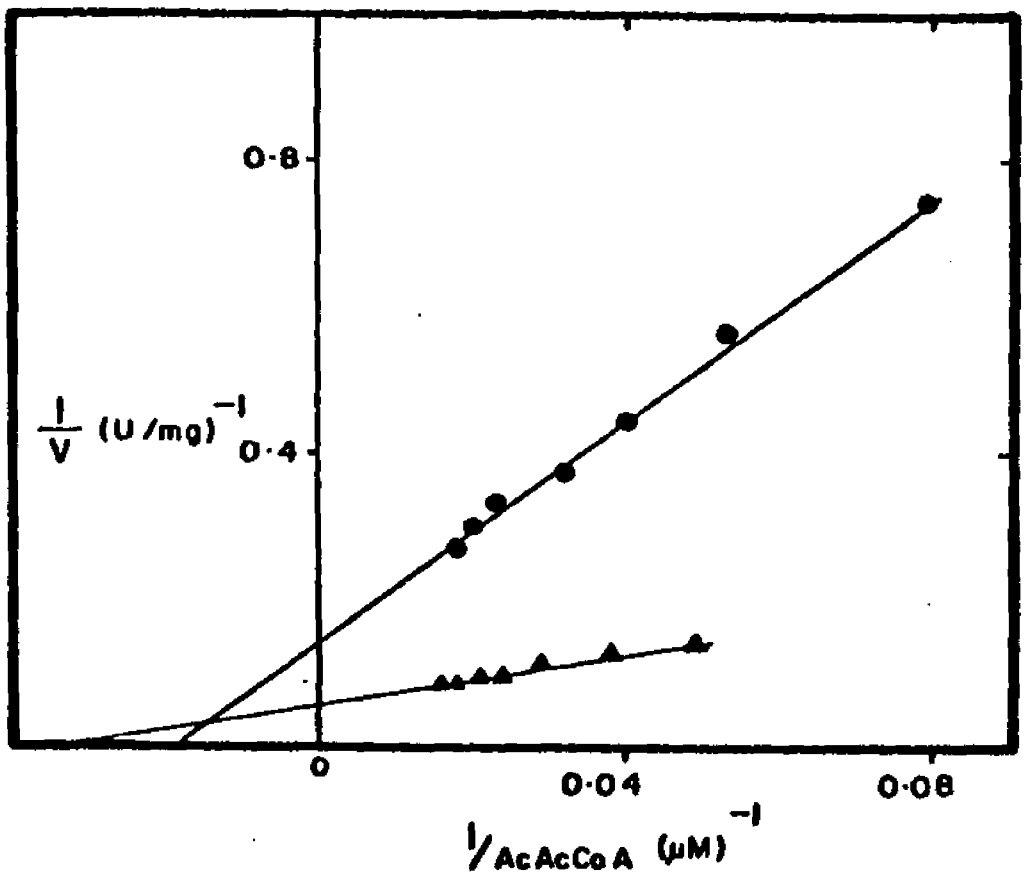


Figure 13

wild-type and mutant complexes. The $K_m$ values for substrates of the other two component enzymes are either identical or close enough to be considered unaffected by the mutation. However, the $K_m$ value for NADH obtained with the mutant complex is significantly lower than that determined with the wild-type enzyme (see Table VI). The main difference between the mutant and wild-type fatty acid oxidation complexes is the much lower maximal velocity of the mutant L-3-hydroxyacyl-CoA dehydrogenase. Although the specific activity of this enzyme increases with increasing chain length of the substrate, it remains dramatically lower than that of the wild-type complex.

Immunological Study of the Fatty Acid Oxidation Complexes. The purified fatty acid oxidation complexes were compared immunologically by the double diffusion technique of Ouchterlony (57). The complexes from E. coli strains K-12-YMel and fadB64 cross-reacted with antibodies raised against the purified complex from E. coli B (see Fig. 14). Since the main antigen-antibody precipitin lines fused perfectly, it seems that the three complexes are immunologically identical or very similar. There appears to be a weak precipitin line in front of the main line. The antigen giving rise to this precipitate may be lower molecular weight material that I have observed on polyacrylamide gradient gels of the native complex (data not shown) and that I attribute to partially degraded or dissociated complex.

I have evaluated the possibility of having an inactive fatty acid oxidation complex or a fragment of the complex present in the fad5

Table VI. Kinetic Constants of Some of the Component Enzymes of Fatty Acid Oxidation Complexes from *E. coli*.

| Enzyme | Substrate | K-12-YMel | | *fadB64* | |
|---|---|---|---|---|---|
| | | $K_m$ (μM) | $V_{max}$ (μmol/min/mg) | $K_m$ (μM) | $V_{max}$ (μmol/min/mg) |
| Enoyl-CoA hydratase | Crontonyl-CoA | 23,40 | 34,32 | 27,32 | 107,108 |
| L-3-Hydroxyacyl-CoA dehydrogenase | 3-Ketodecanoyl-CoA | 9,13 | 24 | 9,15 | 3.2,2.9 |
| | NADH | 24 | 24 | 8,10 | 3.3 |
| 3-Ketoacyl-CoA thiolase | Acetoacetyl-CoA | 54,68 | 6,7 | 30,39 | 18,20 |

Figure 14. Ouchterlony immunodiffusion analysis of fatty acid oxidation complexes from E. coli. Antibody (210 µg) raised against the pure complex from E. coli B was placed in the center well. Purified fatty acid oxidation complexes were applied to the outer wells. E. coli B complex (65 µg) to wells B and E, E. coli K-12 YMel complex (68 µg) to wells C and F, and E. coli fadB64 complex (40 µg) to wells A and D.

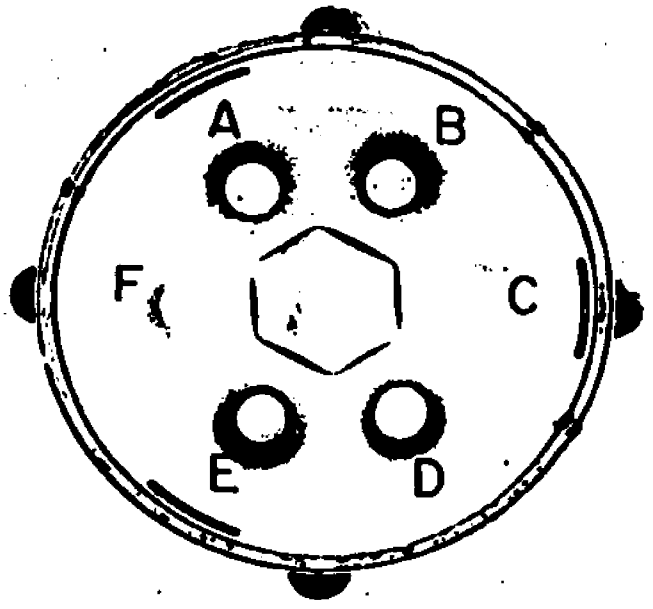


Figure 14

mutant. In this study antibodies raised against the fatty acid oxidation complex isolated from E. coli B cell were used. If a fragment of the complex or a complete but inactive fatty acid oxidation complex should be present, an immunoprecipitate should form. The analysis by SDS gel electrophoresis of a precipitate formed upon addition of antibodies to fad5 homogenate showed only the presence of IgG and a small amount of proteins with molecular weight higher than 79,000. This finding led to the tentative conclusion that the precipitated proteins are most likely not related to the complex because no protein with a molecular weight of either 78,000 or 42,000 was observed.

Inactivation of the Fatty Acid Oxidation Complex by Tris-Hydrochloride. The multienzyme complex though heat stable (39,40) is surprisingly unstable in Tris-hydrochloride buffer. A systematic study of this phenomenon revealed that all component enzymes of the complex were inactivated by Tris-hydrochloride (Fig. 15). The half times for the inactivations were 2 minutes for thiolase and 4 to 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 (Table VII). However, in 0.2M potassium phosphate, pH 8, all of the component enzymes of the complex are very stable.

The protection by substrates of the fatty acid oxidation complex against inactivation by Tris-hydrochloride was studied. Acetoacetyl-CoA at 1 mM concentration did not affect significantly the rate of inactivation of any of the three component enzymes listed in Table VIII.

Figure 15. Effects of Tris-hydrochloride and potassium phosphate on the activities of the multienzyme complex of fatty acid oxidation from E. coli B. Purified complex (27 μg/ml) at 0°C in the presence of 1M 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: (o) thiolase activity with acetoacetyl-CoA; (●), 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-$\Delta^3$-trans-$\Delta^2$-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.

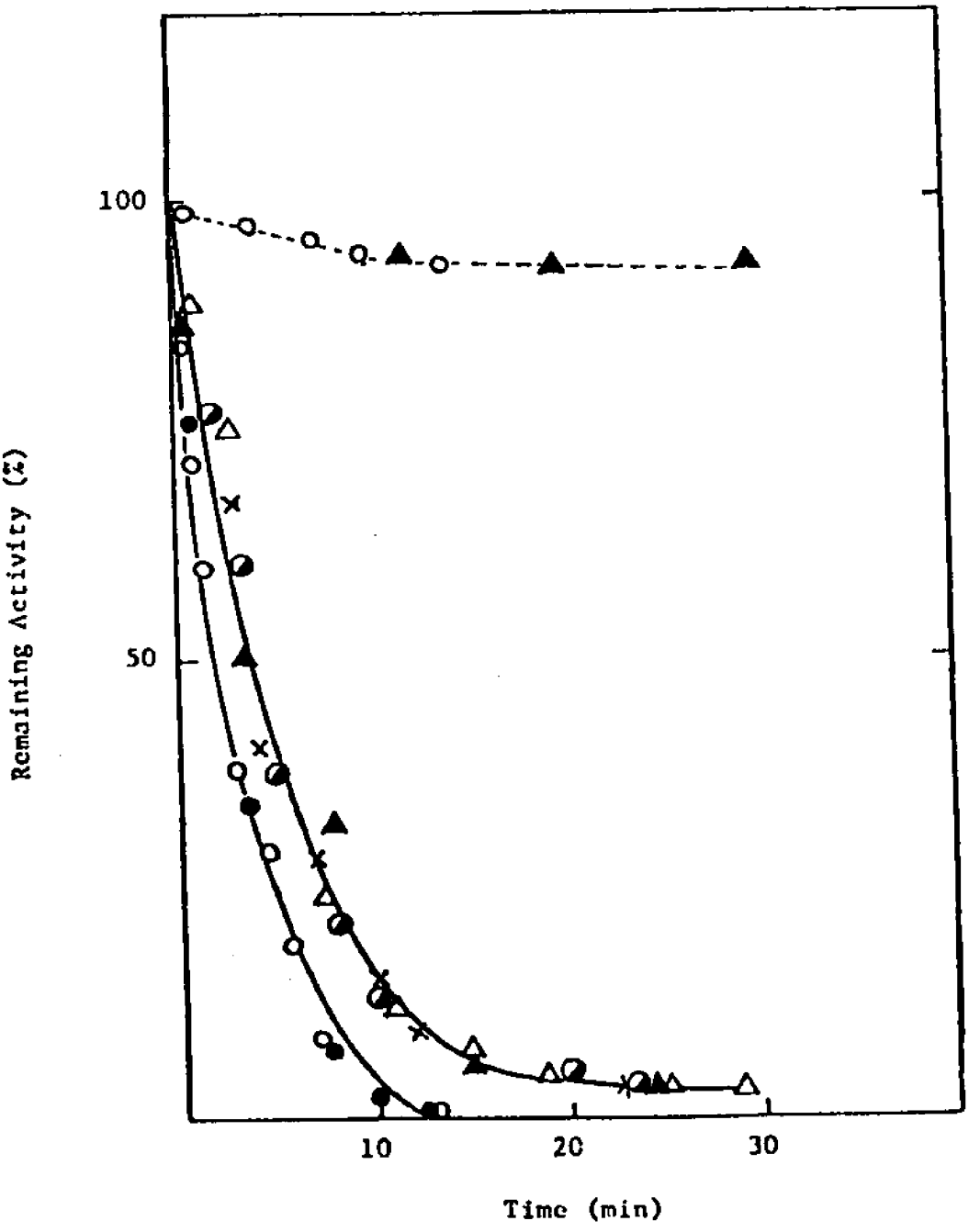


Figure 15

TABLE VII

Effect of Tris-hydrochloride on the stability of the multienzyme complex of fatty acid oxidation from *E. coli* B. Standard deviations are given for those data that are based on more than two independent measurements. The number of observations on which the data are based are indicated in parenthesis.

| Enzyme | Half-time for inactivation (min) |
|---|---|
| Enoyl-CoA hydratase | 4.75 ± 0.9 (4) |
| L-3-Hydroxyacyl-CoA dehydrogenase | 4.1 ± 0.9 (4) |
| 3-Ketoacyl-CoA thiolase | 2 ± 0.2 (6) |
| *cis*-$\Delta^3$-*trans*-$\Delta^2$-Enoyl-CoA isomerase | 4.25 (2) |
| 3-Hydroxyacyl-CoA epimerase | 4 (2) |

However, crotonyl-CoA protected slightly crotonase as well as L-3-hydroxyacyl-CoA dehydrogenase whereas its presence did not affect the inactivation of thiolase Table VIII). The most effective protection was observed in the presence of NADH (NAD in the case of thiolase) which greatly reduced the rate of inactivation of all three component enzymes listed in Table VII.

Inactivation of the Fatty Acid Oxidation Complex by Acetic Anhydride. The inhibition of enoyl-CoA hydratase and L-3-hydroxy-acyl-CoA dehydrogenase by acetic anhydride was studied. Initial data demonstrated the rapid inactivation of enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase by acetic anhydride (Fig. 16). Only enoyl-CoA hydratase was significantly protected against this inactivation in the presence of 1 mM crotonyl-CoA (Fig. 16). This finding prompted an experiment in which the specific labeling of enoyl-CoA hydratase with [1-$^{14}$C]acetic anhydride was attempted. For this purpose the complex was first separated from sulfhydryl reagents by rapid centrifugation-gel filtration (59). The complex was then incubated with cold acetic anhydride in the presence of 1 mM crotonyl-CoA for 3 minutes and rapidly separated from the incubation medium by rapid centrifugation-gel filtration.

The complex thus pretreated was allowed to react for 2 minutes with [1-$^{14}$C] acetic anhydride in the presence and absence of 1 mM crotonyl-CoA. The radioactively-labeled complex was then separated

TABLE VIII

Protection of the Fatty Acid Oxidation Complex Against Inactivation by Tris-Hydrochloride.

| Enzyme | $\tau_{1/2}$ (min) No addition | (+) Acetoacetyl-CoA (1 mM) | (+) Crotonyl-CoA (1 mM) | (+) NADH (1 mM) |
|---|---|---|---|---|
| Enoyl-CoA hydratase | 5 | 6 | 17 | 60 |
| L-3-Hydroxyacyl-CoA dehydrogenase | 5 | 10 | 13 | 40 |
| 3-Ketoacyl-CoA thiolase | 3 | 3[a] | 3 | 20[b] |

[a]From Dr. Pawar's Ph.D. dissertation (60).

[b]Data with $NAD^+$; from Dr. Pawar's Ph.D. dissertation (60).

Figure 16. Effect of acetic anhydride on the fatty acid oxidation complex from E. coli B. Enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities were determined as a function of time. Enoyl-CoA hydratase activity in the absence (Δ) and the presence (▲) of 1 mM crotonyl-CoA. L-3-Hydroxyacyl-CoA dehydrogenase activity in the absence (O) and presence (●) of 1 mM crotonyl-CoA. For experimental details see under "Materials and Methods".

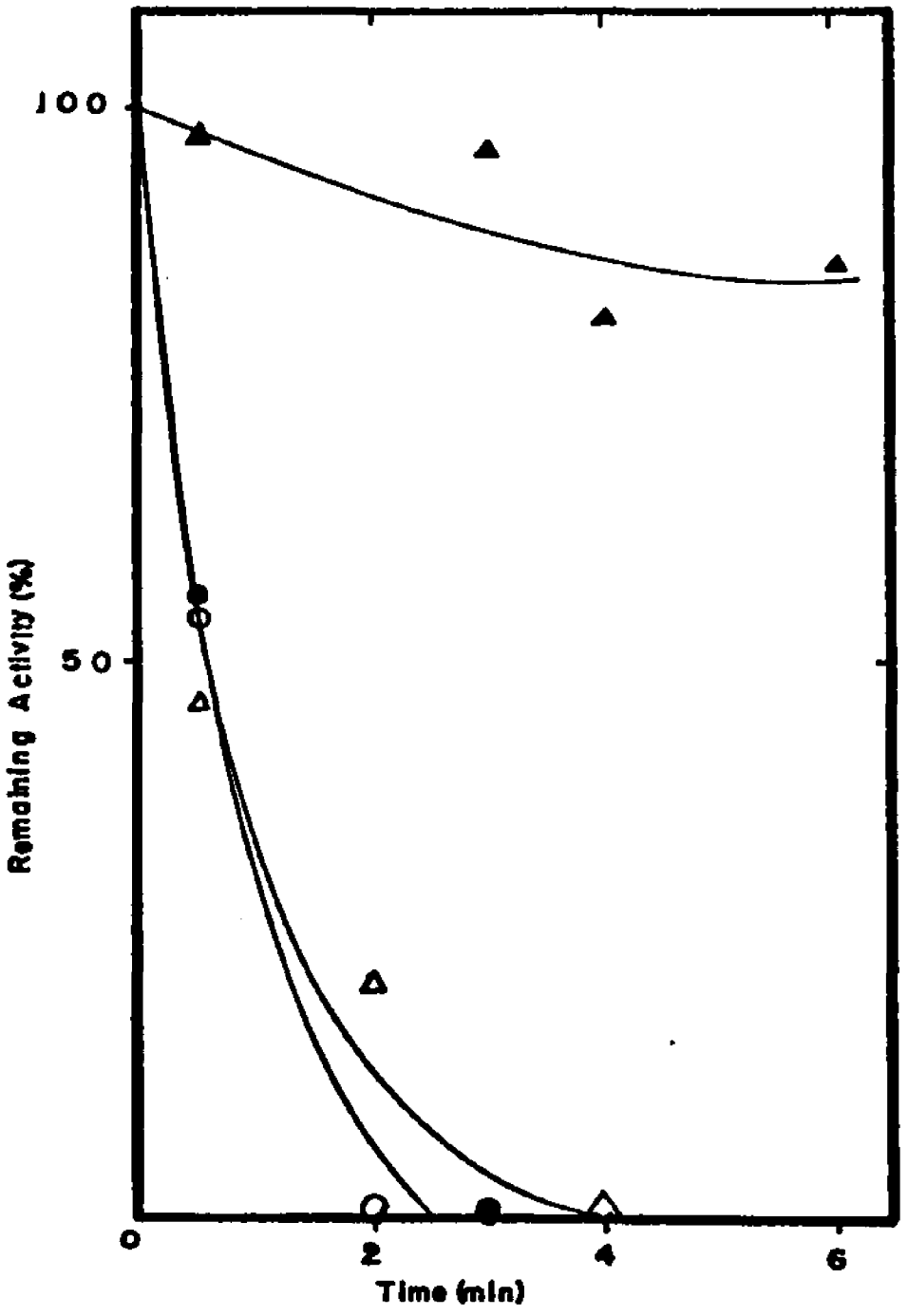


Figure 16

from the incubation medium by centrifugation-gel filtration (see flow diagram in Fig. 17). The remaining activity after each step is shown in Table IX. 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. Although both subunits were labeled (see Fig. 18), the degree of labeling was greater in the absence of crotonyl-CoA. Unfortunately, the ratio of labeling in the two subunits was the same in the presence and absence of crotonyl-CoA. Thus, no information was gained regarding the subunit location of enoyl-CoA hydratase. It is interesting to note that cis-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA isomerase was not affected by the reaction with acetic anhydride.

Comparison of the *E. coli* Fatty Acid Oxidation Complex with the Bifunctional Enzyme From Rat Liver Peroxisomes and with Enoyl-CoA Hydratase from Beef Liver. Enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase of peroxisomes (61) and glyoxisomes (7) have been shown to be associated with a single polypeptide. The association of cis-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase with this bifunctional enzyme has never been tested. A pure preparation of the bifunctional enzyme from rat liver peroxisomes (supplied by Dr. Hashimoto of Shinshu University, Japan) was compared with the complex of fatty acid oxidation from *E. coli* B and with beef liver enoyl-CoA hydratase. The results of this comparison are shown in Table X. The enoyl-CoA hydratase and L-3-hydroxyacyl-CoA

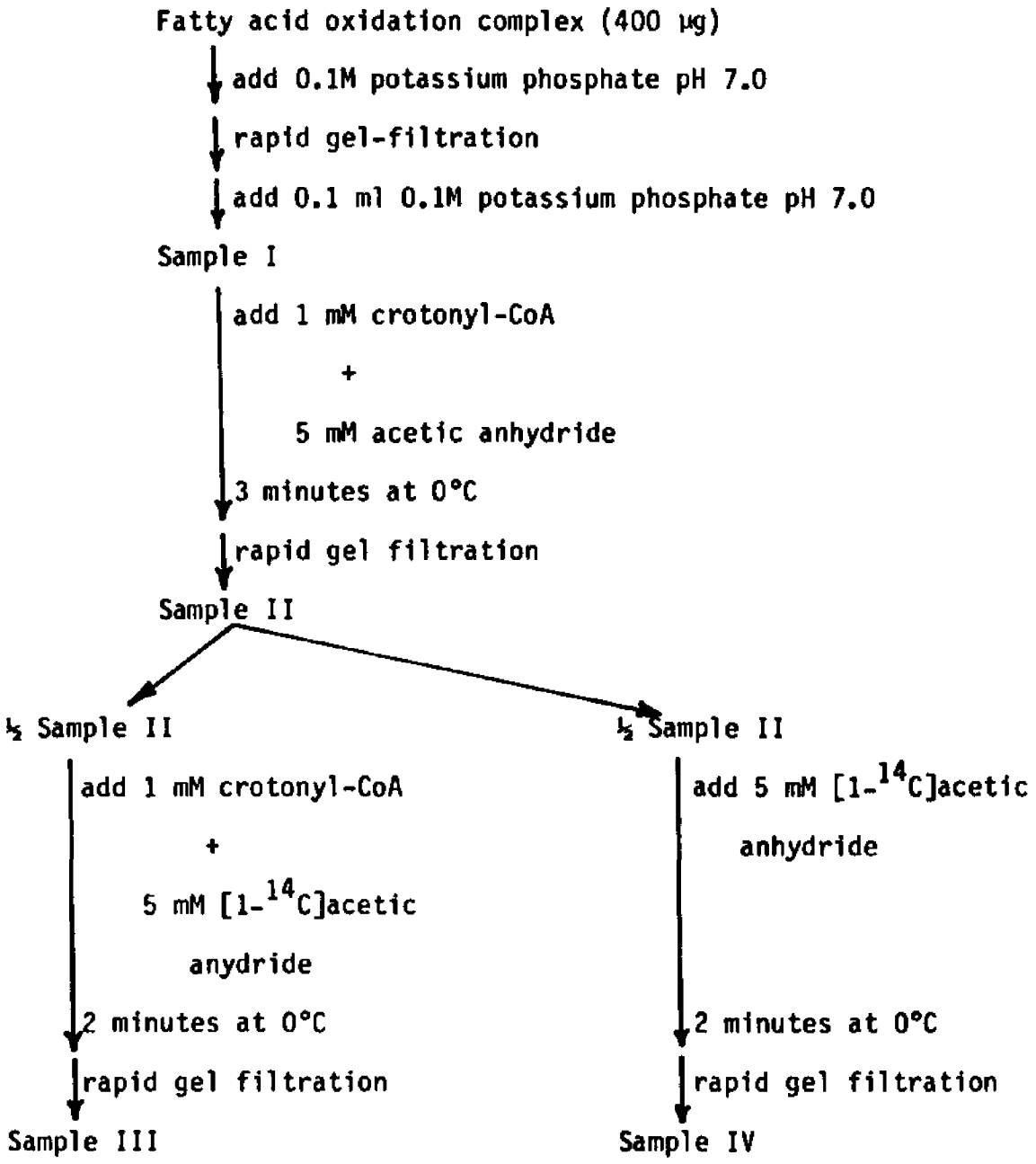


Figure 17. Labeling the Fatty Acid Oxidation Complex with [1-$^{14}$C]Acetic Anhydride.

TABLE IX

Inhibition of the Enzymes of the Fatty Acid Oxidation Complex by Acetic Anhydride

| Enzymes | Remaining Activity (U/mg) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Sample-I | (%) | Sample-II | (%) | Sample III | (%) | Sample IV | (%) |
| Enoyl-CoA hydratase | 167 | 100 | 42.3 | 25.3 | 40 | 24 | 10.4 | 6.2 |
| L-3-Hydroxyacyl-CoA dehydrogenase | 55.8 | 100 | 1.5 | 2.7 | 0 | 0 | 0 | 0 |
| 3-Ketoacyl-CoA Thiolase | 0.2 | 100 | 0 | 0 | 0 | 0 | 0 | 0 |
| cis-$\Delta^3$-trans-$\Delta^2$enoyl-CoA isomerase | 2.5 | 100 | 2.7 | 108 | 3.1 | 124 | 2 | 80 |
| 3-Hydroxyacyl-CoA epimerase | 0.72 | 100 | 0 | 0 | 0 | 0 | 0 | 0 |

Figure 18. Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate of the $^{14}C$-labeled fatty acid oxidation complex. Complex labeled in the presence (A), and in the absence (B) of crotonyl-CoA. For experimental details see under "Materials and Methods".

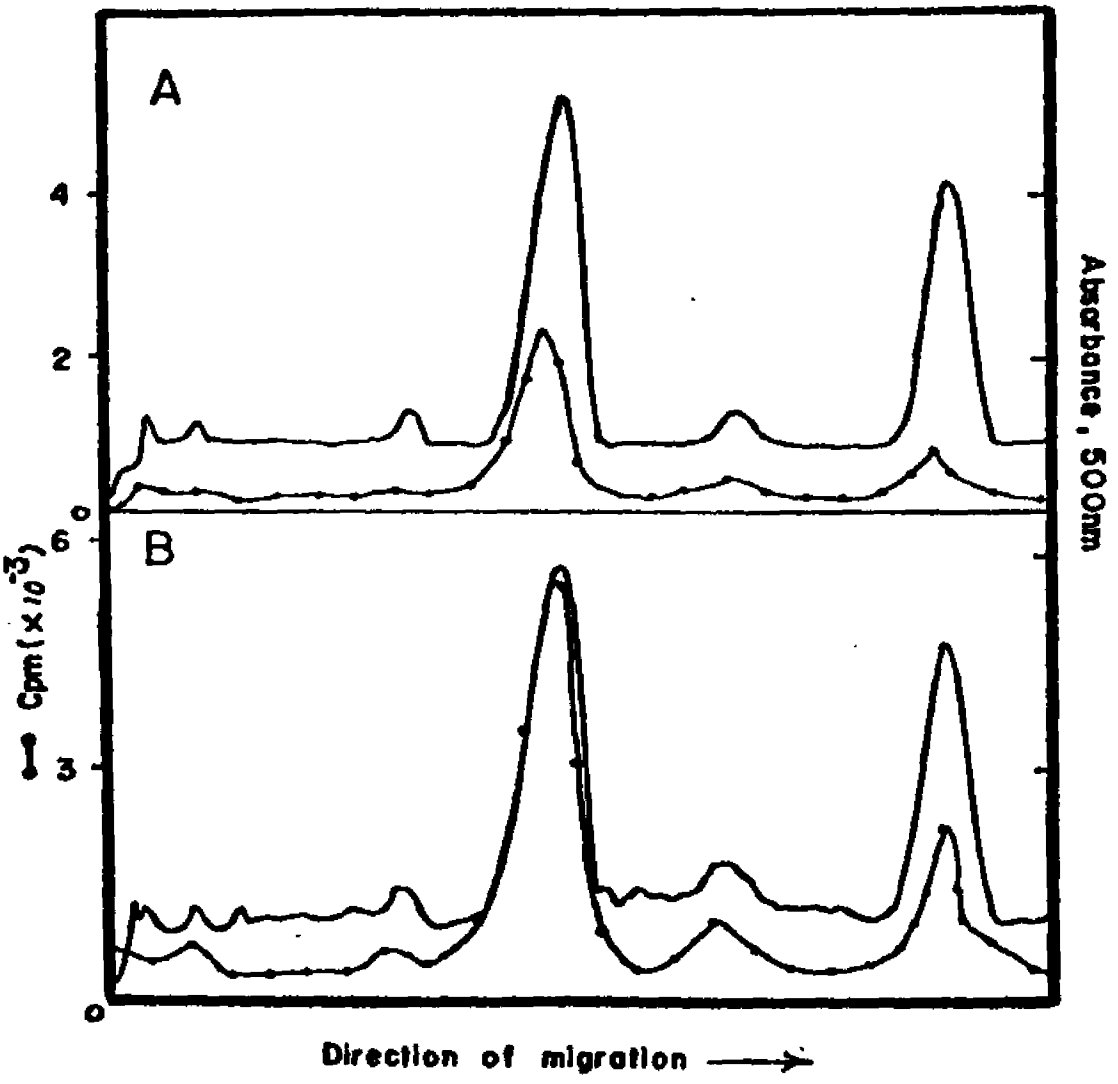
A
B
4
2
0
6
3
0
Cpm (x 10^-3)
Absorbance, 500nm
Direction of migration

Figure 18

TABLE X

Enzyme Activities Associated with Bifunctional β-Oxidation Enzyme (BOE) From Rat Liver Peroxisomes, with the Fatty Acid Oxidation Complex (FAOC) from E. coli and with Beef Liver Enoyl-CoA Hydratase (EH).*

| Enzyme | Substrate | Sp. Act. (μmol/min/mg) Rat Liver Peroxisomal BOE | E. coli FAOC | Beef Liver EH |
|---|---|---|---|---|
| Enoyl-CoA hydratase | Crotonyl-CoA | 221 | 115 | 1285 |
| L-3-Hydroxyacyl-CoA dehydrogenase | Acetoacetyl-CoA | 16 | 20 | 0.14 |
| cis-$\Delta^3$-trans-$\Delta^2$-Enoyl-CoA isomerase | cis-$\Delta^3$-Octenoyl-CoA | 0.3 | 8 | 0.03 |
| 3-Hydroxyacyl-CoA epimerase | D-3-Hydroxydodecanoyl-CoA | 0.001 | 2.4 | 0.001 |

*For experimental details see under "Materials and Methods".

dehydrogenase activities of the peroxisomal enzyme are comparable to those of the fatty acid oxidation complex. The cis-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase activities in this peroxisomal enzyme preparation are either very low or close to zero. The activities of dehydrogenase, cis-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase associated with beef liver crotonase are very low or close to zero. From this study I conclude that the bifunctional enzyme of rat liver peroxisomes contains neither isomerase nor epimerase activities. Additionally crotonase does not exhibit a cis-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA isomerase activity as has been reported by Stern (62).

# DISCUSSION

This study of the E. coli mutant fadB64 was initiated for the purpose of determining the number of enzymes with L-3-hydroxyacyl-CoA dehydrogenase activity present in E. coli and in the hope of obtaining active subunits or fragments of the multienzyme complex of fatty acid oxidation. The mutant fadA30, reported to have low levels of 3-ketoacyl-CoA thiolase was also used to study the effect of mutations on the structure of the fatty acid oxidation complex. When the complexes were subjected to chromatography on phosphocellulose, the observed purification patterns were identical to that of the wild-type complex. The subunit composition of the complex from fadB64 determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and its immunological behavior indicated that the mutant complex is structurally very similar to the wild-type complexes from E. coli K-12 YMel and B.

An interesting observation about long chain enoyl-CoA hydratase has been made during the course of this study. The presence of a long chain enoyl-CoA hydratase separate from crotonase was first reported by Beadle et al., (42). Pawar et al., (41) later showed that this long chain enoyl-CoA hydratase is not part of the fatty acid oxidation complex. In an induction study of short and long chain enoyl-CoA hydratase, Beadle et al., (42) showed that the two hydratases are being induced at different rates. This observation suggests that the gene for long chain crotonase is not part of the ABC operon. In this study I have found a fadABC mutant (RS 3084)

that exhibits the parental level of long-chain enoyl-CoA hydratase but is devoid of the activities associated with the fatty acid oxidation complex. In contrast fad mutants fad5 as well as RS 3110 and RS 3112 are devoid of both the short-chain and the long-chain enoyl-CoA hydratase activities. This observation suggests that the gene for long-chain enoyl-CoA hydratase is part of the fadABC operon but that its gene product is not part of the fatty acid oxidation complex.

The existence of polymeric forms of the fatty acid oxidation complex was detected and studied by use of gradient polyacrylamide gel electrophoresis. Besides the monomeric form, a dimer with an estimate molecular weight 580,000 was observed. The trimeric form of the complex molecular weight of 820,000 can also be detected (40). This study indicates that the dimer exhibits all five component enzymes activities associated with complex. Beadle et al. have also reported that high molecular weight aggregates of the complex are present in E. coli extracts. However, the physiological significance of the aggregation of this multienzyme complex needs to be established.

A detailed study of the fatty acid oxidation complex from mutant fadB64 revealed it to be structurally very similar to the wild-type complexes from either E. coli strains K-12 YMel or B. However, the specific activities of the complex from E. coli K-12-YMel, with the exception of 3-hydroxyacyl-CoA, were 2.5-times lower than those of the mutant complex. These differences between the two complexes in part due to a lower degree of purification achieved for the complex from the parental strain. Although the activitity differences remain

partially unexplained, I believe that they are a result of the isolation procedure and do not reflect an inherent difference between these two complexes beyond their different 3-hydroxyacyl-CoA dehydrogenase activities. The residual medium-chain and long-chain L-3-hydroxyacyl-CoA dehydrogenase activities are associated with the fatty acid oxidation complex. The main consequence of the mutation is the greatly reduced L-3-hydroxyacyl-CoA dehydrogenase activity which dramatically increases with increasing chain length of the substrate. However, even with longer chain substrates the dehydrogenase of the mutant complex is dramatically less active than the dehydrogenase of the wild-type complex. Since the $K_m$ value for NADH and 3-keto-decanoyl-CoA determined with the mutant dehydrogenase are lower or equal to the values obtained with the wild-type complex, the reduced dehydrogenase activity of the mutant is not due to a lower degree of saturation of the enzyme with either NADH or substrate. The major consequence of the mutation is a greatly reduced maximal velocity of the dehydrogenase which may be due to an impaired hydride transfer, the key step in the reaction catalyzed by the enzyme. An important finding is the very strong dependence of the mutant dehydrogenase activity on the chain length of the substrate. This observation suggests to me that the hydrocarbon chain beyond carbon 3 of the substrate is essential for the productive positioning of the substrate on the enzyme. The hydrocarbon side chain may be responsible for bringing the 3-keto group or 3-hydroxymethyl group of the substrate close to the coenzyme so that the hydride transfer can occur.

If the hydrocarbon chain is just a methyl group as in acetoacetyl-CoA or 3-hydroxybutyryl-CoA, the redox reaction does not occur at a measurable rate. However, with a n-propyl or n-heptyl group extending from carbon 3, 0.2% and 5%, respectively, of the wild-type activities are observed. Thus, it appears that several carbons of the hydrocarbon side chain contribute to the productive positioning of the substrate at the active site of the mutant L-3-hydroxyacyl-CoA dehydrogenase. In contrast, the dehydrogenase of the wild-type complex productively binds acetoacetyl-CoA which has only a single methyl group beyond carbon 3.

A completely different interpretation of the above data has to be considered, if two L-3-hydroxyacyl-CoA dehydrogenases with different chain length specificities were associated with the complex. If so, the mutation could have resulted in the inactivation of a short-chain 3-hydroxyacyl-CoA dehydrogenase without affecting a long-chain dehydrogenase. However, no evidence for the presence of two dehydrogenase on the complex has so far been obtained. In addition, the location of more than five component enzymes on the $\alpha$ and $\beta$ subunits with a combined molecular weight of 120,000 is not very likely. Hence, I suggest that the mutation has resulted in the complete inactivation of the only L-3-hydroxyacyl-CoA dehydrogenase of the complex toward acetoacetyl-CoA and in its partial inactivation toward longer-chain substrates.

During the course of these studies, I observed a rapid and parallel inactivation of all component enzymes of the fatty acid oxidation

complex by Tris-hydrochloride. It was also noted that thiolase was inactivated at a faster rate than were the other component enzymes of the complex. In fact, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, cis-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase were inactivated at approximately the same rate. The half-time of inactivation for these four enzymes was found to be 4-5 min whereas the half-time of thiolase was 2 min. In addition, when the protection of the complex against inactivation by Tris-hydrochloride was studied, crotonyl-CoA was found to protect enoyl-CoA hydratase as well as L-3-hydroxyacyl-CoA dehydrogenase; NADH and $NAD^+$ on the other hand protected enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase as well as thiolase. Since L-3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase, in contrast to thiolase, were inactivated by Tris-hydrochloride at the same rate and protected against this inactivation by crotonyl-CoA, the former two enzymes may be located on one subunit which does not contain thiolase. Since thiolase was found to be associated with the 42,000- dalton subunit (41), I suggest that enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase are located on the large subunit which has a molecular weight of 78,000.

Attempts to specifically label the active site of enoyl-CoA hydratase with [1-$^{14}$C]acetic anhydride failed. Although crotonyl-CoA specifically protects enoyl-CoA hydratase against inactivation by acetic anhydride both subunits were labeled nonspecifically by [1-$^{14}$C]acetic anhydride, even after prelabeling the complex with unlabeled acetic anhydride. Due to this extensive and nonspecific

acetylation of the complex the subunit location of enoyl-CoA hydratase could not be definitely determined.

Osumi and Hashimoto (61) reported the association of enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase with one polypeptide in rate liver peroxisomes. A similar observation was made by Kindle and Frevert (7) with glyoxisomes. However, the possible assocation of cis-$\Delta^3$-trans-$\Delta^2$-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase with this bifunctional enzyme has never been tested. I have measured isomerase and epimerase activities of the bifunctional enzyme. The levels of isomerase and epimerase in this preparation of bifunctional enzyme were found to be so low that these two enzymes are clearly not part of the bifunctional enzyme. At the same time a preparation of bovine liver enoyl-CoA hydratase was assayed for isomerase and epimerase. Because the observed levels of these two auxiliary β-oxidation enzymes were extremely low, they are not associated with enoyl-CoA hydratase. This observation disproves a claim by Stern (62) that isomerase is associated with enoyl-CoA hydratase.

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