A STUDY OF FATTY ACID OXIDATION IN *ESCHERICHIA COLI*: ISOLATION OF A MULTI-ENZYME COMPLEX OF FATTY ACID OXIDATION AND PURIFICATION AND PROPERTIES OF THIOLASES

by

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ABSTRACT

A multi-enzyme complex of fatty acid oxidation was isolated from Escherichia coli B cells and was purified to near homogeneity by a simple two-step procedure. The complex exhibited thiolase (EC2.3.1.16), enoyl-CoA hydratase (EC4.2.1.17), and 3-hydroxyacyl-CoA dehydrogenase (EC1.1.1.35) activities towards short-, medium-and long-chain substrates. The complex was shown to have a molecular weight of approximately 300,000 and is apparently composed of two types of subunits with molecular weights of 78,000 and 42,000.

The presence of only one thiolase activity (thiolase I) in wild-type E.coli induced for enzymes of β-oxidation was demonstrated. A different thiolase (thiolase II) was shown to be present in a mutant constitutive for the enzymes of butyrate degradation. The two thiolases were purified to near homogeneity and were found to be associated with different proteins as shown by gel electrophoresis. Thiolase I, which is a component enzyme of the multi-enzyme complex, was active on 3-ketoacyl-CoA derivatives containing 4 to 16 carbons, but exhibited optimal activity with medium-chain substrates, whereas thiolase II was shown to be specific for acetoacetyl-CoA. The molecular weight of thiolase II was estimated to be 175,000 in the absence and 41,500 in the presence of sodium dodecyl sulfate. Hence it appears that this thiolase is composed of four subunits. The pH optimum for thiolase I was found to be approximately 8 when assayed with both acetoacetyl-CoA and 3-ketodecanoyl-CoA. Thiolase II showed a pH optimum of 8.2. The maximal velocities for the thiolytic cleavage of acetoacetyl-CoA and 3-ketodecanoyl-CoA by thiolase I were estimated to be 1.2 and 27.7 μmoles/min/mg of

protein, respectively, and that for thiolase II as 187.5 μmoles/min/mg of protein. The Km values for acetoacetyl-CoA observed with thiolase I and II were 31μM and 7.5μM respectively and the values determined for CoA were 20μM and 7.6μM respectively. Thiolase I in dilute solution proved to be much less stable than thiolase II and showed in contrast to thiolase II an absolute requirement for a thiol reagent.

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INTRODUCTION I

The degradation of fatty acids through β-oxidation, first suggested by Knoop in 1904, involves a sequence of four reactions in which two carbon atoms are removed from a fatty acid as acetyl-CoA. The steps of this pathway as established by the groups of Lynen, Green, Ochoa and others in mammalian tissues are (see Fig. 1): 1) activation of the fatty acid to its coenzyme A (CoA) thioester by acyl-CoA synthetase (EC 6.2.1.3); 2) dehydrogenation of the acyl-CoA to the trans-enoyl-CoA by acyl-CoA dehydrogenase (EC 1.3.99.3); 3) hydration of the α, β-unsaturated-acyl-CoA to the L(+)-β-hydroxyacyl-CoA by enoyl-CoA hydratase (crotonase) (EC4.2.1.17); 4) oxidation of the β- hydroxy-acyl-CoA to the β - ketoacyl-CoA by β-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35); and 5) thiolytic cleavage of the β-ketoacyl-CoA derivative to acetyl-CoA and an acyl-CoA two carbons shorter in length by thiolase (EC 2.3.1.16) (1). The resulting acyl-CoA compounds then pass through the four β -oxidation reactions listed under 2) to 5) until the fatty acid is completely degraded to acetate units.

Despite the importance of understanding the regulation of fatty acid oxidation, little is known about the control of this metabolic pathway in eukaryotic cells and about the in vivo organization of the enzymes of β-oxidation. Work with rat liver mitochondria (2, 3) indicates that these enzymes are either attached to the inner membrane or located in the matrix space. Drysdale and Lardy (4) have been unsuccessful in demonstrating the accumulation of acyl-CoA intermediates

during the conversion of octanoyl-CoA to acetyl-CoA and suggested that the true intermediates of β-oxidation may be enzyme bound. Similar conclusions were reached by Garland, Shepherd and Yates (5) who studied the oxidation of palmityl-CoA in intact rat liver mitochondria. The absence of detectable amounts of intermediates in fatty acid oxidation led to the statement of Drysdale and Lardy (4) in 1953 that "it is equally possible that the acyl-CoA could transfer its fatty acid to a group on the enzyme before oxidation...." resembling the situation in fatty acid synthesis where the intermediates involved are enzyme bound as thiol esters to one or more of the proteins involved (6). Alternatively, the low concentrations of intermediates in fatty acid oxidation may be due to their movement from one enzyme to another without release into the medium. Thus, the suggestion that the enzymes of β -oxidation exist in a highly ordered arrangement in vivo is based on earlier observations made during studies of fatty acid oxidation.

Although studies with bacteria have helped to clarify the mechanism of fatty acid synthesis (7,8), there have been few studies of fatty acid oxidation in bacterial systems. Besides the early work with Clostridium kluyveri by Barker, Stadtman and Kennedy (9, 10) who studied short-chain fatty acid metabolism and the possible intermediates involved in butyrate oxidation, information regarding β-oxidation in microorganisms is relatively scarce. The first hint of the inducibility of the enzymes of fatty acids in E.coli came from the work of Silliker and Rittenberg (11) but later studies (12, 13) failed to show the presence of β-oxidation enzymes in extracts of E.coli as well as in those of other microorganisms.

The recent work by Overath et al. (14) and Weeks et al. (15) has demonstrated the presence of the β-oxidation enzymes in *E.coli* and their co-ordinate induction when cells were grown on long-chain fatty acids as the sole carbon source. Further genetic work by Overath and coworkers (16) and the subsequent isolation of mutants of fatty acid oxidation (16) has led to the suggestion that genes of thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase form an operon and that fatty acid oxidation in *E.coli* is regulated at the transcriptional-translational level. In view of the extensive genetic work done by Overath and the availability of fatty acid oxidation mutants and because of the great contribution that bacterial genetics has made in understanding metabolic mechanisms, this project was undertaken in the hope that the study of enzymes of β -oxidation from *E.coli* would provide some insight into the organization of these enzymes as well as into the regulation of fatty acid oxidation.

In the first part of this thesis the isolation and purification of a multi-enzyme complex of fatty acid oxidation from *E.coli* B cells is reported. This multi-enzyme complex exhibits thiolase, enoyl-CoA hydratase and 3-hydroxyacl-CoA dehydrogenase activities toward short-, medium-, and long-chain substrates.

MATERIALS AND METHODS I

Chemicals. CoA, NAD^+, NADH, acetyl-CoA and n-decanoyl-CoA were purchased from PL-Biochemicals. Ethyl chloroformate, triethylamine, diketene, pyruvic acid and trans-$\Delta^{2,3}$-decenoic acid were obtained from Aldrich Chemical Co. trans-$\Delta^{2,3}$-Hexadecenoic acid and apoferritin were purchased from Miles Laboratories, Inc. L-3 Hydroxyacyl-CoA dehydrogenase (EC1.1.1.35) was bought from Boehringer Mannheim Corp. Lactate dehydrogenase was purchased from Worthington Biochemicals Corp. Sepharose 6B and Sephadex G-200 were bought from Pharmacia, Inc. ATP, glutmate dehydrogenase, phosphorylase A, phosphorylase B, bovine serum albumin, catalase, p-iodonitrotetrazolium chloride and phenazine methosulfate were purchased from Sigma Chemical Co. All other chemicals were of reagent grade. Diketene and ethylchloroformate were distilled before use whereas all other chemicals were used without further purification. Enoyl-CoA hydratase was isolated from beef liver according to the procedure of Steinman and Hill (17).

Synthesis of substrates. trans-$\Delta^{2,3}$-Hexenoic acid, trans-$\Delta^{2,3}$-octenoic acid and trans-$\Delta^{2,3}$-dodecenoic acid were synthesized by reacting malonic acid in the presence of pyridine with n-butyraldehyde, n-hexanal and n-decanal respectively, by following the procedure of Linestead et al (18). The CoA derivative of $\Delta^{2,3}$-hexenoic acid and its longer-chain homologues were prepared by reacting their mixed anhydrides with CoA, as described by Schulz (19). The concentrations of the resulting $\Delta^{2,3}$-enoyl-CoA substrates were determined by the method of Ellman (20) after cleaving the thioester bond with hydroxylamine at

pH7. The 3-ketoacyl-CoA substrates, other than acetoacetyl-CoA, were synthesized enzymatically from their respective $\Delta^{2,3}$-enoyl CoA derivatives in the presence of enoyl-CoA hydratase and3-hydroxyacyl-CoA dehydrogenase as described by Feigenbaum and Schulz (21). Acetoacetyl-CoA was prepared from diketene and CoA following the method of Seubert (22).

Organism. E.coli B ATCC 11775 cells were grown in M-9 mineral salts medium with oleate as the sole carbon source, as described by Overath et al (16). These cells were grown with shaking to the late exponential growth phase (absorbance of 1.7 to 1.8 at 420 nm, measured on a Gilford spectrophotometer, model 240), harvested, and stored at -20C.

Purification of the multi-enzyme complex of fatty acid oxidation. The crude homogenate was prepared by suspending E.coli B cells induced for enzymes of β-oxidation (20g) in 40 ml of 10 mM potassium phosphate (pH 7.0) containing 10mM 2-mercaptoethanol.and 25% (vol/vol) glycerol and by sonically treating it for 4 min at 0-5°C. All further operations were performed at 0 to 5°C unless otherwise stated. The resulting crude homogenate was centrifuged for 60 min at 31,300x g and was then subjected to a 10-min heat treatment at 60°C. The heat treated homogenate was centrifuged at 31,300xg for 30 min and dialyzed overnight against 1 liter of 20 mM potassium phosphate (pH6.6) containing 25% (vol/vol) glycerol, and 10mM 2-mercaptoethanol. This dialysate was applied to a phosphocellulose column (5.0 X 32cm) previously equilibrated with the dialysis buffer. The column was washed with the same buffer until no

more ultraviolet-absorbing material was eluted. The column was then developed with a phosphate gradient prepared from 2l of 20mM potassium phosphate (pH6.6) containing 10mM 2-mercaptoethanol and 25% (vol/vol) glycerol, and from 2l of 500 mM potassium phosphate (pH6.6) containing 10mM 2-mercaptoethanol and 25% (vol/vol) glycerol. Fractions of 20ml each were collected and assayed for thiolase, 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities as described under "Protein and Enzyme Determinations". The fractions containing these enzyme activities were pooled and concentrated by ultrafiltration with an Amicon concentrator (PM-10 membrane) to approximately 5ml. The protein was stored at -70°C. When the enzymes were rechromatographed, 5ml (9mg) of the material purified by chromatography on phosphocellulose was applied to a second phosphocellulose column (1.2x45cm) previously equilibrated with 0.1M potassium phosphate (pH6.6) containing 10mM 2-mercaptoethanol and 25% (vol/vol) glycerol after being dialyzed overnight against the column equilibrating buffer. The column was washed with this buffer until no more ultraviolet-absorbing material was eluted and the multi-enzyme complex was eluted with a phosphate gradient prepared from 500 ml of 0.1M potassium phosphate (pH6.6) containing 2-mercaptoethanol and 25% (vol/vol) glycerol and 500 ml of 0.4M potassium phosphate (pH6.6) containing 2-mercaptoethanol and 25% (vol/vol) glycerol. Fractions (10 ml) were collected, assayed for enzyme activities and concentrated as described for the first phosphocellulose column. Samples were stored at -70°C. Data for this purification procedure are presented in Table 1.

Protein and enzyme determinations. Protein concentrations were determined by the method of Lowry et al (23). Thiolase activities were assayed by following spectrophotometrically the decrease in absorbance at 303 nm due to the disappearance of the Mg^{+2} - enolate complex of the 3-ketoacyl-CoA substrates as has been previously described (21). The extinction coefficients used in calculating thiolase activities were: acetoacetyl-CoA, $16.9 \times 10^3 cm^{-1} M^{-1}$; 3-ketohexanoyl-CoA $15.6 \times 10^3 cm^{-1} M^{-1}$; 3-ketodecanoyl-CoA $13.5 \times 10^3 cm^{-1} M^{-1}$ (24). The latter value was also used as the extinction coefficient for 3-ketododecanoyl-CoA and 3-ketohexadecanoyl-CoA. The extinction coefficient used in calculating thiolase activity with 3-ketooctanoyl-CoA was $14.5 \times 10^3 cm^{-1} M^{-1}$. The assay of 3-hydroxyacyl-CoA dehydrogenase was based on the substrate-dependent oxidation of NADH which was followed by measuring the change in absorbance at 340nm at 25°C (15). Enoyl-CoA hydratase was assayed by following spectrophotometrically the hydration of $\Delta^{2,3}$ - enoyl-CoA substrates at 263nm as described by Schulz (19). Units of activity are expressed as μ moles per min. The acyl-CoA synthetase activity was measured by using the assay of Kornberg & Pricer as described by Overath (16). The spectrophotometric assay of acyl-CoA dehydrogenase was performed by measuring at 492 nm the reduction of iodonitrotetrazolium chloride in the presence of phenazine methosulfate, similar to the procedure of Dommes and Kunau (25).

Disc gel electrophoresis. Disc gel electrophoresis of the multienzyme complex of fatty acid oxidation was performed with standard 7.5% polyacrylamide gels at 15°C and pH 8.5, as described by Davis (26), except that 10mm 2-mercaptoethanol was added to the separating gel and

tris-(hydroxmethyl) aminomethane-glycine buffer. Gels were stained for protein with Coomassie brilliant blue or Fast Green, destained with 7% acetic acid, and scanned at 600nm. When the gels were assayed for enzyme activities, duplicate unstained gels were sliced and each slice was incubated for 12 hrs.at 5°C in 0.2 ml of 0.05M tris-(hydroxymethyl) aminomethane-HCL buffer (pH8.1) containing 25% (vol/vol) glycerol, bovine serum albumin (1mg/ml) and 2mM dithiothreitol. Samples were assayed for enzyme activities as described under "Protein and enzyme determinations". Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate was carried out as described by Weber and Osborn (27). Analytical gradient gel electrophoresis was performed on 4%-30% polyacrylamide gradient slab gels (Pharmacia Fine Chemicals, Inc.) in the presence of Tris-boric acid (pH8.35) at 10°C and 125V for 20 hrs. on a Pharmacia GE-4 electrophoresis apparatus.

Molecular weight determinations. Molecular weight estimations were carried out on a standardized Sephadex G-200 column following the procedure of Andrews (28) and a similar manner on a standardized Sepharose 6B column.

Analytical ultracentrifugation. Analytical ultracentrifucation was carried out with a Beckman Model E analytical ultracentrifuge equipped with Schlieren optics. Sedimentation velocity runs were performed at 25°C using an AN-D rotor at a speed of 40,000 rpm. Pictures were taken every four minutes. The multi-enzyme complex was dialyzed against 0.2M Tris-Cl (pH8.35) containing 10mM 2-mercaptoethanol which was used during the ultracentrifugation run. The final protein concentration was 5mg/ml. The protein was run in a double sector capillary type cell so that small volumes could be employed. The sedimentation coefficient ($S_{25}o$) was determined by

plotting the natural log of r(cm), the distance of the protein from the center of the rotor, versus time (sec.). The slope of this line (m) is used to calculate the S_{250} value where $m=\omega^2 r$ and ω= angular velocity of the rotor in radians/sec. The sedimentation coefficient was corrected to that value for H_2O at 20°C ($S_{20,w}$) by using the following equation (29):

$$S_{20,w} = \frac{(1-\bar{v}\rho)_{20,w}\eta_{T,b}}{(1-\bar{v}\rho)_{T,b}\,\eta_{20w}} \times S_{T,b}$$

where

$\eta_{T,b}$ = solvent viscosity at 25°C

$\eta_{20,w}$ = viscosity of H_2O at 20°C

$\bar{v}$ = partial specific volume for protein solution

$\rho_{T,b}$ = density of solvent at 25°C

ρ_{20w} = density of H_2O at 20°C

$S_{T,b}$ = sedimentation coefficient in solvent at 25°C

Values used were;

$\bar{v}$=0.74 cm^3/g (approximation of average protein solutions, Dr. D. Sloan)

$\rho_{T,b}$ = 1.00436g/ml (calculated by weighing known volume of solvent)

$\rho_{20,w}$=0.99676g/ml (standard value in Handbook of Chemistry and Physics)

$S_{T,b}$ = 11.50×10^{-13} sec (calculated from slope of line in Fig. 5)

$\frac{\eta_{T,b}}{\eta_{20,w}}$ = 1.080 (approximated from extrapolation of data supplied by Dr. D. Sloan)

Estimations of the molecular weight were made using the generalized equations of Halsall (30): $\log S_{20,w} = \bar{3}.383\pm0.044+2/3 \log M$, where S_{20w} = sedimentation coefficient obtained in H_2O at 20°C and M= molecular weight.

RESULTS I

During the purification of the enzymes of fatty acid oxidation from E.coli B cells, on phosphocellulose, it was observed that thiolase, 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase cochromatographed (Fig. 2). The enzyme preparation obtained after phosphocellulose chromatography was active with short-and medium-chain substrates of all three enzymes. The results of the simple two step purification, as summarized in Table 1, show 33 to 60-fold purification of the three enzymes in approximately 25% yield. The reasons for the variations in fold purification from one enzyme to another and between short- and medium-chain activities of one enzyme have not yet been elucidated. However, an unequal inactivation of the three enzymes and the removal of additional thiolase, enoyl-CoA hydratase or 3-hydroxyacyl-CoA dehydrogenase activities from the enzyme complex during its purification may account for these variations. Unequal degrees of inactivation of the component enzymes of the purified complex during dialysis and DEAE-cellulose chromatography has been observed. Also, the removal of a separate medium-chain enoyl-CoA hydratase activity from the complex has been detected in both the forerun and 1M potassium phosphate wash during phosphocellulose chromatography. Although no separate thiolase and 3-hydroxyacyl-CoA dehydrogenase activities were observed during phosphocellulose chromatography, it is possible that such activities may have gone undetected or may have been lost due to inactivation. In addition, the low activity of thiolase towards acetoacetyl-CoA made it difficult to determine accurately the ratio of short-chain to medium-chain activities.

Rechromatographing a purified enzyme preparation on a second phosphocellulose column resulted in a 60% loss of the activities of all three component enzymes with no substantial increase in their specific activities. Since this second purification step on phosphocellulose did not give rise to increased specific activities of thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, it was not routinely included in the isolation procedure of the multi-enzyme complex.

Since it is possible that the three enzymes cochromatographed as a result of the heat treatment, this step was omitted during the purification of one enzyme preparation. This preparation as judged by phosphocellulose chromatography, polyacrylamide gradient gel electrophoresis and gel electrophoresis in the presence of sodium dodecyl sulfate was identical to preparations which had been heat treated. The purified enzyme preparations which had been subjected to heat treatment was apparently devoid of acyl-CoA synthetase and acyl-CoA dehydrogenase activities, but the effect of the heat treatment on these two activities has not yet been studied.

The purity of the fatty acid oxidation enzymes after phosphocellulose chromatography was evaluated by polyacrylamide disc gel electrophoresis. As can be seen in Fig.3, a single protein band was observed which was associated with short-and medium-chain activities of thiolase, enoyl -CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase. A single band was also observed when the enzyme was subjected to electrophoresis on a polyacrylamide gradient gel. Additionally, the chromatography on Sepharose 6B showed the cofiltration of these three enzyme activities (see Fig. 4).

Pictures of a sedimentation velocity run of the multi-enzyme complex showed a single peak (see Fig. 5) thus denoting the presence of a single protein. The fact that thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase cochromatographed on phosphocellulose and Sepharose 6B and comigrated during polyacrylamide gel electrophoresis and during ultracentrifugation suggests the existence of a multi-enzyme complex of fatty acid oxidation in E.coli B.

The native molecular weight of this complex as determined by gel filtration on Sephadex G-200 (Fig. 6A) and Sepharose 6B (Fig. 7) was estimated to be 320,000. When subjected to polyacrylamide gradient gel electrophoresis, the complex behaved as a single protein with a molecular weight of 270,000 when compared to the mobilities of standard proteins on the same gel (Fig. 6B). Sedimentation velocity data obtained with the multi-enzyme complex gave a S_{20} value of 12.69 (see Fig. 5) which when analyzed by the method of Halsall (30) yielded on estimated native molecular weight of327,000 (See Materials and Methods I). This high molecular weight obtained by four methods lends support to the proposal that thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase exist as a multi-enzyme complex of fatty acid oxidation.

The subunit pattern of the enzyme complex (shown in Fig. 8A) as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis in the presence and absence of 2-mercaptoethanol shows two main protein bands corresponding to molecular weights of 78,000 (I) and 42,000 (II). When the enzyme preparation was rechromatographed on a second phosphocellulose column,the specific activities of all three enzymes remained virtually unchanged, whereas the subunit pattern showed a decrease of

band III as illustrated in Fig 8B. Thus, the change in subunit pattern indicates that band III is not an inherent part of the complex. The appearance of a minor band in front of band I (see Fig. 8B) may be an artifact since it was not observed in the protein pattern of the original preparation (see Fig. 8A). The multi-enzyme complex of fatty acid oxidation, therefore appears to be comprised of two types of subunits of molecular weights 78,000 and 42,000 which are not covalently linked.

When the purified complex was subjected to DEAE-cellulose chromatography, evidence of a breakdown of the multi-enzyme complex was apparent. At least two protein fractions containing thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase were observed (see Fig. 9). The specific activities associated with peak I were approximately three times those associated with peak II (see Table 2). When samples corresponding to peaks I and II were analyzed by gradient gel electrophoresis both preparations showed a main band corresponding to a molecular weight of 270,000 whereas only the protein corresponding to peak II exhibited two definite additional bands corresponding to a molecular weight of 165,000 and 64,000. Hence it appears that the complex under the conditions of DEAE-cellulose chromatography partially dissociated.

Further attempts to dissociate the complex with choline chloride or by exposure to low ionic strength buffer or to high pH resulted in extensive inactivation without apparent dissociation. Treatment with 2M urea also inactivated the complex but caused as well dissociation of the complex yielding proteins with molecular weights of 110,000 and 78,000 as judged by a gradient gel electrophoresis. One of these

proteins may be the same as the 78,000 molecular weight subunit observed on sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The substrate specificities of the three component enzymes of the complex were determined spectrophotometrically at pH 8. Thiolase and enoyl-CoA hydratase, but not 3-hydroxyacyl-CoA dehydrogenase, were assayed by the standard procedures described under "Materials and Methods I." The 3-hydroxyacyl-CoA dehydrogenase activities were followed in this experiment by measuring the rate of dehydrogenation of 3-hydroxyacyl-CoA substrates because the enzyme catalyzes the reaction in this direction during the in vivo oxidation of fatty acids. It should be noted that the activities of the dehydrogenase obtained with this assay are different from and generally much lower than the activities observed with the standard assay. As shown in Fig. 10, all enzymes were active on substrates over a broad range of chain lengths. The activity of enoyl-CoA hydratase is highest with short-chain substrates, specifically with crotonyl-CoA and $\Delta^{2,3}$-hexenoyl-CoA, but decreases continuously with increasing chain length of the substrates. In contrast, thiolase and 3-hydroxyacyl-CoA dehydrogenase exhibit both their highest activities with the medium-chain substrates. Since long-chain acyl-CoA derivatives are good detergents and therefore cause the inactivation of many enzymes, it is possible that thiolase and 3-hydroxyacyl-CoA dehydrogenase are most active under in vivo conditions with long-chain substrates. Fig. 10 also demonstrates that the slowest reaction measured with the complex was the thiolytic cleavage of acetoacetyl-CoA which may be the rate-limiting step in β-oxidation unless the activities of the acyl-CoA dehydrogenases are even lower.

DISCUSSION I

The presence of an enzyme complex of fatty acid oxidation in *E. coli* B cells, induced for the enzymes of β-oxidation, was detected in the process of isolating and purifying thiolase from these cells (see Part II). Despite various purification procedures, the subunit pattern of this thiolase preparation repeatedly showed two main bands on sodium dodecyl sulfate polyacrylamide gels and the native molecular weight of this preparation was estimated to be approximately 300,000. Since thiolases from various sources (31-33) were reported to have molecular weights around 160,000 and to be composed of four identical subunits with molecular weights of approximately 40,000, the high values obtained for the *E. coli* B thiolase preparation from both native and subunit molecular weight data seemed unexplanable. In light of these findings, the purified thiolase preparation was assayed for other enzymes of β-oxidation and was shown to contain substantial enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities. Since these enzyme activities remained associated when subjected to a variety of separation techniques, it is concluded that thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase exist as a multi-enzyme complex which is apparently devoid of acyl-CoA synthetase and acyl-CoA dehydrogenase activities.

In view of these findings, it is interesting to note that the genes for thiolase, enoyl-CoA hydratase, and 3-hydroxyacyl-CoA dehydrogenase (17), but not those for acyl-CoA synthetase, (17) and the acyl-CoA dehydrogenases (34), are closely linked and perhaps form an operon named the "fad" operon by Overath (35). Since thiolase, enoyl-CoA hydratase and

3-hydroxyacyl-CoA dehydrogenase are linked genetically and in view of the fact that their coordinate inductibility has been demonstrated in E.coli cells grown on long-chain fatty acids (16), it is possible that the "fad" operon assures the synthesis of these three enzymes in amounts necessary for the correct assembly of the multi-enzyme complex. If the same multi-enzyme complex of fatty acid oxidation also exists in E.coli K_{12} cells, an explanation of the nature of the fad-5 mutation, which is characterized by the lack of thiolase, enoyl-CoA hydratase, and 3-hydroxyacyl-CoA dehydrogenase activities (35), can be provided. In this explanation, it is assumed a mutation involving these genes may lead to the formation of gene products which cannot be incorporated into the multi-enzyme complex thus preventing its formation with the result that the activities of all three component enzymes are lost. In addition, it is possible that the fad-5 mutation is caused by a deletion of the structural genes of these three enzymes.

Although the native molecular weight of the multi-enzyme complex has been estimated and the presence of two types of subunits was demonstrated, the quaternary structure of the complex has not been definitely established. This is due to the fact that the molar ratio of the two subunits cannot be exactly determined by sodium dodecyl sulfate gel electrophoresis because different proteins do not stain equally well (A. Pramanik, unpublished observations.) Given a native molecular weight of approximately 320,000 and subunit molecular weights of 78,000 (α) and 42,000 (β), the following quaternary structures are possible: $\alpha\beta_6$, $\alpha_2\beta_4$, $\alpha_3\beta_2$. The subunit structure of $\alpha_2\beta_4$ best agrees with the molar ratios of the two subunits observed on sodium dodecyl sulfate gel electrophoresis. Throughout this study it has been assumed that each

of the two main bands, which were observed when the complex was subjected to sodium dodecyl sulfate gel electrophoresis represented a homogeneous protein. If this assumption is correct, it must be concluded that two of three enzyme activities of the complex are located on one polypeptide chain. Such situations are known to exist in bacterial systems such as in E.coli DNA polymerase I (36) and E.coli DNA polymerase II (37, 38). In addition to both these multi-functional proteins, DNA polymerase III of E.coli consists of two types of polypeptide chains, one of 140,000 daltons and one of 40,000 daltons (39) and is associated with three different enzymatic activities (40), a picture very similar to that of the multi-enzyme complex of fatty acid oxidation in E.coli B cells. A situation where one polypeptide contains a multiple of enzymatic activities is, therefore, not impossible but because the conclusion is unexpected, further verification of the quaternary structure of the complex is needed.

Activity measurements with the multi-enzyme complex revealed that the component enzymes were active with substrates of all chain lengths. Similar to the mammalian enzymes, enoyl-CoA hydratase (41, 42) exhibited the maximal activity with short-chain substrates whereas the highest thiolase (24,43) and 3-hydroxyacyl-CoA dehydrogenase (44,45) activities were observed with medium-chain substrates. The fact that the complex is composed of three enzyme activities active with all chain-length substrates but composed of only two types of polypeptides, leads to the suggestions that only one enzyme each catalyzes the hydration, dehydrogenation, and thiolytic cleavage over the whole chain-length range. In light of the observations by Klein et al. (46) that

mutants deficient in acetoacetyl-CoA thiolase or 3-hydroxybutyryl-CoA dehydrogenase activities can partially or even totally degrade long-chain fatty acids, one must assume that the minimal remaining activities of the fatty acid oxidation enzymes exhibited by these mutants (16) is sufficient to degrade long-chain fatty acids. In addition, because these enzymatic activities were assayed by Klein et al. only with short-chain substrates (46), the ability of mutants deficient in thiolase and 3-hydroxyacyl-CoA dehydrogenase to oxidize long-chain fatty acids may not be surprising considering the higher activities of these enzymes towards long-chain than towards short-chain substrates. It is, however, possible that extremely labile thiolase and 3-hydroxyacyl-CoA dehydrogenase activities have so far gone undetected. The observation of a separate medium-chain enoyl-CoA hydratase activity remains to be confirmed but may indicate that more than one enoyl-CoA hydratase are present in E.coli.

The isolation of three enzymes of β-oxidation in the form of a multi-enzyme complex provides the opportunity to compare the catalytic efficiency of an integrated enzyme system versus that of an enzyme mixture. Furthermore, a detailed study of the quaternary structure of the complex will provide an understanding of the in vivo organization of the enzymes of β-oxidation. It is anticipated that a detailed picture of the E.coli fatty acid oxidation system will be helpful in elucidating the organization and regulation of the mitochondrial β-oxidation systems of mammals.

INTRODUCTION II

Thiolase, the last enzyme in the fatty acid oxidative pathway, catalyzes the thiolytic cleavage of 3-ketoacyl-CoA thioesters to acetyl-CoA and saturated acyl-CoA esters shorter by two carbon atoms:

$$R\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}SCoA + CoASH \rightarrow R\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}SCoA + CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-}SCoA$$

, where R= saturated carbon chain. A thiolase specific for acetoacetyl-CoA, crystallized from pig heart and extensively studied by Gehring et al. (47), contains an essential cysteine residue at the active site which participates in the formation of an acyl-enzyme intermediate. Recent work with the yeast thiolases (32) demonstrated that borohydride inhibits enzymatic activity in the presence of acetoacetyl-CoA. A mechanism for the thiolytic cleavage has been proposed which suggests the involvement of an amino group besides the thiol group in catalysis.

The wide distribution of thiolases in nature reflects the ubiquitous occurence of the fatty acid oxidation cycle. Among bacteria, Pseudomonas putida (48), Rhodosprillum rubrum (13) and Clostridia (13) were found to contain thiolase in addition to E.coli cells induced for enzymes of fatty acid oxidation (14, 48). Yeast contains two forms of thiolase (32) one of which is present in the cytosol whereas the other is located in the mitochondria. Thiolases have been shown to be present in all mammalian tissues that have been examined. Liver, heart, kidney and rat adrenals (24) are examples of tissues with high thiolase activity. Recent reports have demonstrated the presence of multiple forms of thiolase in rat, ox and avian liver (24, 49,33) and that thiolase activities are located both in mitochondria and in the cytoplasm as had previously been reported for yeast. This recent work on the multiplicity of thiolases

has led to the proposal that mitochondrial thiolases with broad chain length specificities are active in fatty acid oxidation (24,43) whereas acetoacetyl-CoA thiolases located in liver mitochondria are involved in ketone body formation (24, 33, 50), and those located in mitochondria of extrahepatic tissues are involved in acetoacetate utilization (24). The cytosylic acetoacetyl-CoA thiolase, absent from muscle tissue (24), has been implicated in cholesterol biosynthesis (33,51,52). Its tissue distribution agrees well with the capacity of these tissues to produce cholesterol. Fig. 11 illustrates the possible metabolic roles of different thiolases.

The existence of more than one thiolase activity in E.coli has been the subject of recent work (35). The co-inducibility of the enzymes of fatty acid oxidation in E.coli and the existence of their genes in the form of a "fad" (fatty acid degradation) operon was demonstrated by Overath et al (16). Further genetic studies by these workers (35) showed that in the presence of acetoacetate, E.coli thiolase and acetoacetyl-CoA: acetate CoA-transferase activities were stimulated by a factor of 3000, and that the structural genes for these enzymes were closely linked together with a regulatory gene forming an "ato" operon (acetoacetate degradation). Genetic studies proved this operon to be located on the E.coli chromosome map at a point different from the location of the "fad" operon and thus the ability of E.coli to synthesize two thiolases from two distinct structural genes was suggested (35):
thiolase I, synthesized by the genes of the "fad" operon and involved in fatty acid oxidation and thiolase II coded for by a gene on the "ato" operon and required for butyrate degradation.Growth curves of different constructed mutants (35) showed that thiolase I is not required for butyrate degradation in the presence of thiolase II but that thiolase II

could not replace thiolase I in long-chain fatty acid oxidation. Thiolase I, however, can partially replace thiolase II in butyrate degradation illustrating that these two thiolase activities are inter-replaceable during butyrate degradation. These conclusions led to the suggestion that thiolase I must have a broad substrate specifity whereas thiolase II must be specific for short-chain substrates.

This study was therefore undertaken to establish the number of thiolases synthesized in E.coli in response to induction by fatty acids and by acetoacetate and to compare their catalytic and physical properties. In addition, the recently established multiplicity of thiolases in higher organisms can provide some further insight into the evolutionary development of these enzymes by comparing these multiple thiolases and discussing their possible roles in metabolism. By studying both the catalytic and physical properties of E.coli thiolases, whose functions are known, one may conclude that thiolases in higher organisms having similar properties as the E.coli thiolases may have similar metabolic roles. In this part of this thesis the isolation and purification of thiolase I and thiolase II are reported. Thiolase I was isolated from wild type E.coli induced for the enzymes of fatty acid oxidation whereas thiolase II was obtained from a constitutive mutant able to degrade butyrate. In agreement with the work of Overath, thiolase I was found to be an enzyme acting on short-, medium- and long-chain substrates whereas thiolase II is an acetoacetyl-CoA specific enzyme.

MATERIALS AND METHODS II

Materials and chemicals. New Zealand white rabbits were purchased from Marland Breeding Farms, New Jersey. Immunodiffusion plates and Freund's adjuvant were bought from Cappel Laboratories. N-ethyl-maleimide was purchased from Sigma Chemical Co. and α-iodoacetamide was bought from Calbiochem. All other materials and chemicals are the same as listed under "Materials and Methods I". *E.coli* mutant, fad-5-fad-16-atoC^{c}49 was obtained from P. Overath.

Isolation of anti-thiolase II antibodies. Two New Zealand white rabbits (3-4kg) were injected subcutaneously into two footpads each with 0.5 mg of thiolase II(3.26 mg/ml) which had previously been dialyzed extensively against saline solution (0.9% NaCl) adjusted to pH7.2-7.4, and then emulsified in a 1:1 ratio with Freund's complete adjuvant. After seven days, a booster injection of 1 mg of thiolase(4 mg/ml) was similarly injected into each of two footpads of the same rabbits. The booster injection of protein was previously dialyzed against saline and emulsified in a 1:1 ratio with Freund's incomplete adjuvant. Veinal bleeding was performed 10 days later at which time 25-50 ml of blood was collected in centrifuge tubes. The whole blood was cooled for 30 minutes after which red blood clots were loosened from the walls of the tubes with a wooden spatula and the resulting serum was spun at 17000xg for 20 min at 4^{0}C and stored at -20^{o}C. Purification of the anti-thiolase II antibodies was performed on a DEAE-cellulose column according to a published procedure (53). A control rabbit was injected with a saline solution emulsified with Freund's adjuvant and the resulting

serum was used as a control in immunodiffusion experiments.

Immunodiffusion Qualitative immunodiffusion was performed on immunodiffusion agar slabs. The enzyme solution was placed in the center well while varying amounts of serum were added to the five outer wells. After overnight incubation at 0-5°C, an antigen-antibody reaction was demonstrated by the presence of a precipitate. The antibody concentration was assumed to be equal to the known concentration of thiolase when the precipitation band was midway between the antigen and antibody wells.

pH optima. The pH optima of thiolases I and II were determined by spectrophotometric assays as described under "Materials and Methods I". The extinction coefficient used for acetoacetyl-CoA at various pH values were obtained by extrapolating the data of Stern (54) to match the conditions of this assay (25mM Mg^{+2}; λ = 303 nm). The values thus obtained are: pH7.4, $7.47 \times 10^3 cm^{-1} M^{-1}$; pH7.8, $14.94 \times 10^3 cm^{-1} M^{-1}$; pH8.1, $23.47 \times 10^3 cm^{-1} M^{-1}$; pH8.45, $27.74 \times 10^3 cm^{-1} M^{-1}$; pH8.55, $28.45 \times 10^3 cm^{-1} M^{-1}$;

All other methods are described under "Material and Methods, I".

RESULTS AND DISCUSSION II

Thiolase I, isolated from induced wild-type E.coli and thiolase II isolated from a constitutive E.coli mutant fad-5-fad-16atoC^{C}49 were purified by the same two step procedure. Since thiolase I is a component enzyme of the multi-enzyme complex of fatty acid oxidation, the purification procedure of the complex as outlined under "Materials and Methods I" represents also the purification procedure of thiolases I and II.

Thiolase I and thiolase II were eluted from phosphocellulose with approximately 0.2M potassium phosphate (Fig. 2 and Fig. 12). In the case of thiolase I, which in contrast to thiolase II is also active on long-chain substrates, activities with acetoacetyl-CoA and 3-ketodecanoyl-CoA were associated with the same fractions. The results of these purification procedures are summarized in Tables 1 and 3A. As previously discussed, the rechromatography of the thiolase I activity on a second phosphocellulose column did not result in an increase in specific activity and was therefore not included in the routine isolation procedure. In the case of thiolase II, chromatography of 20g of cells on phosphocellulose (see Table 3B) resulted in a lower specific activity and lower purity than that obtained with only 2g of cells (see Table 3A). Rechromatography of this large preparation on a second phosphocellulose column yielded a homogenous protein but of lower specific activity (Table 3B). This decrease in specific activity may possibly be due to inactivation as a result of keeping the enzyme in dilute solution over an extended period.

The purities of the two thiolases were evaluated by polyacrylamide disc gel electrophoresis. As can be seen in Fig. 13B, one main protein

band was observed for thiolase I which was associated with short- and medium-chain activities and a single protein band was observed for thiolase II, which was associated with only acetoacetyl-CoA thiolytic activity (Fig. 13A). When thiolases I and II, whose relative mobilities were 0.23 and 0.40 respectively, were examined on the same gel, a good separation of these two enzymes was achieved (Fig. 13C) and the relative mobilities observed agreed well with those which had been determined when the enzymes were subjected to electrophoresis on separate gels. Because all acetoacetyl-CoA thiolytic activity of thiolase I was associated with one band (Fig. 13B) which migrated differently from thiolase II, it is concluded that no thiolase II was present in the thiolase I preparation. This fact, as well as the observation that the ratios of activities with acetoacetyl-CoA and 3-ketodecanoyl-CoA remained fairly constant throughout the purification, indicates that only one thiolase (thiolase I) is present in E.coli B cells induced for enzymes of fatty acid oxidation and that this enzyme is active on short- and long-chain substrates.

The native molecular weights of thiolase I and thiolase II were determined using similar procedures. As described previously (see Results, Part I), thiolase I was associated with a multi-enzyme complex of fatty acid oxidation whose native molecular weight has been estimated to be 320,000 by gel filtration and 270,000 by gradient gel electrophoresis. As seen in Fig. 6A, chromatography of thiolase II on a standardized Sephadex G-200 column yielded an estimated molecular weight of 175,000. When subjected to polyacrylamide gradient gel electrophoresis, thiolase II behaved as a single protein with a molecular

weight of 155,000 when compared to the mobilities of standard proteins run on the same gel (Fig. 6B). The subunit pattern of the multi-enzyme complex as discussed above indicates the presence of two proteins with molecular weights of 78,000 and 42,000 (see Fig. 8). When thiolase II was subjected to sodium dodecyl sulfate gel electrophoresis in the presence and absence of 2-mercaptoethanol only one protein band corresponding to a molecular weight of 41,500 was observed. Since both the native and subunit molecular weights estimated for thiolase II agree well with values obtained previously with other thiolases (31-33), it is assumed that thiolase II consists of four identical subunits which are not linked through covalent bonds.

The substrate specificities of thiolases I and II were determined by use of the spectrophotometric assay described previously (see "Materials and Methods I"). The substrate specificities of thiolase I has been presented in "Results, Part I." Thiolase II, which is induced by acetoacetate (35) was shown to be specific for acetoacetyl-CoA. Since the activity of thiolase II towards 3-ketohexanoyl-CoA was found to be less than 1% of that observed with acetoacetyl-CoA, it is concluded that thiolase II is an acetoacetyl-CoA thiolase. In view of the possibility that the rate-limiting step in β-oxidation is the thiolytic cleavage of acetoacetyl-CoA (see Discussion, Part I), it may be possible that acetoacetyl-CoA accumulates and in turn causes the induction of thiolase II. However, during the course of this study thiolase II was not detected in wild-type E. coli B cells induced for fatty acid oxidation.

When the kinetic parameters of thiolases I and II were studied, reaction rates of both enzymes were shown to be linear with respect to

enzyme concentrations (Fig. 14). Substrate inhibition was not observed for thiolase I or II at acetoacetyl-CoA and CoA concentrations up to 33μM and 100μM respectively (Fig. 15 and 16). Thiolase I was inhibited at 3-ketodecanoyl-CoA concentrations of above 20μM possibly due to the detergent effect of the long-chain fatty acyl-CoA compound (see "Results, Part I"). By plotting rates of thiolytic cleavage versus CoA and acetoacetyl-CoA concentrations, respectively, on reciprocal coordinates (see Fig. 17) the following kinetic parameters of thiolase II were determined: Vmax of 187.5^{U}/mg with acetoacetyl-CoA as substrates and apparent Km values of 7.6μM for CoA and 7.5μM for acetoacetyl-CoA. The Vmax (187.5^{U}/mg) of thiolase II was found to be lower than the original specific activity of the same preparation (220^{U}/mg, see Fig. 38), a finding which reflects the inactivation of the enzyme upon storage. Similar kinetic studies with thiolase I yielded a Vmax of 1.2^{U}/mg with acetoacetyl-CoA as the variable substrate and apparent Km values of 20μM and 31μM for CoASH and acetoacetyl-CoA respectively (Fig. 18). The apparent Km for 3-ketodecanoyl-CoA was too low (<2μM) to be determined accurately with the optical assay. The Vmax for thiolase I with β-ketodecanoyl-CoA as a substrate was estimated to be 27.7^{U}/mg. The lower Vmax values as compared to the specific activity for thiolase I shown in Table I may be due to the inactivation of this enzyme upon storage.

To determine the optimal pH for both thiolase I and thiolase II, their rates of reactions were determined between pH 6-10. The optimal pH for thiolase I with acetoacetyl-CoA as the substrate was pH 7.8 whereas the optimal pH for the thiolytic cleavage of 3-ketodecanoyl-CoA proved to

be in the range from pH 8.1 to 8.55 (Fig. 19). A similar study with thiolase II showed an optimal pH of 8.2 (Fig. 19).

The effect of dilution on enzyme activity was used to study the stability of thiolases I and II. As illustrated in Fig. 20, thiolase I was totally inactivated within 130 minutes when diluted in a Tris buffer (pH 8.1) containing glycerol (25% vol/vol), bovine serum albumin (1^{mg}/ml) and 2-mercaptoethanol (10mM) to a final concentration of 2.25 µg/ml. When compared to the activity of the undiluted enzyme, an immediate loss of 50% of the activity was observed when the enzyme was diluted in the buffer in the presence of the potential stabilizers. This rapid initial inactivation was followed by a continuous but slower loss of activity. The omission of any one of the three stabilizers resulted in an increase rate of inactivation. The rapid inactivation of thiolase I in dilute solution made it difficult to assay this enzyme and may have led to an underestimation of its specific activity. Thiolase II proved to be stable in dilute solution even at a concentration as low as 1.3 µg/ml, although in the absence of a sulfhydryl reagent an initial loss of 50% of activity was observed (see Fig. 20). The activity of thiolase II in this diluted form persisted for 24 hours even in the absence of dithiothreitol.

The greater stability of thiolase II as compared to that of thiolase I could also be observed when the activities of the stock solutions kept at 5^{o}c were followed over an extended period of time. As seen in Fig. 21, thiolase I showed an approximate 50% decrease in activity within 33 days even when stored in the presence of 10mM 2-mercaptoethanol whereas thiolase II retained 73% of its activity after

41 days of storage under the same conditions. The loss in activity of thiolase II was later shown to be due to the oxidation of the sulfhydryl reagent and the stability of both enzymes was greatly increased when stored at -70°C. Under this condition no significant loss in activity of either enzyme was observed within one month.

Thiolase I exhibited an absolute requirement for a sulfhydryl reagent as evidenced by an immediate and total loss of activity when thiolase I activity was assayed in the absence of 2-mercaptoethanol. The reason for detecting thiolase I activity when the enzyme was diluted in the absence of a thiol reagent (see Fig. 20) was evidently due to the fact that all diluted enzyme preparations were assayed in a buffer containing 2-mercaptoethanol (see assay conditions under "Material and Methods I"). In contrast, thiolase II showed no change in activity when assayed in the absence of a sulfhydryl reagent.

The inhibition of thiolase II activity by known sulfhydryl group inhibitors is illustrated in Table 4. Complete loss of thiolase activity in the presence of N-ethyl-maleimide (5×10^{-3} M) was observed after 15 minutes. While 70% of the activity was lost during incubation with iodoacetamide (1×10^{-3} M), complete substrate protection of thiolase II against inactivation by iodoacetamide was seen in the presence of 0.93mM acetyl-CoA. This data agrees well with the work of Gehring et al (47) who reported a strong inhibition of pig heart thiolase by thiol inhibitors and protection against this inhibition by acetyl-CoA. Corresponding inhibitions experiments could not be performed with thiolase I due to its absolute requirement for 2-mercaptoethanol during the assay. However, this fact itself leads to the suggestion that thiolase I similarly to thiolase II contains an essential sulfhydryl group.

Initially, one of the aims in studying E.coli thiolases was to examine the similarities between thiolasesI and II and the short-chain and general thiolases isolated from pig heart in the hope of obtaining evidence for the evolution of various thiolases from the same ancestral gene. The formation of antibodies against thiolase II was undertaken in order to determine if any antibody-reactions would be observed between antibodies to thiolase II and thiolases of these two systems. The result of immunodiffusion on agar slabs showed positive recognition of thiolase II by its own antibody but no cross-reactivity between thiolase II antibodies and thiolase I or the pig heart thiolases. Hence, it is concluded that possible structural similarities between the thiolases of E.coli or between thiolases of E.coli and pig heart are not sufficient to be detected by an antibody-antigen reaction.

The role of thiolases in both the cleavage and formation of acetoacetyl-CoA and the recent evidence of the occurrence of multiple thiolases in higher organisms have led investigators to suggest possible different metabolic roles for these isozymes (see Fig. 11 and Table 5). Cytosylic acetoacetyl-CoA thiolases found in yeast (32, 52) avian liver (33), rat liver and many other tissues (24,49,51) but absent from muscle tissue (24) have been proposed to be involved in choiesterol synthesis. The high Km values exhibited by this yeast thiolase toward acetoacetyl-CoA (32), and the strong inhibition of thiolase activity by increasing concentrations of either acetoacetyl-CoA and/or CoA (32)agree well with this suggested role for the cytosylic thiolases. The kinetic parameters of these enzymes together with their localization in the cytosol indicates their prime role is in supplying acetoacetyl-CoA as a substrate for the 3-hydroxy-3-methylglutaryl-CoA

synthase in the first step towards cholesterol biosynthesis. In support of this role, Clinkenbeard et al (55) have demonstrated that the cytoplasmic thiolases from chicken and rat liver are subject to cholesterol feedback control.

Acetoacetyl-CoA thiolases, have been reported in the mitochondria of yeast (52), pig heart, muscle, (31, 47) avian liver (33) rat liver and other tissues (24, 49, 50) and are proposed to be involved in either ketone body utilization or formation. The yeast mitochondrial acetoacetyl-CoA thiolase which exhibits a low Km value for acetoacetyl-CoA and CoA (32) may be responsible for acetoacetate utilization. Although cytosylic thiolases are not present in significant quantities in muscle (24), this tissue does contain substantial amounts of mitochondrial thiolases. Heart tissue contains both a general substrate thiolase as well as a mitochondrial acetoacetyl-CoA thiolase (24) whereas the thiolase present in skeletal muscle such as hind-leg muscle is mainly an acetoacetyl-CoA specific enzyme (24). The widespread occurence of mitochondrial acetoacetyl-CoA thiolases in muscle, a tissue which uses acetoacetate as the preferential metabolic fuel (56, 57) and the good correlation between 3-oxoacid-CoA transferase (the first enzyme in acetoacetate metabolism) and these thiolases (24) all support the role of this enzyme in extrahepatic ketone body utilization. The presence of high activities of a mitochondrial acetoacetyl-CoA thiolase in liver (24), a tissue devoid of 3-oxoacid-CoA transferase and known to be almost exclusive in synthesizing acetoacetate (58) indicates that this enzyme is involved in acetoacetyl-CoA formation , the first step in ketogenesis. This thiolase has been localized within the matrix

compartment of the mitochondria and is one of the 3-hydroxy-3-methyl-glutaryl-CoA cycle enzymes responsible for ketogenesis (59). The metabolic function of a thiolase which is active on a broad range of substrates and which has been crystallized from ox liver (43) is indicated by both its mitochondrial location and broad substrate specificity to be the thiolase involved in β-oxidation.

The known function of thiolase I in fatty acid oxidation and that of thiolase II in butyrate and acetoacetate degradation agree well with their chain length specificities presented in this thesis. The specificity of thiolase II towards acetoacetyl-CoA, reflected in both its low Km and high Vmax values, resembles the yeast thiolase reported to be involved in acetoacetate utilization (52). In addition, the similarity between the mitochondrial acetoacetyl-CoA thiolase of pig heart and thiolase II in both native and subunit molecular weight (see Table 5) suggests that the pig heart acetoacetyl-CoA thiolase is involved in ketone body utilization. The broad-chain length specificity of thiolase I is typical of thiolases thought to be involved in fatty acid oxidation (43). Recent work on the mitochondrial general substrate specific thiolase from pig heart (Staack, H., unpublished data) indicates the higher activity of this enzyme toward medium-chain substrates is due to both a very low Km (<2μM) toward β-ketodecanoyl-CoA and high Vmax (67 U/mg), and thus resembles thiolase I in its kinetic parameters and suggested role in fatty acid oxidation.

Although thiolases I and II are not synthesized at the same time when the E.coli bacterium is grown on oleate as the sole carbon source, the existence of the genetic information for these two enzymes in a

primitive system such as E.coli indicates that multiple thiolases of different metabolic functions are not the result of evolutionary development in higher organisms. The similarity between the E.coli thiolases and the mitochondrial thiolases of higher organisms in their kinetic properties and functiuns supports the theory that the bacterial thiolases were maintained through evolution as mitochondrial thiolases. Since E.coli does not synthesize cholesterol, the cytoplasmic thiolase proposed to be involved in cholesterogenesis may be an additional thiolase that evolved in higher organisms.

This report describes the isolation of two distinct thiolases from E.coli cells. The differences in their chain length specificities, kinetic parameters, native and subunit molecular weights, pH optima and stabilities and the good correlation between their substrate specificities and proposed functions indicate that acetoacetyl-CoA thiolase (thiolase II) which was mapped at the "ato" operon and the general thiolase (thiolase I) who gene is located on the "fad" operon on the E.coli chromosome map (35) are two physically and functionally distinct enzymes.

Table 1. Summary of the purification of the multi-enzyme complex of fatty acid oxidation≠

	Sp. act. with C_4 substrates* (μmoles/min per mg of protein)			Sp. act. with C_{10} substrates+ (μmoles/min per mg of protein)			Total Protein (mg)
	Thiolase	Enoyl-CoA hydratase	3-Hydroxyacyl-CoA dehydrogenase	Thiolase	Enoyl-CoA hydratase	3-Hydroxyacyl-CoA dehydrogenase	
Crude Homogenate	0.037	1.79	1.05	0.98	0.48	0.93	1447.0
Heat Treated	0.12	3.24	4.68	3.47	0.78	4.04	390.1
Phosphocellulose	1.99	59.00	49.30	40.00	29.00	33.65	9.2
Purification (fold)	54	33	47	41	60	36	
Yield (%)	34.5	21.0	30.0	26.5	36.0	23.0	

*Enzyme activities were determined as described under "Materials and Method I", using acetoacetyl-CoA as the substrate for thiolase and 3-hydroxyacyl-CoA dehydrogenase and crotonyl-CoA as the substrate for enoyl-CoA hydratase.

+Enzyme activities were determined as described under "Materials and Methods I" using 3-ketodecanoyl-CoA as the substrate for thiolase and 3-hydroxyacyl-CoA dehydrogenase and $\Delta^{2,3}$-decenoyl-CoA as the substrate for enoyl-CoA hydratase.

≠ Details of the purification procedure are given under "Materials and Methods I".

Table 2. Chromatography of the multi-enzyme complex of fatty acid oxidation on DEAE-cellulose

	Sp. act. with C_4 substrates* (μmoles/min per mg of protein)			Sp. act. with C_{10} substrates+ (μmoles/min per mg of protein)		
	Thiolase	Enoyl-CoA hydratase	3-Hydroxyacyl-CoA dehydrogenase	Thiolase	Enoyl-CoA hydratase	3-Hydroxyacyl-CoA dehydrogenase
Peak #1	1.64	46.88	26.50	16.45	12.97	undetermined
Peak #2	0.44	15.37	12.57	6.00	4.57	undetermined

*Enzyme activities were determined as described under "Materials and Methods I" using acetoacetyl-CoA as the substrate for thiolase and 3-hydroxyacyl-CoA dehydrogenase and crotonyl-CoA as the substrate for enoyl-CoA hydratase.

+Enzyme activities were described under "Materials and Methods I" using 3-ketodecanoyl-CoA as the substrate for thiolase and 3-hydroxyacyl-CoA dehydrogenase and $\Delta^{2,3}$-decenoyl-Co-A as the substrate for enoyl-CoA hydratase.

Table 3. Purification of thiolase II

Purification step	Total activity* (μmol/min)	Total protein (mg)	Sp. act. (μmol/min per mg of protein)	Purification (fold)
A.				
Crude Homogenate	1207	101	12.0	1
Heat Treated	1155	73.7	15.7	1.3
Phosphocellulose	498	0.98	506	42.4
B.				
Phosphocellulose #1	2658	12.0	220.8	25.5
Phosphocellulose #2	890	7.56	117.7	

* Enzyme activity was determined as described under "Materials and Methods I" using acetoacetyl-CoA as substrate.

A. Purification procedure of thiolase II using 2g of cells.

B. Purification of thiolase II by phosphocellulose chromatography using 20g of cells. Refer to "Results and Discussion II."

Table 4. Inhibition of thiolase II

Reagent Added	% activity remaining after 15 min.
no addition	100%
$5x10^{-4}$M N-ethyl-maleimide	5%
$5x10^{-3}$M N-ethyl-maleimide	0%
$5x10^{-4}$M Iodoacetamide	35%
$1x10^{-4}$M Iodoacetamide	29%
$1x10^{-4}$M Iodoacetamide + 0.93mM Acetyl-CoA	100%

Thiolase II (6.3μg/ml) was incubated with inhibitors at indicated concentrations and kept for 15 min at 0-5°C. The reaction was stopped by the addition of excess 2-mercaptoethanol as described by Gehring et al.(56). The enzyme was assayed in a total volume of 0.6 ml in the presence of 22μM acetoacetyl-CoA and 75μM CoA as described under "Materials and Methods I." Percentage activity remaining was calculated based on enzyme assays done in the absence of inhibitor.

Table 5. Properties of various thiolases

Tissue and Localization	Substrate* Specificity	Native Molecular Weight	Subunit Molecular Weight	Kinetic* Parameters	Suggested Metabolic Role	Reference
E. coli	C_4	175,000	41,500	K_m=7.5μM (C4) K_m=7.6μM (CoA)	Acetoacetate utilization	this thesis
	C_4-C_{16}	320,000 (complex)		K_m=31μM (C4) K_m=<2μM (C10) K_m=20μM (CoA)	Fatty Acid Oxidation	this thesis
Yeast Cytoplasm	C_4	140,000		K_m=370μM (C4) K_m=160μM (CoA)	Cholesterol Synthesis	32, 52
Mitochondria	C_4	65,000		K_m=20μM(C4) K_m=<1μM (CoA)	Acetoacetate Utilization	32, 52
Pig Heart Mitochondria	C_4[a,b]	170,000[a]	42,000[a]	K_m^b=11.8μM(C4) K_m^b=51.3μM(CoA)	Acetoacetate Utilization	47[a] Staack, H.[b] (unpublished data)
Mitochondria	C_4-C_{16}[b]	200,000[b]	46,000[b]	K_m^b=16.1μM (C4) K_m^b=<2μM (C10) K_m^b=9.1μM (CoA)	Fatty Acid Oxidation	
Avian Liver Cytoplasm	C_4	169,000	41,000	K_m=38.4μM (C4) K_m=6.4μM (CoA)	Cholesterol Synthesis	33
Mitochondria	C_4				Acetoacetate formation	33

Table 5 (Continued)

Tissue and Localization	Substrate* Specificity	Native Molecular Weight	Subunit Molecular Weight	Kinetic* Parameters	Suggested Metabolic Role	Reference
Rat Liver Cytoplasm	C_4	170,000	44,000	K_m=33μM (C_4) K_m=15μM (CoA)	Cholesterol Synthesis	51
Mitochondria	C_4			K_m^a=7μM (C_4) K_m^a=21μM(CoA) K_m^b=6.2μM (Acetyl-CoA)	Acetoacetate formation	24[a] 50[b]
Mitochondria	C_4-C_{10}			K_m=10μM (C_4) K_m=18μM (CoA)	Fatty Acid Oxidation	24
Ox Liver Cytoplasm	C_4			K_m=20μM (C_4) K_m=7μM (CoA)	Cholesterol Synthesis	49
Mitochondria	C_4			K_m=10μM (C_4) K_m=29μM (CoA)	Acetoacetate formation	49
Mitochondria	C_4-C_{16}[a]			K_m^b=10μM (C_4) K_m^b=10μM (CoA)	Fatty Acid Oxidation	43[a] 49[b]

*Abbrevations: C_4, Acetoacetyl-CoA; C_{10}, β-ketodecanoyl-CoA; C_{16}, β-ketohexadecanoyl-CoA.

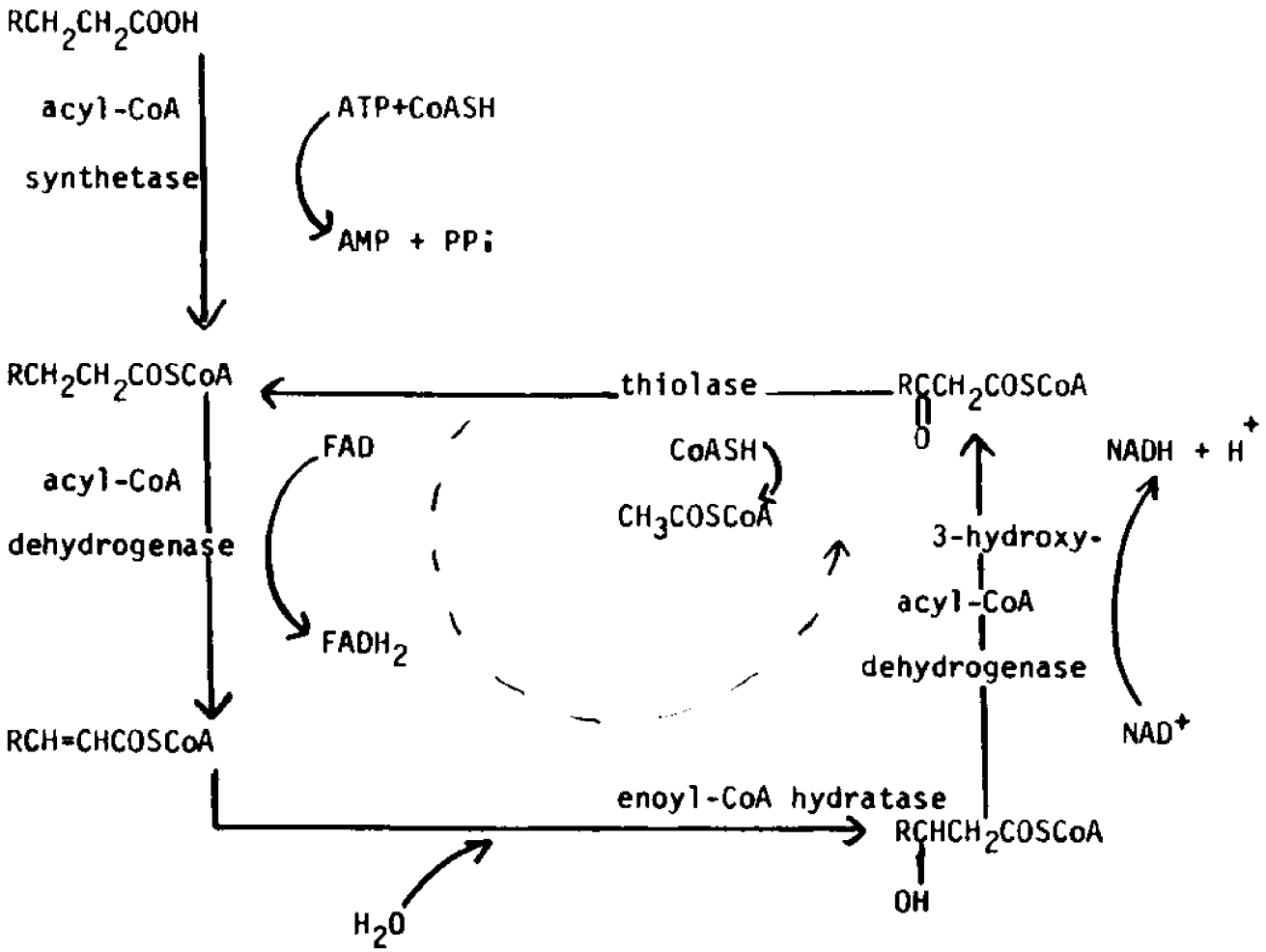


Figure 1. Pathway of β-oxidation of fatty acids

Legends

Figure 2. Purification of enzymes of fatty acid oxidation by chromatography on phosphocellulose. Symbols: O, thiolase activity with 3-ketodecanoyl-CoA; △, 3 hydroxyacyl-CoA dehydrogenase activity with acetoacetyl-CoA; ●, enoyl-CoA hydratase activity with crotonyl-CoA. The absorbance at 280 nm is not shown because the amount of protein eluted with the potassium phosphate gradient was very small. A phosphocellulose column (1.2 x 45cm) was developed as described under "Materials and Methods I".

Figure 2

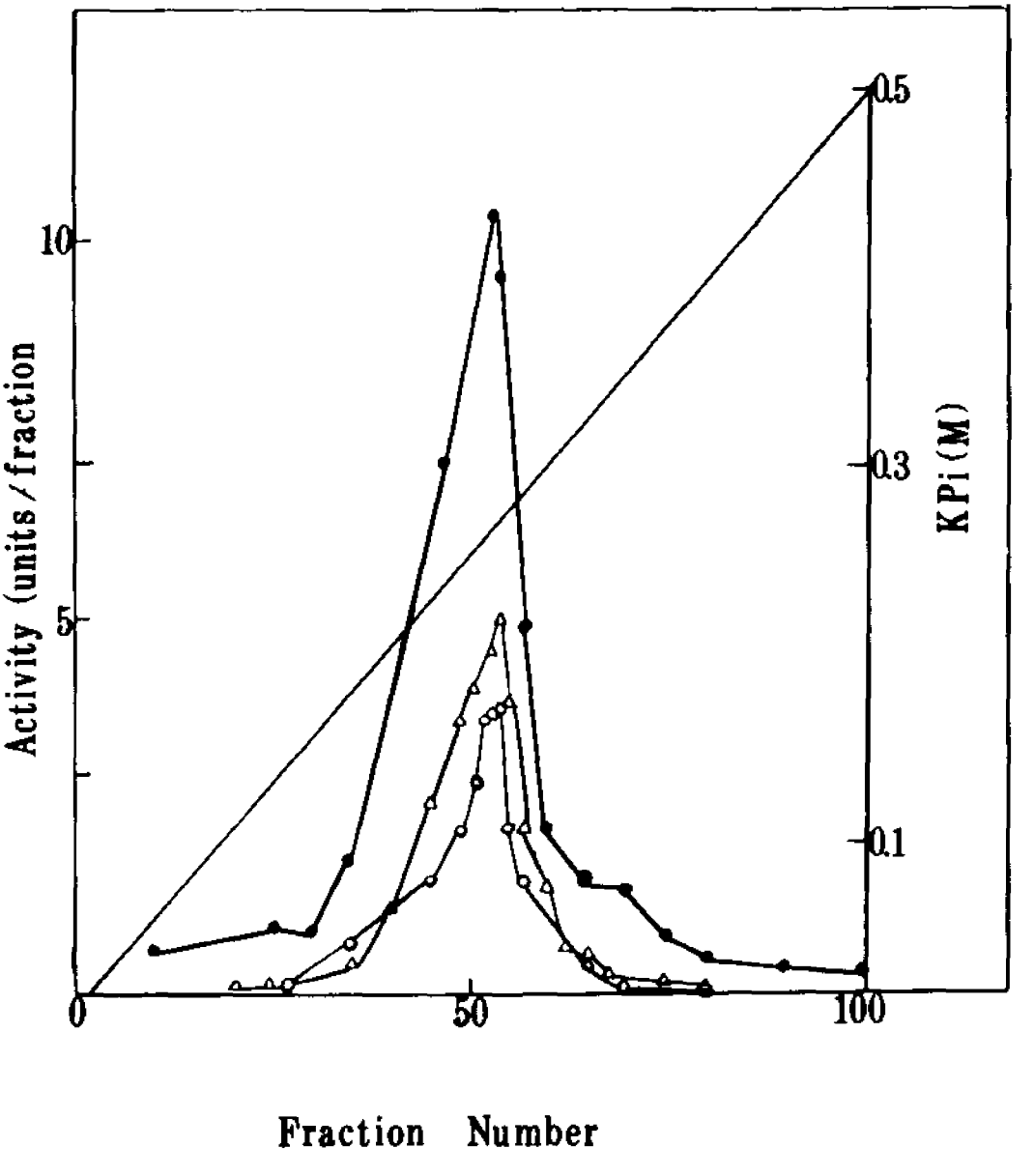

0.5
10
0.3
KPi (M)
Activity (units / fraction
5
0.1
0
50
100
Fraction Number

Figure 3. Disc gel electrophoresis of the multi-enzyme complex of fatty acid oxidation.

Gels were developed and stained for protein and duplicate unstained gels were sliced and assayed for activity as described under "Materials and MethodsI". All slices were assayed for thiolase activity and those slices corresponding to the protein peak were assayed for enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities. Hatched bars indicate relative activities of the three enzymes. The activities in the peak fraction when assayed with C_{10} substrates were: thiolase, 78 mU/fraction, enoyl-CoA hydratase, 140 mU/fraction and 3-hydroxyacyl-CoA dehydrogenase, 112 mU/fraction. The ratios of C_4 to C_{10} activities for all three enzymes were approximately the same as observed with the purified enzymes. Gels were pre-electrophoresed for 30 min at 3mA per gel. If this step was omitted, a second significant band was observed, similar to the picture shown in Fig. 13B.

Figure 3

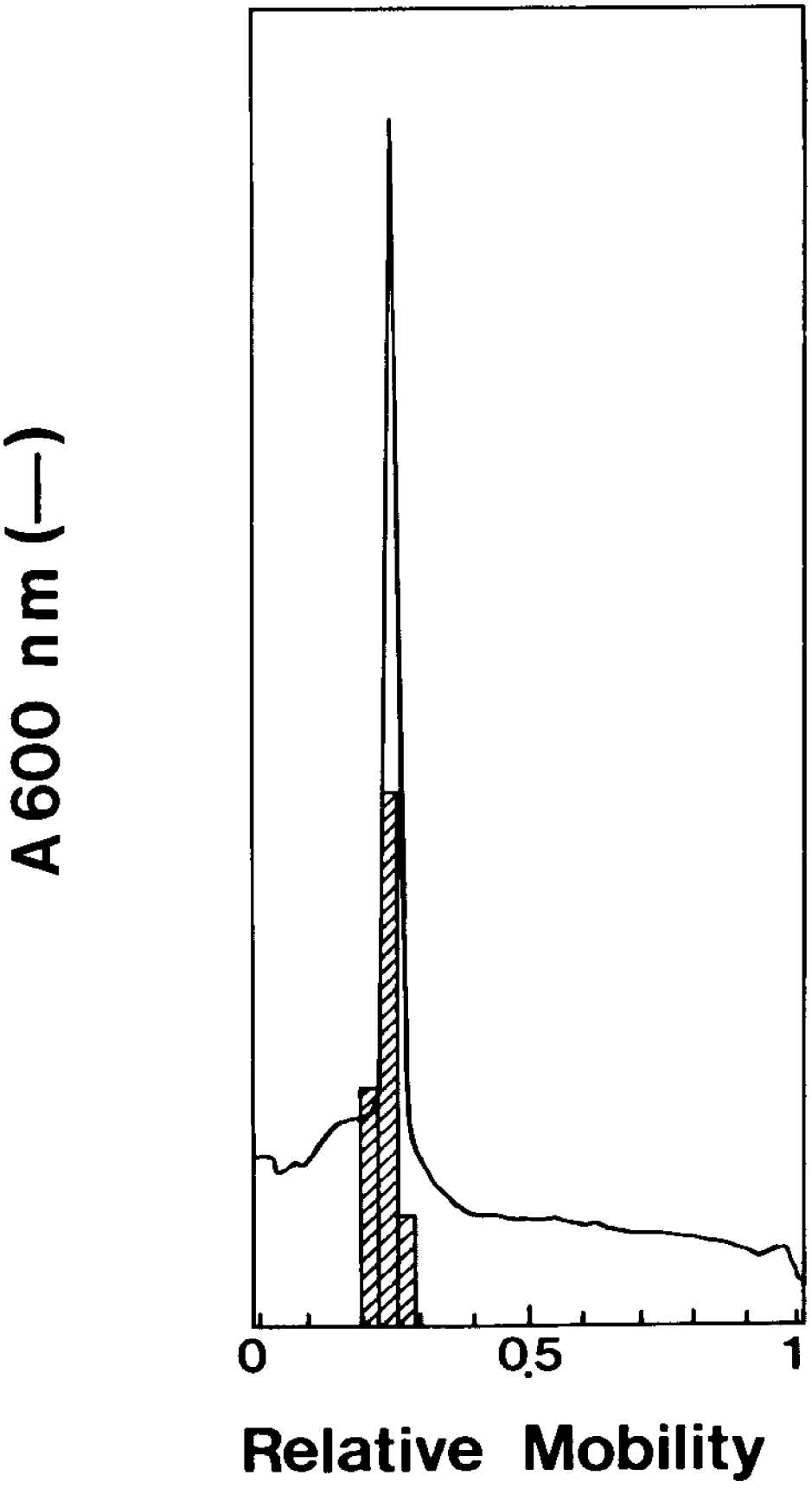


Figure 4. Gel filtration of the multienzyme complex of fatty acid oxidation on Sepharose 6B. Symbols: O, thiolase activity with acetoacetyl-CoA; △, 3-hydroxyacyl-CoA dehydrogenase activity with acetoacetyl-CoA; ●, enoyl-CoA hydratase activity with crotonyl-CoA. A Sepharose 6B column (2.6 x 77 cm) was equilibrated with 0.05 M potassium phosphate (pH 7) containing 0.1 M NaCl and 10 mM 2-mercaptoethanol. The void volume was determined with blue dextran. Proteins were extensively dialyzed against the column buffer before their application on the column. Fractions were collected (1.84 ml) and the elution volumes of the standards were determined by measuring peaks of absorbance at 280 nm. Standards used were: glutamate dehydrogenase, phosphorylase A, catalase, phosphorylase B, lactate dehydrogenase, and bovine serum albumin. Those fractions necessary for determining the elution volume of the complex were assayed for the three enzyme activities.

Figure 4

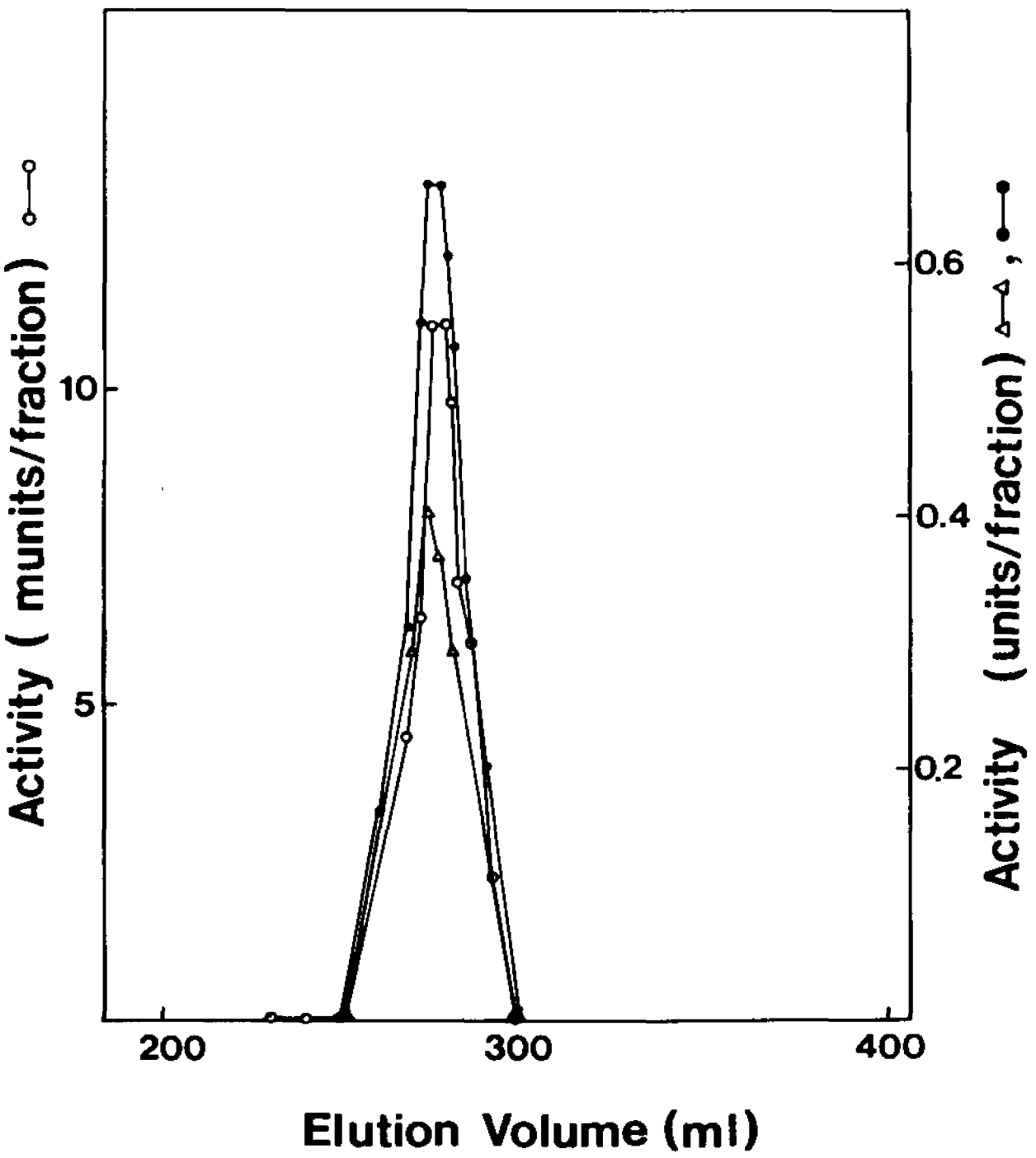

Activity (munits/fraction)
10
5
0.6
0.4
0.2
Activity (units/fraction)
200
300
400
Elution Volume (ml)

Figure 5. Sedimentation velocity of the multi-enzyme complex as determined by ultracentrifugation. Inset are photographs of Schlieren patterns of the complex obtained 32, 36 and 40 min. after the beginning of centrifugation at 40,000 RPM. Direction of sedimentation is from right to left. Details of procedure are explained under "Materials and Methods I".

Figure 5

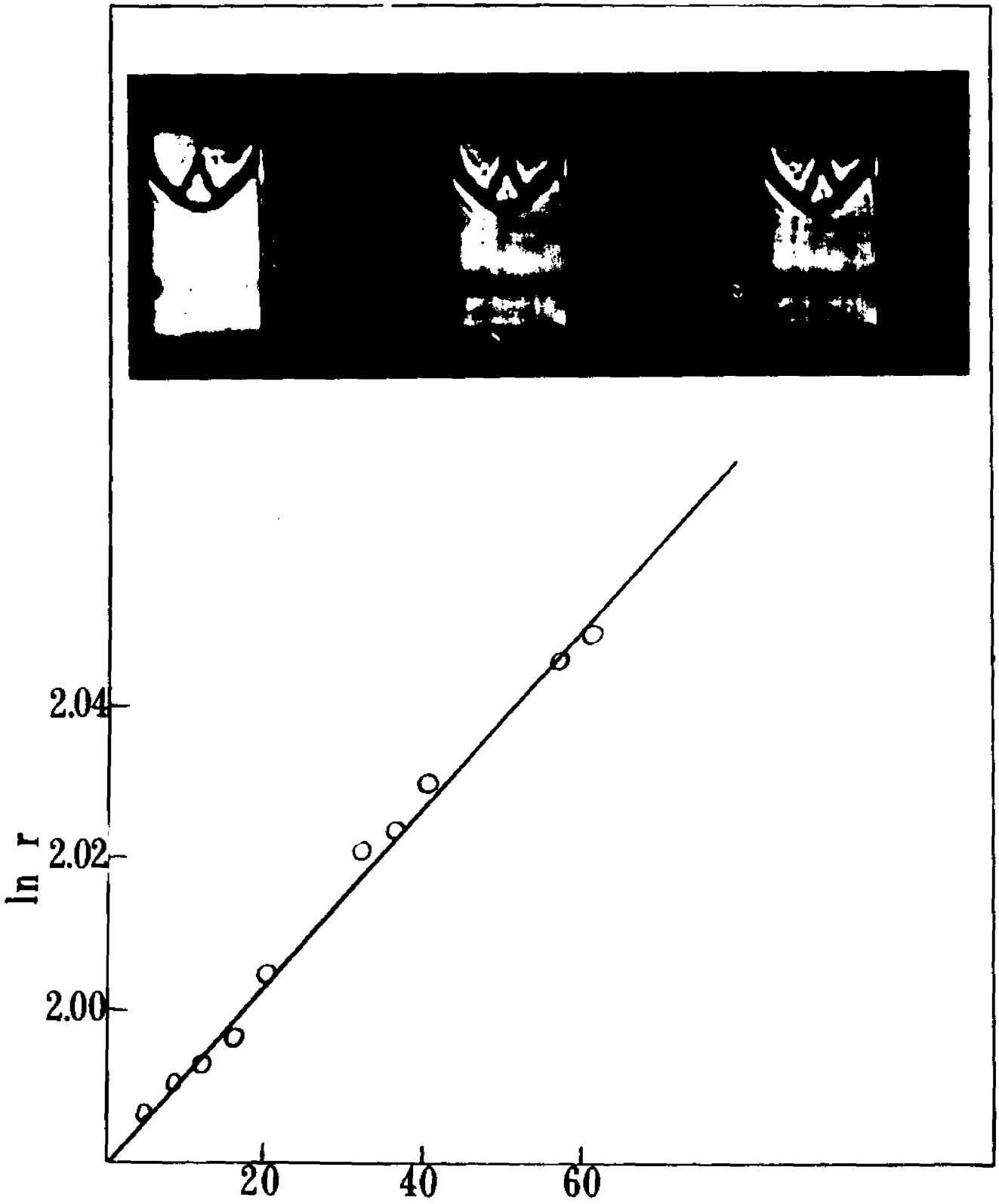


Figure 6A. Molecular weight determination of the multi-enzyme complex of fatty acid oxidation and thiolase II on Sephadex G-200.

A Sephadex G-200 column (2.6x58cm) was equilibrated with 0.05M potassium phosphate (pH) containing 0.1M Nacl and 10mM 2-mercaptoethanol. The void volume was determined with blue dextran. The following standards were used: O, phosphorylase B; □, catalase; △, lactate dehydrogenase; X, bovine serum albumin. All standards (10mg/ml) were applied to the column in 2 ml samples and their elution volumes were determined by measuring absorbances at 280nm. A 2 ml sample of the multi-enzyme complex (4mg) and thiolase II (3mg) were applied separately to the column and their elution volumes were determined by the thiolase assay. Arrow I indicates position of the multi-enzyme complex and arrow II indicates position of thiolase II.

Figure 6B. Molecular weight determination of the multi-enzyme complex of fatty acid oxidation and thiolase II by gradient gel electrophoresis 20μg of each protein were applied to the slab gel. The following standards were used on the same gel: O, apoferritin; □, catalase; △, lactate dehydrogenase; X, bovine serum albumin. Arrow I indicates the position of the multi-enzyme complex; arrow II indicates position of thiolase II.

Figure 6

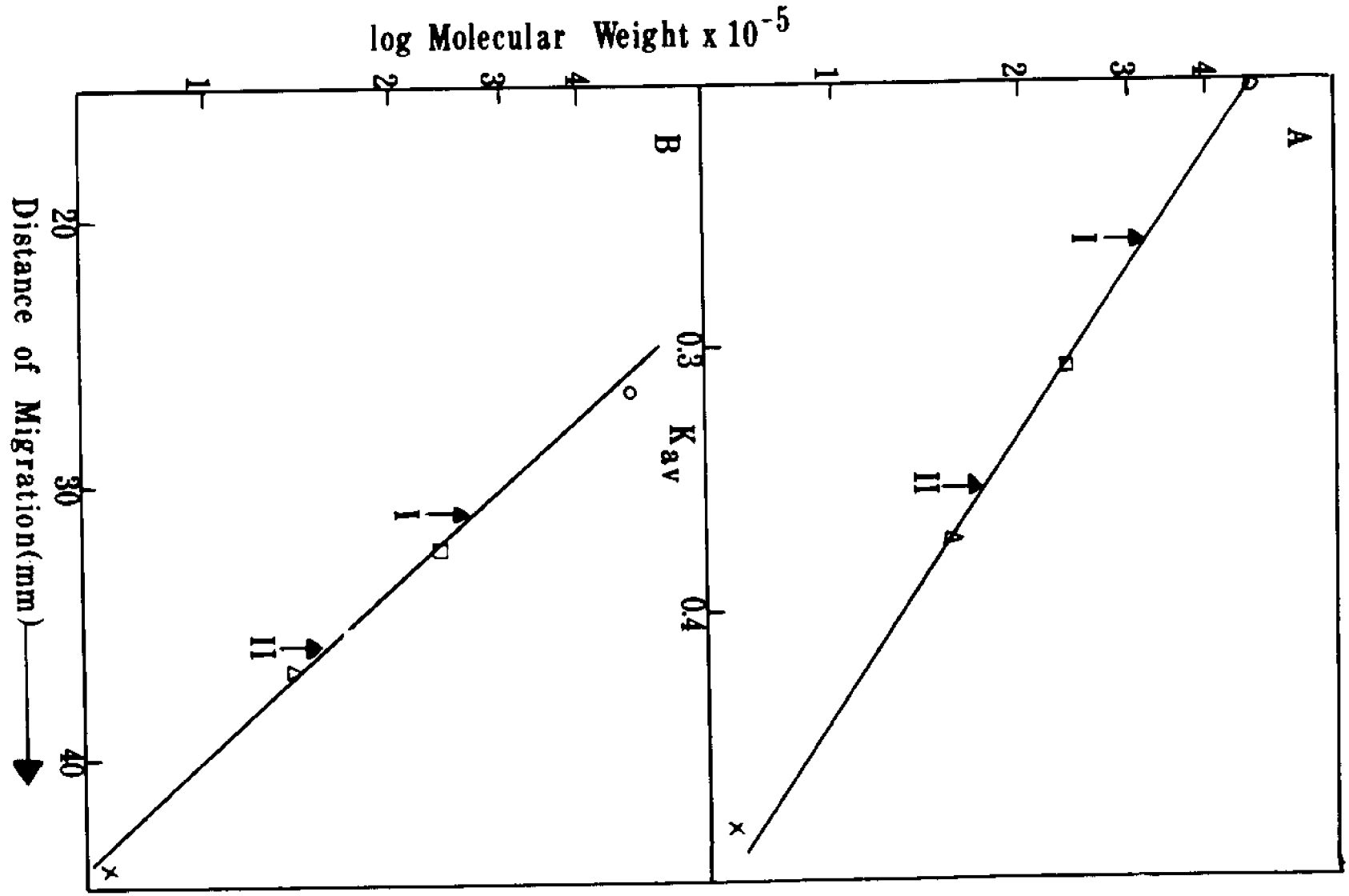
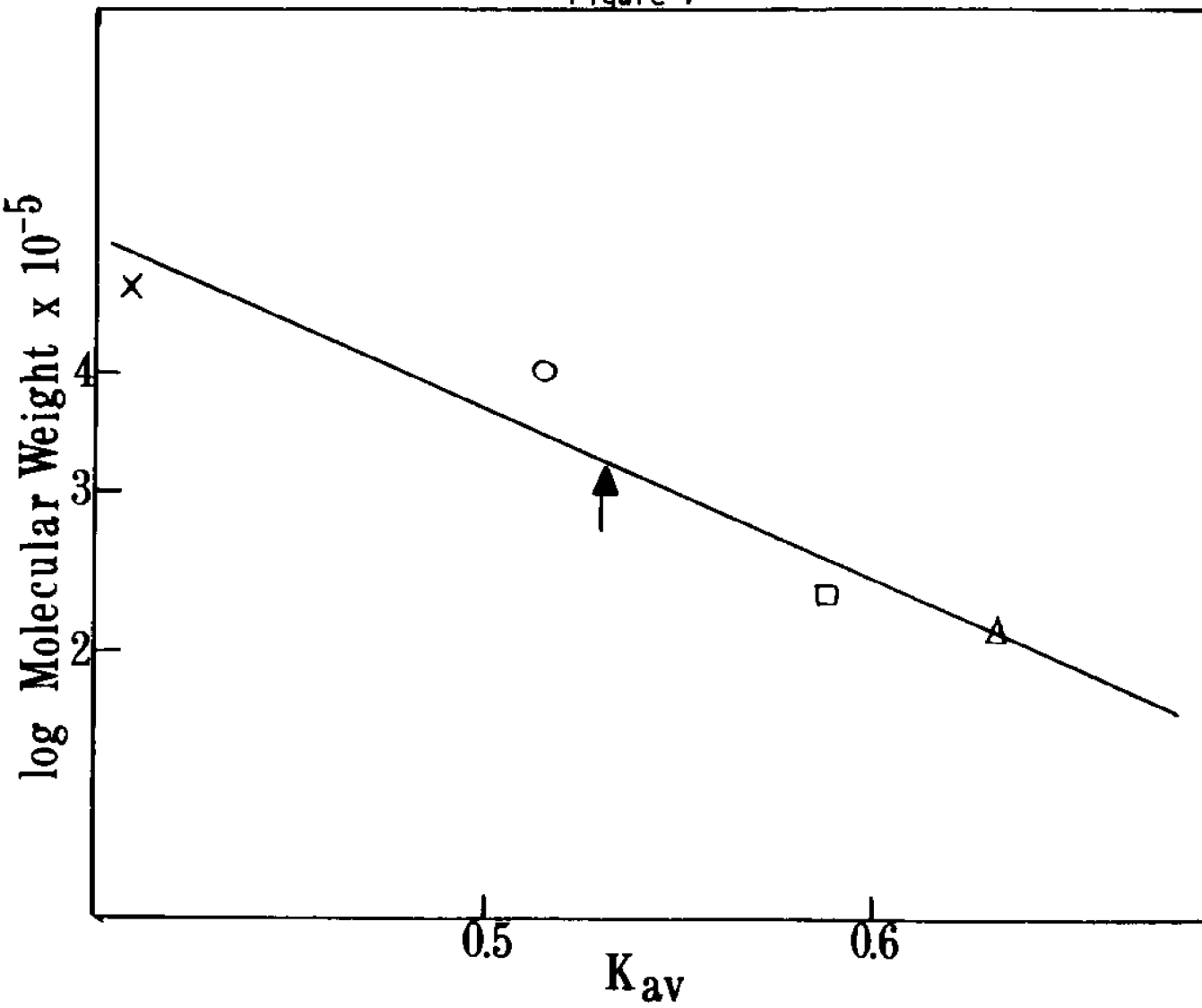

log Molecular Weight x 10^{-5}
A
B
I
II
K_{av}
Distance of Migration(mm)

Figure 7



Molecular weight determination of the multi-enzyme complex of fatty acid oxidation on Sepharose 6B. The following standards were used: X, glutamate dehydrogenase; O, phosphorylase A; □, catalase; △, phosphorylase B. All standards (10mg/ml) were applied to the column in 1 ml samples and their elution volumes were determined by measuring absorbances at 280nm. A 1 ml sample of the multi-enzyme complex (1.8mg/ml) was applied to the column and those fractions necessary for determining the elution volume of the complex were assayed for the three enzyme activities. For details of the Sepharose 6B column see Legend to Fig. 5. Arrow indicates the position of the multi-enzyme complex

Figure 8. Disc gel electrophoresis of the multi-enzyme complex of fatty acid oxidation in the presence of sodium dodecyl sulfate on a 10% polyacrylamide gel.

A. Enzyme preparation from the first phosphocellulose column.

B. Enzyme preparation from the second phosphocellulose column. The proteins were incubated at 37°C for 2 hours in 0.01 M sodium phosphate (pH 7.0), 10% in sodium dodecyl sulfate and 1% in 2-mercaptoethanol. The protein concentrations in the incubation mixtures were 1 mg/ml and 5-10 μg of each protein were applied to each gel.

Figure 8

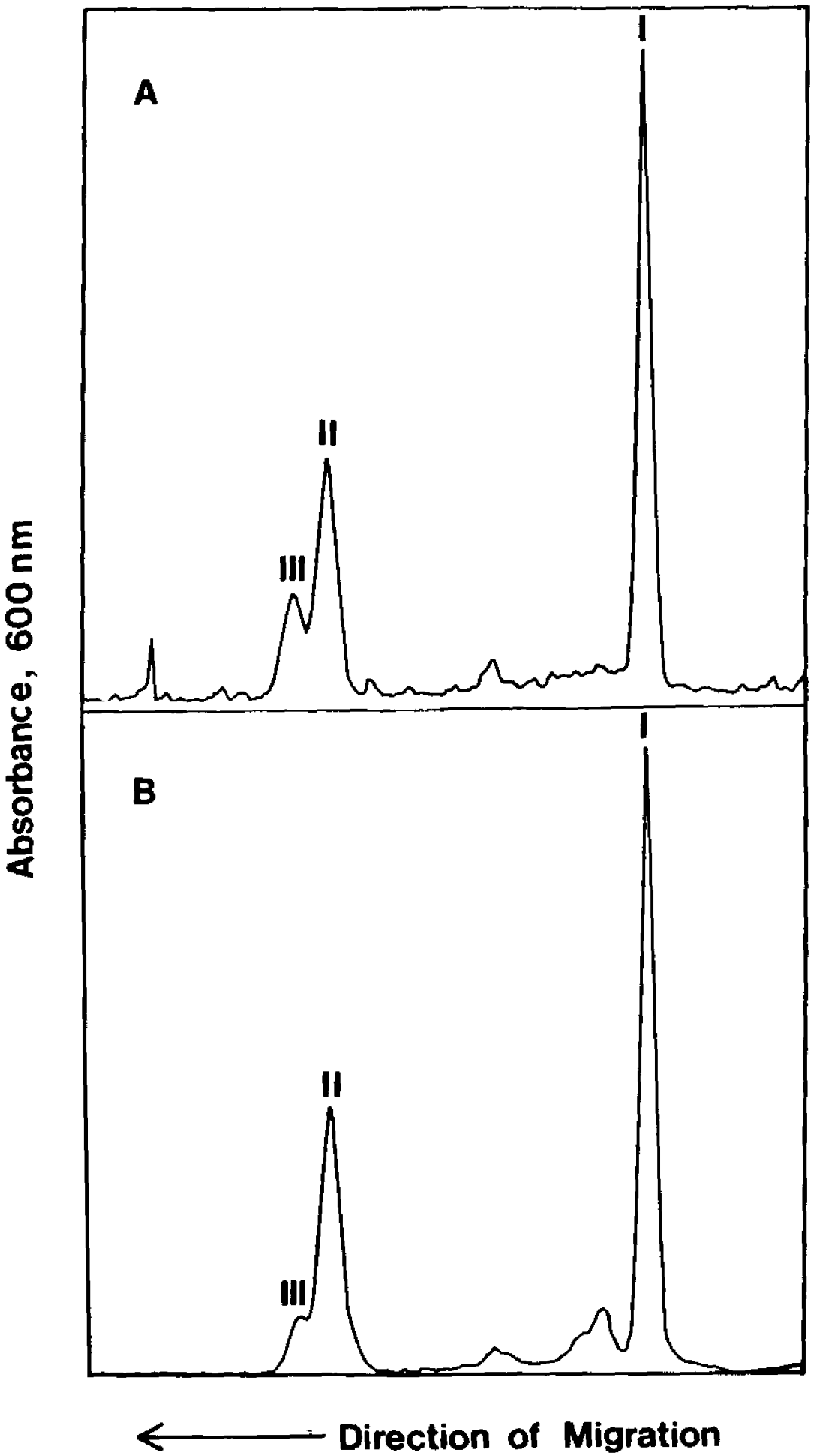


Figure 9. DEAE-cellulose chromatography of the multi-enzyme complex. Symbols: △, thiolase I activity assayed with acetoacetyl-CoA; X, thiolase I activity assayed with 3-ketodecanoyl-CoA. DEAE-cellulose chromatography was performed by pre-equilibrating a DEAE-cellulose column (1.2x22cm) with 10mM potassium phosphate (pH 7.3) containing 10mM 2-mercaptoethanol and 25% vol/vol glycerol. A purified preparation of the multi-enzyme complex (1.8mg)was dialyzed against 90mM potassium phosphate (pH 7.3) containing 10mM 2-mercaptoethanol and 25%(vol/vol) glycerol and was applied to the pre-equilibrated DEAE-cellulose column. The column was washed with the equilibrating buffer until no more ultraviolet-absorbing material was eluted. The column was then developed with a phosphate gradient prepared from 75 ml of 90mM potassium phosphate (pH7.3) containing 10mM 2-mercaptoethanol and 25% (vol/vol) glycerol, and from 75ml of 250mM potassium phosphate (pH7.3) containing 10mM 2-mercaptothanol and 25% (vol/vol) glycerol. Fractions of 3ml each were collected and assayed for thiolase activity as described under "Material and Methods I". The fraction containing enzyme activities were pooled and concentrated by ultracentrifugation with an Amicon concentrate PM-10 membrane to approximately 1.4ml. Concentrated fractions were assayed for thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase as described under "Materials and Methods I".

Figure 9

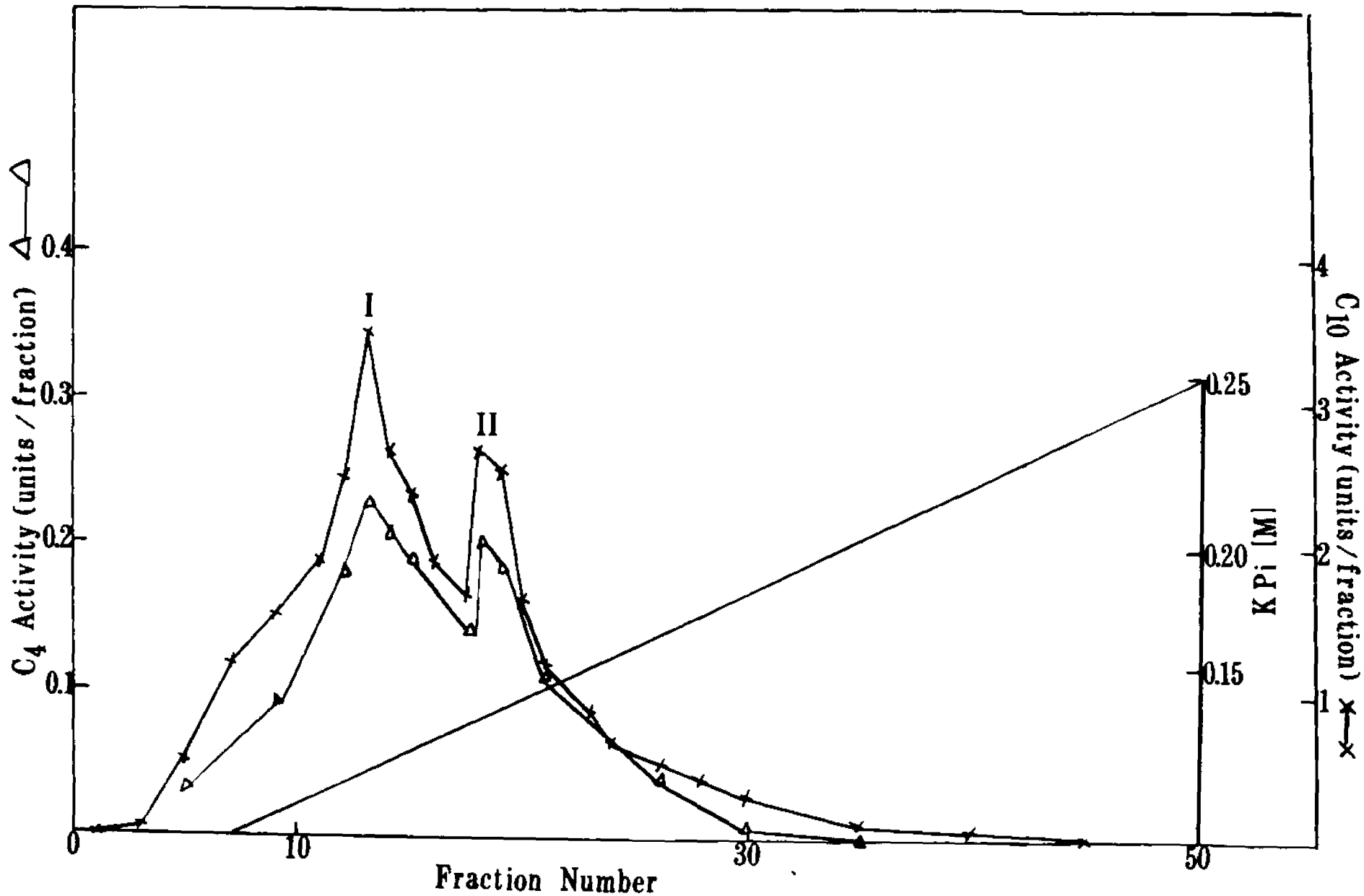

C4 Activity (units / fraction)
0.4
0.3
0.2
0.1
I
II
0
10
30
50
Fraction Number
0.25
0.20
0.15
K Pi [M]
C10 Activity (units/fraction)
4
3
2
1

Figure 10. Chain length specificities of the component enzymes of the multi-enzyme complex of fatty acid oxidation. Symbols: solid bars, thiolase activity; open bars, enoyl-CoA hydratase activity; hatched bars, 3-hydroxyacyl-CoA dehydrogenase activity. Thiolase and enoyl-CoA hydratase were assayed as described under "Materials and Methods I". 3-Hydroxyacyl-CoA dehydrogenase was assayed at pH 8 by spectrophotometrically following the reduction of NAD^+ at 340 nm in the presence of the 3-L-hydroxyacyl-CoA substrates formed from the corresponding $\Delta^{2,3}$-enoyl-CoA derivatives by the action of enoyl-CoA hydratase. A typical assay contained 50 μmoles of potassium phosphate buffer, pH 8, 0.2 mg bovine serum albumin, 1μmole of 2-mercaptoethanol, 150 nmoles CoA, 270 nmoles NAD^+, 10 nmoles $\Delta^{2,3}$-enoyl-CoA and enzyme in a total volume of 0.6 ml. The hydration of the enoyl-CoA compound was allowed to come to equilibrium in the absence of NAD^+. The assay was then started by the addition of NAD^+. The presence of CoA in the assay mixture resulted in the thiolytic cleavage of the reaction products, 3-ketoacyl-CoA derivatives, which otherwise inhibited the 3-hydroxyacyl-CoA dehydrogenase.

Figure 10

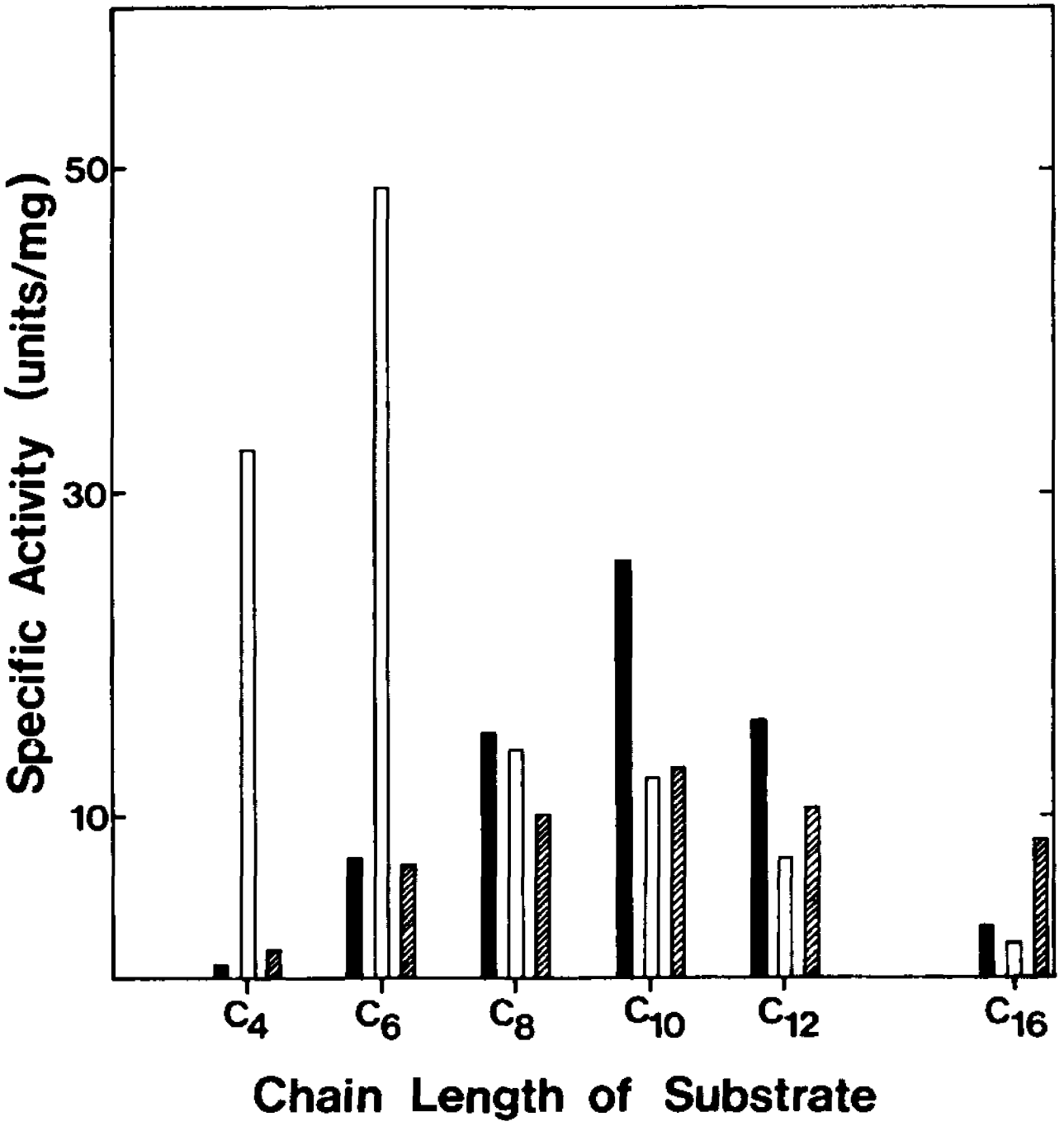

Specific Activity (units/mg)
50
30
10
C4
C6
C8
C10
C12
C16
Chain Length of Substrate

Figure 11. Possible metabolic roles of multiple thiolases

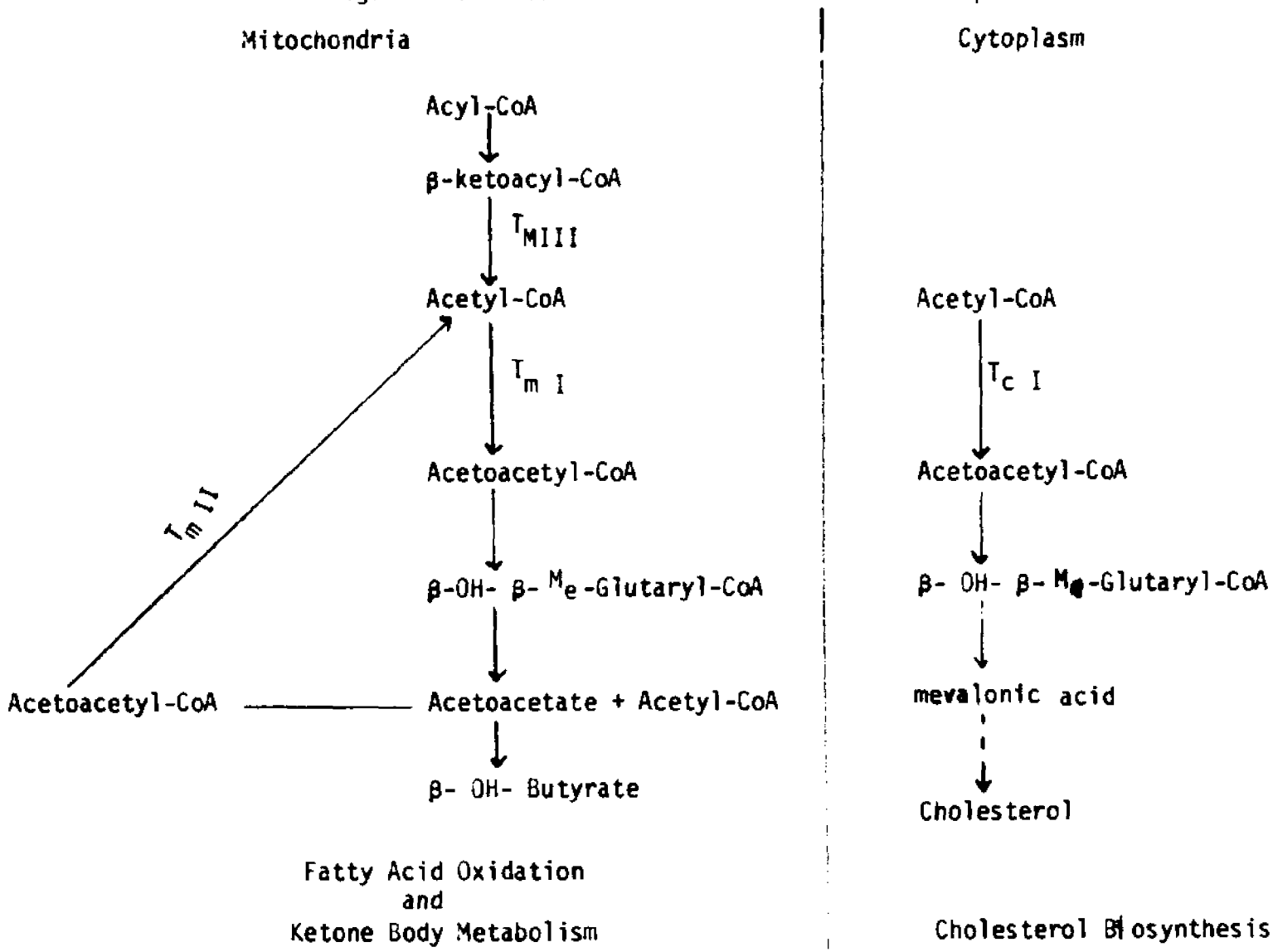


Figure 11. Possible Metabolic Roles of Multiple Thiolases

Abbreviations:

$T_{m\ I}$ = Acetoacetyl-CoA mitochondrial thiolase associated with ketone body formation

$T_{m\ II}$ = Acetoacetyl-CoA mitochondrial thiolase associated with ketone body utilization

$T_{m\ III}$ = General-Substrate mitochondrial thiolase associated with β-oxidation

$T_{c\ I}$ = Acetoacetyl-CoA cytoplasmic thiolase associated with cholesterol biosynthesis.

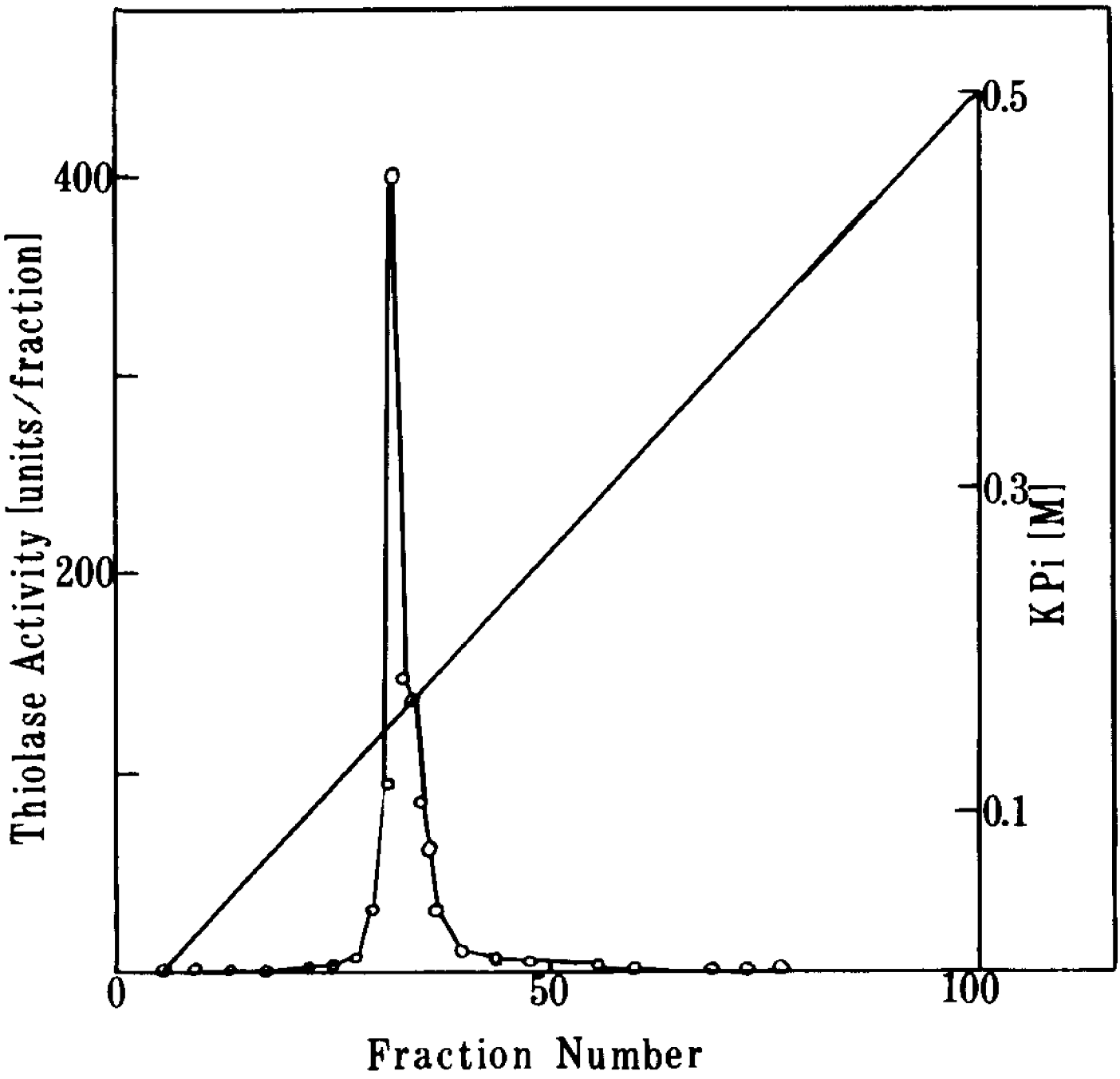


Figure 12. Purification of thiolase II by chromatography on phosphocellulose. Thiolase II activity was determined with acetoacetyl-CoA. A phosphocellulose column (1.2x45cm) was developed as described under "Materials and Methods I". The absorbance at 280nm is not shown because the amount of protein eluted with the potassium phosphate gradient was very small.

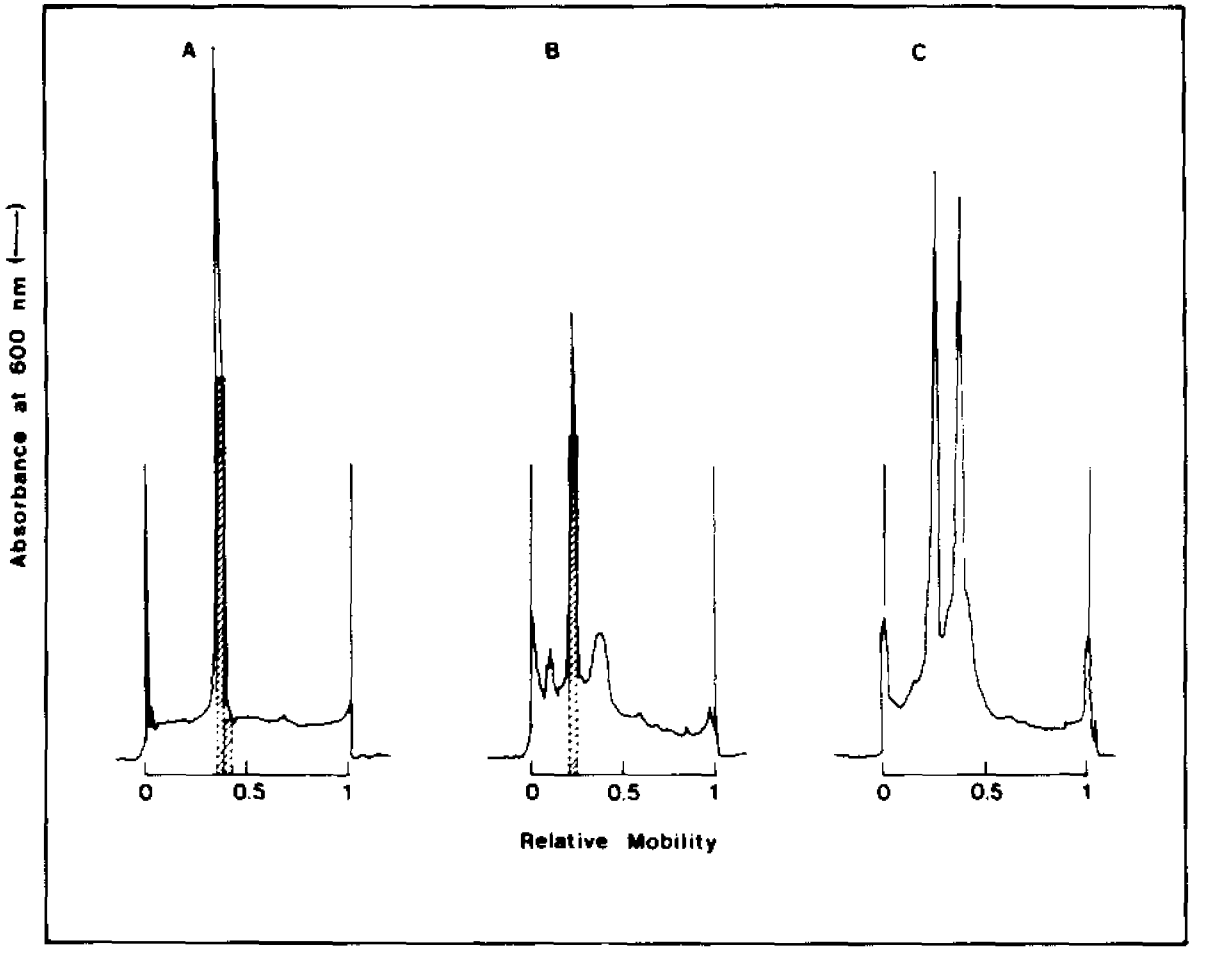
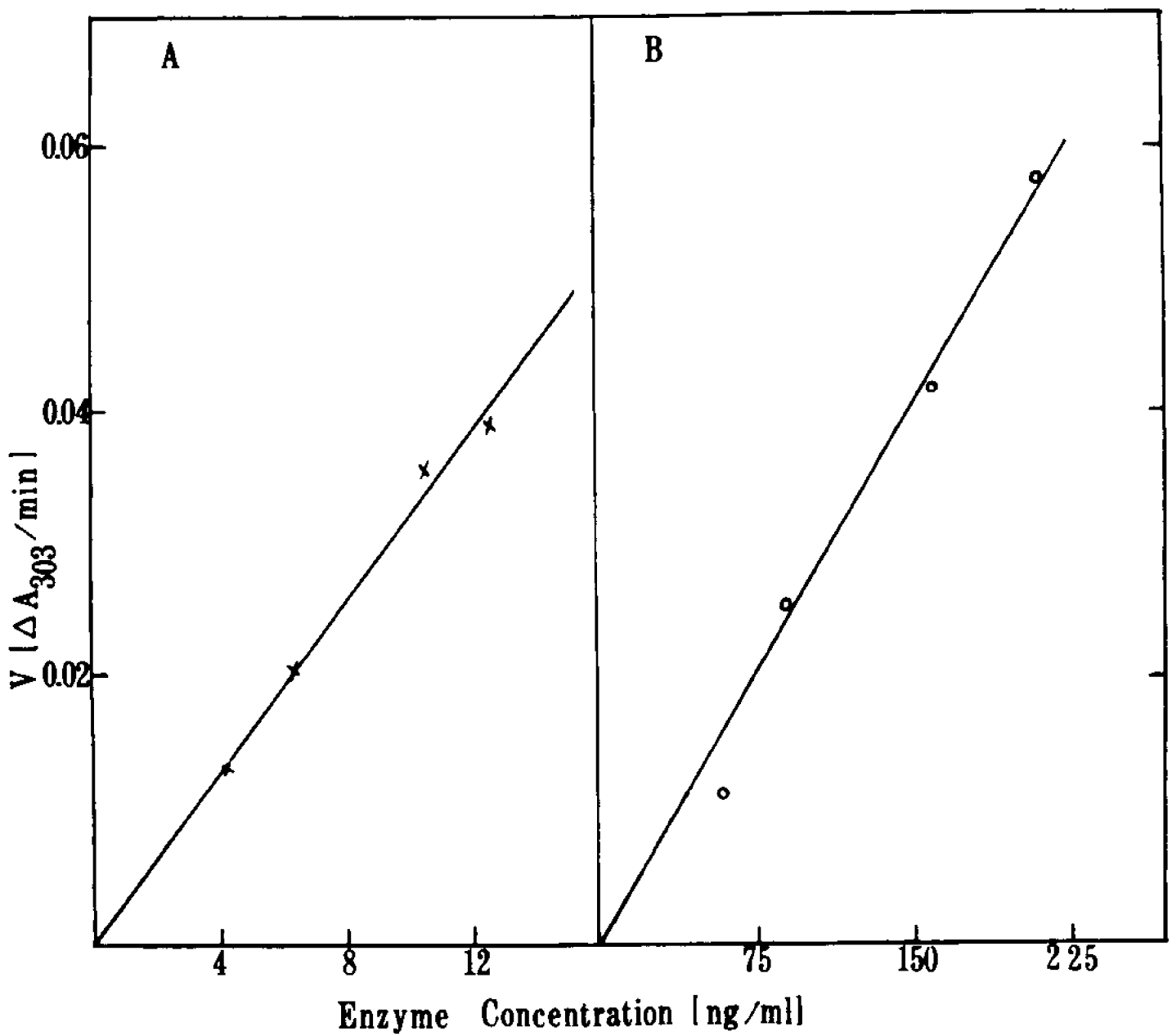


Figure 13

Disc gel electrophoresis of thiolase I and thiolase II. (A), Thiolase II; (B), thiolase I; (C), thiolases I and II. The positions of the thiolytic activities are indicated by hatched bars. (A) Activity with acetoacetyl-CoA; (B), activity with acetoacetyl-CoA and β-ketodecanoyl-CoA.

Figure 14. The rate of the thiolase reaction as a function of the enzyme concentration. A. Thiolase II. B. Multi-enzyme complex. All assays were performed in a total volume of 0.6 ml as described under "Materials and Methods II".

Figure 14


A
B
0.06
0.04
0.02
V [ΔA303/min]
4
8
12
75
150
225
Enzyme Concentration [ng/ml]

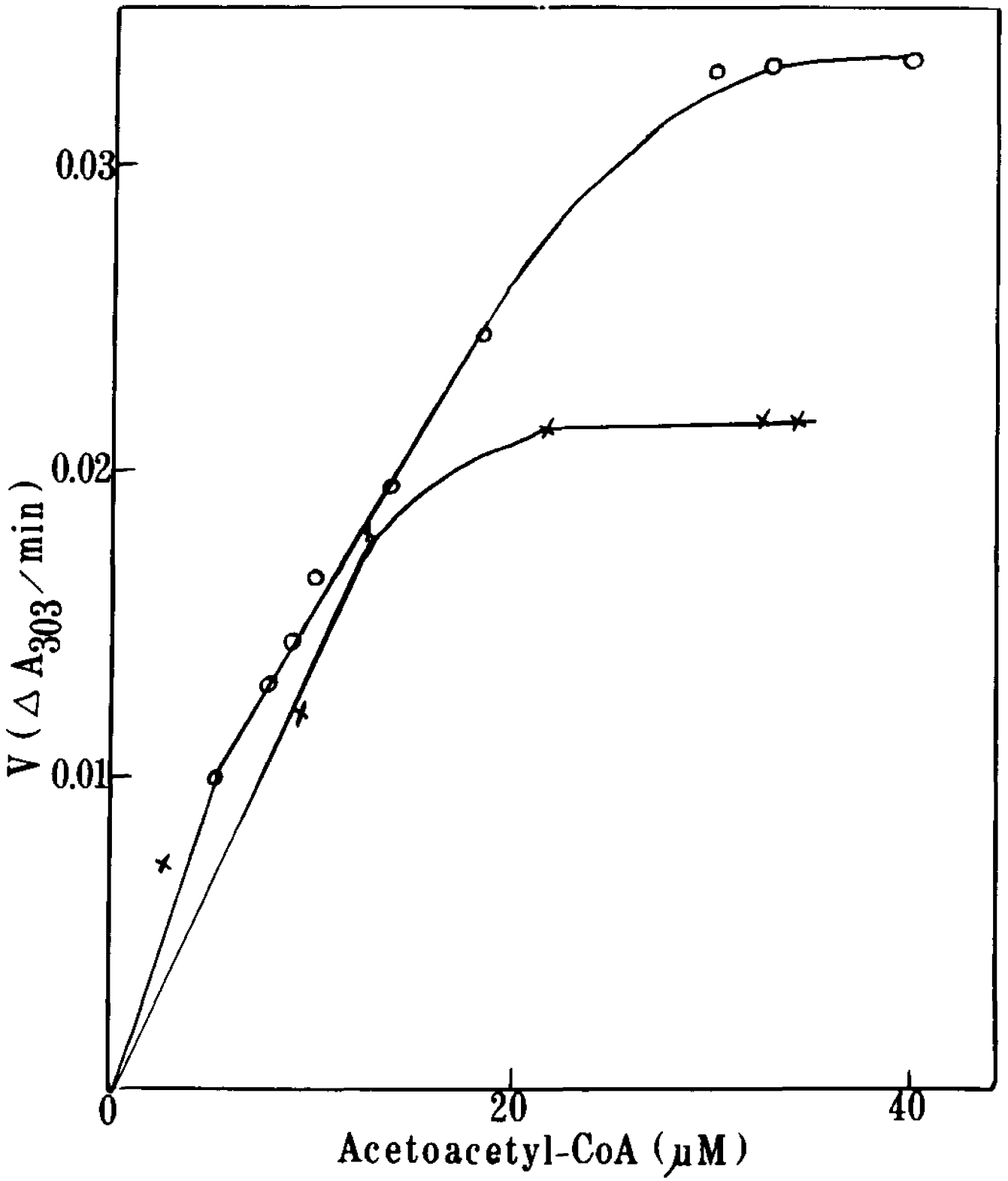


Figure 15

The rate of the thiolytic cleavage of acetoacetyl-CoA as a function of acetoacetyl-CoA concentration. Symbols: O, multi-enzyme complex; X, thiolase II. All assays were performed in a total volume of 0.6ml at a constant CoA concentration of 90μM and using the indicated amounts of acetoacetyl-CoA. The assay contained 2.25 μg of the multi-enzyme complex or 3.8 ng of thiolase II.

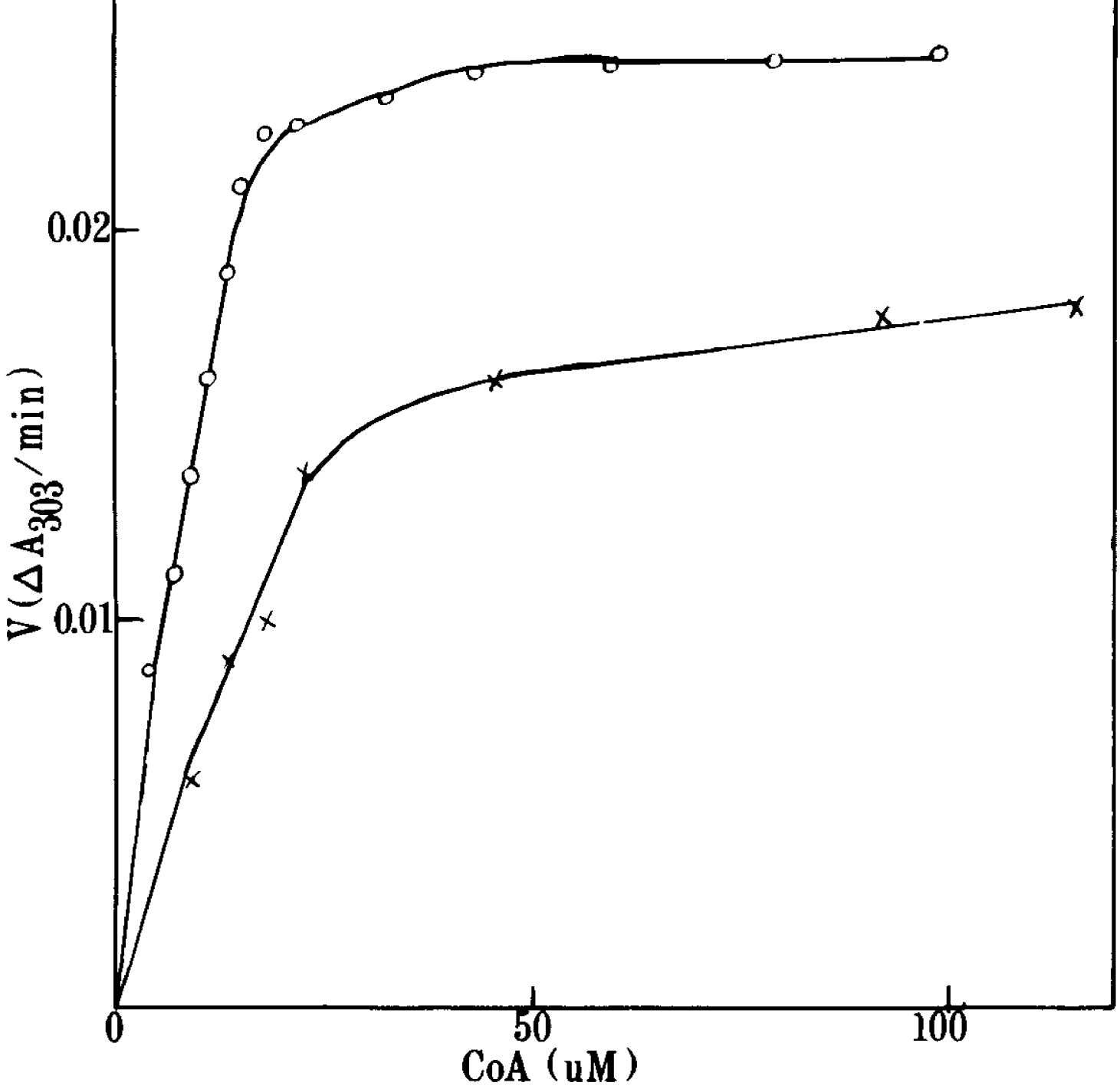


Figure 16

The rate of the thiolytic cleavage of acetoacetyl-CoA as a function of CoA concentration. Symbols: O, multi-enzyme complex; X, thiolase II. All assays were performed in a total volume of 0.6 ml containing 38 μM acetoacetyl-CoA when assaying the multi-enzyme complex and 28 μM acetoacetyl-CoA when assaying thiolase II using the indicated amounts of CoA. The assay contained 2.25 μg of the multi-enzyme complex or 3.8 ng of thiolase II.

Figure 17. Double reciprocal plot of the rate of thiolase II reaction versus substrate concentration. A. varying CoA concentrations at a fixed concentration of 40μM acetoacetyl-CoA. B. varying acetoacetyl-CoA concentrations at a fixed concentration of 90μM CoA. Each assay contained 6 ng of enzyme and was done in a total volume of 0.6 ml as described in "Materials and Methods I".

Figure 17

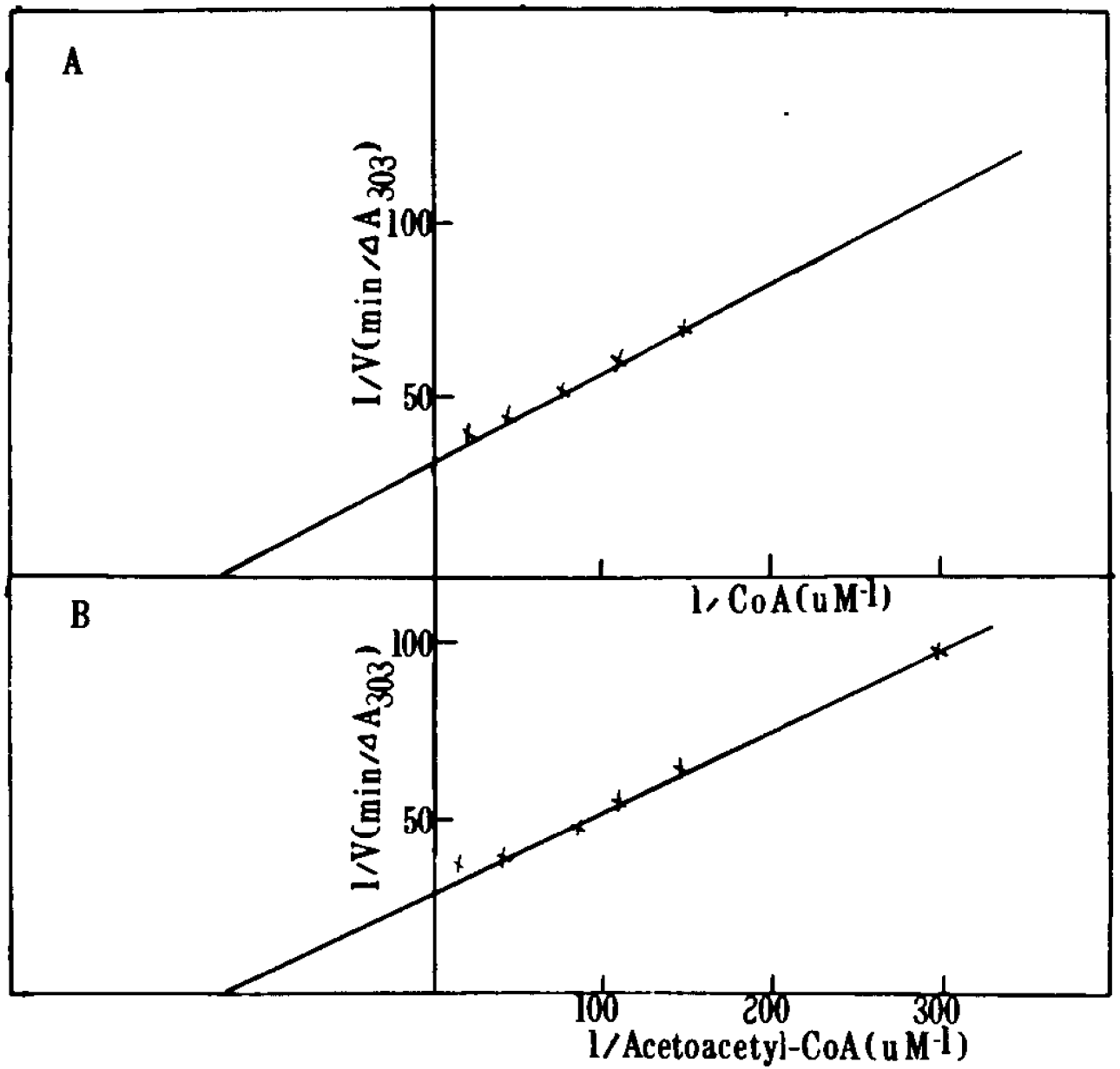

A
1/V(min/ΔA303)
100
50
1/CoA(uM-1)
B
1/V(min/ΔA303)
100
50
100
200
300
1/Acetoacetyl-CoA(uM-1)

Figure 18. Double reciprocal plot of the rate of thiolase I reaction versus substrate concentration. A. varying CoA concentrations at a fixed concentration of 39μM acetoacetyl-CoA using 14μg of enzyme per assay. B. varying acetoacetyl-CoA concentrations at a fixed concentration of 90μM CoA using 2.2 μg of enzyme per assay. C. varying 3-ketodecanoyl-CoA at a fixed concentration of 90μM CoA using 4.5 μg of enzyme per assay. Each assay was done in a total volume of 0.6 ml as described in "Materials and Methods I".

Figure 18

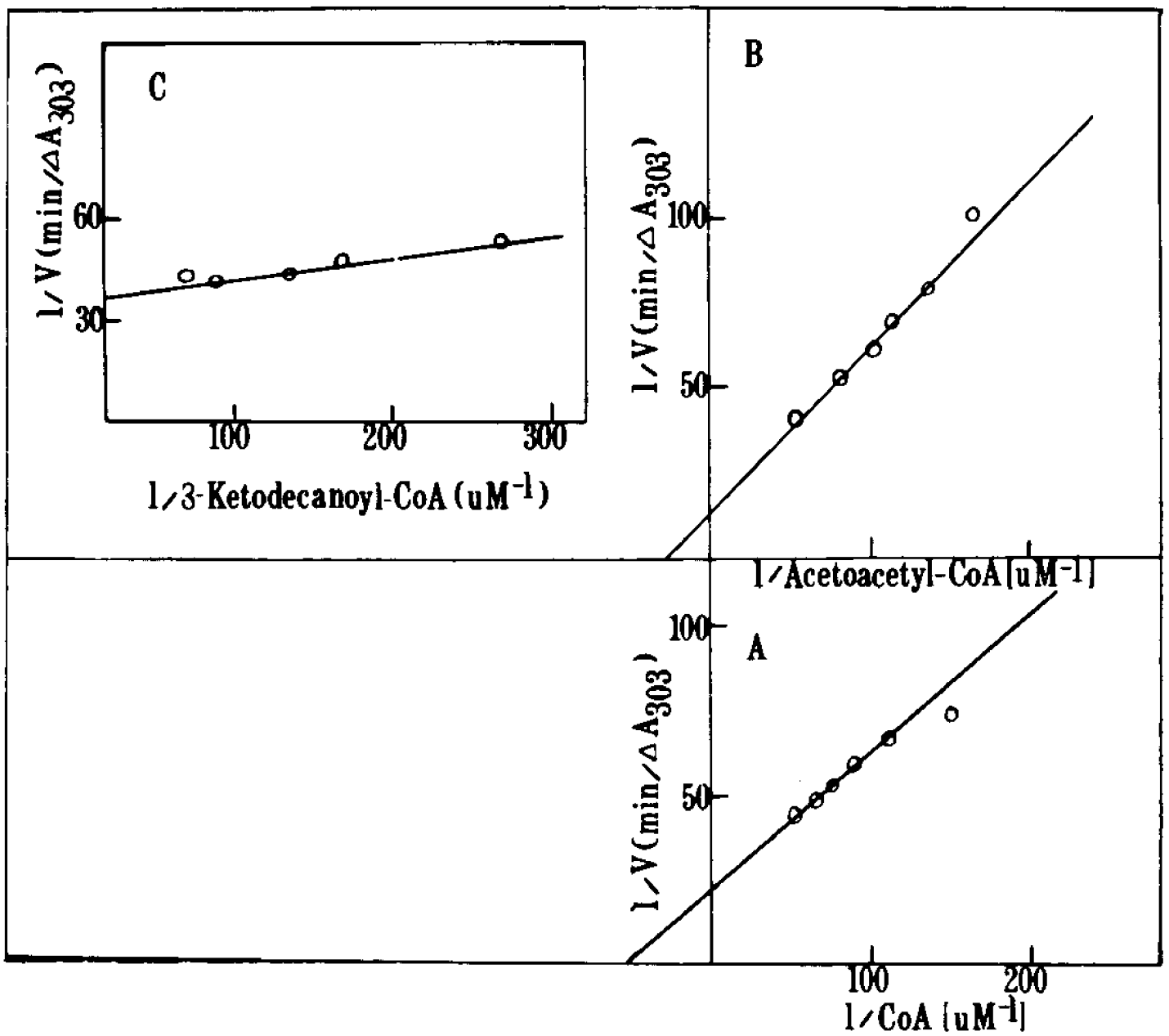

C
1/V(min/ΔA303)
60
30
100
200
300
1/3-Ketodecanoyl-CoA (uM-1)
B
1/V(min/ΔA303)
100
50
1/Acetoacetyl-CoA [uM-1]
A
1/V(min/ΔA303)
100
50
100
200
1/CoA [uM-1]

Figure 19. pH optima of thiolases I and II. A. pH optimum of thiolase I when assayed with acetoacetyl-CoA and 4.5µg of enzyme. B. pH optimum of thiolase I when assayed with 3-ketodecanoyl-CoA and 45 µg of enzyme. C. pH optimum of thiolase II when assayed with acetoacetyl-CoA and 8µg of enzyme. Buffers used were potassium phosphate, O, and Tris-HCl, Δ. The assay mixture was in a total volume of 0.6 ml, 100mM of the indicated buffer, 20µM acetoacetyl-CoA or 10µM 3-ketodecanoyl-CoA, 50µM CoA, and enzyme.

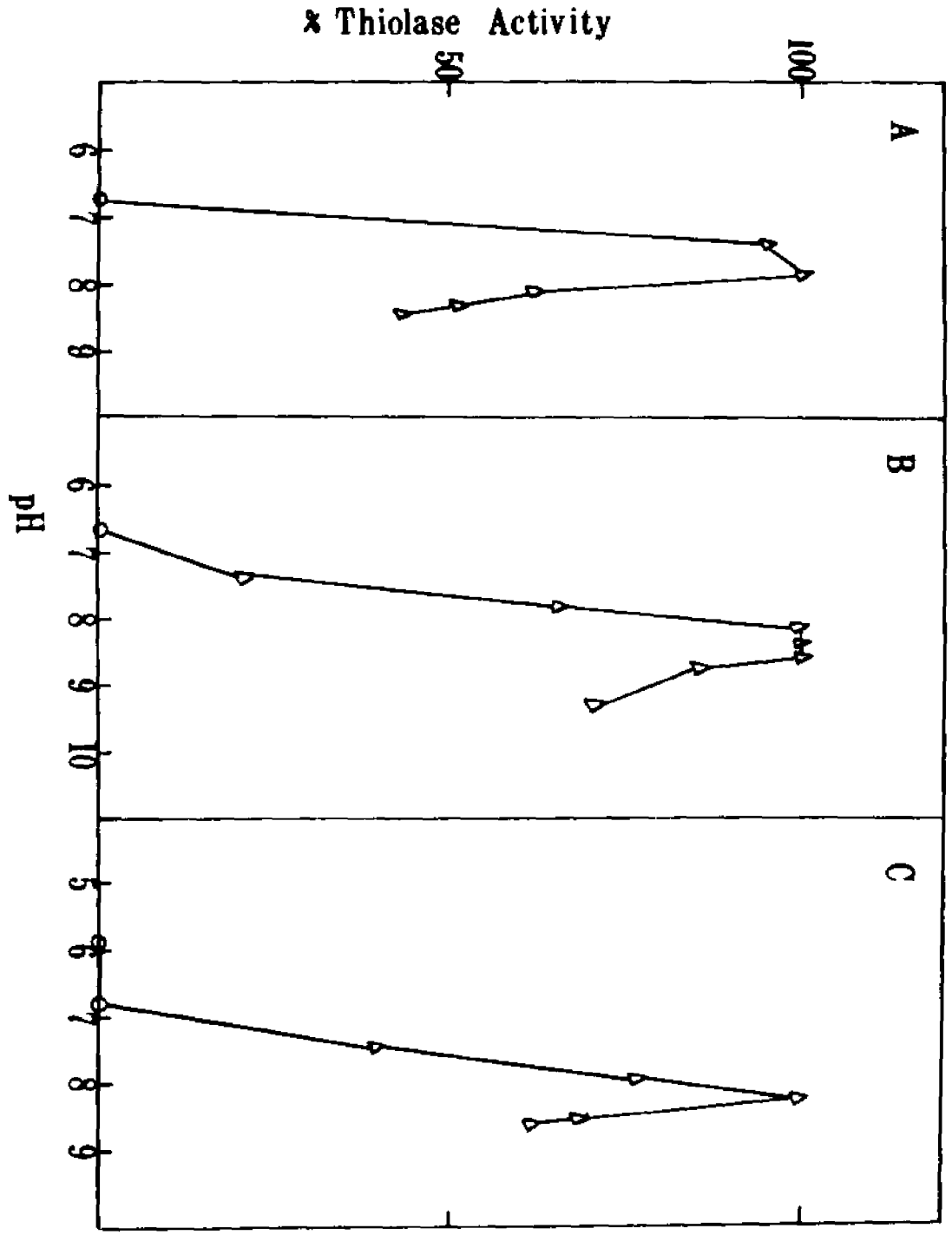


Figure 19

Figure 20. Stability of thiolase I and II upon dilution. Thiolase I was diluted to a concentration of 2.25 μg/ml in the following buffers, kept at 0.5°C and assayed at various times: △, 1M Tris-HCl (pH 8.0) containing 10mM 2-mercaptoethanol, 1 mg/ml bovine serum albumin and 25% (v/v) glycerol; ○, 1M Tris-HCl (pH 8.1) containing 10mM 2-mercaptoethanol; ●, 1M Tris-HCl (pH 8.1) containing 25% (v/v) glycerol; X, 1M Tris-HCl (pH 8.1). The assay was in a total volume of 0.6 ml and contained 9 μM 3-ketodecanoyl-CoA, 50μM CoA, 0.9μg of enzyme and assay buffer as described under "Materials and Methods I". Thiolase II was diluted to 1.26μg/ml in the following buffers, kept at 0-5°C and assayed at various times: □, 1M Tris-HCl (pH 8.1), 10mM 2-mercaptoethanol, 1mg/ml bovine albumin and 25% (v/v) glycerol; ■, 1M Tris-HCl (pH 8.1). The assay contained in a total volume a/ 0.6 ml 20μM acetoacetyl-CoA, 50μM CoA, 4.8 ng of thiolase II and assay buffer as described under "Materials and Methods I".

Figure 20

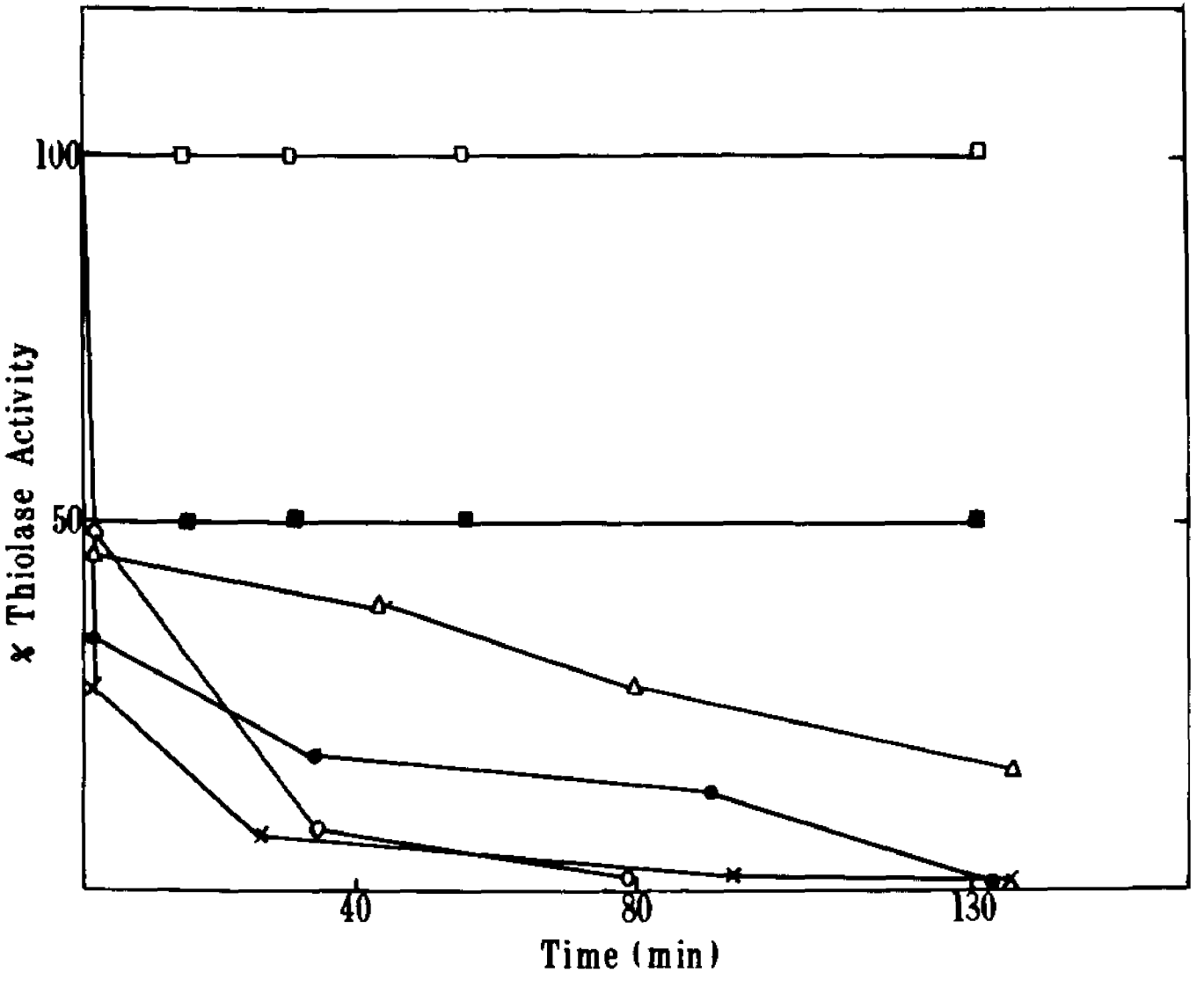
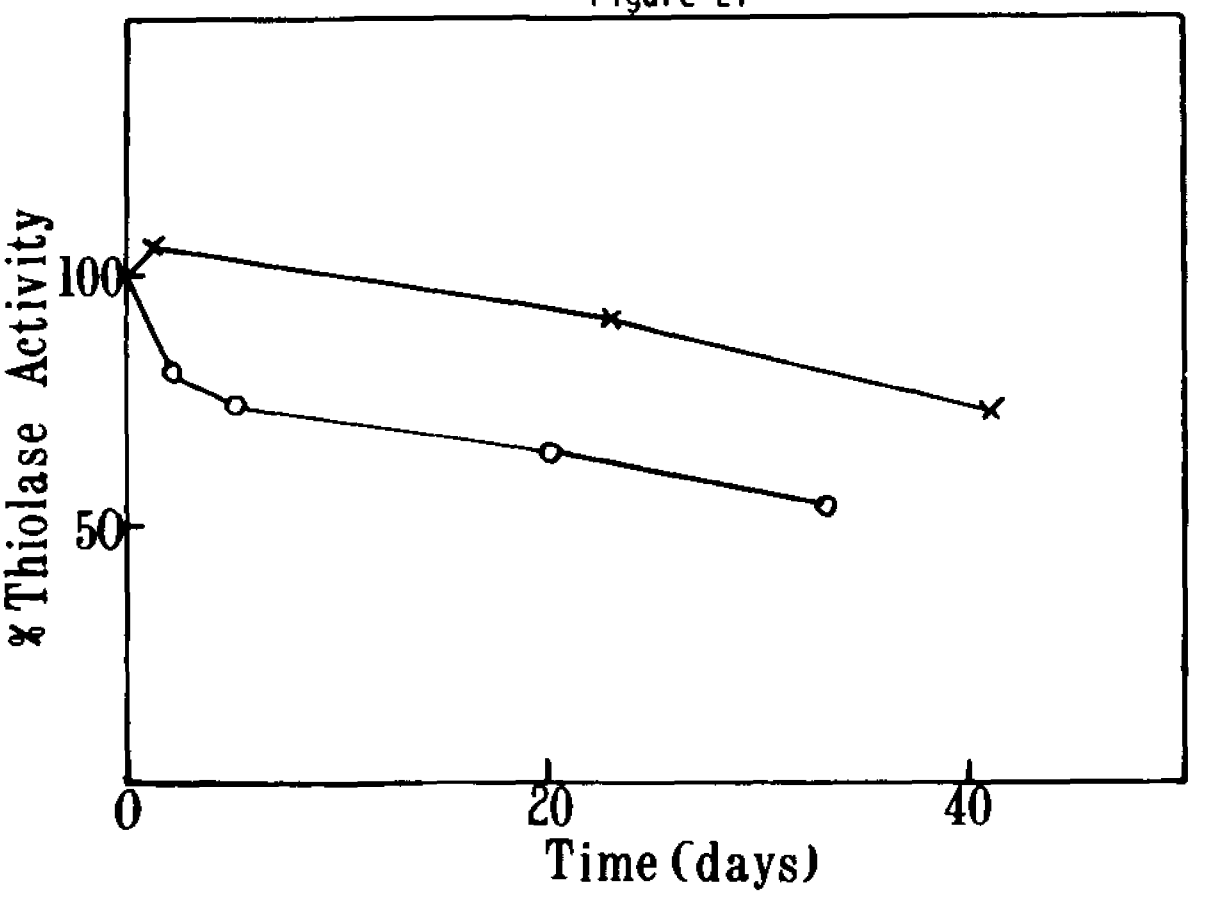


Figure 21



Stability of thiolase I and II stock solutions upon storage at 0-5°C. Thiolase II (X) was stored at 1.26 mg/ml in 0.2M potassium phosphate (pH 6.6) containing 10mM 2-mercaptoethanol, and 25% (v/v) glycerol. The assay contained in a total volume of 0.6 ml 3.8 ng of enzyme, 40μM acetoacetyl-CoA, 90μM CoA and assay buffer as described under "Materials and Methods I". Thiolase I (O) was stored at 0.45 mg/ml under the same conditions as thiolase II. The assay is as described above except 2.25μg of of thiolase I was added to the assay mixture containing 30μM acetoacetyl-CoA and 50μM CoA.

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