FATTY ACID OXIDATION IN HEART :

A Study of Enoyl-CoA Hydratases and of the Rate-Determining Step of Fatty Acid Oxidation

by

JIM CHIMING FONG

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Executive Officer

Abstract

A short chain enoyl-CoA hydratase (crotonase) from pig heart has been purified to apparent homogeneity. The enzyme has an estimated native molecular weight of 155,000 and appears to be composed of six subunits of molecular weight 27,300. A study of the kinetic properties of the enzyme revealed that the maximal velocity decreases nearly linearly with increasing chain length of the substrates from 1,670 units/mg with crotonyl-CoA to 40 units/mg with hexadecenoyl-CoA. However, the same K_m values of 30 μM were obtained for all substrates except for crotonyl-CoA for which a value of 13 μM was determined.

Since the presence of both crotonase and long chain enoyl-CoA hydratase in pig heart has been reported earlier, the presence of the same two enoyl-CoA hydratases in various tissues of several animals was investigated by sequential extraction and chromatography on hydroxylapatite of tissue homogenates. The simultaneous occurrence of both types of enoyl-CoA hydratase in tissues of pig and guinea pig has thus been established. It is proposed that the complementary actions of the two enoyl-CoA hydratases assure a high rate of hydration of enoyl-CoA intermediates of all chain lengths in fatty acid oxidation.

In the study of the rate-determining step of fatty acid oxidation the hypoglycemic compound 4-pentenoate was used to inhibit fatty acid oxidation in coupled rat heart mitochondria. It was found that this compound in contrast to n-pentanoate caused the inhibition of 3-ketoacyl-CoA thiolase and of acetoacetyl-CoA thiolase but did not affect any of the other enzymes of β-oxidation. A time study demonstrated that the inhibition of the two thiolases paralleled the inhibition of palmitoylcarnitine-supported respiration. Since respiration supported by either palmitoyl-CoA or octanoate was inhibited by 4-pentenoate in a nearly identical fashion while pyruvate-dependent respiration was only slightly inhibited and to the same degree as it was affected by n-pentanoate, it is concluded that under the conditions used in this study the thiolase-catalyzed step in fatty acid oxidation is rate-limiting or at least is as slow as other steps are. It is therefore suggested that fatty acid oxidation in heart may be controlled via the regulation of 3-ketoacyl-CoA thiolase. Other slow steps identified by <u>in vitro</u> enzyme assays are those catalyzed by 3-hydroxyacyl-CoA dehydrogenase and carnitine palmitoyltransferase.

Additionally, 4-pentenoate was found to cause the parallel inhibitions of acetylcarnitine-supported respiration and of carnitine acetyltransferase in coupled

rat heart mitochondria. This study led also to the conclusion that the specific and pronounced inhibition of fatty acid oxidation by 4-pentenoate is due to the inhibition of 3-ketoacyl-CoA thiolase.

To My Parents and My Wife

ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. Horst Schulz whose constant guidance, continued support, and indispensable advice made this all possible.

I am thankful also to Dr. Thomas Haines for many helpful discussions, and to Alvin Stern who kindly prepared the electron micrographs of mitochondria.

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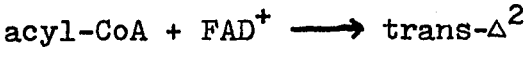
PART ONE

A Study of Enoyl-CoA Hydratases from Pig Heart

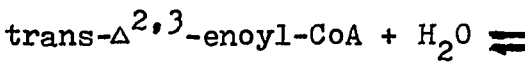
Introduction

In mammalian mitochondria, fatty acids in the form of their CoA esters are degraded by a sequence of four reactions referred to as the β-oxidation cycle. The reactions of this pathway and the enzymes involved in it are listed below:

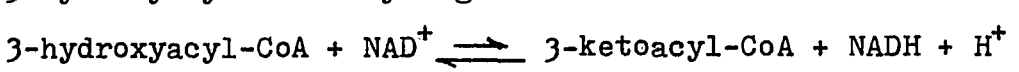
1) Acyl-CoA dehydrogenase



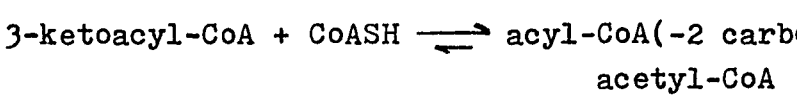
2) Enoyl-CoA hydratase



3) 3-Hydroxyacyl-CoA dehydrogenase



4) Thiolase



The shortened acyl-CoA will then be recycled from 1) through 4) until the fatty acid is completely degraded into acetyl-CoA units. The acetyl-CoA so formed will be further oxidized via the citric acid cycle to provide the energy required by the living cell.

Enoyl-CoA hydratase (EC 4.2.1.17), or crotonase, as the second enzyme in the sequence of β-oxidation, catalyzes the reversible hydration of trans-$\Delta^{2,3}$-enoyl-CoA substrates to their corresponding L-3-hydroxyacyl-CoA derivatives (1). The enzyme was first purified from bovine liver and obtained in crystalline form by Stern (2). It acts on a broad spectrum of structural analogs of crotonyl-CoA as long as the compounds possess $\Delta^{2,3}$-double bonds (3) and it hydrates the trans- as well as the cis-enoyl-CoA derivatives to the corresponding L- and D-3-hydroxy compounds respectively (4). However, the enzyme appears to have a strict requirement for thioesters which retain the nucleotide portion of the CoA moiety (1). Although it can hydrate crotonylpantetheine (1), the rate of hydration is only about 1% of that measured with crotonyl-CoA. Recently, the chain length specificity of the liver crotonase was established by Waterson and Hill (5) who showed that this enzyme catalyzes the hydration of CoA derivatives of $\Delta^{2,3}$-enoic acids containing 4 to 16 carbons (5). However, the V_{max} for this series decreases with increasing chain length with the result that the activity with hexadecenoyl-CoA is less than 1% of that measured with crotonyl-CoA.

Crotonase was assumed to be the only enoyl-CoA hydratase present in mitochondria (6) until recently when a long chain enoyl-CoA hydratase was isolated from

pig heart (7). The latter enzyme catalyzes the hydration of medium and long chain trans-$\Delta^{2,3}$-enoyl-CoA substrates to their corresponding L-3-hydroxy derivatives but it is virtually inactive toward crotonyl-CoA. The highest V_{max} was observed with $\Delta^{2,3}$-octenoyl-CoA, while longer chain substrates gave progressively decreasing values. Since this enzyme is localized in mitochondria, it appears reasonable to assume that it participates in the β-oxidation of fatty acids.

In order to elucidate the function of long chain enoyl-CoA hydratase in the fatty acid oxidation, it is essential to fully characterize short chain enoyl-CoA hydratase, or crotonase, which is also present in mitochondria of pig heart (7). In this part, I will report the purification of pig heart crotonase which has kinetic properties different from those of the bovine liver enzyme, whereas the physical properties of the two enzymes are very similar (8). Results from this (8) and a previous study (7) suggest that crotonase and long chain enoyl-CoA hydratase complement each other thereby assuring a high rate of hydration of all enoyl-CoA intermediates formed during the oxidation of long chain fatty acids (8). Additionally, I will present evidence that both crotonase and long chain enoyl-CoA hydratase are present in pig liver as well as in guinea pig tissues (8).

Experimental Procedures

Materials- Coenzyme A and NAD^+ were obtained from P-L Biochemicals,Inc. N-Methylmaleimide and bovine serum albumin were purchased from Sigma Chemical Co. Sodium p-chloromercuribenzoate and α-iodoacetamide were bought from Calbiochem. Ethyl chloroformate, triethylamine, 2-decenoic acid and 2-octenoic acid were obtained from Aldrich Chemical Co. 2-Hexadecenoic acid was purchased from Miles Laboratories,Inc. L-3-Hydroxyacyl-CoA dehydrogenase and crotonic anhydride were bought from Boehringer and Sons and Eastman Kodak, Co., respectively. All other chemicals were of reagent grade. Trans-$\Delta^{2,3}$-hexenoic acid and trans-$\Delta^{2,3}$-dodecenoic acid were synthesized by reacting malonic acid in the presence of pyridine with n-butyraldehyde and n-decanal respectively, according to a procedure by Linestead et al.(9). Polyacrylamide gradient gels were purchased from Pharmacia Fine Chemicals,Inc. Guinea pigs (350-400 g) were obtained from Marland Breeding Farms,Inc. Fresh beef heart, beef liver, pig heart and pig liver were bought from Max Insel Cohen, Co. For longer periods of time they were stored at - 20^{o}.

Preparation of Substrates - Crotonyl-CoA was prepared according to the procedure of Weeks and Wakil (10). All other trans-$\Delta^{2,3}$-enoyl-CoA substrates were synthesized by the method of Goldman and Vagelos (11). Acetoacetyl-CoA was prepared according to the method of Seubert (12). The concentrations of all CoA derivatives were determined by the method of Ellman (13) after cleaving the thioester bond with hydroxylamine at pH 7.

Enzyme and Protein Assays - Enoyl-CoA hydratase activities were routinely measured by following the decrease in absorbance at 263 nm due to the hydration of $\Delta^{2,3}$-double bond of the enoyl-CoA substrate on a Gilford recording spectrophotometer, Model 240 (direct method) as described by Stern (2). Molar extinction coefficients (E_{263}) of 6,700 $cm^{-1}M^{-1}$ were used for $\Delta^{2,3}$-enoyl-CoA substrates of all chain lengths. A standard assay mixture contained 0.2 M potassium phosphate (pH 8), 1.5 μM bovine serum albumin and 30 μM $\Delta^{2,3}$-enoyl-CoA. One unit of activity is defined as a micromole of substrate converted to product per min. In some instances, a combined assay was used as reported earlier (7). A standard assay mixture contained 0.1 M Tris-HCl (pH 9), 0.1 M KCl, 1.5 μM bovine serum albumin, 120 μM NAD^+, 30 μM $\Delta^{2,3}$-enoyl-CoA and L-3-hydroxyacyl-CoA dehydrogenase. Protein concentrations were determined by the method of Lowry et al. (14).

Purification of Pig Heart Crotonase- Frozen pig hearts (700 g) were cut into pieces and forced through a meat grinder. Batches of one third of the material were blended together with 500 ml of cold acetone (-5°) each in a Warring blender twice for 1 min. The resulting acetone suspensions were combined and filtered. The solid retentate was washed twice with 500 ml of cold ether (-5°) each. Residual ether was then removed under vacuum. A total of 230 g of acetone powder were thus obtained. The acetone powder was immediately extracted with 800 ml of 0.02 M potassium phosphate (pH 6.3) (all the phosphate buffers mentioned in the description of the purification procedure contained 5 mM mercaptoethanol and 10% glycerol unless otherwise indicated) under stirring for 8 h (all experiments were performed at 4° unless otherwise indicated). Insoluble material was removed by centrifugation for 45 min at 30,000 x g. The clear supernatant was dialyzed against 7.5 liters of 0.01 M potassium phosphate (pH 6.3) for 8 h and the resulting precipitate was removed by centrifugation. The supernatant was then applied to a phosphocellulose column (4.0 x 44 cm) which had been previously equilibrated with 0.01 M potassium phosphate (pH 6.3). The column was washed with the same buffer until ultraviolet absorbing material ceased to be eluted and was

then developed with 0.2 M potassium phosphate ($_p$H 6.3). A total volume of 1150 ml was collected which contained 95% of the applied crotonase activity and was concentrated in an Amicon concentrator (PM-30 membrane) to a final concentration of 7.7 mg of protein/ml. The concentrate was extensively dialyzed against 0.01 M potassium phosphate ($_p$H 6.3) and applied to a second phosphocellulose column (5.0 x 36 cm) which had been equilibrated with 0.01 M potassium phosphate. The column was extensively washed with 0.02 M potassium phosphate ($_p$H 6.3) and then developed with a gradient made up of 2 liters each of 0.02 M potassium phosphate ($_p$H 6.3) and 0.2 M potassium phosphate ($_p$H 6.3). Fractions of 16 ml were collected and assayed for crotonase activity with crotonyl-CoA as the substrate. Fractions with high activity were pooled and concentrated in an Amicon concentrator (PM-10 membrane). The concentrate was directly applied to a hydroxylapatite column (2.5 x 15 cm) which had been previously equilibrated with 0.2 M potassium phosphate ($_p$H 6.3). The column was washed with 4 column volumes of 0.2 M potassium phosphate ($_p$H 6.3) and then 2 column volumes of 0.35 M potassium phosphate ($_p$H 6.3). The column was developed with a gradient made up of 300 ml each of 0.35 M potassium phosphate ($_p$H 6.3) and 0.7 M potassium phosphate (pH 6.3). Fractions of 6 ml were collected at a flow rate of 60 ml/h. The fractions

with highest crotonase activity were pooled and concentrated to a final concentration of 2.4 mg of protein/ml. Solid $(NH_4)_2SO_4$ was added to the concentrated crotonase preparation to give 50% saturation. The preparation was kept at 4°. The activity of this preparation remained constant for several months after 70% of the original activity was lost during the first 3 days. The results of this purification are summarized in Table 1.1.

Disc Electrophoresis - Disc electrophoresis was performed on 7.5% polyacrylamide gels at 15° and at pH 8.5 as described by Ornstein (15) and Davis (16). The method of Shapiro et al.(17) as modified by Weber and Osborn (18) was used for polyacrylamide disc gel electrophoresis of proteins in the presence of sodium dodecyl sulfate. Gradient gel electrophoresis was performed on 4% to 30% polyacrylamide gradient slab gels at pH 8.35 for 20 h at 15° on a Pharmacia GE-4 electrophoresis apparatus.

Preparation of Mitochondria - Guinea pig mitochondria were prepared by a procedure similar to that described by Johnson and Lardy (19). Guinea pigs weighing from 350 to 400 g were killed. The livers were removed, cut into small pieces, and washed with cold 0.25 M sucrose solution until the wash solution remained clear. The liver pieces were then homogenized (three passes) in cold 0.25 M

sucrose (8 ml of sucrose solution/g of tissue) with a motor-driven Teflon-glass homogenizer. The nuclear fraction and debris were removed by centrifugation for 5 min at 950 x g. The resulting supernatant was then centrifuged for 7 to 9 min at 10,000 x g to sediment the mitochondria. The mitochondrial pellet was suspended in an equal volume of 0.25 M sucrose, re-homogenized by hand with the same pestle, and centrifuged for 7 to 9 min at 10,000 x g. This washing procedure was repeated twice until the supernatant was free of crotonase activity. The final mitochondrial pellet was stored at -20°.

Sequential Extraction of Enoyl-CoA Hydratases - All extractions were performed by homogenization with a PT 10 ST Polytron homogenizer (Brinkman Instrument,Inc.) at high speed (position 8) for 30 s at 4°. Except for mitochondria, all tissues (either frozen or fresh) were cut into small pieces before the first extraction. After each extraction, the homogenate was centrifuged for 10 min at 30,000 x g and the supernatant was assayed for enoyl-CoA hydratase activity. The precipitate was re-extracted by following the above procedure.

Separation of Enoyl-CoA Hydratases on Hydroxylapatite- Frozen pig liver (27g) was homogenized with a PT 10 ST Polytron homogenizer in 0.02 M potassium phosphate (pH6.3), 5 mM mercaptoethanol, 20% glycerol and centrifuged for 20 min at 30,000 x g. The resulting precipitate

was sequentially extracted with the above buffer once and with 0.1 M potassium phosphate (pH 9) and 5 mM mercaptoethanol twice. The pH of the supernatant obtained after the last extraction was adjusted with 1 N HCl to 6.3. Residual solid material was removed by centrifugation and the resulting clear solution was applied to a hydroxylapatite column (1.2 x 25 cm) which had been previously equilibrated with 0.1 M potassium phosphate (pH 6.3), 5 mM mercaptoethanol and 10% glycerol. The column was washed with 6 column volumes of the starting buffer and was then developed with a gradient made up of 150 ml each of 0.1 M potassium phosphate (pH 6.3), 5 mM mercaptoethanol, 10% glycerol and 0.3 M potassium phosphate (pH 6.3), 5 mM mercaptoethanol, 10% glycerol. Fractions of 5.8 ml were collected and assayed for enoyl-CoA hydratase activities with crotonyl-CoA, decenoyl-CoA and hexadecenoyl-CoA as substrates. The column was further developed with a second gradient made up of 70 ml each of 0.3 M potassium phosphate (pH6.3), 5 mM mercaptoethanol, 10% glycerol and 0.7 M potassium phosphate (pH 6.3), 5 mM mercaptoethanol, and 10% glycerol. Fractions of 3 ml were collected and assayed for enoyl-CoA hydratase activities with the substrates mentioned above.

Results

Purification and Stability of Crotonase - Crotonase was extracted from pig heart acetone powder and purified by chromatography on phosphocellulose and hydroxylapatite as summarized in Table 1. The first phosphocellulose column served as a means of reducing the amount of extracted protein to a manageable level while the key to the ultimate purification of the enzyme was the chromatography on hydroxylapatite. By the above procedure crotonase was purified nearly 800-fold in 42% yield. The purified enzyme was found to be free of thiolase and 3-hydroxylacyl-CoA dehydrogenase activities. Polyacrylamide disc gel electrophoresis revealed the presence of only one protein band which coincided with the enoyl-CoA hydratase activity peak (see Fig.1). However, when crotonase was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, only 90% of the protein banded in one position, a finding which indicated the presence of small amounts of impurities.

During the initial stages of the purification, it was observed that pig heart crotonase in contrast to the bovine liver enzyme was not very stable. However, a significant stabilization of the enzyme was achieved when 10% glycerol was present in all buffers used

Table I Purification of Pig Heart Crotonase[a]

Step	Total protein	Total activity	Specific activity	Purification	Yield
	mg	μmol/min	μmol/min/mg	-fold	%
Acetone powder extract	19,800	34,400	1.74	1	100
Phosphocellulose(batch)	3,358	27,000	8.04	4.6	78
Phosphocellulose(gradient)	560	25,600	45.7	26.3	74
Hydroxylapatite	11	14,570	1,334	767	42

a. From 700 g of pig heart.

Fig.1 Polyacrylamide Gel Electrophoresis of Pig Heart Crotonase

Disc electrophoresis was performed with two identical 7.5% polyacrylamide gels at pH 8.5. After electrophoresis, one was stained with Coomassie Blue R and was scanned at 550 nm, the other one was sliced, extracted with 0.2 M potassium phosphate (pH 6.3), 10 mM mercaptoethanol, 20% glycerol by homogenization with a PT 10 ST Polytron homogenizer at high speed (position 8) for 30 s and assayed for enoyl-CoA hydratase activity with crotonyl-CoA as the substrate.

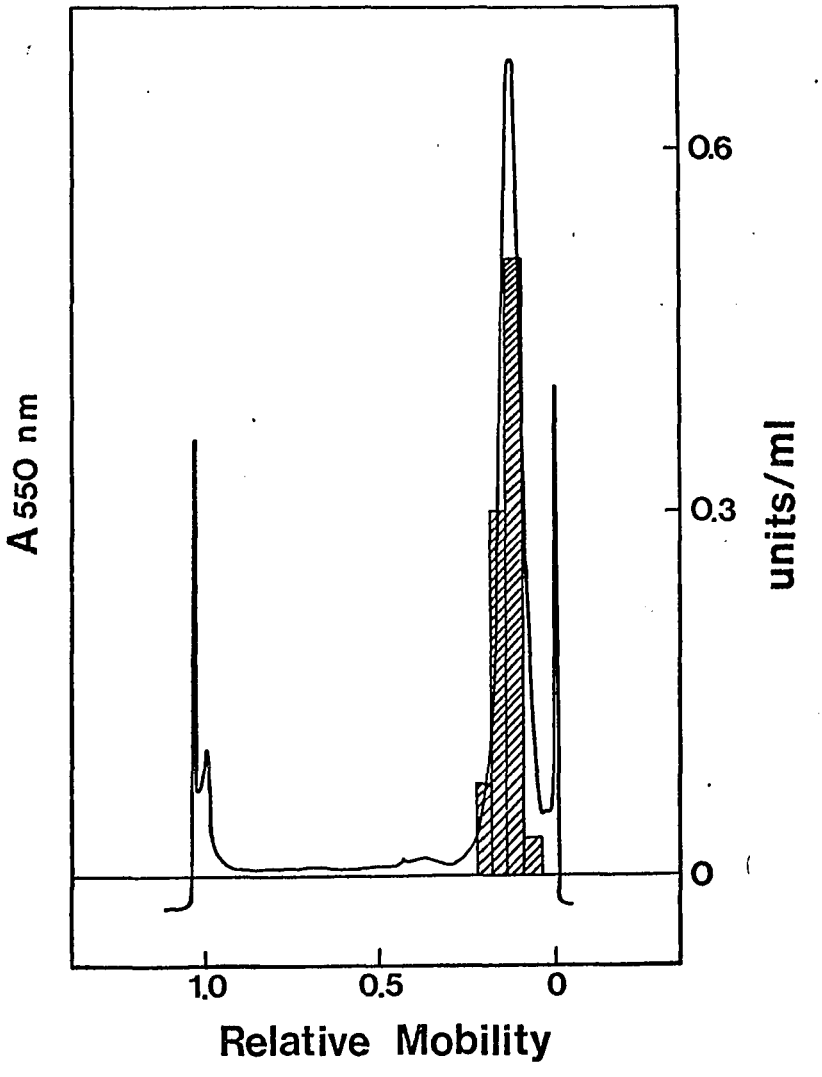


Fig. 1

during the purification procedure. The purified enzyme, when stored in the elution buffer after hydroxylapatite chromatography (0.55 M potassium phosphate (pH 6.3), 5 mM mercaptoethanol, 10% glycerol) to which $(NH_4)_2SO_4$ had been added to 50% saturation, was found to be stable for months after a rapid initial loss of 70% of its activity. To further assess the influence of the various components of the storage buffer on the stability of crotonase, the decrease in enzyme activity was determined as a function of time after dialysis against several buffers missing one or more of the components of the storage buffer. As shown in Table II , a high concentration of potassium phosphate was most effective in decreasing the rate of inactivation while mercapto-ethanol and glycerol by themselves were not sufficient to prevent the inactivation of the enzyme. It is concluded that maintaining a high ionic strength, followed in effectiveness by high protein concentration and the addition of glycerol, is the best method of stabilizing the enzyme. This conclusion also explains why hydroxyl-apatite chromatography, which permitted the application of the enzyme preparation at relatively high concentration of phosphate buffer, resulted in the purification of the active enzyme in good yield.

Table II Stability of Crotonase Under Various Conditions

Samples of crotonase taken from the preparation stored in the presence of ammonium sulfate (see "Experimental Procedures") were dialyzed against various buffers as indicated below and assayed for enoyl-CoA hydratase activity with crotonyl-CoA as the substrate (direct method as described under "Experimental Procedures"). The activities of the samples were determined again after 5 and 25 days of storage at 4°. Buffer: A, 0.1 M potassium phosphate (pH 6.3), 10% glycerol and 5 mM mercaptoethanol; B, 0.02 M potassium phosphate (pH 6.3), 10% glycerol and 5 mM mercaptoethanol; C, 0.02 M potassium phosphate (pH 6.3) and 5 mM mercaptoethanol; D, 0.02 M potassium phosphate (pH 6.3).

Days	Remaining activity			
	Buffer A	Buffer B	Buffer C	Buffer D
		%		
0	100	100	100	100
5	99	37	23	15
25	70	1	0	0

Physical and Kinetic Properties of Crotonase - The molecular weight of native pig heart crotonase was estimated by electrophoresis on a 4% to 30% polyacrylamide gradient gel. As shown in Fig. 2 (upper line), the four standards fell on a straight line which was used to estimate a molecular weight of 155,000 for crotonase. Polyacrylamide disc gel electrophoresis of crotonase in the presence of sodium dodecyl sulfate gave a subunit molecular weight of 27,300 (see Fig. 2, lower line). Thus it is suggested that pig heart crotonase is composed of six subunits. These data are very similar to those obtained with bovine liver crotonase for which a native molecular weight of 164,000 and a subunit molecular weight of 27,300 have been determined (20). The chain length specificity of pig heart crotonase was studied with a number of CoA derivatives of even-numbered $\Delta^{2,3}$-enoic acids having 4 to 16 carbons. The V_{max} and K_m values for each substrates were calculated from Lineweaver-Burk plots and are summarized in Table III . The V_{max} values are progressively decreasing with increasing chain length of the substrate while the K_m values are virtually the same for all substrates (30 μM) with the exception of crotonyl-CoA for which a value of 13 μM was determined. Two types of assays were used for critical kinetic measurements:

Fig. 2 Native and Subunit Molecular Weights of Crotonase

Molecular weight of native crotonase was determined by 4 to 30% polyacrylamide gradient gel electrophoresis. Subunit molecular weight was determined by sodium dodecyl sulfate electrophoresis (see "Experimental Procedures"). Standards: 1, apoferritin; 2, catalase; 3, lactate dehydrogenase; 4, bovine serum albumin; 5, ovalbumin; 6, pepsin; 7, α-chymotrypsinogen A; 8, myoglobin; 9, cytochrome C.

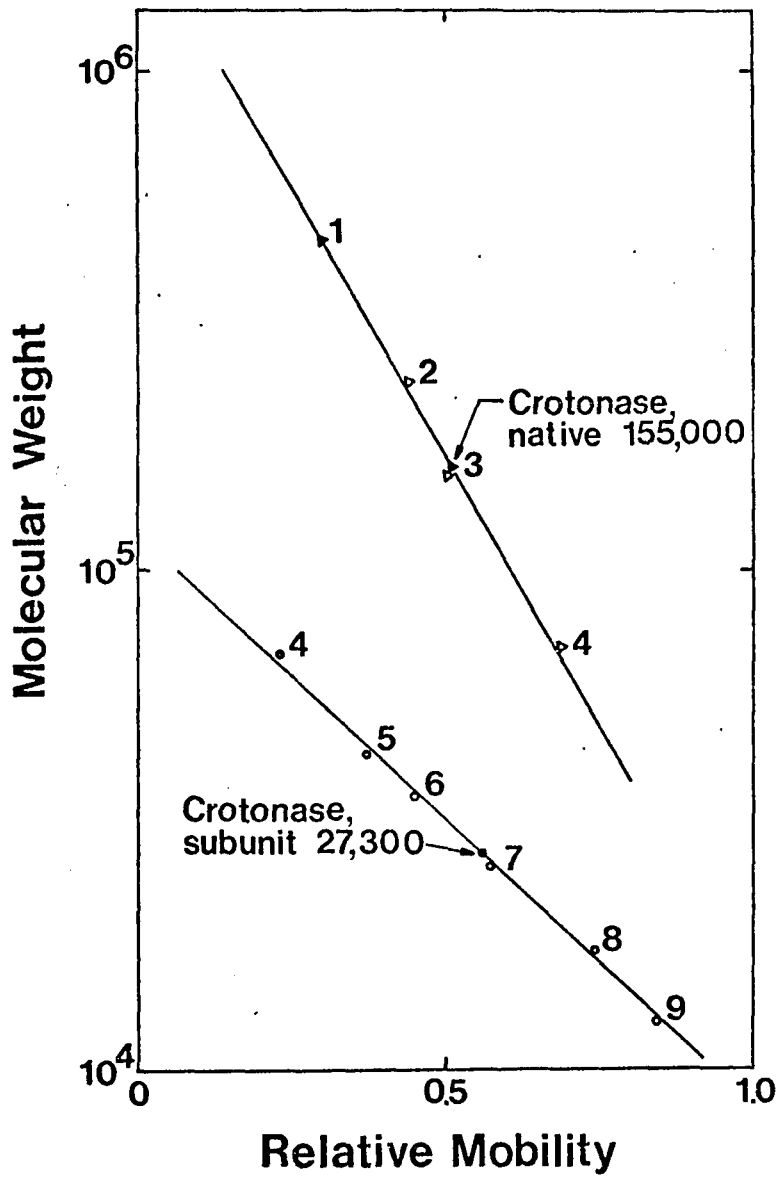

Molecular Weight
10^6
10^5
10^4
0
0.5
1.0
Relative Mobility
1
2
3
4
4
5
6
7
8
9
Crotonase, native 155,000
Crotonase, subunit 27,300

Fig. 2

Table III Substrate Specificity

Substrate	K_m	V_{max}	Relative V_{max}
	μM	μmol/min/mg	%
Crotonyl-CoA	13	1,670	100
$\Delta^{2,3}$-Hexenoyl-CoA	29	1,280	77
$\Delta^{2,3}$-Octenoyl-CoA	29	910	54
$\Delta^{2,3}$-Decenoyl-CoA	29	540	32
$\Delta^{2,3}$-Dodecenoyl-CoA	30	160	9.6
$\Delta^{2,3}$-Hexadecenoyl-CoA	30	40	2.4

the direct assay method in which the decrease in absorbance at 263 nm was monitored and the combined assay in which the amount of formed L-3-hydroxyacyl-CoA was determined by the reduction of NAD^+ in the presence of L-3-hydroxyacyl-CoA dehydrogenase. Both assay methods gave principally identical results except for kinetic measurements at high substrate concentrations where the direct method led to an underestimation of rates due to the nonlinear relationship between absorbance and concentration. Thus it was concluded that the strong substrate inhibition observed with the direct assay method at high substrate concentrations was an artifact. The pH optimum of pig heart crotonase was found to be 8.5, which is the same value reported for the long chain enoyl-CoA hydratase from the same organ (7).

Inhibition Studies - The pig heart crotonase, similar to the bovine liver enzyme, is competitively inhibited by acetoacetyl-CoA with an apparent K_I value of 14 μM. Since this inhibition appears to be due to the binding of the enolate form of acetoacetyl-CoA to the active site of crotonase (5), the apparent K_I value should be highly pH-dependent, a fact which might explain the difference between the K_I value of 14 μM obtained with pig heart crotonase at pH 8 and the value of 30 μM determined with bovine liver crotonase at pH 7.5 (5).

Additionally, the effect of sulfhydryl inhibitors on the activity of pig heart crotonase was investigated. When the enzyme was preincubated for 10 min at 0° and pH 8 in the presence of 1 mM (0.1 mM) p-chloromercuribenzoate or in the presence of 10 mM (5 mM) N-methylmaleimide, 100% (11%) or 19% (13%) inhibition was observed, respectively. Increasing the preincubation temperature from 0° to 25° resulted in 52% (42%) inhibition in the presence of 10 mM (5 mM) N-methylmaleimide. The inhibition by iodoacetamide was less pronounced. A 20% inhibition was observed when crotonase was preincubated for 20 min with 10 mM iodoacetamide at 25° and pH 8. These data suggest that pig heart crotonase contains one or more thiol groups which can not be modified without causing the inactivation of the enzyme. This finding is similar to observations made with the long chain pig heart enoyl-CoA hydratase (7) and with the bovine liver crotonase (5). Extensive work with the latter enzyme led to the conclusion that none of the thiol groups are directly involved in the catalytic event but that their modification interfered with the binding of the substrate to the enzyme (21). A similar situation may exist in pig heart crotonase.

Presence of Two Enoyl-CoA Hydratases in Other Tissues and Animals - After having demonstrated that both a short chain and a long chain enoyl-CoA hydratase are present in pig heart, it was questioned whether other organs, as for example pig liver, also contain two enoyl-CoA hydratases. To study this problem, the simple method of sequential extraction of the two enoyl-CoA hydratases was developed. This method is based on the observation that the long chain enoyl-CoA hydratase of pig heart is much more tightly associated with the particulate fraction of the homogenate than is crotonase. When pig heart homogenate was extracted with several buffers, the ratios of enoyl-CoA hydratase activities based on measurements with crotonyl-CoA, $\Delta^{2,3}$-decenoyl-CoA and $\Delta^{2,3}$-hexadecenoyl-CoA clearly indicated the presence of at least two enoyl-CoA hydratases with different chain length specificities (see Table IV). Crotonase is easily extracted at neutral pH and in the presence of glycerol whereas long chain enoyl-CoA hydratase is efficiently solubilized at high ionic strength, high pH (pH 9), and in the absence of glycerol. In fact, when the activity ratios measured in the first extract are compared with the relative V_{max} values of pure crotonase, listed in Table III , it is obvious that the first extraction step removes crotonase which is

Table IV Sequential Extraction of Enoyl-CoA Hydratases

Tissues or mitochondria were extracted, centrifuged and assayed for enoyl-CoA hydratase activities as described under "Experimental Procedures". Sequence of extractions: Step 1, with 0.02 M potassium phosphate (pH 6.3), 20% glycerol and 5 mM mercaptoethanol; Step 2, with 0.1 M potassium phosphate (pH 7), 10% glycerol and 5 mM mercaptoethanol; Step 3, with 0.2 M potassium phosphate (pH 9) and 5 mM mercaptoethanol; Step 4, with same as Step 3.

Step	Relative activities[a]									
	Pig heart			Pig liver			Guinea pig heart		Guinea pig liver mitochondria	
	C_4	C_{10}	C_{16}	C_4	C_{10}	C_{16}	C_4	C_{10}	C_4	C_{10}
1	28	8	1	37	10	1	5.6	1	4.9	1
2	3	5.6	1	15	8	1	1.6	1	1.4	1
3	1	6	2	5.2	6.5	1			1	1.5
4				2.6	5	1				

a. Enoyl-CoA hydratase activities were determined with crotonyl-CoA (C_4), $\Delta^{2,3}$-decenoyl-CoA (C_{10}), and $\Delta^{2,3}$-hexadecenoyl-CoA (C_{16}).

virtually devoid of long chain enoyl-CoA hydratase. When the same technique was applied to a liver homogenate, similar results were obtained which suggest that both crotonase and a long chain enoyl-CoA hydratase are also present in pig liver. In order to determine that the sequential extraction technique is a valid method in assessing the presence of the two enoyl-CoA hydratases, an extract enriched with respect to long chain enoyl-CoA hydratase was chromatographed on a hydroxylapatite column. Two enoyl-CoA hydratase activity peaks were identified (see Fig.3). Based on the activity ratios, the second peak, which was eluted at higher potassium phosphate concentration, was due to crotonase. Assuming that the two enoyl-CoA hydratases of pig liver and pig heart have identical properties, the first hydratase peak must represent a combination of crotonase and long chain enoyl-CoA hydratase. Since it was recently reported that liver peroxisomes also contain crotonase activity (21a), the question concerning the number and location of enoyl-CoA hydratases in liver remains unresolved.

In order to demonstrate that the presence of two enoyl-CoA hydratases was not limited to pig organs, heart and liver mitochondria from guinea pig were sequentially extracted as described above. As shown in Table IV, the activity ratios clearly suggest that both types of enoyl-CoA hydratases are also present in guinea pig. Moreover, the fact that guinea

Fig. 3 Separation of Long Chain Enoyl-CoA Hydratase and Crotonase on Hydroxylapatite

The experiment was performed as described under "Experimental Procedures". Enoyl-CoA hydratase activities were assayed by the direct method as described under "Experimental Procedures" with crotonyl-CoA (C4), $\Delta^{2,3}$-decenoyl-CoA (C10) and $\Delta^{2,3}$-hexadecenoyl-CoA (C16).

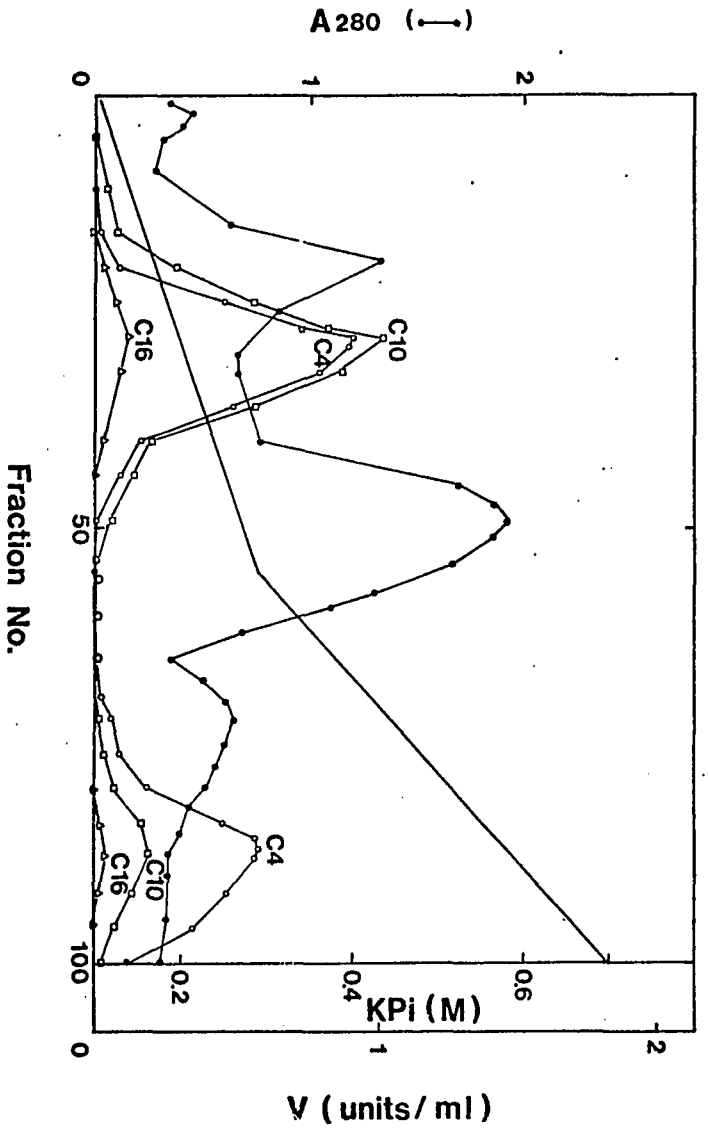

A280 (⟷)
0
1
2
Fraction No.
50
100
C16
C4
C10
C4
C16
C10
0.2
0.4
0.6
KPi (M)
0
1
2
V (units/ml)

Fig. 3

pig liver mitochondria were used in this experiment demonstrates that both enoyl-CoA hydratases are located in mitochondria as has been shown for pig heart (7).

Enoyl-CoA Hydratase Activities in Various Mammals and Organs - Enoyl-CoA hydratase activities were determined with crotonyl-CoA, $\Delta^{2,3}$-decenoyl-CoA and $\Delta^{2,3}$-hexadecenoyl-CoA in several organs of pig, beef and guinea pig as summarized in Table V . In all these animals, the crotonase activity with crotonyl-CoA as substrate was 5- 10 times higher in liver than in heart. In guinea pig crotonase activity was highest in liver, followed by kidney, heart and skeletal muscle. Assuming that the C_{16}: C_4 ratio for crotonase is somewhere between 0.7% as found for the bovine liver enzyme and 2.4% as determined for the pig heart enzyme, the ratio of activities with short, medium and long chain substrates suggest that most, if not all, tissues contain two enoyl-CoA hydratases with different chain length specificities. This conclusion also applies to bovine heart and bovine liver for which the presence of a separate long chain enoyl-CoA hydratase has not yet been established.

Table V. Tissue Distribution of Enoyl-CoA Hydratases

Fresh tissues were homogenized in 0.1 M potassium phosphate (pH 7) and 5 mM mercaptoethanol (5 ml/g of tissue) with a PT 10 ST Polytron homogenizer at high speed (position 8) for 30 s. Extracts were assayed for enoyl-CoA hydratase activities as described under "Experimental Procedures".

Tissue	Enoyl-CoA hydratase activities								
	Pig			Beef			Guinea pig		
	C_4	C_{10}	C_{16}	C_4	C_{10}	C_{16}	C_4	C_{10}	C_{16}
	μmol/min/g wet tissue								
Heart	63	72	23	145	33	7	35	22	5
Liver	481	215	34	717	235	35	378	218	9
Kidney							224	166	11
Skeletal muscle							22	12	1

a. C_4, crotonyl-Coa; C_{10}, $\Delta^{2,3}$-decenoyl-CoA; C_{16}, $\Delta^{2,3}$-hexadecenoyl-CoA.

Discussion

The purification of pig heart crotonase to homogeneity or near homogeneity provides the opportunity to compare its properties with those of bovine liver crotonase, the only other mammalian short chain enoyl-CoA hydratase which has been extensively studied. The physical properties of the two crotonases, specifically their native and subunit molecular weights, are virtually identical. Additionally, both enzymes exhibit similar sensitivities towards sulfhydryl inhibitors and towards the competitive inhibitor acetoacetyl-CoA. However, the two enzymes differ significantly with regard to their stabilities and their kinetic properties. The bovine liver enzyme appears to be stable under a variety of conditions even in dilute form, while the pig heart enzyme requires high salt concentration and/or glycerol for the maintenance of its activity. A surprising difference appears when the kinetic properties (K_m, V_{max}) of the two crotonases are compared. Whereas the K_m values for crotonyl-CoA are similar for both enzymes (20 μM for bovine liver crotonase, 13 μM for pig heart crotonase), the K_m values for all longer chain substrates are approximately 30 μM with the pig heart enzyme in contrast to values between 250 μM and 500 μM observed with the bovine liver enzyme. Additionally, the V_{max}

values of bovine liver enzyme show a steeper decrease with increasing chain length of the substrates compared to the pig heart enzyme. As a result of this, the pig heart enzyme is significantly more active with $\Delta^{2,3}$-hexadecenoyl-CoA than is bovine liver crotonase (40 units/mg versus 14 units/mg) while the liver enzyme shows a slightly higher activity with crotonyl-CoA when compared to pig heart crotonase (2070 units/mg versus 1670 units/mg). Thus, when both the V_{max} and K_m values are considered, the bovine liver enzyme compared to the pig heart enzyme has a dramatically lower capacity to catalyze the hydration of long chain enoyl-CoA intermediates in fatty acid oxidation especially if the in vivo concentrations of the intermediates are only in the low micromolar range.

The above discussion of the chain length specificities of the two crotonases raises the question whether another enoyl-CoA hydratase, highly active towards long chain substrates, is necessary to complement crotonase in order to assure the efficient catalysis of all enoyl-CoA intermediates of fatty acid oxidation. This question has been definitely answered with regard to pig heart muscle where the presence of a long chain enoyl-CoA hydratase has been demonstrated (7).

In this report it is shown that long chain enoyl-CoA hydratase is possibly present in pig liver and most likely in guinea pig heart and liver as well. It is concluded that the occurrence of two enoyl-CoA hydratases, one of which is most active on short chain substrates whereas the other is highly active on medium and long chain substrates, is common for various tissues of several mammals. Attempts to identify a long chain enoyl-CoA hydratase in either bovine heart or liver did not produce convincing results. If the two enoyl-CoA hydratases are present in these tissues, it appears that their properties are very similar thus making a separation by the two methods used in this study difficult. However, the fact that the ratio of enoyl-CoA hydratase activities measured with crotonyl-CoA versus $\Delta^{2,3}$-hexadecenoyl-CoA is 20:1 when determined with a crude bovine liver homogenate but is 148:1 when measured with purified crotonase, suggests that another enzyme, more active on long chain substrates, must be present in bovine liver. Hence it is probable that beef liver and heart also contain two enoyl-CoA hydratases with different but complementary chain length specificities.

Since it was shown that the long chain enoyl-CoA hydratase of pig heart (7) and possibly that of guinea pig liver (Table IV) are present in mitochondria, it is assumed that the long chain enoyl-CoA hydratase and crotonase

cooperate in the hydration of intermediates of fatty acid oxidation. Based on the total activities measured with several substrates (see Table V) and based on the kinetic data reported for crotonase (see Table III) and long chain enoyl-CoA hydratase (7), it has been calculated in which manner the two pig heart enzymes complement each other in catalyzing the hydration of substrates of various chain lengths(Fig.4). Obviously, the presence of the two enzyme in pig heart mitochondria assures a high rate of hydration of minimally 23 units/g of wet tissue over the whole range of substrates. This high rate of hydration makes it highly improbable that the hydration step could ever become rate-limiting in fatty acid oxidation. Since additionally the long chain enoyl-CoA hydratase of pig heart is not inhibited by acetoacetyl-CoA, it is suggested that the proposed regulation of fatty acid oxidation via an inhibition of crotonase by acetoacetyl-CoA as suggested by Waterson and Hill (5) is not effective in pig heart and most likely not in other mammalian tissues. In Fig. 5, the complementary activities of crotonase and long chain enoyl-CoA hydratase in pig liver are shown. In calculating these data the assumption was made that the kinetic properties of crotonase and long chain enoyl-CoA hydratase from pig liver and heart are identical. The contribution of the long chain enzyme to the total activity

Fig. 4 Contribution of Crotonase and Long Chain Enoyl-CoA Hydratase to the Total Hydratase Activity in Pig Heart

The data of this graph were calculated by using the values for the total enoyl-CoA hydratase activities presented in Table V and by using the kinetic parameters of crotonase (Table III) and of long chain enoyl-CoA hydratase (7).

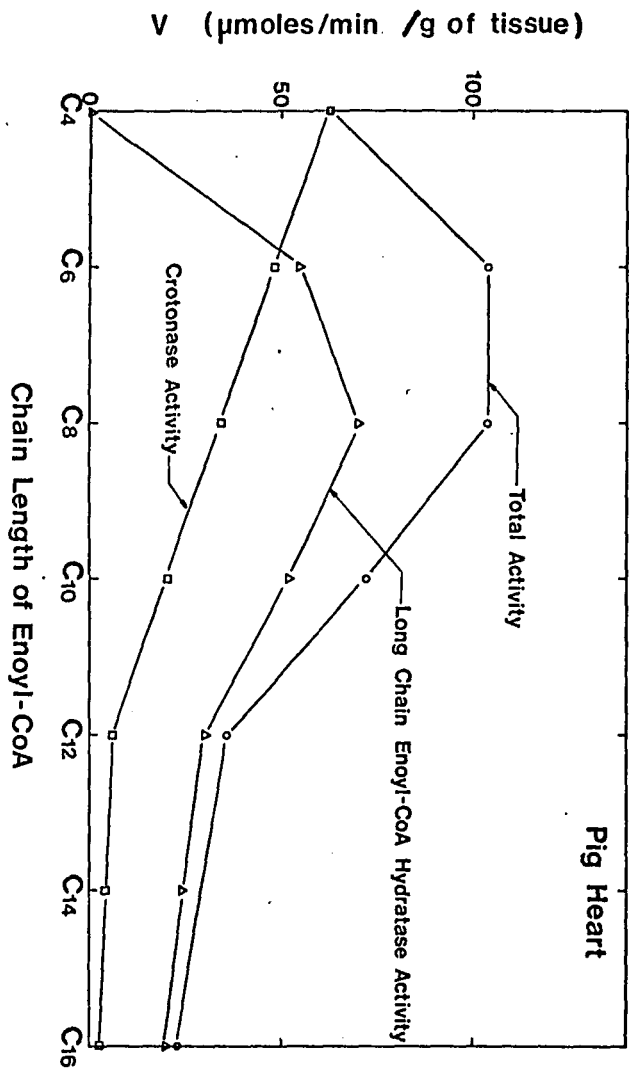

V (μmoles/min /g of tissue)
0
50
100
C4
C6
C8
C10
C12
C14
C16
Chain Length of Enoyl-CoA
Crotonase Activity
Total Activity
Long Chain Enoyl-CoA Hydratase Activity
Pig Heart

Fig. 4

Fig. 5 Contribution of Crotonase and Long Chain Enoyl-CoA Hydratase to the Total Hydratase Activity in Pig Liver

The data were obtained as explained in the legend to Fig. 4.

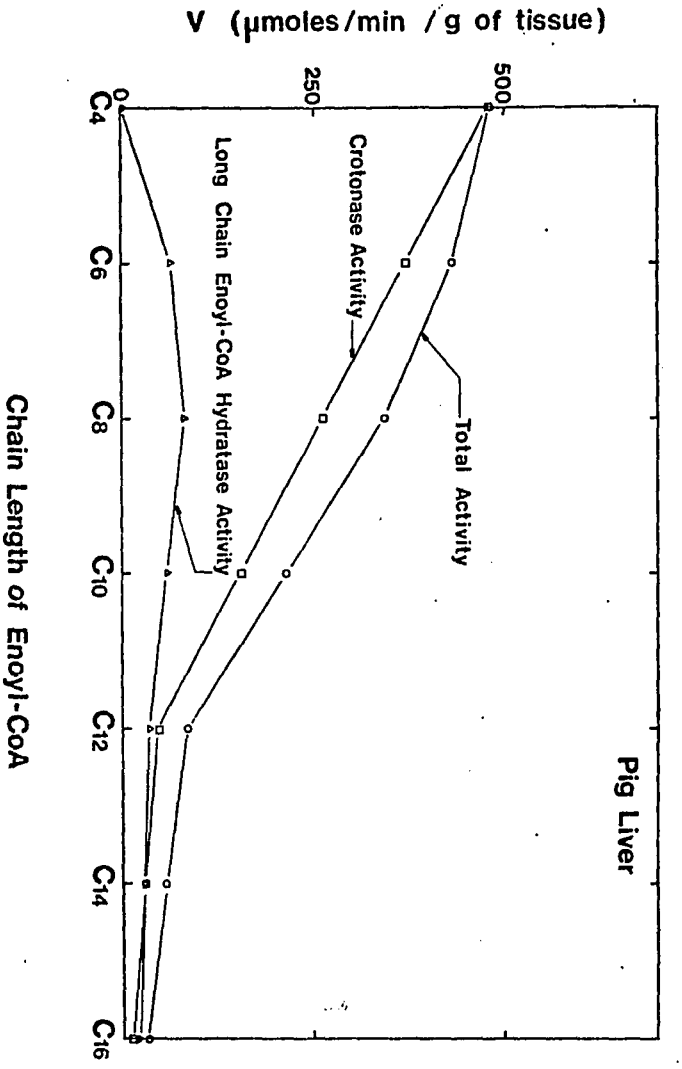

V (µmoles/min / g of tissue)
0
250
500
C4
C6
C8
C10
C12
C14
C16
Chain Length of Enoyl-CoA
Long Chain Enoyl-CoA Hydratase Activity
Crotonase Activity
Total Activity
Pig Liver

Fig. 5

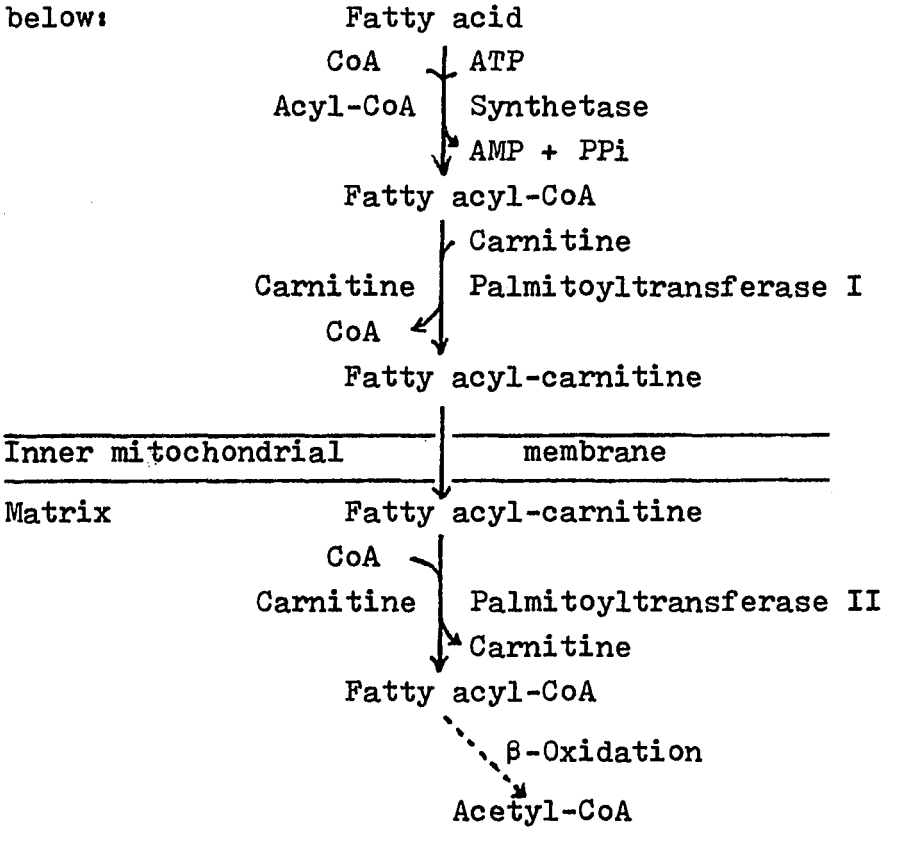
is less striking than in heart but is certainly important for the hydration of long chain substrates. It should be pointed out that the total activities of long chain enoyl-CoA hydratase in liver and heart are approximately the same. Most outstanding is the unusually high activity in liver towards short chain substrates, especially towards crotonyl-CoA. The physiological significance of the exceptionally high crotonase activity in liver which is due to a high concentration of crotonase in this tissue is not known. One possible explanation is derived from the known central function of liver in amino acid metabolism which requires the presence of crotonase for the hydration of metabolites of valine and isoleucine (22). This suggestion is supported by the observation that both liver and kidney which actively metabolize amino acids show high crotonase activities whereas in heart and skeletal muscle the crotonase concentration is approximately 10 times lower. However, it is also possible that the high concentration of crotonase in liver reflects its dual location both in mitochondria and peroxisomes (21a).

PART TWO

The Rate-Determining Step of Fatty Acid Oxidation

Introduction

Fatty acids which are a major fuel in heart muscle are mainly degraded in mitochondria by β-oxidation as outlined in PART ONE. The enzymes of β-oxidation are present in the matrix space of mitochondria which is enclosed by the inner mitochondrial membrane. Since this membrane is impermeable to free long chain fatty acids as well as to their CoA derivatives, long chain fatty acid can enter the matrix only by the process illustrated below:



The fatty acyl-carnitine, formed from free fatty acid by two consecutive enzyme-catalyzed reactions, can cross the inner mitochondrial membrane and is then converted back to fatty acyl-CoA which is oxidized by the enzymes of the β-oxidation process.

Although the sequence of reactions by which long chain fatty acids are degraded has been elucidated many years ago and the enzymes involved in this process have been studied (6), the regulation of this important metabolic pathway remains poorly understood. This lack of understanding is partly due to conflicting observations and suggestions regarding the rate-limiting step of the overall process. Reactions thought to be the rate-determining include those catalyzed by acyl-CoA synthetase, acyl-CoA dehydrogenase and carnitine palmitoyltransferase (23). However, more recently it has been concluded that carnitine palmitoyltransferase and acyl-CoA synthetase are unlikely to limit fatty acid oxidation (24).

In order to elucidate this unclear situation and to establish the modes of regulation of fatty acid oxidation in heart, the rate-limiting step of fatty acid oxidation in heart was reinvestigated. The determination of the rate-limiting step was attempted by measuring and comparing in a heart mitochondria homogenate the total activities of all enzymes of fatty acid oxidation with substrates of different chain lengths. However, more

promising seems to be an approach in which the effect of the specific inhibition of one of the enzymes on the rate of the complete pathway is evaluated. If the enzyme under investigation catalyzes the rate-limiting step of the reaction sequence, the inhibition of the pathway should parallel the inhibition of the enzyme. A possible specific inhibitor of one of the enzymes of fatty acid oxidation is the hypoglycemic compound 4-pentenoate which is thought to cause hypoglycemia by impairing gluconeogenesis secondarily to inhibiting fatty acid oxidation (25). Though this view has been generally accepted, the mechanism of the inhibition of fatty acid oxidation by 4-pentenoate remains unresolved. On the one hand, Bressler and coworkers suggested that metabolites of 4-pentenoate sequester cellular CoA and carnitine as inert-acyl-derivatives and thus impair fatty acid oxidation (26); on the other hand, Holland and Sherratt (27) contended that the primary effect of 4-pentenoate is the specific inhibition of one of the enzymes of fatty acid oxidation by a unique metabolite of this compound. The finding of Holland et al. (28) that 2,4-pentadienoyl-CoA, a unique metabolite of 4-pentenoate, specifically inhibited thiolase prompted me to use 4-pentenoate to investigate the rate-limiting step of fatty acid oxidation.

As a result of this study, it is concluded that several reactions of the pathway of fatty acid oxidation, including those catalyzed by carnitine palmitoyltransferase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, may proceed at comparable rates and that the inhibition of any of these reactions would possibly lead to an inhibition of β-oxidation. This study also establishes that the inhibition of fatty acid oxidation by 4-pentenoate is due to the specific inhibition of 3-ketoacyl-CoA thiolase.

Experimental Procedures

Materials - Acetyl-CoA, butyryl-CoA, octanoyl-CoA, decanoyl-CoA, palmitoyl-CoA, NAD^+ and NADH were purchased from P-L Biochemicals,Inc. CoASH, N-methylmaleimide, octanoic acid, ADP, D,L-3-hydroxybutyric acid and l-malate were obtained from Sigma Chemical Co. Pyruvic acid, $\Delta^{2,3}$-decenoic acid and diketene were bought from Aldrich Chemical Co. and 4-pentenoic acid from Fluka,A.-G.,Switzerland. n-Pentanoic acid was obtained from City Chemical Co. and was distilled before use. 3-Hydroxyacyl-CoA dehydrogenase was purchased from Boehringer Mannheim Corp. Pig heart 3-ketoacyl-CoA thiolase was a gift from H.Staack. (-)Carnitine, acetyl-(-)carnitine, octanoyl-(-)carnitine and palmitoyl-(-)carnitine were generously provided by Dr. K. Brendel. Crotonyl-CoA (10) and acetoacetyl-CoA (11) were prepared according to standard procedures. D,L-3-Hydroxydecanoic acid was synthesized by reduction with $NaBH_4$ of ethyl 3-ketodecanoate prepared according to an established procedure (29) followed by hydrolysis. The CoA derivatives of D,L-3-hydroxybutyric acid, D,L-3-hydroxydecanoic acid and $\Delta^{2,3}$-decenoic acid were synthesized from the corresponding free acid and CoA by the method of Goldman and Vagelos (11). 3-Ketodecanoyl-CoA was prepared enzymatically from $\Delta^{2,3}$-decenoyl-CoA by following the procedure of Seubert et al.(30). The concentrations of all acyl-CoA

compounds except for that of 3-ketodecanoyl-CoA were determined by the method of Ellman (13) after cleaving the thioester bond with hydroxylamine at pH 7. The concentration of 3-ketodecanoyl-CoA was measured by following the oxidation of NADH in the presence of 3-hydroxyacyl-CoA dehydrogenase.

Enzyme and Protein Assays - All assays were performed at 25°. The carnitine acyltransferases were assayed spectrophotometrically as described by Bieber et al.(31). The assay mixtures contained 0.11 M Tris-HCl (pH 8), 0.09% Triton X-100, 1 mM EDTA, 0.11 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 7 μM bovine serum albumin and 30 μM palmitoyl-CoA or 0.13 mM acetyl-CoA for the assay of carnitine palmitoyltransferase and carnitine acetyltransferase respectively. The reaction was started by the addition of (-)carnitine to a final concentration of 2 mM.

The acyl-CoA dehydrogenases were assayed by recording the reduction of 2,6-dichlorophenolindophenol at 600 nm with phenazine methosulfate as the primary electron acceptor (32). The assay mixtures contained 0.1 M potassium phosphate (pH 7), 0.09% Triton X-100, 0.17 mM 2,6-dichlorophenolindophenol, 0.2 mM N-methylmaleimide and 30 μM butyryl-CoA or 50 μM decanoyl-CoA or 50 μM hexadecanoyl-CoA. The reaction was started by the addition of phenazine methosulfate to a final concentration of 0.77 mM. A molar extinction coefficient of

20,600 $cm^{-1}M^{-1}$ was used to calculate rates.

The enoyl-CoA hydratases were measured spectrophotometrically at 263 nm as described (2) except that the assay mixture contained 0.2 M potassium phosphate (pH 8), 1.5 μM bovine serum albumin, 0.06% Triton X-100 and 30 μM crotonyl-CoA or 30 μM $\Delta^{2,3}$-decenoyl-CoA.

3-Hydroxyacyl-CoA dehydrogenase was assayed spectrophotometrically at 340 nm by either recording the reduction of NAD^+ or the oxidation of NADH. In the backward reaction (oxidation of NADH), the standard assay mixture contained 0.05 M potassium phosphate (pH 7), 0.06% Triton X-100, 0.12 mM NADH and 2.7 μM bovine serum albumin. The reaction was started by the addition of acetoacetyl-CoA to a final concentration of 30 μM. In the forward reaction (reduction of NAD^+), the assay mixture contained 0.2 M potassium phosphate (pH 8), 0.06% Triton X-100, 0.23 mM NAD^+, 1.3 μM bovine serum albumin, 0.13 mM CoASH and 3-ketoacyl-CoA thiolase. The reaction was started by the addition of either 3-hydroxybutyryl-CoA or 3-hydroxydecanoyl-CoA to a final concentration of 30 μM and 10 μM respectively.

The activity of thiolase was routinely determined by following spectrophotometrically the disappearance of the Mg^{2+}-enolate complex at 303 nm. The reaction mixture contained 0.1 M Tris-HCl (pH 8), 25 mM $MgCl_2$,

30 mM KCl, 0.06% Triton X-100, 7 μM bovine serum albumin and 30 μM acetoacetyl-CoA or 10 μM 3-ketodecanoyl-CoA. The reaction was started by the addition of CoASH to a final concentration of 0.13 mM. Molar extinction coefficient of 18,000 $cm^{-1}M^{-1}$ and 13,800 $cm^{-1}M^{-1}$ were used to calculate the rates determined with acetoacetyl-CoA and 3-ketodecanoyl-CoA respectively. Protein concentrations were determined by the biuret method (33).

Activity Measurements of Acetoacetyl-CoA Thiolase and of 3-Ketoacyl-CoA Thiolase in Rat Heart Mitochondria - Following chromatography on hydroxylapatite with a potassium phosphate gradient (pH 6.3) from 0.05 to 0.7 M, acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase were separated by chromatography on phosphocellulose (34). Acetoacetyl-CoA thiolase thus obtained was only active with acetoacetyl-CoA and was stimulated by K^+ 3.2-fold whereas 3-ketoacyl-CoA thiolase was found to be 2.5 times more active with 3-ketodecanoyl-CoA than with acetoacetyl-CoA and was not affected by K^+. Based on these findings, it was possible to calculate that three fourths of the acetoacetyl-CoA thiolase activity of 0.8 μmol per min and mg of protein observed in a homogenate of rat heart mitochondria was due to acetoacetyl-CoA thiolase whereas only one fourth was due to 3-ketoacyl-CoA thiolase.

Isolation of Mitochondria - Mitochondria were prepared from fed male albino rats (250- 300 g) according to the procedure of Chappell and Hansford (35).

Measurements of Oxygen Uptake by Mitochondria - Oxygen consumption was monitored at 25° by means of a Clark oxygen electrode. A standard basal incubation mixture contained 0.11 M KCl, 33 mM Tris (pH 7.4), 2 mM potassium phosphate (pH 7.4), 2 mM $MgCl_2$, 0.1 mM ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid, 28 μM bovine serum albumin, 1 mM ADP, 0.5 mM l-malate, and 2.5 mM (-)carnitine. Mitochondria (1 mg of protein) were routinely preincubated for 1 min in the mixture before the reaction was started by the addition of either a fatty acid, pyruvate, fatty acyl-CoA or fatty acyl carnitine as indicated in the tables or legends to figures.

Measurements of Enzyme Activities in Mitochondria Preincubated in the Presence of n-Pentanoate or 4-Pentenoate - Mitochondria (1- 3 mg of protein) were added to a standard basal incubation mixture as described above. n-Pentanoate (0.1 mM) or 4-pentenoate (0.1 mM) was added at time 0. Aliquots of 30 to 50 μl were removed from the mixture after 20 s, 50 s, 110 s and 170 s and were frozen within 7 s in a methanol- dry ice mixture. After the mitochondrial suspension was thawed, 2 to 20 μl were used to assay various enzymes of fatty acid oxidation. The presence of 0.06% Triton X-100 in the assay mixture assured disruption of the mitochondria.

Results

Activities of the Enzymes of Fatty Acid Oxidation in Rat Heart Mitochondria - The enzymes of β-oxidation were assayed at pH 8 in the forward direction with short chain and medium chain substrates except for carnitine palmitoyltransferase for which only the activity with palmitoyl-CoA was determined. The acyl-CoA dehydrogenases were not included in this evaluation because their *in vitro* activities, which are measured with artificial electron acceptors, are not representative of their *in vivo* catalytic efficiencies. Since only 3-ketoacyl-CoA thiolase of the two thiolases present in heart mitochondria is assumed to participate in β-oxidation (36), the thiolytic activity of acetoacetyl-CoA thiolase is listed separately and is not considered in this assessment of the rate-limiting step of fatty acid oxidation. When comparing the enzyme activities listed in Table VI., it is obvious that the combined activities of the two enoyl-CoA hydratases (7, 8) are at least one order of magnitude higher than those of 3-ketoacyl-CoA thiolase or 3-hydroxyacyl-CoA dehydrogenase. And even though the activities of the two enoyl-CoA hydratases decrease with increasing chain length of the substrates (7, 8), the rate of hydration of $\Delta^{2,3}$-hexadecenoyl-CoA would still be higher than the

Table VI Activities of Several Enzymes of Fatty Acid Oxidation. Assays were performed as described under "Experimental Procedures".

Enzymes	Specific Activity		
	C_4[a]	C_{10}[a]	C_{16}[a]
	μmol/min/mg protein		
Carnitine palmitoyltransferase			0.024
Enoyl-CoA hydratase	14.8	5.8	
3-Hydroxyacyl-CoA dehydrogenase[b]	0.21	0.23	
3-Ketoacyl-CoA thiolase [c]	0.21	0.46	
Acetoacetyl-CoA thiolase[c]	0.60	0.00	

a. C_4, C_{10} and C_{16} refer to the chain lengths of the acyl-CoA's required for assaying the various enzymes

b. 3-Hydroxyacyl-CoA dehydrogenase was assayed in the forward direction with 3-hydroxybutyryl-CoA and 3-hydroxydecanoyl-CoA as substrates

c. 3-Ketoacyl-CoA thiolase and acetoactyl-CoA thiolase activities were calculated as described under "Experimental Procedures".

rates of the corresponding reactions catalyzed by 3-ketoacyl-CoA thiolase (36) and by 3-hydroxyacyl-CoA dehydrogenase (37). When comparing the activity of carnitine palmitoyltransferase with those of the other enzymes of β-oxidation, the activity of the former enzyme must be multiplied by seven because the complete degradation of a palmitoyl residue requires its passing seven-times through the β-oxidation cycle. Hence it seems that the activities of carnitine palmitoyltransferase, 3-ketoacyl-CoA thiolase and 3-hydroxyacyl-CoA dehydrogenase are nearly equal and are lower than the activities of the other listed enzymes. If one assumes that the activities listed in Table VI closely reflect the in vivo activities of these enzymes, then inhibition of either carnitine palmitoyltransferase, 3-hydroxyacyl-CoA dehydrogenase or 3-ketoacyl-CoA thiolase should result in an inhibition of fatty acid oxidation in rat heart mitochondria.

Years ago it was observed that intermediates formed during the oxidation of fatty acids do not accumulate in mitochondria (38). This finding has been taken as suggestive evidence for the existence of the enzymes of β-oxidation in an organized form, possibly as a multienzyme complex. However, the non-accumulation of intermediates may simply be due to the efficient operation

at equal rates of the enzymes of β-oxidation at low substrate levels. Low K_m values of 1 to 30 μM determined for the various substrates of acyl-CoA dehydrogenases (39), enoyl-CoA hydratases (7,8) and 3-ketoacyl-CoA thiolase (36) attest to the efficient operation of these enzymes at low substrate concentrations whereas K_m values of 168 to 340 μM obtained for 3-hydroxyacyl-CoA dehydrogenase (37,40) appear to be out of line. Therefore the K_m values were re-determined for 3-hydroxybutyryl-CoA and 3-hydroxydecanoyl-CoA in the reactions catalyzed by 3-hydroxyacyl-CoA dehydrogenase. The measurements were performed at pH 8 instead of the usual pH 9-10 and in the presence of 3-ketoacyl-CoA thiolase and CoASH to assure the removal of the formed 3-ketoacyl-CoA compounds which are very effective product inhibitors (40). The K_m value obtained under these conditions for 3-hydroxybutyryl-CoA was 14 μM as compared to values of 310 μM (37) and 168 μM (40) reported previously. The K_m value for 3-hydroxydecanoyl-CoA was found to be 3 μM and the value for 3-ketodecanoyl-CoA in the backward reaction was 18 μM which is similar to a value of 12 μM previously determined for acetoacetyl-CoA (40).

The Effects of 4-Pentenoate and n-Pentanoate on the Oxidation of Palmitoyl-carnitine and on the Enzymes of Fatty Acid Oxidation in Rat Heart Mitochondria - The effect of the hypoglycemic compound 4-pentenoate, a known inhibitor of fatty acid oxidation (25), on the enzymes of fatty acid oxidation was studied with coupled rat heart mitochondria under conditions at which the compound is metabolized. As shown in Table VII, the two thiolases were strongly inhibited whereas none of the other enzymes was significantly affected. Since the inhibition was observed despite the extensive dilution of the mitochondrial suspension by the assay mixtures, it is concluded that the inhibition was irreversible. In order to evaluate the relationship between the inhibitions of the thiolases and the inhibition of palmitoyl-carnitine-supported respiration, rat heart mitochondria were preincubated for various periods of time with either 4-pentenoate or n-pentanoate and were assayed with respect to their thiolase activities and for their capacity to oxidize palmitoylcarnitine. As shown in Fig.6A , the rate at which palmitoylcarnitine was oxidized decreased within 3 min to 20% of its original value whereas n-pentanoate caused only a slight inhibition. Similar results were obtained in the absence of carnitine. The same samples were used to determine the

Table VII Effect of 4-Pentenoate on Enzymes of Fatty Acid Oxidation. After mitochondria were preincubated for 3 min in the presence of 4-pentenoate, aliquots of the mitochondrial suspension were taken for the enzyme assays as described under "Experimental Procedures".

Enzyme	Substrate	Control[a]	4-Pentenoate	Remaining activity
		µmol/min/mg protein		%
Carnitine palmitoyltransferase	palmitoyl-CoA	0.023	0.021	92
Acyl-CoA dehydrogenase	butyryl-CoA	0.013	0.013	100
	decanoyl-CoA	0.005	0.005	100
	palmitoyl-CoA	0.007	0.007	100
3-Hydroxyacyl-CoA dehydrogenase	acetoacetyl-CoA	1.44	1.30	90
Enoyl-CoA hydratase	$\Delta^{2,3}$-decenoyl-CoA	2.06	1.90	92
Thiolase	acetoacetyl-CoA	0.50	0.16	32
	3-ketodecanoyl-CoA	0.24	0.08	33

a. Activities measured in the absence of 4-pentenoate.

Fig.6 A <u>Inhibition of the Oxidation of Palmitoyl-(-)carnitine by 4-Pentenoate in Coupled Rat Heart Mitochondria</u>.

In a series of experiments, mitochondria (1 mg protein) were preincubated with 0.1 mM 4-pentenoate (Δ^4-C_5, o—o) or 0.1 mM n-pentanoate (n-C_5, • —— •) in 2 ml of basal medium described under "Experimental Procedures" at 25° for various time as indicated on the abscissa. Palmitoyl-(-)carnitine (30 μM) was then added to start the reaction. The rate of oxygen uptake was monitored with a Clark oxygen electrode. Results are expressed as percentage of the rates determined in the presence of 4-pentenoate or n-pentanoate versus that measured in the absence of these compounds.

Fig.6 B <u>Inhibition of Thiolase by 4-Pentenoate in Coupled Rat Heart Mitochondria</u>

Mitochondria were preincubated with 0.1 mM 4-pentenoate (Δ^4-C_5) or o.1 mM n-pentanoate (n-C_5) as described in the legend to Fig.2.1A. After various preincubation times aliquots were assayed for thiolase with acetoacetyl-CoA (▲— ▲, •— •) or 3-ketodecanoyl-CoA (o — o) as substrates. For details see "Experimental Procedures".

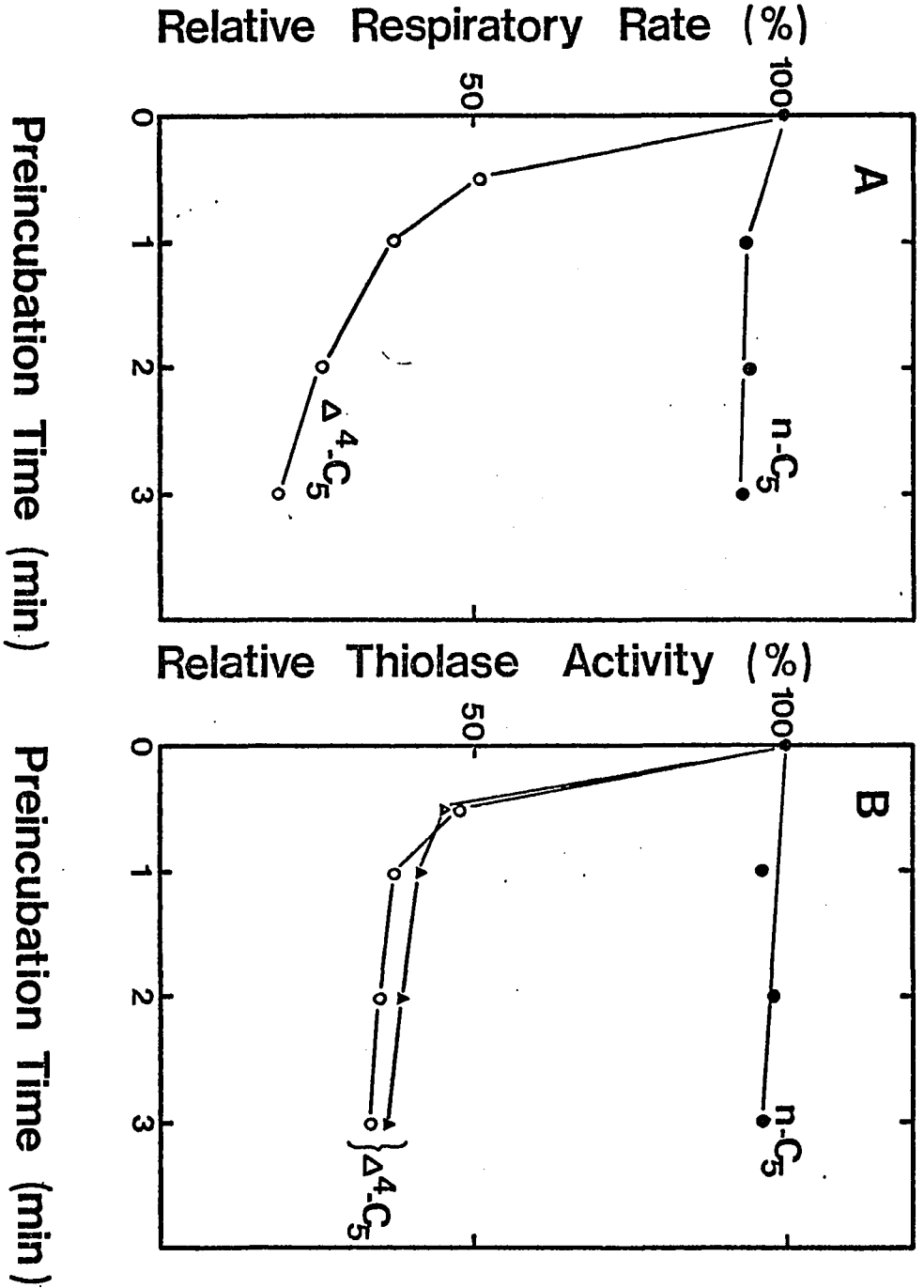

Relative Respiratory Rate (%)
100
50
0
1
2
3
A
Δ4-C5
n-C5
Preincubation Time (min)
Relative Thiolase Activity (%)
100
50
0
1
2
3
B
Δ4-C5
n-C5
Preincubation Time (min)

Fig. 6

remaining thiolase activities and the data shown in Fig. 6B demonstrate that the inhibition of the thiolases initially paralleled the inhibition of fatty acid oxidation but became less severe than the inhibition of oxidation after several minutes of preincubation with 4-pentenoate. Again, n-pentanoate did not affect the thiolase activities. Since the acetoacetyl-CoA and 3-ketodecanoyl-CoA thiolytic activities decreased in a virtually identical fashion, it is concluded that the two thiolases present in rat heart mitochondria, 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase, were inactivated to the same extent.

In contrast, no significant inhibition of the oxidation of palmitoylcarnitine by 4-pentenoate was observed in the presence of malonate (see Fig. 7). Since malonate inhibites the citric acid cycle, it may also cause a decrease in the formation of the inhibitory metabolite of 4-pentenoate with the result that the thiolases may not be at all or less severely inhibited. To test this possibility, thiolase activities were determined in rat heart mitochondria preincubated in the presence of 4-pentenoate plus

Fig. 7 The Effect of 4-Pentenoate and n-Pentanoate on the Oxidation of Palmitoyl-(-)carnitine in the Presence of Malonate in Coupled Rat Heart Mitochondria

Mitochondria were preincubated with o.1 mM 4-pentenoate (Δ-C_5) or 0.1 mM n-pentanoate (n-C_5) in 2 ml of basal medium(described under "Experimental Procedures")which also contained 16 mM malonate at 25^{o} for various time as indicated on the abscissa. Palmitoyl-(-)carnitine (30 μM) was then added to start the reaction.

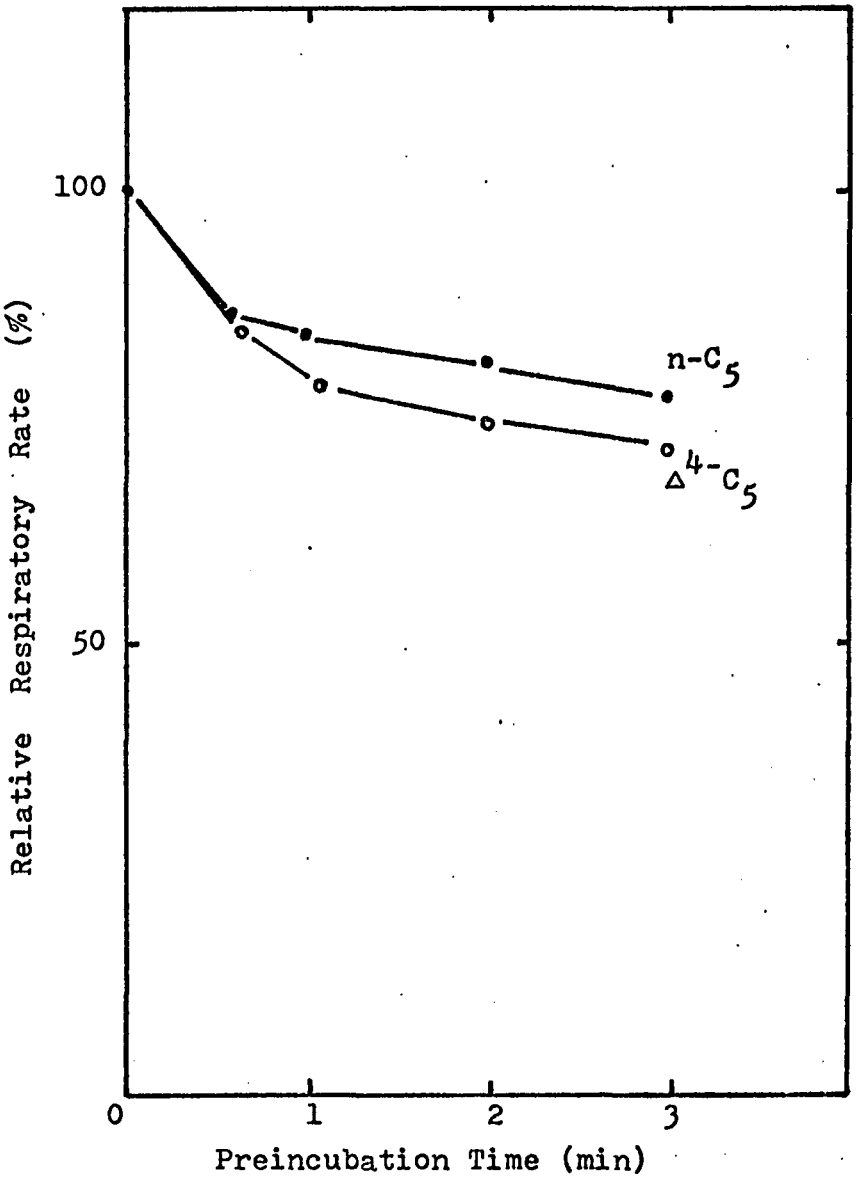

100
50
0
1
2
3
Relative Respiratory Rate (%)
Preincubation Time (min)
n-C5
Δ4-C5

Fig. 7

malonate. The results showed that both acetoacetyl-CoA and 3-ketodecanoyl-CoA thiolytic activities decreased to about 45% of its original level after 3 min of preincubation. Thus the reason for the reduced inhibition of palmitoylcarnitine oxidation by 4-pentenoate in the presence of malonate remains unexplained. A possible interpretation is that in heart mitochondria the oxidation of palmitoylcarnitine in the presence of malonate is no longer limited by the rate of β-oxidation but instead by the rate of the citric acid cycle.

Effect of 4-Pentenoate and n-Pentanoate on the Mitochondrial Oxidation of other Substrates - The oxidations of both palmitoyl-CoA and octanoate in rat heart mitochondria were strongly inhibited by 4-pentenoate but were only slightly affected by n-pentanoate (see Fig. 8A). The inhibition pattern for palmitoyl-CoA-supported respiration is similar to that obtained with palmitoylcarnitine as substrate (see Fig. 6A and 8A). Because of this finding and because the rates of oxidation with palmitoyl-CoA and palmitoylcarnitine are similar (see Table VIII), it is concluded that the reaction catalyzed by carnitine palmitoyltransferase was not rate-limiting

Fig. 8A <u>Inhibition of the Oxidation of Palmitoyl-CoA and Octanoate by 4-Pentenoate in Coupled Rat Heart Mitochondria</u>

Mitochondria were preincubated with 0.1 mM 4-pentenoate (Δ^4-C_5) or o.1 mM n-pentanoate (n-C_5) as described under "Experimental Procedures". After variou preincubation times palmitoyl-CoA (——) or octanoate (----) were added to give final concentration of 15 μM and 0.15 mM respectively and the rates of oxygen uptake were determined.

Fig. 8B <u>The Effect of 4-Pentenoate and n-Pentanoate on the Oxidation of Pyruvate in Coupled Rat Heart Mitochondria</u>

Mitochondria were preincubated with 0.1 mM 4-pentenoate (Δ^4-C_5) or o.1 mM n-pentanoate (n-C_5) as described under "Experimental Procedures". After various preincubation times, 7 mM pyruvate was added to initiate the reaction.

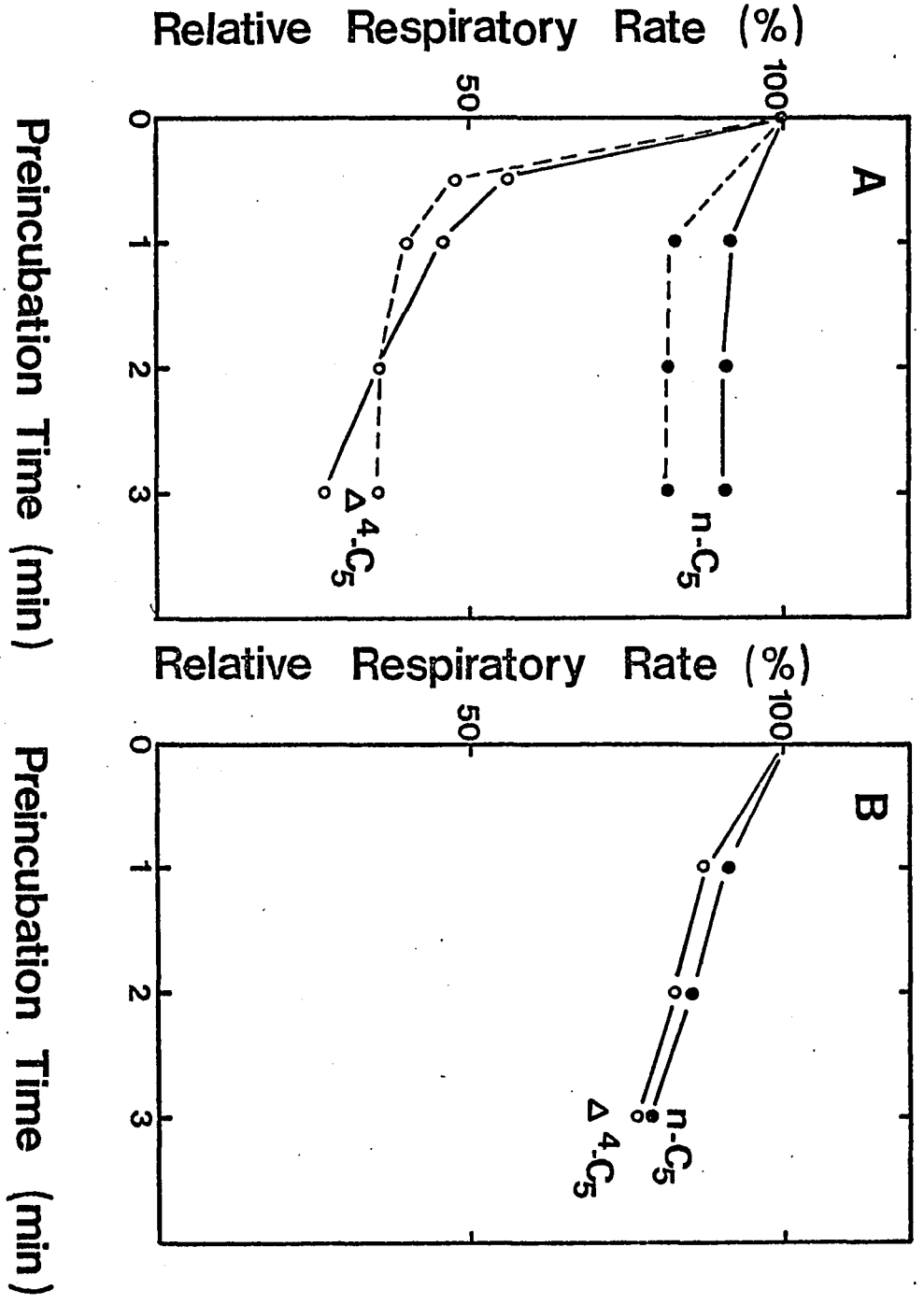

Relative Respiratory Rate (%)
50
100
0
1
2
3
A
Δ 4-C5
n-C5
Preincubation Time (min)
Relative Respiratory Rate (%)
50
100
0
1
2
3
B
n-C5
Δ 4-C5
Preincubation Time (min)

Fig. 8

Table VIII Oxygen Uptake by Rat Heart Mitochondria in the Presence of Various Substrates. Oxygen uptake measurements are described under "Experimental Procedures".

Substrates		Oxygen uptake[a]
		ng atom O_2/min/mg protein
Palmitoyl-(-)carnitine	(30μM)	214 ± 25 (12)
Palmitoyl-CoA	(15μM)	184 ± 10 (3)
Octanoyl-CoA	(40μM)	118 ± 14 (2)
Octanoate	(100μM)	110 ± 11 (4)
Acetyl-(-)carnitine	(2.5mM)	105 ± 11 (8)
Pyruvate	(7mM)	236 ± 30 (4)

a. Values are means ± S.E. Figures in parentheses are the number of observations with samples from different animals.

in fatty acid oxidation in rat heart mitochondria under the conditions used in this investigation. Since the oxidation of octanoate, whose transport is not carnitine-dependent, was strongly inhibited in the presence of 4-pentenoate, it is also concluded that the translocation of palmitoylcarnitine across the inner mitochondrial membrane was not rate-limiting under conditions used in this study. Additionally, the parallel inhibitions of thiolase and octanoate oxidation in the presence of 4-pentenoate suggest that the activation of octanoate to octanoyl-CoA proceeded more rapidly than the thiolytic cleavage of its 3-keto-acyl-CoA degradation products.

In contrast, the oxidation of pyruvate was only slightly inhibited in the presence of 4-pentenoate and only to the same extent as was caused by n-pentanoate (see Fig. 8B). The omission of (-)carnitine had little effect on the inhibition of pyruvate oxidation by 4-pentenoate. Since higher respiratory rates were observed with pyruvate than with palmitoylcarnitine as substrate (see Table VIII), and since 4-pentenoate led to the inhibition of palmitoylcarnitine oxidation but affected only slightly pyruvate oxidation, it is concluded that the citric acid cycle did not limit the rate of fatty acid oxidation in rat heart mitochondria under the conditions of this study. This conclusion

is in agreement with that of Pande(24). However, it remains unexplained why an incubation for 3 min with either 4-pentenoate or n-pentanoate resulted in a 70-80% inhibition of pyruvate oxidation in liver mitochondria (41,42), whereas in heart mitochondria only a 25% inhibition was observed. A possible explanation is that heart mitochondria have much higher citric acid cycle activity than have liver mitochondria.

Inhibition of Carnitine Acetyltransferase by 4-Pentenoate- When the effect of 4-pentenoate on the citric acid cycle was studied with acetylcarnitine as a substrate, a significant inhibition of the respiratory rate was observed (see Fig. 9A). Clearly, the inhibition pattern shown in Fig. 9A is different from those seen in Fig. 6A or 8A . Since 4-pentenoate had caused only a slight and nonspecific inhibition of the citric acid cycle, carnitine acetyltransferase was suspected of being the site of this inhibition. This was found to be correct when the activity of carnitine acetyltransferase was determined as a function of the preincubation time of mitochondria with 4-pentenoate (see Fig. 9 B). Since the respiratory rate observed with pyruvate was higher than that obtained with acetylcarnitine (see Table VIII) and since the inhibition patterns of carnitine acetyltransferase and acetylcarnitine-supperted respiration were parallel (see Fig. 9 A and 9 B), it is

Fig. 9A Inhibition of the Oxidation of Acetyl-(-)-carnitine by 4-Pentenoate in Coupled Rat Heart Mitochondria

Mitochondria were preincubated with 0.1 mM 4-pentenoate (Δ^4-C_5) or 0.1 mM n-pentanoate (n-C_5) as described under "Experimental Procedures". After various preincubation times, 2.5 mM acetyl-(-)carnitine was added to initiate the reaction.

Fig. 9B Inhibition of Carnitine Acetyltransferase by 4-Pentenoate in Coupled Rat Heart Mitochondria

Mitochondria were preincubated with 0.1 mM 4-pentenoate (Δ^4-C_5) or 0.1 mM n-pentanoate (n-C_5) as described under "Experimental Procedures". After various preincubation times, 30 to 50 µl of the mitochondrial suspension were withdrawn, rapidly frozen, disrupted with Triton X-100 and assayed for carnitine acetyltransferase as described under "Experimental Procedures".

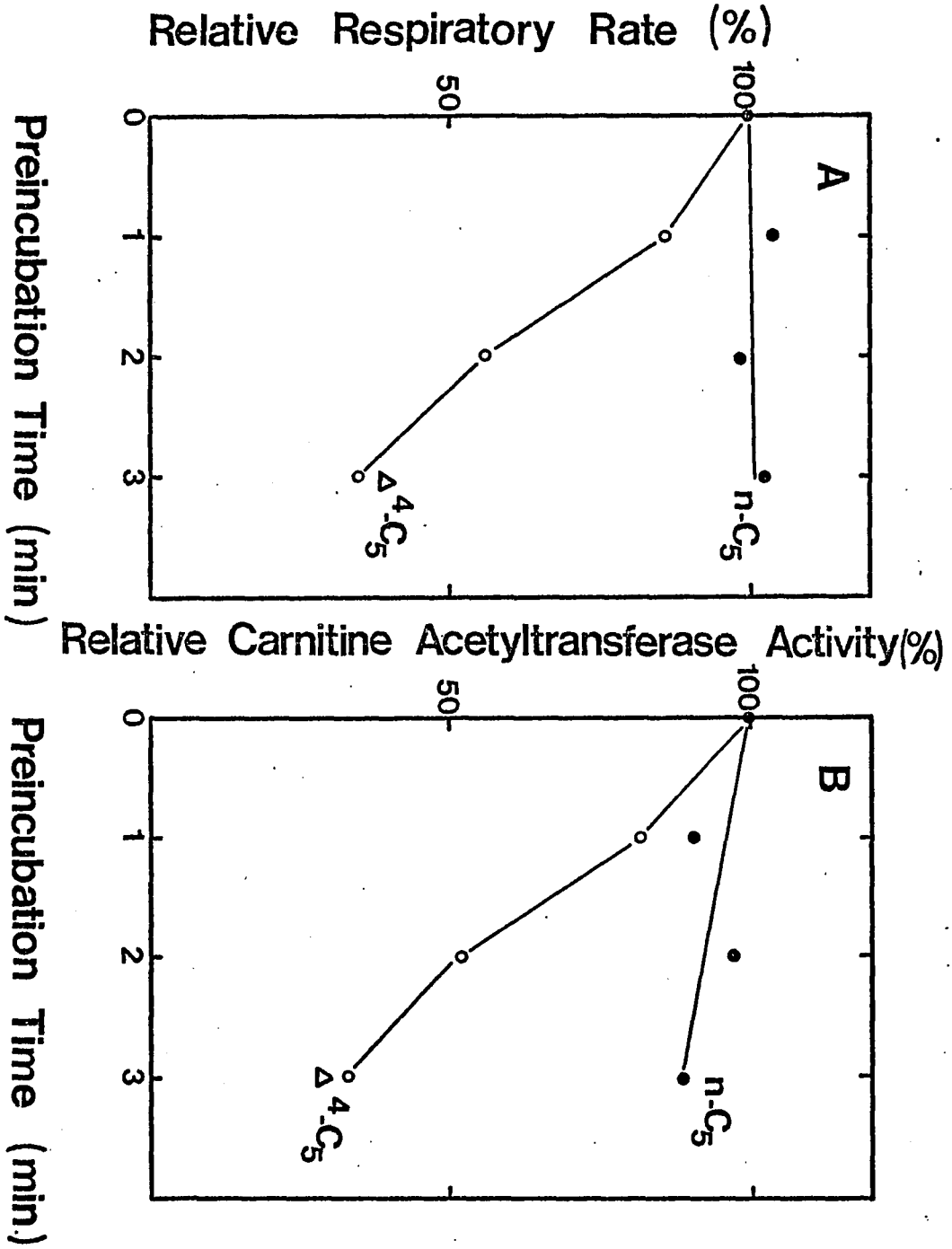

Relative Respiratory Rate (%)
50
100
0
1
2
3
A
Δ4-C5
n-C5
Preincubation Time (min)
Relative Carnitine Acetyltransferase Activity(%)
50
100
0
1
2
3
B
Δ4-C5
n-C5
Preincubation Time (min)

Fig. 9

suggested that carnitine acetyltransferase under the conditions of this study catalyzed the rate-limiting step in the oxidation of acetylcarnitine in rat heart mitochondria.

Discussion

As described in PART ONE, CoA derivatives of fatty acids are degraded in mitochondria through the actions of four types of enzymes, i.e., acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and thiolase. For each reaction except for that catalyzed by 3-hydroxyacyl-CoA dehydrogenase several enzymes which differ in their chain length specificities have been reported to exist. In addition to the reported existence of three acyl-CoA dehydrogenases in liver mitochondria (6), there exist in heart mitochondria two enoyl-CoA hydratases (see PART ONE) and two thiolases, one specific for acetoacetyl-CoA and one active on substrates of different chain lengths (34). As described in PART ONE, chromatography on hydroxylapatite proved to be a successful method of separating the two enoyl-CoA hydratases present in the pig liver (see Fig. 3). Furthermore, this method proved also to be a good one for separating most of the enzymes of β-oxidation of heart. As shown in Fig. 10 there are two enoyl-CoA hydratases, two thiolases and one 3-hydroxyacyl-CoA dehydrogenase present in pig heart. Since this study deals also with rat heart, a homogenate prepared from this organ was chromatographed on hydroxylapatite with principally the same result (see Fig. 11). Based on this as well as on other observations it is concluded that the operation of the pathway of β-oxidation in heart muscle requires the presence of at least two acyl-CoA dehydrogenases(43), two enoyl-CoA hydratases, one

Fig. 10 Separation by Chromatography on Hydroxylapatite of the Enzymes of β-Oxidation from Pig Heart

Frozen pig heart (60 g) was homogenized with a PT 10 ST Polytron homogenizer in 0.1 M potassium phosphate (pH 8.5) and 10 mM mercaptoethanol, and centrifuged for 20 min at 30,000 x g. The pH of the supernatant was then adjusted with 1 N HCl to 6.3. Residual solid material was removed by centrifugation and the resulting clear solution was applied to a hydroxylapatite column (2.5 x 20 cm) which had been previously equilibrated with 0.1 M potassium phosphate (pH 6.3), 10 mM mercaptoethanol and 10% glycerol. The column was then developed with two consecutive potassium phosphate gradients (pH 6.3), 0.1- 0.3 M and 0.3- 0.7 M respectively. Fractions were collected and assayed for enzymes of β-oxidation. HDH, β-hydroxyacyl-CoA dehydrogenase; LEH, long chain enoyl-CoA hydratase; SEH, short chain enoyl-CoA hydratase; TH I, 3-ketoacyl-CoA thiolase; TH II, acetoacetyl-CoA thiolase. C4 and C10 refer to the chain lengths of the substrates used in the assays.

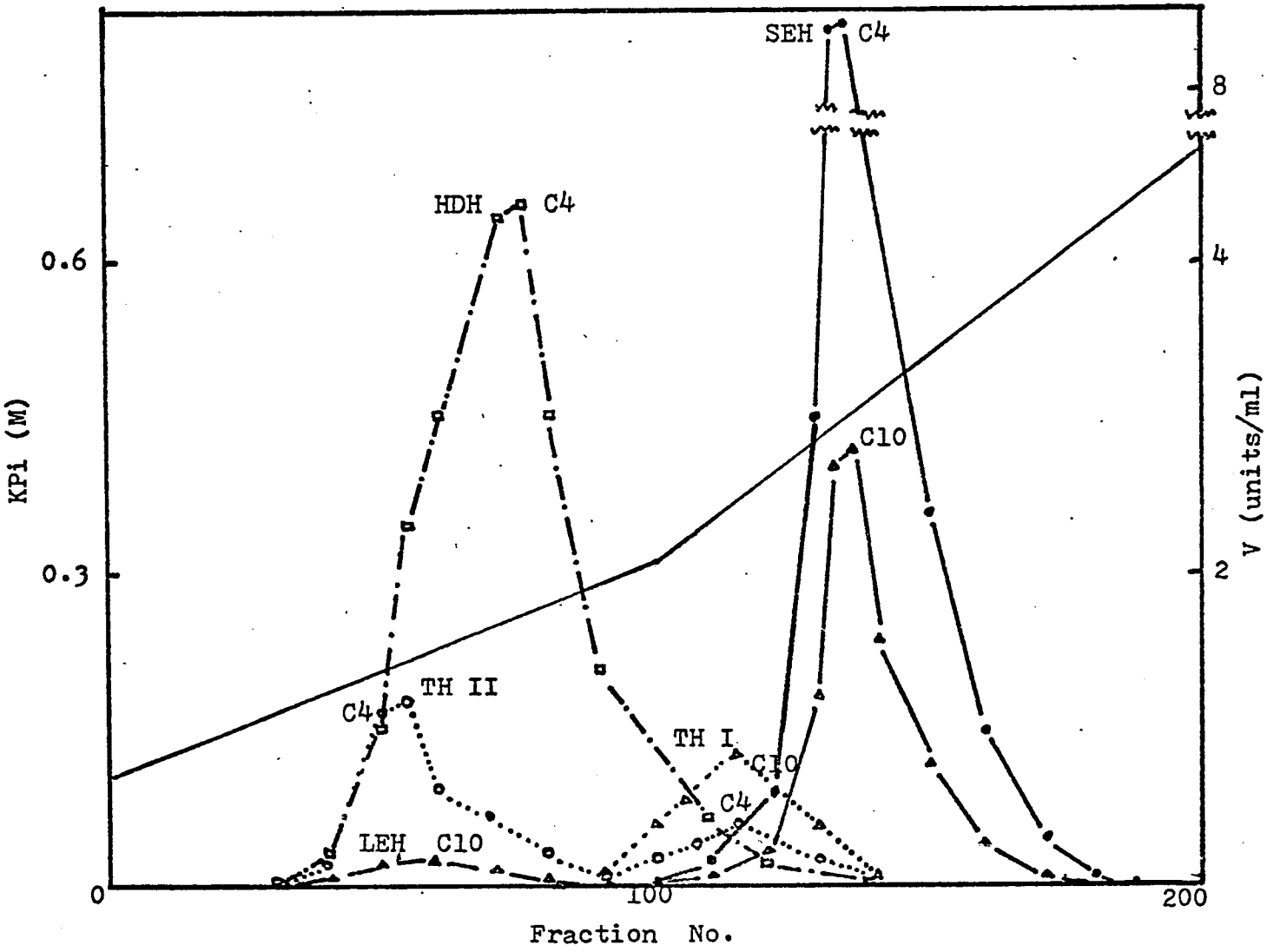

SEH C4
HDH C4
C10
TH II
C4
TH I
C10
C4
LEH C10
0.6
0.3
0
KPi (M)
8
4
2
V (units/ml)
100
200
Fraction No.

Fig. 10

Fig. 11 Separation by Chromatography on Hydroxylapatite of the Enzymes of β-Oxidation from Rat Heart

Fresh rat heart (2 g) was homogenized with a PT 10 ST Polytron homogenizer in 0.05 M potassium phosphate (pH 7) and 5 mM mercaptoethanol, and centrifuged for 20 min at 30,000 x g. The pH of the supernatant was then adjusted with 1 N HCl to 6.3. Residual solid material was removed by centrifugation and the resulting clear solution was applied to a hydroxylapatite column (1.2 x 18 cm) which had been previously equilibrated with 0.05 M potassium phosphate (pH 6.3), 5 mM mercaptoethanol and 10% glycerol. The column was then developed with two consecutive potassium phosphate gradients (pH 6.3), 0.05- 0.3 M and 0.3- 0.7 M respectively. Fractions were collected and assayed for enzymes of β-oxidation. The symbols used are those listed in the legend to Fig. 10.

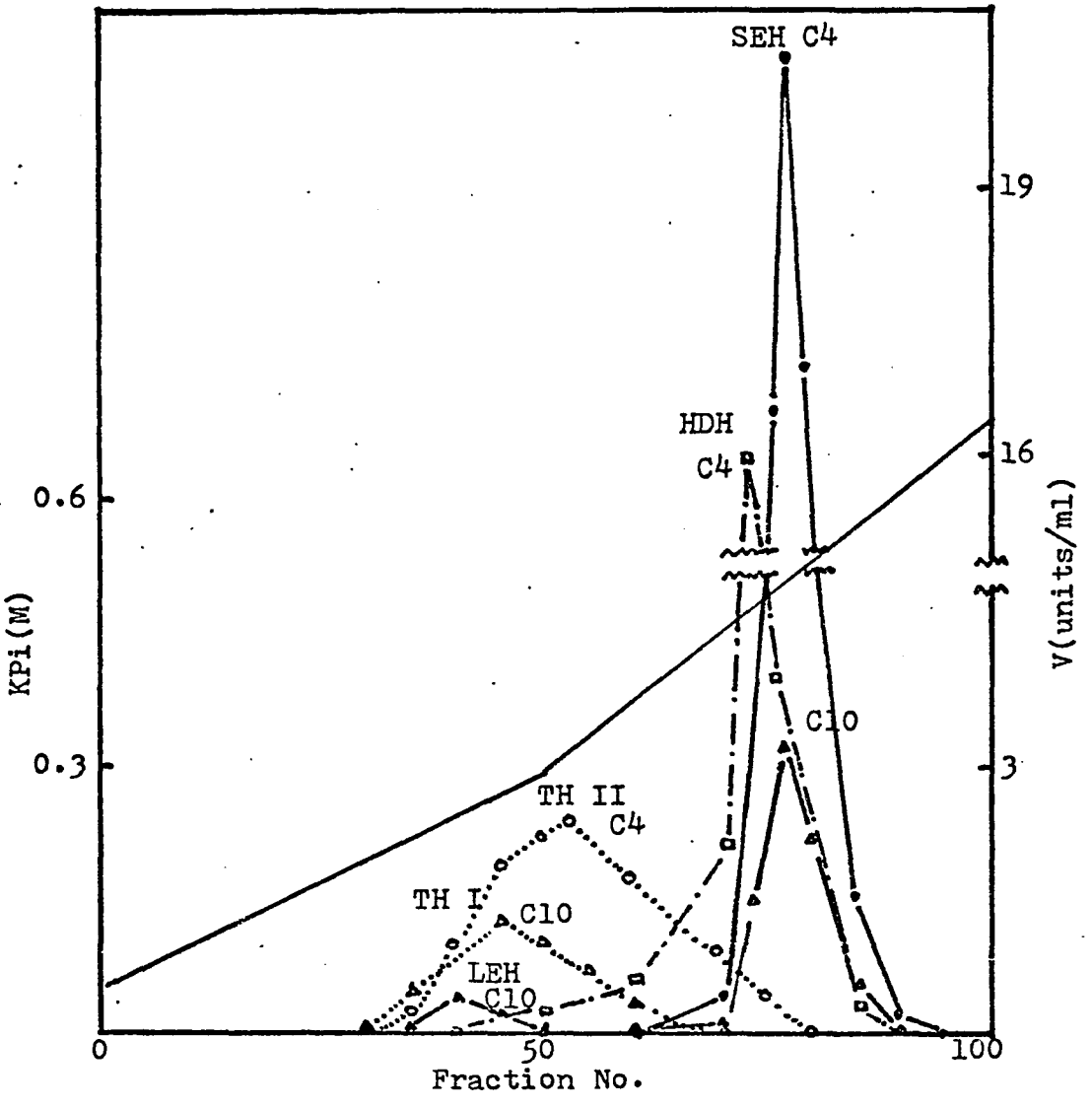

SEH C4
HDH
C4
C10
TH II
C4
TH I
C10
LEH
C10
0.6
0.3
KPi(M)
19
16
3
V(units/ml)
0
50
100
Fraction No.

Fig. 11

3-hydroxyacyl-CoA dehydrogenase, and one thiolase. Only 3-ketoacyl-CoA thiolase is assumed to be involved in β-oxidation because acetoacetyl-CoA thiolase is believed to function only in ketone body degradation (36).

In vitro measurements of the activities of several of the enzymes of fatty acid oxidation suggest that three reactions, those catalyzed by 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase and carnitine palmitoyltransferase, may proceed at equal but limiting rates (see Table VI). However, since the activities of these enzymes in undamaged mitochondria are unknown and since several of the enzymes cannot be assayed under physiologically relevant conditions, the direct measurements of the enzymes do not provide a definite answer regarding the rate-limiting step of the complete pathway. Therefore, it was decided to specifically inhibit an enzyme suspected of catalyzing the rate-limiting step of fatty acid oxidation and to assess the effect of this inhibition on the rate of fatty acid oxidation in coupled rat heart mitochondria. 4-Pentenoate was chosen as a possible inhibitor because one of its metabolites, 2,4-pentadienoyl-CoA, has been shown to be an effective inhibitior of thiolase (28). Preincubation of rat heart mitochondria with 4-pentenoate resulted in the parallel inhibition of the two thiolases and of the oxidation of palmitoyl-CoA, palmitoylcarnitine and octanoate while pyruvate-supported respiration was only slightly and nonspecifically inhibited. These

findings lead to the conclusion that the reaction catalyzed by thiolase is the rate-limiting step in the oxidation of palmitoyl-CoA and possibly of palmitate because in vitro measurements have shown that the activity of palmitoyl-CoA synthetase in rat heart is higher than that of carnitine palmitoyltransferase(24). Since the in vitro activities of carnitine palmitoyltransferase and 3-hydroxyacyl-CoA dehydrogenase were found to be as low as that of 3-ketoacyl-CoA thiolase (see Table VI), it is possible that the inhibition of any of these three enzymes would result in an inhibition of fatty acid oxidation. However, this conclusion may be without basis if the activities of the enzymes of β-oxidation are significantly altered due to their existence as a multienzyme complex. Such a complex has been isolated from E.coli (44) and thus may also be present in mitochondria of higher organisms. If the enzymes of β-oxidation are organized in a multi-enzyme complex, intermediates of fatty acid oxidation may move from one active site to the next without being released into the surrounding medium. Such a situation would explain why intermediates formed during fatty acid oxidation do not accumulate in mitochondria (38) and such an arrangement would make it possible that the inhibition of any of the participating enzyme would result in inhibition of the overall pathway.

Since none of the enzymes of fatty acid oxidation is known to be regulated allosterically or by covalent modification, substrate availability or product inhibition are possible control mechanisms. Thus the activity of 3-ketoacyl-CoA thiolase may be regulated by the availability of free CoASH, the concentration of which decreases dramatically during state 4 oxidation of palmitoylcarnitine or octanoate in rabbit heart mitochondria (45). This regulatory mode of 3-ketoacyl-CoA thiolase is of special interest in conjunction with the proposed control of pyruvate dehydrogenase by the intramitochondrial acetyl-CoA/CoASH ratio (46). Other proposals concerning the control of fatty acid oxidation as for example via the suggested regulations of 3-hydroxyacyl-CoA dehydrogenase by the mitochondrial NAD^+/NADH ratio (47), of carnitine palmitoyltransferase by the cytosolic carnitine concentration (48) and of palmitoyl-CoA synthetase by the cytosolic CoASH concentration (49) are possible but need to be evaluated more vigorously.

An interesting result of this study is the elucidation of how fatty acid oxidation is inhibited by the hypoglycemic compound 4-pentenoate. The observed inhibitions of the two thiolases which paralleled the inhibition of palmitoylearnitine-supported respiration leads to the conclusion that the inhibition of

3-ketoacyl-CoA thiolase is the cause for the inhibition of fatty acid oxidation. This conclusion is in agreement with Sherratt's hypothesis that a metabolite of 4-pentenoate causes the inhibion of fatty acid oxidation by inhibiting one of the enzymes of this pathway (50) but it contradicts the explanation forwarded by Bressler and coworkers that the inhibition of this process is due to the sequestration of CoA and carnitine by inert metabolites of 4-pentenoate (26). However, the inhibition of the oxidation of palmitoylcarnitine by 4-pentenoate beyond the level of inhibition of thiolase (compare the 20% remaining respiratory rate in Fig. 6A to the 35% remaining thiolase activity in Fig. 6B at 3 min preincubation time) is possibly caused by the sequestration of CoA in a metabolically inactive form.

Although Holland et al. (28) had shown that 2,4-pentadienoyl-CoA, a metabolite of 4-pentenoate, caused the inhibition of isolated acetoacetyl-CoA thiolase, they were unable to demonstrate an inhibition of thiolase after preincubation of rat liver mitochondria with 4-pentenoate (51). Since the experiments of this study were performed with rat heart mitochondria, it has been tested for and observed that 4-pentenoate caused the inhibition of thiolase in rat liver mitochondria (see Fig. 12A and 12B). With liver mitochondria the presence of malonate had little effect on the inhibition patterns.

Fig. 12A Inhibition of the Oxidation of Palmitoyl-(-)carnitine by 4-Pentenoate in Coupled Rat Liver Mitochondria

In a series of experiments, mitochondria (6 mg of protein) were preincubated with 0.1 mM 4-pentenoate (Δ^4-C_5) or 0.1 mM n-pentanoate (n-C_5) in 2 ml of incubation medium containing 0.1 M KCl, 20 mM Tris (pH 7.4), 4 mM potassium phosphate (pH 7.4), 4 mM $MgCl_2$, 0.1 mM ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid, 2 mM ADP and 10 mM malonate at 25° for various time as indicated on the abscissa. Palmitoyl-(-)carnitine (30 μM) was then added to start the reaction.

Fig. 12B Inhibition of Thiolases by 4-Pentenoate in Coupled Rat Liver Mitochondria

Mitochondria were preincubated with 0.1 mM 4-pentenoate (Δ^4-C_5) or 0.1 mM n-pentanoate (n-C_5) as described in the legend to Fig. 12A. After various preincubation times, aliquots were assayed for thiolase with acetoacetyl-CoA (▲- ▲, ●- ●) or 3-ketodecanoyl-CoA (o- o) as substrates.

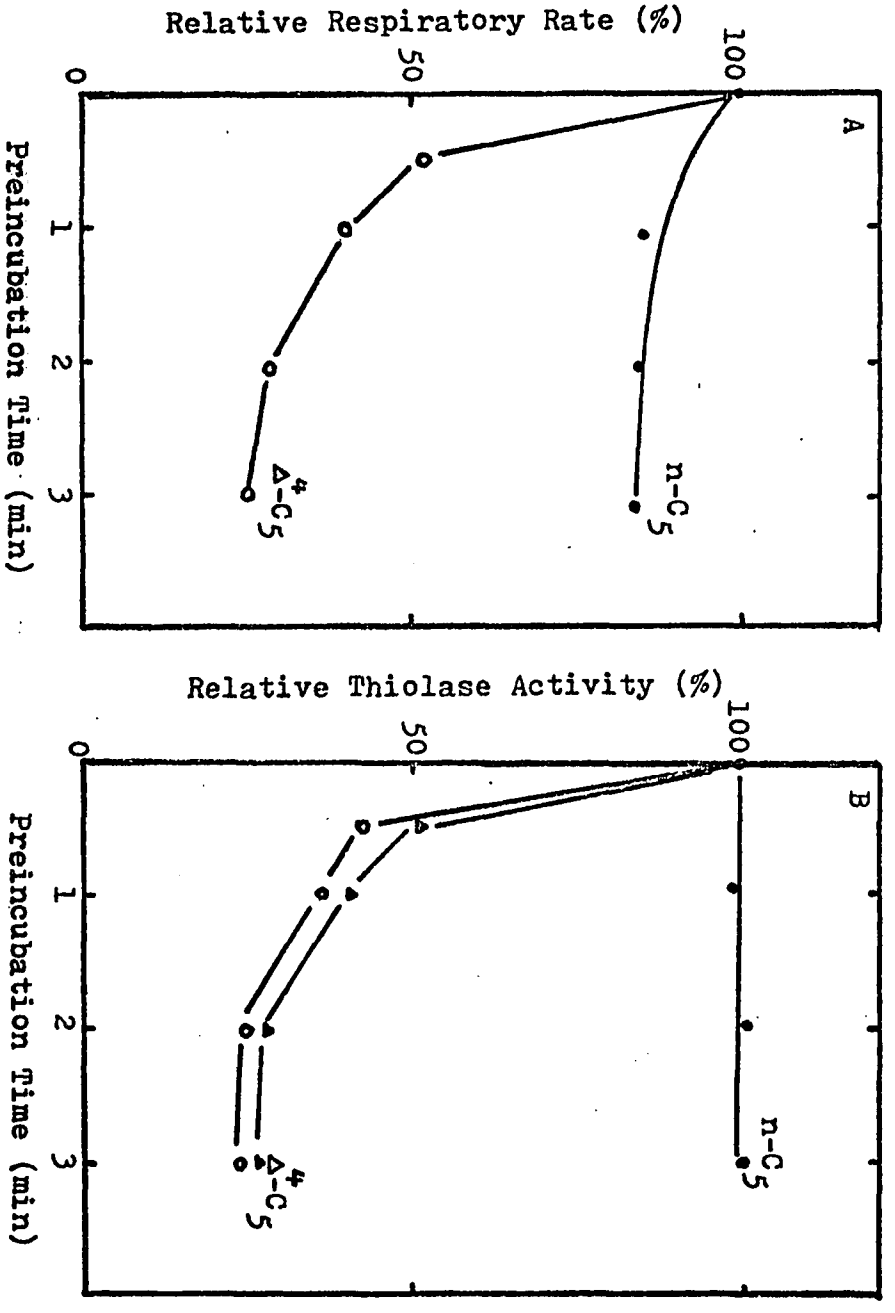

Relative Respiratory Rate (%)
0
50
100
A
Preincubation Time (min)
1
2
3
Δ^4-C_5
n-C_5
Relative Thiolase Activity (%)
0
50
100
B
Preincubation Time (min)
1
2
3
Δ^4-C_5
n-C_5

Fig. 12

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