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REGULATION OF THIOLASES AND CONTROL OF FATTY ACID OXIDATION IN HEART

*City University of New York*

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# REGULATION OF THIOLASES

# AND

# CONTROL OF FATTY ACID OXIDATION IN HEART

by

YETUNDE O. OLOWE

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1981

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# INTRODUCTION

Fatty acids are important substrates for mitochondrial respiration in many tissues, especially in the mammalian heart. The major source of fatty acids is the adipose tissue, where they are stored in the form of triglycerides. Triglycerides are first hydrolyzed to non-esterified fatty acids which can be transported from the adipose tissue to the peripheral tissues. Since long-chain fatty acids are relatively insoluble in an aqueous medium, they are transported as a complex with serum albumin. Triglyceride hydrolysis in adipose tissue occurs in a sequential manner in which fatty acids are removed one at a time. The process is catalyzed by a series of lipases. The removal of the first fatty acid by triglyceride lipase, which is believed to be rate-limiting (1), is under control by insulin, glucagon, and other hormones.

The mobilization of fatty acids from adipose tissue depends not only on the rate of lipolysis but also on the rate of re-esterification of fatty acids to form triglycerides. The rate of esterification exceeds that of lipolysis when excess fatty acids are available. When the supply of carbohydrate is limited, however, during stress, starvation or prolonged exercise, rapid mobilization of fatty acid is required to meet the need for fuel. The secretion of glucagon in response to low levels of glucose promotes the release of fatty acids and lipolysis predominates. Epinephrine has the same lipolytic effect on adipose tissue as glucagon (2). The

lipolytic effect is a consequence of an increase in the cAMP content of adipose tissue via stimulation of adenylate cyclase (3). The effects of insulin and prostaglandin $E_1$ ($PGE_1$) are antilipolytic. Insulin inhibits basal and epinephrine stimulated lipolysis (4) and $PGE_1$ inhibits lipolysis in adipose tissue (5). A recent investigation demonstrated the inhibition of lipolysis by $PGE_1$ in the heart (6).

In addition to fatty acids derived from triglycerides of adipose tissue, the heart can also use lipoprotein triglycerides as a source of free fatty acids. Free fatty acids are hydrolysed from plasma triglycerides by the action of lipoprotein lipase located on the capillary endothelium. The activity of lipoprotein lipase is dependent on the hormonal and nutritional state of the animal (7). Insulin induces a rise in activity, and epinephrine, ACTH, growth hormone, and thyroid-stimulating hormone decrease the activity. The hormonal response of lipoprotein lipase is in general opposite to the response of hormone-sensitive triglyceride lipase. Although it is established that triglyceride lipase activity is mediated through changes in cAMP levels, the effect of cAMP on lipoprotein lipase has not yet been elucidated (8).

Triglyceride lipase has been shown to exist in two enzymatic interconvertible forms; an active phosphorylated form and an inactive dephosphorylated form (9). cAMP stimulates protein kinases in adipose tissue and thereby causes phosphorylation of the lipase (10).

The circulating albumin-bound non-esterified fatty acids are absorbed into the cell of peripheral tissues where they are activated and used as respiratory fuels. The enzymes that activate the fatty acids are a group of transferases collectively referred to as acyl-CoA synthetases (EC 6.2.1.3) (11, 12, 13, 14, 15). Butyryl-CoA synthetase and octanoyl-CoA synthetase activate short- and medium-chain fatty acids respectively: Longer chain fatty acids, from lauric acid to docosanoic (behenic) acid, are activated by an enzyme named palmitoyl-CoA synthetase or long-chain acyl-CoA synthetase. The intracellular distribution is not the same for all activating enzymes. Butyryl-CoA synthetase as well as octanoyl-CoA synthetase appear to be located in the mitochondrial matrix (11, 12). Apparently short-chain and medium-chain fatty acids move into mitochondria as free fatty acid where they are activated. This situation probably accounts for the fact that mitochondrial oxidation of short- and medium-chain fatty acids does not depend on the presence of carnitine (14). Palmitoyl-CoA synthetase activity in heart is mainly located on the sarcoplasmic reticulum and outer mitochondrial membrane (14, 16, 17). Since fatty acids are activated in the extramitochondrial space, and since CoA as well as its derivatives cannot cross the inner mitochondrial membrane, a specific uptake mechanism for long chain acyl groups is required. The uptake and thus the oxidation of long chain fatty acids is completely dependent on the presence of carnitine. The

transport of long chain acyl groups into mitochondria involves the transfer of acyl groups from CoA to carnitine by carnitine palmitoyl transferase I. Acylcarnitines enter the matrix space via acylcarnitine translocase which catalyzes the 1:1 exchange of acylcarnitine for carnitine, acetylcarnitine or acylcarnitine (18, 19). After crossing the inner mitochondrial membrane the acyl unit is transferred back to CoA by carnitine palmitoyl transferase II (20). The carnitine palmitoyl transferase I is located on the outer surface of the inner mitochondrial membrane and carnitine palmitoyl-transferase II is on the inner surface of inner mitochondrial membrane.

Once present in the mitochondrial matrix fatty acyl-CoA's can enter the B oxidation system (21) as outlined below:

1) Acyl-CoA + $FAD^+ \rightleftharpoons$ 2-enoyl-CoA + $FADH_2$

2) 2-enoyl-CoA + $H_2O \rightleftharpoons$ 3-hydroxyacyl-CoA

3) 3-hydroxyacyl-CoA + $NAD^+ \rightleftharpoons$ 3-ketoacyl-CoA + NADH + $H^+$

4) 3-ketoacyl-CoA + CoASH $\rightleftharpoons$ acyl-CoA(-2C) + acetyl-CoA

The fatty acyl-CoA molecule loses first two hydrogens which are transferred to flavin adenine dinucleotide (FAD) by the enzyme fatty acyl-CoA dehydrogenase (EC 1.3.99.3). Then a molecule of water is added across the carbon-carbon double bond by the enzyme enoyl-CoA hydratase (EC 4.2.1.17). The product, L-3-hydroxyacyl-CoA is dehydrogenated by the NAD-linked L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35). The resulting 3-ketoacyl-CoA is cleaved thiolytically by

3-ketoacyl-CoA thiolase (EC 2.3.1.16) to produce one molecule of acetyl-CoA and one molecule of a fatty acyl-CoA whose hydrocarbon chain is two carbon atoms shorter than the original fatty acid. The intermediates of this pathway, fatty enoyl-CoA, L-3-hydroxyacyl-CoA and 3-ketoacyl-CoA do not normally accumulate in mitochondria, a finding which has led to the suggestion that all fatty acid intermediates are enzyme-bound and channeled from one active site to the next without being first released into the medium.

Although the pathway of fatty acid oxidation is very well established, the mechanism of regulation of this pathway has not yet been definitely established. To designate a reaction as regulatory, it must be rate-limiting. Suggestions about the rate-controlling step of B oxidation have been mainly derived from studies of enzyme activities in isolated mitochondria or mitochondrial extracts. Enzymes whose activities are found to be high are likely to catalyze equilibrium reactions and thus are not considered to be regulatory enzymes. Enzymes with lower activities may catalyze a non-equilibrium reaction and are therefore possibly subject to regulation. Each of the reactions in fatty acid oxidation that has been proposed to be rate-limiting can thus be a site of regulation of the overall pathway.

Acyl-CoA synthetase has been considered to be rate-limiting, and may be regulated under physiological conditions by palmitoyl-CoA. Palmitoyl-CoA has been

reported to inhibit the synthetase (22) by increasing the $K_m$ for CoASH. The synthetase may also be regulated by the cytosolic CoASH concentration which may fluctuate in parallel with the rate of respiration (23). The reaction catalyzed by carnitine-palmitoyl transferase has been considered to be rate-limiting in liver mitochondria. This hypothesis is based on the observation that the rate of respiration supported by palmitoylcarnitine in liver mitochondria is twice that determined with palmitoyl-CoA plus carnitine. If carnitine palmitoyltransferase is rate-limiting in fatty acid oxidation it may be subject to regulation by the cytosolic carnitine concentration (24). More recently the inhibition of carnitine palmitoyltransferase I by malonyl-CoA and the possible effect of malonyl-CoA on the oxidation of fatty acid in liver mitochondria have been investigated (25). The enzyme may be regulated in liver by malonyl-CoA but not in heart, where malonyl-CoA is not synthesized. Pande (26) has concluded that in heart mitochondria either the capacity to translocate acyl units into the matrix space or B oxidation limits the rate of fatty acid oxidation. It has also been proposed that acyl-CoA dehydrogenase may be rate-limiting. Based on <u>in vitro</u> assays acyl-CoA dehydrogenase appears to be 10-times less active than subsequent enzymes in the pathway (27). However, it is possible that the <u>in vitro</u> assays of this group of enzymes yield unrealistically low activity values, because artificial electron acceptors are utilized

in assaying them. The regulation of 3-hydroxyacyl-CoA dehydrogenase may also result in the control of B oxidation. A high NADH/NAD ration inhibits 3-hydroxyacyl-CoA dehydrogenase as well as fatty acid oxidation and results in an accumulation of 3-hydroxyacyl-CoA compounds (13). Most recently it was suggested that the thiolase-catalyzed reaction may be rate-limiting in B oxidation or may be as slow as other steps are (28).

Although no consensus exists as to the regulation of fatty acid oxidation, it is generally accepted that the rate of utilization of fatty acids is dependent on both the plasma concentration of free fatty acids and on the energy demands of the tissue. The plasma concentration of fatty acid depends mainly on the mobilization of fatty acids from adipose tissue by triglyceride lipase which is regulated hormonally via phosphorylation and dephosphorylation. In isolated perfused hearts the rate of acetyl-CoA oxidation by the citric acid cycle limits the rate of fatty acid utilization at low levels of ADP (29, 30), a finding which suggests that oxidative phosphorylation may be rate limiting.

The heart oxidizes ketone bodies in addition to glucose and fatty acids. Acetoacetate, 3-hydroxybutyrate and acetone are collectively referred to as ketone bodies. Acetone is formed by a spontaneous decarboxylation of acetoacetate, and has no physiological function. Ketone bodies are produced by the liver. In the carbohydrate fed state (low ratio glucagon to insulin), the direction of fatty acid

metabolism in liver is primarily toward synthesis and the ketone body concentration is low. However, during starvation or when the carbohydrate concentration is low there is an elevation of the glucagon to insulin ratio which leads to increased fatty acid oxidation and ketogenesis. The two main ketone bodies, acetoacetate and D-3-hydroxybutyrate, can be interconverted in the liver and then diffuse into the blood to supply the extrahepatic tissues with energy substrate. Either of these fuels can enter the mitochondria where they are degraded to acetyl-CoA. Ketone bodies enter the mitochondria as anions via a carrier similar or identical to that used by pyruvate (31). Ketone bodies can also cross the mitochondrial membranes by diffusion as undissociated acids (31, 32, 33). The enzymes concerned with the oxidation of ketone bodies are present in mitochondria. 3-Hydroxybutyrate is converted to acetoacetate by 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30). No degradation of ketone bodies takes place prior to their activation. For the activation of acetoacetate, the heart muscle is well equipped because it contains high levels of 3-ketoacid-CoA transferase (EC 2.8.3.5) (34,35). The absence of this enzyme in liver assures the utilization of ketone bodies in extrahepatic tissues only. Acetoacetyl-CoA formed is then subjected to thiolytic cleavage. The equilibrium of the transferase reaction favors acetoacetate formation (34). However, the equilibrium of the acetoacetyl-CoA thiolase (EC 2.3.1.9) catalyzed reaction is toward the formation of

acetyl-CoA. It is the coupling of the thiolase to the transferase that allows the conversion of acetoacetate to acetyl-CoA to proceed more readily than the reverse reaction.

The pathways of ketone body production and utilization have been extensively studied, but very little is known about the control of the extrahepatic utilization of ketone bodies. Since the major role of ketone bodies is to supply an alternate fuel to glucose for peripheral tissues, it is logical that the concentration of ketone bodies in the circulation increases in parallel with the decrease in supply of carbohydrate. For example, during periods of extreme starvation or diabetes mellitus, the concentration of ketone bodies reaches very high values. There could be two obvious reasons for this, either there is a decrease in the rate of utilization by peripheral tissues or an increase in ketone body production by the liver.

Various investigators using tissue slice preparations (36, 37), perfused organs (38, 39) and other methods, have concluded that the activities of enzymes involved in ketone body degradation under circumstances of enhanced hepatic production and peripheral uptake of acetoacetate and 3-hydroxybutyrate are essentially unaltered. Apparently the utilization of ketone bodies is not controlled by a variation in enzyme activities. The rate of ketone body utilization appears to be simply a function of its concentration in blood (38, 40). Factors other than ketone body

concentration in blood may be involved in determining the rate of uptake by tissues. Ketone bodies have been found to inhibit the release of non-esterified fatty acids from adipose tissue and hence they regulate their own production as well as the availability of non-esterified fatty acids for oxidation in peripheral tissues. In order to spare carbohydrate, ketone bodies inhibit pyruvate oxidation. Incubation of mitochondria with ketone bodies decreases the amount of extractable pyruvate dehydrogenase in the active form (41). A likely explanation is that the increased [acetyl-CoA/CoASH] ratio in mitochondria due to acetoacetate metabolism may activate pyruvate dehydrogenase kinase to cause increased phosphorylation and a decrease in the activity of pyruvate dehydrogenase (42,43). Whereas insulin and glucagon play a role in fatty acid oxidation and ketone body formation (25), the only reports of insulin affecting ketone body utilization are conflicting (38,44).

The mobilization, distribution and oxidation of fatty acids are integrated with the utilization of carbohydrates. All these pathways yield acetyl-CoA, which is further oxidized by the tricarboxylic acid cycle to $H_2O$ and $CO_2$. While the rate-controlling steps for carbohydrate metabolism have been well established, those for fatty acid oxidation and ketone body utilization still need to be determined.

The subject of my thesis is to investigate the regulation of both fatty acid oxidation and ketone body degradation. In order to identify possible sites of regulation,

it is necessary to determine the rate-limiting steps of these pathways.

In general, two approaches have been used for the identification of rate controlling reactions in intact cells. These methods include firstly, the correlation of rates of individual reactions in a pathway with rates of flux through the pathway. The second involves the detection of reactions whose levels of substrates, products and cofactors within the tissue are far removed from levels expected from the thermodynamic equilibrium of the reaction. For accurate interpretation of results, substrates and products must be contained within a single compartment. In this regard the compartmentation of substrates and products between mitochondria and cytoplasmic spaces make meaningful measurement difficult. Therefore I have used a different method of approach for identifying the rate-limiting step in B oxidation. The method adopted involves the use of a specific inhibitor of an enzyme suspected of catalyzing a rate-limiting step of the pathway. The rate of inhibition of the overall pathway is compared with the rate of inactivation of the suspected enzyme. This approach was used to evaluate the possible rate-limiting nature of the thiolase-catalyzed reactions in fatty acid oxidation and ketone body degradation in heart. Subsequently I have studied the regulation of 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase from pig heart by mitochondrial metabolites and co-enzymes as well as by phosphorylation and dephosphorylation.

## EXPERIMENTAL PROCEDURES

Materials - CoASH, NAD, NADH, butyryl-CoA, decanoyl-CoA, and palmitoyl-CoA, acetyl-CoA, ADP, ATP, were purchased from P-L Biochemicals, Inc. [$^3$H]CoASH was bought from New England Nuclear. Octanoic acid, DL-3-hydroxybutyric acid, lithium acetoacetate, L-malate and 3-hydroxyacyl-CoA dehydrogenase were obtained from Sigma Chemical Co. Sodium pyruvate, 2-decenoic acid and dikene were bought from Aldrich Chemical Co. DL(-)carnitine was provided by Dr. K. Brendel, University of Arizona College of Medicine. 4-Bromocrotonic acid was obtained by chemical synthesis (45). Mitochondrial 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase were isolated from pig heart and purified as described by Staack et al.(46). Crotonyl-CoA (47) and acetoacetyl-CoA (48) were prepared according to standard procedures. DL-3-Hydroxybutyryl-CoA, 2-decenoyl-CoA, 4-bromocrotonyl-CoA and 4-bromocrotonyl-[$^3$H]CoA were synthesized from the corresponding free acids and CoASH by the method of Goldman and Vagelos (49). 3-Ketodecanoyl-CoA, 3-keto-4-bromobutyryl-CoA, and 3-keto-4-bromobutyrl-[$^3$H]CoA were prepared enzymatically from 2-decenoyl-CoA, 4-bromocrotonyl-CoA and 4-bromocrotonyl-[$^3$H]CoA respectively by the procedure of Seubert et al. (50). Succinyl-CoA was prepared from succinic anhydride and CoASH as described by Simon and Shemin (51). The concentrations of all CoA derivatives except for 3-ketodecanoyl-CoA and 3-keto-4-bromobutyryl-CoA

were determined by the method of Ellman (52) after cleaving the thioester bond with hydroxylamine at pH 7. The concentrations of 3-ketodecanoyl-CoA and 3-keto-4-bromobutyryl-CoA were measured by following the oxidation of NADH at 340 nm in the presence of 3-hydroxyacyl-CoA dehydrogenase at pH 7.

Enzyme Assays - All enzyme assays were performed at 25°C. Enzyme activities were determined spectrophotometrically on a Gilford recording spectrophotometer. Acyl-CoA dehydrogenase [EC 1.3.99.2] and [EC 1.3.99.3] were assayed spectrophotometrically at 600nm as described in principle by Hoskins (53). The assay mixture contained 0.1M $KP_i$ (pH 7.6), 28 uM 2,6-dichlorophenolindophenol, 0.65mM phenazinemethosulfate, 20uM acyl-CoA, 0.2mM N-ethylmaleimide, 0.45mM KCN and 0.09%Triton X-100. The reaction was initiated by the addition of phenazinemethosulfate. Enoyl-CoA hydratase [EC 4.2.1.17] was measured spectrophotometrically at 263nm as described (54), except that the assay mixture contained 0.2M $KP_i$ (pH 8), bovine serum albumin (0.1 mg/ml), 0.06% Triton X-100 and either 30uM crotonyl-CoA or 30 uM 2-decenoyl-CoA. 3-Hydroxyacyl-CoA dehydrogenase [EC 1.1.1.35] was assayed spectrophotometrically at 340nm. The standard assay mixture contained 0.05M $KP_i$ (pH 7), 0.06% Triton X-100, 0.12mM NADH, and bovine serum albumin (0.11 mg/ml). The reaction was started by the addition of acetoacetyl-CoA to a final concentration of 30uM. The activities of both thiolases [EC 2.3.1.9 and EC 2.3.1.16] were determined by following spectrophotometrically the disappearance of the

$Mg^{2+}$-enolate complex at 303nm as described by Lynen and Ochoa (55). The reaction mixture contained 0.1M Tris-HCl (pH 8.2), 25mM $MgCl_2$, 30mM KCl, 0.06% Triton X-100, bovine serum albumin (0.13 mg/ml), 70 uM CoASH and either 33 uM acetoacetyl-CoA or 10 uM 3-ketodecanoyl-CoA. Triton X-100 was excluded when the activity of pure thiolase was assayed. Molar extinction coefficients of 21,400 and 13,900 $cm^{-1}M^{-1}$ were used to calculate the rates determined with aceto-acetyl-CoA and 3-ketodecanoyl-CoA respectively. 3-Hydroxybutyrate dehydrogenase [EC 1.1.1.30] was assayed spectrophotometrically as described by Lehninger (56). The assay mixture contained 50mM Tris-HCl (pH 8.2), 12.5mM $MgCl_2$, 15mM KCl, 50mM nicotinamide, 10mM cysteine, 2mM NAD, 29mM 3-hydroxybutyrate, 50uM KCN and 20uM antimycin. Optimal activity of 3-hydroxybutyrate dehydrogenase in mitochondrial homogenate was obtained only in the presence of nicotinamide and cysteine. The reaction was initiated by the addition of 3-hydroxybutyrate. 3-Ketoacid-CoA transferase [EC 2.8.3.5] was assayed as described by Stern (57). The assay mixture contained 67mM Tris-$SO_4$ (pH 8.1), 5mM $MgSO_4$, 10mM succinate and 30 uM acetoacetyl-CoA. The disappearance of acetoacetyl-CoA $Mg^{2+}$-enolate was measured spectrophotometrically at 303nm. A molar extinction coefficient of 9,000 $cm^{-1}$ $M^{-1}$ was used to calculate transferase activities. A unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1umol of substrate to product per min.

Isolation of Mitochondria and Preparation of Mitochondrial Extracts - Heart mitochondria were isolated by the procedure of Chappell and Hansford (58). The isolation buffer contained 0.21M mannitol, 0.07M sucrose, 5mM Tris-HCl (pH 7.4) and 1mM EGTA.[1] Protein concentrations were determined by the biuret method (59). For the preparation of a soluble mitochoncrial extract, mitochondria (1mg/ml) were suspended in 0.1M Tris-HCl (pH 8.2) containing 25mM $MgCl_2$, 30mM KCl, 20% glycerol and 10mM mercaptoethanol. The mitochondrial suspension was sonicated at 5° for 45 sec. with a Branson sonifier (Model W-185E) equipped with a microtip. The resulting mixture was centrifuged at 100,000 X g for 15 min. The protein concentration of the supernatant was determined by the method Lowry (60).

Measurement of Oxygen Uptake by Mitochondria - Mitochondria (0.5 to 1 mg/ml) were incubated in 1.9 ml of a basal isotonic medium containing 0.11M KCl, 33mM Tris-HCl (pH 7.4), 2mM $KP_i$, 2mM $MgCl_2$ and 0.1mM EGTA. To this suspension were added bovine serum albumin (0.5 mg/ml), and either 0.5mM L-malate or 2.5 uM 2-ketoglutarate. Respiration rates were measured polarographically by use of a Clark oxygen electrode with either pyruvate (2.5mM), palmitoylcarnitine (30uM), octanoate (100uM), acetoacetate (1mM) or 3-hydroxybutyrate (1mM) as substrates. Rates of respiration observed with these substrates are listed in Table 1. Bovine serum

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[1]EGTA - ethylene glyco-bis (B-aminoethylether)-N,N'-tetra acetic acid.

Table I

Oxygen uptake by rat heart mitochondria. Oxygen uptake measurements are described under "Experimental Procedures."

| Substrates | Concentration | Oxygen uptake (ng atoms $O_2$/min/mg protein) |
|---|---|---|
| Palmitoylcarnitine | 30 uM | 218 ± 16 |
| Pyruvate | 2.5 mM | 225 ± 35 |
| Octanoate | 100 uM | 86 ± 2 |
| Acetoacetate | 1 mM | 74 ± 21 |
| 3-Hydroxybutyrate | 1 mM | 65 ± 11 |

albumin was not included when pyruvate served as substrate.

Determination of Enzyme Activities in Mitochondria Preincubated with 4-Bromocrotonic Acid - Mitochondria were preincubated with varying concentrations of 4-bromocrotonic acid (0-20 uM) for 1, 2 or 3 min. Aliquots of the mitochondrial suspension (50 ul-200 ul) were rapidly frozen in dry-ice and stored at-76°C until enzyme activities were assayed as de-described above. To insure the complete disruption of mitochondria, Triton X-100 (0.06%) was added to all assay mixtures except when 3-hydroxybutyrate dehydrogenase was assayed.

Binding Study - Purified pig heart 3-ketoacyl-CoA thiolase (3 ug) in 0.1 ml of 0.7M Tris-HCl (pH 8.2) containing 10% glycerol and 10mM mercaptoethanol was incubated with either 8uM 4-bromocrotonyl-[$^3$H]CoA (45,000 cpm) or 8 uM 3-keto-4-bromobutyryl-[$^3$H]CoA (55,000 cpm) for 20 min at 25°C. The reaction mixture was then rapidly filtered through Sephadex G-50 fine (1 ml) equilibrated with the incubation buffer as described in principle by Penefsky (61). Thiolase activities were determied before and after filtration. The radioactivity associated with thiolase was determined by liquid scintillation counting.

Measurement of Thiolase Activities at Various Respiratory States - Coupled rat heart mitochondria (0.5 mg/ml) were incubated with a substrate (100 uM palmitoylcarnitine, or 2.5mM pyruvate or 1mM acetoacetate) in a basal isotonic medium containing 0.11M KCl, 33mM Tris-HCl (pH 7.4), 2mM KPi, 2mM

$MgCl_2$ and 0.1mM EGTA for 5 min. at 25°C. 10mM glucose, 1mM ATP and 2U/ml hexokinase were added to provide a continuous regeneration of ADP in order to maintain a state 3 condition. Bovine serum albumin (0.5 mg/ml) was included when palmitoylcarnitine served as a substrate to prevent its inhibition of respiration. Malate (0.5mM) served as a primer to enhance respiration rates when using palmitoylcarnitine or pyruvate as substrates. 2-Ketoglutarate (0.5mM) was used when acetoacetate served as a respiratory substrate. The incubation of mitochondria with palmitoylcarnitine was performed in the presence and absence of 20mM malonate. To maintain state 4 respiration, ATP was omitted. After 5 min. of incubation, samples (20 ul-50 ul) were frozen in dry ice-methanol mixture. Aliquots of 10ul were thawed and assayed for acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase with acetoacetyl-CoA and 3-ketodecanoyl-CoA as substrates. Triton X-100 (0.05%) was present in the assay mixture to ensure the complete disruption of mitochondria.

Precipitation of 3-Ketoacyl-CoA Thiolase with Antibodies to Purified Pig Heart 3-Ketoacyl-CoA Thiolase - Coupled rat heart mitochondria (9 mg containing 40 ug 3-ketoacyl-CoA thiolase) were incubated with 100 uM palmitoylcarnitine or 10mM pyruvate and 10 uCi $^{32}P_i$ (50nmol) in 0.5 ml of a medium containing 1mM ATP, 10mM glucose, hexokinase (2 U/ml) and 0.5mM malate. To maintain mitochondria at state 4 respiration either ATP or hexokinase was deleted. The experiment was stopped after 5 min by the addition of 1mM

KCN. The mixture was centrifuged at maximum speed of the tabletop centrifuge (International Equipment Co., Model CL) and the supernatant was discarded. The pellet was washed twice more with the basal isotonic medium to remove unbound radioactive phosphate. The mitochondrial pellet was finally suspended in 0.5 ml of basal medium containing 20mM NaF and 0.05% Triton X-100. The mitochondria were disrupted by repeated freezing and thawing in a dry ice-methanol mixture. The mixture was then centrifuged at 120,000 x g for 45 min. The supernatant was collected and re-centrifuged. 400 ug of antibody was added to the final supernatant and incubated for 30 min. The mixture was centrifuged at 120,000 x g for 45 min and the pellet was washed twice. The recovered antigen-antibody complex was analyzed by SDS[2] gel electrophoresis. In control experiment no antibody was added. SDS gels were run in duplicates, and both were stained for protein with Coomasie Blue. They were first scanned at 500nm and then sliced into segments of 1mm. Each slice was incubated in 0.5ml of a 9:1 mixture of NCS reagent (Amersham/Searle) and $H_2O$ for 2 hrs. at 50°C according to the method of Diedrich et al. (62). The radioactivity of the resulting samples was determined by liquid scintillation counting.

Gel Electrophoresis - Polyacrylamide disc gel electrophoresis in the presence of SDS was performed at pH 8.3 according to the method of Laemmli (77). Protein samples (10 ug) were

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[2]SDS - Sodium dodecyl sulfate.

dissolved in 0.2 ml buffer containing 0.625M Tris (pH 6.8), 2% SDS, 10% glycerol, 5% mercaptoethanol and 0.001% bromophenol blue. Protein was dissociated by immersing samples in boiling water for 5 min. The standards used were catalase (57,500), chymotrypsinogen A (25,700), bovine serum albumin (68,000) and lactic dehydrogenase (35,000). Electrophoresis was performed at 3mA per gel for 3.5-4.5 hr. Protein was fixed by incubating gels in 50% trichloracetic acid (TCA) solution for 30 min. Gels were then stained in 0.1% Coomassie Blue in 50% TCA solution for 1 hr. at 37°C. Destaining for several hours in acetic acid (7%) in the presence of pure virgin wool resulted in the removal of background stain.

# RESULTS

The Effect of 4-Bromocrotonic Acid on Fatty Acid Oxidation in Rat Heart Mitochondria - The regulation of fatty acid oxidation is still poorly understood partly because the rate-limiting step of this pathway has not yet been definitely determined. It has previously been shown that 4-pentenoic acid inhibits fatty acid oxidation in rat heart mitochondria by specifically inactivating thiolase (28). Since the rates of inactivation of thiolase and of fatty acid oxidation were found to be nearly identical, it was suggested that the thiolase-catalyzed reaction may be rate-limiting in B oxidation or may be as slow as other steps are (28). Unfortunately, attempts to evaluate the rate of the thiolytic cleavage relative to the rate of ketone body metabolism in rat heart mitochondria by the same approach failed because 4-pentenoic acid is a good respiratory substrate in the presence of 2-ketoglutarate which was used in place of malate to stimulate respiration supported by ketone bodies. 2-Bromooctanoic acid, an effective and specific inhibitor of thiolase and thus of palmitoylcarnitine-supported respiration in rat liver mitochondria (64, 65), was ineffective in rat heart mitochondria. Thus various compounds were tested for their ability to specifically inactivate thiolase and consequently inhibit respiration supported by either palmitoylcarnitine or ketone bodies in rat heart mitochondria. 4-Bromocrotonic acid was found to be such an inhibitor.

Both palmitoylcarnitine and pyruvate support high rates

of respiration in isolated rat heart mitochondria as illustrated in Figs. 1A and 1C. When mitochondria were preincubated for 3 min in the presence of 20 uM 4-bromocrotonic acid, respiration supported by palmitoylcarnitine was completely inhibited (see Fig. 1B), whereas pyruvate-dependent respiration was unaffected (see Fig. 1D). This observation is indicative of the specific inhibition of fatty acid oxidation by 4-bromocrotonic acid. Since this compound inhibited also respiration supported by octanoic acid (Fig.2), which enters mitochondria in a carnitine-independent manner, the site at which the inhibitor blocks fatty acid oxidation must be within the B oxidation cycle. In order to identify the site of inhibition, I have assayed the enzymes of B oxidation present in a supernatant of disrupted mitochondria preincubated with 20 uM 4-bromocrotonic acid for 3 min. As shown in Table II, both thiolases present in rat heart mitochondria were completely inactivated, whereas none of the other oxidation enzymes was significantly affected. Thus, it is concluded that the observed inhibition of fatty acid oxidation by 4-bromocrotonic acid is a consequence of the inactivation of 3-ketoacyl-CoA thiolase. This inhibition was not observed with mitochondria uncoupled with 30uM 2,4-dinitrophenol. It is therefore likely that the inhibitor must first be activated by conversion to its CoA derivative and possibly must be further metabolized before it becomes inhibitory to thiolase. It is interesting to note that 4-bromobutyric acid did not inhibit respiration supported by

Fig. 1. Effect of 4-bromocrotonic acid on respiration supported by either palmitoylcarnitine or pyruvate in coupled rat heart mitochondria. For experimental details see under "Experimental Procedures." $C_{16}$-cn, palmitoylcarnitine (30 uM); 4BrCt, 4-bromocrotonic acid (20 uM); pyr, pyruvate (2.5 mM). The numbers represent the rates of respiration in ng-atoms $O_2$/min and mg of protein.

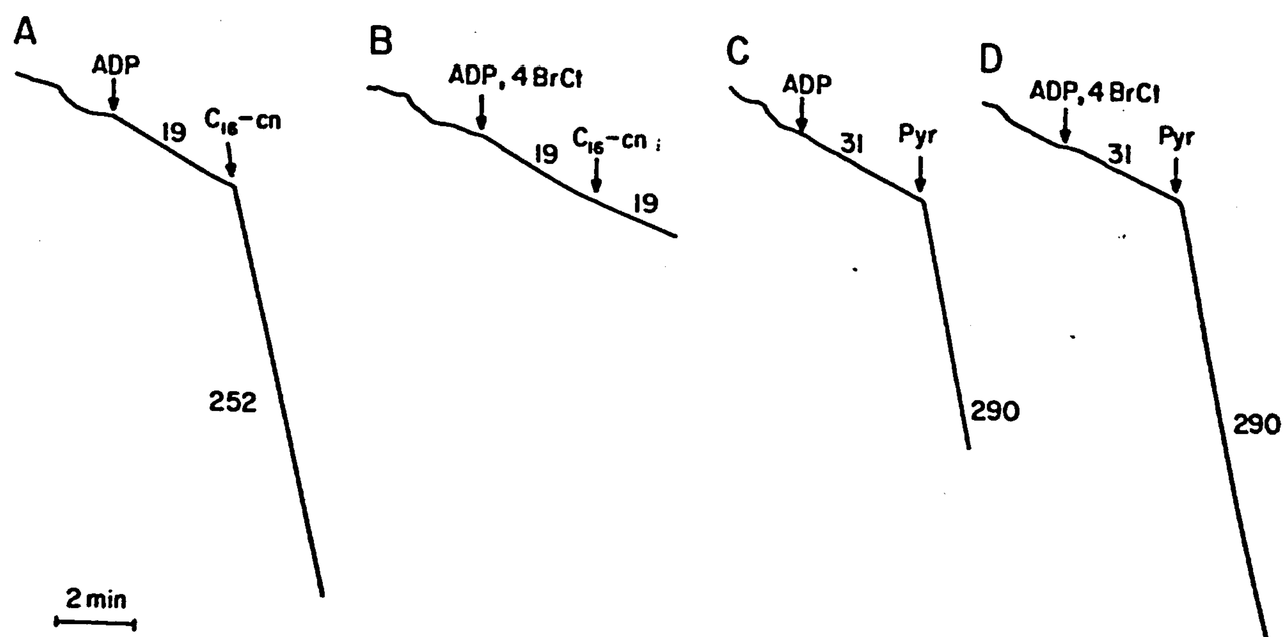



Fig. 1

Fig. 2. Effect of 4-bromocrotonic acid on respiration supported by octanoate in coupled rat heart mitochondria. For experimental details see under "Experimental Procedures." Octanoate (100 uM); 4BrCt, 4-bromocrotonic acid (20 uM). The numbers represent the rates of respiration in ng-atom $O_2$/min and 2 mg of protein.

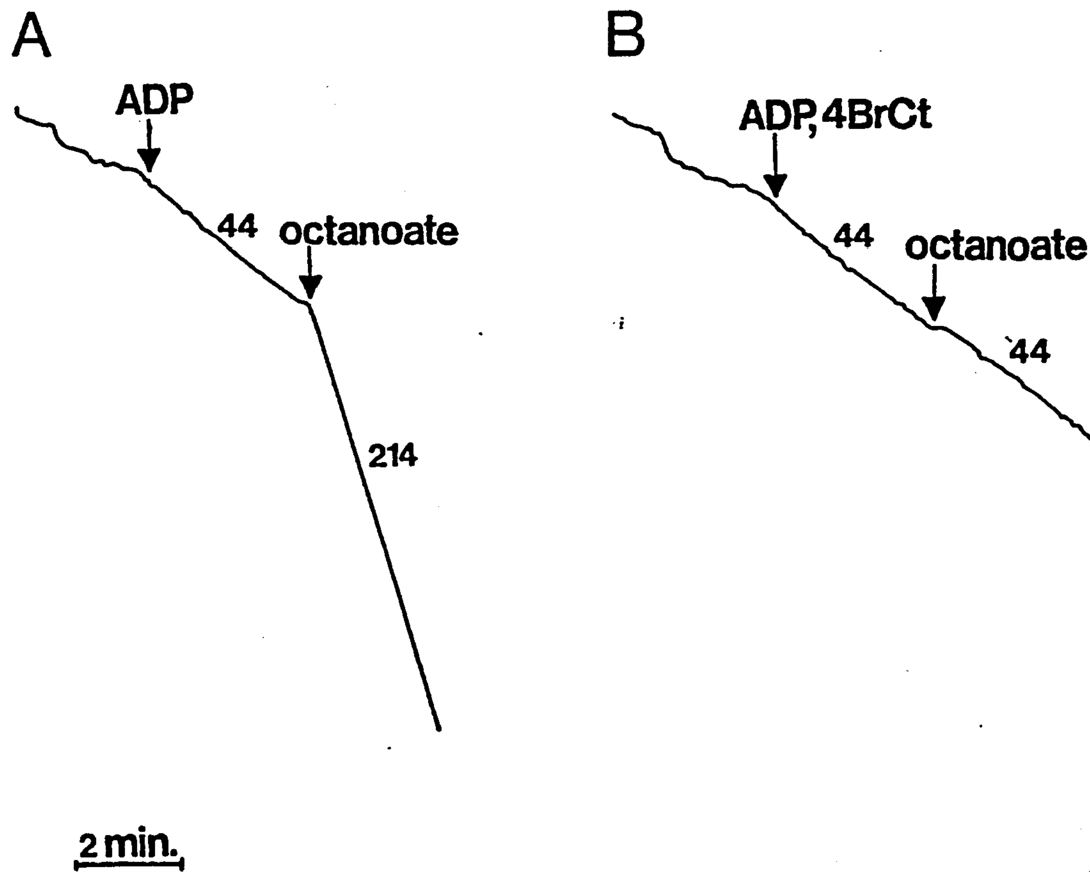



Fig. 2

Table II

Effect of 4-bromocrotonic acid on enzymes of fatty acid oxidation. Mitochondria were preincubated for 3 min with 20uM 4-bromocrotonic acid and were assayed for the enzymes of B oxidation as described under "Experimental Procedures."

| Enzyme | Substrate | Control[a] | + 4-Bromocrotonic Acid | Remaining Activity |
|---|---|---|---|---|
| | | umol/min/mg protein | | % |
| Acyl-CoA dehydrogenase | Butyryl-CoA | 0.072 | 0.072 | 100 |
| | Decanoyl-CoA | 0.064 | 0.064 | 100 |
| | Palmitoyl-CoA | 0.086 | 0.086 | 100 |
| Enoyl-CoA hydratase | Crotonyl-CoA | 2.12 | 2.12 | 100 |
| | 2-Decenoyl-CoA | 1.33 | 1.22 | 92 |
| 3-Hydroxyacyl-CoA dehydrogenase | Acetoacetyl-CoA | 0.915 | 0.915 | 100 |
| Thiolase | Acetoacetyl-CoA | 0.40 | 0 | 0 |
| | 3-Ketodecanoyl-CoA | 0.38 | 0 | 0 |

[a]Activities measured in the absence of 4-bromocrotonic acid.

palmitoylcarnitine most likely due to the observed inactivity of butyryl-CoA dehydrogenase toward 4-bromobutyryl-CoA.

The inhibition of palmitoylcarnitine-supported respiration and the inactivation of the two thiolases were studied as a function of the concentration of 4-bromocrotonic acid. As illustrated in Fig. 3A acetoacetyl-CoA thiolase, which is believed to function only in ketone body degradation, was inhibited more rapidly ($I_{50}$ = 2uM) than was 3-ketoacyl-CoA thiolase ($I_{50}$ = 4 uM) which functions in fatty acid oxidation. The latter enzyme is completely inhibited by 15uM 4-bromocrotonic acid. The inhibition curve obtained for 3-ketoacyl-CoA thiolase parallels the inhibition pattern observed for palmitoylcarnitine-dependent respiration (see Fig. 3A). This finding supports the previous conclusion (28) that the thiolase-catalyzed reaction may be rate-limiting in fatty acid oxidation or at least may be as slow as other steps are. The inhibition of respiration by 4-bromocrotonic acid is also a function of time (see Fig. 3B). In the presence of 20 uM inhibitor respiration is completely inhibited within 2 min.

The Effect of 4-Bromocrotonic Acid on Ketone Body Degradation in Rat Heart Mitochondria - The effect of 4-bromocrotonic acid on respiration supported by either 3-hydroxybutyrate or acetoacetate was studied with coupled rat heart mitochondria. As can be seen in Fig. 4 the inhibitor at a concentration of 20 uM and after 3 min of preincubation caused the complete inhibition of acetoacetate-supported respiration, whereas

Fig. 3A. Inhibitions of palmitoylcarnitine-supported respiration, 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase as a function of the concentration of 4-bromocrotonic acid. Coupled rat heart mitochondria were preincubated for 3 min with the inhibitor and assayed for palmitoylcarnitine-dependent respiration (■), pyruvate-dependent respiration (▲), 3-ketoacyl-CoA thiolase (●) and acetoacetyl-CoA (O) as described under "Experimental Procedures."

B. Inhibition of palmitoylcarnitine-supported respiration by 4-bromocrotonic acid as a function of the preincubation time. Coupled rat heart mitochondria were preincubated with 20 uM 4-bromocrotonic acid and assayed for palmitoylcarnitine-supported respiration (■) and pyruvate-supported respiration (▲) as described under "Experimental Procedures."

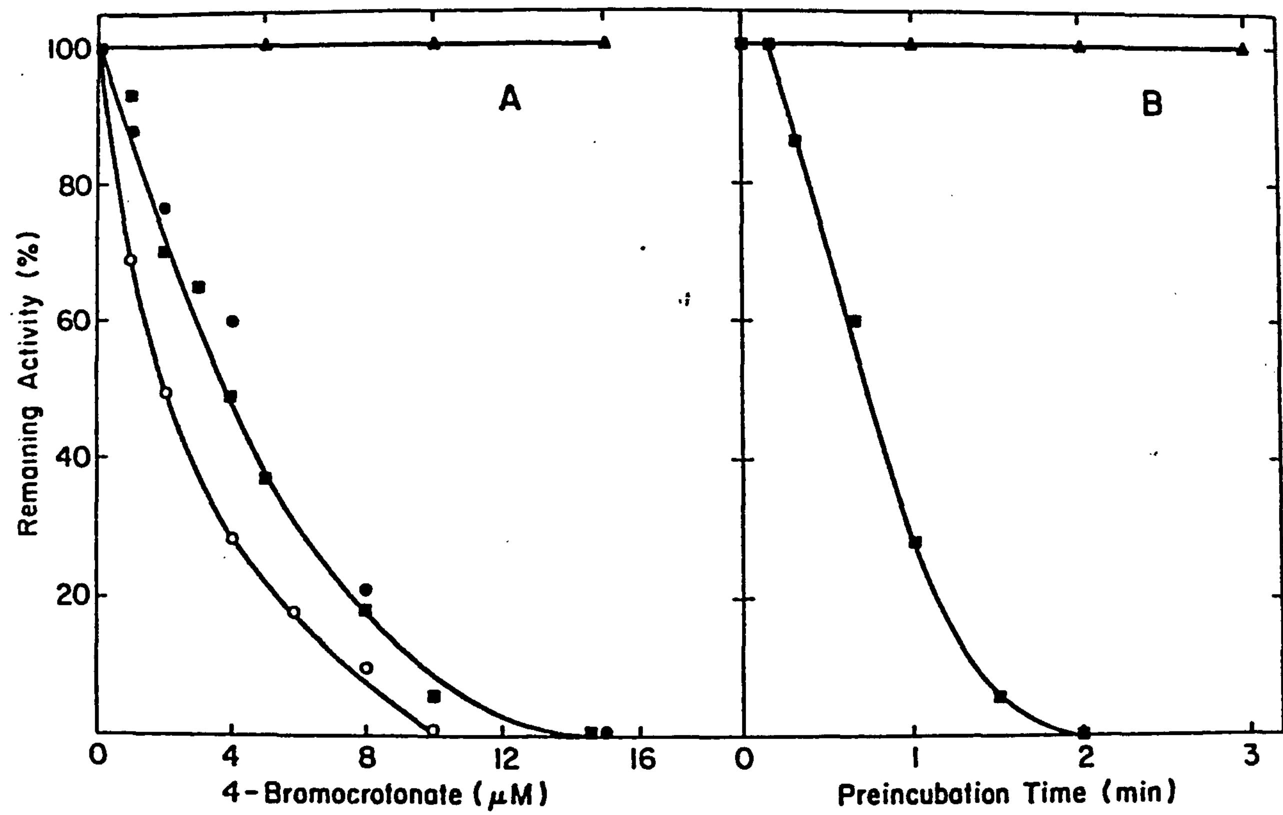



Fig. 3

Fig. 4. Effect of 4-bromocrotonic acid on respiration supported by ketone bodies in coupled rat heart mitochondria. For experimental details see under "Experimental Procedures." $BOHC_4$, 3-hydroxybutyrate (1 mM); AcAcOH, acetoacetate (1mM); 4BrCt, 4-bromocrotonic acid (20 uM). The numbers represent the rates of respiration in ng-atom $O_2$/min and 2 mg of protein.

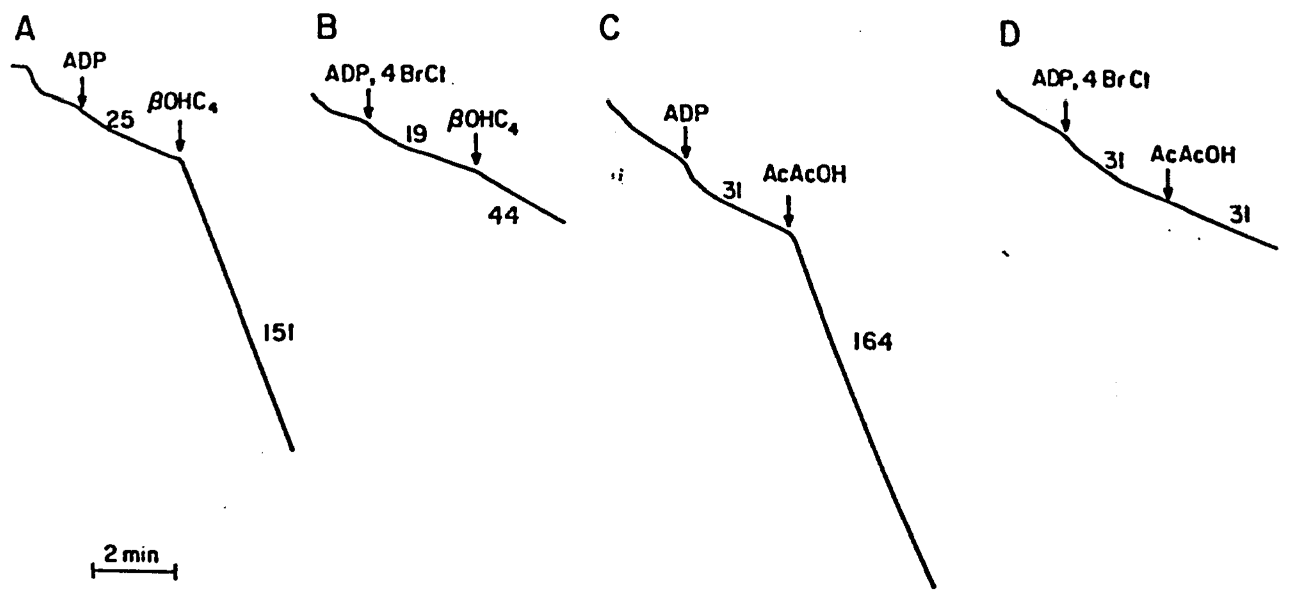



Fig. 4

respiration sustained by 3-hydroxybutyrate was reduced by 80%. Assays of individual enzymes of ketone body degradation proved that only acetoacetyl-CoA thiolase was inhibited (see Table III). The inhibition of acetoacetyl-CoA thiolase and of respiration supported by 3-hydroxybutyrate as a function of the 4-bromocrotonate concentration is shown in Fig. 5A. Clearly acetoacetyl-CoA thiolase at low concentrations of the inhibitor is more severely inhibited than is respiration. The residual rate of respiration not sensitive to 4-bromocrotonate is probably sustained by NADH formed during the dehydrogenation of 3-hydroxybutyrate to acetoacetate. The inactivation of acetoacetyl-CoA thiolase by 4-bromocrotonic acid is also more rapid than is the inhibition of respiration supported by acetoacetate (see Fig. 5B). Although the difference between the inhibition of the enzyme and that of the overall process was slight, it was reproducible and significant.

<u>Metabolism of 4-Bromocrotonic Acid in Rat Heart Mitochondria</u> - The observation that 4-bromocrotonic acid inhibits the two thiolases and consequently fatty acid oxidation in coupled, but not in uncoupled rat heart mitochondria, suggests that the acid must be activated in an energy-dependent reaction before it becomes inhibitory. 4-Bromocrotonmic acid is most likely converted to 4-bromocrotonyl-CoA by medium chain acyl-CoA synthetase which is located in the mitochondrial matrix (66). 4-Bromocrotonyl-CoA has been shown to be an inhibitor of isolated thiolase (67). When a supernatant of

Table III

Effect of 4-bromocrotonic acid on the enzymes of ketone body degradation. Mitochondria were preincubated for 3 min with 20 uM 4-bromocrotonic acid and assayed for the enzymes of ketone body degradation as described under "Experimental Procedures."

| Enzyme | Substrate | Control[a] | + 4-Bromocrotonic Acid | Remaining Activity |
|---|---|---|---|---|
| | | umol/min/mg protein | | % |
| Acetoacetyl-CoA thiolase | Acetoacetyl-CoA | 0.52 | 0 | 0 |
| 3-Ketoacid-CoA transferase | Succinate Acetoacetyl-CoA | 1.67 | 1.67 | 100 |
| 3-Hydroxybutyrate dehydrogenase | 3-Hydroxybutyrate | 0.021 | 0.021 | 100 |

[a] Activities measured in the absence of 4-bromocrotonic acid.

Fig. 5. Inhibitions of acetoacetyl-CoA thiolase and respiration supported by either 3-hydroxybutyrate or acetoacetate as a function of the concentration of 4-bromocrotonic acid. Coupled rat heart mitochondria were preincubated for 3 min with the inhibitor and assayed as described under "Experimental Procedures." A, 3-hydroxybutyrate-dependent respiration (■); acetoacetyl-CoA thiolase (●). B, acetoacetate-dependent respiration (▲); acetoacetyl-CoA thiolase (●).

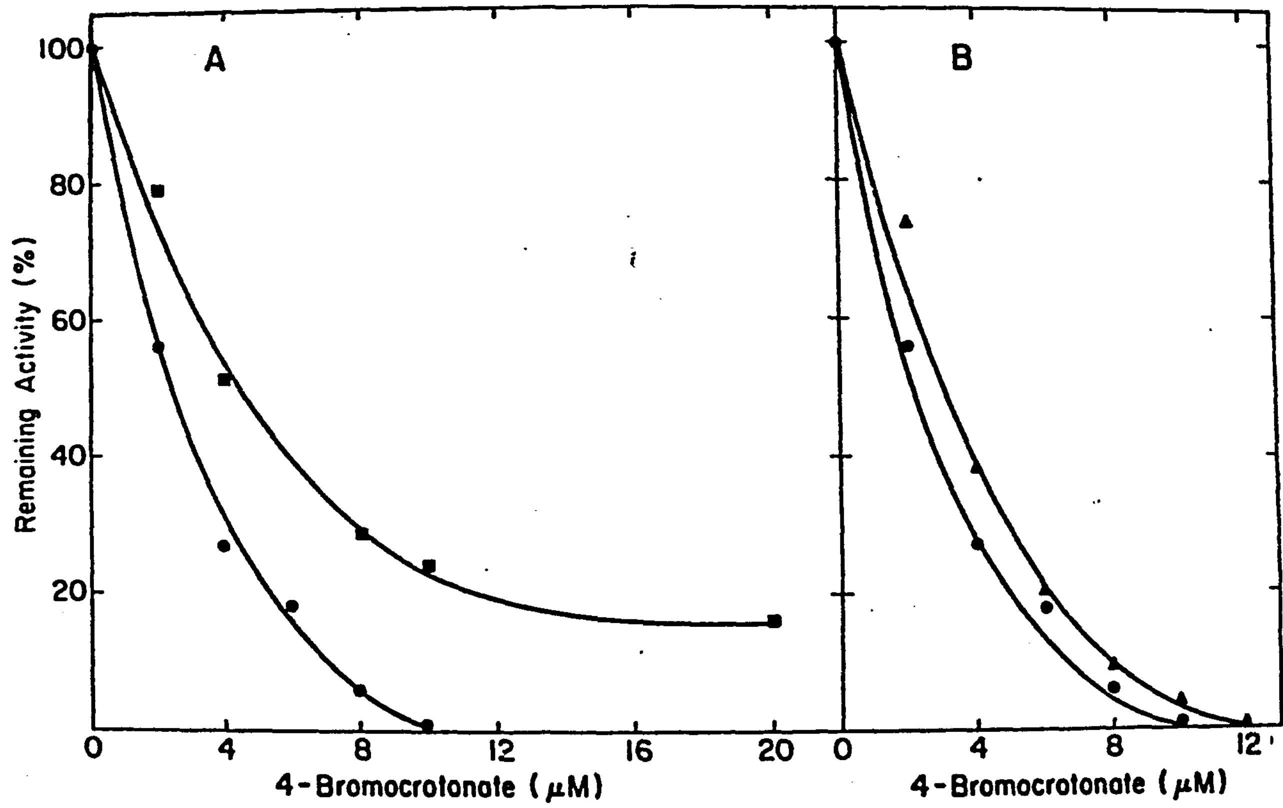



Fig. 5

disrupted rat heart mitochondria was incubated with 20 uM 4-bromocrotonyl-CoA, a partial inhibition of thiolase activities was detected with either acetoacetyl-CoA (see Fig. 6A) or 3-ketodecanoyl-CoA (see Fig. 6B) as substrates. However, when additionally NAD was present in the incubation mixture, the inhibition of the two thiolase activities was much more rapid and complete within 15 min. This observation suggests that 4-bromocrotonyl-CoA is metabolized, most likely via B oxidation, and that one of its metabolites is an efficient inhibitor of both thiolases present in heart mitochondria. Experiments with purified enzymes demonstrated that 4-bromocrotonyl-CoA is acted upon by crotonase (See Fig. 7) as has been reported by Steinman and Hill (68). The rapid hydration of 4-bromocrotonyl-CoA to 3-hydroxy-4-bromocrotonyl-CoA by crotonase may be the reason why the inhibition of thiolase present in a mitochondrial extract stops after a few minutes (see Fig. 6). The product of the crotonase-catalyzed reaction, presumably L-3-hydroxy-4-bromobutyryl-CoA, is a substrate for L-3-hydroxyacyl-CoA dehydrogenase as evidenced by the formation of NADH (see Fig. 7). The expected formation of both NADH and 3-keto-4-bromobutyryl-CoA is illustrated in Fig. 8A, curve 1, which shows the overlapping absorbance peaks of NADH and the $Mg^{2+}$-enolate complex of 3-keto-4-bromoburyryl-CoA. Addition of EDTA to this solution resulted in a large decrease in absorbance due to the disappearance of the $Mg^{2+}$-enolate complex (see Fig. 8A, curve 2). When 3-keto-4-bromobutyryl-CoA was generated

Fig. 6. Inactivation of thiolases present in an extract of rat heart mitochondria. Soluble mitochondrial proteins (0.2 mg/ml) in 2 ml of 0.2 M Tris-HCl (pH 8.2) containing 25 mM $MgCl_2$, 30 mM KCl, 20% glycerol and 10 mM mercaptoethanol were incubated in the presence of either 20 uM 4-bromocrotonyl-CoA (▲) or 20 uM 4-bromocrotonyl-CoA plus 0.23 mM NAD (●). Aliquots were assayed for thiolase activities with acetoacetyl-CoA (A) or 3-ketodecanoyl-CoA (B) as substrates as described under "Experimental Procedures."

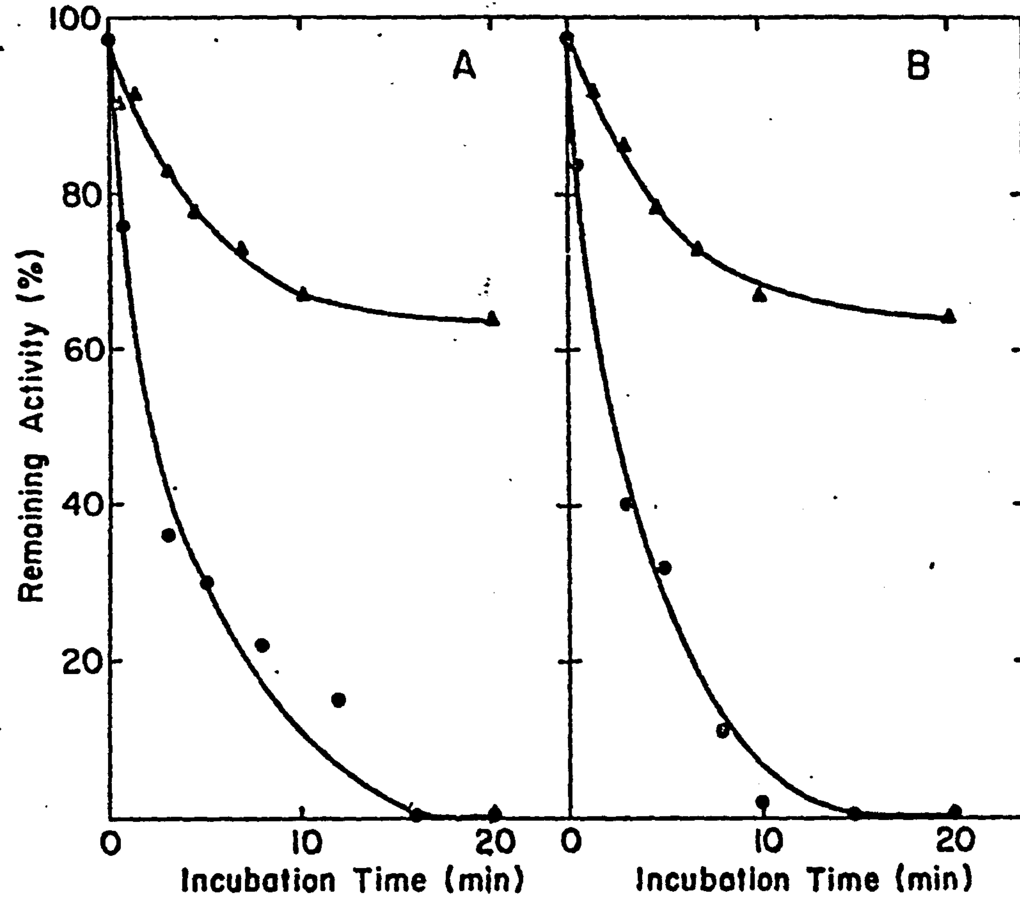



Fig. 6

Fig. 7. Spectrophotometric evidence for the conversion of 4-bromocrotonyl-CoA to 3-keto-4-bromobutyryl-CoA. The reaction was started by the addition of crotonase (4mU) to 4-bromocrotonyl-CoA (11 nmoles) in 0.6 ml of 0.1 M Tris-HCl (pH 8.2) containing 25 mM $MgCl_2$ and 30 mM KCl. After completion of the hydration reaction NAD (0.14 umol) and 3-hydroxyacyl-CoA dehydrogenase (1.5 U) were added to the assay mixture.

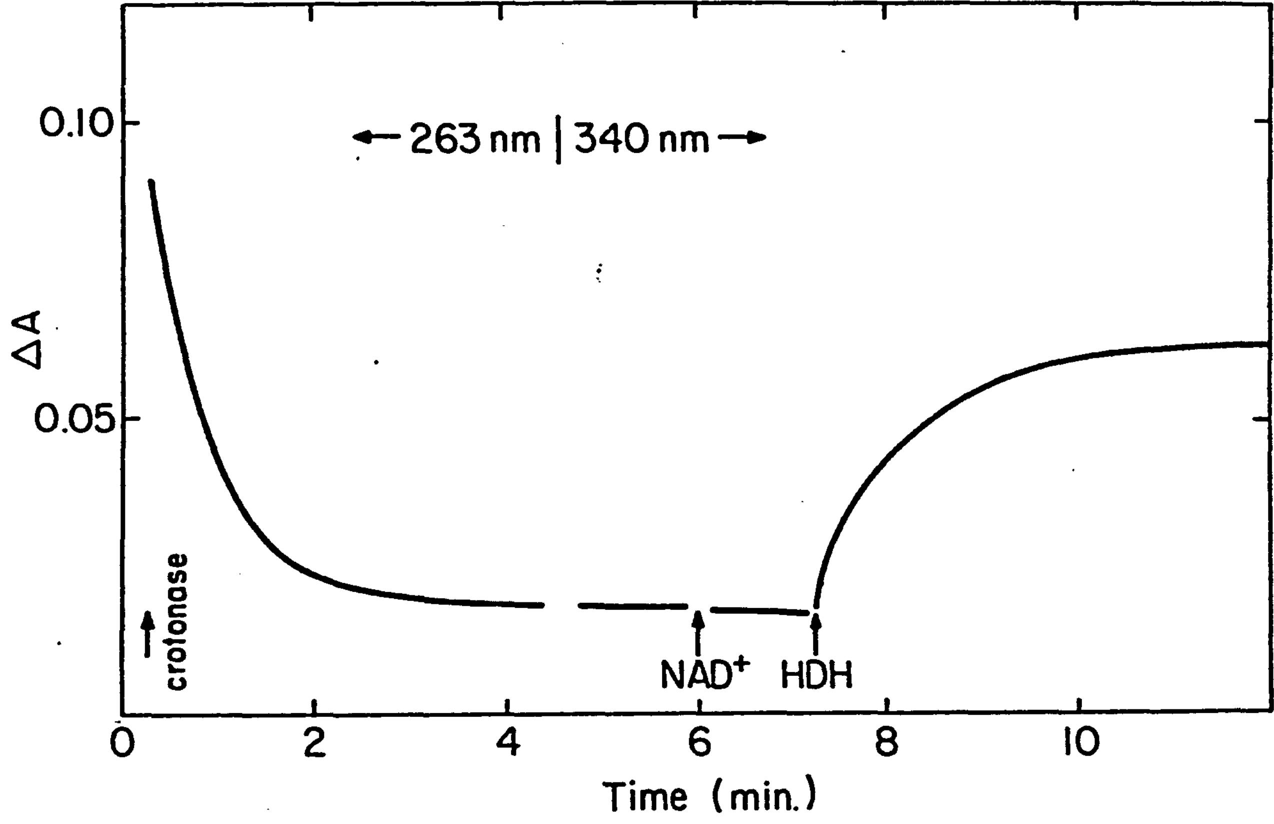



Fig. 7

Fig. 8 The spectrum of $Mg^{2+}$-enolate complex of 3-keto-4-bromobutyryl-CoA. A: (1) The spectrum obtained when 4-bromocrotonyl-CoA (18 nmol) in 1 ml of 0.1 M Tris-HCl (pH 8.2) containing 25 mM $MgCl_2$, and 30 mM KCl was incubated with crotonase (7 mU), NAD (0.23 umol) and 3-hydroxyacyl-CoA dehydrogenase (2.4 U). (2) The spectrum after addition of EDTA (45 umol) to the above described reaction mixture. B: The spectrum obtained when the reaction mixture described under A (1) contained additionally sodium pyruvate (1 umol) and lactate dehydrogenase (4 U) to reoxidize NADH. (1) 10 min, (2) 20 min and (3) 60 min after the start of the reaction.

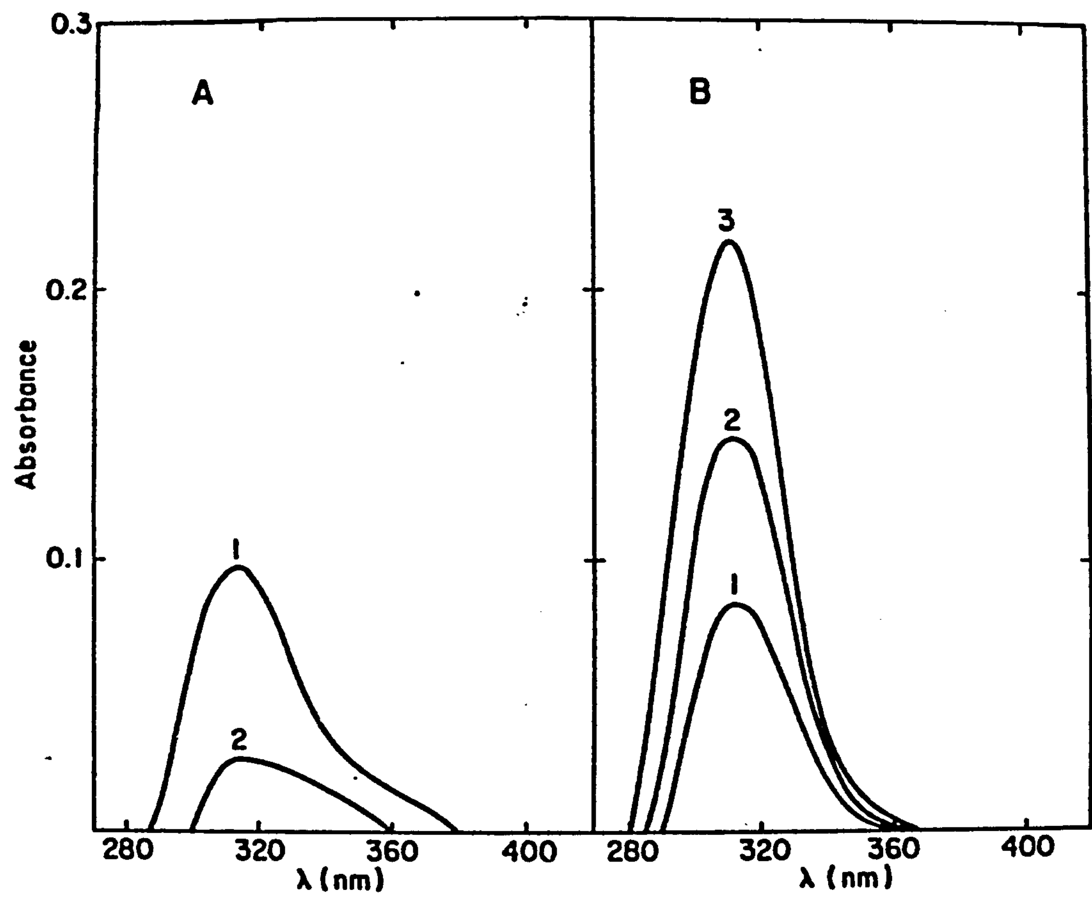


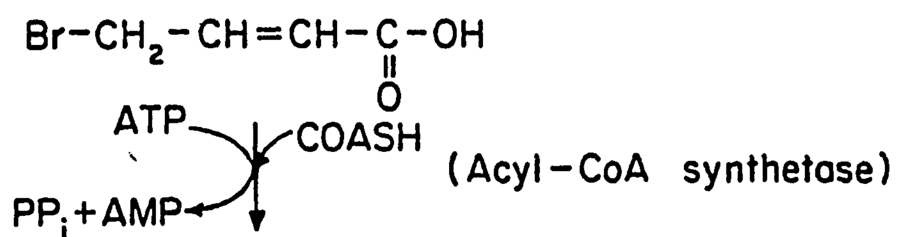

Fig. 8

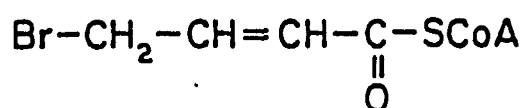
enzymatically from 4-bromocrotonyl-CoA in the presence of pyruvate and lactate dehydrogenase to re-oxidize NADH, the spectrum of only the $Mg^{2+}$-enolate complex of 3-keto-4-bromobutyryl-CoA was observed. As shown in Fig. 8B the formation of 3-keto-4-bromobutyryl-CoA is time-dependent and the maximum absorbance of its $Mg^{2+}$-enolate complex is observed at 310 nm. Since the absorbance maximum of the corresponding complex of acetoacetyl-CoA is at 303 nm (57), the bromine substituent causes a red shift of 7 nm. In Fig. 9 is the summarized outline regarding the metabolism of 4-bromocrotonic acid in rat heart mitochondria. 4-bromocrotonic acid is converted to its CoA ester, hydrated by crotonase and dehydrogenated by 3-hydroxyacyl-CoA dehydrogenase to 3-keto-4-bromobutyryl-CoA which is an effective inhibitor of both acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase. When 20 uM bromoacetyl-CoA, a possible thiolytic cleavage product of 3-keto-4-bromobutyryl-CoA was incubated with a mitochondrial extract, neither of the two thiolases was inhibited.

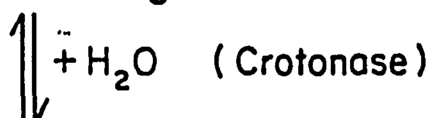
Mechanism of Inhibition of Thiolases by 3-Keto-4-bromobutyryl-CoA - When purified 3-ketoacyl-CoA thiolase was preincubated and assayed in the presence of 2 uM 3-keto-4-bromobutyryl-CoA, its activity decreased by two thirds in less than 10 seconds. The enzyme was completely inactivated within 20 min (see Fig. 10A). In contrast, 2 uM 4-bromocrotonyl-CoA caused only a slight inhibition which did not increase with time (see Fig. 10A). A similar result was obtained when 3-ketoacyl-CoA

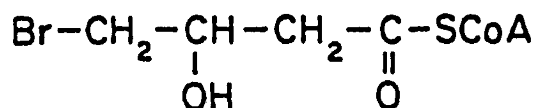
Fig. 9. Proposed metabolism of 4-bromocrotonic acid in rat heart mitochondria.

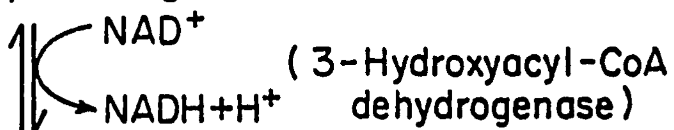
## Metabolism of 4 - Bromocrotonic Acid



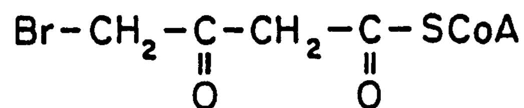



Fig. 10 Inhibition of purified rat heart 3-ketoacyl-CoA thiolase by metabolites of 4-bromocrotonic acid. A: 3-keto-acyl-CoA thiolase (0.19 ug) in 0.6 ml of 0.1 M Tris-HCl (pH 8.2) containing 25 mM $MgCl_2$ and 30 mM KCl was incubated in the presence of 2 uM 4-bromocrotonyl-CoA (O) or 2 uM 3-keto-4-bromobutyryl-CoA (●) and assayed as described under "Experimental Procedures" in the presence of 2 uM bromocrotonyl-CoA or 2 uM 3-keto-4-bromobutyryl-CoA respectively. B: 3-Keto-acyl-CoA thiolase (3 ug) in 0.1 ml of 0.7 M Tris-HCl (pH 8.2), containing 10% glycerol and 10 mM mercaptoethanol was incubated in the presence of either 6 uM 3-keto-4-bromobutyryl-CoA (●), or 6 uM 3-keto-4-bromobutyryl-CoA plus 1 mM acetoacetyl-CoA (Δ) or no addition (O). The enzyme was assayed as described under "Experimental Procedures."

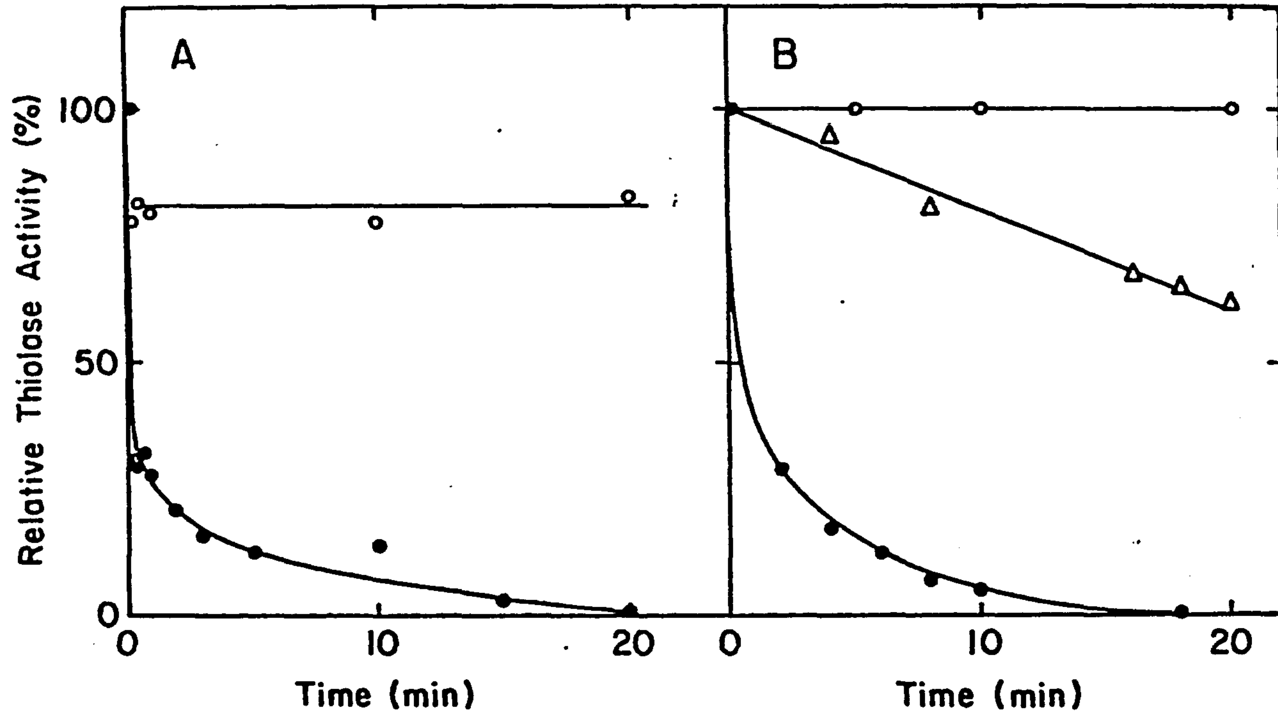



Fig. 10

thiolase was preincubated with 6 uM 3-keto-4-bromobutyryl-CoA but assayed in the presence of only 50 nM of the inhibitor (see Fig. 10B). Most important was the observation that 1 mM acetoacetyl-CoA effectively protected 3-ketoacyl-CoA thiolase against inactivation by 6 uM 3-keto-4-bromobutyryl-CoA (see Fig. 10B). Thus the inhibitor seems to inactivate thiolase after first binding to the active site of the enzyme. This conclusion is not surprising because the inhibitor has all the structural elements of a substrate for thiolase and is highly effective at low concentrations. The time-dependence of the inhibition of thiolase by 3-keto-4-bromobutyryl-CoA suggests that the inhibition is a consequence of a covalent modification of the enzyme. To provide direct evidence for this hypothesis 3-ketoacyl-CoA thiolase was inactivated in the presence of 3-keto-4-bromobutyryl-[$^3$H]CoA. After separating thiolase from the incubation medium by rapid filtration through Sephadex G-50, no radioactivity was found to be associated with the enzyme which remained inactive (Table IV). Hence, the complete inhibitor molecule is not attached to the inactivated enzyme. However it is possible that the part of 3-keto-4-bromobutyryl-[$^3$H]CoA which is not radioactively labeled remains bound to the inactive enzyme.. When 3-keto-4-bromobutyryl-CoA was incubated with CoASH and a large amount of purified pig heart 3-keto-acyl-CoA thiolase (3.8 ug) no evidence for a measurable thiolytic cleavage of the inhibitor was obtained.

Table IV

Inactivation of 3-Ketoacyl-CoA Thiolase by 3-Keto-4-bromobutyryl-CoA

Enzyme was incubated with the indicated compound for 20 min. before filtration. The reaction mixture was filtered through Sephadex as described under "Experimental Procedures".

| | E | E-4-BrCt-CoA[a] | E-I[a] | 4-BrCt-CoA[b] |
|---|---|---|---|---|
| Activity (u/mg) before filtration | 8.67 | 8.67 | 0 | |
| after filtration | 5.72 | 5.72 | 0 | |
| Counts before filtration (cpm) | | 44,771 | 55,570 | |
| Counts after filtration (cpm) | 43 | 484 | 267 | 220 |

a) E, 3-ketoacyl-CoA thiolase; 4-BrCt-CoA, 4-bromocrotonyl-CoA; I, 3-keto-4-bromobutyryl-CoA.

b) Control in absence of enzyme.

DISCUSSION

4-Bromocrotonic acid was found to be an effective inhibitor of respiration supported by either fatty acids or ketone bodies. Since pyruvate oxidation was unaffected by this inhibitor, neither the tricarboxylic acid cycle nor oxidative phosphorylation are inhibited by 4-bromocrotonic acid. Also the levels of CoASH and NAD are not lowered dramatically or otherwise pyruvate oxidation would be inhibited. The impaired oxidation of octanoate, which enters mitochondria independent of carnitine, does not agree with the mitochondrial uptake ot fatty acids as the primary site of inhibition. Consequently one of the steps of B oxidation must be the site of inhibition. When the enzymes of B oxidation were assayed, only 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase, which function in fatty acid oxidation and ketone body degradation respectively, were found to be inactivated by 4-bromocrotonic acid. Thus, it is concluded that specific inactivation of the thiolases is the cause for the inhibitions of fatty acid oxidation and ketone body degradation in rat heart mitochondria.

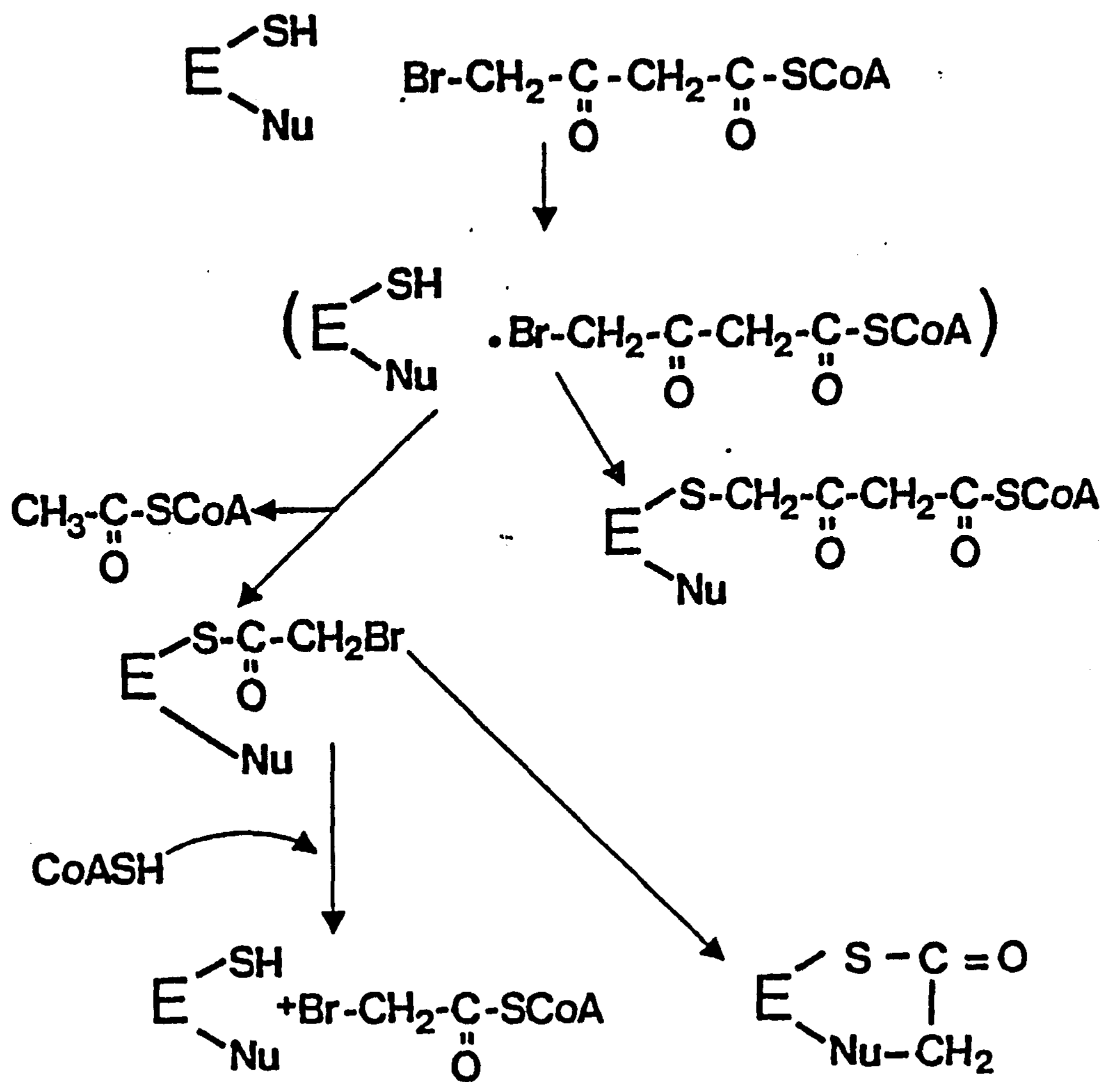
The repeated observation of a lag phase in the 4-bromocrotonate-dependent inhibition of respiration (see Fig. 3B) led to the suspicion that 4-bromocrotonic acid must first be metabolized before it becomes inhibitory to thiolase. Since the inhibitor affected respiration in coupled, but not in uncoupled mitochondria, it is most likely first converted intramitochondrially to its CoA derivative in an ATP-dependent

reaction. The resulting 4-bromocrotonyl-CoA is rapidly hydrated by crotonase, presumably to L-3-hydroxy-4-bromobutyryl-CoA, which in turn is dehydrogenated by NAD in the presence of L-3-hydroxyacyl-CoA dehydrogenase to yield 3-keto-4-bromobutyryl-CoA. Of the metabolites of 4-bromocrotonic acid, 4-bromocrotonyl-CoA has been reported to be an inhibitor of purified thiolase (67). 3-Keto-4-bromobutyryl-CoA is expected to be an even more effective inhibitor because it should bind to thiolase as a substrate and should be very susceptible to a nucleophilic substitution at the $\gamma$ carbon. The results demonstrate that 3-keto-4-bromobutyryl-CoA of all metabolites of 4-bromocrotonic acid is by far the most effective inhibitor of thiolase.

The most likely mechanisms by which 3-keto-4-bromobutyryl-CoA inhibits thiolase are shown in Fig. 11. 3-Keto-4-bromobutyryl-CoA binds first noncovalently to thiolase at its active site as suggested by the observed protection against inhibition in the presence of acetoacetyl-CoA. However, the inhibition of thiolase is not just a consequence of a noncovalent interaction with the inhibitor, because filtration of thiolase preincubated with 3-keto-4-bromobutyryl-[$^3$H]CoA through Sephadex G-50 yielded the still inactive enzyme devoid of radioactivity. The same experiment also eliminated the possibility that a covalent enzyme-inhibitor complex may have been formed via a simple nucleophilic displacement of bromine by either the sulfydryl group

Fig. 11. Proposed mechanism of 3-ketoacyl-CoA thiolase inhibition by 3-keto-4-bromobutyryl-CoA.

Fig. 11



or another nucleophilic group present at the active site. Thus, most like thiolase cleaves the inhibitor to yield the bromoacetylated form of the enzyme. The resulting form of thiolase is inactive because (a) it is very slowly or not at all deacylated, (b) it is not deacylated and additionally it undergoes a substitution reaction in which bromine is displaced by a nucleophilic group present at the active site of the enzyme (c) it is deacylated but it also undergoes the substitution reaction mentioned in (b). Since no evidence for a measurable thiolytic cleavage of 3-keto-4-bromobutyryl-CoA was obtained, even when a larger amount of thiolase was used, it appears that the deacylation reaction does not take place at a measurable rate. Therefore, the probable mechanism of inhibition of thiolase by 3-keto-4-bromobutyryl-CoA is one of those listed in (a) and (b).

The availability of a compound which specifically inhibits both thiolases present in coupled rat heart mitochondria, has provided the opportunity to determine whether rates of fatty acid oxidation and ketone body degradation in isolated mitochondria are determined by the activities of 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase respectively. In a multienzyme system, in which intermediates are not channeled from one active site to the next, partial inactivation of the enzyme that catalyzes the rate-limiting step would result in a proportional inhibition of the overall pathway. In contrast, partial inactivation of an enzyme that catalyzes an equilibrium reaction would result

in a lesser or no inhibition of the pathway. The observation of parallel inactivations of 3-ketoacyl-CoA thiolase and palmitoylcarnitine-supported respiration agrees with the previous suggestion that the thiolase-catalyzed reaction is either rate-limiting in B oxidation or at least is as slow as other reactions are (28). Although this hypothesis agrees with the observation, it is not sufficiently supported by it, if channeling of B oxidation intermediates occurs as has been suggested (69).

A comparison of the rate, at which acetoacetate-supported respiration is inhibited by 4-bromocrotonic acid, with the rate of inactivation of acetoacetyl-CoA thiolase leads to the conclusion that this step is not rate-limiting in ketone body degradation. The incomplete inhibition of 3-hydroxybutyrate-supported respiration by 4-bromocrotonic acid is most likely due to the unaffected oxidation of 3-hydroxybutyrate to acetoacetate. Continuation of this process requires the mitochondrial uptake of 3-hydroxybutyrate and efflux of acetoacetate to be at least as rapid as the dehydrogenation of 3-hydroxybutyrate is, which was observed to be 12.5 nmol/min and mg of protein. If in uninhibited mitochondria 3-hydroxybutyrate is completely oxidized to $CO_2$, the observed rate of respiration corresponds to a rate of 3-hydroxybutyrate breakdown of 7 nmol/min and mg of protein. Since this value is lower than the values of 12.5 nmol/min and mg of protein observed with mitochondria containing inactive thiolase, both the uptake and dehydrogenation of 3-hydroxybutyrate are not rate-limiting in ketone

body degradation in normal heart mitochondria. Since the thiolase-catalyzed step does not seem to be rate-limiting either and since the capacity of rat heart mitochondria to oxidize acetate units greatly exceeds their ability to degrade ketone bodies, the activation of acetoacetate catalyzed by 3-ketoacid-CoA transferase appears to be the rate-limiting step in ketone body degradation in heart. The rate of acetoacetate activation and consequently that of the overall pathway is most likely regulated by the availability of succinyl-CoA as has been suggested by Hatefi and Fakouhi (70).

## THE REGULATION OF THIOLASES

Since 3-ketoacyl-CoA thiolase catalyzes the rate-limiting step in B oxidation or at least one of several slow steps, it is possible that B oxidation is controlled via the regulation of this enzyme.

## RESULTS

Regulators of Pig Heart Thiolases - In order to evaluate the possible regulation of thiolases, I have studied the effect of various coenzymes and metabolites on the activities of purified 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase from pig heart. The CoASH concentrations used were based on the data of Hansford and Johnson (71), who determined that the CoASH content during oxidation of palmitoylcarnitine by rabbit mitochondria varies from about 20 uM at state 4 respiration to 200 uM at state 3 respiration. The concentration of acetoacetyl-CoA was kept at a near saturating value of 33 uM. The concentrations of metabolites and coenzymes were chosen to be as high or higher than their intramitochondrial concentrations. Results obtained for some metabolites and coenzymes are shown in Table V. Of all compounds tested, acetyl-CoA was the most effective inhibitor of 3-ketoacyl-CoA thiolase and was as effective as 3-hydroxybutyryl-CoA in inhibiting acetoacetyl-CoA thiolase. The observed inhibition of the two thiolases was expected because acetyl-CoA is the product of the thiolase-catalyzed reaction. However, unexpected were the different sensitivities of the two thiolases towards acetyl-CoA (see Fig. 12). 3-Ketoacyl-

Table V

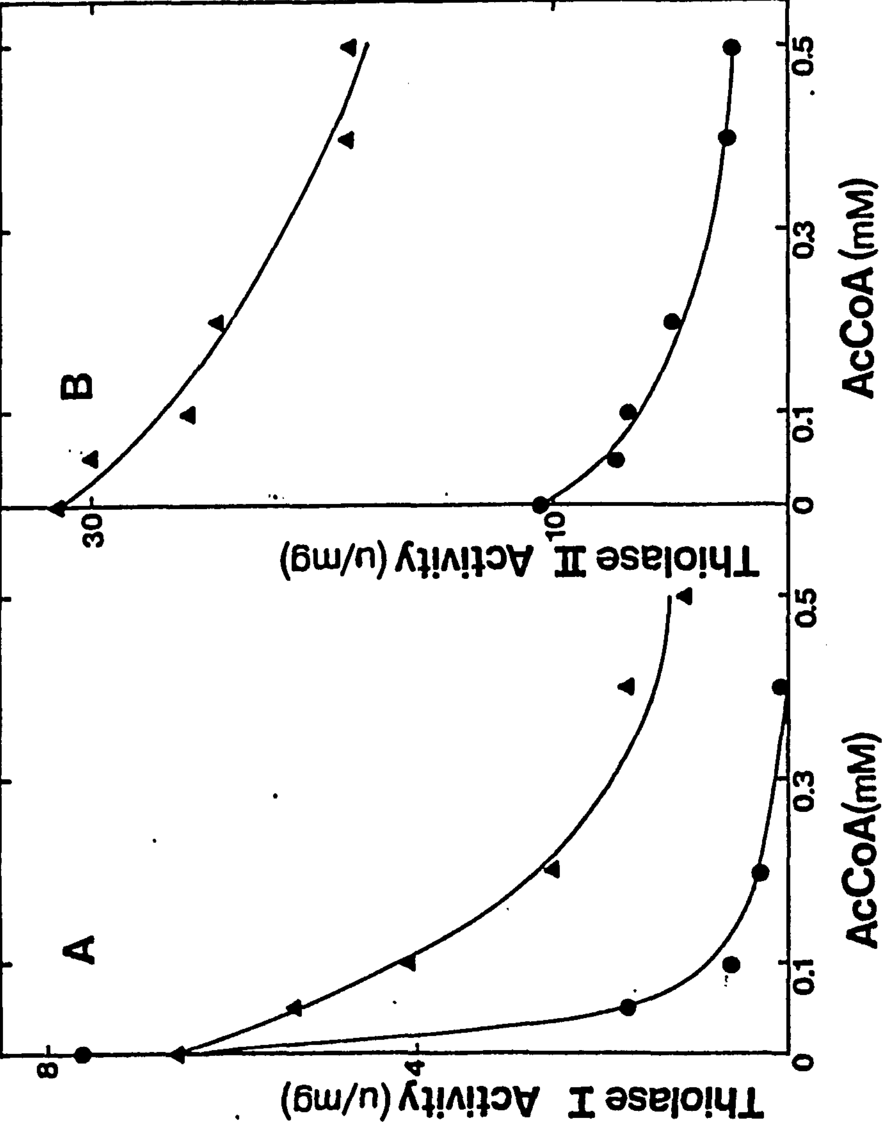
Effects of some mitochondrial coenzymes and metabolites on the activities of 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase. Assays were performed as described under "Experimental Procedures" with 33 uM acetoacetyl-CoA as substrate.

| Coenzymes and metabolites | Concentration | Remaining Activity (%)[a] | | | |
|---|---|---|---|---|---|
| | | 3-Ketoacyl-CoA Thiolase | | Acetoacetyl-CoA Thiolase | |
| | mM | 20 uM CoA | 200 uM CoA | 20 uM CoA | 200 uM CoA |
| | | % | | | |
| Acetyl-CoA | 0.02 | 49 | 92 | 89 | 96 |
| Acetyl-CoA | 0.5 | 0 | 17 | 30 | 62 |
| 3-Hydroxybutyryl-CoA | 0.5 | 46 | | 28 | |
| Decanoyl-CoA | 0.05 | 64 | 68 | 92 | 91 |
| Decanoyl-CoA | 0.2 | 24 | 52 | 72 | 73 |
| Succinyl-CoA | 0.5 | 100[b] | 102 | 113 | 73[b] |
| ADP | 10 | 89 | 104 | 63 | 88 |
| ATP | 10 | 88 | 103 | 51 | 73 |
| Citrate | 10 | 126 | 134 | 47 | 72 |
| Malate | 10 | 121 | 127 | 68 | 87 |

(a) Activity in the absence of any coenzymes or metabolites represents 100% activity.
(b) Values at 100 uM of CoA.

Fig. 12. Inhibition of 3-ketoacyl-CoA thiolase (A) and of acetoacetyl-CoA thiolase (B) by acetyl-CoA (AcCoA) at two concentrations of CoASH. (●) 20 uM CoASH; (▲) 200 uM CoASH. The substrate was acetoacetyl-CoA (33 uM).

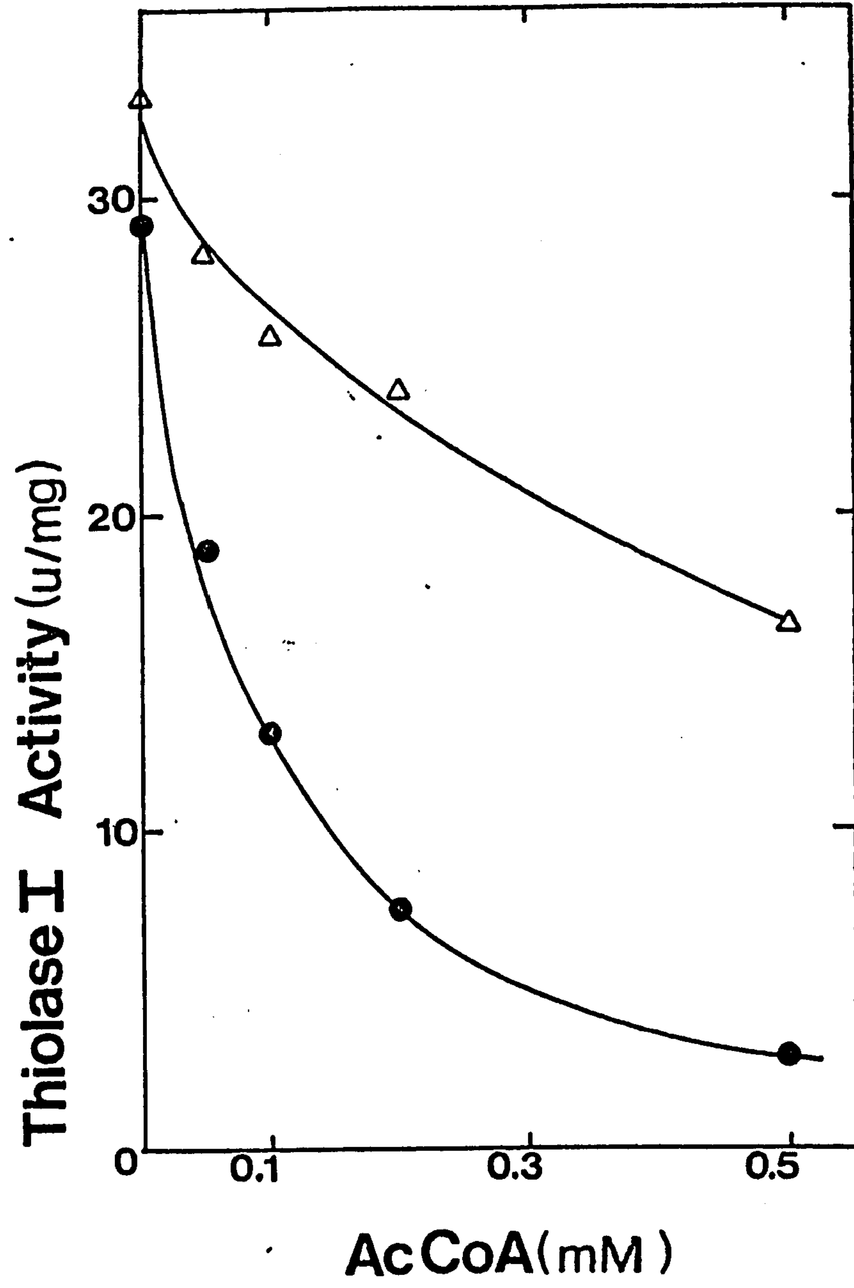
Fig. 12



CoA thiolase was completely inhibited at concentrations of acetyl-CoA (0.4mM) and CoASH (20 uM) which may prevail intramitochondrially at state 4 respiration supported by palmitoylcarnitine (71). In contrast, acetoacetyl-CoA thiolase at the same concentrations of CoASH and acetyl-CoA was only partially inhibited (Fig. 12B). At a higher concentration of CoASH (200 uM), which may approximate the intramitochondrial concentration of CoASH at state-3 respiration supported by palmitoylcarnitine (71), both thiolases were only partially inhibited even when the concentration of acetyl-CoA was 0.5mM. However, at all conditions tested, 3-ketoacyl-CoA thiolase was more severely inhibited by acetyl-CoA than was acetoacetyl-CoA thiolase. An increase in the concentration of CoASH from 20 uM to 200uM resulted in a large increase in activity with acetoacetyl-CoA thiolase but not with 3-ketoacyl-CoA thiolase (see Fig. 12), because the $K_m$ for CoASH with the former enzyme is 51.3 uM in contrast to 8.7 uM determined with 3-ketoacyl-CoA thiolase (72). Acetyl-CoA inhibited 3-ketoacyl-CoA thiolase also when 3-ketodecanoyl-CoA served as a substrate (see Fig. 13). However, the inhibition was less pronounced than that seen with acetoacetyl-CoA as a substrate. 3-Keto-acyl-CoA thiolase was inhibited by longer-chain acyl-CoA compounds as for example decanoyl-CoA (see Table IV). However, the inhibition was less pronounced than that caused by acetyl-CoA (see Table V and Fig. 12). Acetoacetyl-CoA

Fig. 13. The inhibition of 3-ketoacyl-CoA thiolase by acetyl-CoA at two concentrations of CoASH. ( ● ) 20 uM CoASH; ( Δ ) 200 uM CoASH. 10 uM of 3-ketodecanoyl-CoA was used as substrate.

Fig. 13



thiolase in contrast, was only slightly inhibited by decanoyl-CoA and apparently in a non-specific fashion because the magnitude of the effect was independent of the CoASH concentration. The lack of a strong and specific effect of decanoyl-CoA on acetoacetyl-CoA thiolase is consistent with the inability of this enzyme to act on long chain substrates (73). The decrease in acetoacetyl-CoA thiolase activity caused by 0.2mM decanoyl-CoA may be due to a non-specific inhibition caused by the detergent properties of decanoyl-CoA. DL-3-Hydroxybutyryl-CoA (Fig. 14), which is structurally related to the substrate acetoacetyl-CoA, inhibited acetoacetyl-CoA thiolase significantly ($K_i$ = 0.18mM), but had only a slight effect on 3-ketoacyl-CoA thiolase ($K_i$ = 0.48mM). Since under normal conditions the concentrations of intermediates of fatty acid oxidation, including 3-hydroxyacyl-CoA compounds, in mitochondria is low (69, 74), the regulation of thiolases by 3-hydroxybutyryl-CoA does not seem to be a likely one. Succinyl-CoA, which in contrast to intermediates of fatty acid oxidation, is present intramitochondrially in substantial amounts (71), did not affect the activity of 3-ketoacyl-CoA thiolase, and stimulated acetoacetyl-CoA thiolase at low (20 uM) but inhibited moderately at a higher (100 uM) concentration of CoASH. This strange pattern may be attributable to the labile character of succinyl-CoA at alkaline pH at which the assay was performed. Succinyl-CoA which is stable at acidic pH (pH 1), is hydrolyzed under alkaline conditions. The resulting

Fig. 14. The inhibition of 3-ketoacyl-CoA thiolase (A) and acetoacetyl-CoA thiolase (B) by 3-hydroxybutyryl-CoA. Data are plotted according to Lineweaver-Burk. (O) no inhibitor; (●) 0.5mM DL-3-hydroxybutyryl-CoA.

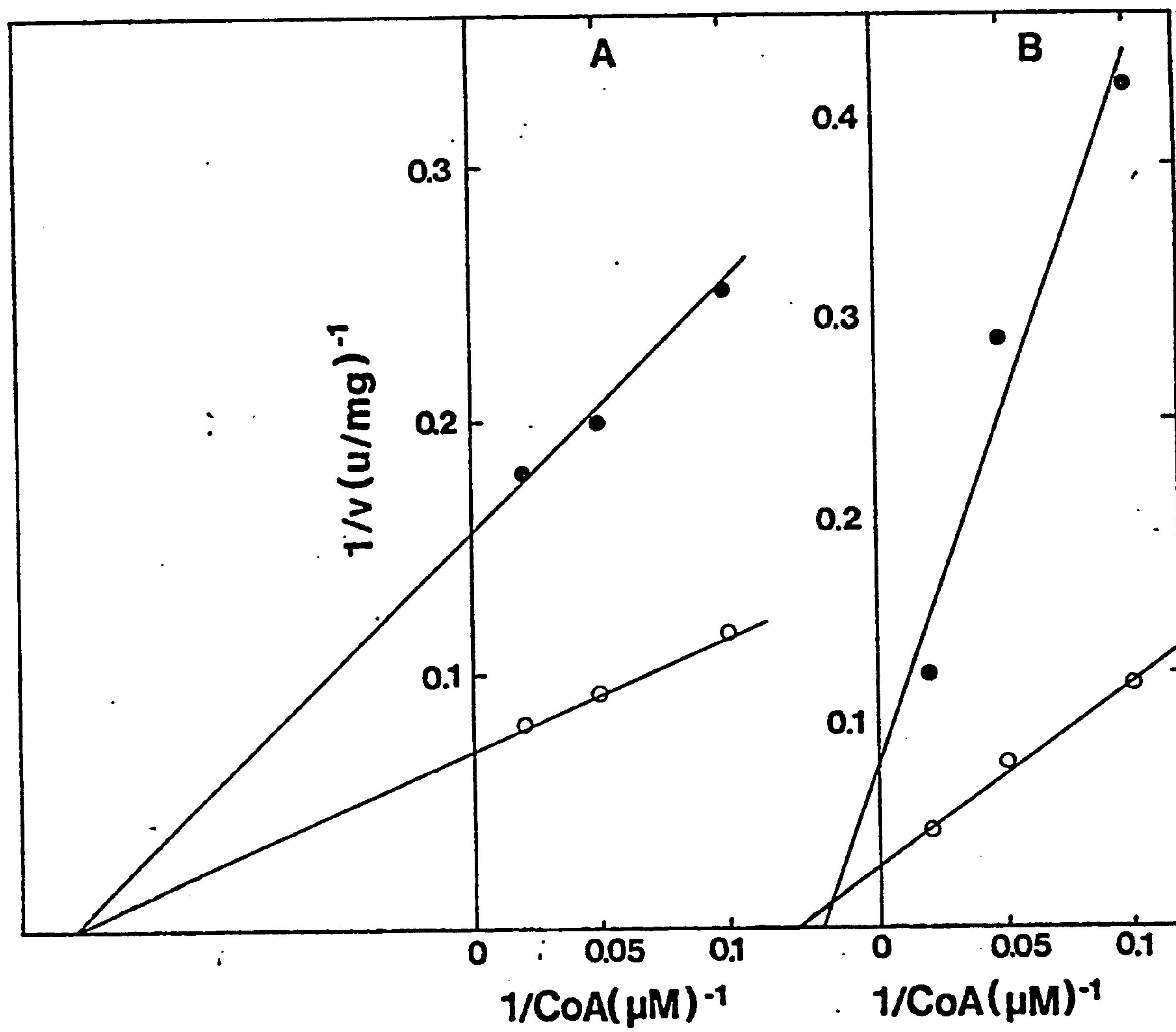



Fig. 14

increase in the concentration of free CoASH may have caused at low levels of CoASH the observed stimulation of acetoacetyl-CoA thiolase which has a $K_m$ for CoASH of 51.3 uM without stimulating 3-ketoacyl-CoA thiolase which has a $K_m$ of only 8.7 uM for CoASH. Since ATP and ADP, citrate and malate affected the thiolases only moderately at 10mM concentrations, these effects are not considered physiologically significant. ATP and ADP inhibited acetoacetyl-CoA thiolase slightly, but were virtually without effect with 3-ketoacyl-CoA thiolase. An inhibition of acetoacetyl-CoA thiolase by ATP at 20 uM CoASH was only observed at an ATP concentration above 4mM (Fig. 15). It is interesting to note that the inhibitions caused by citrate (Fig. 16) and malate (Fig. 17) appear to be competitive with respect to CoASH. $K_i$ values of 5mM and 14mM were determined for citrate and malate respectively. Other metabolites and coenzymes which did not affect the activity of either thiolases significantly at 10mM concentrations, were succinate, fumarate, pyruvate, acetoacetate, 3-hydroxybutyrate, and AMP. DL-Carnitine at 5mM, NADH at 0.5mM and $NAD^+$, NADPH, $NADP^+$ at 1mM concentrations each were also without influence on the two thiolases. Although the inhibition of acetoacetyl-CoA thiolase by 2-ketoglutarate has been reported by Gehring et al. (73), no such effect was observed in the present study as long as the $Mg^{2+}$ concentration in the assay mixture was sufficient to complex both 2-ketoglutarate and acetoacetyl-CoA.

Fig. 15. Inhibition of acetoacetyl-CoA thiolase by ATP at two concentrations of CoASH, (●) 20 uM CoASH; (Δ) 200 uM CoASH. Data are plotted according to Dixon.

Fig. 15

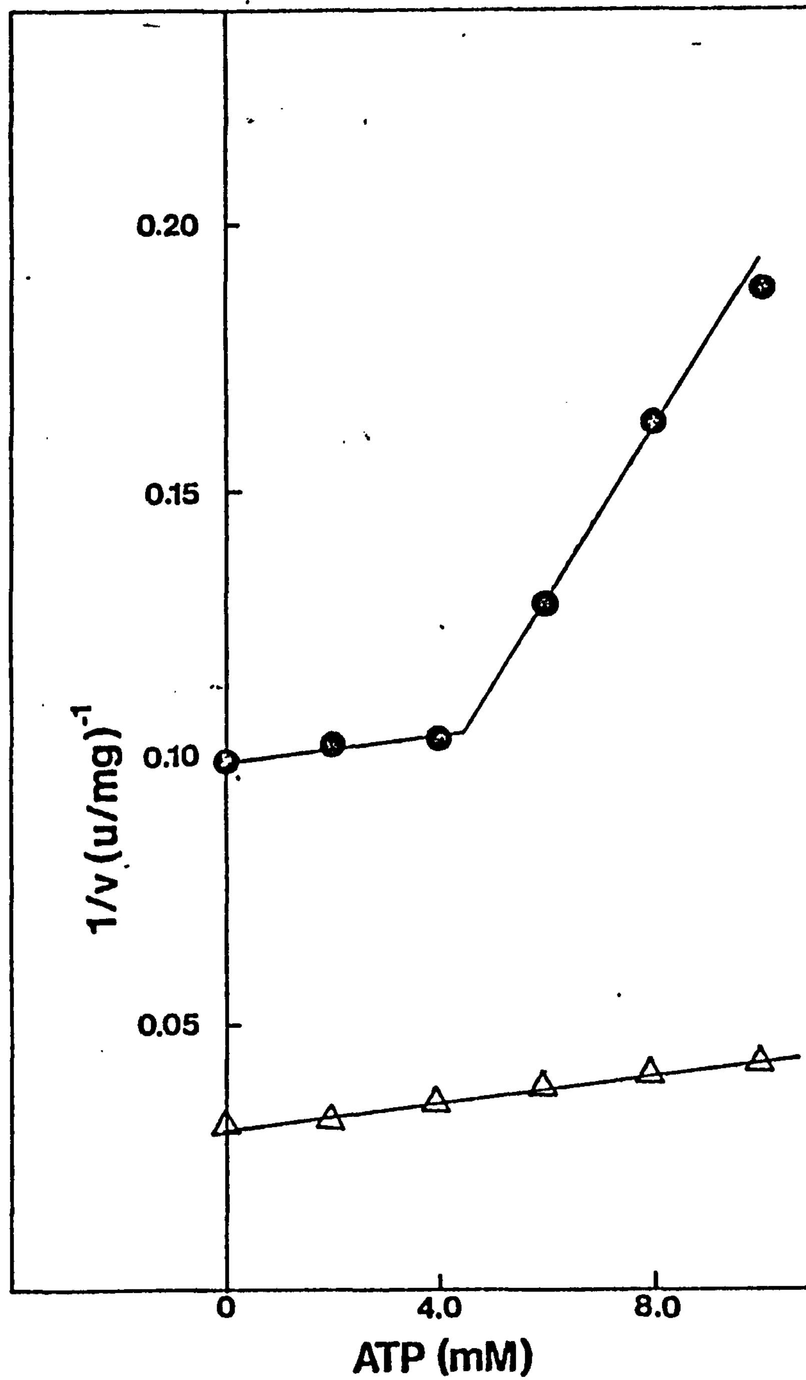


Fig. 16. Inhibition of acetoacetyl-CoA thiolase by 10mM citrate. Data plotted according to Lineweaver-Burk. (●) No citrate; (▲) 10mM citrate.

Fig. 16

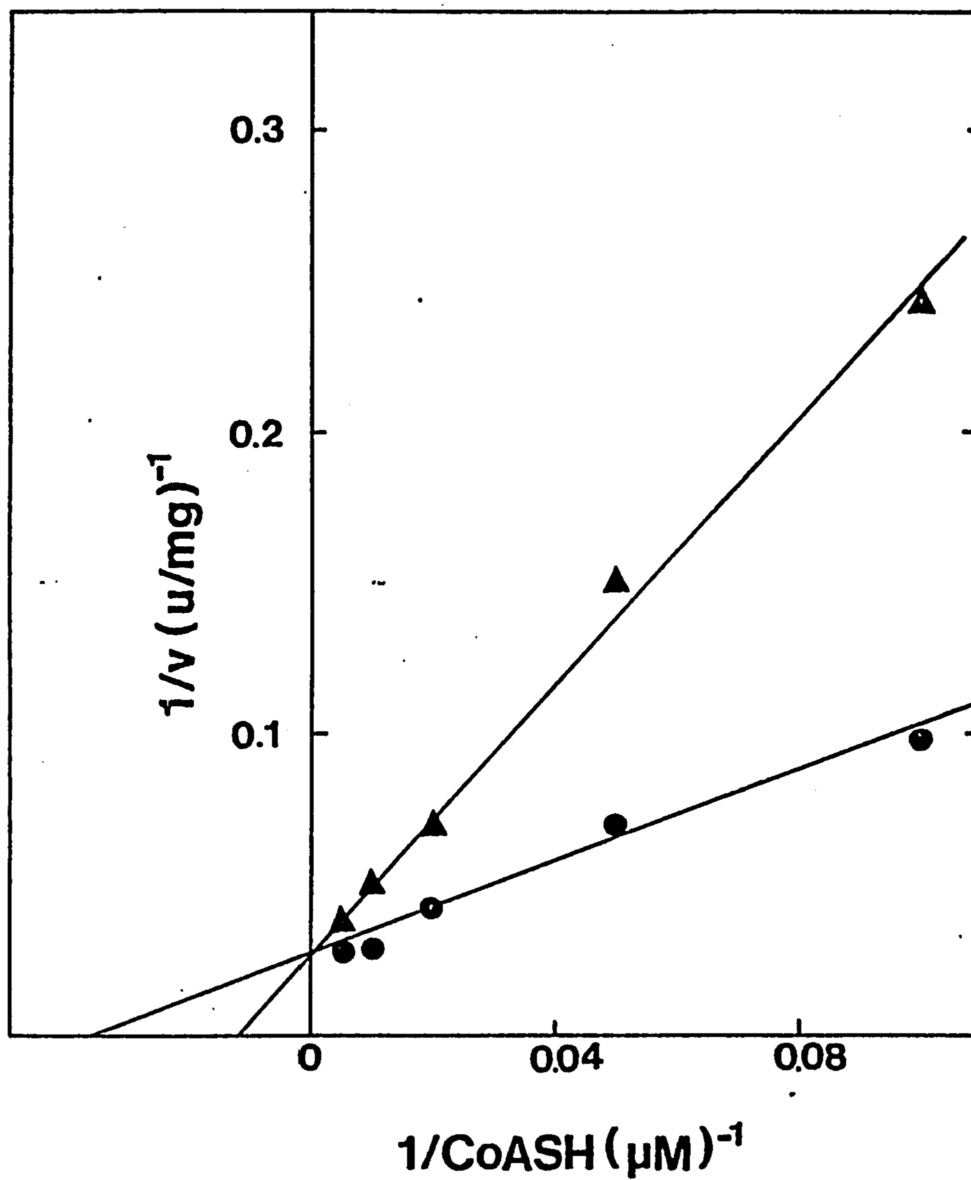
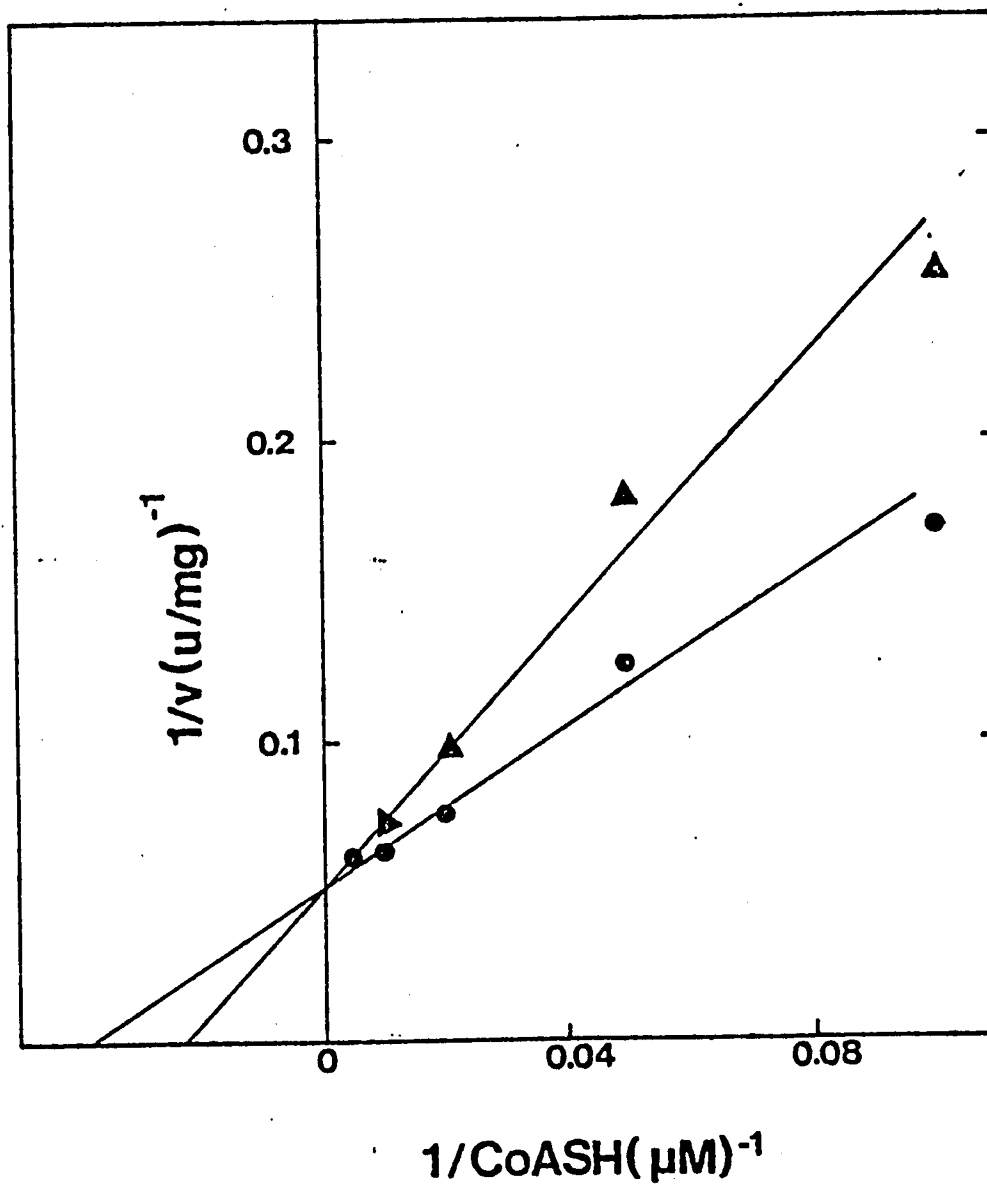


Fig. 17. Inhibition of acetoacetyl-CoA thiolase by malate. Data are plotted according to Lineweaver-Burk. (●) No malate; (▲) 10mM malate.

Fig. 17



Inhibition of Thiolases by Acetyl-CoA - When the activity of acetoacetyl-CoA thiolase was measured at several concentrations of CoASH over a range of acetyl-CoA from 0 - 0.5mM, the Dixon plot, shown in Fig. 18, was obtained. The inhibition was found to be linear over the concentration range of acetyl-CoA used in this experiment, and the graphically estimated $K_i$ was determined to be 125 uM. An identical $K_i$ value was obtained from a Lineweaver-Burk plot (Fig. 19), which also indicated that the inhibition by acetyl-CoA is non-competitive with respect to CoASH. Since the thiolase-catalyzed cleavage of acetoacetyl-CoA yields two molecules of acetyl-CoA, which are presumed to be released from the enzyme sequentially, the simple linear inhibition pattern represents a surprising finding. This observation suggests that either one of the two enzyme forms, to which acetyl-CoA can bind (the free enzyme or the acylated enzyme), has a very low affinity for acetyl-CoA or more likely that the affinities of both enzyme forms for acetyl-CoA are identical or nearly identical. 3-Ketoacyl-CoA thiolase, in contrast to acetoacetyl-CoA thiolase, was inhibited by acetyl-CoA in a non-linear fashion. The lines of the Dixon plot shown in Fig. 20 are parabolic at low but linear at high concentrations of CoASH. The parabolic inhibition lines reflect possibly the binding of acetyl-CoA to two enzyme forms with different affinities. If so, acetyl-CoA at low concentrations should bind to only one enzyme form. The apparent linear inhibition observed at concentrations of CoASH up

Fig. 18. Inhibition of acetoacetyl-CoA thiolase by acetyl-CoA (AcCoA) at different concentrations of CoASH. (⊗) 20uM; (●)50 uM; (Δ)100 uM; (■)200 uM. Data are plotted accordinng to Dixon. Acetoacetyl-CoA (33 uM) served as a substrate.

Fig. 18

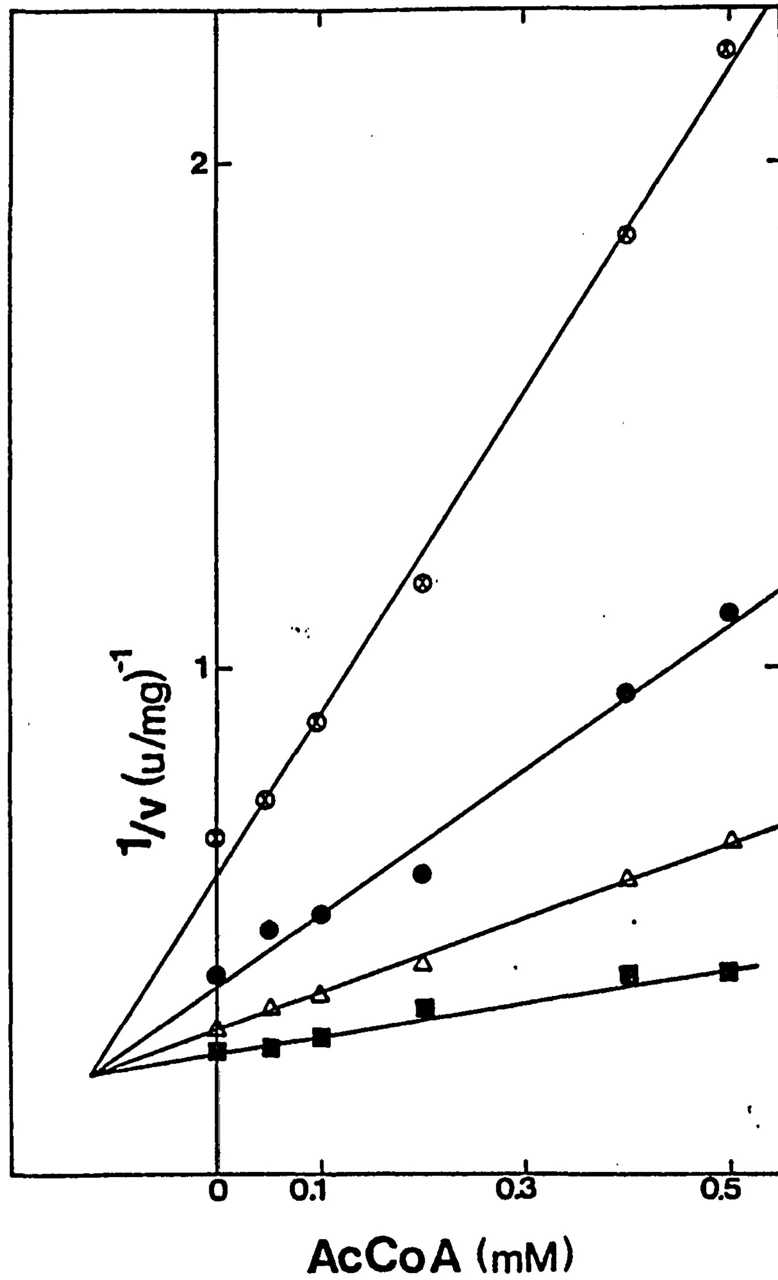


Fig. 19. Inhibition of acetoacetyl-CoA thiolase by acetyl-CoA at varied concentrations of CoASH and fixed concentrations of acetyl-CoA. (●) No acetyl-CoA; (O) 0.05mM acetyl-CoA; (▲) 0.1mM acetyl-CoA; (□) 0.2mM acetyl-CoA; (△) 0.4mM acetyl-CoA; (■) 0.5mM acetyl-CoA. Inset: Replot of slopes versus the concentration of acetyl-CoA.

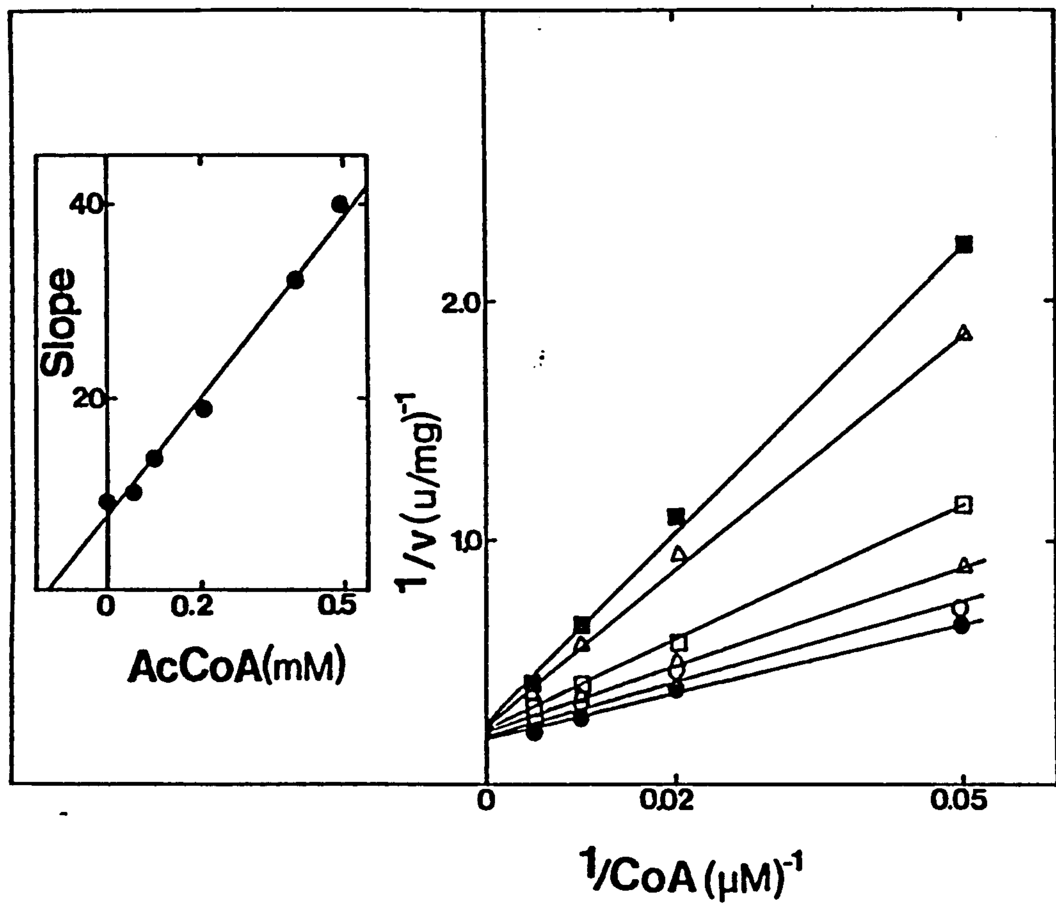



Fig. 19

Fig. 20. Inhibition of 3-ketoacyl-CoA thiolase by acetyl-CoA at different fixed concentrations of CoASH A: (Δ) 20 uM CoASH; (●) 50 uM CoASH; (□) 100 uM CoASH; (▲) 200 uM CoASH B: (●) 10 uM CoASH; (Δ) 20 uM CoASH; (0) 50 uM CoASH; (□) 100 uM CoASH. Acetoacetyl-CoA (33 uM) served as substrate.

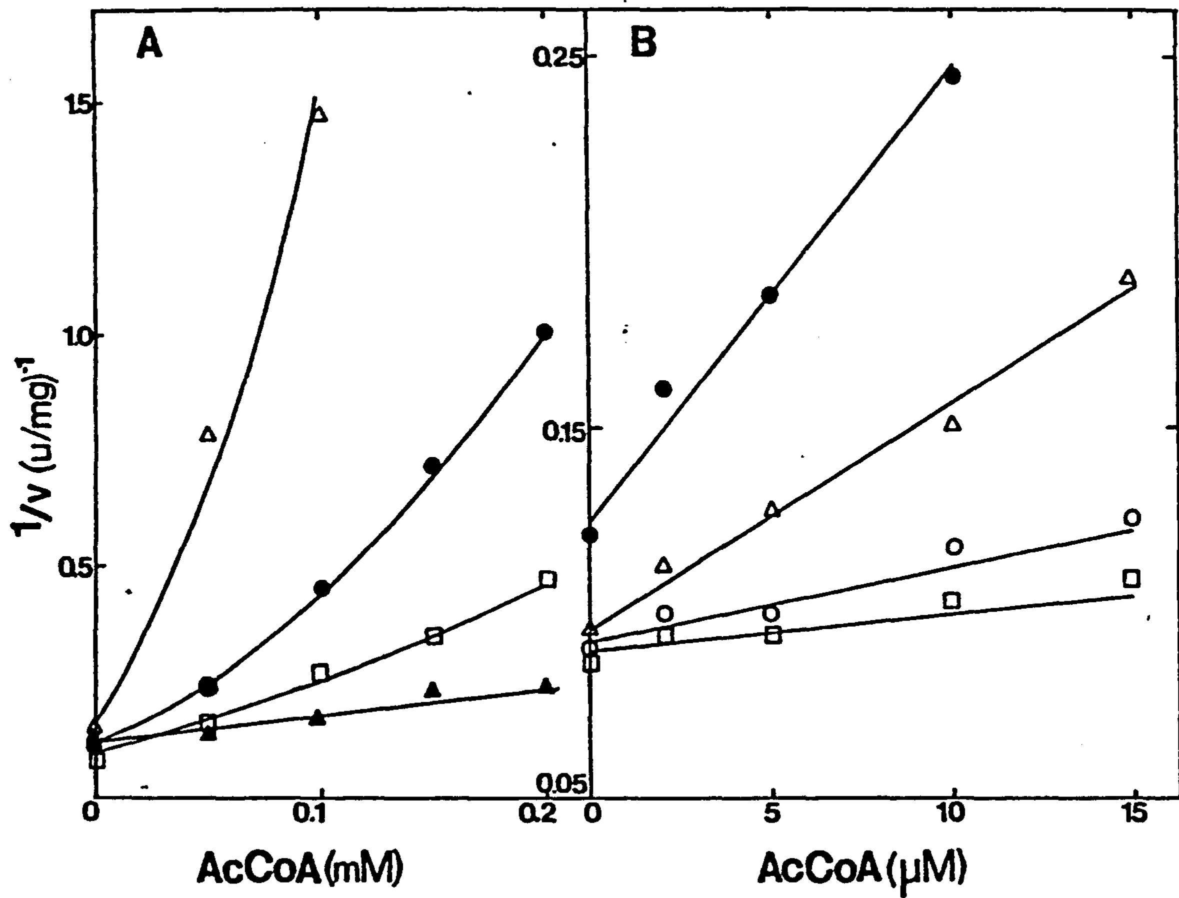


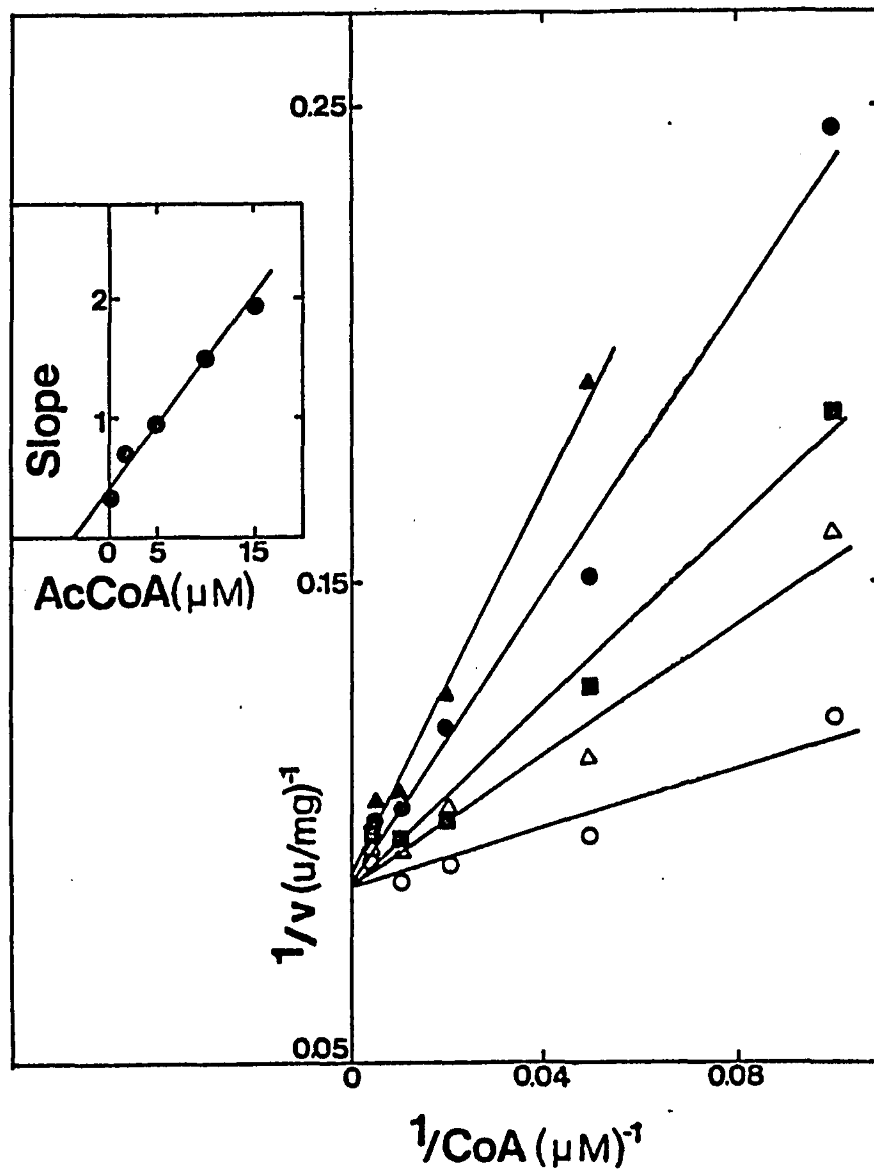

Fig. 20

to 10uM (see Fig. 20B) lends support to this possible explanation. The Lineweaver-Burk plot shown in Fig. 21 suggests that the inhibition of 3-ketoacyl-CoA thiolase by acetyl-CoA is competitive with respect to CoASH. A $K_i$ of 3.9 uM for acetyl-CoA was obtained from a replot of slopes versus acetyl-CoA concentrations (see inset of Fig. 21). A definite interpretation of the inhibition by acetyl-CoA requires an understanding of the kinetic mechanism which has not been determined for the two pig heart thiolases. However, for a discussion of the possible regulation of the two mitochondrial thiolases and thus of fatty acid oxidation, it is adequate to recognize the dramatically different sensitivities of the two enzymes toward acetyl-CoA. These different sensitivities are reflected by the high affinity inhibition constant of 3.9 uM for 3-ketoacyl-CoA thiolase and by the 30-times larger $K_i$ of 125 uM obtained with acetoacetyl-CoA thiolase.

<u>The possible Regulation of Rat Heart 3-Ketoacyl-CoA Thiolase by Phosphorylation - Dephosphorylation</u> - Pyruvate dehydrogenase and thiolase catalyze the final step of carbohydrate oxidation and the terminal step of fatty acid oxidation respectively. Both reactions provide acetyl-CoA which serves as a fuel for the tricarboxylic acid cycle. I have provided evidence for the possible regulation of thiolase similar to pyruvate dehydrogenase by the acetyl-CoA/CoASH ratio. I have also examined the possibility that thiolase, like pyruvate dehydrogenase may be subject to reversible covalent modifi-

Fig. 21. Inhibition of 3-ketoacyl-CoA thiolase by acetyl-CoA at varied concentrations of CoASH and fixed concentrations of acetyl-CoA. Acetyl-CoA: (0) none; (Δ) 2 uM; (■) 5 uM; (●) 10 uM; (▲) 15 uM. Acetoacetyl-CoA served as substrate. Inset: Replot of slopes versus the concentration of acetyl-CoA.

Fig. 21



cation by phosphorylation.

In order to detect phosphorylation of an enzyme, two parameters which may reflect the degree of phosphorylation, for example enzyme activity and incorporation of $^{32}P$ phosphate, were measured. The enzyme activity and $^{32}P$ incorporation were measured at state 3 and state 4 respiration. At state 3, the ADP concentration is high and oxidative phosphorylation is optimal. At state 4 the level of ADP is low and oxidative phosphorylation is minimal. When an extract of mitochondria preincubated with palmitoylcarnitine in the presence and absence of malonate was assayed for thiolase, no significant difference in enzyme activity was observed (see Table VI). There was also no change in enzyme activity when either acetoacetate or pyruvate served as respiratory substrates.

In order to determine phosphorylation of thiolase, mitochondria were incubated with $^{32}P$ phosphate and 3-ketoacyl-CoA thiolase was isolated by immuno-precipitation. The resulting antigen-antibody complex was subjected to SDS gel electrophoresis and the $^{32}P$ distribution was determined. This experiment was performed with two samples obtained from mitochondria preincubated at state 3 and state 4 respiration. No significant level of radioactivity was associated with the protein band or any other portion of the gel. When compared with standard gels on which either 3-ketoacyl-CoA thiolase or antibodies to this thiolase had been run separately, the slower-and the faster-moving bands

Table VI

Assay of Mitochondrial Extract for Thiolase Activity

Mitochondria (P/O) 2.8, RCR 5-8) were incubated for 5 min with palmitoylcarnitine (100 uM), malate (0.5mM) and the additional compounds listed below. Thiolase activities were measured as detailed under "Experimental Procedure."

| Compound | Specific Activity Umol/min/mg protein | |
|---|---|---|
| | $C_4$[a] | $C_{10}$[a] |
| No addition | 0.56 | 0.68 |
| + ATP | 0.58 | 0.68 |
| + 20mM malonate | 0.61 | 0.70 |
| + ATP + 20mM malonate | 0.53 | 0.67 |

[a] $C_4$, $C_{10}$ refer to the chain length of 3-ketoacyl-CoA's used for assaying thiolase activities.

were determined to correspond to the heavy chain of IgG (53,000) (75) and to the 3-ketoacyl-CoA thiolase subunit (46,000) (72) respectively (see Fig. 22).

## DISCUSSION

The rate at which fatty acids are oxidized by heart is dependent on the plasma concentration of free fatty acids as well as on the energy demand of the muscle (30). At sufficiently high levels of free fatty acids, the rate is only dependent on the energy demand of the tissue (30). However, no generally accepted view has emerged regarding the feedback mechanism by which the rate of fatty acid oxidation is adjusted to the energy demand of the cell. Studies with perfused hearts and isolated mitochondria have demonstrated that the ratios of NADH/$NAD^+$ and acetyl-CoA/CoASH increase in response to decreases in the energy demand as a consequence of a lower workload imposed on the isolated heart (30) or due to limiting amounts of ADP in the case of isolated mitochondria (71). The increase in the acetyl-CoA/CoASH ratio reflects an inhibition of the citric acid cycle which in turn should lead to a decrease in the rate of fatty acid oxidation. Based on the finding presented in this thesis it is suggested that the feedback connection between citric acid cycle and B oxidation cycle is provided by the acetyl-CoA/CoASH ratio which determines the activity of 3-ketoacyl-CoA thiolase (see Fig. 23). This suggestion takes into account the fact that 3-ketoacyl-CoA thiolase may be rate-limiting in B oxidation or at least is as slow as the

Fig. 22. Analysis of the antibody: 3-ketoacyl-CoA thiolase complex. The trace shows a spectrophotometric scan of a gel stained for protein. When compared with control gels on which either 3-ketoacyl-CoA thiolase or antibody to 3-ketoacyl-CoA thiolase had been run separately, it was concluded that the slower moving band (I) corresponds to the heavy chain of anti-3-ketoacyl-CoA thiolase (53,000) and the faster-moving band (II) corresponds to the subunit of 3-ketoacyl-CoA thiolase (46,000).

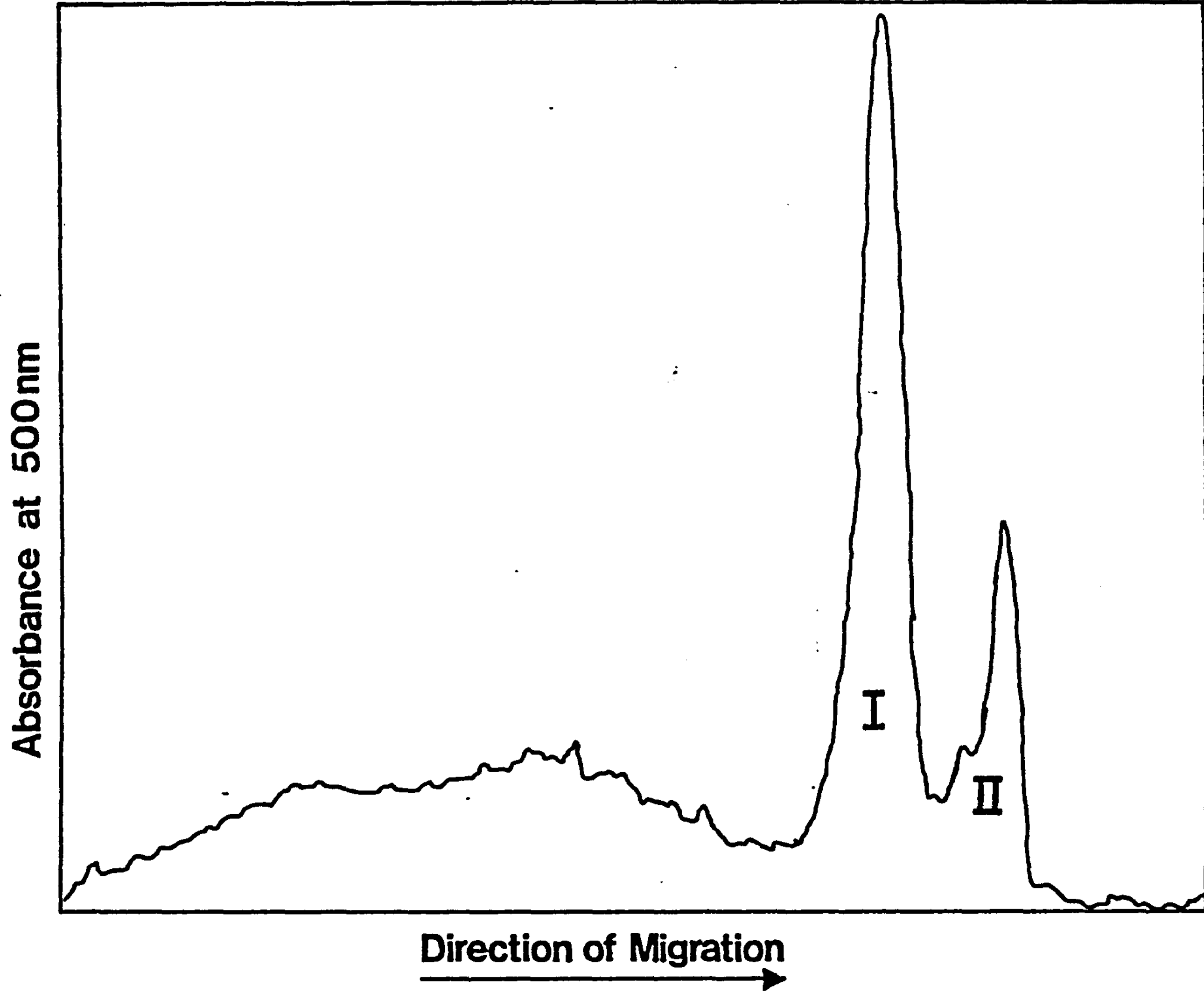



Fig. 22

other reactions are (28). An inhibition of 3-ketoacyl-CoA thiolase could result in an accumulation of CoASH derivatives of 3-keto acids or more likely of 3-hydroxy acids and thus of 2-enoic acids because the equilibrium favors the conversion of 3-ketoacyl-CoA compounds to the corresponding hydroxy derivatives especially when the mitochondrial concentration of NADH is high. Although the metabolites of fatty acids, except for saturated acyl-CoA compounds, do not accumulate in mitochondria when B oxidation proceeds rapidly (69), measurable amounts of hydroxy acids and enoic acids have been observed when mitochondria were incubated with palmitoylcarnitine in the presence of rotenone and carnitine (69). Hence it is possible that a decrease in the energy demand which leads to elevated acetyl-CoA levels, secondarily results in an accumulation of fatty acid metabolites. Since acetoacetyl-CoA and crotonyl-CoA are effective inhibitors of acyl-CoA dehydrogenase (76), fatty acid metabolites may function as feedback inhibitors of the first step of B oxidation. If the enzymes of B oxidation exist in higher organisms in the form of a multienzyme complex as they do in Escherichia coli (63), the intermediates of fatty acid oxidation may incompletely or not at all equilibrate with the surrounding medium and consequently their effective concentrations at the putative complex may even be higher than indicated by their intramitochondrial concentration. The data presented here additionally suggest that at periods of decreased energy demand ketone body degradation will be less

severely inhibited than fatty acid oxidation. This conclusion is based on the observation that acetoacetyl-CoA thiolase, which is presumed to function in ketone body degradation (72), is less severely inhibited by acetyl-CoA than is 3-ketoacyl-CoA thiolase. This regulatiory aspect could possibly assure the preferential utilization of ketone bodies by heart under such metabolic conditions as starvation or diabetes.

In summary it is proposed that in heart the rate of fatty acid oxidation is tuned to the rate at which the citric acid cycle operates via a feedback regulation of 3-ketoacyl-CoA thiolase by the acetyl-CoA/CoASH ratio. It is interesting to note that this regulatory mechanism resembles one of the mechanisms by which pyruvate dehydrogenase is controlled. However, an evaluation of the possible regulation of 3-ketoacyl-CoA thiolase by phosphorylation-dephosphorylation similar to that of pyruvate dehydrogenase did not provide any evidence for this regulatory mechanism.

Fig. 23. Diagramatic representation of the integrated pathways of B oxidation, ketone body degradation, TCA cycle and oxidative phosphorylation.

## REGULATION OF FATTY ACID OXIDATION IN HEART

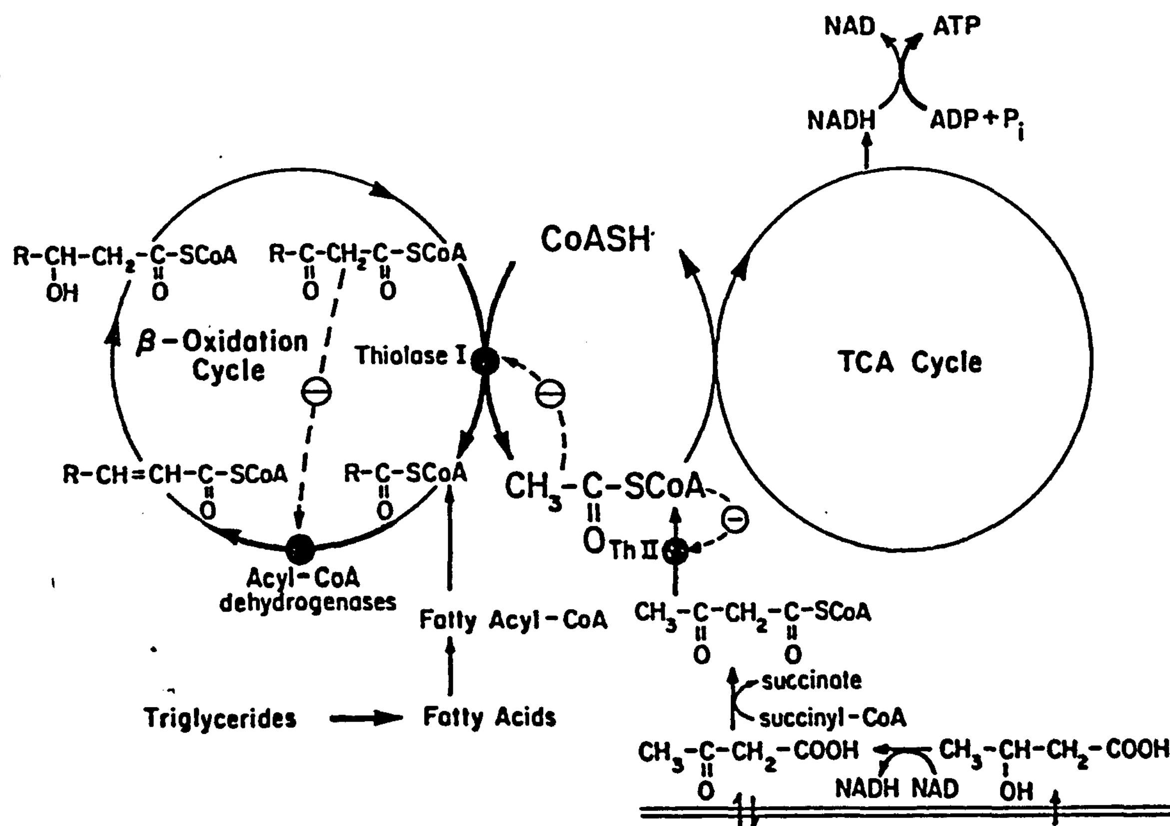



Fig. 23

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