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Aspects of Mitochondrial Oxidative Metabolism

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ASPECTS OF MITOCHONDRIAL OXIDATIVE METABOLISM

City University of New York PH.D. 1985

ASPECTS OF MITOCHONDRIAL OXIDATIVE METABOLISM

by

Leslie Kushner

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.

1985

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

ASPECTS OF MITOCHONDRIAL OXIDATIVE METABOLISM

by

Leslie Kushner

Adviser: Dr. Horst Schulz

The effects of metabolic intermediates and coenzymes on the activities of fatty acyl-CoA synthetase and carnitine palmitoyltransferase (CPT) in rat heart mitochondria were studied. ADP and palmitoyl-*l*-carnitine were weak inhibitors of fatty acyl-CoA synthetase. CPT A was inhibited 59.3% by 100 μM succinyl-CoA. However, succinate thiokinase activity in rat heart cytosol was too low to maintain a succinyl-CoA concentration sufficient to affect the activity of CPT A. There was no indication that fatty acid oxidation in rat heart mitochondria is controlled via the regulation of either CPT A or fatty acyl-CoA synthetase.

The dependence of the oxidation of unsaturated fatty acids containing *cis* double bonds on the presence of 3-hydroxyacyl-CoA epimerase was investigated. The specific activity of epimerase in rat heart mitochondria was found to be significantly lower than the rate of linoleoyl-CoA oxidation. Thus, it is the NADPH-dependent 2,4-dienoyl-CoA reductase, not the 3-hydroxyacyl-CoA epimerase, which functions in the oxidation of polyunsaturated fatty acids. [C.-H. Chu, L. Kushner, D. Cuebas, and H. Schulz, *Biochem. Biophys. Res. Comm.*, vol. 118, pp. 162-167, 1984]

3-Mercaptopropanoyl-CoA and S-acetyl-3-mercaptopropanoyl-CoA, physiological metabolites of the known convulsant 3-mercaptopropanoic acid, were examined for their effects on purified pyruvate dehydrogenase complex from porcine and bovine heart. Both 3-mercaptopropanoyl-CoA and S-acetyl-3-mercaptopropanoyl-CoA were found to be inhibitors of this enzyme. Under optimal conditions, 50% inhibition was obtained at 12.6 μM 3-mercaptopropanoyl-CoA or 5.2 μM S-acetyl-3-mercaptopropanoyl-CoA while 100 μM of the latter compound gave 90% inhibition. The inhibition observed with S-acetyl-3-mercaptopropanoyl-CoA was demonstrated to be irreversible. Maximal inhibition of the complex and its component enzymes was observed when it was reacted with the inhibitor under conditions which promote reduction of the endogenous lipoate. [L. Kushner, D. Cuebas, and H. Schulz, *Fed. Proc.*, vol. 43(7), pp. 1870, 1984]

Preliminary results indicate that S-acetyl-3-mercaptopropanoyl-CoA formed intramitochondrially inhibits pyruvate-supported respiration in rat heart and liver mitochondria.

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ABBREVIATIONS

ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CoA	coenzyme A
CoASH	reduced coenzyme A
CPT	carnitine palmitoyltransferase (EC 2.3.1.2)
DCPIP	2,6-dichlorophenolindophenol
DEAE	diethylamino ethyl
DTNB	5,5'-dithio-*bis*-(2-nitrobenzoic acid)
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol *bis*-(β-aminoethyl ether)N,N'-tetraacetic acid
FAD	flavin adenine dinucleotide
FADH	reduced flavin adenine dinucleotide
GABA	γ-amino butyric acid
GTP	guanosine 5'-triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
IG	immunoglobulin
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PDH	pyruvate dehydrogenase
RCR	respiratory control ratio

THF	tetrahydrofuran
TPP	thiamin pyrophosphate
TRIS	tris (hydroxymethyl) amino methane

INTRODUCTION

Oxidation of fats and carbohydrates is a major mechanism by which cells obtain energy. In glycolysis, glucose is broken down to pyruvate in a series of reactions which occur in the cytosol. The pyruvate formed enters the mitochondria to be converted to acetyl-CoA by the pyruvate dehydrogenase complex. Fatty acids are degraded to acetyl-CoA in mitochondria via the β-oxidation pathway. The acetyl-CoA thus formed may either enter the TCA cycle to provide additional cellular energy or the citrate derived from the acetyl-CoA may leave the mitochondria and eventually supply acetate units which serve as building blocks for fatty acid synthesis. The relative activities of each of these pathways depends on the tissue being studied and the physiological state of the organism. These two factors play a role in regulation of key enzymes of fatty acid and carbohydrate oxidation.

In mammalian liver, there is a reciprocal relationship between fatty acid and carbohydrate metabolism.[1] In the carbohydrate-fed state, carbohydrates are oxidized and fatty acid synthesis occurs, while fatty acid oxidation is suppressed. In the carbohydrate-starved state, fatty acid synthesis is suppressed, while gluconeogenesis and fatty acid oxidation are the primary metabolic activities.[2] A major point in regulation of these metabolic pathways is thought to be the carnitine acyltransferase (CPT)(EC 2.3.1.21) system which is located in the inner mitochondrial membrane.[1]

In mammalian heart, fatty acids are the main substrate for energy metabolism,[3] although glycolysis does occur. *De novo* fatty acid synthesis is not thought to be a major metabolic pathway in heart. However, some fatty acid elongation and complex lipid synthesis does occur in this tissue. Regulation of

these processes in heart tissue is not completely understood.

In tissues which oxidize fatty acids, the long-chain fatty acids in the cytosol must be activated by the enzyme fatty acyl-coenzyme A synthetase (EC 6.2.1.3), which is located in the outer mitochondrial membrane[4,5], the endoplasmic reticulum[6,7], and probably the peroxisome. The activated fatty acyl moiety can either be channeled into lipid biosynthesis or β-oxidation.[8] In order for β-oxidation to occur, the fatty acyl moiety must cross the inner mitochondrial membrane. For long-chain fatty acyl groups, this process is facilitated by the carnitine acyltransferase system. It has been suggested that fatty acid activation and/or the carnitine acyltransferase reactions function as points of regulation in mammalian heart tissue.

Unsaturated fatty acids are also degraded by β-oxidation in both liver and heart tissue. However, there is controversy over the enzymatic requirements for the shifting or removal of the double bonds and over the subcellular location of the enzymes involved. The subcellular location of the enzymes is important since compartmentalization would have a bearing on the regulation of this metabolic process.

All tissues have the capacity for glycolysis. The final product of this pathway, pyruvate, is either reduced to lactate or oxidized in mitochondria. The pyruvate dehydrogenase complex is a key regulatory enzyme of the oxidative metabolism of pyruvate in the mitochondria. There is a reciprocal relationship between the oxidation of carbohydrates and fatty acids.[9] This reciprocity is dependent on the synthetic needs of the cell, the energy demand of the cell, the availability of the different substrates, and the accumulation of

products. An increase in the intramitochondrial NADH/NAD^+ ratio will inhibit the TCA cycle[10] , the 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) reaction[10] of β-oxidation, and the pyruvate dehydrogenase complex.[11, 12, 13] An increase in the acetyl-CoA/CoASH ratio will inhibit both the 3-ketoacyl-CoA thiolase (EC 2.3.1.16) reaction[14] of β-oxidation and the pyruvate dehydrogenase reaction.[11, 12, 13] Factors which affect the key enzyme, pyruvate dehydrogenase complex, have an important bearing on the carbohydrate metabolism of all tissues. However, this is most critical in the brain, which has little, if any, capacity for β-oxidation and derives most of its metabolic energy from glucose oxidation.[15, 16, 17]

Regulation of Fatty Acyl-CoA Synthetase and Carnitine Palmitoyltransferase of Mammalian Heart Mitochondria

Fatty acids are the main substrates for energy metabolism in heart muscle.[3] Although the metabolic steps of β-oxidation are well established, the regulation of this pathway in heart muscle is not yet fully understood.

Long-chain fatty acids in the cytosol must be activated by the enzyme fatty acyl-coenzyme A synthetase (EC 6.2.1.3), which is located in the outer mitochondrial membrane.[4, 5] This process may facilitate the translocation of activated fatty acids across the outer membrane.[18] In heart tissue, the activated fatty acyl moiety can either be channeled into lipid biosynthesis or β-oxidation.[8] In order for β-oxidation to occur, the fatty acyl moiety must cross the inner mitochondrial membrane. For long-chain fatty acyl groups, this process is facilitated by the carnitine acyltransferase (CPT) (EC 2.3.1.21) system.[19, 20, 21]

There are two CPT enzymes in the inner mitochondrial membrane[20, 22, 23, 24, 25, 26, 27, 28, 29] : CPT A[25, 26, 27] or I[28, 29] is loosely attached to the outer surface of the inner mitochondrial membrane; CPT B[25, 26, 27] or II[28, 29] is located on the inner surface of the inner mitochondrial membrane (see Figure 1). Hoppel[19] presents a convincing argument that the CPT I and II of Kopec and Fritz[28, 29] are both CPT B.

Kopec and Fritz[29] prepared antibodies to purified CPT I. One preparation, IG-I, inhibited the activity of only purified CPT I. Another preparation, IG-II, inhibited the activity of both purified CPT I and partially purified CPT II. Brosnan et al.,[20] showed that IG-II of Kopec and Fritz[29] inhibited the CPT reaction on both the external and internal surface of the inner mitochondrial membranes by 60% and 88%, respectively, while IG-I inhibited the CPT reaction only on the external surface of the inner mitochondrial membrane, also by 60%. They concluded that the two CPT reactions are not catalyzed by identical enzymes. If, as Hoppel[19] believes, the CPT I and II of Kopec and Fritz[28, 29] are both CPT B, then two further conclusions are possible: although both CPT enzymes are identical (or have some identical regions), the CPT A is more exposed to the external milieu than CPT B, thus exposing an additional antigenic determinant; or that the CPT B transverses the membrane and is exposed on both sides of the membrane. This last possibility is unlikely since trypsinization of the digitonin "mitoplast" left the CPT B activity intact.[30]

Although CPT A and CPT B have the same catalytic activity (both enzymes catalyze the forward and reverse reactions[31]), kinetic differences between the two enzymes have been demonstrated.[27] The CPT from beef heart mitochondria was purified as a single protein.[32, 33, 34] In addition, Bremer et

al.,[35] present evidence that there are no distinct inner and outer CPT enzymes. When the inner and outer CPT were purified from rat liver mitochondria separately, they were shown to be kinetically and immunologically identical and have identical molecular weights.[30] However, these same researchers demonstrated that there are differences between the inner and outer CPT in the intact mitochondrial membrane.[30] It is likely that CPT A and CPT B are identical enzymes, altered in some way by their different locations in the mitochondrial membrane.

It has been suggested that fatty acid activation[36, 37] or the carnitine acyltransferase reactions may function as points of regulation for β-oxidation in mammalian heart tissue.

In the perfused rat heart, at low levels of ventricular pressure, an increase in the concentration of palmitate in the perfusate causes a proportional increase in the rate of palmitate uptake. This is associated with a slight increase in tissue levels of long-chain acyl-CoA, acyl-carnitine, acetyl-CoA, and acetylcarnitine. When the concentration of palmitate is increased above 0.6 mM, the rate of uptake does not increase further. In addition, the rate of oxygen consumption and $^{14}CO_2$ production from labelled palmitate remains constant. However, large amounts of acyl-CoA and acyl-carnitine derivatives accumulate in the tissue. While the mass action ratio for CPT remains constant as the palmitate concentration is increased, the mass action ratio of carnitine acetyl transferase shifts toward acetyl-CoA formation. Under these conditions, it appears that at palmitate concentrations in the perfusate between 0 and 0.6 mM, β-oxidation is limited by the rate of palmitate uptake or activation. At higher levels of palmitate, the rate of fatty acid uptake is limited by the

availability of free CoASH and/or carnitine which are required for the fatty acyl-CoA synthetase and the carnitine acyl transferase reactions, respectively. This finding is supported by the fact that acetyl-CoA oxidation through the TCA cycle is not accelerated under conditions of high exogenous palmitate concentration and also by the fact that the tissue ratios of acetyl-CoA/CoASH and acetylcarnitine/carnitine continue to increase.[38]

Availability of carnitine may be a factor in limiting the rate of CPT A, while availability of CoASH may be a factor in limiting the rate of either the fatty acyl-CoA synthetase or CPT B. However, the effect of substrate availability on these enzymes may not be the limiting factor for β-oxidation since the products of both reactions accumulate in the tissue. The subcellular locations of the metabolites measured and their concentration changes in each subcellular compartment (cytosol and mitochondria) under the conditions studied are more significant than whole tissue levels in determining the participation of CPT in limiting β-oxidation and in determining whether the CPT enzymes could be controlled by changes in substrate availability.

Cytosolic and mitochondrial levels of CoASH and carnitine in rat heart and liver tissue have been estimated.[37] Nearly all of the heart tissue CoASH was mitochondrially located, and 92% of this was free CoASH. In the liver, 30% of the free CoASH was located in the cytosol. In both tissues it was determined that 100% of the free carnitine was in the cytosol. This is probably an overestimation due to the loss of free carnitine during the preparation of mitochondria. The carnitine acylcarnitine translocase facilitates the equilibration of free carnitine across the mitochondrial inner membrane.[39] While this would function to maintain the same carnitine concentration in the cytosolic

and mitochondrial compartments of the cell, it would also allow carnitine loss from mitochondria during the preparation of this organelle.

The maximal concentration of free CoASH in the cytosol was determined to be 22 μM, but it would probably always be less due to the presence of some acyl-CoA.[37] The Km for CoASH for the fatty acyl-CoA synthetase is 7 μM but is 24 μM in the presence of 8 μM palmitoyl-CoA.[37]

These data do suggest, however, that the availability of free CoASH in the cytosol of heart tissue could be a limiting factor for fatty acid activation, and thus, β-oxidation. There is evidence that the availability of free CoASH in the cytosol of brown adipose tissue is a limiting factor for fatty acid activation in that tissue.[40]

When acetyl-CoA oxidation in heart tissue is increased by increasing ventricular pressure, the rate of palmitate uptake and β-oxidation is also increased.[38] This is associated with a decrease in tissue levels of fatty acids, acyl-CoA, acetyl-CoA, and acetyl-carnitine, and with an increase in tissue levels of acyl-carnitine, free CoASH, and free carnitine.[38] When palmitate is replaced by octanoate, which is able to cross the inner mitochondrial membrane bypassing both activation and the transfer to carnitine[41] , oxidation is fast enough to maintain high tissue levels of acetyl-CoA. This occurs even when fatty acid oxidation is accelerated by increased ventricular pressure.[38] It can be inferred that the rate of β-oxidation may be limited by the availability of substrate inside the mitochondria, which is dependent on the activation and transferase rates.

Palmitoyl-CoA is an inhibitor of fatty acyl-CoA synthetase, with a K_i of 5 μM. The inhibition can be relieved by addition of CoASH or albumin.[37] Since, *in vivo*, most of the cytosolic palmitoyl-CoA is probably bound by protein, and since the carnitine level in the cytosol is quite high[37] , thereby tending to drive any palmitoyl-CoA formed to palmitoyl-carnitine, the inhibition of fatty acid activation by palmitoyl-CoA is probably not physiologically significant. An exception would be in the ischemic heart, where the cytosolic concentration of acyl-CoA is estimated to rise as high as 17 μM.[37]

The other products of the fatty acyl-CoA synthetase reaction, AMP and pyrophosphate, were found to be inhibitory at 1 mM. Acetyl-CoA, acetylcarnitine, NAD^+, NADH, glucose-6-phosphate, pyruvate, and TCA cycle intermediates do not inhibit the reaction at 1 mM.[37]

Rat liver microsomal palmitate activation is inhibited by 5 mM AMP, ADP, and cyclic-AMP by 48%, 40%, and 40%, respectively.[42] Rat liver mitochondrial palmitate activation is also inhibited by AMP and pyrophosphate.[43]

Total activity of the fatty acyl-CoA synthetase in various tissues, including heart mitochondria, has been shown to be greater than that required to support the highest rates of β-oxidation and lipid biosynthesis.[10, 44] However, Normann et al.,[36] found the fatty acyl-CoA synthetase of intact rat heart mitochondria to have a four-fold lower activity than the CPT A. Regulation of fatty acid activation *in vivo*, may make this the rate-controlling step of β-oxidation. The stimulation of palmitate oxidation in rat skeletal muscle mitochondria caused by salicylic acid was due to the effect of this compound on the fatty acyl-CoA synthetase.[45] The fact that modification of the fatty acid

activation step affects the entire β-oxidation pathway implicates this as the rate limiting step. While it is likely that availability of cytosolic CoASH is a means of physiological regulation of this enzyme[37] (for opposing view, see Pande, 1971[44] , 1972[46]), other means of regulation have not been fully investigated.

Examination of fatty acyl-CoA synthetase has been done primarily on tissue other than heart, because it was thought that heart mitochondria did not possess this enzyme. Recently, it was demonstrated that lack of enzyme activity was due only to the method of preparation of rat heart mitochondria.[4, 42] The protease, nagase was included in the preparation. This enzyme selectively inactivates fatty acyl-CoA synthetase without an effect on the other enzymes of β-oxidation.[4]

Possible inhibitors of fatty acyl-CoA synthetase include ketone bodies, glycolytic intermediates, and ADP. These compounds were tested for their effect on fatty acyl-CoA synthetase in rat heart mitochondria isolated without nagase.

Palmitoyl-carnitine is also a likely inhibitor of fatty acyl-CoA synthetase. In addition to its structural similarity to palmitate, the concentration of long chain acyl-carnitines has been demonstrated to increase in heart tissue during reduced oxidative metabolism.[38] In order to study the effect of palmitoyl-carnitine on fatty acyl-CoA synthetase, an attempt was made to partially purify the enzyme, i.e. to separate fatty acyl-CoA synthetase activity from carnitine palmitoyltransferase activity. This was undertaken because in rat heart mitochondria the specific activity of both enzymes is the same. Since

palmitoyl-CoA has been shown to inhibit the synthetase,[37] CPT operating in the reverse direction may cause an apparent inhibition due to accumulation of the CoA derivative - an inhibition which would be indistinguishable from that due to the added carnitine derivative.

It has been suggested that some factor in the cytosol stimulates the activity of the fatty acyl-CoA synthetase.[47, 48] Aas and Dale[2] have shown that refeeding of carbohydrate-starved rats causes a decrease in the fatty acid activation rate in liver and heart. Since the changes observed in liver are associated with hormonal changes, the possibility exists that the effector of fatty acyl-CoA synthetase is a kinase or phosphatase. Streptozotocin-induced diabetes caused a reduced rate of β-oxidation and fatty acyl-CoA synthetase activity in rat brown adipose tissue.[49] In addition, administration of O-tetradecanoylphorbol 13-acetate (TPA) decreased fatty acyl-CoA synthetase activity in rat adipocytes.[50] This compound is known to exert its effects on protein kinase C.[51, 52] Noradrenaline also caused a decrease in fatty acyl-CoA synthetase activity in adipose tissue.[50] Cyclic AMP, an activator of certain protein kinases, stimulated β-oxidation in rat hepatocytes.[53] The possibility of activation or inactivation of rat heart fatty acyl-CoA synthetase by phosphorylation-dephosphorylation was investigated.

In mammalian liver, there is a relationship between fatty acid and carbohydrate metabolism.[1] In the carbohydrate-fed state, carbohydrates are utilized and fatty acid synthesis occurs, while fatty acid oxidation is suppressed. In this state the tissue level of malonyl-CoA is high. In the carbohydrate-starved state, fatty acid synthesis and malonyl-CoA production is suppressed, while fatty acid oxidation, gluconeogenesis, and ketogenesis are the primary

metabolic activities. Under these conditions, CPT activity in liver is increased.[2] In fact, it has been shown that the outer CPT activity increases to a much greater extent than total CPT activity.[54] Simultaneously, hepatic carnitine levels and fatty acyl-CoA levels increase. It has been demonstrated that high levels of carnitine[55] and fatty acyl-CoA[1] stimulate β-oxidation, while high levels of malonyl-CoA inhibit β-oxidation.[56] The site of malonyl-CoA inhibition has been identified as the CPT A.[56, 57, 58, 59] Recent evidence indicates that total CPT may be sensitive to malonyl-CoA inhibition.[35, 60] In addition, sensitivity of CPT A,[54, 61, 62, 63, 64] ketogenesis,[65] and palmitate oxidation[66, 67] to malonyl-CoA changes with the physiological state of the organism. Whether this is due to a change in the CPT A protein or a movement of CPT B to exposure on the outer surface[54] is not known. Other evidence indicates that the changes in sensitivity of CPT to malonyl-CoA inhibition do not depend on a change in the CPT enzyme itself, but on some other "regulatory" component of the membrane[35, 60, 68] and may actually involve the synthesis of some new protein.[69] The existence of a membrane regulatory component is supported by recent data which indicate that the malonyl-CoA binding site for rat liver, heart, and skeletal muscle mitochondria is distinct from the active site of CPT A.[70] In any case, such changes in kinetics indicate that this is a regulated enzyme.

Carbohydrate-starvation[54, 62, 64, 71, 72] and streptozotocin-induction of ketosis and diabetes[61, 73] in rats have the same effect of diminishing the sensitivity of liver mitochondria CPT A to malonyl-CoA. Treatment of diabetic animals with insulin not only reverses ketosis, but increases the sensitivity of CPT A to malonyl-CoA.[73] This indicates that this enzyme or regulation of this enzyme is under hormonal control.

The evidence that CPT is a point of regulation for β-oxidation in mammalian heart tissue is not as convincing.

Studies done to estimate cytosolic carnitine concentration[37] reveal that this substrate is probably not limiting to the CPT A reaction in heart tissue.

Fong and Schulz[74] demonstrated that the activities of CPT, 3-ketoacyl-CoA thiolase, and 3-hydroxyacyl-CoA dehydrogenase are nearly equal and lower than other enzymes of β-oxidation in rat heart mitochondria. They concluded that inhibition of any of these enzymes could result in inhibition of β-oxidation.

On the other hand, Pande[44] showed that mitochondria from red and white skeletal muscle of rabbit and heart and liver of rat respired as rapidly with palmitoyl-CoA and carnitine as substrates as with palmitoyl-carnitine. This indicates that CPT A does not limit β-oxidation under normal conditions. In addition, CPT activity in rat heart and liver mitochondria and in skeletal muscle mitochondria expressed in nmoles palmitoyl-carnitine formed/min/mg protein was always greater than β-oxidation expressed in nmoles palmitoyl group consumed/min/mg protein. However, the method used to assay for CPT activity did not distinguish between CPT A and CPT B, nor was the buffer used isoosmotic with the mitochondrial matrix. Therefore, values obtained for CPT activity probably reflect the sum of CPT A and CPT B activity.

It is still possible that accumulation of some metabolite in the cytosol has an inhibitory effect on CPT A, and thereby affects the rate of β-oxidation by limiting the amount of substrate reaching the mitochondrial compartment.

This seems to be the case for liver, where it has been shown that the rate of fatty acid synthesis can be hormonally modulated. Control of this process using glucagon showed a positive correlation between the rate of fatty acid synthesis and tissue malonyl-CoA concentration.[75, 76] Both of these parameters correlated inversely with long-chain acyl-carnitine concentration and the fatty acid oxidation rate.[76] In addition, it was shown that about 60% of the total CPT was sensitive to malonyl-CoA inhibition, while the remainder was totally resistant. The response of the sensitive CPT to increasing malonyl-CoA concentration was paralleled by the effect on palmitate and palmitoyl-CoA oxidation, but showed no effect on the oxidation of palmitoyl-carnitine.[57]

The CPT of heart mitochondria,[77] brown adipose tissue mitochondria, [78] muscle mitochondria,[79] and brain mitochondria,[80] is also inhibited by malonyl-CoA. In fact, CPT A from heart, muscle and adipose tissue was shown to be about 50 times more sensitive to malonyl-CoA than is the CPT A from liver. By adjusting the assay mixture with KCl and sucrose to be isoosmotic with the mitochondrial matrix, Saggerson[77] demonstrated that, in intact heart mitochondria, 93% of the overt CPT activity could be inhibited by malonyl-CoA. Thus, it is the CPT A that is malonyl-CoA sensitive.

Since fatty acid synthesis is a major metabolic pathway in liver and it has already been demonstrated that tissue levels of malonyl-CoA fluctuate, it is likely that malonyl-CoA accumulates in the cytosol of this tissue and inhibits CPT A. However, heart tissue does not actively synthesize fatty acids. Thus, it would be unlikely that malonyl-CoA appears in the cytosol of heart tissue in significant concentration. This has been disputed by McGarry et al.,[81] who estimated total tissue contents of malonyl-CoA to be 2-8 nmol/g wet wt in

liver, heart and skeletal muscle of fed rats. In addition, the complex kinetic changes observed concerning CPT sensitivity to malonyl-CoA in rat liver mitochondria are not seen in intact rat heart mitochondria,[35] nor are they seen in brain mitochondria.[82]

Aas and Dale[2] observed changes in CPT activity in liver during fasting and refeeding, but not in heart. Therefore, it is unlikely that the CPT A of rat heart mitochondria is regulated by the same mechanism as that of rat liver mitochondria.

Possible inhibitors of CPT A include ketone bodies, glycolytic intermediates, ADP, ATP, NAD^+, NADH. and carnitine derivatives. These compounds were tested for their effects on the CPT activity in rat heart mitochondria. In additon, CoASH derivatives with structural similarities to malonyl-CoA have been tested for their effects on CPT A activity in intact rat heart mitochondria. The mitochondria were kept intact by adjusting the osmolarity of the assay mixture with KCl and sucrose to be isoosmotic with the mitochondrial matrix. The intactness of the mitochondria is significant when examining the effect of CoASH derivatives on CPT A for several reasons: at the low level of inhibitor used, a significant amount can be lost due to the thioesterase activity present in the mitochondrial matrix [83] , thus masking the inhibition; and it has been demonstrated that malonyl-CoA only inhibits the membrane-associated enzyme and not the solubilized enzyme,[57, 59] although this has been disputed.[35] If other CoASH derivatives act similarly to malonyl-CoA, then the intactness of the mitochondrial membrane must be ensured in order to observe the inhibition.

Oxidation of Unsaturated Fatty Acids in Mammalian Heart and Liver Mitochondria

Although the metabolic steps of β-oxidation of saturated fatty acids is well known, the oxidation pathway of unsaturated fatty acids containing *cis* double bonds has not been fully elucidated. The generally accepted pathway requires two enzymes, *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase (EC 5.3.3.8) and 3-hydroxyacyl-CoA epimerase (EC 5.1.2.3), in addition to the normal β-oxidation enzymes for the degradation of unsaturated fatty acids (see Figure 2A).[84] The isomerase catalyzes the conversion of 3-*cis*-enoyl-CoA intermediates to the 2-*trans* isomer[85] which can be further degraded by β-oxidation. The epimerase catalyzes the epimerization of D-3-hydroxyacyl-CoA esters to the corresponding L-hydroxy isomers[84] , which can be completely degraded by the normal β-oxidation enzymes. Kunau and Dommes [86] have presented evidence that the oxidation of 4-*cis*-decenoyl-CoA follows a different pathway requiring 2,4-dienoyl-CoA reductase. Acyl-CoA dehydrogenase II catalyzes the conversion of 4-*cis*-decenoyl-CoA to 2-*trans*,4-*cis*-decadienoyl-CoA. This compound is reduced to 3-decenoyl-CoA by the NADPH-dependent 2,4-dienoyl-CoA reductase[86, 87] ,(see Figure 2B). The 3-decenoyl-CoA thus formed is isomerized to 2-*trans*-decenoyl-CoA by the *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase. This later compound can be further degraded by the normal enzymes of β-oxidation. In addition, it was demonstrated that *cis*-4-decenoyl-CoA is degraded by beef liver mitochondria to octanoyl-CoA without the formation of *cis*-2-octanoyl-CoA[86] , an intermediate expected by the pathway of Stoffel and Caesar.[84]

More recently, Cuebas and Schulz[88] demonstrated that 2-*trans*,4-*cis*-decadienoyl-CoA, a metabolite of linoleate, cannot be degraded by the pathway proposed by Stoffel and Caesar.[84] It was shown that while 2-*trans*,4-*trans*-decadienoyl-CoA is rapidly degraded via β-oxidation, a mixture of 2-*trans*,4-*cis*-decadienoyl-CoA and 2-*cis*,4-*cis*-decadienoyl-CoA can not be degraded via β-oxidation by either rat heart mitochondria or a reconstituted β-oxidation system even when the crotonase concentration was increased 10 fold over the physiological level sufficient for the oxidation of 2-*trans*,4-*trans*-decadienoyl-CoA.[88] In addition, all three isomers of 2,4-decadienoyl-CoA were substrates for the NADPH-dependent 2,4-dienoyl-CoA reductase.[88] The pathway proposed by Cuebas and Schulz[88] for the degradation of linoleic acid is presented in Figure 2B. The product of the reaction catalyzed by 2,4-dienoyl-CoA reductase is 3-decenoyl-CoA[86, 89] which can be isomerized to 2-*trans*-decenoyl-CoA by the *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase.[85] 2-*trans*-Decenoyl-CoA can then be completely degraded by the normal β-oxidation pathway.

Stoffel and Caesar[84] have demonstrated that the *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase and the 3-hydroxyacyl-CoA epimerase are present in both rat liver and rat heart mitochondria in addition to the normal β-oxidation enzymes. In our laboratory, we have only been able to show an extremely low level of the epimerase activity in any mammalian heart tissue examined.[90] It was not clear whether the epimerase activity observed in heart tissue could support the level of linoleic acid degradation expected by this tissue. In order to further investigate this point, oxidation of palmitoyl-CoA, oleoyl-CoA and linoleoyl-CoA were compared in rat heart and rat liver mitochondria. These same mitochondrial preparations were examined for *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase, 2,4-dienoyl-CoA reductase, and 3-hydroxyacyl-CoA epimerase

activities by Mr. Chin-Hung Chu.[91]

Inhibition of Pyruvate Dehydrogenase Complex by Metabolites of 3-Mercaptopropanoic Acid

The convulsant, 3-mercaptopropanoic acid, is believed to cause seizures by interfering with the metabolism of the inhibitory neurotransmitter, γ-aminobutyric acid (GABA), thereby causing a reduction in brain GABA concentration.[92] 3-Mercaptopropanoic acid has been shown to reversibly inhibit glutamate decarboxylase [93, 94, 95] and to activate GABA aminotransferase.[95] It has also been suggested that 3-mercaptopropanoic acid disrupts energy metabolism in rat brain mitochondria, possibly by affecting the utilization or formation of ATP.[96] Sabbagh et al.,[97] have shown 3-mercaptopropanoic acid to be a potent inhibitor of fatty acid oxidation in rat heart mitochondria. Apparently, metabolites of 3-mercaptopropanoic acid reversibly inhibit one or several of the β-oxidation enzymes.[98]

It has been demonstrated that 3-mercaptopropanoic acid is activated in rat heart mitochondria. The 3-mercaptopropanoyl-CoA thus formed is an effective substrate for thiolase (EC 2.3.1.9 and EC 2.3.1.16). When 3-mercaptopropanoyl-CoA substitutes for CoASH in the thiolytic cleavage of acetoacetyl-CoA, S-acetyl-3-mercaptopropanoyl-CoA is formed (Figure 3).[98]

Since β-oxidation is not a major pathway in brain[15, 16, 99], the observed inhibition of this pathway in 3-mercaptopropanoic acid-treated rat heart mitochondria could not explain the onset of seizures in 3-mercaptopropanoic acid-treated rats. Oxidation of glucose is the major pathway of energy production

in the brain[15, 17, 100] and inhibition of the key enzyme, pyruvate dehydrogenase, in this pathway would have a profound effect on energy metabolism in this tissue. In addition, there is a close dependence of acetylcholine synthesis on the pyruvate dehydrogenase reaction.[101, 102, 103] Since ketone bodies are oxidized by brain tissue in proportion to their concentration in the blood[16, 104], the presence of acetoacetyl-CoA in the brain exposed to 3-mercaptopropanoic acid may result in the production of S-acetyl-3-mercaptopropanoyl-CoA, intramitochondrially.

In view of this information, the effects of 3-mercaptopropanoyl-CoA and S-acetyl-3-mercaptopropanoyl-CoA on pyruvate dehydrogenase complex and its component enzymes were investigated. In addition, the effects of S-acetyl-3-mercaptopropanoic acid and 3-mercaptopropanoic acid on pyruvate-supported respiration in rat heart and liver mitochondria were studied.

Aim of Research

Understanding the regulation of β-oxidation in mammalian heart is the major research aim of this laboratory. Since fatty acid activation and the translocation of fatty acyl groups into the mitochondria via the carnitine acyltransferase system are the first two committed steps of β-oxidation, it is possible that this pathway is controlled by the regulation of either the fatty acyl-CoA synthetase or one of the CPT enzymes. Hence, I have studied the effects of metabolites and coenzymes on the activities of these two enzymes, especially the effects of malonyl-CoA and its structural analogs on the activity of CPT A. In addition, I have examined the possibility that the fatty acyl-CoA synthetase is regulated via phosphorylation-dephosphorylation. The results of these

studies gave no indication that fatty acid oxidation in rat heart mitochondria is controlled via the regulation of either CPT or fatty acyl-CoA synthetase.

Because of my experience in the isolation of mitochondria and measurement of β-oxidation, a new project was undertaken to demonstrate that the rate of oxidation of linoleoyl-CoA in rat heart mitochondria was too high to be dependent on the low level of epimerase found in that organelle.

When I completed the first part of my thesis research, other members of our laboratory had demonstrated that 3-mercaptopropanoic acid, a known convulsive agent, is a potent inhibitor of fatty acid oxidation in rat heart mitochndria.[97] Since β-oxidation is not a major pathway in brain[15, 16, 99] , the observed inhibition of this pathway could not explain the onset of seizures in 3-mercaptopropanoic acid-treated rats. When it was demonstrated, in our laboratory, that 3-mercaptopropanoic acid is activated and that the 3-mercaptopropanoyl-CoA thus formed is an effective substrate for thiolase[98] , the possibility that 3-mercaptopropanoyl-CoA may act as a substrate or inhibitor of pyruvate dehydrogenase occurred to me and this study was then undertaken. In addition, inhibition of the pyruvate dehydrogenase reaction by S-acetyl-3-mercapopropanoyl-CoA (the product of the thiolase reaction) was investigated. Pyruvate dehydrogenase was selected for this study because of its key role in the energy metabolism of brain.

EXPERIMENTAL PROCEDURES

Materials

Most organic chemicals and the pyruvate dehydrogenase complex were purchased from Sigma Chemical Co. NAD^+, NADH, CoASH, and CoA derivatives not synthesized in our laboratory were purchased from P-L Biochemicals. The enzyme, nagase, was purchased from the Enzyme Development Corp., New York, N.Y. Citrate synthase was purchased from Boehringer-Mannheim Corp. Radiolabelled compounds were purchased from New England Nuclear. Digitonin was purchased from Calbiochem. Diketene and DCPIP were purchased from Aldrich Chemical Co. Oleic acid and linoleic acid were purchased from Supelco, Inc. L-Carnitine and palmitoyl-L-carnitine were the generous gifts of Dr. K. Brendel, University of Arizona College of Medicine. S-Acetyl-3-mercaptopropanoic acid and *bis*[2-carboxyethyl]disulfide were synthesized in our laboratory by Dean Cuebas. Pig heart 3-ketoacyl-CoA thiolase (EC 2.3.1.16) was purified by Mr. Chin-Hung Chu according to published procedures.[105]

Preparation of Rat Heart Mitochondria

Mitochondria were isolated from the hearts of adult male albino rats by the following method. The beating hearts were removed from two ether-anesthetized rats and placed in ice-cold MST buffer[106] (210 mM mannitol, 70 mM sucrose, 5 mM Tris, 1 mM EGTA, adjusted to pH 7.4 with HCl). The hearts were cut open, cleaned of blood vessels and washed free of blood in ice-cold MST buffer. The hearts were finely minced. The suspension was stirred

continuously for 9 min at 0°C with 6 mg of nagase, a general protease used to soften the heart tissue by degrading some of the connective tissue protein. This step was omitted if the mitochondria were to be used for the fatty acyl-CoA synthetase reaction. The resulting suspension was transferred to a glass homogenization tube. After dilution to approximately 30 ml in ice-cold MST buffer, the suspension was homogenized by 5 strokes of a loose-fitting Teflon pestle spinning at 750 rpm. If nagase was used, the homogenate was centrifuged at 20,000 rpm in an SS-34 rotor of a Sorvall Centrifuge for 7 min at 5°C. The pellet was resuspended in about 30 ml of ice-cold MST buffer with a rubber policeman and homogenized by one stroke of the glass-Teflon tissue homogenizer. The resulting suspension was centrifuged twice at 10,000 rpm in an SS-34 rotor of a Sorvall Centrifuge for 7 min at 5°C. When nagase was not used, the homogenate was centrifuged at 10,000 rpm in an SS-34 rotor of a Sorvall Centrifuge for 7 min at 5°C. The pellet, each time, was resuspended in about 30 ml ice-cold MST buffer with a rubber policeman and homogenized by one stroke of the glass-Teflon tissue homogenizer. The resulting suspension was centrifuged at 2,300 rpm in an SS-34 rotor of a Sorvall Centrifuge for 5 min at 5°C. The supernatant was decanted through two layers of cheesecloth into 15 ml Corex tubes. These tubes were centrifuged at 8,000 rpm in an SS-34 rotor of a Sorvall Centrifuge for 7 min at 5°C. The mitochondrial pellets were resuspended into a small volume of ice-cold MST buffer using a rubber policeman. Mitochondria prepared in this way were used fresh or stored frozen at -80°C and used after thawing.

Integrity of the mitochondria isolated was assessed by determining the respiratory control ratio (RCR) and NADH dehydrogenase activity by means of oxygen consumption monitored at 25°C using a Clark oxygen electrode and

Gilson Oxygraph. The standard incubation mixture contained in 2 ml, 0.11 M KCl, 33 mM Tris, 2 mM KP*i*, 2 mM $MgCl_2$, 0.1 mM EGTA, pH adjusted to 7.4 with HCl, and mitochondria (1 mg protein). For measuring the RCR, the incubation mixture also contained 1 mg/ml BSA, 0.5 mM *l*-malate, and 30 μM palmitoyl-*l*-carnitine. The state III respiration was initiated by the addition of 0.5 μmoles of ADP. State IV respiration was taken as the rate of oxygen consumption after ADP depletion. The RCR is the ratio of state III to state IV respiration.

The NADH dehydrogenase reaction was initiated by the addition of 0.5 μmoles of NADH to the standard incubation mixture. A conversion factor of 1 nanoatom of oxygen consumed per nanomole of NADH oxidized was used.

Preparation of Rat Liver Mitochondria

Rat liver mitochondria were prepared from the livers of adult male albino rats by the following method. The liver of one ether-anesthetized rat was removed and placed in ice-cold STE buffer (0.25 M sucrose, 5.0 mM Tris, 1 mM EGTA, adjusted to pH 7.4 with HCl). The liver was cleaned of large blood vessels and washed free of blood in ice-cold STE buffer. After finely mincing, 2-3 g of the tissue was transferred to a glass homogenization tube and diluted to 35 ml in ice-cold STE buffer. This suspension was homogenized by 2 strokes of a loose-fitting Teflon pestle spinning at 750 rpm. This was repeated until all of the tissue suspension was homogenized. The homogenate was centrifuged at 2,300 rpm in an SS-34 rotor of a Sorvall Centrifuge for 10 min at 5°C. The supernatant was decanted through two layers of cheesecloth into fresh centrifuge tubes. These tubes were centrifuged at 10,000 rpm in an SS-

34 rotor of a Sorvall Centrifuge for 10 min at 5°C. The supernatants were discarded and the white fluffy layers were washed off the mitochondrial pellets. The pellets were resuspended into ice-cold STE buffer using a rubber policeman and transferred to 15 ml Corex tubes. These tubes were centrifuged at 9,000 rpm in an SS-34 rotor of a Sorvall Centrifuge for 10 min at 5°C. The pellets were again washed and resuspended and finally centrifuged at 8,000 rpm for 10 min. The final mitochondrial pellets were resuspended into a small volume of ice-cold STE buffer.

Preparation of Bovine Heart Mitochondria[32]

Bovine heart mitochondria were prepared from fresh bovine heart stored several hours on ice. The entire procedure was performed at 4°C. Ventricular tissue was cleaned of all fat and connective tissue and cut into small pieces which were passed through a meat grinder. Portions of ground tissue (60 g) were blended at high speed in a Waring blender for 45s in 500 ml Buffer A (0.25 M sucrose, 5.0 mM HEPES, 0.25 mM EDTA, pH 7.7) followed by homogenization in 60 ml aliquots for 30s with a Polytron tissue homogenizer set at 33% maximal speed. The homogenized samples were pooled and centrifuged for 15 min at 500 x g, 15,000 x g, 500 x g, 11,000 x g, and 7,000 x g. High-speed pellets were resuspended in isotonic buffer (0.25 M sucrose, 2.5 mM HEPES, 0.25 mM EDTA, pH 7.5) by two strokes of a glass-Teflon tissue homoginizer with a loose-fitting plunger set at 750 rpm. The 7,000 x g pellet was resuspended in a small volume of isotonic buffer using a rubber policeman and dispensed into test tubes which were stored at -80°C.

Preparation of Rat Heart Tissue Homogenate[37]

Homogenate was made from the hearts of adult male albino rats. The beating hearts were removed from ether-anesthetized rats and placed in ice-cold buffer (180 mM KCl, 10 mM Tris, 10 mM EDTA, 0.5 % (w/v) BSA, pH 7.6). The atria and blood vessels were cleaned from the ventricular tissue, which was then washed free of blood in fresh buffer. The tissue was minced, then transferred to 6 ml ice-cold buffer in a 30 ml centrifuge tube and homogenized on ice with a Polytron tissue homogenizer with rheostat at 33% maximal speed for two 5-s periods. The homogenate was diluted in ice-cold buffer and homogenized in a glass-Teflon homogenizer with a loose-fitting pestle.

Subcellular Fractionation of Rat Heart Tissue

Method I

Hearts were obtained and minced as described on page 20. The finely minced hearts were transferred to a glass homogenization tube and diluted to approximately 30 ml in ice-cold MST buffer. The suspension was homogenized by 5 strokes of a loose-fitting Teflon pestle spinning at 750 rpm. The homogenate was centrifuged at 2,300 rpm in an SS-34 rotor of a Sorvall Centrifuge for 5 min at 5°C. A portion of the supernatant was retained as the whole heart homogenate. The remainder of the supernatant was centrifuged at 44,000 rpm in a Type-50 rotor of a Spinco Ultracentrifuge for 1 hr at 5°C. The high-speed supernatant was retained as the cytoplasmic fraction. The high-speed pellet was resuspended in a small volume of MST buffer and retained as the extracytoplasmal fraction. The three fractions were stored

overnight at -20°C. After thawing, the whole heart homogenate and the extracytoplasmic fraction were sonicated using the microtip of a probe-type sonicator for 5 x 5 sec at 4°C. The sonicated fractions were centrifuged at 44,000 rpm in a Type-50 rotor of a Spinco Ultracentrifuge for 1 hr at 5°C. The pellet was discarded.

Method II

A homogenate of the rat heart tissue was obtained as in Method I. The homogenate was centrifuged at 10,000 rpm in an SS-34 rotor of a Sorvall Centrifuge for 7 min at 5°C. The supernatant was retained as the cytoplasmic fraction. The pellet was resuspended in about 30 ml ice-cold MST buffer with a rubber policeman and homogenized by one stroke of a glass-Teflon tissue homogenizer. The resulting suspension was centrifuged at 2,300 rpm in an SS-34 rotor of a Sorvall Centrifuge for 5 min at 5°C. The supernatant was decanted through two layers of cheesecloth into 15 ml Corex tubes. These tubes were centrifuged at 8,000 rpm in an SS-34 rotor of a Sorvall Centrifuge for 7 min at 5°C. The mitochondrial pellets were resuspended into a small volume of ice-cold MST buffer using a rubber policeman. The mitochondrial fraction was sonicated using the microtip of a probe-type sonicator for 5 x 5 sec. Both the sonicated mitochondrial fraction and the cytoplasmic fraction were centrifuged at 44,000 rpm in a Type-50 rotor of a Spinco Ultracentrifuge for 1 hr an 5°C. The pellets were discarded.

Measurement of Protein Concentration

Protein concentration was determined by either the Biuret Method[107] or the Lowry Method[108] using BSA as standard.

Measurement of Oxygen Uptake by Mitochondria

Oxygen consumption was monitored at 25°C by means of a Clark oxygen electrode and Gilson Oxygraph. The standard incubation mixture for rat heart mitochondria contained 0.11 M KCl, 33 mM Tris (pH 7.4), 2 mM KP*i*, 2 mM $MgCl_2$, 0.1 mM EGTA, and mitochondria (0.5 mg protein/ml). The standard incubation mixture for rat liver mitochondria contained 0.1 M KCl, 20 mM Tris (pH 7.4), 4 mM KP*i* (pH 7.4), 4 mM $MgCl_2$, 0.1 mM EGTA, and mitochondria (1 mg protein/ml). When palmitoyl-*l*-carnitine (30 μM) was used as substrate, 14.7 μM BSA was included in the incubation mixture. When fatty acyl-CoA (15 μM) was used as substrate, 14.7 μM BSA, 0.5 mM L-malate, and 13 mM *l*-carnitine was included in the incubation mixture. In addition, either 0.5 mM malate or 5 mM α-ketoglutarate, was also included in the incubation mixture. After preincubation of the mixture for about 1 min, the reaction was started by the addition of ADP (final concentration, 1 mM) and substrate. Respiratory Control Ratios (RCR) were measured using 30 μM palmitoyl-*l*-carnitine as substrate, in the absence of carnitine.

Preparation of Malonyl-CoA

Reduced coenzyme A, Li salt (12.5 mg), S-malonyl-N-capryl cysteamine (17.4 mg), and sodium bicarbonate (42 mg) were placed in a test tube to which

was added 1 ml of distilled water. This was left at room temperature for 1 hr, then refluxed with ether for 30 min. The pH of the solution was adjusted to 1.0 with 2 N H_2SO_4. The solution was extracted with ether for 30 min and adjusted to pH 5.0 with 1.0 N KOH. Residual ether was removed under a stream of nitrogen.

Preparation of Acetoacetyl-CoA [109]

Reduced CoA, Li salt (8 mg), and 3 μl of diketene were added to 1 ml of 0.1 M potassium bicarbonate. This solution was stirred under a stream of nitrogen for 20 min at room temperature. The pH was adjusted to 3-4 with concentrated HCl.

Preparation of Succinyl-CoA [110]

Reduced CoA, Li salt (7 mg), was dissolved in 1 ml of ice-cold distilled water. Succinic anhydride (85.38 mg) was added and the pH adjusted to 8.0 with 71.66 mg sodium bicarbonate. The mixture was kept in an ice bath for 30 min with frequent shaking. The pH was brought to 2.0. The resulting solution was stored at -20°C.

For succinyl-CoA solution devoid of CoASH the following procedure was performed at 0°C under nitrogen. Potassium bicarbonate (25 mg) was dissolved in 1 ml of ice-cold distilled water. The solution was evacuated with a stream of nitrogen prior to addition of 7 mg of reduced CoA, Li salt. Succinic anhydride (20 mg) was dissolved in 3 ml of dry THF and the resulting solution was added (6-8 drops at a time) to the CoASH solution until no yellow

color was observed when a drop of the solution was added to a drop of Ellman's reagent.[111] The pH of the solution was brought to 1.0 and the THF removed by rotary evaporation under reduced pressure. The pH was brought to 2.0 and the resulting solution was stored at -20°C.

Preparation of Oleoyl-CoA and Linoleoyl-CoA

Oleoyl-CoA and linoleoyl-CoA were prepared with the assistance of Dean Cuebas by the mixed anhydride method of Goldman and Vagelos.[112] During this procedure, all operations were carried out under nitrogen. The final aqueous solutions were stored under nitrogen in sealed ampules at -76°C.

Preparation of 3-Mercaptopropanoyl-CoA

The mixed anhydride method of Goldman and Vagelos[112] was used to synthesize the CoA derivatives of *bis*-[2-carboxyethyl]-disulfide. The products formed were separated on a DEAE cellulose column by gradient elution with 0 to 0.3 M LiCl, pH 4.0. The fractions containing 2-carboxyethyl-disulfidepropanoyl-CoA were identified spectrophotometrically in a Gilford Spectrophotometer by measuring absorbance at 260 nm and 235 nm. The appropriate fractions were pooled and concentrated to 3.3 ml on an Amicon Diaflo Ultrafiltration membrane, YC 05, using 50 psi N_2. The purity of the product as determined by assessing the concentration of both adenosine and thioester. That of adenosine was measured spectrophotometrically at an absorbance of 260 nm; that of thioester was measured by the method of Ellman.[111] Theoretically, the ratio of adenosine to thioester bond should be 1:1.

This solution was acidified to pH 1-2 with HCl and purged with nitrogen. The disulfide linkage was reduced with 40-60 mg zinc dust with constant vortexing under nitrogen until the Ellman Test [111] revealed that the reaction had gone to completion. The solution was filtered to remove excess zinc dust, and applied to a DEAE cellulose column. Gradient elution with 0 to 0.3 M LiCl, pH 4.0, that had been purged with N_2 was performed. The fractions containing 3-mercaptopropanoyl-CoA were identified spectrophotometrically by measuring the absorbance at 260 nm. The appropriate fractions were pooled and concentrated to approximately 3.5 ml on an Amicon Diaflo Ultrafiltration membrane, YC 05, using 50 psi N_2. The purity of the product was determined by assessing the concentration of adenosine, free sulfhydryl group and thioester. That of adenosine was measured spectrophotometrically at 260 nm; that of free sulfhydryl group and thioester were measured by the method of Ellman.[111] Theoretically, the ratio of adenosine to free sulfydryl group, to thioester bond should be 1:1:1. By this method, the solution was determined to contain 88% 3-mercaptopropanoyl-CoA and 12% dimerized 3-mercaptopropanoyl-CoA. The product was purged with nitrogen and stored at -80°C. Prior to use, the solution was thawed and the concentration reassessed.

Preparation of S-Acetyl-3-Mercaptopropanoyl-CoA

S-Acetyl-3-mercaptopropanoyl-CoA was synthesized by the mixed anhydride method of Goldman and Vagelos.[112] The crude product thus formed was purified on a DEAE cellulose column by gradient elution with 0 to 0.3 M LiCl, pH 4.0. The fractions containing S-acetyl-3-mercaptopropanoyl-CoA were identified spectrophotometrically by measuring absorbance at 260 nm and 235 nm. The appropriate fractions were pooled and concentrated to 3.5 ml on an

Amicon Diaflo Ultrafiltration membrane, YC 05, using 50 psi N_2. The purity of the product was determined by two methods. Spectrophotometrically, the concentration of adenosine was determined by measuring the absorbance at 260 nm, and the concentration of thioester was measured by the method of Ellman.[111] Theoretically, the ratio of adenosine to thioester bond should be 1:2. The compound was always found to be greater than 99% pure by this method. HPLC was also used to assess purity of the product, using a Waters Microbondapak C18 column and eluting with a gradient of 0.05 M ammonium phosphate buffer, pH 5.5, containing 15-30% methanol, in 3 min at a rate of 1 ml/min. This method revealed three contaminants, with the major component, S-acetyl-3-mercaptopropanoyl-CoA always representing at least 88% of the CoA-containing material.

Preparation of *d,l*-Dihydrolipoamide

Dihydrolipoamide was synthesized by the reduction of *d,l*-lipoamide by the method of Reed et al..[113] A suspension of 200 mg *d,l*-lipoamide in 5 ml methanol/water, 4/1 (v/v), was stirred at 0°C. A solution of 200 mg sodium borohydride in 1 ml water was cooled and added to the lipoamide suspension while stirring. The reaction mixture was stirred until it became clear and colorless. The solution was acidified with HCl and the methanol was evaporated under a stream of nitrogen. The aqueous solution remaining was extracted 5 times with 15 ml of chloroform. The resulting chloroform solution was evaporated to dryness under reduced pressure. The residue was recrystallized from a solution of petroleum ether and acetone to yield 46.91 mg of white plates; m.p. 63.5-65.5°C. A solution of 4.49 mM dihydrolipoamide yielded 8.44 mM free sulfhydryl group based on the Ellman's

determination.[111]

Ellman's Determination[111] of the Concentration of Coenzyme A Derivatives

Hydroxylamine will cleave the thioester bond of a coenzyme A derivative. The resulting free CoASH will react with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) to produce 5-thio-2-nitrobenzoate which absorbs light strongly at 412 nm with a molar extinction coefficient of 13,600 $cm^{-1}M^{-1}$ at pH 8.0.[114]

A small aliquot of the derivative (5-20 μl) was placed in a cuvette with 20 μl of 2 M hydroxylamine, pH 7.0. This was allowed to stand at room temperature for 5 min. To this was added 10 μl of Ellman's reagent, 300 μl of 0.1 M potassium phosphate buffer (pH 8.0), and distilled water to a total volume of 1 ml. Absorbance at 412 nm was read in a Gilson Spectrophotometer. The value obtained was corrected for free CoASH in the solution by performing the Ellman's determination without the addition of hydroxylamine.

Assay for Palmitoyl-CoA Synthetase Activity

Activity was measured based on the rate of production of [U-^{14}C]-palmitoyl-CoA from [U-^{14}C]-palmitate.[6] The assay system contained 0.1 M Tris, 4 mM $MgCl_2$, 5 mM reduced glutathione, 2 mM KCN, 5 mM malonate, 5 mM ATP, 0.15 mM CoASH, 0.02 mM BSA, and 0.1 mM [U-^{14}C]-palmitate (0.45 mCi/mmole) in a volume of 1 ml.

The reaction was started by the addition of 0.25-0.30 mg of protein. The samples were incubated in a 37°C water bath and the reaction was stopped by the addition of 4 ml of Dole reagent [115] (isopropyl alcohol/n-heptane/1 N H_2SO_4, 40/10/1, v/v/v). Control tubes had Dole reagent added prior to addition of protein. To each tube, 1.4 ml of distilled water and 2.4 ml of heptane were added. The sample was vortexed and centrifuged. The upper layer, containing the unreacted palmitate, was removed. The lower aqueous layer was washed four times with 2.4 ml of heptane. An aliquot of the lower aqueous layer, containing [^{14}C]-palmitoyl-CoA, was placed in 10 ml of Bray's solution and assayed for radioactivity by liquid scintillation counting in a Beckman Scintillation Counter.

Spectrophotometric Assay for Carnitine Palmitoyltransferase Activity[116]

The assay was based on measuring the initial rates of RSH formation using the DTNB reaction. The product, 5-thio-2-nitrobenzoate, absorbs light strongly at 412 nm with a molar extinction coefficient of 13,600 $cm^{-1}M^{-1}$ at pH 8.0.[114]

The assay system contained 116 mM Tris-Cl (pH 8.0), 0.09 % Triton X-100, 0.035 mM palmitoyl-CoA, 0.11 mM DTNB, 1.1 mM *l*-carnitine, 1.1 mM EDTA* , in a volume of 1 ml.

The reaction was started by the addition of protein. Absorbance at 412 nm was monitored over a time period of 2-5 min. The rate of free RSH

EDTA was demonstrated to be unnecessary for maximal activity and was omitted in several assays.

formation by competing reactions was assayed by omission of carnitine from the assay mixture. This value was subtracted from that determined in the carnitine-containing mixture in order to determine the rate of CPT-generated CoASH formation.

Radiochemical Assay for Carnitine Palmitoyltransferase Activity[77, 78]

Rat heart mitochondrial protein (0.05 mg) was preincubated at room temperature for 2 min in 1 ml containing 210 mM sucrose, 30 mM KCl, 50 mM Tris-HCl or MOPS-KOH, pH 7.4, 1 mM EGTA, BSA (1.3 mg/ml), 40 μmoles of *l*-carnitine containing 0.6-1.0 μCi *d,l*-[Me-^{14}C]-carnitine or 1.1 μCi *d,l*-[Me-^{3}H]-carnitine and 40 μM palmitoyl-CoA. The reaction was terminated after 2-4 min with 1 ml of ice-cold 1.2 M HCl followed by 2 ml of water-saturated butanol. After vortexing and brief centrifugation, the butanol layer was washed with 5 ml of butanol-saturated water. An aliquot of the butanol layer was taken for liquid scintillation counting in 5 ml of Scintiverse II (Fisher Scientific) using a Beckman Liquid Scintillation Counter.

Hydrolysis of CoASH Derivatives Under Assay Conditions

The assay was based on measuring the rates of RSH formation using the 5,5'-dithio-*bis*-(2-nitrobenzoic acid) reaction as described on page 31, under the conditions used in the radiochemical assay for carnitine palmitoyltransferase.

Rat heart mitochondria (0.05 mg) was added to 1 ml containing 210 mM sucrose, 30 mM KCl, 50 mM Tris-HCl or MOPS-KOH (pH 7.4), 1 mM EGTA, 1.3 mg/ml BSA, 100 μM CoASH derivative (palmitoyl-CoA was omitted).

Absorbance at 412 nm was measured for 2 min. *l*-Carnitine (0.4 μmoles) was added and the absorbance at 412 nm was measured for at least 2 min. Initial rates of hydrolysis of CoASH derivatives were determined for the preincubation period (without carnitine) and the reaction period (with carnitine).

Separation of Palmitoyl-CoA Synthetase Activity from CPT Activity

This procedure was based on the method described[117] for the separation of the outer mitochondrial membrane from the "mitoplast".

A stock solution of 1.0% digitonin in isotonic beef heart mitochondria isolation buffer was prepared by dissolving the digitonin in hot (almost boiling) buffer. After cooling to room temperature, BSA (50 mg/ml) was added to the solution. A volume of beef heart mitochondria (100 mg/ml protein) was placed in a pre-cooled vial on ice, to which an equal volume of the digitonin stock solution was added. The mixture was stirred gently with a magnetic stirrer for 15 min on ice. The solution was diluted with 3 volumes of buffer and centrifuged at 9,000 x g in the SS-34 rotor of the Sorvall Centrifuge at 4°C for 10 min. The supernatant, which should contain the outer membrane vesicles and intermembrane components[117] , was retained. The pellet was brought to one half the original volume with buffer and centrifuged at 9,000 x g in the SS-34 rotor of the Sorvall Centrifuge at 4°C for 10 min. The resulting supernatant was combined with the first supernatant, and the pellet was discarded.

The supernatant obtained was treated in one of two ways. Either the protein was precipitated out of solution by ammonium sulfate precipitation at 4°C followed by centrifugation, or the protein was brought out of solution by

ultracentrifugation at 144,000 x g for 1 hr in a Spinco ultracentrifuge. The pellets were resuspended in a small amount of buffer and stored at -80°C.

Assay for Succinate Thiokinase (EC 6.2.1.4)[118]

Activity was measured based on the rate of production of succinyl-CoA from succinate and reduced CoASH. Tris-succinate, pH 7.4, was prepared as a solution containing 0.1 M succinate and 0.22 M Tris. The assay system, 1 ml in volume, contained 0.11 M Tris, 0.05 M succinate, 0.01 M $MgCl_2$, 0.10 mM GTP, and 0.10 mM CoASH. The reaction was initiated by the addition of protein. The reactions were allowed to proceed at room temperature for 5, 20, and 40 min, at which times a 250 μl aliquot was removed to 25 μl of 1.2 N HCl to stop the reaction. Controls were prepared by adding the appropriate amount of protein to a 250 μl assay system which had already been acidified. After 10 min at pH 1, the solutions were brought to pH 3 with 10 μl of 1 M KOH.

When the assay was repeated on subcellular fractions obtained by Method II, it was performed in a volume of 250 μl containing, in addition to the usual components, 0.10 mM ATP. The reaction was stopped after 5 min by the addition of 40 μl of 1.2 N HCl. After 10 min at pH 1, the solutions were brought to pH 3 with 15 μl of 1 M KOH.

Succinyl-CoA content of each solution was determined by reversed-phase HPLC using a Waters Microbondapak C18 column with absorbance detection at 254 nm. A gradient elution system was employed using 5-9% methanol in 0.5 M ammonium phosphate buffer, pH 5.5, and a rate of 1.5 ml/min. Peak area was measured.

Assay for Citrate Synthase (EC 4.1.3.7)[119]

The assay was based on measuring the initial rates of RSH formation using the 5,5'-dithio-*bis*-(2-nitrobenzoic acid) reaction of Ellman.[111]

The assay system, 1 ml in volume, contained 0.1M Tris-HCl (pH 8.1), 0.1 mM DTNB, 0.3 mM acetyl-CoA, and 0.5 mM oxaloacetate. The enzyme source was added to the solution containing Tris, DTNB, and acetyl-CoA. The reaction was initiated by addition of oxaloacetate. Absorbance at 412 nm was monitored over a time period of 2-5 min.

Assay for Enoyl-CoA Hydratase (EC 4.2.1.17)[120]

The assay mixture contained, in a volume of 1 ml, 0.35 M Tris-HCl (pH 7.4), and 85 μM crotonyl-CoA. The reaction was initiated by the addition of enzyme source. Absorbance change at 280 nm was measured in a Gilford Spectrophotometer.

Assay for 3-Hydroxyacyl-CoA Dehydrogenase (EC 1.1.1.35)[116]

Oxidation of NADH was measured spectrophotometrically at 340 nm in a Gilford Spectrophotometer. The assay mixture contained, in a volume of 1 ml, 0.35 M Tris-HCl (pH 7.0), 0.1 mM NADH, and 50 μM acetoacetyl-CoA. The reaction was initiated by addition of enzyme source.

Activity of Pyruvate Dehydrogenase Complex

Pyruvate dehydrogenase complex was assayed spectrophotometrically at 30°C as described by Pettit and Reed.[121] The assay mixture contained, in a volume of 1 ml, 50 mM KP*i* (pH 7.4), 1.0 mM $MgCl_2$, 2.5 mM TPP, 65 μM CoASH, 16 μM dithiothreitol* , 2.0 mM sodium pyruvate (Sigma type II), 2.5 mM NAD^+, and the enzyme. Absorbance changes were monitored at 340 nm in a Gilford Spectophotometer.

Activity of Pyruvate Dehydrogenase (EC 1.2.4.1)

Pyruvate dehydrogenase was measured spectrophotometrically by the method of Lowe et al.[122] , in an assay involving reduction of 2,6-dichlorophenolindophenol. The reaction mixture contained, in a volume of 1 ml, 0.1 M KP*i* (pH 7.0), 100 μM or 50 μM dichlorophenolindophenol, 0.2 mM TPP, 1 mM $MgCl_2$, 50 mM sodium pyruvate (Sigma type II), and the enzyme. The reduction of 2,6-dichlorophenolindophenol was measured spectrophotometrically at 600 nm in a Gilford Spectrophotometer.

This enzyme was also measured spectrophotometrically in an assay involving the reduction of ferricyanide. The reaction mixture contained, in a volume of 1 ml, 0.1 M KP*i* (pH 6.5), 18 or 1.8 mM potassium ferricyanide, 0.14 mM TPP, 0.71 mM $CaCl_2$, 36 mM sodium pyruvate (Sigma type II), 1.4 mg/ml BSA, and the enzyme. The reduction of ferricyanide was measured spectrophotometrically at 410 nm in a Gilford Spectrophotometer.[123]

Dithiothreitol was demonstrated to be unnecessary for maximal activity and was therefore omitted in many of the assays.

Activity of Pyruvate Dehydrogenase - Lipoate Acetyl Transferase

The combined pyruvate dehydrogenase - lipoate acetyl transferase activity was assayed by measuring the amount of acetyl-CoA produced. This was quantitated by converting it to citrate and CoASH.

$$\text{pyruvate + lipoamide + CoASH} \longrightarrow \text{acetyl-CoA + dihydrolipoamide}$$

$$\text{acetyl-CoA + oxaloacetate} \xrightarrow{\text{citrate synthase}} \text{citrate + CoASH}$$

The reaction mixture contained, in a volume of 2.5 ml, 50 mM Tris-HCl (pH 7.4), 1.0 mM $MgCl_2$, 0.2 mM TPP, 65 μM CoASH, 2.0 mM sodium pyruvate (Sigma type II), 0.4 mM lipoamide, and the enzyme. The reaction was stopped at various time intervals by addition of a 0.5 ml aliquot of the reaction mixture to 0.5 ml of the citrate synthase reaction mixture containing 150 mM Tris-HCl (pH 8.1), 0.8 mM DTNB, and the citrate synthase. The reaction was initiated by the addition of 5.0 μmoles of oxaloacetate and allowed to go to completion. The absorbance at 412 nm, an absorbance maxima for 5-thio-2-nitrobenzoate which is released by the reaction of DTNB with free sulfhydryl groups[114] , was measured spectrophotometrically in a Gilford Spectrophotometer prior to the addition of oxaloacetate and after the reaction had gone to completion. The difference between the two values was used to determine the amount of acetyl-CoA produced.

Activity of Lipoamide Dehydrogenase

Lipoamide dehydrogenase activity was assayed in both the forward and reverse direction by the method of Hayakawa et al..[124] For the forward direction, the reaction mixture contained 50 mM KP*i* (pH 6.5), 0.1 mM NAD^+, 0.4 mM dihydrolipoamide, 1.25 mM EDTA, and the enzyme, in a final volume of 1 ml. For the reverse direction, the reaction mixture contained 50 mM KP*i* (pH 6.5), 0.1 mM NADH, 0.4 mM lipoamide, 1.25 mM EDTA, and the enzyme, in a final volume of 1 ml. The disappearance and formation of NADH were measured spectrophotometrically at 340 nm in a Gilford Spectrophotometer.

Rapid Gel Filtration of Pyruvate Dehydrogenase Complex

Rapid gel filtration of the enzyme was performed by applying 0.1 ml of sample to a 1 ml Sephadex G-50 column which had been washed with 50 mM KP*i* buffer (pH 7.4) containing BSA (10 mg/ml) and from which the void volume had been removed by centrifugation. The eluate was quickly collected by centrifugation of the charged column for 2 min in a clinical centrifuge. Samples were prepared, each containing in 0.2 ml, pyruvate dehydrogenase (0.085 mg protein), 50 mM KP*i* (pH 7.56), 1 mM $MgCl_2$, 0.2 mM TPP, 66.5 μm CoASH, 2 mM sodium pyruvate (Sigma Type II), ±235.25 μM S-acetyl-3-mercaptopropanoyl-CoA, and incubated at room temperature for 15 min. Just prior to centrifugation, BSA was added to the sample to give a final concentration of 0.3% BSA. Aliquots of 20 μl were assayed immediately while 100 μl of each sample was subjected to rapid gel filtration and then assayed for pyruvate dehydrogenase activity.

NADH-Dependent Acetylation of the Pyruvate Dehydrogenase Complex

The NADH-dependent acetylation of the pyruvate dehydrogenase complex by [1-^{14}C]-acetyl-CoA, was performed by the method of Collins and Reed.[125]

The reaction mixture contained 0.2 - 0.22 mg pyruvate dehydrogenase complex, 50 mM KPi, pH 7.0, 1mM $MgCl_2$, 0.2 mM TPP, 0.1 mM NADH, and ± S-acetyl-3-mercaptopropanoyl-CoA. This was preincubated at room temperature for 3 min prior to initiation of the reaction with 10μl of 6.25 mM [1-^{14}C]-acetyl-CoA, 10 μCi/ml, to bring the volume of the reaction mixture to a total of 250 μl. Aliquots of 50 μl were removed from the reaction mixture at 5 min and 10 min and applied to squares (2x2 cm) of Whatman No. 3MM filter paper. The papers were washed four times with 20 ml of ice-cold 10% trichloroacetic acid with shaking for 10 min each time. This was followed by washing twice with 20 ml of ice-cold ethanol with shaking for 5 min, followed by washing twice with 20 ml of ice-cold ether at room temperature with shaking. The papers were placed in 5 ml of Scintiverse II (Fisher Scientific) and counted in a Beckman Scintillation Counter.

Effect of Enzymatically Prepared S-Acetyl-3-Mercaptopropanoyl-CoA on the Pyruvate Dehydrogenase Complex

S-Acetyl-3-mercaptopropanoyl-CoA was synthesized enzymatically using 3-mercaptopropanoyl-CoA as a substrate for 3-ketoacyl-CoA thiolase. Varying concentrations of 3-mercaptopropanoyl-CoA were added to a cuvette containing 33 μM acetoacetyl-CoA, 1.0 mM $MgCl_2$, BSA (0.1 mg/ml), 0.1 M Tris-HCl, pH 8.0, and thiolase (33 mU/ml) in 1 ml. The reaction was

observed to go close to completion by monitoring the change in absorbance at 303 nm. The reaction was further pulled to completion by conversion of the acetyl-CoA to citrate, catalyzed by citrate synthase.

The resulting solution containing S-acetyl-3-mercaptopropanoyl-CoA was made to contain 2.5 mM TPP, 130 μM CoASH, 2.0 mM sodium pyruvate (Sigma Type II), and the pyruvate dehydrogenase enzyme. After 3 min incubation at 30°C, the reaction was initiated by the addition of 10 μl of 0.25 M NAD^+ and the change in absorbance at 340 nm was measured using a Gilford Spectrophotometer.

The control reaction included CoASH instead of 3-mercaptopropanoyl-CoA in the thiolase reaction.

In assessing the effects of 3-mercaptopropanoyl-CoA and chemically synthesized S-acetyl-3-mercaptopropanoyl-CoA on pyruvate dehydrogenase under these conditions, they were added to the assay mixture subsequent to the citrate synthase reaction.

RESULTS AND DISCUSSION

Regulation of Fatty Acyl-CoA Synthetase and Carnitine Palmitoyltransferase

As indicated in Table 1, likely effectors of palmitoyl-CoA synthetase activity were tested at levels which are likely to be above their concentrations in heart cytosol. Ketone bodies, glycolytic intermediates, NAD^+, and NADH had no effect on the enzyme. 5 mM ADP was found to inhibit synthetase activity by 41%. This was the same level of inhibition found for the rat liver microsomal enzyme.[42] Octanoylcarnitine, at a concentration of 1 mM, was found to have no effect on the enzyme.

Palmitoylcarnitine was shown to have a significant effect on the activity of palmitoyl-CoA synthetase (Table 2). Palmitoylcarnitine at 1 mM inhibited the enzyme by 45%. As shown in Table 2 and Figure 4, raising levels of this carnitine derivative from 0 to 2 mM had an increasingly inhibitory effect on the enzyme. Inhibition of palmitoyl-CoA synthetase by palmitoylcarnitine never exceeded 45%.

Carnitine palmitoyltransferase activity in rat heart mitochondria was determined to fall in the range of 11.4 to 15.0 nmol/min/mg protein, depending on the particular preparation. Since this activity was comparable to or in excess of the activity of the synthetase, it was considered that the CPT enzyme, acting in the reverse direction, could be masking the inhibitory effect by decreasing the effective concentration of palmitoyl-carnitine in the assay mixture. Alternatively, the same reaction could serve to increase the concentration

of palmitoyl-CoA, which itself is an inhibitor of the synthetase.[37]

An attempt was made to separate palmitoyl-CoA synthetase activity from carnitine palmitoyltransferase activity of beef heart mitochondria by digitonin fractionation. Digitonin selectively removes the outer membrane of liver mitochondria, without an apparent effect on the inner mitochondrial membrane.[117] Because palmitoyl-CoA synthetase is located on the outer membrane[4,5] and CPT on the inner membrane[25,26,27,28,29], it was expected that these activities could be separated by the digitonin fractionation procedure. It was further expected that the palmitoyl-CoA synthetase activity would be recovered in the "outer membrane plus intermembrane" fraction and that this fraction would be devoid of CPT activity. As shown in Table 3, the results were not as expected. Significant CPT activity was recovered in the "outer membrane" fraction. This is probably due to the fact that carnitine palmitoyltransferase A is loosely associated with the outer surface of the inner mitochondrial membrane and was easily removed by the perturbation. Other researchers have also had difficulties separating these two activities by membrane fractionation techniques.[126,127] In fact, Bergstrom and Reitz[30] demonstrated that 25% of rat liver mitochondrial CPT was easily released and solubilized by digitonin treatment. Hoppel and Tomec[27] used this technique to release CPT A from the membrane. In addition, this technique was developed for use with liver mitochondria and it is not known if it is effective with heart mitochondria.

The possibility of covalent modification of the palmitoyl-CoA synthetase by phosphorylation-dephosphorylation was investigated. Since such modification would be caused by a cytosolic protein such as protein kinase or protein phosphatase, assays were performed using whole heart tissue

homogenate. Prior to assay for palmitoyl-CoA synthetase activity, the homogenate was preincubated at 25°C with compounds known to regulate the activities of various protein kinases or phosphatases. Assays were performed with and without the potential effectors included in the assay mixture.

A number of substrate proteins for Ca^{2+}-dependent protein kinases have been identified in the cytosolic and mitochondrial fractions of guinea pig heart.[128] These protein kinases were shown to be either phospholipid- or calmodulin-sensitive. Phosphorylation of one mitochondrial protein was augmented by calmodulin in the presence or absence of added $CaCl_2$.[128] In addition, phosphorylation of a cytosolic and a mitochondrial protein catalyzed by a calmodulin-sensitive Ca^{2+}-dependent protein kinase was inhibited by palmitoylcarnitine.[128]

ATP has been shown to stimulate phosphorylation of acetyl-CoA carboxylase via a protein kinase, in the absence of additional regulators.[129, 130] Many protein kinases are cyclic-AMP-dependent[131, 132], sometimes requiring preincubation with cyclic-AMP prior to the addition of ATP.[133] Fluoride, a known inhibitor of phosphatase,[129] was also examined.

The results, shown in Table 4, indicate that regulators of protein kinases or protein phosphatases may cause either a slight stimulation or a slight inhibition of palmitoyl-CoA synthetase. These effects, however, are too small to indicate regulation of the enzyme via phosphorylation-dephosphorylation.

As indicated in Table 5, likely effectors of CPT activity were tested at levels equal to or greater than their maximal concentrations in heart cytosol.

Pyruvate, lactate, NAD^+, and NADH were shown to have an insignificant, if slightly stimulatory, effect on the enzyme. β-Hydroxybutyrate, fructose-1,6-diphosphate, ADP, ATP, acetyl-carnitine, and octanoyl-carnitine were shown to be slightly inhibitory. Pyruvate and lactate were also shown to have a slightly stimulatory effect on CPT A (Table 6).

Mitochondria used in the assay for CPT A were kept intact by maintaining the osmolarity of the assay mixture as described. Respiratory control ratios, using palmitoylcarnitine as substrate, and NADH dehydrogenase activities were taken as indicators of intactness. Only mitochondrial preparations with a respiratory control ratio greater than 4.0 and NADH dehydrogenase activity less than 50 nmol/min/mg protein were used for the CPT A assays. In addition, there was very little loss (less than 2%) of CoASH derivatives under the assay conditions (Table 7). This indicates that the CoASH derivatives were not exposed to thioesterase which is present in the mitochondrial matrix.[83]

As expected, malonyl-CoA is a potent inhibitor of rat heart CPT A. Approximately 40% inhibition is obtained with 1 μM malonyl-CoA under the conditions used (Table 8, Figure 5). Saggerson[77] found 50% inhibition of rat heart CPT A with 1-2 μM malonyl-CoA. Also consistent with the findings of Saggerson[77] was the fact that Triton X-100 could abolish the inhibitory effects of malonyl-CoA on this enzyme (Table 6). This effect has also been demonstrated with the liver enzyme.[57]

Since the presence of malonyl-CoA in the cytosol of heart tissue is not clearly established, its inhibitory effect on CPT A may not be physiologically significant. Other CoASH derivatives with structural similarities to malonyl-

CoA were tested for their effect on this enzyme. Of those CoASH derivatives examined, only succinyl-CoA had an effect on the enzyme comparable to that of malonyl-CoA (Table 6). At 100 μM, malonyl-CoA inhibited CPT A activity by 69.5%, while succinyl-CoA inhibited CPT A activity by 59.3% (Table 9). At 100 μM, acetyl-CoA did not inhibit CPT A activity, while acetoacetyl-CoA and methylmalonyl-CoA inhibited the enzyme by 5.8% and 23.0%, respectively. This agrees with the results of Mills et al.,[134, 135] who reported that, for rat liver, heart, and skeletal muscle mitochondrial CPT A, the inhibitory potency of CoASH derivatives followed the order malonyl-CoA > succinyl-CoA > methylmalonyl-CoA > propanoyl-CoA > acetyl-CoA. McGarry et al.,[57] demonstrated that at 10 μM CoASH, acetyl-CoA, propanoyl-CoA, and methylmalonyl-CoA did not inhibit rat liver CPT, while malonyl-CoA inhibited this enzyme by 40%. This indicates that it is important for the inhibitory compound to have a straight chain carbon skeleton with a carboxyl group on the terminal carbon in order for it to exert a significant effect on the enzyme.

Succinyl-CoA is not as potent an inhibitor of rat heart CPT A as malonyl-CoA. While 1 μM malonyl-CoA inhibits this enzyme by 40%, succinyl-CoA has no effect at this concentration (Table 8, Figure 5). As shown in Table 8 and Figure 5, increasing the concentration of succinyl-CoA from 1 to 100 μM had an increasingly inhibitory effect of the enzyme. However, inhibition by succinyl-CoA was always less than the inhibition by the same concentration of malonyl-CoA.

Since the inhibition of rat heart CPT A by succinyl-CoA was significant, it was considered that succinyl-CoA might be an inhibitor *in vivo*. In order for

succinyl-CoA to be a physiological inhibitor of the CPT A, it must be present in the cytosol and its concentration must change in response to metabolic changes. Since, succinyl-CoA does not cross the mitochondrial membrane, any succinyl-CoA in the cytosol must be produced in that subcellular compartment. In order to determine whether succinyl-CoA can be formed in the cytosol, heart tissue was fractionated into cytosolic and extra-cytosolic fractions or cytosolic and mitochondrial fractions. Each subcellular fraction was assayed for succinate thiokinase. Enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and citrate synthase activities were measured as mitochondrial matrix marker enzymes. As shown in Table 10 and 11, the cytosolic fraction does exhibit succinate thiokinase activity. However, the percent of total succinate thiokinase activity in the cytosol falls in the same range as that for the three mitochondrial matrix marker enzymes present in the cytosol. Thus, the level of succinate thiokinase activity in the cytosolic fraction of rat heart is not significantly different than the level expected due to leakage of mitochondrial matrix enzymes. The leakage of mitochondrial enzymes is much higher for heart than for other tissues because harsher treatment is required for subcellular fractionation due to the toughness of the heart tissue. Based on these results it is unlikely that the cytosol of rat heart contains a high enough succinate thiokinase activity to maintain a level of succinyl-CoA inhibitory to the CPT A enzyme.

Since there was no effector of either the fatty acyl-CoA synthetase or the CPT enzymes which inhibited at a level deemed to be physiologically significant or was demonstrated to be in the cytosol, further kinetic studies of the observed inhibitions were not undertaken.

Although much evidence exists for the regulation of β-oxidation via the CPT enzyme or fatty acyl-CoA synthetase enzyme in tissues other than heart, it appears that these enzymes are not points of regulation for β-oxidation in heart mitochondria.

Aas et al.,[2] found that, under nutritional conditions (carbohydrate-starved or fat-fed) which increase CPT activity levels in rat liver, neither CPT activity nor fatty acyl-CoA synthetase activity is affected in heart. They suggested that regulation of fatty acid metabolism in heart is regulated by availability of substrates.

Oxidation of Unsaturated Fatty Acids in Mammalian Heart and Liver Mitochondria

As shown in Table 12, the rates of oxidation of palmitoyl-CoA, oleoyl-CoA and linoleoyl-CoA in rat liver mitochondria are approximately the same. Christopherson and Bremer[136] , found the rates of oxidation of palmitoyl-carnitine and oleoyl-carnitine in liver mitochondria to be the same, whereas that of linoleoyl-carnitine was slightly lower and decreased with time. Reid and Husbands[137] found that the oxidation rate of oleate and linoleate was higher than that of palmitate in rat liver mitochondria. The rate of oxidation of linoleoyl-CoA in rat heart mitochondria is slightly less than that observed using palmitoyl-CoA or oleoyl-CoA as substrate. This agrees with the results of Christopherson and Bremer[136] for oxidation of the corresponding carnitine derivatives. Lawson and Holman[138] showed a slightly higher rate of oxidation of linoleoyl-CoA as compared to oleoyl-CoA in rat heart mitochondria. Brown adipose tissue mitochondria also show similar oxidation rates with palmitoyl-

and linoleoyl-carnitine.[139]

Assuming complete oxidation of the substrate in rat heart mitochondria, it was possible to calculate the nmoles of substrate consumed/min/mg protein (Table 13). Comparing these values to the specific activities of the three enzymes studied in this tissue (Table 14)[91] , it is evident that the levels of *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase are in excess of that required for the oxidation of linoleoyl-CoA via the pathway proposed by Cuebas and Schulz.[88] 3-Hydroxyacyl-CoA epimerase shows a greater specific activity with 3-D-hydroxydodecanoyl-CoA than with 3-D-hydroxyoctanoyl-CoA as substrate.[91] If the specific activity for the epimerase in heart mitochondria using the C-12 substrate (3.42 nmol/min/mg protein) is compared to the specific activity for linoleoyl-CoA oxidation (3.06 nmol consumed/min/mg protein) in heart mitochondria, it would appear that this level of enzyme is sufficient to support oxidation of linoleoyl-CoA in heart mitochondria via the pathway proposed by Stoffel and Caesar.[84] However, according to this pathway, oxidation of linoleoyl-CoA would result in the formation of 3-D-hydroxyoctanoyl-CoA as an intermediate and not the 3-D-hydroxydodecanoyl-CoA (Figure 2A). The specific activity of the epimerase in rat heart mitochondria using 3-D-hydroxyoctanoyl-CoA as substrate (2.0 nmol/min/mg protein)[91] is significantly lower than what is required to explain the level of oxidation of linoleoyl-CoA in this tissue. Thus, it may be concluded that at least a portion of the linoleoyl-CoA is oxidized by a pathway other than the one proposed by Stoffel and Caesar[84] in rat heart mitochondria and that the 3-hydroxyacyl-CoA epimerase is not necessary for the degradation of linoleoyl-CoA.

Although 3-hydroxyacyl-CoA epimerase has been reported to exist in significant amounts in liver mitochondria[84, 91, 140] a rigorous study of the subcellular location of this enzyme in rat liver has shown it to be associated with peroxisomes, and not present in mitochondria.[141] Therefore, the epimerase cannot function in mitochondrial β-oxidation of unsaturated fatty acids.

Inhibition of Pyruvate Dehydrogenase Complex by Derivatives of 3-Mercaptopropanoic Acid

Since 3-mercaptopropanoyl-CoA was able to substitute for CoASH in the thiolase reaction[98] , it was thought that this compound might also be a substrate for pyruvate dehydrogenase. When CoASH was excluded from the assay mixture and the reaction was initiated with 3-mercaptopropanoyl-CoA (to 65 μM), the reaction rate and extent observed could be totally accounted for by some small amount of CoASH known to be present in the 3-mercaptopropanoyl-CoA solution. Thus, it was assumed that 3-mercaptopropanoyl-CoA could not substitute for CoASH in the pyruvate dehydrogenase reaction.

However, pyruvate dehydrogenase complex can utilize α-ketobutyrate as substrate to produce propanoyl-CoA which is an end-product inhibitor of this enzyme.[12] Thus, it was expected that 3-mercaptopropanoyl-CoA would inhibit the pyruvate dehydrogenase complex.

Inhibition of pyruvate dehydrogenase by 3-mercaptopropanoyl-CoA was demonstrated to be 50% at 12.6 μM, and approached a maximum of 80% inhibition at a concentration of 100 μM (Figure 6A). The degree of inhibition

caused by 3-mercaptopropanoyl-CoA was found to be a function of time, but was demonstrated to be about 50% complete at approximately 1 min (Figure 6B).

The normal product of pyruvate dehydrogenase is acetyl-CoA. It is well known that acetyl-CoA exerts end-product inhibition on this enzyme.[142] Since it has been demonstrated that 3-mercaptopropanoic acid is converted to S-acetyl-3-mercaptopropanoyl-CoA by mitochondrial enzymes,[98] it was expected that the S-acetyl-3-mercaptopropanoyl-CoA thus formed would inhibit mitochondrial respiration by inhibiting the key enzyme, pyruvate dehydrogenase.

Under optimal conditions, 5.2 μM S-acetyl-3-mercaptopropanoyl-CoA inhibited the enzyme by 50%, while 100 μM of this compound gave 90% inhibition (Figure 6A). The degree of inhibition caused by S-acetyl-3-mercaptopropanoyl-CoA was found to be a function of time, yet was greater than 50% complete after only 5 seconds (Figure 6B).

The reversibility of the inhibition by S-acetyl-3-mercaptopropanoyl-CoA was investigated by subjecting the inhibited enzyme complex to rapid gel filtration on Sephadex G-50. If the inhibition is reversible, this process could restore enzyme activity through removal of the inhibitor.

As can be seen in Table 15, rapid gel filtration did not result in the reactivation of the inhibited enzyme. Thus, the inhibition is irreversible. Although only approximately 50% of the enzyme activity was recovered during this process, the activity in the inhibited sample relative to the control was the same prior to and subsequent to filtration and therefore indicated no recovery

of activity. In the absence of BSA all enzyme activity is lost, while increasing the BSA content of the applied sample and washing the column with BSA increased the activity recovered. Therefore, it is presumed that loss of enzyme activity during rapid gel filtration was due to a loss of protein on the column. Since activity was not restored to the inhibited enzyme by rapid gel filtration, the eluate was tested for the presence of S-acetyl-3-mercaptopropanoyl-CoA by HPLC. It was determined that only 8.4% of the applied S-acetyl-3-mercaptopropanoyl-CoA remained in the eluate. This meant that the assay mixture for this sample contained 0.8 μM S-acetyl-3-mercaptopropanoyl-CoA. This concentration of inhibitor could not account for the observed inhibition. In fact, if the inhibition were reversible, we would expect recovery of the enzyme activity to almost 100%.

Upon examination of S-acetyl-3-mercaptopropanoyl-CoA by HPLC techniques, it was discovered that the solution contained three major contaminants: acetyl-CoA, 3.8 ± 1.8%; dimer CoA, 0.8 ± 0.4%; and an unidentified component, 8.1 ± 1.1%. Thus, the S-acetyl-3-mercaptopropanoyl-CoA was 88.3 ± 0.1% pure. Attempts to purify the S-acetyl-3-mercaptopropanoyl-CoA by preparative HPLC resulted in a preparation which did not exhibit the inhibitory potency of the original solution, although none of the other peaks could account for the observed inhibition. In an effort to confirm that S-acetyl-3-mercaptopropanoyl-CoA was the inhibitor, it was synthesized enzymatically via the thiolase reaction. The inhibitory potency of enzymatically-synthesized S-acetyl-3-mercaptopropanoyl-CoA was compared to that of chemically-synthesized S-acetyl-3-mercaptopropanoyl-CoA and 3-mercaptopropanoyl-CoA. As can be seen in Table 16, inhibition of the pyruvate dehydrogenase complex is approximately the same for enzymatically and chemically

synthesized S-acetyl-3-mercaptopropanoyl-CoA. A somewhat lesser degree of inhibition was observed here than was previously evidenced (Figure 6). It is likely this is due to the higher concentration of protein, especially BSA (which is known to bind certain CoA derivatives) and thiolase (which probably also binds 3-mercaptopropanoyl-CoA and S-acetyl-3-mercaptopropanoyl-CoA, the substrate and product, respectively, in the thiolase reaction).

Although citrate has been demonstrated to inhibit the pyruvate dehydrogenase reaction,[12, 143] the maximum amount of citrate (60 μM) which might be produced under these reaction conditions was demonstrated to have no effect on the activity of pyruvate dehydrogenase.

The extent of inhibition observed with both 3-mercaptopropanoyl-CoA and S-acetyl-3-mercaptopropanoyl-CoA was dependent on the preincubation conditions (Figure 7, 8, Table 17, 18). Maximal inhibition was observed when the enzyme was reacted with the inhibitor in the presence of either pyruvate and CoASH or NADH. These two conditions tend to promote reduction of the lipoate moiety of the enzyme. Somewhat less inhibition was observed when only CoASH, or CoASH and pyruvate in the absence of TPP were in the preincubation mixture. Probably under these conditions CoASH alone could promote reduction of the lipoate moiety. The least inhibition occurred under those conditions which promote oxidation of the lipoate moiety of the enzyme, i.e. when NAD^+ was in the preincubation mixture. It is suggested that inhibition by 3-mercaptopropanoyl-CoA and S-acetyl-3-mercaptopropanoyl-CoA requires the lipoate moiety of the enzyme to be in the reduced state. Under other conditions, the presence of inhibitor caused the reaction rate to decrease with time. Addition of CoASH or pyruvate to the system after inhibition of

the enzyme had no effect on reaction rate. Addition of fresh enzyme to the system restored the reaction rate to a rate somewhat less than the control values, indicating that the assay system was intact and that there was no accumulation of inhibitory product in the mixture. At some point in the pyruvate dehydrogenase reaction, the reduced lipoate is made available. This may account for the observed increase in inhibition during the course of the reaction.

In general, it was found that inhibition of pyruvate dehydrogenase complex, in the absence of substrates in the preincubation mixture varied substantially from enzyme preparation to preparation. Since the degree of oxidation or reduction of lipoate moieties is not altered by the preincubation conditions in this case , it is assumed that the degree of observed inhibition reflects the degree of reduction of the lipoate moieties of the enzyme preparation.

One notable difference between inhibition by the two compounds bears mentioning. The enzyme was, to a small degree, protected from inhibition by S-acetyl-3-mercaptopropanoyl-CoA when pyruvate was included in the preincubation mixture. Under this condition, the lipoate would be acetylated and might not be as accessible to modification as in the reduced state. Under the same conditions, however, inhibition by 3-mercaptopropanoyl-CoA was enhanced somewhat. Apparently the acetylation of the lipoate moiety enhances inhibition by 3-mercaptopropanoyl-CoA. One possible explanation is that the 3-mercaptopropanoyl-CoA might participate, to some small degree, in the lipoate acetyltransferase (E2) reaction to produce S-acetyl-3-mercaptopropanoyl-CoA and reduced lipoate. 3-Mercaptopropanoyl-CoA would thus inhibit the enzyme via this additional mechanism.

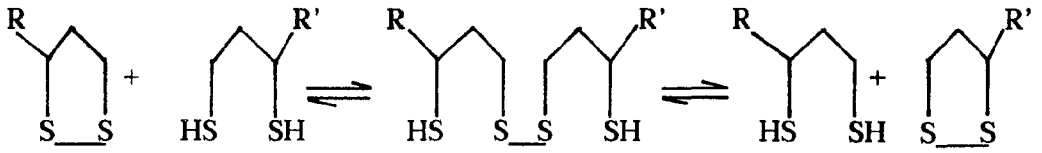
The pyruvate dehydrogenase complex contains three enzymes; pyruvate dehydrogenase (EC 1.2.4.1), lipoate acetyl transferase (EC 2.3.1.12), and dihydrolipoyl dehydrogenase (EC 1.6.4.3). The reactions catalyzed by the enzymes in the complex are illustrated in Figure 9. The reaction catalyzed by the complex has an absolute requirement for pyruvate, CoASH, and NAD^+. Thus, the overall reaction can be initiated by the addition of one or more of any of these substrates. The order of addition of these substrates may have an effect on the state of the enzyme, particularly on the lipoate portion of the lipoate acetyl transferase. In addition, it can be seen that the process of the reactions brings the lipoate moiety through three states; the acetyl-bound state, the oxidized state, and the reduced state.

Inhibition of the component enzymes of the pyruvate dehydrogenase complex by S-acetyl-3-mercaptopropanoyl-CoA was also maximal under preincubation conditions, which tend to promote reduction of the lipoate moiety.

Assessing the effect of S-acetyl-3-mercaptopropanoyl-CoA on pyruvate dehydrogenase, the first component enzyme of the complex, proved impossible. Due to the slow rate of the reaction, it was not possible to assay the enzyme by the method involving reduction of DCPIP. This method has been shown to cause the progressive inhibition of the pig heart pyruvate dehydrogenase component by modification of enzyme-bound TPP.[144] The method employed was based on the reduction of ferricyanide and yielded a specific activity of 0.21 μmol/hr/mg protein. This value is comparable to that of 5.7 μmol/hr/mg protein reported by Hayakawa et al., [124] who used slightly different conditions, which may have enhanced the activity of the enzyme. In any case, the enzyme activity was too low for inhibition to be effectively examined.

The second component enzyme of the complex, lipoate acetyl transferase, is usually assayed by the determination of heat-stable thioester formed in the reaction of constantly generated acetyl-CoA with dihydrolipoamide in the absence of CoASH. This assay could not be employed because the inhibitor molecule, S-acetyl-3-mercaptopropanoyl-CoA, is a heat-stable thioester. In addition, the inhibitor molecule is thought to act by forming a heat-stable thioester with dihydrolipoate or dihydrolipoamide.

The first and second component enzymes of the pyruvate dehydrogenase complex were assayed together by measuring, via the citrate synthase reaction, the amount of acetyl-CoA produced. As shown in Table 19, when lipoamide was absent from the preincubation mixture, but pyruvate was present, the inhibition was significantly greater than under other conditions. This condition tended to promote the reduction of the endogenous lipoamide during the preincubation period, thus making it available for reaction with the inhibitor. It should be noted that exogenous lipoamide cannot participate in these reactions directly.[113] It can, however, drive the reaction by oxidizing enzyme-bound dihydrolipoate in the absence of NAD^+ by the following reaction sequence:



Thus, endogenous lipoate will be regenerated for reaction with pyruvate. The fact that inhibition was nearly the same in the presence or absence of lipoamide, when pyruvate was omitted during preincubation, suggests that most of

the endogenous, enzyme-bound lipoate was in the oxidized state. Including pyruvate in the preincubation, but omitting lipoamide, would tend to generate endogenous enzyme-bound dihydrolipoate in the process of the reaction. Inhibition was enhanced under these conditions, suggesting that reduced endogenous lipoate is necessary for the inhibitor to exert its effect.

The third component enzyme of the pyruvate dehydrogenase complex, dihydrolipoyl dehydrogenase, was assayed in both the forward and reverse direction by measuring the appearance or disappearance of NADH. Exogenous dihydrolipoamide or lipoamide can be, and was, used as substrate for the forward or reverse reaction, respectively. Because, under conditions where the inhibition of the pyruvate dehydrogenase complex was minimal, inhibition increased with time (Figure 8), it was thought that some process of the reaction (possibly making reduced lipoate available) contributed to the inhibition. By assaying the dehydrogenase enzyme with exogenous lipoamide, the step thought to be critical to inhibition (enzymatically catalyzed binding of S-acetyl-3-mercaptopropanoyl-CoA to reduced endogenous lipoate) was bypassed. Thus, it was expected that S-acetyl-3-mercaptopropanoyl-CoA would have no effect on this enzyme.

Contrary to expectation, Table 20 shows that dihydrolipoyl dehydrogenase was inhibited in both directions under certain conditions. In the forward direction, the enzyme was inhibited only when dihydrolipoamide was in the preincubation mixture together with the inhibitor and when NAD^+ was used to initiate the reaction. In the reverse direction, inhibition only occurred when NADH was included in the preincubation mixture with the inhibitor and lipoamide was used to initiate the reaction. These data are not inconsistent

with the hypothesis that S-acetyl-3-mercaptopropanoyl-CoA reacts irreversibly with endogenous dihydrolipoate. However, the fact that inhibition of the dehydrogenase portion of the enzyme still occurs in the presence of exogenous lipoamide indicates that the inactivation of endogenous lipoate cannot totally explain the observed inhibition of the pyruvate dehydrogenase complex. One possible explanation is apparent from the fact that inhibition of the dehydrogenase was only seen under those conditions which tended to promote reduction of endogenous lipoate. In fact, under conditions which promoted oxidation of endogenous lipoate, no significant inhibition was observed. The hypothesis could be expanded to include the possibility that covalently modified endogenous lipoate may compete for the lipoamide site on the dehydrogenase. This modified endogenous dihydrolipoate would be a very effective competitive inhibitor compared to covalently modified exogenous lipoamide, because of its close proximity to the active site of the enzyme.

However, it cannot be ruled out that the S-acetyl-3-mercaptopropanoyl-CoA directly inhibits the dihydrolipoyl dehydrogenase reaction. Several inhibitors of this enzyme are known.[145, 146] In the *E. coli* pyruvate dehydrogenase complex, *p*-[(bromoacetyl)-amino]phenyl arsenoxide binds first to the reduced lipoyl residues (via its arsenoxide moiety) on the lipoate acetyltransferase. This is followed by an irreversible alkylation of histidine and cysteine in the active site of the dihydrolipoyl dehydrogenase, resulting in inhibition of this reaction and the overall reaction of the pyruvate dehydrogenase complex.[145] Pig heart dihydrolipoyl dehydrogenase is inhibited by 1,3-*bis*-(2-chloroethyl)-1-nitrosourea.[146] This inhibition is dependent on the reduction of a cystine disulfide located within the active site of the enzyme. The inhibition can be prevented by preincubation of the enzyme with lipoamide. Thus, inhibition of

dihydrolipoyl dehydrogenase by S-acetyl-3-mercaptopropanoyl-CoA may follow a similar pattern in that this component enzyme is inhibited under reducing conditions, and the inhibition is prevented by preincubation of the enzyme with lipoamide. This, however, would have to be in addition to the inhibition of the E1E2 portion of the pyruvate dehydrogenase complex which was demonstrated (Table 19).

The site of inhibition by S-acetyl-3-mercaptopropanoyl-CoA was further investigated by examining its effect on the incorporation of radioactively labelled acetyl groups from acetyl-CoA into the pyruvate dehydrogenase complex. In the presence of NADH, the lipoate moiety of the lipoate acetyltransferase (E2) is reduced via the lipoamide dehydrogenase (E3) reaction. In the reverse reaction, the reduced lipoate can be acetylated from the product, acetyl-CoA. Since mammalian pyruvate dehydrogenase complex contains a dihydrolipoyl transacetylase core consisting of 60 polypeptide chains each containing one molecule of covalently bound lipoic acid,[147] the upper limit for acetyl groups bound per pyruvate dehydrogenase complex is 60. If the acetyl-CoA binding site is blocked by the inhibitor or the inhibitor itself reacts with the reduced lipoate, the acetylation of the pyruvate dehydrogenase complex will be inhibited. It must be pointed out that there is another binding site for acetyl-CoA, demonstrated for the *E. coli* enzyme[148, 149, 150, 151] which is on the pyruvate dehydrogenase (E1) and is a site for allosteric inhibition of the E1 reaction.[150, 151, 152] However, binding of acetyl-CoA to this site occurs in the presence or absence of NADH and can therefore be accounted for by measuring acetyl-CoA incorporation in the absence of NADH. As can be seen in Table 21, S-acetyl-3-mercaptopropanoyl-CoA does inhibit incorporation of the acetyl group from the acetyl-CoA into the enzyme, and the degree of inhibition

increases with increasing inhibitor concentration. That the inhibition requires a greater concentration of inhibitor than in the pyruvate dehydrogenase complex assay can be explained by the greater concentration of enzyme protein used. These results lend support to the hypothesis that the enzyme is reacting with the inhibitor. In fact, in an experiment where BSA was included in the reaction in an amount ten times that of pyruvate dehydrogenase complex, inhibition was significantly reduced, but still occurred and increased with increasing S-acetyl-3-mercaptopropanoyl-CoA concentration.

The data strongly suggest that S-acetyl-3-mercaptopropanoyl-CoA inhibits the pyruvate dehydrogenase complex by donating the S-acetyl-3-mercaptopropanoyl group to the dihydrolipoate moiety of the enzyme to form S-acetyl-3-mercaptopropanoyl lipoate, thereby making the lipoate unavailable to accept an acetyl group in the normal reaction. This would be similar to the irreversible inhibition of the *E. coli* pyruvate dehydrogenase complex by 2-bromopyruvate. This inhibition is due to the reductive bromoacetylation of lipoyl moieties of the E2 subunit.[153, 154] It is suggested, in addition, that S-acetyl-3-mercaptopropanoyl-CoA is formed intramitochondrially under certain conditions and, once formed, inhibits pyruvate-supported respiration by direct inhibition of the pyruvate dehydrogenase complex.

Some preliminary work has been done to examine the possibility that S-acetyl-3-mercaptopropanoyl-CoA formed intramitochondrially affects pyruvate-supported respiration. Since 3-mercaptopropanoic acid is known to be activated in rat heart mitochondria[98] and to inhibit palmitoyl-*l*-carnitine-supported respiration[97] , its effect was compared to that of S-acetyl-3-mercaptopropanoic acid. As can be seen in Table 22, S-acetyl-3-

mercaptopropanoic acid was comparable to 3-mercaptopropanoic acid in its ability to inhibit both palmitoyl-*l*-carnitine- and pyruvate-supported respiration in both rat heart and rat liver mitochondria. One unusual aspect of these data is that pyruvate-supported respiration was inhibited significantly in rat liver mitochondria, but not in rat heart mitochondria. This is consistent with the observation that propanoate (known to be activated to propanoyl-CoA which inhibits pyruvate dehydrogenase) inhibits flux through the pyruvate dehydrogenase in perfused rat liver, but not rat heart.[155] This may have to do with the difference in the activation enzymes of these tissues and thus the difference in intramitochondrial concentration of the CoA derivative maintained in each of these tissues.

The data in this table indicate that S-acetyl-3-mercaptopropanoyl-CoA is formed intramitochondrially and is as potent an inhibitor as the metabolites of 3-mercaptopropanoic acid. The fact that S-acetyl-3-mercaptopropanoic acid was also a potent inhibitor of palmitoyl-*l*-carnitine-supported respiration and that the pattern of inhibition so closely paralleled that of 3-mercaptopropanoic acid led to the suspicion that perhaps the acetyl group is cleaved from the S-acetyl-3-mercaptopropanoic acid to form 3-mercaptopropanoic acid and/or its derivatives. If this were the case, the data would be the same because inhibition would be due to 3-mercaptopropanoic acid metabolites. To avoid this confusion, work was pursued in a manner that was considered to result in the formation of S-acetyl-3-mercaptopropanoic acid intramitochondrially without the introduction of S-acetyl-3-mercaptopropanoic acid. One way in which this could be accomplished in rat heart mitochondria was to supply acetoacetate as substrate so that acetoacetyl-CoA would be formed intramitochondrially. In the presence of 3-mercaptopropanoyl-CoA, thiolase would utilize both

substrates to form, as product, S-acetyl-3-mercaptopropanoyl-CoA.[98]

The data from using this strategy are presented in Table 23. As can be seen in this table, pyruvate-supported respiration in rat heart mitochondria is not inhibited by 3-mercaptopropanoic acid when malate is used as primer. Pyruvate-supported respiration is inhibited, however, when acetoacetate (20 μM) is being metabolized. This would indicate that S-acetyl-3-mercaptopropanoyl-CoA is formed intramitochondrially and inhibits pyruvate-supported respiration. However, pyruvate-supported respiration is inhibited to the same extent in the absence of acetoacetate, when α-ketoglutarate is used as primer. This may mean that α-ketoglutarate somehow provides the acetyl group involved in the acetylation of 3-mercaptopropanoyl-CoA.

As stated previously, both 3-mercaptopropanoyl-CoA and S-acetyl-3-mercaptopropanoyl-CoA inhibit the pyruvate dehydrogenase complex. Under optimal conditions, 50% inhibition was obtained at 12.6 μM 3-mercaptopropanoyl-CoA or 5.2 μM S-Acetyl-3-mercaptopropanoyl-CoA. In addition, inhibition by S-acetyl-3-mercaptopropanoyl-CoA was greater than 50% complete in 5 seconds. While that caused by 3-mercaptopropanoyl-CoA was 50% complete in approximately 1 min. Both inhibitors appear to be more potent under conditions where the endogenous lipoate moiety of the enzyme is reduced.

Table 1. **Effects of Metabolites and Coenzymes on the Fatty Acyl-CoA Synthetase of Rat Heart Mitochondria**

Effectors (nmol/min/mg protein)		Specific Activity (%)	Relative Activity
	None	11 ±1	100
1	mM octanoylcarnitine	11 ±3	104
5	mM acetoacetate	10 ±1	96
10	mM DL-β-hydroxybutyrate	10 ±1	93
	None	10 ±4	100
10	mM pyruvate	11 ±1	115
1	mM NAD^+	11 ±1	109
1	mM NADH	11 ±2	116
	None	7 ±2	100
10	mM Lactate	7.8 ±0.1	114.5
0.1	mM fructose-1,6-diphosphate	7 ±1	107
5	mM ADP	4.0 ±0.2	58.7

Values are means ±S.E. The values are based on three assays with one preparation of mitochondria. Each grouping used a different mitochondrial preparation. The concentration of each effector was selected such that it was likely to be equal to or greater than its concentration in rat heart cytosol. This was estimated from the literature values of the acylcarnitine[a], acetoacetate[b], β-hydroxybutyrate[b], pyruvate[b], NAD^+[c], NADH[c], lactate[b], fructose-1,6-diphosphate[b], and ADP[d] content of heart tissue in μmole/g fresh wt. and the value of 2 ml/g dry tissue[e] for the cytosolic space in heart muscle. Additional details of the experiment are described under "Experimental Procedures".

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[a] M. M. Abdel-Kader, and G. Wolf in *Recent Research on Carnitine*, ed. G. Wolf, p. 147, M.I.T. Press, Cambridge, Mass., 1965.

[b] O. Kraupp, L. Adler-Kastner, H. Niessner, and B. Plank, *Eur. J. Biochem.*, vol. 2, p. 197, 1967.

[c] H. B. Burch, O. H. Lowry, and P. von Dippe, *J. Biol. Chem.*, vol. 238, p. 2838, 1963.

[d] P. Arese, A. Bosia, and G. Pescarmona, *unpublished results*, in *Methods in Enzymatic Analysis*, ed. H. U. Bergmeyer, and K. Gawehn, vol. 4, p. 2295, Academic Press, Inc., N.Y., 1974.

[e] H. E. Morgan, D. M. Regen, and C. R. Park, *J. Biol. Chem.*, vol. 239, pp. 369-374, 1964.

Table 2. **Effect of Palmitoylcarnitine on Rat Heart Mitochondria Fatty Acyl-CoA Synthetase**

Concentration (mM)	Specific Activity (nmol/min/mg protein)	% Inhibition
0	13 ± 1	0
0.020	11 ± 3	17
0.20	10 ± 1	25
1.00	7 ± 1	45
0	12 ± 1	0
0.2	10 ± 1	17
0.5	9 ± 2	24
2.0	7 ± 1	37

Values are means ±S.E. The values are based on three assays with one preparation of mitochondria. Each grouping of data used a different mitochondrial preparation. Additional details of the experiment are described under "Experimental Procedures".

Table 3. **Separation of Outer Mitochondrial Membrane and Intermembrane Fraction From Inner Membrane Fraction of Beef Heart Mitochondria by Digitonin Treatment**

Fatty Acyl-CoA Synthetase		
Specific Activity of Mitochondria	8.2	nmol/min/mg protein
Activity of "outer" fraction	28.4	nmol/min/ml
Total Activity of mitochondria used	791	nmol/min
Total Activity Recovered in "outer" fraction	44	nmol/min
Carnitine Palmitoyltransferase		
Specific Activity of Mitochondria	4.4	nmol/min/mg protein
Specific Activity of "outer" fraction	2.1	nmol/min/mg protein
Total Activity in "outer" fraction	20.2	nmol/min

Carnitine palmitoyltransferase activity was assayed by the spectrophotometric method. Details of the experiment are described under "Experimental Procedures".

Table 4. **Effect of Regulators of Protein Kinases or Phosphatases on the Activity of Fatty Acyl-CoA Synthetase**

Experiments were performed using heart tissue homogenate. Specific activity is expressed in nmol/min/mg protein. Values are means ± S. E. Figures in parentheses are the number of observation with samples from different animals. Each sample was assayed a minimum of two times. Other details of the experiment are described under "Experimental Procedures".

	Effectors Included in Preincubation Mixture Only		Effectors Included in Preincubation Mixture and Assay Mixture	
	Specific Activity	% Stimulation	Specific Activity	% Stimulation
Preincubation at 25°C for 20 min in:				
100 mM Tris-HCl buffer, pH 7.4	15.4 ± 0.5(4)	0	15.4 ± 0.5(4)	0
Buffer, 5 mM Mg-ATP, 0.25 mM $CaCl_2$	16.8 ± 1.1(3)	9.1	17.5 ± 1.1(3)	13.7
Buffer, 5 mM Mg-ATP, calmodulin (1μg/mg protein)	18.5 ± 1.1(3)	20.5	18.9 ± 1.0(3)	23.1
Buffer, 5 mM Mg-ATP, 0.25 mM $CaCl_2$, calmodulin (1 μg/mg protein)	19.0 ± 0.5(3)	23.5	17.6 ± 0.7(3)	14.4
Buffer	12.6 ± 0.4(5)	0	12.8 ± 0.4(5)	0
Buffer, 5 mM Mg-ATP, 25 μM $CaCl_2$	12.5 ± 2.0(4)	0	12.3 ± 1.1(4)	0
Buffer, 5 mM Mg-ATP, 1 mM EGTA	13.1 ± 0.5(3)	2.9	13.2 ± 0.6(3)	3.7
Buffer, 5 mM Mg-ATP, 25 μM $CaCl_2$, 1 mM EGTA	13.8 ± 0.4(5)	8.5	13.0 ± 1.6(5)	2.0
Preincubation at 25°C for 30 min in:				
Buffer	13.1 ± 1.0(4)	0	13.2 ± 1.0(4)	0
Buffer, 0.1 mM cAMP, 5 mM $MgCl_2$	13.4 ± 1.0(4)	2.8	12.7 ± 0.6(4)	0
Buffer, 0.1 mM cAMP, 5 mM Mg-ATP	13.6 ± 1.9(4)	4.0	14.7 ± 1.0(4)	12.3
Buffer, 0.1 mM cAMP, 5 mM Mg-ATP added after 15 min	14.0 ± 0.2(3)	7.5	13.8 ± 0.2(3)	5.7
Preincubation at 25°C for 20 min in:				
Buffer	13.0 ± 1.8(5)	0	13.0 ± 1.8(5)	0
Buffer, 5 mM ATP/$MgCl_2$	12.2 ± 1.4(5)	6.1	12.2 ± 1.4(5)	6.1
Buffer, 5 mM ATP/$MgCl_2$, 50 mM KF	12.1 ± 1.1(5)	7.1	12.1 ± 1.0(3)	6.8
Buffer, 50 mM KF	12.7 ± 0.6(5)	2.7	12.7 ± 0.3(3)	2.7

Table 5. **Effecs of Metabolites and Coenzymes on Carnitine Palmitoyltransferase from Rat Heart Mitochondria**

Carnitine palmitoyltransferase activity was assayed by the radiochemical method. Values are means ±S.E. Figures in parentheses are the number of observations with samples from different animals. Each sample was assayed a minimum of two times. The concentration of each effector was selected such that it was likely to be equal to or greater than its concentration in rat heart cytosol. This was estimated from the literature values of the β-hydroxybutyrate[a], ATP[a], ADP[b], acetylcarnitine[c], fructose-1,6-diphosphate[a], NAD^+[d], NADH[d], pyruvate[a], lactate[a], and acylcarnitine[e] content of heart tissue in μmole/g fresh wt. and the value of 2 ml/g dry tissue[f] for the cytosolic space in heart muscle. Other details of the experiment are described under "Experimental Procedures".

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[a] O. Kraup, L. Adler-Kastner, H. Niessner, and B. Plank, *Eur. J. Biochem.*, vol. 2, p. 197, 1967.

[b] P. Arese, A. Bosia, and G. Pescarmona, *unpublished results*, in *Methods of Enzymatic Analysis*, ed. H. U. Bergmeyer, and K. Gawehn, vol. 4, p. 2295, Academic Press, Inc., N. Y., 1974.

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[f] H. E. Morgan, D. M. Regen, and C. R. Park, *J. Biol. Chem.*, vol. 239, pp. 369-374, 1964.

Table 5. **Effects of Metabolites and Coenzymes on Carnitine Palmitoyltransferase from Rat Heart Mitochondria**

Effectors	Specific Activity (nmol/min/mg protein)	% Inhibition
None	14 (1)	
10 mM β-hydroxybutyrate	13 (1)	12
None	12 ± 1(2)	
5 mM ATP	10 ± 0(2)	18 ± 1
5 mM ADP	8 ± 1(2)	32 ± 5
None	11 ± 0(3)	
1 mM acetyl-*l*-carnitine	11 ± 3(2)	6 ±28
0.1 mM fructose-1,6-diphosphate	10 ± 1(3)	15 ±10
1 mM NAD^+	12 ± 2(3)	
1 mM NADH	12 ± 1(3)	
None	16 ± 5(4)	
10 mM pyruvate	18 ± 1(4)	
10 mM lactate	17 ± 4(4)	
1 mM octanoyl-*l*-carnitine	15 ± 2(3)	6 ±12
10 mM β-hydroxybutyrate	15 ± 1(4)	8 ± 8

Table 6. **Effects of Metabolites on Carnitine Palmitoyltransferase A of Rat Heart Mitochondria**

Effectors	Specific Activity (nmol/min/mg protein)	% Inhibition
None	12.5 (1)	
100 μM malonyl-CoA	5.1 (1)	59.4
100 μM methylmalonyl-CoA	10.1 (1)	18.6
100 μm acetyl-CoA	13.6 (1)	
None	7.7 (1)	
100 μM malonyl-CoA	3.1 (1)	59.8
100 μM methylmalonyl-CoA	7.0 (1)	9.6
100 μM acetyl-CoA	9.7 (1)	
100 μM acetoacetyl-CoA	8.4 (1)	
None	9.0 (1)	
100 μM malonyl-CoA	3.6 (1)	59.7
100 μM succinyl-CoA	4.0 (1)	55.4
None	13.4 (1)	
100 μM succinyl-CoA	7.0 (1)	52.2
100 μM acetoacetyl-CoA	13.7 (1)	
100 μM acetyl-CoA	13.4 (1)	
100 μM methylmalonyl-CoA	11.2 (1)	16.6
10 mM pyruvate	16.9 (1)	
10 mM lactate	17.2 (1)	
None	16.1 (1)	
100 μM malonyl-CoA	2.1 (1)	86.9
100 μM malonyl-CoA + Triton X-100	13.4 (1)	16.3
0.09% Triton X-100	12.6 (1)	21.4
None	11.4 $\pm$ 1.0(3)	
100 μM malonyl-CoA	0.7 $\pm$ 0.0(3)	93.6
100 μM succinyl-CoA	3.5 $\pm$ 0.8(3)	69.0
100 μM acetoacetyl-CoA	8.0 $\pm$ 1.1(3)	29.8
100 μM acetyl-CoA	9.3 $\pm$ 1.1(3)	18.3
100 μM methylmalonyl-CoA	6.0 $\pm$ 0.8(3)	47.0

CPT A activity was assayed using the radiochemical method. Values are means $\pm$ S. E. Figures in parentheses are the number of observations with samples from different animals. Each sample was assayed a minimum of two times. Other details of the experiment are described in "Experimental Procedures".

Table 7. **Loss of CoASH Derivatives During Time and Conditions of the Assay for Carnitine Palmitoyltransferase A Activity**

Derivative (100 μM)	Specific Activity nmol/min/0.05 mg protein	
	without carnitine	with carnitine
succinyl-CoA	0.44	1.25
malonyl-CoA	0	0.19
acetyl-CoA	0	0.15
acetoacetyl-CoA	0.15	0
methylmalonyl-CoA	0	0

Values are based on two assays with one preparation of mitochondria. Other details of the experiment are described under "Experimental Procedures".

Table 8. **Relationship Between Concentration of Malonyl-CoA or Succinyl-CoA and Degree of Inhibition of Carnitine Palmitoyltransferase A**

Inhibitor	Concentration (μM)	Specific Activity (nmol/min/mg protein)	% of Control Activity
None		10.4 ± 2.2	100
Malonyl-CoA	100	3.3 ± 0.1	31
	10	4.6 ± 0.9	44
	1	6.1 ± 0.1	59
	0.1	7.8 ± 2.8	76
	0.01	10.8 ± 2.8	103
Succinyl-CoA	100	4.7 ± 1.2	45
	10	7.6 ± 0.1	72
	1	10.8 ± 0.0	104
	0.1	11.1 ± 0.7	107
	0.01	10.4 ± 1.5	99

Carnitine palmitoyltransferase A activity was assayed by the radiochemical method. Values are means ± S. E. Values are based on assays with two different preparations of mitochondria. Other details of the experiment are described under "Experimental Procedures".

Table 9. **Inhibition of Rat Heart Mitochondrial Carnitine Palmitoyltransferase A by Malonyl-CoA and Structurally Related Compounds**

Inhibitor (100 μM)	Inhibition (%)
malonyl-CoA	70 ± 15
succinyl-CoA	59 ± 0
acetyl-CoA	-4 ± 20
acetoacetyl-CoA	6 ± 27
methylmalonyl-CoA	23 ± 16

Carnitine palmitoyltransferase A activity was assayed by the radiochemical method. Values are based on the experimental results presented in Table 6. Other details of the experiment are described under "Experimental Procedures".

Table 10. **Enzyme Activities of Subcellular Fractions Obtained by Method I**

	Time (min)	Specific Activity (μmol/min/mg)	Total Activity (μmol/min)	% of Combined Activity
Succinate Thiokinase				
whole heart homogenate	0	0		
	5	6.8	46.1	100
	20	3.4		
	40	1.3		
cytoplasm	0	0		
	5	2.0	12.8	27.8
	20	0.9		
	40	0.7		
extracytoplasmic fraction	0	0		
	5	27.3	39.0	84.4
	20	8.5		
Enoyl-CoA Hydratase				
cytoplasm		1.0 ± 0.2	6.5	9.8
extracytoplasmic fraction		41 ± 9	59	90
3-Hydroxyacyl-CoA Dehydrogenase				
cytoplasm		0.2 ± 0.0	1.0	29.9
extracytoplasmic fraction		2 ± 1	2	70
Citrate Synthase				
cytoplasm		0.3 ± 0.0	1.9	18.3
extraclytoplasmic fraction		6.1 ± 1.1	8.6	81.7

Values are mean ± S. E. The values for succinate thiokinase are based on one assay. The values for the other enzymes are based on three assays. All assays were performed on the same preparation of subcellular fractions. Other details of the experiment are included in "Experimental Procedures".

Table 11. Enzyme Activities of Subcellular Fractions Obtained by Method II

	Specific Activity (μmol/min/mg)	Total Activity (μmol/min)	% of Combined Activity
Succinate Thiokinase			
cytosol	2.7×10^{-3}	50.8×10^{-3}	41.5
mitochondria	76.4×10^{-3}	71.7×10^{-3}	58.5
Enoyl-CoA Hydratase			
cytosol	1.0 ± 0.1	19.6	33.3
mitochondria	41 ± 4	39	67
3-Hydroxyacyl-CoA Dehydrogenase			
cytosol	0.2 ± 0.0	3.0	44.5
mitochondria	4.0 ± 0.2	3.8	55.5
Citrate Synthase			
cytosol	0.3 ± 0.0	5.7	40.9
mitochondria	8.8 ± 0.6	8.2	59.1

Values are mean ± S. E. The values for succinate thiokinase are based on two assays. The values for the other enzymes are based on three assays. All assays were performed on the same preparation of subcellular fractions. Other details of the experiment are described under "Experimental Procedures".

Table 12. **Rates of Respiration Supported by Fatty Acid Oxidation in Rat Liver and Rat Heart Mitochondria**

(Data is expressed in nanoatoms O_2 consumed/min/mg protein)

Substrate	Rat Heart	Rat Liver
Palmitoyl-CoA	167.1 ± 18.8	73.7 ± 9.7
Oleoyl-CoA	174.0 ± 26.1	78.7 ± 8.9
Lineoyl-CoA	154.1 ± 23.9	73.9 ± 8.4
RCR	4.3 ± 0.6	4.9 ± 0.9

Values are means ± S. E. The values are based on three assays each with three different preparations of mitochondria. Other details of the experiment can be found under "Experimental Procedures".

Table 13. **Substrate Utilization by Rat Heart Mitochondria**

Substrate	Specific Activity (nmol consumed/min/mg protein)
Palmitoyl-CoA	3.6 ± 0.4
Oleoyl-CoA	3.4 ± 0.5
Linoleoyl-CoA	3.1 ± 0.5

Values are means ± S. E. The values are based on three assays each with three different preparations of mitochondria. Other details of the experiment can be found under "Experimental Procedures".

Table 14. Activities of Auxiliary Enzymes of β-Oxidation Required for the Degradation of Polyunsaturated Fatty Acids in Rat Liver and Rat Heart Mitochondria[92]

Enzyme	Rat Heart	Rat Liver
3-Hydroxyacyl-CoA epimerase		
C-12 substrate	3.4 ± 0.5(3)	84.9 ± 8.4(7)
C-8 substrate	2.0 ± 0.3(3)	79.3 ± 14.4(5)
cis-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase	745 ±93 (3)	721 ±134 (3)
2,4-Dienoyl-CoA reductase	27.4 ± 5.1(3)	73.6 ± 17.0(5)

Data is expressed in nmol/min/mg protein. Values are means ± S. E. Figures in parentheses are the number of observations with samples from different animals. Each sample was assayed a minimum of three times.

Table 15. **Recovery of Pyruvate Dehydrogenase Complex by Rapid Gel Filtration**

	% volume recovered	% activity recovered	% of control activity prefiltration	post-filtration
Control	85 ± 4	46 ± 7	100	100
Inhibited	88 ± 8	49 ± 16	11.2 ± 2	12 ± 3

% S-acetyl-3-mercaptopropanoyl-CoA remaining in eluate = 8.4 %

Concentration of S-acetyl-3-mercaptopropanoyl-CoA in eluate = 19.8 μM

Concentration of S-acetyl-3-mercaptopropanoylCoA in assay = 0.8 μM

Details of the experiment can be found under "Experimental Procedures".

Table 16. **Inhibition of Pyruvate Dehydrogenase Complex by Enzymatically Synthesized S-Acetyl-3-Mercaptopropanoyl-CoA as Compared to Chemically Synthesized S-Acetyl-3-Mercaptopropanoyl-CoA and 3-Mercaptopropanoyl-CoA**

	Pyruvate Dehydrogenase Complex Activity (% of control)		
Concentration of Inhibitor (μM)	S-Acetyl-3-Mercaptopropanoyl-CoA		3-Mercaptopropanoyl-CoA
	Enzymatically Synthesized	Chemically Synthesized	
26.7	35 ± 2	35 ± 1	47 ± 5
13.6	49 ± 10	56 ± 7	62 ± 5
6.8	68 ± 1	87	86 ± 18

The S-Acetyl-3-Mercaptopropanoyl-CoA was enzymatically synthesized via the thiolase reaction as described under "Experimental Procedures". The control reaction included CoASH instead of 3-mercaptopropanoyl-CoA in the thiolase reaction. Chemically synthesized 3-mercaptopropanoyl-CoA and S-acetyl-3-mercaptopropanoyl-CoA were added to the assay mixture just prior to the preincubation period. The enzyme was preincubated for 3 min at 30°C in the presence of KP_i, $MgCl_2$, TPP, CoASH, and pyruvate. The reaction was initiated by the addition of NAD^+. Other details of the experiment are described under "Experimental Procedures".

Table 17. Inhibition of Pyruvate Dehydrogenase Complex by 3-Mercaptopropanoyl-CoA as a Function of Different Preincubation Conditions

Preincubation Components	Initiation Components	Initial Velocity (μmol/min/mg protein)		% of Control
		Control	Inhibited	
TPP, CoASH, pyruvate	NAD^+	1.5 ± 0.3	0.3 ± 0.1	6.5
TPP, CoASH	NAD^+, pyruvate	2.7 ± 0.3	0.6 ± 0.1	21.1
TPP, NADH	NAD^+, pyruvate, CoASH	1.6 ± 0.2	0.2 ± 0.1	5.8
TPP, pyruvate, NAD^+	CoASH	2.0 ± 0.2	1.1 ± 0.2	54.4
TPP, CoASH, NAD^+	pyruvate	2.1 ± 0.2	1.2 ± 0.6	72.5
TPP	NAD^+, pyruvate, CoASH	1.9 ± 0.3	0.5 ± 0.2	28.3
TPP, pyruvate	NAD^+, CoASH	1.8 ± 0.5	0.3 ± 0.1	17.1
TPP, NAD^+	pyruvate, CoASH	2.2 ± 0.1	1.7 ± 0.1	79.1
CoASH, pyruvate	TPP, NAD^+	2.2 ± 0.5	0.4 ± 0.1	19.5

The enzyme was preincubated for 3 min at 30°C in the presence of KP*i*, $MgCl_2$, and various components of the assay mixture. The reaction was initiated by the addition of those assay components not present during the preincubation. The 3-mercaptopropanoyl-CoA, when included in the preincubation mixture, was at a concentration of 30.5 μM. Other details of the experiment can be found under "Experimental Procedures".

Table 18. Inhibition of Pyruvate Dehydrogenase Complex by S-Acetyl-3-Mercaptopropanoyl-CoA as a Function of Different Preincubation Conditions

Preincubation Components	Initiation Components	Initial Velocity (μmol/min/mg protein)		% of Control
		Control	Inhibited	
TPP, CoASH, pyruvate	NAD^+	2.7 ± 0.3	0.2 ± 0.1	8.2
TPP, CoASH	NAD^+, pyruvate	5.1 ± 0.4	0.5 ± 0.1	9.4
TPP, pyruvate, NAD^+	CoASH	2.5 ± 0.4	1.3 ± 0.0	51.2
TPP, CoASH, NAD^+	pyruvate	3.3 ± 0.3	1.8 ± 0.6	53.7
TPP	NAD^+, pyruvate, CoASH	3.3 ± 0.2	0.6 ± 0.1	16.8
TPP, pyruvate	NAD^+, CoASH	3.7 ± 0.3	1.2 ± 0.5	33.3
TPP, NAD^+	pyruvate, CoASH	4.7 ± 0.8	2.8 ± 0.7	58.8
CoASH, pyruvate	TPP, NAD^+	5.0 ± 0.6	0.6 ± 0.1	12.7

The enzyme was preincubated for 3 min at 30°C in the presence of KP*i*, $MgCl_2$, and various components of the assay mixture. The reaction was initiated by the addition of those assay components not present during the preincubation. The S-acetyl-3-mercaptopropanoyl-CoA, when included in the preincubation mixture, was at a concentration of 67.5 μM. Other details of the experiment can be found under "Experimental Procedures".

Table 19. Inhibition of Pyruvate Dehydrogenase - Lipoate Acetyltransferase

The enzyme was preincubated for 3 min at room temperature in the presence of TRIS-HCl, $MgCl_2$, TPP, and CoASH. Either pyruvate, lipoamide or neither were included in the preincubation mixture. The reaction was initiated by the addition of either pyruvate, lipoamide or both as indicated. Acetyl-CoA produced was measured via the citrate synthase reaction. Other experimental details can be found under "Experimental Procedures".

I = S-Acetyl-3-Mercaptopropanoyl-CoA (67.5 μM)

Preincubation Conditions	Initiating Component	Specific Activity (μmol/min/mg)	Relative Activity (%)
lipoamide	pyruvate	1.2 ± 0.2	100
lipoamide, I	pyruvate	0.8 ± 0.2	72
pyruvate	lipoamide	1.1 ± 0.2	100
pyruvate, I	lipoamide	0.4 ± 0.1	39
	pyruvate, lipoamide	1.0 ± 0.2	100
I	pyruvate, lipoamide	0.7 ± 0.2	68

Table 20. Inhibition of Dihydrolipoyl Dehydrogenase

The enzyme protein was preincubated for 3 minutes at 30°C in the presence of KP_i and one substrate. The reaction was initiated by the addition of the second substrate. A_{340} was measured spectrophotometrically. Other experimental details can be found under "Experimental Procedures".

I = S-Acetyl-3-Mercaptopropanoyl-CoA (67.5 μM)

$$\text{Dihydrolipoamide} + NAD^+ \rightleftharpoons \text{Lipoamide} + NADH + H^+$$

Preincubation Conditions	Initiating Component	Specific Activity (μmol/min/mg)	Relative Activity
Dihydrolipoamide	NAD^+	6.8 ± 1.1	100
Dihydrolipoamide, I	NAD^+	3.3 ± 0.8	47.0 ± 5.3
NAD^+	Dihydrolipoamide	5.6 ± 0.1	100
NAD^+, I	Dihydrolipoamide	4.9 ± 0.3	88.1 ± 6.0
Lipoamide	NADH	5.7 ± 0.8	100
Lipoamide, I	NADH	5.4 ± 1.3	94.4 ± 18.3
NADH	Lipoamide	5.8 ± 0.2	100
NADH, I	Lipoamide	3.1 ± 0.2	53.0 ± 3.8

Table 21. **Inhibition of [1-^{14}C]-Acetyl Group Incorporation into the Pyruvate Dehydrogenase Complex**

Experiment I

Inhibitor Concentration (μM)	Acetyl Bound/PDH Complex mol/mol	Acetyl Bound % of Control
0	50.7	100
47.8	31.0	61.1
980.7	4.3	8.5

Experiment II

Inhibitor Concentration (μM)	Acetyl Bound/PDH Complex mol/mol	Acetyl Bound % of Control
0	34.5	100
47.8	21.9	63.6
119.6	17.8	51.7
239.2	7.1	20.7
478.4	1.5	5.2

Experiment I utilized porcine pyruvate dehydrogenase complex. Experiment II utilized the bovine enzyme. The enzyme (0.2-0.22 mg protein) was preincubated at room temperature for 3 min. The preincubation mixture contained KP*i*, $MgCl_2$, TPP, NADH, and ± S-acetyl-3-mercaptopropanoyl-CoA (the inhibitor). The reaction was initiated with [1-^{14}C]-acetyl-CoA. Other details of the experiment are described in "Experimental Procedures".

Table 22. **Inhibition of Palmitoylcartine- and Pyruvate-Supported Respiration by 3-Mercaptopropanoic Acid and S-Acetyl-3-Mercaptopropanoic Acid**

The mitochondria were preincubated 1 min in the presence of malate at which time the inhibitor (~ 800 μM) was added. ADP was added to the chamber 3 min after malate. The substrate was added to the chamber 4 min after malate. Values are means ± S. E. The values are based on at least two assays for each preparation of mitochondria. Figures in parentheses indicate the number of mitochondrial preparations studied. All preparations of mitochondria had RCR values larger than 4, using palmitoylcarnitine as substrate. Other details of the experiment are described under "Experimental Procedures".

Rat Liver Mitochondria	
Palmitoylcarnitine-Supported Respiration	
Inhibitor	Respiration Rate (% of Control)
3-mercaptopropanoic acid	40 ± 11(4)
S-acetyl-3-mercaptopropanoic acid	32 ± 14(2)
Pyruvate-Supported Respiration	
Inhibitor	Respiration Rate (% of Control)
3-mercaptopropanoic acid	57 ± 8(4)
S-acetyl-3-mercaptopropanoic acid	56 ± 20(2)
Rat Heart Mitochondria	
Palmitoylcarnitine-Supported Respiration	
Inhibitor	Respiration Rate (% of Control)
3-mercaptopropanoic acid	17 ± 15(3)
S-acetyl-3-mercaptopropanoic acid	27 (1)
Pyruvate-Supported Respiration	
Inhibitor	Respiration Rate (% of Control)
3-mercaptopropanoic acid	104 ± 0(2)
S-acetyl-3-mercaptopropanoic acid	99 (1)

Table 23. Inhibition of Pyruvate-Supported Respiration in Rat Heart Mitochondria by 3-Mercaptopropanoic Acid in the Presence of Acetoacetate

The mitochondria were preincubated with primer. 3-Mercaptopropanoic acid (final concentration was 840.7 μM) was added to the chamber 30 sec after the addition of primer. Acetoacetate was added to the chamber 1 min after the addition of primer. Substrate was added to the chamber 4.5 min after the addition of primer. Values are means ± S. E. The values are based on at least two assays for each preparation of mitochondria. Figures in parentheses indicate the number of mitochondrial preparations studied. All preparations of mitochondria had RCR values larger than 4, using palmitoylcarnitine as substrate. Other details of the experiment are described under "Experimental Procedures".

Conditions			Rate of Respiration (natom O/min/mg)		% of Control
Primer	Other Additions	Substrate	Control	Inhibited	
malate		palmitoyl-carnitine	105.6 ± 10.7(2)	7.4 ± 3.2(2)	6.6
malate		pyruvate	274.2 (1)	202.3 (1)	81.8
α-KG[a]			67.4 ± 0.0(2)	67.4 ± 0.0(1)	100.0
α-KG	acetoacetate		80.9 ± 0.0(2)	103.4 ± 0.0(2)	127.8
α-KG	acetoacetate	pyruvate	238.2 ± 0.0(2)	105.6 ± 3.2(2)	44.3
α-KG		pyruvate	209.0 ± 3.2(2)	96.7 ± 3.2(1)	46.2

a α-KG = α-ketoglutarate

Figure 1. **Schematic Representation of Fatty Acid Activation and Transport Across the Mitochondrial Membranes**

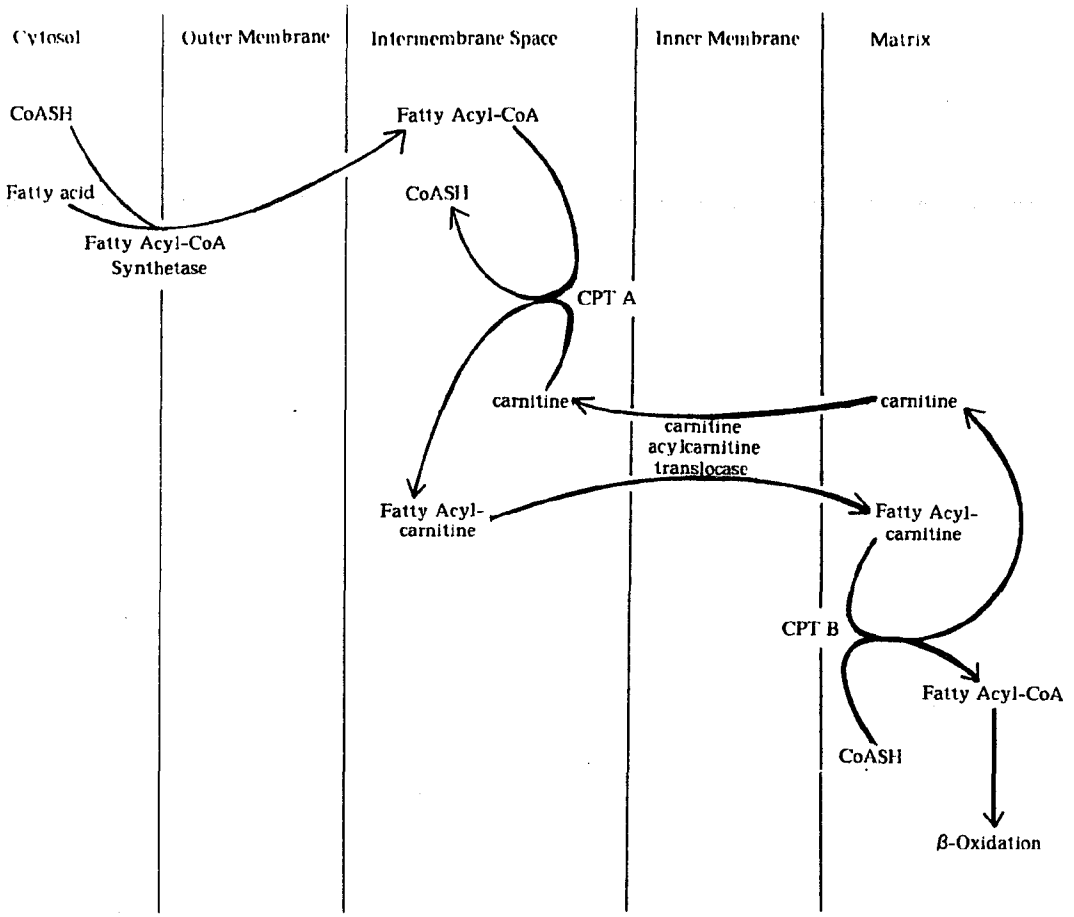


Figure 2. **Pathway of Linoleic Acid Degradation**

A, pathway proposed by Stoffel and Caesar[85]. B, modified pathway supported by findings published by Kunau and Dommes[87], as well as Cuebas and Schulz[89]. Reactions catalyzed by: (1) acyl-CoA dehydrogenase; (2) enoyl-CoA hydratase; (3) L-3-hydroxyacyl-CoA dehydrogenase; (4) 3-ketoacyl-CoA thiolase; (5) 3-hydroxyacyl-CoA epimerase; (6) *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase; (7) 2,4-dienoyl-CoA reductase.

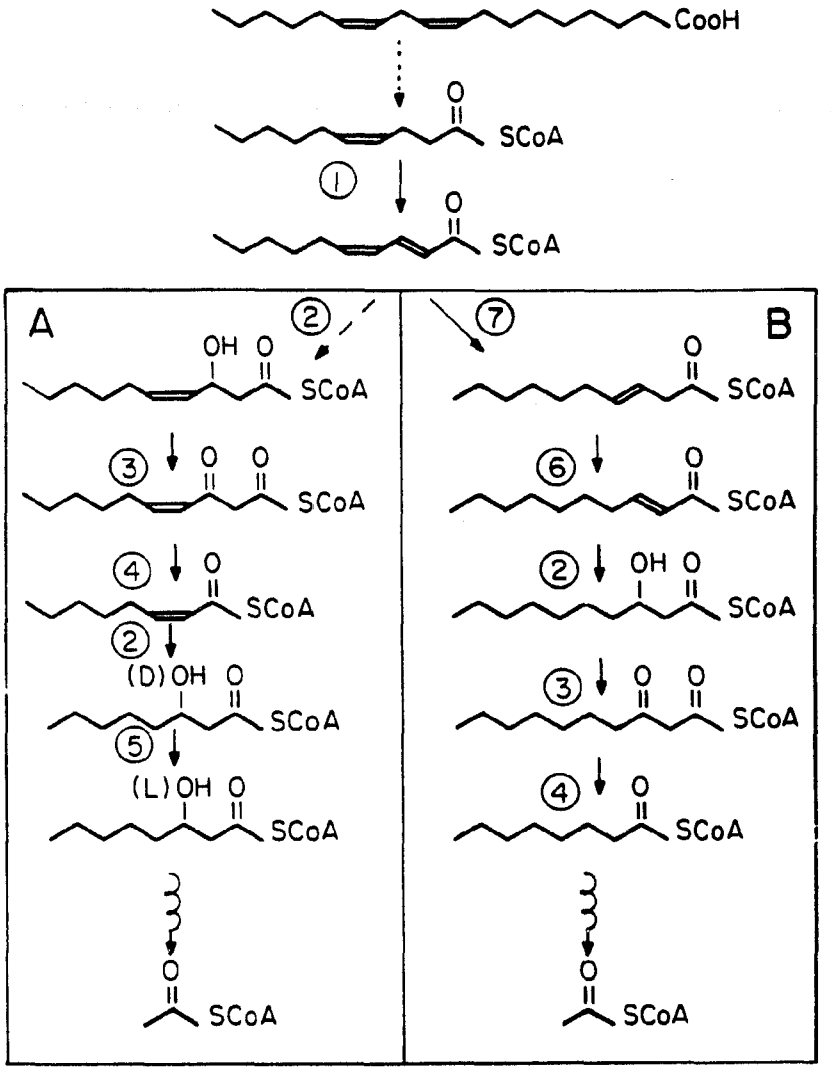


Figure 3. **Mitochondrial Metabolism of 3-Mercaptopropanoic Acid**

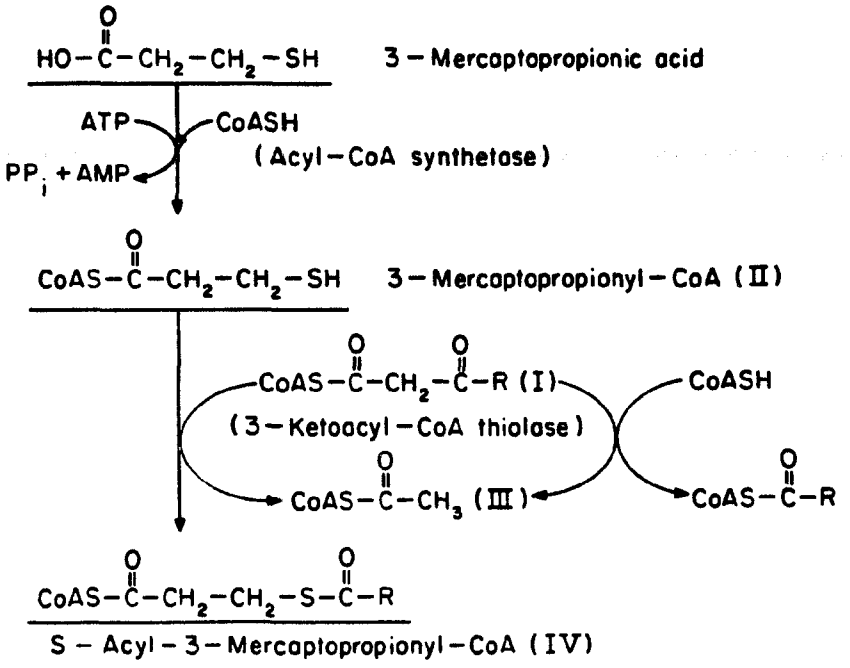


Figure 4. **Effect of Palmitoylcarnitine on Activity of Palmitoyl-CoA Synthetase in Rat Heart Mitochondria**
The figure was generated from the experimental data in Table 2. The curve is a theoretical curve based on linear regression analysis. Other details of the experiment can be found under "Experimental Procedures".

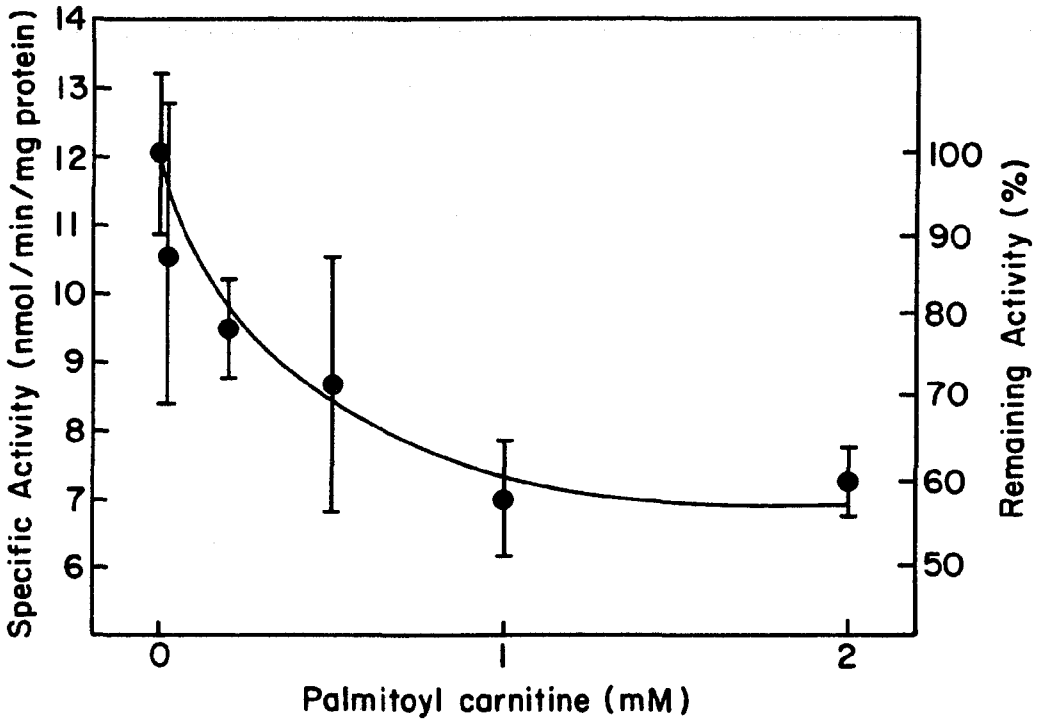


Figure 5. **Concentration Dependence of Inhibition by Malonyl-CoA and Succinyl-CoA of Carnitine Palmitoyltransferase A**
CPT A activity was assayed by the radiochemical method. The filled circles indicate malonyl-CoA. The opened circles indicate succinyl-CoA. The figure was generated from the data in Table 8. Other details of the experiment can be found under "Experimental Procedures".

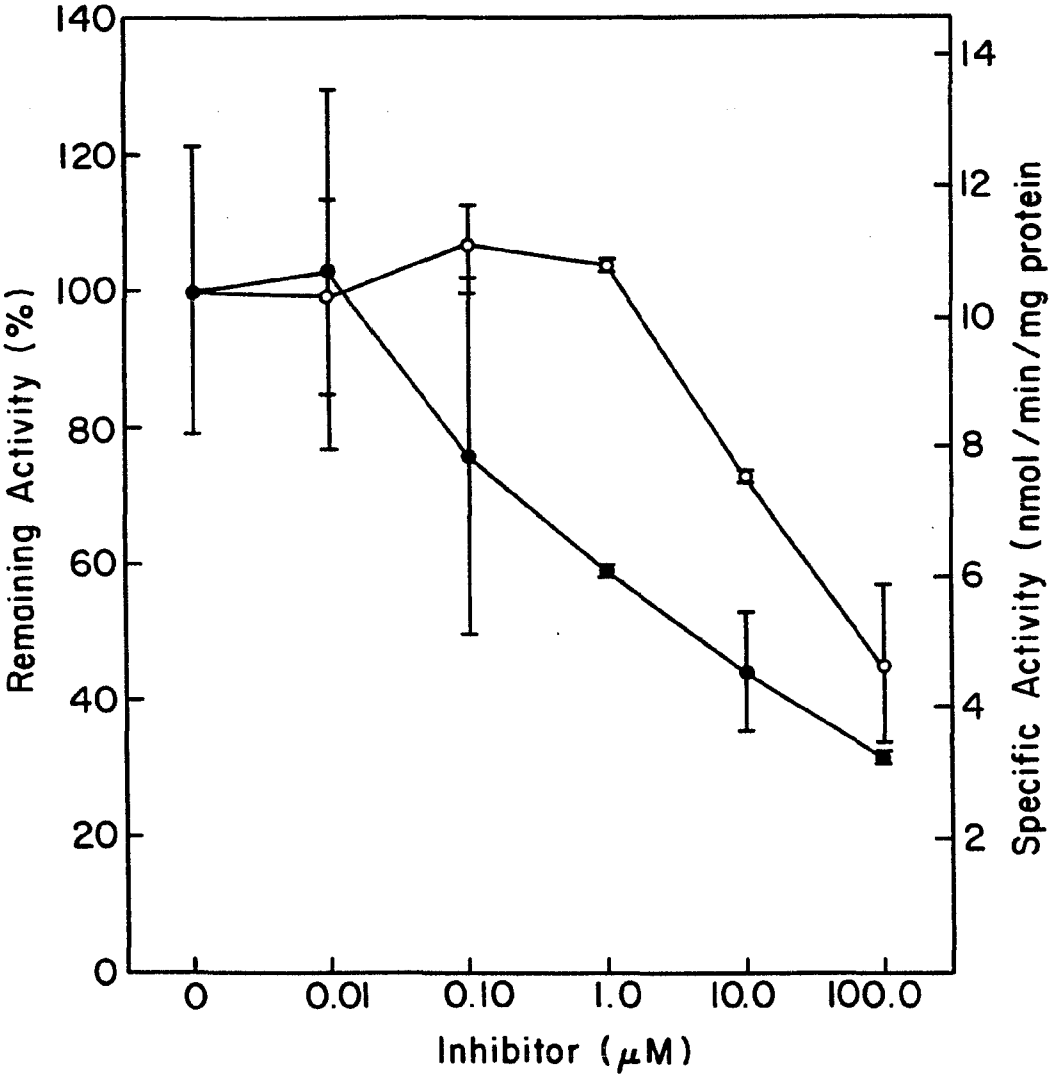


Figure 6. **Inhibition of Pyruvate Dehydrogenase Complex by 3-Mercaptopropanoyl-CoA and S-Acetyl-3-Mercaptopropanoyl-CoA**
A, As a Function of Concentration
B, As a Function of Time

A, The enzyme was preincubated for 3 min at 30°C in the presence of KP*i*, $MgCl_2$, TPP, CoASH, pyruvate, and the inhibitor. The reaction was initiated with NAD^+.
B, The enzyme was preincubated for 3 min at 30°C in the presence of KP*i*, $MgCl_2$, TPP, CoASH, and pyruvate. The inhibitor was added to provide for preincubation for the lengths of time indicated.
The solid line indicates S-acetyl-3-mercaptopropanoyl-CoA. The dashed line indicates 3-mercaptopropanoyl-CoA. Each value represents the mean of a minimum of three assays. The curves are theoretical curves generated by linear regresion analysis.

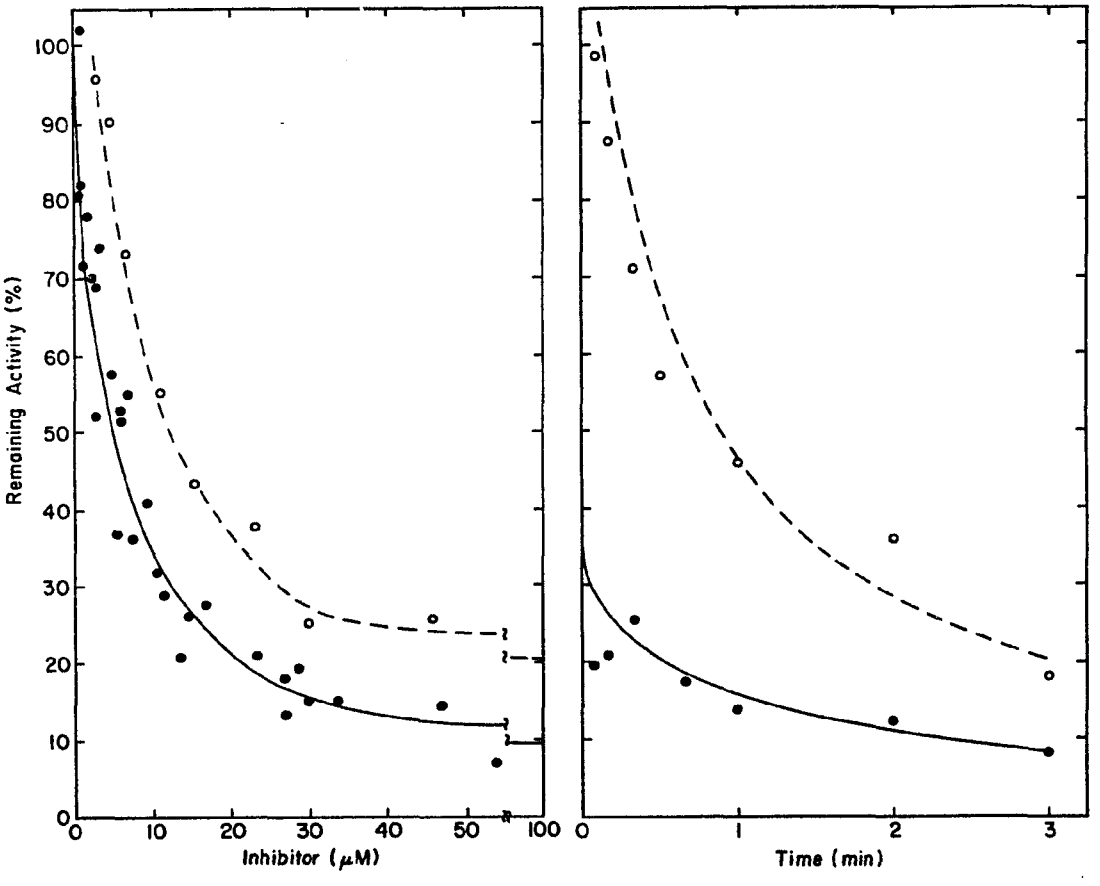


Figure 7. **Inhibition of Pyruvate Dehydrogenase by 3-Mercaptopropanoyl-CoA as a Function of Different Preincubation Conditions**

The enzyme was preincubated for 3 min at 30°C in the presence of KP_i, $MgCl_2$, and various components of the assay mixture. The reaction was initiated by the addition of those assay components not present during the preincubation. The dashed line indicates the presence of 30.5 μM 3-mercaptopropanoyl-CoA in the preincubation mixture.

Symbol	Preincubation Components	Initiating Components
●	TPP, CoASH, Pyruvate	NAD^+
o	TPP, NADH	CoASH, Pyruvate, NAD^+
x	TPP, NAD^+, CoASH	Pyruvate
x	TPP, NAD^+	CoASH ,Pyruvate
◘	TPP, Pyruvate	NAD^+, CoASH
△	TPP, CoASH	NAD^+, Pyruvate
△	TPP, Pyruvate, NAD^+	CoASH
△	TPP	NAD^+, Pyruvate, CoASH
△	CoASH, Pyruvate	TPP, NAD^+

Figure 7.

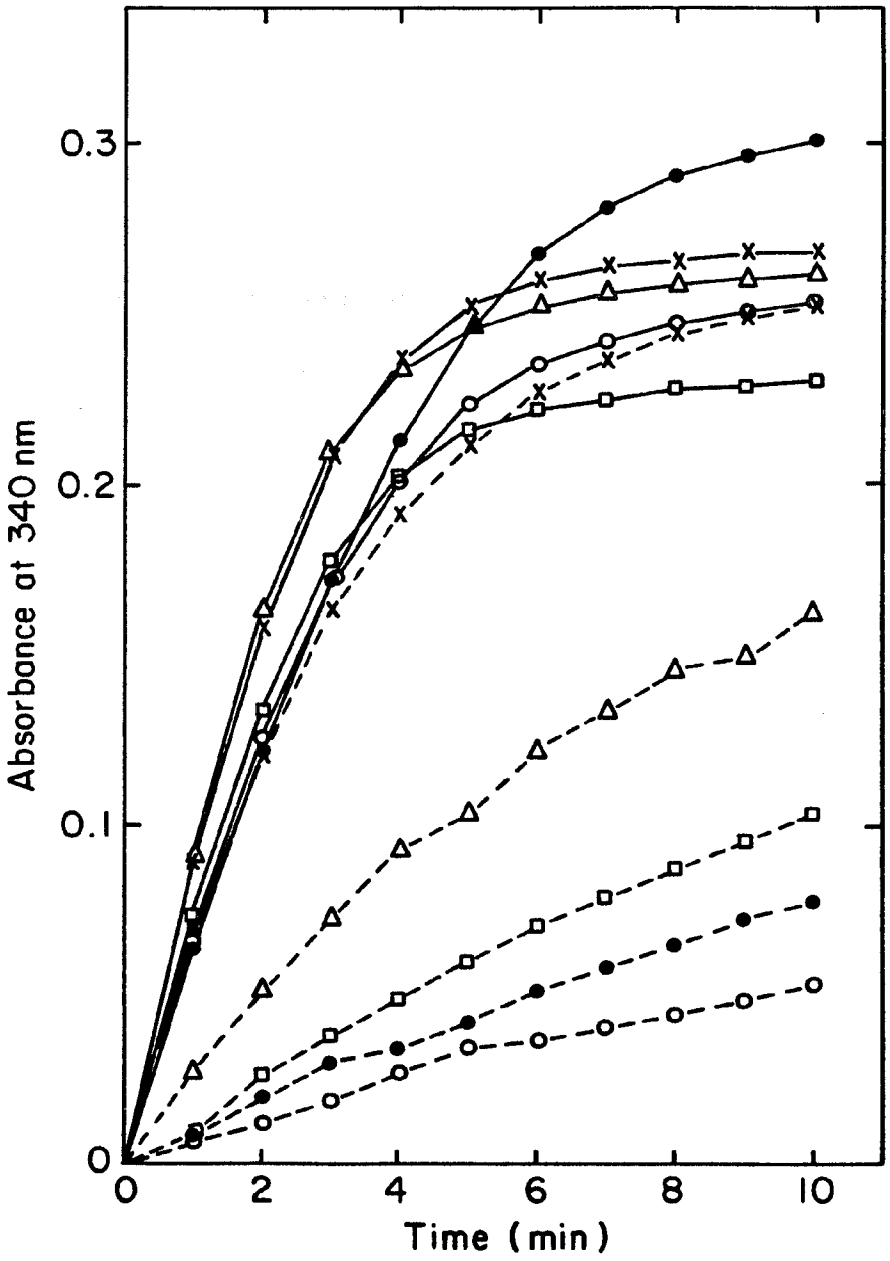


Figure 8. Inhibition of Pyruvate Dehydrogenase by S-Acetyl-3-Mercaptopropanoyl-CoA as a Function of Different Preincubation Conditions

The enzyme was preincubated for 3 min at 30°C in the presence of KP_i, $MgCl_2$, and various components of the assay mixture. The reaction was initiated by the addition of those assay components not present during the preincubation. The dashed line indicates the presence of 67.5 μM S-acetyl-3-mercaptopropanoyl-CoA in the preincubation mixture.

Symbol	Preincubation Components	Initiation Components
●	TPP, CoASH, Pyruvate	NAD^+
o	TPP, NADH	CoASH, Pyruvate, NAD^+
□	TPP, CoASH	Pyruvate, NAD^+
×	TPP, NAD^+	Pyruvate, CoASH
△	TPP, Pyruvate, NAD^+	CoASH
	TPP, CoASH, NAD^+	Pyruvate
	TPP	NAD^+, Pyruvate, CoASH
	TPP, pyruvate	NAD^+, CoASH
	CoASH, Pyruvate	TPP, NAD^+

Figure 8.

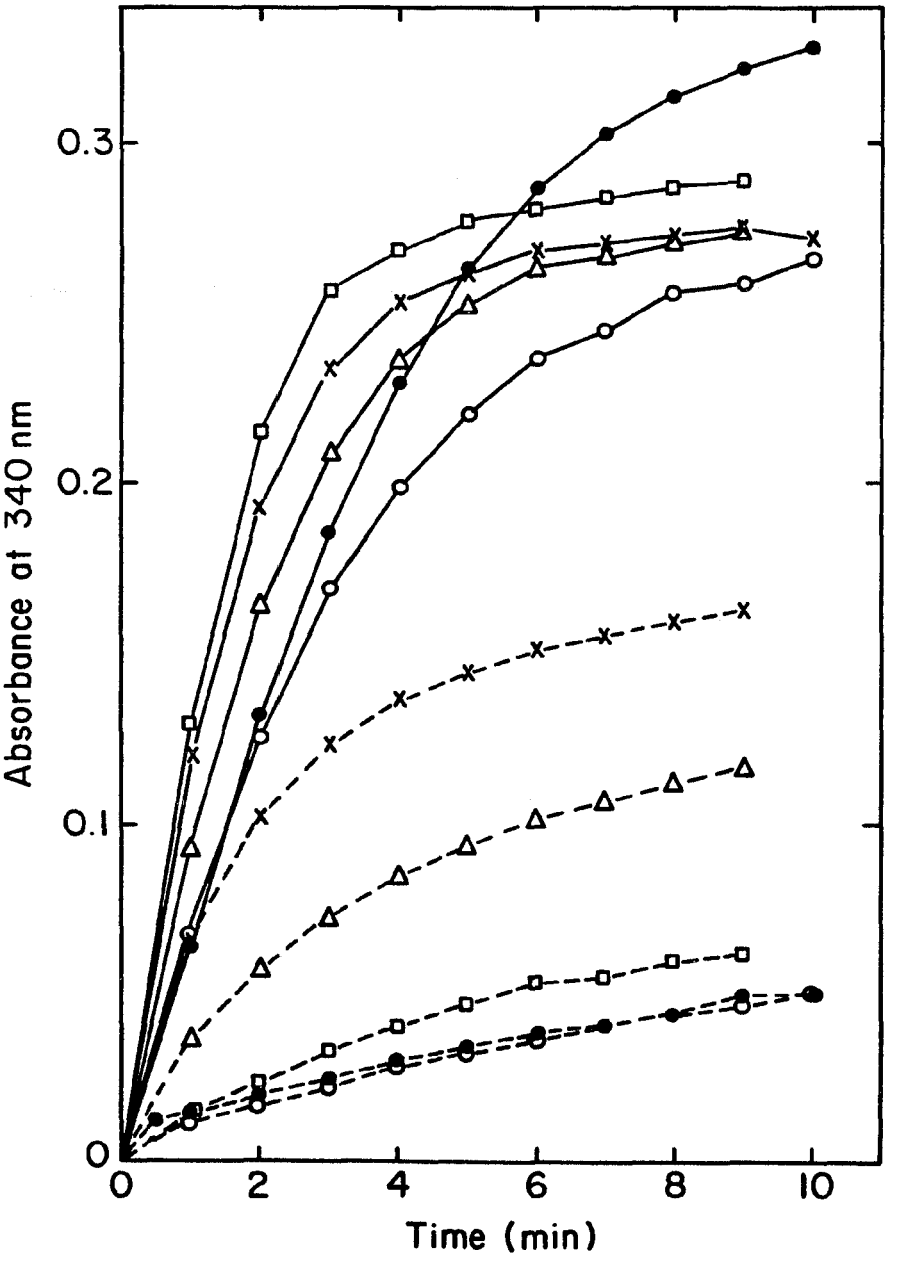
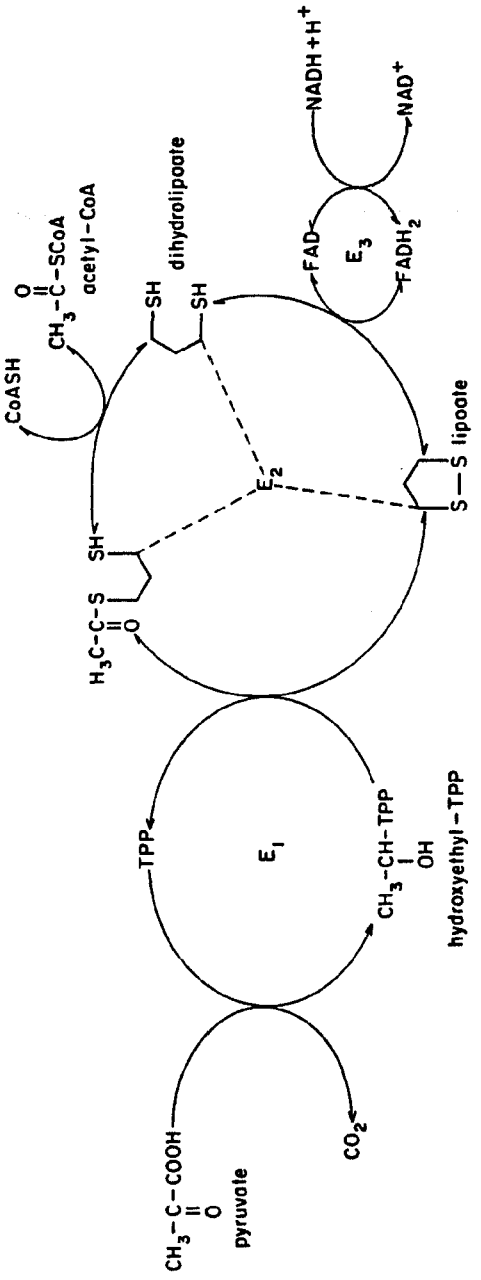


Figure 9. **Schematic Representation of the Pyruvate Dehydrogenase Complex**
E1 = Pyruvate Dehydrogenase
E2 = Lipoate Acetyl Transferase
E3 = Dihydrolipoyl Dehydrogenase



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