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by

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ABSTRACT

PEROXISOMAL FATTY ACID OXIDATION IN TETRAHYMENA AND IN RAT LIVER

by

Daniel J. Hryb

Adviser: Professor James F. Hogg

This thesis reports the presence of a new enzyme for fatty acid oxidation, the fatty acyl-CoA oxidase, in Tetrahymena and rat liver. The oxidase activity (a fatty acyl-CoA-dependent H_2O_2 producing activity) was assayed by using a modified and much improved method to assay H_2O_2-producing oxidases. The oxidase assay involves the continuous spectrophotometric measurement of the reaction product, H_2O_2, by coupling it to the oxidation of a chromogen (o-dianisidine or a p-hydroxybenzoic acid and 4-aminoantipyrine mixture) by horseradish peroxidase. There was no catalase inhibition of the oxidase activity as long as the catalase/peroxidase ratio of activities was lower than 10.

The oxidase in Tetrahymena was extracted from lyophilized cells with 50% (v/v) glycerol, which was required to stabilize the enzyme, and characterized. The oxidase from Tetrahymena is a FAD-dependent enzyme, has a pH maximum of 8.0-9.0, low affinity for acyl-CoA's, and high affinity for oxygen. The oxidase from cells grown on the standard medium

(proteose-peptone-glucose-acetate) showed a chain length specificity for short-chain length acyl-CoA substrates (C_4-C_8) and it was not able to oxidize any acyl-CoA compound of chain length greater than C_8. However, if Tetrahymena cells are grown in an oleate-rich medium (proteose-peptone-glucose-Tween 80), the chain length specificity of the oxidase becomes much broader (C_4-C_{20}) and their activity increases by several fold over the cells grown in the standard medium. Assay of subcellular fractions indicate the oxidase to be a peroxisomal enzyme. The fatty acyl-CoA dehydrogenase was shown to be present in the mitochondria. β-oxidation also occurs in the peroxisome.

The β-oxidation system for fatty acids and the novel fatty acyl-CoA oxidase activity of rat liver peroxisomes were characterized in terms of assay requirements and of chain length specificity of substrate. The rat liver oxidase is a FAD-dependent enzyme, has low affinity for acyl-CoA, high affinity for oxygen, and it also is a membrane-bound enzyme. The oxidase, as well as the peroxisomal β-oxidation system showed a specificity for medium to long-chain length acyl-CoA's (C_{10}-C_{16}) as substrates, with a well defined peak of activity at C_{12}. Their specific activities were similar and, in each case, oleoyl-CoA was a better substrate than stearoyl-CoA. The mitochondrial fatty acyl-CoA dehydrogenase activity showed a broader chain

length specificity (C_4-C_{16}), with the shorter-chain length substrates being more active and no preference being shown for oleoyl-CoA over stearoyl-CoA. Using palmitoyl-CoA as substrate, the total enzymatic activity for the first step of β-oxidation in the mitochondrion was 7.7-fold greater than for the corresponding step in the peroxisome (11% of the total oxidation by peroxisomes).

A hypothesis is presented which postulates that the energy of peroxisomal oxidations (respiration) is conserved (or used by the cell) by the establishment of a thermal-gradient in such a way that it can do several different kinds of work: 1) heating of the cell and/or organism (thermogenesis), 2) the movement of metabolites and organized structures within the cytosol (cytoplasmic streaming), and the mechanical work of conformational changes in the peroxisomal membrane or intrinsic proteins.

... sin otra luz y guia
sino la que en el corazon ardia.

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PART II

PROLOGUE

No one can take from us the joy of the first becoming aware of something, the so-called discovery. But if we also demand the honor, it can be utterly spoiled for us, for we are usually not the first. What does discovery mean, and who can say that he has discovered this or that? After all it's pure idiocy to brag about priority, for it's simply unconscious conceit, not to admit frankly that one is a plagiarist.

--GOETHE

Fatty acids are one of the principal fuels of oxidative metabolism in higher organisms. They play a central role as an energy-rich fuel in higher animals and plants since large amounts can be stored in cells in the form of triacylglycerols. Triacylglycerols have a high energy content, about 9 Kcal/g and are accumulated in nearly anhydrous form as intracellular fat droplets. On the other hand, the other principal fuel of oxidative metabolism, glycogen (or starch), can yield only about 4 Kcal/g, and is found in a highly hydrated form. Thus fatty acids can be stored in a highly concentrated and efficient manner. Fatty acids provide 40% of the total fuel requirements in man on a normal diet and during fasting they become the sole source of energy.

The quest for the understanding of the mechanism of fatty acid oxidation began with the experiments of the German biochemist F. Knoop in 1904 (1) on hippuric acid formation from phenyl fatty acids fed to rabbits. Phenyl-substituted fatty acids (substituted at the omega-carbon or methyl end) yielded benzoic acid when the fatty acid chain was odd-numbered, regardless of the chain length and phenylacetic acid when the chain was even. These two end products were isolated from the rabbit's urine as their conjugates with glycine (hippuric acid and phenylaceturic acid, respectively). These results suggested that these ω-phenyl fatty acids were being degraded by an oxidative removal of successive two-carbon fragments

starting from the carboxyl end. Knoop interpreted his results in terms of a theory of β-oxidation of fatty acids, by which fatty acids are oxidized at the β-carbon to yield a β-keto acid, which then undergoes cleavage to form acetic acid and a fatty acid shorter by two carbon atoms--a theory that proved to be eventually correct in every detail, but 50 years was needed before the definitive proofs were provided.

Embden (2) continued the study of fatty acid oxidation by making the transition from the whole animal to a perfused liver preparation and Jowett and Quastel (3) from perfused liver to liver slices. However, efforts to demonstrate fatty acid oxidation in cell-free extracts of animal tissues were unsuccessful for many years. Then Leloir and Munoz (4) finally were able to demonstrate the oxidation of fatty acids in an extremely labile cell-free system. By 1949 Grafflin and Green (5) and Kennedy and Lehninger (6) showed that the oxidation of fatty acids supposedly occurs exclusively in the mitochondrion. From the above studies on fatty acid oxidation in simpler systems, several relationships became apparent to the above investigators; the fatty acids had to be first activated by ATP in order to undergo oxidation and their oxidation was inextricably tied up with mitochondrial respiration and oxidative phosphorylation. The next major lead in this continuing story came from the discovery of a new coenzyme

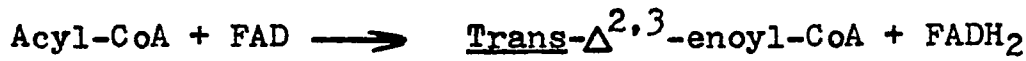
by Lipmann (7,8) and Nachmansohn (9), which was essential for the generation of active acetate. The discovery of Coenzyme A by Lipmann, set the stage for Lynen's (10) proof that active acetate was identical with acetyl-Coenzyme A. He found that the ATP-dependent activation of fatty acids involves their esterification with the thiol group of Coenzyme A to yield acyl-CoA and that all subsequent oxidative steps take place with the fatty acid in the form of its CoA thioester.

As the quest for the complete characterization of the β-oxidation system neared its end, two independent approaches were followed to the ultimate objective of the identification and isolation of the enzymes involved in the β-oxidation of fatty acids. The Madison group led by D. E. Green relied on the activating enzyme of Mahler et al. (11) to prepare Coenzyme A esters of unsaturated acids and β-hydroxyacids, which were then used for the one-by-one identification and isolation of the four different enzymes of the fatty acid oxidation system (12,13,14). At the same time a parallel development took place in the Munich laboratory of F. Lynen. Lynen's group chemically prepared acyl esters of pantotheine rather than CoA. Although low in activity, these synthetic substrates were used also to identify and characterize the principal enzymes involved in fatty acid oxidation (15,16,17).

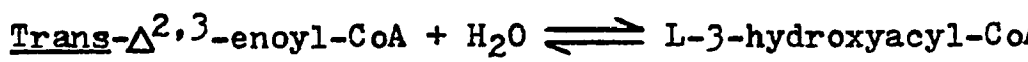
After the various enzymes catalyzing the successive steps in the oxidation of fatty acids were identified, isolated and

characterized by Green, Lynen and Ochoa, the following picture of the β-oxidation cycle emerged (shown in schematic form bellow):

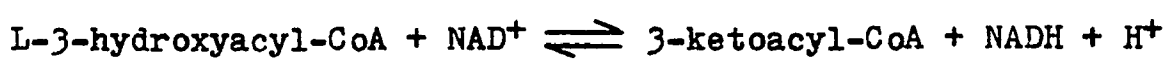
1) Acyl-CoA dehydrogenase(s)



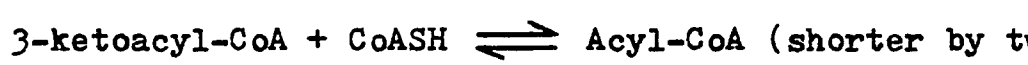
2) Enoyl-CoA hydratase(s)



3) L-3-Hydroxyacyl-CoA dehydrogenase(s)



4) Thiolase(s)



Before the fatty acid can be oxidized by the β-oxidation cycle in the mitochondria, it is first enzymatically activated by the fatty acyl-CoA synthetase(s) to yield a fatty acyl-CoA thioester. The fatty acyl-CoA is then transported across the mitochondrial membrane by a carnitine-dependent process (carnitine acyltransferase)(85). The subsequent oxidation of the fatty acyl-CoA then takes place entirely in the mitochondrial matrix. The fatty acyl-CoA is first dehydrogenated to yield the trans-$\Delta^{2,3}$-enoyl-CoA by acyl-CoA dehydrogenase(s), a FAD-dependent enzyme which then transfers the electrons to a electron transfering flavoprotein (ETF); which in turn transfers the electrons to coenzyme Q of the electron transport chain.

The trans-$\Delta^{2,3}$-enoyl-CoA intermediate is enzymatically hydrated by enoyl-CoA hydratase(s) to L-3-hydroxyacyl-CoA, this intermediate is then dehydrogenated in the next step by the NAD^+-specific L-3-hydroxyacyl-CoA dehydrogenase(s) to yield the 3-ketoacyl-CoA. The 3-ketoacyl-CoA is then cleaved in the last step of the cycle by thiolase(s) to yield acetyl-CoA and a fatty acyl-CoA having two fewer carbon atoms, which then becomes the new substrate for the first dehydrogenation step of the β-oxidation cycle. The fatty acyl-CoA is thus oxidized until it has been totally degrated to acetyl-CoA's.

By 1963--sixty years after the initial discoveries of Knoop--the full story of fatty acid oxidation could be told; or was it really the full story? Aside from such minor oxidative pathways as alpha- and omega-oxidation of fatty acids there recently has been discovered another major pathway for the oxidation of fatty acids--the peroxisomal β-oxidation pathway. This new and continuing story of fatty acid oxidation has not been fully told because it is not yet finished. However, its beginings will be recapitulated here because this thesis was part of it.

The story of fatty acid oxidation, part II, began in 1969 when Cooper and Beevers (18) published their classical paper on β-oxidation in glyoxysomes (peroxisomes) from castor bean endosperm. Cooper and Beevers found that in the endosperm of germinating castor bean seedlings, the system oxidizing

fatty acyl-CoA and its derivatives is located in the peroxisomes and not in the mitochondria. Peroxisomes are cytoplasmic oxidative organelles that are characterized by their content of H_2O_2-producing oxidases (such as L-a-hydroxyacid oxidase, D-amino acid oxidase and urate oxidase) and H_2O_2-decomposing catalase (19). The peroxisomes from castor bean endosperms (glyoxysomes) also contain the enzymes of the glyoxylate cycle (20), which permit the net conversion of acetyl-CoA derived from lipid into hexose. The peroxisomal fatty acid oxidation activity was measured as the palmitoyl-CoA-dependent NAD+ reducing activity in the presence of KCN (18). Cooper and Beevers reported that the peroxisomal β-oxidation pathway resembles that of the mitochondria except for the first enzyme. They suggested that the first dehydrogenase in the peroxisomal system transfers its electrons directly to O_2, producing H_2O_2. However, except for some indirect evidence of the presence of this new enzyme in fatty acid oxidation, Cooper and Beevers were not able to directly assay this enzyme, and so it remained until I entered into this story.

When I started to work in Dr. J. F. Hogg's laboratory, my initial research interest was the study of gluconeogenesis from fats in the ciliated protozoan Tetrahymena pyriformis. Gluconeogenesis from fats in Tetrahymena is induced during the transition from the log phase of growth to the stationary phase (51,53) or during the static conditions brought about by lowering oxygen tension (21,22). Alternatively, an

increase in gluconeogenesis also occurs when cells are shaken under an atmosphere of low oxygen tension (22). It is of interest to note that, while the increase capacity for gluconeogenesis is acquired when cells are under nearly anaerobic conditions, maximal rates of synthesis occur when the cells are subsequently returned to aerobic conditions (51). Therefore it seemed clear to us that oxygen tension was the key factor associated with the induction, and ultimately with the optimal rates of synthesis of glycogen from fats. Since peroxisomal respiration (catalyzed by H_2O_2-producing oxidases and catalase) was subject to regulation by oxygen tension (19), the study of its function became the focus of our research. It was thus that I came to be interested in peroxisomal oxidases and their physiological functions; more specifically, in the generation of acetyl-CoA from fats to support the peroxisomal glyoxylate bypass. Searching for a better way to assay H_2O_2-producing oxidases (L-a-hydroxy acid oxidase and D-amino acid oxidase), we used Hugget and Nixon's coupled enzyme system (glucose oxidase-horseradish peroxidase-o-dianisidine) for determining blood glucose and modified it in such a way that it could be used as a continuous spectrophotometric method to assay the enzymatic activity of any H_2O_2-producing oxidase.

At about this time (1973-74) two papers were published in which several of the enzymes involved in fatty acid oxi-

dation; fatty acyl-CoA synthetase, L-3-hydroxyacyl-CoA dehydrogenase, and thiolase, were found in the peroxisomes of Tetrahymena (24) and Euglena (25). These authors also tried to measure the overall peroxisomal β-oxidation pathway using Cooper and Beevers' assay (18), but were unsuccessful.

Stimulated by Cooper and Beevers' paper and under the guidance of Professor Hogg, I turned my attention to the search for the peroxisomal β-oxidation pathway and its novel enzyme, the fatty acyl-CoA oxidase in Tetrahymena. In the spring of 1975, using the spectrophotometric oxidase assay method that had been worked out for lactate oxidase (unpublished results with J. F. Hogg and E. Wajnberg), I was finally able to assay the fatty acyl-CoA oxidase activity. The oxidase was extracted from lyophilized Tetrahymena cells with 50% (v/v) glycerol, which was required to stabilize the enzyme, and characterized. The oxidase from cells grown under normal conditions showed a chain length specificity towards short-chain fatty acids; with C_4-CoA being 100% and C_{10}-CoA, 0%. However, if the cells were grown on a lipid-rich medium (Tween 80), the chain length specificity of the oxidase shifted towards the higher-chain length substrates and the total activity increased by several fold. This indicates that the oxidase, and presumably the peroxisomal β-oxidation system, is inducible by fatty acids in the diet. The oxidase was found to have a low affinity for acyl-CoA's and a high affinity for oxygen. The nature of the activation

of lipid gluconeogenesis by lowered oxygenation, still remained an unexplained phenomenon. Assay of subcellular fractions indicated the oxidase to be a peroxisomal enzyme. β-oxidation was also shown to occur in the peroxisome. Part of this work was then presented at the 67th annual meeting of the American Society of Biological Chemists, San Fransisco, June 6-10, 1976 and published as an abstract (26).

At this same meeting, Lazarow (27) claimed that he was able to measure H_2O_2 production from palmitoyl-CoA in rat liver peroxisomes; however, neither here nor in later publications did he ever give any particulars of the assay. At about the same time, Lazarow and de Duve (28) published a paper in which they reported the presence of a fatty acyl-CoA oxidizing system (Cooper and Beevers' system) in the peroxisomes of rat liver. They also reported that the activity of this system is markedly enhanced (10 fold) by clofibrate (2-(p-chlorophenoxy)-2-methylpropionic acid ethyl ester), a hypolipidemic drug which causes peroxisomal proliferation (86). In a later publication, Lazarow (29) reported that in addition to clofibrate, tibric acid and Wy-14,643, all known hypolipidemic drugs, induced an 11-to-18 fold increase in the capacity of the livers of male rats, to oxidize palmitoyl-CoA. This was attributed to an enhancement of the rat liver peroxisomal β-oxidation activity.

Lazarow's last and most ambitious paper was published in

1978 (30). He reported the presence of crotonase, L-3-hydroxybutyryl-CoA dehydrogenase and thiolase in rat liver peroxisomes. These peroxisomes were relatively inactive in the oxidation of short-chain fatty acyl-CoA compounds, and appeared to be involved in the β-oxidation of long-chain fatty acids. It is of interest to note that even though he experimentally showed that a purified peroxisomal fraction could not oxidize butyryl-CoA, the substrates used in the assay of the above enzymes were all β-oxidation products of butyryl-CoA; crotonyl-CoA and acetoacetyl-CoA. Also, the oxidation of palmitoyl-CoA by this purified peroxisomal fraction gave a stoichiometry of 1:5:5 (C_{16}-CoA:C_2-CoA:NADH), implying that the peroxisome could not complete an oxidation that it initiates. Yet, Lazarow claimed that the bulk of palmitoyl-CoA oxidation in rat liver occurs in the peroxisome. And lastly two comments about this paper; first, contrary to repeated claims in previous papers (27,28) of having measured the fatty acyl-CoA oxidase activity, this enzyme was not mentioned in this paper, and second, all experiments were done with rats treated with clofibrate, without any comparisons of activities to normal rats.

Shortly after I was able to measure the enzymatic activity of the fatty acyl-CoA oxidase in Tetrahymena, I tried to measure this activity in rat liver also but without any success. In the spring of 1978 we found out that the chromogen

used in the original oxidase assay does not work in rat tissues. After replacing the original chromogen (o-dianisidine) with another one (p-hydroxybenzoic acid plus 4-aminoantipyrine) (31), I was finally able to measure the enzymatic activity of the fatty acyl-CoA oxidase in rat liver. Using the peroxisomal fraction from rat liver (de Duve's fraction L (32)), the fatty acyl-CoA oxidase and the β-oxidation system were characterized in terms of assay requirements and of chain length specificity of substrate. The oxidase, as well as the β-oxidation system showed a specificity for medium to long-chain length acyl-CoA's (C_{10}-C_{16}), with a well defined peak of activity at C_{12}. The specific activities were similar and, in each case, oleoyl-CoA was a better substrate than stearoyl-CoA. This indicated that the oxidase is the rate-limiting enzyme in the peroxisomal β-oxidation system and that this system has a preference towards medium to long-chain length mono-unsaturated fatty acyl-CoA's. The mitochondrial fatty acyl-CoA dehydrogenase was also studied and its activity showed a broader chain length range (C_4-C_{16}), with the shorter-chain length substrates being the more active and no preference being shown for oleolyl- over stearoyl-CoA. Based upon the total activities of the two initiating enzymes (the oxidase and the dehydrogenase), peroxisomal oxidation of palmitoyl-CoA accounted for at least 11% of the total C_{16}-CoA oxidation in the rat liver. Further studies on the peroxisomal oxidase showed that it is a membrane-bound enzyme and that it has a high affinity

for oxygen. Some of these results were published in April of 1979 (33).

In 1978, while I was extending my work to the rat liver oxidase, there began to be published a stream of papers on the peroxisomal β-oxidation system, one which still continues unabated today. By far the most prolific of the research groups is headed by T. Hashimoto in Japan. In 1978, Hashimoto's group was able to measure and characterize the peroxisomal β-oxidation system and the fatty acyl-CoA oxidase in rat liver (34,35,36). They showed that both activities are induced by di-(2-ethylhexyl)phthalate (DEHP), a widely used plasticizer which causes peroxisomal proliferation. Osumi and Hashimoto's (36) results on the rat liver fatty acyl-CoA oxidase differed from my results (33), specifically in terms of assay conditions and chain length specificity. Their results show the oxidase to have a broader chain length range (C_{10}-C_{22}), with a peak at C_{16}. However, it should be noted that their work was done with livers from rats treated with DEHP, while mine was done with normal rat livers. Osumi and Hashimoto (37) next reported the mitochondrial/peroxisomal distribution of β-oxidation enzymes; enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase, in rat liver and their induction by the administration of DEHP to rats. Unlike Lazarow, who used 4-carbon chain substrates (30) to study these enzymes, Osumi and Hashimoto used 4-, 8-, and 16-carbon chain length

substrates. All of the enzymatic activities were elevated by the DEHP treatment, however the extent of the increases were larger when longer-chain length substrates were used. Osumi and Hashimoto (37) suggested that different L-3-hydroxyacyl-CoA dehydrogenases and 3-ketoacyl-CoA thiolases are located in mitochondria and peroxisomes, with the peroxisomal enzymes being more active with longer-chain length substrates. This work was extended significantly when Osumi and Hashimoto (38) reported the occurrence of two L-3-hydroxyacyl-CoA dehydrogenases in rat liver, one peroxisomal and the other mitochondrial. These dehydrogenases differed in substrate specificity, stability, behavior in CM-cellulose chromatography, and antigenic properties. However, both isozymes were induced by the administration of DEHP to rats (38). Osumi and Hashimoto (39) were also able to show that the peroxisomal enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase are located in the same protein molecule. The purified multifunctional protein corresponded to a peroxisome-specific polypeptide with a molecular weight of 80,000 (39). Reddy and Kumar (40) had already demonstrated that a peroxisome-specific polypeptide with a molecular weight of 80,000 increased in the livers of rats when treated with various hypolipidemic drugs. Lastly, the presence of a new 3-ketoacyl-CoA thiolase in rat liver peroxisomes has been reported by Hashimoto's group (41). The novel peroxisomal 3-ketoacyl-CoA thiolase was distinguished from

the mitochondrial general 3-ketoacyl-CoA thiolase and the cytoplasmic acetoacetyl-CoA thiolase by the following criteria: DEAE-cellulose chromatography, phosphocellulose chromatography, immunochemical titrations, and substrate specificity (41).

Since the discovery of fatty acid oxidation by liver peroxisomes (28), some controversy has arisen about the relative importance of peroxisomes and mitochondria in overall fatty acid oxidation up to the point that the role of the mitochondria has been seriously questioned by Lazarow (30). Several groups of researchers have addressed themselves to this question and began to compare carefully the rates of fatty acid oxidation by both organelles. Surprisingly, Lazarow who questioned the role of the mitochondrion in liver fatty acid oxidation, has never compared both organelles in their abilities to oxidize fatty acids.

A preliminary report by Krahling et al. (42), estimates that the ratio of palmitoyl-CoA oxidation by the mitochondrial fraction relative to the peroxisomal fraction from livers of male rats is 3.2 (24% of the total oxidation by peroxisomes). In a later and more extensive report, Krahling et al. (43) reported that peroxisomal β-oxidation of palmitoyl-CoA accounted for at least 10% of the total β-oxidation activity in the young rat liver. However, the peroxisomal activity became 30% of the total in the livers of adult female and 20% in the adult male due to a decrease in mitochondrial β-oxidation after 2

weeks of age. These results seem to be in approximate agreement with my results (33) and in total disagreement with Lazarow's claim (30) of peroxisomal predominance in liver fatty acid oxidation.

Mannaerts et al. (44) studied mitochondrial and peroxisomal fatty acid oxidation in livers from control and clofibrate-treated rats. They found that clofibrate treatment increased mitochondrial and peroxisomal oxidation 2- and 6- to 8-fold, respectively. They also calculated that the contribution of the peroxisome to fatty acid oxidation was less than 10% both in livers from control and clofibrate-treated animals (44). The fact that clofibrate treatment seems to enhance mitochondrial β-oxidation has also been previously reported. Christiansen et al. (45) showed that clofibrate in the diet results in a significant stimulation of β-oxidation by the mitochondria, especially for the shorter-chain length fatty acids (below C_{14}).

These results point out that the mitochondrial β-oxidation system is also stimulated by clofibrate feeding, but not to the great extent that was observed for the peroxisomal β-oxidation system. On the other hand, it is not yet clear as to just how much of the total fatty acid oxidation that occurs in the liver is performed by the peroxisomal β-oxidation system, since the above results are not in good agreement.

My initial observations that the peroxisomal β-oxidation

system is more active towards mono-unsaturated fatty acids over the corresponding saturated fatty acids (33) has recently been confirmed in clofibrate-treated rats. Osmundsen et al. (46) suggested that in clofibrate-treated rats, peroxisomes play a significant role in the cellular oxidation of long-chain mono-unsaturated fatty acids. In these peroxisomes, the rates of oxidation of mono-unsaturated fatty acyl-CoA's were higher (10-fold) than for the corresponding saturated fatty acyl-CoA's (46).

Neat et al. (47) and Ishii et al. (48) showed that the feeding of high-fat diets to rats gave a 2- to 8-fold increase in the total liver peroxisomal β-oxidation activity. Ishii and Suga (49) have also shown that acetylsalicylic acid (aspirin) has clofibrate-like effects. Feeding of diets containing 1% (w/w) aspirin to rats caused a 4-fold enhancement of liver peroxisomal β-oxidation. Another remarkable effect noticed by Suga's group (48,49), was that both high-fat diet and aspirin feeding provoked a marked proliferation of hepatic peroxisomes and an increase in quantity of the peroxisomal-specific polypeptide (M. W. of 80,000). It is ofcourse noteworthy that the effects of high-fat diets and aspirin feeding resemble those of clofibrate. Recently Reddy et al. (50) have suggested that clofibrate and other hepatic peroxisomal proliferators are carcinogenic; they have in fact called these chemicals, a new type of carcinogenic agent. Thus one must ask the following questions: Can high-fat diets and the taking of

large amounts of aspirin lead to cancer of the liver? Is there an association between the induction of a hepatocarcinoma and the appearance of a peroxisomal-specific, 80,000 M. W. polypeptide?

Having described the initial and intense history of the peroxisomal β-oxidation system, and the small role that my thesis work played in it, I will now describe my research work in detail. This thesis is divided into two parts. Part I describes the work on the fatty acyl-CoA oxidase of Tetrahymena pyriformis, and part II deals with the peroxisomal and mitochondrial β-oxidation systems in rat liver.

PART I

THE FATTY ACYL-CoA OXIDASE OF *TETRAHYMENA PYRIFORMIS*: A NEW ENZYME FOR FATTY ACID OXIDATION.

INTRODUCTION

Under appropriate conditions, strains of the ciliated protozoan Tetrahymena pyriformis develop the capacity of rapidly converting lipids to glycogen (51,52). Hogg and Kornberg (53) reported that Tetrahymena cells capable of high rates of glycogen synthesis from lipids contained higher levels of the key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, than did cells with only limited or no gluconeogenic capacity. In addition, both enzymes were induced by the addition of acetate to a synthetic medium low in ketogenic amino acids. Furthermore they reported the association of the glyoxylate cycle enzymes in what they believed to be a novel type of mitochondrion, one biosynthetic in function. These findings suggested that the anaplerotic function necessary for gluconeogenesis from fats required not only that isocitrate lyase and malate synthase be present in large amounts but also that they be sequestered in a cytoplasmic organelle distinct from the mitochondrion. In later studies on the subcellular distribution of enzymes in Tetrahymena, Muller, Hogg and de Duve (54) separated from Tetrahymena homogenates on sucrose gradients a particulate fraction containing most of the L-a-hydroxy acid oxidase, catalase, isocitrate lyase, malate synthase, and approximately one-half of the NADP-linked isocitrate dehydrogenase. By analogy with liver pero-

xisomes the protozoal glyoxylate cycle granules were termed peroxisomes. The major impact of this work was that a peroxisome with a known function in gluconeogenesis had been found. The presence of the glyoxylate bypass enzymes in Tetrahymena peroxisomes suggests that these peroxisomes are roughly intermediate in enzymatic composition between mammalian peroxisomes which do not contain these enzymes and plant "glyoxysomes" which do contain the entire glyoxylate cycle. "Glyoxysomes" are peroxisomes found in germinating fatty seedlings. They play a necessary role in the conversion of stored lipids to carbohydrates during the germination of fatty seedlings (20). They contain not only all the enzymes of the glyoxylate cycle; citrate synthase, aconitase, isocitrate lyase, malate synthase and malate dehydroenase, but also urate oxidase, L-a-hydroxy acid oxidase and catalase (55).

Euglena is another acellular organism that readily utilizes acetate or lipids as sole carbon and energy source for growth by what is usually assumed to be the glyoxylate pathway. In this organism the glyoxylate cycle enzymes are localized in the glyoxysomes (microbodies) (56).

The term peroxisome was originally given to any microbody-like cytoplasmic organelle characterized by the association of H_2O_2-producing oxidases with H_2O_2-destroying catalase (19). However, when Breidenbach and Beevers (20) discovered that in germinating fat seedlings, the enzymes

of the glyoxylate cycle were specifically associated with an organelle that was distinct from the mitochondria, they hastily named this organelle "the glyoxysome". Only later, after careful studies did they find that these "glyoxysomes" also contained enzymes that characterize the peroxisome, namely; urate oxidase, L-a-hydroxy acid oxidase and catalase (55). Nevertheless, on the basis of the presumably greater significance of the glyoxylate cycle in the plant organellar function, Beevers has continued to insist on the term "glyoxysome" in preference to peroxisome.

In Tetrahymena, castor bean endosperm and Euglena, the evidence indicating that most acetyl units are consumed in the peroxisome in vivo, raised the question of how transport of acetyl-CoA from the mitochondrion, where it was supposedly generated, was brought about. That is, in making a case for the peroxisomes of these organisms to be the exclusive site of succinate formation from acetyl-CoA through the glyoxylate pathway, one major problem remained to be clarified. What is the source of acetyl-CoA for the glyoxylate bypass? Can the peroxisome generate acetyl-CoA or must it depend upon the mitochondrion? This problem was clearly solved when Cooper and Beevers (18) discovered that the generation of acetyl-CoA from the β-oxidation of fatty acids occurs in the peroxisome of castor bean endosperm. Addition of palmitoyl-CoA to peroxisomes resulted in O_2 uptake, NADH accumulation and acetyl-CoA

production, in the ratios, 0.5:1:1. KCN had no effect on NADH accumulation, but it doubled the rate of O_2 uptake (18). These findings were consistent with their suggestion that the first dehydrogenase in the peroxisomal β-oxidation cycle transfers its electrons directly to O_2, thereby producing H_2O_2, which is then broken down by the catalase present in the peroxisome.

Blum (24) and Graves and Becker (25) next reported the presence of fatty acyl-CoA synthetase, L-3-hydroxybutyryl-CoA dehydrogenase and thiolase in the peroxisomes from Tetrahymena and Euglena, respectively. This new evidence suggested that in Tetrahymena and Euglena, a peroxisomal β-oxidation pathway could be responsible for the formation of the acetyl-CoA which is needed for the glyoxylate pathway. However, the demonstration of the novel enzyme (the oxidase) as well as the operation of the complete β-oxidation cycle as shown for the peroxisomes of castor bean seedlings (18) remained elusive.

Using an improved method to assay H_2O_2-producing oxidases, the new enzyme in fatty acid oxidation, the fatty acyl-CoA oxidase, was assayed and characterized in Tetrahymena extracts. Some of the properties of this new enzyme were studied and its peroxisomal localization was determined. The new β-oxidation system also was measured as per Cooper and Beevers (18) and localized to the peroxisome.

MATERIALS AND METHODS

Growth of cells. Tetrahymena pyriformis, strain E, originally obtained from A. M. Elliot, was cultivated axenically as described by Hogg and Kornberg (53). The culture medium contained 1% (w/v) proteose-peptone (Difco), 0.1% glucose, 0.1% sodium acetate, 0.1% dipotassium phosphate, and 0.01% yeast extract (Difco); the pH was adjusted to 7.2 with HCl. Stock cultures were kept in 19-mm screw cap test tubes containing 10 ml of this medium. Mass cultures were grown in flat cylindrical flasks, 7 inches in diameter, provided with a side neck (Jobling No. 1420 culture flasks, Laboratory Glassblowers Company, Sands, High Wycomb, Bucks, England), containing 500 ml of medium. These flasks were inoculated with a 1% volume (5 ml) of a rapidly growing stock culture. All cultures were grown without shaking at 25° C for 4-7 days before harvesting. As it was shown by Wagner (51, 52), cultures at this time are in the stationary phase, glucose is exhausted, and the cells have a high glyconeogenetic capacity. The cells were harvested in 500 ml batches with a modified plankton centrifuge (57) and washed once with an equal volume of dilute Ringer phosphate solution (58). The sedimented cells were either taken up in a minimum volume of dilute Ringer phosphate solution and used to prepare lyophilized cells or they were washed once with cold 0.25 M man-

nitol solution, resuspended in 0.25 M mannitol and used for cell fractionation.

Extraction of cells. The cell suspension in dilute Ringer phosphate solution was adjusted to a density of 10% (v/v). Cell concentration was estimated by centrifuging at 1000 x g for 10 min in a Constable protein tube. The cell suspension was frozen rapidly in dry ice-acetone and dried at high vacuum in an all glass Kontes lyophilizer and stored at -20° C. Stable extracts were prepared by the addition of cold 50% (v/v) glycerol (1 ml/10 mg of dry powder) to the freeze-dried cell powder and grinding in a Brendler homogenizer (A. H. Thomas) for one minute at 1000 rpm. The homogenate was centrifuged at 30,000 x g for 20 min in a Sorvall RC-2 centrifuge with a SS-34 fixed-angle rotor. The clear extract obtained was used for all enzyme studies unless otherwise indicated. The presence of dried buffer salts (from the dilute Ringer phosphate solution) in the lyophilized cells is essential to an efficient extraction of enzyme activities.

Fractionation of cells. Cell suspensions in 0.25 M mannitol were adjusted to a density of 5% (v/v). The cell suspension was chilled in ice and then passed slowly through a pre-chilled fritted-glass filter (SF 113, pore size 20 to 30 microns, obtained from Laboratory Glassblowers Company) under light suction. The cell homogenates were fractionated by two different centrifugational methods.

Fractionation of the cell homogenate by differential centrifugation was performed as described by Hogg and Kornberg (53). The homogenate was centrifuged at 2000 x g for 10 min in a Sorvall RC-2 centrifuge with a SS-34 rotor. The supernatant liquid layer (S) was transferred to a new centrifuge tube and the original homogenate volume was restored by the addition of cold 0.25 M mannitol to the sediment. Gentle stirring with a spatula allowed the resuspension of a bulky, jelly-like layer of sediment (P1). The tightly-packed pellet remaining (P_n) was resuspended in mannitol by vigorous stirring. All fractions were centrifuged again at 2000 x g for 10 min, with the resultant separation of a small amount of P1 fraction from S, and of P_n fraction from P1. Usually, the S fraction (cytosol and microsomes) was diluted to the original homogenate volume, while the P1 fraction (mostly peroxisomes) and the P_n fraction (mostly mitochondria) were resuspended in 0.25 M mannitol to 1/4 of the original homogenate volume.

Fractionation of cell homogenates by discontinuous sucrose density-gradient centrifugation was performed as follows: A discontinuous sucrose density-gradient was prepared by pipetting into a 38 ml centrifuge tube (polyallomer, 1" x 3½") the following sucrose solutions; 7.0 ml of 60% (w/w), 4.0 ml each of 50%, 48%, 46%, 44%, 42%, and 40% (w/w) sucrose. The discontinuous sucrose density-gradient was allowed to stand at 4° C for 5 hours prior to the loading of the homo-

genate. The homogenate (6.0 ml) was pipetted on top of the sucrose gradient and centrifuged at 26,000 rpm (120,000 x g) for 2 hours in a swinging bucket rotor (Beckman SW 27) using the Beckman model L2-50 ultracentrifuge. After the centrifugation, the bottom of the centrifuge tube was pierced (Fractionator, model No. FS 101, Hoefer Scientific Instruments, San Francisco, Ca.) and 9 fractions were collected drop-wise; the first fraction was 5 ml in volume and the next eight fractions were 4 ml each. In this way each fraction collected, except the first (bottom) and last (top), represented a density interface.

Enzyme assays. The fatty acyl-CoA oxidase activity was assayed by measuring the acyl-CoA dependent H_2O_2 production, using a spectrophotometric modification of the Hugget and Nixon method (23) for determining blood glucose with glucose oxidase. The oxidase was assayed at 30° C in 100 mM potassium phosphate buffer, pH 7.6, containing 10 U/ml horseradish peroxidase, 378 μM o-dianisidine.2HCl, 50 μM FAD, and the enzyme preparation. The reaction was started by adding butyryl-CoA (unless otherwise indicated) to a final concentration of 100 μM. The increase in absorbance at 436 nm was monitored in a Gilford 240 recording spectrophotometer. The millimolar extinction coefficient of the chromogen was experimentally calculated, with a standard solution of H_2O_2, to be 7.9 cm^2/ μ mole at pH 7.6.

The peroxisomal β-oxidation assay used was a slight modification of the method described by Cooper and Beevers (18) which measures the acyl-CoA-dependent NADH production. The assay was performed at 30° C in 100 mM potassium phosphate buffer, pH 7.6, containing 200 μM CoA, 200 μM 3-acetylpyridine-NAD, 50 μM FAD, and the enzyme preparation. The reaction was started by adding butyryl-CoA to a final concentration of 100 μM. The increase in absorbance at 365 nm was monitored. An extinction coefficient for 3-acetylpyridine-NADH of 9.1 $cm^2/\mu mole$ was used. Whenever the above two enzyme assays were performed on particulate enzyme preparations, Triton X-100 was added to the assay mixture at a final concentration of 0.1% (v/v).

Catalase was assayed by the initial rate of decomposition of 12.5 mM H_2O_2 as measured by the decrease in absorbancy at 240 nm (59). The recorder was set at 1.0 absorbance per full scale and a chart speed of 1 inch per minute. To the cuvette was added 2.9 ml of a solution containing 66 mM potassium phosphate buffer at pH 7.0 and 12.5 mM H_2O_2 (solution prepared by adding 0.16 ml of a 31% (v/v) H_2O_2 solution to 100 ml of buffer; absorbance of the H_2O_2 solution *vs* the buffer should be 0.50 ± 0.01), and 0.1 ml of a 2% (v/v) Triton X-100 solution. The reaction was initiated by adding 5-50 microliter of enzyme preparation. The decrease in absorbance for the first 30 sec was used to calculate ΔA/min. An extinction coefficient for H_2O_2 of 0.040 $cm^2/\mu mole$ was used.

The succinate dehydrogenase assay used was a slight modification of the method described by Muller, Hogg and de Duve (54). The dehydrogenase was assayed at 30° C in 66 mM sodium-potassium phosphate buffer, pH 7.4, containing 66 μM 2,6-dichlorophenolindophenol (DCPIP), 1.0 mM phenazine methosulfate (PMS), 2.0 mM KCN, 0.067% (v/v) Triton X-100, and the enzyme preparation. The reaction was started by adding sodium succinate to a final concentration of 20 mM. The decrease in absorbance at 600 nm was monitored. An extinction coefficient for DCPIP of 19.1 $cm^2/\mu mole$ was used (60). A similar method was used in the assay of fatty acyl-CoA dehydrogenase activity, except that 50 μM FAD and 200 μg/ml of crystalline bovine serum albumin (BSA) were also included in the assay mixture and the reaction started by the addition of lauroyl-CoA to a final concentration of 66 μM.

Experiments on the effect of changing the oxygen concentration on the rate of the oxidase reaction were carried out by first incubating on ice, in a 100 ml flask, the oxidase assay mixture (minus enzyme, plus substrate) while flushing this mixture with the desired oxygen-nitrogen mixture for 20 min. A 3.0 ml aliquot of the equilibrated reaction mixture was then removed and placed in a Thunberg cuvette that contained the enzyme (glycerol extract) in the side arm. The cuvette was then flushed with the desired O_2/N_2 mixture by repeated evacuation and refilling. The cuvette was sealed

after the last flushing and the reaction initiated by tipping the enzyme extract from the side arm into the reaction mixture.

Protein concentration was determined by the method of Lowry *et al.* (61) after the protein had been precipitated with 0.5 N perchloric acid and then washed with 0.5 N perchloric acid followed by absolute ethanol. Crystallized and lyophilized BSA (Sigma) was used as the standard.

Materials. Horseradish peroxidase (E. C. 1.11.1.7), HPOD (1000 I.U./mg) and HPOFF (3000 I.U./mg), was purchased from Worthington Biochemical Corporation, Freehold, New Jersey. Horseradish peroxidase, Type II, FAD, FMN, DCPIP, PMS, CoA, p-hydroxybenzoic acid, 4-aminoantipyrine, 3-acetylpyridine-NAD, Triton X-100, and Tween 80 (polyoxyethylene sorbitan mono-oleate) were purchased from Sigma Chemical Co., St. Louis, Mo. The several acyl-CoA compounds and DL-butyrylcarnitine.chloride were purchased from P-L Biochemicals, Milwaukee, Wis. All other chemicals were of analytical reagent grade.

RESULTS

Characterization of the fatty acyl-CoA oxidase activity. A continuous spectrophotometric assay method for measuring the activity of H_2O_2-producing oxidases was developed from the original colorimetric method of Hugget and Nixon (23) for determining blood glucose. The assay involves the continuous spectrophotometric measurement of the reaction product, H_2O_2, by coupling it to the enzymatic oxidation of a chromogen, o-dianisidine, by horseradish peroxidase. This assay method was used to detect and to measure the activity of the fatty acyl-CoA oxidase in Tetrahymena pyriformis.

Table 1 shows that the fatty acyl-CoA oxidase activity is dependent upon enzyme source, butyryl-CoA, and FAD. The oxidase assay is also dependent on peroxidase and the chromogen. It is clear that the oxidase from the glycerol extracts displayed little activity without added FAD since a 5-fold increase in activity upon addition of FAD was observed. FMN could not replace the FAD. When the oxidase activity was assayed in a particulate preparation (the P1 fraction), the omission of FAD caused only a 40-50% loss in activity (data not shown). These data suggest that the oxidase is a flavoprotein with the prosthetic group, FAD, weakly bound to the apoenzyme. It was also observed that better

TABLE 1. Characterization of the fatty acyl-CoA oxidase assay.

Complete assay[a]	100% (41.4 nmoles/min/mg protein)
- enzyme source	0
- C_4-CoA	0
- peroxidase	0
+ 2X peroxidase	101
- chromogen[b]	0
+ 2X chromogen	95
- FAD	18
+ 2X FAD	107
- FAD, + 75 μM FMN	18
- C_4-CoA, + 200 μM DL-C_4-carnitine.Cl	0
- C_4-CoA, + 0.15-1.5 mM ammonium butyrate	0

a- for assay conditions see Materials and Methods.
b- 378 μM o-dianisidine.2HCl.

rates were obtained when the FAD was preincubated with the enzyme prior to the addition of the substrate. This indicated the formation of an apoenzyme-substrate complex, which hindered the binding of FAD to the apoenzyme. The addition of BSA (200 µg/ml) to the assay mixture did not have any effect on the oxidase rate with butyryl-CoA as the substrate. From table 1 it can be seen that DL-butyrylcarnitine.Cl and ammonium butyrate cannot serve as substrates for the oxidase; the CoA thioester is absolutely required.

The assay method depends on the coupling of the H_2O_2 produced by the oxidase reaction to the oxidation of o-dianisidine by horseradish peroxidase. The presence of catalase, in varying amounts in cell extracts and peroxisomal fractions, can interfere with this reaction. The catalase interference can be eliminated by the addition of sodium azide, a catalase inhibitor, to the assay mixture (62). However, sodium azide is a very powerful respiratory inhibitor and it may well have some unknown inhibitory effects on the oxidase assay. A better method of eliminating the catalase interference was found by increasing the amount of peroxidase in the assay mixture. Table 2 shows the effect of catalase on the measurement of a constant amount (80 n-moles) of H_2O_2 by the peroxidase-chromogen assay. As seen in table 2, catalase begins to interfere (20% inhibition) in the assay when the ratio of activities of catalase to

TABLE 2. The effect of catalase on the measurement of H_2O_2 by the peroxidase-chromogen assay system.

Complete assay[a]	100% (80 nmoles of oxidized chromogen)
+ Catalase :	
1.0 U/ml	99
2.5 U/ml	98
5.0 U/ml	94
10 U/ml	95
50 U/ml	80
100 U/ml	67
500 U/ml	23
1000 U/ml	12
5000 U/ml	0
1000 U/ml, + 100 U/ml peroxidase	100

a- 50 mM potassium phosphate buffer, pH 7.0, containing 1 U/ml peroxidase and 378 μM o-dianisidine.2HCl. The reaction was started by adding H_2O_2 to a final concentration of 0.08 mM. The total change in absorption at 436 nm was measured. The final volume of the assay mixture was 1.0 ml.

peroxidase is brought to 50 or greater. However, this interference was totally eliminated when the amount of peroxidase used was increased to produce a catalase/peroxidase ratio of 10 or less. Having studied the nature of the catalase interference and its reversal in a model system, the effect of catalase inhibitors and catalase, on the actual oxidase assay was then investigated (table 3). Adding sodium azide and 3-amino 1,2,4-triazole at levels sufficient to inhibit the catalase present in the glycerol extracts (100% and 85% inhibition, respectively) does not increase the oxidase rate; if anything, there was a slight inhibition of the rate. Catalase added to the reaction mixture again begins to inhibit the oxidase reaction rate (20% inhibition) when the catalase/peroxidase activities ratio is 50 or greater; the same ratio observed in the model system (see table 2). By increasing the peroxidase content of the assay mixture from the normal 10 U/ml to 500 U/ml, the inhibitory effect of catalase on the oxidase rate was eliminated completely (table 3). The catalase content of Tetrahymena cell extracts and particulate preparations was never higher than 10-20 U/ml. Therefore a peroxidase content of 10 U/ml was routinely used in all the oxidase assays performed.

Very late in the progress of this work, the chromogen, o-dianisidine, was reported to be a possible carcinogenic agent. Therefore, a p-hydroxybenzoic acid-4-aminoanti-

TABLE 3. The effect of catalase inhibitors and catalase on the oxidase assay.

	Peroxidase concentration	
	10 U/ml	500 U/ml
Complete assay[a]	100% (42.9 nmoles/min/mg protein)	100% (42.7)
+ 30 mM 3-amino 1,2,4-triazole[b]	90	--
+ 1 mM sodium azide[c]	93	--
+ catalase:		
10 U/ml	98	--
100 U/ml	95	--
500 U/ml	81	95
1000 U/ml	76	99
5000 U/ml	52	104

a- for assay conditions see Materials and Methods; the endogenous catalase content was 6 U.
b- the endogenous catalase was inhibited 85%.
c- the endogenous catalase was inhibited 100%.

pyrine mixture, which is known to be another chromogenic substrate for horseradish peroxidase (31), was tried and compared to o-dianisidine. Table 4 shows that, if o-dianisidine (chromogen A) is replaced in the oxidase assay by the p-hydroxybenzoic acid plus 4-aminoantipyrine mixture (chromogen B) at the concentrations used for the rat liver oxidase assays (33), chromogen B had 73% of the effectiveness of chromogen A. If the concentration of chromogen B was cut in half, it was almost as effective as chromogen A. Apparently chromogen B is a better substrate for horseradish peroxidase than chromogen A, since it inhibits the oxidation of A (table 4) whereas the converse is not true. However, chromogen A is a more sensitive chromogen due to its higher extinction coefficient. In this work, o-dianisidine continued to be the chromogen of choice due to its higher sensitivity. But it was handled with care, due to its probable carcinogenic properties. No doubt that by the time this thesis is published, chromogen B also will be classified as a carcinogen by the FDA.

The effect of protein concentration and pH on the rate of the oxidase reaction.

The fatty acyl-CoA oxidase reaction rate was proportional to the amount of protein added (glycerol extract) in the range of 0-100 µg/ml (figure 1). At higher amounts of protein, some inhibition was observed. This was probably due

TABLE 4. The use of p-hydroxybenzoic acid plus 4-amino-antipyrine as a chromogen in the oxidase assay.

	Chromogen A[a]	Chromogen B[b]
Complete assay	100% (84.4 nmoles/min/mg protein)	100% (61.2)
+ chromogen B	15	--
+ chromogen A	--	90
½ chromogen B	--	124

ratios of chromogens B/A = 73% ; ½B/A = 90%

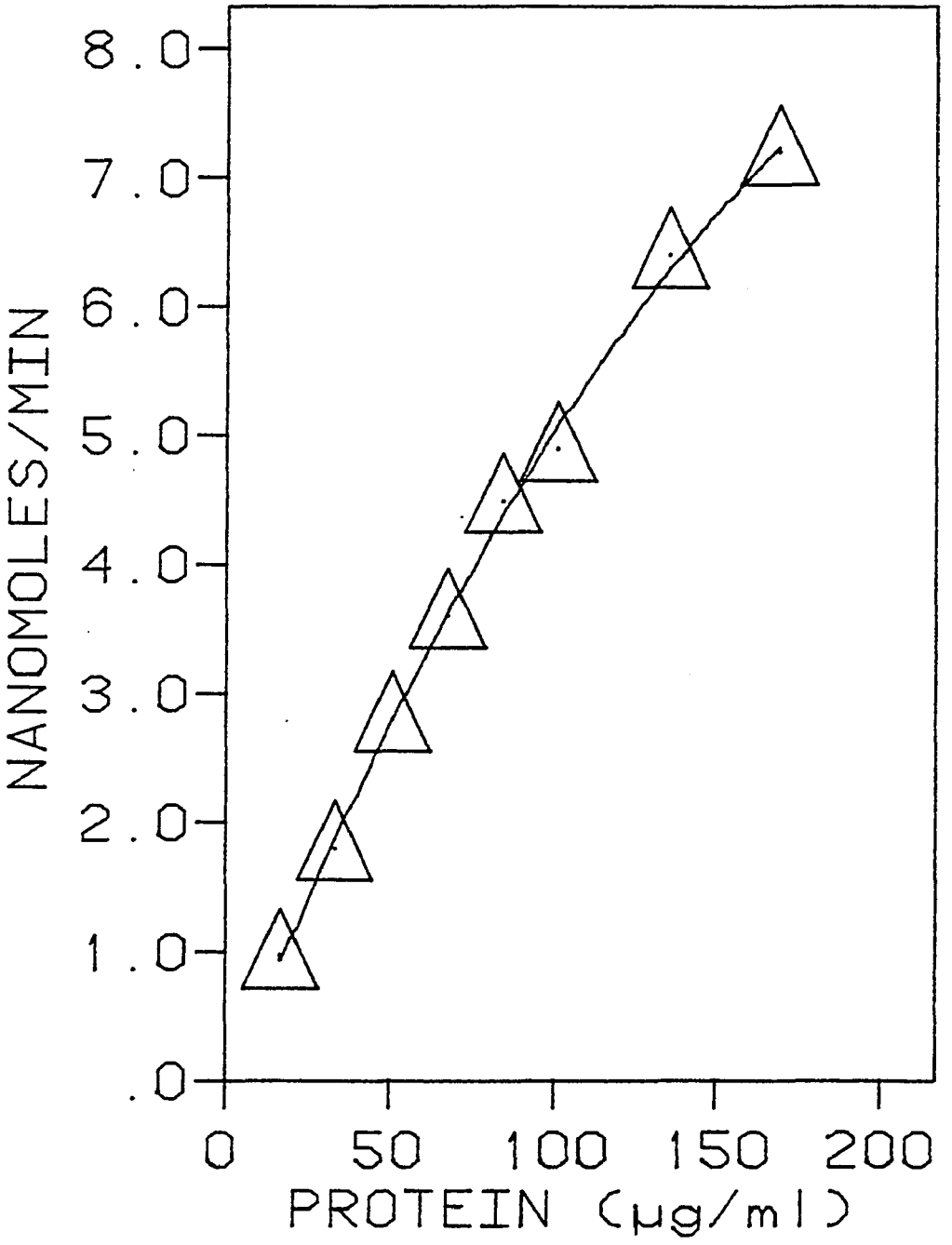
---

a- 378 μM o-dianisidine.2HCl; assay measured at 436 nm using an extinction coefficient of 7.9 $cm^2/\mu mole$.

b- 25 mM p-hydroxybenzoic acid plus 1.0 mM 4-amino-antipyrine; assay measured at 500 nm using an extinction coefficient of 5.1 $cm^2/\mu mole$.

FIGURE 1. Effect of enzyme protein concentration on the rate of the oxidase reaction. Assay conditions were as described in Materials and Methods. The glycerol extract (Δ) was used as the enzyme source and 100 μM butyryl-CoA as the substrate.

FIGURE 1.


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
.0
NANOMOLES/MIN
0
50
100
150
200
PROTEIN (µg/ml)

to the increasing concentration of glycerol (from the extract) in the reaction mixture.

The oxidase has a very sharp pH profile, with a pH maximum of 8.0-9.0 in phosphate buffer (figure 2). Tris-buffer inhibited the oxidase reaction.

Stoichiometry of the oxidase reaction. The stoichiometry of the fatty acyl-CoA oxidase reaction as well as for the peroxisomal β-oxidation system was determined using butyryl-CoA as the substrate. Table 5 shows that glycerol extracts oxidized butyryl-CoA with the formation of H_2O_2 and NADH (measured as 3-acetylpyridine-NADH), in a 1:1:1 stoichiometry. Presumably the oxidase catalyzes the oxidation of acyl-CoA substrates to their corresponding trans-$\Delta^{2,3}$-enoyl-CoA derivatives and H_2O_2 as evidenced by the ability of the oxidation products (trans-$\Delta^{2,3}$-enoyl-CoA's) to serve as substrates for the enoyl-CoA hydratase and for L-3-hydroxyacyl-CoA dehydrogenase. The reduction of one mole of oxygen and one mole of 3-acetylpyridine-NAD per mole of butyryl-CoA was observed (table 5).

Stability of the oxidase. The fatty acyl-CoA oxidase from Tetrahymena is a very unstable enzyme. Aqueous glycerol (50% v/v), a "cosmostatic agent", was the only solvent into which the oxidase could be extracted with any reasonable assurance of stability. Table 6 shows that extracts of dried cells prepared with 0.25 M mannitol, 0.05 M phosphate

FIGURE 2. Effect of pH on the rate of the oxidase reaction. The activity was assayed as described in Materials and Methods, except that the pH of the 100 mM potassium phosphate buffer (Δ) was varied as indicated. The glycerol extract was used as the enzyme source and 100 μM butyryl-CoA as the substrate.

FIGURE 2.

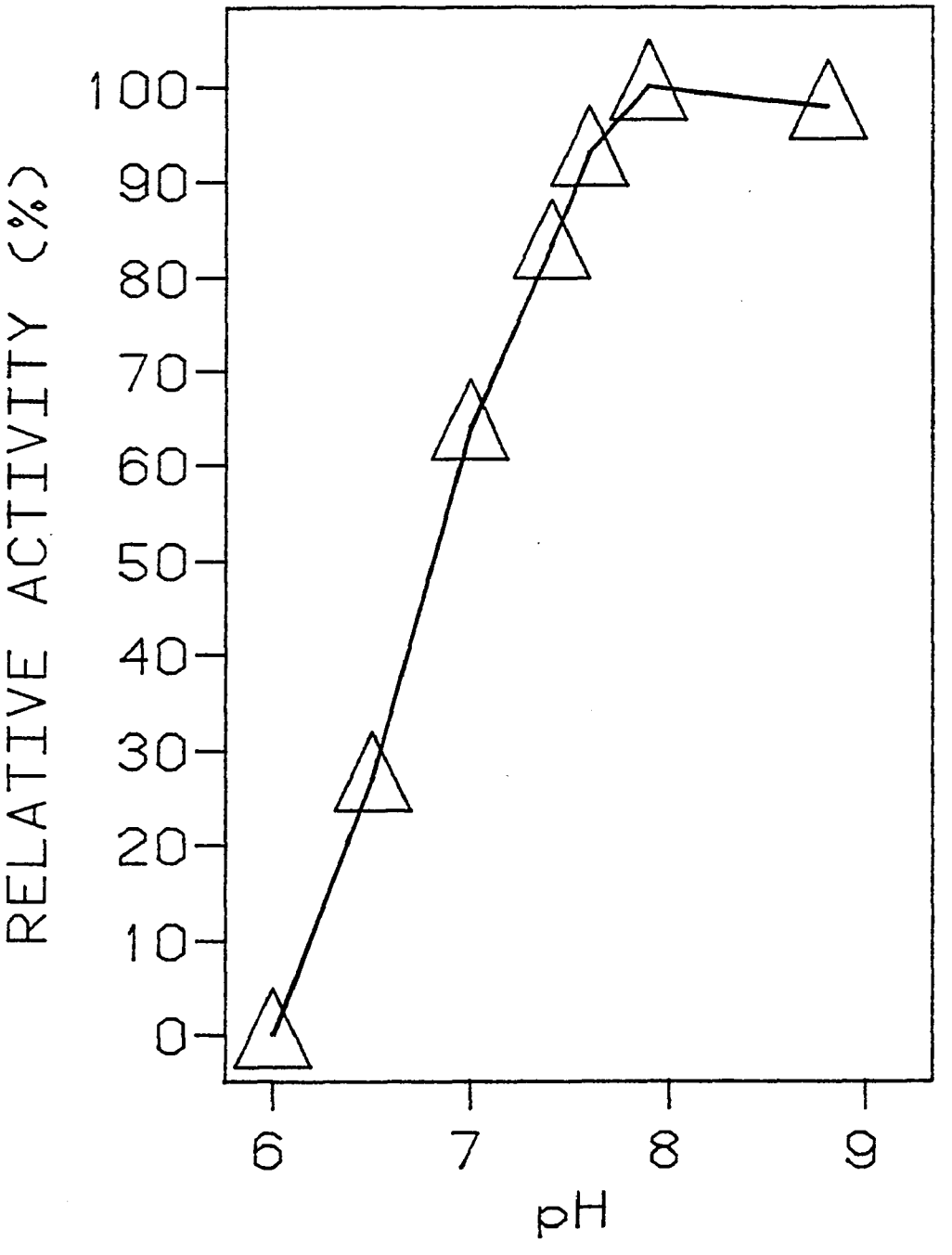


TABLE 5. Stoichiometry for the oxidase and for β-oxidation.

Substrate	Oxidase action[a] (nmoles of H_2O_2 formed)	β-oxidation[a] (nmoles of 3-acetylpyridine-NADH formed)
Butyryl-CoA added:		
10 nmoles	11.1	10.1
20 nmoles	22.1	17.8

a- for assay conditions see Materials and Methods.

TABLE 6. Stability of the oxidase at 0° C in different solvents[a].

Time (hrs)	50% (v/v) glycerol	0.25M D-mannitol	0.05M phosphate buffer, pH 7.4	distilled water
0	100% (42.5 nmoles/ min/mg protein)	100% (37.4)	100% (22.1)	100% (35.3)
24	100	40	25	26
48	90	17	11	10
72	87	10	0	6
96	76	8	0	0

a- the extracts were prepared from lyophilized cells by homogenizing 10 mg of dry cells per ml of solvent in a Brendler homogenizer and centrifuging at 30,000Xg for 20 minutes. As described in Materials and Methods, the cells were lyophilized as a 10% (v/v) suspension in dilute Ringer phosphate buffer. Therefore, the extracting solvent in each case will contain the original buffer salts that are essential to the solubilization of the enzyme.

buffer, pH 7.4, or distilled water as solvent, lost their enzymatic activity (oxidase) within 48 hours. The oxidase extracted into 50% (v/v) glycerol lost only 24% of the original activity after 4 days at 0° C, but if the extract is kept at -20° C, the activity is stable indefinitely. Significant purification of the oxidase was precluded by the viscocity of the glycerol extracts. However, the glycerol extraction afforded some purification of the oxidase (2-4 fold) over other solvents and the homogenate.

Kinetic properties of the oxidase. The effect of changing the acyl-CoA concentration on the oxidase reaction velocity is shown in figure 3. Of interest is the fact that there is no substrate inhibition shown by any substrate as the chain length of the substrate was increased from butyryl-CoA, to octanoyl-CoA; not even at high concentrations and in the absence of BSA. In figure 4 the initial velocities versus concentrations were plotted on reciprocal coordinates for the several substrates. The kinetic parameters (derived from figure 4) for several acyl-CoA substrates of different chain lengths are listed in table 7. K_m values were the same (120 μM) for the several substrates, regardless of their chain length. The highest maximal velocity was observed with butyryl-CoA as the substrate while longer chain length substrates gave progressively decreasing values of Vmax. Activity was undetectable for chain length greater

FIGURE 3. Effect of acyl-CoA concentration and chain length on the rate of the oxidase reaction. The assay conditions were as described in Materials and Methods, except that butyryl-CoA (◇), hexanoyl-CoA (△), and octanoyl-CoA (□) were used as substrates at the indicated concentrations. The glycerol extract was used as the enzyme source.

FIGURE 3.

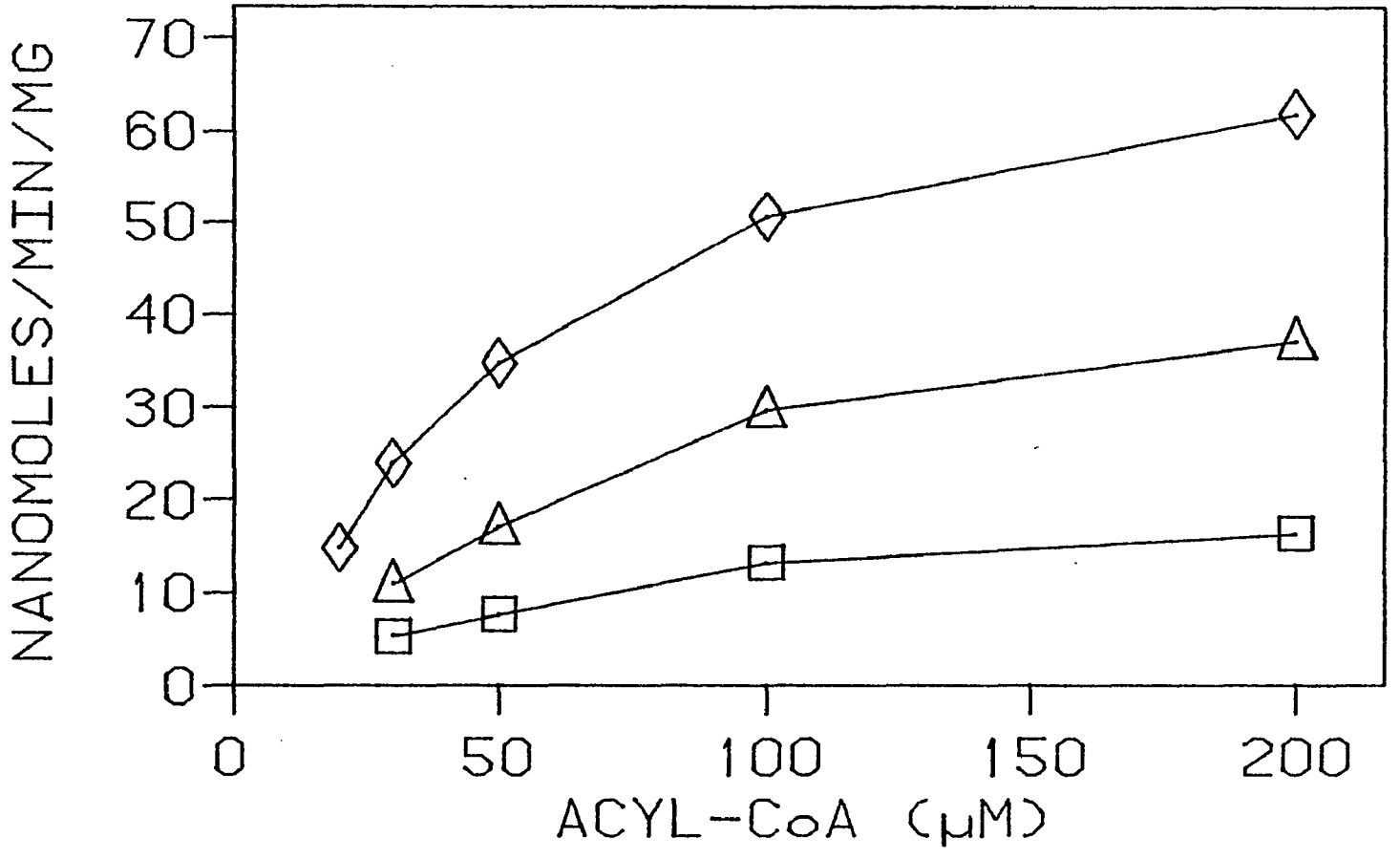

NANOMOLES/MIN/MG
70
60
50
40
30
20
10
0
0
50
100
150
200
ACYL-CoA (μM)

FIGURE 4. Double reciprocal plot of the oxidase activity versus acyl-CoA concentrations. These are the Lineweaver-Burke plots of the data presented in figure 3. Substrates used were: butyryl-CoA (◇), hexanoyl-CoA (△), and octanoyl-CoA (□).

FIGURE 4.

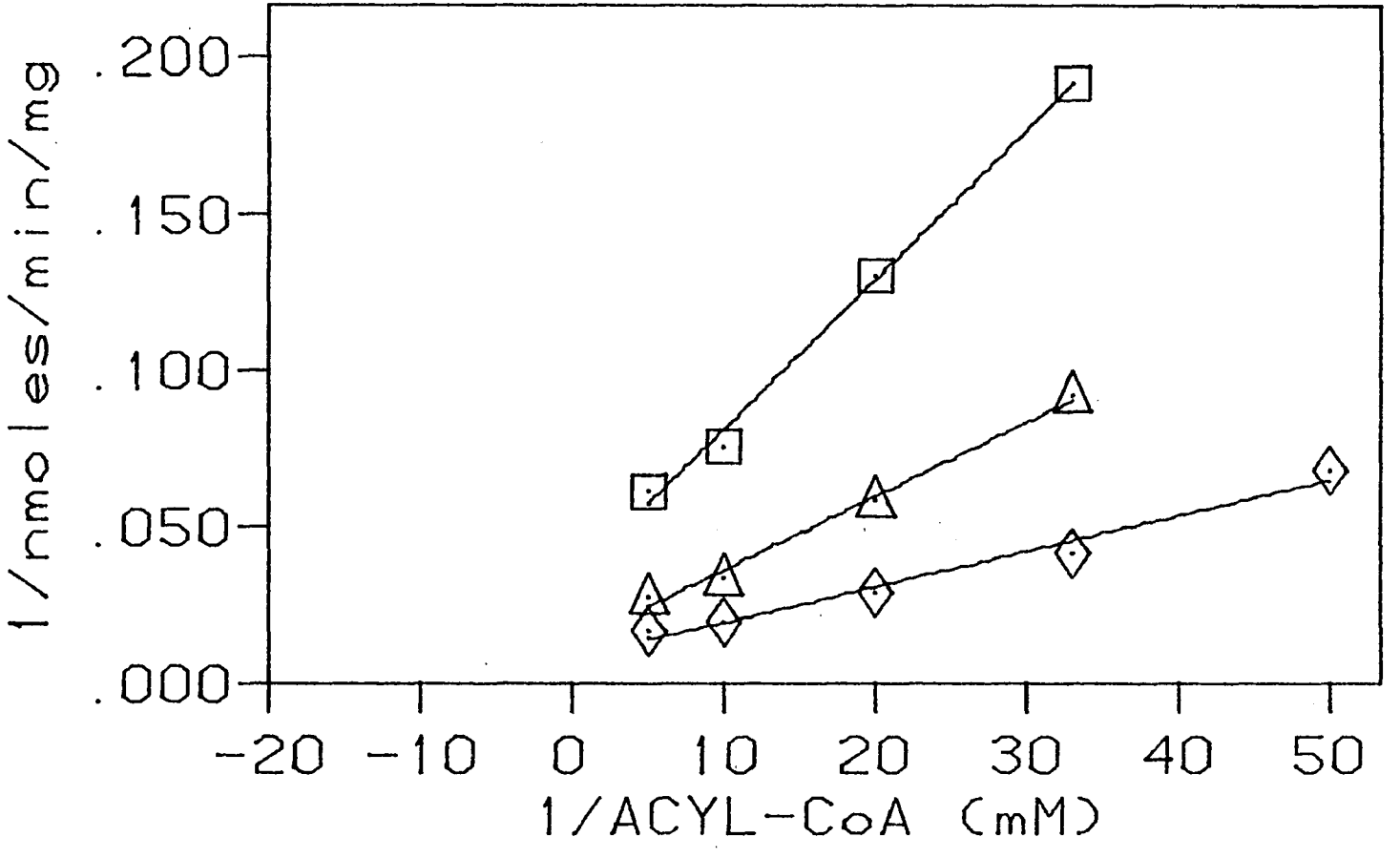

1/nmoles/min/mg
.200
.150
.100
.050
.000
-20
-10
0
10
20
30
40
50
1/ACYL-CoA (mM)

TABLE 7. Kinetic parameters of the oxidase[a].

Substrate	Km	Vmax	Relative Vmax
C_4-CoA	120 µM	110 mU/mg	100
C_6-CoA	120 µM	59 mU/mg	54
C_8-CoA	120 µM	26 mU/mg	24

a- these parameters were derived from figure 4.

than octanoyl-CoA.

The effect of oxygen on the rate of the oxidase reaction. The effect of changing the concentration of the other substrate, oxygen, on the reaction rate of the oxidase is shown in table 8. Lowering the oxygen content of the gas phase from 100% (v/v) to either 2.3% or 1.0%, decreases the oxidase activity by only 20 to 22%, respectively. This suggests that the fatty acyl-CoA oxidase has a high affinity for oxygen. It was not possible to completely eliminate oxygen from the reaction mixture, because this would have involved the use of urate and urate oxidase in the reaction mixture to remove residual oxygen--H_2O_2 is the byproduct of this reaction.

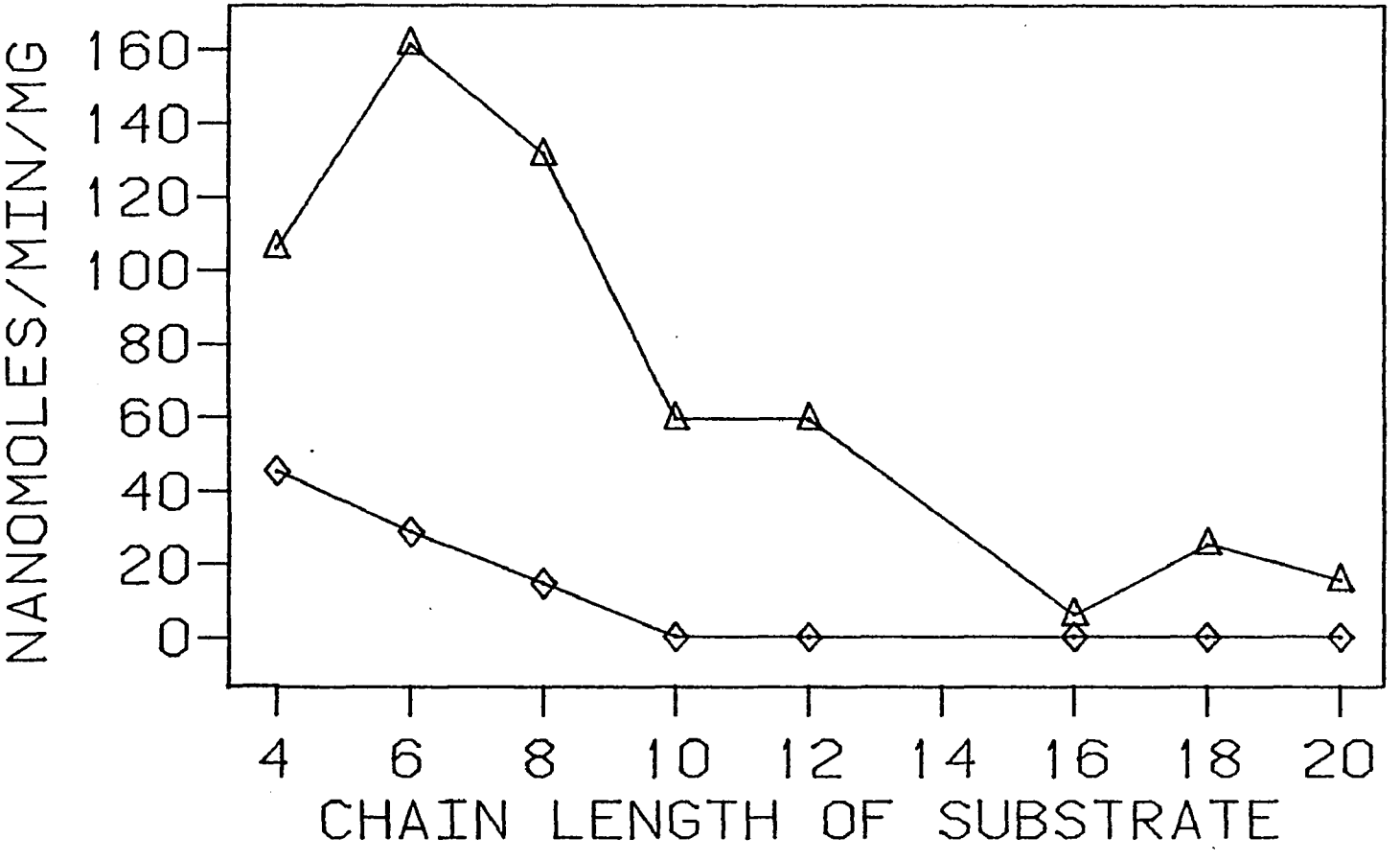
The chain length specificity of the oxidase: effect of Tween 80 in the growth medium. In the prior study of chain length specificity of the fatty acyl-CoA oxidase, the enzyme extracted from the cells of stationary phase cultures grown on the PPGA medium exhibited greatest activity on butyryl-CoA, with progressively lower activity declining to undetectable on substrates of chain length exceeding C_8 (table 7; figures 3, 4, and 5). However, when 1% (w/v) Tween 80 replaced acetate in the culture medium (63), a marked change in specific activities and in chain length specificity was found. The oxidase activity extracted from cells grown on the oleate-rich medium (PPGT) shows a broad

TABLE 8. The effect of changing the oxygen content in the gas phase on the rate of the oxidase reaction.

Oxygen in gas phase (O_2/N_2 mixtures)	Relative activity	
	Exp. A	Exp. B
100%	100%	100%
21% (air)	98	97
2.3%	80	--
1.0%	--	78

FIGURE 5. Chain length specificity of the fatty acyl-CoA oxidase from proteose-peptone-glucose-acetate (PPGA) (◇) and proteose-peptone-glucose-Tween-80 (PPGT) (Δ) grown Tetrahymena. The PPGT medium was identical to the standard PPGA media, except 1% (w/v) Tween-80 replaced the acetate (63). Cells inoculated in PPGT medium were grown, harvested, and lyophilized as indicated under Materials and Methods for PPGA cells. The oxidase activity was assayed as described in Materials and Methods using glycerol extracts as the enzyme source and 100 μM saturated acyl-CoA's of the indicated chain length as substrates. All assays using extracts prepared from PPGT grown cells were done in the presence of 200 μg/ml BSA.

FIGURE 5.


NANOMOLES/MIN/MG
160
140
120
100
80
60
40
20
0
4
6
8
10
12
14
16
18
20
CHAIN LENGTH OF SUBSTRATE

chain length specificity (C_4-C_{20}) (figure 5). The activity was highest towards the C_4-C_{12} substrates. In comparison to the oxidase from PPGA grown cells, the PPGT-oxidase showed a shift of specificity towards higher chain length of substrate and also the rates for C_4-, C_6-, and C_8-CoA increased by 3-, 6-, and 12-fold, respectively. The PPGT-oxidase was assayed in the presence of 200 μg/ml BSA, otherwise there was substrate inhibition. Surprisingly, this oxidase showed no preference for oleoyl-CoA over stearoyl-CoA (data not shown).

Subcellular distribution of the fatty acyl-CoA oxidase. Fractionation of cellular homogenates by differential centrifugation was carried out by a procedure described by Hogg and Kornberg (53). Catalase was used as the peroxisomal marker enzyme, while succinate dehydrogenase was the mitochondrial marker. As shown in table 9, the distribution of the fatty acyl-CoA oxidase activity was similar to that of catalase, with the highest activity of each being found in the peroxisomal fraction (P1). On the other hand, the pattern of distribution of the mitochondrial fatty acyl-CoA dehydrogenase was similar to that of succinate dehydrogenase. The specific activity distribution of the butyryl-CoA-dependent NAD^+ reduction (Cooper and Beevers' (18) peroxisomal β-oxidation assay) was similar to that of the oxidase and catalase, with the highest activity being in the pero-

TABLE 9. Subcellular fractionation of Tetrahymena homogenates by differential centrifugation.

	Cell fraction		
	Pn (mitochondrial)	P1 (peroxisomal)	S (soluble and microsomal)
Enzyme	nmoles/min/mg protein		
Catalase	180,000	360,000	47,000
Fatty acyl-CoA oxidase	35.8	54.9	9.5
β-oxidation	5.6	7.5	0.0
Succinate dehydrogenase	116	87.5	0.0
Fatty acyl-CoA dehydrogenase	31.0	23.0	0.0
Protein (% of total)	12%	28%	60%

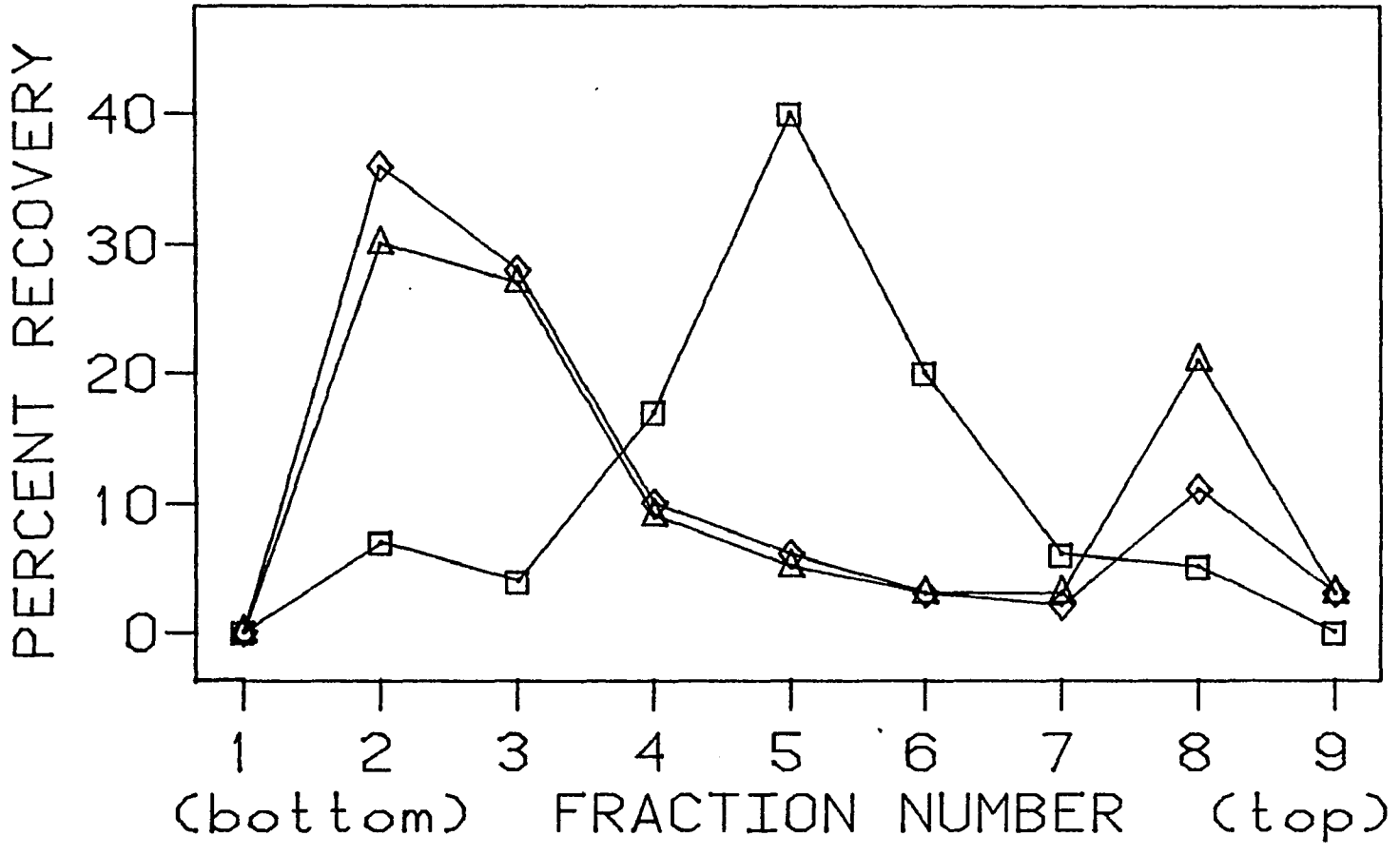
xisomal fraction (P1).

The results of the discontinuous sucrose density-gradient centrifugation of Tetrahymena homogenates are shown in figure 6. From the distribution patterns of catalase and succinate dehydrogenase, it was obvious that peroxisomes and mitochondria were concentrated in fractions 2-3 and 5, respectively. The oxidase showed a distribution pattern identical to that of catalase.

The results presented in table 9 and figure 6 show conclusively that the fatty acyl-CoA oxidase is sharply localized in the peroxisomes and that a very significant proportion of β-oxidation of fatty acyl-CoA compounds occurs in the peroxisome.

FIGURE 6. Subcellular fractionation of Tetrahymena homogenates by discontinuous sucrose density-gradient centrifugation. Cells were homogenized and fractionated as described in Materials and Methods. Enzymatic assays were carried out as described in Materials and Methods and their activities were recovered 100% ± 10, with respect to the original homogenate. Figure shows the distribution of the mitochondrial marker enzyme, succinate dehydrogenase (□); the peroxisomal marker enzyme, catalase (△); and the fatty acyl-CoA oxidase (◇), using butyryl-CoA as the substrate.

FIGURE 6.


PERCENT RECOVERY
40
30
20
10
0
1
2
3
4
5
6
7
8
9
(bottom) FRACTION NUMBER (top)

DISCUSSION

This thesis reports the presence of a new enzyme for fatty acid oxidation, the fatty acyl-CoA oxidase, in Tetrahymena pyriformis. The oxidase activity (a fatty acyl-CoA-dependent H_2O_2 producing activity) was assayed in Tetrahymena by using an improved method to assay oxidases. The oxidase assay involves the continuous spectrophotometric measurement of the reaction product, H_2O_2, by coupling it to the oxidation of o-dianisidine by horseradish peroxidase. Catalase interference was overcome by using high amounts of peroxidase; there was no catalase inhibition of the oxidase activity as long as the catalase/peroxidase ratio of activities was lower than 10. The advantages of this peroxidase-chromogen method for oxidase activity determination are clear. By using it, it is possible to measure the enzyme activity in tissue homogenates and peroxisomal preparations, irrespectiveof the levels of catalase also present in them. This assay method also permits the measurement of the activities of a variety of H_2O_2-producing oxidases by simply changing the buffer and substrate.

The fatty acyl-CoA oxidase as well as the fatty acyl-CoA-dependent NAD+ reducing activity were found to be localized in the peroxisomes of Tetrahymena (table 9 and figure 6). The pronounced stimulation of the oxidase by FAD

suggests that it is a flavoprotein. FMN cannot replace the FAD.

Kinetic studies on the fatty acyl-CoA oxidase indicate that it has a relatively low affinity for the acyl-CoA substrates (K_m= 120 μM) and a high affinity for oxygen. In contrast, the mitochondrial fatty acyl-CoA dehydrogenases have a very high affinity for the acyl-CoA substrates (K_m of 2-5 μM) (64). The finding that the oxidase has a high affinity for oxygen seems to be somewhat unusual in that it is commonly believed that peroxisomal H_2O_2-producing oxidases have a very low affinity for oxygen (19). These kinetic parameters suggest that the peroxisomal fatty acyl-CoA oxidase, as well as the β-oxidation system, could be limited by acyl-CoA availability rather than by the cellular oxygen tension.

From the stoichiometric studies of the fatty acyl-CoA oxidase reaction as well as of the β-oxidation reaction, it is concluded that the oxidase catalyzes the oxidation of butyryl-CoA to the corresponding crotonyl-CoA in the presence of molecular oxygen, which serves as the electron acceptor, with the concomitant production of H_2O_2. The overall metabolic sequence that the data support can be summarized as follows:

1) Fatty acyl-CoA + O_2 $\xrightarrow{\text{FAD}}$ enoyl-CoA + $\underline{H_2O_2}$

2) Enoyl-CoA + H_2O $\longrightarrow$ β-hydroxyacyl-CoA

3) β-hydroxyacyl-CoA + NAD^+ → β-ketoacyl-CoA + NADH + H^+

The production of acetyl-CoA from acyl-CoA in Tetrahymena peroxisomes was not measured experimentally. However, Cooper and Beevers (18) have already shown that for every mole of NADH produced in the peroxisome of castor bean endosperm from acyl-CoA, one mole of acetyl-CoA also accumulated. This also has been shown to be true in the rat liver peroxisomal β-oxidation system (30). Therefore it can be assumed that the same holds true in the peroxisomal β-oxidation system of Tetrahymena.

The fatty acyl-CoA oxidase extracted from Tetrahymena cells grown on the standard PPGA medium showed a chain length specificity for short-chain length acyl-CoA substrates (C_4-C_8) and it was not able to oxidize any acyl-CoA compound of chain length greater than C_8. However, if Tetrahymena cells are grown in an oleate-rich medium (PPGT), the chain length specificity of the oxidase becomes much broader (C_4-C_{20}) and increases in activity by several fold over the oxidase from PPGA-grown cells (figure 5). This indicates that the peroxisomal fatty acyl-CoA oxidase, and presumably the peroxisomal β-oxidation system, are inducible by growth on a high-fat medium (diet). Figure 5 indicate that the fatty acyl-CoA oxidase extracted from PPGT-grown cells is not really one enzyme, but three; a short-chain acyl-CoA oxidase (maximum activity with C_6), a medium-chain

oxidase (maximum activity with C_{12}), and a long-chain oxidase (maximum activity with C_{18}). This observation is supported in a preliminary study by the appearance of corresponding oxidase bands in polyacrylamide gel electrophoresis of Tetrahymena extracts (unpublished results with L. May). Thus, if the peroxisomal fatty acyl-CoA oxidase is not one enzyme, but several (three), as is the case with the mitochondrial fatty acyl-CoA dehydrogenase (64), their induction would easily be controlled by substrate availability; all (three) of the oxidase isozymes need not be expressed at once. Therefore the peroxisomal β-oxidation pathway is very adaptable to environmental changes, such as diet.

The results presented above have shown that Tetrahymena peroxisomes have a fatty acyl-CoA oxidizing system (β-oxidation) such as the one previously identified in the peroxisomes of germinating fatty seedlings (18) and quite recently, in rat liver (28). T. pyriformis also has a mitochondrial β-oxidation pathway, as evidenced by the presence of the fatty acyl-CoA dehydrogenase in the mitochondrial fraction (table 9) and by the differential inhibition of the β-oxidation pathway by malonate (65).

The relative roles played by the mitochondrion and peroxisome in fatty acid oxidation in vivo, as well as the contributions of these β-oxidation systems to organellar and cellular functions, are perhaps the main questions

raised by the discovery and description of the peroxisomal fatty acid oxidizing system.

The relative contribution of the mitochondrion and peroxisome to the total cellular fatty acid oxidation in in Tetrahymena has not been fully determined experimentally. However, since the peroxisomal β-oxidation system is an inducible system for fatty acid oxidation, its contribution to the total cellular fatty acid oxidation would depend upon the environment to which the cells are exposed. The higher the fat content in the diet, presumably the greater will be the peroxisomal contribution to fatty acid oxidation. It has recently been shown that the feeding of high-fat diets to rats also give a several fold increase in the total liver peroxisomal β-oxidation activity (47,48).

The question of the roles played by the mitochondrial and peroxisomal β-oxidation systems in organellar and cellular functions can best be answered by examining the products of these fatty acid oxidative systems and determining to what useful purpose these oxidation products can be put in the cell.

The action of the mitochondrial β-oxidation system on acyl-CoA produces $FADH_2$, NADH and acetyl-CoA. The acetyl-CoA produced is mainly used to fuel the mitochondrial tricarboxylic acid cycle, while the $FADH_2$ and NADH are oxidized by the electron transport system, their potential energies

being conserved in ATP by the oxidative phosphorylation process in the mitochondria.

In Tetrahymena, peroxisomal β-oxidation of acyl-CoA's can also produce NADH and acetyl-CoA, but unlike the mitochondrial system, H_2O_2 is produced. The acetyl-CoA produced by the peroxisomal β-oxidation cycle in Tetrahymena can serve as one of the two fuels for the glyoxylate bypass enzymes present in the peroxisomes and thus it may stimulate the glyoxylate cycle and gluconeogenesis. The relative rates of peroxisomal β-oxidation and of cellular gluconeogenesis (7.5 nmoles/min/mg protein, table 9 and 5.0-8.3 nmoles glucose/min/mg protein (22), respectively) indicate that the peroxisomal β-oxidation system can adequately supply the acetyl-CoA needed for an efficient gluconeogenetic process from fats.

The NADH secreted by the peroxisomes can establish the high cytosolic NADH/NAD ratio that is needed in order for gluconeogenesis to proceed efficiently. Also the high NADH/NAD ratio would slow down considerably any glycolytic process that would act to reverse gluconeogenesis. Therefore these two products of the peroxisomal β-oxidation pathway can be used in concert by Tetrahymena (and possibly also in rat liver) for an efficient gluconeogenetic process. The source of isocitrate, the other fuel for the glyoxylate bypass, remains an unsolved mystery.

PART II

PEROXISOMAL AND MITOCHONDRIAL β-OXIDATION IN RAT LIVER

INTRODUCTION

There are now reports on the presence of a fatty acyl-CoA oxidizing system in the peroxisomes of castor bean endosperm (18), Tetrahymena (26), rat liver (28), yeast (66), brown adipose tissue (67), and human liver (68).

The peroxisomal fatty acid oxidizing system also appears to proceed through a mechanism of β-oxidation (18,30, 37,66). Like mitochondrial β-oxidation, it generates acetyl-CoA through successive steps of dehydrogenation, hydration, dehydrogenation, and thiolitic cleavage (30,37,38,39, 41). However, in contrast to the mitochondrial β-oxidation system, the first dehydrogenation step in the peroxisomal β-oxidation system appears to involve the direct reduction of O_2 to H_2O_2 by a flavoprotein oxidase (18).

The novel enzyme involved in the first oxidative reaction (dehydrogenation) in the peroxisomal β-oxidation cycle, a fatty acyl-CoA oxidase, was first assayed and characterized by Hryb and Hogg using extracts from Tetrahymena (26). The presence of the fatty acyl-CoA oxidase also has been reported in rat livers (36,69,70), human liver (68), and yeast (71). The oxidase has been partially purified (20-30 fold) from livers of rats treated with either DEHP (36), or Nafenopin (69,70), both peroxisomal proliferators and hypolipidemic agents, and some of its properties described.

The chain length specificity of the peroxisomal β-oxidation system in rat liver has also been reported (30,46). However, these investigations were performed with rats fed clofibrate and without any comparison to normal rats. The results of the present work on the rat liver fatty acyl-CoA oxidase and the peroxisomal β-oxidation system differ, in terms of assay conditions, chain length specificities and in the use of livers of normal rats, from the above investigations (30,36,46,69,70).

In the present study, using the peroxisomal fraction from rat liver (de Duve's fraction L (32)), the fatty acyl-CoA oxidase and the peroxisomal β-oxidation activities were characterized chiefly in terms of assay conditions and chain length specificities. The chain length specificity of the mitochondrial fatty acyl-CoA dehydrogenase(s) was also re-examined and found to be somewhat different from previously published data (64,72). The chain length specificities of the mitochondrial dehydrogenase and the peroxisomal oxidase show a partial complementarity, with the mitochondrial system being the more active towards short-chain length substrates and the peroxisomal system being the more active with medium to long-chain length substrates.

MATERIALS AND METHODS

Preparation of particulate fractions. The peroxisomal (L) and mitochondrial (M) fractions were prepared from the livers of male albino rats (Sprague-Dawley) according to the procedure of de Duve et al. (32). The rats were fed ad libitum on Purina lab chow, and starved overnight previous to sacrifice. The fatty acyl-CoA oxidase activity was found to be localized in the peroxisomal fraction (L).

Enzyme assays. The fatty acyl-CoA oxidase activity was assayed by measuring the acyl-CoA-dependent H_2O_2 production using a modification of the method described by Hryb and Hogg (26). The oxidase was assayed at 30° C in 50 mM potassium phosphate buffer, pH 7.6, containing 500 U/ml horseradish peroxidase, 25 mM p-hydroxybenzoic acid, 1.0 mM 4-aminoantipyrine (31), 50 μM FAD, 200 μg/ml BSA, 0.05% (v/v) Triton X-100, and the particulate preparation (L). The reaction was started by adding the acyl-CoA to a final concentration of 50 μM. The increase in absorbance at 500 nm was monitored in a Gilford 2400 recording spectrophotometer. The extinction coefficient of the chromogen was experimentally calculated, with a standard solution of H_2O_2, to be 5.1 $cm^2/\mu mole$ at pH 7.6.

The fatty acyl-CoA dehydrogenase assay used was a modification of the method described by Hoskins (73). The dehy-

drogenase was assayed at 30° C in 66 mM potassium phosphate buffer, pH 6.8, containing 66 μM DCPIP, PMS (1.0, 0.5, and 0.25 mM), 1mM KCN, 200 μg/ml BSA, 0.067% (v/v) Triton X-100, and the particulate preparation (M). The reaction was started by adding the acyl-CoA to a final concentration of 50 μM. The decrease in absorbance at 600 nm was monitored. An extinction coefficient for DCPIP of 19.1 cm^2/μmole was used (60).

The peroxisomal β-oxidation assay used was a modification of the methods described by Cooper and Beevers (18) and Lazarow and de Duve (28). The assay was performed at 30° C in 50 mM potassium phosphate buffer, pH 7.4, containing 200 μM NAD+, 100 μM CoA, 50 μM FAD, 1.0 mM KCN, 12 mM dithiothreitol (DTT), 200 μg/ml BSA, 0.025% (v/v) Triton X-100, and the particulate preparation (L). The reaction was started by adding the acyl-CoA to a final concentration of 50 μM. The increase in absorbance at 340 nm was monitored.

Catalase was assayed by the initial rate of disappearance of 12.5 mM H_2O_2 as measured by the decrease in absorbancy at 240 nm (see part I of this thesis).

The intraperoxisomal distribution of catalase and the fatty acyl-CoA oxidase was determined by sonicating the peroxisomal fraction (L) with a Branson Sonifier Cell Disruptor (model W140D) at a power setting of 6 for 40 seconds

(in 10 sec intervals, with two min intermediate cooling periods on ice) using a $\frac{1}{2}$ inch horn. After assaying the sonic homogenate, it was centrifuged at 30,000 x g for 30 min and the supernate transferred to a fresh tube. The pellet was resuspended in 0.25 sucrose and made equal in volume to the supernate, whereupon both fractions were assayed. The recovery of each enzyme was within 100% $\pm$ 10, with respect to the sonicate.

Protein concentration was determined by a biuret method (74) after the protein was precipitated and washed with 0.5N perchloric acid. Crystallized and lyophilized BSA was used as the standard.

Materials. Horseradish peroxidase, Type II (E.C. 1.11.1.7), FAD, FMN, p-hydroxybenzoic acid, 4-aminoantipyrine, DCPIP, PMS, NAD+, NADP+, CoA, DTT, BSA, and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, Mo. Acyl-CoA's and DL-C_{12}-carnitine.chloride were purchased from P-L Biochemicals, Milwaukee, Wis. Sprague-Dawley rats were obtained from Charles River Breeding Labs., Wilmington, Mass. All other chemicals were of analytical reagent grade.

RESULTS

Characterization of the peroxisomal fatty acyl-CoA oxidase activity of rat liver. Table 1A shows that the fatty acyl-CoA oxidase activity is clearly dependent on enzyme source, palmitoyl-CoA, peroxidase, and chromogen. The high concentration of catalase present in the rat liver peroxisome does not interfere with the assay because of the high activity of peroxidase present in the assay; the catalase/peroxidase ratio of activities was less than 10 (26, part I of this thesis). Adding sodium azide to the assay mixture to inhibit the peroxisomal catalase does not increase the oxidase rate, as it should, if there was any interference by catalase (62). With respect to our previous work on the oxidase in Tetrahymena (26, part I of this thesis), o-dianisidine could not act here as the chromogen in the rat liver oxidase assay. Therefore it was replaced with a p-hydroxybenzoic acid plus 4-aminoantipyrine mixture (31) (also see table 4, part I of this thesis). The omission of BSA from the assay does not alter the activity of the oxidase. However, the rate curve for the assay did not remain linear beyond 2 min in the absence of BSA. The omission of Triton X-100 did not alter the activity of the oxidase but in its absence there was some optical interference from particle agglutination. The rat liver fatty acyl-CoA oxida-

TABLE 1A. Requirements for activity of the peroxisomal fatty acyl-CoA oxidase of rat liver: palmitoyl-CoA as substrate.

Complete assay[a]	100% (7.9 nmoles/min/mg protein)
- enzyme source	0
- C_{16}-CoA	0
- peroxidase	0
+ 2X peroxidase	100
- chromogen[b]	0
+ 2X chromogen	83
½ chromogen	101
- FAD	70
+ 2X FAD	100
- FAD, + 75 μM FMN	66
- BSA	89
- Triton X-100	99
+ 1.0 mM NaN_3	90

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a- for assay conditions see Materials and Methods.
b- 25 mM p-hydroxybenzoic acid plus 1.0 mM 4-aminoantipyrine.

se is an FAD dependent enzyme; FMN could not substitute for the FAD.

Substituting DL-C_{12}-carnitine.chloride for the C_{12}-CoA did not produce a reaction (table 1B). However, if CoA was also added to the test, a small response was observed. These results indicate that the fatty acyl-CoA oxidase has an absolute requirement for the fatty acid to be in the form of its CoA thioester, and suggest the presence of a medium-chain length carnitine acyltransferase in the rat liver peroxisome. Alternatively, the carnitine acyltransferase activity could be due to mitochondrial contamination.

The fatty acyl-CoA oxidase reaction rate was proportional to the amount of peroxisomal protein added in the range of 0-200 μg/ml, regardless of substrate used (figure 1). Higher levels of peroxisomal protein caused a discernible inhibitory effect. However, this observed inhibition was not caused by increasing levels of catalase, since doubling of the peroxidase content did not eliminate or reduce the inhibition.

The oxidase has a pH maximum of 8.0-9.0 (figure 2). Tris-HCl buffer can serve as the buffer in the assay just as well as phosphate.

The stoichiometry for formation of H_2O_2 from palmitoyl-CoA by the fatty acyl-CoA oxidase is shown in table 2. As can be seen, there is not an exact 1:1 stoichiometry between palmitoyl-CoA oxidized and O_2 reduced to H_2O_2, but H_2O_2

TABLE 1B. Requirements for activity of the peroxisomal fatty acyl-CoA oxidase of rat liver: lauroyl-CoA as substrate.

Complete assay[a]	100% (13.3 nmoles/min/mg protein)
- C_{12}-CoA, + 100 μM DL-C_{12}-carnitine.Cl	0
- C_{12}-CoA, + 100 μM CoA, + 100 μM DL-C_{12}-carnitine.Cl	24

a- for assay conditions see Materials and Methods.

FIGURE 1. Effect of protein concentration on the rate of the fatty acyl-CoA oxidase reaction. Assay conditions were as described in Materials and Methods. The rat liver peroxisomal fraction (L) was used as the enzyme source at the indicated concentrations, and lauroyl-CoA (△) and palmitoyl-CoA (◇) as the substrates.

FIGURE 1.

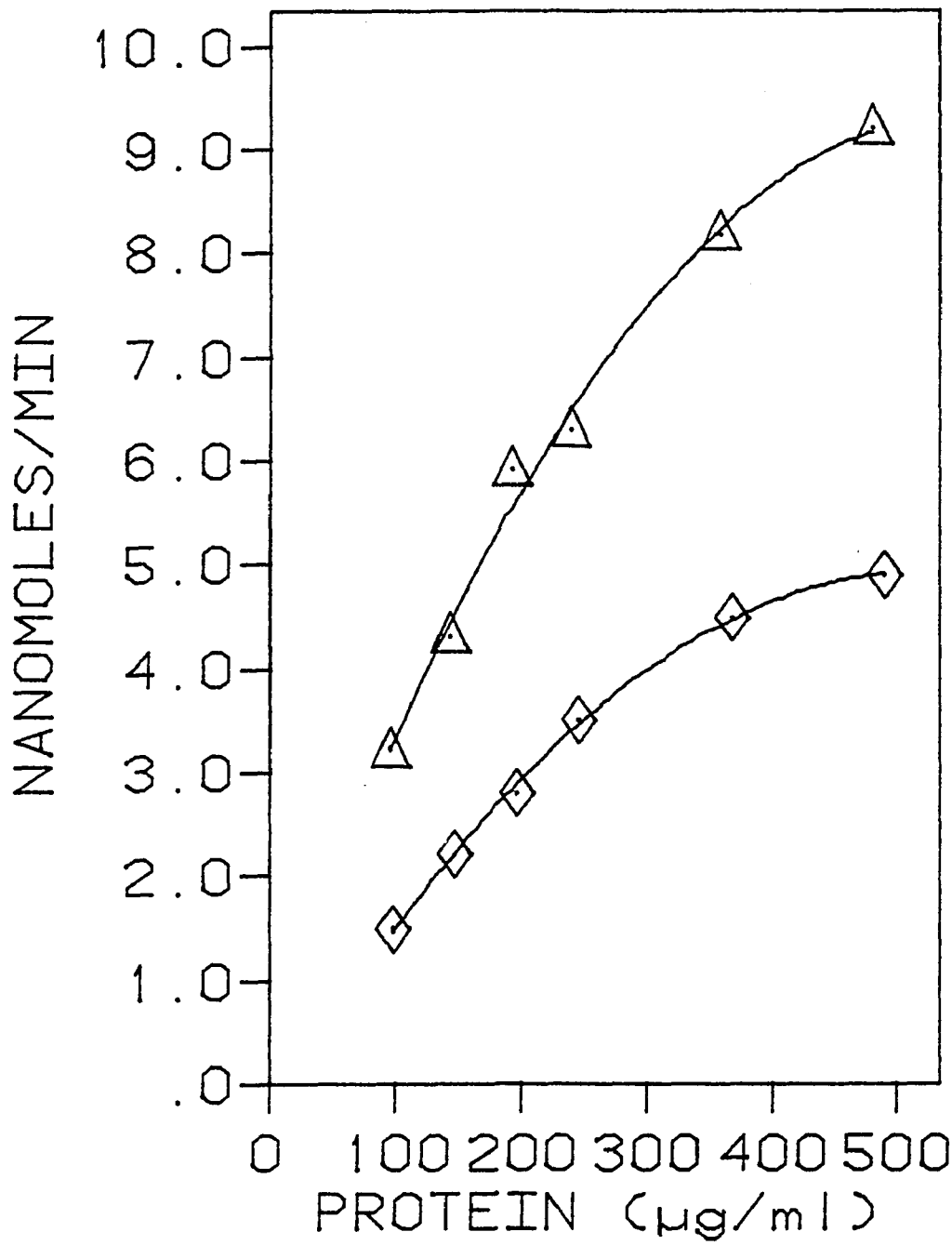

10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
.0
NANOMOLES/MIN
0
100
200
300
400
500
PROTEIN (µg/ml)

FIGURE 2. Effect of pH on the rate of the fatty acyl-CoA oxidase reaction. Assay conditions were as described in Materials and Methods, except that the pH of the buffers used were varied as indicated. The rat liver peroxisomal fraction (L) was used as the enzyme source and palmitoyl-CoA as the substrate. The buffers used at a final concentration of 50 mM were; potassium phosphate (△) and Tris-HCl (□).

FIGURE 2.

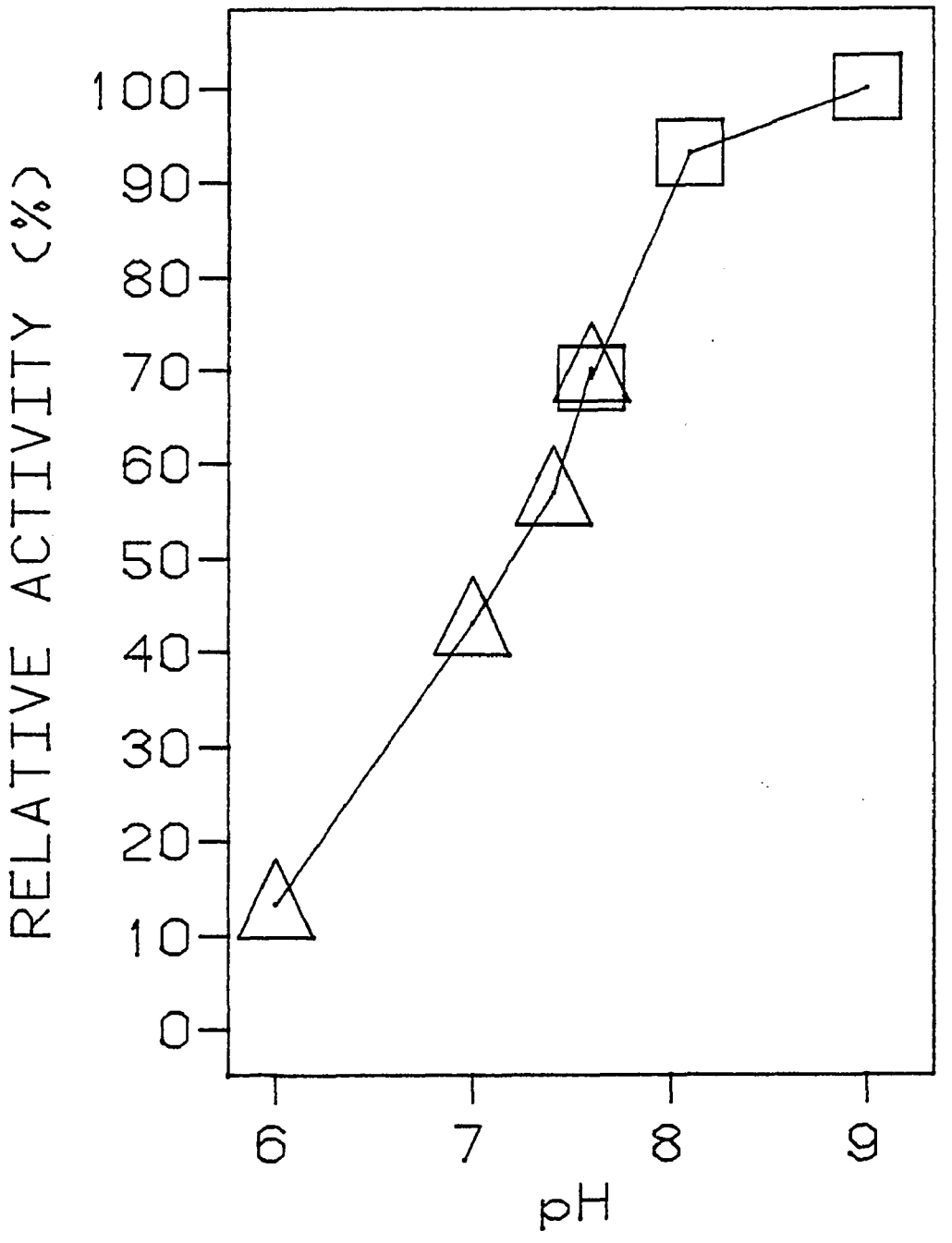


TABLE 2. Stoichiometric formation of H_2O_2 from palmitoyl-CoA by the fatty acyl-CoA oxidase.

Palmitoyl-CoA added (nanomoles)	H_2O_2 formed (nanomoles)
10	6.8
20	14.1

production doubles as substrate doubles.

The effect of acyl-CoA concentration and of oxygen content in the gas phase on the rate of the fatty acyl-CoA oxidase reaction. The effect of changing the levels of the two substrates (acyl-CoA and oxygen) on the reaction rate of the oxidase was studied (figures 3, 4 and 5). Figure 3 shows that no substrate inhibition occurs with either lauroyl-CoA or palmitoyl-CoA. As a matter of fact, even at 100 μM substrate concentration, the oxidase was not completely saturated. The oxidase does not exhibit typical Michaelis-Menten kinetics, thus Lineweaver-Burk plots could not be constructed from the data of figure 3. However, an approximate K_m value of 30 μM, for both substrates (C_{12}- and C_{16}-CoA), was estimated from figure 3 (from ½ Vmax).

The effect of changing the oxygen content in the gas phase on the rate of the fatty acyl-CoA oxidase reaction is shown in figures 4 and 5. Lowering the oxygen content of the gas phase from 100% to 1.0% (v/v) decreased the oxidase activity by only 40%. The K_m for oxygen, determined from figure 5, is approximately 0.8% (v/v). The oxygen tension in living cells (human hepatocytes) is known to be about 23 mm Hg (3% (v/v)). Thus the oxidase has a K_m value for oxygen which is well below the actual oxygen content in cells. This indicates that the fatty acyl-CoA oxidase has a very high affinity for oxygen in comparison to other flavo-

FIGURE 3. Effect of acyl-CoA concentration on the rate of the fatty acyl-CoA oxidase reaction. The assay conditions were as described in Materials and Methods, except that lauroyl-CoA (△) and palmitoyl-CoA (◇) were used as substrates at the indicated concentrations. The rat liver peroxisomal fraction (L) was used as the enzyme source.

FIGURE 3.

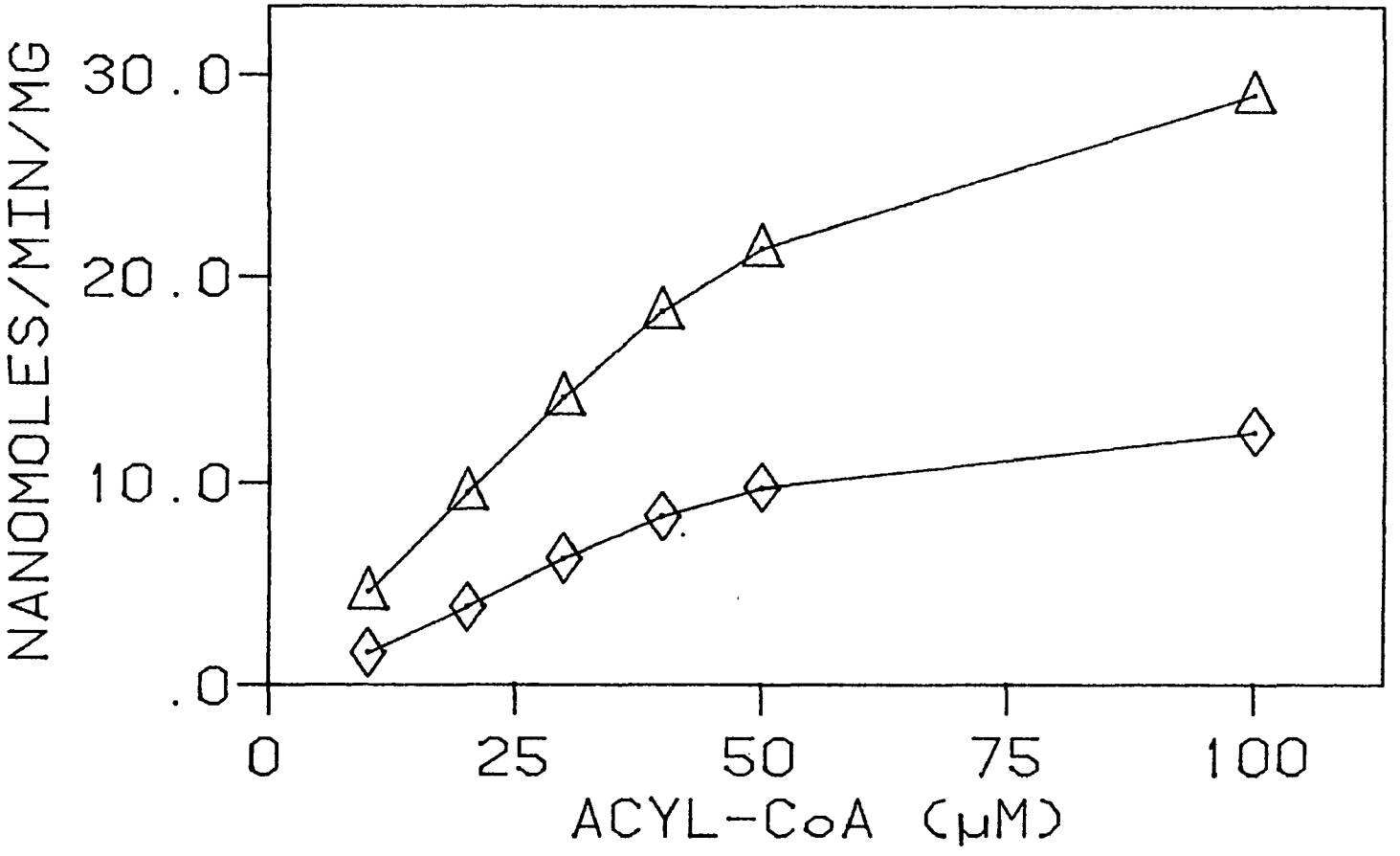


FIGURE 4. The effect of changing the oxygen content in the gas phase on the rate of the fatty acyl-CoA oxidase reaction. The assay conditions were as described in Materials and Methods. The oxidase assay mixture (minus the palmitoyl-CoA, plus the rat liver peroxisomal fraction (L)), in a final volume of 12 ml, was incubated on ice in a 100 ml flask. This assay mixture was then flushed with the desired oxygen-nitrogen mixture for 20 minutes. A 3.0 ml reaction mixture aliquot was removed and placed in a Thunberg cuvette, containing the palmitoyl-CoA in the side arm. The cuvette was equilibrated with the desired oxygen-nitrogen mixture by repeated evacuation and flushings. The cuvette was sealed after the last flushing (3^{rd}) and the reaction was initiated by tipping the palmitoyl-CoA (final concentration of 50 μM) from the side arm into the reaction mixture. Oxygen concentrations of 1.0%, 2.3%, 10%, 21% (air), 50%, and 100% (v/v) were tested.

FIGURE 4.

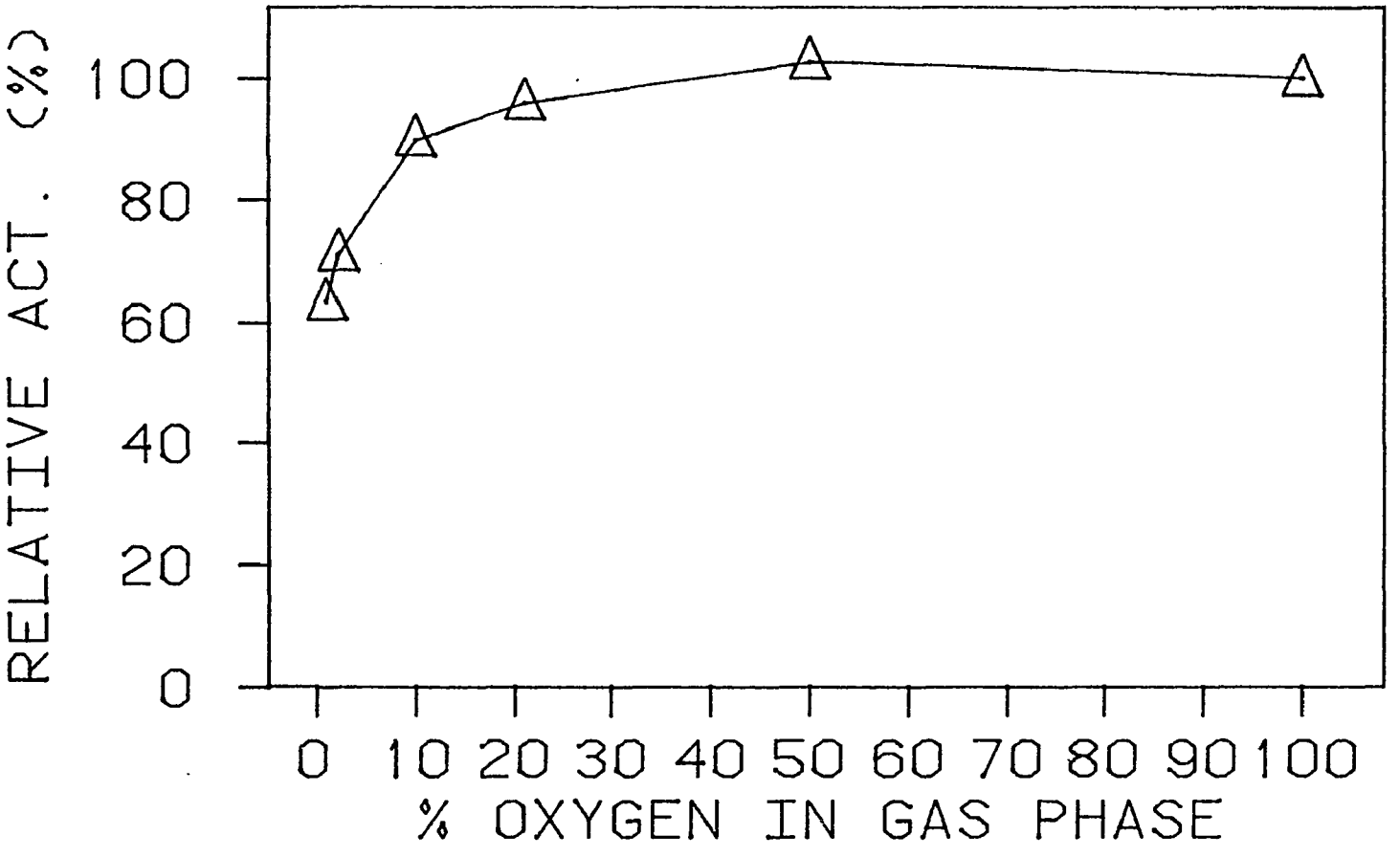
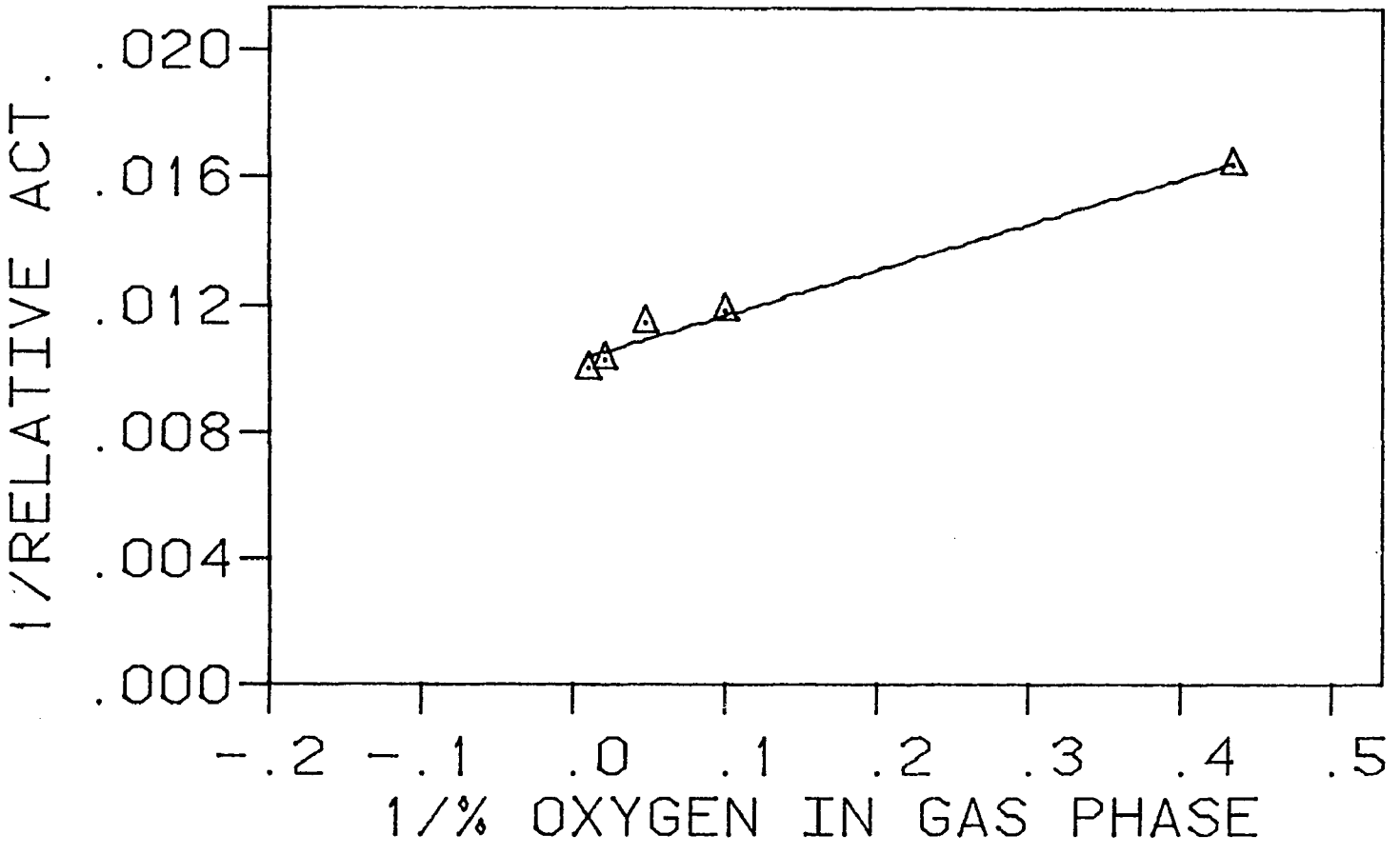

RELATIVE ACT. (%)
100
80
60
40
20
0
0 10 20 30 40 50 60 70 80 90 100
% OXYGEN IN GAS PHASE

FIGURE 5. Double reciprocal plot of the fatty acyl-CoA oxidase activity versus oxygen concentration in the gas phase. This is the Lineweaver-Burke plot of the data presented in figure 4.

FIGURE 5.


1/RELATIVE ACT.
.020
.016
.012
.008
.004
.000
-.2
-.1
.0
.1
.2
.3
.4
.5
1/% OXYGEN IN GAS PHASE

protein oxidases. Therefore the intracellular concentration of acyl-CoA's, rather than the oxygen tension, may regulate the rate of the fatty acyl-CoA oxidase reaction.

The intraperoxisomal localization of catalase and fatty acyl-CoA oxidase of rat liver. In order to determine the intraperoxisomal distribution of the fatty acyl-CoA oxidase, it was first necessary to demonstrate that sonicating the organelles caused the peroxisomal-limiting membrane to rupture and spill its liquid contents (the peroxisol). The centrifugation of such sonicates would thus afford a separation of the peroxisol from the peroxisomal membrane. Catalase has already been shown to be a soluble matrix (peroxisol) enzyme in the rat liver peroxisome (75,76). Therefore it was used here as a marker enzyme for the peroxisol.

Table 3A shows the effect of Triton X-100 on the measurable activity of the peroxisomal enzymes catalase and fatty acyl-CoA oxidase when the detergent is added to the intact peroxisomes. Catalase shows a 2.2-fold increase in activity, indicating that its activity is limited by a membrane, i.e., it is a latent enzyme. On the other hand, the oxidase shows no increase in activity or no latency behavior. Table 3B shows the effect of 40 second sonication of the intact peroxisome on the activity of catalase and fatty acyl-CoA oxidase. As with the detergent treatment, catalase shows a 2.2-fold increase in activity, while the oxidase shows a

TABLE 3. The effect of Triton X-100 and 40 second ultrasonic treatment on the activity of the peroxisomal enzymes, catalase and fatty acyl-CoA oxidase.

A- The effect of Triton X-100 on the activity of the peroxisomal enzymes

Enzyme	$\frac{\text{Act. with Triton X-100}}{\text{Act. without Triton X-100}} \times 100\%$
Catalase[a]	224%
Fatty acyl-CoA oxidase[b]	101%

B- The effect of 40 second ultrasonic treatment on the activity of the peroxisomal enzymes

Enzyme[c]	$\frac{\text{Act. with sonication}}{\text{Act. without sonication}} \times 100\%$
Catalase	215%
Fatty acyl-CoA oxidase	73%

a- 0.067% (v/v) Triton X-100.
b- 0.050% (v/v) Triton X-100.
c- Triton X-100 was omitted from all assays.

slight decrease (27%) in activity. Therefore, the results presented in table 3 indicate that access to catalase is limited by the peroxisomal membrane, while the fatty acyl-CoA oxidase does not display such latent behavior. Also these results demonstrate that sonication of the intact peroxisomes for 40 seconds ruptures the peroxisomal membrane, spilling the soluble content of the peroxisome.

The distribution of catalase and fatty acyl-CoA oxidase after 30,000 x g centrifugation of the 40 second sonically treated peroxisomal fraction is shown in table 4. As shown in table 4, 93% of the total catalase activity of the sonically disrupted peroxisomes resides in the 30,000 x g supernate, while only 41% of the fatty acyl-CoA oxidase is there. Alternatively, about 60% of the oxidase activity was sedimented. The peroxisolic (or soluble matrix) localization of catalase has been confirmed (75,76), and the data indicate also that the intraperoxisomal distribution of the oxidase is opposite to that of catalase, i.e., it is a membrane-bound enzyme. The fact that 41% of the oxidase activity was solubilized, indicates either that it is not bound very tighly to the peroxisomal membrane or that the sonication treatment proved a little harsh.

Characterization of the peroxisomal β-oxidation system of rat liver. Table 5A shows the effect of cofactor omission on the peroxisomal β-oxidation system. The fatty acyl-

TABLE 4. Distribution of catalase and fatty acyl-CoA oxidase after 30,000 X g centrifugation of the 40 second sonically treated peroxisomal fraction.

Enzyme[a]	% of total activity of sonicated peroxisomes remaining in 30,000 X g supernate
Catalase	93%
Fatty acyl-CoA oxidase	41%

a- Triton X-100 was used in all the assays. Recovery of each enzyme after centrifugation was within 100% ± 10, with respect to the sonicate.

TABLE 5A. Characterization of the peroxisomal β-oxidation system of rat liver: palmitoyl-CoA as substrate.

Complete assay[a]	100% (15.1 nmoles/min/mg protein)
- enzyme source	0
- C_{16}-CoA	0
- NAD+	0
- NAD+, + 200 μM NADP+	7
- CoA	96
- FAD	78
- KCN	89
- DTT	100
- BSA	102

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a- for assay conditions see Materials and Methods.

CoA oxidizing activity was dependent on enzyme source, palmitoyl-CoA and NAD+. NADP+ could not substitute for the NAD+. FAD was required for optimal activity. The omission of CoA, KCN, DTT, and BSA had little or no effect on the activity. Their omissions however, did affect the linearity of the assay response curve.

If C_{12}-CoA is replaced by DL-C_{12}-carnitine.Cl (table 5B), a high rate of reaction is still observed. However, if CoA is omitted from the reaction mixture, there then no activity is observed. Therefore the data shown here support those presented in table 1B, which taken together, indicate that the peroxisomal β-oxidation system has a absolute requirement for the fatty acid to be in the form of its CoA thioester. These results also point to the presence of a carnitine lauroyltransferase in the rat liver peroxisome. Markwell et al. (77) have reported the presence of carnitine acetyltransferase and carnitine octanoyltransferase in rat liver peroxisomes. However, the carnitine lauroyltransferase activity could be due to mitochondrial contamination.

Chain length specificities for acyl-CoA substrates of the peroxisomal and mitochondrial fatty acyl-CoA oxidizing systems of rat liver. The chain length specificities for acyl-CoA substrates (50 μM final concentration) of the peroxisomal fatty acyl-CoA oxidase and the β-oxidation system, as well as the mitochondrial fatty acyl-CoA dehydrogenase

TABLE 5B. Characterization of the peroxisomal β-oxidation system of rat liver: lauroyl-CoA as substrate.

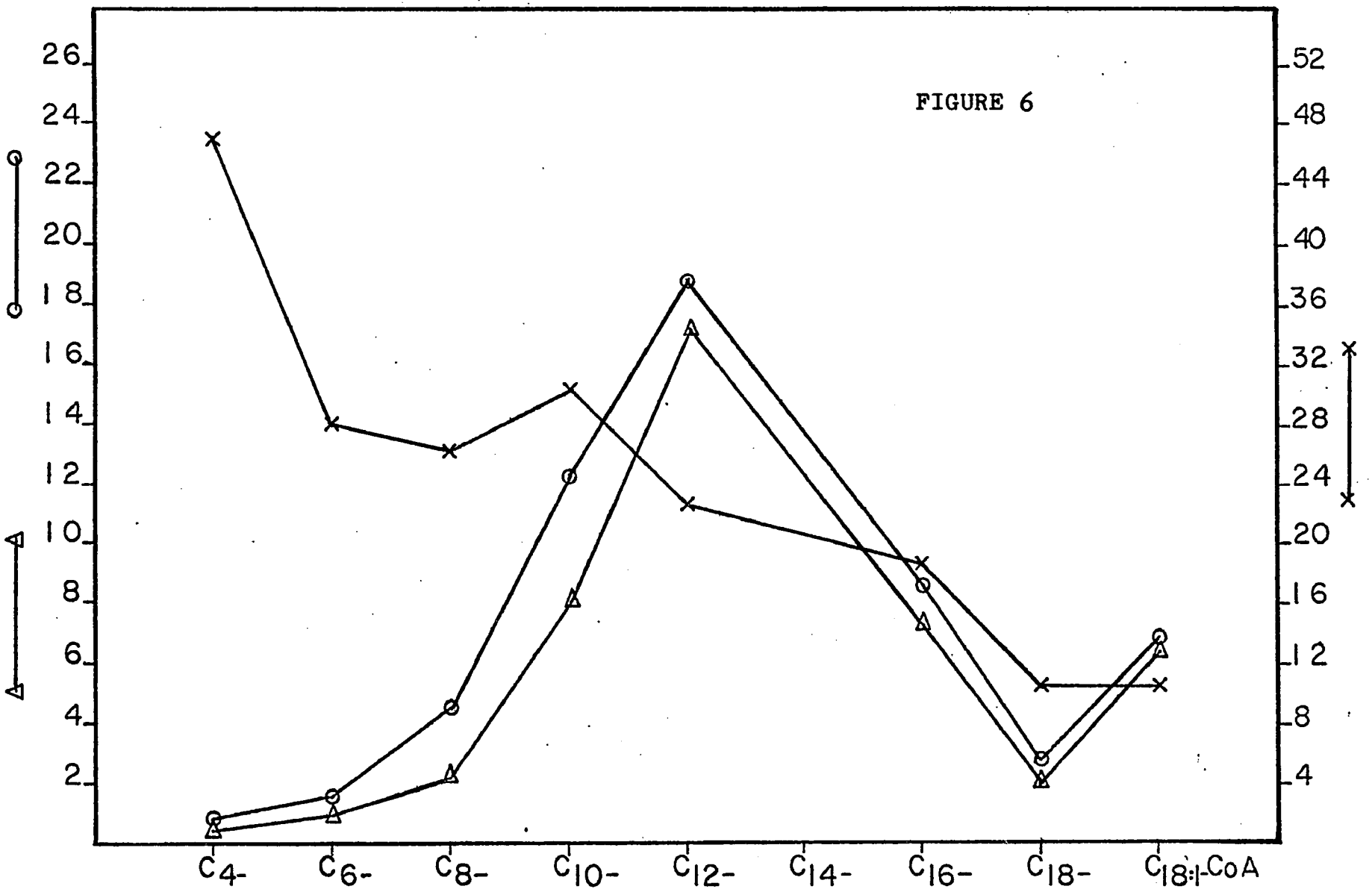
Complete assay[a]	100% (32.0 nmoles/min/mg protein)
- C_{12}-CoA, + 100 μM DL-C_{12}-carnitine.Cl	73
- C_{12}-CoA, - CoA, + 100 μM DL-C_{12}-carnitine.Cl	0

a- for assay conditions see Materials and Methods.

(calculated at Vmax PMS) were studied (figure 6). The peroxisomal fatty acyl-CoA oxidase shows the ability to oxidize medium to long-chain length acyl-CoA's (C_{10}-C_{16}). However, there is a very definite peak of activity at C_{12} (figure 6). This is in contrast with Osumi and Hashimoto's (36) data on the chain length specificity of the oxidase. Their results show the oxidase to have a broader chain length range (C_{10}-C_{22}), with a peak at C_{16}. Their oxidase however, was obtained from rats treated with DEHP, a peroxisomal proliferator which caused a great increase in the oxidase activity.

The chain length specificity of the peroxisomal β-oxidation system is identical to that of the fatty acyl-CoA oxidase; C_{10}-C_{16}, with a peak of activity at C_{12}. The specific activities in the two assays are also very similar and, in each case, oleoyl-CoA was a better substrate (3-fold better) than stearoyl-CoA (figure 6). Thus these data indicate that the oxidase is the rate-limiting enzyme in the peroxisomal β-oxidation system. The chain length specificity of the peroxisomal β-oxidation system in livers of rats treated with clofibrate also has been reported (30,46). Those results show the chain length specificity to have shifted towards the higher-chain length substrates, with a peak at C_{14}-C_{16}. In addition, Osmundsen *et al.* (46) have reported that, in the peroxisomes from the livers of clofibrate-treated rats, the rates of oxidation of mono-unsaturated

FIGURE 6. Chain length specificities for acyl-CoA substrates of the peroxisomal fatty acyl-CoA oxidase (Δ), the peroxisomal β-oxidation system (O), and the mitochondrial fatty acyl-CoA dehydrogenase (X). The rat liver peroxisomal (L) and mitochondrial (M) fractions were used as enzyme sources, and 50 μM acyl-CoA's of the indicated chain lengths as substrates. All other assay conditions were as described in Materials and Methods. The rates are given in nanomoles/min/mg of protein, with the dehydrogenase values being obtained by extrapolation to PMS saturation.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20
16
12
8
4
C4-
C6-
C8-
C10-
C12-
C14-
C16-
C18-
C18:1-CoA

FIGURE 6

fatty acyl-CoA's were higher (10-fold) than for the corresponding saturated fatty acyl-CoA's.

The chain length specificity of the mitochondrial fatty acyl-CoA dehydrogenase(s) shows a broader range (C_4-C_{16}), with the shorter-chain length substrate being the more active and no preference shown for oleoyl-CoA over stearoyl-CoA (figure 6). The peak activity observed at C_4 is unusual in that it is contrary to previously published data (64,72). Our experiments on the dehydrogenase were performed utilizing the same amount of mitochondrial protein and the same substrate concentration throughout the series, in order that the ratio of substrate to the three fatty acyl-CoA dehydrogenases present in the mitochondria would be the same for all the acyl-CoA's tested. Although the data are given in terms of Vmax PMS, the same chain length specificity pattern was obtained at each PMS level.

The total enzymatic activities (per gram of liver) for the oxidation of acyl-CoA's by the mitochondrial fatty acyl-CoA dehydrogenase and the peroxisomal fatty acyl-CoA oxidase are shown in table 6. If the mitochondrial dehydrogenase and the peroxisomal oxidase are taken to be the rate-limiting step in their respective β-oxidation systems, then the ratio of their activities for the oxidation of palmitoyl-CoA (820 and 106 nmoles/min/g liver, respectively) tells us that the rate of palmitoyl-CoA oxidation in the total mito-

TABLE 6. Total enzymatic activities for the oxidation of acyl-CoA's by the mitochondrial fatty acyl-CoA dehydrogenase and the peroxisomal fatty acyl-CoA oxidase.

Substrate[a]	Mitochondrial fraction (fatty acyl-CoA dehydrogenase)	Peroxisomal fraction (fatty acyl-CoA oxidase)	Mitochondria / Peroxisome
	nmoles/min/g liver	nmoles/min/g liver	
C_4-CoA	1902	6.0	b
C_6-CoA	1094	13.0	b
C_8-CoA	1080	31.0	35.0
C_{10}-CoA	1118	116.4	9.6
C_{12}-CoA	942	250.0	3.8
C_{16}-CoA	820	106.0	7.7
C_{18}-CoA	440	29.4	15.0
$C_{18:1}$-CoA	452	93.0	4.9

a- the final concentration of acyl-CoA's was 50 μM.
b- the peroxisomal oxidation is negligible compared to the mitochondria.

chondrial fraction is 7.7 times that in the peroxisomal fraction. In other words, the peroxisome contributes 11% of the total palmitoyl-CoA oxidation in the normal rat liver. However, as the data shown in table 6 indicates, the contribution of the peroxisome to the total liver fatty acid oxidation varies from 0% to 21%, depending on the fatty acid that is being oxidized in the liver, its chain length and degree of unsaturation being major factors.

DISCUSSION

Using an improved assay method for oxidases, the novel enzyme for the first oxidative reaction (dehydrogenation) in the peroxisomal β-oxidation pathway of rat liver, the fatty acyl-CoA oxidase, has been characterized. The results presented here show that the fatty acyl-CoA oxidase does not follow typical Michaelis-Menten kinetics for acyl-CoA's, it has a very high affinity for oxygen, and it is a membrane-bound enzyme.

Both the fatty acyl-CoA oxidase and the peroxisomal β-oxidation system showed a specificity towards medium to long-chain length acyl-CoA's (C_{10}-C_{16}) as substrates. The specific activities in both assays were similar and both activities showed a preference for long-chain mono-unsaturated fatty acyl-CoA's over the corresponding saturated acyl-CoA's. Thus the substrate specificity of the peroxisomal β-oxidation pathway is determined by the fatty acyl-CoA oxidase.

There is little information on the mechanism of control of the transport of fatty acyl-CoA's into and out of peroxisomes. The transport of acetyl-CoA out of the peroxisome may be regulated by the carnitine acetyltransferase present in the peroxisome (77). However, the transport of acyl-CoA into the peroxisome may be regulated by the fatty acyl-CoA

oxidase by virtue of its membrane localization; the acyl-CoA need not completely traverse the peroxisomal membrane in order to be oxidized by the oxidase. The membrane localization of the oxidase could also imply that the oxidase, together with the bifunctional enzyme, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (39), and thiolase (41) could exist as a multienzyme (β-oxidation) complex in or on (inside part) the peroxisomal membrane.

My results of figure 6 suggest that in the adult rat liver, the fatty acyl-CoA oxidase is the rate-limiting enzyme of the peroxisomal β-oxidation pathway, and that this peroxisomal oxidase could in turn be limited by acyl-CoA availability rather than by the cellular oxygen tension (figures 3, 4, and 5). This of course is contrary to current dogma; heretofore it had been thought that peroxisomal oxidations, unlike those of the mitochondria (19), were limited by the oxygen tension.

The rat liver fatty acyl-CoA oxidase and the oxidase from Tetrahymena have several properties in common; they both are FAD-dependent enzymes; have low affinities for the acyl-CoA's; high affinity for oxygen; and are inducible.

The participation of peroxisomes in the β-oxidation of fatty acids in the rat liver, as well as in other tissues, is now well established (18,26,28,66,67,68). The rat liver peroxisomal β-oxidation system has been shown to be enhanced

(induced) several fold by the treatment with peroxisome-proliferating hypolipidemic drugs; clofibrate (28), Nafenopin (69), and DEHP (34). These results have led the above authors to claim that the well known hypolipidemic action of the above drugs was caused by the enhancement of the peroxisomal β-oxidation system, thus ascribing to the peroxisome a role in the maintenance of low levels of serum lipids. However, it is also clear that mitochondrial fatty acid oxidation is stimulated by clofibrate feeding. This effect is primarily expressed with fatty acids shorter than C_{14} (45). Nevertheless, what seems clear, regardless of which organelle is the primary beneficiary of clofibrate, is that the induction not only increases greatly the ability to oxidize fatty acids but also, it produces a shift in chain length specificity. After clofibrate treatment, the mitochondrial specificity shifts towards the lower-chain length acyl-CoA's (45), while the peroxisomal specificity shifts towards the longer-chain length acyl-CoA's (30) (using figure 6 to represent the norm). Thus the partial complementarity shown by the mitochondrial and peroxisomal fatty acid oxidizing systems (figure 6) is enhanced.

Before the discovery of the peroxisomal β-oxidation system in rat liver (28), it was generally believed that the β-oxidation of fatty acids was exclusively a mitochondrial function in mammalian cells. Since this discovery, however,

the role of the mitochondria in the overall fatty acid oxidation in the liver has been seriously questioned (30). After the initial excitement of discovery, however, cooler heads have prevailed, thus resulting in a more critical view on the role of the peroxisome in fatty acid oxidation. What is not yet clear is the extent to which peroxisomes supplement (or complement) mitochondrial fatty acid oxidation. My results indicate that peroxisomal oxidation of palmitoyl-CoA accounted for at least 11% of the total palmitoyl-CoA oxidation in the adult male rat liver. This value is in approximate agreement with those reported by Krahling *et al.* (42,43), who reported that peroxisomal β-oxidation of palmitoyl-CoA accounted for 10% of the total β-oxidation activity in the young rat liver. The figure became 30% of the total in the liver of the adult female and 20% in the adult male (43). However, the results presented in this thesis are in complete disagreement with Lazarow's claim (30) that the bulk of palmitoyl-CoA oxidation in rat liver occurs in the peroxisome. My results, as well as those of Krahling *et al.* (42,43) were obtained with normal rats, while Lazarow (30) used rats treated with clofibrate. On the other hand, Mannaerts *et al.* (44) have reported that the contribution of the peroxisome to fatty acid oxidation is less than 10% both in livers from control and clofibrate-treated rats.

The results presented here lead me to conclude that in normal rats the peroxisomal β-oxidation system plays a subservient role to the mitochondrial system in the oxidation of fatty acids by the liver. I submit that for the completion of the oxidation of a medium to long-chain fatty acid initiated in the peroxisome, there must be participation by the mitochondrion. This is clearly shown by their chain length specificities (figure 6). Only the mitochondrion can oxidize fatty acids of less than C_8 chain length, i.e., the mitochondrion must complete an oxidation initiated in the peroxisome. This is also shown to be true by Lazarow's (30) data where the oxidation of palmitoyl-CoA by a purified peroxisomal fraction gave a stoichiometry of 1:5:5 (C_{16}-CoA: C_2-CoA:NADH). Thus the auxiliary function of the peroxisomal β-oxidation system is to assist in the mitochondrial β-oxidation system whenever, and for whatever reason, long-chain fatty acyl-CoA's would excessively accumulate in the cell; in particular in the oxidation of those that are less desirable as substrates for mitochondrial β-oxidation. Thus the products of the peroxisomal β-oxidation pathway (short-chain acyl-CoA's) should be ideally suited for subsequent oxidation by the mitochondrial β-oxidation pathway (figure 6). The presence of short to medium-chain carnitine acyltransferase(s) in the peroxisome (77, and tables 1B and 5B) would thus function to convert the short-chain acyl-CoA's

(and acetyl-CoA) leaving the peroxisome into their corresponding carnitine derivatives, thereby serving a dual function of conserving CoA within the peroxisome, and speeding the transport (across the cytosol) and uptake of these short-chain fatty acids into the mitochondrion.

The acetyl-CoA and NADH produced by the peroxisomal β-oxidation pathway would serve to create an environment in the cell cytosol conducive to gluconeogenesis; the acetyl-CoA activating pyruvate carboxylase and the NADH maintaining the higher cytosolic NADH/NAD ratio needed for high gluconeogenetic rates.

Very recently Goodman et al. (78) have reported the presence of the glyoxylate bypass enzymes (isocitrate lyase and malate synthase), as well as the peroxisomal β-oxidation pathway in the peroxisomes of toad urinary bladder epithelial cells. This paper also reported net gluconeogenesis from palmitate and its stimulation by aldosterone. It is of course very interesting to speculate as to whether the rat liver peroxisome also contains the glyoxylate bypass enzymes which have upto date remained undetected there. If they should be present there, the functional role of the peroxisomal β-oxidation system in gluconeogenesis would then become undisputable, as is the case with Tetrahymena (26,53, and part I of this thesis).

EPILOGUE

If you have an important point to make, don't try to be subtle or clever. Use a pile driver. Hit the point once. Then come back and hit it again. Then hit it a third time--a tremendous whack.

Winston Churchill

The conservation of energy of peroxisomal oxidations by the establishment of a thermal-gradient: A hypothesis.

Peroxisomes have been widely recognized as cellular organelles for many years (19,79,80). As the name suggest, peroxisomal function has long been characterized as involving the production and degradation of hydrogen peroxide, with the peroxisomal oxidases being active in the formation of this metabolite and catalase catalyzing its destruction (19).

Oshino et al. (81) have observed peroxisomal H_2O_2 production when rat livers were perfused with urate and glycolate (substrates for urate oxidase and L-a-hydroxy acid oxidase, respectively), and demonstrated that this constituted nearly half of the total liver respiration. Thus they concluded that biological oxidations of considerable physiological significance are possible in the peroxisome. Therefore, constant production of H_2O_2 in the peroxisome is now established as a normal part of liver metabolism (as well as in other tissues) (80). Although our knowledge of the endogenous substrates of the peroxisome is still very limited, the recent discovery of the peroxisomal fatty acyl-CoA oxidase (26,33,36) and the β-oxidation pathway that this novel oxidase initiates (18,26,28,33), have begun to clarify the cellular functions of the peroxisome.

The generation of H_2O_2 by peroxisomal H_2O_2-producing

oxidases has been much too readily dismissed as the formation of a toxic and wasteful byproduct, that is rapidly removed from the scene by the action of catalase. It has been suggested that the only evolutionary advantage in the presence of catalase is to protect the cell (organism) against the toxic effects of H_2O_2 (19,82). Indeed, de Duve (82,83) has claimed that the respiratory chain of peroxisomes ($O_2 \longrightarrow H_2O_2 \longrightarrow H_2O$, catalyzed by oxidases and catalase) is of a very primitive nature, since it carries no provision for the conservation of energy and catalyzes an essentially wasteful form of respiration. Thus it can be (and has been) argued that the first oxidative step in the peroxisomal β-oxidation system, that is catalyzed by the fatty acyl-CoA oxidase, as well as all the other oxidations catalyzed by peroxisomal H_2O_2-producing oxidases, is totally wasteful insofar as free energy conservation is concerned (80,84). However, this may not be so. It should be pointed out that peroxisomal respiration could, despite its seemingly wasteful nature, be energetically useful.

I would like to submit that a part of the energy of peroxisomal oxidations is conserved in the form of H_2O_2 (analogous to ATP formation in mitochondrial oxidations). The action of catalase on the H_2O_2 releases heat ($\Delta H_o'$ for $H_2O_2 \longrightarrow H_2O + \frac{1}{2}O_2$ is -26 Kcal/mole) into the peroxisol (inner liquid portion of the peroxisome). The peroxisomal mem-

brane serves as a thermal barrier (lipids are good heat insulators) separating the relatively hotter peroxisol from the cytosol, thus creating a thermal-gradient. This thermal-gradient can be put to use by the cell in several constructive and energetically useful manners as follows:

A) Thermogenesis. The steady and controlled heat flow out of the peroxisome and into the cytosol can be used in the heating of the cell (or organism). Thus in analogy to the mitochondria ("the powerhouse of the cell"), the peroxisome may be termed "the furnace of the cell".

B) Thermal currents. The thermal-gradient across the peroxisomal membrane could create thermal currents (or convection currents) in the cell cytosol. These thermal currents emanating from the surface of the peroxisome could be the driving force for cellular cytoplasmic streaming. Thus the peroxisome would be involved in powering the movement of intracellular components within the cell.

C) Conformational changes. The thermal-gradient could create conformational or structural changes in the peroxisomal membrane and/or in proteins embeded within the peroxisomal membrane (intrinsic proteins). The thermal-gradient across the peroxisomal membrane could result in energy-dependent shifts in the number or location of weak bonds (hydrogen bonds, hydrophobic interactions) maintaining the three-dimensional conformation of the membrane or intrinsic

proteins. Thus the "energized" conformational state could be used in the transport of metabolites across the peroxisomal membrane (the transport of acyl-CoA's into the peroxisome), simultaneously undergoing a reversion to its original low-energy conformational state.

It is generally believed that life originated as an anaerobic proccess, and remained so until the appearance of free oxygen in the primitive atmosphere. As living organisms adapted to the presence of oxygen in the environment, two distinct lines of primitive cells appeared. One line (a primitive aerobic bacterium) would have owed its success to an enhanced ability to extract energy from a given amount of food (oxidative phosphorylation), the other to a greater capacity to capture and utilize food (primitive phagocytes containing lysosomes and peroxisomes). This primitive phagocytic organism of relatively large size, and dependent on peroxisomes for respiration (and the ability to extract some kind of energy from phagocytized food), later became the host of the symbiotic ancestors of the mitochondria (symbiotic adoption of the primitive aerobic bacterium), and eventually evolved to form eukaryotic cells. Once the symbiotic relationship became established, some sort of evolutionary competition between the two respiratory particles took place. In general, one would expect the mitochondria to win in such a contest on the grounds of greater kinetic

and thermodynamic efficiency. However, special functions of the peroxisome may have favoured retention of parts of the ancestral peroxisome, thus explaining the present properties and distribution of these particles. Although all known types of peroxisomes do not have a ubiquitous enzymatic complement, two facts are becoming apparent; first, fatty acids are oxidized by peroxisomes, thus fats appear to be the principal fuel in peroxisomal respirations, and second, the most constant component of all peroxisomes is the presence of high concentration of catalase, together with some kind of H_2O_2-producing oxidase. Therefore, the simple respiratory chain of peroxisomes (catalyzed by oxidases and catalase) must play an important physiological role in the cells in which they are found. The hypothesis presented above speculates on the nature of the physiological function of the peroxisomal respiratory system. The hypothesis postulates that the energy of the respiratory chain of peroxisomes is conserved by the establishment of a thermal-gradient in such a way that it can do several different kinds of work: 1) heating of the cell and/or organism (thermogenesis), 2) the movement of metabolites and organized structures within the cell cytosol (cytoplasmic streaming), and 3) the mechanical work of conformational changes in the peroxisomal membrane or peroxisomal intrinsic proteins.

To term the respiratory chain of peroxisomes primitive

in contrast to the cytochrome system of respiration (mitochondrial), however, appears inappropiate. Both functions developed in response to the presence of free oxygen in the primitive atmosphere, both are necessary to the cellular existence in eukaryots, and both must be regulated to meet internal needs and external stresses.

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