Order Number 8820839

The effect of inhibitors and growth hormone on fatty acid oxidation in rat myocytes

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THE EFFECT OF INHIBITORS AND GROWTH HORMONE ON FATTY ACID OXIDATION IN RAT MYOCYTES

by

Salah Abdel-aleem

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

1988

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

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ABSTRACT

The Effect of Inhibitors and Growth Hormone on Fatty Acid Oxidation In Rat Myocytes

by

Salah Abdel-aleem

Advisor: Porfessor Horst Schulz

The effects of 4-bromocrotonic acid, 2-bromopalmitic acid, 3-mercaptopropionic acid, 4-pentenoic acid, and 2-tetradecylglycidic acid on the oxidation of palmitate, octanoate, and pyruvate in adult rat myocytes were studied. Since all of these compounds inhibit the oxidation of palmitate but not of pyruvate, they are specific inhibitors of fatty acid oxidation. Fifty percent inhibition of palmitate oxidation was obtained when myocytes were preincubated for 10 min with 0.1 μM 2-tetradecylglycidic acid, 60 μM 4-bromocrotonic acid, 60μM 2-bromopalmitic acid, 100 μM 3-mercaptopropionic acid, or 100 μM 4-pentenoic acid. Removal of the inhibitors from the medium after preincubation relieved inhibitions caused by 3-mercaptopropionic acid or 4-pentenoic acid but did not reverse the effects of the other inhibitors. This study leads to the conclusion that 2-tetradecylglycidic

acid is the compound of choice for inhibiting the mitochondrial uptake of fatty acids and thereby their oxidation while 4-bromocrotonic acid is the best irreversible inhibitor of mitochondrial β-oxidation.

The effect of growth hormone on the oxidation of oleate and docosahexaenoate was studied in rat heart mitochondria and myocytes isolated from control rats, hypophysectomized rats and hypophysectomized rats treated with growth hormone. Although growth hormone treatment increased the activity of 2,4-dienoyl-CoA reductase 2-fold, the rate at which docosahexaenoate and docosahexaenoylcarnitine are oxidized in control rats were similar to rates observed with hypophysectomized rats or hypophysectomized rats treated with growth hormone. This study suggests that the reaction catalyzed by 2,4-dienoyl-CoA reductase is not rate-limiting in the oxidation of polyunsaturated fatty acids.

To: Maro, Omar and Tarek

ACKNOWLEDGEMENTS

I would like to acknowledge the assistance and encouragement of my thesis committee members. I would like to thank my colleagues for their help and assistance. I am very grateful to my thesis supervisor Dr. Horst Schulz for his assistance, patience and encouragemnt.

Table of Contents

Table of Contents (continued)

LIST OF TABLES

LIST OF FIGURES

List of Figures (continued)

ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
AMP	adenosine 5'-monophosphate
BSA	bovine serum albumin
CoA	coenzyme A
CoASH	reduced coenzyme A
CPT	carnitine palmitoyltransferase
FAD	flavine adenine dinucleotide
FADH	reduced flavin adenine dinucleotide
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
GH	growth hormone

INTRODUCTION

In mammalian heart, fatty acids are the main substrate for energy metabolism (1), even though glycolysis does occur. De novo fatty acid synthesis is not thought to be a major metabolic pathway in heart, whereas fatty acid elongation desaturation and complex lipid synthesis take place in this tissue. Regulation of these processes is not fully understood.

In tissues which oxidize long-chain fatty acids, these acids must be activated in the cytosol by the enzyme fatty acyl-CoA synthetase (EC 6.2.1.3), which is located in the outer mitochondrial membrane (2,3) and the endoplasmic reticulum (4,5). The activated fatty acyl moiety can either be channeled into lipid biosynthesis or β-oxidation (6). In order for β-oxidation to occur, the fatty acyl moiety must cross the inner mitochondrial membrane. For long-chain fatty acyl groups, this process is facilitated by the carnitine-acyltransferase-uptake system. The first step in the β-oxidation cycle is the dehydrogenation of acyl-CoA thioester which is catalyzed by acyl-CoA dehydrogenases (EC 1.3.99.3 and EC 1.3.99.2). The reaction product, 2-trans-enoyl-CoA, is hydrated by enoyl-CoA hydratase (EC 4.2.1.17) to yield L-3-hydroxyacyl-CoA. The second dehydrogenation reaction, by which the 3-hydroxy

group is converted to a keto group, is catalyzed by 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35). The final step is the cleavage of 3-ketoacyl-CoA by 3-ketoacyl-CoA thiolase (EC 2.3.1.16) to yield acetyl-CoA and chain shortened acyl-CoA which undergoes further cycles of β-oxidation until it is completely degraded to acetyl-CoA (Fig. 2).

Oxidation of Unsaturated Fatty Acids in Mammalian Heart and Liver Mitochondria

Although the steps of β-oxidation by which saturated fatty acids are degraded are well known, the oxidation pathway of unsaturated fatty acid containing cis double bonds had not been fully elucidated until recently. According to the generally accepted pathway two enzymes are required, 3-cis,2-trans-enoyl-CoA isomerase (EC 5.3.3.8) and 3-hydroxyacyl-CoA epimerase (EC 5.1.2.3), in addition to the β-oxidation enzymes necessary for the degradation of saturated fatty acids (Fig. 3A) (7). The isomerase catalyzes the conversion of 3-cis-enoyl-CoA intermediates to the 2-trans isomers (8) which can be further degraded by β-oxidation. The epimerase catalyzes the epimerization of D-3-hydroxyacyl-CoA esters to the corresponding L-hydroxy isomers (7), which can be completely degraded by the

β-oxidation enzymes. Kunau and Dommes (9) have presented evidence that the oxidation of 4-cis-decenoyl-CoA, a presumed intermediate of linoleate oxidation follows a different pathway requiring 2,4-dienoyl-CoA reductase. Acyl-CoA dehydrogenase catalyzes the conversion of 4-cis-decenoyl-CoA to 2-trans,4-cis-decadienoyl-CoA. This compound is reduced to 3-decenoyl-CoA by the NADPH-dependent 2,4-dienoyl-CoA reductase (9,10) (Fig. 3B). 3-Decenoyl-CoA thus formed is isomerized to 2-trans-decenoyl-CoA by the 3-cis,2-trans-enoyl-CoA isomerase. The latter compound can be degraded completely by passing 4-times through the β-oxidation cycle.

More recently, Cuebas and Schulz (11) demonstrated that 2-trans,4-cis-decadienoyl-CoA can not be degraded by the pathway proposed by Stoffel and Caesar (7). It was shown that 2-trans,4-trans-decadienoyl-CoA was rapidly degraded via β-oxidation, whereas a mixture of 2-trans,4-cis-decadienoyl-CoA and 2-cis,4-cis-decadienoyl-CoA was not degraded via β-oxidation by either rat heart mitochondria or a reconstituted β-oxidation system even when the crotonase concentration was increased 10-fold over the physiological level sufficient for the oxdiation of 2-trans,4-trans-decadienoyl-CoA (11). However, all three isomers of 2,4-decadienoyl-CoA were substrates for the NADPH-dependent 2,4-dienoyl-CoA reductase (11). The

pathway proposed by Cuebas and Schulz (11) for the degradation of linoleic acid is presented in Fig. 3 B. The product of the reaction catalyzed by 2,4-dienoyl-CoA reductase is 3-decenoyl-CoA (9,12) which can be isomerized to 2-trans-decenoyl-CoA by the 3-cis,2-trans-enoyl-CoA isomerase (8). 2-trans-Decenoyl-CoA can then be completely degraded by the normal β-oxidation pathway.

Stoffel and Caesar (7) have demonstrated that the 3-cis,2-trans-enoyl-CoA isomerase and the 3-hydroxyacyl-CoA epimerase are present in both rat liver and rat heart mitochondria in addition to the normal β-oxidation enzymes. However Chu et al. (13) have only been able to detect an extremely low level of the epimerase activity in any mammalian heart tissue examined. It was not clear whether the epimerase activity observed in heart tissue could support the level of linoleic acid degradation expected by this tissue. In order to further investigate this point, oxidation of palmitoyl-CoA, oleoyl-CoA and linoleoyl-CoA were compared in rat heart and rat liver mitochondria. The same mitochondrial preparations were assayed for 3-cis,2-trans-enoyl-CoA isomerase, 2,4-dienoyl-CoA reductase, and 3-hydroxyacyl-CoA epimerase activities by Chu et al. (14) who concluded that the epimerase activity present in heart is insufficient to sustain the observed oxidation of linoleate.

Inhibitors of Fatty Acid Oxidation

Studies of fatty acid oxidation, especially of its regulation, have been greatly aided by the availability of specific inhibitors which have also been investigated for their use as oral hypoglycemic agents. Three reactions of the β-oxidation pathway are inhibited by these inhibitors; those catalyzed by acyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolases, and carnitine palmitoyltransferase I (CPT I). These inhibitors are carboxylic acids and they must be converted to their coenzyme A thioesters and in most cases metabolized through the β-oxidation pathway to compounds that bind reversibly or irreversibly to the active site of the target enzyme, thereby inhibiting it. Generally these inhibitors have structural similarity to fatty acids, in addition they have a functional or potentially reactive group which upon metabolic conversion will produce a highly reactive and active site-directed inhibitor.

Inhibitors of Acyl-CoA Dehydrogenase

The first inhibitor of fatty acid oxidation to be studied was hypoglycin, the toxic principle of the unripe arillus of the ackee fruit, which is believed to cause hypoglycemia as a consequence of inhibiting β-oxidation (15-

17). Hypoglycin is converted by transamination to methylenecyclopropylpyruvic acid which is converted intramitochondrially to methylenecyclopropylacetyl-CoA (15). The latter is a strong inhibitor of butyryl-CoA dehydrogenase (17-18). Studies with isolated liver mitochondria suggested that methylenecyclopropylacetyl-CoA inhibits butyryl-CoA dehydrogenase by forming a convalent adduct with the FAD cofactor of the enzyme (19).

3-Mercaptopropionic acid, a known convulsant agent, has been found to inhibit fatty acid oxidation in isolated rat heart mitochondria (20). 3-Mercaptopropionic acid is converted intramitochondrially to 3-mercaptopropionoyl-CoA (21), which in the presence of thiolases is metabolized to S-acyl-3-mercaptopropionoyl-CoA thioesters (21), which inhibit reversibly long-chain and short-chain acyl-CoA dehydrogenases (20).

2-Mercaptoacetic acid has been found to inhibit hepatic fatty acid oxidation possibly by inhibiting acyl-CoA dehydrogenase (22,23).

Inhibitors of Thiolases

The search for structurally simpler analogs of hypoglycin led to the identification of 4-pentenoic acid which inhibits fatty acid oxidation in rats (16), the

perfused rat liver (24), rat liver mitochondria (17) and rat heart mitochondria (25). 4-Pentenoic acid also induces ketosis (26). Studies with isolated rat heart mitochondria suggested that 4-pentenoic acid is metabolized via a major pathway and a minor pathway (27,28). The major and minor pathways share the activation of 4-pentenoic acid to 4-pentenoyl-CoA and the dehydrogenation of 4-pentenoyl-CoA to 2,4-pentadienoyl-CoA by butyryl-CoA dehydrogenase. 2,4-Pentadienoyl-CoA is converted by the NADPH-dependent 2,4-dienoyl-CoA reductase of the major pathway to 3-pentenoyl-CoA which is isomerized to 2-pentenoyl-CoA and then oxidized via β-oxidation to propionyl-CoA and acetyl-CoA (27,28). The minor pathway involves the direct β-oxidation of 2,4-pentadienoyl-CoA to acryloyl-CoA and acetyl-CoA (28). An intermediate in the minor pathway is 3-keto-4-pentenoyl-CoA which is a reversible as well as an irreversible inhibitor of 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase (28). This hypothesis is supported by the observation that increasing the activity of 2,4-dienoyl-CoA reductase by clofibrate feeding and thereby the capacity of the major pathway (29), causes a decrease in the toxic effect of 4-pentenoate.

Another compound which inhibits 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase and thus the oxidation of fatty acids is 4-bromocrotonic acid (30). Studies

with isolated rat heart mitochondria, suggested that 4-bromocrotonic acid is converted intramitochondrially to 4-bromocrotonyl-CoA which is metabolized via β-oxidation to 3-keto-4-bromobutyryl-CoA which inactivates irreversibly both 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase. 4-Bromocrotonic acid at micromolar concentrations, has been found to inhibit fatty acid oxidation in the perfused rat heart (31).

A third inhibitor of thiolases and fatty acid oxidation is 2-bromooctanoic acid. This inhibitor is effective at micromolar concentrations in isolated rat liver mitochondria where it completely inhibits 3-ketoacyl-CoA thiolase (32). 2-Bromooctanoic acid is activated intramitochondrially to 2-bromooctanoyl-CoA and then converted via β-oxidation to 2-bromo-3-ketooctanoyl-CoA which causes the irreversible inactivation of both thiolases. 2-Bromooctanoic acid has also been found to inhibit fatty acid oxidation and ketogenesis in the perfused rat liver (33).

Inhibitors of Carnitine Palmitoyltransferase I

2-Tetradecylglycidic acid, at low micromolar concentrations, inhibits fatty acid oxidation in rat hepatocytes, liver mitochondria and the perfused heart (34). The inhibition of gluconeogenesis due to 2-

tetradecylglycidic acid seems to be secondary to the inhibition of fatty acid oxidation (35). Since tetradecylglycidic acid inhibits the oxidation of palmitate, but not of palmitoylcarnitine or octanoate (35), the inhibitory target was assumed to be carnitine palmitoyltransferase I. Recently, it was shown that 2-tetradecylglycidic acid and 2-bromopalmitic acid bind to the mitochondrial membranes from liver and muscle at a common locus in such a way that the percentage occupancy of this site is directly proportional to the extent of inhibition of CPT I (36). 2-Tetradecylglycidic acid is converted intracellularly into 2-tetradecylglycidyl-CoA which is a powerful and irreversible inhibitor of CPT I (37).

2-Bromopalmitic acid inhibits the oxidation of palmitate, but not of palmitoylcarnitine in rat hepatocytes (38). Whereas, in liver mitochondria 2-bromopalmitoyl-CoA strongly inhibits the oxidation of palmitoyl-CoA, but not of palmitoylcarnitine, pyruvate, or hexaenoate, suggesting that carnitine palmitoyltransferase I is the inhibitory target of this compound (39).

More recently aminocarnitine and acylaminocarnitines, as for example palmitoylaminocarnitine, have been identified as reversible inhibitors of carnitine palmitoyltransferase I and thus of fatty acid oxidation (40,41).

Regulation of Mitochondrial Fatty Acid Oxidation In Heart (42)

In heart tissue the concentration of free fatty acids and the energy demand of the tissue are the main factors that control the oxidation of fatty acids except at high levels of free fatty acids, the rate becomes dependent only on the energy demand of the tissue. Studies with perfused hearts and isolated mitochondria under conditions of reduced energy demand have demonstrated that the concentration of NADH and acetyl-CoA increase and those of NAD^+ and CoASH decrease. An increase in the ratio of NADH to NAD^+ in mitochondria causes inhibition of the tricarboxylic acid cycle and consequently an increase in the ratio of acetyl-CoA to CoASH. Acetyl-CoA, NADH, and $FADH_2$ are products of fatty acid oxidation, and their accumulation may inhibit β-oxidation. Since 3-hydroxy fatty acids accumulate when oxidative phosphorylation is inhibited in isolated mitochondria, the first degradation step in β-oxidation is apparently not the primary site of regulation. Kinetic studies with purified 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase have revealed their severe inhibition by NADH and acetyl-CoA respectively (76). When the mitochondrial activities of these two enzymes are calculated at low and high concentrations of

NADH and acetyl-CoA, it appears that 3-ketoacyl-CoA thiolase may be the primary site at which β-oxidation is regulated (76). However, the influx of reducing equivalents into mitochondria could also cause an inhibition of β-oxidation.

Effects of Growth Hormone on Carbohydrate and Lipid Metabolism

Growth hormone (GH) is synthesized and secreted by the anterior pituitary in man and most vertebrates (43). Human growth hormone (hGH) is a single-chain protein which is composed of 191 amino acid residues. It has a molecular weight of 21,500 and contains two disulfide bridges. It shows significant sequence homology with human placental lactogen (hPL) and prolactin (PRL) with which it shares 161 out of 191 amino acid residues.

Minutes after GH is injected into animals, the liver ceases to release glucose and the blood sugar concentration falls (44). No acute elevations of insulin levels are causing the hypoglycemic response, even though GH affects secretions of insulin and pancreatic polypeptides (45). Nevertheless, some prior exposure to insulin is necessary (46). Within 30 min glucose levels return to or exceed the normal range, presumably due to compensatory

release of hyperglycemic hormones.

Glucose uptake increases within 20 min after GH is presented in vitro to skeletal muscle from hypophysectomized animals. Although muscle uptake of glucose is responsible for most of the early hypoglycemic effects of insulin, it probably makes little contribution to the blood sugar changes that rapidly follow GH injection into normal animals. Such stimulation is not seen with tissues taken from either normal or GH pretreated hypophysectomized animals. In addition, hypophysectomized tissues become refractory to this stimulation within an hour, after which they take up glucose at lower rates than tissues not subjected to the hormone.

GH may participate in glucose homeostasis under normal conditions. GH-deficient humans and hypophysectomized animals become hypoglycemic under fasting conditions. Antibodies directed against GH can depress sugar levels of normal animals. Moderate dosages of GH decrease glucose tolerance within 2 hours in healthy humans. Chronic overdosage leads to sustained hyperglycemia in laboratory animals and delayed effects include secretion of insulin, changes in hormone receptor numbers and hormone affinities of receptors (47).

When GH is given in moderate dosages, it increases glycogen storage in skeletal muscle. Studies with fatty

cells indicate that the hormone antagonizes the glycogenolytic effects of other regulators and thereby augments glycogen synthesis.

When GH is administered to food-deprived hypophysectomized animals, the animals have higher blood glucose levels than untreated controls. They also display better protein conservation while losing more fat. Since GH is lipolytic (48), it is possible that elevated free fatty acid concentrations in the blood plasma reduce glucose uptake by skeletal muscle cells. However, at moderate dosages GH has not been found to elevate plasma fatty acid concentrations (47).

Some evidence for early, transient stimulation of glucose uptake by adipocytes of hypophysectomized animals has been obtained (49). However, adipocytes isolated from hypophysectomized animals, show transport rates of glucose at maximal levels which can not be further stimulated by insulin. GH administration normalizes the basal rate of glucose uptake as well as restoring sensitivity to insulin. It has therefore been suggested that GH induces or activates a factor that limits glucose uptake under physiological conditions (50).

It is probable that insulin-like effects of GH are not important under normal conditions, since GH is secreted and it invokes refractoriness. However, stress usually inhibits GH secretion in rats, and tissues from normal,

stressed rats display the antilipolytic response (51).

It has been shown that stimulation of mitochondrial protein synthesis (52) and changes in the composition of mitochondrial phospholipids are caused by GH (53). Specifically, GH treatment of hypophysectomized rats results in incorporation of more polyunsaturated fatty acids, such as docosahexaenoic acid, into mitochondrial phospholipids (53). The authors suggested that this alteration of mitochondrial phospholipids results in an increased membrane fluidity which in turn may result in activity changes of some membrane-bound enzymes (53). Specific examples include increases in the activities of NADH dehydrogenase and the energy dependent transhydrogenase as a result of GH treatment of hypophysectomized rats.

Recently, Clejan and Schulz (54), demonstrated that GH stimulates rates of respiration supported by polyunsaturated fatty acylcarnitines in mitochondria isolated from hypophysectomized rats treated with GH. Also it has been shown that, the activity of 2,4-dienoyl-CoA reductase increased threefold in these mitochondrial preparations.

AIM OF RESEARCH

Although the number of available inhibitors of fatty acid oxidation has increased significantly over the last few years, choosing the best one for a given study is complicated by the fact that they were evaluated by use of different biological systems. This study is aimed at establishing the effectiveness of most of the known inhibitors of fatty acid oxidation in adult rat myocytes. In addition it attempted to answer some unresolved questions about the mechanisms of action of these compounds.

The finding that growth hormone stimulates rates of respiration supported by polyunsaturated fatty acylcarnitines and that it causes a parallel increase of 2,4-dienoyl-CoA reductase activity in rat liver mitochondria, prompted us to study the effect of growth hormone on the oxidation of docosahexaenoic acid in myocytes isolated from hypophysectomized animals treated with growth hormone. This study may clarify some aspects of regulation of oxidation of polyunsaturated fatty acids in heart.

MATERIALS AND METHODS

Materials

[1-^{14}C]Palmitic acid, [1-^{14}C]octanoic acid, and [2-^{14}C] pyruvate were obtained from Amersham, whereas, [10-^{14}C]oleic acid, and 4,7,10,13,16,19-[1-^{14}C]docosahexaenoic acid were purchased from New England Nuclear. Collagenase (type II) was obtained from Worthington, whereas Sigma was the source of bovine growth hormone, hyaluronidase (type I), bovine serum albumin (essentially free of fatty acids IV), 3-mercaptopropionic acid, ADP, palmitoylcarnitine, and most standard biochemicals. NAD+, NADH, CoASH were purchased from P-L Biochemicals. Crontonyl-CoA (55), acetoacetyl CoA (56), 3-ketooctanoyl-CoA (57), and 2-trans,4-trans-decadienoyl-CoA (11) were synthesized according to published procedures. 2-Bromopalmitic acid and 4-pentenoic acid were purchased from Fluka A. G., Switzerland. 4-Bromocrotonic acid was synthesized as described [58]. 2-Tetradecylglycidic acid were provided by Dr. John Lowenstein, Brandeis University. Hypoglycin was a gift of Dr. E. A. Kean, University of the West Indies. Fisher Scientific was the source of Scinti Verse II. Docosahexaenoylcarnitine was a gift of Dr. S. Clejan, City Hospital Center at Elmhurst and Mount

Sinai School of Medicine.

Animals and Hormone Treatment

Male Sprague - Dawley rats were obtained from Taconic Farms, Inc., Germantown, N.Y. and maintained on Purina laboratory chow ad libitum. Control rats weighed 200-250 g. while hypophysectomized rats weighed 150-175 g. Hypophysectomized rats were provided with 5% dextrose solution instead of water. Hypophysectomized rats were kept for at least 3 weeks in our animal unit, then growth hormone, 0.1 IU/100 g. body weight, was injected subcutaneously daily for 7 days (59). Hypophysectomized rats injected with growth hormone gained approximately 15 grams per seven days of hormone injection while untreated hypophysectomized rats did not gain any weight during this time.

Isolation of Myocytes

Adult rat myocytes were isolated by the method of Frangakis et al. (60) except that the hearts were perfused with a Ca^{2+}-free Krebs-Henseleit buffer containing 118 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$ and 0.93 mM KH_2PO_4. Hearts rapidly excised from adult rats were perfused in a

Langendorff apparatus with a pressure equal to 60 mm Hg. After the heart was devoid of blood, the perfusion medium was replaced with medium containing 1 mg/ml of collagenase type II, 1 mg/ml hyaluronidase Type I and 0.03% bovine albumin. The enzymic perfusion medium was recirculated at 37°C for 15 min or until the heart become flaccid. The heart was placed in a 25 ml buffer, divided into 8 pieces, incubated for 15 min at 37°C with constant shaking. The process of incubating and decanting was repeated 3-4 times until nearly all of the tissues had been disaggregated into individual cells. The cells were twice resuspended in 40 ml of enzyme-free buffer and allowed to settle. Intact cells settled within 15 min, whereas broken or damaged cells did not and were easily removed by aspiration (Fig. 1). The viability of myocytes isolated by this procedure was 80-90% as judged by trypan blue exclusion. The cells oxidized palmitic acid linearly for up to 60 min (Fig. 5).

Metabolic Studies with Myocytes

Myocytes (3 mg protein) suspended in 0.9 ml of Krebs-Henseleit buffer containing 118 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 0.93 mM KH_2PO_4 and 0.5 mM $CaCl_2$ were placed in a 25 ml Erlenmeyer flask. To this cell

suspension was added 20 µl of an inhibitor solution to give the desired concentration of the inhibitor. Stock solutions of inhibitors were prepared by dissolving compounds with short or medium hydrocarbon chains like 4-bromocrotonic acid, 3-mercaptopropionic acid, 4-pentenoic acid, 2-bromooctanoic acid, and hypoglycin in the cell suspension buffer. The pH was kept at 7.4 by the addition of KOH. The molar ratio of 2-tetradecylglycidic acid or 2-bromopalmitate to BSA was 4:1. After preincubating the myocytes with one of the inhibitors for 10 min at 37°C under constant shaking, 0.1 ml of a metabolic substrate was added to the cell suspension to give a final concentration of 0.2 mM[1-^{14}C]-palmitic acid (2.5 x 10^5 dpm), 0.2 mM[10-^{14}C]-oleic acid (2.5 x 10^5 dpm), 0.2 mM [1-^{14}C]-docosahexaenoic acid (2.4 x 10^5 dpm), 0.2 mM [1-^{14}C]-octanoate (3.2 x 10^5 dpm) or 2 mM [2-^{14}C]-pyruvate (1.8 x 10^5 dpm).

Stock solutions of the substrates were prepared by dissolving sodium pyruvate or octanoic acid in the cell suspension buffer and by dissolving palmitic acid, oleic acid or docosahexaenoic acid in a solution of defatted bovine serum albumin in the cell suspension buffer. The molar ratio of the fatty acid substrate to albumin was 2:1 (Fig. 4). The Erlenmeyer flask was then closed with a rubber septum to which a plastic center well was attached. The incubation was continued under shaking at 37°C for 30

min or as indicated. After that time, 0.4 ml of 1 M hyamine hydroxide was injected through the spectum into the center well, and the reaction was terminated by injecting 0.3 ml of 70% perchloric acid through the septum into the incubation medium. The flask was shaken continuously for 2 hr at 37°C at which time the plastic center well was removed, placed into a scintillation vial containing 4 ml of Scinti Verse II, and counted in a liquid scintillation counter. Control experiments with $NaH^{14}CO_3$ added to the cell suspension proved that the release of $^{14}CO_2$ was complete 1 hr after the addition of perchloric acid (Table 2).

Isolation of Mitochondria and Oxygen Uptake Measurement

Mitochondria were isolated from the hearts of adult rats by the following method. The beating hearts were removed and placed in ice-cold MST (61) (210 mM mannitol, 70 mM sucrose, 5 mM Tris, 1 mM EGTA, adjusted to pH 7.4 with HCl). The hearts were cut open, cleaned of blood vessels and washed free of blood in ice-cold MST. The hearts were finely minced. The suspension was stirred continously for 10 min at 0°C with 3 mg of Nagase/heart, a general protease used to soften the heart tissue by degrading some of the connective tissue protein. The

resulting suspension was transferred to a glass homogenization tube. After dilution to approximately 30 ml in ice-cold MST, the suspension was homogenized by 5 strokes of a loose-fitting Teflon pestle spinning at 750 rpm. The homogenate was centrifuged at 20,000 rpm in an SS-34 rotor of a Sorvall centrifuge for 7 min at 4°C. The pellet was resuspended in about 30 ml of ice-cold MST with a rubber policeman and homogenized by one stroke of the glass-Teflon tissue homogenizer. The resulting suspension was centrifuged at 10,000 rpm in an SS-34 rotor for 7 min at 4°C. The pellet, each time, was resuspended in about 30 ml ice-cold MST with a rubber policeman and homogenized by one stroke of the glass-Teflon tissue homogenizer. The resulting suspension was centrifuged at 2,300 rpm in an SS-34 rotor of a Sorvall centrifuge for 5 min at 4°C. The supernatant was decanted through two layers of cheesecloth into 15 ml Corex tubes. These tubes were centrifuged at 8,000 rpm in an SS-34 rotor for 7 min at 4°C. The mitochondrial pellets were resuspended into a small volume of ice-cold MST using a rubber policeman. Mitochondria prepared in this way were used fresh or stored frozen at -80°C and used after thawing.

For oxygen uptake measurements, mitochondria (0.5 to 1 mg/ml) were incubated in 1.9 ml of a basal isotonic

medium containing 0.11 M KCl, 33 mM Tris-HCl (pH 7.4), 2 mM KP_i, 2 mM $MgCl_2$, and 0.1 mM ethyleneglycol bis(β-aminoethylether)-N,N,N'N'-tetracetic acid. To this suspension were added bovine serum albumin (0.5 mg/ml), and 0.5 mM L-malate. Respiration rates were measured polarographically by use of a Clark oxygen electrode with either palmitoylcarnitine (30 μM) or docosahexaenoylcarnitine (30 μM) in the presence of 0.5 mM ADP.

Enzyme Assays

All enzyme assays were performed at 25°C. Enzyme activities were determined spectrophotometrically on a Gilford recording spectrophotometer. Enoyl-CoA hydratase was measured spectrophotometrically at 263 nm as described (62). rotonyl-CoA (30 μM) served as a substrate. 3-Hydroxyacyl-CoA dehydrogenase was assayed spectrophotometrically at 340 nm. The standard assay mixture contained 0.05 M Kp_i (pH 7), 0.06% Triton X-100, 0.12 mM NADH, and bovine serum albumin (0.11 mg/ml). The reaction was started by the addition of acetoacetyl-CoA to a final concentration of 30 M. The activities of both thiolases were determined by following spectrophotometrically the disappearence of the Mg^{2+}-enolate complex at 303 nm. The reaction mixture contained 0.1 M Tris HCl (pH 8.2), 25 mM $MgCl_2$, 30 mM KCl,

0.06% Triton X-100, bovine serum albumin (0.13 mg/ml), 70 M CoASH and extinction coefficient of 21,000 and 14,400 cm^{-1} M^{-1} were used to calculate the rates determined with acetoacetyl-CoA (50 μM) and 3-ketooctanoyl-CoA (30 μM), respectively (63). 2,4-Dienoyl-CoA reductase was assayed by recording the decrease in absorbance due to the oxidation of NADPH (9). An assay mixture contained 48 mM Kp_i (pH 7.4), 60 μM NADPH and 30 μM 2-trans,4-trans-decadienoyl-CoA. Protein concentrations were determined by Lowry et al. method (64).

Determination of the Concentration of Coenzyme A Derivatives (65)

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

A small aliquot of the derivative (5-20 μl) was placed in a cuvette with 20 μl of 2 M hydroxylamine, pH 7.0. This was allowed to stand at room temperature for 5 min, to this solution was added 10 μl of Ellman's reagent, 300 μl of 0.1 M KP_i (pH 8), and distilled water to a total volume

of 1 ml. Absorbance at 412 nm was read in a Gilford spectrophotometer. The value obtained was corrected for free CoASH in the solution by performing the Ellman's determination without the addition of hydroxylamine.

RESULTS

I. Fatty Acid Oxidation in Rat Heart Myocytes

For measuring their oxidation fatty acids were complexed with bovine serum albumin and added to a suspension of rat myocytes. Fig. 4 shows that the formation of a palmitate: bovine serum albumin complex with a molar ratio of 2:1 required an incubation time of 90 min at 37°C with constant gentle shaking.

Myocytes oxidized palmitate, present at a concentration of 0.2 mM, linearly for at least 60 min. The observed rate of oxidation of palmitate oxidation was 0.045 nmol/mg protein/min (Fig. 5). Octanoate and pyruvate were intially oxidized at higher rates than that obtained with palmitate but their rates leveled off after approximately 10 min and approached zero after 30 min (see Table 1).

When [1-^{14}C]-palmitic acid was used as a substrate by myocytes, approximately 90% of the radioactive label was found in the CO_2 fraction, whereas the radioactivity in the acid-soluble fraction, accounted only for 10% of the radioactivity (Fig. 5). The radioactive acid-soluble fraction consists mostly of CoA and carnitine esters, of intermediates of the tricarboxylic acid cycle and of

amino acids.

Addition of 5 mM glucose to the cell suspensions had no effect on the oxidation of palmitate (Table 3). Also addition of 2,4-dinitrophenol (DNP) at a concentration between 10^{-7} and 10^{-5}M did not stimulate the oxidation of palmitate (Table 3). Myocytes isolated from rats starved for 24 hours showed almost the same rate observed with cells from fed animals (Table 3).

Control experiments with $NaH^{14}CO_3$ added to the cell suspension proved that the release of $^{14}CO_2$ was complete one hour after the addition of perchloric acid (see Table 2).

II. Evaluation of Inhibitors of Fatty Acid Oxidation in Rat Myocytes

Seven known inhibitors of fatty acid oxidation, listed in Table 4, were tested for their potency in inhibiting the oxidation of palmitate by rat myocytes. The most effective of all the inhibitors was 2-tetradecylglycidic acid, which at a concentration of 0.1 mM caused almost complete inhibition of palmitate oxidation. Also very effective were 2-bromopalmitic acid, 4-bromocrotonic acid, 3-mercaptopropionic acid. Less effective was 4-pentenoic acid which at a concentration of 0.5 mM reduced palmitate

oxidation by almost two-thirds. Hypoglycin, which slightly stimulated fatty acid oxidation, and 2-bromooctanoic acid, which at a concentration of 0.5 mM inhibited this pathway by only 10%, were considered ineffective in rat myocytes and thus were not studied any further.

The extent to which fatty acid oxidation was inhibited by 2-bromopalmitic acid, 4-bromocrotonic acid, 3-mercaptopropionic acid, or 4-pentenoic acid was a function of the preincubating time (Fig. 7). With all compounds optimal inhibitions were obtained after 8 min of preincubation, although some of the short-chain inhibitors seemed to cause maximal effects in shorter time periods.

The effects of the inhibitors on the oxidation rate of palmitate, octanoate, and pyruvate in rat myocytes were studied as a function of the inhibitor concentrations. 2-Tetradecylglycidic acid was the most effective of the five inhibitors. At a concentration of 0.5 μM it caused a decrease in palmitate oxidation by 80%, while pyruvate oxidation remained unaffected (Fig. 8A). However, the inhibitor at concentrations of up to 5 μM was found to stimulate octanoate oxidation by up to 50%, while at an inhibitor concentration of 10 μM the rate of oxidation was back to control levels.

2-Bromopalmitic acid inhibited both palmitate and octanoate oxidation. On the other hand, pyruvate oxidation

was found to be stimulated by 2-bromopalmitic acid (Fig. 8B).

4-Bromocrotonic acid inhibited the oxidation of palmitate and less severely that of octanoate while hardly or not at all affecting pyruvate oxidation (Fig. 8C).

As expected, 4-pentenoic acid caused the inhibition of palmitate oxidation without affecting pyruvate oxidation (Fig. 8D). Surprisingly, the oxidation of octanoate was not inhibited by 4-pentenoic acid.

Finally, 3-mercaptopropionic acid, was found to inhibit palmitate oxidation without having any effect on octanoate or pyruvate oxidation (Fig. 8E).

To determine whether the inhibitions caused by these compounds are reversible or not, myocytes were preincubated with one of the inhibitors for 10 min after which time the cell suspension medium was replaced by inhibitor-free buffer and palmitate oxidation was intiated. As seen in Fig. 9, the inhibition caused by 2-tetradecylglycidic acid, 2-bromopalmitic acid, and 4-bromocrotonic acid persisted after removal of the inhibitors from the incubation medium. In contrast, 3-mercaptopropionic acid, and 4-pentenoic acid caused little inhibition after their removal from the preincubation medium. Thus the first three seem to cause the irreversible inhibition of fatty acid oxidation, whereas 3-mercaptopropionic acid and 4-pentenoic acid appear to be

reversible inhibitors.

III. Effect of Growth Hormone on the Oxidation of Fatty Acids

This part of my project was aimed at determining the effect of growth hormone treatment on the oxidation of polyunsaturated fatty acids in rat heart which is an organ with a much simpler metabolism than is liver and thus more suitable for studying the effects of hormone on fatty acid oxidation.

Rates at which myocytes oxidize oleate or docosahexaenoate were measured as a function of the substrate concentration (see Fig. 10). Values of K_m and V_{max} were determined with both fatty acids. K_m values for oleate and docosahexaenoate were 20 μM and 40 μM, respectively, whereas the V_{max} value with docosahexaenoate were slightly lower (0.075 nmol/mg protein/min) than the value observed with oleate (0.09 nmol/mg protein/min).

Rates of oxidation of oleate and docosahexaenoate in myocytes isolated from control rats, hypophysectomized rats, and hypophysectomized rats treated with growth hormone are shown in Table 5. The rate of oxidation of oleate is 50-70% higher than that of docosahexaenoate in all three

animal groups (control, hypophysectomized, and hypophysectomized treated with growth hormone). Growth hormone treatment of hypophysectomized rats does not have any significant effect on the oxidation rate of either oleate or docosahexaenoate. On the other hand, the oxidation rate of oleate in hypophysectomized animals is 30-40% higher than that of control or hypophysectomized animals treated with growth hormone (Table 5).

Although the oxidation of polyunsaturated fatty acids was not stimulated by growth hormone treatment in rat heart, I wanted to determine whether or not the activities of other enzymes of fatty acid oxidation were affected by the hormone. Thus, several β-oxidation enzymes, including 2,4-dienoyl-CoA reductase, were assayed in mitochondria isolated from control rats, hypophysectomized rats, and hypophysectomized rats treated with growth hormone (Table 7). The specific activities of 2,4-dienoyl-CoA reductase is reduced by 40% in hypophysectomized rats, but increased twofold over normal rats upon growth hormone treatment. In contrast the specific activities of enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase, changed insignificantly in hypophysectomized rats and hypophysectomized rats treated with growth hormone. Therefore, similar to rat liver, growth hormone treatment of hypophy-

sectomized rats caused an increase in the activity of 2,4-dienoyl-CoA reductase. However, in contrast to rat liver, the increase in the activity of this enzyme did not result in a stimulation of docosahexaenoate oxidation.

In an attempt to establish if a step prior to β-oxidation may be slow and thus prevent detecting a relationship between the oxidation of docosahexaenoate and the activity of 2,4-dienoyl-CoA reductase, I measured fatty acid oxidation with isolated mitochondria. Table 6 presents rates of respiration supported by either palmitoylcarnitine or docosahexaenoylcarnitine in rat heart mitochondria isolated from control rats, hypophysectomized rats, and hypophysectomized rats treated with growth hormone. Rates of respiration supported by docosahexaenoylcarnitine are slightly lower than that of palmitoylcarnitine in all three animal groups (control, hypophysectomized, and hypophysectomized rats treated with growth hormone). However, growth hormone treatment of hypophysectomized rats did not result in a stimulation of respiration supported by docosahexaenoylcarnitine.

DISCUSSION

Palmitate and other long-chain fatty acids were offered to myocytes complexed to serum albumin to mimic the physiological situation of fatty acid delivery to tissues, and to enable the solubilization of larger quantities of free fatty acids (66).

Myocytes oxidized 0.2 mM palmitate linearly for up to 60 min at a rate of 2.7 nmol/mg protein/hour. The observed rates of oxidation of palmitate and oleate were similar and reproducible from experiment to experiment. Although it was difficult to compare this rate of palmitate oxidation with published results due to changes in experimental conditions, the value determined here is close to an oxidation rate of 2.85 nmol/mg protein/45 min (67) reported in a similar study when myocytes were incubated with 0.4 mM oleate. On the other hand, octanoate and pyruvate were oxidized at initially higher rates than that of palmitate; their rates of oxidation were 4.7 and 17.6 nmol/mg protein/10 min, respectively. Rates of oxidation of octanoate and pyruvate levelled off after approximately 10 min of incubation of myocytes with either 0.2 mM octanoate or 2mM pyruvate. A possible explanation of this observation is the availability of substrate for oxidation. Availability of fatty acids for oxidation may be limited because

transport of long-chain fatty acids through the cell membrane, at low concentrations of fatty acids, is a carrier-mediated process (68). Also the transport of long-chain fatty acids across the inner mitochondrial membrane is facilitated by the carnitine palmitoyltransferase system. In contrast to long-chain fatty acids, medium-chain fatty acids, and pyruvate may enter myocytes rapidly and consequently their uptake may possibly not restrict their metabolism.

Since 90% of the radioactivity released during the oxidation of the palmitic acid was associated with CO_2 and the acid-soluble radioactivity accounted only for 10% of the ^{14}C released, the oxidation rates are based only on the amount of released CO_2.

The observation that the presence of 5mM glucose in the incubation medium had no effect on palmitate oxidation confirms the results of earlier, studies with perfused rat hearts (69) and cultured cardiac cells from chick embryo (70).

Seven known inhibitors of fatty acid oxidation, for which the mechanisms of inhibition are known or reasonably well established, were tested for their effectiveness in inhibiting palmitate oxidation in adult rat myocytes. The most effective of all inhibitors was 2-tetradecylglycidic acid. Also very effective were 2-bromopalmitic acid, 4-bromocrotonic acid, and 3-mercaptopropionic acid.

Less effective was 4-pentenoic acid. Hypoglycin, and 2-bromooctanoic acid were found to be ineffective in inhibiting fatty acid oxidation in myocytes. Especially interesting was the ineffectiveness of 2-bromooctanoic acid because it has been shown to be an excellent inhibitor of fatty acid oxidation in the perfused rat liver and in isolated rat liver mitochondria (71). Since the inhibitor is also ineffective with coupled rat heart mitochondria (30), it is possible that in heart this compound is not metabolized to 2-bromo-3-ketooctanoyl-CoA which is an irreversible inhibitor of 3-ketoacyl-CoA thiolase. The reason for 2-bromooctanoic acid not being metabolized in heart mitochondria may be the inability of the intramitochondrial acyl-CoA synthetase to activate this substituted fatty acid. This explanation is supported by published data which indicate that the medium-chain acyl-CoA synthetase of heart mitochondria has a much narrower substrate specifity than the liver enzyme (72,73).

2-Tetradecylglycidic acid was the most effective of the five inhibitors. At a concentration of 0.5 μM it caused a decrease in palmitate oxidation of 80%, while pyruvate oxidation remained unaffected. However in contrast to published reports (35,74), the inhibitor at concentrations of up to 5 μM was found to stimulate octanoate oxidation by up to 50%, while at an inhibitor concentration

of 10 μM the rate of oxidation was back to control levels. Since myocytes are expected to also oxidize endogenous and thus unlabeled fatty acids derived from intracellular triglycerides, an inhibition of their mitochondrial uptake could result in a stimulation of octanoate oxidation. The return of octanoate oxidation to control levels at a concentration of 10 μM 2-tetradecylglycidic acid may be the consequence of the inhibitor directly affecting the oxidation of medium-chain fatty acids.

2-Bromopalmitic acid, which after conversion to its CoA thioester, acts as an inhibitor of carnitine palmitoyltransferase I (39), inhibited both palmitate and octanoate oxidations. The unexpected inhibition of octanoate oxidation may be due to the inhibitor entering mitochondria and affecting octanoate activation or β-oxidation. Since pyruvate oxidation was not inhibited, depletion of the cofactors CoASH and NAD^+, necessary for the oxidation of both pyruvate and fatty acids, cannot be the reason for the observed octanoate inhibition. Also, inhibition of acetyl-CoA metabolism, which is formed from fatty acids as well as pyruvate, can be ruled out as the cause for octanoate inhibition. Although 2-bromopalmitoylcarnitine is known to enter mitochondria and cause an inhibition of β-oxidation (39), it is not clear how the acid can be converted to its carnitine derivative and pass the inner

mitochondrial membrane if it causes the irreversible inactivation of carnitine palmitoyltransferase I. Clearly, the effects of 2-bromopalmitic acid on fatty acid oxidation need to be studied further.

The observed stimulation of pyruvate oxidation by 2-bromopalmitic acid was unexpected. In a previous study (39) with rat liver mitochondria, 2-bromopalmitoyl-CoA was found to have no effect on pyruvate oxidation, while 2-bromopalmitoylcarnitine was observed to cause an inhibition of pyruvate-supported respiration which could be partially reserved by the addition of carnitine. The stimulation of pyruvate oxidation may be the consequence of 2-bromopalmitic acid inhibiting the oxidation of endogenous fatty acids derived from myocardial triglycerides, thereby allowing more pyruvate to be degraded. However, if this hypothesis were correct, 2-tetradecylglycidic acid should also stimulate pyruvate oxidation which it does not. Thus, I can not explain satisfactorily the observed stimulation of pyruvate oxidation by 2-bromopalmitic acid.

4-Bromocrotonic acid, an inhibitor of both 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase (30), inhibited the oxidation of palmitate and less severly that of octanoate while hardly or not at all affecting pyruvate oxidation. These results agree with the published reports

of the effects of 4-bromocrotonic acid on fatty acid oxidation in rat heart mitochondria (30), and in the perfused rat heart (31).

4-Pentenoic acid caused the expected inhibition of palmitate oxidation without affecting pyruvate oxidation. Surprisingly, the oxidation of octanoate was not inhibited even though the suggested irreversible inhibition of 3-ketoacyl-CoA thiolase by a metabolite of 4-pentenoate (75, 28) should repress octanoate oxidation.

Finally, 3-mercaptopropionic acid, which so far has been studied only with rat heart mitochondria (20), was found to also inhibit palmitate oxidation in rat myocytes. The virtual absence of any effect on octanoate and pyruvate oxidations agrees with the proposal that intramitochondrially formed S-acyl-3-mercaptopropionyl-CoA thioesters reversibly inhibit long-chain acyl-CoA dehydrogenase and thereby the β-oxidation of long-chain fatty acids (20). Thus, the partial inhibition of octanoate oxidation observed with isolated mitochondria (20) is most likely a consequence of octanoate and 3-mercaptopropionic acid competing for the same intramitochondrial acyl-CoA synthetase.

In an attempt to confirm the proposed mechanisms by which these inhibitors affect fatty acid oxidation, the reversibility of the inhibition of palmitate oxidation in rat myocytes was studied. The inhibitions caused by 2-

tetradecylglycidic acid, 2-bromopalmitic acid, and 4-bromocrotonic acid persisted after removal of the inhibitors from the incubation medium. Thus, inhibitions caused by these compounds seems to be irreversible, as suggested by previous studies (30,34,39). In contrast, 3-mercaptopropionic acid, which was reported to inhibit β-oxidation reversibly (20), caused little inhibition after its removal from the preincubation medium.

A surprising finding was the almost complete reversibility of the 4-pentenoate-induced inhibition of fatty acid oxidation in myocytes. This result contradicts the reported irreversible inhibition of 3-ketoacyl-CoA thiolase and thus β-oxidation in coupled heart mitochondria (75). However, 4-pentenoate is metabolized by two pathways, only one of which, the minor one, yields a metabolite that inhibits 3-ketoacyl-CoA thiolase reversibly as well as irreversibly (28). If the intramitochondrial concentrations of 4-pentenoate in isolated mitochondria and myocytes differ significantly, the flux of the inhibitor through the minor pathway could be affected, thereby giving rise to different patterns of inhibitions.

This work demonstrates that rates of oxidation of oleate and docosahexaenoate in hypophysectomized rats are 30-40% higher than rates in control or hypophysectomized rats treated with growth hormone. One possible explana-

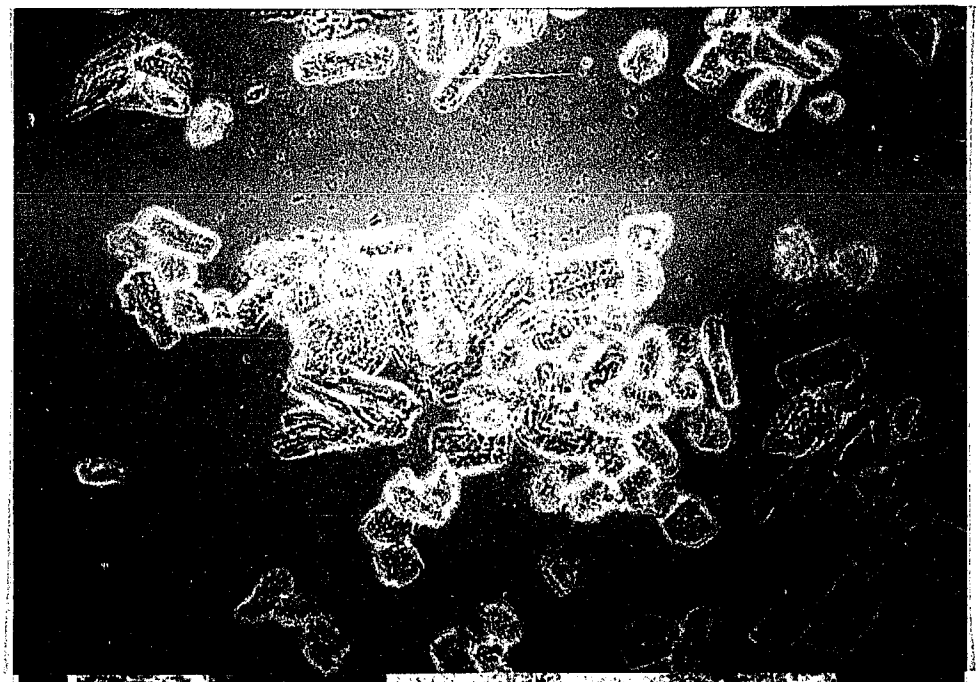
tion is that growth hormone which stimulates lipolysis in adipose tissue (48), may have the same effect in myocardial tissue. If so, the hormone would cause the release of fatty acids from endogenous triglycerides, and the fatty acid so released would be oxidized together with radioactive oleate and docosahexaenoate, consequently giving rise to lower oxidation rates of the externally added radioactive oleate and docosahexaenoate in control and hypophysectomized rats treated with growth hormone as compared to hypophysectomized rats.

Growth hormone treatment of hypophysectomized rats had little, if no effect on the oxidation rates observed with either oleate or docosahexaenoate. These results were supported by measuring the rates of respiration supported by either palmitoylcarnitine or docosahexaenoylcarnitine in heart mitochondria isolated from control, hypophysectomized rats, and hypophysectomized rats treated with growth hormone.

However, the specific activity of 2,4-dienoyl-CoA reductase is reduced by 40% in hypophysectomized rats, but increased twofold as compared to normal rats upon growth hormone treatment. This finding is expected because growth hormone did cause an increase in the activity of the same enzyme in rat liver mitochondria (54). Surprising is the fact that, in contrast to liver mitochondria, the rate of

oxidation of docosahexaenoylcarnitine remained unchanged upon growth hormone treatment. One possible explanation is that 2,4-dienoyl-CoA reductase does not catalyze the rate controlling reaction in the oxidation of polyunsaturated fatty acids in heart.

(a)



(b)

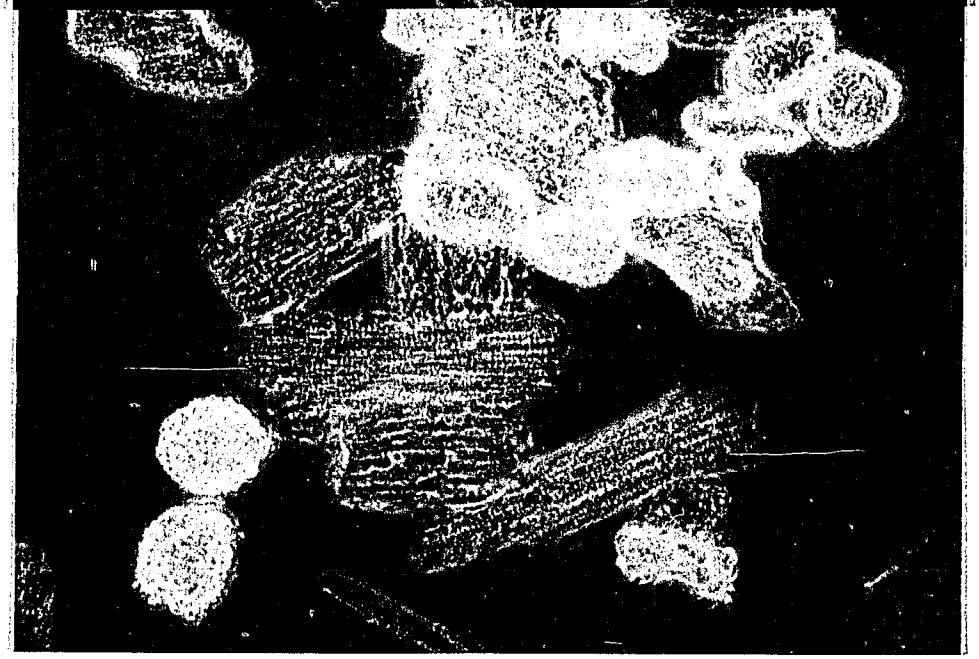


Figure 1. Light micrographs of dissociated cardiac myocytes from adult rat ventricles. Magnification at 160 x (a) and 400 x (b).

Figure 2. Schematic representation of the uptake and oxidation of fatty acids.

AS:	Acyl-CoA synthetase
CPT:	Carnitine palmitoyltransferase
AD:	Acyl-CoA dehydrogenase
EH:	Enoyl-CoA hydratase
HAD:	3-Hydroxyacyl-CoA dehydrogenase
TH:	3-Ketoacyl-CoA thiolase
TL:	Translocase

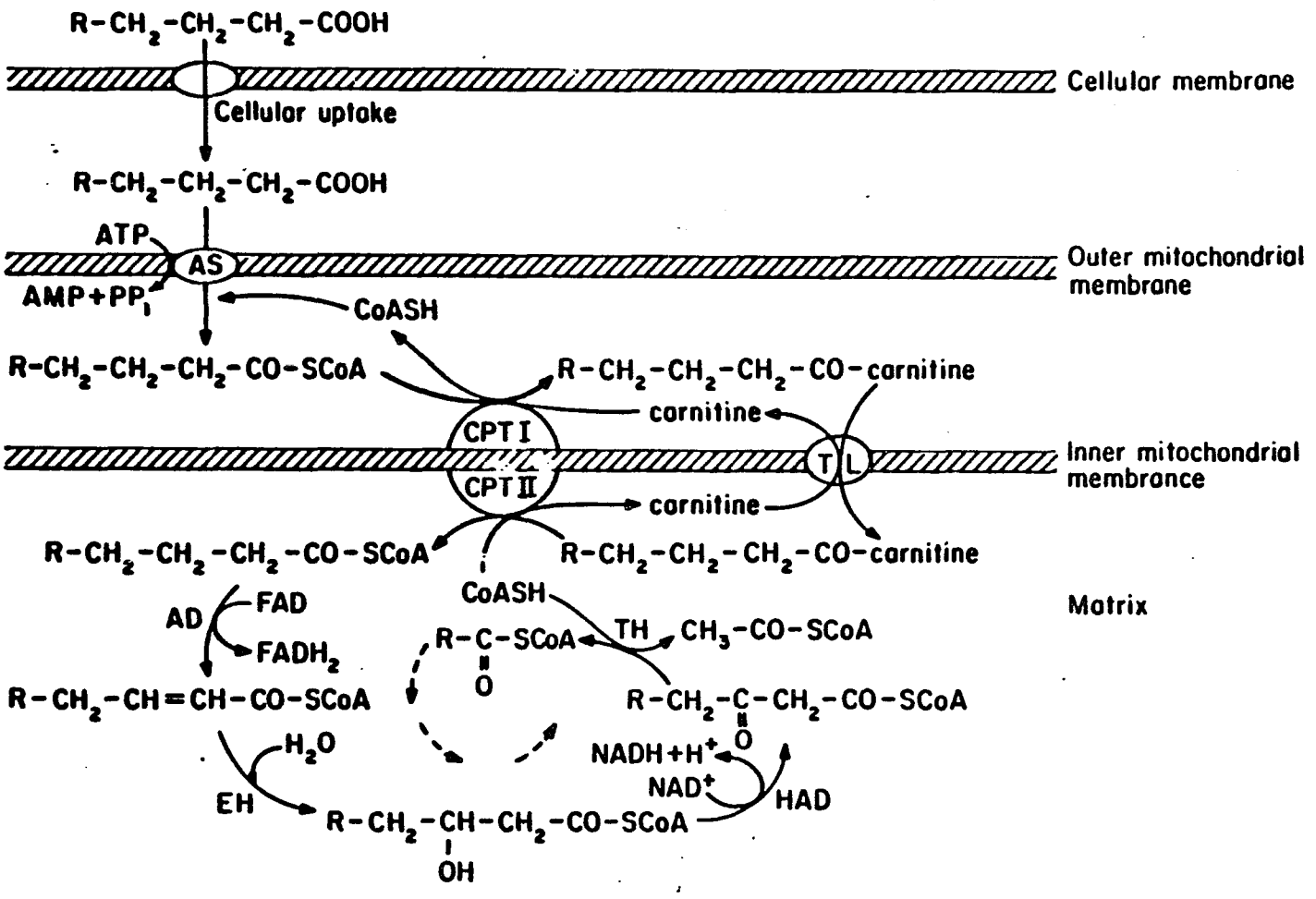
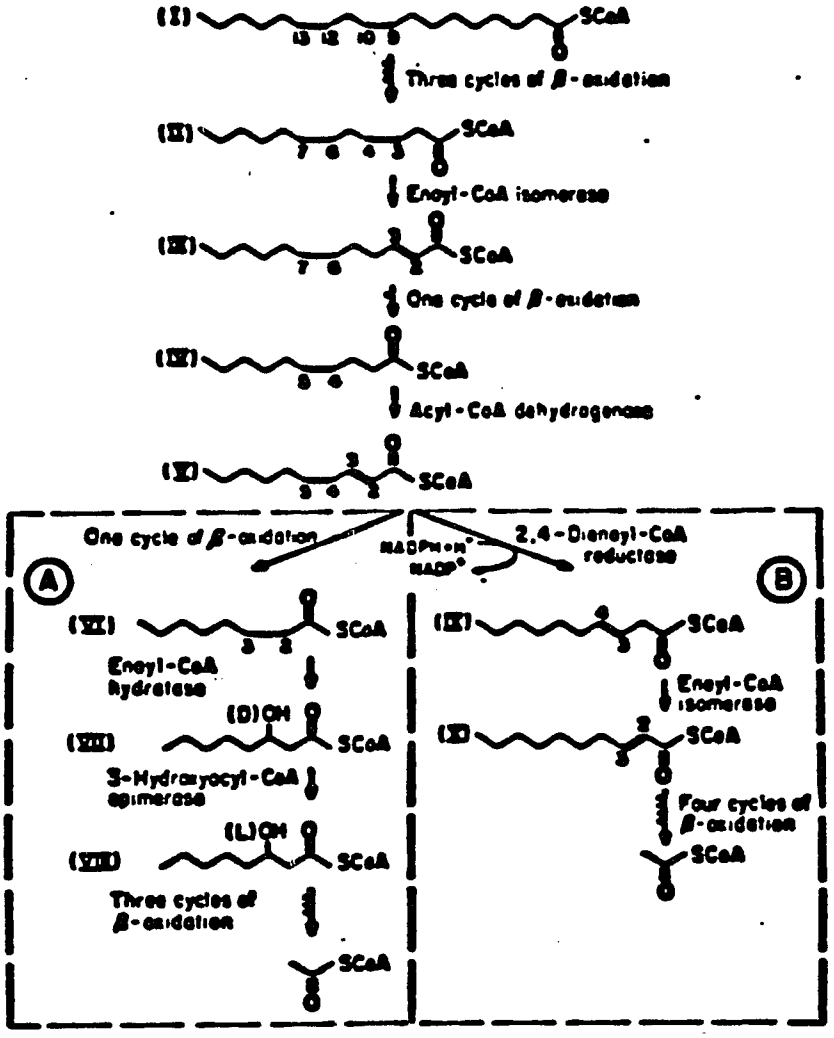

R-CH2-CH2-CH2-COOH
Cellular membrane
Cellular uptake
R-CH2-CH2-CH2-COOH
ATP
AS
AMP+PP1
Outer mitochondrial membrane
CoASH
R-CH2-CH2-CH2-CO-SCoA
R-CH2-CH2-CH2-CO-carnitine
carnitine
CPT I
CPT II
T
L
Inner mitochondrial membrance
carnitine
R-CH2-CH2-CH2-CO-SCoA
R-CH2-CH2-CH2-CO-carnitine
CoASH
Matrix
AD
FAD
FADH2
R-C-SCoA
O
TH
CH3-CO-SCoA
R-CH2-CH=CH-CO-SCoA
R-CH2-C-CH2-CO-SCoA
O
H2O
NADH+H+
NAD+
HAD
EH
R-CH2-CH-CH2-CO-SCoA
OH

Figure 3. Pathway of linoleic acid degradation.
A, Pathway proposed by Stoffel and Caesar (7).
B, modified pathway supported by findings published by Kunau and Dommes (9), as well as Cuebas and Schulz (11).



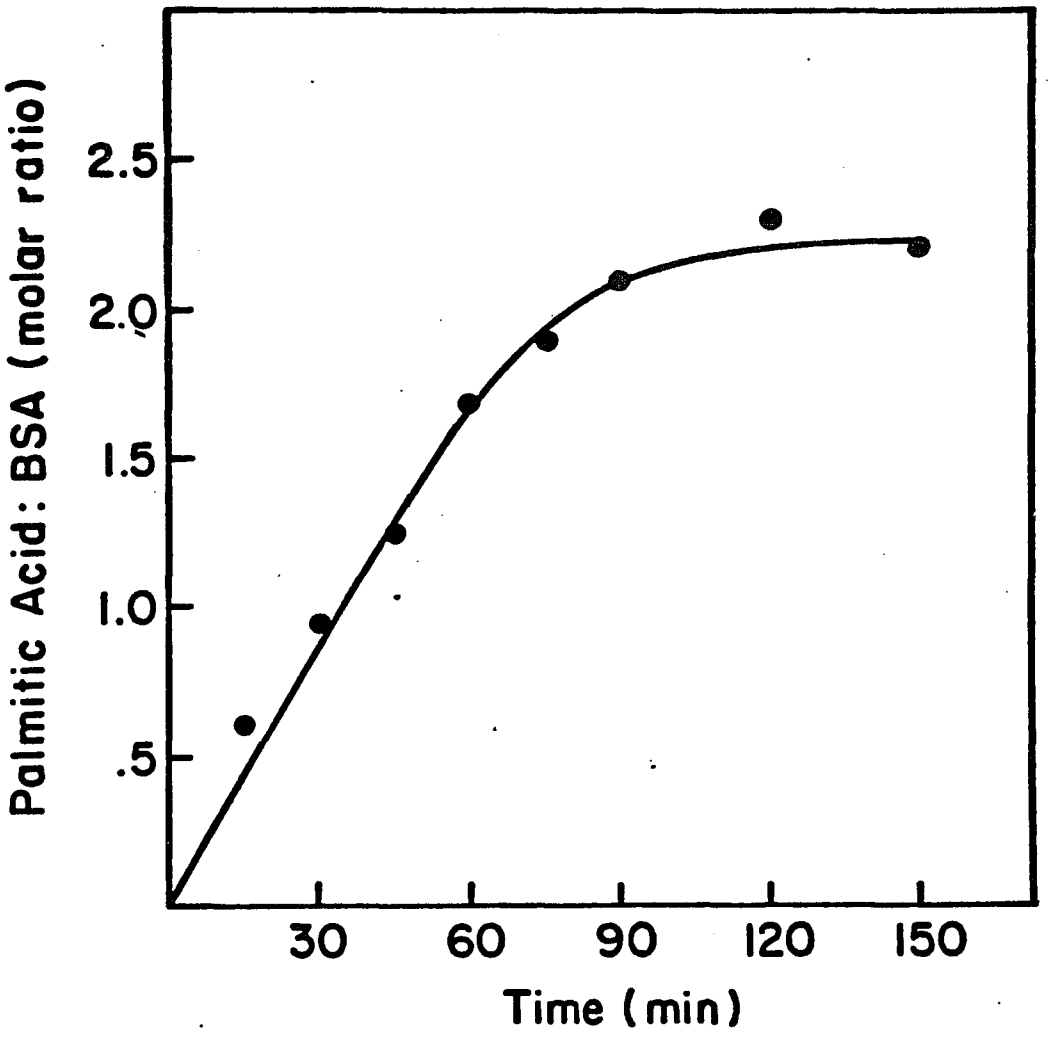


Figure 4. Time course of palmitate binding to bovine serum albumin (BSA).

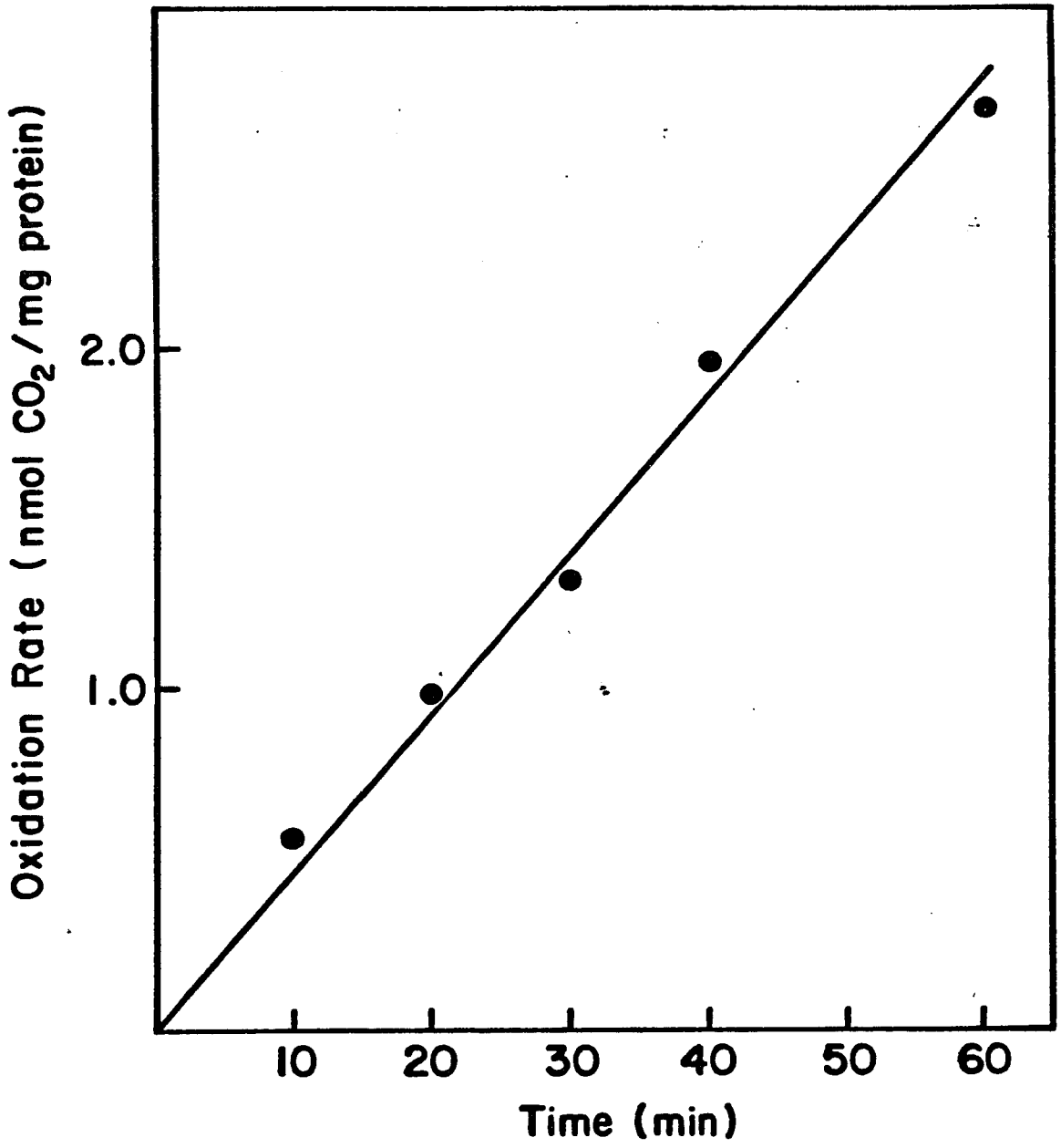


Figure 5. Oxidation of palmitic acid in rat myocytes as a function of time.

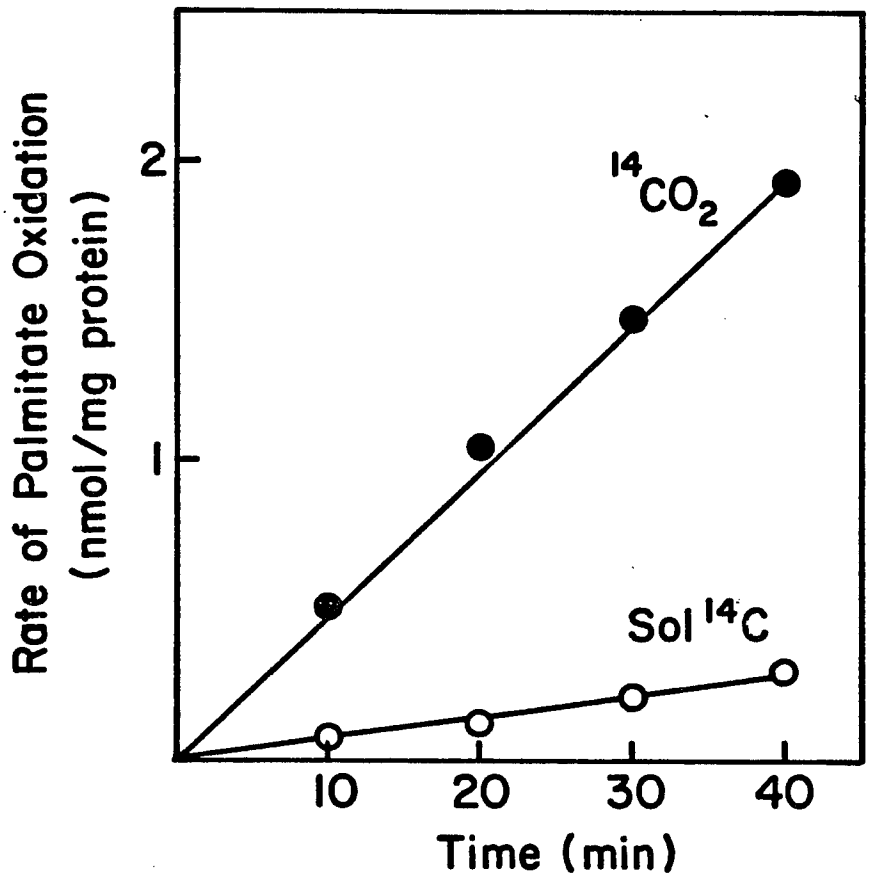


Figure 6. Release of $^{14}CO_2$ and acid-soluble radioactivity (Sol ^{14}C) during palmitate oxidation.

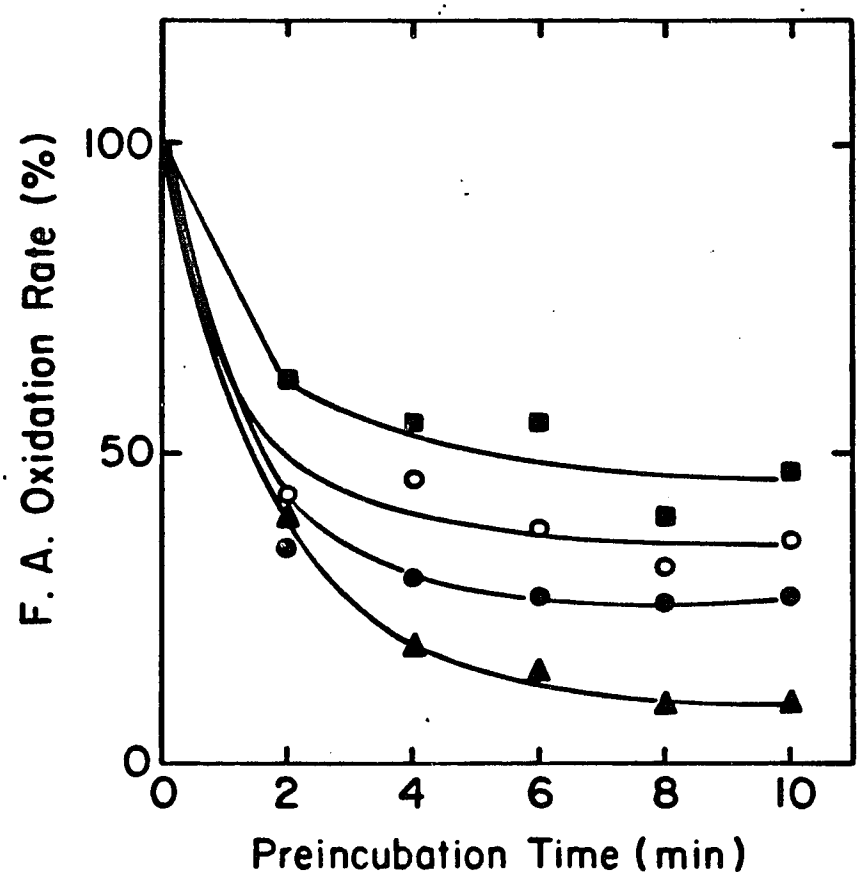
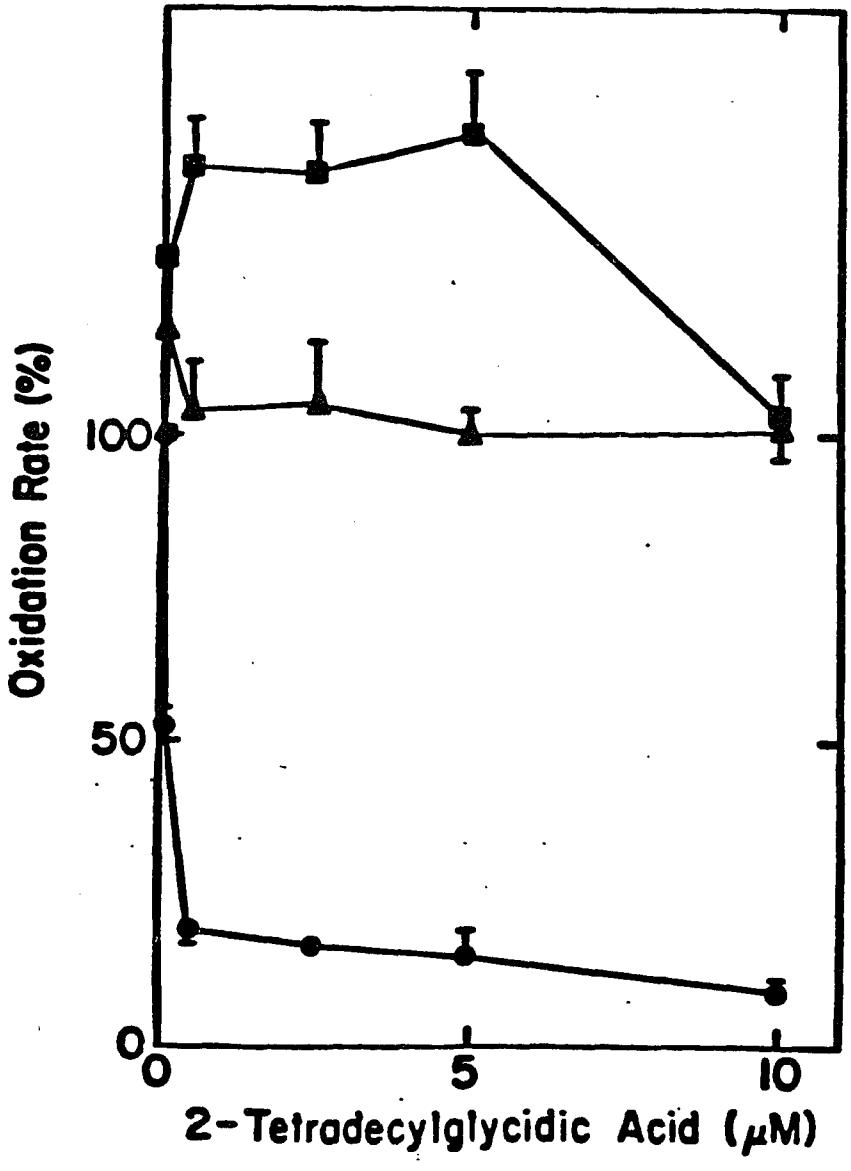


Figure 7. Inhibition of fatty acid oxidation by several inhibitors as a function of the preincubation time. Myocytes were preincubated for the indicated periods of time with 120 μM 2-bromopalmitic acid (▲), 125 μM 4-bromocrotonic acid (●), 300 μM 3-mercaptopropionic acid (○), or 300 μM 4-pentenoic acid (■) before fatty acid oxidation was initiated by the addition of [1-^{14}C]palmitate as detailed under Materials and Methods.

Figure 8. Effects of inhibitors of fatty acid oxidation on the oxidations of palmitate, octanoate, and pyruvate by rat myocytes. A, 2-tetradecylglycidic acid; B, 2-bromopalmitic acid; C, 4-bromocrotonic acid; D, 4-pentenoic acid; E, 3-mercaptopropionic acid. Palmitate (●); octanoate (■), and pyruvate (▲) served as metabolic substrates as described in detail under Materials and Methods.



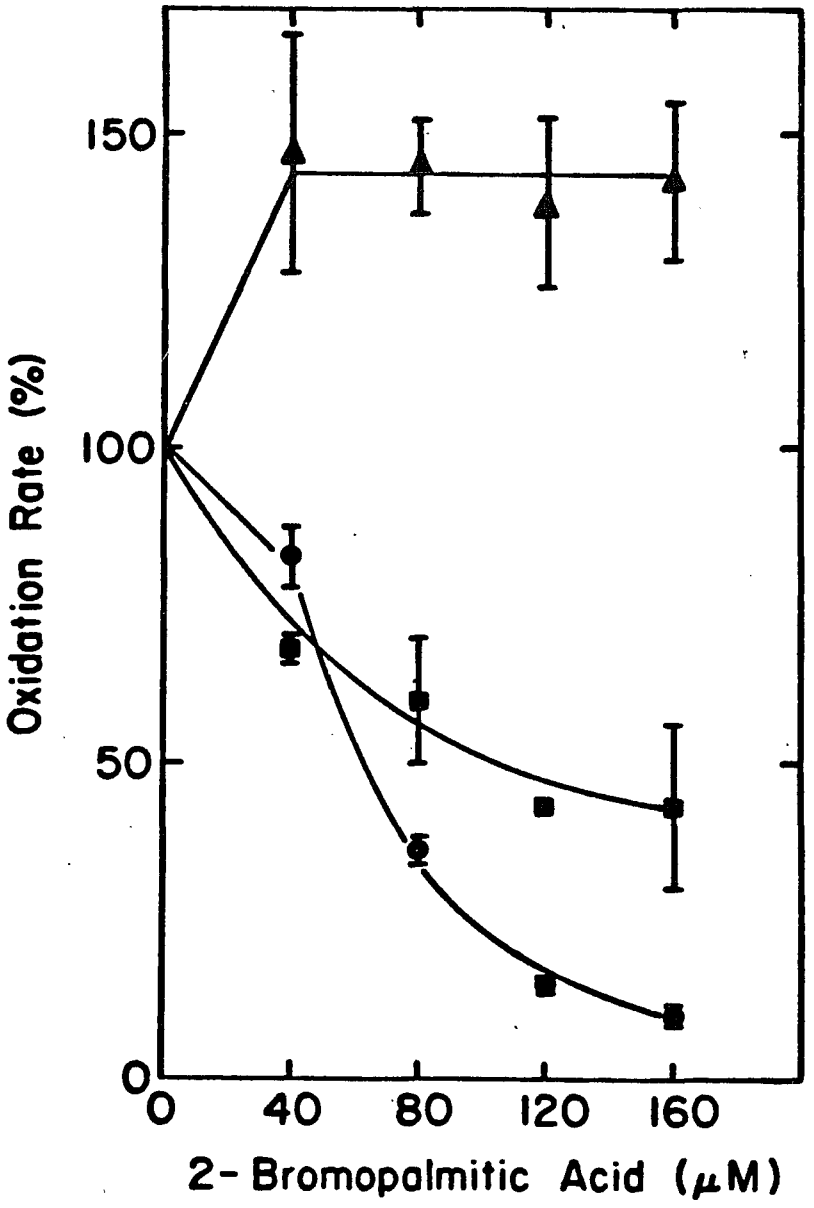

150
100
50
0
Oxidation Rate (%)
0
40
80
120
160
2-Bromopalmitic Acid (μM)

Figure 8B.

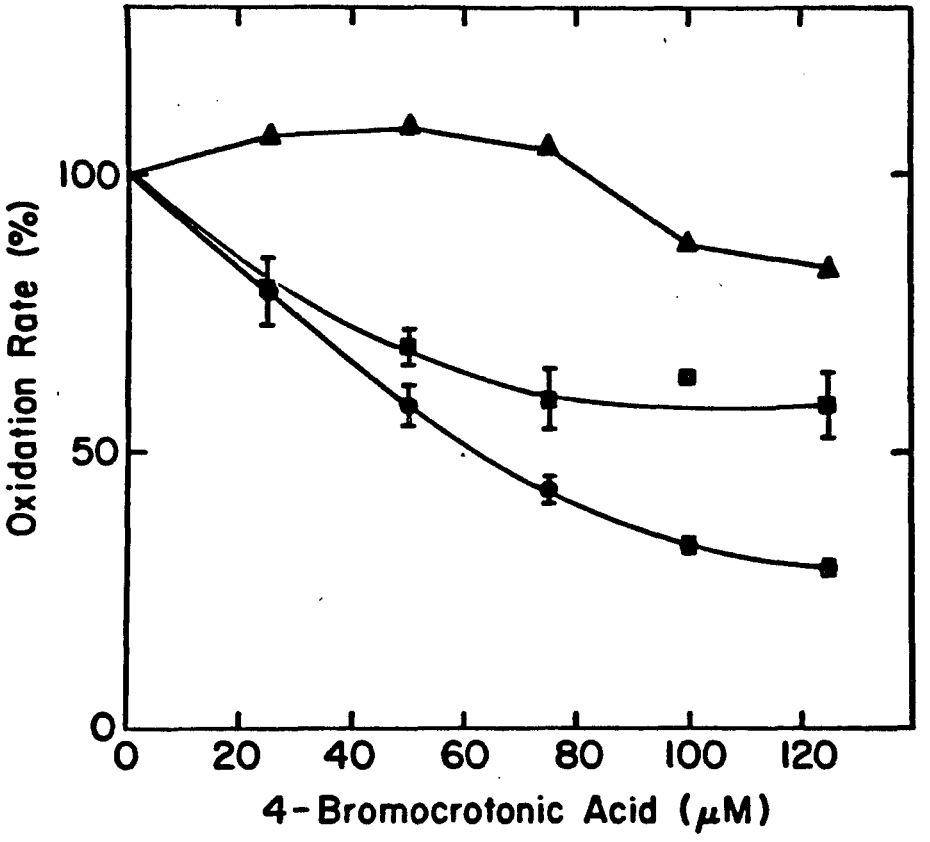

Oxidation Rate (%)
100
50
0
0
20
40
60
80
100
120
4-Bromocrotonic Acid (μM)

Figure 8C.

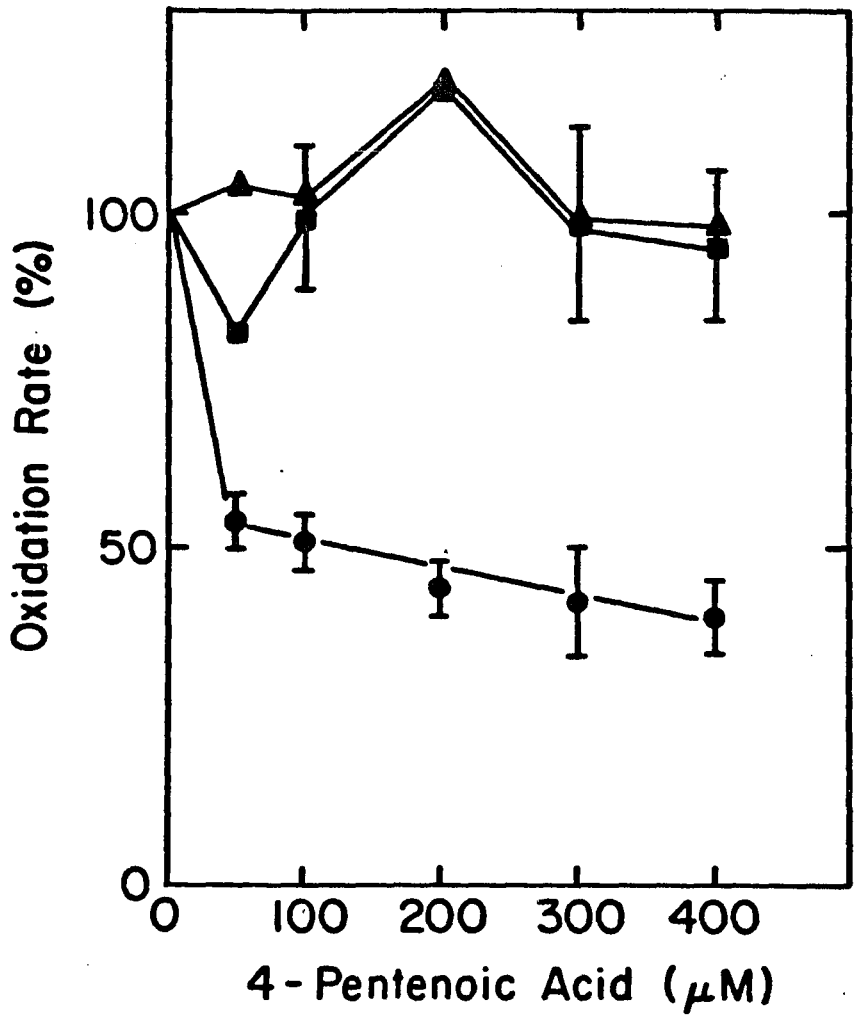


Figure 8D.

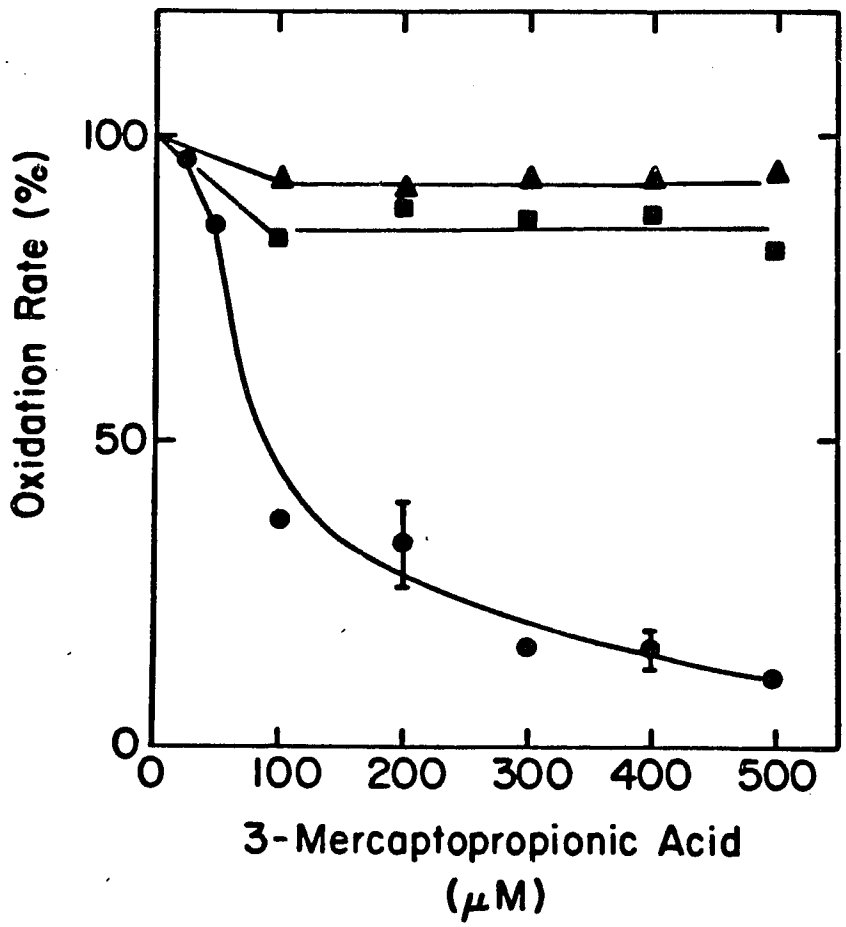

Oxidation Rate (%)
100
50
0
0
100
200
300
400
500
3-Mercaptopropionic Acid
(μM)

Figure 8E.

Figure 9. Effects of inhibitors of fatty acid oxidation on palmitate oxidation in rat myocytes preincubated with the inhibitors followed by their removal from the incubation medium. Myocytes were preincubated for 10 min with 5 μM 2-tetradecylglycidic acid (□), 120 μM 2-bromopalmitic acid (■), 125 μM 4-bromocrotonic acid (▲), 300 μM 3-mercaptopropionic acid (○), 300 μM 4-pentenoic acid (△), or no inhibitor (●). After preincubation the cell suspension medium was replaced with an inhibitor-free medium. Fatty acid oxidation was initiated by the addition of 0.2 mM [1-^{14}C]palmitate and allowed to proceed for 40 min.

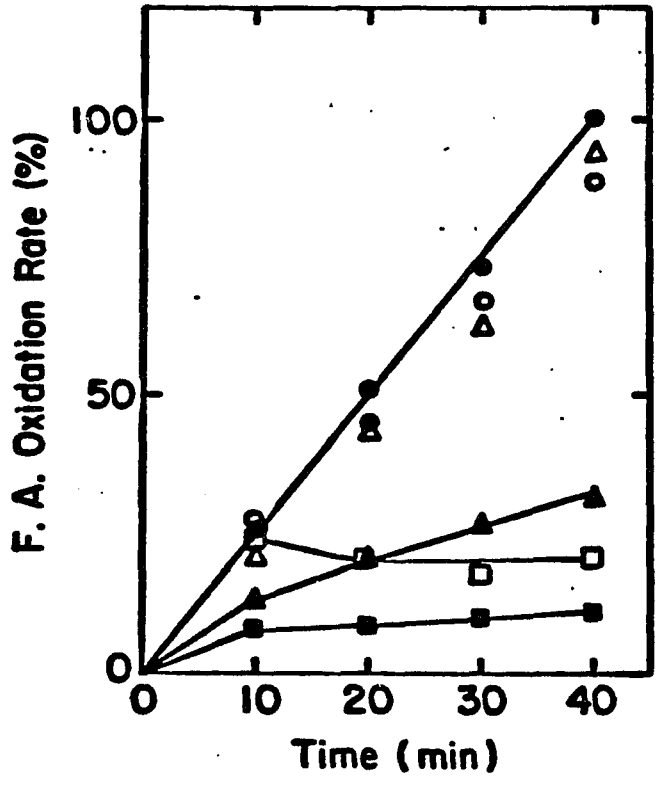


Figure 10. Oxidation of oleic acid and docosahexaenoic acid in rat myocytes.

Myocytes were incubated with various concentration of oleic acid (O—O) or docosahexaenoic acid (●—●) for 30 min at 37°C. Insect data are plotted on double reciprocal coordinates.

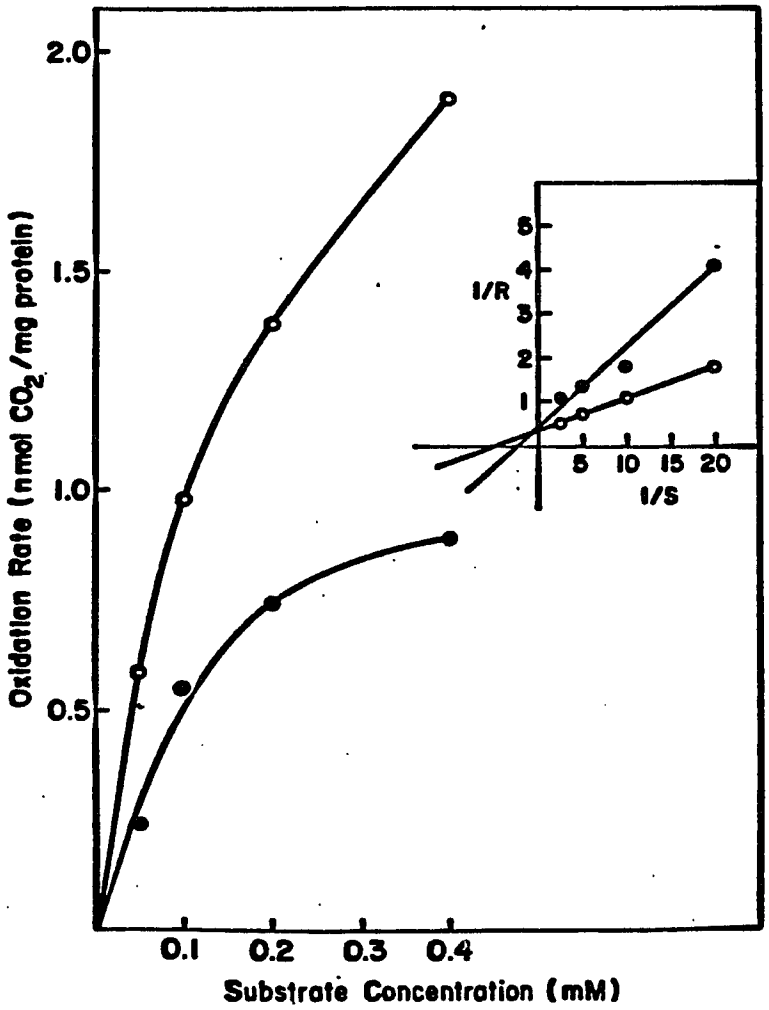


Table 1. Oxidation of palmitate, octanoate, and pyruvate in rat myocytes.[a]

Time (min)	Oxidation Rate (nmol Co_2/mg ptotein)[b]		
	Palmitate	Octanoate	Pyruvate
10	0.59±0.07 (4)	4.7 ±0.87 (4)	17.56±2.6 (4)
20	0.99±0.08 (4)	5.34±0.09 (4)	19.60±1.4 (4)
30	1.38±0.13 (4)	5.82±0.38 (4)	24.88±2.5 (4)
40	1.93±0.12 (4)	5.92±0.40 (4)	23.40±2.2 (4)

[a] Myocytes were incubated with 0.2 mM palmitate, 0.2 mM octanoate, or 2 mM pyruvate for 10,20,30, and 40 min at 37°C under constant shaking.

[b] Values are means ± SD, number in parentheses gives the number of experimental measurements.

Table 2. Release of $^{14}CO_2$ from $NaH^{14}CO_3$

Time (min)	dpm
20	576,400
40	787,400
60	858,800
80	858,7o0
100	859,600

50 µl $NaH^{14}CO_3$ (856500 dpm) were added to 0.95 ml cell suspension, then 0.3 ml of 70% perchloric acid was injected into the incubation medium. Subsequently 0.3 ml of 1 M hyamine hydroxide was injected through the septum into the center well. The flasks were shaken continously for 20,30,60,80,100 min at 37°C at which time the plastic center well was removed, placed into a scintillation vial containing 4 ml of Scinti Verse II, and counted in a liquid scintillation counter.

Table 3. The effect of glucose, 2,4-dinitrophenol and starvation on the oxidation of palmitate.

Addition or Treatment	Rate of Palmitate Oxidation[a]
	nmol CO_2/mg protein/30 min
None	1.42 ± 0.14 (3)
5 mM Glucose	1.39 ± 0.13 (3)
Starvation for 24 hours	1.35 ± 0.16 (3)
10 μM DNP	1.40 ± 0.14 (3)
1 μM DNP	1.37 ± 0.16 (3)
0.1 μM DNP	1.39 ± 0.18 (3)

[a] Values are means ± SD. Numbers in parenthesis give the number of experimental measurements

Table 4. Effects of inhibitors of fatty acid oxidation on the rate of palmitate oxidation in adult rat myocytes.

Inhibitors (I)	Rate of palmitate oxidation[a] (% of control) 0.1 mM I	0.5 mM I
Hypoglycin	114.3 ± 0.4 (3)	115.8 ± 10.4 (4)
4-Pentenoic acid	50.3 ± 2.9 (3)	37.2 ± 0.4 (3)
2-Bromooctanoic acid	98.0 ± 7.1 (4)	89.4 ± 3.9 (4)
4-Bromocrotonic acid	35.3 ± 1.3 (3)	14.7 ± 1.2 (3)
3-Mercaptopropionic acid	48.1 ± 6.1 (4)	13.2 ± 0.9 (3)
2-Bromopalmitic acid	38.7 ± 4.7 (3)	1.7 ± 0.1 (3)
2-Tetradecylglycidic acid	4 ± 0.3 (3)	

[a] Myocytes were preincubated without (control) or with inhibitors (I) for 10 min at 37°C and then allowed to react with 0.2 mM palmitic acid for 30 min at 37°C. Values are means ± S.D. Numbers in parentheses give the number of measurements on which the values are based.

Table 5. Rates of oxidation of oleic acid and docosahexaenoic acid in rat myocytes.

Treatment	Oxidation rates (n mol CO_2/mg protein/30 min)[a]			
	50 M C18:1[b]	200 M C18:1[b]	50 M C22:6[b]	200 M C22:6[b]
Normal Control	0.58 ± 0.004 (3)	1.29 ± 0.08 (4)	0.166 ± 0.002 (3)	0.725 ± 0.14 (4)
Hypophys.	0.93 ± 0.11 (3)	2.06 ± 0.04 (4)	0.24 ± 0.01 (3)	0.66 ± 0.07 (4)
Hypophys. + GH	0.44 ± 0.02 (3)	1.36 ± 0.21 (3)	0.13 ± 0.01 (3)	0.69 ± 0.02 (3)

[a] Values are means ± SD. Numbers in parentheses give the number of myocyte preparations used. Each preparation was measured three times.

[b] C18:1, Oleic acid, C22:6, docosahexaenoic acid.

Table 6. Rates of respiration supported by acylcarnitines in rat heart mitochondria.

Acylcarnitine	Rates of Respiration		
	Control	Hypophysectomized	Growth hormone treated
	ng atom O/min/mg protein		
Palmitoylcarnitine	208	148	185
Docosahexaenoylcarnitine	193	147	141

Table 7. Specific activities of β-oxidation enzymes in rat heart mitochondria.[a]

Enzyme	Substrate	Specific activities (nmol/min/mg protein)[b] Control[c]	Hypophys.[c]	Hypophys. + GH[c]
2,4-Decadienoyl-CoA reductase	2,4-Decadienoyl-CoA	12.6±1.3	7.4±0.9	24.6±3.3
Enoyl-CoA hydratase	Crotonyl-CoA	4,130±500	4,530±870	5,260±1,000
3-Hydroxyacyl-CoA dehydrogenase	Acetoacetyl-CoA	2,230±350	1,880±120	1,550±100
3-Ketoacyl-CoA thiolase	Acetoacetyl-CoA	428± 38	395± 41	377± 50
	3-Ketooctanoyl-CoA	562± 90	416± 50	307± 30

[a] Assay conditions are described under Material and Methods.

[b] Values are means ± SD; numbers in parentheses give the number of mitochondrial preparations used. Each preparation was assayed three times.

[c] Control, normal rats; Hypophys, hypophysectomized rats; Hypophys. + GH, hypophysectomized rats treated with growth hormone.

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