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THE REGULATION OF FATTY ACID
OXIDATION IN BOVINE MAMMARY TISSUE

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THE REGULATION OF FATTY ACID OXIDATION IN BOVINE MAMMARY TISSUE

By

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ABSTRACT

THE REGULATION OF FATTY ACID
OXIDATION IN BOVINE MAMMARY TISSUE

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Oxidation of fatty acid was studied in bovine mammary tissue slices in order to evaluate their potential contribution to energy metabolism. Mammary tissue slices were incubated in bicarbonate buffer with palmitate[1-^{14}C], and $^{14}CO_2$ was counted as an index of oxidation. Lipids were extracted from the tissue and esterified lipids determined by thin-layer chromatography.

Rates of palmitate oxidation increased with time of incubation in mammary tissue. This phenomenon is not an artifact of the incubation system or due to substrate solubility, as rates of palmitate oxidation were constant in rat kidney cortex slices. Preincubating mammary tissue with or without unlabelled palmitate revealed that increasing rates of palmitate oxidation is not due to utilization of endogenous fatty acids.

Fatty acid oxidation in mammary tissue is probably not controlled by tissue fatty acid concentration. Palmitate at 0.26 mM, equivalent to arterial fatty acid concentration and less than tissue fatty acid concentration, gave maximal

rates of oxidation. Half-maximal rates of oxidation were obtained at 0.1 mM palmitate. Rates of palmitate oxidation increased with time at all concentrations tested.

Rates of fatty acid oxidation decreased with increasing chain length: acetate > octanoate > palmitate or oleate. Rates of oxidation of only long-chain fatty acids increased over time, which could not be explained by carnitine palmitoyltransferase (CPT) activity. The β-oxidation enzymes may restrict fatty acid oxidation as oxidation of palmitate [1-^{14}C] > palmitate[U-^{14}C].

Acetate inhibited palmitate oxidation (75%) but not esterification, suggesting that acetate inhibits palmitate oxidation by substrate competition at the mitochondrial level or via malonyl-CoA inhibition of CPT. Rates of palmitate oxidation increased with time in the presence of acetate.

Glucose inhibited palmitate oxidation (67%) and stimulated palmitate esterification. Low palmitoyl-CoA levels would favor glyceride synthesis over oxidation, since the apparent K_m for palmitoyl-CoA of the glycerol 3-phosphate (G3P) acyltransferases is lower than that of CPT. Thus, glucose presumably diverts palmitate from oxidation to glycero-lipids. In some experiments, rates of palmitate oxidation increased over time in the presence of glucose, which could not be explained by glucose depletion from the media. Decreasing rates of glycero-lipid formation over time could quantitatively account for increasing rates of palmitate oxidation.

Clofenapate, a glyceride synthesis inhibitor, decreased triacylglycerol formation, increased intracellular fatty acid accumulation, and marginally increased palmitate oxidation. However, rates of palmitate oxidation increased 180% in the presence of carnitine and clofenapate, suggesting the existence of multiple controls of palmitate oxidation.

Glucose did not affect octanoate oxidation, but stimulated octanoate esterification, possibly resulting from separate microsomal and mitochondrial pools of octanoate. Octanoate activated in the mitochondria is not accessible to acylation of G3P. Octanoate activated at the microsomes is accessible to acylation of G3P.

Fasting appeared to decrease the absolute rates of palmitate oxidation. However, fatty acid oxidation as a proportion of the total oxidative metabolism was not determined and may have increased.

Therefore, fatty acid esterification and oxidation seem to compete for available acyl-CoA with esterification being favored. Also, acetate inhibits palmitate oxidation presumably by substrate competition at the mitochondrial level or via malonyl-CoA inhibition of CPT. It can be estimated that long-chain fatty acids can potentially account for 6-10% of the oxidative metabolism of mammary tissue.

DEDICATION

To Niki and Debi

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INTRODUCTION

Milk synthesis is a very energy-demanding process. High-producing dairy cows during peak of lactation are often in a state of negative energy balance. The concentration of fat in milk is nearly constant throughout lactation, and milk fat represents a loss of energy to the cow. Therefore, by increasing the oxidative metabolism of fatty acids in the mammary gland more energy would be available for the production of more milk and milk protein. This would be especially useful to high-producing cows at peak of lactation, since production may be limited by the extent of energy deprivation. However, Annison et al. (1967) could not detect the occurrence of fatty acid oxidation in the mammary gland of fed goats.

Thus, in order to assess the feasability of stimulating fatty acid oxidation in the mammary gland, two questions were raised at the onset. To what extent does mammary tissue from fed lactating cows oxidize fatty acid, and what factor(s) regulates fatty acid oxidation in bovine mammary tissue?

LITERATURE REVIEW

This review will discuss the regulation of fatty acid oxidation in mammalian tissues. A brief introduction on the modification of dietary lipids in the gastrointestinal tract of ruminants is in order, since these modified lipids will eventually serve as an energy source for some tissues. Characterization of the pathways involved in the metabolism of absorbed fatty acids and their regulation will follow. This review will be centered on the regulation of fatty acid oxidation in non-ruminant organs, as little is known of fatty acid oxidation in the ruminant mammary gland. In conclusion, long-chain fatty acids as a possible source of energy for the mammary gland will be discussed, integrating the previous aspects of this review.

LIPID ABSORPTION AND DIGESTION

Practical dairy rations normally contain less than 4% fat. Most of the fatty acids in the diet are in glyceride combination and are unsaturated (Garton, 1960). Ruminal microorganisms extensively modify the dietary lipids, by hydrolysis of the glycerides, fermentation of the liberated glycerol, and hydrogenation of the unsaturated fatty acids (Garton, 1960). As a result, ruminant tissue lipids contain a large proportion of saturated fatty acids even though the

fatty acids found in pasture grasses are mainly polyunsaturated.

Ruminal Biohydrogenation of Unsaturated Fatty Acids

Attention was first directed towards the influence of the rumen on dietary lipids by Reiser (1951), who showed that when linseed oil was incubated with sheep rumen contents, the linolenic acid content was reduced from approximately 30% to 5%. This was attributed to hydrogenation of linolenate by rumen bacteria. Mills et al. (1970) studied the *in vitro* hydrogenation of linolenic, linoleic and oleic acids by a Gram-negative rumen micrococcus. Positional and geometrical isomers of the fatty acids were formed as intermediates. These isomers are detectable in ruminant depot fat and milk (Garton, 1960). Polan et al. (1964) found a synergism between *Butyrivibrio fibrisolvens*, *Peptostreptococcus elsdenii* and a *Selenomonas* species for the biohydrogenation of linolenic acid to stearic acid. Rumen protozoa are also able to hydrogenate linoleic acid to stearic acid (Chalupa and Kutches, 1967). Thus, rumen microorganisms reduce dietary unsaturated fatty acids to their saturated isomers which eventually pass to the intestines for absorption.

Fatty Acid Absorption

In the ruminant nonesterified fatty acids are the major class of lipids appearing in the small intestine. The fatty acids found in the intestinal lumen of the ruminant are associated with solid surfaces. The transfer of fatty acids

from solid surfaces into micellar solution is accomplished by biliary and pancreatic secretions (Harrison and Leat, 1972). Ruminant bile is composed of cholesterol, fatty acids and phospholipids. The predominant phospholipid is phosphatidyl choline (Leat, 1965). Lysophosphatidyl choline and fatty acids, arising from the action of pancreatic phospholipases on phosphatidyl choline, act in concert with the bile salts to solubilize the fatty acids into a micellar phase (Harrison and Leat, 1975). The major site of fatty acid absorption is the ileum (Leat, 1965).

After the fatty acids of the micellar dispersion are absorbed by the intestinal mucosal cell, triglycerides are synthesized predominantly by the glycerol-3-phosphate pathway (Cunningham and Leat, 1969). The monoglyceride pathway is present in adult sheep (Cunningham and Leat, 1969), but it would be of little importance quantitatively since virtually no monoglyceride is absorbed from the intestines of ruminants (Harrison and Leat, 1975).

Therefore, fatty acid absorption by the intestinal mucosal cells in ruminants involves fatty acid solubilization by biliary and pancreatic secretions, uptake by the mucosal cells, and esterification to glycerol-3-phosphate in the mucosal cell.

Removal and Transport of Absorbed Lipids

The long-chain fatty acids enter the circulatory system in the form of chylomicrons or very low density lipoproteins (VLDL), density less than 1.006, from the digestive tract

via the thoracic duct. The VLDL is absorbed by the intestinal lymph, and passes into the thoracic duct via the intestinal duct (Felinski et al., 1964; Palmquist, 1976). In ruminants, triglyceride accounts for approximately 70% of lymph lipids and phospholipids as 20% (Palmquist, 1976). In sheep the flow of bile phosphatidyl choline is theoretically sufficient to account for most of the lymph phospholipids (Harrison and Leat, 1975). The results of Palmquist and Mattos (1978) suggests that 76% of absorbed lipid is taken up directly by the mammary gland of the lactating cow. In the liver the fatty acids derived from blood are reesterified to glycerol. The majority of the triglycerides then re-enter the plasma as VLDL ($d < 1.006$) and low density lipoprotein ($1.006 < d < 1.040$)(Palmquist, 1976).

THE PATHWAYS AND CONTROL OF FATTY ACID UTILIZATION

Cellular Uptake of Blood Fatty Acids

Blood fatty acids, which are of quantitative importance for cellular energy production in some tissues, exist in two compartments: nonesterified fatty acid bound to albumin and triglyceride fatty acids in a lipid-protein complex (VLDL and other lipoproteins). In mammalian species the majority of these fatty acids are of long-chain length (Spector, 1971).

Approximately 80% of blood triglycerides are completely hydrolyzed during uptake by the mammary gland (Emery, 1973). This hydrolysis is catalyzed by the enzyme lipoprotein lipase (LPL)(EC 3.1.1.3). As a result, fatty acid concentration in

capillary fluid is increased. The process is partially reversible giving a degree of equilibration between plasma triglycerides and fatty acids (Emery, 1973). The activity of lipoprotein lipase is modified by apoprotein fractions of lipoprotein C. Apo C-II and maybe C-I activate LPL and apo C-III inhibits activity (Jensen and Pitas, 1976). Super et al. (1976) noted that activator concentration is related to the concentration of plasma total lipids in cows. Bovine mammary LPL activity is also responsive to diet and hormones. Mammary lipoprotein lipase activity is decreased in cows fed a milk fat depressing ration (Emery, 1973). Prolactin and prolactin plus insulin enhances LPL activity in bovine mammary tissue explants (Emery, 1973). Fasting, diabetes, exercise and epinephrine treatment increase LPL activity in rat heart (Neely and Morgan, 1974). In summation, the lipoprotein lipase enzyme is of quantitative importance for tissue uptake of fatty acid from blood triglyceride, especially in the mammary gland, and is highly regulable.

Circulating nonesterified fatty acid is transported as a tightly bound, non-covalent complex with albumin (Spector, 1971). The binding is reversible and some fatty acid exists free in solution in equilibrium with albumin-bound fatty acid. Tissue uptake of nonesterified fatty acid is not dependent upon metabolic energy or mediated enzymatically. In order to be absorbed the circulating fatty acid must dissociate from the carrier protein. Cells rapidly take up fatty acid and this alters the steady state distribution between bound and

unbound fatty acid, which causes additional amounts of fatty acid to dissociate from albumin (Spector, 1971).

The rate of fatty acid uptake is a function of plasma concentration in intact animals (Neely and Morgan, 1974) and in *in vitro* systems (Eaton and Steinburg, 1961). However, in *in vitro* systems uptake can be affected by factors other than simply concentration. Ockner et al. (1972) isolated a cytoplasmic low molecular weight protein termed fatty acid binding protein (FABP) or Z protein, which binds fatty acids. FABP has a high affinity for fatty acids, and the relative binding increases with increasing chain length. FABP is present in the cytoplasm of rat liver, myocardium, skeletal muscle, intestinal mucosa, adipose tissue and kidney (Mishkin et al., 1972). Flavispidic acid competes with fatty acid for the FABP and decreases oleate uptake in isolated rat hepatocytes (Wu-Rideout et al., 1976). Uptake of fatty acid in rat aorta pieces is dependent on fatty acid chain length, decreasing as chain length increases (Hashimoto and Dayton, 1971). Glucose enhances palmitate uptake in the perfused rat heart (Shipp, 1964). However, the rate of uptake was unaffected by fasting in a perfused rat liver system (McGarry and Foster, 1971). Kidneys possess a unique pathway for fatty uptake, which is dependent on potassium. Indeed, palmitate uptake is enhanced in rat renal cortex slices by addition of potassium (Wagner and Heinemann, 1977). Even though tissue uptake of fatty acid bound to albumin is not dependent on metabolic energy or mediated enzymatically, nevertheless

some studies suggest that nonesterified fatty acid uptake is regulated.

Fatty Acid Activation

The following reaction accounts for activation of fatty acids in mammalian tissues:

ATP + fatty acid + CoASH $\longleftrightarrow$ acyl-CoA + AMP + PPi

The reaction is catalyzed by acyl-CoA synthetases (acid:CoA ligases, (AMP forming) EC 6.2.1.1-3). It requires Mg^{2+} and is readily reversible (Groot et al., 1976). The reaction involves formation of an enzyme-bound acyl adenylate intermediate. Pyrophosphate hydrolysis, catalyzed by inorganic pyrophosphatase (EC 3.6.1.1), shifts the equilibrium in favor of acyl-CoA formation. In addition to the ATP-dependent fatty acid activation, a mammalian acyl-CoA synthetase specific for GTP catalyzes the reaction:

GTP + fatty acid + CoASH $\longleftrightarrow$ acyl-CoA + GDP + Pi

The enzyme is also Mg^{2+}-dependent (Groot et al., 1976). However, this enzyme is of little physiological significance (Neely and Morgan, 1974). Thus, fatty acid activation is an enzymatic process dependent on metabolic energy.

Enzymes that activate short-, medium-, and long-chain fatty acids have been described (Groot et al., 1976). Acetyl-CoA synthetase is present in all tissues and it also activates propionate. Quraishi and Cook (1972) found that about two-thirds of the enzyme activity is localized in the cytoplasm and one-third in the mitochondria of bovine heart and mammary gland, in kidney equally divided, and in lung and liver

predominantly in the mitochondria. However, in the liver the activity of acetyl-CoA hydrolase is greater than the synthetase. Groot (1976) isolated a propionyl-CoA synthetase from guinea pig liver mitochondria. Acetate, propionate and butyrate are activated, but specificity for propionate is highest. Butyryl-CoA synthetase, an enzyme with the highest V_{max} toward butyrate, has been purified from beef heart mitochondria (Webster et al., 1965).

A medium-chain acyl-CoA synthetase has been purified from beef liver particles (Mahler et al., 1953). This enzyme has broad substrate specificities, but the K_m for octanoate is the lowest (0.15 mM). Medium-chain fatty acids (MCFA) are activated exclusively in the mitochondrial matrix (Aas and Bremer, 1968). Long-chain fatty acids (LCFA) can be activated by this enzyme, which could explain the carnitine-independent LCFA fatty acid oxidation found by Van Tol and Hülsmann (1970). In heart mitochondria LCFA oxidation is totally carnitine-dependent.

A long-chain acyl-CoA synthetase is present in microsomes and the outer mitochondrial membrane of mammalian tissues. This enzyme has broad substrate specificities with a higher specificity for palmitate (Groot et al., 1976). In rat liver microsomes the enzyme has K_m values of 1-3 μM, 7.2-9.5 μM and 0.29-0.4 mM for LCFA, CoASH and ATP (Groot et al., 1976). In isolated rat liver mitochondria the K_m values are 50 μM and 7.0 μM for palmitate and ATP (Van Tol and Hülsmann, 1970). Thus, enzymes specific for activation of short-, medium-, and long-chain fatty acids exist and their intracellular

localization is of metabolic importance.

Pande (1973) found that low concentrations of palmitoyl-CoA inhibit the activity of long-chain acyl-CoA synthetase in a reversible manner. The inhibition was competitive with respect to CoA. The reported apparent K_i for palmitoyl-CoA was nearly equal to the apparent K_m for CoA. This mechanism would serve to control intracellular levels of long-chain acyl-CoA esters within desirable limits.

Adenosine and AMP are potent competitive inhibitors with respect to ATP (Van Tol and Hülsmann, 1970). Levels of AMP are increased and LCFA oxidation is impaired in hypoxic hearts, thus AMP may have an energy-sparing effect and may limit the undesirable accumulation of long-chain acyl-CoA esters (Neely and Morgan, 1974). On the contrary, AMP has been shown to accelerate activation and oxidation of short- and medium-chain fatty acids in a rat heart homogenate (Neely and Morgan, 1974). Chagoya de Sanchez et al. (1977) administered high doses of adenosine, and found decreased rates of hepatic LCFA oxidation via an inhibition of the extramitochondrial acyl-CoA synthetase.

Pande (1971) revealed that the ability of isolated mitochondria from several rat tissues to activate LCFA was much greater than that required to support maximum rates of fatty acid oxidation, and concluded that this enzyme does not limit fatty acid oxidation. Aas and Daae (1971) measured rates of LCFA activation in liver, heart and adipose tissue of rats fed a stock diet, fasted, fasted refed-fat, fasted

refed-carbohydrate, and fat-fed. LCFA activation was slightly depressed only in the liver of the carbohydrate-refed rats. Thus, long-chain acyl-CoA synthetase is a constitutive enzyme in mammals.

Glyceride Synthesis

Activated fatty acids, acyl-CoA esters, are at a branchpoint between acylation to sn-glycerol-3-phosphate (G3P) or carnitine. Patton (1975) found an intensely labelled pool of phosphatidic acid in mammary tissue within 10 minutes after an IV injection of P-32. Askew et al. (1971a) showed that monopalmitin was an ineffective acyl acceptor in bovine mammary homogenates, while palmitate, stearate, oleate and linoleate were esterified to G3P at rates consistent with their concentration in milk (Askew et al., 1971b). These results suggest that the G3P pathway is the major pathway for the formation of milk fat triglycerides. Also, since triglycerides account for approximately 98% of milk fat, this section will be limited to discussing the controls of triglyceride synthesis via the G3P pathway.

Triglyceride synthesis by the G3P pathway is shown in Figure 1. Glucose serves as the source of G3P in mammary tissue, since glycerol kinase levels are low (Baldwin and Milligan, 1966). Phosphatidic acid synthesis proceeds through a sequential acylation of G3P mediated by two distinct acyltransferases (Monroy et al., 1973; Yamashita and Numa, 1972). The enzyme phosphatidate phosphohydrolase converts phosphatic acid to diacylglycerol, which is subsequently

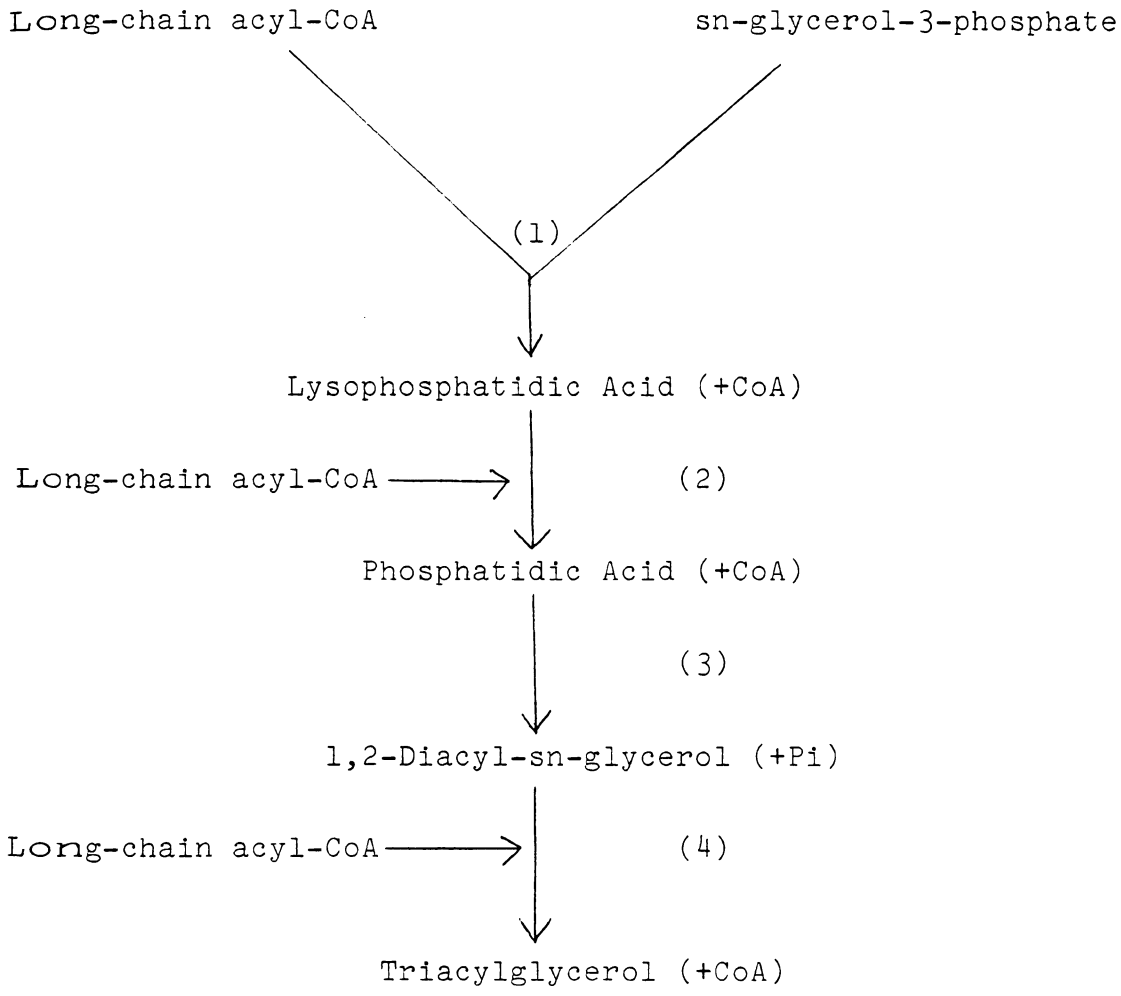


Figure 1. Pathway for the biosynthesis of triacylcerol. (1) acyl-CoA:sn-glycerol-3-phosphate O-acyltransferase (EC 2.3.1.15); (2) acyl-CoA:sn-monoacylglycerol-3-phosphate O-acyltransferase; (3) L-α-phosphatidate phosphohydrolase (EC 3.1.3.4); and (4) acyl-CoA:sn-diacylglycerol O-acyltransferase (EC 2.3.1.20).

acylated to triacylglycerol. The acyltransferases exist both in the microsomes and mitochondria of rat liver (Shephard and Hübscher, 1969; Zborowski and Wojtczak, 1969) and mammary gland (Pynadath and Kumar, 1964). Phosphatidate phosphohydrolase is distributed between the soluble and microsomal fractions (Sturton et al., 1978). Phosphatidic acid may be deacylated by the microsomal enzyme phospholipase A (Sturton et al., 1978). The first two acyl transferases may not be evenly distributed between the mitochondrial and microsomal fractions, as lysophosphatidic acid is the principle product formed from the acylation of G3P by palmitoyl-carnitine in rat liver mitochondria while phosphatidic acid is principally formed in microsomes (Daae, 1972). Thus, triglyceride synthesis by the G3P pathway in microsomes and mitochondria occurs by sequential acylation of G3P to form phosphatidic acid, hydrolysis of phosphatidate followed by acylation of the resultant diglyceride to form triglyceride.

The enzyme of triacylglycerol synthesis are modified by a number of effectors. Magnesium ions have been shown to inhibit the first acylation step in rat liver microsomes (Fallon and Lamb, 1968) and the second acylation reaction in bovine mammary gland (Kinsella, 1976) and to activate phosphohydrolase in rat adipocytes (Moller et al., 1977) guinea pig mammary gland (Kuhn, 1967) and bovine mammary gland (Marshall and Knudsen, 1977). However, partially purified glycerophosphate acyltransferase from rat liver mitochondria

requires Ca^{2+}, which can be substituted by Mg^{2+}, Mn^{2+}, and Co^{2+} (Yamashita and Numa, 1972). Phosphatidic acid inhibits the first acylation reaction, thus regulating its own formation (Monroy et al., 1973; Shapiro and Tzur, 1968). The inhibitory effects of palmitoyl-CoA on acylation are relieved by albumin (Gross and Kinsella, 1974). Daae (1972) showed that the mitochondrial acylation of G3P was more sensitive to inhibition by acyl-carnitines than the microsomal system. The regulation of glyceride synthesis by G3P will be discussed in a later section.

Synthesis of fatty acids occurs on a soluble acyl carrier protein (ACP) and the products of this process are acyl thioesters of ACP. ACP has been shown to transfer its acyl group to G3P in E. coli and clostriduim butyricum (Goldfine and Ailhaud, 1971). Palmitoyl-CoA competes effectively with palmitoyl-ACP for the sn-2 position of the glyceride molecule. The significance of this system in mammalian tissues is unknown.

Glyceride synthesis in particulate systems is stimulated by addition of a particle-free supernatant (PFS). Vavrecka et al. (1969) attributed this stimulation to the presence of phosphatidate phosphohydrolase in the soluble fraction. Serum proteins can bind the products of synthesis, thereby removing product inhibition (Shapiro and Tzur, 1968). Brindley et al. (1967) found that unsaturated LCFA stimulate glyceride synthesis, and attributed the stimulatory action of PFS to the presence of unsaturated fatty acids, which would

allow synthesis of a more balanced product. Thus, the stimulatory effect of PFS on glyceride synthesis may be a result of enzyme (phosphatidate phosphohydrolase) addition, unsaturated LCFA, or simply removal of end-product.

Fatty acids tend to be utilized differently depending upon fatty acid chain length and degree of unsaturation. Slakey and Lands (1968) discovered a non-random distribution of fatty acids between the 1- and 3-positions on triacylglycerol in rat liver. The esterification of fatty acids at each position proceeds with a specificity that is not correlated with the composition of the other positions of the molecule. The fatty acid specificity for the formation of milk triacylglycerols is particularly intriguing. Palmitoyl-CoA is the preferred substrate for the initial acylation of G3P, and the rate of palmitoyl-CoA acylation is 8 to 10 times greater that with myristoyl-, stearoyl-, or oleoyl-CoA. However, with 1-palmitoyl sn-glycero-3-P as an acyl acceptor all acyl-CoA esters were easily esterified (Kinsella and Gross, 1973). Short-chain fatty acids, synthesized in the mammary epithelial cell, are preferentially esterified to position sn-3 of milk fat triglycerides (Breckenridge and Kuksis, 1969). Oleic acid produced from desaturation of stearic acid <u>in situ</u> is also preferentially esterified to the third position, and desaturase activity is correlated with the rate of triglyceride synthesis (Kinsella, 1972). Results of Askew et al. (1971a) suggested that ability to acylate the sn-3 position in a bovine mammary

homogenate is difficult to achieve. However, Marshall and Knudsen (1977) found that microsomal 1,2-diacylglycerol acyltransferase from bovine mammary gland incorporated equal molar amounts of diglyceride, short-chain acyl-CoA or palmitoyl-CoA. Thus, glyceride synthesis may be controlled in part by substrate specificity.

The addition of sugars to the diet increases the formation of liver triglycerides (Lamb and Fallon, 1974). This increase is correlated temporally with an increase in microsomal and soluble phosphatidate phosphohydrolase. Under similiar dietary conditions, the ratio of phosphohydrolase: deacylase activity is enhanced (Sturton et al., 1978). These results suggest that the rate at which phosphatidate is converted to diacylglycerol or recycled back to G3P partly controls hepatic triacylglycerol synthesis.

Carnitine Acyltransferase

The stimulatory action of carnitine on fatty acid oxidation is well documented. Carnitine is an obligatory requirement for oxidation of long-chain fatty acids in isolated mitochondria of heart and skeletal muscle (Bode and Klingenburg, 1964), heart homogenate (Passeron et al., 1968), and heart slices (Fritz, 1964). Liver and kidney mitochondria can oxidize LCFA without carnitine, however, the oxidation of their corresponding acyl-carnitine esters is much greater (Bode and Klingenburg, 1964). Since fatty acyl-CoA is impermeable to the mitochondrial inner-membrane, acyl-CoA esters are compartmentalized in cells between the

extramitochondrial compartment, site of fatty acid activation, and the intramitochondrial compartment, site of fatty acid oxidation (Beattie, 1968). In order to traverse this impermeable barrier acyl-carnitine derivatives, which may be permeable to this barrier, are formed from acyl-CoA and carnitine. This transfer is catalyzed by carnitine acyltransferase (also called carnitine palmitoyltransferase, CPT) as presented in Figure 2.

Two pools of carnitine palmitoyltransferase (CPT-I and CPT-II) activities exist in mitochondria. CPT-I is associated with the outer membrane or the outer aspect of the inner membrane, and CPT-II is tightly bound to the inner membrane and available only to CoA in the mitochondrial matrix. CPT-II shows greater chain-length substrate specificity for the transfer of long-chain acyl groups from acyl-carnitine derivatives to CoA, while CPT-I shows broad chain-length substrate specificity (Kopec and Fritz, 1973; Yates and Garland, 1970; West et al., 1971; Brosnan and Fritz, 1971).

Some now believe that the mitochondrial inner-membrane is as impermeable to carnitine and acyl-carnitine esters as to CoA and its esters. A process of exchange diffusion between carnitine and acyl-carnitine across the mitochondrial inner-membrane, analogous to that causing ATP-ADP exchange, has been proposed for the transfer of acyl units (Ramsay and Tubbs, 1974; Pande, 1975). This transport is facilitated by the presence of a translocase system in mitochondria. Using isopycnic sucrose density gradient centrifugation methods, CPT is exclusively mitochondrial (Markwell et al.,

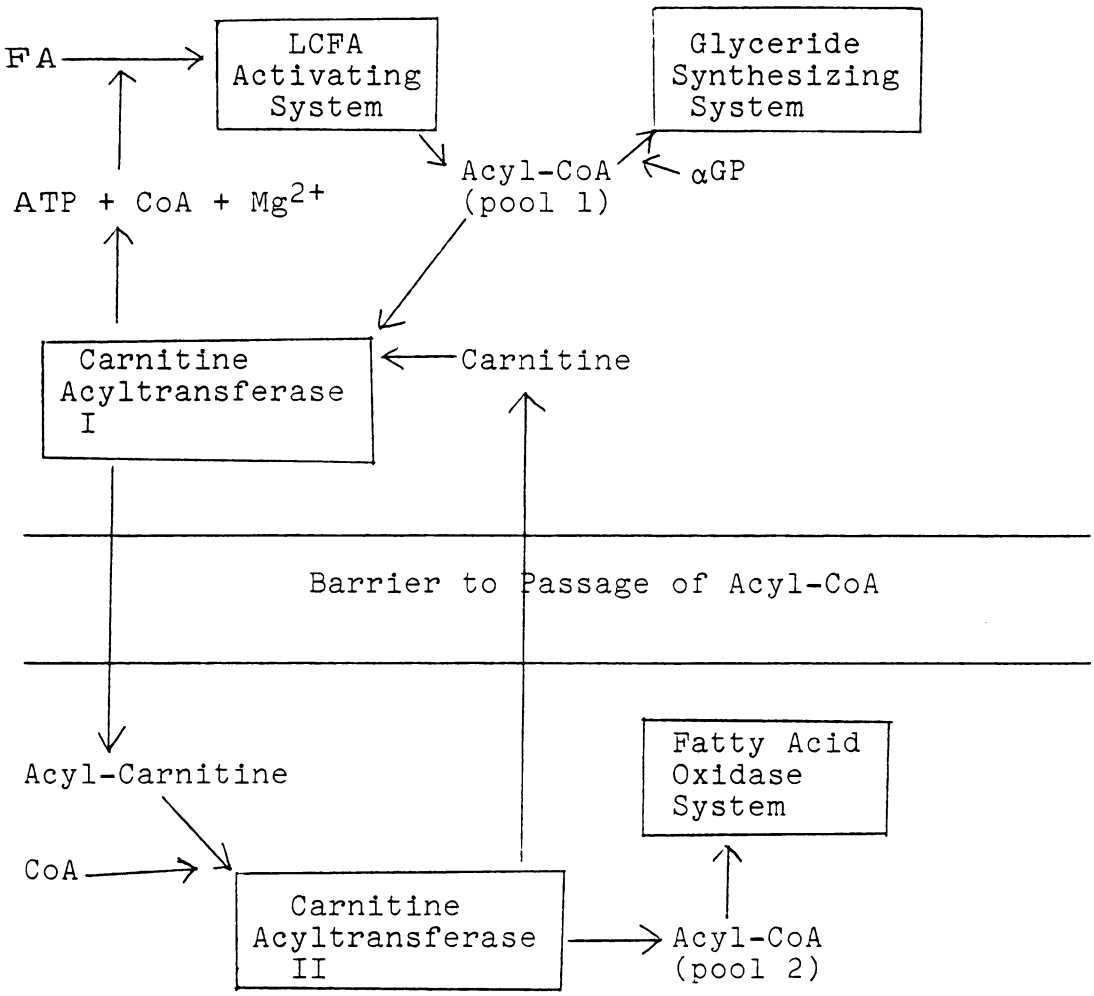


Figure 2. Mechanism for the transport of fatty acyl-Coa esters across the mitochondrial inner-membrane via the enzyme palmitoyl-CoA: L-carnitine O-palmitoyltransferase (trivial name carnitine palmitoyltransferase (CPT) EC 2.3. 1.21)(Fritz and Yue, 1963).

1973).

A carnitine acetyltransferase was extracted from pig heart and partially purified (Fritz et al., 1963). The substrates, acetyl-CoA, propionyl-CoA, and butyryl-CoA react at approximately equal rates, while activity with palmitoyl-CoA is nil. This enzyme was postulated to facilitate acetyl-CoA movement across mitochondrial membranes (Fritz and Yue, 1964). Snoswell and Henderson (1970) postulated that the presence of carnitine acetyltransferase in large amounts in sheep liver allows the "acetyl pressure" in the starved condition to be shifted from the vital CoA system to the carnitine system. Acetyl-carnitine would then serve as a storage form of acetyl-CoA, thus making more CoA available for gluconeogenesis from propionate. Carnitine acetyl-transferase is located in mitochondrial and microsomal fractions (Markwell et al., 1973; Markwell and Bieber, 1976). Microsomal carnitine acetyltransferase could be involved in providing a source of acetyl at sites of acetylation reactions.

A partially purified preparation of carnitine palmitoyltransferase revealed the existence of an additional protein fraction, which displayed a high substrate specificity towards the transfer of medium-chain acyl-carnitine derivatives (Kopec and Fritz, 1971). This fraction was tentatively designated carnitine octanoyltransferase. Clofibrate, a hypolipidemic drug, causes variable increases in carnitine acyltransferase activities in liver dependent on the chain length of the substrates. Factors for increase in specific

activity are 5.3, 2.8 and 1.7 for the transfer of short-chain, medium-chain and long-chain acyl-carnitine derivatives (Solberg et al., 1972). Therefore, three separate carnitine acyltransferases exist in mitochondria. When attaching biological significance to the short- and medium-chain acyltransferases, one must keep in mind that short-chain and medium-chain fatty acids are activated in the matrix of the mitochondria. Thus, the carnitine acyltransferase step would be bypassed. The fact that the effect of carnitine is more pronounced in the oxidation of LCFA lends credence to this point (Bremer, 1962).

Shepherd et al. (1966) discovered that isolated rat liver mitochondria respire faster in the presence of palmitoyl-carnitine than with palmitoyl-CoA plus carnitine as substrates. This led to the tentative hypothesis that the CPT enzyme is rate-limiting for fatty acid oxidation. On the contrary, by using much lower substrate concentrations equal rates of respiration are obtained with palmitoyl-carnitine versus palmitoyl-CoA plus carnitine (Pande, 1971). In addition, several investigators have shown that CPT activity is several-fold greater than the maximum ability for β-oxidation of palmitoyl-carnitine in isolated mitochondrial preparations (Bremer and Norum, 1967b; Pande, 1971; Cederbaum et al., 1975; Van Tol and Hulsmann, 1969). In vitro studies showed that palmitoyl-carnitine at a concentration of 2 μM, well below tissue concentrations of acid-insoluble carnitine (255-426 Mμ moles/g of liver)(Pearson and Tubbs, 1967), resulted in maximum oxygen uptake rates (Bremer and Norum, 1967a), and

high concentrations of carnitine inhibit palmitoyl-carnitine transferase at low palmitoyl-CoA levels (Bremer and Norum, 1967a). With this in mind it is being accepted that CPT is not rate-limiting for the oxidation of fatty acids.

In support of this latter concept, the CPT reaction is freely reversible in vitro (Norum, 1964), and if there is sufficient CPT in the cell, the ratio of long-chain acyl-CoA/CoA will vary with the ratio of long-chain acyl-carnitine/carnitine (Bremer, 1967). Indeed, long-chain acyl-CoA and acyl-carnitine tissue levels are increased during fasting, fasted refed-fat and diabetes (Tubbs and Garland, 1964; Bohmer, 1967; Greenbaum et al., 1971). Therefore, the ratios of long-chain acyl-CoA/CoA and long-chain acyl-carnitine/carnitine are in equilibrium in vivo. Long-chain acyl-CoA increased from 53 to 110 μM in livers of rats after fasting (Tubbs and Garland, 1964), and long-chain acyl-carnitine increased from 70 to 200 μM in hearts of rats after fasting (Marquis and Fritz, 1964). These results suggest that the capacity of the CPT enzyme does not limit fatty acid oxidation. The β-oxidation process itself may be limiting presumably via the availability of free CoA for the formation of intramitochondrial long-chain acyl-CoA, since long-chain acyl-carnitine accumulates (Bremer, 1967).

The K_m for palmitoyl-CoA for the CPT enzyme is 31 μM (Norum, 1964), and a concentration of 53 μM for long-chain acyl-CoA in liver has been reported (Tubbs and Garland, 1964). Thus, the concentration of long-chain acyl-CoA may play a

regulatory role in the formation of palmitoyl-carnitine. Bremer and Norum (1967b) found that palmitoyl-CoA is a competitive inhibitor of carnitine for CPT *in vitro*, but inhibition did not occur with protein-bound palmitoyl-CoA as one would expect to find in the cell. A number of studies do lend support for the original hypothesis that CPT activity may be rate-limiting *in vivo*. The results of Norum (1965) suggests that CPT activity is enhanced by fasting, fat-feeding, and diabetes. The increase in activity is not due to *de vovo* enzyme synthesis, but activation of preformed protein.

Rates of ketogenesis in perfused livers from fed rats diminished with oleic acid and octanoylcarnitine as substrates in comparison to fasting, whereas octanoic acid supported high rates of ketogenesis irrespective of the nutritional state of the donor animal (McGarry and Foster, 1974). This suggests that CPT-I is not coupled to CPT-II, and the ability of both LCFA and the medium-chain carnitine ester to gain entry into the mitochondria is restricted in the fed state. An inhibitor of carnitine acyltranserase, (+)-decanoyl-carnitine, decreases ketogenesis *in vivo* (McGarry and Foster, 1973) and changes the pattern of oleate metabolism in livers from that of fasted rats to that demonstrated by livers from normal animals (oleate is virtually completely esterified)(McGarry et al., 1973). The authors concluded that no fundamental defect exists in the triglyceride-synthesizing capability of the liver in the fasted state, and that fatty acid oxidation is under strict dietary and hormonal control exerted primarily

by regulation of an early step in the oxidation sequence, probably the carnitine acyltransferase. The relationship between fatty acid oxidation and esterification will be discussed later in more detail.

New and exciting evidence suggests that malonyl-CoA, an intermediate in fatty acid synthesis, at physiological concentrations inhibits ketogenesis from oleate and the site of inhibition is carnitine acyltransferase (McGarry et al., 1977). Subsequently, malonyl-CoA was found to be a competitive inhibitor of ketogenesis in isolated hepatocytes with a K_i of 2 nmol/g wet weight of cells (malonyl-CoA content in hepatocytes from meal-fed rats was 14.8 nmols/g wet weight of cells)(Cook et al., 1978). This suggests that fatty acid synthesis and oxidation are incompatible.

The β-oxidation Pathway

β-oxidation is the main catabolic pathway for fatty acids (Spector, 1971; Wakil, 1970; Green and Allman, 1968). This pathway, as shown in Figure 3, derives its name from the fact that the oxidative attack occurs at carbon atom 3 of the fatty acid, the β carbon atom. An acyl-CoA ester is degraded by successive cleavage of 2-carbon atom fragments from the carboxyl end of the fatty acid chain. The enzymes that catalyze these reactions are located in the mitochondrial matrix in close association with the inner-membrane (Beattie, 1968).

The acyl-CoA dehydrogenase catalyzes the oxidation of saturated acyl-CoA derivatives to the corresponding trans-α,β-

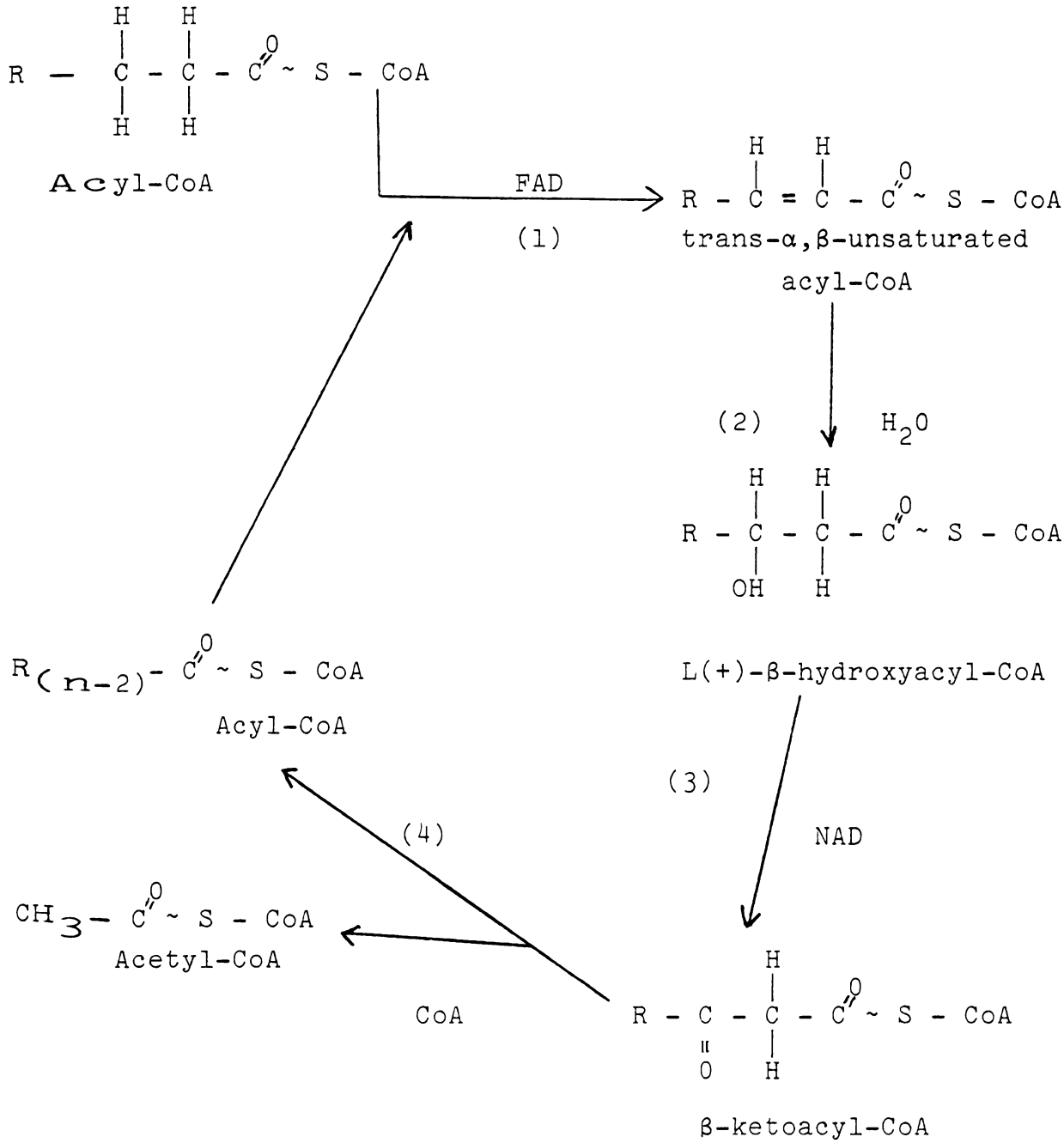


Figure 3. The β-oxidation pathway. (1) acyl-CoA dehydrogenase (acyl-CoA: FAD oxidoreductase, EC 1.3.2.2); (2) enoyl-CoA hydrase (L-3-hydroxyacyl-CoA hydrolase, EC 4.2.1.17); (3) L-β-hydroxyacyl-CoA dehydrogenase (L-3-hydroxyacyl-CoA: NAD oxidoreductase, EC 1.1.1.35); and (4) β-ketoacyl-CoA thiolase (acyl-CoA: acetyl-CoA acyltransferase, EC 2.3.1.16).

unsaturated derivative. Three enzymes specific for short-, medium-, and long-chain acyl-CoAs have been isolated. Therefore, fatty acyl-CoA derivatives of chain lengths C_4-C_{20} are readily oxidized to their corresponding α,β-unsaturated derivatives (Wakil, 1970). The binding of the substrate by the enzyme is essentially irreversible, and the K_m of the most effective substrate is about 1 μM (Green and Allman, 1968). The acyl-CoA dehydrogenases are flavoproteins containing flavin adenine dinucleotides (FAD) as prosthetic groups. The electrons from the substrate reduce the FAD to $FADH_2$, which are then transferred to another FAD-containing flavoprotein, the electron transferring flavoprotein, which is directly linked to cytochrome b of the mitochondrial electron transport system (Wakil, 1970). The trans α,β-unsaturated acyl-CoA ester is subsequently stereospecifically hydrated by enoyl-CoA hydrase (also referred to as crotonase) to the L(+)-β-hydroxyacyl-CoA derivative. Rate of reaction decreases markedly with increasing chain length. The V_{max} with crotonyl-CoA (the C_4 derivative) is approximately 150-fold greater than with the C16 derivative as substrate (Waterson and Hill, 1972).

The L(+)-β-hydroxyacyl-CoA derivative is oxidized by NAD in the presence of the L-β-hydroxyacyl-CoA dehydrogenase to the corresponding β-ketoacyl-CoA derivative. The enzyme is specific for the L-form, and is active on the various chain-length fatty acyl derivatives (Wakil, 1970).

The last step in the thiolytic cleavage of the β-ketoacyl-CoA to acetyl-CoA and a saturated acyl-$CoA_{(n-2)}$. The thiolase

enzyme has broad substrate specificity, and reacts equally with β-ketoacyl-CoAs of various chain lengths. Although the thiolase reaction is reversible, the equilibrium (K_{eq} = 6 x 10^4) greatly favors acetyl-CoA formation (Wakil, 1970).

When acyl-CoA derivatives enter the β-oxidation pathway, their oxidation was once thought to commence without regulation. However, studies now indirectly suggest the contrary. Intact liver mitochondria oxidizing labelled palmitate (Lopes-Cardozo et al., 1978) or labelled palmitoyl-carnitine (Stanley and Tubbs, 1974) accumulate substantial amounts of saturated acyl-CoA intermediates. The amount of these intermediates decreases with increasing chain length of the substrate (Stanley and Tubbs, 1975). The acyl-CoA intermediates accumulate slowly, while acetyl-CoA is produced linearly with time. These separate pools of "free acyl-CoA" intermediates arise via "leakage" from the true intermediates of β-oxidation, which are restricted in amount and have priveleged access to the enzymes of β-oxidation (Stanley and Tubbs, 1975). The quantity of intermediates formed depended on total flux of acyl units through β-oxidation. These results indicate that acyl-CoA dehydrogenase is rate limiting in vivo, or it may reflect some degree of organization of the enzymes of β-oxidation. Korsrud et al. (1977) found that the V_{max} of the acyl-CoA dehydrogenase decreases with increasing chain length, which indicates a possible control point for the oxidation of long-chain fatty acids. In accordance, intact liver mitochondria oxidizing octanoate did

not accumulate intermediates of the β-oxidation pathway (Stewart et al., 1973).

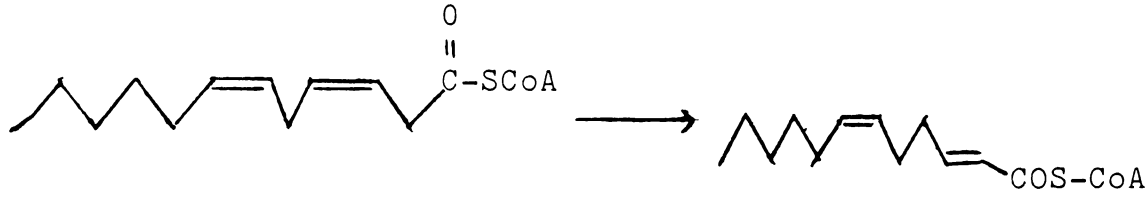
Bremer and Wojtczak (1972) revealed that a high NADH/NAD ratio blocked β-oxidation in liver mitochondria with the subsequent accumulation of β-hydroxypalmitoyl-carnitine. It was concluded that the NAD-linked oxidation of the β-hydroxypalmitoyl-carnitine is more easily suppressed than the flavoprotein-linked oxidation of palmitoyl-CoA.

Acetoacetyl-CoA strongly inhibits the hydroxyacyl-CoA dehydrogenase (Schifferdecker and Schulz, 1974) and enoyl-CoA hydrase enzymes (Waterson and Hill, 1972). Inhibition is non-competitive with respect to dehydrogenation but competitive with respect to hydration. The inhibition of enoyl-CoA hydrase is particularly interesting. Due to a combination of its cascading substrate specificity and its marked susceptibility to acetoacetyl-CoA, enoyl-CoA hydrase could be rate limiting for the oxidation of long-chain substrates, but not medium- and short-chain acyl-CoA substrates (Waterson and Hill, 1972).

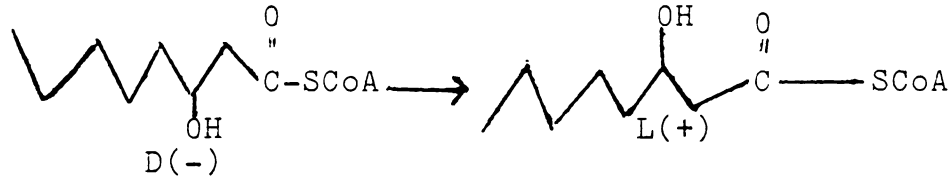
Pent-4-enoic acid is a hypoglycemic compound structurally related to the active metabolite of hypoglycin from the fruit of the ackee. Pent-4-enoic acid is a potent inhibitor of fatty acid oxidation in intact mitochochondria by its specific inhibition of the thiolase reaction (Holland et al., 1973).

Oxidation of Unsaturated and Odd-Chain Fatty Acids

The oxidation of unsaturated fatty acids by the β—oxidation pathway involves two additional enzymic reactions, isomerization and epimerization (Wakil, 1970). The oxidation of linoleic acid, for example, involves successive cleavage of acetyl units via β-oxidation to the formation of cis-$\Delta^{3,6}$-C12-CoA. This compound is then isomerized to the trans-Δ^{2}-enoyl derivative by Δ^{3}-cis-Δ^{2}-trans-enoyl-CoA, as shown in the following reaction:



The product enters the β-oxidation scheme at the enoyl-CoA hydrase step and undergoes two cycles of β-oxidation, forming cis-Δ^{2}-octenoyl-CoA. This is then hydrated by enoyl-CoA hydrase to the D-β-hydroxyacyl-CoA epimerase to the L(+) antipode, as shown in the following reaction:



The L—β-hydroxyacyl-CoA is the substrate for L-β-hydroxyacyl-CoA dehydrogenase shown in Figure 3. The isomerase and epimerase enzymes most likely do not regulate oxidation to any extent, since palmitate, stearate, oleate, linoleate, and linolenate are oxidized at equivalent rates in isolated mitochondria in the presence of carnitine (Lopes-Cardozo and and Van Den Bergh, 1974a).

Odd-chain fatty acids exist in nature in relatively low concentrations. They are oxidized by the β-oxidation pathway to acetyl-CoA and one equivalent of propionyl-CoA. Propionyl-CoA is then converted to succinyl-CoA (Wakil, 1970).

ω—Oxidation of Fatty Acids

Fatty acid oxidation via the ω-oxidation pathway has received a renewed interest. Bjorkhem (1976) demonstrated that ω-oxidation of stearic acid in liver is enhanced by fasting, and dicarboxylic acid administration to starved or diabetic rats lowers the concentration of blood ketones (Wada and Usami, 1977; Wada et al., 1971).

Hemmelgarn et al. (1977) estimated that 40% of the initial oxidation of fatty acids in the nonketotic diabetic rat is via ω-oxidation and the remainder is β-oxidation, while Wada and Usami (1977) estimate that about 15% of the fatty acids are subjected to ω-oxidation and then β-oxidation in fasted or diabetic rats. Thus, a discussion of the mechanism of ω-oxidation and its implication is warranted.

The ω-oxidation pathway is shown in Figure 4. The omega methyl group of fatty acid is directly hydroxylated in the liver microsomal fraction. The reaction requires NADP and O_2 and is catalyzed by omega fatty acid hydroxylase (Robbins, 1968). The omega hydroxy fatty acid is oxidized to the omega keto fatty acid by a liver-soluble supernatant fraction requiring NAD (Robbins, 1968). Bjorkhem (1973) attributes the stimulatory action of the supernatant fraction to the presence of alcohol dehydrogenase, which could protect the omega-hyroxylase from product inhibition by oxidation of

the omega-hydroxy fatty acid. Finally, the omega keto fatty acid is oxidized to the dicarboxylic acid by a liver-soluble supernatant fraction requiring NAD (Robbins, 1968). The hexadecanedioic acid (dicarboxylic acid) can be activated by rat liver mitochondria in presence of CoA, ATP, and Mg^{2+}. A carnitine ester is formed by the action of hexadecanoyl-CoA: carnitine O-hexadecanoyltransferase (EC 2.3.1.-). The rate of this reaction is an order of magnitude lower than the rate of formation of palmitoyl-carnitine, and is increased by fasting, diabetes, and clofibrate feeding. However, O_2 uptake with hexadecandioyl-carnitine as the substrate is low and transitory in liver and heart mitochondria (Pettersen, 1973).

The four terminal carbon atoms of fatty acids become aceto acetyl-CoA during β-oxidation, while those of dicarboxylic acids become succinyl-CoA. Wada et al. (1971) speculated that the significance of ω-oxidation is to produce succinyl-CoA from fatty acids in the mitochondria. Succinyl-CoA production facilitates the citric acid cycle and diverts acetyl-CoA from ketogenesis to oxidation. A net synthesis of succinyl-CoA could also result in a net synthesis of glucose. Hemmelgarn et al, (1977) suggested the ω-oxidation could possibly function to detoxify long-chain fatty acids, since it is a microsomal process.

Peroxisomal Fatty Acid Oxidation

A new development in fatty acid metabolism is the discovery of fatty acid oxidation in peroxisomes. Rat liver peroxisomes oxidize palmitoyl-CoA to acetyl-CoA reducing O_2 to

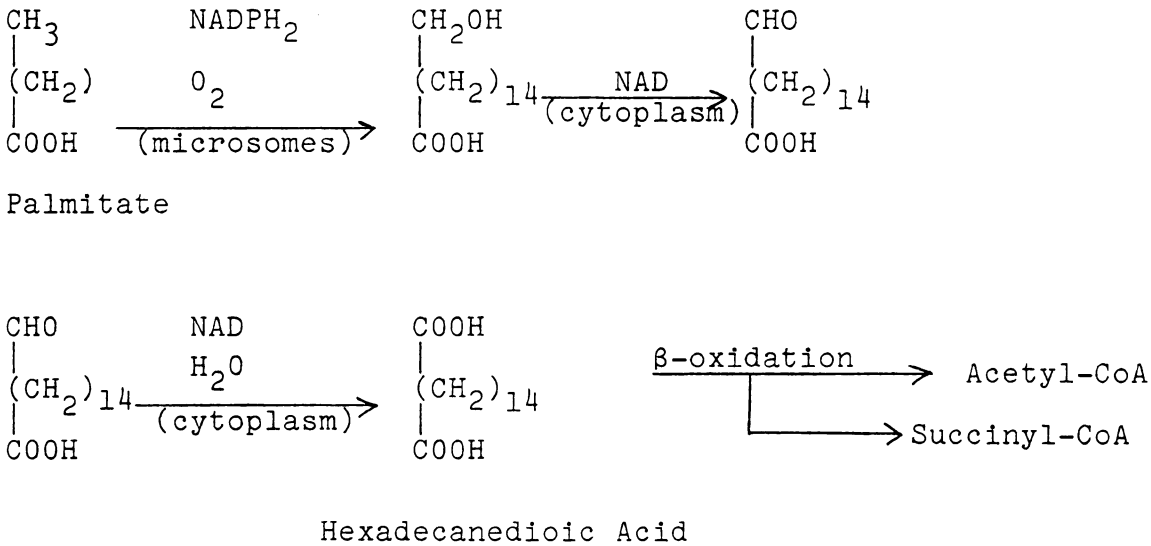


Figure 4. ω-oxidation pathway of fatty acids.

H_2O_2 and three moles of NAD (Lazarow and De Duve, 1976; Lazarow, 1977). The activity of this system is increased approximately one order of magnitude by feeding hypolipidemic drugs (clofibrate, Wy-14,643, and tibric acid). These drugs induce a marked proliferation of peroxisomes, while only stimulating catalase activity 50%. Catalase can constitute as much as 16% of the peroxisomal protein. Lazarow (1978) has recently discovered the presence of the β-oxidation enzymes in a purified peroxisomal preparation. The system has a high substrate specificity for long-chain acyl-CoA and is inactive towards butyryl-CoA. Palmitoyl-CoA is oxidized to acetyl-CoA. Thus, the oxidation of palmitoyl-CoA in peroxisomes involves the enzymes of β-oxidation and incomplete cleavage of palmitoyl-CoA to three acetyl-CoAs.

Relationship Between β-oxidation and the Citric Acid Cycle

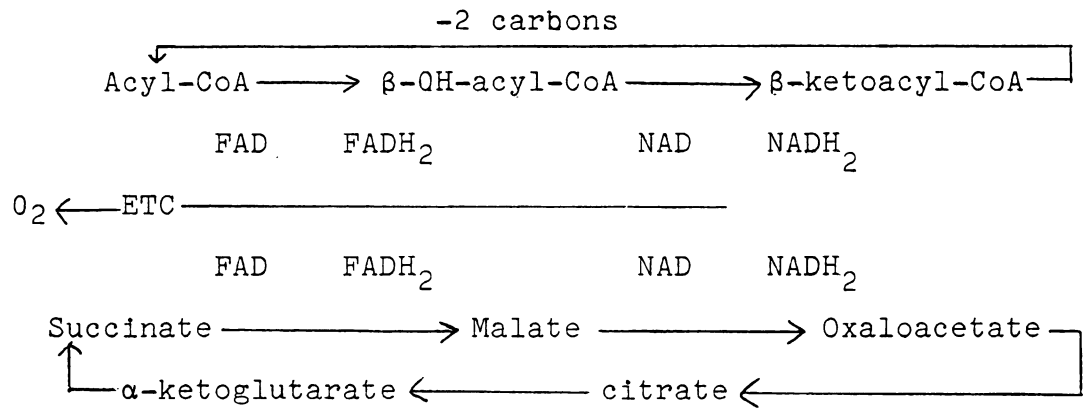
The complete oxidation of fatty acids to CO_2 involves a concerted operation between the β-oxidation and citric acid cycle pathways. Acetyl-CoA formed from β-oxidation of fatty acids is at a branchpoint between ketogenesis and oxidation via the citric acid cycle (TCA). The rate of ketogenesis in liver is correlated with fatty acid concentration, while complete oxidation to CO_2 is moderately affected (Ontko and Jackson, 1964). This implies that the capacity of the TCA cycle limits acetyl-CoA oxidation, and ketosis is a result of increased fatty acid flux to the liver. However, kidney cortex slices oxidized palmitate completely to CO_2 at all concentrations tested (Lee et al., 1962). Thus, TCA cycle

capacity for oxidation of acetyl-CoA appears to vary among organs.

As rat liver oxidizes fatty acids the intramitochondrial NADH/NAD ratio rises. The substrate pairs malate/oxaloacetate and NAD/NADH are in equilibrium, and an increased NADH/NAD ratio would favor malate formation at the expense of oxaloacetate. Thus, a lack of oxaloacetate would divert acetyl-CoA from citrate synthesis to acetoacetate synthesis (Lopes-Cardozo and Van Den Bergh, 1974b; Wieland, 1968). Van Tol (1970) showed that carnitine addition to isolated liver mitochondria enhanced ketogenesis and inhibited CO_2 production from palmitate, which resulted in an increased NADH/NAD ratio a decreased TCA cycle activity and lowered intramitochondrial oxaloacetate levels. Williamson et al. (1969) perfused livers with oleate and found increased rates of ketogenesis, but flux through the citrate synthase reaction was unaltered by oleate. Oleate did increase flux of oxalacetate from the mitochondria to the cytoplasm. The intramitochondrial oxalacetate concentrations were much less than the K_m for the citrate synthase reaction. So fatty acid oxidation did not alter TCA cycle flux, but diverted excess oxalacetate formation (from enhanced pyruvate carboxylation) to the cytoplasm for gluconeogenesis, in effect keeping oxalacetate concentrations sub-optimal for citrate synthase.

Succinate inhibits fatty acid oxidation in isolated liver mitochondria (Bremer, 1967). The interaction of succinate with β-oxidation was summarized by Bremer and Wojtczak (1972)

as shown in the following scheme:



Succinate and acyl-CoA compete at the flavoprotein level. Succinate can suppress β-oxidation independent of a high NADH/NAD ratio. Both the acyl-CoA and succinate dehydrogenations are followed by NAD-linked dehydrogenations, and accumulation of NADH prevents their oxidation. Malate dehydrogenation is more displaced to the hydroxy side than β-OH-acyl-CoA dehydrogenation and will be relatively more inhibited by NADH.

Under conditions of relatively high ATP/ADP ratio in the cell the electron transport chain will be rate limiting, and mutual inhibitions of the two cycles will be observed. Huxtable and Wakil (1971) found maximum palmitate oxidation at an energy charge of 0.65 in mitochondrial incubations. Since the β-oxidation cycle is less subject to suppression it will tend to dominate under conditions of increased acyl-carnitine availability, resulting in acetyl-CoA accumulation with a concomitant decreased rate of oxaloacetate formation.

Partitioning of Fatty Acids Between Oxidation and Esterification.

It is well documented that the concentration of fatty acids in the milieu is a major determinant of the rate of fatty acid utilization. However, enhanced uptake of fatty acid by the liver is not sufficient in itself to initiate maximum ketogenesis. Fasting increases fatty acid oxidation and decreases fatty acid incorporation into glycerides in liver slices (Rubenstein and Rubenstein, 1966), in hemidiaphragm pieces (Fritz and Kaplan, 1961), liver cells (Ontko, 1972), and in perfused livers (McGarry and Foster, 1971), but in perfused hearts from rats previously starved fatty acid incorporation into glycerides is elevated (Neely and Morgan, 1974). Fasting markedly enhances fatty acid oxidation in the goat mammary gland, but marginally decreases milk fat production (Annison et al., 1968). It appears likely that a major determinant of the rate of oxidation is competition for the fatty acid substrates between the β-oxidation and glyceride synthesis pathways.

McGarry and Foster (1971) perfused livers from fasted and nonfasted rats with labelled oleate and octanoate. Fasting markedly enhanced ketogenesis and depressed esterification from oleate, while octanoate metabolism was minimally affected. Octanoate is not directly used for glyceride synthesis in the rat liver. However, de novo synthesized fatty acids (C_2-C_{16}) are used for glyceride synthesis in ruminant mammary tissue (Breckenridge and Kuksis, 1969). Antiketogenic compounds (fructose, glycerol,

lactate and ethanol) in the perfusate reversed oleate metabolism from that observed in the fasting state. The major effect of these compounds was to promote esterification which was quantitatively sufficient to account for the diminished production of labelled ketones. These antiketogenic agents did increase the concentration of glycerol-3-phosphate (G3P) in livers from fasted rats. However, the concentration of G3P was not altered by fasting alone when compared to controls, even though the rate of esterification increased five-fold in the fed state.

It is of interest to note that glucose in the perfusate had no effect on any parameter measured. Glucose plus insulin does enhance palmitate esterification in the diaphragm muscle (Bodel et al., 1962), but not in kidney cortex slices (Lee et al., 1962) and heart slices (Fritz, 1964). The glycerol effect was confirmed by Ontko (1972). Prager and Ontko (1976) have since shown that fructose inhibits fatty acid oxidation by competitive substrate inhibition. Sorbitol has antiketogenic effects in liver, presumably via formation of G3P (Loten et al., 1966; Exton and Edson, 1964).

Glucagon increased palmitate oxidation and decreased palmitate incorporation into triglyceride in liver from rats previously fasted-refed carbohydrate. Glucagon enlarges the content of long-chain acyl-CoA which may directly inhibit the glyceride synthesizing enzymes (Christiansen, 1977). Glucagon may exert its effect through cAMP (Klausner et al., 1978). Fatty acid flux through the carnitine palmitoyltransferase

reaction is enhanced by glucagon, which is partly mediated by elevated tissue carnitine concentrations with glucagon treatment (McGarry et al., 1975). However, an increased cellular concentration of carnitine could not account for the altered fatty acid metabolism in liver cells from fasted rats (Christiansen et al., 1976).

The distribution of fatty acid between oxidation and esterification is likely to be influenced by the relative activities of the carnitine palmitoyltransferase and glycerophosphate acyltransferases enzymes. Starvation or fat-feeding decreased glycerophosphate acyltransferase activity in rat liver only and increased carnitine palmitoyl-transferase activity in liver and heart. Carbohydrate re-feeding reversed these enzyme changes (Aas and Daae, 1971). Thus, these enzymes may establish the metabolic fate of fatty acids in the liver. No corresponding enzyme changes occurred in adipose tissue indicating that the regulation of fatty acid metabolism in adipose tissue and heart is mainly mediated through the availability of fatty acids and competing sub-strates (Aas and Daae, 1971). At low acyl-CoA concentrations the acylation of glycerophosphate is the preferred reaction, probably because this reaction has the lower K_m for palmitoyl-CoA (Borrebaek et al., 1976). In agreement, dietary conditions that enhance glyceride synthesis also reduce tissue levels of acyl-CoA esters.

Thus, the partitioning of fatty acids between oxidation and esterification may be affected by addition of precursors for G3P and the relative activities of the carnitine and G3P

acyltransferases. The apparent K_m's for G3P for glycerophosphate acylation in liver mitochondria and microsomes are 0.5 and 1.7 mM (Van Tol, 1974), while the concentration of G3P in liver is about 85 µM (Greenbaum et al., 1971). So increases in G3P concentration should augment glyceride synthesis. Also, the relative activities of the carnitine and glycerophosphate acyltransferases are important in this partitioning mechanism, especially in organs which are both highly lipogenic and oxidative, such as the liver. Organs such as heart and kidney have very low rates of glycerophosphate acylation in comparison to carnitine acylation, while adipose is the reverse (Borrebaek et al., 1976). Again, in these organs fatty acid concentration in the milieu is the major determinant of utilization in the cell. Both enzymes demonstrate high activity in the liver.

Flavaspidic acid, an inhibitor of fatty acid for the fatty acid binding protein, depresses oleate esterification but increases oxidation in rat hepatocytes (Wu-Rideout et al., 1976). Octanoate utilization is not altered. This suggests that as fatty acids are taken-up by the cell, their primary fate is esterification. However, this may simply be an unphysiological effect of binding by protein, since albumin addition lowers long-chain fatty acid oxidation (Lopes-Cardozo and Van Den Bergh, 1974a).

POSSIBLE CONTROL POINTS OF LONG-CHAIN FATTY ACID OXIDATION IN RUMINANT MAMMARY TISSUE

Annison and coworkers (1964, 1967, and 1968) provided quantitative data in an elaborate series of experiments on the metabolism of substrates by the mammary gland of lactating ruminants. These data were obtained by using isotope dilution coupled with measurements of milk secretion rate, mammary blood flow, and arteriovenous (AV) differences of substrates across the mammary gland. These data are summarized in Table 1. Acetate and glucose are of primary importance for oxidative metabolism in the whole animal and the mammary gland. Fatty acids are important oxidative substrates in the fasted animal. Glucose uptake by the udder was 60-85% of the total glucose entering the circulation, and lactose accounted for the greatest proportion of the glucose uptake. Acetate, besides being a substrate for oxidative metabolism, and β-hydroxybutyrate are the precursors for the C_4 to C_{16} fatty acids synthesized de novo in the mammary gland. It is interesting to note that glucose, acetate and plasma fatty acids account for less than 50% of the total CO_2 output by the udder of the fed goat. Acetate and glucose alone accounted for 30-75% of the total CO_2 from the udders of fed cows (Bickerstaffe et al., 1974).

These data imply that acetate is the preferred oxidative substrate in the mammary gland, and that fasting releases inhibition of long-chain fatty acid oxidation. However, indirect evidence suggests that long-chain fatty acids may

be important oxidative substrates in the mammary gland of the fed ruminant. The total long-chain acyl-carnitine level (41 μM) in mammary tissue of lactating goats is higher than in skeletal muscle, heart, kidney cortex and liver (Snoswell and Linzell, 1975). Also, carnitine palmitoyltransferase activity is significant in mammary tissue from lactating ewes (Snoswell and Linzell, 1975). Thus, the level of long-chain acyl-carnitine in mammary tissue is higher than the level in tissues in which fatty acids are quantitatively important oxidative substrates.

The data in Table 1 suggest several possible points of control of long-chain fatty acid oxidation in mammary tissue. Acetate, the preferred oxidative substrate, could inhibit long-chain fatty acid oxidation via substrate competition, either by entry into the citric acid cycle or competition for available CoA. Acetate inhibits palmitate oxidation in rat skeletal muscle, presumably via competition between the two for available CoA (Karlsson et al., 1977).

Another possible control would simply be an increased substrate availability to the udder, since starvation markedly enhances plasma fatty acid uptake. This would seem unlikely, since the mammary gland also actively extracts fatty acids from blood triglycerides. The rate of plasma triglyceride uptake by the udder of fed cows is about 200 mg/min (Bickerstaffe et al., 1974), and mammary AV differences of plasma triglycerides in the fasted goat are 30% of those of fed goats (Annison et al., 1968). Askew et al. (1971a) found

Table 1.--Mammary Metabolism of Glucose, Acetate, and Fatty Acids in Fed and Fasting Lactating Goats.

Substrate	Entry Rate[1]	Udder Uptake[2]	Contribution of Substrate to CO_2(%)		% of Uptake Oxidized by Udder
			Whole Animal	Udder	
Glucose (Fed)[3]	4.2	69.3	10	39	27
(Fasted)[5]	1.3	13.4	4	9	7
Acetate (Fed)[3]	5.7	32.5	29	27	49
(Fasted)[5]	1.2	1.6	10	11	63
Palmitate (Fed)[4]	0.4	<0.1	<1	<1	-
(Fasted)[5]	6.8	3.0	8	8	25
Stearate (Fed)[4]	0.2	<0.1	<1	<1	-
(Fasted)[5]	0.9	5.7	6	4	6
Oleate (Fed)[4]	0.3	<0.1	<1	<1	-
(Fasted)[5]	3.2	4.3	9	6	8

[1]Entry rate expressed as mg/min-kg body weight.

[2]Udder uptake expressed as mg/min-kg tissue.

[3]Data from Annison and Linzell (1964).

[4]Data from Annison et al. (1967).

[5]Data from Annison et al. (1968).

fatty acid concentrations of 4 mM in bovine mammary tissue. So it appears unlikely that fatty acid is in limited supply for fatty acid oxidation.

Plasma fatty acids are quantitatively transferred to milk triglyceride fatty acids, and blood glucose is the precursor of the glycerol in milk triglycerides. Thus, long-chain fatty acid oxidation in mammary tissue may be limited by the competing glyceride synthesis pathway. However, long-chain fatty acid oxidation in mammary tissue of the goat is stimulated by fasting, but milk fat secretion is 81% of that in the fed animal (Annison et al., 1968). Thus, during fasting when fatty acid oxidation is stimulated, fatty acid esterification to G3P remains relatively constant.

Assuming that the carnitine palmitoyltransferase (CPT) enzyme in rat liver mitochondria is similar to that of mammary gland, the possibility exists that carnitine may be limiting for fatty acid oxidation in the mammary gland. The apparent K_m for carnitine is 0.25 mM for the liver mitochondrial CPT enzyme (Bremer and Norum, 1967a). Free carnitine concentrations of 0.1 mM in cow's milk and 0.25 mM in goat mammary tissue have been reported (Snoswell and Linzell, 1975). Thus, mammary tissue levels of free carnitine may not be optimal for carnitine palmitoyltransferase activity.

CONCLUDING REMARKS

The control of fatty acid oxidation is multi-faceted. Availability of fatty acid is a major determinant on the rate of fatty acid oxidation in some tissues, such as kidney. In

organs such as liver with appreciable rates of glycerophosphate and carnitine acylation, competition between oxidation and esterification determines the intracellular fate of fatty acids. The possibility of the carnitine transport mechanism as a rate-limiting step is still controversial. In ketogenic organs fatty acid oxidation is coupled to the citric acid cycle, and the capacity of the citric acid cycle can determine the ultimate fate of the end-product of β-oxidation. Just where the control(s) of fatty acid oxidation in the bovine mammary gland lies is presently unknown.

MATERIALS AND METHODS

PROCEDURE FOR MEASURING FATTY ACID OXIDATION AND ESTERIFICATION IN BOVINE MAMMARY TISSUE SLICES

Preparation of Buffer and Radioactive Substrates for Incubation

Tissue slices were incubated in Krebs-Ringer bicarbonate (KRB) buffer (Umbreit et al., 1972). The buffers were made the night before use, and pH was adjusted to 7.4 prior to collection of tissue. The composition of KRB buffer used for incubation is shown in Appendix Table 1. The $CaCl_2$ concentration was halved relative to that recommended by Umbreit et al. (1972), due to its insolubility at recommended concentrations. The incubation buffer contained bovine serum albumin (BSA Fraction V, fatty acid-poor, Sigma Chemical Co.). It was determined that palmitate oxidation was not affected by varying the BSA concentration in the media. In consideration of the four strong fatty acid binding sites on BSA and that in the majority of published studies on fatty acid oxidation the molar ratio of fatty acid:BSA ranges from 2.0 to 7.0, it was decided to keep the fatty acid:BSA molar ratio constant at 4.0.

Stock solutions of palmitate[1-^{14}C], palmitate[U-^{14}C], and oleate[1-^{14}C](free acids, Amersham Corp.) were stored in benzene at 4°, octanoate[1-^{14}C](sodium salt, Amersham Corp.) in absolute ethanol at 4°, and acetate[1-^{14}C](sodium salt, Amersham Corp.) in distilled water at -15°.

Palmitate and oleate were prepared for incubation as follows: 1) 1.0 ml of labelled fatty acid in benzene (about 0.01 mCi) was dried under N_2; 2) unlabelled fatty acid (free acid, Sigma Chem. Co.) was added to desired concentration; and 3) the mixture was dissolved in 1.0 ml of absolute ethanol and stored at 4° prior to use. A 0.01 ml aliquot of the fatty acid in ethanol solution was added to 3 ml of KRB buffer in 25 ml flasks in a shaking water bath (37°, 60 cycles/min, Dubnoff Metabolic Shaker). The flasks were then gassed for 10-15 sec with O_2:CO_2 (95:5) and capped with rubber stoppers fitted with plastic center wells (Kontes of Vineland, New Jersey) containing a fluted filter paper 2 mm^2. Gassing the flasks with O_2:CO_2 prior to incubation stimulated palmitate oxidation (about 25%) at 180 and 240 min of incubation. The flasks were then left shaking for approximately one hour to allow dissolution of fatty acid.

Originally, palmitate was added to the incubation as an ammonium salt according to Ontko and Jackson (1964). However, using ethanol as a carrier for fatty acid was easier, and rates of palmitate oxidation were equivalent (Appendix Table 2). Also, palmitate oxidation was not affected by increasing volumes of ethanol.

Tissue Collection and Preparation for Incubation

Mammary tissue pieces, weighing 30 to 50 g, were excised from udders of lactating Holstein cows killed by a bolt gun. The tissue pieces were immediately placed in an insulated flask containing warm (37°) KRB buffer (2 units oxytocin/100 ml, Sigma Chem. Co.). Tissue was then rinsed in warm KRB

buffer (no oxytocin), cubed, trimed to minimize non-parenchymal tissue, and sliced using a Stadie-Riggs microtome. The Stadie-Riggs was grooved to give slices of approximately 0.5 mm in thickness. The slices were placed in a common flask containing warm buffer, then rinsed several times with warm buffer. The data in Appendix Table 3 show that collecting and rinsing tissue in warm buffer as opposed to cold buffer enhanced palmitate oxidation.

Slices were gently blotted to remove excess fluid, trimed to minimize non-parenchymal tissue, weighed (usually 60 to 80 mg) on a Mettler balance, then added to incubation flasks to commence incubation. Rates of palmitate oxidation replicated well with tissue slices ranging from 40 to 100 mg. Treatments within experiments were run in quadruplicate flasks, and values were averaged.

Determination of CO_2

Production of CO_2 was corrected against blanks (flasks incubated for 0 min with tissue). Incubation was terminated after various times by injecting 0.3 ml of 5 N H_2SO_4 into the media, followed by injection of 0.3 ml of methyl benzathonium hydroxide (tradename Hyamine Hydroxide, Sigma Chem. Co.) into the center well to trap CO_2. The flasks were shaken for an additional hour, after which the center well and its contents were transferred to scintillation vials. Samples were counted in a Nuclear-Chicago model 720 liquid scintillation counter for two-ten minute counts in 10 ml of Aqueous Counting

Scintillant (ACS, Amersham Corp.). Counting efficiency was 87.5%, using [^{14}C] benzoic acid (New England Nuclear) as an internal standard, and 82.5% in the presence of 0.3 ml of Hyamine Hydroxide. The rate of palmitate [1-^{14}C] conversion to $^{14}CO_2$ was calculated as follows:

$$\underset{\text{(pmoles/mg-min)}}{^{14}CO_2} = \frac{\text{(dpm in sample)} - \text{(dpm in blank)}}{\text{(specific activity added)(mg tissue)(min)}}$$

Radioactivity in the blanks was 10-50% of that obtained in samples incubated for the shorter incubation times, and approximately 10% of that obtained for the longer incubation times.

To determine concentration of glucose remaining in incubation media after CO_2 collection, 1.0 ml of media was neutralized and analyzed for glucose by using a coupled enzymatic assay (kit no. 115-A, Sigma Chem. Co.).

Source of Animals

All of the animals used in this study were lactating Holstein cows either from the university herd or a local abattoir. The cows from the university herd could be divided into two groups. One group consisted of cows killed in early lactation (10 to 16 days postpartum) from two separate physiology experiments. The other group of cows from the university herd were cull cows. These cows were in middle to late lactation and culled for various reasons, mostly infertility and mastitis.

Briefly, the physiology experiments involved: 1) prepartum injections of an ergot derivative or an ergot derivative

plus prolactin and 2) serum hormone patterns in cows nursing calves. Rates of palmitate oxidation for all groups of cows are summarized in Appendix Table 4. Palmitate oxidation was not affected by any of the treatments in the physiology experiments and these cows are grouped as early lactation.

Lipid Extraction of the Tissue Slices

After the center wells were transferred to scintillations vials, the tissue slices from quadruplicate flasks were pooled and rinsed four times in distilled water to remove "loosely attached" fatty acids. The tissue slices were then extracted for lipids by the method of Folch et al. (1957). Tissue slices were homogenized in chloroform:methanol (2:1, 2 ml per tissue slice) with a Polytron homogenizer (Brinkman Instruments). The homogenate was filtered, and the homogenizer and filter paper washed twice with 5 ml of chloroform:methanol (2:1). The volume of the total extract was recorded and a 5.0 ml aliquot was counted and counting efficiency was 75%, and the efficiency of recovery of palmitate [1-^{14}C] by the Folch method was 95%.

The rate of uptake was calculated as follows:

$$\text{Uptake (pmoles/mg-min)} = \frac{\left[\begin{array}{l}\text{dpm in total}\\ \text{extract of}\\ \text{sample}\end{array}\right] - \left[\begin{array}{l}\text{dpm in total}\\ \text{extract of}\\ \text{blank}\end{array}\right]}{(\text{specific activity added})(\text{mg})(\text{min})} + \begin{array}{l}\text{rate of}\\ CO_2\\ \text{production}\end{array}$$

Total recovery of dpm as CO_2 plus tissue plus media was 93-98% of that added.

To the lipid extract 0.2 volumes of 0.05 M KCl were added, vortexed three times, and centrifuged (IEC model K

centrifuge, Damon/IEC Division). The upper phase (water phase) was drawn off with a disposable Pasteur pipet, and the chloroform phase (lower phase) was washed again. The two upper phases were combined, volume recorded, and a 0.5 ml aliquot counted in 10 ml of ACS. Counting efficiency of 0.5 ml of the upper phase was 79.3%. Accumulation of water-soluble intermediates was less than 1% of the total activity in tissue, and therefore, is not presented. Excess anhydrous Na_2SO_4 was added to the chloroform phase to remove "trapped" water. The chloroform solution was centrifuged, the supernatant drawn off into scintillation vials, dried under N_2, and resuspended in 0.3 to 0.5 ml of chloroform:methanol (2:1) for thin-layer chromatography.

Fatty Acid Esterification

Esterified fatty acid fractions were determined by thin-layer chromatography (TLC). A 30-50 µl aliquot of the lipid extract in chloroform:methanol was spotted on silica gel-60 TLC plates (0.25 mm gel thickness, precoated on glass, E.M. Merck, Co.). The TLC plates were developed 50 to 60 min in a glass tank with hexane:diethyl ether:glacial acetic acid (70:30:2). A neutral lipid standard mix (Sigma Chem. Co.) containing monoglyceride, 1,2- and 1,3-diglycerides, fatty acid, and triglyceride was co-chromatographed. The plates were then dried and sprayed with 0.2% dichloroflourescein in ethanol for visualization. The spots were scraped-off and counted in 10 ml of ACS to determine percentage distribution. Since size of the spots varied, quenching and efficiency of elution of radioactivity from the gel was determined. Spots

of varying but known size and radioactivity were dried, sprayed, scraped-off, and counted. Quenching was nil and recovery of radioactivity was essentially 100%.

Rates of accumulation for each lipid fraction were calculated as follows:

$$\text{Rate (pmols/mg-min)} = \frac{(\text{dpm in total extract})(\%\text{ of radioactivity on TLC plate})}{(\text{specific activity added})(\text{mg tissue})(\text{min})}$$

The rates for fatty acid were corrected against blanks, since fatty acid was the only labelled lipid in the blanks.

Incubation media, from an experiment with tissue slices incubated in the presence of glucose and palmitate[1-^{14}C] for 60 and 180 min, was extracted for lipids and the extract was chromatographed. Fatty acid was the only lipid fraction detected, thus data reported in the text apply to tissue slices only.

CARNITINE PALMITOYLTRANSFERASE ACTIVITY (CPT)

CPT activity was assayed spectrophotometrically in mammary mitochondria according to Bieber et al. (1972). This assay is based on the principle that DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) traps coenzyme A liberated from palmitoyl-CoA by the action of CPT.

Sources of Reagents

Tris (Tris hydroxymethyl aminomethane), EDTA (ethylenediamine tetraacetic acid), D-mannitol, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, Triton X-100, DTNB, and S-palmitoyl-CoA (free acid) were supplied by Sigma Chemical Co., sucrose by Mallinckrodt, NaEDTA by Fisher

Scientific Co., and sodium bicarbonate by J.T. Baker Chemical Co. L(-)-carnitine was a generous gift of Dr. L. L. Bieber of the Biochemistry Department of Michigan State University.

Isolation of Mitochondria

Mammary mitochondria from three cows were assayed for CPT with varying concentrations of carnitine and palmitoyl-CoA, in order to determine appropriate substrate concentrations.

Mammary tissue was excised from lactating cows at a local abattoir and placed in cold (4°) buffer (0.25 M sucrose, 0.2 mM EDTA, pH 7.5 with 150 mM Tris buffer). Tissue was finely minced with scissors and homogenized in 10 volumes (wt/v) of buffer in a Ten-Broeck ground, glass homogenizer. Homogenates were centrifuged at 4° for 12 min at 750 x g. The supernatant was centrifuged at 4° for 12 min at 6700 x g. The pellet was resuspended and the 750 x g and 6700 x g centrifugations repeated twice. The mitochondrial pellet was suspended in 5 ml of buffer (70 mM sucrose, 220 mM mannitol, 2 mM HEPES buffer, pH 7-4, 1 mM EDTA) and stored frozen.

For isolation of mitochondria from tissue slices the above procedure was modified somewhat. Since mitochondrial yield was low from slices, the 750 x g and 6700 x g centrifugations were not repeated. The mitochondrial pellet, however, was washed 3 times with cold homogenization buffer, resuspended in 0.3 ml of suspension buffer, and stored frozen.

CPT Assay

Stock solutions of reagents (1% Triton X-100, 100 mM l(-)-carnitine, 0.7 mM palmitoyl-CoA, and 2.5 mM DTNB in 0.01 M $NaHCO_3$ pH 7.0 were prepared and stored frozen. A stock solution of buffer (2.3 M Tris, 0.022 M NaEDTA, pH 8.0) was prepared the day of assay. Reaction was started by adding 100 µl of a reagent and buffer mix solution, water, and mitochondrial suspension (kept on ice). Final reaction volume was 200 µl and the reaction was monitored at 412 nm (Gilford Spectrophotometer model 2400-S) at room temperature. For the CPT assay in tissue slice mitochondria 60 µl of water and 40 µl of mitochondria were added to the cuvette. The final concentrations of reagents during the assay were: 116 mM Tris-HCl 0.1 mM NaEDTA, 0.1% Triton X-100, 35 µM palmitoyl-CoA, 0.25 mM DTNB, and 1.25 mM l(-)-carnitine. The change in optical density was determined for the first minute of the reaction, because the assay is linear for only 2-5 min at 25°.

To determine palmitoyl-CoA hydrolase activity, duplicate samples were assayed in the absence of carnitine. CPT activity was obtained by subtracting the initial rate of formation of CoA in the absence of carnitine from the rate in the presence of carnitine, an extinction coefficient of 13,600 for DTNB was used to determine enzyme rates.

Enzyme activity was linear with protein concentrations to 0.24 mg/ml. Protein was determined by the method of Lowrey et al. (1951).

STATISTICAL ANALYSIS

Experiments were blocked according to treatment and time. Statistical evaluations were by analysis of variance, orthogonal contrasts and t-tests. For some sets of data there was mild heterogeneity of variance even after log transformation. However, standard statistical tests are known to be robust against departures from the assumption of equal variance.

RESULTS AND DISCUSSION

When investigating controls *in vitro* on a particular metabolic pathway, it is imperative to establish maximum reaction conditions for the incubation system to be used. As previously stated, palmitate oxidation per mg tissue was not affected by amount of tissue, BSA, and ethanol in the incubation media within limits used, and only slightly enhanced by gassing the media prior to incubation. However, the rates of palmitate oxidation in mammary tissue slices were found to increase in a curvilinear fashion over time, shown in Table 2. The values shown are rates of palmitate oxidation obtained at the particular incubation time. Thus, mammary tissue can oxidize fatty acid, but rates of oxidation increase over time of incubation *in vitro*.

Using typical rates of palmitate oxidation at 180 min for mammary tissue slices obtained from cows in which total udder weight was determined, it was estimated that palmitate oxidation could potentially account for 15 ml of O_2 consumption by the whole udder per min using an RQ of 0.70 for the oxidation of lipids. This rate of O_2 consumption is 6.2% of the rate of O_2 uptake (243 ml/min) by the udder of fed lactating cows (Bickerstaffe et al., 1974). Using higher rates of palmitate oxidation, fatty acids could potentially account for 10% of the oxidative metabolism of mammary tissue. Thus, fatty acid

oxidation can account for a significant proportion of the overall energy metabolism in the mammary gland of a lactating cow.

Table 2.--Palmitate Oxidation Versus Time of Incubation in Bovine Mammary Tissue Slices.

	Incubation Time (min)				
	30	60	120	180	240
Palmitate[1] to CO_2[2]	0.4*	0.9	1.5	1.9	2.9
SD[3]	0.3	0.5	0.9	1.0	1.4
N[4]	(5)	(12)	(10)	(12)	(6)

*Rates at 180>30 ($P<0.10$), 240>30 ($P<0.01$), 180>60 ($P<0.05$), and 240>60 ($P<0.01$).

[1]Palmitate at 0.26 mM.

[2]Rates expressed as pmoles/mg-min.

[3]Standard deviation.

[4]Number of replicates.

As mentioned, rates of palmitate oxidation increase with time of incubation, and further investigation of this time effect may provide insight concerning regulation of fatty acid oxidation in the mammary gland of the cow. However, certain questions must first be adequately recognized and answered. Is the effect of incubation time on palmitate oxidation a matter of substrate solubility or an artifact of the incubation system? Are endogenous pools of fatty acid utilized in preference to the exogenous pools?

In order to determine if this increasing oxidation rate with time was unique to mammary tissue or an artifact of the

incubation system, the rate of palmitate oxidation was determined in rat kidney cortex slices in the same manner as that determined for mammary tissue slices. The data in Table 3 show that rates of palmitate oxidation in rat kidney cortex slices were constant over time. The maximum rate of palmitate oxidation (2.9 pmoles/mg-min) observed in mammary tissue slices (Table 2) is comparable to rates found in rat kidney cortex slices (3.5-4.2 pmoles/mg-min). Barac-Nieto (1976) found that rat kidney cortex slices oxidize 0.98 mM palmitate at a constant rate of 8.3 pmoles/mg-min, which is comparable to the rates shown in Table 3 with 0.26 mM palmitate. Thus, increasing rates of palmitate oxidation with increasing time of incubation is not an artifact of the incubation conditions, and mammary tissue slices oxidize palmitate at rates similar to those of rat kidney cortex slices.

Table 3.--Palmitate Oxidation Versus Time of Incubation in Rat Kidney Cortex Slices.

	Time				
	30	60	120	180	240
Palmitate[1] to CO_2[2]	3.5	3.5	5.0	4.2	3.9
SD[3]	0.5	0.3	1.5	0.2	-
N[4]	(2)	(2)	(2)	(2)	(1)

[1]Palmitate at 0.26 mM
[2]Rates expressed as pmoles/mg-min.
[3]Standard deviation.
[4]Number of replicates.

The data in Tables 2 and 3 were taken from experiments in which palmitate was added to start the incubation. Palmitate is insoluble in aqueous media, and dissolution of fatty acid by binding to protein is not an immediate process. Hence, one can envision that as palmitate becomes available with time in a physically suitable form to the tissue, its rate of oxidation will increase. The results shown in Table 3 and data presented in the remainder of the text from experiments in which approximately one hour was allowed for binding of palmitate to protein in the media prior to start of incubation by adding tissue show that palmitate oxidation is enhanced by incubation time. Thus, the effect of incubation time can not be explained by substrate solubility.

It should be noted that data in Tables 2 and 3 were from tissue slices that were weighed at the end of the incubation. The data presented in the remainder of the text are from experiments in which the tissue slices were weighed prior to start of incubation. Thus, these values may not be directly comparable to those obtained in the following experiments.

The effect of substrate concentration on palmitate oxidation in mammary tissue slices is shown in Table 4. A palmitate concentration of 0.26 mM gave maximum rates of oxidation at both times. This level of palmitate would correspond to arterial plasma fatty acid concentrations in the fed cow (Bickerstaffe et al., 1974). Also, rate of palmitate oxidation doubled between 60 and 180 min at all concentrations tested. Half-maximal velocity was reached

at 0.1 mM palmitate for both times. Therefore, maximal rates of palmitate oxidation are obtained with physiological fatty acid concentrations (0.26 mM) at both times, and the effect of incubation time is independent of this substrate concentration which normally would be expected in mammary tissue. Thus, it seems that in mammary tissue fatty acid oxidation is not regulated by fatty acid concentration.

Table 4.--Palmitate Oxidation Versus Palmitate Concentration.

Concentration	Time	
	60	180
	--pmoles/mg-min--	
0.065 mM	0.3^{1}	0.6^{a}
0.13	0.5^{x}	1.2^{by}
0.26	0.8^{x}	1.8^{cy}
0.33	0.8^{x}	1.8^{cy}

^{abc}Means in columns with different superscripts are different $P<0.10$.

^{xy}Means in rows with different superscripts are different $P<0.02$.

^{1}Standard error of difference between means is 0.26, and number of replicates is 4.

A comparison was made of the effect of fatty acid chain length on fatty acid oxidation as revealed in Table 5. Palmitate, oleate and octanoate oxidations were determined simultaneously in four experiments, while the rate reported for acetate oxidation was from three independent experiments. The rates reported here are not on an equivalent carbon-atom basis. The data clearly show that oxidation decreases, as

chain length increases up to C16. Acetate is oxidized at substantially greater rates than is palmitate, oleate or octanoate, which agrees with Bickerstaffe et al (1974) who found that acetate is the preferred oxidative substrate in the bovine mammary gland. Swenson and Dimick (1974) found substantial rates of medium-chain fatty acid oxidation in the goat mammary gland. Annison et al. (1967) could not detect oxidation of long-chain fatty acids in the mammary gland of the fed goat. Palmitate and oleate are oxidized at equal rates irrespective of chain length and unsaturation. The oxidation of octanoate and acetate marginally changes with time, while oxidation of palmitate and oleate is doubled by incubation time.

Table 5.--Fatty Acid Oxidation Versus Chain Length.

Fatty Acid[1]	Time	
	60	180
	--pmoles/mg-min---	
Palmitate	0.7[2a]	1.3[a]
Oleate	0.7[a]	1.3[a]
Octanoate	9.1[b]	11.3[b]
Acetate	49.7[3]	57.7[3]

[ab]Means in columns with different superscripts are different P<0.01.

[1]Palmitate, oleate, and octanoate at 0.26 mM, acetate at 0.6 mM.

[2]Standard error of difference between means is 0.46, and number of replicates is 4 for palmitate, oleate, and octanoate.

[3]Rates are average of three separate experiments, standard deviations are 17.9 and 17.8 for 60 and 180 min.

The differences in rates of oxidation with different chain can be explained by a number of possible reasons. Short-chain and medium-chain fatty acids can be activated in the intramitochondrial-matrix space, thereby bypassing the carnitine mediated transport mechanism. Whereas, long-chain fatty acid oxidation is carnitine dependent, since they are activated on the outside of the inner-mitochondrial membrane. This suggests that carnitine palmitoyltransferase activity limits fatty acid oxidation. Also, long-chain fatty acids by virtue of their site of activation are accessible to the competing esterification pathway. The higher rates of octanoate oxidation and/or the lack of time effect may indicate regulation of long-chain fatty acid oxidation at the level of β-oxidation, since the results of others suggest that long-chain fatty acid oxidation is regulated by the β-oxidation enzymes (Korsrud et al., 1977; Stewart et al., 1973; Waterson and Hill, 1972). In addition, long-chain fatty acid oxidation may be regulated at the peroxisomal level. In summation, short- and medium-chain fatty acids are oxidized at much greater rates than long-chain fatty acids, and increasing oxidation with time is unique to long-chain fatty acids.

As previously mentioned, utilization of endogenous fatty acids with time of incubation could account for increasing rates of palmitate oxidation. Mammary tissue fatty acid concentrations of 4 mM have been reported (Askew et al., 1971a). To test this hypothesis, mammary tissue slices were preincubated for various times in buffer without substrate,

afterwhich palmitate was added. The data are shown in Table 6. The rate of ^{14}C-palmitate oxidation doubled between 60 and 180 min in the tissue slices preincubated for 0 min. However, preincubation of tissue slices for 60 min maximally stimulated palmitate oxidation and abolished the effect of time on palmitate oxidation.

Table 6.--Effect of Preincubating Mammary Tissue Slices in Buffer with No Substrate on Palmitate Oxidation.

Preincubation	Time 60*	180**
	--pmoles/mg-min----	
0 min	0.7[1a]	1.4[b]
30	1.0[a]	1.9[b]
60	1.5	1.7
90	1.1	1.4

*At 60 min 1.5>0.7 ($P<0.01$).

**At 180 min 1.9>1.4 ($P<0.05$).

[ab]Means in rows with different superscripts are different $P<0.01$.

[1]Standard error of difference between means is 0.21, and number of replicates is 3.

It should be noted here that determination of CO_2 specific activity over time would yield more definitive answers to the above hypothesis. However, attempts to determine the CO_2 specific activity in earlier unrelated experiments using NaOH as a CO_2 trap were unsuccessful.

To test the validity of the previous hypothesis in another manner, mammary tissue slices were again preincubated for

various times, but unlabelled palmitate was present during preincubation after which palmitate [1-^{14}C] was added. The presence of palmitate during preincubation should block palmitate oxidation by replenishing endogenous pools of fatty acid. The results are shown in Table 7. Rate of palmitate oxidation tripled between 60 and 180 min with 0 min preincubation. However, preincubation of the tissue slices for 60 min with unlabelled substrate maximally stimulated palmitate oxidation and abolished the effect of incubation time. Thus, increasing rates of palmitate oxidation are independent of the presence of substrate prior to incubation. This would negate the hypothesis that utilization of endogenous fatty acids limits ^{14}C-palmitate oxidation in vitro.

Table 7.--Effect of Preincubating Mammary Tissue Slices With Unlabelled Palmitate on Palmitate Oxidation.

Preincubation	Time	
	60*	180
	-----pmoles/mg-min---	
0 min	0.6[1a]	1.8[b]
30	1.4	2.0
60	2.1	2.0
90	2.2	2.0

*At 60 min 2.1>0.6 ($P<0.05$).

[ab]Means in rows with different superscripts are different $P<0.05$.

[1]Standard error of difference between means is 0.39, and number of replicates is 2.

In summation, the observed effect of incubation time on fatty acid oxidation can not be explained by substrate solubility, concentration, or presence of endogenous fatty acids, and is unique to bovine mammary tissue when compared to the rat kidney.

Experiments were designed to indirectly test whether the entire palmitate molecule is oxidized to CO_2 in mammary tissue slices. This hypothesis was evaluated by comparing the oxidation of palmitate[1-^{14}C] to palmitate[U-^{14}C]. If fatty acid oxidation is limited by the β-oxidation enzymes in the mitochondria and/or peroxisomes, then greater rates of oxidation with palmitate[1-^{14}C] in comparison to palmitate [U-^{14}C] would be expected. If flux through the citric acid cycle limits palmitate oxidation to CO_2, then a greater rate of accumulation of non-acid labile, water-soluble intermediates (mostly ketone bodies and citric acid cycle intermediates) would result from the oxidation of palmitate[U-^{14}C] versus palmitate[1-^{14}C]. The data in Table 8 indeed reveal that oxidation of palmitate[1-^{14}C] is greater than palmitate[U-^{14}C]. Also, time of incubation appears to have a more pronounced effect on palmitate[U-^{14}C] oxidation, nearly a three-fold increase. Rates of accumulation of non-acid labile, water-soluble intermediates are low with both palmitate isotopes. Differences between the two isotopes in rates of uptake are statistically insignificant.

Thus, it appears that the enzymes of β-oxidation may limit palmitate oxidation. In agreement, Stanley and Tubbs (1975)

found substantial amounts of saturated acyl-CoA intermediates in liver mitochondria oxidizing palmitoyl-carnitine, and concluded that acyl-CoA dehydrogenase may be rate-limiting for fatty acid oxidation. Harper and Saggerson (1976) showed that in isolated rat adipocytes three palmitate isotopes were oxidized at different rates and in the following order: palmitate[1-^{14}C] > palmitate[U-^{14}C] > palmitate[16-^{14}C]. Waterson and Hill (1972) showed that enoyl-CoA hydrase demonstrates much greater substrate specificity for short- and medium-chain substrates than for long-chain substrates. This coupled with the data in Table 5, which shows greatly enhanced rates of octanoate oxidation as opposed to palmitate and oleate, suggests that the β-oxidation enzymes are rate-limiting.

Long-chain fatty acyl-CoA is at a metabolic branchpoint between the pathways of esterification and oxidation. The effect of metabolites, which serve as precursors to glycerol-3-phosphate (G3P),on fatty acid oxidation is well documented in non-ruminant tissues. In general, when one pathway is favored it is at the expense of the other pathway. In the bovine blood glucose is the precursor for G3P in the mammary gland. Therefore, stimulation of fatty acid esterification should influence fatty acid oxidation. The influence of glucose on fatty acid oxidation was investigated, and the results are shown in Table 9. Glucose at a physiological concentration (2.8 mM = 50 mg/100 ml) markedly inhibits palmitate oxidation, and is especially effective when palmitate oxidation is maximally stimulated by doubling its

Table 8.--Oxidation of Palmitate[1-^{14}C] Versus Palmitate[U-^{14}C).

	CO_2[1]		Uptake[1,2]		WS[1,3]	
Isotope	60	180	60	180	60	180
	pmoles/mg-min					
Palmitate[1-^{14}C][4]	0.7^{ax}	1.2^{ay}	16.4^{x}	9.1^{y}	0.1	<0.1
Palmitate[U-^{14}C]	0.3^{bx}	0.8^{by}	11.5	8.8	0.1	<0.1

[ab]Means in columns with different superscripts are different $P<0.01$.

[xy]Means in rows with different superscripts are different $P<0.05$.

[1]Standard errors of difference between means for CO_2, uptake, and WS are 0.11, 2.74, and 0.16, and number of replicates is 4.

[2]Uptake = rate of accumulation of activity in tissue + rate of CO_2 production.

[3]WS = water-soluble intermediates.

[4]Palmitate at 0.26 mM.

concentration. In agreement, glucose causes a 50% reduction in palmitate oxidation in chick embryo heart cells (Rosenthal and Warshaw, 1973), and glucose plus insulin blocks palmitate oxidation in rat hemi-diaphragm pieces (Fritz and Kaplan, 1961). Glucose apparently blocks fatty acid oxidation by supplying more G3P, which in turn would deplete the fatty acyl-CoA pool available for oxidation. Reimer et al. (1975) showed that increased G3P levels derived from an increased glucose turnover, due to insulin, stimulates fatty acid esterification. Rates of palmitate oxidation increase over time, although not statistically significant, with glucose present. It is unlikely that this is due to a depletion of glucose from the media as incubation progresses, as 1.4 mM glucose remains in the media after 180 min. The results in Table 10 show that palmitate oxidation is maximally inhibited by glucose at 1.4 mM in the media. Thus, rates of palmitate oxidation tend to increase with time even in the presence of glucose, which can not be explained on the basis of glucose concentration. Accordingly, acetate may inhibit long-chain fatty acid oxidation in the mammary gland by competing with fatty acids for available coenzyme A in the mitochondrial-matrix space, entry into the citric acid cycle, or at the oxidative phosphorylation level. Henceforth, this will be termed substrate competition at the mitochondrial level. Acetate may also compete with fatty acids for available coenzyme A in the cytoplasm. Also, acetate may inhibit fatty acid oxidation via malonyl-CoA which inhibits carnitine palmitoyltransferase activity.

Table 9.--Effect of Glucose on Palmitate Oxidation.

Treatments	Time 60	180*
	--------pmoles/mg-min-------	
0.13 mM Palmitate	0.6[a1]	1.3[b]
0.13 mM P + Glucose[2]	0.2	0.5
0.26 mM Palmitate	0.9[a]	2.2[b]
0.26 mM P + Glucose	0.3	0.6
	(2.2)[3]	(1.4)[3]

*At 180 min 1.3> 0.5 ($P<0.10$), 2.2> 0.6 ($P<0.01$), and 2.2> 1.3 ($P<0.10$).

[ab]Means in rows with different superscripts are different $P<0.05$.

[1]Standard error of difference between means is 0.31, and number of replicates is 4.

[2]Glucose at 2.8 mM (Sigma Chem. Co.)

[3]Concentration of glucose in mM remaining in media at end of incubation.

In terms of metabolic energy in the ruminant mammary gland, acetate is quantitatively the most important source (Bickerstaffe et al., 1974). Hence, the effect of acetate on palmitate oxidation in mammary tissue slices was studied, and the results are shown in Table 11. Acetate inhibits palmitate oxidation by 40% at both times, and doubling the acetate concentration from 0.6 to 1.2 mM resulted in no further inhibition. In agreement, acetate inhibited palmitate in isolated rat liver mitochondria (Cederbaum and Rubin, 1975). Karlsson et al. (1977) discovered that acetate inhibits palmitate oxidation in the perfused hindquarter of resting rats, and suggested that acetate and palmitate compete for the available pool of free CoA in the mitochondria. Thus, acetate at physiological concentrations inhibits palmitate oxidation in bovine mammary tissue slices.

Table 10.--Effect of Glucose Concentration on Palmitate Oxidation.

	[Glucose] mM						
	0	0.7	1.4	2.8	4.2	5.6	8.4
Palmitate[1,2] to CO_2	1.3	0.9	0.8	0.8	0.8	0.7	0.8
SD	0.6	0.3	0.1	0.1	0.1	0.2	0.2

[1]Rates of palmitate oxidation determined at 180 min only, and number of replicates is 2. Rates expressed as pmoles/mg-min.
[2]Palmitate at 0.26 mM.

Table 11.--Effect of Acetate on Palmitate Oxidation.

Treatments	Time 60	180
	----pmoles/mg-min-------	
Palmitate[1]	0.8[3a]	2.0[bx]
P + 0.6 mM Acetate[2]	0.5[a]	1.2[by]
P + 1.2 mM Acetate	0.5[a]	1.2[by]

[ab]Means in rows with different superscripts are different P<0.05.

[xy]Means in columns with different superscripts are different P<0.05.

[1]Palmitate at 0.26 mM.

[2]Acetate from Sigma Chem. Co.

[3]Standard error of difference between means is 0.27, and number of replicates is 4.

Acetate and β-hydroxybutyrate supply carbon for the synthesis of fatty acids in ruminant mammary gland. Malonyl-CoA, an intermediate in fatty acid synthesis, blocks ketogenesis in the rat liver by inhibiting the carnitine palmitoyltransferase enzyme (Cook et al., 1978; McGarry et al., 1977). The formation of malonyl-CoA is catalyzed by the enzyme acetyl-CoA carboxylase, which is inhibited by the drug TOFA (5-(tetradecyloxy)-2-furoic acid)(Cook et al., 1978). Therefore, an experiment was designed to evaluate if acetate exerts its effect on palmitate oxidation via a build-up of malonyl-CoA. The results in Table 12 show that acetate blocks palmitate oxidation and the drug TOFA is ineffective in relieving this inhibition. This experiment was not replicated.

The data in Table 12 implies that acetate does not inhibit palmitate oxidation via malonyl-CoA. However, the extent to which TOFA entered the cells and specifically inhibited acetyl-CoA carboxylase activity was not determined. Rates of palmitate oxidation increase with time in the presence of acetate, as shown in Tables 11 and 12. Accordingly, the results obtained in two independent experiments are consistent with a malonyl-CoA inhibition of palmitate oxidation, as the ratio of rate of acetate oxidation to rate of fatty acid accumulation from acetate increased from 5.3 at 60 min to 9.2 at 180 min. Therefore, as less acetate is being utilized for fatty acid synthesis, more palmitate is being oxidized. However, cause and effect has not been established.

Also, by using an acetate oxidation rate of 50 pmoles/mg-min (equivalent to 0.9 μmoles/100 mg tissue in 180 min), it can be estimated that enough acetate would be present at 180 min with the 1.2 mM acetate treatment (Table 11) to invoke a maximum acetate inhibition on palmitate oxidation. Thus, increasing rates of palmitate oxidation in the presence of acetate is probably not a result of acetate depletion from the media.

As alluded to previously, glucose may be depressing fatty acid oxidation by supplying more G3P, thereby decreasing fatty acyl-CoA available for oxidation by increasing esterification. The effect of acetate on fatty acid oxidation was alluded to as a competition between palmitate and acetate for the available pool of free CoA in the cytoplasm. Thus, acetate

should reduce both palmitate oxidation and esterification. If acetates inhibits palmitate oxidation by substrate competition at the mitochondrial level or via malonyl-CoA inhibition of CPT, then it should not affect palmitate esterification. It was also of interest to determine whether glucose and acetate were additive in inhibiting palmitate oxidation, since both of these substrates are of primary importance in mammary gland metabolism. Therefore, rates of palmitate oxidation and esterification were simultaneously measured in mammary tissue slices incubated with glucose and acetate. Also, rates of palmitate oxidation and esterification were determined over time of incubation, in order to determine if changing rates of palmitate esterification.

Table 12.--Effect of Acetate and TOFA on Palmitate Oxidation.

Treatments	Time	
	60	180
	-------pmoles/mg-min------	
Palmitate	0.4[1]	1.2
P + Acetate[2]	0.3	0.5
P + TOFA[3]	0.4	1.1
P + A + TOFA	0.3	0.5

[1]Means from one experiment.

[2]Palmitate and acetate at 0.26 and 0.6 mM.

[3]TOFA (5-(tetrodecyloxy)-2-furoic acid) at 0.1 mM, was a generous gift of Merrell National Labs of Cincinnati, Ohio.

The effects of glucose and acetate on palmitate utilization are shown in Table 13. To be concise, the reported rates of accumulation of the various glycero-lipid fractions are the means for each treatment over time. The complete data are reported in Appendix Table 6. Rates of palmitate uptake and conversion to phospholipid (PL), monoacylglycerol (MG), diacylglycerol (DG), neutral lipid (NL, sum of mono-, di-, and triglyceride), glycero-lipid (GL, NL plus PL), and intracellular fatty acid (FA) decrease with incubation time in the presence of glucose. Acetate has similar effects on uptake, MG, and FA. Rate of DG formation decreases with time in the presence of glucose plus acetate.

As shown in Table 13, palmitate oxidation was typically enhanced by incubation time. Glucose and acetate are potent inhibitors of palmitate oxidation, and they demonstrate no additive effect on oxidation. Acetate was just as effective as glucose in inhibiting oxidation in these experiments. Rates of palmitate oxidation tend to increase with time, although statistically insignificant, in the presence of acetate. Thus, glucose and acetate again are inhibiting palmitate oxidation with no additive effect of the two existing, and rates of palmitate oxidation remain low throughout the entire incubation in the presence of glucose and acetate.

The decrease in palmitate oxidation in the presence of glucose was associated with a concomitant increase in palmitate esterification. Rao and Abraham (1975) also found similar changes in palmitate utilization with glucose in mammary tissue slices from lactating mice. The increased conversion

Table 13.--Effect of Glucose and Acetate on Palmitate Oxidation and Esterification.

Treatments	CO_2[1] 60	CO_2[1] 120	CO_2[1] 180	Uptake[1,2]	PL[1,2]	MG[1,2]	DG[1,2]	TG[1,2]	NL[1,2]	GL[1,2]	FA[1,2,3]
	pmoles/mg-min										
Palmitate	0.7^{ax}	1.3^{ay}	1.5^{ay}	12.7	0.7^{a}	0.2^{a}	1.0^{a}	7.8	9.1^{d}	9.7^{a}	1.5
P + Glucose	0.3^{b}	0.4^{b}	0.4^{b}	15.7	1.3^{b}	0.8^{b}	3.3^{b}	6.9	11.0^{e}	12.3^{b}	2.8
P + Acetate	0.2^{b}	0.4^{b}	0.4^{b}	12.7	0.8^{a}	0.2^{a}	1.0^{a}	7.6	8.9^{d}	9.6^{a}	2.4
P + G + A[4]	0.2^{b}	0.3^{b}	0.4^{b}	14.9	1.2^{b}	0.6^{b}	2.4^{b}	8.1	11.1^{e}	12.3^{b}	2.0

[ab]Means in columns with different superscripts are different $P<0.01$.

[xy]Means in rows with different superscripts are different $P<0.01$.

[de]Means in columns with different superscripts are different $P<0.05$.

[1]Standard errors of difference between means for CO_2, Uptake, PL, MG, DG, TG, NL, GL, and FA are 0.10, 1.03, 0.07, 0.06, 0.29, 0.55, 0.69, 0.74, and 0.55, and number of replicates is 6.

[2]Uptake is the sum of CO_2 and total activity on the TLC plate. PL (phospholipid, is what remained at origin on TLC plate but designated PL since PL do not migrate from origin with system used). MG (monoacylglycerol), DG (diacylglycerol), TG (triacylglycerol), NL (neutral lipid, MG + DG + TG), GL (glycero-lipid, NL + PL), and FA (fatty acid).

[3]Rate of FA accumulation corrected against blanks, since blanks were known to contain only FA.

[4]Palmitate, glucose, and acetate at 0.26, 2.8, and 0.6 mM.

of palmitate into neutral lipids and glycero-lipids is quantitatively sufficient to account for the depressed rates of palmitate oxidation. Palmitoyl-CoA molecules are at a branchpoint between esterification to G3P or carnitine. The apparent K_m for palmitoyl-CoA for carnitine palmitoyltransferase in bovine mammary tissue is 21 μM as shown in Figure 5a, however, Kinsella and Gross (1973) found an apparent K_m of 4 μM palmitoyl-CoA for the acyl-CoA:G3P acyltransferase. Thus, both enzymes exhibit very high affinities for palmitoyl-CoA but that of G3P acyltransferase is higher. In turn, at relatively low palmitoyl-CoA concentrations esterification would be the favored route. This is consistent with the data in Table 13 in which rates of esterification are much greater than those of oxidation. Also, the apparent K_m for G3P is 0.26 mM for G3P acyltransferase (Kinsella and Gross, 1973), and lactating bovine mammary tissue levels of 0.14 mM for G3P have been reported (Baldwin and Cheng, 1969). So it would be expected that supplying precursors to G3P in the media would enhance fatty acid esterification. Levels of G3P doubled in mammary tissue slices from lactating mice when incubated with glucose (Rao and Abraham, 1975). In summation, glucose seems to inhibit palmitate oxidation by diverting palmitate to glycero-lipids. Alternatively, glucose metabolism in mammary tissue would increase the NADPH/NADP ratio, which may inhibit peroxisomal fatty acid oxidation. Although, the extent of fatty acid oxidation in the peroxisomes of mammary tissue is unknown.

It is of particular interest to note the effect of glucose on rates of palmitate conversion to the various lipid fractions. Glucose augmented the conversion of palmitate to PL, MG, and DG, but not TG (triacylglycerol). Rate of TG formation accounted for approximately 56 and 86% of the rate of total glycero-lipid formation in the presence and absence of glucose. This would suggest that maximum stimulation of TG formation is limited by some factor(s). The data of Kinsella (1972) suggests that TG synthesis in the bovine mammary gland is limited by oleic acid availability. Short- and medium-chain fatty acids are preferentially esterified to the sn-3 position of the TG molecule, and TG synthesis in a bovine mammary microsomal preparation was stimulated by increasing butyryl-CoA but not palmitoyl-CoA concentrations (Marshall and Knudsen, 1977). Glucose causes a "build-up" of partial glycerides, MG and DG, and maximum formation of TG in the tissue slices may be limited by supply of fatty acids that are preferentially esterified to the sn-3 position.

In support of the hypothesis that acetate is blocking palmitate oxidation by substrate competition at the mitochondrial level or via malonyl-CoA inhibition on CPT, the data in Table 13 show that acetate inhibits palmitate oxidation but rates of palmitate uptake and esterification are unaltered. Glucose and acetate are not additive in blocking oxidation and rates of palmitate esterification are similar to those found with glucose alone. Therefore, acetate appears to inhibit palmitate oxidation by substrate competition.

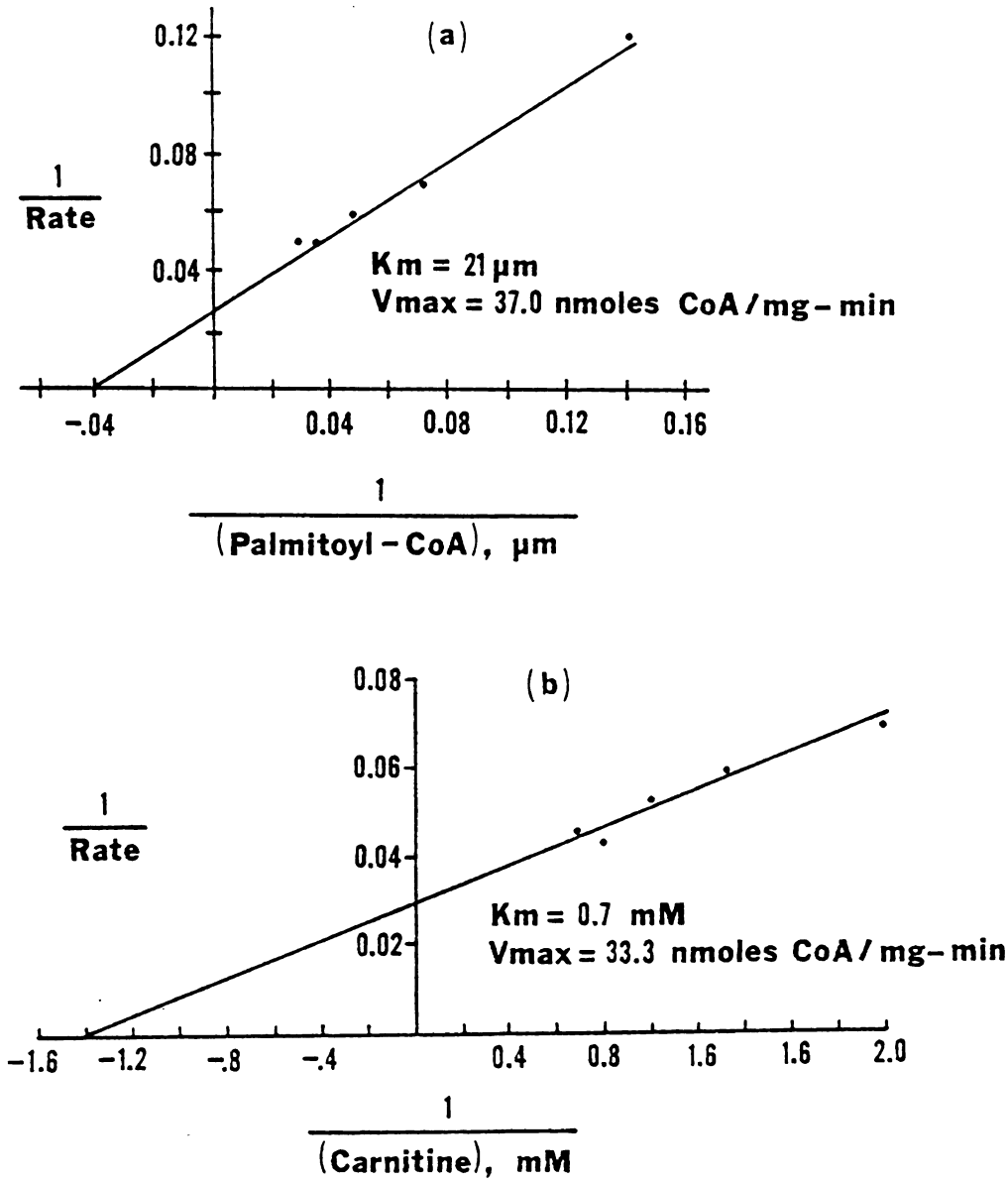


Figure 5.--Double-receprocal plots of substrate concentration versus carnitine palmitoyltransferase activity in mammary mitochondria.

The rates of glycero-lipid accumulation tend to decline over time. One can envision that as less palmitoyl-CoA is being esterified to G3P more palmitoyl-CoA would be available for oxidation, resulting in increasing rates of oxidation with time. The shaded areas in Figure 6 represent the deviation from linearity over time in the formation of glycero-lipid and CO_2. The decline in glycero-lipid formation could quantitatively account for the increasing rates of palmitate oxidation in the absence of glucose.

By the same reasoning, rates of palmitate oxidation should increase even more with time in the presence of glucose, since rates of glycero-lipid formation decrease with time ($P<0.05$) in the presence of glucose more than in its absence. However, rates of CO_2 production were less than predicted by the presumed increase in palmitoyl-CoA availability. However, increasing rates of palmitate oxidation over time is consistent with the hypothetical limitation of oxidation of palmitate esterification.

Medium-chain fatty acids can be activated in the intra-mitochondrial space, and the resultant CoA esters are not accessible to acylation of G3P. Since fatty acid esterification is an extra-mitochondrial process, glucose should not affect medium-chain fatty oxidation. Therefore, mammary tissue slices were incubated with octanoate[1-^{14}C] with or without glucose and rates of octanoate oxidation and esterification were determined. The data are shown in Table 14. The rates of octanoate esterification shown in Table 14 are the means of

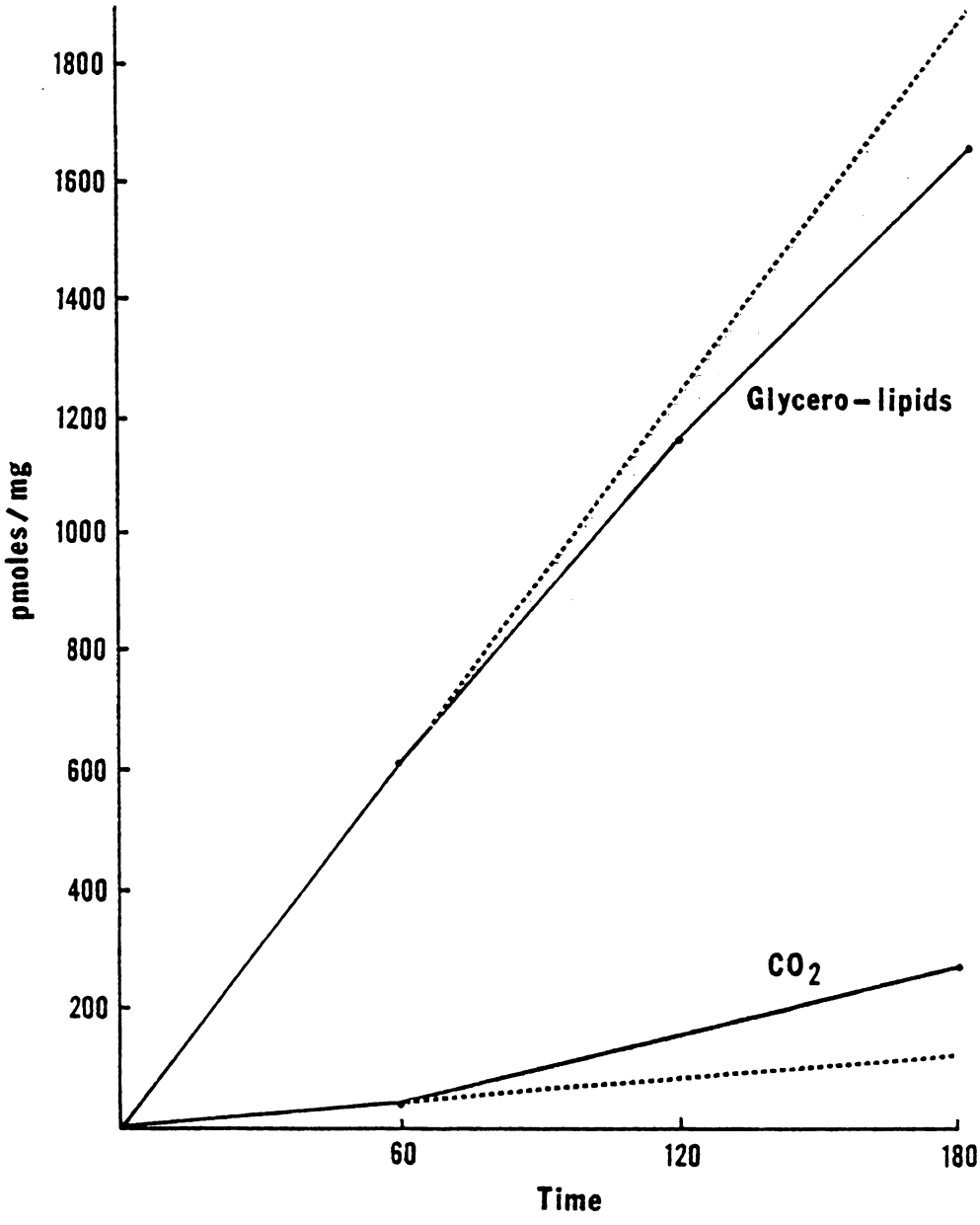


Figure 6.--Conversion of palmitate to CO_2 and glycero-lipids versus time. The shaded areas represent the deviation from linearity over time in the formation of glycero-lipid and CO_2.

rates obtained at 60 and 180 min, and the complete data is presented in Appendix Table 6. The results reveal that glucose does not influence octanoate oxidation, nor do rates of octanoate oxidation change with time. Contrarily, glucose markedly enhances octanoate esterification in all of the lipid fractions, and the rates of glycero-lipid accumulation with octanoate are similar to those found with palmitate (Table 13). Marshall and Knudsen (1977) found that microsomal bound 1,2-diacylglycerol acyltransferase from mammary tissue of lactating cows incorporates equal molar amounts of butyryl-CoA, hexanoyl-CoA, or palmitoyl-CoA to microsomal-bound diglyceride. In addition, McGarry and Foster (1971) demonstrated that octanoate oxidation and esterification in the isolated perfused rat liver is unaffected by antiketogenic compounds. Thus, the ability of the mammary gland to esterify large quantities of short- and medium-chain fatty acids is unique. The predominant lipid fraction synthesized is TG, which is expected since medium-chain fatty acids are preferentially acylated to the sn-3 position (Breckenridge and Kuksis, 1969) and is the final step in TG formation (Marshall and Knudsen, 1977). Therefore, glucose does not block octanoate oxidation but enhances octanoate esterification. These results suggest the existence of two large segregated pools of octanoyl-CoA at two sites of activation, microsomes and intramitochondria. Glucose, by virtue of its ability to supply G3P, presumably increases octanoate esterification at the microsomal site only.

Table 14.--Effect of Glucose on Octanoate Oxidation and Esterification.

Treatments	CO_2[1] 60	CO_2[1] 180	Uptake[1]	PL[1]	MG[1]	DG[1]	TG[1]	NL[1]	GL[1]	FA[1,2]
	pmoles/mg-min									
Octanoate	8.8	10.2	15.8	0.3[a]	0.1[a]	0.5[a]	7.8[d]	8.4[d]	8.7[d]	-2.4
0 + Glucose[3]	9.6	10.3	17.8	0.7[b]	0.2[b]	1.4[b]	11.2[e]	12.8[e]	13.5[e]	-5.5

[ab]Means in columns with different superscripts are different $P<0.001$.

[de]Means in columns with different superscripts are different $P<0.05$.

[1]Standard errors of difference between means for CO_2, Uptake, PL, MG, DG, TG, NL, GL, and FA are 1.48, 1.62, 0.05, 0.02, 0.12, 1.21, 1.23, 1.24, and 2.27, and number of replicates is 4.

[2]Most of added octanoate was utilized, so the following equation was used to calculate the rate of accumulation of intracellular fatty acid:

$$\text{Rate (pmoles/mg-min)} = \frac{(\text{total dpm in tissue})(\%\ \text{FA on TLC plate}) - (\text{total dpm in blank})\left[\frac{\text{final dpm}^*}{\text{initial dpm}}\right]}{(\text{specific activity added})(\text{mg tissue})(\text{min})}$$

*final dpm = initial dpm - CO_2

[3]Octanoate and Glucose at 0.26 and 2.8 mM.

As previously mentioned, rates of octanoate as opposed to palmitate oxidation changes very little with incubation time and inclusion of glucose in the media. Also, octanoate is oxidized at much greater rates than palmitate. Since octanoate oxidation bypasses the carnitine palmitoyltransferase (CPT) enzyme reaction, this suggests that carnitine palmitoyltransferase activity limits palmitate oxidation. Furthermore, the possibility that glucose depresses CPT activity *in vitro* was raised. To examine this possibility, mammary tissue slices were incubated with palmitate in the presence or absence of glucose, after which mitochondria were isolated from the tissue slices and assayed for CPT activity. The results in Table 15 show that carnitine palmitoyltransferase activity is not influenced by incubation time or glucose, although this does not prove that the effective activity *in vitro* was not changed. However, it can be estimated that the CPT activities are much greater than maximal rates of palmitate oxidation, after equating the amount of mitochondrial protein recovered to the original tissue slice weight. Therefore, limitation of palmitate oxidation by CPT activity does not seem likely.

It has been shown that as rates of palmitate esterification increase, rates of palmitate oxidation decrease. Accordingly, blocking palmitate esterification by artificial means should result in concomitant changes in palmitate oxidation. Clofenapate, a hypolipidemic drug and a derivative of clofibrate, effectively reduced glyceride synthesis in rat liver

Table 15.--Effect of Incubation Time and Glucose on Carnitine Palmitoyltransferase Activity in Mammary Tissue Slices.

Treatments	Time 0	60	180
Palmitate	10.15[1,2]	8.82	7.63
P + Glucose[3]	N.D.[4]	8.09	7.35

[1]Rates expressed as nmoles of CoA formed/mg mitochondrial protein-min.

[2]Standard error of difference between means is 3.17, and number of replicates for palmitate is 5 and P + glucose is 2.

[3]Palmitate and glucose at 0.26 and 2.8 mM.

[4]Rates were not determined.

homogenates and slices, (Brindley and Bowley, 1975). Clofenapate at 0.5 mM reduced the incorporation of [^{3}H]glycerol into lipid fractions by 50% in liver slices (Brindley and Bowley, 1975). Therefore, the effect of blocking palmitate esterification by inclusion of 0.5 mM clofenapate into the media on palmitate oxidation was investigated. Results from preliminary experiments showed that clofenapate at concentrations greater than 0.5 mM was relatively insoluble in aqueous media and may be toxic at long incubation times. The effects of clofenapate on palmitate utilization are shown in Table 16. The data are taken from experiments in which mammary tissue slices were incubated for 60 min only. Clofenapate significantly reduced TG formation, and formation of neutral lipid and glycero-lipid tended to be depressed by clofenapate although differences were statistically insignificant. Glucose

tended to enhance palmitate esterification, although the rates observed with PL, MG, DG, NL, and GL were not statistically different from the palmitate control treatment. Clofenapate was effective in decreasing TG, neutral-lipid and glycero-lipid formation in the presence of glucose. Clofenapate is particularly effective in decreasing rates of TG accumulation both in the absence or presence of glucose. This is compatible with results of Brindley and Bowley (1975) who show that 0.6 mM clofenapate produces a 50% inhibition of diacylglycerol acyltransferase activity. A trend ($P \approx 0.25$) exists toward increasing palmitate oxidation with clofenapate. Clofenapate is marginally effective in relieving the glucose inhibition on palmitate oxidation. Of the five experiments in which CO_2 was measured one tissue sample was believed to be atypical, since it did not respond to glucose treatment. When CO_2 rates of this experiment are deleted, the respective rates of CO_2 production are 0.6, 0.1, 0.8, and 0.4 for palmitate, P plus glucose, P plus clofenapate, and P plus glucose plus clofenapate. In conclusion, decreasing fatty acid esterification by artificial means tends to augment palmitate oxidation and relieve the glucose suppression on fatty acid oxidation.

From the results in Table 16 it is evident that the clofenapate treatments markedly increase the proportion of activity remaining in the cells as fatty acid. This fraction could be mostly fatty acyl-CoA, since triglyceride synthesis is depressed by clofenapate. If the drug is causing a "build-up" of fatty acyl-CoA, then other factor(s) must be limiting

Table 16.--Effect of Clofenapate and Glucose on Palmitate Oxidation and Esterification.

Treatments	CO_2	Uptake[1]	PL[1]	MG[1]	DG[1]	TG[1]	NL[1]	GL[1]	FA[1]
	pmoles/mg-min[2]								
Palmitate	0.5[a]	10.5[a]	0.9	0.1	1.2	6.4[d]	7.8	8.7	1.1[d]
P + Glucose	0.2[b]	13.6[b]	1.7	0.4	3.0	6.9[d]	10.3	11.9	1.0[d]
P + Clofenapate[3]	0.7[a]	12.5[ab]	1.5	0.3	1.8	3.5[e]	5.6	7.0	4.5[e]
P + G + C[4]	0.3[b]	11.4[ab]	1.6	0.6	3.3	1.7[e]	5.6	7.2	3.7[de]

[ab]Means in columns with different superscripts are different $P<0.10$.

[de]Means in columns with different superscripts are different $P<0.05$.

[1]Standard errors of difference between means for CO_2, Uptake, PL, MG, DG, TG, NL, GL, and FA are 0.07, 0.71, 0.21, 0.15, 0.53, 0.86, 1.20, 1.39, and 0.82 and number of replicates is 5 for CO_2 and 3 for other parameters.

[2]Determined at 60 min only.

[3]Clofenapate a generous gift of Imperial Chemical Industries, Ltd., of London, England.

[4]Palmitate, glucose, and clofenapate at 0.26, 2.8, and 0.5 mM.

fatty acid oxidation besides simply acyl-CoA availability, because palmitate oxidation is marginally increased by drug treatment. Free carnitine concentrations of 0.1 mM in cow's milk and 0.25 mM in goat mammary tissue have been found (Snoswell and Linzell, 1975). The apparent K_m for carnitine for the CPT enzyme is 0.70 mM as shown in Figure 5b. Accordingly, carnitine addition should stimulate palmitate oxidation. Thus, tissue slices were incubated in the presence or absence of glucose, clofenapate, and 2.0 mM l-carnitine and CO_2 from palmitate was measured at 60 min only. The experiment was only replicated twice. Borrebaek et al. (1976) found that carnitine could not compete with G3P for the available acyl-CoA. Unfortunately, the data in Table 17 neither supports or refutes the findings of Borrebaek et al. (1976). The data, however, show that carnitine addition increases palmitate oxidation. These results suggest that fatty acyl-CoA availability and carnitine levels are limiting for maximum rates of palmitate oxidation. On the contrary, a previous experiment revealed that carnitine stimulated palmitate oxidation at 60 min but not at 180 min (data not shown). Therefore, if endogenous carnitine is in limited supply for palmitate oxidation in mammary tissue slices, rates of palmitate oxidation should not increase with time unless de novo carnitine synthesis is occurring.

Bremer and Wojtczak (1972) discovered that high NADH/NAD ratios decreased fatty acid oxidation in liver mitochondria. Hence, it was of interest to determine the effect of the cellular redox state on palmitate oxidation in mammary tissue

Table 17.--Effect of Carnitine, Glucose, and Clofenapate on Palmitate Oxidation.

Treatments	-Carnitine	+Carnitine[1]*
	-------pmoles/mg-min----------	
Palmitate	0.5[2]	0.7
P + Glucose	0.1[a]	1.0[b]
P + Clofenapate	0.7[a]	1.4[b]
P + G + C	0.3	0.4

*1.4>0.7 ($P<0.05$), 1.4>0.4 ($P<0.05$), 1.0>0.4 ($P<0.10$).

[ab]Means in rows with different superscripts are different $P<0.05$.

[1]L-carnitine at 2.0 mM, a generous gift of Dr. L. L. Bieber, Biochemistry Department, Michigan State University.

[2]Standard error of difference between means is 0.16, and number of replicates is 2.

slices. This was approached in two ways. In one manner, the influence of β-hydroxybutyrate (βOHB) on palmitate oxidation was studied since the enzyme βOHB dehydrogenase is a mitochondrial enzyme in bovine mammary tissue (Bauman et al., 1970), and the other manner was by addition of l-lactate since lactate dehydrogenase is a cytoplasmic enzyme.

Rates of palmitate oxidation were unaffected by 0.2 mM and 0.4 mM βOHB, as shown in Table 18. Thus, it would appear that palmitate oxidation is insensitive to mitochondrial redox state. However, βOHB dehydrogenase activity and βOHB and acetoacetate levels were not determined. βOHB also serves as a primer for fatty acid synthesis in mammary tissue. Thus, the extent to which βOHB entered the mitochondria and effectively altered the redox state was unknown.

Table 18.--Effect of β-hydroxybutyrate on Palmitate Oxidation.

Treatments	Time	
	60	180
	------pmoles/mg-min-----	
Palmitate	1.1[a1]	2.0[b]
P + 0.2 mM βOHB[3]	1.2[a]	1.8[b]
P + 0.4 mM βOHB	1.4[a]	1.9[b]

[ab]Means in rows with different superscripts are different P<0.02.

[1]Standard error of difference between means is 0.18, and number of replicates is 4.

[3]βOHB from Sigma Chem. Co. and palmitate at 0.26 mM.

Lactate, however, markedly inhibits palmitate oxidation, shown in Table 19. The rates of palmitate esterification are averages of rates obtained at 60 and 180 min and the complete data is presented in Appendix Table 7. Since these experiments were replicated only twice, statistically significant differences between treatment means were difficult to achieve, but trends do exist for increasing esterification rates with lactate. Rate of palmitate oxidation tends to increase with time in presence of lactate, and rates of palmitate esterification to glycero-lipid sharpely decrease between 60 and 180 min (15.3 vs 11.5). Palmitate oxidation is typically enhanced by time in the absence of lactate and glucose. This is in spite of increasing rates of palmitate esterification (rates for glycero-lipid at 60 and 180 min are 7.7 and 9.6). The inclusion of glucose was for comparative purposes and the effects of glucose on oxidation and esterification of

Table 19.--Effect of Lactate and Glucose on Palmitate Oxidation and Esterification.

Treatments	CO_2[1] 60	CO_2[1] 180	Uptake[1]	PL[1]	MG[1]	DG[1]	TG[1]	NL[1]	GL[1]	FA[1]
	pmoles/mg-min									
Palmitate	0.6^{x}	1.4^{ay}	10.1	1.4	0.3	1.1^{d}	6.0	7.4^{d}	8.8^{d}	-0.2
P + Lactate[2]	0.3	0.6^{b}	14.0	1.7	0.3	2.3^{de}	9.3	11.9^{de}	13.6^{de}	-0.3
P + Glucose	0.3	0.4^{b}	15.5	1.9	0.3	3.1^{e}	10.5	13.9^{e}	15.8^{e}	-0.9

^{ab}Means in columns with different superscripts are different $P<0.05$.

^{xy}Means in rows with different superscripts are different $P<0.02$.

^{de}Means in columns with different superscripts are different $P<0.10$.

[1]Standard errors of difference between means for CO_2, Uptake, PL, MG, DG, TG, NL, GL, and FA, are 0.23, 2.28, 0.33, 0.05, 0.66, 2.03, 2.43, 2.47, and 0.86 and number of replicates is 2.

[2]L-lactate at 2.0 mM, from Sigma Chem. Co., palmitate and glucose at 0.26 and 2.8 mM.

palmitate were similar to previous experiments (Table 13). However, rates of palmitate esterification were slightly higher with glucose than lactate. McGarry and Foster (1971) also found that rates of oleate incorporation into lipids increased and rates of ketogenesis from oleate decreased in rat livers perfused with oleate and lactate. In conclusion, the results suggest that the cellular redox state is of minor importance in regulation of fatty acid oxidation. When these results are coupled with data previously presented, it can be concluded that stimulation of fatty acid esterification inhibits fatty acid oxidation. Lactate appears to inhibit palmitate oxidation not by changing redox state but by stimulation of palmitate esterification.

It was of interest to determine if mammary tissue slices from fasted lactating cows oxidized greater amounts of palmitate than those from fed lactating cows. Serum fatty acid concentrations were used as a guideline for the length of the fasting period. Serum fatty acid levels from five cows fasted up to 96 hr were highest at 72 hr post-feeding (data not presented). Thus, four cows culled from the university herd were fasted 72 hr prior to slaughter, and rates of palmitate oxidation were compared to those obtained from culled cows on feed up to time of slaughter. All cows were in middle to late lactation. The results in Table 20 show that fasting actually tends to lower palmitate oxidation. On the contrary, Annison et al. (1968) found greatly enhanced rates of palmitate oxidation in the infused mammary gland of

fasted lactating goats. Mammary uptake of glucose and acetate is decreased by fasting (Annison et al., 1968). Thus, CO_2 specific activity may have been increased in mammary tissue from fasted cows, indicating that fatty acids provided an increased proportion of the oxidative metabolism in mammary tissue.

Table 20.--Effect of Fasting on Palmitate Oxidation in Mammary Tissue Slices.

Treatments	Time	
	60	180
	----pmoles/mg-min-----	
Fed[1]	0.6 ± 0.5[3]	0.9 ± 0.4
Fasted[2]	0.2 ± 0.1	0.5 ± 0.3

[1]Culled cows on feed up to time of slaughter, n=11.
[2]Culled cows fasted 72 hr prior to slaughter, n=4.
[3]Rates are means ± standard deviations.

Milk and milk fat productions were measured in two of the fasted cows, shown in Table 21. Milk production declined with fasting, but milk fat production was unaltered. Thus, the rates of palmitate oxidation obtained with fasting may be a consequence of an unaltered ability of the tissue to esterify fatty acid.

Table 21.--Effect of Fasting on Milk and Milk Fat Production.

	Day of Fast					
	-3	-2	-1	+1	+2	+3
	kg/day					
Milk	26.5[1]	25.8	26.1	28.9	15.0	9.1
Milk Fat	0.7	0.7	0.7	1.0	0.9	0.6

[1]Average of two values.

CONCLUSIONS

Rates of palmitate oxidation increased with time of incubation. This increasing rate of palmitate oxidation is not due to substrate solubility in the incubation media or depletion of endogenous fatty acid, and is not an artifact of the incubation system as rate of palmitate oxidation was constant in rat kidney cortex slices.

Fatty acid oxidation in mammary tissue does not appear to be controlled by tissue fatty acid concentration. Rates of fatty acid oxidation are dependent on fatty acid chain length: acetate > octanoate > palmitate or oleate. Palmitate at 0.26 mM which is the arterial plasma fatty acid concentration, gave maximum rates of oxidation, and half-maximal rates of oxidation were obtained at 0.1 mM palmitate. Rates of palmitate oxidation increased with time of incubation at all concentrations of palmitate tested.

Also, increasing rates of fatty acid oxidation with time are unique to long-chain fatty acids, which can not be explained by activity of carnitine palmitoyltransferase, as enzyme activity was constant over time and maximal enzyme activity was greater than rates of palmitate oxidation. Rates of palmitate[1-^{14}C] oxidation were greater than rates of palmitate[U-^{14}C] oxidation, suggesting that the β-oxidation enzymes may limit fatty acid oxidation.

Rates of palmitate oxidation are greatly diminished by acetate and glucose at physiological concentrations. Acetate inhibits palmitate oxidation but not palmitate esterification. This suggests that acetate inhibits palmitate oxidation by substrate competition at the mitochondrial level or via malonyl-CoA inhibition of carnitine palmitoyltransferase, and not by competition with palmitate for available CoA in the cytoplasm. TOFA, an inhibitor of acetyl-CoA carboxylase, did not relieve acetate inhibition of palmitate oxidation, suggesting that acetate is not affecting palmitate oxidation via malonyl-CoA. However, the extent to which TOFA altered intracellular metabolism was not determined. In some experiments, rates of palmitate oxidation increased with time in the presence of acetate, which could not be explained by acetate depletion from the media.

Glucose inhibits palmitate oxidation and markedly enhances palmitate esterification to G3P. In some experiments, rates of palmitate oxidation tend to increase with time in the presence of glucose, which apparently is not due to glucose depletion from the media as the glucose concentration remaining in the media is sufficient to induce maximum depression of palmitate oxidation by glucose. At low palmitoyl-CoA levels acylation of G3P would be favored over that of carnitine, since the apparent K_m of 21 μM palmitoyl-CoA for carnitine palmitoyltransferase is greater than the apparent K_m for 4 μM palmitoyl-CoA for G3P acyltransferase as found by Kinsella and Gross (1973). Thus, glucose

inhibits palmitate oxidation presumably by decreasing fatty acyl-CoA available for oxidation by stimulating palmitate esterification of G3P.

Glucose does not affect octanoate oxidation, even though octanoate esterification to G3P was stimulated. This could be a result of two relatively large pools of octanoate at the microsomes and mitochondria. Octanoate activated at the microsomes is accessible to acylation of G3P. Octanoate activated in the mitochondria is not accessible to acylation of G3P.

Rates of glycero-lipid formation from palmitate tend to decrease with time of incubation. The decreasing rates of glycero-lipid formation could quantitatively account for increasing rates of palmitate oxidation with time.

Clofenapate, an inhibitor of glyceride synthesis in rat liver, was used as a model for studying how modifying palmitate esterification would affect oxidation. Clofenapate decreased rates of glycero-lipid accumulation and marginally stimulated palmitate oxidation. Clofenapate was also partially effective in relieving glucose inhibition of palmitate oxidation. Clofenapate enhanced the accumulation of intracellular fatty acid, which was assumed to be composed of mostly acyl-CoA esters. Addition of carnitine stimulated palmitate oxidation in tissue slices incubated in the presence or absence of clofenapate, suggesting that carnitine levels are limiting to fatty acid oxidation. However, this would be contradictory to data showing rates of palmitate oxidation increasing with time.

β-hydroxybutyrate addition did not affect palmitate oxidation. Lactate decreased palmitate oxidation, but stimulated palmitate esterification. Thus, lactate, a potential G3P precursor, decreased palmitate oxidation presumably by stimulating esterification. The extent to which cellular redox state was influenced by β-hydroxybutyrate and lactate was not determined.

Rates of palmitate oxidation in mammary tissue slices from cows fasted for 72 hr were lower than those obtained from fed cows. Milk fat production was not affected by fasting, suggesting that lack of stimulatory effect of fasting on palmitate oxidation is a result of constant glyceride synthesis. Fatty acid oxidation as a proportion of the total oxidative metabolism was not determined and may have increased.

In summation, the pathways of fatty acid esterification and oxidation seem to compete for available acyl-CoA with esterification being favored. Increasing rates of palmitate oxidation with time could be explained by simultaneously decreasing rates of glycero-lipid formation. Also, acetate inhibits palmitate oxidation presumably by substrate competition at the mitochondrial level or by malonyl-CoA inhibition of carnitine palmitoyltransferase. It can be estimated that long-chain fatty acids can supply as much as 6-10% of the oxidative metabolism of mammary tissue.

APPENDICES

Table A1.--Composition of Incubation Media.

Volume	Solution
100.0 ml	0.154 M NaCl
4.0	0.154 M KCl
1.5	0.11 M CaCl
1.0	0.154 M KH_2PO_4
1.0	0.154 M $MgSO_4 7H_2O$
21.0	0.154 M $NaHCO_3$ (gas with CO_2 for 1 hour)[2]
	BSA[1]
	50 mg Penicillin/liter[2]
	50 mg Streptomycin/liter[2]
	1.0 ml Fungizone/liter[3]

[1]Molar ratio of FA:BSA kept constant at 4.0.
[2]Purchased from Sigma Chemical Co.
[3]Purchased from Grand Island Biological Co.

Table A2.--The Effect of Ethanol on Palmitate Oxidation.

Treatments:[2]		Time[1] 60	120	180
		-----pmoles/mg-min-----		
Expt. I				
NH_4-Palmitate			2.4	
Palm. in 10 µl Ethanol			2.6	
Expt. II				
Vol. Ethanol:	2 µl	0.4		0.8
	6 µl	0.4		1.0
	10 µl	0.4		1.1
Expt. III				
Vol. Ethanol:	10 µl	0.7	1.2	1.2
	20 µl	0.7	1.1	1.3
	30 µl	0.7	1.0	1.0

[1]Times of incubation were 60, 120, and 180 min and the rates of palmitate oxidation shown are those obtained at the particular incubation time.

[2]Each experiment represents one tissue sample.

Table A3.--The Effect of Collecting and Rinsing Tissue in Warm Versus Cold Buffer on Palmitate Oxidation.

Treatments	Time	
	60	180
	--pmoles/mg-min--	
Cold	0.2[1]	0.3
Warm	0.3	0.7

[1]Average of two experiments.

Table A4.--Palmitate Oxidation With Source of Cows.

Source of Cows	Time 60	120	180
	----------pmoles/mg-min----------		
Abattoir	0.6 ± 0.28[1] (18)	0.9 ± 0.53 (4)	1.2 ± 0.45 (18)
Early Lactation	0.8 ± 0.42 (28)	1.1 ± 0.35 (12)	1.6 ± 0.78 (27)
MSU Culls	0.6 ± 0.45 (11)		0.9 ± 0.44 (11)

[1]Rates are means ± standard deviations and number in parenthesis is the number of replicates.

Table A5.--Effect of Glucose and Acetate on Palmitate Oxidation and Esterification.

Treatment	CO_2[1]			Uptake[1]			PL[1]		
	60	120	180	60	120	180	60	120	180
	pmoles/mg-min								
Palmitate	0.7	1.3	1.5	13.6[g]	12.5	12.0	0.6[a]	0.7[a]	0.6[a]
P + Glucose	0.3	0.4	0.4	19.1[hj]	15.1[k]	12.9[k]	1.6[bd]	1.2[be]	1.2[be]
P + Acetate	0.2	0.4	0.4	14.7[gd]	13.2[de]	10.0[e]	0.8[a]	0.8[a]	0.8[a]
P + G + A[2]	0.2	0.3	0.4	16.0[gh]	14.9	13.8	1.3[b]	1.1[b]	1.1[b]

Table A5.--(cont'd.)

Treatment	MG[1]			DG[1]			TG[1]		
	60	120	180	60	120	180	60	120	180
	pmoles/mg-min								
Palmitate	0.3[a]	0.2[a]	0.2[a]	1.7[a]	0.8[a]	0.6[a]	7.6	8.0	7.8
P + Glucose	1.0[bd]	0.8[bde]	0.6[be]	4.9[bd]	2.8[be]	2.2[be]	6.6	7.2	6.7
P + Acetate	0.4[ad]	0.2[ade]	0.1[ae]	1.5[a]	0.9[a]	0.7[a]	7.6	8.2	7.0
P + G + A[2]	0.7[c]	0.5[c]	0.5[b]	3.2[cd]	2.3[bde]	1.8[be]	7.5	8.4	8.4

Table A5.--(cont'd.)

Treatment	NL[1]			GL[1]			FA[1]		
	60	120	180	60	120	180	60	120	180
	pmoles/mg-min								
Palmitate	9.6	9.1	8.6	10.3^{a}	9.7	9.2^{gh}	2.2	1.4	0.9
P + Glucose	12.5^{d}	10.7^{de}	9.5^{e}	14.1^{bd}	11.9^{de}	10.6^{ghe}	4.3^{d}	2.4^{de}	1.6^{e}
P + Acetate	9.6	9.3	7.8	10.3^{a}	10.1	8.6^{g}	3.8^{d}	2.5^{de}	0.8^{e}
P + G + A[2]	11.4	11.2	10.7	12.6^{ab}	12.3	11.7^{h}	2.8	2.0	1.3

^{abc}Means in columns with different superscripts are different $P<0.05$.

^{def}Means in rows with different superscripts are different $P<0.05$.

^{ghi}Means in columns with different superscripts are different $P<0.10$.

^{jkl}Means in rows with different superscripts are different $P<0.10$.

[1]Standard errors of difference between means for CO_2, Uptake, PL, MG, DG, TG, NL, GL, and FA are 0.10, 1.77, 0.13, 0.10, 0.49, 0.95, 1.19, 1.28, 0.96 and number of replicates is 6.

[2]Palmitate, glucose and acetate at 0.26, 2.8, and 0.6 mM.

Table A6.--Effect of Glucose on Octanoate Oxidation and Esterification.

Treatments	CO_2[1]		Uptake[1]		PL[1]		MG[1]		DG[1]	
	60	180	60	180	60	180	60	180	60	180
	pmoles/mg-min									
Octanoate	8.8	10.2	15.9	15.7	0.3^{c}	0.2^{c}	0.1^{ac}	$<0.1^{bc}$	0.7^{ce}	0.3^{cf}
0 + Glucose[2]	9.6	10.3	17.3	18.4	0.7^{d}	0.6^{d}	0.2^{ad}	0.1^{bd}	2.0^{ad}	0.9^{bd}

Table A6.--(cont'd.)

Treatments	TG[1]		NL[1]		GL[1]		FA[1]	
	60	180	60	180	60	180	60	180
	pmoles/mg-min							
Octanoate	9.2^{g}	6.3	10.0^{gi}	6.6^{kj}	10.4^{ci}	6.8^{jk}	-3.5	-1.4
0 + Glucose[2]	13.5^{eh}	8.9^{f}	15.6^{ah}	9.8^{bl}	16.4^{ad}	10.5^{bl}	-8.4^{i}	-2.5^{j}

^{ab}Means in rows with different superscripts are different P<0.01.

^{cd}Means in columns with different superscripts are different P<0.01.

^{ef}Means in rows with different superscripts are different P<0.05.

^{gh}Means in columns with different superscripts are different P<0.05.

^{ij}Means in rows with different superscripts are different P<0.10.

^{kl}Means in columns with different superscripts are different P<0.10.

[1]Standard errors of difference between means for CO_2, Uptake, PL, MG, DG, TG, NL, GL, and FA are 1.48, 2.29, 0.07, 0.03, 0.17, 1.72, 1.73, 1.76, 3.22, and number of replicates is 4.

[2]Octanoate and glucose at 0.26 and 2.8 mM.

Table A7.--Effect of Lactate and Glucose on Palmitate Oxidation and Esterification.

Treatments	CO_2[1]		Uptake[1]		PL[1]		MG[1]		DG[1]	
	60	180	60	180	60	180	60	180	60	180
	pmoles/mg-min									
Palmitate	0.6^{c}	1.4^{ad}	7.9^{g}	12.2	1.4	1.4	0.4^{c}	0.1^{de}	1.5^{e}	0.7
P + Lactate[2]	0.3	0.6^{b}	15.1^{h}	12.9	1.7	1.7	0.4^{c}	0.2^{def}	2.9^{ef}	1.6
P + Glucose[2]	0.3	0.4^{b}	16.0^{h}	15.0	1.9	1.8	0.3	0.3^{f}	4.0^{fi}	2.2^{j}

Table A7.--(cont'd.)

Treatments	TG[1]		NL[1]		GL[1]		FA[1]	
	60	180	60	180	60	180	60	180
	pmoles/mg-min							
Palmitate	4.6[g]	7.4	6.3[eg]	8.2	7.7[eg]	9.6	-1.2[i]	0.9[j]
P + Lactate[2]	10.4[h]	8.2	13.7[h]	9.9	15.3[h]	11.5	-1.1	0.5
P + Glucose[2]	10.9[h]	10.1	15.2[f]	12.6	17.0[f]	14.4	-1.6	-0.2

[ab]Means in columns with different superscripts are different $P<0.05$.

[cd]Means in rows with different superscripts are different $P<0.05$.

[ef]Means in columns with different superscripts are different $P<0.10$.

[gh]Means in columns with different superscripts are different $P<0.20$.

[ij]Means in rows with different superscripts are different $P<0.20$.

[1]Standard errors of difference between means for CO_2, Uptake, PL, MG, DG, TG, NL, GL, and FA are 0.23, 3.23, 0.47, 0.07, 0.94, 2.87, 3.43, 3.49, and 1.2, and number of replicates is 2.

[2]Palmitate, lactate, and glucose at 0.26, 2.0, and 2.8 mM.

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