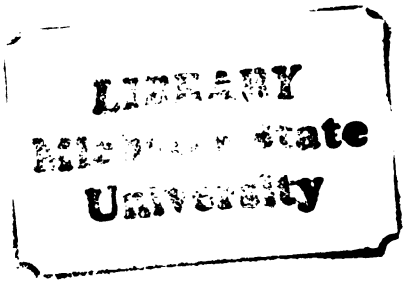

This is to certify that the

thesis entitled

CONTROL MECHANISMS OF FATTY ACID OXIDATION IN BOVINE LIVER

presented by

BARRY WILLIAM JESSE

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Animal Science and Institute of Nutrition

Major professor
Dr. J. William Thomas

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CONTROL MECHANISMS OF FATTY ACID OXIDATION IN BOVINE LIVER

By

Barry William Jesse

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ABSTRACT

CONTROL MECHANISMS OF FATTY ACID OXIDATION IN BOVINE LIVER

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Barry William Jesse

Fatty acid oxidation by bovine liver slices and isolated bovine liver mitochondria was examined to determine regulatory sites of fatty acid oxidation. Conversion of 1-^{14}C-palmitate to $^{14}CO_2$ and ^{14}C-acid-soluble metabolites (ASM) was used as a measure of palmitate oxidation. Total ASM was determined by high-performance liquid chromatography to consist primarily of ketones and acetate.

Palmitate concentrations from .5 to 2 mM had little effect on palmitate oxidation by bovine liver slices. dl-Carnitine (2 mM) maximally stimulated palmitate oxidation. Palmitate oxidation was linear with respect to amount of tissue, over the range from 40 to 210 mg wet slice weight, and incubation time, up to 60 minutes. Octanoate was oxidized by liver slices at ten times the rate of palmitate. Peroxisomes were estimated to contribute a minimum of 6 to 7% of total fatty acid oxidation in liver slices.

Feeding a restricted roughage/high concentrate ration to lactating cows resulted in marked inhibition of palmitate oxidation to ASM with little effect on oxidation to CO_2. The palmitate oxidizing capacity of liver slices from early lactation cows increased with time postpartum, reaching maximum at 42 days postpartum. Long-term fasting had relatively little effect on palmitate oxidation to ASM by liver slices, although oxidation to CO_2 was decreased.

Glucose, insulin, propionate and lactate all inhibited palmitate oxidation by liver slices, presumably by increasing palmitate esterification. Clofenapate, an esterification inhibitor, prevented propionate-induced inhibition of palmitate oxidation by liver slices. Acetate inhibited palmitate oxidation by liver slices, possibly by inhibition of carnitine palmitoyl transferase I (CPT I) following conversion to malonyl-CoA. Malonyl-CoA potently inhibited palmitate oxidation by isolated bovine liver mitochondria, with an I_{50} of .3 μM. Dibutyryl cAMP inhibited palmitate oxidation by rat and bovine liver slices. This effect appeared to be an artifact of the liver slice incubation system.

Bovine liver mitochondrial CPT exhibited palmitoyl-CoA and l-carnitine Km values of 11.5 μM and .59 mM, respectively. Dibutyryl cAMP treatment of liver slices had little effect on mitochondrial CPT kinetic parameters.

The suggestion was made that *in vivo* the rumen fermentation products acetate, propionate, β-hydroxybutyrate and butyrate could serve as inhibitors of hepatic long-chain fatty acid oxidation.

To Elizabeth Ann Jesse

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LIST OF ABBREVIATIONS

AcAc: Acetoacetic Acid

Acetyl-CoA: Acetyl Coenzyme A

AMP: Adenosine-5'-Monophosphate

ASM: Acid-Soluble Metabolites

ATP: Adenosine-5'-Triphosphate

BHBA: β-Hydroxybutyric Acid

BSA: Bovine Serum Albumin

Bt_2cAMP: Dibutyryl Adenosine-3',5'-Cyclic Monophosphate

cAMP: Adenosine-3',5'-Cyclic Monophosphate

CAT: Carnitine Acyltransferase

CoASH: Free Coenzyme A

CPT: Carnitine Palmitoyl Transferase

DG: Diacylglycerol

DNP: Dinitrophenol

DTNB: 5,5'-Dithiobis-(2-Nitrobenzoic Acid)

FA: Fatty Acid

FABP: Fatty Acid Binding Protein

FA-Carnitine: Long-chain Fatty Acyl-carnitine

FA-CoA: Long-chain Fatty Acyl-Coenzyme A

FAD: Flavin Adenine Dinucleotide

FFA: Free Fatty Acid

G3P: sn-Glycerol-3-Phosphate

HMG-CoA: β-Hydroxy, β-Methyl-Glutaryl-Coenzyme A

HPLC: High-Performance Liquid Chromatography

I_{50}: Inhibitor concentration which produces one-half of the maximum inhibition of a reaction

K_m: Substrate concentration which results in one-half maximal reaction velocity

KRB: Krebs-Ringer Bicarbonate Buffer

Malonyl-CoA: Malonyl-Coenzyme A

MW: Molecular Weight

NAD(H): Nicotinamide Adenine Dinucleotide

NADP(H): Nicotinamide Adenine Dinucleotide Phosphate

OAA: Oxaloacetic Acid

PA: Phosphatidic Acid

PEPCK: Phosphoenolpyruvate Carboxykinase

Pi: Inorganic Phosphate

PL: Phospholipid

PPi: Inorganic Pyrophosphate

TCA Cycle: Tricaboxylic Acid Cycle

TG: Triacylglycerol

TML: Trimethyllysine

Vmax: Maximum reaction velocity catalyzed by an enzyme

INTRODUCTION

The central role played by liver as a nutrient processing and distribution center has long been appreciated. This role is due to the unique position of the liver, relative to the circulatory system and digestive tract, which forces nutrients to flow through the liver prior to contact with other major organ systems. Liver can also reprocess and recycle nutrients derived from the metabolism of various body tissues, e.g. lactate from muscle, or nonesterified fatty acids from adipose tissue. That liver serves the same basic purpose in both ruminant and nonruminant animals is consistent with much of the functional similarity of liver observed between the two species. For example, the liver of both species can be a major site of fatty acid esterification, with the subsequent formation and secretion of lipoproteins. In addition, the livers of both species have the capacity to oxidize large amounts of long-chain fatty acids, producing carbon dioxide and ketone bodies. Despite this, several aspects of metabolic function differ substantially between the ruminant and nonruminant liver. Rat liver, the nonruminant species typically discussed, is a major site of _de novo_ fatty acid synthesis, in

contrast to bovine or ovine liver where this process is severely limited. Also, bovine liver is in a continuous gluconeogenic state, whereas gluconeogenesis occurs only during post-absorptive and fasting states in the rat.

This same type of pattern of occurrence is also observed with regard to hepatic fatty acid oxidation and ketogenesis, and results directly from the two-fold function fatty acid oxidation appears to serve in both rat and bovine liver. First, ketogenesis provides an alternative to glucose as an energy supply to extrahepatic tissues when glucose is in short supply, thus sparing glucose for more vital functions within the organism. Second, and perhaps more importantly, fatty acid oxidation to carbon dioxide and ketone bodies serves a permissive role relative to gluconeogenesis, allowing maximal gluconeogenic rates to occur when required by the organism. The importance of hepatic fatty acid oxidation and ketogenesis to the bovine, which is in a continuous gluconeogenic state, becomes readily apparent. This is especially true in the high-producing dairy cow, where the demand for glucose can be great.

In the rat the question is what changes the liver from a net glycolytic/lipogenic state immediately after feeding to a gluconeogenic/ketogenic state when fasted? Ultimately, the answer lies in the change in insulin: glucagon ratio in response to changing nutrient

availability, which induces the switch in hepatic metabolism as the animal moves from a fed state, with a high insulin:glucagon ratio, to a fasted condition, with a low insulin:glucagon ratio. Many of the molecular and biochemical changes occurring within the rat liver cell have been well-characterized, although some controversy exists as to the relative importance of some of these changes, e.g. the changing sensitivity of mitochondrial carnitine palmitoyl transferase to malonyl-CoA inhibition in liver from rats in differing physiological states. The situation in the bovine is remarkably different, because the insulin:glucagon ratio is always relatively low, even during the fed state. The question for the ruminant, then, is what prevents uncontrolled hepatic fatty acid oxidation and ketogenesis from occurring in a typically functioning cow, and what changes can occur to allow a cow, especially a high-producing animal, to enter a condition of pathological ketogenesis? This thesis describes, first, an *in vitro* system for the measurement of bovine hepatic fatty acid oxidation rates, and second, a series of experiments undertaken to determine potential factors involved in the regulation of hepatic fatty acid oxidation.

REVIEW OF THE LITERATURE

Our knowledge of whole body lipid metabolism and of many of the interactions occurring among various tissues is extensive, however the available information concerning biochemical events involved in the control of ruminant lipid metabolism is much more limited. The pathways of fatty acid metabolism are known to be the same within rat and cow liver, but major differences exist between the two species with respect to digestive physiology and the end-products of digestion, suggesting that regulation of those pathways is also likely to be different. This review will examine the information currently available concerning ruminant hepatic fatty acid metabolism, and will draw extensively on examples from the nonruminant literature for discussion of topics not previously examined, but of potential importance, for the regulation of ruminant hepatic fatty acid metabolism. In addition, the biochemical basis for bovine lactational ketosis will be addressed. Finally, the relationship of hepatic fatty acid metabolism to whole body metabolism in the ruminant and nonruminant will be discussed.

Hepatic de novo Fatty Acid Synthesis

Unlike the rat and most other nonruminant species, ruminant liver is not a major site for de novo fatty acid synthesis, although limited synthesis does occur. Ultimately, this adaptation in the ruminant is probably due to the continual requirement for hepatic gluconeogenesis, because most dietary carbohydrate is fermented to volatile fatty acids within the rumen with the result that usually little glucose is absorbed from the gut. Low rates of fatty acid synthesis in the ruminant liver have been attributed to competition for cytoplasmic oxaloacetate (OAA) between the gluconeogenic and lipogenic processes (Bell, 1980). These two processes, however, are mutually exclusive in all species examined, presumably due to the modulation of enzyme activity within the liver in response to hormonal action (Newsholme and Start, 1976), so that gluconeogenesis can more effectively compete for cytoplasmic OAA, thus limiting de novo fatty acid synthesis rates.

Fatty acid synthesis requires a cytoplasmic source of acetyl-coenzyme A (acetyl-CoA). In the nonruminant animal this is derived primarily from dietary carbohydrate. Glucose carbon flows through the glycolytic sequence, and is finally converted to pyruvate. Pyruvate enters the mitochondrial matrix, and, under the action of the pyruvate dehydrogenase complex, is converted to acetyl-CoA. Although a number of potential avenues exist for the

translocation of intramitochondrially-generated acetyl-CoA to the cytoplasm, the ATP citrate lyase pathway is generally accepted as the major source of cytoplasmic acetyl-CoA for fatty acid synthesis (Newsholme and Start, 1976). Mitochondrial acetyl-CoA condenses with OAA in the matrix to form citrate, which can be transported from the matrix into the cytoplasm. Acetyl-CoA and OAA are regenerated from citrate in the cytoplasm by ATP citrate lyase, and fatty acid synthesis may proceed with this newly reformed acetyl-CoA as a carbon source. Oxaloacetate can reenter the mitochondria directly as OAA, or as pyruvate, following the action of the NAD- and NADP-malate dehydrogenases on OAA.

Glucose is only sparingly used as a carbon source for fatty acid synthesis by ruminant liver (and adipose tissue, also), which is not surprising in view of the low glucose availability and the constant gluconeogenic condition in which the liver operates. Traditionally, this limited use has been attributed to the low activity of ATP citrate lyase and NADP-malate dehydrogenase in the ruminant relative to the nonruminant (Hanson and Ballard, 1967, 1968). Activity of these enzymes in the liver apparently changes little during development from the fetus to the mature ruminant (Bell, 1980). Intravenous or intra-abomasal glucose infusions, however, were shown to increase the activity of hepatic ATP citrate lyase and

NADP-malate dehydrogenase (Ballard et al., 1972). Dietary manipulations resulting in increased glucose absorption from the gut reportedly had little effect on these enzyme activities (Bell, 1980), but glucose-6-phosphate dehydrogenase activity, an enzyme involved in generation of NADPH for fatty acid synthesis, was increased when calves with functional rumens were fed a high concentrate compared to a pelleted dried grass diet (Pearce and Unsworth, 1980). Despite some relatively large increases in activity, however, the greatest activity achieved for either ATP citrate lyase or NADP-malate dehydrogenase in ruminant liver was well below corresponding values for these enzymes in the rat (Bell, 1980).

Recently, the traditional concepts of the factors limiting glucose utilization for fatty acid synthesis in the ruminant have been challenged. This challenge was mounted because of the discovery that lactate and pyruvate could be incorporated more rapidly into fatty acid than either glucose or acetate (Prior, 1978; Whitehurst et al., 1978). (These results were obtained with ruminant adipose tissue, but should also be applicable to the small amount of fatty acid synthesized in the liver.) Since lactate- and pyruvate-carbon would be metabolized in a manner identical to glucose-carbon from the level of pyruvate onward, these results indicated that ATP citrate lyase activity was not the limiting factor for glucose

incorporation into fatty acid in the ruminant. An examination of the original data on which this concept was based clearly indicates that ATP citrate lyase and NADP-malate dehydrogenase activities were several hundred-fold greater than the fatty acid synthetic rates observed in both ruminant liver and adipose tissue (Hanson and Ballard, 1967, 1968). At present, the factor limiting glucose utilization in the ruminant is unknown, although various investigators have suggested insufficiencies in hexokinase, phosphofructokinase, pyruvate kinase or pyruvate dehydrogenase activities as potential factors (Bell, 1980). Of these, the most likely candidate appears to be pyruvate dehydrogenase because, although lactate and pyruvate could serve as substrates for fatty acid synthesis, this only occurred at supraphysiological concentrations of lactate and pyruvate (Whitehurst et al., 1978), suggesting that at physiological concentrations pyruvate conversion to acetyl-CoA only occurs at low rates. At present, no information appears to be available concerning pyruvate dehydrogenase activity in ruminant liver, however, some evidence exists suggesting that pyruvate dehydrogenase activity is limiting to glucose utilization in the lactating ruminant mammary gland (Read et al., 1977), so that a similar situation could exist in the ruminant liver. Low pyruvate dehydrogenase activity in ruminant liver would have profound implications

for utilization of glucogenic substrates entering the gluconeogenic pathway at the level of pyruvate, since a low pyruvate dehydrogenase activity would force partitioning of pyruvate towards OAA formation for gluconeogenesis, away from acetyl-CoA formation and lipogenesis.

The primary carbon source used for energy production and fatty acid synthesis in the ruminant is acetate, and the small amount of fatty acids produced by the ruminant liver is probably derived mainly from acetate. Acetate can be activated to acetyl-CoA by acetyl-CoA synthetase both in the cytoplasm or the mitochondrial matrix (Ballard and Hanson, 1967; Snoswell and Koundakjian, 1972), and so could be utilized for fatty acid synthesis either directly or via the ATP citrate lyase route. Acetyl-CoA synthetase activity is generally regarded as being relatively low in ruminant liver, especially with respect to other short-chain acyl-CoA synthetases found in the liver (Bell, 1980; Ricks and Cook, 1981a). Various researchers, however, have demonstrated that ruminant liver can absorb 15-20% of the acetate supplied to the liver, and that, if completely oxidized, this acetate could potentially account for up to 20% of the oxygen consumed by the adult ruminant liver (Pethick et al., 1981; Thompson et al., 1975). Thus, although low relative extrahepatic tissues, acetate utilization is not

insignificant in the ruminant liver, and could contribute to the limited fatty acid synthesis occurring in the liver.

Besides the acetyl-CoA requirement as a carbon source, fatty acid synthesis also requires reducing equivalents in the form of NADPH. As was the case for ATP citrate lyase, NADP-malate dehydrogenase was once thought to be a potentially limiting factor for glucose utilization in ruminant fatty acid synthesis, based on the relatively low activity of the enzyme in ruminant relative to nonruminant liver and adipose tissue (Hanson and Ballard, 1967, 1968). Subsequent research has demonstrated that NADP-malate dehydrogenase does play an active part in ruminant fatty acid synthesis (Prior et al., 1981; Smith and Prior, 1981). Reducing equivalents may also be generated in the pentose phosphate shunt (Bauman, 1976; Bauman et al., 1970; Smith, 1983) and the isocitrate dehydrogenase pathway (Bauman, 1976; Bauman et al., 1970). All three sources of NADPH have been demonstrated to be of importance for ruminant fatty acid synthesis within adipose tissue, and could potentially supply reducing equivalents for hepatic fatty acid synthesis.

Quantitatively, hepatic fatty acid synthesis is minor when compared to the total de novo fatty acid synthesis which can occur in ruminant adipose tissue or

lactating mammary gland. Nevertheless, the fatty acid synthesis which does occur could potentially play an important role in regulating hepatic long-chain fatty acid oxidation for the ruminant by providing a source malonyl-CoA. Malonyl-CoA has been demonstrated to play a major role in limiting fatty acid oxidation in nonruminant liver (McGarry and Foster, 1980), and will be discussed in greater detail.

Hepatic Free Fatty Acid Uptake

Nonesterified, or free, fatty acids which are found in the circulation result from either lipolysis of triacylglycerol stored in adipose tissue, or the action of lipoprotein lipase on circulating lipoproteins. Various investigators (Baird et al., 1977; Bergman et al., 1971; Katz and Bergman, 1969; Thompson and Darling, 1975; Thompson et al., 1975) have demonstrated the ability of ovine and bovine liver to absorb large quantities of FFA from the blood. Since little fatty acid synthesis occurs, this FFA uptake is the major source of fatty acids utilized by the liver. Free fatty acids can furnish a large proportion of the energy metabolized by the ruminant, particularly the high-producing dairy cow (Emery, 1980). In spite of this, few investigations have examined hepatic FFA uptake in the ruminant. Methodological difficulties may have limited these types of experiments. Only one

study is available which has examined hepatic FFA uptake with respect to whole body FFA flux (Bergman et al., 1971). These investigators found that liver of conscious fed sheep took up from the circulation 25% of the total daily FFA pool.

The most widely utilized experimental model in the study of hepatic FFA uptake has been the isolated perfused rat liver. Free fatty acid uptake has been found to be generally proportional to the FFA concentration in the blood, at least up to concentrations of 2 to 3 mM (Heimberg et al., 1974). Typically, rat blood contains .1-.2 mM FFA in the fed state, and .5-.7 mM during fasting. Concentrations ranging from 1 to 2 mM, however, can be observed during pathological conditions such as diabetic ketoacidosis. Similar concentrations are observed in ruminant blood during corresponding conditions (Bergman, 1971), and various researchers have noted that, as in rat liver, ruminant liver FFA uptake is proportional to blood FFA concentrations up to 2 mM (Katz and Bergman, 1969; Thompson and Darling, 1975; Thompson et al., 1975). Over these concentration ranges, which were obtained in sheep under a wide range of physiological conditions, including fasting (3 days) and alloxan-induced diabetes, a near constant proportion (~10%) of the FFA presented to the sheep liver was extracted by the liver. Similar results were calculated for the fed, lactating dairy cow using data

published by Lomax and Baird (1983). When lactating dairy cows were fasted for six days, however, the calculated proportion of FFA extracted by the liver increased to 20%, in contrast to the sheep. This difference could be partially accounted for by the shorter time (1-3 days) of fast imposed on the sheep (Katz and Bergman, 1969; Thompson et al., 1975), or may indicate that bovine liver in the fasted state is more efficient than ovine liver for FFA uptake. The 20% proportion extracted is close to that measured for conscious intact nonruminants (Basso and Havel, 1970). One factor which has been advanced to account for the lower proportion of FFA extracted by ruminant liver compared to nonruminant liver is the larger proportion of the cardiac output (i.e. blood flow) received by the ruminant liver, resulting simply in a greater presentation of FFA to the ruminant liver (Hales, 1973; Bell, 1980). This may not apply under all conditions, however, as Thompson et al. (1975) found that sheep liver received a proportion of the cardiac output (~25%) similar to that of the nonruminant.

Although total FFA uptake from the blood is relatively constant in the ruminant liver, uptake of individual fatty acids, such as palmitate, stearate and oleate (the major FFA in ruminant blood), can be variable (Thompson and Darling, 1975; Thompson et al., 1975). Uptake rate of individual fatty acids is proportional

to the degree of unsaturation of the fatty acids, and inversely related to fatty acid chain length, so that uptake rate followed the order: oleate > palmitate > stearate. Stearate uptake is low and variable relative to palmitate and oleate, and, unlike the other FFA, is not proportional to blood stearate concentrations. Similar results were observed in the perfused rat liver (Soler-Argilaga et al., 1973), and may explain various anomalies observed with respect to stearate metabolism. The cause for the discrimination by liver between palmitate and oleate on the one hand and stearate on the other is currently unknown.

Free fatty acids are carried in the blood as noncovalent complexes with serum albumin. Each serum albumin molecule contains a total of six high energy binding sites for FFA, equally divided into two distinct classes (Spector et al., 1969). The three primary binding sites possess an apparent equilibrium association constant (k', at 37°C, pH 7.4) of $10^6 M^{-1}$, while k' for the three secondary sites is $10^5 M^{-1}$. A large number of tertiary binding sites ($k' = 10^3 M^{-1}$), estimated to number in excess of 60 sites, are also present. Because of the number of available binding sites and their relatively high affinity, blood will contain a large population of bound FFA molecules in equilibrium with a much smaller population of unbound FFA. The end result is that a much greater

amount of FFA can be carried in the blood than is indicated by the solubility of these long-chain fatty acids in simple aqueous solutions. Dissociation from serum albumin has generally been considered the rate-limiting step for FFA uptake (Soler-Argilaga et al., 1974), although more recent reports suggest that penetration of the plasma membrane by FFA is the more likely rate-limiting site (Abumrad et al., 1981). In either case, unbound FFA concentration is a major determinant of uptake rates, and will be influenced largely by the fatty acid:albumin ratio.

The actual mechanism of FFA uptake is currently an area of active research. A recent report utilizing perfused rat liver (Weisiger et al., 1981) suggested that binding of the fatty acid:albumin complex to a specific albumin receptor on the plasma membrane was a required step prior to FFA dissociation and subsequent uptake. This concept has been disputed by Abumrad et al. (1981), who, using isolated rat adipocytes, found no difference in the kinetics of FFA uptake rates when the fatty acid:albumin ratio was varied by two different procedures: 1) holding fatty acid concentration constant while varying the albumin concentration, and 2) holding albumin concentration constant while varying the fatty acid concentration. For a given fatty acid concentration, faster FFA uptakes would be observed in the presence of

a greater albumin concentration, if albumin binding were an obligatory event preceding FFA uptake. This was not observed, however indicating that albumin binding to plasma membrane is not required for FFA uptake. The concentration of unbound FFA was the major determinant of FFA uptake rates.

The mechanism by which FFA penetrate the plasma membrane is currently unknown. DeGrella and Light (1980, a,b) provided evidence for the simple diffusion of FFA across the plasma membrane of isolated rat heart myocytes. Using a kinetic analysis, FFA uptake rates could be resolved into saturable and nonsaturable components. The nonsaturable component was attributed to fatty acid accumulation into a pool of free fatty acid (presumably protein-bound) within the cell, while the saturable component was thought to correspond to a smaller pool of fatty acid which was converted to metabolic products, i.e. this was the immediate precursor pool for fatty acid metabolism. These authors argued that FFA uptake rate was limited by the rate of fatty acid metabolism within the cell. Unbound fatty acid concentrations (as high as 10-30 μM) used in these studies, however, were well above physiological concentrations. In isolated rat adipocytes Abumrad et al. (1981) also found evidence for simple diffusion of FFA through the plasma membrane at high concentrations (15 μM) of unbound fatty acid. Using

very short incubation times (15 seconds) and physiological unbound fatty acid concentrations (<1 μM), however, a phloretin-inhibitable, saturable fatty acid uptake was observed. (Phloretin is a polyphenolic compound which binds specifically to membrane-associated transport molecules, such as the glucose transporter.) Based on these and other observations, these authors concluded that at low physiological unbound fatty acid concentrations, FFA uptake occurs via a carrier-mediated mechanism, presumably protein in nature, while at higher concentrations diffusion of FFA through the plasma membrane occurs. Similar research has yet to be extended to other species.

Hepatic Fatty Acid Metabolism

Fatty Acid Binding Protein

Free fatty acids within the liver cell encounter an aqueous environment similar to that of plasma, i.e. a highly polar medium in which fatty acids exhibit limited solubility. In a manner analogous to the binding of FFA to albumin in the plasma, cytoplasmic fatty acids can bind to intracellular proteins. Although nonspecific fatty acid binding to cellular proteins can take place, the principal binding appears to occur to a specific class of 12,000 MW proteins (Mishkin *et al.*, 1972; Ockner *et al.*, 1972). These proteins were originally identified in the liver due to their ability to bind various dyes, e.g.

bromsulphophthalein, and received the designation Z-protein (Litwack et al., 1971). Z-protein has been identified in the cytoplasm of a number of rat tissues besides the liver, including kidney, myocardium, skeletal muscle, intestinal epithelium and adipose tissue. Binding of long-chain fatty acid, usually oleic acid, by Z-protein occurs with an affinity similar to that of the high energy sites of serum albumin ($Km = 2.8X10^{-6}M$; Mishkin et al., 1972). Subsequent to the discovery of Z-protein, a fatty acid binding protein (FABP) was characterized with an identical tissue distribution and similar dye- and fatty acid-binding characteristics to Z-protein (Ockner et al., 1972). Based on immunological characteristics and amino acid composition, the FABP and Z-proteins characterized from the rat liver appear to be identical polypeptides (Ockner et al., 1972), and suggests that a similar identity may exist between FABP and Z-protein within the other tissues examined. Not only is FABP ubiquitously distributed, but, at least within the liver, can also comprise up to 5% of the total cytoplasmic protein (Ockner et al., 1982).

A growing body of evidence suggests that binding of hydrophobic metabolites by specific intracellular proteins may play an important role in the subsequent metabolism of those compounds (Ockner et al., 1982). Such a role has been suggested for FABP in fatty acid

metabolism, and is supported by two main lines of evidence. First, a good correlation has been found between FABP concentrations within a tissue and rates of FA uptake and metabolism by that tissue under a variety of conditions. In the rat intestine, for example, FABP concentrations are greater in the jejunum compared to the ileum, and in villi compared to crypts, i.e. areas of active fat absorption. In addition, intestinal mucosa from high-fat fed rats contains more FABP than mucosa from low-fat fed rats (Ockner and Manning, 1974). Clofibrate administration has been demonstrated to increase both hepatic fatty acid uptake and FABP content (Fleischner et al., 1975; Renaud et al., 1978). Binding activity of FABP is also reportedly increased in liver of the obese Zucker rat, a condition in which the total FA flux is also increased relative to the nonobese rat (Morrow et al., 1979).

The second line of evidence concerns the ability of FABP in vitro to influence the activity of various enzyme reactions concerned with FA metabolism. Fatty acid binding protein has been demonstrated to stimulate the activity of rat intestinal fatty acyl-CoA (FA-CoA) synthetase (Ockner and Manning, 1976) and diacylglycerol (DG) acyltransferase (O'Doherty and Kuksis, 1975), as well as several hepatic enzymes, namely glycerophosphate acyltransferase (Mishkin and Turcotte, 1974), microsomal (Wu-Rideout et al., 1976) and mitochondrial FA-CoA

synthetase (Burnett et al., 1979), DG acyltransferase (O'Doherty and Kuksis, 1975), and peroxisomal fatty acid oxidation (Appelkvist and Dallner, 1980). In addition, FABP can overcome fatty acid- or FA-CoA-induced inhibition of acetyl-CoA carboxylase (Lunzer et al., 1977) and mitochondrial adenine nucleotide transport (Barbour and Chan, 1979). In all of these cases binding of fatty acid to FABP is required for alterations in enzyme activity to be observed. Inclusion of flavaspidic acid, a competitive inhibitor of fatty acid binding to FABP, prevents FABP-induced alterations in enzyme activity (Wu-Rideout et al., 1976). The ability of FABP to stimulate enzyme activity is a specific effect attributable to FABP, and is not due simply to nonspecific binding of fatty acid to protein. For example, if FABP is replaced by albumin in an in vitro system, qualitatively similar results are obtained, but enzyme activity is stimulated to only a fraction of the amount observed with FABP (Mishkin and Turcotte, 1974; Ockner and Manning, 1976; O'Doherty and Kuksis, 1975). The stimulatory effects of FABP on enzyme activity are dose dependent, and large changes in activity are often observed in response to small changes of FABP within the physiological range of FABP concentrations (Barbour and Chen, 1979; Lunzer et al., 1977; Mishkin and Turcotte, 1974; Ockner and Manning, 1976; O'Doherty and Kuksis, 1975;

Iritani et al., 1980). These observations, plus the large contribution of FABP to total hepatic cytoplasmic protein, give strong support to the suggestion that FABP is an important factor in regulation of fatty acid metabolism. Conversely, this could also mean that fatty acid metabolism must be a very important aspect of cell function, since the cell devotes such a large portion of its protein content to this phenomenon.

More recent research supports the concept that FABP does play a role in fatty acid metabolism by favoring esterification of long-chain fatty acids at the expense of fatty acid oxidation. This was demonstrated using hepatocytes isolated from fed rats. Inclusion in the incubation media of flavaspidic acid, a competitive inhibitor of fatty acid binding to FABP, inhibited both oleic acid uptake from the media and its subsequent esterification, but stimulated oleic acid oxidation to $C0_2$ and ketone bodies (Wu-Rideout et al., 1976). (Oxidation of octanoic acid, which does not undergo esterification in rat liver and is activated within the mitochondrial matrix, was not effected by flavaspidic acid addition.) Since the mechanism of action of flavaspidic acid is inhibition of fatty acid binding to FABP, these results demonstrate the potential importance of FABP in the partitioning of long-chain fatty acid towards esterification and away from oxidation, perhaps

by compartmentalization of fatty acid at the sites of esterification within the cell (Ockner and Manning, 1976). Because most fatty acid esterification occurs in the cytoplasm, cytoplasmic extracts from rat livers exhibiting active esterification rates should have a greater capacity to bind fatty acid than similar extracts made with livers from fasted rats, if FABP is involved in partitioning of fatty acids towards esterification. Such a difference was observed by Iritani et al. (1980), who found a greater fraction of added ^{14}C-palmitoyl-CoA bound in the supernate of a liver homogenate from fasted rats refed a fat-free diet than was bound by an identical liver preparation from fasted rats. These authors also found greater amounts of labelled palmitoyl-CoA bound to a FABP-fraction in the supernate from the fasted rats refed a fat-free diet than from the fasted rats, but were unable to distinguish between an increase in the amount of FABP, or an increase in the affinity of fatty acid binding to the protein, as the cause of the increased association of labelled palmitoyl-CoA with the FABP-fraction. A recent report suggests an increase in total FABP as the cause of the increased binding of labelled palmitoyl-CoA (Bass et al., 1982).

At present, the mechanism by which FABP stimulates enzyme activity is unknown. (Relief of fatty acid-/FA-CoA-induced inhibition of acetyl-CoA carboxylase and

mitochondrial adenine nucleotide transport may be due simply to binding and removal from inhibitory sites on the enzymes of fatty acid/FA-CoA by the FABP (Barbour and Chan, 1979; Lunzer et al., 1977).) Recently, a newly discovered property of FABP isolated from pig heart has been reported, self-aggregation of the 12,000 MW monomer to form oligomeric structures (Fournier and Rahim, 1983; Fournier et al., 1983). At least four distinct molecular species of FABP were noted, based on their differential fatty acid binding capacity. Small changes in FABP concentration can cause major changes in the aggregation state of FABP. The results of these studies were used to construct a mathematical model to predict the effect of FABP aggregation state on the activity of a hypothetical membrane-bound, fatty acid-requiring enzyme. This model predicted that alterations in FABP aggregation state, in response to changing FABP concentrations, could result in major changes in activity of the hypothetical enzyme. Activity alterations appeared to result from the ability of FABP to place fatty acid (or FA-CoA) in close proximity to the model enzyme, thereby allowing faster reaction rates than would occur if fatty acid were forced to diffuse through an aqueous environment to reach the enzyme. These results have yet to be extended to the in vivo situation.

Up to this time FABP does not appear to have been isolated from any ruminant species. In view of the postulated importance of FABP for stimulation of fatty acid esterification, future investigations of FABP in the ruminant liver appear warranted, since ruminant liver can be a major site of esterification (e.g. in a high-producing dairy cow).

Fatty Acid Activation

Before fatty acid metabolism can begin within the liver, the fatty acid must undergo activation, i.e. esterification with free coenzyme A (CoASH) to form the more reactive FA-CoA. The general reaction

$$RCOOH + CoASH + ATP \longleftrightarrow RC(=O)-S-CoA + AMP + PPi$$

is readily reversible, but due to the rapid destruction of PPi within the cell, the formation of FA-CoA is favored (Groot et al., 1976). The enzymes which catalyze this reaction can be broadly classified as short-, medium-, or long-chain FA-CoA synthetases, depending upon the acyl chain-length specificity of the enzyme (Groot et al., 1976).

Due to the importance of short-chain fatty acids in the energy metabolism of the ruminant (short-chain fatty acids are the main energy source to the animal produced in the rumen fermentation), the short-chain FA-CoA synthetases have received considerable attention.

Much of the work examining short-chain FA-CoA synthetases in ruminant liver has utilized extracts of liver homogenate or of liver mitochondria as an enzyme source (e.g. Ash and Baird, 1973; Cook et al., 1969; Quraishi and Cook, 1972; Scholte et al., 1971). This has led to some confusion as to the number and specificities of FA-CoA synthetases present in ruminant liver, because no enzyme purification was attempted to these studies. Based on the ability of these extracts to activate acetate, propionate and butyrate (propionate > butyrate >> acetate), and interactions among these short-chain fatty acids (e.g. propionate inhibition of acetate and butyrate activation), ruminant liver was postulated to contain a propionate-specific FA-CoA synthetase, and a FA-CoA synthetase specific for butyrate and other medium-chain fatty acids. These results appeared to correspond well with uptake and utilization of short-chain fatty acids by ruminant liver (Bell, 1980).

Various FA-CoA synthetases have, however, been isolated from ruminant liver. Mahler and Wakil (1953) isolated a fatty acid activating enzyme with a broad substrate specificity from beef liver mitochondria. Evidence for a butyryl-CoA synthetase has also been published (Killenberg et al., 1971). Recently, the isolation of propionyl-CoA synthetase, and evidence suggesting the existence of both a butyryl-CoA and a valeryl-CoA synthetase, in bovine liver mitochondria

was published (Ricks and Cook, 1981a). These reports are compatible with the observed pattern of short-chain fatty acid activation by various liver extract preparations.

To date, although various researchers have assayed ruminant liver preparations for acetyl-CoA synthetase activity (Ash and Baird, 1973; Cook et al., 1969; Quraishi and Cook, 1972), no isolation of the liver enzyme has been reported. Acetyl-CoA synthetase has been isolated from bovine heart (Campagnari and Webster, 1963; Ricks and Cook, 1981b), kidney (Ricks and Cook, 1981c) and lactating mammary gland (Qureshi and Cook, 1975), and lactating goat mammary gland (Cook et al., 1975). Short-chain and medium-chain activating enzymes are located primarily within the mitochondrial matrix (Groot et al., 1976), but acetyl-CoA synthetase activity is low in bovine liver mitochondria (Quraishi and Cook, 1972; Ricks and Cook, 1981a). What acetyl-CoA synthetase activity is present in bovine liver mitochondria has been attributed to activation of acetate by propionyl-CoA synthetase (Ricks and Cook, 1981a). Acetyl-CoA synthetase in ruminant liver may not follow the traditional pattern of cellular distribution, however, and appears to be located predominantly in liver cytoplasm (Ballard and Hanson, 1967; Snoswell and Koundakjian, 1972). This acetyl-CoA synthetase activity can account for the observation that ruminant liver can potentially absorb and utilize 15-20%

of the acetate presented to the liver (Pethick et al., 1981; Thompson et al., 1975). Cytoplasmically produced acetyl-CoA could be used directly for fatty acid synthesis in the cytoplasm without passing through the mitochondrial acetyl-CoA pool. The pattern of short-chain activating enzymes found in the ruminant liver allows the liver near exclusive utilization of propionate from the rumen fermentation for gluconeogenesis, while sparing acetate for use by extrahepatic tissues (Ricks and Cook, 1981a).

Medium-chain fatty acid metabolism has not been investigated to any degree in the ruminant liver. Based on the reports of Mahler and Wakil (1953), and Ricks and Cook (1981a), ruminant liver probably has the capacity to activate medium-chain fatty acids, although the importance of this in vivo is unknown.

Little research has been done with the long-chain FA-CoA synthetases in ruminant liver. In liver of all species examined, however, these enzymes are found primarily in the microsomes and the outer mitochondrial membrane, with perhaps some activity in the peroxisomes (Groot et al., 1976). These locations are consistent with the functions of long-chain FA-CoA synthetase: providing FA-CoA for esterification and oxidation. Esterification occurs in the microsomes and outer mitochondrial membrane, while oxidation takes place within the mitochondria and peroxisomes, in close spatial proximity to the locations

of FA-CoA generation. (Some evidence has been presented suggesting that the microsomal FA-CoA synthetase may be part of a multienzyme complex involved in triacylglycerol (TG) formation (Groot et al., 1976).) Long-chain FA-CoA synthetase activity is not thought to be rate-limiting for either esterification (Lloyd-Davies and Brindley, 1973) or oxidation (Pande, 1971). The intracellular localization of long-chain FA-CoA synthetase corresponds with the reported distribution of FABP in rat liver (Capron et al., 1979), giving further support to the concept that FABP has an important role in FA metabolism. No a priori evidence exists to suggest that long-chain FA-CoA synthetase functions differently in the ruminant than in the nonruminant.

Fatty Acid Esterification and Lipoprotein Metabolism

Fatty acid esterification is a general term applied to the process of incorporating acyl groups from FA-CoA into triacylglycerols (TG) and phospholipids (PL). Esterification is the major pathway competing with oxidation for utilization of FA-CoA in the liver. Thus, esterification and oxidation generally tend to be regulated in a reciprocal manner, so that high esterification rates in a fed animal are accompanied by low oxidation rates, and vice versa in the fasted state (Newsholme and Start, 1976).

Bell and Coleman (1980) have recently reviewed the reactions involved in fatty acid esterification and glycerolipid synthesis. Most of the information available concerning fatty acid esterification has been obtained from the nonruminant. Studies of ruminant hepatic fatty acid esterification are limited. The major route of fatty acid esterification appears to be via the sn-glycerol-3-phosphate (G3P) pathway discussed by Kennedy (1961; Figure 1). The acyl moiety of two FA-CoA are sequentially incorporated onto G3P to produce phosphatidic acid (PA). Phosphatidic acid can be used directly for phospholipid (PL) synthesis, or can be converted to DG. Diacylglycerol may also be used for PL synthesis. Alternatively, a third acyl group may be incorporated to form TG. The enzymes which catalyze these reactions appear to be located primarily within the microsomal fraction of the liver (Bell and Coleman, 1980; Daae, 1973), with some evidence suggesting more specifically the rough endoplasmic reticulum (Stein and Stein, 1967, 1971). Some activity, however, notably the G3P and acyl-G3P acyltransferases and various enzymes of PL formation, is also found in the outer mitochondrial membrane (Bell and Coleman, 1980; Daae, 1973; van den Bosch, 1974).

In the fed nonruminant, FA-CoA used for esterification is derived primarily from de novo fatty acid synthesis (Newsholme and Start, 1976). This contrasts with the

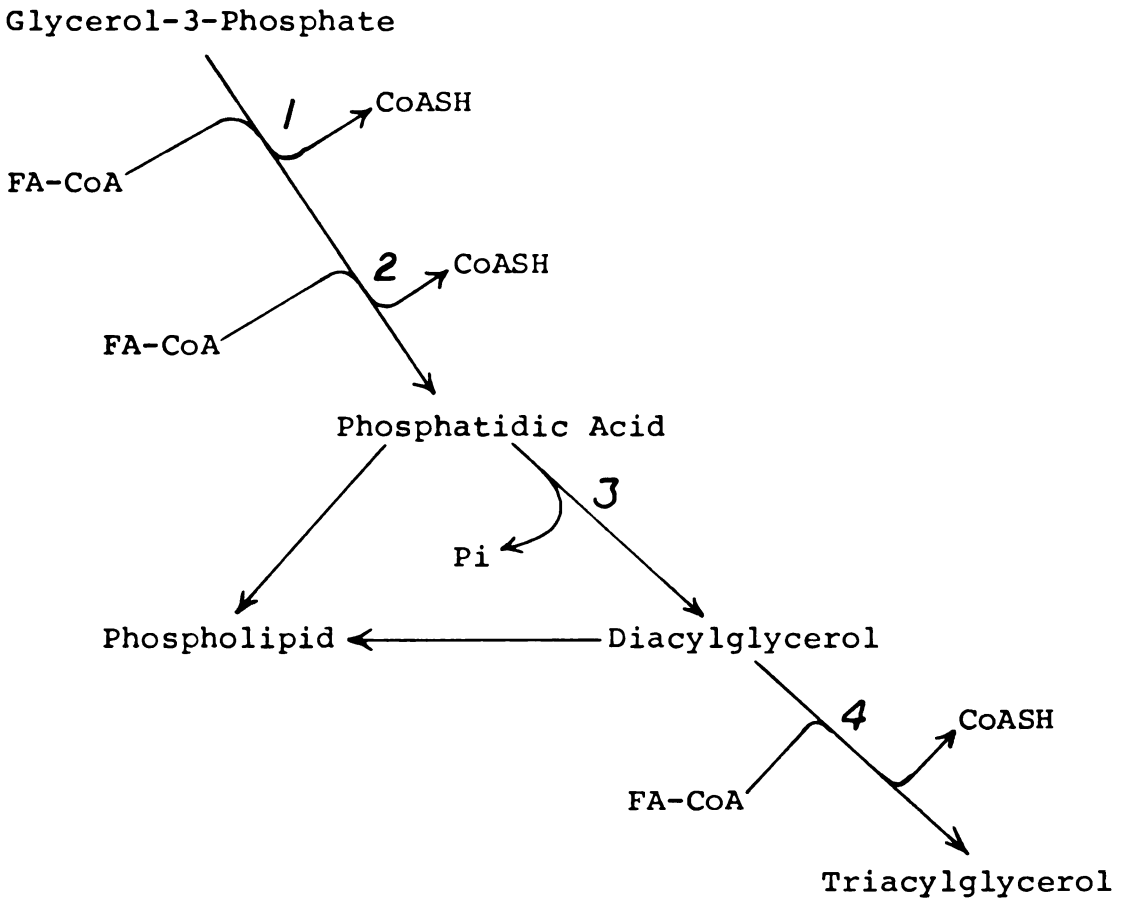


Figure 1.--The glycerol-3-phosphate pathway of phospholipid and triacylglycerol synthesis.

The reactions of the glycerol-3-phosphate pathway are catalyzed by:

1) acyl-CoA:sn-glycerol-3-phosphate 0-acyltransferase
2) acyl-CoA:sn-monoacylglycerol-3-phosphate 0-acyltransferase
3) L-α-phosphatidic acid phosphohydrolase
4) acyl-CoA:sn-diacylglycerol 0-acyltransferase

FA-CoA = Long-chain fatty acyl-coenzyme
CoASH = Free coenzyme A

situation in liver from the fed or fasted ruminant, where, because of the inherently low de novo fatty acid synthesis, esterified fatty acids are derived almost exclusively from FFA absorbed from the blood (West and Annison, 1964; Bell, 1980). Under some circumstances where exceptionally large (>1 mM) FFA concentrations are encountered, such as during a prolonged fast or pathological conditions (e.g. diabetic ketoacidosis), nonruminant liver can utilize absorbed FFA for esterification (Newsholme and Start, 1976). Glycerol-3-phosphate, the backbone of TG and PL molecules, is formed both during glycolysis and gluconeogenesis, and in the pentose phosphate cycle. In addition, glycerol can be derived from the metabolism of extrahepatic tissues, e.g. lipolysis in adipose tissue (Bergman, 1968). Glycerol is activated to G3P by the enzyme glycerol kinase, which has been isolated and purified from beef liver (Grunnett and Lundquist, 1967). Glycerol metabolism has been studied in the sheep (Bergman et al., 1968). Under a wide variety of metabolic conditions (fed, fasted, pregnancy toxemic), about 40% of the circulating glycerol carbon was converted into compounds other than glucose and $C0_2$, some of which were presumably glycerolipid in nature.

Glycerol-3-phosphate acylation is not a random process with respect to either the order with which the free hydroxyls of G3P are acylated, or to the composition of the incorporated fatty acyl groups. Acylation occurs

first at the sn-1 position of G3P, forming lysophosphatidic acid, followed by acylation at the sn-2 carbon (Bell and Coleman, 1980; Daae, 1973; van den Bosch, 1974). Rat liver mitochondria exhibited a preference for incorporation of saturated fatty acid, especially palmitic acid, into the sn-1 position of G3P, a preference not shown by rat liver microsomes (Bell and Coleman, 1980; Daae, 1973). Rat liver microsomes, on the other hand, preferred to incorporate unsaturated fatty acid into the sn-2 position of lysophosphatidic acid (Bell and Coleman, 1980). Different products were also synthesized by these rat liver fractions, the microsomes producing mainly phosphatidic acid and the mitochondria synthesizing lysophosphatidic acid (Daae, 1973). The specificity with which different fatty acids are incorporated into the sn-1 and sn-2 positions of ruminant liver glycerolipids has not been reported, but bovine liver homogenates and ovine liver slices have been noted to display a definite preference for the overall incorporation of palmitic acid into TG and stearic acid into PL (Benson and Emery, 1971; Payne and Masters, 1971). Calf liver microsomes produce mainly PA and mitochondria form primarily lysophosphatidic acid, a pattern qualitatively similar to that of the corresponding rat liver fractions, although the effects are not as pronounced (Daae, 1973). These observations, different products formed and, at least in rat liver, different fatty acid

specificities, for the microsomes and mitochondria, have led to the suggestion that the mitochondrial acyltransferases are important for determination of the final fatty acid composition of hepatic glycerolipids, particularly of the PL (Bell and Coleman, 1980; van den Bosch, 1974). A mitochondrially-associated lysophospholipase has been identified in bovine liver mitochondria, prompting speculation of a similar role for mitochondria in determining the fatty acid composition of bovine hepatic glycerolipids (de Jong et al., 1974; van den Bosch and de Jong, 1975).

Although the G3P-pathway is thought to be the major route of TG and PL synthesis, various researchers have occasionally proposed alternate pathways for glycerolipid formation in which dihydroxyacetone phosphate (DHAP) or glyceraldehyde-3-phosphate acts as acyl acceptor. Fatty acid esterification to DHAP has been observed in rat liver microsomes and peroxisomes (Bell and Coleman, 1980; Hajra et al., 1979). The microsomal DHAP-utilizing activity has been dismissed as merely an alternate catalytic function of the microsomal G3P acyltransferase, based on kinetic and other considerations, such as pH dependence, acyl-CoA chain length specificity, and susceptibility to detergents and proteases (Bell and Coleman, 1980). The peroxisomal DHAP acyltransferase appears to be distinct from G3P acyltransferase, based on

subcellular distribution patterns (Hajra et al., 1979). Other enzymes involved in DHAP metabolism have also been identified in rat liver, specifically glycerophosphate dehydrogenase and acyl-DHAP:NADPH oxidoreductase (Hajra et al., 1979; Tolbert, 1981). These enzymes for DHAP utilization in the peroxisomes are apparently involved in ether glycerolipid synthesis, rather than TG or PL formation (van den Bosch, 1974).

Regulation of TG formation has been postulated to occur at two levels: substrate availability and modulation of enzyme activity (Hübscher, 1970). Availability of intracellular G3P has long been thought to be a major factor for regulating hepatic fatty acid esterification rates. Tzur et al. (1964) demonstrated that liver G3P concentrations were decreased in fasted or epinephrine-treated rats, conditions generally associated with decreased esterification rates. Lund et al. (1980) reported that glycerol addition to the media of hepatocytes isolated from fasted rats increased fatty acid esterification at the expense of oxidation without altering the overall rate of fatty acid uptake. These results demonstrated that under a given set of conditions alterations in G3P concentrations could induce corresponding alterations in fatty acid esterification. Glycerol-3-phosphate concentration is not, however, the only factor involved in regulation of esterification rates.

In his review on glyceride metabolism, Hübscher (1970) cited evidence that fatty acid esterification by perfused hearts from alloxan-diabetic rats was higher than expected, based on lower cellular G3P concentrations. This anomoly was apparently due to an increased flux rate through the G3P pool, so that the cellular G3P concentration was not a valid estimate of G3P availability. When ethanol was added to the perfusate of livers isolated from control and cortisol-treated rats, only a small increase in esterification rates occurred (Pikkukangas et al., 1982), despite a marked increase in cellular G3P concentrations (Schimassek et al., 1971). Thus, increasing G3P concentrations above that typically observed in fed rat liver had little effect on fatty acid esterification. Most researchers generally agree that while G3P availability can influence fatty acid esterification rates in a given situation, G3P availability is not the primary factor involved in regulation of esterification rates (Pikkukangas et al., 1982; Wirthensohn et al., 1980).

Fatty acid availability can have a profound effect on esterification rates. In the presence of glycerol (1 mM) and glucose (10 mM), esterification rates increased in hepatocytes isolated from fed or fasted rats when oleic acid (a representative long-chain fatty acid) concentrations in the media were increased from 0 to 2 mM (Pikkukangas et al., 1982). Fatty acid esterification

rates by hepatocytes from fasted rats were actually faster than rates in hepatocytes isolated from fed rats. These results have been confirmed (Wirthensohn et al., 1980). Even in the absence of added glucose, significant fatty acid esterification occurred in fasted rat hepatocytes in the presence of high (2 mM) fatty acid concentrations (Pelech et al., 1983). These results could explain the fat infiltration of the liver which occurs in fasted or ketotic dairy cows (Bell, 1980; Bergman, 1971). Under these circumstances the liver is absorbing more fatty acid than can be disposed of through oxidation. Esterification of the surplus fatty acid would prevent potential toxic effects to the liver.

Modulation of the activity of various enzymes involved in glycerolipid synthesis is the primary mechanism for regulation of hepatic fatty acid esterification. In general, the overall process of fatty acid esterification in rat liver is stimulated by insulin and inhibited by glucagon (Beynen, 1982). Glucocorticoids have also been reported to stimulate both esterification rates and secretion of very low density lipoproteins in rat liver (VLDL; Glenny and Brindley, 1978; Lawson et al., 1981; Reaven et al., 1974). A number of the enzymes involved in glycerolipid biosynthesis have been examined for potential response to hormonal treatment. Glycerol-3-phosphate acyltransferase (Figure 1) in perfused liver

from fasted rats is reportedly stimulated by insulin (Bates et al., 1977), while glucagon inhibits G3P acyltransferase in hepatocytes isolated from fed rats (Sugden et al., 1980). These results suggest that G3P acyltransferase is subject to phosphorylation/dephosphorylation control for short-term regulation (Geelen et al., 1980). The importance of these modifications of G3P acyltransferase activity in vivo specifically to TG synthesis has yet to be assessed, since regulation of this enzyme would influence both TG and PL synthesis (Figure 1).

Phosphatidic acid phosphohydrolase has also been proposed as a regulatory enzyme for TG synthesis (Figure 1). Cortisol and dexamethasone have both been demonstrated to increase PA phosphohydrolase activity (Jennings et al., 1981; Pikkukangas et al., 1982), but FA esterification rates did not increase in parallel with PA phosphohydrolase activity. These results indicate that PA phophohydrolase is not rate-limiting to TG synthesis. As was the case for G3P acyltransferase, PA phosphohydrolase does not represent a unique branch-point between TG and PL synthesis, so that the importance of modulation of this enzyme activity specifically for TG synthesis can be questioned.

Diacylglycerol acyltransferase represents the first unique reaction of the G3P-pathway for TG synthesis, and as such may be a controlling factor in TG formation

(Figure 1). Glucagon reportedly decreases DG acyltransferase in hepatocytes isolated from fed rats (Haagsman et al., 1981). In addition, DG acyltransferase can be reversibly activated and inactivated in vitro in a manner consistent with, but not conclusive of, a phosphorylation/dephosphorylation mechanism (Haagsman et al., 1982). Further support for the regulatory nature of DG acyltransferase in TG synthesis was provided by Hillmar et al. (1983), who demonstrated induction of this enzyme in primary rat hepatocyte cultures in response to various fatty acids. Addition of oleic acid (.5 mM) in the presence of glucose, lactate and insulin, increased DG acyltransferase activity by 191% and nearly doubled the cellular content of TG during the course of a 72 hour incubation. Other long-chain fatty acids produced qualitatively similar results, but the responses were not as dramatic. The ability of fatty acid to increase DG acyltransferase activity was attributed to induction of enzyme synthesis, since cyclohexamide could prevent the increase in enzyme activity (Hillmar et al., 1983). Taken together, these results indicate that DG acyltransferase is an important regulatory factor for TG synthesis. While G3P acyltransferase and PA phosphohydrolase catalyze reactions which are not unique to TG synthesis, regulation of these enzymes may also be involved in TG synthesis,

since high TG production rates would also require high G3P acyltransferase and PA phosphohydrolase activity.

Triacylglycerols synthesized within the liver are usually secreted by the liver as VLDL. During periods when TG formation exceeds TG secretion, however, TG accumulates within the liver as neutral lipid. This neutral lipid appears to be stored within the liver as membrane-bound vesicles within the cytoplasm (Debeer et al., 1979, 1982; Mooney and Lane, 1981). Triacylglycerols stored in this manner cannot be directly incorporated into lipoprotein, but must first undergo lipolysis by a hepatic TG lipase. The consequently released fatty acids become available for either a second round of esterification or oxidation. These results have been demonstrated in hepatocytes from rat (Debeer et al., 1979, 1982) and chicken (Mooney and Lane, 1981). Indirect evidence suggests that ruminant liver also contains a hepatic TG lipase (Bergman et al., 1971). In rat liver, hepatic TG lipase is located within the lysosomes (Debeer et al., 1979). Treatment of isolated rat hepatocytes with the lysosomotropic agents chloroquine or methylamine, which decrease the cellular content of lysosomes, inhibited the metabolism of endogenously derived fatty acid. Hepatic TG lipase, unlike the hormone sensitive lipase of adipose tissue, does not appear to be under direct hormonal control, i.e. via a

phosphorylation/dephosphorylation mechanism (Debeer et al., 1979). Glucagon may indirectly regulate hepatic TG lipase, however, by inducing formation of autophagosomes, membrane-bounded vacuoles which undergo fusion with lysosomes (Debeer et al., 1982). This mechanism would not alter activity of the lipase, but would facilitate presentation of the TG substrate to the lipase. Such a mechanism could be involved in the regression of the fatty infiltration of the bovine liver which occurs during the periparturient period, or during lactational ketosis.

The process of VLDL-TG secretion involves three processes: TG synthesis, assembly of TG into lipoprotein particles, and the subsequent release of VLDL from the liver. Insulin and glucagon influence hepatic VLDL secretion in the same manner as they effect TG synthesis, i.e. insulin stimulates and glucagon inhibits VLDL secretion (Beynen, 1982). Beynen et al. (1981) attempted to determine at which point in the secretion process these hormones produced their effects. The results were, as expected, that insulin stimulated VLDL secretion and glucagon inhibited secretion from isolated rat hepatocytes. When the isolated hepatocytes were preincubated with tritiated water, however, neither insulin nor glucagon addition had an effect on the rate of release of the pre-labelled TG from the hepatocytes. These authors concluded that insulin and glucagon indirectly regulate

VLDL secretion by altering rate of TG synthesis, and possibly by the availability of suitable apoproteins. These two possibilities could not be distinguished.

The assembly of TG into VLDL prior to release from the liver has not been studied in ruminant liver. Metabolism of ruminant plasma lipoproteins has recently been reviewed (Kris-Etherton and Etherton, 1982), and is similar to that of the nonruminant (Brumby and Welch, 1970). Thus, hepatic VLDL assembly in the ruminant probably differs little from the nonruminant. Based on chemical composition and electronmicroscopic studies, VLDL assembly begins in the microsomes, probably at the transition between the smooth and rough endoplasmic reticulum, where TG first begins associating with protein. Nascent lipoprotein particles then migrate to the Golgi apparatus for maturation and secretion via exocytosis (Chapman et al., 1973; Bell, 1980).

Concentrations of VLDL in ruminant plasma are lower than those of nonruminant plasma, but ruminant VLDL contain a higher proportion of TG (Kris-Etherton and Etherton, 1982). Circulating plasma TG concentrations in the nonpregnant, nonlactating ruminant may be only one-tenth of the concentration found in swine plasma, but may increase to a higher proportion of the nonruminant TG concentration in the lactating or pregnant ruminant (Kris-Etherton and Etherton, 1982). Despite these

relatively lower plasma VLDL and TG concentrations, plasma lipoproteins play a significant role in the lipid metabolism of the lactating bovine mammary gland. Glascock and Welch (1974) and Glascock et al. (1966) have demonstrated that up to 50%, by weight, of milk TG fatty acid was derived from plasma lipoprotein TG fatty acid. (Under normal conditions net uptake of plasma FFA by mammary gland does not occur (Bickerstaffe et al., 1974).) These observations demonstrate the importance of hepatic FA esterification and lipoprotein release to the high-producing dairy cow. Ruminant hepatic lipoprotein metabolism should prove a fertile and productive area of future research.

Mitochondrial Fatty Acid Oxidation

Fatty acid oxidation within the mitochondria is the second major fate of fatty acid in liver. In a typical nonruminant consuming a high-carbohydrate diet, hepatic mitochondrial fatty acid oxidation occurs exclusively during the post-absorptive and fasting states, while hepatic fatty acid synthesis is confined to the fed (absorptive) state. Malonyl-CoA, the first committed intermediate in fatty acid synthesis, has been proposed as a key component in this reciprocal control of fatty acid oxidation and synthesis (McGarry and Foster, 1980), although this view has been challenged (Brass and Hoppel, 1978). Some interesting questions are apparent concerning

malonyl-CoA as a regulatory mechanism of fatty acid oxidation in the ruminant liver, where fatty acid synthesis rates, and presumably malonyl-CoA concentrations, are low even in the fed animal (Ballard and Hanson, 1967).

Fatty acid oxidation occurs predominantly via β-oxidation, the enzymes of which are located within the mitochondrial matrix. β-Oxidation consists of a sequence of four different reactions which have been collectively termed the "fatty acid oxidase spiral" (Figure 2; Fritz, 1961). Fatty acyl-CoA within the matrix undergoes α, β-dehydrogenation by a flavin-linked enzyme, followed by hydration of the double-bond to yield a β-hydroxy-FA-CoA. A second dehydrogenation, catalyzed by a NAD-linked enzyme, yields the β-keto-FA-CoA derivative, which subsequently undergoes thiolytic cleavage to yield acetyl-CoA and a FA-CoA two carbon units shorter than initially. For example, palmitoyl-CoA would undergo seven spirals of β-oxidation and ultimately yield eight acetyl-CoA. Acetyl-CoA could enter the tricarboxylic acid (TCA) cycle for complete oxidation to CO_2, or could be utilized for ketogenesis. Nonruminant liver mitochondria contain several different acyl-CoA dehydrogenases and β-ketoacyl-CoA thiolases, each with a different acyl chain-length specificity (Fritz, 1961). These results were confirmed in bovine liver, which was found to contain

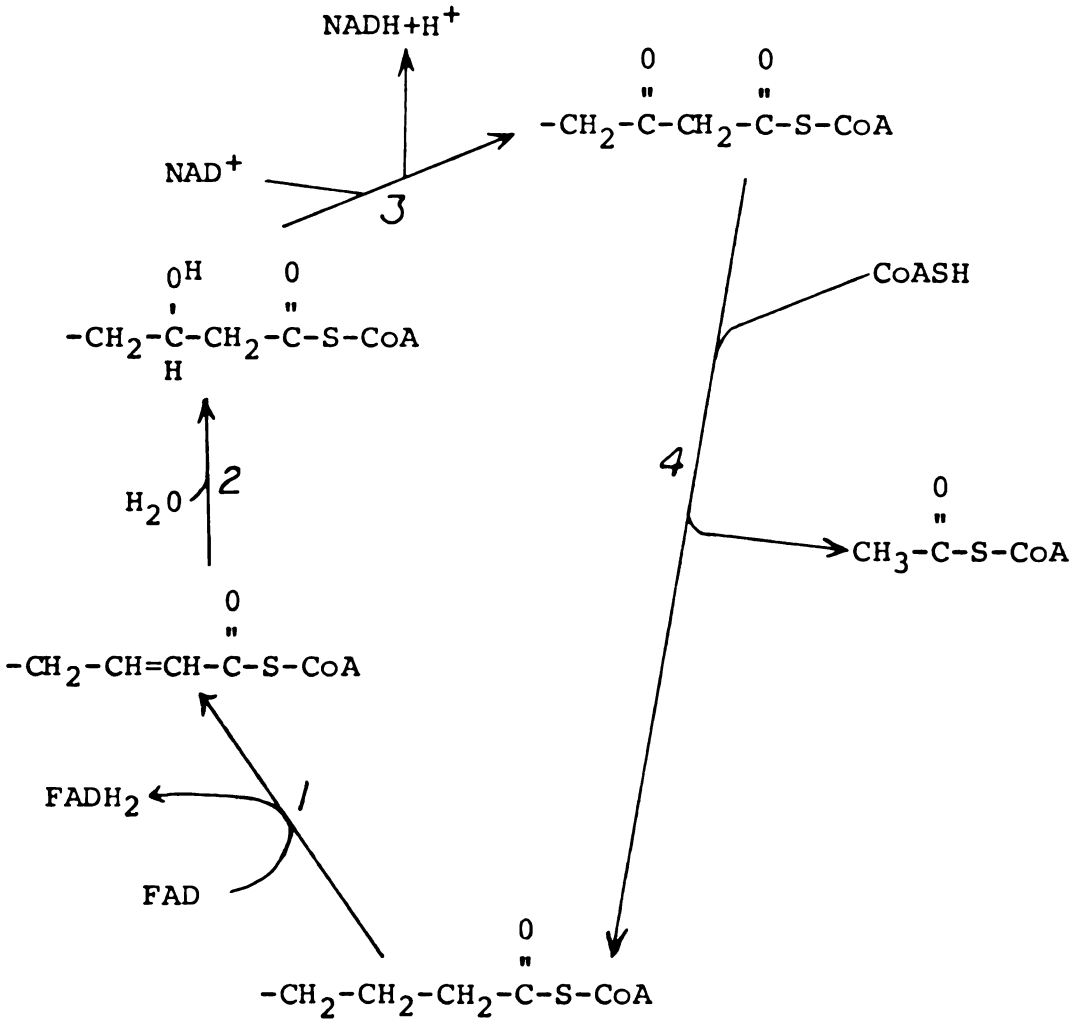


Figure 2.--The β-oxidation pathway

The reactions of this pathway are catalyzed by:

1) acyl-CoA dehydrogenase
2) enoyl-CoA hydrase
3) β-hydroxyacyl-CoA dehydrogenase
4) β-ketoacyl-CoA thiolase

Abbreviations are:

NAD = Nicotinamide Adenine Dinucleotide
FAD = Flavin Adenine Dinucleotide
CoASH = Free Coenzyme A

three different acyl-CoA dehydrogenases, with specificities for short-, medium- and long-chain acyl-CoA (Davidson and Schultz, 1982). In heart and skeletal muscle the rate of β-oxidation is regulated by the energy demand placed on the muscle, so that acetyl-CoA production is matched by acetyl-CoA utilization in the TCA cycle (Hochachka et al., 1977). Such regulation of β-oxidation probably may not occur in the liver, since acetyl-CoA production routinely exceeds utilization in the TCA cycle during a fast or pathological ketosis.

Fatty acids entering the mitochondrial matrix for oxidation must first penetrate the inner mitochondrial membrane, which is impermeable to acyl-CoA derivatives. Since short- and medium-chain fatty acids are activated within the mitochondrial matrix (Groot et al., 1976), these acids can readily penetrate the inner membrane and undergo oxidation. Ruminant liver mitochondria can readily oxidize short- and medium-chain fatty acids, albeit at lower rates than nonruminant mitochondria (Koundakjian and Snoswell, 1970; Mayfield et al., 1965). Acetate oxidation by isolated sheep liver mitochondria is inhibited in the presence of propionate and/or butyrate (Smith, 1971). These results are in agreement with the data of Ricks and Cook (1981a) who found that propionate and butyrate could inhibit acetate activation by bovine liver mitochondria.

Long-chain fatty acids undergo activation in the cytoplasm, and so are separated from the site of β-oxidation by the CoASH-impermeable inner membrane. Long-chain fatty acyl-CoA cannot cross the inner membrane unassisted, but are transported across the membrane by the carnitine palmitoyl transferase (CPT) system, so named because of its specificity for long-chain FA-CoA (Hoppel, 1982). Carnitine palmitoyl transferase catalyzes the reversible exchange of long-chain acyl-groups from CoASH to carnitine, as noted by this reaction

$$\text{FA-CoA + l-carnitine} \overset{\text{CPT}}{\longleftrightarrow} \text{FA-l-carnitine + CoASH.}$$

In the nonruminant, long-chain fatty acid oxidation rates in the liver are proportional to blood FFA concentrations, and thus rates of fatty acid uptake and subsequent activation to FA-CoA (Heimberg et al., 1974). Several studies in ruminant animals have demonstrated a linear relationship between blood FFA concentrations and both rate of FFA entry (West and Annison, 1964; Pethick et al., 1983) and rate of whole-body fatty acid oxidation (Pethick et al., 1983), so that a relationship similar to that in the nonruminant between FFA concentrations and rates of hepatic fatty acid oxidation probably occurs in the ruminant.

Generally, reports of hepatic fatty acid oxidation in the ruminant are more limited than in the nonruminant. Connelly et al. (1964) found that the isolated goat liver

perfused with ^{14}C-palmitic acid did not release $^{14}CO_2$, but did produce significant amounts of ^{14}C-labelled ketone bodies. Isolated sheep liver mitochondria oxidized FA-carnitine at less than one-third the rate found with rat liver mitochondria (Koundakjian and Snoswell, 1970). More recently, isolated sheep hepatocytes were also found to oxidize palmitate to ketone bodies at about one-third the rate of rat hepatocytes (Lomax et al., 1983a; Kean and Pogson, 1979; Whitelaw and Williamson, 1977). (Ketone body production by the isolated sheep hepatocytes was similar to in vivo hepatic ketogenesis in the sheep (Lomax et al., 1983a; Katz and Bergman, 1969).) The difference in fatty acid oxidation between rat and sheep liver preparations is surprising in that similar carnitine palmitoyl transferase activities have been reported for rat and sheep liver mitochondria (Snoswell and Henderson, 1970), indicating that factors other than fatty acid transport capacity into the mitochondria are limiting to hepatic fatty acid oxidation in the ruminant.

The catalytic function of CPT and l-carnitine in the transport of FA-CoA across the inner mitochondrial membrane was first proposed by Fritz and Yue (1963). These authors noted that palmitoyl-carnitine stimulated respiration rates of isolated heart muscle mitochondria to a much greater extent than did palmitoyl-CoA, but addition of l-carnitine plus palmitoyl-CoA induced

mitochondrial respiration rates comparable to those obtained with palmitoyl-carnitine. Fritz and Yue (1963) proposed that CPT at the inner mitochondrial membrane catalyzed the formation of palmitoyl-carnitine from palmitoyl-CoA. Palmitoyl-carnitine would then pass through the CoASH-impermeable inner membrane and enter the matrix, where a second CPT would catalyze the reverse reaction, reforming palmitoyl-CoA. This basic concept, use of an acyl-carnitine to penetrate the CoASH-impermeable inner membrane, has remained essentially unchanged, although some details have been modified in light of subsequent research. Carnitine palmitoyl transferase is now known to be confined exclusively to the inner mitochondrial membrane (Brosnan et al., 1973; Hoppel and Tomec, 1972; Kopec and Fritz, 1971). Generation of palmitoyl-carnitine and palmitoyl-CoA occurs via different CPT activities at the outer and inner surfaces, respectively, of the inner mitochondrial membrane (Hoppel, 1982). This proposition is supported by the finding that part of the CPT activity is readily extractable from the mitochondria, while the remaining activity is much more tenaciously bound to the inner membrane, reflecting, perhaps, the outer and inner locations of the enzyme (Hoppel and Tomec, 1972; West et al., 1971). Several systems of nomenclature have been devised to describe the CPT system. In this review, CPT I and CPT II will

refer to the carnitine palmitoyl transferase activity located at the outer and inner surfaces, respectively, of the inner mitochondrial membrane (McGarry and Foster, 1980).

Inner mitochondrial membrane contains a carnitine: acylcarnitine translocase which can facilitate movement of acylcarnitine into the matrix. This enzyme, identified in rat heart (Idell-Wenger, 1981) and liver mitochondria (Parvin and Pande, 1979), catalyzes the exchange of acylcarnitine in the intermembrane space with l-carnitine in the matrix. Carnitine:acylcarnitine translocase activity in rat liver increases during fasting and alloxan-diabetes, conditions associated with increased fatty acid oxidation rates (Parvin and Pande, 1979; Zammit, 1980). Physiological concentrations of short-chain acylcarnitines can inhibit the carnitine:carnitine exchange catalyzed by the translocase (Idell-Wenger, 1981). Both sets of findings support a potential physiological role for carnitine:acylcarnitine translocase in transport of acylcarnitine across the inner mitochondrial membrane. The relative importance of the translocase for the transport of short-, branched- and medium-chain acyl-carnitine in contrast to transport of long-chain acylcarnitine has yet to be established. Hoppel (1982) has suggested that the carnitine:acylcarnitine translocase may be of relatively lesser importance for long-chain

acylcarnitine since CPT I and CPT II are located in close proximity on opposite sides of the inner mitochondrial membrane, a situation not observed for the short- and medium-chain carnitine acyltransferases (Bieber et al., 1982). The current concept of the carnitine palmitoyl transferase system is presented in Figure 3.

In view of the ready reversibility of the carnitine palmitoyl transferase reaction (Bergstrom and Reitz, 1980; Kopec and Fritz, 1971) the following questions often arise: (1) Are CPT I and CPT II identical enzymes simply located at different positions on the inner mitochondrial membrane? (2) Are CPT I and CPT II isozymes, the difference in spatial distribution on the inner mitochondrial membrane reflecting an inherent structural difference between the two activities? To answer these questions, a number of researchers have attempted the isolation and purification of CPT I and CPT II from liver mitochondria of the rat (Hoppel and Tomec, 1972; McGarry et al., 1978b) and the bovine (Brosnan et al., 1973; Edwards, 1977; Kopec and Fritz, 1971, 1973; Norum, 1964; West et al., 1971). Hoppel (1982) reviewed these early investigations and concluded that much of the information was probably untrustworthy, as many of the studies had used conditions during the purification process which are now known to result in the loss of CPT I from the inner membrane, yet still reported results for two carnitine palmitoyl

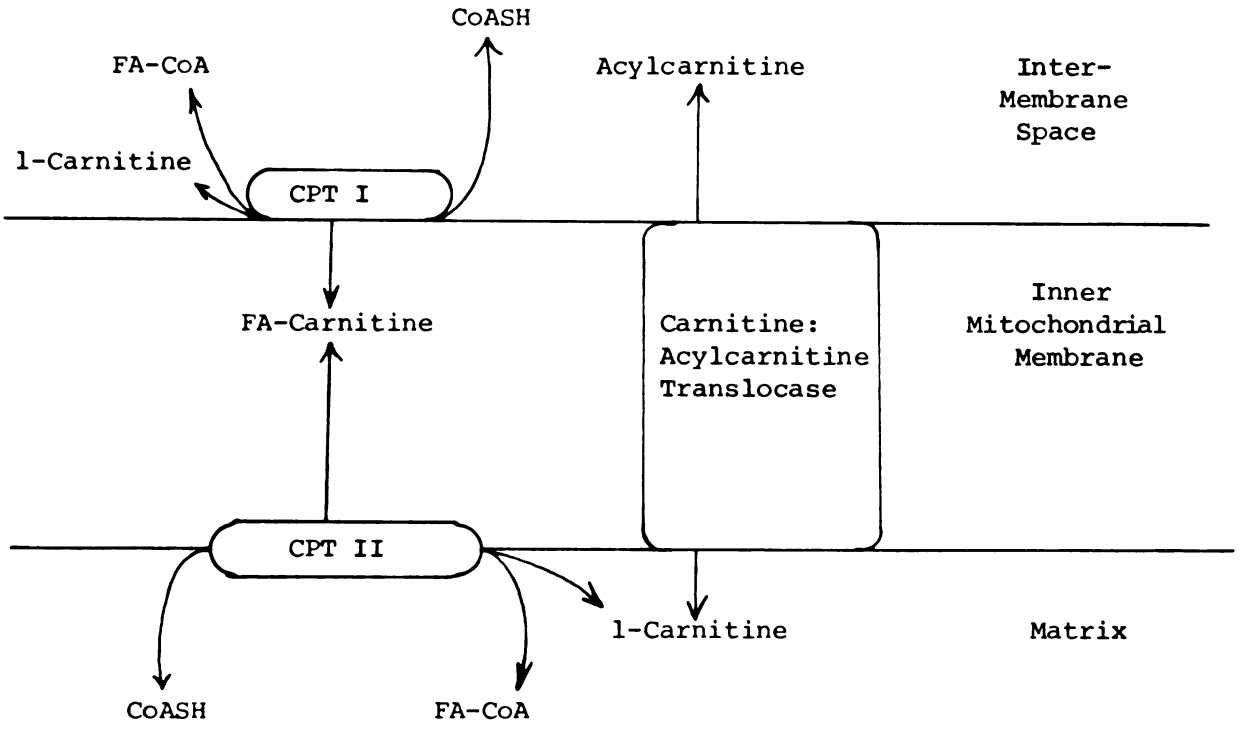


Figure 3.--The carnitine palmitoyl transferase and carnitine:acylcarnitine translocase systems for transport of acylcarnitine across the inner mitochondrial membrane.

Abbreviations are:

FA-CoA	= Long-chain Fatty AcylCoA
FA-Carnitine	= Long-chain Fatty Acylcarnitine
CoASH	= Free Coenzyme A
CPT	= Carnitine Palmitoyl Transferase

transferase activities. A more recent study took great care in the removal of CPT I from the inner membrane of rat liver mitochondria using digitonin (Bergstrom and Reitz, 1980). Subsequently, identical procedures were used for the purification of both CPT I and CPT II. The two enzyme activities displayed remarkable similarity with respect to kinetic parameters, molecular weight, and immunological crossreactivity, suggesting that if CPT I and CPT II are isozymes, they must share a great degree of homology. These researchers also found evidence suggesting the involvement of *in situ* factors within the inner mitochondrial membrane which can cause dramatic changes in catalytic properties of the enzymes. This observation is in agreement with the investigations of McGarry *et al.* (1978b), who found CPT I lost its sensitivity to malonyl-CoA inhibition when removed from the inner membrane.

The research of Bergstrom and Reitz (1980) demonstrated a degree of similarity between CPT I and CPT II of such magnitude that one might almost conclude that the two enzymes were identical. Hoppel (1982), however, discusses information indicating that the two enzymes are distinct polypeptides. Muscle mitochondria isolated from an individual with a lipid storage myopathy could readily oxidize palmitoyl-carnitine, but not palmitoyl-CoA plus l-carnitine, indicating the absence

of CPT I activity (Hofstetler et al., 1978). This disorder apparently had a genetic basis, inferring that CPT I and CPT II were the products of distinct genes, and as such were isozymes rather than identical polypeptides. Alternatively, a genetic defect could alter the interaction between CPT I and the inner membrane, perhaps by altering the unknown in situ factors discussed by Bergstrom and Reitz (1980). At present, available data are unable to distinguish between the identity or unique nature of CPT I and CPT II. If the activities should prove to be isozymes, however, the data of Bergstrom and Reitz (1980) indicate they must share a high degree of homology.

Carnitine palmitoyl transferase I has often been proposed as the rate-limiting, and thus the regulatory, reaction of fatty acid oxidation (e.g. McGarry et al., 1978b; McGarry and Foster, 1980). That CPT I is bound to the inner mitochondrial membrane and facilitates movement of FA-CoA from the intermembrane space, through the otherwise impermeable inner membrane, and into the matrix, provides a certain teleological basis for this argument. Although some researchers have disputed this proposal (Hoppel, 1982), most reports support the regulatory nature of the CPT I reaction for fatty acid oxidation. Shepherd et al. (1966) found that oxygen consumption by isolated rat liver mitochondria was two to three times greater in the presence of palmitoyl-carnitine than it was with

either palmitate or palmitoyl-CoA in the presence of appropriate cofactors. (Palmitate was oxidized as rapidly as palmitoyl-CoA, indicating that palmitoyl-CoA synthetase was not rate-limiting.) Normann et al. (1978) made similar observations on the relative activities of CPT I and CPT II by monitoring changes in the reduction potential of acyl-CoA dehydrogenase flavoproteins in the presence of palmitoyl-carnitine or palmitoyl-CoA plus l-carnitine. In the presence of palmitoyl-carnitine, CPT II activity was two to three orders of magnitude greater than CPT I activity in the presence of palmitoyl-CoA plus l-carnitine. Total carnitine palmitoyl transferase activity has been reported to increase in rat liver during situations associated with high rates of fatty acid oxidation, such as a prolonged fast, diabetes, or consumption of high fat diets (Norum, 1965), providing further support for the regulatory nature of CPT I in fatty acid oxidation.

As the regulatory enzyme of fatty acid oxidation, changes in CPT I activity would lead to changes in the overall rate of fatty acid oxidation. Various factors which could influence CPT I activity have been investigated over the years. Availability of carnitine has been thought to play a regulatory role in CPT I activity. Hepatic carnitine concentrations are increased during periods of active fatty acid oxidation, supporting a regulatory role for carnitine (Brass and Hoppel, 1980b; McGarry et al.,

1975; Pearson and Tubbs, 1967; Snoswell and Henderson, 1970; Snoswell and Koundakjian, 1972). Carnitine addition to liver preparations from fed rats, however, will not increase fatty acid oxidation to rates comparable to those in liver preparations from fasted rats (Christiansen et al., 1976; McGarry and Foster, 1980; McGarry et al., 1975). Compared to the nonlactating rat, markedly higher carnitine concentrations are found in the liver of the lactating rat, yet hepatic fatty acid oxidation rates are lower in the lactating than in the nonlactating rat, whether the comparison is made between fed or fasted animals (Robles-Valdez et al., 1976). Thus, increased carnitine availability, while necessary for increased fatty acid oxidation, is not a sufficient condition. Other factors must be involved in the regulation of fatty acid oxidation.

Carnitine palmitoyl transferase activity displays long-term regulatory properties, and adapts to conditions requiring high rates of fatty acid oxidation (Norum, 1965). Carnitine palmitoyl transferase I also displays short-term regulatory properties. Short-term regulation generally refers to either reversible covalent modification, e.g. phosphorylation/dephosphorylation, of an enzyme, or to noncovalent interaction of small molecules, such as competitive inhibitors or allosteric effectors, with the enzyme. Indirect evidence suggests that CPT I may undergo

covalent modification during short-term regulation. Various researchers have reported that liver preparations from fasted rats displayed an inherently greater capacity for fatty acid oxidation than identical preparations from fed rats (e.g. Christiansen et al., 1976; McGarry and Foster, 1980). Part of this increased capacity could be attributed to an increase in carnitine palmitoyl transferase specific activity in the fasted rats (Norum, 1965). Protein synthesis inhibitors had no effect on this change in carnitine palmitoyl transferase activity, indicating that the increase in activity was due to activation of pre-existing enzyme, and not synthesis of new enzyme protein. The increase in carnitine palmitoyl transferase activity in the fasted rat probably resulted from increased glucagon and decreased insulin concentrations in the circulation, and the consequent decrease in the insulin:glucagon ratio, conditions associated with high rates of hepatic fatty acid oxidation (Newsholme and Start, 1976). The activity of a number of regulatory enzymes in other metabolic pathways is well known to be modified by changes in the insulin:glucagon ratio (Cohen, 1980a), many by a phosphorylation/dephosphorylation mechanism. Recently, Harano et al. (1982) demonstrated that short-term addition of glucagon to primary cultures of hepatocytes from fed rats caused an increase in rates of fatty acid oxidation and ketogenesis. This was accomplished, at least in part, by a 50% reduction in the

Km of CPT I for palmitoyl-CoA, thus leading to increased CPT I activity. Based on the known mode of glucagon action in activating various protein kinases within a cell (Newsholme and Start, 1976), and that covalent modification typically results in changes of substrate Km values of the modified enzyme (e.g. Engstrom, 1980), covalent modification of CPT I, presumably by phosphorylation/dephosphorylation, could certainly be suspected as a control mechanism of CPT I. Alternatively, covalent modification could occur via modification of sulfhydryl groups within the enzyme. Sulfhydryl group modification has recently been recognized as a potential regulatory mechanism, similar to the classical phosphorylation/dephosphorylation mechanism, of pyruvate dehydrogenase kinase (Pettit et al., 1982) and acetyl-CoA hydrolase (Namboodiri et al., 1980). A recent report has implicated sulfhydryl group modification as a possible explanation for the decreased CPT I sensitivity to malonyl-CoA inhibition in liver mitochondria from fasted rats (Zammit, 1983).

Direct, noncovalent interaction of small ligand molecules with enzymes is a second type of short-term regulatory mechanism. Allosteric control, in which a ligand binds to a regulatory site on an enzyme distinct from the active site, has never been reported for CPT I. Competition between substrate and ligand for binding to

the active site resulting in inhibition of enzyme activity, as occurs during classical feedback inhibition, is another mechanism of short-term enzyme regulation. Such a role has been proposed for malonyl-CoA, a competitive inhibitor of palmitoyl-CoA binding to CPT I (McGarry and Foster, 1980; Mills et al., 1983). Malonyl-CoA is the first committed intermediate in fatty acid synthesis, produced by the carboxylation of acetyl-CoA via the enzyme acetyl-CoA carboxylase (Newsholme and Start, 1976). Fatty acid synthesis and oxidation constitute a potential energetically-wasteful futile cycle. Synthesis and oxidation of fatty acids are thus under a reciprocal control mechanism, as occurs with other well-known potential futile cycles, e.g. glycogen synthesis/ glycogenolysis and glycolysis/gluconeogenesis (Newsholme and Start, 1976). Inhibition of fatty acid oxidation by the first committed intermediate of fatty acid synthesis represents an elegant solution to the problem of regulating these opposing metabolic pathways.

Available data strongly support a major role for malonyl-CoA in the regulation of CPT I activity, and thus of fatty acid oxidation, in rat liver. Fatty acid synthesis rate is directly proportional, and FA oxidation rate is inversely related, to hepatic malonyl-CoA concentrations (McGarry et al., 1979). Following a high carbohydrate meal fatty acid synthesis occurs at high rates in rat

liver, while fatty acid oxidation proceeds at high rates during the post-absorptive and fasted states, conditions associated with high and low hepatic malonyl-CoA concentrations, respectively. Malonyl-CoA inhibits fatty acid oxidation by isolated liver mitochondria from fed rats with an I_{50} (inhibitor concentration which produces a 50% inhibition of activity) of about 2 μM, within the range of hepatic malonyl-CoA concentrations found *in vivo* in fed and fasted rats (McGarry and Foster, 1979; McGarry *et al.*, 1978a,b). Malonyl-CoA will not inhibit palmitoyl-carnitine oxidation, indicating that its site of action is the CPT I reaction, rather than CPT II.

Further support for the regulatory role played by malonyl-CoA in fatty acid oxidation is provided by the reports of hormonally-induced changes in hepatic malonyl-CoA concentrations. Insulin administration to hepatocytes isolated from fasted rats stimulates fatty acid synthesis rates and increases cellular malonyl-CoA concentrations, while concomitantly decreasing fatty acid oxidation rates (McGarry and Foster, 1979; McGarry *et al.*, 1978b). Glucagon, on the other hand induces diametrically opposite changes in hepatocytes from fed rats. Thus, malonyl-CoA concentrations in the liver change in the directions required for malonyl-CoA to regulate fatty acid oxidation. Some investigators have questioned the importance of malonyl-CoA as a regulatory

factor (e.g. Brass and Hoppel, 1978), but the bulk of currently available data strongly supports a regulatory role for malonyl-CoA in fatty acid oxidation (McGarry and Foster, 1980). At the other extreme, McGarry and Foster have often minimized the importance of other factors, e.g. activation of CPT I (Harano et al., 1982) and carnitine:acylcarnitine transferase (Parsin and Pande, 1979), in the regulation of fatty acid oxidation. Ultimately, all of these factors may be important for fine-tuning hepatic fatty acid oxidation rates.

Some controversy has surrounded determination of an exact I_{50} value for malonyl-CoA in the CPT I reaction. Following the initial report of a 2 μM I_{50} for malonyl-CoA (McGarry and Foster, 1978b), other investigators reported malonyl-CoA I_{50} values ranging from 20 μM to more than 100 μM for the inhibition of mitochondrial fatty acid oxidation (Cook et al., 1980; Ontko and Johns, 1980). These high values are well above physiological concentrations of malonyl-CoA and were used as a partial argument refuting the regulatory role of malonyl-CoA. McGarry and Foster (1981) gave methodological reasons to reject these reported high values. High mitochondrial protein concentrations (>10 mg/assay) and relatively long incubation periods (10-20 minutes) may have resulted in the loss of malonyl-CoA during the incubation, since mitochondria contain a specific malonyl-CoA decarboxylase (Kim and

Kolattukudy, 1978), and malonyl-CoA itself may undergo spontaneous decarboxylation. The result would be artificially high I_{50} values for malonyl-CoA.

An observation initially overlooked in the controversy surrounding the absolute I_{50} value of malonyl-CoA was that CPT I in liver mitochondria from fed rats was more sensitive to malonyl-CoA inhibition, i.e. had a lower malonyl-CoA I_{50}, than were liver mitochondria from fasted rats (Cook et al., 1980; Saggerson and Carpenter, 1981a,b; Ontko and Johns, 1980). A certain metabolic logic is implicit in these results, in that CPT I is more sensitive to malonyl-CoA inhibition during the fed state, when fatty acid oxidation should be minimal, than during the fasted state. (Thyroxine treatment of rats appears to have a similar effect to fasting, and somewhat decreases hepatic mitochondrial CPT I sensitivity to malonyl-CoA inhibition (Stakkestad and Bremer, 1982).) A strong inverse relationship was found between hepatic malonyl-CoA concentration and the malonyl-CoA I_{50} for CPT I, i.e. higher malonyl-CoA concentrations were associated with lower I_{50} values (Robinson and Zammit, 1982). Recently published evidence suggests that modification of sulfhydryl groups within the enzyme may be responsible for the variation in malonyl-CoA I_{50} values observed between liver mitochondria from fed and fasted rats. 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) can

prevent malonyl-CoA induced inhibition of rat liver mitochondrial CPT I (Saggerson and Carpenter, 1982a). This densensitization of CPT I to malonyl-CoA inhibition can be reversed by incubating DTNB-treated mitochondria with near-physiological concentrations of malonyl-CoA prior to assay for CPT I activity (Zammit, 1983). As discussed earlier in this section, modification of sulfhydryl groups could regulate metabolism in a manner analogous to phosphorylation/dephosphorylation control. Whatever the mechanism by which sensitivity of mitochondrial CPT I to malonyl-CoA inhibition is altered, the result appears to allow changing malonyl-CoA concentrations to more precisely modulate CPT I activity in response to changing environmental conditions. One can speculate that this sulfhydryl group effect may be related to the loss of sensitivity to malonyl-CoA inhibition observed when CPT I is removed from the inner mitochondrial membrane (McGarry et al., 1978b).

Malonyl-CoA inhibition of hepatic CPT I subsequently led to investigations of the ability of malonyl-CoA to inhibit mitochondrial CPT I from other rat tissues. Malonyl-CoA was found to be a potent inhibitor of mitochondrial CPT I in lactating mammary gland, white and brown adipose tissue, kidney cortex, and heart and skeletal muscle (Mills et al., 1983; Saggerson, 1982; Saggerson and Carpenter, 1981a, 1982b, 1983). These

results are surprising since none of these tissues exhibit both fatty acid oxidative and synthetic capacities to the degree displayed by liver. An inverse relationship was noted across tissues between mitochondrial CPT I sensitivity to malonyl-CoA inhibition and the l-carnitine Km in the CPT I reaction, although the palmitoyl-CoA Km was essentially unchanged across tissues (McGarry et al., 1983). The physiological significance of CPT I sensitivity to malonyl-CoA inhibition in extrahepatic tissues is currently unknown, but malonyl-CoA I_{50} values for these tissues is within the range of malonyl-CoA concentrations which have been observed in these tissues (McGarry et al., 1983), suggesting that a regulatory role for malonyl-CoA in extrahepatic tissues may also exist.

Other short-chain acyl-CoA compounds have also been examined for their potential to inhibit CPT I activity. In the original report of McGarry et al. (1978b) only malonyl-CoA produced significant inhibition of CPT I. Subsequent experiments (Mills et al., 1983) demonstrated that other short-chain acyl-CoA derivatives (propionyl-CoA, methylmalonyl-CoA, succinyl-CoA, acetyl-CoA, and CoASH) could also inhibit CPT I activity, albeit to a lesser degree than malonyl-CoA. These compounds were least effective with CPT I in rat liver mitochondria, but heart and skeletal muscle mitochondria proved much more sensitive to inhibition by some of these compounds,

methylmalonyl-CoA and succinyl-CoA proving nearly as effective as malonyl-CoA. Again, the significance of these effects *in vivo* is unknown, but these findings suggest that regulation of CPT I may be much more complex than previously expected.

Except for a single report that malonyl-CoA was an ineffective inhibitor of bovine hepatic CPT I (Edwards and Edwards, 1981), no work has apparently addressed this area of the regulation of fatty acid oxidation in ruminant species. This report is probably invalid, however, since these investigators used a partially purified preparation of beef liver CPT I to assay for malonyl-CoA induced inhibition, a situation known to result in loss of CPT I sensitivity to malonyl-CoA inhibition (McGarry *et al.*, 1978b). Thus, the question of malonyl-CoA regulation of ruminant CPT I and fatty acid oxidation remains to be answered.

Carnitine Metabolism

Except for its catalytic role in mitochondrial fatty acid oxidation, this discussion has ignored carnitine metabolism. The precursor of l-(-)-carnitine biosynthesis is trimethyllysine (TML). Mammalian systems apparently do not contain enzymes for methylation of free lysine, but methylate lysine post-translationally, i.e. after incorporation into protein. Thus, TML in mammals is

derived from proteolysis during the course of daily protein turnover (Broquist, 1982). This seemingly roundabout pathway for producing the first committed intermediate of carnitine biosynthesis is thought to allow carnitine biosynthesis to take advantage of the continuity of protein turnover, and the consequent continuous supply of TML (Rebouche, 1982).

The pathway of carnitine biosynthesis is illustrated in Figure 4. Trimethyllysine is converted to carnitine by four different enzymatic reactions: hydroxylation to yield β-hydroxy-TML, aldolytic cleavage to yield γ-butyrobetaine aldehyde with concomitant release of glycine, dehydrogenation to form γ-butyrobetaine, and a second hydroxylation yielding the final product, l-(-)-carnitine (Broquist, 1982; Henderson et al., 1982). The enzymes responsible for TML conversion to -butyrobetaine can apparently be found in all tissues studied to date, at least in the rat and human (Rebouche, 1982). γ-Butyrobetaine hydroxylase, the enzyme responsible for carnitine synthesis from γ-butyrobetaine, is found in the liver, but displays some species specificity with respect to distribution among other tissues (Rebouche, 1982). Tissues which contain γ-butyrobetaine hydroxylase can synthesize carnitine, but when the hydroxylase activity is absent, γ-butyrobetaine is exported to the liver for carnitine production. Kidney participates in a unique manner in

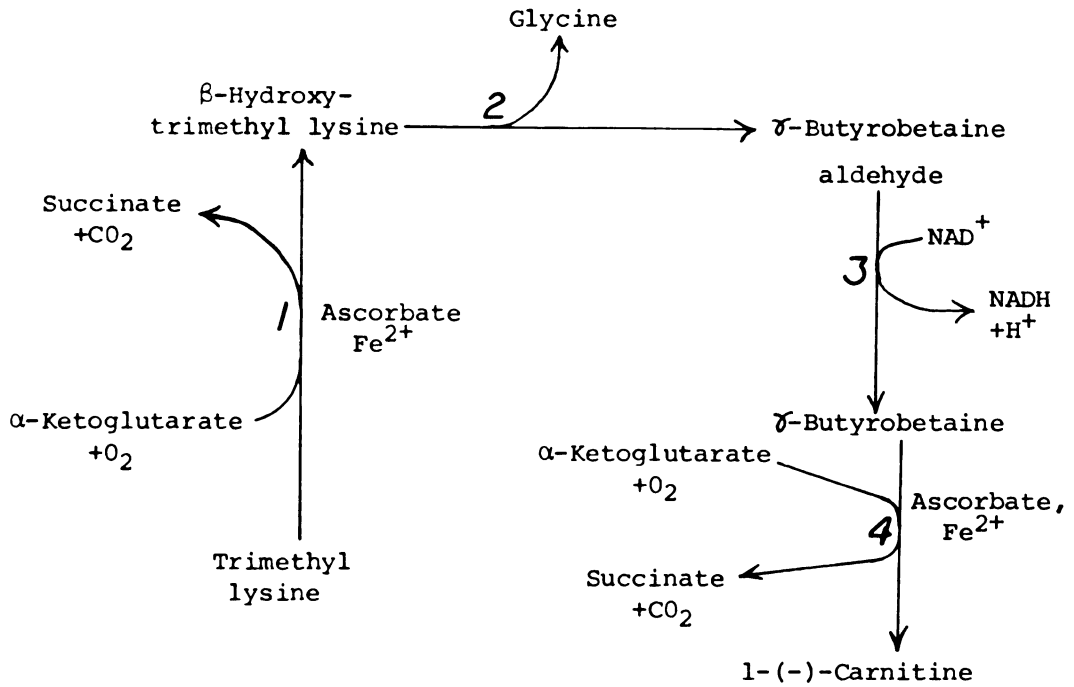


Figure 4.--The pathway of l-(-)-carnitine biosynthesis.

Enzymes are:

1) Trimethyllysine hydroxylase
2) β-Hydroxy-trimethyllysine aldolase
3) γ-Butyrobetaine aldehyde dehydrogenase
4) γ-Butyrobetaine hydroxylase

carnitine metabolism by scavenging TML from the circulation and, depending upon the species-specific presence or absence of γ-butyrobetaine hydroxylase, releasing γ-butyrobetaine or carnitine back into the circulation (Broquist, 1982; Rebouche, 1982). Bovine liver and kidney reportedly contain all the enzymes required for carnitine biosynthesis (Kondo et al., 1981).

Little information is currently available concerning the regulation of carnitine biosynthesis (Broquist, 1982). Since TML is continuously produced by a totally unrelated mechanism, any potential regulatory mechanism of carnitine synthesis would have to be effective within the biosynthetic pathway between TML and carnitine. Feedback inhibition by carnitine on its synthesis is apparently not involved, since dietary supplementation with carnitine had no effect on rates of carnitine synthesis in rats, despite increased urinary carnitine excretion (Rebouche, 1982). Some evidence is available suggesting that carnitine biosynthesis may be developmentally regulated. Hepatic carnitine synthesis is higher in weanling and adult rats than in fetal or neonatal rats (Robles-Valdez et al., 1976). Hormonal regulation of carnitine biosynthesis has not been extensively studied. Only thyroxine has been demonstrated to directly effect carnitine synthesis (Rebouche, 1982; Pande and Parvin, 1980). Dietary thyroxine administration increased hepatic γ-butyrobetaine hydroxylase activity

nearly two-fold. Hepatic carnitine content can change, however, in response to changing metabolic conditions, perhaps reflecting the changing hormonal milieu. Fasting or alloxan-diabetes, conditions associated with a low insulin:glucagon ratio and concomitantly high FFA concentrations and rates of fatty acid oxidation, induce large increases in both the total hepatic carnitine concentration and the proportion of acyl-carnitine (Brass and Hoppel, 1980b; McGarry et al., 1975; Pearson and Tubbs, 1967; Snoswell and Henderson, 1970; Snoswell and Koundakjian, 1972). On the other hand, inhibition of fatty acid oxidation in neonatal rats by oral administration of tetradecylglycidic acid caused a marked increase in total hepatic carnitine, primarily as free carnitine, with no apparent change in circulating hormones (Frost and Wells, 1982). At present the cause of this increased hepatic carnitine content is unknown. The additional carnitine could have resulted from either increased hepatic synthesis, or increased hepatic uptake of carnitine from the blood. One suggestive observation from these studies is that hepatic carnitine content increased during periods when fatty acid and FA-CoA concentrations within the liver would also have been high, indicating that increased intracellular fatty acid concentrations could have triggered whatever mechanism was responsible for increasing carnitine concentrations. Further research

is required to obtain a complete understanding of the mechanisms regulating carnitine biosynthesis.

A number of tissues including liver often contain, in addition to long-chain acyl-carnitine, large quantities of short-, medium- and branched-chain acyl-carnitines, produced from the acyl-CoA derivatives by carnitine acyltransferases (CAT) with appropriate chain-length specificities (Bieber et al., 1982). Rat liver CATs have been the most extensively characterized, but carnitine acetyltransferase and carnitine octanoyltransferase have been reported in both ovine and bovine liver (Bieber et al., 1982; Snoswell and Henderson, 1970; Snoswell and Koundakjian, 1972). These short- and medium-chain CATs have a wide distribution within the liver cells, and have been found in the peroxisomes and the endoplasmic reticulum, as well as in the mitochondria (Bieber et al., 1982; Miyazawa et al., 1983). The discovery of these short- and medium-chain acyl-carnitines and CATs in turn revealed a number of potential roles for carnitine in metabolism besides its traditional use to facilitate long-chain fatty acid oxidation.

Carnitine appears to be necessary to shuttle the chain-shortened products of peroxisomal β-oxidation from the peroxisomes into the mitochondria for complete oxidation (Bieber et al., 1982; Tolbert, 1981). During periods of intense fatty acid oxidation, acetyl-CoA can be

generated so rapidly within the matrix that CoASH could potentially become limiting to β-oxidation and other CoASH-requiring reactions in the mitochondrial matrix. Carnitine "buffers" the mitochondrial CoASH-pool against these drastic changes by participating in the conversion of acetyl-CoA to acetyl-carnitine, with the concomitant release of CoASH (Bieber et al., 1982; Brass and Hoppel, 1980a,b; Pearson and Tubbs, 1967; Snoswell and Henderson, 1970; Snoswell and Koundakjian, 1972). Additional roles for carnitine have been postulated in branched-chain amino acid metabolism and certain biosynthetic reactions (Bieber et al., 1982), but these have not been as widely investigated.

Of these potential sites for carnitine utilization, only the ability of carnitine to buffer the mitochondrial CoASH-pool has been extensively investigated in the ruminant. Sheep liver behaves in much the same way as rat liver with respect to acetyl-carnitine formation (Snoswell and Henderson, 1970; Snoswell and Koundakjian, 1972). Bovine liver, however, functioned differently. When liver from lactating cows was compared with that from nonlactating cows, the hepatic content of total acid-soluble (i.e. short-chain) carnitine was actually lower in liver from the lactating cows despite a markedly higher acetyl-CoA/CoASH ratio (Snoswell et al., 1978). Further study will be needed to clarify species

differences and the extent to which carnitine participates in these alternate areas of metabolism.

Peroxisomal Fatty Acid Oxidation

Peroxisomes are small, membrane-bound vesicles formed as buds from the smooth endoplasmic reticulum and found in the cytoplasm of nearly all tissues (Tolbert, 1981). Peroxisomes contain a number of metabolic pathways which are near duplicates of pathways found elsewhere in the cell. The existence of a peroxisomal system for β-oxidation of long-chain fatty acid is a relatively recent discovery (Tolbert, 1981). This system has been studied the most extensively in rat liver, although the peroxisomes of many tissues in many species may at times demonstrate the capacity to oxidize long-chain fatty acids. Peroxisomal β-oxidation has been estimated to potentially account for as much as 10% of the total hepatic fatty acid oxidative capacity (Mannaerts et al., 1979).

Aside from the obvious difference in intracellular location, peroxisomal β-oxidation differs from its mitochondrial counterpart in several respects. Peroxisomal β-oxidation is catalyzed by enzymes different from those within the mitochondria (Tolbert, 1981; Hayashi, 1981). The initial reaction is catalyzed by a flavin-linked acyl-CoA oxidase (instead of a dehydrogenase). The products of this reaction are hydrogen peroxide and the

α,β-unsaturated FA-CoA. The two subsequent reactions appear to be catalyzed by the same bifunctional polypeptide, in contrast to the separate enzymatic activities of mitochondria. These activities catalyze the formation of the β-hydroxy-FA-CoA, and the subsequent NAD^+-dependent dehydrogenation to β-keto-FA-CoA. Finally, a thiolase catalyzes release of acetyl-CoA and a chain-shortened FA-CoA which is available for another cycle of β-oxidation. The peroxisomal thiolase and acyl-CoA oxidase reactions are specific for FA-CoA of greater than C_8 chain-length, so that only long-chain fatty acids undergo peroxisomal β-oxidation (Tolbert, 1981; Inestrosa et al., 1979). Acetyl-CoA, produced during each spiral of β-oxidation, and octanoyl-CoA, which can not be oxidized further through peroxisomal β-oxidation, are converted to the corresponding acyl-carnitine esters by peroxisomal CATs (Bieber, 1982; Tolbert, 1981). Acetyl-carnitine and octanoyl-carnitine may shuttle to the mitochondria for complete oxidation, or may be exported from the liver for use by extrahepatic tissues or urinary excretion.

Long-chain FA-CoA, the substrate for peroxisomal β-oxidation, can enter apparently intact peroxisomes via a carnitine-independent transport mechanism (Osmundsen, 1982). (Peroxisomal β-oxidation is not, however, totally independent of carnitine, since the final products formed are acyl-carnitine derivatives.) Peroxisomes also contain

a long-chain acyl-CoA synthetase, apparently located on the cytoplasmic face of the peroxisome and representing 6-7% of the total hepatic long-chain acyl-CoA synthetase capacity (Krisans et al., 1980; Mannaerts et al.,1982), as well as a significant (~16%) proportion of the total cellular coenzyme A (van Broekhoven et al., 1981). Peroxisomal CoASH is available for the thiolase reaction, but is not accessible to the acyl-CoA synthetase reaction (Mannaerts et al., 1982), indicating that cytoplasmic CoASH is required for peroxisomal acyl-CoA formation. The absolute activity of the peroxisomal acyl-CoA synthetase has been estimated to provide sufficient long-chain FA-CoA to maintain maximal peroxisomal β-oxidation rates (Krisans et al., 1980), although fatty acid availability within the liver may dictate the relative importance of peroxisomally-produced acyl-CoA versus acyl-CoA synthesized at other locations within the cell.

Peroxisomes do not contain an electron transport chain. Oxidized nicotinamide adenine dinucleotide can, however, be regenerated by several other reactions within the peroxisomes which can substitute for the absent electron transport system, e.g. G3P dehydrogenase. Also, addition of α-keto acids, such as oxaloacetate and pyruvate, to in vitro incubations can accelerate peroxisomal β-oxidation, apparently by allowing regeneration of peroxisomal NAD^+ (Osmundsen, 1982; Osmundsen and

Neat, 1979). The lack of an electron transport chain allows rat liver peroxisomal β-oxidation to proceed under conditions which totally inhibit mitochondrial β-oxidation, e.g. the presence of cyanide (Inestrosa et al., 1979; Tolbert, 1981). This characteristic displays some species specificity, because chicken liver peroxisomal β-oxidation is sensitive to cyanide inhibition (Ishii et al., 1983), although factors causing this species difference are currently unknown. (Sufficient amounts of cyanide can inhibit rat liver peroxisomal catalase, the enzyme responsible for hydrogen peroxide degradation, leading to hydrogen peroxide buildup within the peroxisome and eventual inhibition of peroxisomal β-oxidation.) Whereas the peroxisomal coenzyme A-pool is separate and distinct from the remaining cellular coenzyme A, peroxisomal nicotinamide adenine dinucleotides may be able to traverse the peroxisomal membrane (Mannaerts et al., 1982). Regeneration of NAD^+ may not necessarily have to occur within the peroxisomes.

Rat liver peroxisomes rapidly proliferate in response to dietary administration of hypolipidemic agents such as clofibrate or di-(2-ethylhexyl)-phthalate (Mannaerts et al., 1979; Miyazawa et al., 1980). Peroxisomal β-oxidation enzyme activities increased markedly in conjunction with peroxisomal proliferation. Feeding high fat diets (30%) can also lead to peroxisomal

proliferation and increased activity of peroxisomal β-oxidation enzymes (Ishii et al., 1980). These findings suggest a role for peroxisomal β-oxidation in hepatic, and perhaps other organ, fatty acid metabolism. Information concerning possible mechanisms for regulation of peroxisomal β-oxidation is sparse. Tolbert (1981) has suggested that hormonal factors may be involved, since male and female rats differ both in the hepatic content of peroxisomal enzymes and in the response of those enzymes to clofibrate administration. The CoASH/acyl-CoA ratio may be an important factor in the regulation of peroxisomal β-oxidation (Osmundsen, 1982; Osmundsen and Neat, 1979). These researchers found that CoASH concentrations as low as 100-200 μM markedly inhibited palmitoyl-CoA and myristoyl-CoA oxidation by seemingly intact peroxisomes, but had little effect on elaidoyl-CoA or erucoyl-CoA oxidation. The latter two fatty acids are poorly metabolized by mitochondria, and the suggestion was made that these acids would typically be oxidized in the peroxisomes, while the more common long-chain fatty acids, such as palmitic, oleic and myristic acids, would only undergo peroxisomal β-oxidation during periods of high FA-CoA and low CoASH availability, as occurs during fasting or high fat feeding (Osmundsen, 1982). In this view, peroxisomal β-oxidation would primarily represent a relief valve, oxidizing typical fatty acids during periods when fatty

acid availability exceeds mitochondrial β-oxidation capacity. Further research will be needed to clarify the role of peroxisomal β-oxidation in overall hepatic fatty acid metabolism.

No reports are currently available which have examined peroxisomal β-oxidation in ruminant liver. Initial investigations could follow three lines of research, determining whether (1) ruminant hepatic peroxisomes are capable of β-oxidation, changes in activity (2) during the peripartum period when the liver generally becomes infiltrated with fat, and (3) during peak lactation, and perhaps lactational ketosis, periods when fatty acids are actively mobilized from adipose tissue.

Ketogenesis

One aspect of fatty acid metabolism which has received much research attention is ketogenesis, the production of β-hydroxybutyrate (BHBA) and acetoacetate (AcAc) using acetyl-CoA derived from the β-oxidation of fatty acid. Ketogenic rates are proportional to fatty acid oxidation rates, and are thus regulated by the same factors which regulate fatty acid oxidation. (Unless referring specifically to CO_2 production, the term "fatty acid oxidation" will be used to describe collectively both CO_2 and ketone production from fatty acid.)

Generally, high ketogenic rates are associated with high gluconeogenic rates, as occurs during fasting or diabetic ketoacidosis (Ferré et al., 1981; Flatt, 1972; Newsholme and Start, 1976). Ketones can serve as an alternate energy source to glucose in extrahepatic tissues during these times of glucose scarcity, sparing both glucose, for use by tissues with a near-absolute demand for glucose, and body protein, by reducing demand for glucogenic amino acids. Ketogenesis by ruminant liver occurs even during the fed state (Baird et al., 1977, 1979; Connolly et al., 1964; Yamdagni and Schultz, 1969) is a logical result of the continuous requirement for hepatic gluconeogenesis.

Although ketone bodies have occasionally been referred to as the partial oxidation products of fatty acid, ketogenesis is in reality a biosynthetic process utilizing acetyl-CoA derived primarily from β-oxidation. The major route of ketone production is the β-hydroxy, β-methyl-glutaryl-CoA (HMG-CoA) pathway (Figure 5). Two molecules of acetyl-CoA condense to form acetoacetyl-CoA (AcAc CoA), with the liberation of CoASH, the reaction catalyzed by acetyl-CoA acetyltransferase (acetoacetyl-CoA thiolase). A third acetyl-CoA can be added to AcAc CoA by HMG-CoA synthase in an irreversible reaction to yield HMG-CoA, again liberating CoASH. The following reaction, cleavage of HMG-CoA to AcAc and acetyl-CoA, is catalyzed by HMG-CoA lyase, another irreversible reaction. These

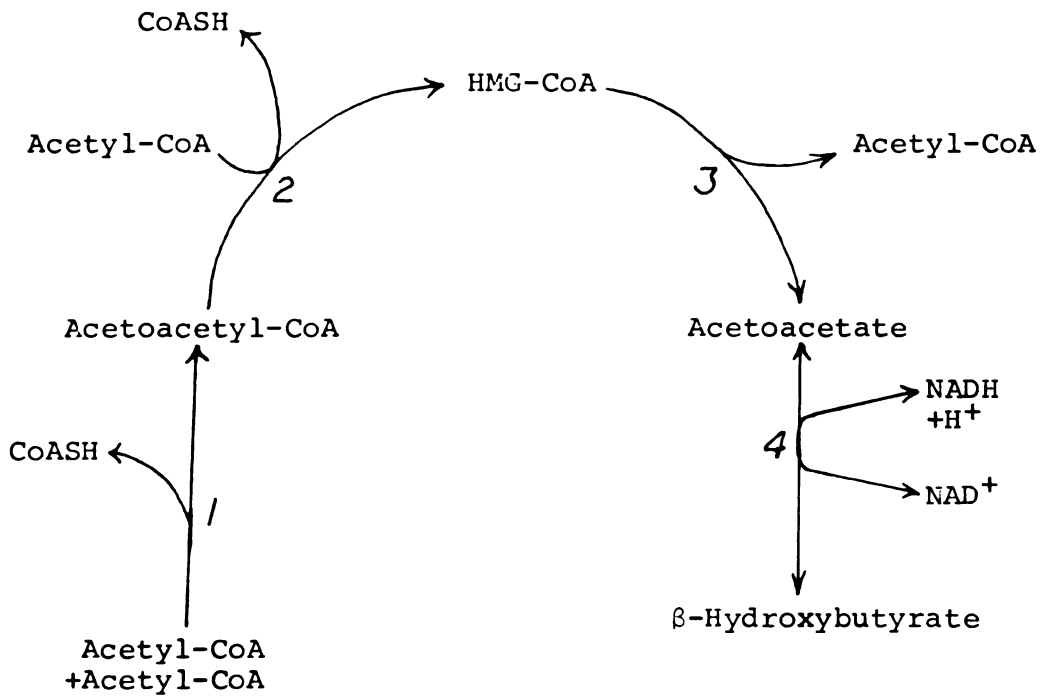


Figure 5.--The β-hydroxy, β-methyl-glutaryl-CoA pathway of ketogenesis.

The enzymes of acetoacetate and β-hydroxybutyrate formation are:

1) Acetyl-CoA acetyltransferase (Acetoacetyl-CoA thiolase)
2) HMG-CoA synthase
3) HMG-CoA lyase
4) β-Hydroxybutyrate dehydrogenase

HMG-CoA = β-hydroxy, β-methyl-glutaryl-CoA

three enzymes are found primarily within the mitochondrial matrix of rat and bovine liver (Baird et al., 1970; Mulder and van den Bergh, 1981; Williamson et al., 1968). The activity of these enzymes is of comparable magnitude between rat and bovine liver. (Some HMG-CoA synthase activity can be found in liver cytoplasm of both species, but appears to be involved with cholesterol synthesis rather than ketogenesis (Lopes-Cardozo et al., 1975).) Acetoacetate can also be produced via direct deacylation of AcAc CoA. Previous research has indicated that AcAc CoA deacylase is present in the liver at only 20% or less of the activity of the HMG-CoA pathway (Newsholme and Start, 1976; Williamson et al., 1968), and more recent data suggests that this pathway takes essentially no part in hepatic ketogenesis (Brady et al., 1982).

β-Hydroxybutyrate dehydrogenase catalyzes the reversible dehydrogenation of AcAc to BHBA. This enzyme is not actually a part of the HMG-CoA pathway, but plays an important role in determining the relative amounts of AcAc and BHBA produced during ketogenesis. Essentially all BHBA dehydrogenase activity in rat liver is confined to the mitochondrial matrix (Koundakjian and Snoswell, 1970), whereas BHBA dehydrogenase is essentially a cytoplasmic enzyme in ruminant liver (Koundakjian and Snoswell, 1970; Watson and Lindsay, 1972). (Ballard et al. (1969) attempted to estimate the mitochondrial content of

various metabolites using the procedure of Krebs and Veech (1969), which assumes that BHBA dehydrogenase is confined to the mitochondrial matrix. That assumption is not valid in ruminant liver, thus the results of Ballard *et al.* (1969) are not valid.) Ruminant liver contains much less BHBA dehydrogenase activity than does rat liver (Koundakjian and Snoswell, 1970). Some investigators have suggested that these low reported values for ruminant hepatic BHBA dehydrogenase activity can not account for observed *in vivo* hepatic BHBA production rates (Bell, 1980). Watson and Lindsay (1972) have concluded otherwise, based on a comparison between BHBA dehydrogenase activity which they measured in sheep liver and *in vivo* BHBA production by sheep liver calculated from data reported by Katz and Bergman (1969).

Rumen epithelium also contains an active HMG-CoA pathway for ketone production (Baird *et al.*, 1970; Bush and Milligan, 1971), which in the fed ruminant can account for over half of the total ketone production (Baird *et al.*, 1979). In contrast to hepatic ketogenesis, rumen ketogenesis utilizes primarily butyrate from the rumen fermentation as a source of acetyl-CoA rather than long-chain fatty acid (Ramsey and Davis, 1965). Because of the substrate utilized, rumen ketogenesis is highest in the fed animal, and all but disappears in the fasted ruminant (Baird *et al.*, 1979; Katz and Bergman, 1969).

β-Hydroxybutyrate dehydrogenase activity is also much higher in rumen epithelium than in liver (Koundakjian and Snoswell, 1970). The decreased BHBA:AcAc ratio found in the blood of fasted versus fed ruminants primarily reflects the decreased importance of ruminal ketogenesis in the fasted animal (e.g. Baird et al., 1979), rather than a shift in the reduction potential of the liver.

Mammalian ketosis can be classified into two general categories: physiological and pathological ketosis (Krebs, 1970; Lopes-Cardozo et al., 1975). During physiological ketosis production of ketone bodies equals their rate of utilization. Such a state exists in a fed ruminant, or in a fasted animal (ruminant or nonruminant). In contrast, ketone production far exceeds utilization in a pathological state of ketosis. Blood ketone concentrations can exceed 2 mM, and unused ketones are excreted in the urine. Diabetic ketoacidosis, bovine lactational ketosis and ovine pregnancy toxemia are all examples of pathological ketosis. Mechanisms regulating the onset and rate of ketogenesis, and the transition from physiological to pathological ketosis, have been investigated for several decades, but a complete understanding of these ketogenic regulatory mechanisms is still not available. One aspect of ketogenic regulation, modulation of HMG-CoA pathway activity, has received relatively little attention. This lack of research has

been attributed to the difficulty involved in characterizing the enzymes of the HMG-CoA pathway, since these enzymes are relatively unstable in semi-purified form (Menahan et al., 1981). Most investigators generally agree, however, that the HMG-CoA pathway is not subject to long-term regulation, i.e. the total amount of enzyme protein does not change with changes in metabolic state (Baird et al., 1970; Menahan et al., 1981; Menke and Huth, 1980; Watson and Lindsay, 1972; Williamson et al., 1968).

More recently, modulation of HMG-CoA pathway enzyme activity via short-term regulation has been suggested to be involved in ketogenic regulation. Acetyl-CoA acetyltransferase reportedly exists within rat liver in two different forms exhibiting different activities (Menke and Huth, 1980). Whether or not the relative amounts of these two forms of acetyl-CoA acetyltransferase change during transitions between metabolic states is currently the subject of debate (Menke and Huth, 1980; Huth and Menke, 1982), so that the potential role these enzyme forms may play in ketogenic regulation is unknown. Acetyl-CoA acetyltransferase activity can be modulated by changes in the mitochondrial matrix acetyl-CoA:CoASH ratio (Menke and Huth, 1980). This characteristic allows acetyl-CoA acetyltransferase activity to increase in response to increases in acetyl-CoA:CoASH ratio, as would occur during active β-oxidation (Menahan et al., 1981).

Modulation of HMG-CoA synthase activity has also been examined for its potential in ketogenic regulation. Increases in acetyl-CoA concentration produce increases in HMG-CoA synthase activity (Menahan et al., 1981). Surprisingly, AcAc CoA concentrations within the physiological range found in rat liver (1-10 μM) can inhibit HMG-CoA synthase (K_I for AcAc CoA=6-10 μM; Menahan et al., 1981). These researchers found that AcAc CoA concentrations within the mitochondria actually decreased during ketoacidosis, however, relieving HMG-CoA synthase inhibition and allowing a marked increase in ketogenesis. Further investigations will be required to fully delineate the extent to which modulation of enzyme activity can effect ketogenic rate through the HMG-CoA pathway.

Traditionally, research into ketogenic regulation has focused on the partitioning of acetyl-CoA between ketogenesis and the TCA cycle (Krebs, 1970). Availability of oxaloacetate (OAA) and rate of the citrate synthase reaction would, in this view, determine the relative partitioning of acetyl-CoA between these two fates. The greater the rate at which acetyl-CoA is incorporated into the TCA cycle, the lower the ketogenic rate. The importance of OAA availability as an absolute determinant of ketogenic rate is now being questioned (Lopes-Cardozo et al., 1975; McGarry and Foster, 1980). Although oxaloacetate concentrations can decrease in ketotic liver, be it ruminant or

nonruminant, the decrease may actually be in response to, rather than the cause of, increased fatty acid oxidation rates (Siess et al., 1982). Increased fatty acid oxidation rates will increase the mitochondrial matrix NADH/NAD ratio, resulting in a shift in malate dehydrogenase equilibrium away from OAA. At a given rate of β-oxidation and acetyl-CoA production, however, ketogenesis can be decreased in response to increases in mitochondrial OAA availability, within the limits of citrate synthase activity (Christiansen, 1979; Nosadini et al., 1980; O'Donnell and Freedland, 1980). The concensus currently forming is that rate of fatty acid oxidation and acetyl-CoA production is the major factor determining ketogenic rate within the liver (Lopes-Cardozo et al., 1975; McGarry and Foster, 1980; Williamson, 1979), although other factors, such as OAA availability or the presence of competitive oxidative substrates, could be involved in fine-tuning ketogenic rates. The factors discussed with regard to regulation of fatty acid oxidation, e.g. fatty acid availability, CPT I activity, and malonyl-CoA concentrations, would also regulate ketogenesis. In this more contemporary view, ketogenesis represents an overflow of acetyl-CoA from the TCA cycle, and would be stimulated when acetyl-CoA production exceeds the capacity for citrate synthesis, due either to limited OAA availability

or exceeding the maximum citrate synthase activity (Lopes-Cardozo et al., 1975).

An early observation in metabolic regulation was the association between hepatic ketogenesis and gluconeogenesis during periods of carbohydrate insufficiency, such as starvation, diabetes or consumption of a high fat/low carbohydrate diet. A large number of investigations, conducted primarily in the rat, have examined the relationship between hepatic ketogenesis and gluconeogenesis, and have concluded that at least a minimal ketogenic rate is required in order for maximal gluconeogenic rates to occur. (The converse, i.e. a minimal gluconeogenesis for maximal ketogenesis, also appears to be valid (Flatt, 1972).) This conclusion is based on a number of observations in both the rat and other species. Addition of long-chain fatty acids to the perfusate of livers isolated from 48 hour starved rats increased not only ketogenic, but also gluconeogenic rates (Söling and Kleineke, 1976). Similar observations were made with hepatocytes isolated from one day old rats (Ferré et al., 1981). Liver glycogen stores in neonatal rats are rapidly depleted, so that an active hepatic gluconeogenesis is established soon after birth to maintain blood glucose. Hepatic ketogenesis increases in conjunction with the establishment of gluconeogenesis. When oleic or octanoic acids were added to hepatocytes isolated from

one day old rats, gluconeogenic rates from lactate plus pyruvate increased in parallel with increasing ketogenic rates. (Maximum gluconeogenic rates in response to the added fatty acid were attained before maximal ketogenic rates were achieved.) In contrast to the effect of added fatty acid, inhibition of fatty acid oxidation by decanoyl-(+)-carnitine decreased both ketogenesis and gluconeogenesis by hepatocytes isolated from 48 hour fasted rats (Söling and Kleineke, 1976). These investigations were extended *in vivo* to the neonatal rat. Administration of pent-4-enoate, a fatty acid oxidation inhibitor, to suckling newborn rats induced a decrease in blood concentrations of not only ketones, but also of glucose (Pégorier *et al.*, 1977). Since glucose utilization was found to be unaffected, it was concluded that glucose production had decreased. A two-fold mechanism has been suggested for these effects of fatty acid oxidation and ketogenesis on gluconeogenesis in rat liver involving, first, activation of pyruvate carboxylase within the mitochondrial matrix in response to increased acetyl-CoA concentrations, and second, an increase in the cytoplasmic NADH/NAD ratio, shifting the glyceraldehyde-3-phosphate dehydrogenase equilibrium towards gluconeogenesis (Ferré *et al.*, 1979).

Phosphoenolpyruvate carboxykinase (PEPCK) is a predominantly cytoplasmic enzyme in rat liver (Söling and

Kleineke, 1976). In most other species, including ruminants, PEPCK is more evenly distributed between the cytoplasm and mitochondria (Söling and Kleineke, 1976; Ballard et al., 1969). Because PEPCK has a different intracellular distribution between the species, the routes by which carbon and reducing equivalents for gluconeogenesis are transported out of the mitochondria are also different. Thus, for those species with significant amounts of mitochondrial PEPCK, the rat may not be the most appropriate experimental model in which to study ketogenic/gluconeogenic interrelationships (Söling and Kleineke, 1976). A case in point is the response of hepatic gluconeogenesis to added fatty acid. Gluconeogenesis in perfused livers from 48 hour fasted guinea pigs actually decreased in response to oleic acid addition to the perfusion medium, despite an increased ketogenic rate (Söling and Kleineke, 1976). This contrasts with results observed in the rat, and has led various authors to conclude that fatty acid oxidation and ketogenesis do not play a regulatory role for gluconeogenesis in species containing significant amounts of mitochondrial PEPCK.

Recent investigations with the guinea pig suggest, however, that hepatic ketogenesis exerts a necessary permissive effect on gluconeogenesis. These experiments examined the effect of 2-tetradecylglycidic acid on

hepatic ketogenesis and gluconeogenesis in perfused guinea pig liver. 2-Tetradecylglycidic acid is an oral hypoglycemic agent (Tutwiler et al., 1978) whose only apparent site of action is the CPT I reaction of fatty acid oxidation (Tutwiler and Dellevigne, 1979). Addition of 2-tetradecylglycidic acid to the medium perfusing livers isolated from 48 hour fasted guinea pigs totally eliminated ketogenesis from endogenous substrates and markedly inhibited gluconeogenesis from added lactate plus pyruvate (Tutwiler and Brentzel, 1982). Upon addition of octanoic acid to the 2-tetradecylglycidic acid-containing medium, gluconeogenic rates were restored to near initial values. Since octanoic acid is activated within the mitochondrial matrix and does not require CPT I for uptake into the mitochondria, the authors concluded that inhibition of the oxidation of endogenous long-chain fatty acid by 2-tetradecylglycidic acid was responsible for the decreased gluconeogenesis. Thus, even in species which contain significant mitochondrial PEPCK, a certain amount of fatty acid oxidation and ketogenesis is apparently required to allow maximum gluconeogenic rates to occur.

The relationship between ketogenesis and gluconeogenesis has yet to be investigated to any degree in the ruminant. The continuous requirement for hepatic gluconeogenesis in these animals, and the observation that hepatic ketogenesis is also a continuous process,

suggests that a similar relationship between the two processes may exist in the ruminant as in the nonruminant. Some limited experimental evidence also supports such a relationship. One report is available which found that methylmalonic acid inhibited both gluconeogenesis from propionic acid and palmitic acid oxidation by bovine liver slices (Wahle et al., 1982), although cause and effect remain to be established. In addition, when serial liver biopsies were obtained from lactating Holstein cows, gluconeogenic and ketogenic capacities of slices made from the biopsies exhibited parallel changes in activity over time post partum (Aiello and Herbein, 1983). This should prove to be a fruitful area of future research.

Endogenous Acetate Production

Production of acetate from endogenous sources within the body, in contrast to acetate production in the rumen fermentation, has received much research attention but is still little understood. Annison and White (1962) performed one of the earliest studies in which endogenous acetate production was measured as a fraction of the total production. These researchers estimated that in the fed sheep endogenous acetate from all sources accounted for 25% of the total acetate entry rate. Bergman and Wolff (1971) found similar values (20%) for fed sheep, but in fasted sheep endogenous acetate contribution to total

acetate entry rate increased to 80%. Several investigators have attempted, with moderate success, to localize the site(s) of endogenous acetate production. The liver is a major contributor, accounting for about 20% of endogenous acetate production (Bergman and Wolff, 1971). Other researchers have also reported on the ability of liver to produce endogenous acetate (Costa et al., 1976; Lomax and Baird, 1983; Snoswell et al., 1978). Other potential sites of endogenous acetate production in the ruminant include cardiac muscle (Knowles et al., 1974) and the hind-limb (Pethick and Lindsay, 1978), where acetate production could be occurring in both skeletal muscle and adipose tissue. Endogenous acetate production has also been observed in rat liver (Seufert et al., 1974).

The sources from which endogenous acetate is derived have also not been completely defined. Palmquist (1972) estimated that palmitate supplied 2% and 16% of the plasma acetate carbon in the fed and fasted sheep, respectively. In addition, some amino acids may also supply endogenous acetate during their catabolism (König et al., 1981). Potentially, any compound which produces acetyl-CoA during the course of its metabolism may contribute to endogenous acetate production. (Although early research suggested that acetate was produced from acetyl-carnitine via acetyl-carnitine hydrolase (Costa et al., 1976; Costa and Snoswell, 1975a,b), subsequent

investigations refuted this and demonstrated that acetyl-CoA was the immediate precursor of that acetate production (Snoswell and Tubbs, 1978).)

The importance of endogenous acetate production to metabolism has yet to be established. Endogenous acetate has been suggested to play a role analogous to that of ketones by redistributing oxidizable substrate throughout the body (Knowles et al., 1974). Alternatively, endogenous acetate production has been explained as a mechanism for relieving "acetyl pressure", i.e. the buildup of acetyl-CoA and the increased acetyl-CoA:CoASH ratio during periods of high fatty acid oxidation rates (Costa et al., 1976; Costa and Snoswell, 1975a; Snoswell et al., 1978). More recently, however, other research suggests little or no relationship between fatty acid oxidation rates and endogenous acetate production (Lomax and Baird, 1983; Pethick et al., 1981; Snoswell et al., 1981). Further research, more accurately defining sites and sources of acetate production, and characteristics of the enzymes involved, will be needed before the importance of endogenous acetate to metabolism can be defined. To date, only one report exists describing the isolation (from rat liver) and purification to homogeneity of an acetyl-CoA hydrolase (Prass et al., 1980). Activity of this enzyme proved highly sensitive to modulation by various nucleotides, suggesting the potential for in vivo regulation. In view of the potential

futile cycle and consequent energy loss possible due to the activities of acetyl-CoA hydrolase and acetyl-CoA synthetase, some form of reciprocal regulation of these enzymes would be required.

Pathological Ketosis in the Ruminant

Under some circumstances the continuous physiological ketosis observed in ruminant liver (e.g. Lomax and Baird, 1983) can become a pathological ketosis. High-producing dairy cows in early lactation and twin-pregnant ewes late in gestation are most susceptible to this condition, termed bovine lactational ketosis or ovine pregnancy toxemia, respectively (Bell, 1980). Pathological ketosis is characterized by both hypoglycemia and hyperketonemia, and is accompanied by elevated blood FFA concentrations, fatty infiltration of the liver and loss of liver glycogen, and a progressive appetite loss (Baird, 1982; Bell, 1980). Ruminant pathological (or clinical) ketosis, especially bovine lactational ketosis, has been the subject of a large number of reviews (e.g. Baird, 1981a,b, 1982; Bell, 1980; Bergman, 1971; Kronfeld, 1971, 1982; Schultz, 1971). Most investigators now agree that the plethora of signs associated with ketosis are initiated not by hyperketonemia but by the hypoglycemia. ("Ketosis" in this discussion will specifically refer to this clinical syndrome of hypoglycemia accompanied by hyperketonemia and the other associated signs.)

Ketosis can be a confusing topic of discussion since many different events can result in the pathological syndrome. Kronfeld (1982) has attempted to standardize the discussion with respect to lactational ketosis, with which the remainder of this discussion will be concerned, by defining four different classes of clinical ketosis: primary and secondary underfeeding ketosis, which result, respectively, either from not feeding sufficient quantities of feed, or from the simple inability of the cow to eat sufficient amounts of feed, generally due to appetite loss secondary to another disease condition; alimentary or ketogenic ketosis, in which the diet contains a high concentration of ketogenic precursors; and finally, spontaneous ketosis, in which the cow becomes ketotic for no apparent reason other than high milk production. The latter class is most typically associated with the term lactational ketosis, and of the four classes has been the most controversial with respect not only to the cause of its occurrence, but also as to its very existence (Kronfeld, 1982). Some investigators have commented that most cases of so-called spontaneous ketosis may simply be secondary underfeeding ketosis in which the cause of the depressed feed intake has not been identified. This interpretation is disputed by other investigators who point out that depressed feed intake is generally not observed until some time after the onset of hypoglycemia

and hyperketonemia (Kronfeld, 1971). Part of the difficulty may lie in identifying cows in the early stages of spontaneous ketosis, as these animals have often reverted to a secondary underfeeding ketosis by the time they become available for observation (Kronfeld, 1971).

Lactational ketosis appears to result from a unique combination of circumstances which occur during early lactation. The mammary gland in early lactation appears to have a priority over other body tissues for nutrient utilization. In addition, peak milk production usually occurs between four and nine weeks into lactation, but feed and energy intake reach a maximum about eight to twelve weeks into lactation. Over this time period, despite what may still be a considerable feed intake, the high-producing dairy cow is in a negative energy balance, and is forced to mobilize body reserves to meet the demands of the lactating mammary gland. This adaptation has been termed homeorhesis, described as the metabolic coordination of various tissues to support a given physiological state, such as lactation (Bauman and Currie, 1980). (In contrast, homeostasis is described as the maintenance of constant conditions within the internal environment of an organism.) The priority given the mammary gland over other body tissues for nutrient utilization may be attributed to changes in the circulating hormonal milieu of the lactating dairy cow. In healthy cows the following changes occur

during early lactation to support milk production. Insulin concentrations decrease during early lactation to the lowest values observed in any physiological state (Hart et al., 1980; Lomax et al., 1979; Vasilatos and Wangsness, 1981), encouraging lipolysis and fatty acid mobilization from adipose tissue. Low insulin concentrations may also confer priority for glucose utilization to the mammary gland. Mammary tissue apparently does not require insulin for glucose uptake (Laarveld et al., 1981), placing the mammary gland at a competitive advantage for glucose utilization to insulin-sensitive tissues. Insulin:glucagon ratio is also at its lowest value during this period, primarily due to the decrease in insulin concentrations, further favoring adipose tissue lipolysis (Brockman, 1979; de Boer et al., 1983). Growth hormone, which may actively promote lipid mobilization during periods of negative energy balance, in contrast to insulin attains its highest observed concentrations during early lactation (Vasilatos and Wangsness, 1981). Thus, a major factor involved in the development of pathological ketosis, mobilization of fatty acid from adipose tissue, is already actively occurring. Despite this, FFA concentrations remain relatively low, within the range of .30 to .60 mM, with the higher concentrations found in higher producing cows (Baird, 1981a, 1982). (Typically, blood FFA concentrations are .1 to .3 mM in the nonlactating cow, but

can exceed 1 mM in the clinically ketotic animal (Bergman, 1971; Baird et al., 1979).) This may partially account for the observed association between high milk production and a certain degree of ketone accumulation in the blood of otherwise healthy cows (Emery and Williams, 1964). These cows are often referred to as subclinically, or borderline, ketotic animals. Since the ruminant liver typically produces ketones, the increased substrate (i.e. FFA) availability due to fatty acid mobilization from adipose tissue is merely accelerating somewhat the ketogenic rate. Increased blood ketone concentrations also follow simply from the increased ketogenic capacity of the liver during early lactation (Aiello and Herbein, 1983).

Blood concentrations of glucose and FFA are inversely related (Bergman, 1971), so that the elevated FFA concentrations observed in healthy early lactation cows is consistent with the lower blood glucose concentrations found in these same animals (Baird, 1981b). A decrease in blood glucose induces increased adipose tissue lipolysis, resulting in higher FFA concentrations in the blood. The onset of a marked hypoglycemia (Baird, 1982) is necessary, however, for the massive fatty acid mobilization associated with pathological ketosis, with blood FFA concentrations in the range of 1 to 2 mM (Bergman, 1971). Hypoglycemia results from a decreased

carbohydrate status in the susceptible cow, and is ultimately caused by the attempt to maintain milk production in the face of a glucose shortage (Baird, 1981b). Although blood glucose concentrations are decreased, some evidence suggests that glucose entry rate may remain unchanged in the early stages of spontaneous ketosis (Kronfed, 1971), and does not decrease until the loss of appetite diminishes the absorption of glucogenic precursors from the rumen. Hormonal changes during ketosis have not been studied in detail, but some information is available indicating that insulin concentrations are decreased further during spontaneous ketosis (Schwalm and Schultz, 1976), further contributing to adipose tissue lipolysis and fatty acid mobilization.

The potential fates for FFA absorbed by the liver, i.e. esterification, oxidation to CO_2, or ketogenesis, have already been discussed. During ketosis in the nonruminant, the proportion of the FFA absorbed by the liver which is utilized for ketogenesis increases (McGarry and Foster, 1980). Based on limited observations a similar result, i.e. increased partitioning of FFA towards ketogenesis, appears to occur in the ketotic ruminant (Bergman, 1971), although the basis for increased partitioning of fatty acid into ketogenesis has yet to be established in the ruminant. The ability of a decreased carbohydrate status to influence utilization of FFA within the liver

has been extensively discussed (e.g. Baird, 1982). Decreased OAA concentrations within the liver have often been cited as a factor in the increased hepatic ketogenesis of the ketotic ruminant. Oxaloacetate concentrations in the whole-liver (mitochondrial OAA concentrations have yet to be determined for ruminant liver) are known to decrease during starvation-induced ketosis, as do the concentrations of other glucogenic intermediates (Baird et al., 1979). On the other hand, Ballard et al. (1968) observed no difference in hepatic OAA concentrations between spontaneously-ketotic and healthy cows, despite decreased concentrations of lactate, pyruvate, malate and citrate in the ketotic liver. These results are in agreement with the concept that hepatic OAA concentrations are not the major determinant of ketogenic rates (Siess et al., 1982). (These results also demonstrate the problems encountered when using fasted cows as an experimental model for spontaneous ketosis.)

The major competing fate to ketogenesis for use of FFA in the liver is esterification, and partitioning of fatty acid away from esterification could increase ketogenic rates. This would occur, however, only when FFA concentrations were low, and hence limiting for both esterification and ketogenesis. Since hyperketonemia only occurs when FFA concentrations become elevated (Bergman, 1971), decreased fatty acid esterification

would not seem a likely factor in the increased partitioning of fatty acid towards ketogenesis, at least in the early stages of ketosis prior to loss of appetite. This is supported by the observation that ketosis in the cow is generally accompanied by fatty infiltration of the liver (Bell, 1980). This fat is primarily neutral lipid, indicating that the esterification pathway is functional in the ketotic ruminant liver.

Other factors which could potentially alter the proportion of fatty acid partitioned toward ketogenesis, e.g. carnitine availability, CPT activity and the presence of malonyl-CoA, have not been examined in the bovine suffering from spontaneous lactational ketosis. Carnitine availability may prove of particular importance, based on observations in the sheep. Induction of ketosis via alloxan-diabetes, a condition with many similarities to lactational ketosis, resulted in a marked increase in the free carnitine and acyl-carnitine in sheep liver (Snoswell and Koundakjian, 1972). Increased carnitine availability would by its nature result in greater partitioning of fatty acid towards oxidation and ketogenesis.

Ultimately, the increase in proportion of fatty acid undergoing oxidation during ketosis may simply be due to the increase in circulating FFA concentrations resulting from hypoglycemia. Greater quantities of fatty acid would be available for oxidation, and may

themselves induce the increase in hepatic carnitine content and the increased ketogenic rates (Frost and Wells, 1982). Such a relationship could be involved in the self-limiting nature of lactational ketosis, in contrast to diabetic ketoacidosis and pregnancy toxemia. Milk production decreases during the course of lactational ketosis, resulting in a decreased energy requirement for the cow (Baird, 1982). Consequently, the demand for glucose declines and blood glucose concentrations increase, resulting in decreased rates of fatty acid mobilization from adipose tissue. With FFA absorption by liver occurring at a lower rate, hepatic ketogenesis would decrease, as presumably would hepatic carnitine content. Ketones themselves, i.e. BHBA and AcAc, may also be involved in the self-limiting nature of lactational ketosis by directly inhibiting adipose tissue lipolysis (Metz et al., 1974). Despite the large amount of research undertaken to determine the causes of pathological ketosis in the ruminant, a complete understanding of all of the background factors which predispose an animal to ketosis has not been forthcoming. An increased understanding of the nature of pathological ketosis should in turn lead to a better understanding of ruminant energy metabolism.

Metabolic Regulation in the Ruminant and Nonruminant

Many aspects of the hormonal control of fatty acid oxidation and ketogenesis within the liver have been examined in the preceding discussion. This section will summarize much of that information, and will make specific comparisons between the ruminant and nonruminant to highlight their differences and similarities in respect to metabolic regulation. The discussion will concern primarily the liver, but some aspects of whole-body metabolism will also be examined. Many reviewers have indicated that the typical ruminant absorbs little glucose from the digestive tract due to the fermentation of dietary carbohydrate within the rumen (e.g. Bell, 1980). Consequently, a ruminant animal is in a continuous state of gluconeogenesis in order to meet the demands of various organs (e.g. nervous system, mammary gland, fetus) which require glucose (Katz and Bergman, 1969). Since the liver is the primary site of gluconeogenesis, all metabolic adaptations by the ruminant liver will occur against this backdrop of continuous gluconeogenesis. Maintenance of glucose homeostasis is central to maintaining energy homeostasis in both the ruminant and nonruminant. The maintenance of energy homeostasis is a complex process involving many levels of control, in both the short- and long-term. To simplify matters, only the roles played by

insulin and glucagon during the short-term responses to feeding and fasting will be discussed.

A typical nonruminant possesses a digestive tract with minimal storage capacity within the stomach (Church and Pond, 1974), so that the stomach is empty within a few hours of feeding, and the major part of the intestinal digestive process is complete. The result is a feeding pattern which produces discrete time periods of nutrient absorption from the gastrointestinal tract, interspersed with periods of nonabsorption. This is true even in species like the rat which can exhibit "nibbling" behavior, i.e. the consumption of a large number of small meals. Nibbling tends to smooth out nutrient passage rates through the gastrointestinal tract, but does not totally eliminate periods of nonabsorption. A nonruminant consuming a high carbohydrate diet derives most of its energy from glucose. Since much more glucose is absorbed following a meal than the nonruminant immediately requires, the organism must store the excess, as glycogen or triacylglycerol, against a future period of energy shortage.

After a meal secretion rates of insulin increase, while those of glucagon decrease. These changes occur very rapidly (within minutes), and may be due to the increased glucose availability, or perhaps to the very act of feeding itself (Unger, 1971). The result is an increase in the circulating insulin:glucagon ratio,

which serves to promote storage of the energy surplus. Within the liver this is accomplished through an increase in the rates of glycogen synthesis, glycolysis and fatty acid synthesis and esterification, while glycogenolysis, gluconeogenesis and fatty acid oxidation rates are decreased. Many of the key enzymes of these pathways are controlled by a phosphorylation/dephosphorylation mechanism (Cohen, 1980b). The increased insulin:glucagon ratio promotes energy storage by inducing dephosphorylation of regulatory enzymes involved in these pathways, in part by decreasing intracellular cyclic AMP (cAMP) concentrations, in part by stimulation of phosphoprotein phosphatase (Cohen, 1980b). The glycogen synthase/glycogen phosphorylase system and its associated enzyme cascade system has been well-characterized, at least in nonruminant liver (Cohen, 1980a). Dephosphorylation activates glycogen synthase and glycogen synthesis, but inactivates glycogen phosphorylase and thus glycogen breakdown. Glycolytic rate may be increased due to enzyme modulation at a number of different points. Fructose-2,6-bisphosphate activates phosphofructokinase and concomitantly inhibits fructose-1,6-bisphosphate and gluconeogenesis (Hers and van Schaftingen, 1982). Intracellular fructose-2,6-bisphosphate concentrations increase in response to the activation of fructose-6-phosphate 2-kinase and inactivation of fructose-2,6-bisphosphatase as a result of dephosphorylation (Hers and

van Schaftingen, 1982). In addition, both pyruvate kinase and pyruvate dehydrogenase appear to be activated by a dephosphorylation mechanism (Engstrom, 1980; Popp et al., 1980; Pratt et al., 1979; Pratt and Roche, 1979). An increased glycolytic rate indirectly supports increased fatty acid synthesis by enhancing citrate synthesis, and ultimately acetyl-CoA formation, the precursor for fatty acid synthesis. Acetyl-CoA carboxylase, the rate-limiting enzyme for fatty acid synthesis, is also activated by dephosphorylation (Hardie, 1980; Kim, 1979), causing both increased malonyl-CoA concentrations within the cell and fatty acid synthesis rates. In turn malonyl-CoA inhibits fatty acid oxidation (McGarry and Foster, 1980). Carnitine palmitoyl transferase I and carnitine:acylcarnitine translocase activities may also be reduced in the presence of high insulin:glucagon ratios, although the mechanism for these changes is currently unknown (Harano et al., 1982; Idell-Wenger, 1981).

As the digestion process nears completion, the organism begins the transition to the post-absorptive state. In response to the decreased flow of absorbed nutrients into the body, insulin secretion falls and glucagon secretion increases over a period of several hours (Ruderman et al., 1976), resulting in the characteristically low insulin:glucagon ratio of the post-absorptive and ultimately the fasted states (Unger, 1971).

Consequently, the cellular cAMP concentrations increase, leading to phosphorylation of the liver enzyme systems just discussed. The result is a reversal of the metabolic set of the liver, giving net glycogenolysis, gluconeogenesis, and fatty acid oxidation and ketogenesis, thus ensuring a supply of energy substrate to extrahepatic tissues. The most important aspect of nonruminant hepatic metabolism illustrated by this discussion is the radical change in metabolic set which can occur within the nonruminant liver over relatively short periods of time in response to a changing hormonal environment.

The ruminant animal differs from the nonruminant in a number of important respects, all of which result from the presence of a rumen and the associated microbial fermentation. Whereas the nonruminant has discrete periods of nutrient absorption following a meal, a ruminant consuming feed on a regular schedule is always in an absorptive state, even when feeding occurs as infrequently as once a day. Rumen fermentation and nutrient absorption rates increase somewhat a short time after feeding, but as long as 24 hours post-feeding significant amounts of feed material are still undergoing fermentation within the rumen, or cecum and large intestine, with subsequent absorption of the fermentation endproducts. The rumen fermentation also alters the type of nutrients eventually absorbed by the ruminant. Typically, dietary carbohydrate

is fermented within the rumen to short-chain volatile fatty acids, primarily acetate, propionate and butyrate. This process occurs especially rapidly for starches and soluble sugars, so that little glucose is absorbed by the ruminant. This situation may be modified to some degree in special cases such as feedlot steers and high-producing dairy cows that consume large portions of their diet as starch. Whether fed or fasted, the ruminant liver is in a continuous state of gluconeogenesis to ensure a continuous supply of glucose for those extrahepatic tissues which require glucose for normal functioning.

Compared to the nonruminant, relatively little is known concerning hormonal regulation of metabolism in the ruminant. Pertinent information has been summarized in four review articles (Bassett, 1975, 1978; Brockman, 1979; Trenkle, 1981). Insulin and glucagon appear to fulfill much the same purpose in the ruminant as in the nonruminant. Recent data indicates that ruminant (sheep and goat) liver contains insulin and glucagon receptors, and that the receptor numbers may change over the long-term in response to varying metabolic conditions (Gill and Hart, 1980, 1981). As in the nonruminant, receptor numbers were inversely related to plasma hormone concentrations. Ruminant liver may also be an important site for hormone degradation (Brockman et al., 1976). Factors which modulate hormone secretion and so alter circulating

hormone concentrations, have not been as well characterized in the ruminant as in the nonruminant. Long-term adaptation to plane of nutrition and dietary composition can alter glucagon and insulin secretion in the ruminant (Bassett et al., 1971; Gill and Hart, 1981). Recent evidence suggests that physiological concentrations of propionate and butyrate are important regulators of insulin and glucagon secretion (Brockman, 1982). Also, the mere act of feed ingestion may alter insulin and glucagon secretion (Bassett, 1978).

Much as in the nonruminant, insulin and glucagon interact to maintain plasma glucose concentrations, although insulin secretion in the ruminant appears to be related more to glucose entry rate than to plasma glucose concentrations (Bassett et al., 1971). This interaction between insulin and glucagon is demonstrated by the results obtained in vivo during either insulin or glucagon infusions. Intravenous infusion of insulin into sheep decreased plasma glucose, apparently by stimulating glucose utilization in extrahepatic tissues (Brockman, 1983; Brockman et al., 1975). Glucagon concentrations increased during insulin infusion, presumably because of the decrease in glucose concentrations. When insulin plus glucose were infused, which maintained plasma glucose concentrations, glucagon concentrations were unaffected (Brockman et al., 1975). Glucagon infusions, on the other

hand, increased glucose availability by stimulating hepatic gluconeogenesis, resulting in an increase in insulin concentrations (Bassett, 1971; Brockman and Bergman, 1975). These results, consistent with observations in the nonruminant, demonstrate that in the ruminant glucagon appears to act primarily on the liver and insulin on extrahepatic tissues (Brockman and Bergman, 1975; Brockman et al., 1975). Glucagon may, however, stimulate lipolysis in ruminant adipose tissue (Brockman, 1976; Brockman et al., 1975), while insulin may inhibit hepatic gluconeogenesis and glucose production (Bassett, 1978; Brockman, 1983).

Relative to the nonruminant, the ruminant animal always has a low circulating insulin:glucagon ratio (Bassett, 1975), which serves to maintain hepatic gluconeogenic rates. Upon feeding, insulin secretion increases in the ruminant as occurs in the nonruminant (Bassett, 1975, 1978; Trenkle, 1981). In contrast to the nonruminant, glucagon secretion increases in parallel with insulin secretion, maintaining the relatively low insulin:glucagon ratio (Bassett, 1975, 1978; Trenkle, 1981). Dual secretion of insulin and glucagon after feeding permits insulin to stimulate utilization of incoming nutrients (e.g. acetate, amino acids, glucose) by extrahepatic tissues, while glucagon stimulates hepatic gluconeogenesis from the influx of propionate. This summary agrees with the observation that hepatic

gluconeogenesis and glucose production reach maximal rates two to four hours after feeding (Katz and Bergman, 1969; Kronfeld et al., 1969), corresponding to maximal ruminal propionate absorption rates and glucagon concentrations (Bassett, 1975; Trenkle, 1981). Although the insulin:glucagon ratio remains constantly low relative to the nonruminant, the ratio does change somewhat in the ruminant, albeit within a much narrower range than in the nonruminant (Bassett, 1975). The insulin:glucagon ratio is highest after feeding and lowest during a prolonged fast. Both insulin and glucagon secretion rates decline during fasting, but insulin decreases to a greater extent. (Glucagon in the ruminant is not a homogeneous entity, but consists of both pancreatic and gut-derived glucagon-like immunoreactivity. Glucagon-like immunoreactivity can apparently account for the vast majority of circulating glucagon activity in the ruminant (Gill and Hart, 1981). Little is known about the glucagon-like immunoreactivity, but it appears to have much the same effect on metabolism as does pancreatic glucagon.)

Metabolism in the nonruminant liver is controlled in large part by the phosphorylation or dephosphorylation of key regulatory enzymes (Cohen, 1980a). Covalent modification via phosphorylation/dephosphorylation as a regulatory mechanism of ruminant metabolism has not yet been examined. Pyruvate dehydrogenase kinase has been isolated and purified from bovine liver and kidney,

however, demonstrating that ruminant metabolism could potentially be regulated by a phosphorylation/dephosphorylation mechanism (Pratt and Roche, 1979; Pratt et al., 1979). Changing insulin:glucagon ratios in the nonruminant induce alterations in the degree of enzyme phosphorylation within the liver, resulting in alterations of hepatic metabolic activity. In view of the relatively narrow range through which the insulin:glucagon ratio changes in the ruminant (Bassett, 1975), the importance for metabolic regulation of subsequent changes in enzyme phosphorylation state in the ruminant could be questioned.

With regard to regulation of fatty acid oxidation, ruminant liver exhibits some striking contrasts to nonruminant liver. For example, liver from the fed rat has an inherently lesser capacity for fatty acid oxidation and ketogenesis than liver from the fasted rat, a difference which can not be overcome even with addition of excess carnitine (Christiansen et al., 1976; McGarry and Foster, 1980). Fatty acid oxidation in the presence of carnitine by isolated sheep hepatocytes, on the other hand, was the same whether the liver donor had been fed or fasted (Lomax et al., 1983b). Glucagon or dibutyryl cAMP (Bt2cAMP) can stimulate fatty acid oxidation by liver preparations from fed rats to rates comparable to those observed in fasted rats (Christiansen, 1979; Harano et al., 1982; McGarry and Foster, 1980). Both glucagon and

Bt2cAMP, however, were unable to stimulate fatty acid oxidation by hepatocytes from fed sheep. These results, consistent with the continuous presence of a relatively low insulin:glucagon ratio in the ruminant (Bassett, 1975), raise questions with respect to regulation of fatty acid oxidation in the ruminant. In the nonruminant liver, a decreased insulin:glucagon ratio stimulates fatty acid oxidation by relieving malonyl-CoA inhibition of CPT I (McGarry and Foster, 1980), activating CPT I (Harano et al., 1983), increasing hepatic carnitine content, and partitioning fatty acid away from esterification (McGarry and Foster, 1980). The importance of these effects for regulation of fatty acid oxidation in ruminant liver, in the presence of continuously low insulin:glucagon ratios, has yet to be determined.

MATERIALS AND METHODS

This section describes the procedures used to obtain quantitative measurements of fatty acid oxidation by rat and bovine liver tissue during *in vitro* incubation.

Source of Tissue

Bovine liver was obtained from two different sources, either the Michigan State University Meats Laboratory or Large Animal Clinic. In the Meats Laboratory, cattle were stunned with a captive-bolt gun and exsanguinated. Liver samples were generally removed from the animals within 15 to 20 minutes of the time of exsanguination. Samples were obtained from beef breed animals (cows and steers) as well as nonlactating Holsteins (heifers and cows). A small number of samples were also obtained from Holstein calves. Liver from the Large Animal Clinic was obtained via biopsy. Liver biopsies (3 to 10 gms) were removed under local anesthesia through an incision between the 13th and 14th ribs. Samples were obtained from both lactating (10-30 Kg/day) and nonlactating Holstein cows. Except for experiments which specifically involved a long-term fast, all animals had been fed within 4 to 14 hours of the time of liver retrieval. Beef breed

animals were slaughtered for use in carcass evaluation classes, prior to which they had been consuming typical growing or finishing rations at the University farms. Holstein cows were from the University herd. Many of these animals were culled due to low production or reproductive problems. Lactating cows had been previously assigned to one of two experiments: either a physiology experiment, which involved slaughter of the cows at preselected times following parturition, or a nutrition experiment, in which an experimental drug for the modification of rumen fermentation was fed. Holstein heifers (200-450 Kg BW) were from a physiology experiment which examined the effect of different photoperiods (16 hours light:8 hours dark vs. 8 hours light:16 hours dark) on growth. Holstein calves (5-8 weeks old) had been used in nutritional experiments testing the use of various protein sources in milk replacers.

Sprague-Dawley rats were obtained from a local breeding colony (Spartan Laboratory Animals, Inc.). Most experiments used virgin female rats of 150 to 200 gms body weight. Fatty acid oxidation rates were also compared among rats of various ages: weanling (~40 gms BW), young (100-130 gms BW), and aged rats (750-1000 gms BW). Rats were maintained on either Purina or Wayne Feeds Laboratory Chow, and were either fed, or fasted for 48 hrs, prior to decapitation.

Tissue Preparation

Upon retrieval bovine liver samples were cut into strips (approximately 1 cm X 2 cm X 8 cm) and placed into ice-cold Krebs-Ringer bicarbonate buffer (KRB), pH 7.4 (Appendix Table 1; Umbreit et al., 1964). Biopsy samples, already of these dimensions, and rat liver samples were placed directly into ice-cold KRB. The KRB, prepared less than 12 hours previously, was oxygenated, checked for pH, and sealed immediately prior to use (with rat liver), or to departure from the laboratory (with bovine liver). When strips arrived in the laboratory they were cut into blocks (1 cm X 2 cm X 2 cm), trimmed to remove nonparenchymal tissue, and sliced with a Stadie-Riggs microtome grooved to produce slices about .5 mm thick. These operations were conducted on ice. Liver slices were kept in ice-cold KRB until weighed for the incubation, 5-60 minutes from the time of slicing. In general, liver slices from slaughter tissue were placed into incubation media within 30-80 minutes of the time of exsanguination. Slices from biopsied liver began incubating within 18-35 minutes of the time of removal from the cow. Rat liver slices were incubating within 5-20 minutes from the time of decapitation.

Hepatocyte Preparation

Isolated rat and bovine hepatocytes were obtained from the Animal Toxicology Laboratory in the Department of Animal Science. Rat hepatocytes were isolated by the procedure of Berry and Friend (1969), as modified by Seglen (1972, 1973a,b). This method was adapted to bovine liver (Forsell et al., 1984). A brief description of the procedure follows. The caudate lobe was removed from cattle slaughtered at the Meats Laboratory within 15-20 minutes of the time of exsanguination. Immediately the lobe was perfused with ice-cold perfusion buffer (Ca/Mg-free, HEPES-buffered, pH 7.4) using a large syringe inserted into exposed arteries and veins. A small piece (20 gms) was cut from the lobe, keeping as much of the capsule intact as possible and minimizing the cut surface area. Hepatocytes were isolated from this liver piece following perfusion with a collagenase solution. Based on trypan blue exclusion, average viability of isolated hepatocytes was about 80%.

Fatty Acid Oxidation Studies - Liver Slices and Isolated Hepatocytes

Radiochemicals/Biochemicals/ Chemicals

Solutions of 1- and U-^{14}C-palmitic acid, 1-^{14}C-oleic acid (in toluene, Amersham), 1-^{14}C-octanoic acid (in ethanol) and crystalline 7-^{14}C-benzoic acid (New England

Nuclear) were stored as purchased at -20°C. Solutions of 1-^{14}C-acetic acid and 3-^{14}C-β-hydroxybutyric acid (in ethanol, New England Nuclear) were stored as purchased at 4°C. Malonyl-CoA (lithium salt) was purchased from P-L Biochemicals and stored at -20°C. All remaining biochemicals, hormones and antibiotics were from Sigma. Other chemicals used were of the highest grade commercially available.

Media Preparation

All media and buffer were prepared with glass-distilled, deionized water (dd H_2O) within 12 hours of use. A five-fold concentrated KRB (Appendix Table 1), adjusted to pH 7.4 with O_2:CO_2 (95:5) using a gas dispersion tube (Kimble, Model #12C), served as the basis for the incubation media. Appropriate volumes of substrates, antibiotics and effectors were added to the KRB, which was then diluted to a given volume with dd H_2O (see Appendix Table 2 for example of incubation media preparation). Later incubations also contained 25 mM HEPES, pH 7.4. Aliquots (3.00 ml) of incubation media were pipetted into 25 ml erlenmeyer flasks. This method entailed the preparation of a separate incubation media for each effector, or combination of effectors, tested within a given experiment. Alternatively, a single stock media was prepared containing all ingredients except the

effectors to be studied in a given experiment. A volume (2.50 ml) of this stock media was pippetted into the incubation flasks, and aliquots (.50 ml) containing the desired amounts of the effectors of interest were then added to the flasks to give a final volume of 3.00 mls. (See Appendix Table 3 for example of incubation media preparation by this method.)

Flasks of incubation media were prepared prior to slaughter of animals. When tissue retrieval from an animal would occur more than 3-4 hours after media preparation, all flasks were temporarily stored at 4°C. Approximately 1-2 hours prior to tissue collection, incubation flasks were placed in a 37°C water bath of a Dubnoff metabolic shaker (Precision Scientific). Media pH remained within the range of 7.35 to 7.45 after warming to 37°C.

Substrate preparation of ^{14}C-labelled fatty acids consisted of the following procedure: 1) an aliquot of ^{14}C-labelled fatty acid, as purchased, was placed into a volumetric flask and taken to dryness under N_2; 2) appropriate amounts of unlabelled fatty acid were added to the flask to give a final specific activity of 250-300 dpm/nmole; 3) bovine serum albumin (BSA), dissolved in 10 mM KH_2PO_4,pH 7.4 and filtered through a .8 micron filter (Millipore), was added to the flask; 4) the solution was gently warmed and stirred (Sybron/Thermolyne Model

Nuova 7 heating stir plate) until dissolution of the fatty acid, which occurred almost immediately for octanoic acid (free acid) or oleic acid (potassium salt), but required 3-4 hours for palmitic acid (sodium salt). Final concentrations of fatty acid and BSA in these stock substrate solutions were 5 mM and 1.25 mM, respectively (fatty acid:BSA ratio = 4). All stock solutions were stored frozen at -20°C and were freshly prepared every 4-6 weeks. Generally, .6 μCi of ^{14}C-fatty acid was added per incubation flask.

Incubation Procedure

Liver slices were blotted, trimmed further to minimize nonparenchymal tissue, weighed on a double-pan torsion balance (Federal Pacific Electric Co., Precision Balance Model LG), and placed into incubation flasks to begin incubations. The airspace above the media was gassed for 15 seconds with O_2:CO_2 (95:5), the flasks were sealed with rubber serum caps containing suspended plastic center-wells (Kontes), and placed in a 37°C water bath of a Dubnoff metabolic shaker oscillating at 60 cycles/minute. All treatments were performed in quadruplicate. Incubations were terminated by injection of 3.0 ml of 3M perchloric acid (PCA), for the determination of total acid-soluble metabolites. Following acidification .3 ml of methylbenzothonium

hydroxide (tradename Hyamine Hydroxide, Sigma) was injected into the center-wells, which contained a 2 cm^2 fluted filter paper, and the incubation continued for an additional hour to trap CO_2.

In a limited number of studies, liver slices were preincubated in HEPES-supplemented KRB with or without dibutyrylcyclic AMP (Bt_2cAMP, 1 mM). Slices were weighed, placed in preincubation flasks, gassed with O_2:CO_2 (15 seconds) and preincubated for 30 minutes in a 37°C metabolic shaker. After the preincubation, slices were removed, blotted and transferred to incubation flasks. All subsequent operations were as above. Preincubation media was stored at -20°C until assayed for glucose (Sigma, Kit #510).

Incubation of isolated hepatocytes followed a similar procedure, except for the following changes. First, 2.00 ml of a two-fold concentrated incubation media were pipetted into incubation flasks. Incubations were initiated by addition of 2.0 ml of hepatocyte suspension (5-10 $X10^6$ viable cells/ml, 10-20 mg dry weight/ml), and were terminated by injection of 4.0 ml of 3M PCA.

Determination of $^{14}CO_2$ and ^{14}C-Acid-Soluble Metabolites

Following the time period for trapping CO_2, PCA-treated flasks were placed on ice for 15 minutes. Center-wells from all flasks were transferred to scintillation vials (20 ml, polyethylene, Rochester

Scientific, or Research Products, Inc.) for liquid scintillation counting to determine $^{14}CO_2$. The contents of PCA-treated flasks were swirled, transferred to disposable culture tubes (16 X 125 mm) and centrifuged (Damon/IEC, Model K centrifuge) at 60 xg for 20 minutes to remove precipitated protein. A .5 ml aliquot of the supernate was removed for scintillation counting to determine ^{14}C-acid-soluble metabolites (ASM). Three milliliters of the remaining supernate were neutralized with .3 ml of 3M K_2CO_3, placed on ice for 15 minutes, centrifuged as above to remove precipitated $KClO_4$ and stored at -20°C. Perchloric acid precipitation of media containing 1-^{14}C-acetate or 3-^{14}C-β-hydroxybutyrate (BHBA) and unlabelled palmitate:BSA gave 97-98% recovery of labelled acetate and BHBA. About 1% of the labelled acetate and none of the labelled BHBA appeared in the center-wells.

Supernates from incubations using 1-^{14}C-octanoate as substrate were treated as follows to remove the considerable quantities of PCA-soluble octanoate prior to determination of ^{14}C-ASM. Bonded-phase C18 Sep-Pak cartridges (Waters Associates) were wetted with 8 ml methanol. Methanol was displaced by injecting 10 ml dd H_2O through the cartridge. One milliliter of supernate was injected into the cartridge, discarding the first .6 ml of effluent and collecting the remainder. Finally,

an additional 1.5 ml of dd H_2O was injected, the effluent collected and pooled, adjusted to known final volume, and a .5 ml aliquot removed for scintillation counting and ^{14}C-ASM determination. This treatment quantitatively removed added 1-^{14}C-octanoate, and gave 85% recoveries of standard 1-^{14}C-acetate and 3-^{14}C-BHBA.

Samples, i.e. .5 ml aliquots of PCA-treated media or center-wells with their contents, were counted (Nuclear Chicago Model Mark I, or Searle Analytic Isocap Model 300) for two-10 minute counting periods in the presence of 10 ml of aqueous counting scintillant (Amersham, ACS). A channels ratio standard quench curve to determine sample counting efficiencies was constructed using a series of vials containing known amounts of 7-^{14}C-benzoic acid and increasing amounts of chloroform (Neame and Homewood, 1974). Counting efficiencies ranged from 76-83% for $^{14}CO_2$ samples containing .3 ml Hyamine Hydroxide, and from 83 to 88% for ^{14}C-ASM in .5 ml of PCA-treated media. Sample counts per minute were converted to disintegrations per minute (dpm) based on sample counting efficiency. Oxidation rates of ^{14}C-fatty acid to $^{14}CO_2$ and ^{14}C-ASM were calculated as follows:

$$\text{pmoles of fatty acid oxidized} \cdot \text{min}^{-1} \cdot (\text{mg wet tissue weight})^{-1} = \frac{\text{dpm in Sample} - \text{dpm in Blank}}{(\text{Specific Activity})(\text{Incubation Time})(\text{Wet Tissue Weight})} \times \frac{1000 \text{ pmoles}}{\text{nmole}} \times \text{Dilution Factor},$$

where Specific Activity = dpm $\cdot$ (nmole fatty acid)$^{-1}$
Incubation Time = minutes
Wet Tissue Weight = mg
Dilution Factor = correction for acid-dilution and media volume, giving total dpm produced during incubation.

Blank flasks were acidified immediately before addition of liver slices or hepatocytes at zero time.

High Performance Liquid Chromatography (HPLC) of Acid-Soluble Metabolites

A Waters Associates HPLC system, consisting of a Model M45 solvent delivery system, U6K universal injector, RCM-100 radial compression module equipped with a bonded-phase C18 cartridge (5 mm X 10 cm), and Model 441 ultraviolet absorbance detector set at a wavelength of 214 nm, was used to characterize the perchloric acid-soluble metabolites. Chromatographic band elution was displayed on a strip chart recorder (Sargent-Welch, Model SR). Mobile phase (.01 M NaH_2PO_4, pH 3.0) was isocratically pumped through the system at a flow-rate of 1.0 ml/min. Twenty-five or 100 µl aliquots of neutralized PCA-extract (reacidified to pH2 with dilute H_3PO_4 to increase band resolution) were injected into the system. The detector

effluent corresponding to acetate- and BHBA-containing bands, as determined by the retention time of standard acetate and BHBA, was collected into individual scintillation vials, dried and counted as above. Recoveries of known amounts of labelled acetate and BHBA were essentially 100%.

Gluconeogenesis Studies - Liver Slices and Isolated Hepatocytes

Studies of gluconeogenesis by liver slices and isolated hepatocytes were conducted in the Animal Toxicology Laboratory of the Department of Animal Science. Control media was Earle's balanced salt solution, pH 7.4, without glucose or phosphate, and supplemented to give final concentrations of 10 mM sodium acetate and 0.2 mM Bt_2cAMP. Gluconeogenesis media contained final concentrations of 2 mM lysine, 1 mM pyruvate and either 10 mM lactate, or 10 mM propionate. Incubations were similar to those for determining fatty acid oxidation. Hepatocytes (2 ml) or slices were placed into 30 ml culture flasks, gassed with O_2:CO_2 and incubated in a 37°C shaking water bath. Concentrated PCA (70%, 120 µl) was added to terminate the incubations. Culture flasks were chilled on ice for 15 minutes, followed by centrifugation (900 xg). Glucose content of the supernate was determined as above for fatty acid oxidation preincubation media. Glucose production rates were corrected against zero time controls.

Net gluconeogenesis rates were corrected against endogenous rates of glucose production in the absence of added substrate.

Fatty Acid Oxidation Studies - Liver Mitochondria

Mitochondrial Isolation

Mitochondria used to study fatty acid oxidation were isolated from a known mass of liver. All operations were carried out on ice. The outer membrane capsule was removed, and the remaining liver weighed and finely minced with a sharp razor blade. The liver mince was rinsed three times with isolation buffer (70 mM sucrose, 220 mM mannitol, 1 mM EDTA, and 2 mM HEPES, pH 7.4) before transfer to a 125 ml erlenmeyer flask. Three to four volumes of isolation buffer were added per volume of liver mince. Liver was homogenized with a Polytron Model ST tissue homogenizer on a setting of 4.5 for a total of 45 seconds (three-15 second periods). The liver homogenate was diluted 1:2 with isolation buffer (1 part homogenate +1 part buffer) and filtered sequentially through 2, 4, 6 and 8 layers of cheesecloth. The final volume of the total homogenate was recorded, an aliquot removed for protein analysis (biuret procedure), and the remainder apportioned into 50 ml polyethylene centrifuge tubes.

The total homogenate was centrifuged for 10 minutes at 650 xg in a Model RC2-B Superspeed centrifuge equipped with a model SS-34 rotor (Sorvall). The pellet was discarded and the supernate recentrifuged at 7,000 xg for 15 minutes. The supernate was discarded and the mitochondrial pellet resuspended in isolation buffer to approximately one-half the original volume of the total homogenate. The 650 xg and 7,000 xg centrifugations were repeated twice, with the mitochondrial pellet resuspended in isolation buffer to one quarter the original volume of total homogenate after the first repeat centrifugations. The final mitochondrial pellet was resuspended in 150 mM KCl to give a final concentration of about 6.25 mg mitochondrial protein/ml. Mitochondrial protein yields ranged between 15 and 20 mg protein/g liver. Mitochondria not used for fatty acid oxidation studies were recentrifuged at 7,000 xg for 15 minutes, resuspended in 10 mM KH_2PO_4, pH 7.0, 1 mM dithiothreitol, and stored frozen at -20°C.

Incubation Procedure

Studies of fatty acid oxidation with isolated liver mitochondria were similar to those with liver slices or isolated hepatocytes, and were essentially identical to the conditions of McGarry and Foster (1981). The buffer system was a modified KRB in which the proportions of NaCl and KCl were reversed, and supplemented with 25 mM HEPES, pH 7.4.

Initially, 2.2 ml of media were pipetted into 25 ml erlenmeyer flasks, followed by 0.1 ml additions of 150 mM KCl with or without appropriate amounts of the effectors of interest. As with liver slice incubation media, mitochondrial incubation media was refrigerated at 4°C if incubations were scheduled to begin more than 3-4 hours after media preparation. After warming to room temperature, incubation flasks were gassed for 15 seconds with O_2:CO_2 10-15 minutes before beginning incubations. When malonyl-CoA was the effector under study, 0.1 ml additions of malonyl-CoA, freshly prepared in ice-cold 150 mM KCl, were made at this time.

Incubations were begun by the addition of 0.2 ml of mitochondrial suspension (about 1.25 mg of mitochondrial protein), the flasks regassed with O_2:CO_2 (5 seconds for 0 and 1 minute incubations, 10 seconds for 2 minute incubations, 15 seconds for all others), sealed and placed in a 30°C shaking water bath. Final concentrations of standard incubation components were: 1-^{14}C-palmitic acid, 35 μM, 7259 dpm/nmole (.29 μCi per flask); BSA, .7% (fatty acid:BSA molar ratio = 0.3); l-carnitine, 100 μM; ATP, 4 mM; ADP, 1 mM; CoASH, 50 μM; reduced glutathione, 250 μM. Incubations were terminated by the addition of 2.5 ml of 1M PCA. All subsequent operations in preparation for counting were identical to those for liver slice incubations.

Carnitine Palmitoyltransferase Assay

Carnitine palmitoyltransferase activity was measured in liver mitochondria isolated as above, except that the final resuspension was made in isolation buffer instead of 150 mM KCl. In addition, CPT activity was also determined in mitochondria isolated from liver slices which had been preincubated in the presence or absence of Bt_2cAMP. Mitochondria from liver slices were isolated by a procedure essentially identical to that above, except for the following changes. Liver slices (500-650 mg wet weight) were homogenized, as they were, in graduated 30 ml test tubes containing 10 ml of isolation buffer. The homogenate was diluted 1:4 with isolation buffer, and filtered through 2 layers of cheesecloth. Due to the low mitochondrial yields (about 10 mg mitochondrial protein/g liver slices), the 650 xg and 7,000 xg centrifugations were repeated only once. The final mitochondrial pellet was resuspended in isolation buffer to give about 1.8 mg mitochondrial protein/ml, and was stored at -20°C until assayed for CPT activity.

Carnitine palmitoyltransferase activity was assayed at room temperature essentially by the method of Bieber *et al*. (1972). A description of the reagent solutions used follows. Palmitoyl-CoA was suspended in dd H_2O and solubilized by dropwise addition of 10 mM $NaHCO_3$ to give a final concentration of 0.7 mM. Solutions of palmitoyl-CoA,

Triton X-100 (1%), l-(-)-carnitine (100 mM), and dithiobisnitrobenzoic acid (DTNB, 2.5 mM in 10 mM $NaHCO_3$, pH 7.0) were stored at -20°C until use. A stock buffer mix (22 mM EDTA, 1.15 M Tris-HCl, pH 8.0) was made fresh daily. The assay measures the initial rate of total CoASH release from palmitoyl-CoA by the reaction of CoASH with DTNB. Two assay cuvettes are employed. Cuvette one contains l-carnitine and measures total CoASH release. This includes both the l-carnitine-dependent CoASH release from palmitoyl-CoA by the CPT reaction and the l-carnitine-independent CoASH release in all other CoASH-forming reactions, e.g. palmitoyl-CoA hydrolase and other nonspecific deacylases. Cuvette two is identical to cuvette one except for the absence of l-carnitine, and thus measures only the l-carnitine-independent CoASH release. Carnitine palmitoyl transferase activity was measured as the difference in rate of CoASH release between cuvettes one and two. The reaction was monitored at 412 nm using a Gilford Model 2400S recording spectrophotometer. A molar extinction coefficient of 13,600 was used for all calculations.

Cuvette one contained, in a final volume of 1.00 ml, 0.1% Triton X-100, 115 mM Tris-HCl, pH 8.0, 2.2 mM EDTA, .25 mM DTNB and the small volume of isolation buffer containing the mitochondrial suspension. Palmitoyl-CoA concentrations were varied from 2.80 to 47.6 μM in the

presence of 2.5 mM l-carnitine, while l-carnitine concentrations were varied from .25 to 8.00 mM in the presence of 28.0 μM palmitoyl-CoA. Cuvette two was identical except for the omission of l-carnitine. Reactions were started by the addition of 5 to 50 μl of mitochondrial suspension, usually 15 μl. The cuvette contents were mixed immediately, and monitoring of the reaction began as quickly as possible following mitochondrial addition. In general, the reaction is linear for only two to three minutes, thus requiring rapid manipulations. For the determination of Km values for palmitoyl-CoA and l-carnitine and the V max of the bovine hepatic CPT reaction, the initial reaction velocity data were analyzed using the Hanes-Woolf derivation of the Michaelis-Menten equation (Segel, 1976).

Statistical Analysis

Experiments were blocked according to tissue, treatment and time. Statistical evaluations were by analysis of variance and appropriate t-tests. Evaluations were conducted using the Genstat V statistical package (Lawes Agricultural Trust, Rothamsted Experimental Station).

RESULTS AND DISCUSSION

Due to its relative simplicity and widely reported use in a variety of metabolic studies with both rat and bovine tissues, Krebs-Ringer bicarbonate (KRB) buffer was selected as the basis for the incubation media in these studies. The incubation system was designed to measure the oxidation by liver slices of ^{14}C-labelled palmitic acid to $^{14}CO_2$ and ^{14}C-acid-soluble metabolites (ASM). The ^{14}C-ASM from a number of experiments were later characterized by high-performance liquid chromatography (HPLC). Recovery of added standard 1-^{14}C-acetate and 3-^{14}C-BHBA from HPLC analyses averaged between 95% and 97%. About 16% of the label in the ^{14}C-ASM was associated with the acetate fraction and 54% of the label with the BHBA fraction, based on the HPLC analyses. Acetoacetate was not determined in these chromatographic analyses, but if a BHBA:AcAc ratio of 5.9:1 is assumed for bovine hepatic ketogenesis, based on net production of BHBA and AcAc across the bovine liver (Lomax and Baird, 1983), AcAc could potentially account for about 9% of the label in the ^{14}C-ASM. Thus, about 63% of the label in ^{14}C-ASM was present as ketone bodies. The unaccounted label could be present as glucose (Singh *et al.*, 1982) or other

intermediates of the glycolytic pathway and TCA cycle, due to randomization of label through the TCA cycle.

Due to the ready availability of laboratory rats, in contrast to cows, initial studies were conducted with rat liver slices. Liver slices from weanling or young adult rats oxidized palmitic acid considerably faster than liver slices from mature rats (Table 1). Palmitate oxidation rates by the weanling and young adult rat liver slices were of comparable magnitude to the values given by Krebs et al. (1969). These results demonstrated the suitability of this incubation system for the determination of palmitic acid oxidation rates by the liver. All succeeding experiments with rat liver slices used young adult female rats (100-200 gms body weight) as liver donors.

Bovine liver slices were made from liver samples obtained either at slaughter or via biopsy. In general, liver slices from biopsy samples oxidized palmitic acid at faster rates than did slices from slaughter samples (Table 2). Frequently, however, slices from slaughter liver samples oxidized palmitic acid at rates comparable to those of slices from biopsy samples. The difference usually observed in palmitate oxidation rates between liver samples obtained at slaughter and via biopsy may be attributable to the length of time the liver remains in the animal following exsanguination before sampling occurs.

TABLE 1.--Influence of Rat Age on Palmitate Oxidation by Liver Slices.

	Palmitate Oxidized To			
	CO_2		Total Acid-Soluble Metabolites	
Incubation Time	1 Hr	3 Hr	1 Hr	3 Hr
	------- pmoles · min^{-1} · mg wet wt^{-1} -------			
Age/Body Weight				
Weanling/~40 gm	7.07±.18	6.04±.18	15.0±.37	13.6±.33
Young Adult/100-130 gm	5.10±.20	3.76±.19	11.2±.37	7.71±.36
Mature/750-1000 gm	.27±.03	.44±.05	2.33±.25	2.65±.24

Values are means ± S.E.M. for quadruplicate liver slices from 12 weanling and young adult rats and 3 mature rats. Weanling rats had been fasted for 24 hours and young adult and mature rats for 48 hours prior to experiment. Incubation procedures were as described in Methods. Media contained final concentrations of 2 mM palmitate, .5 mM BSA and 2 mM dl-carnitine.

TABLE 2.--Palmitate Oxidation by Bovine Liver Slices from Liver Obtained at Slaughter or Via Biopsy.

Liver Obtained At	Palmitate Oxidized To	
	CO_2	Total Acid-Soluble Metabolites
	- pmoles · min^{-1} · mg wet wt^{-1} -	
Slaughter	.493±.04	1.93±.10
Biopsy	1.17±.05	3.74±.15

Values are means ± S.E.M. for quadruplicate liver slices of five livers obtained at slaughter and eight liver samples obtained via biopsy from different cows. Incubation procedures were as described in Methods. Media contained final concentrations of 1 mM palmitate, .25 mM BSA and 2 mM dl-carnitine. Slices were incubated for 180 minutes.

Palmitate oxidation rates in this study (Table 2) are comparable to published values for palmitate oxidation by bovine and ovine liver slices (Mesbah and Baldwin, 1983; Taylor and Jackson, 1968), but represent only 5-10% of the *in vivo* fatty acid oxidation rate observed in bovine liver, based on calculations from data reported by Lomax and Baird (1983). Similar to the rat, mature bovine liver slices oxidized palmitic acid at slower rates than did slices from younger calf liver (Table 3), but the differences were not as dramatic nor as regular as observed with the rat.

TABLE 3.--Influence of Bovine Age on Palmitate Oxidation by Liver Slices.

	Palmitate Oxidized To			
	CO_2		Total Acid-Soluble Metabolites	
Incubation Time	1 Hr	3 Hr	1 Hr	3 Hr
	-------- pmoles · min^{-1} · mg wet wt^{-1} --------			
Age				
8-12 weeks, milk fed	1.16±.14	1.43±.12	4.48±.24	4.91±.59
9-12 months, 115-160 Kg Body Weight	---	.352±.025	---	1.36±.10
Mature, >24 mos	.609±.191	.607±.030	2.04±.12	2.93±.11

Values are means ± S.E.M. for quadruplicate liver slices of livers obtained from four calves (8-12 weeks), eight heifers (9-12 months) and seven mature cows. All animals were nonlactating Holsteins. Liver samples were obtained at slaughter, and liver slices incubated as described in Methods. Media contained final concentrations of 1 mM palmitate, .25 mM BSA and 2 mM dl-carnitine.

Covariate analysis (Appendix Table 4) indicated a highly significant relationship between liver slice wet weight and palmitate oxidation to CO_2 ($p<.001$) and ASM ($p<.001$) for liver slices from 40 to 210 mg wet weight. Significant differences were also found in the ability to oxidize palmitic acid among liver samples ($p<.01$ for CO_2 and $p<.025$ for ASM). These differences could in part be due to varying periods of elapsed time between exsanguination and liver removal from the carcass for the different liver samples. Alternatively, some of these liver samples may have had an inherently greater capacity for fatty acid oxidation, perhaps because of physiological or genetic considerations. Liver slices used in experiments were usually 100 to 180 mg wet weight. Results of a typical experiment (Figure 6) demonstrate linearity of palmitate oxidation over this range of liver slice wet weight.

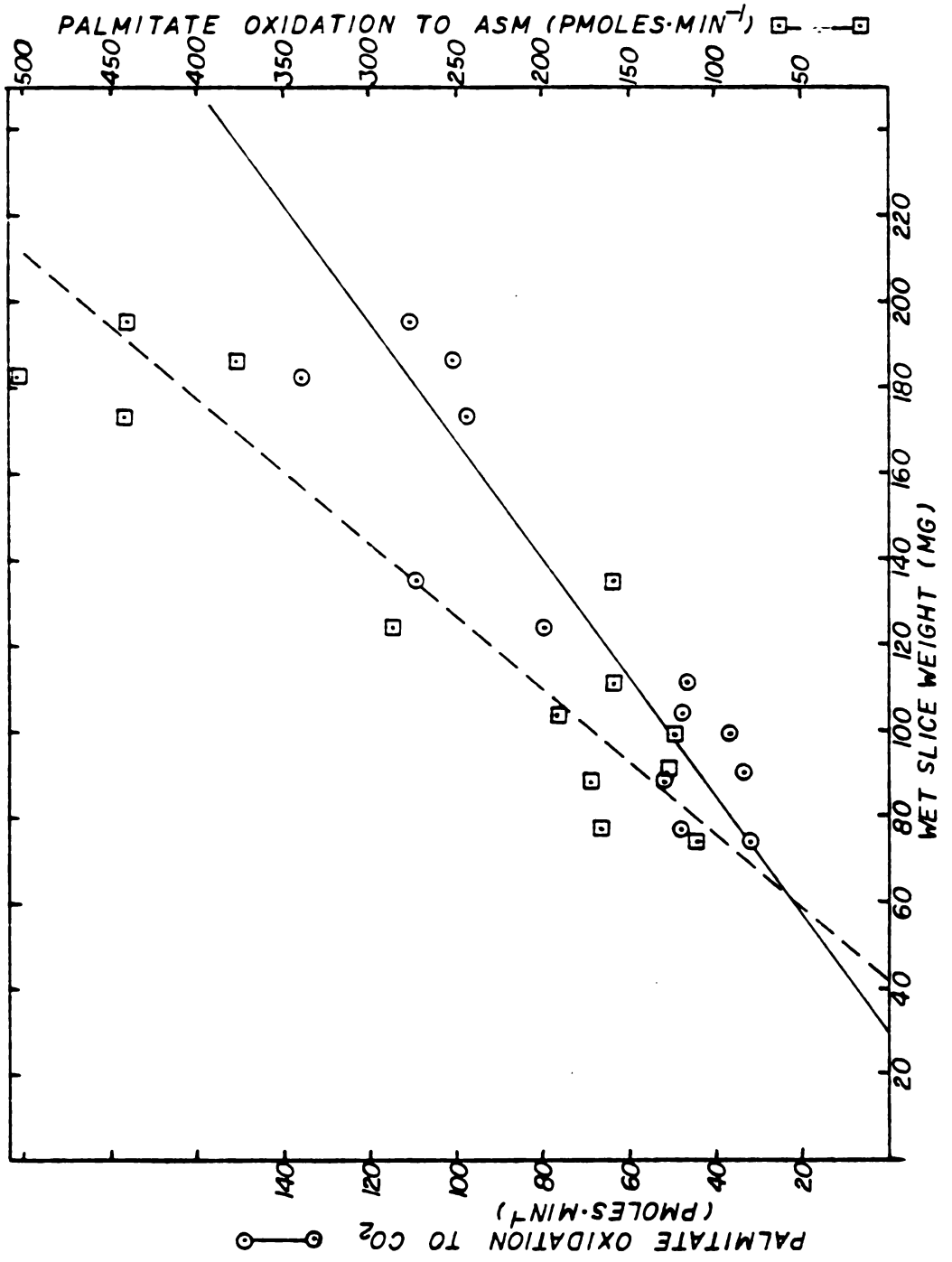
A potential problem encountered with bovine liver not found in rat liver is the possible effect that site of tissue sampling may have on palmitate oxidation rates. Rat livers were generally in the range of 3 to 6 gm, and were almost completely used during the preparation and slicing processes. Bovine liver, on the other hand, may be 6 to 7 Kg. A sample of only 250 to 300 gm may be obtained at slaughter, while only 10 to 12 gm can be obtained at biopsy. These relatively small samples may not be truly representative of the whole bovine liver.

Figure 6.--Relationship between palmitate oxidation by bovine liver slices and slice wet weight.

Data are results of one typical experiment. Each point represents a single observation made on one liver slice. Slices were incubated for 180 minutes as described in Methods. Media contained final concentrations of 1 mM palmitate, .25 mM BSA and 2 mM dl-carnitine. Linear regression equations calculated for palmitate oxidation to CO_2 and ASM were

$$Y = .7276X - 20.72, \quad r^2 = .7991,$$
$$\text{and } Y = 2.961X - 124.5, \quad r^2 = .8349,$$

respectively, where X= mg slice wet weight, and Y= pmoles palmitate oxidized$\cdot min^{-1}$. Complete statistical analysis is presented in Appendix Table 4.


PALMITATE OXIDATION TO ASM (PMOLES·MIN⁻¹)
500
450
400
350
300
250
200
150
100
50
WET SLICE WEIGHT (MG)
20
40
60
80
100
120
140
160
180
200
220
PALMITATE OXIDATION TO CO2
(PMOLES·MIN⁻¹)
140
120
100
80
60
40
20

To address this issue, samples were removed from the periportal and the lobular regions of the hepatic diaphrammatic lobe, i.e. the major liver lobe. The periportal region was defined as the area of the diaphrammatic lobe closest to the point of interconnection among the various liver lobes and the hepatic blood supply, while the lobular region was the area on the diaphrammatic lobe farthest removed from the periportal region. Little difference existed in palmitate oxidation by liver slices from these two hepatic regions (Table 4). All liver samples in subsequent experiments, whether obtained at slaughter or via biopsy, were from the lobular region of the diaphrammatic lobe. This experiment did not eliminate the possibility, however, that other areas of the bovine liver could exhibit different behavior with respect to fatty acid oxidation rates.

Palmitic acid is a major component of ruminant blood FFA, constituting as much as 25% of the total FFA (Bickerstaffe et al., 1974), and so was chosen as the fatty acid substrate for these experiments. Obtaining an aqueous solution of palmitic acid can be difficult, because of the hydrophobic nature of palmitic acid. Aqueous-based media containing labelled palmitic acid were prepared by two different methods. The first media, designated 4:1*, was as described in Materials and Methods. The second media (4:1S) was prepared in a manner identical

TABLE 4.--Palmitate Oxidation by Bovine Liver Slices from Two Regions of the Liver.

	Palmitate Oxidized To			
	CO_2		Total Acid-Soluble Metabolites	
Incubation Time	1 Hr	3 Hr	1 Hr	3 Hr
	pmoles · min^{-1} · mg wet wt^{-1}			
Sampling Site				
Periportal	.367±.031	.359±.031	.874±.093	.550±.054
Lobular	.543±.051	.392±.030	1.17±.12	.551±.057

Values are means ± S.E.M. for quadruplicate liver slices from two livers. Samples were collected from the periportal and lobular regions (defined in the text) of the diaphrammatic lobe of each liver. Incubation procedures were as described in Methods. Media contained final concentrations of 2 mM palmitate and .5 mM BSA. No dl-carnitine was added.

to 4:1*, except that palmitic acid dissolved in ethanol was used instead of the palmitic acid:BSA mixture. Media was pipetted into individual incubation flasks, followed by the addition of 10 µl of ethanol containing labelled palmitic acid of known specific activity. Palmitate completely dissolved in the incubation media during the 60 minute preincubation preceding addition of liver slices to the flask. Palmitate oxidation rates were slightly faster with 4:1* media (Table 5). In view of the faster oxidation rates and the greater ease of preparation, 4:1* became the standard method of substrate presentation.

Fatty acid availability is thought to play a major role in regulating hepatic oxidation rates in vivo, so that increased in vitro palmitate oxidation rates by liver slices would be expected in response to increased palmitate concentrations in the media. Palmitate oxidation rates by bovine liver slices changed little in response to increasing palmitate concentrations (Table 6). High palmitic acid concentrations (2.0 mM) appeared to inhibit oxidation slightly, suggesting a toxic effect of the high fatty acid concentration on the liver slices (Newsholme and Start, 1976). This series of incubations did not contain added carnitine, however, so that the liver slices may have been limiting in carnitine and thus been unable to respond to the higher plamitic acid concentrations. A 1.0 mM concentration of palmitic

TABLE 5.--Oxidation by Rat Liver Slices of Palmitate Prepared in Two Different Methods.

	Palmitate Oxidized To			
	CO_2		Total Acid-Soluble Metabolites	
Incubation Time	1 Hr	3 Hr	1 Hr	3 Hr
	pmoles · min^{-1} · mg wet wt^{-1}			
Method of Palmitate Preparation				
4:1*	3.10±.22	2.97±.29	13.0±.96	7.69±.66
4:1S	1.32±.09	1.24±.12	10.0±.92	6.42±.46

Values are means ± S.E.M. of quadruplicate liver slices from five livers. Substrate preparation is described in detail in the text, but involved addition of either a palmitate:BSA complex (4:1*) or palmitate in ethanol (4:1S) to media. Incubation procedures were as described in Methods. Media contained final concentrations of 2 mM palmitate, .5 mM BSA and 1 mM dl-carnitine.

TABLE 6.--Palmitate Oxidation by Bovine Liver Slices in the Presence of Increasing Palmitate Concentrations.

	Palmitate Oxidized To			
	CO_2		Total Acid-Soluble Metabolites	
Incubation Time	1 Hr	3 Hr	1 Hr	3 Hr
[Palmitate] mM	pmoles · min^{-1} · mg wet wt^{-1}			
.5	.111±.012	.189±.018	.239±.050	.232±.038
1.0	.155±.009	.206±.010	.269±.024	.243±.022
1.5	.149±.015	.196±.008	.248±.090	.222±.026
2.0	.162±.010	.186±.008	.187±.045	.206±.028

Values are means ± S.E.M. of quadruplicate liver slices from three livers. Incubation procedures were as described in Methods. Palmitate:BSA ratio was maintained at 4:1, resulting in BSA concentrations of .125, .25, .375 and .5 mM. No dl-carnitine was added.

acid was selected as the standard concentration in subsequent experiments, since this FFA concentration is often observed in fasted or ketotic cows and sheep (Bergman, 1971).

Addition of increasing dl-carnitine concentrations to the incubation media stimulated palmitate oxidation rates by bovine liver slices, with 2 mM dl-carnitine giving maximal stimulation (Table 7). Since l-carnitine is the naturally occurring carnitine isomer, a comparison was made between the ability of l- and dl-carnitine to stimulate palmitate oxidation. Little difference was found in palmitate oxidation rates in the presence of equal amounts of l-carnitine supplied as either l- or dl-carnitine (Table 7). Thus, 2 mM dl-carnitine was routinely utilized in subsequent experiments to provide maximum stimulation of palmitate oxidation, since it was the least costly of the two carnitine isomers.

Palmitate oxidation was also stimulated when the incubation flasks were gassed for 15 seconds with O_2:CO_2 (95:5) immediately following addition of a liver slice to an incubation flask (Table 8). Oxygen:CO_2 was forced into the airspace above the media within individual incubation flasks. Gassing was incorporated into the standard incubation procedure. An additional 15 second period of O_2:CO_2 gassing at the start of the 60 minute preincubation

TABLE 7.--Palmitate Oxidation by Bovine Liver Slices in the Presence of Carnitine: Carnitine Concentration Dependence and Comparison Between l- and dl-Carnitine.

Addition		Palmitate Oxidized To	
		CO_2	Total Acid-Soluble Metabolites
	mM	- pmoles · min^{-1} · mg wet wt^{-1} -	
None		.209±.009	.301±.035
dl-Carnitine	1	.306±.024	1.01±.13
	2	.311±.024	1.17±.14
	4	.287±.017	1.15±.12
	8	.285±.022	1.25±.14
None		.595±.032	1.29±.08
dl-Carnitine	2	1.54±.20	5.89±.13
l-Carnitine	1	1.61±.18	6.35±.32

Values are means ± S.E.M. for quadruplicate liver slices from seven livers for the determination of dl-carnitine concentration dependence and two livers for the comparison between l- and dl-carnitine. Incubation times were 180 minutes and 60 minutes for the two experiments, respectively. Media contained final concentrations of 1 mM palmitate and .25 mM BSA. All other conditions were as described in Methods.

TABLE 8.--Palmitate Oxidation by Bovine Liver Slices with and without Gassing of Incubation Flasks with Oxygen:Carbon Dioxide.

Treatment	Palmitate Oxidized To	
	CO_2	Total Acid-Soluble Metabolites
	- pmoles · min^{-1} · mg wet wt^{-1} -	
No Gas	.158±.017	.829±.081
Gas	.371±.048	1.35±.18

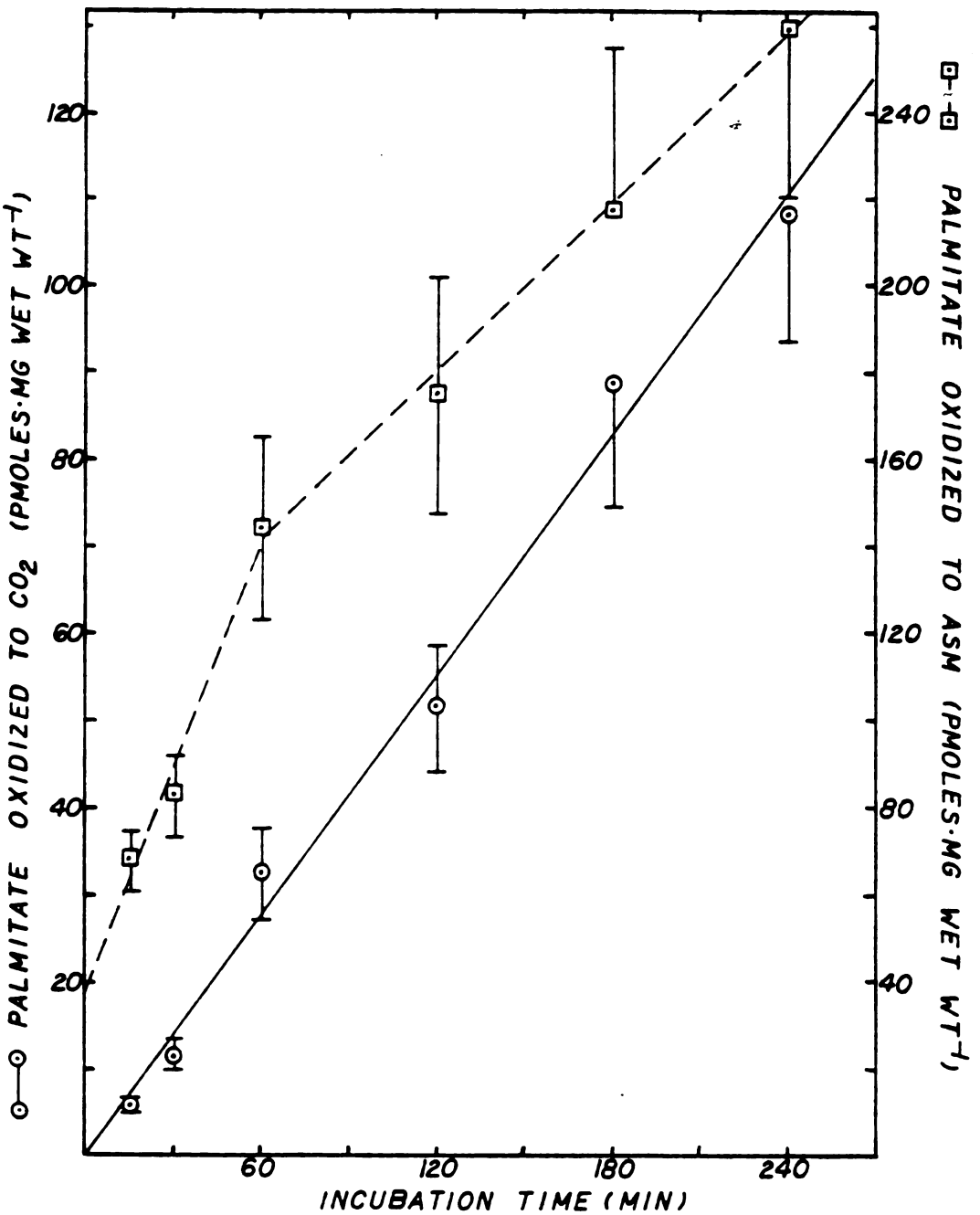
Values are means ± S.E.M. of quadruplicate liver slices from four livers. Oxygen:CO_2 (95:5) was blown for 15 seconds into the airspace above the media within the incubation flasks immediately after addition of liver slices, and the flasks instantaneously sealed with rubber serum stoppers. Media contained final concentrations of 1 mM palmitate, .25 mM BSA and 2 mM dl-carnitine. Incubation time was 180 minutes, and all other conditions as described in Methods.

before addition of liver slices to the flasks produced no further stimulation of palmitate oxidation rates.

To assess the linearity of palmitate oxidation with respect to time, the time-course of palmitate oxidation by bovine liver slices was analyzed using orthogonal polynomial contrasts (Appendix Table 5). Palmitate oxidation to CO_2 exhibited a significant ($p<.001$) linear effect over the time period from 15 to 240 minutes (Figure 7), while palmitate oxidation to ASM displayed both significant linear ($p<.001$) and quadratic ($p<.05$) effects. Over the time period from 15 to

Figure 7.--Time-course of palmitate oxidation by bovine liver slices.

Each point represents the mean ± S.E.M. for quadruplicate liver slices from four livers. Slices were incubated for the indicated time periods as described in Methods. Media contained final concentrations of 1 mM palmitate, .25 mM BSA and 2 mM dl-carnitine. Complete statistical analysis is presented in Appendix Table 5.


PALMITATE OXIDIZED TO CO_2 (PMOLES·MG WET WT^{-1})
PALMITATE OXIDIZED TO ASM (PMOLES·MG WET WT^{-1})
INCUBATION TIME (MIN)
20
40
60
80
100
120
40
80
120
160
200
240
60
120
180
240

60 minutes, palmitate oxidation to ASM was linear (Figure 7). From 60 to 240 minutes palmitate oxidation to ASM continued at a linear, albeit reduced, rate. Significant liver ($p<.001$) and liver by time ($p<.001$) effects were also observed for palmitate oxidation to both CO_2 and ASM (Appendix Table 5), further corroborating the variability in ability to oxidize palmitic acid among individual liver samples.

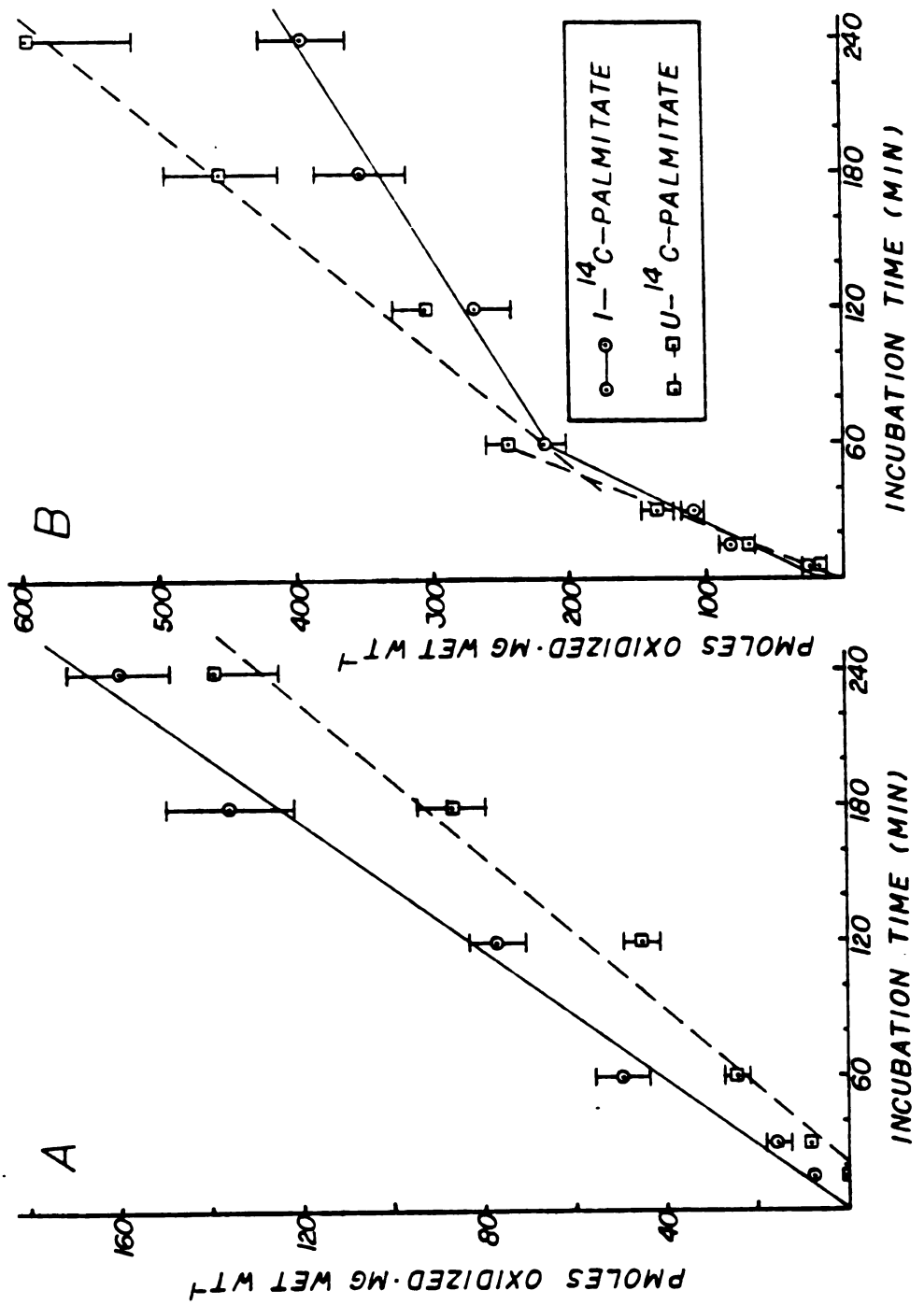
A comparison was made between the oxidation rates of 1-^{14}C- and U-^{14}C-palmitate to assess the extent to which the palmitate molecule was oxidized by bovine liver slices. Stanley and Tubbs (1975) demonstrated the accumulation of saturated acyl-CoA intermediates in rat liver mitochondria which were oxidizing palmitoyl-carnitine, the acyl-CoA intermediates presumably resulting from the incomplete oxidation of palmitate through β-oxidation. Palmitate [1-^{14}C] should be oxidized at a greater rate than U-^{14}C-palmitate, the difference in oxidation rates giving some indication of the completeness of palmitate oxidation through β-oxidation. Bovine liver slices oxidized 1-^{14}C-palmitate to CO_2 at greater rates ($p<.001$) than U-^{14}C-palmitate over a 240 minute incubation, with average values of 65.5 and 43.1 pmoles·mg^{-1} for the 1-^{14}C- and U-^{14}C-palmitate, respectively (Appendix Table 6). During the same incubation, however, U-^{14}C-palmitate oxidation to ASM (269 pmoles·mg^{-1}) was greater

($p<.001$) than 1-^{14}C-palmitate oxidation (207 pmoles·mg^{-1}). Oxidation of both the 1-^{14}C- and U-^{14}C-palmitate to CO_2 was linear over the entire incubation period (Figure 8A). Linear oxidation rates to ASM were also observed for both labelled palmitates through 60 minutes of incubation, with little apparent difference in oxidation rates (Figure 8B). After 60 minutes of incubation, oxidation of both labelled substrates continued at linear, albeit reduced, rates. Palmitate [1-^{14}C] oxidation to ASM decreased more than did U-^{14}C-palmitate oxidation, however, resulting in the greater overall oxidation rates to ASM for U-^{14}C-palmitate. The greater oxidation of 1-^{14}C-palmitate to CO_2 is in agreement with previously published results (Harper and Saggerson, 1976). Reasons for the (seemingly anomalous) greater oxidation of U-^{14}C-palmitate to ASM are not clear, although it should be emphasized that these observations of 1-^{14}C- and U-^{14}C-palmitate oxidation are based on relatively limited data.

The possibility that peroxisomal β-oxidation may contribute to total hepatic fatty acid oxidation has not been investigated in bovine liver. The potential contribution that peroxisomal β-oxidation may make to bovine hepatic fatty acid oxidation was estimated by the inclusion of KCN in the incubation media. In rat liver, KCN will completely inhibit mitochondrial β-oxidation by inhibiting electron transport, but

Figure 8.--Time-course of 1-^{14}C-palmitate and U-^{14}C-palmitate oxidation by bovine liver slices.

(A) Palmitate oxidation to CO_2. (B) Palmitate oxidation to acid-soluble metabolites. Each point represents the mean ± S.E.M. for quadruplicate liver slices from two livers. Slices were incubated for the indicated time periods as described in Methods. Media contained final concentrations of 1 mM palmitate, .25 mM BSA and 2 mM dl-carnitine. Complete statistical analysis is presented in Appendix Table 6.


A
B
PMOLES OXIDIZED·MG WET WT⁻¹
INCUBATION TIME (MIN)
40
80
120
160
100
200
300
400
500
600
60
120
180
240
l—14C-PALMITATE
U-14C-PALMITATE

will not affect peroxisomal β-oxidation (Tolbert, 1980). Cyanide completely inhibited $^{14}CO_2$ formation by bovine liver slices, indicating the total inhibition of mitochondrial β-oxidation (Table 9). Some palmitate oxidation to ASM (.38 pmoles·min^{-1}.mg wet wt^{-1}) occurred in the presence of KCN, amounting to 6 to 7% of the palmitate conversion to ASM observed in the absence of KCN. These results suggested that bovine liver peroxisomes may contribute to the total hepatic fatty acid oxidation to ASM.

Palmitate oxidation was examined in a number of different in vitro bovine liver preparations for comparison to oxidation by liver slices. Isolated hepatocytes

TABLE 9.--Palmitate Oxidation by Bovine Liver Slices in the Presence or Absence of Potassium Cyanide.

	Palmitate Oxidized To	
Additions	CO_2	Total Acid-Soluble Metabolites
	- pmoles · min^{-1} · mg wet wt^{-1} -	
None	.88±.11	5.65±.69
KCN	N.D.	.38±.11

Values are means ± S.E.M. of quadruplicate liver slices from two livers. Media contained final concentrations of 1 mM palmitate, .25 mM BSA, 2 mM dl-carnitine and, when present, 2 mM KCN. Incubation conditions were as described in Methods. Incubation time was 60 minutes. N.D. = Not Detectable.

have been widely utilized for metabolic studies in the rat, but only recently have similar reports appeared using ruminant hepatocytes (Clark et al., 1976; Forsell et al., 1984; Lomax et al., 1983a,b; Pocius et al., 1983). Both palmitate oxidation and gluconeogenesis were determined in several preparations of bovine hepatocytes and slices made from the same liver. Isolated hepatocytes oxidized palmitic acid at greater rates than liver slices (Table 10). Bovine hepatocytes in this study (Table 10) oxidized palmitic acid at only one-tenth the rate reported for sheep hepatocytes (Lomax et al., 1983a,b). The difference in palmitate oxidation rates between bovine hepatocytes and liver slices (~3.4-fold) was not as large as expected. A 12.6-fold difference in long-chain fatty acid oxidation rates was reported between rat liver slices and perfused rat liver, a system which gives similar results to isolated rat hepatocytes (Krebs et al., 1969). In contrast to the difference in palmitate oxidation rates, similar glucose production and gluconeogenic rates were exhibited by bovine liver slices and hepatocytes (Table 11). Gluconeogenic rates from propionate and lactate plus pyruvate by bovine hepatocytes were of comparable magnitude to those observed in lamb hepatocytes (Clark et al., 1976). Glucose production and gluconeogenic rates observed in this study with bovine liver slices (Table 11) were similar to rates reported by Mesbah and Baldwin (1983)

TABLE 10.--Comparison of Palmitate Oxidation Rates Between Bovine Liver Slices and Isolated Bovine Hepatocytes.

Liver Preparation	Palmitate Oxidized To	
	CO_2	Total Acid-Soluble Metabolites
	-- pmoles $\cdot$ min^{-1} $\cdot$ mg wet wt^{-1} ---	
Slices - wet wt basis	.337±.042	1.10±.19
dry wt basis	3.32±.58	11.6±2.4
Hepatocytes - dry wt basis	14.1±2.5	39.0±5.9

Values are means ± S.E.M. for quadruplicate incubations of liver slices and isolated hepatocytes from five livers. Slices and hepatocytes were prepared from the same livers. Media contained final concentrations of 1 mM palmitate, .25 mM BSA and 2 mM dl-carnitine. Incubation time was 180 minutes.

TABLE 11.--Comparison of Gluconeogenic Rates Between Bovine Liver Slices and Isolated Bovine Hepatocytes.

Addition	Concentration	Liver Preparation	
		Slices	Hepatocytes
	mM	μmoles glucose released·min^{-1}·mg dry wt^{-1}	
None		5.28	4.54
Lactate + Pyruvate[a]	10+1	.924	1.18
Propionate[a]	10	2.18	.57

Values are means of two experiments, except for liver slices plus propionate, with only one experiment. Incubation conditions were as described in Methods. Slices and hepatocytes were prepared from the same liver. Incubation time was 60 minutes.

[a]Net gluconeogenic rates from the added substrate corrected for glucose production rates observed in the absence of added substrate.

for bovine liver slices. That bovine liver slices were unable to oxidize palmitic acid as rapidly as did isolated hepatocytes, but exhibited similar gluconeogenic rates to the hepatocytes, may be due to the greater ease with which propionate, lactate and pyruvate diffused into the liver slice compared to long-chain fatty acids.

A second series of experiments compared palmitate oxidation by liver slices with liver snips. Liver snips are small (a few cubic millimeters in volume) organized pieces of tissue which, unlike liver slices, can be utilized for both short- and long-term incubations (Pollard and Dutton, 1982). Liver snips were prepared by the following procedure: (1) a section was cut from a block of liver using a Stadie-Riggs microtome set to give .5 mm thick sections, individual liver sections were then (2) weighed on a double-pan torsion balance, (3) laid flat on a teflon board, and (4) cut into liver snips approximately 1 to 2 mm square with a scalpel, and (5) the liver snips were transferred into incubation flasks. All other procedures were identical to the liver slice incubations. Palmitate oxidation by the liver snips was inferior to that of liver slices (Table 12). The marked increase in surface area available for fatty acid penetration which could have resulted in greater oxidation rates for the liver snips may have been offset by the additional trauma of preparation. Liver

TABLE 12.--Comparison of Palmitate Oxidation Rates Between Bovine Liver Slices and Liver Snips.

Liver Preparation	Palmitate Oxidized To	
	CO_2	Total Acid-Soluble Metabolites
	- pmoles · min^{-1} · mg wet wt^{-1} -	
Slices	.730±.088	3.80±.53
Snips	.347±.035	1.84±.27

Values are means ± S.E.M. for quadruplicate incubations of liver slices or snips from five livers. Slices and snips were prepared from the same livers. Procedure for liver snip preparation is discussed in the text. Media contained final concentrations of 1 mM palmitate, .25 mM BSA and 2 mM dl-carnitine. Incubation time was for 60 to 120 minutes.

snips could be placed into long-term culture under proper conditions, however, and might exhibit some recovery in ability to oxidize long-chain fatty acids.

Regulation of fatty acid oxidation and ketogenesis has been widely studied in isolated rat liver mitochondria (e.g. Lopes-Cardozo et al., 1975), but relatively little information is available concerning ruminant liver mitochondria. Palmitate oxidation by isolated bovine liver mitochondria was linear from 2 to 15 minutes of incubation (Figure 9), but proved highly variable among different mitochondrial preparations. Bovine liver mitochondria actively oxidized palmitic acid (Figure 9,

Figure 9.--Time-course of palmitate oxidation by isolated bovine liver mitochondria.

Each point represents the mean ± S.E.M. for triplicate incubations of mitochondrial preparations from three livers. Mitochondria were incubated for the indicated times as described in Methods. Complete statistical analysis is presented in Appendix Table 7.

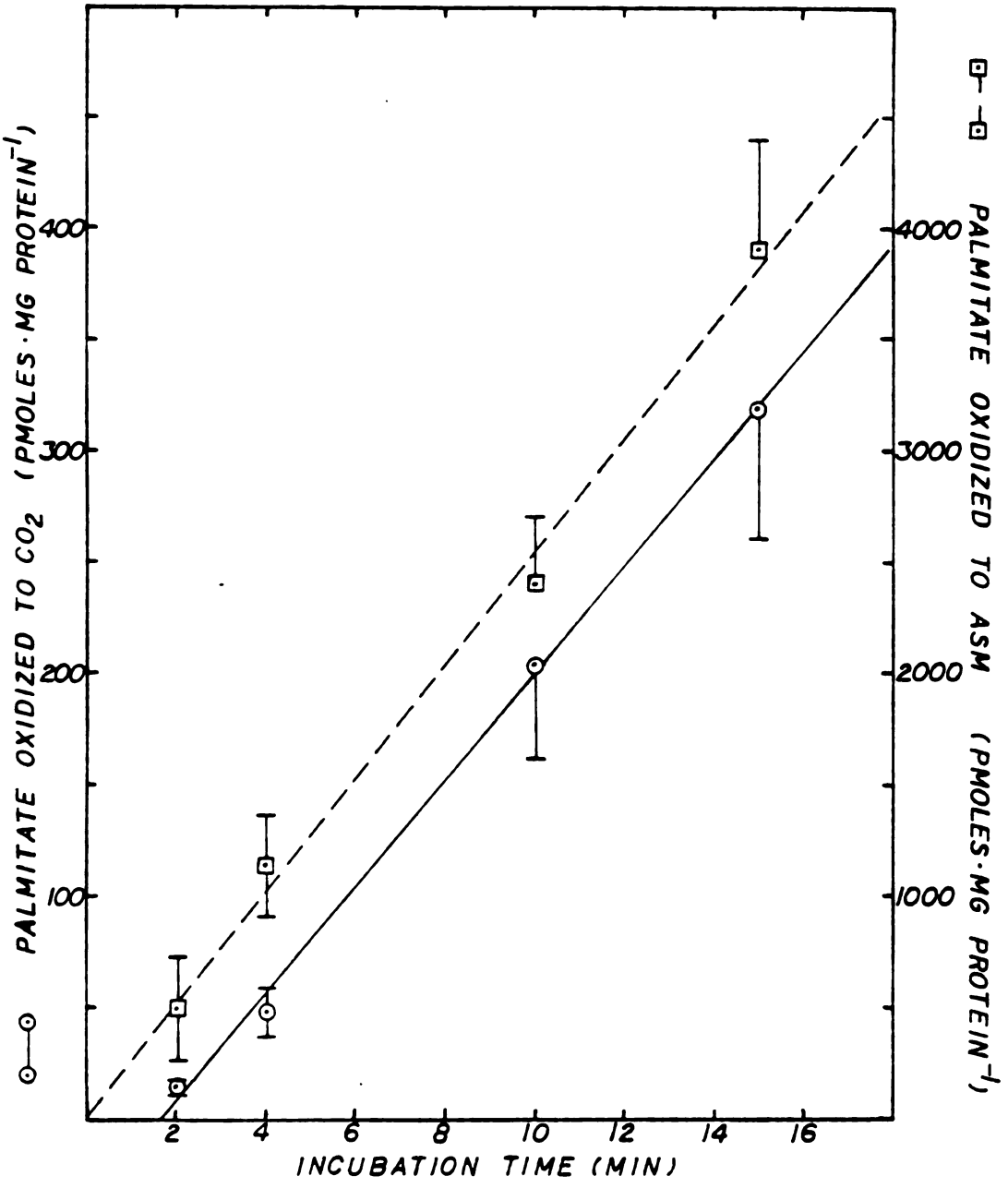

PALMITATE OXIDIZED TO CO_2 (PMOLES·MG PROTEIN^{-1})
PALMITATE OXIDIZED TO ASM (PMOLES·MG PROTEIN^{-1})
100
200
300
400
1000
2000
3000
4000
2
4
6
8
10
12
14
16
INCUBATION TIME (MIN)

Table 13), in contrast to results obtained with sheep liver mitochondria (Koundakjian and Snoswell, 1970). Palmitate oxidation by rat liver mitochondria was greater than that by bovine liver mitochondria, but the differences were not as pronounced as those observed between rat and sheep liver mitochondria (Koundakjian and Snoswell, 1970).

Dinitrophenol (DNP) is an uncoupler of mitochondrial oxidative phosphorylation. Treatment of rat liver mitochondria with DNP results in an increased mitochondrial respiration rate and a decreased energy charge within the mitochondrial matrix. Potential changes of palmitate oxidation in response to DNP treatment were examined in bovine liver slices and isolated liver mitochondria.

TABLE 13.--Comparison of Palmitate Oxidation Rates Between Bovine and Rat Liver Mitochondria.

Mitochondrial Source	Palmitate Oxidized To	
	CO_2	Total Acid-Soluble Metabolites
	pmoles·min^{-1}·mg mitochondrial protein^{-1}	
Bovine	10.8±2.2	260.0±48.5
Rat	20.0±2.2	318.2±8.8

Values are means ± S.E.M. of triplicate incubations for mitochondria from three bovine livers and pooled mitochondria from three rat livers. Media contained final concentrations of 35 µM palmitate, .7% BSA, 100 µM l-carnitine, 4 mM ATP, 1 mM ADP, 50 µM CoASH and 250 µM reduced glutathione. Each flask contained 1.25 mg mitochondrial protein. Mitochondria were incubated for four minutes as described in Methods.

Dinitrophenol proved to be a potent inhibitor of palmitate oxidation by bovine liver slices (Table 14). Acid-soluble metabolite formation from palmitate was inhibited at all DNP concentrations, while palmitate oxidation to CO_2 was decreased by all concentrations except .3 mM DNP, this latter an apparently anomalous response. The anticipated response to DNP treatment was to have been an increase in palmitate oxidation rates. Since DNP uncouples oxidative phosphorylation, reducing the energy charge within the mitochondrial matrix, ultimately the cellular energy charge would be reduced. The end result would be reduced ATP availability for fatty acid activation and inhibition

TABLE 14.--Palmitate Oxidation by Bovine Liver Slices in the Presence of Increasing Concentrations of Dinitrophenol.

		Palmitate Oxidized To	
Addition		CO_2	Total Acid-Soluble Metabolites
	mM	- pmoles · min^{-1} · mg wet wt^{-1} -	
None		1.16±.05	7.36±.33
DNP	.1	1.03±.09	7.01±.39
	.3	1.44±.17	6.30±.48
	.5	.75±.04	1.36±.04

Values are means ± S.E.M. of quadruplicate liver slices from one liver. Media contained final concentrations of 1 mM palmitate, .25 mM BSA and 2 mM dl-carnitine, while incubation time was 60 minutes.

of fatty acid oxidation. Isolated bovine liver mitochondria were incubated in the presence of exogenous ATP, providing for palmitate activation in the presence of DNP. Dinitrophenol had no effect on palmitate oxidation to CO_2 by isolated bovine liver mitochondria (Table 15), while palmitate oxidation to ASM was actually stimulated at low DNP concentrations. Higher DNP concentrations (.12 mM) decreased ASM formation to near control values. These results suggest that factors other than a simple decrease in mitochondrial energetic state are apparently involved in regulation of hepatic mitochondrial fatty acid oxidation.

Several different in vivo manipulations of the liver donor animals were examined for potential effects on subsequent palmitate oxidation by liver slices. The manipulations included fasting, changes in dietary composition, and liver sampling at increasing time intervals from parturition. Liver preparations from the fasted rat display an inherently greater capacity for fatty acid oxidation than do similar preparations from fed rats (McGarry and Foster, 1980). Liver biopsies were obtained from five Holstein cows before and after a seven day fast (one cow was fasted for five days) to determine potential effects of fasting on bovine hepatic fatty acid oxidation. Palmitate oxidation to CO_2 by bovine liver slices was actually decreased after a

TABLE 15.--Palmitate Oxidation by Bovine Liver Mitochondria in the Presence of Exogenous ATP and Increasing Concentrations of Dinitrophenol.

Addition		Palmitate Oxidized To	
		CO_2	Total Acid-Soluble Metabolites
		- pmoles·min^{-1}·mg mitochondrial protein^{-1} -	
None		12.5±3.1	345±36
DNP	.03	12.0±3.5	476±78
	.06	11.2±2.5	419±49
	.12	12.0±3.6	384±46

Values are means ± S.E.M. of triplicate incubations of mitochondria isolated from two livers. Media contained final concentrations of 35 µM palmitate, .7% BSA, 100 µM l-carnitine, 4 mM ATP, 1 mM ADP, 50 µM CoASH and 250 µM glutathione. Each flask contained 1.25 mg mitochondrial protein. Mitochondria were incubated for four minutes as described in Methods.

prolonged fast (Table 16), possibly due to decreased availability of TCA cycle intermediates. This has been observed in bovine liver under similar circumstances (Baird et al., 1979). Oxidation of palmitic acid to ASM, however, was essentially unchanged following prolonged fasting. Liver samples from the fasted cows were noticeably paler in color, indicating some degree of fatty infiltration. Insufficient amounts of tissue precluded an assay for liver fat content. Sufficient liver was obtained from three cows before and after the fast for determination of hepatic protein content. No change in protein content was observed in the liver in response to the fast. Hepatic protein contents were 222 and 228 mg protein/gm wet weight for liver before and after fasting, respectively, so that correction of palmitate oxidation rates to account for alteration of hepatic fat content would not alter the results (Table 16). These data are supported by observations with isolated sheep hepatocytes, where palmitate oxidation in the presence of carnitine was the same whether the hepatocytes had been isolated from fed or fasted sheep (Lomax et al., 1983a).

Lipid metabolism in the high-producing dairy cow can be drastically modified by changing the dietary composition from that of a typical production ration, with a roughage:concentrate ratio of 40:60 or greater,

TABLE 16.--Palmitate Oxidation by Liver Slices from Fed and Fasted Cows.

	Palmitate Oxidized To			
	CO_2		Total Acid-Soluble Metabolites	
Incubation Time	1 Hr	3 Hr	1 Hr	3 Hr
	pmoles $\cdot$ min^{-1} $\cdot$ mg wet wt^{-1}			
State				
Fed	1.32±.02	1.25±.10	5.87±.27	3.84±.16
Fasted	.789±.004	.528±.029	5.69±.32	3.27±.19

Values are means ± S.E.M. for quadruplicate liver slices from five livers. Liver samples were obtained via biopsy from the same cows before and after a five to seven day fast. Milk production was 13.1 and 4.4 Kg/day pre- and post-fast, respectively. Media contained final concentrations of 1 mM palmitate, .25 mM BSA and 2 mM dl-carnitine. Incubation conditions were as described in Methods.

to that of a restricted roughage ration, where the roughage:concentrate ratio may be 20:80 or less. The most obvious manifestation of the changes in lipid metabolism is a decrease in milk fat concentrations (Bell, 1980). Other less obvious modifications include decreases in the proportions of saturated and concomitant increases in proportions of unsaturated fatty acids in the blood, mammary tissue and milk lipids, increased lipoprotein lipase and glycerolipid synthesizing activities in adipose tissue, and a general increase in the flux of fatty acids towards adipose tissue and away from the mammary gland (Askew et al., 1971; Benson et al., 1972). Such drastic changes in whole-body fatty acid metabolism might also be expected to produce alterations in hepatic fatty acid metabolism. To investigate this possibility, liver biopsies were obtained from four lactating Holstein cows before and after the cows had been accustomed to a restricted roughage, high concentrate ration. The cows had been consuming the restricted roughage ration for approximately three weeks prior to the second biopsy. The restricted roughage ration consisted of (dry matter basis) 1.2 Kg alfalfa hay, 1.6 Kg corn silage and 14.3 Kg of a concentrate mix (Table 17). One cow was in early lactation, producing 30.7 Kg milk with a fat test of 3.6%, which changed to 26.0 Kg milk testing 2.9% fat following three weeks of restricted roughage feeding. The other

TABLE 17.--Composition of the Concentrate Mix Fed to Induce Milk Fat Depression in Lactating Holstein Cows.

Ingredient	% of Dry Matter
High Moisture Shelled Corn	70.0
Soybean Meal - 44%	14.0
Commercial Protein Supplement - 18%	14.0
Dicalcium Phosphate	.75
Limestone	.75
Trace Mineralized Salt	.50
	100.0

Calculated net energy for lactation of this concentrate mix was 1.78 Mcal/Kg.

three cows were in late lactation, producing less than 16.5 Kg milk/day, and exhibited little change in milk fat test in response to restricted roughage feeding. Calculated energy requirements were 24.1 and 20.8 Mcal/day for the cows before and after the ration changeover. Energy intake was calculated only for the restricted roughage ration (29.6 Mcal/day). Palmitate oxidation to $C0_2$ by liver slices was essentially unchanged by dietary treatment, but oxidation to ASM was markedly depressed after feeding the restricted roughage, high concentrate ration (Table 18). Similar results were reported by Aiello and Herbein (1983), who observed

TABLE 18.--Palmitate Oxidation by Liver Slices from Cows Before and After Feeding a Restricted Roughage Ration

	Palmitate Oxidized To			
	CO_2		Total Acid-Soluble Metabolites	
Incubation Time	1 Hr	3 Hr	1 Hr	3 Hr
Ration	-------- pmoles · min^{-1} · mg wet wt^{-1} ---------			
Typical	1.42±.07	1.21±.12	6.50±.29	4.02±.23
Restricted Roughage	1.31±.05	1.26±.07	4.88±.18	3.20±.10

Values are means ± S.E.M. for quadruplicate liver slices from four livers. Liver samples were obtained via biopsy from the same cows before and after adaptation to the restricted roughage/high concentrate ration. Daily milk production and fat test were 18.4 Kg and 4.6%, and 15.6 Kg and 3.8%, respectively, before and after adaptation to the ration. Media contained final concentrations of 1 mM palmitate, .25 mM BSA and 2 mM dl-carnitine.

decreased ketogenesis by liver slices from early lactation Holstein cows following adaptation to a restricted roughage, high concentrate ration.

High-producing dairy cows can encounter a severe negative energy balance during early lactation when maximal milk production is attained but energy intake is still limiting. Susceptibility to spontaneous ketosis is also greatest at this time. Little information is available, however, concerning potential changes in the hepatic fatty acid oxidative capacity during this period. Liver samples were obtained at slaughter from lactating Holstein cows at predetermined times after parturition (days 28, 42 and 56). Palmitate oxidation rates were highest at 42 days postpartum (Table 19). Similar results were obtained by Aiello and Herbein (1983), although these researchers found maximum palmitate oxidation rates by bovine liver slices at 30 days postpartum. These results are in contrast to those of Whitelaw and Williamson (1977), who found an inherently lower capacity for fatty acid oxidation and ketogenesis in liver from rats at peak lactation than by liver from rats in other physiological states. Maximum gluconeogenic rates by bovine liver slices were also observed at 30 days postpartum (Aiello and Herbein, 1983), suggesting a possible relationship between ketogenesis and gluconeogenesis in the bovine similar to that observed

TABLE 19.--Palmitate Oxidation by Liver Slices from Lactating Holstein Cows Slaughtered at Different Times Postpartum.

Days Postpartum	Palmitate Oxidized To	
	CO_2	Total Acid-Soluble Metabolites
	-- pmoles $\cdot$ min^{-1} $\cdot$ mg wet wt^{-1} --	
28	.186±.006	.202±.017
42	.286±.018	.481±.061
56	.294±.026	.394±.022

Values are means ± S.E.M. of quadruplicate liver slices for livers obtained from two cows each at 28 and 42 days postpartum and from four cows at 56 days postpartum. Media contained final concentrations of 1 mM palmitate and .25 mM BSA. No dl-carnitine was included. Slices were incubated for 180 minutes as described in Methods.

in the rat and guinea pig liver (Tutwiler and Brentzel, 1982; Tutwiler and Dellevigne, 1979). If ketogenesis does exert a permissive effect on gluconeogenesis in the bovine liver, then the increased hepatic ketogenic capacity during early lactation could be in response to the high demand for glucose at this time, and the consequent increase in hepatic gluconeogenic capacity.

Partitioning between esterification and glycerolipid synthesis on the one hand, and transport into mitochondria for oxidation represents a major branch-point for FA-CoA utilization in the liver. Accordingly, a number of treatments which have been demonstrated to

increase fatty acid esterification rates in rat liver were examined for their potential effect on palmitate oxidation rates in bovine liver slices. Glucose inhibited palmitate oxidation to both CO_2 and ASM (Table 20), presumably by increasing intracellular concentrations of G3P, thereby increasing palmitate esterification rates and decreasing palmitate availability for oxidation. Insulin also decreased palmitate oxidation rates, possibly by increasing the activity of glycerol acyltransferases, or increasing glycolytic activity and thereby intracellular G3P concentrations. The combination of insulin plus glucose

TABLE 20.--Palmitate Oxidation by Bovine Liver Slices Incubated With and Without Glucose, Insulin or a Combination of Glucose Plus Insulin.

Addition	Palmitate Oxidized To	
	CO_2	Total Acid-Soluble Metabolites
	-- pmoles $\cdot$ min^{-1} $\cdot$ mg wet wt^{-1} --	
None	.610±.025	2.14±.17
Glucose	.446±.022	1.76±.11
Insulin	.446±.018	1.65±.07
Glucose + Insulin	.389±.020	1.44±.09

Values are means ± S.E.M. of quadruplicate liver slices from five livers. Media contained final concentrations of 1 mM palmitate, .25 mM BSA, 2 mM dl-carnitine, and, when present, 10 mM glucose and .1 U/ml insulin. Incubation time was 180 minutes.

proved a more effective inhibitor of palmitate oxidation than the individual treatments, although the effects were not strictly additive. These results indicate that in the absence of glucagon, insulin can inhibit fatty acid oxidation in a manner apparently analogous to its effect in the nonruminant. In addition, despite the gluconeogenic set of the bovine liver, glucose can be metabolized and inhibit palmitate oxidation.

Propionate, a major gluconeogenic precursor in the ruminant, also proved an effective inhibitor of palmitate oxidation (Table 21). Since propionate carbon would be metabolized through the gluconeogenic pathway to produce

TABLE 21.--Palmitate Oxidation by Bovine Liver Slices in the Presence or Absence of Propionate, Clofenapate or Propionate Plus Clofenepate.

Additions	Palmitate Oxidized To	
	CO_2	Total Acid-Soluble Metabolites
	pmoles $\cdot$ min^{-1} $\cdot$ mg wet wt^{-1}	
None	.564±.020	2.60±.18
Propionate	.310±.017	2.04±.19
Clofenapate	.772±.102	3.59±.77
Propionate + Clofenapate	.602±.098	3.44±.72

Values are means ± S.E.M. of quadruplicate liver slices from three livers. Media contained final concentrations of 1 mM palmitate, .25 mM BSA, 2 mM dl-carnitine, and, when present, 10 mM propionate and .5 mM clofenapate. Incubation time was 180 minutes.

glucose, propionate would contribute to intracellular G3P and could thereby increase palmitate esterification and inhibit oxidation. If increased esterification rates can decrease fatty acid oxidation by limiting fatty acid availability, then inhibition of esterification should increase fatty acid oxidation by increasing fatty acid availability. The effect of clofenepate, a metabolic inhibitor which specifically blocks fatty acid esterification (Brindley and Bowley, 1975), was thus examined for its effect on fatty acid oxidation rates. Addition of clofenepate to the incubation media stimulated palmitate oxidation to both CO_2 and ASM. By inhibiting fatty acid esterification clofenepate apparently increased fatty acid availability for oxidation. Clofenepate addition could almost completely overcome the propionate-induced inhibition of fatty acid oxidation (Table 21). Propionate addition in the presence of clofenepate had essentially no effect on palmitate oxidation to ASM, although oxidation to CO_2 was reduced somewhat below the value observed in the presence of clofenepate alone. These results indicate that a major site of action for propionate inhibition of fatty acid oxidation occurs at the branch-point partitioning fatty acid between esterification and oxidation. Propionate is activated and undergoes its initial metabolism in the mitochondrial matrix (Ricks and Cook, 1981a), and thus has the potential for a direct interaction with fatty acid

oxidation. For example, various intermediates resulting from propionate metabolism, such as methylmalonyl-CoA or succinyl-CoA, could act within the mitochondrial matrix to inhibit palmitate oxidation. To examine this possibility, the effect of increasing propionate concentrations on palmitate oxidation by isolated bovine liver mitochondria was studied. Propionate concentrations as high as 10 mM had no effect on mitochondrial palmitate oxidation (Table 22). Thus, propionate inhibition of palmitate oxidation is probably confined to the cytoplasmic branch-point between esterification and oxidation.

Lactic acid in the nonruminant can be a major gluconeogenic precursor, and is a very potent antiketogenic agent. Lactate is metabolized via pyruvate and pyruvate carboxylase to produce OAA enroute to glucose formation. Fatty acid oxidation could be inhibited by lactate at the level of G3P and fatty acid esterification, as already discussed. Alternatively, palmitate oxidation to ASM could be decreased by lactate in response to increased mitochondrial OAA concentrations and a concomitant increase in acetyl-CoA entry into the TCA cycle and CO_2 production. Lactate proved surprisingly ineffective as an inhibitor of palmitate oxidation by bovine liver slices (Table 23), inhibiting both CO_2 and ASM production from palmitate by about 10%. That palmitate oxidation to both CO_2 and ASM was inhibited to a similar degree indicates that lactate

TABLE 22.--Palmitate Oxidation by Isolated Bovine Liver Mitochondria in the Presence of Increasing Propionate Concentrations.

	Palmitate Oxidized To	
[Propionate]	CO_2	Total Acid-Soluble Metabolites
mM	pmoles·min^{-1}·mg mitochondrial protein^{-1}	
0	10.8±2.2	260±48.5
.5	11.5±2.3	324±86.9
1	11.1±2.5	285±73.8
2	11.1±2.2	250±53.0
5	11.0±2.4	294±67.4
10	8.99±2.7	321±88.2

Values are means ± S.E.M. for triplicate incubations of mitochondrial preparations from three livers. Media contained final concentrations of 35 µM palmitate, .7% BSA, 100 µM l-carnitine, 4 mM ATP, 1 mM ADP, 50 µM CoASH and 250 µM reduced glutathione. Flasks contained 1.25 mg mitochondrial protein, and were incubated for four minutes as described in Methods.

TABLE 23.--Palmitate Oxidation by Bovine Liver Slices in the Presence or Absence of l-Lactate or Acetate.

Additions	Palmitate Oxidized To	
	CO_2	Total Acid-Soluble Metabolites
	-- pmoles · min^{-1} · mg wet wt^{-1} --	
None	1.42±.15	5.02±.44
l-Lactate	1.28±.15	4.45±.33
Acetate	1.21±.12	4.00±.33

Values are means ± S.E.M. of quadruplicate liver slices from four livers. Media contained final concentrations of 1 mM palmitate, .25 mM BSA, 2 mM dl-carnitine, and, where present, 10 mM l-lactate and 10 mM acetate. Incubation time was 60 minutes.

apparently had little effect on palmitate oxidation at the mitochondrial level. Thus, the inhibitory effect lactate displays toward palmitate oxidation probably occurred at the level of fatty acid esterification. Other researchers have also commented on the relative ineffectiveness of lactate as an antiketogenic agent in ruminant liver (Lomax et al., 1983a). These researchers noted that both lactate and propionate stimulated $^{14}CO_2$ production from labelled palmitate while inhibiting ketogenesis by isolated sheep hepatocytes, in contrast to the inhibition both compounds exhibited toward palmitate oxidation to CO_2 by bovine liver slices (Tables 21 and 23).

Palmitate oxidation can be inhibited at the mitochondrial level by competitive oxidation, i.e. by the metabolism of compounds which are rapidly converted to acetyl-CoA within the mitochondrial matrix, and thus compete with fatty acid for CoASH and reduced cofactors (O'Donnell and Freedland, 1980). Acetate, which can be activated both within the mitochondrial matrix and the cytoplasm, inhibited palmitate oxidation to CO_2 by 15% and to ASM by 20% (Table 23), proving as nearly effective an inhibitor of palmitate oxidation as glucose or propionate. Acetate is generally thought to be sparingly utilized by the ruminant liver, but as much as 15% to 20% of the acetate supplied to the liver can be absorbed and utilized by the liver (Pethick et al., 1981; Thompson et al., 1975). The near-physiological acetate concentration (10 mM) used in this study suggests that acetate could play a role in the in vivo regulation of long-chain fatty acid oxidation.

Medium-chain fatty acids are activated within the mitochondrial matrix and thus do not require the carnitine acyltransferase system for transport across the inner mitochondrial membrane. Medium-chain fatty acids such as octanoate are oxidized at much greater rates by rat liver preparations than are long-chain fatty acids (Krebs et al., 1969). Before making a similar comparison in bovine liver, the potential effect of carnitine on octanoate oxidation by bovine liver slices was examined, since the incubation

media routinely contained 2 mM dl-carnitine. Carnitine addition to a final concentration of 4 mM induced a slight decrease in octanoate oxidation to ASM, and a marked inhibition of CO_2 production (Table 24). In the presence of 2 mM carnitine, octanoate oxidation to CO_2 was relatively unaffected compared to oxidation in the presence of 4 mM carnitine. Stimulation of endogenous long-chain fatty acid oxidation by carnitine addition might account for part of the decrease in octanoate oxidation, in which case the minimal effect of carnitine on octanoate oxidation to ASM suggests that endogenous long-chain fatty acid oxidation probably makes only a minor contribution to

TABLE 24.--Octanoate Oxidation by Bovine Liver Slices in the Presence of Increasing Carnitine Concentrations.

	Octanoate Oxidized To	
[dl-Carnitine]	CO_2	Total Acid-Soluble Metabolites
mM	- pmoles · min^{-1} · mg wet wt^{-1} -	
0	10.6±.70	72.5±3.1
1	10.8±.64	64.4±5.9
2	8.80±.95	62.3±5.6
4	5.94±.29	63.9±4.7

Values are means ± S.E.M. of quadruplicate liver slices from two livers. Media contained final concentrations of 1 mM octanoate and .25 mM BSA. Incubation time was 60 minutes, and all other conditions were as described in Methods.

total fatty acid oxidation in the liver slices. The greater effect of carnitine on octanoate oxidation to CO_2 is more difficult to explain, but may be attributable to increased acetyl-carnitine formation, decreasing acetyl-CoA availability for entry into the TCA cycle.

Bovine liver slices oxidized octanoate to CO_2 and ASM at much greater rates than the long-chain fatty acids palmitate and oleate (Table 25). Octanoate oxidation rates were almost ten times faster than the rates observed with the long-chain fatty acids. Even larger differences were observed in oxidation rates between octanoate and palmitate with rat liver slices (Table 26), but these results were

TABLE 25.--Oxidation of Octanoate, Palmitate and Oleate by Bovine Liver Slices.

	Fatty Acid Oxidized To	
Acid	CO_2	Total Acid-Soluble Metabolites
	-- pmoles $\cdot$ min^{-1} $\cdot$ mg wet wt^{-1} --	
Octanoate	10.5±.99	62.4±4.8
Palmitate	1.13±.06	6.33±.60
Oleate	1.08±.04	6.66±.69

Values are means ± S.E.M. of quadruplicate liver slices from three livers. Media contained final concentrations of 1 mM fatty acid, .25 mM BSA and 2 mM dl-carnitine. Incubation time was 60 minutes, and all other conditions were as described in Methods.

TABLE 26.--Oxidation of Octanoate and Palmitate by Rat Liver Slices.

	Fatty Acid Oxidized To			
	CO_2		Total Acid-Soluble Metabolites	
Incubation Time	1 Hr	3 Hr	1 Hr	3 Hr
Fatty Acid	pmoles · min^{-1} · mg wet wt^{-1}			
Octanoate	2.73±.59	4.41±.37	27.5±3.4	16.1±1.5
Palmitate	.212±.017	.262±.028	1.74±.12	1.85±.19

Values are means ± S.E.M. of quadruplicate liver slices from two livers. Media contained final concentrations of 1 mM fatty acid, .25 mM BSA and 1 or 2 mM dl-carnitine. All other conditions were as described in Methods.

obtained early during the incubation system validation period and may not be totally reliable. Krebs et al. (1969) reported results for rat liver slices similar to those obtained here with bovine liver slices, i.e. a 7- to 8-fold greater rate of oxidation with octanoate than with palmitate or oleate. Less than a two-fold difference, however, was observed between octanoate and oleate oxidation rates by perfused rat livers (Krebs et al., 1969). Octanoate oxidation by bovine liver slices (62.4 $pmoles \cdot min^{-1} \cdot mg^{-1}$) are comparable to reported values for rat liver slices (97.5 $pmoles \cdot min^{-1} \cdot mg^{-1}$), which in turn were about one-fourth the rate observed in perfused rat liver (Krebs et al., 1969). A perfused liver system closely mimicks in vivo substrate delivery conditions, and this is probably the primary reason for the relatively low long-chain fatty acid oxidation and ketogenic rates observed in liver slices compared to perfused liver. (Isolated liver cells are comparable to the perfused liver in this regard.) The long-chain fatty acids would have more difficulty diffusing to the interior of a liver slice than through the plasma membranes of individual hepatocytes in the perfused liver. Octanoate is much more soluble in aqueous systems than long-chain fatty acids, and thus octanoate oxidation rates are not as sensitive to changes in liver preparation (i.e. slice or perfused liver) as are long-chain fatty acids.

Liver preparations obtained from fasted rats have an inherently greater capacity for fatty acid oxidation than do similar preparations obtained from fed rats (McGarry and Foster, 1980). Treatment with glucagon or Bt_2cAMP, however, can convert liver preparations from fed rats to a fatty acid oxidizing, ketogenic condition similar to that observed in the fasted rat (Christiansen, 1979; Harano et al., 1982). Bovine liver slices from fed cows were incubated with either glucagon or Bt_2cAMP to determine what effect these agents would have on palmitate oxidation rates. Palmitate oxidation was inhibited by both glucagon and Bt_2cAMP in either the presence or absence of carnitine (Table 27). Dibutyryl cAMP concentrations as low as 10^{-7} M effectively inhibited palmitate oxidation to both CO_2 and ASM by bovine liver slices (Table 28). These results were totally unexpected, and led to an investigation of the effects of Bt_2cAMP and glucagon on palmitate oxidation by rat liver slices and isolated hepatocytes. In agreement with previously published results (Harano et al., 1982; McGarry et al., 1979), palmitate oxidation by hepatocytes isolated from a fed rat was stimulated by both glucagon and Bt_2cAMP (Table 29). In contrast, Bt_2cAMP in the presence or absence of carnitine inhibited palmitate oxidation by liver slices from fed rats (Table 30). This data suggests that glucagon- or Bt_2cAMP-induced inhibition

TABLE 27.--Palmitate Oxidation by Bovine Liver Slices in the Presence or Absence of Glucagon or Dibutyryl cAMP.

Additions	Treatment	Palmitate Oxidized To	
		CO_2	Total Acid-Soluble Metabolites
		pmoles · min^{-1} · mg wet wt^{-1}	
None	Control	.199±.015	.318±.036
	Glucagon	.161±.019	.240±.039
	Bt_2cAMP	.119±.013	.205±.045
dl-Carnitine	Control	.499±.042	2.45±.33
	Glucagon	.402±.051	1.91±.33
	Bt_2cAMP	.358±.030	1.81±.31

Values are means ± S.E.M. of quadruplicate liver slices from four livers. Media contained final concentrations of 1 mM palmitate, .25 mM BSA, and, where present, 2 mM dl-carnitine, 1 mM Bt_2cAMP and 1 mM glucagon. Incubation times were from 60-180 minutes. All other conditions were as described in Methods.

TABLE 28.--Palmitate Oxidation by Bovine Liver Slices in the Presence of Increasing Dibutyryl cAMP Concentrations.

	Palmitate Oxidized To	
[Bt_2cAMP]	CO_2	Total Acid-Soluble Metabolites
Molarity	- pmoles · min^{-1} · mg wet wt^{-1} -	
0	.807±.075	4.28±.26
10^{-7}	.504±.069	2.85±.22
10^{-6}	.553±.069	3.28±.24
10^{-5}	.560±.057	3.24±.22
10^{-4}	.633±.096	3.34±.21
10^{-3}	.644±.099	3.42±.35

Values are means ± S.E.M. of quadruplicate liver slices from two livers. Media contained final concentrations of 1 mM palmitate, .25 mM BSA and 2 mM dl-carnitine. Incubation time was 60 minutes.

TABLE 29.--Palmitate Oxidation by Isolated Rat Hepatocytes in the Presence or Absence of Glucagon or Dibutyryl cAMP.

Additions	Palmitate Oxidized To	
	CO_2	Total Acid-Soluble Metabolites
	-- pmoles $\cdot$ min^{-1} $\cdot$ mg dry wt^{-1} --	
None	41.0±1.1	136.0±3.0
Glucagon	44.7±1.6	156.6±2.6
Bt_2cAMP	46.4±3.0	152.4±6.9

Values are means ± S.E.M. of quadruplicate incubations for one preparation of hepatocytes from a fed rat. Media contained final concentrations of 2 mM palmitate, .5 mM BSA, 2 mM dl-carnitine, and, when present, 1 mM glucagon and 1 mM Bt_2cAMP. Incubation time was 60 minutes.

TABLE 30.--Palmitate Oxidation by Rat Liver Slices in the Presence or Absence of Dibutyryl cAMP

Additions	Treatment	Palmitate Oxidized To	
		CO_2	Total Acid-Soluble Metabolites
		pmoles $\cdot$ min^{-1} $\cdot$ mg wet wt^{-1}	
None	Control	1.40±.09	3.33±.19
	Bt_2cAMP	1.11±.08	2.71±.16
dl-Carnitine	Control	1.94±.17	5.40±.40
	Bt_2cAMP	1.56±.09	3.95±.22

Values are means ± S.E.M. of quadruplicate liver slices for three livers from fed rats. Media contained final concentrations of 1 mM palmitate, .25 mM BSA, and, when present, 2 mM dl-carnitine and 1 mM Bt_2cAMP. Incubation time was 60 minutes.

of palmitate oxidation by liver slices is an artifact of the liver slice incubation system.

Since the isolated hepatocytes and liver slices were both obtained from fed rats, both rat liver preparations possessed the capacity to respond to glucagon or Bt_2cAMP with an increase in palmitate oxidation rates. The question arises, then, as to what potential differences exist between isolated hepatocytes and liver slices which could account for the diametrically opposed response of these liver preparations towards glucagon or Bt_2cAMP. One potential difference between these liver preparations exists with respect to glycogen content. Rat hepatocytes reportedly lose a significant proportion of their glycogen stores during the course of isolation and preparation. Because shorter time-periods are involved in the preparation of liver slices, a higher glycogen content should be found in the slices than in the hepatocytes. Liver glycogen mobilization can be stimulated by glucagon or Bt_2cAMP, resulting in increased cellular glucose-6-phosphate concentrations (Cohen, 1980b). A greater glycogen concentration in the rat liver slices could result in higher glucose-6-phosphate concentrations within the individual cells of the liver slice, and lead to inhibition of palmitate oxidation in a manner analogous to glucose addition. (Glucose is metabolized via glucose-6-phosphate.) Nearly identical amounts of glycogen can be stored in

bovine and rat liver (Bahnak and Gold, 1982; Baird, 1981), so that a similar mechanism, i.e. mobilization of glycogen stores, could potentially account for Bt_2cAMP-induced inhibition of palmitate oxidation by bovine liver slices. Endogenous glucose release from bovine liver slices was stimulated by Bt_2cAMP treatment (Table 31). Concurrently, palmitate oxidation by slices from the same liver was inhibited by Bt_2cAMP, suggesting that increased intracellular glucose availability could have been responsible for the observed inhibition of palmitate oxidation.

If mobilization of liver glycogen stores is responsible for Bt_2cAMP-induced inhibition of palmitate oxidation by bovine liver slices, depletion of liver glycogen should relieve the inhibition. Accordingly, in an attempt to deplete hepatic glycogen stores, bovine liver slices were preincubated for 30 minutes in the presence or absence of Bt_2cAMP prior to determination of palmitate oxidation rates. Palmitate oxidation by bovine liver slices without preincubation was inhibited by Bt_2cAMP, in agreement with previous observations (Table 32). Preincubation of liver slices, whether in the presence or absence of Bt_2cAMP, resulted in marked inhibition of palmitate oxidation in the subsequent incubation. Inclusion of Bt_2cAMP in the incubation media had little effect on palmitate oxidation by bovine liver slices following preincubation. The cause of the decreased

TABLE 31.--Endogenous Glucose Release and Palmitate Oxidation by Bovine Liver Slices in the Presence or Absence of Dibutyryl cAMP.

Additions	Endogenous Glucose Release	Palmitate Oxidized To	
		CO_2	Total Acid-Soluble Metabolites
	nmoles·30 min^{-1}·mg wet wt^{-1}	- pmoles·min^{-1}·mg wet wt^{-1} -	
None	19.3±1.2	.82±.05	3.07±.40
Bt_2cAMP	23.9±2.2	.60±.04	2.38±.37

Values are means ± S.E.M. of quadruplicate liver slices from four livers. Glucose release was measured in liver slices incubated (30 minutes) in KRB with or without 1 mM Bt_2cAMP, and no other additions. Palmitate oxidation was determined with different liver slices as previously described. Media contained final concentrations of 1 mM palmitate, .25 mM BSA, 2 mM dl-carnitine, and when present, 1 mM Bt_2cAMP. Incubation time was 60 minutes.

TABLE 32.--Palmitate Oxidation by Bovine Liver Slices Preincubated with and without Dibutyryl cAMP.

	Palmitate Oxidized To			
	CO_2		Total Acid-Soluble Metabolites	
Incubation Media	Control	Bt_2cAMP	Control	Bt_2cAMP
Treatment	pmoles · min^{-1} · mg wet wt^{-1}			
No preincubation	.888±.056	.683±.059	4.28±.26	3.42±.35
Preincubation w/oBt_2cAMP	.095±.015	.082±.012	.243±.048	.476±.095
Preincubation w/Bt_2cAMP	.111±.015	.082±.016	.819±.182	.418±.076

Values are means ± S.E.M. of quadruplicate liver slices from two livers. Palmitate oxidation was determined on bovine liver slices as previously, or on liver slices which had been preincubated for 30 minutes with or without 1 mM Bt_2cAMP. Incubation and preincubation procedures were as described in Methods. Media contained final concentrations of 1 mM palmitate, .25 mM BSA, 2 mM dl-carnitine, and, when present, 1 mM Bt_2cAMP. Incubation time was 60 minutes.

palmitate oxidation by bovine liver slices following preincubation is currently unknown. One possibility is that a 30 minute preincubation in the absence of an added energy source could result in the death of the liver slice, although this seems unlikely. A more plausible explanation is that the absence of added fatty acid resulted in the mobilization of endogenous fatty acid stores, resulting in dilution of ^{14}C-labelled palmitate during the incubation period. Thus, total palmitate oxidation rates by the preincubated liver slices could have been as great as that found in slices without preincubation, but would have been underestimated due to the lower rate of ^{14}C-palmitate oxidation to $^{14}CO_2$ and ^{14}C-ASM. This research was not pursued further. Future investigations will be needed to define the mechanisms by which Bt_2cAMP and preincubation inhibit palmitate oxidation by bovine liver slices.

A number of investigations examined various characteristics of the bovine hepatic carnitine palmitoyl transferase (CPT) system, the enzyme system responsible for transport of FA-CoA across the inner mitochondrial membrane. Kinetic constants for the bovine hepatic CPT reaction were calculated from initial reaction velocities determined by the procedure of Bieber et al. (1972). Initial reaction velocity for the CPT-catalyzed release of CoASH was linear with respect to amount of added mitochondrial protein, from .0415 to .415 mg of protein

(Figure 10). A typical Hanes-Woolf plot for the determination of palmitoyl-CoA and l-carnitine Km values in the CPT reaction from one mitochondrial preparation are presented in Figure 11. Results from three different mitochondrial preparations gave mean Km values of 11.5±1.7 μM and .591±.151 mM for palmitoyl-CoA and l-carnitine, respectively, while V_{max} was 42.6±9.4 nmoles CoASH released·min^{-1}·(mg mitochondrial protein)$^{-1}$. The palmitoyl-CoA Km for bovine liver CPT was comparable to the value (13 μM) for rat mitochondrial CPT isolated from glucagon-treated hepatocytes (Harano et al., 1982), while the l-carnitine Km was almost twice as great as the value (.32 mM) for rat liver CPT (McGarry et al., 1983). Bovine hepatic mitochondrial CPT activity was greater than both rat and sheep liver CPT (Harano et al., 1982; McGarry et al., 1983; Snoswell and Henderson, 1970). The kinetic constants obtained for bovine liver CPT are comparable to previously published results for bovine mammary mitochondrial CPT (Dimenna and Emery, 1980).

Recently, a report demonstrated that treatment with glucagon of hepatocytes isolated from fed rats caused a dramatic decrease of the Km for palmitoyl-CoA in the CPT reaction within mitochondria isolated from the treated hepatocytes (Harano et al., 1982). A similar experiment was conducted with liver slices from fed cows. The liver slices were preincubated in the presence or absence of

Figure 10.--Relationship between bovine hepatic mitochondrial carnitine palmitoyl transferase reaction velocity and amount of added mitochondrial protein.

Results of one typical experiment. Each point represents the mean ± S.E.M. for triplicate determinations of carnitine palmitoyl transferase activity. Reaction velocities were determined as described in Methods in the presence of 28.0 μM palmitoyl-CoA and 2.5 mM l-carnitine. The calculated linear regression for carnitine palmitoyl transferase reaction velocity on mitochondrial protein was

$$Y = 15.93X + .4090,\ r^2 = .9729,$$

where Y = reaction velocity, nmoles CoASH released·min^{-1}
and X = mitochondrial protein, mg.

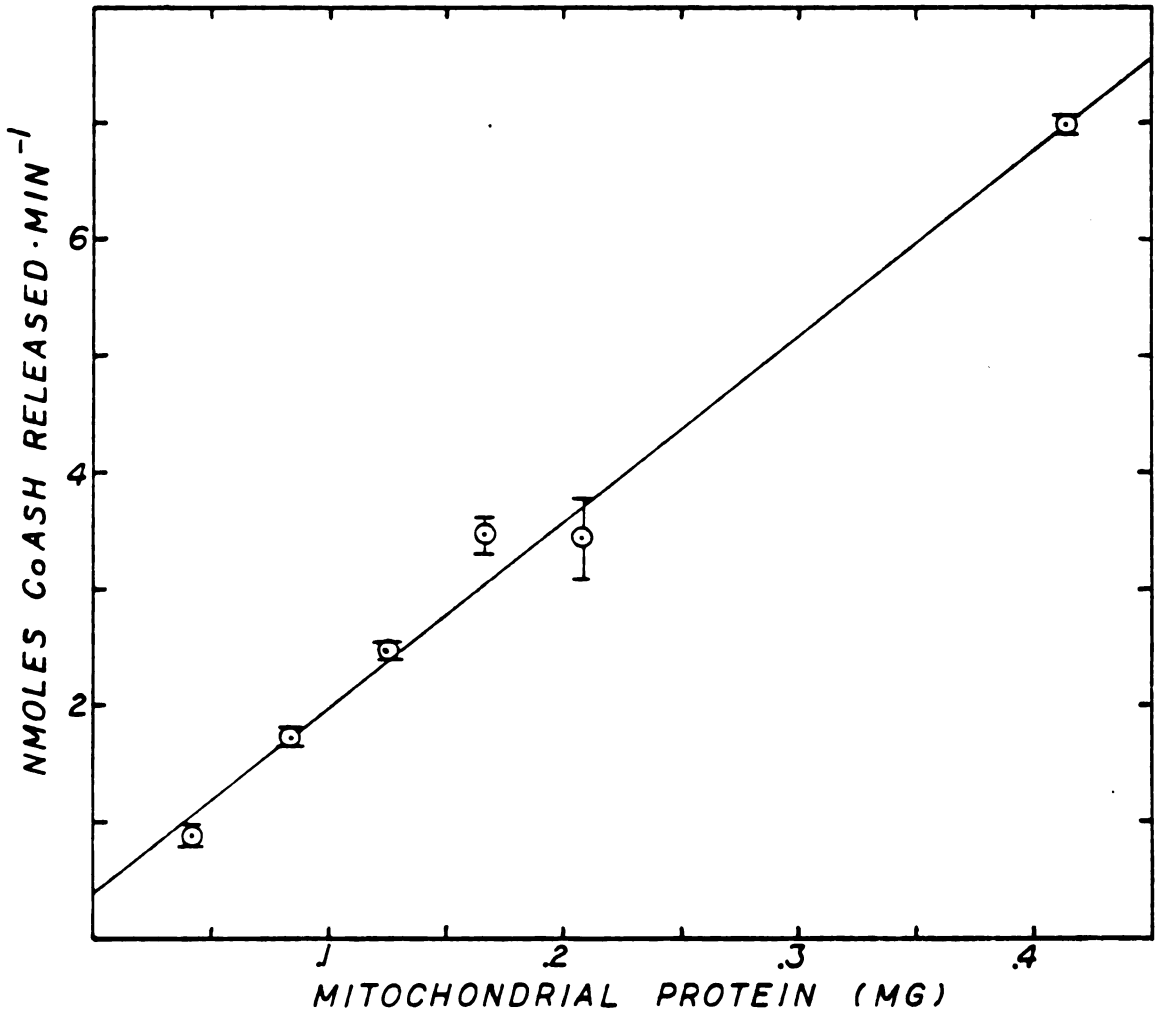
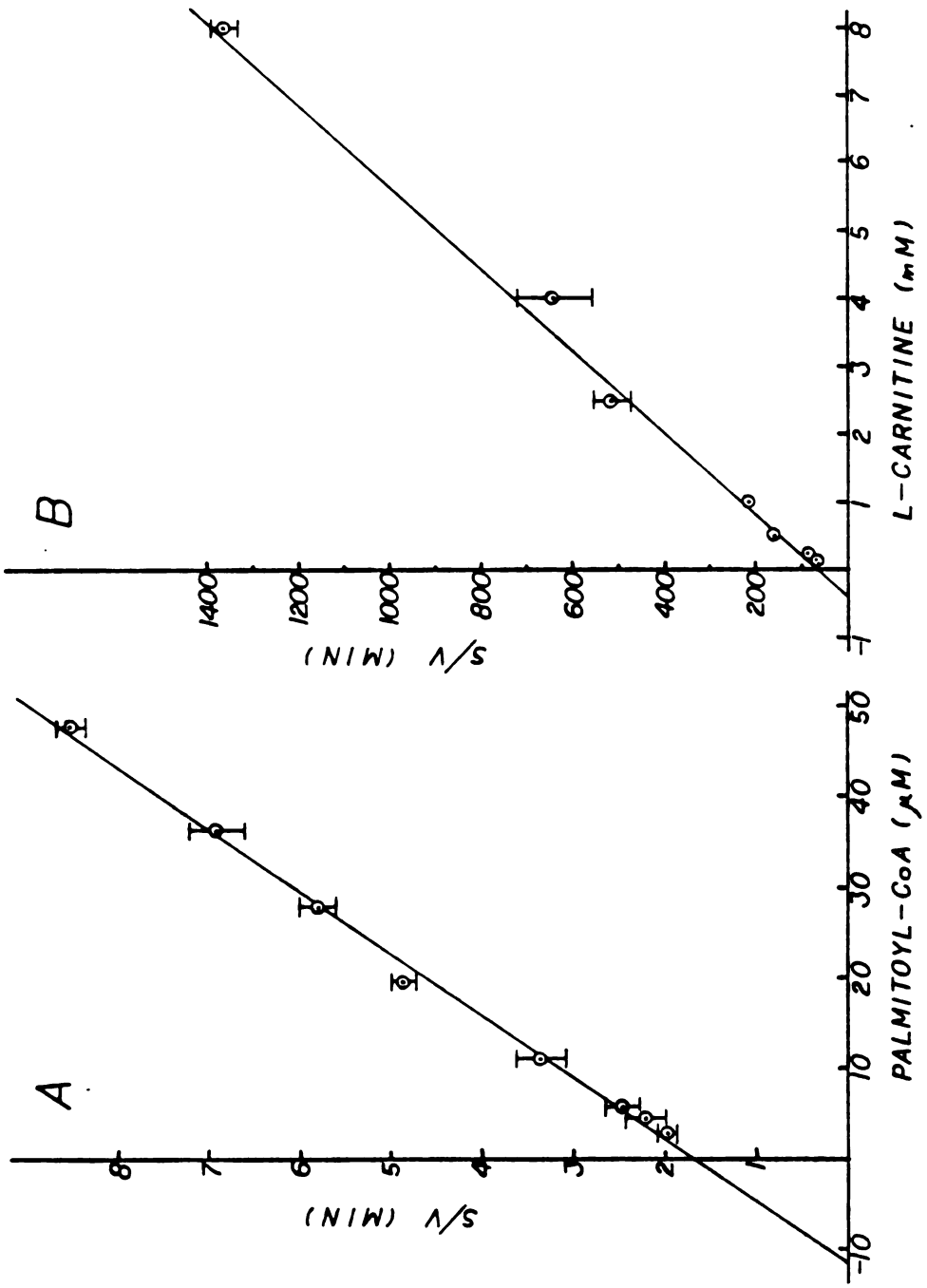

NMOLES CoASH RELEASED · MIN⁻¹
6
4
2
.1
.2
.3
.4
MITOCHONDRIAL PROTEIN (MG)

Figure 11.--A representative Hanes-Woolf plot for the determination of Km values for palmitoyl-CoA and l-carnitine in the bovine liver carnitine palmitoyl transferase reaction.

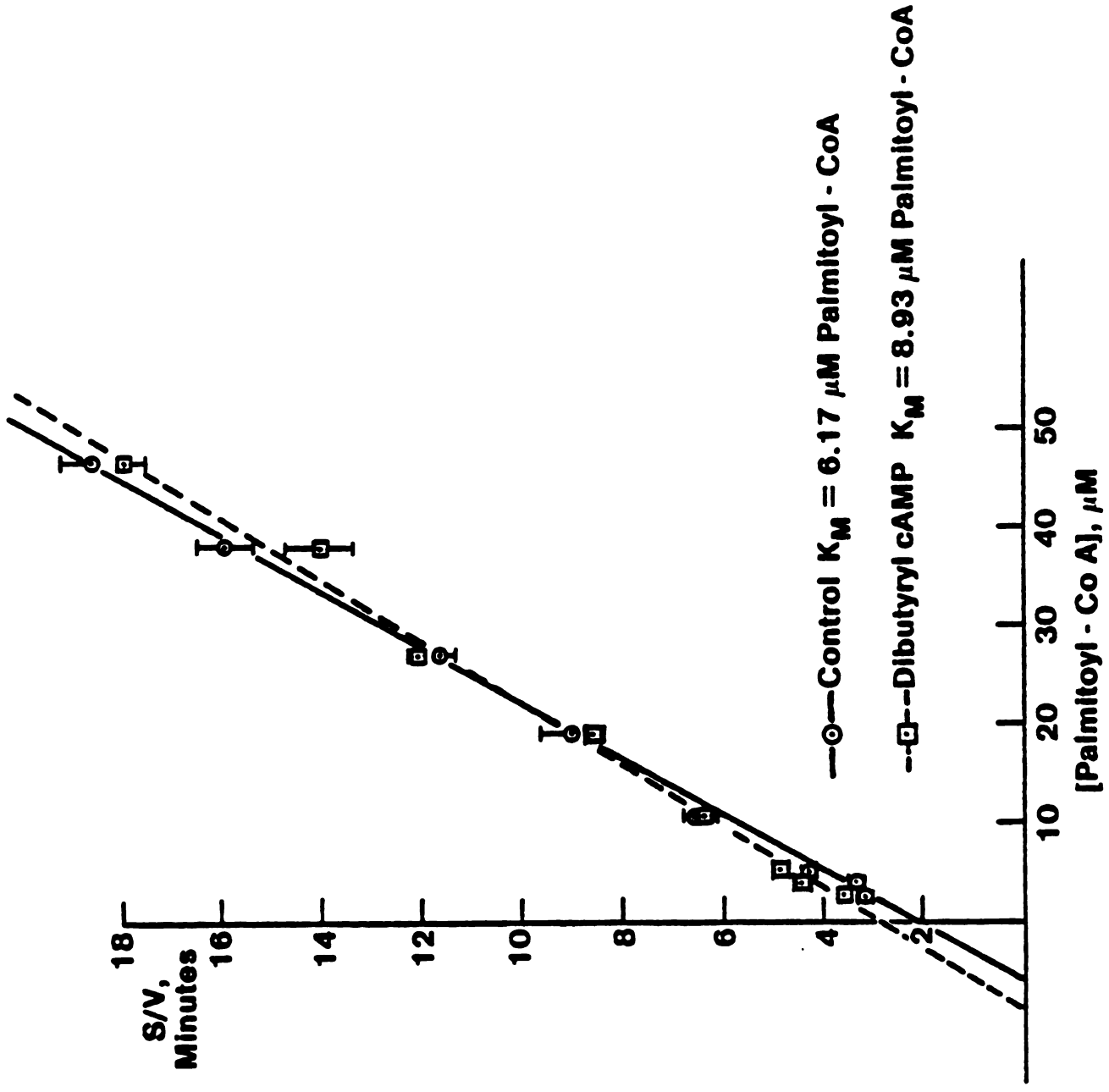
Carnitine palmitoyl transferase was assayed as described in Methods using .125 mg mitochondrial protein. (A) Determination of palmitoyl-CoA Km in the CPT reaction. Assays were conducted in the presence of 2.5 mM l-carnitine. Each point represents the mean ± S.E.M. for three to five determinations. Palmitoyl-CoA Km and CPT V_{max} were 11.8 μM and 55.4 nmoles·min^{-1}·mg^{-1}, respectively. (B) Determination of l-carnitine Km in the CPT reaction. Assays were conducted in the presence of 28.0 μM palmitoyl-CoA. Each point represents the mean ± S.E.M. for three determinations. l-Carnitine Km and CPT V_{max} were .384 mM and 48.3 nmoles·min^{-1}·mg^{-1}, respectively.


A
B
S/V (MIN)
PALMITOYL-CoA (μM)
L-CARNITINE (mM)

Bt_2cAMP, mitochondria were isolated from the liver slices, and the mitochondrial CPT reaction assayed to determine if preincubation with Bt_2cAMP induced a change in palmitoyl-CoA Km. Results of a typical experiment are illustrated in Figure 12. Treatment of liver slices with Bt_2cAMP resulted in essentially no change in kinetic parameters of the CPT reaction. In three such experiments, mean values for the palmitoyl-CoA Km and V_{max} of the mitochondrial CPT reaction were 6.50±.99 μM and 39.4±2.9 nmoles CoASH released·min^{-1}·(mg mitochondrial protein)$^{-1}$, and 8.31±.33 μM and 43.4±2.8 nmoles CoASH released·min^{-1}·(mg mitochondrial protein)$^{-1}$, in the control and Bt_2cAMP-treated slices, respectively. Insufficient mitochondrial protein was available for determination of a value for l-carnitine Km. The cause of the lower palmitoyl-CoA Km observed for CPT assayed in mitochondria isolated from liver slices in contrast to that observed in mitochondria isolated from a liver homogenate is unknown. Unfortunately, the proper contol, i.e. assay of CPT in mitochondria isolated from liver slices without preincubation, was not determined. The absence of a marked change in palmitoyl-CoA Km in the CPT assay for bovine liver mitochondria following treatment with Bt_2cAMP contrasts with observations in rat liver (Harano et al., 1982), but is in agreement with a) the inability of starvation to increase fatty acid oxidation rates by bovine liver slices (Table 16) or by isolated

Figure 12.--A representative Hanes-Woolf plot for the determination of the palmitoyl-CoA Km in the carnitine palmitoyl transferase reaction of mitochondria isolated from bovine liver slices following treatment with dibutyryl cAMP.

Bovine liver slices were preincubated for 30 minutes in KRB with or without 1 mM Bt_2cAMP. Subsequently, mitochondria were isolated from the slices and assayed for CPT activity as described in Methods. Each point represents the mean ± S.E.M. for two to five determinations. Assays were conducted in the presence of 2.5 mM l-carnitine, and .082 or .064 mg mitochondrial protein for the control and Bt_2cAMP-treated mitochondria, respectively.


S/V, Minutes
18
16
14
12
10
8
6
4
2
10
20
30
40
50
[Palmitoyl - Co A], μM
—⊙—Control K_M = 6.17 μM Palmitoyl - CoA
--⊡--Dibutyryl cAMP K_M = 8.93 μM Palmitoyl - CoA

ovine hepatocytes (Lomax et al., 1983a), and b) the inability of glucagon or Bt_2cAMP to stimulate fatty acid oxidation by hepatocytes isolated from fed sheep (Lomax et al., 1983b). These results suggest that the enzymatic capacity of ruminant liver to oxidize fatty acid is maximally stimulated at all times, in contrast to the situation observed in the nonruminant. Such a hypothesis is supported by the observed insulin:glucagon ratio in the ruminant circulation, which remains relatively low at all times (Bassett, 1975). The ability of a fast to increase the oxidative capacity of rat liver is thought to be the result of the transition from a high insulin:glucagon ratio during the fed state to a low insulin:glucagon ratio during the fasted state (McGarry and Foster, 1980).

A key element in the regulation of rat liver fatty acid oxidation is thought to be the decrease in intracellular malonyl-CoA concentrations which occurs in the fasted rat (McGarry and Foster, 1980). Since ruminant liver does not undergo transitions between fatty acid synthesizing and fatty acid oxidizing states, and is not a major site of fatty acid synthesis, malonyl-CoA would not be expected to play a significant role in regulation of bovine hepatic fatty acid oxidation. Malonyl-CoA, however, proved a potent inhibitor of palmitate oxidation by isolated bovine liver mitochondria (Table 33). The I_{50} calculated for malonyl-CoA inhibition of bovine liver mitochondrial

TABLE 33.--Palmitate Oxidation by Isolated Bovine Liver Mitochondria in the Presence of Increasing Malonyl-CoA Concentrations.

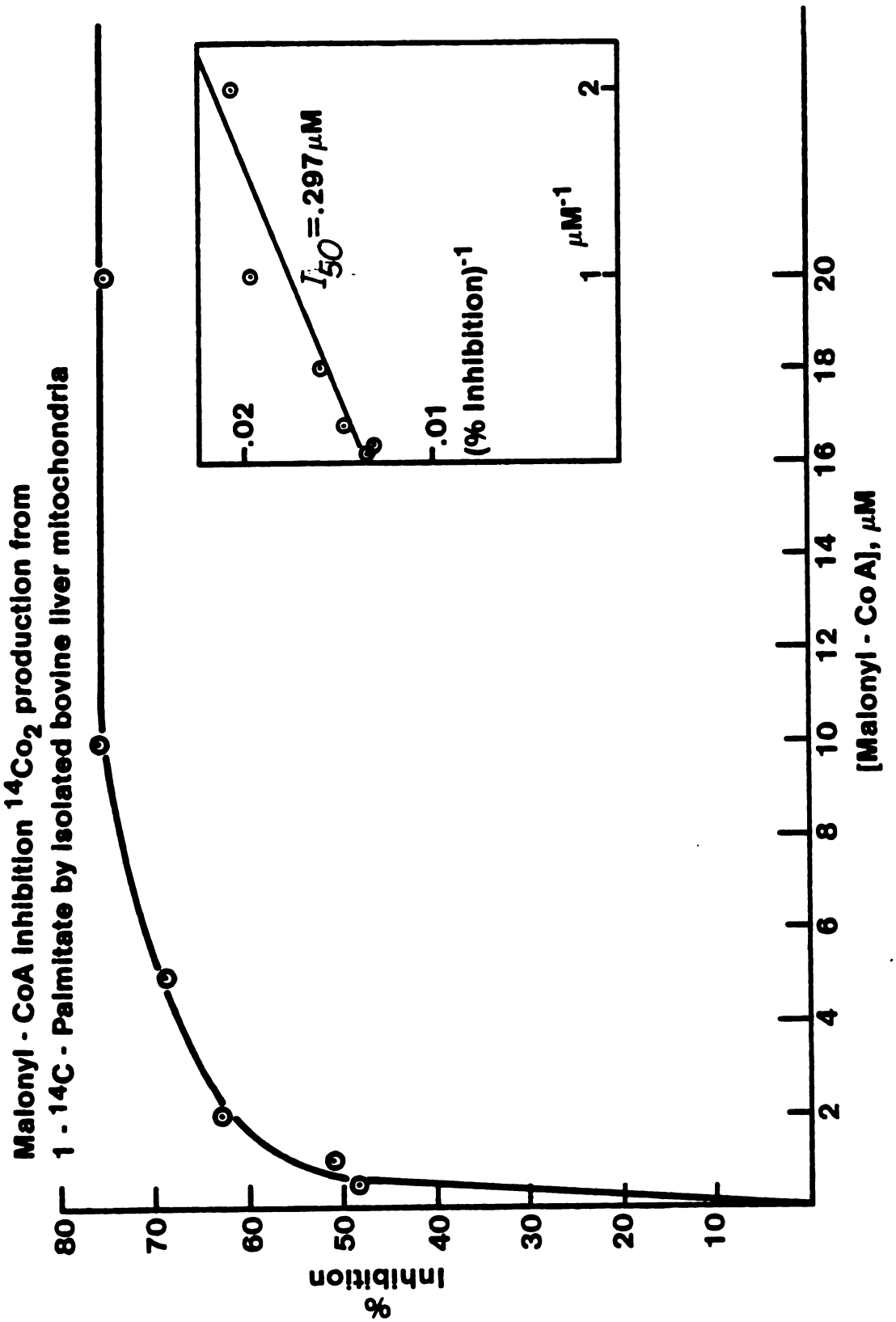
[Malonyl-CoA]	Palmitate Oxidation to CO_2	% Inhibition
μM	pmoles·min^{-1}· mg mitochondrial protein^{-1}	
0	10.8±2.2	
.5	5.59±.38	48.3
1	5.31±.65	50.8
2	4.03±2.00	62.7
5	3.39±.53	68.6
10	2.61±.43	75.9
20	2.71±.47	74.9

Values are means ± S.E.M. for triplicate incubations of mitochondrial preparations from three livers. Media contained final concentrations of 35 μM palmitate, .7% BSA, 100 μM l-carnitine, 4 mM ATP, 1 mM ADP, 50 μM CoASH and 250 μM reduced glutathione. Flasks contained 1.25 mg mitochondrial protein, and were incubated for four minutes as described in Methods. Data is plotted in Figure 13.

palmitate oxidation was about .3 μM (Figure 13), a value well below the lowest reported I_{50} for malonyl-CoA inhibition of rat liver mitochondrial fatty acid oxidation (2 μM; McGarry et al., 1978b). Ruminant liver can synthesize fatty acids, albeit at a relatively low rate, and must accordingly possess acetyl-CoA carboxylase for malonyl-CoA production, although malonyl-CoA concentrations have not yet been reported for ruminant liver. Further research will be needed to determine a) hepatic malonyl-CoA concentrations in the ruminant, b) relationship between ruminant hepatic malonyl-CoA concentrations and I_{50} for malonyl-CoA inhibition of mitochondrial fatty acid oxidation and CPT activity, and c) changes in hepatic content of malonyl-CoA with changing physiological status of the ruminant. These factors will need to be determined in order to assess the physiological importance of malonyl-CoA for the regulation of ruminant hepatic fatty acid oxidation.

Figure 13.--Malonyl-CoA inhibition of palmitate oxidation to CO_2 by isolated bovine liver mitochondria.

Isolated bovine liver mitochondria were incubated in the presence of increasing malonyl-CoA concentrations as described in Methods. Data are plotted from Table 33.


Malonyl - CoA Inhibition $^{14}Co_2$ production from
1 - ^{14}C - Palmitate by isolated bovine liver mitochondria
% Inhibition
80
70
60
50
40
30
20
10
2
4
6
8
10
12
14
16
18
20
[Malonyl - Co A], μM
.02
.01
(% Inhibition)$^{-1}$
I_{50}=.297μM
1
2
μM^{-1}

GENERAL DISCUSSION

The laboratory rat, because of ready availability and a relatively uniform genetic composition, has become a model system for the study of nonruminant metabolic regulation. Tissue slice systems, particularly for the easily sliced rat liver, were some of the earliest incubation systems designed for *in vitro* study of metabolic regulation within intact cells (Umbreit *et al.*, 1964). In recent years the rat liver slice has been supplanted by both the perfused liver and especially by isolated hepatocytes as *in vitro* incubation systems for metabolic experiments. Despite the ease and simplicity with which liver slices can be produced and incubated, at least two major disadvantages have lead to the downfall of the liver slice for metabolic studies of the rat. First, liver slices are not suitable for long-term incubations, as are isolated hepatocytes. Second, reaction rates of various metabolic pathways estimated from *in vitro* incubations with liver slices are generally only a fraction of the rates estimated from *in vivo* observations, while reaction rates with either perfused liver or isolated hepatocytes more nearly approximate *in vivo* metabolic rates. One potential cause of these lower rates,

i.e. decreased ability of substrate to diffuse into the liver slice, has already been discussed with regard to the difference between octanoate and palmitate oxidation rates with liver slices (Tables 25 and 26).

Despite these shortcomings, liver slices generally behave in a manner qualitatively similar to the *in vivo* situation. Thus, alteration of metabolic activity in a liver slice in response to an agent should approximate *in vivo* changes in hepatic metabolic activity to the same agent. The inhibition of fatty acid oxidation by Bt_2cAMP in both rat and bovine liver slices observed in this study (Tables 27 and 30) appears to be a major exception to this generalization. In the ruminant, tissue slices have been used extensively for the study of liver, adipose tissue, pancreas and mammary gland metabolism. Due perhaps to the investigators inability to arrange for rapid removal of liver from the animal, techniques for the isolation of ruminant hepatocytes are only now coming into use. Use of isolated cell preparations for the study of ruminant intermediary metabolism should increase.

In contrast to the nonruminant liver, which cycles between glycolytic/fatty acid synthetic and gluconeogenic/ketogenic states, corresponding to fed and fasted states, respectively (Newsholme and Start, 1976), ruminant liver is in a continuous state of gluconeogenesis and ketogenesis (Baird *et al.*, 1979). Since only small amounts of glucose

are usually absorbed from the digestive tract, the result is an insulin:glucagon ratio which remains relatively low at all times (Bassett, 1975). The low insulin:glucagon ratio in turn is responsible for the continuous hepatic gluconeogenesis necessary to satisfy the glucose demands of extrahepatic tissues. Thus, all metabolic regulation within the ruminant liver occurs in relationship to this requirement for continuous gluconeogenesis.

The transition from a fed to a fasted state in a nonruminant is accompanied by a marked decrease in the insulin:glucagon ratio, triggering the change in metabolic set of the liver to a gluconeogenic/ketogenic condition. This changeover is accomplished by alteration of the activity of various regulatory enzymes within the liver in response to the decreased insulin:glucagon ratio, and is mediated directly or indirectly via covalent modification, i.e. via a phosphorylation/dephosphorylation mechanism (Newsholme and Start, 1976). The result is an inherently greater capacity for fatty acid oxidation in liver from a fasted rat than from a fed rat (McGarry and Foster, 1980). Similar results were observed in the present study. Palmitate oxidation rate following 60 minutes of incubation in the presence of carnitine was almost twice as great by liver slices from fasted young adult rats (Table 1) than from fed young adult rats (Table 30).

Since the insulin:glucagon ratio remains relatively low at all times in the ruminant, hepatic regulatory enzymes would not be expected to undergo the cyclical changes in covalent modification, and thus the activity modulation, observed in nonruminant liver. This hypothesis in turn suggests that the capacity for fatty acid oxidation should also remain relatively constant in liver from fed or fasted ruminants. Experimental observations in the current study tend to support this hypothesis. Despite a decrease in palmitate oxidation to CO_2 as the result of a long-term fast, perhaps because of a depletion of TCA cycle intermediates (Baird et al., 1979), palmitate oxidation to ASM was essentially unchanged following the fast (Table 16). In addition, CPT activity in mitochondria isolated from fed bovine liver slices was not activated by treatment of the liver slices with Bt_2cAMP (Figure 12), in contrast to the activation of CPT which occurred in mitochondria isolated from glucagon-treated hepatocytes of a fed rat (Harano et al., 1982). The results of the current study are in agreement with the observations of Lomax et al. (1983a,b) who noted that 1) palmitate oxidation by hepatocytes isolated from fed sheep was not stimulated by either glucagon or Bt_2cAMP, and 2) palmitate oxidation in the presence of exogenous carnitine was not different between hepatocytes isolated from either fed or fasted sheep. Collectively, these

observations suggest that the enzymatic capacity for mitochondrial transport and oxidation of fatty acids in the ruminant liver may not be subject to modulation via covalent modification to the degree found in nonruminant liver. (This suggestion does not exclude modulation of ruminant hepatic enzyme activity by mechanisms such as competitive inhibition or allosteric activation.) Dibutyryl cAMP, however, can increase glucose release from bovine liver slices (Table 31), indicating that some metabolic pathways (e.g. glycogenolysis and gluconeogenesis) in ruminant liver may be subject to classical regulation via covalent modification. Such results are not inconsistent with observations in nonruminant liver, in which various metabolic pathways are known to exhibit differential sensitivities to glucagon-induced modulation of enzyme activity (Soling and Kleineke, 1976). Further research will be required to clarify the role that covalent modification plays in the regulation of ruminant hepatic metabolism.

In view of the low insulin:glucagon ratio typically present in the ruminant circulation, the ability of insulin to inhibit palmitate oxidation by bovine liver slices is not unexpected (Table 20). Insulin-mediated inhibition of palmitate oxidation in this situation would be analogous to the ability of insulin to inhibit fatty acid oxidation by liver preparations derived from fasted rats, a

comparable situation to the ruminant with respect to circulating insulin and glucagon concentrations (McGarry and Foster, 1980). The ability of insulin to inhibit palmitate oxidation in bovine liver could result from any of the mechanisms previously demonstrated to inhibit rat liver fatty acid oxidation, such as increased esterification rates, inactivation of CPT, or possibly through increased malonyl-CoA concentrations. The ability of insulin to inhibit bovine hepatic fatty acid oxidation could have potential significance *in vivo*. Feeding large amounts of concentrates has been demonstrated to increase average circulating insulin concentrations (Jenny and Polan, 1975; Jenny *et al.*, 1974). Increased insulin concentrations, presumably producing a higher insulin:glucagon ratio, may be partially responsible for the lower palmitate oxidation rates in liver slices from cows consuming a restricted roughage/high concentrate ration (Table 18), although this particular effect may more likely represent a long-term adaptation as opposed to a short-term regulatory effect.

Although ketogenesis is a continuous process in ruminant liver (Bergman, 1971; Lomax and Baird, 1983), some data suggests that a larger proportion of the FFA absorbed by the liver is oxidized in a fasted or pathologically ketotic ruminant than undergoes oxidation in a fed ruminant (Bergman, 1971). As just discussed, modulation of enzyme activity via covalent modification

may not be a mechanism available to the ruminant liver to account for the increased proportion of fatty acid undergoing oxidation. The question then arises as to what other factors could potentially account for an increased partitioning of fatty acid towards oxidation in the fasted or ketotic ruminant. At one time, carnitine was thought to play a regulatory role in nonruminant fatty acid oxidation because of the obligatory relationship between carnitine and fatty acid transport into mitochondria. Within the rat liver carnitine concentrations increase during a fast or other situations associated with high rates of fatty acid oxidation (e.g. Snoswell and Henderson, 1970), suggesting a regulatory nature for carnitine. Increased hepatic carnitine alone, however, cannot account for the increased fatty acid oxidation observed in rat liver upon fasting, since carnitine addition to liver preparations from fed rats does not stimulate fatty acid oxidation to the degree observed in fasted rats (Tables 1, 30; Christiansen et al.,1976; McGarry and Foster, 1980). Thus, increased hepatic carnitine concentration has been described as being a necessary but not sufficient condition for the increased rate of fatty acid oxidation in rat liver during a fast.

Carnitine stimulated palmitate oxidation by liver slices from fed cows (Table 7). In contrast to the rat, however, palmitate oxidation in the presence of carnitine

by bovine liver slices (Table 16) or isolated sheep hepatocytes (Lomax et al., 1982, 1983b) was the same whether the donor animal had been fed or fasted. Thus, carnitine availability may play a more direct role in regulation of hepatic fatty acid oxidation in the ruminant than in the nonruminant. Hepatic carnitine concentrations in the ruminant have been observed to increase during a fast, similar to observations in the rat (Snoswell and Henderson, 1970). The factors responsible for this increase in hepatic carnitine content are currently unknown (Rebouche, 1982), but clearly require additional investigation to clarify the role of carnitine in regulation of ruminant hepatic fatty acid oxidation.

Decreased intracellular malonyl-CoA concentrations, and the resulting relief of CPT I from malonyl-CoA-induced inhibition, have been implicated as major factors for the increased partitioning of fatty acid towards oxidation in liver from fasted rats (McGarry and Foster, 1980). Inactivation of acetyl-CoA carboxylase in response to decreased insulin:glucagon ratios during fasting is thought to be the primary factor leading to decreased malonyl-CoA concentrations. The importance of malonyl-CoA for regulation of ruminant hepatic fatty acid oxidation can be questioned in a number of respects, most particularly because ruminant liver is not a major site of fatty acid

synthesis and does not experience the marked shifts in metabolic set observed in nonruminant liver.

Circumstantial evidence, however, suggests a potential role for malonyl-CoA in regulation of ruminant hepatic fatty acid oxidation. While not a major site of fatty acid synthesis, fatty acids can nevertheless be synthesized in ruminant liver, albeit at a low rate (Hanson and Ballard, 1967), indicating that some malonyl-CoA must be present in ruminant liver, although at lower concentrations than in nonruminant liver. In view of these considerations, a physiologically relevant role for malonyl-CoA in the ruminant liver would demand a lower I_{50} value for malonyl-CoA inhibition of mitochondrial fatty acid oxidation in ruminant liver than in rat liver. This was observed to be the case (Figure 13). Malonyl-CoA inhibited bovine liver mitochondrial palmitate oxidation with an I_{50} (~.3 μM) which is about one-tenth of the lowest reported value for rat liver mitochondria (~2 μM; McGarry et al., 1978b).

Another suggestive piece of evidence is the relatively high Km (~.6 mM) of l-carnitine in the bovine hepatic CPT reaction (Figure 5), compared to values of about .2 mM and .7 mM for rat liver and skeletal muscle CPT, respectively (Mills et al., 1983). In rat tissues, carnitine palmitoyl transferases which had the highest l-carnitine Km also proved the most sensitive to

malonyl-CoA inhibition. Thus, skeletal muscle mitochondrial CPT with the highest l-carnitine Km of the tissues examined exhibited the lowest malonyl-CoA I_{50} (Mills et al., 1983). Bovine liver mitochondrial CPT fits into this pattern, exhibiting both a relatively high l-carnitine Km and low malonyl-CoA I_{50}. In view of the fatty acid synthesizing ability of bovine liver, malonyl-CoA more likely is involved in the regulation of fatty acid oxidation in bovine liver than in rat skeletal muscle which does not synthesize fatty acid. In vivo determinations of ruminant hepatic malonyl-CoA concentrations, and potential changes of these concentrations in response to changing physiological conditions, will be required before a definitive conclusion can be made regarding malonyl-CoA as a regulatory factor of fatty acid oxidation in the ruminant liver. Malonyl-CoA-induced inhibition of CPT could partially account for acetate inhibition of palmitate oxidation (Table 23), since acetate could be activated and converted to malonyl-CoA in the cytoplasm. The increased absorption of acetate following feeding could thus act via malonyl-CoA to inhibit ruminant hepatic fatty acid oxidation in a manner analogous to the increased glucose availability in the nonruminant after a meal.

Within nonruminant liver modulation of fatty acid oxidation rates appear to result mainly from modulation of the rate at which fatty acids are transported into the

mitochondria, while alterations in fatty acid esterification, the major competing pathway for fatty acid utilization within the liver, are of lesser importance (McGarry and Foster, 1980). Under ketogenic conditions within the rat liver, however, various antiketogenic agents can inhibit fatty acid oxidation and ketogenesis by increasing fatty acid utilization for esterification (Christiansen, 1979). In view of the continuous ketogenesis within ruminant liver, alteration of fatty acid esterification rates may be a more important modulator of fatty acid oxidation in the ruminant than in the nonruminant. A major portion of the inhibitory effect which glucose, insulin, propionate and lactate exert on palmitate oxidation rates in bovine liver slices could be accounted for by this mechanism (Tables 20, 21 and 23). Such a conclusion is supported by results of other investigations with both ruminant and nonruminant liver (Christiansen, 1979; Lomax et al., 1982, 1983b).

That partitioning of palmitate between esterification and oxidation could function in the regulation of fatty acid oxidation in bovine liver was more clearly demonstrated by the action of clofenapate on palmitate oxidation (Table 21). Clofenapate is a metabolic inhibitor which specifically blocks fatty acid esterification in rat liver (Brindley and Bowley, 1975). Palmitate oxidation was markedly stimulated in bovine liver slices by

clofenapate addition, presumably because fatty acid which would have undergone esterification became available for oxidation. The palmitoyl-CoA Km determined in the current study for bovine liver CPT (6-12 μM) is within the range reported for rat liver G3P acyltransferase (Yamada and Okuyama, 1978). If similar palmitoyl-CoA Km values exist for bovine liver G3P acyltransferase, the results would suggest that mitochondrial fatty acid oxidation could effectively compete with cytoplasmic esterification for use of fatty acid within the liver cell. Thus, modulation of esterification rates could act effectively to alter fatty acid oxidation.

The relative inability of lactate to inhibit palmitate oxidation by bovine liver slices (Table 23) contrasts to the very effective lactate-induced inhibition of ketogenesis by isolated rat and ovine hepatocytes (Christiansen, 1979; Lomax et al., 1983b). With the present data, a definitive conclusion as to the cause of this differential behavior cannot be made. One possible explanation could be a higher cytoplasmic NADH/NAD ratio in bovine liver slices than in the isolated cells, limiting the conversion of lactate to pyruvate. Production of both $^{14}CO_2$ and ^{14}C-ASM from labelled palmitate was decreased in the presence of lactate during the current study, but in situations where lactate exhibits marked inhibition of ketogenesis, $^{14}CO_2$ production is actually increased

(Christiansen, 1979; Lomax et al., 1983b). An explanation for this discrepancy might also provide insight into the relative inability of lactate to inhibit palmitate oxidation in bovine liver slices.

Propionate effectively inhibited palmitate oxidation by bovine liver slices (Table 21). With isolated sheep hepatocytes propionate proved a more effective inhibitor of ketogenesis from palmitate than other traditional antiketogenic agents, e.g. fructose, glycerol and lactate (Lomax et al., 1983b). Propionate is activated within the mitochondrial matrix, and is metabolized to methylmalonyl-CoA before entry into the TCA cycle as succinyl-CoA. Because of a structural similarity to malonyl-CoA, one could speculate as to a potential functional similarity between methylmalonyl-CoA and malonyl-CoA, with methylmalonyl-CoA inhibiting CPT II in a manner analogous to malonyl-CoA inhibition of CPT I. Other researchers have also made similar speculations, suggesting that methylmalonyl-CoA, succinyl-CoA or some other TCA cycle intermediate resulting from propionate metabolism could limit fatty acid oxidation by inhibiting a matrix-located reaction, possibly CPT II, involved in fatty acid oxidation (Baird, 1982; Lomax et al., 1983b). Methylmalonyl-CoA has been demonstrated to inhibit CPT in rat liver, heart and skeletal muscle (Mills et al., 1983), providing some support to this speculation. Other data

obtained during the present study, however, appears to rule out a mitochondrial site of action for propionate-induced inhibition of palmitate oxidation. Addition of clofenapate, which acts specifically to inhibit fatty acid esterification, almost completely overcame the propionate-induced inhibition of palmitate oxidation by bovine liver slices (Table 21). Direct addition of propionate to isolated bovine liver mitochondria, however, had little effect on palmitate oxidation by the mitochondria (Table 22). These results indicate that the major site of action of propionate inhibition of palmitate oxidation in ruminant liver is most likely at the point of fatty acid esterification.

The importance of propionate-induced inhibition of fatty acid oxidation *in vivo* could be questioned, since the propionate concentrations used in the current study (10 mM) are well above the physiological propionate concentrations found in portal blood (.3-.4 mM; Baird *et al.*, 1975; Lomax and Baird, 1983). Propionate concentrations as low as .8 mM, however, have reportedly produced 50% of the maximum propionate-induced inhibition of ketogenesis by isolated sheep hepatocytes (Lomax *et al.*, 1983b), suggesting that propionate could be physiologically important for the regulation of long-chain fatty acid oxidation.

Near-physiological concentrations of acetate also inhibited palmitate oxidation by bovine liver slices (Table 23), also suggesting a potential role *in vivo* for acetate in the regulation of fatty acid oxidation. Acetate-induced inhibition of palmitate oxidation could have occurred via a number of mechanisms, including direct competition as an oxidizable substrate within the mitochondrial matrix, or inhibition of CPT I following conversion to malonyl-CoA. Recently published information suggests that acetyl-CoA itself may directly inhibit CPT I (McCormick *et al.*, 1983). Since these potential mechanisms of acetate inhibition of palmitate oxidation are not mediated at the level of fatty acid esterification, a combination of acetate plus propionate should prove a more effective fatty acid oxidation inhibitor than either short-chain acid alone. Unfortunately, this combination was not examined for its potential effect on palmitate oxidation.

Neither BHBA nor butyrate were examined for potential inhibition of palmitate oxidation by bovine liver slices. β-Hydroxybutyrate has been demonstrated to inhibit ketogenesis from oleate in isolated rat liver mitochondria (Roeder *et al.*, 1982), perhaps by inhibiting AcAc synthesis. Also, infusion of BHBA into chronically catheterized sheep inhibited hepatic ketogenesis, an effect which was not due to increased insulin concentrations

(Fernandez and Heitmann, 1983). Butyrate can be readily metabolized by ruminant liver (Lomax and Baird, 1983; Baird et al., 1977), and could conceivably inhibit palmitate oxidation by substrate competition within the mitochondrial matrix.

Thus, a situation exists where the principal products of the rumen fermentation, i.e. acetate, propionate, BHBA and butyrate, could potentially limit the oxidation of long-chain fatty acids within the liver of the fed ruminant. In a fasted ruminant, production of these short-chain acids within the rumen is reduced, and consequently the blood concentrations are also reduced, relieving the inhibition of hepatic fatty acid oxidation. Hepatic carnitine content increases during a fast (Snoswell and Henderson, 1970), which coupled with the decrease in short-chain fatty acids and increased FFA mobilization, would result in the increased fatty acid oxidation and ketogenesis observed in the fasted ruminant (Bergman, 1971). If hormonally-induced modulation of hepatic fatty acid oxidation is of relatively minor importance to the ruminant compared to the nonruminant, the ability of rumen fermentation products to regulate fatty acid oxidation in the liver would represent a unique adaptation by the ruminant to directly utilize the products of digestion as indicators of physiological status.

SUMMARY AND CONCLUSIONS

Greater rates of palmitate oxidation were observed with liver slices from younger animals, either rats or calves, than from older animals. The difference between age groups was not as pronounced for bovine liver slices as for rat liver slices. In general, rat liver slices oxidized palmitate at much greater rates than did bovine liver slices. Slices made from bovine liver obtained via biopsy oxidized palmitate at greater rates than did slices obtained at slaughter, in some cases oxidizing palmitate at nearly one-half the rate of rat liver slices. Palmitate oxidation by isolated rat liver mitochondria was only 1- to 2-fold greater than palmitate oxidation by isolated bovine liver mitochondria. These results suggest that much of the difference observed in palmitate oxidation between rat and bovine liver slices may be attributable to the length of time required in order to obtain a bovine liver sample at slaughter, the major source of bovine liver. The site on the bovine liver from which a sample was obtained had little effect on subsequent palmitate oxidation rates by liver slices.

At the concentrations examined (.5 to 2 mM), palmitate concentration had little effect on palmitate

oxidation rates by bovine liver slices in the absence of added carnitine. Addition of dl-carnitine stimulated palmitate oxidation, with 2 mM dl-carnitine producing maximum stimulation. Little difference was observed in the ability of l- and dl-carnitine to stimulate palmitate oxidation when equimolar amounts of l-carnitine were supplied. Palmitate oxidation rates were linear with respect to amount of tissue, for liver slices from 40 to 210 mg wet weight. Palmitate oxidation to CO_2 was linear with respect to incubation time, for periods up to 240 minutes. Linear rates of palmitate oxidation to ASM with respect to time were observed for 60 minutes. After 60 minutes palmitate oxidation rates to ASM decreased, but continued in a linear fashion up to 240 minutes. Oxidation of 1-^{14}C-palmitate to CO_2 was greater than U-^{14}C-palmitate, but oxidation of U-^{14}C-palmitate to ASM was greater than 1-^{14}C-palmitate. Octanoate was oxidized by bovine liver slices at about ten times the rate of palmitate.

Cyanide completely inhibited palmitate oxidation to CO_2 by bovine liver slices, but oxidation to ASM continued at 6 to 7% of the rate observed in the absence of cyanide. Since cyanide completely inhibits mitochondrial β-oxidation, but has variable effects on peroxisomal β-oxidation, peroxisomes can be estimated to contribute a minimum of 6 to 7% of the total fatty acid oxidation occurring in bovine liver slices.

Palmitate oxidation by bovine liver slices occurred at one-third to one-quarter the rate observed with isolated bovine hepatocytes. Endogenous glucose release and gluconeogenesis from propionate or lactate plus pyruvate, however, occurred at comparable rates between the two types of liver preparations. These results suggested that the superior palmitate oxidizing capacity exhibited by isolated bovine hepatocytes may in large part have been due to the greater ability of palmitate to enter individual hepatocytes than to diffuse into a liver slice. This concept was supported by the observation that bovine and rat liver slices oxidized octanoate at rates 10-fold or more greater than palmitate. Only a 2- to 4-fold difference in oxidation rates of these two acids is reportedly observed with perfused rat liver or isolated rat and ovine hepatocytes, indicating that palmitate diffusion into liver slices is limited relative to octanoate diffusion.

Palmitate oxidation by bovine liver slices could be altered by <u>in vivo</u> manipulation of the host animal prior to removal of a liver sample. Feeding a restricted roughage/high concentrate ration to lactating cows resulted in little change in palmitate oxidation by liver slices to CO_2, but markedly inhibited oxidation to ASM. When liver was obtained from lactating cows at different times postpartum, the ability of bovine liver slices to oxidize

palmitate appeared to increase with time from parturition, reaching maximum values around 42 days postpartum. Long-term fasting of cows had relatively little effect on palmitate oxidation to ASM by liver slices, although oxidation to CO_2 was decreased.

Glucose, insulin, propionate and lactate all inhibited palmitate oxidation by bovine liver slices. Presumably the inhibition of oxidation resulted from stimulation of palmitate esterification and the consequent decrease in palmitate availability for oxidation. Propionate had no effect on palmitate oxidation by isolated bovine liver mitochondria. Clofenapate, a metabolic inhibitor which specifically blocks fatty acid esterification, stimulated palmitate oxidation, most likely by increasing palmitate availability for oxidation. Clofenapate addition almost totally overcame the propionate-induced inhibition of palmitate oxidation. These results indicated that propionate inhibition of palmitate oxidation most likely occurs at the level of palmitate esterification.

Acetate also inhibited palmitate oxidation by bovine liver slices. Acetate could have acted as a competitive oxidative substrate to inhibit palmitate oxidation. Alternatively, acetate could have been converted to malonyl-CoA, inhibiting carnitine palmitoyl transferase I (CPT I) and consequently palmitate oxidation.

Malonyl-CoA potently inhibited palmitate oxidation by isolated bovine liver mitochondria, exhibiting an I_{50} value of about .3 μM.

Dibutyryl cAMP (Bt_2cAMP) inhibited palmitate oxidation by rat and bovine liver slices, but stimulated palmitate oxidation by isolated rat hepatocytes. These results suggested that Bt_2cAMP-induced inhibition of palmitate oxidation by liver slices was most likely an artifact of the liver slice incubation system. Endogenous glucose release from bovine liver slices was increased by Bt_2cAMP, perhaps by stimulating glycogen hydrolysis. Thus, the immediate cause of decreased palmitate oxidation by liver slices in response to Bt_2AMP may have been an increase in intracellular glucose availability. Preincubation of bovine liver slices with or without Bt_2cAMP, in an attempt to exhaust cellular glycogen reserves and so prevent Bt_2cAMP-induced inhibition of palmitate oxidation, resulted in marked inhibition of subsequent palmitate oxidation by the liver slices, perhaps due to mobilization of endogenous fatty acid.

Bovine liver mitochondrial CPT exhibited Km values for palmitoyl-CoA and l-carnitine of 11.5 μM and .59 mM, respectively. These values are comparable to those reported for bovine mammary CPT. When CPT was assayed in mitochondria isolated from bovine liver slices instead of a liver homogenate, palmitoyl-CoA Km values were

decreased to between 6.5 and 8.3 μM. Treatment of bovine liver slices with Bt_2cAMP prior to mitochondrial isolation had little effect on the kinetic parameters of CPT. With a Km for palmitoyl-CoA between 6.5 and 11.5 μM, bovine liver CPT could effectively compete with fatty acid esterification for use of cytoplasmic long-chain fatty acyl-CoA.

The suggestion was made that in vivo the principle products of the rumen fermentation, i.e. acetate, propionate, β-hydroxybutyrate and butyrate, could serve to effectively inhibit long-chain fatty acid oxidation in the fed ruminant. These metabolites could potentially inhibit fatty acid oxidation at a number of sites: 1) by increasing fatty acid esterification, 2) by inhibiting mitochondrial CPT I, 3) by competitive oxidation, and 4) by inhibiting acetoacetate synthesis. The possibility that ruminant hepatic ketogenesis is active in the fed state was also discussed.

APPENDICES

APPENDIX TABLE 1

Composition of Krebs-Ringer Bicarbonate Buffer

Component Solutions	Volume	Concentration	
		5-Fold	1-Fold
	ml	Molarity	
NaCl	100.0	.5923	.1188
KCl	4.0	.0237	.0047
KH_2PO_4	1.0	.0059	.0012
$MgSO_4 \cdot 7H_2O$	1.0	.0059	.0012
$NaHCO_3$	21.0	.1244	.0249
Total	130.0	.7522	.1508

Initial concentrations of all component solutions was .77 M. When solutions are mixed together in the volumes indicated, the final concentrations of each component are those indicated in the 5-fold concentration column. This serves as the basis for the incubation media, and when all incubation media ingredients plus an appropriate volume of ddH_2O are added (giving a 5-fold dilution) the final component concentrations are those found in the 1-fold column above. Liver samples were collected in ice-cold 1-fold concentrated KRB, diluted from the 5-fold concentrated KRB with ddH_2O. Sodium bicarbonate is gassed for one hour before preparing KRB. The pH of either concentration of KRB may be adjusted by gassing with either 100% CO_2 (decreases pH) or 95:5 O_2:CO_2 (increases pH). Final pH was adjusted to 7.4.

APPENDIX TABLE 2

Example of the Preparation of Three Different Incubation Media for Use in One Experiment Utilizing Liver Slices

Ingredient	Initial Concentration	Media			Final Concentration
		Control	Lactate	Acetate	
		Volume, ml			
KRB	5X	10.00	10.00	10.00	1X
*Palmitate:BSA	5 mM:1.25 mM	10.00	10.00	10.00	1 mM:0.25 mM
dl-Carnitine	40 mM	2.50	2.50	2.50	2 mM
l-Lactate	50 mM		10.00		10 mM
Acetate	50 mM			10.00	10 mM
Streptomycin-SO_4	1 mg/ml	2.50	2.50	2.50	.05 mg/ml
Penicillin G	1 mg/ml	2.50	2.50	2.50	.05 mg/ml
HEPES, pH 7.4	250 mM	5.00	5.00	5.00	25 mM
ddH_2O		17.50	7.50	7.50	
Total		50.00	50.00	50.00	

*Specific Activity = 250-300 dpm/ml; Fatty Acid:BSA Ratio = 4.0

Three ml of media pipetted into individual incubation flasks.

APPENDIX TABLE 3

Example of the Preparation of One Stock Incubation Media for Use in One Experiment Utilizing Liver Slices

Ingredient	Initial Concentration	Volume	Stock Concentration	Final Concentration
KRB	5X	15.00 ml	1.2X	1X
*Palmitate:BSA	5 mM:1.25 mM	15.00	1.2 mM:0.3 mM	1 mM:0.25 mM
dl-Carnitine	40 mM	3.75	2.4 mM	2 mM
Streptomycin-SO_4	1 mg/ml	3.75	.06 mg/ml	.05 mg/ml
Penicillin G	1 mg/ml	3.75	.06 mg/ml	.05 mg/ml
HEPES, pH 7.4	250 mM	7.50	30 mM	25 mM
ddH_2O		13.75		
		62.50		

*Specific Activity = 250-300 dpm/nmole; Fatty Acid:BSA Ratio = 4.0

Aliquots (2.50 ml) of stock media, with the stock concentrations listed above, are pipetted into incubation flasks. Addition of 0.50 ml ddH_2O or effector solution to the flasks will give the desired final concentrations listed. For example, if a final propionate concentration of 10 mM is desired, addition of .50 ml of 60 mM propionate to 2.50 ml stock media will produce the appropriate final concentrations of all ingredients and effectors.

APPENDIX TABLE 4

Analysis of Covariance of Palmitate Oxidation to CO_2 and Acid-Soluble Metabolites by Bovine Liver Slices Using Liver Slice Wet Weight as Covariate

Variate = CO_2

Source of Variation	df	Sums of Squares	Mean Square	F-value	Significance of F
Liver	3	12,458.7	4,152.9	4.961	p<.01
Covariate	1	71,739.7	71,739.7	85.699	p<.001
Residual	62	51,901.2	837.1		
Total	66	136,099.6			

Variate = Acid-soluble metabolites

Source of Variation	df	Sums of Squares	Mean Square	F-value	Significance of F
Liver	3	180,696	60,232	4.007	p<.025
Covariate	1	1,217,255	1,217,255	80.983	p<.001
Residual	63	946,929	15,031		
Total	67	2,344,880			

Palmitate oxidation to CO_2 and ASM was determined for a total of 68 liver slices from four different livers as described in Methods. Wet slice weights ranged from 40 to 210 mg. Media contained final concentrations of 1 mM palmitate, .25 mM BSA and 2 mM dl-carnitine. Incubation time was from 120 to 180 minutes. Data from one liver sample are given in Figure 6.

APPENDIX TABLE 5

Analysis of Variance of the Time-Course of Palmitate Oxidation to CO_2 and Acid-Soluble Metabolites by Bovine Liver Slices

Variate = CO_2

Source of Variation	df	Sums of Squares	Mean Square	F-value	Significance of F
Liver	3	62,800.3	20,933.4	33.44	p<.001
Time	5	146,872.9	29,374.6	46.93	p<.001
Linear	1	143,615.3	143,615.3	229.42	p<.001
Quadratic	1	463.1	463.1	.740	NS
Cubic	1	663.1	663.1	1.059	NS
Deviations	2	2,131.3	1,065.7	1.702	NS
Liver by Time	15	45,135.5	3,009.0	4.807	p<.001
Residual	68	42,567.5	626.0		
Total	91	297,276.2			

Appendix Table 5.--Continued.

Variate = Acid-soluble metabolites

Source of Variation	df	Sums of Squares	Mean Square	F-value	Significance of F
Liver	3	637,407	212,469	126.22	p<.001
Time	5	448,550	89,710	53.29	p<.001
Linear	1	431,668	431,668	256.44	p<.001
Quadratic	1	7,438	7,438	4.419	p<.05
Cubic	1	5,025	5,025	2.985	NS
Deviations	2	4,418	2,209	1.312	NS
Liver by Time	15	280,206	18,680	11.10	p<.001
Residual	63	106,047	1,683		
Total	86	1,472,210			

Palmitate oxidation to CO_2 and ASM was determined for a total of 96 liver slices as described in Methods. Quadruplicate incubations of slices from four different livers were conducted at each of six incubation times (15, 30, 60, 120, 180 and 240 minutes). Four values were missing from the CO_2 analysis and nine from the acid-soluble metabolite analysis. Media contained final concentrations of 1 mM palmitate, .25 mM BSA and 2 mM dl-carnitine. The time-course of palmitate oxidation to CO_2 and ASM is illustrated in Figure 7.

APPENDIX TABLE 6

Analysis of Variance of 1-^{14}C-Palmitate and U-^{14}C-Palmitate Oxidation to CO_2 and Acid-Soluble Metabolites by Bovine Liver Slices

Variate = CO_2

Source of Variation	df	Sums of Squares	Mean Square	F-value	Significance of F
Liver	1	477.4	477.4	.794	NS
Label	1	14,072	14,072	23.41	$p<.001$
Time	6	335,541	55,924	93.03	$p<.001$
Liver by Label	1	1,111.7	1,111.7	1.849	NS
Liver by Time	6	9,453.2	1,575.5	2.621	$p<.05$
Label by Time	6	10,882	1,813.7	3.017	$p<.025$
Liver by Label by Time	6	1,828.5	304.8	.507	NS
Residual	79	47,489	601.1		
Total	106	420,855			

Appendix Table 6.--Continued

Variate = Acid-soluble metabolites

Source of Variation	df	Sums of Squares	Mean Square	F-value	Significance of F
Liver	1	39,130	39,130	10.41	p<.005
Label	1	108,058	108,058	28.75	p<.001
Time	6	3,154,782	525,797	139.9	p<.001
Liver by Label	1	1,151	1,151	.306	NS
Liver by Time	6	248,322	41,387	11.01	p<.001
Label by Time	6	211,725	35,287	9.39	p<.001
Liver by Label by Time	5	39,083	7,817	2.08	NS
Residual	74	278,105	3,758		
Total	100	4,080,355			

Oxidation of 1-^{14}C- and U-^{14}C-palmitate to CO_2 and ASM was determined for a total of 56 liver slices for each labelled palmitic acid. Quadruplicate incubations of slices from two different livers were conducted at each of seven incubation times (5, 15, 30, 60, 120, 180 and 240 minutes). Five and eleven values were missing from the CO_2 and acid-soluble metabolite analyses, respectively. Incubation conditions were as described in Methods. Media contained final concentrations of 1 mM palmitate, .25 mM BSA and 2 mM dl-carnitine. Time-course of 1-^{14}C- and U-^{14}C-palmitate oxidation to CO_2 and ASM is presented in Figure 8.

APPENDIX TABLE 7

Analysis of Variance of the Time-Course of Palmitate Oxidation by Isolated Bovine Liver Mitochondria

Variate = CO_2

Source	df	Sums of Squares	Mean Squares	F-value	Significance of F-value
Mitochondria	2	222,332.6	111,166.3	321.5	$p<.001$
Time	3	545,222.2	181,740.7	525.6	$p<.001$
Mitochondria by Time	6	145,776.3	24,296.0	70.27	$p<.001$
Residual	24	8,298.1	345.75		
Total	35	921,629.2			

Variate = Acid-soluble metabolites

Source	df	Sums of Squares	Mean Squares	F-value	Significance of F-value
Mitochondria	2	24,271,092.3	12,135,546.1	150.7	$p<.001$
Time	3	61,431,199.3	20,477,066.4	254.3	$p<.001$
Mitochondria by Time	6	5,796,068.8	966,011.5	12.00	$p<.001$
Residual	24	1,932,543.5	80,522.6		
Total	35	93,430,903.9			

Oxidation of palmitate to CO_2 and ASM was determined by triplicate incubations of mitochondrial preparations from three livers. Incubations were for 2, 4, 10 and 15 minutes as described in Methods. Media contained 35 µM palmitate, .7% BSA, 100 µM l-carnitine, 4 mM ATP, 1 mM ADP, 50 µM CoASH and 250 µM reduced glutathione. Each flask contained 1.25 mg mitochondrial protein. The time-course of palmitate oxidation by isolated bovine liver mitochondria is presented in Figure 9.

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