8-1983

The role of B [Beta]-hydroxybutyrate in the regulation of ketogenesis in sheep

Juan Marcos Fernandez

To the Graduate Council:

I am submitting herewith a thesis written by Juan Marcos Fernandez entitled "The role of B [Beta]-hydroxybutyrate in the regulation of ketogenesis in sheep." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Richard N. Heitmann, Major Professor

We have read this thesis and recommend its acceptance:

H. G. Kattesh, J. T. Smith

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a thesis written by Juan Marcos Fernandez entitled "The Role of β-Hydroxybutyrate in the Regulation of Ketogenesis in Sheep." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Richard N. Heitmann, Major Professor

We have read this thesis
and recommend its acceptance:

H. G. Kattesh

John T. Smith

Accepted for the Council:

The Graduate School

THE ROLE OF β-HYDROXYBUTYRATE IN THE REGULATION OF KETOGENESIS IN SHEEP

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Juan Marcos Fernandez

August 1983

ACKNOWLEDGMENTS

The author wishes to express his most sincere appreciation and respect to his major advisor, Dr. Richard N. Heitmann, for his valuable advice, assistance, patience, encouragement, and friendship throughout the course of this study.

Appreciation is expressed to the members of his graduate committee, Dr. H.G. Kattesh and Dr. J.T. Smith, for their valuable advice and understanding, and reviewing the manuscript.

The author wishes to express his utmost appreciation and love to his wife, Sheila:

"For your help throughout the course of this study; for your sincere and whole-hearted interest in this project; for giving up your weekends and Christmas vacation; for your enlightening and reassuring words at times when all seemed lost; and most importantly, for the love, patience, and understanding ... thank you."

The author wishes to express his deep sense of gratitude to the following fellow graduate students without whom this study would not have been possible:

Aaron Cole and Ron Mann for their help during surgery, experimentation, and maintenance of the experimental animals;

Mark Britton, Joan Hembree, and Milton Orr for their assistance during surgery and experimentation;

Christopher K. Reynolds for establishing and running the insulin radioimmunoassay and aiding in surgery;

Victor Fulgoni for his advice and help on the statistical analysis;

and to those fellow students and friends which made the author's stay at the University of Tennessee a little more enjoyable.

Appreciation is expressed to Alma and Nancy Ramsey for their attentiveness and help in the production of this thesis.

The author is indebted to his family for their continual and sincere support throughout his young career. In particular, the author wishes to acknowledge the following family members as a way of showing his gratitude: Jose and Josefa A. de Castaneda; Jose "Pepin" Tabares; Ofelia T. Fernandez; Juan "Pancho" and Concha Ramos; Ignacio and Blanca Varela; his sister Josephine "Pepita", and his brothers Aure, Cesar and Jose.

ABSTRACT

Eleven ewes weighing between 55 and 65 kg were randomly placed in one of three experimental categories: normal (n=5); diabetic insulin-treated, DIT (n=3); and diabetic 72-hour-untreated, DUT (n=3). Animals were rendered diabetic pharmacologically via intravenous doses of alloxan (50 mg/kg). The femoral artery and vein, along with the portal, hepatic, and mesenteric veins were cannulated immediately prior to each experiment. Experiments were therefore conducted in anesthetized (sodium pentobarbital) animals following surgery. Mesenteric infusion of para-aminohippuric acid (PAH) was used to determine whole blood flow rates across the splanchnic tissues. Three sets of preinfusion or control samples were obtained at 15-minute intervals immediately followed by continuous infusion of β-hydroxybutyrate into the caudal vena cava with four subsequent serial whole blood samples obtained from the femoral artery, and portal and hepatic veins at 30-minute intervals. The whole blood samples were analyzed for β-hydroxybutyrate, acetoacetate, and PAH, and plasma analyzed for free fatty acids and insulin.

As expected, free fatty acid levels were depressed by β-hydroxybutyrate infusion in the normal, DIT, and DUT animals. Since there was a concomitant decrease in net hepatic and total splanchnic uptake of free fatty acids, the concentration changes must have been due to decreased peripheral tissue lipolysis. Infusion of β-hydroxybutyrate increased pancreatic insulin release, but there was a corresponding

hepatic uptake of the hormone, and thus, no net change in total splanchnic insulin flux was observed. In the normal and DIT, but not the DUT sheep, a net increase in total splanchnic acetoacetate uptake and a concomitant decrease in net total splanchnic β-hydroxybutyrate release during infusion of the ketone body was observed. From these results it was concluded that the effects of β-hydroxybutyrate infusion on ketone body and free fatty acid concentrations and fluxes may be mediated at the insulin receptor site and not by increased pancreatic insulin production.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION

Ketosis is a metabolic disorder in which large amounts of ketone bodies -- D-β-hydroxybutyrate, acetoacetate, and acetone -- are produced due to impaired carbohydrate or lipid metabolism. It reflects excessive ketone body production due to a negative nutritional state, which is observed when the nutrient demands far exceed nutrient intake (Hibbitt, 1979), or to hormonal factors, especially the endocrine pancreas, in the partitioning of nutrients during different levels of production (Brockman, 1979; Bauman and Currie, 1980). Baird (1982) suggested that in the U.S. and Western Europe the incidence of the disorder lies within the range of 2 to 15%, and that it is basically observed in susceptible high-producing dairy cows between the second and seventh week of lactation and in twin-pregnant sheep during the latter third of gestation. In addition, the milk yields of dairy cows have steadily increased by approximately 25% in the past 20 years (Hibbitt, 1979). Associated with this increased production is an increase in nutrient demands by the individual cows. This demand frequently exceeds the supply, and in conjunction with inherent hormonal adjustments in response to a negative nutrient state or level of production, there is a mobilization of tissue lipids, protein, and carbohydrate stores (Bauman and Currie, 1980; Hood, 1882). Ketosis in ruminant animals occurs as a direct result of increased tissue lipolysis concomitant to low glucose availability during these

periods when nutritional demands far exceed nutrient intake as a consequence of production (Williamson and Hems, 1978; Brockman, 1979; Hibbitt, 1979).

It has been demonstrated that administration of ketone bodies in fasting animals suppress circulating free fatty acids (Björntorp, 1966; Björntorp and Scherstein, 1967; Heitmann and Metzler, 1983) which are the major precursors of hepatic ketogenesis (Williamson and Whitelaw, 1978; Robinson and Williamson, 1980). This decrease in free fatty acid availability as a result of ketone body administration may be due to direct inhibition of peripheral tissue lipolysis by ketone bodies, or by increased pancreatic insulin release in response to the elevated levels of ketone bodies, which will result in a decreased free fatty acid release by peripheral tissues (as reviewed by Alberti et al., 1978; Hales, et al., 1978; Williamson and Whitelaw, 1978; Brockman, 1979; Robinson and Williamson, 1980; McGarry and Foster, 1980a). Therefore, the objectives in this study were to (1) determine the effect of elevated ketone bodies on circulating free fatty acids; (2) attempt to determine if ketone body auto-regulation is taking place at the site of ketogenesis or during peripheral tissue lipolysis; and (3) investiagte the role(s) of insulin in ketogenesis.

CHAPTER II

LITERATURE REVIEW

Characterization of Ketogenic States

Ruminants will normally produce ketone bodies from the alimentary absorption of butyrate. In addition, a pathological manifestation of ketogenesis — ketosis — can occur as clinical or subclinical ketosis.

Physiological Ketogenesis

Physiological ketogenesis describes ketone body production in the normal, healthy animal as part of its regular homeostatic mechanism. In ruminants, normal whole blood concentrations of acetoacetate and β-hydroxybutyrate fall within the range of 10 to 86 and 180 to 420 μmol/l, respectively (Lindsay and Leat, 1975). While in non-ruminants the liver is the major site of ketone body production and free fatty acids are the major substrate, alimentary ketogenesis from volatile fatty acids is the major source of ketone bodies in these animals (Brockman, 1979; Maynard et al., 1979).

Subclinical Ketosis

Baird (1982) described subclinical ketosis as the ketotic state achieved during carbohydrate insufficiency and fat mobilization in response to a negative energy balance, that is accompanied by detectable hyperketonemia and hypoglycemia. No clinical signs of ketosis are exhibited. Whole blood concentration of total ketone

bodies normally will not exceed 1.5 to 2.0 mmol/l in subclinically ketotic ruminants. Baird (1982) continued to emphasize the importance of this form of ketosis stating that subclinical ketosis may have adverse effects on productivity, and since the condition may remain undetected and, hence, untreated, the effects on productivity may parallel those elicited by clinical ketosis.

Clinical Ketosis

Clinical ketosis is the term used to describe the classical pathological form of ketosis. Signs of clinical ketosis appear abruptly in susceptible animals during periods of high productivity when the energy demands for production far exceed those of nutrient intake. During these periods hepatic ketogenesis appears to be the major contributor of ketone bodies (Brockman, 1979). Symptoms include gradual anorexia, apathy, decreased milk production, rapid loss of body condition, and either a positive reaction to the Rothera test, indicating high levels of acetoacetic acid in the milk, or to Acitest, indicating high levels of ketone bodies in the urine (Stamm, 1975; Hibbit, 1979). The more obvious biochemical features of the condition are hyperketonemia with a minimum blood acetoacetate concentration of 0.5 mM, hypoglycemia, elevated free fatty acids in the blood, depletion of hepatic glycogen stores and several gluconeogenic intermediates, and fatty infiltration of the liver (Baird et al., 1968).

Ketone Body Synthesis

Enzymes of Ketone Body Synthesis

The liver is the primary site of ketogenesis in non-ruminant (Williamson and Hems, 1978) and in ketotic ruminant animals (Brockman, 1979). Therefore, most of the information available on the enzymes involved in the synthesis of ketone bodies and subsequently reported upon here is on hepatic ketogenic pathways, and unless otherwise noted, also reflect extra-hepatic pathways.

Free fatty acids (or nonesterified fatty acids) are the major precursors of ketone bodies (McGarry and Foster, 1980a; Robinson and Williamson, 1980). In the liver, acetyl-CoA produced from the intramitochondrial β-oxidation of fatty acids give rise to ketone bodies via the reactions of the hydroxymethylglutaryl-CoA pathway as outlined by McGarry and Foster (1980a). The reactions that constitute this ketogenic pathway are as follows:

$$2\ \text{Acetyl-CoA} \rightleftharpoons \text{Acetoacetyl-CoA} + \text{CoA-SH} \quad (1)$$

$$\text{Acetoacetyl-CoA} + \text{Acetyl-CoA} \rightarrow \text{Hydroxymethylglutaryl-CoA} + \text{CoA-SH} \quad (2)$$

$$\text{Hydroxymethylglutaryl-CoA} \rightarrow \text{Acetoacetate} + \text{Acetyl-CoA} \quad (3)$$

$$\text{Acetoacetate} + \text{NADH+H}^{+} \rightleftharpoons \beta\text{-Hydroxybutyrate} + \text{NAD}^{+} \quad (4)$$

where the enzymes catalyzing the reactions are (1) acetoacetyl-CoA thiolase (E.C.2.3.1.9); (2) hydroxymethylglutaryl-CoA synthase (E.C.4.1.3.5); (3) hydroxymethylglutaryl-CoA lyase (E.C.4.1.3.4); and (4) D-β-hydroxybutyrate dehydrogenase (E.C.1.1.1.30). In the hydroxymethylglutaryl-CoA pathways of ketogenesis acetyl-CoA is not only the precursor of acetoacetate, but also plays a catalytic role

(Williamson and Hems, 1978). All four enzymes have been found in extrahepatic tissues that are considered to be capable of producing ketone bodies (ic., heart, kidney, and intestine), yet, the enzyme that is considered to be rate-limiting to the pathway, hydroxymethylglutaryl-CoA synthase, is only found in large quantities in the liver (McGarry and Foster, 1969). Williamson et al. (1968) found that there is no appreciable difference in the activity of the rate-limiting enzyme in liver homogenates of normal and 48-hour starved rats, but that a 70% and 140% increase in synthase activity was observed in livers of alloxan-diabetic rats and rats fed on a high-fat diet, respectively. The lyase enzyme showed no significant difference in alloxan-diabetic or starved rats, but a 40% increase in activity was observed in the liver of fat-fed rats.

Another pathway for ketone body synthesis consists of the conversion of acetoacetyl-CoA to acetoacetate via deacylation (Stern and Miller, 1959; Williamson and Hems, 1978; Brady et al., 1982). The reaction is as follows:

$$\text{Acetoacetyl-CoA} \longrightarrow \text{Acetoacetate} + \text{CoA-SH}$$

The question arises as to which is the major pathway of acetoacetate snythesis. Published data indicates that acetoacetate synthesis by direct deacylation accounted for between 11% (Brady et al., 1982: Ohgaku et al., 1982) and 20% (Williamson et a l., 1968) in perfused rat liver and kidney. Brady et al. (1982) stated that in liver the predominate pathway of acetoacetate formation is by way of a hydroxymethylglutaryl intermediate, while in the kidney acetoacetyl-CoA is converted to the ketone body to a large extent by direct deacylation.

Ketone bodies were originally considered to be intermediates of β-oxidation of fatty acids. Yet, the intermediate formed during the oxidation of fatty acids is L-β-hydroxybutyryl-CoA, whereas the free hydroxybutyrate found in the body fluids has the D-configuration (Williamson and Hems, 1978). The enzyme D-β-hydroxybutyrate dehydrogenase, which is responsible for the interconversion of acetoacetate and β-hydroxybutyrate in the presence of nicotinamide adenine dinucleotide (NAD), is specific for free D-β-hydroxybutyric acid, and will not oxidize either stereoisomer of β-hydroxybutyryl-CoA (Lehninger, 1975). Therefore, rumen epithelial production of ketone bodies from absorbed butyrate may proceed as follows:

$$\text{Butyric acid} + \text{CoA-SH} + \text{ATP} \rightleftharpoons \text{Butyryl-CoA} + \text{AMP} + \text{pyrophosphate} \quad (1)$$

$$\text{Butyryl-CoA} + \text{Carnitine} \rightleftharpoons \text{Butyryl-carnitine} + \text{CoA-SH} \quad (2)$$

$$\text{Butyryl-carnitine} + \text{CoA-SH} \rightleftharpoons \text{Butyryl-CoA} + \text{Carnitine} \quad (3)$$

$$\text{Butyryl-CoA} + \text{FAD} \longrightarrow \text{Crotonyl-CoA} + \text{FADH}_2 \quad (4)$$

$$\text{Crotonyl-CoA} + \text{H}_2\text{O} \rightleftharpoons \text{3-Hydroxybutyryl-CoA} \quad (5)$$

$$\text{3-Hydroxybutyryl-CoA} + \text{NAD}^+ \longrightarrow \text{Acetoacetyl-CoA} + \text{NADH} + \text{H}^+ \quad (6)$$

where the enzymes catalyzing the reactions are (1) butyryl-CoA synthetase (E.C.6.2.1.2); (2) carnitine acyltransferase I (E.C.2.3.1.-); (3) carnitine acyltransferase II (E.C.2.3.1.-); (4) acyl-CoA dehydrogenase (E.C.1.3.99.3); (5) enoyl-CoA hydratase (E.C.4.2.1.17); and (6) 3-hydroxyacyl-CoA dehydrogenase (E.C.1.1.1.35). It must be emphasized that even though carnitine facilitates the transport of cytosolic fatty acyl-CoAs into the mitochondrial matrix by being a component of the carnitine acyltransferase enzymes (I and II), it is not

absolutely required for the transport of butyryl-CoA, since it is a four-carbon fatty acid, and hence, is able to permeate the mitochondrial membrane (Metzler, 1977). In general, the longer the fatty acyl chain, the more dependent it is on carnitine acyl transferase for its transport across mitochondrial membranes. Therefore, reactions (2) and (3) may or may not be incorporated in all instances. The acetoacetyl-CoA produced in reaction (6) may be converted to ketone bodies by either direct deacylation or by way of hydroxymethylglutaryl-CoA intermediates, as stated above.

Substrates of Hepatic Ketogenesis

Hepatic ketogenesis is the primary source of ketone bodies in non-ruminants and ketotic ruminants, and is second to alimentary ketogenesis only in normal and healthy ruminants (Katz and Bergman, 1969c; McGarry and Foster, 1969). Starvation of a ruminant animal ceases alimentary ketogenesis from volatile fatty acids and the bulk of the ketone bodies produced now may be accounted for by hepatic ketogenesis from free fatty acids (Katz and Bergman, 1969c; Baird, 1977). Baird (1977) has reported a two-fold increase in hepatic ketone body production after a 48-hour fast. In work done on rats, Williamson et al. (1968) found that starvation did not increase the net activities of hydroxymethylglutaryl-CoA synthase or hydroxymethylglutaryl-CoA lyase, but there was a tendency for the cytoplasmic fraction of the hepatocytes decreased in activity. It is ironic that the cytosolic enzymes increased in their activities relative to the mitochondrial enzymes, since the cytosolic enzymes are involved in cholesterol and lipid biosynthesis, while the mitochondrial enzymes

are involved in the synthesis of ketone bodies, which are considered alternate fuels, and thus, in demand in times of negative energy balance such as starvation. Williamson et al. (1968) suggested that variations in the concentrations of ketogenic enzymes play no major role in the regulation of ketone body formation in starvation, and postulated the possibility that ketone bodies may be synthesized in the cytosol in order to dispose of surplus acetyl-CoA arising when lipogenesis is inhibited.

If the enzymes involved in hepatic ketogenesis (acetoacetyl-CoA thiolase, hydroxymethylglutaryl-CoA synthase and lyase, and β-hydroxybutyrate dehydrogenase) cannot be attributed to the regulation of ketogenesis in starvation, then substrate availability must be a major factor in the regulation of ketone body production. Substrate control of ketogenesis includes free fatty acids, glucose availability, gluconeogenic compounds and intermediates, amino acids, and volatile fatty acids. Since volatile fatty acids are by-products of ruminal fermentation, and thus, the major contributors of alimentary ketogenesis, they are discussed in the section below (see Substrates of Alimentary Ketogenesis).

Roles of free fatty acids. It is recognized that free fatty acids are the major precursors of hepatic ketone bodies (Remesy and Demigne, 1983). In work done by Remesy and Demigne (1983) 50% of circulating free fatty acids were removed by the liver in starved rats and 68% of the free fatty acids absorbed by the liver went toward ketone body production. Therefore, any change in fatty acid availability will result in a corresponding change in ketone body

production. The actual precursors of ketone bodies are the acetyl-CoA moieties obtained as a result of the intramitochondrial β-oxidation of fatty acids, as described above. The rate of fatty acid oxidation is directly proportional to circulating levels of free fatty acids (Van Harken et al., 1969; Boyd et al., 1982). Under circumstances when plasma free fatty acid concentrations are high and the availability of non-fatty acid substrates are low (i.e., starvation and diabetes), there is an increased oxidation of fatty acids due to decreased competitive oxidation (Ontko, 1972). Ontko (1973) again demonstrated this by allowing the oxidation of ethanol to compete with that of fatty acids. The result was that ethanol was oxidized in preference to fatty acids, and, in fact, inhibited fatty acid oxidation.

Trenkle and Kuhlemeier (1966) reported plasma free fatty acid concentrations in sheep of 110,600 and 1320 μM 4, 24 and 48 hours following feeding, respectively, with a concomitant decrease in rumen volatile fatty acid concentrations. No change in blood glucose concentration was noted. Thompson and Darling (1975) determined the proportional levels of the major free fatty acids that occur in ruminant blood plasma and found oleate (18:1) > palmitate (16:0) > stereate (18:0). Since all three of these fatty acids require the same acyl carnitine transferase for their entry into the mitochondria not much difference in their rates of oxidation would be expected. Therefore, any work differentiating among the ketogenic potential of the major circulating free fatty acids at physiological concentrations should not show dramatic differences between them.

Circulating levels of free fatty acids are not only responsible for ketogenesis, but also for lipogenesis. One of the symptoms of

ketosis is a fatty liver (Hibbit, 1979). Henderson et al., (1982) suggested that following a large influx of plasma fatty acids into the ovine liver in diabetes or gestational ketosis, there is a diversion of polyunsaturated fatty acids from phospholipids to triacylglycerols. Their results demonstrated a 15-25% increase in liver triacylglyceride concentration in alloxan-diabetic and ketotic sheep, respectively, and a decrease in hepatic proportion of saturated fatty acids (16:0 and 18:0) and increase in proportion of polyunsaturated fatty acids (18:2, 18:3, and 20:4), particularly in ketotic sheep. When β-oxidation of long-chain fatty acids is pharmacologically inhibited (i.e. 2-tetradecylglycidate) hepatic triacylglycerol and cholesterol secretion is stimulated, while ketone body production is ceased (Ide and Ontko, 1981). In addition, 2-tetradecylglycidate stimulated triacylglycerol secretion to levels near normal by the liver of fasted rats, demonstrating that the comparatively low hepatic very low density lipoprotein secretion in the fasting state was not caused by defects in synthesis, assembly, or secretion, but rather by lack of available fatty acid substrate owing to the activation of the fatty acid oxidation sequence. Ide et al. (1982) demonstrated that pharmacological enhancement of fatty acid oxidation by clofibrate results in a concomitant decrease in the flux of fatty acids going into triacylglycerol synthesis, and subsequently in the formation and secretion of triacylglycerol-rich lipoproteins in the liver. Ogata and Hirasawa (1982) suggested that excess plasma free fatty acids during ketonemia may alter the charge of serum lipoproteins by attaching themselves to the lipoproteins, decreasing their activity and consequently increasing the lipid content of the liver.

Roles of glucogenic substrates. Ketogenesis may be described as an overflow mechanism corresponding to a highly active β-oxidation sequence of fatty acids, and the shortage of glucogenic substrates is certainly important in the decreased capacity of the tricarboxylic acid cycle to utilize acetyl-CoA (Lopez-Cardozo and Van den Bergh, 1972). Hypoglycemia and depleted glycogen stores are common symptoms of ketosis (Kronfeld, 1971; Hibbit, 1979; Baird, 1982). The liver of both fed and fasted ruminants produce about 85% of the body's glucose turnover (Bergman et al., 1974) and stores approximately 3% of its weight as glycogen (Kronfeld, 1960). The remaining 10-15% of the body's glucose turnover rate is produced by the kidneys (Bergman et al., 1974). In a clinical study conducted by Ford and Boyd (1960) the depletion of hepatic glycogen had occurred at the onset of symptoms of ketosis in their animals. They noted that clinical recovery may take place some time before glycogen levels can be normalized. In other studies (Kronfeld, 1960; Kronfeld, 1971) the depletion of liver glycogen was observed in ketotic cows and was found to be directly correlated to plasma glucose ($r=.74$) and inversely correlated to the plasma total ketone body concentration ($r=-.67$). It has been suggested that the general carbohydrate insufficiency theory of ketosis and the supply-demand concept of hypoglycemia, as discussed above, may be supported in experimental starvation ketosis, but that they have not been reliable explanations for cows with spontaneous ketosis (Kronfeld, 1972).

Liver glycogen stores are mobilized in times of carbohydrate or glucose intermediate deficiency. Therefore, any change in the glucogenic

status of the liver will affect both glycogen stores and the metabolism of alternate biological fuels such as fatty acids and ketone bodies. Fatty acid oxidation has been found to be essential for the maintenance of maximal rates of gluconeogenesis in the liver of guinea pigs (Tutwiler and Brentzel, 1982). It has been documented that improving gluconeogenesis concomitantly with increasing fatty acid oxidation will lower blood lactate and β-hydroxybutyrate levels in small-for-gestational-age infants (Sabel et al., 1982). Decreased hepatic concentrations of glucogenic amino acids (especially glutamate, glutamine, and alanine) and glucogenic oxo-acids (especially α-keto-glutarate, pyruvate, oxaloacetate) have been documented in ketotic cows (Baird et al., 1968). Robertson et al. (1960) noted an inversely proportional relationship between blood citrate and pyruvate levels in cases of bovine ketosis possibly due to a lack of oxaloacetate. Indeed, Baird et al. (1968) noted that an increased rate of gluconeogenesis, causing a decrease in the concentration of mitochondrial oxaloacetate could be a major factor in ketogenesis. In further work (Williamson et al., 1969) citrate accumulation was regarded as being regulated by the mitochondrial oxaloacetate concentration, while acetyl-CoA buildup would aid in the process. In ruminants, starvation has a detrimental effect on hepatic concentrations of oxaloacetate, citrate, phosphoenolpyruvate, 2-phosphoglycerate, 3- phosphoglycerate, glucose, glycogen, ATP and NAD^+ concentrations, and decreased activities of enzymes involved in gluconeogenesis (Baird et al., 1972). Mitochondrial concentration of free oxaloacetate has been shown to be inversely correlated with rate of ketogenesis

(Siess et al., 1982). McGarry and Foster (1971) have stated that although the activity of the tricarboxylic acid cycle seems to be depressed in ketotic states, that the cycle itself only exerts a minor modulating influence on the rate of ketogenesis.

Substrates of Alimentary Ketogenesis

Normally, alimentary ketogenesis is the major source of ketone bodies in the ruminant. Rumen microorganisms metabolize the ingested feed (i.e. cellulose, hemicellulose, and starch) and produce volatile fatty acids as the end product of glucose catabolism. According to Maynard et al. (1979) the molar percentages of volatile fatty acids on an all roughage diet are: acetate, 65; propionate, 20; and butyrate, 15. Increasing the level of readily available starch by feeding up to 70% concentrate feed (i.e., corn) can change acetate and propionate to approximately 40 and 37 molar percent, respectively. These volatile fatty acids are absorbed by the rumen wall, and are responsible for 53-80% of the digestible energy in ruminants (Carrol and Hungate, 1954; McCarthy et al., 1958; Bergman et al., 1965).

Of the three major volatile fatty acids acetate is the only volatile fatty acid that is found in appreciable quantities in peripheral circulation. This is due to the fact that only 30% of the acetate absorbed is metabolized by the rumen epithelium (Bergman, 1975). Although there is net hepatic acetate production (Baird, 1977; Bergman, 1975) some of the acetate absorbed is converted to acetyl-CoA in the liver, and consequently may be either oxidized via the tricarboxylic acid cycle or be incorporated into lipid biosynthesis, depending on the energy state of the animal (Bauman, 1976; Hochachka et al., 1977).

Propionic acid is the second most abundant volatile fatty acid in circulation, and is considered to be the only gluconeogenic volatile fatty acid (Maynard et al., 1979). It may be converted into propionyl-CoA and subsequently enter the tricarboxylic acid cycle via succinyl-CoA (Lloyd et al., 1978). Neither of these two volatile fatty acids are considered to be major substrates of ketogenesis. However, Lloyd et al. (1978) suggest that in situations when there is a high acetate to propionate ratio (i.e., an all roughage diet) and depleted hepatic glycogen stores the acetyl-CoA produced from acetate will not be oxidized via the tricarboxylate cycle since its intermediates have been depleted due to a lack of glucogenic substrates (i.e., glycogen and propionyl-CoA). The acetyl-CoA will be diverted toward ketone body production via the hydroxymethylglutaryl-CoA pathway, as described above. However, the consensus of opinion is that ketone body production from acetate is almost negligible (Seto et al., 1955; Pethick et al., 1981; Snoswell et al., 1982).

The major ketogenic volatile fatty acid is butyric acid. The majority of ketone bodies produced by alimentary ketogenesis are derived from the rumen epithelial oxidation of absorbed butyric acid to β-hydroxybutyrate (Brockman, 1979). Approximately 80 to 90% of the butyrate is converted to ketone bodies by the rumen epithelium (Bergman, 1975). Seto et al. (1955) performed <u>in vitro</u> studies to determine the amount of butyrate incorporated into ketone bodies in rumen epithelium. They found that approximately half the butyrate that was consumed by the epithelial cells was converted into ketone bodies. In contrast, approximately 19% of the acetate and 2.5% of the propionate absorbed were determined to be used as ketogenic substrates.

In summary, alimentary ketogenesis is the major source of ketone bodies in non-ketotic ruminants. The majority of the ketone bodies produced are dervied from rumen epithelial metabolism of butyrate. The contribution of acetate toward ketogenesis is considered to be minimal. Propionate, the only gluconeogenic volatile fatty acid of the three, is considered to be non-ketogenic.

Ketone Body Utilization

Enzymes of Ketone Body Utilization

Extrahepatic utilization of ketone bodies for energy production has been extensively reviewed (Williamson and Hems, 1978; McGarry and Foster, 1980; Robinson and Williamson, 1980). Ketone bodies must be converted back into acetyl-CoA before they can be completely oxidized via the tricarboxylic acid cycle, and produce a maximum of 12 ATP per two-carbon acetyl unit, or a maximum of 24 or 27 ATP per mole of acetoacetate or β-hydroxybutyrate, respectively (Lehninger, 1975).

The first step in the oxidation of ketone bodies involves the conversion of acetoacetate to acetoacetyl-CoA. Williamson and Hems (1978) describe two enzymes that may be responsible for this conversion:

(a) 3-Oxoacid-CoA transferase (E.C.2.8.3.5):

$$\text{Acetoacetate} + \text{Succinyl-CoA} \rightleftharpoons \text{Acetoacetyl-CoA} + \text{Succinate}$$

(b) Acetoacetyl-CoA synthetase (E.C.6.2.1.3):

$$\text{Acetoacetate} + \text{CoA-SH} + \text{ATP} \rightarrow \text{Acetoacetyl-CoA} + \text{AMP} + \text{pyrophosphate}$$

the rate limiting enzyme in the utilization of ketone bodies is

3-oxoacid-CoA transferase (McGarry and Foster, 1980). This enzyme is present in significant quantities in most tissues except the liver, which accounts for the inability of the hepatocyte to utilize ketone bodies. Cytosolic acetoacetyl-CoA synthetase was first observed by Stern et al. (1953) but was considered to contribute little or no acetoacetyl-CoA from acetoacetate (Stern, 1971; Buckley and Williamson, 1975). Recently, however, Bergstrom et al. (1982) have reported a cytosolic rat hepatic acetoacetyl-CoA synthetase with 5-13 times the activity of that reported by Stern (1971) and Buckley and Williamson (1975), and have proposed that acetoacetyl-CoA produced from acetoacetate in the presence of cytosolic acetoacetyl-CoA synthetase could account for 33% of the hepatic fatty acid synthesis. Importantly, they noted that the activity of the enzyme developed concomitantly to the development of lipogenesis. In addition, under circumstances conducive to lipogenesis (a positive energy balance), the liver can utilize acetoacetate as a lipogenic substrate, even though it can not utilize ketone bodies as energy sources. The acetoacetyl-CoA will be thiolytically cleaved (acetoacetyl-CoA thiolase) in the presence of reduced coenzyme A to form two moles of acetyl-CoA, which can either be oxidized via the tricarboxylic acid cycle or become incorporated in lipogenesis, dependent of the compartmentalization of the enzyme (Ott and Lachance, 1981; Bergstrom et al., 1982).

Utilization of Ketone Bodies

There is a direct correlation between the rates of appearance and disappearance and the blood concentrations of ketone bodies (Bates et al., 1968). Therefore, increased ketone body production is

paralleled by increased ketone body utilization. Indeed, up to a point an overproduction, rather than an underutilization, of ketone bodies is the major cause of ruminant ketosis (Bergman and Kon, 1964a). Consequently, even though the role of ketone body utilization in the regulation of ketone body metabolism is secondary to ketone body production, it is still considered a significant factor and one worth noting.

Bergman and Kon (1964a) observed that the turnover rate of acetoacetate in ketotic ruminants was directly proportional to the plasma concentration of the ketone body until a maximal concentration of about 10 mg/100 ml was attained. At higher circulating acetoacetate levels acetoacetate turnover rates remained constant at nearly 0.4 $g/kg^{0.75}/hr$ (Bergman and Kon, 1964a, 1964b). Similar results have been attained with non-ruminants (Bates, et al., 1968; McGarry and Foster, 1970) along with observations that turnover rates tended to increase almost two-fold during starvation or alloxan-diabetic ketosis.

The question arises as to which tissues are able to utilize ketone bodies. It is a well documented that the liver is unable to utilize ketone bodies for energy since it lacks the rate limiting ketolytic enzyme, 3-oxoacid-CoA transferase (for reviews see Williamson and Hems, 1978; McGarry and Foster, 1980a; Robinson and Williamson, 1980). Even though the liver is unable to utilize ketone bodies, it plays the central role in the equilibration of the acetoacetate:β-hydroxybutyrate ratio (McGarry et al., 1970). Subsequently, ketone bodies may be utilized only by those tissues having significant 3-oxoacid-CoA transferase for mitochondrial acetyl-CoA production and subsequent TCA activity, or acetoacetyl-CoA synthetase for cytosolic

acetyl-CoA production and subsequent lipogenic activity. Among those tissues able to utilize ketone bodies are skeletal and cardiac muscle (Beatty et al., 1960; Kark et al., 1971), kidneys (Kaufman and Bergman, 1971; Kaufman and Bergman, 1974), mammary gland (Kronfeld et al., 1968), intestinal epithelial tissue (Windmueller and Spaeth, 1978) and rat neural tissue (Bossi et al., 1982; Roeder et al., 1982a; Yeh et al., 1982).

Ketone bodies may be utilized for either energy liberation or as precursors in lipogenesis. In sheep, about half the amount of circulating acetoacetate concentration that is taken up by tissues is oxidized to CO_2, regardless of the actual amount utilized (Bergman and Kon, 1964a). It was also reported that the mean percentage of the total exhaled CO_2 derived from acetoacetate metabolism increased from 2% in normal ewes to a maximum of about 20% in ewes experiencing gestational ketosis. Red muscle slices from rats tended to oxidize β-hydroxybutyrate 19 times as rapidly as white muscle slices, and equal amounts of mitochondria from red muscle oxidize β-hydroxybutyrate 8 times as rapidly as the mitochondrial fraction of white muscle (Kark et al., 1971). This may indicate that the oxidation rate of ketone bodies in rat skeletal muscle may be largely due to differences in mitochondrial enzymatic activities rather than simply increased mitochondrial numbers between red and white muscle. Winder et al. (1975) demonstrated that the skeletal muscle of trained rats can oxidize twice as much ketones as that of sedentary rats during prolonged strenuous exercise. They found three, two and one and a half fold increases in enzymatic activities of β-hydroxybutyrate

dehydrogenase, 3-oxoacid-CoA transferase, and acetoacetyl-CoA thiolase, respectively, in response to the exercise program.

Ketone bodies may be a source of carbons in lipid biosynthesis. Cytosolic acetyl-CoA could be derived from acetoacetate via cytosolic acetoacetyl-CoA synthetase and acetoacetyl-CoA thiolase and be used in the biosynthesis of lipids (Bergstrom et al., 1982). In fact, acetoacetate may be an important substrate for cholesterol biosynthesis since it could be directly incorporated into cholesterol as a four-carbon unit where it will be activated to acetoacetyl-CoA, an intermediate one step into the cholesterol synthesizing pathway (Ott and Lachance, 1981; Koper et al., 1981; Bergstrom, 1982). The role of ketone bodies in lipogenesis is especially important in regards to brain growth and development, where cholesterol made partially from ketone body carbons is incorporated into myelin sheath (Koeper et al., 1981; Roeder et al., 1982a; Yeh et al., 1982). Ironically, adipose tissue is not a major site of lipogenesis from ketone bodies *in vivo* in adult rats (Robinson and Williamson, 1978) or mice (Rous, 1977), but it could occur in the liver due to the presence of cytosolic acetoacetyl-CoA synthetase and thiolase (Bergstrom et al., 1982).

Regulation of Ketogenesis

A number of reviews are available on the regulation of ketogenesis (Alberti et al., 1978; Williamson and Whitelaw, 1978; Brockman, 1979; Fain and Shepherd, 1979; McGarry and Foster, 1979b; Zammit, 1981). Recent publications tend to concentrate on the role of hormones on ketogenesis, and more precisely, the effect on ketogenic substrates by metabolic hormones.

Extrahepatic Regulation of Ketogenesis

The major precursors of ketone bodies are free fatty acids (for review see Williamson and Hems, 1978). Therefore, any change in the circulating level of free fatty acids will have a direct and corresponding effect on ketogenesis. Radloff and Schultz (1966) investigated the effect of various hormones on plasma free fatty acid concentration in goats. In general, they found that growth hormone, the catecholamines, ACTH, glucagon, and glucocorticoids all tended to increase plasma free fatty acid level, while insulin tended to decrease it. It was also noted that, in general, female goats tended to exhibit a greater response to hormonal changes than castrated males of the species. β-Adrenergic components of catecholamines and β-adrenergic agonists have been shown to elevate plasma free fatty acids in cattle, whereas α-adrenergic agonists and dopamine had no apparent effects (Blum et al., 1982). Growth hormone and free fatty acid dirunal patterns have been shown to be positively correlated in fed sheep (Bassett, 1974), even though others have shown that free fatty acids inhibit the release of growth hormone (Blackard et al., 1971) and that a decline in circulating levels of free fatty acids will stimulate growth hormone release in sheep (Hertelendy and Kipnis, 1973). Adrenalectomized rats do not show a change in plasma free fatty acid levels (Cole et al., 1982).

The hormones insulin and glucagon and their effects on lipolytic and ketogenic mechanisms in both the ruminant and non-ruminant have been extensively studied. Insulin is considered to (1) stimulate lipogenesis and inhibit lipolysis, (2) reduce circulating levels of

glucose and inhibit gluconeogenesis, and (3) inhibit proteolysis and stimulate amino acid incorporation into proteins (Bassett, 1975). In a study conducted by Chan and Stern (1982) adipocyte lipoprotein lipase activity per cell was found to be highly correlated with adipocyte cell size and lipid content, and its activity was increased by insulin. Lipoprotein lipase is involved in the uptake of plasma triglycerides into extrahepatic tissues (Garfinkel et al., 1967). Therefore, insulin, being a lipogenic hormone, decreases plasma free fatty acid concentrations, and thus, should decrease the amount of free fatty acid reaching the liver to be oxidized and form ketone bodies. Plasma free fatty acids, products of peripheral tissue lipolysis, are inversely correlated to plasma insulin levels in both non-ruminants (de Bruijne et al., 1981; Howard et al., 1983) and ruminants (Radloff and Schultz, 1966; Bassett, 1971; Brockman, 1976, 1978, 1979). Ingle et al. (1972) indicated that the primary site of fatty acid synthesis in sheep is in adipose tissue rather than in the liver, and insulin effects are only considered to be significant in the peripheral tissues and not in the liver (Bowen, 1964). Hence, when insulin is lacking, fatty acid and triacylglycerol synthesis by the peripheral tissues is hampered, and subsequently there is an increase in circulating fatty acid levels.

Glucagon is produced by the α-cells of the islets of Langerhans in the pancreas, and can basically be considered an antagonist of insulin (for review see Unger and Orci, 1981). Brockman (1976, 1979) indicated that glucagon stimulates fat mobilization from adipocytes of sheep, while Bassett (1971) found that glucagon infusion (at a

rate of 5 μg/min for 2 hr) decreased plasma free fatty acid levels. In other work, Keller et al. (1977) concluded that glucagon does stimulate ketogenesis, but it does so by exerting its effect on the liver rather than increasing lipolysis, and thus, free fatty acids. Moreover, insulin has been shown to suppress the lipolytic effects of a high glucagon concentration (Brockman, 1976). Therefore, it appears that glucagon's role in lipolysis is secondary to that of insulin's, and that its primary site of action is the liver (see below).

Some work has been done on the effect of circulating metabolites on the availability of ketogenic precursers. Foremost and most important is the role of circulating ketone bodies on plasma free fatty acid levels and insulin and glucagon secretion. It has been shown that β-hydroxybutyrate markedly inhibits the norepinephrine-induced increase of plasma free fatty acids and glycerol in anesthetized dogs (Björntorp and Schersten, 1967). Björntorp (1966) indicated that ketone bodies decrease plasma free fatty acids and glycerol by inhibiting the activation of the hormone-sensitive lipase. In addition, the ketone bodies do not seem to have an effect on the deactivation role of the lipase. In a study performed on three-day fasted anesthetized dogs Keller et al. (1977) found that infusion of free fatty acids tended to depress plasma insulin levels, whereas glucagon demonstrated no change in concentration. It was also noted that basal rates of insulin played an important role in restraining the rate of ketogenesis in fasted dogs, whereas basal levels of glucagon did not affect the rate of ketogenesis.

It has been demonstrated that β-hydroxybutyrate can augment somatostatin release from the D-cells of the pancreas (Hermansen, 1982). Since somatostatin inhibits both insulin and glucagon secretion from the β- and α-cells, respectively (Honey et al., 1981; Pace and Tarvin, 1981; Kawai and Unger, 1982), it could prove to be physiologically significant in the etiology of hyperketonemia.

Hepatic Regulation of Ketogenesis

The primary site of acetoacetate and β-hydroxybutyrate production in non-ruminants and ketotic ruminants is the liver (Williamson and Hems, 1978; Brockman, 1979). The liver of starved or diabetic animals is capable of producing ketone bodies better than the liver of normal, fed animals, and it does so at the expense of triacylglyceride production (Ide and Ontko, 1981; Ide et al., 1982). Hepatocytes must then have the capability of adapting to physiological and pathological conditions in a minimal amount of time. This requires a highly regulated set of mechanisms responsive to both hormonal and substrate control.

As reviewed by Zammit (1981) there are two branch points, and thus, two possible regulatory sites in the ketogenic pathway. The first site involves the partition of fatty acyl-CoA between esterification to a glycerol moiety to form triacylglycerols or the formation of fatty acylcarnitine, which is required for mitochondrial uptake and subsequent oxidation of long-chain fatty acyl-CoA. The second branch point involves acetyl-CoA. Acetyl-CoA produced from the intramitochondrial β-oxidation of fatty acids can (1) enter the tricarboxylic acid cycle via the citrate synthase reaction and be

used for the liberation of energy, (2) be translocated and used in the cytosolic synthesis of fatty acids, (3) form acetic acid and certain amino acids, and (4) be used in the formation of ketone bodies (Bergman, 1971).

The metabolic fate of fatty acyl-CoA. Fatty acids are activated to fatty acyl-CoA in the cytosol in the presence of ATP and coenzyme A by the enzyme acyl-CoA synthetase (E.C.6.2.1.3). Fatty acyl-CoA can either be re-esterified in the presence of glycerol to form triacylglycerol, or enter the mitochondria to be oxidized, as stated above. In fact, there is a linear and reciprocal relationship between lipogenesis and fatty acid oxidation (McGarry et al., 1978d). The question arises as to what determines the fate of cytosolic fatty acyl-CoA: will it be used in the synthesis of triacylglycerols, or will it be oxidized in the mitochondria? The answer lies with the enzyme, carnitine acyltransferase (E.C.2.3.1.-). In sheep, carnitine acyltransferase has been found in the mitochondrial fractions of liver, skeletal and cardiac muscle, and kidney (Snoswell and Koundakjian, 1972). Moreover, none was found in the cytosolic fraction. The enzyme is responsible for the translocation of cytosolic fatty acyl-CoA into the mitochondria (for review see McGarry and Foster, 1980a). Later, two forms of the enzyme were identified (Kopec and Fritz, 1973; McGarry and Foster, 1978a):

Carnitine acyltransferase I:

$$\text{Fatty acyl-CoA} + \text{Carnitine} \longrightarrow \text{Fatty acylcarnitine} + \text{CoA-SH}$$

Carnitine acyltransferase II:

$$\text{Fatty acylcarnitine} + \text{CoA-SH} \longrightarrow \text{Fatty acyl-CoA} + \text{carnitine}$$

Carnitine acyltransferase I is presumed to be present on the outer aspect of the inner mitochondrial membrane, whereas carnitine acyltransferase II is presumed to reside on the inner aspect of the inner mitochondrial membrane (McGarry and Foster, 1978a). It has been shown that inhibition of the carnitine acyltransferase reaction involving long-chain fatty acyl-CoA will block fatty acid oxidation and inhibit ketone body production (McGarry et al., 1973, 1974b). Brass and Hoppel (1978) found that increasing the acid-insoluble acylcarnitine in rat liver (acid-insoluble acylcarnitine represents the product of the enzyme) by intravenous doses of ℓ-carnitine to levels resembling those found in the liver of fasted rats did not produce a corresponding increase in β-hydroxybutyrate production, and therefore, is contrary to the theory that the enzyme carnitine acyltransferase is rate-limiting and regulatory for ketogenesis. Nonetheless, the general concensus is that the carnitine acyltransferase reaction is a major regulatory component of hepatic ketogenesis.

As stated above, inhibition of carnitine acyltransferase will suppress fatty acid oxidation, and consequently decrease ketone body synthesis. Malonyl-CoA has been found to be such an inhibitor (McGarry et al., 1977). Malonyl-CoA is widely recognized as an intermediate in fatty acid synthesis, and is derived from acetyl-CoA and bicarbonate by the enzyme acetyl-CoA carboxylase (E.C.6.4.1.2).

The reaction takes place in the cytosol, and requires an ATP and biotin as a cofactor (Lehninger, 1975). Of the total carnitine palmitoyltransferase activity found in rat hepatic mitochondria, half was found to be extremely sensitive to malonyl-CoA while the other half appeared resistant to its inhibitory effects (McGarry et al., 1978a). It has been proposed that the malonyl-CoA-sensitive enzyme is the carnitine acyltransferase I enzyme, which is located on the outer aspect of the inner mitochondrial membrane (McGarry et al., 1977, 1978a, 1978d; Cook et al., 1978). Cook et al. (1980) noted that even though rates of ketogenesis at optimal substrate concentrations were identical in hepatic mitochondria from fed and fasted rats, regulation of ketogenesis in the fasted rats appeared to be due to a lower sensitivity of carnitine palmitoyltransferase to inhibitory malonyl-CoA, rather than by the concentration of malonyl-CoA alone, as observed in the fed rats. Brewer (1981) also demonstrated a decreased sensitivity to malonyl-CoA by carnitine acyltransferase in fasted rats. In addition, the data showed that carnitine acyltransferase I activity almost doubled after fasting, while total carnitine acyltransferase (I and II) only increased about 25%.

During clinical ketosis low insulin, high glucagon:insulin ratio, and hypoglycemia will result in a higher rate of hepatic ketogenesis (see review, Brockman, 1979). The effects of insulin and glucagon on the extrahepatic regulation of ketogenesis has already been discussed above. It was noted that a low plasma insulin concentration would contribute toward hepatic ketogenesis by way of increased free fatty acid availability to the liver, and that glucagon did not appear

to contribute significantly to elevated levels of plasma free fatty acids. Yet, a predominant condition for ketogenesis is a high glucagon: insulin ratio (for reviews see Alberti et al., 1978; Brockman, 1979; McGarry and Foster, 1979b).

Glucagon is the catabolic counterpart of insulin, and is secreted by the α-cells of the islets of Langerhans in the pancreas (for review see Unger and Orci, 1981). It has been shown that in hepatocytes from fed rats glucagon administration causes an acute switch in the metabolic machinery of the liver from fatty acid and triacylglycerol synthesis to fatty acid oxidation (Christiansen, 1977; McGarry et al., 1978d; Declercq et al., 1982). Furthermore, elevated levels of glucagon have been found to increase both hepatic fatty acid oxidation and ketogenesis in rats (Heimberg et al., 1969; McGarry et al., 1975) and in diabetic humans (Liljenquist et al., 1974; Schade and Eaton, 1975). It appears that glucagon or an elevated glucagon:insulin ratio enhances fatty acyl-CoA transport into the mitochondria by carnitine acyltransferase (McGarry et al., 1975). In fact, McGarry et al. (1978d) demonstrated that glucagon enhances hepatic fatty acid oxidation, and consequently the liver's ketogenic potential, by reducing tissue malonyl-CoA concentration. As mentioned above, malonyl-CoA inhibits carnitine acyltransferase I activity, and therefore, inhibits fatty acid oxidation (McGarry and Foster, 1979a).

The metabolic fate of acetyl-CoA. As discussed by Bergman (1971), intramitochondrial acetyl-CoA obtained from fatty acid β-oxidation can either be incorporated into the tricarboxylic acid cycle for

energy liberation or be transported out of the mitochondria and be utilized as a carbon source in the synthesis of amino acids and fatty acids, or become ketone bodies. Consequently, the fate of acetyl-CoA can be another major branch point in the regulation of hepatic ketogenesis.

Generally, acetyl-CoA produced in the mitochondria from the β-oxidation of fatty acids enters the tricarboxylic acid cycle via the citrate synthase (E.C.4.1.3.7) reaction. In fact, fatty acid oxidation appears to be essential for the maintenance of maximal rates of the tricarboxylic acid cycle and gluconeogenesis (Tutwiler and Brentzel, 1982). Yet, if intermediates of the tricarboxylic acid cycle are decreased and there is a negative energy balance, acetyl-CoA will be used toward ketone body synthesis. That appears to be the case in increased ketogenesis. Decreased availability of the tricarboxylic acid cycle intermediate, oxaloacetate, will enchance the use of acetyl-CoA for ketone body synthesis rather than in the formation of citric acid (Williamson et al., 1969; McGarry and Foster, 1971; Siess et al., 1982). Decreased tricarboxylic acid cycle intermediates are observed during hypoglycemia (Kronfeld et al., 1960; Kronfeld, 1971) which in turn may increase hepatic ketogenesis (see Zammit, 1981).

Glucagon regulation of ketogenesis is observed at this branch point also. McGarry et al. (1978d) point out that glucagon may exert its inhibitory effect on tissue malonyl-CoA concentration by partially inhibiting the cytosolic enzyme acetyl-CoA carboxylase (E.C.6.4.1.2) which is responsible for the synthesis of malonyl-CoA

and bicarbonate. Not only will fatty acid synthesis be blocked, but acetyl-CoA concentrations will increase, and thus be available for ketogenic purposes.

CHAPTER III

MATERIALS AND METHODS

Animals

Suffolk crossbred nulliparous ewes weighing between 55 and 65 kg and averaging 18 months of age were used throughout the trial. The animals were kept on pasture at a university farm, and transported to individual indoor 6' x 10' pens at least two weeks prior to surgery. This allowed the animals time to get accustomed to their new surroundings, feed and feeding schedule, and handling preceding the experimental phase of the trial. The ewes were kept in adjoining pens on wood shavings and rarely out of visual contact from other ewes, which is important for the normal development of these gregarious animals.

All animals were fed approximately 800 g of commercially prepared alfalfa pellets per day in two 400 g aliquots at 8:00 AM and 5:00 PM. Water and trace mineralized salt were available ad libitum.

The animal pens were located in an adjoining wing of the surgery room. This room was ventilated by an exhaust fan and three air conditioning units, which also served to cool the room during the summer months. This area was kept warm during the winter months by means of passive heat from the main building's own heating system. Three large silicone glass windows allowed sunlight into the pen areas, but on overcast days, incandescent lighting was used to illuminate the area during the day. All lights were turned off at dusk, and thus the animals closely followed normal environmental lighting.

The ewes were handled often in an attempt to get them accustomed to laboratory personnel, instruments, and noises in order to keep stress level to a minimum once they were on trial.

Following the end of the experiments or in the case of catheter failure, animals not rendered diabetic were returned to the farm. Diabetic animals were sacrificed at the end of the trial.

Catheterization of Blood Vessels

Immediately preceeding each experiment chronic indwelling catheters were introduced into the femoral artery and femoral, mesenteric, portal, and hepatic veins. Catheterization of these vessels allowed for the simultaneous blood sampling from the different sites, the infusion of metabolites as described below in the experimental protocol, and in essence, the study of portal-drained visceral, hepatic, and total splanchnic free fatty acid and ketone body metabolism *in vivo* by virtually isolating these tissues *in situ*.

Preoperative Preparations and Care of Animals

All ewes were fasted 24 hours prior to surgery in an attempt to prevent bloat and other gastrointestinal disturbances, and to allow easier access into the abdominal cavity. Throughout the fasting period, water and trace mineralized salt blocks were readily available. All animals received a preoperative examination in order to verify the animal's health.

Ewes used in the diabetic insulin-treated stage of the trial had their blood glucose measured prior to the surgery. The animals were utilized only if their blood glucose levels fell within the 35 to

65 mg/dl range, implying normal carbohydrate and lipid metabolism. Ewes used in the diabetic insulin-untreated stage of the trial had their daily insulin injections withheld 72 hours prior to the surgery. Measurement of blood glucose was not warranted preceeding surgery in these animals, since an abnormally elevated blood glucose level was part of the experimental protocol.

Surgical packs containing the surgical instruments, drapes, and surgical scrub shirts were autoclaved one day prior to the scheduled surgery. A complete list of the contents of the sterile pack is given in Appendix A. During the earlier experiments Silastic catheters were used and were autoclaved along with the surgical pack. Tygon catheter material was used during the latter experiments, and these were steam sterilized in order to avoid denaturing the tygon polymer.

Anesthesia and Intubation

Sodium pentobarbial (65 mg/ml) was the anesthetic used throughout the study. Pentobarbital was administered through a polyvinyl catheter (0.44" I.D. x 0.065" O.D.) acutely implanted into a jugular vein. Approximately 20 mg/kg body weight were administered during the initial dose to anesthetize the animal and allow for a rapid and safe intubation. Sodium pentobarbital was administered throughout the surgery as determined by the acting anesthesiologist.

Intubation was accomplished immediately following initial anesthesia using a 9 mm medical grade oral endotracheal tube. Intubation is especially important in ruminant animals in order to prevent them from drowning in their own vomitus and/or saliva. Intubation also aids the anesthesiologist in monitoring vital respiratory signs, and

provides the animal with a safe and unobstructive respiratory passage. It should be emphasized that at no time during surgery were the animals connected to a gas or breathing unit.

Leg Surgery

The anesthetized and intubated ewe was secured on the operating table in dorsal recumbency. The inguinal region on the medial side of the right leg was clipped, scrubbed with a povidone-iodine solution, shaved using a common single-blade razor, and drenched with 70% ethanol in preparation for the surgery. The alcohol was allowed to air-dry while the surgical field was aseptically draped.

The technique used in the catheterization of the femoral artery and vein is described by Dougherty (1981). An incision was made in the groove medial to the M. sartorius, and the femoral artery and vein were exposed by blunt dissection. Eighty cm in length catheters of either Silastic (0.44" I.D. x 0.065" O.D.) or Tygon (0.050" I.D. x 0.090" O.D.) material were introduced approximately 20 cm into the femoral artery and femoral vein, so as to reside in the caudal aorta and posterior vena cava, respectively. The catheters were tested, filled with heparinized (1000 U/ml) saline, and securely fastened to tissue. The heparinized catheters were exteriorized with the aid of a sterilized skin needle, and secured on the right rump using a commercial branding paste.

Abdominal Surgery

The anesthetized ewe was secured in left lateral recumbency. The right loin area from the lumbar vertebrae and thirteenth rib to the linea alba was prepared in the same manner as described above for the leg surgery.

Cannulation of portal vein and the left hepatic vein were preformed as described by Katz and Bergman (1969c), with a modification in the insertion of the portal vein catheter. In the early surgeries the portal vein catheter was introduced directly via a purse-string suture as described by the authors, but in latter surgeries the cannulation of the vein was accomplished via a mesenteric vein. A mesenteric vein was exposed and a catheter (0.44" I.D. x 0.065" O.D.) was introduced approximately 35 cm thru the common mesenteric vein and into the portal vein until it was confirmed by palpation to be at the porta hepatis. The catheters were tested and filled with heparinized saline once they were secured in tissue.

A mesenteric vein was cannulated using Silastic (0.44" I.D. x 0.065" O.D.) or Tygon (0.040" I.D. x 0.070" O.D.) catheter material. The 80 cm catheter was introduced 10 cm into the vein and fastened securely to mesentery. The catheter was tested and filled with heparinized saline.

At the completion of the three abdominal vessel cannulations, the catheters were exteriorized using the skin needle. The catheters were wrapped in surgical tape and secured on the ewes back (lumbar region) using branding paste. Positioning of these exteriorized catheters is important in preventing their accidental damage.

Postoperative Care of the Animals

The experiments were performed immediately following surgery as described below in the experimental protocol. Therefore, the experiments were conducted on sodium pentobarbital anesthetized animals immediately following a traumatic event —— a 6 to 8 hour operation. The experiments had to be conducted in such a manner due to poor catheter patency.

This acute preparation allowed for the correction of any catheter problems quickly and efficiently.

Once the experiment had been conducted, the wakening ewe was placed in it pen resting on its sternum, and the endotracheal tube was removed. It was important to keep the animal resting on its sternum and against a prop since ruminants may drown or get pneumonia due to rumen fluid accumulation in the lungs. Therefore, the animal was supervised until it was capable of standing on its own. Approximately 250 g of feed and some roughage were made available in an attempt to stimulate gastrointestinal motility and aid in the recovery process. Water and trace mineralized salt were provided ad libitum.

Combiotic (penicillin G and streptomycin) was administered via intramuscular injections at doses of 5 ml, 5 ml, 4 ml, 4 ml, and 3 ml commencing on the day of the surgery, and during the following 4 days, respectively. The doses were modified in accordance to the animal's recovery and health.

Feed was given at preoperative levels once the sheep had fully recovered. The recovery period lasted an average of 5 days to a week, and during that time feed was available at all times, even though the animals rejected much of the feed.

Maintenance of Catheters

All five catheters were filled with heparin (1000 units/ml) or sodium citrate (60 g/l) once the animals were returned to their pens for recovery. The catheters were checked for patency, flushed with saline, and filled with either anticoagulant in order to prevent clot-

ting or fibril clot formation on catheter tips. Catheters were checked twice daily during the first week to 10 days following surgery, and once a day thereafter.

The majority of the catheters would lose their patency one week post-surgery. Regardless of the catheter material (Silastic or Tygon) and anticoagulant (heparin or sodium citrate) used, most catheters would not bleed past a few days after implantation. It was determined during one of the necropsies that the catheter tips were being sheathed in a cocoon-like manner with plaque-like material. The cause and prevention of this condition is unknown at this time.

Experimental Protocol

A total of eleven experiments (5 normal, 3 diabetic insulin-treated and 3 diabetic untreated) were conducted on anesthetized ewes immediately following surgical cannulation as described above. Animals in the diabetic phases of the trial were rendered diabetic pharmacologically by an intravenous dose of alloxan (50 mg/kg), a selective pancreatic beta cell destroyer (Rerup, 1970), at least one week prior to experimentation. Morning blood glucose measurements and afternoon subcutaneous injections of insulin were used in an attempt to control diabetes. Animals used in the diabetic insulin-treated experiments were required to have morning blood glucose levels between 35 and 65 mg/dl prior to surgery, while those ewes used as diabetic untreated animals had average morning blood glucose concentrations of over 120 mg/dl immediately prior to the surgery. It must be empasized that average whole blood glucose measurements in sheep fall between 55 and 80 mg/dl (Lindsay and Leat, 1975).

Para-aminohippuric acid (1.5% solution as the sodium salt) was continuously infused into a mesenteric vein throughout the entire experiment by means of a screw-driven syringe constant-infusion pump at a rate of 0.764 ml/minute. After an hour of infusion, during which time the para-aminohippuric acid equilibrated with the body fluids, three sets of heparinized blood samples were simultaneously withdrawn from the arterial and hepatic and portal vein catheters over a 1 minute period and at 15 minute intervals. Twenty-three ml of blood were obtained from each site and were prepared for analysis of para-aminohippuric acid, ketone bodies, free fatty acids, and insulin as described below. These samples were control period samples.

Immediately following the last set of control samples a pulse dose of 17-20% β-hydroxybutyrate representing a 30 minute infusion (23 ml) was administered via a the femoral vein catheter, and immediately followed by the continuous infusion of 17-20% β-hydroxybutyrate using the screw-driven syringe constant-infusion pump at a rate of 0.764 ml/min. This concentration and flow resulted in a β-hydroxybutyrate infusion rate of approximately 0.4 $g/kg^{0.75}/hr$ for the 55-65 kg sheep used in these experiments. This is the maximum rate of ketone body utilization as reported by Bergman and Kon (1964a,1964b) and indirectly confirmed by Heitmann and Metzler (1983), and therefore should simulate a ketotic state. Four sets of infusion period samples were obtained and treated as described for the perinfusion period above at 30, 60, 90, and 120 minutes following the β-hydroxybutyrate pulse dose. All samples were kept immerged in an ice-bath, and were treated in accordance with assay procedures as described below.

The animals were sutured and allowed to recover following the experiment. Approximately 250 ml of 5% Dextrose solution were administered intravenously to aid in the recuperation process.

Chemical Analysis

All samples were analyzed for whole blood acetoacetate, β-hydroxybutyrate, and para-aminohippuric acid, and plasma insulin and free fatty acids.

Para-aminohippuric Acid

Whole blood flow rates were determined by the indicator-dilution method using para-aminohippuric acid (PAH) as described by Katz and Bergman (1969a). Each sample was analyzed for PAH, and average blood flow rates for each of the three sampling sites were obtained for each experiment. Whole blood flow rates were used to determine substrate and hormonal net fluxes across vascular beds, as described below in the calculation section.

One ml of freshly sampled blood was pipetted into 5 ml of distilled water and vortexed. Five ml of the blood-water mixture were added to 5 ml of 20% trichloroacetic acid (TCA) and vortexed. The solutions were allowed to stand at room temperature for the duration of the experiment. The blood-TCA mixtures were filtered through Whatman #42 filter paper into numbered vials and stored frozen (-20^{o}C) until analyzed. Samples may be stored indefinitely at this stage.

A stock solution was made using a 1:500 dilution of 1.5% PAH, and was considered to be the 100% standard. Standard solutions of

20%, 40%, 60%, and 80% were prepared from the stock solution. The blank samples consisted of equal volumes of 20% TCA and distilled water.

Two ml of sample filtrate, standard, or blank solution were pipetted into corresponding tubes. The samples and blanks were assayed in duplicate, while standards were assayed as single standards. Approximately 3 boiling chips were added to each tube, and a marble placed on the orifice in order to simulate a reflux unit. The tubes were slowly boiled in a hot water bath for 30 minutes from the time that fine bubbles appeared in the tubes. The tubes were cooled at room temperature, and the boiled filtrate stored frozen (-20^{o}C) in capped scintillation vials awaiting the second stage of the analysis. The filtrate may be stored indefinitely at this stage.

In the final stage of the analysis, 400 µl of 1.2 N HCl were added into tubes containing 2 ml of the sample, standard, or blank solution. Next, 200 µl of freshly prepared $NaNO_2$ solution were pipetted into the tubes and vortexed. Two hundred µl of ammonium sulfamate were pipetted into each tube and vortexed within 3 to 5 minutes, followed by the addition of 200 µl of coupling reagent (N-1 naphthylethylenediamine dihydrochloride, 100 mg/100ml H_2O), which also was added and mixed within 3 to 5 minutes. The tubes were incubated at room temperature for a least 10 minutes to allow for color development. The optical densities of the preparations were read on a spectrophotometer at 540 nm.

Para-aminohippuric acid analysis reagents are listed in Appendix B.

Ketone Bodies

Whole blood acetoacetate and β-hydroxybutyrate concentrations were determined enzymatically using a slightly modified version (Pell, personal communication) of the assay reported by Williamson and Mellanby (1965). Whole blood acetoacetate levels had to be determined on the day of the experiment due to their relatively unstable nature, while β-hydroxybutyrate concentrations were determined within a week of sampling.

Equal 4 ml volumes of freshly sampled blood and 1 M $HClO_4$ were vortexed in a capped conical centrifuge tube. The mixed solutions were kept cold during the experiment in an ice bath. The tubes were centrifuged in a refrigerated unit (4^oC) at approximately 1500 x g for 25 minutes. Five ml of the supernatant were transferred into clean 50 ml wide-mouth plastic centrifuge tubes immerged in an ice bath. The supernatant was neutralized with $HClO_4$ or KOH so that the final pH fell in between 6.4 and 7.6. The $KClO_4$ precipitate that was formed was centrifuged off in a refrigerated unit at approximately 1500 x g for 15-20 minutes. The supernatant was carefully poured into a glass, screw-capped scintillation vial, and the precipitate was discarded. Once the aliquot for the acetoacetate assay was obtained, this vial could be stored frozen (-20^oC) for later β-hydroxybutyrate analysis.

Acetoacetate. Analysis of acetoacetate is based on the 1 to 1 acetoacetate/NADH stoichiometry:

$$\text{Acetoacetate} + \text{NADH} \underset{\text{dehydrogenase}}{\overset{\beta\text{-hydroxybutyrate}}{\rightleftharpoons}} \beta\text{-Hydroxybutyrate} + NAD^+$$

The procedure calculates the amount of NADH converted to NAD^+ by spectrophotometrically measuring NADH disappearance at the 340 nm wavelength following enzyme incubation. Briefly, 0.5 ml of pH 6.8 phosphate buffer was pipetted into 12 x 75 mm disposable polyethylene tubes. To this was added 1.0 ml of the sample preparation described above of the pre-β-hydroxybutyrate infusion period samples of normal and diabetic insulin-treated sheep or 0.5 ml of that of the pre-infusion samples of the diabetic untreated and all of the β-hydroxybutyrate infusion period samples. To each of the latter was added 0.5 ml of distilled, deionized water in order to obtain a final volume of 1.5 ml for all tubes. Finally, 0.05 ml of freshly prepared NADH (5 mg in 6 ml of distilled water) was added rapidly in order to avoid auto-oxidation, and the tubes were vortexed. At 60 second intervals an initial (E_1) reading was made followed by a 0.005 ml addition of β-hydroxybutyrate dehydrogenase after which the tube was vortexed. Each tube was incubated for 30 minutes at room temperature followed by a final (E_2) reading again at 60 second intervals.

Freshly prepared working standards of 0.150, 0.100, 0.075, 0.050, 0.025, and 0.000 mM acetoacetate were deproteinized and neutralized similarly to the whole blood samples, and were analyzed similarly to the normal and diabetic insulin-treated preinfusion samples. A linear regression was calculated for the standards where y equaled (E_1 standard - E_2 standard) — (E_1 blank - E_2 blank) and x equaled the concentration of the standard. The response was linear over the concentrations measured and the average regression line was $y = -0.00090 + 2.7595x$ with an R of 0.9906. Unknowns were estimated by calculating the $\hat{x}$ of (E_1 sample - E_2 sample) — (E_1 blank - E_2 blank).

β-Hydroxybutyrate. Since β-hydroxybutyrate dehydrogenase is a fully reversible enzyme dependent upon substrate concentration, β-hydroxybutyrate analysis is the reverse of that of acetoacetate described above and calculates the amount of NAD^+ converted to NADH by spectrophotometrically measuring NADH appearance following enzyme incubation.

Vials containing the sample preparation supernatant can be stored frozen (-20^oC) for not more than one week. Therefore, determination of β-hydroxybutyrate was done within 7 days of the experiment. The frozen sample preparations should only be thawed once, and that being the day of the assay.

Briefly, 0.05 ml of freshly prepared hydrazine-tris buffer (pH 8.5) was pipetted into 12 x 75 mm disposable polyethylene tubes. To this was added 0.25 ml of normal and diabetic insulin-treated preinfusion sample preparations, along with 0.75 ml of distilled/deionized water, or 0.10 ml of the diabetic untreated preinfusion and normal, diabetic insulin-treated, and diabetic untreated infusion sample preparations, along with 0.90 ml of distilled/deionized water. The final volume was 1.50 ml. Finally, 0.05 ml of freshly prepared NAD^+ (0.03 g in 3 ml of distilled water) was rapidly added, and the tubes vortexed.

The sample preparations were now ready to be read spectrophotometrically at 340 nm. As with the acetoacetate assay, initial (E_1) readings were obtained, 0.005 ml of β-hydroxybutyrate dehydrogenase was added, and the tubes vortexed at 60 seconds intervals. The preparations were allowed to incubate at room temperature for a minimum of 45 minutes before the final (E_2) readings were obtained.

Again, a 60 second interval between sample readings was observed, and samples were read in their corresponding order.

Working standards of 2.0, 1.6, 1.2, 0.8, 0.4, and 0.0 mM β-hydroxybutyrate were prepared on the day of the assay, and deproteinized and neutralized similarly to the whole blood samples as described above. The standard preparations were treated in the same manner as preinfusion sample preparations of normal and diabetic, insulin treated animals. A standard regression line and unknown $\hat{x}$ were calculated as described above for acetoacetate. The average β-hydroxybutyrate regression line was $y = -0.0015 + 0.4409x$ with an R of 0.9988.

Ketone body analysis reagents are listed on Appendix C.

Free Fatty Acids

Plasma free fatty acids were determined by modification of the Trout et al. (1960) assay. Two ml of heparinized plasma were added to 10 ml of extraction mixture in 50 ml separatory funnels. The extraction mixture consisted of 40 parts 2-propanol, 10 parts petroleum ether, and 1 part 1N H_2SO_4. The flasks were shaken vigorously for 30 seconds, and immediately vented to relieve gaseous pressure. Four ml of deionized water and 6 ml of petroleum ether were added to the plasma-extraction mixture solution, and the flasks were shaken for 2 minutes. The flasks were immediately vented. The precipitate was carefully removed and discarded, and 1 ml of 0.05% H_2SO_4 was added to the remaining organic solvent layer. The flasks were shaken for another 2 minutes. The mixtures were allowed to settle and separate out to yield a lower acidic layer and an organic solvent layer, which contained the free fatty acids. The acidic layer was completely discarded,

and 5 ml of the remaining organic layer were transferred to corresponding glass conical centrifuge tubes containing 1 ml of titration-indicator mixture. The tubes were immediately capped to prevent evaporation of the highly volatile solution. The titration-indicator mixture consisted of 0.01% thymol blue in 90% ethanol. The solutions were titrated with 0.0018 N NaOH using a 5 ml microburet with 0.01 ml increments. Nitrogen gas was slowly bubbled into the solution while it was being titrated in order to keep the phases mixed, and to expel any acidification caused by atmospheric CO_2.

The samples were assayed in triplicate in an attempt to compensate for the high inherent error (7-15%) associated with the technique. Separatory funnels were rinsed thoroughly with petroleum ether and allowed to air-dry between runs. Separatory funnels were only reused with the same samples, and any new samples required clean funnels. The blanks were treated in the same manner as the samples, as described above, with the exception of the initial plasma addition. Therefore, the initial 30 second shake of the flask containing the blank consisted of only 10 ml of extraction mixture.

Plasma free fatty acid concentrations were calculated as follow:

$$\frac{\mu\text{eq FFA}}{\text{l plasma}} = \frac{\text{ml NaOH}}{\text{ml organic-solvent titrated}} \times \frac{0.0018 \text{ meq NaOH}}{\text{ml NaOH}}$$

$$\times \frac{1 \text{ meq FFA}}{1 \text{ meq NaOH}} \times \frac{\text{total ml petroleum ether}}{\text{ml plasma}}$$

$$\times \frac{1000 \text{ ml plasma}}{1 \text{ l plasma}} \times \frac{1000 \text{ } \mu\text{eq FFA}}{1 \text{ meq FFA}}$$

A list of the reagents used in the analysis of plasma free fatty acids can be found in Appendix D.

Insulin

Insulin was assayed using a radioimmunoassay procedure developed by Aiello as acknowledged by McNeill et al. (1982), and modified by Reynolds and Miller (Personal communication). A standard double-antibody radioimmunoassay technique utilizing guinea-pig anti-bovine insulin antibody as the first antibody and goat anti-guinea pig IgG as the second antibody was used. A total of 123 tubes were processed per 5 day assay, with samples being assayed in duplicate, and standards, total, non-specific binding, and pooled cow and calf samples conducted in triplicate.

On the first day of the assay 200-500 µl of 1% bovine serum albumin-EDTA-phosphate buffer solution were pipetted rapidly into all but the total count borosilicate tubes (12 x 75 mm), along with 100 µl of guinea pig serum-bovine serum albumin-phosphate buffer solution. Standard solutions (200 µl), plasma samples (400 µl), and first antibody (100 µl) were added to corresponding tubes, with the exception of the total count and non-specific binding tubes. The tubes were immediately vortexed and incubated at 2-4^{o}C for 24 hours. On day two, labelled insulin (^{125}I-Insulin) was prepared, and 100 µl were added to all of the tubes. Again, the tubes were vortexed and incubated at 2-4^{o}C for 24 hours. On the third day of the procedure, the second antibody was prepared and 200 µl were added to all but the total count tubes. The tubes were vortexed and incubated for 48 hours at 2-4^{o}C. On the fifth and final day of the assay, all of the tubes, with the

exception of the total tubes, were centrifuged at 1500 x g for 30-35 minutes in a refrigerated centrifuge (4°C), decanted, and placed upside down to drain on Kimwipes in a foil-lined box for a minumum of 15 minutes. The top half of the tubes were gently swabbed with Kimwipes using a wooden applicator stick. Finally, the tubes were placed in an automatic gamma counter and counted for 1 minute. It must be emphasized that the entire assay was accomplished while the tubes and reagents were kept cool in ice baths.

Materials and reagent preparations are listed in Appendix E. An outline of the protocol can be found in Appendix F.

Calculations

Blood flow rates were calculated as described by Katz and Bergman (1969a). Plasma flows were obtained by correcting for the packed cell volume. Insulin and metabolite net fluxes across tissues were calculated as described by Brockman and Bergman (1975).

Blood flow rates were determined using the Fick Principle: opitical density (O.D.) units infused divided by arteriovenous O.D. unit differences. Therefore:

$$F = \frac{I}{Cv - Ca}$$

where F is the rate of blood flow (ml/min): I is the infusion rate of PAH (mg/min); and Cv and Ca are the concentrations of PAH (mg/ml) in venous and arterial blood, respectively. Blood flow rates were determined for hepatic and portal veins and hepatic arterial flow was calculated by difference.

To calculate ketone body, free fatty acid, and insulin net flux across tissues venoarterial concentration differences were multiplied by blood flow. New fluxes were calculated for portal-drained viscera, hepatic, and total splanchnic vascular beds. Portal-drained visceral (PDV) net flux was calculated as follows:

$$PDV = Fpv\ (Cpv - Ca)$$

where PDV is portal drained visceral net flux (μM/min); Fpv is portal blood flow (1/min) and Cpv and Ca are metabolite concentrations (μM) in the portal vein and femoral artery, respectively.

Hepatic net flux was calculated as follows:

$$HEP = Fpv\ (Chv - Cpv) + Fa\ (Chv - Ca)$$

where HEP is hepatic net flux (μM/min); Fpv is portal blood flow (1/min); Fa is arterial blood flow (1/min): and Chv, Cpv, and Ca are metabolite concentrations (μM) in the hepatic vein, portal vein, and femoral artery, respectively.

Total splanchnic net flux, which is the sum of the portal drained visceral and hepatic net fluxes, can also be calculated using the following formula:

$$TSP = Fhv\ (Chv - Ca)$$

where TSP is total splanchnic net flux (μM/min); Fhv is hepatic blood flow (1/min); and Chv and Ca are metabolite concentrations (μM) in the hepatic venous and arterial blood, respectively.

Insulin net fluxes were calculated in the same manner as described above, except insulin net fluxes were calculated as mU/min.

Statistical Analysis

All data was analyzed by a two-way analysis of variance to detect significant differences due to β-hydroxybutyrate infusion. In addition, a Duncan's New Multiple Range Test was used to determine the significance of differences among normal, diabetic insulin-treated, and diabetic untreated groups. Computations were performed by computer using the Statistical Analysis System (SAS).

CHAPTER IV

RESULTS

Whole Blood Flow Rates

Whole blood flow rates for the portal and hepatic veins and portal to hepatic ratios are given in Table 1. In normal, fed sheep portal flow was 1.7 l/minute and approximately 70% that of the hepatic flow. This is in close agreement with previously reported data on fed sheep (Katz and Bergman, 1969a). However, there was a significant ($P<.05$) 55% decrease in portal flow and 37% decrease ($P<.01$) in portal to hepatic ratio in diabetic insulin-treated sheep. This is contrary to previous work which reported no change in blood flows between fed and insulin-treated, alloxanized sheep (Brockman and Bergman, 1975a). No satisfactory physiological explanation has been found to explain this occurrence. On the other hand, diabetic untreated sheep had similar flow rates and ratios to normal animals.

Ketone Bodies

Acetoacetate

Control and β-hydroxybutyrate infusion period arterial whole blood acetoacetate concentrations and venoarterial differences (μM) are given in Table 2. Control arterial acetoacetate concentrations in the normal and diabetic insulin-treated animals of 35.6 and 46.3 μM, respectively, are representative of normal whole blood acetoacetate

TABLE 1. WHOLE BLOOD FLOW RATES, (l/MIN).

	Portal	Hepatic	$\frac{\text{Portal}}{\text{Hepatic}}$
Normal (n=5)	1.663 ± 0.295[1]	2.375 ± 0.378	0.695 ± 0.018
DIT[2] (n=3)	0.757 ± 0.310*	1.624 ± 0.504	0.441 ± 0.052**
DUT[3] (n=3)	1.354 ± 0.261	2.015 ± 0.154	0.693 ± 0.174

*Significantly different from normal, P<.05.

**Significantly different from normal, P<.01.

[1]Means ± SEM

[2]Diabetic insulin-treated

[3]Diabetic untreated

TABLE 2. ACETOACETATE CONCENTRATIONS AND VENOARTERIAL DIFFERENCES, (μM).

	A[1]	H[2]-A	H-P[3]	P-A
Normal (n=5)				
Control	35.6 ± 5.5[4]	-2.2 ± 5.4	-14.6 ± 5.2*	12.5 + 4.3*
BOHB[5] Infusion	195.5 ± 14.1+	-24.6 ± 8.2*	-63.2 ± 5.9*	38.6 ± 4.6*
DIT[6] (n=3)				
Control	46.3 ± 8.9	16.3 ± 9.1	6.1 ± 11.1	10.2 ± 5.4
BOHB Infusion	213.0 ± 14.6+	-66.6 ± 8.3*	-143.4 ± 14.6*	76.9 ± 10.4*
DUT[7] (n=3)				
Control	411.9 ± 52.5††	28.6 ± 17.4	45.5 ± 31.5	-16.9 ± 14.5
BOHB Infusion	418.9 ± 44.1††	11.3 ± 3.8*	1.9 ± 4.1	9.5 ± 3.6*

*Significantly different from 0, P<.05.

+Significantly different from control, P<.01.

††Significantly different from normal, P<.01.

[1]Caudal aorta

[2]Hepatic vein

[3]Portal vein

[4]Means ± SEM

[5]β-Hydroxybutyrate

[6]Diabetic insulin-treated

[7]Diabetic untreated

levels in sheep (Lindsay and Leat, 1975). The abnormally high level of the ketone body (~412 μM) in the diabetic untreated group is indicative of a diabetic ketoacidotic state. The increases observed in arterial acetoacetate during the infusion of β-hydroxybutyrate is an artifact of the infusion and are of no concern when considered alone, but become relevant when their venoarterial differences are calculated. Venoarterial differences different from zero represent a significant ($P<.05$) uptake (negative values) or release (positive values) of acetoacetate by the respective tissue beds as described above in the calculations. However, venoarterial differences must be used in conjunction with their respective blood flow rates to determine any changes in rate of uptake or release (net flux).

Acetoacetate net fluxes for the control and β-hydroxybutyrate infusion periods are given in Table 3. A significant ($P<.05$) increase in portal-drained visceral release of acetoacetate was observed during the β-hydroxybutyrate infusion period in both the normal and diabetic insulin-treated groups, however, this effect was completely lost in the diabetic untreated animals. Net hepatic uptake of acetoacetate increased during the infusion period ($P<.05$) in these same two groups. Again, the effect was completely lost in the diabetic untreated animals. These changes in portal-drained visceral and hepatic fluxes resulted in an increased net total splanchnic acetoacetate uptake upon β-hydroxybutyrate infusion in the normal and diabetic insulin-treated sheep. Even though there were similar trends observed with the diabetic untreated animals, ketone body infusion in these insulin-lacking individuals did not significantly alter net acetoacetate flux across the tissues studied.

TABLE 3. ACETOACETATE NET FLUX, (μM/MIN)

	PDV[1]	HEP[2]	TSP[3]
Normal (n=5)			
Control	24.7 ± 8.7[4]	-35.8 ± 14.2	-11.1 ± 12.4
BOHB[5] Infusion	67.9 ± 10.1*	-112.0 ± 12.6*	-44.2 ± 16.4
DIT[6] (n=3)			
Control	4.0 ± 5.7	12.5 ± 13.4	16.5 ± 11.1
BOHB Infusion	62.7 ± 16.9*	-177.2 ± 37.6*†	-114.5 ± 22.6*†
DUT[7] (n=3)			
Control	-9.4 ± 13.9†	71.7 ± 49.6†	62.3 ± 36.4†
BOHB Infusion	11.9 ± 5.5†	13.0 ± 8.2†	24.9 ± 7.9†

*Significantly different from control, P<.05.

†Significantly different from normal, P<.05.

[1]Portal-drained viscera

[2]Hepatic

[3]Total splanchnic

[4]Means ± SEM

[5]β-Hydroxybutyrate

[6]Diabetic insulin-treated

[7]Diabetic untreated

β-Hydroxybutyrate

Control and infusion period arterial β-hydroxygutyrate concentrations and venoarterial differences (μM) were determined for all three treatment groups (Table 4). Control arterial β-hydroxybutyrate concentration in the normal animals averaged 565 μM. This value was slightly higher than the normal range of 180-420 μM that has been reported on sheep (Lindsay and Leat, 1975). This slightly elevated β-hydroxybutyrate level may be due to fact that the experiments were conducted immediately following surgery, and thus, after a minimum of 24 hours of fasting, as described above in the surgery section. An elevated arterial β-hydroxybutyrate level (1023 μM) during the control period was noted in the diabetic insulin-treated group. The 24 hour fasting required prior to the surgery alone cannot explain such an elevated β-hydroxybutyrate level. Therefore, another explanation may be that the insulin doses administered to control the diabetes were at suboptimal levels. Ketosis was apparent in the diabetic untreated animals, as evidenced by arterial concentrations of the ketone body in the 14 mM range during the control period. As in the case of acetoacetate, the increased arterial concentrations of β-hydroxybutyrate during β-hydroxybutyrate infusion are artifacts and only the venoarterial differences should be considered. Significant venoarterial differences represented a notable change from zero in blood β-hydroxybutyrate levels across tissues.

β-Hydroxybutyrate net fluxes are shown in Table 5. There was a dramatic ($P<.05$) shift from release (137 μM/min) to uptake (-394 μM/min) of β-hydroxybutyrate by the portal-drained viscera upon infusion of

TABLE 4. β-HYDROXYBUTYRATE CONCENTRATIONS AND VENOARTERIAL DIFFERENCES, (μM).

	A[1]	H[2]-A	H-P[3]	P-A
Normal (n=5)				
Control	565 ± 46[4]	244 ± 41*	155 ± 25*	89 ± 22*
BOHB[5] Infusion	4105 ± 352†	105 ± 111	370 ± 57*	-264 ± 71*
DIT[6] (n=3)				
Control	1023 ± 81	481 ± 46*	426 ± 45*	55 ± 38
BOHB Infusion	4321 ± 249†	353 ± 77*	850 ± 149*	-497 ± 204*
DUT[7] (n=3)				
Control	13771 ± 2033††	754 ± 400	422 ± 381	331 ± 311
BOHB Infusion	19537 ± 1701† ††	822 ± 316†	615 ± 291*	207 ± 309

*Significantly different from 0, $P<.05$.

††Significantly different from control, $P<.05$.

†††Significantly different from normal, $P<.01$.

[1]Caudal aorta

[2]Hepatic vein

[3]Portal vein

[4]Means ± SEM

[5]β-Hydroxybutyrate

[6]Diabetic insulin-treated

[7]Diabetic untreated

TABLE 5. β-HYDROXYBUTYRATE NET FLUX, (μM/MIN).

	PDV[1]	HEP[2]	TSP[3]
Normal (n=5)			
Control	137 ± 28[4]	395 ± 51	532 ± 65
BOHB[5] Infusion	-394 ± 94*	679 ± 127	285 ± 191
DIT[6] (n=3)			
Control	9 ± 26	749 ± 126	758 ± 113
BOHB Infusion	-278 ± 102	881 ± 100	602 ± 140
DUT[7] (n=3)			
Control	450 ± 471	1138 ± 788	1588 ± 859
BOHB Infusion	227 ± 435†	1445 ± 563	1672 ± 681†

*Significantly different from control, P<.05.

†Significantly different from normal, P<.05.

[1]Portal-drained viscera

[2]Hepatic

[3]Total splanchnic

[4]Means ± SEM

[5]β-Hydroxybutyrate

[6]Diabetic insulin-treated

[7]Diabetic untreated

the ketone body. This trend was also observed in the diabetic insulin-treated animals. Although, the diabetic untreated group exhibited decreased portal-drained visceral β-hydroxybutyrate release during the infusion period, it did not shift to uptake as occurred in the other treatment groups. No significant changes in hepatic net flux of β-hydroxybutyrate were observed upon ketone body infusion due to the high errors associated with its analysis. However, in all cases there was always a slight increase in hepatic release. There seemed to be a trend toward decreased total splanchnic release of β-hydroxybutyrate during the infusion period in the normal sheep, undoubtedly due to the switch from portal-drained visceral release to uptake, yet, this trend is absent in both the daibetic insulin-treated and diabetic untreated groups.

Free Fatty Acids

Arterial plasma free fatty acid concentrations and venoarterial differences are given in Table 6. A preinfusion plasma free fatty acid concentration of 872 μM in the normal sheep differs from the 100-600 μM range reported by Lindsay and Leat (1975). As in the case of β-hydroxybutyrate, the elevated plasma free fatty acid level may be due to the fasting period preceeding the surgery, and subsequently, the experiment, since it resembles plasma free fatty acid levels in 24 hour fasted sheep as reported by Heitmann and Metzler (1983). Control levels of arterial plasma free fatty acids in the diabetic insulin-treated animals of 1186 μM also indicate an increased tissue lipolysis due to impaired carbohydrate metabolism in response to inadequate

TABLE 6. FREE FATTY ACID CONCENTRATIONS AND VENOARTERIAL DIFFERENCES, (μM).

	A[1]	H[2]-A	H-P[3]	P-A
Normal (n=5)				
Control	872 ± 83[4]	-132 ± 32*	-203 ± 19*	71 ± 18*
BOHB[5] Infusion	528 ± 55†	-31 ± 18	-93 ± 13*	62 ± 12*
DIT[6] (n=3)				
Control	1186 ± 105††	-258 ± 41*	-270 ± 33*	12 ± 22
BOHB Infusion	684 ± 33†	-54 ± 14*	-123 ± 18*	69 ± 14*
DUT[7] (n=3)				
Control	1933 ± 92††	-285 ± 49*	-333 ± 53*	49 ± 24
BOHB Infusion	1719 ± 85† ††	-217 ± 22*	-234 ± 55*	17 ± 45

*Significantly different from 0, P<.01.

†Significantly different from control, P<.05.

††Significantly different from normal, P<.01.

[1]Caudal aorta

[2]Hepatic vein

[3]Portal vein

[4]Means ± SEM

[5]β-Hydroxybutyrate

[6]Diabetic insulin-treated

[7]Diabetic untreated

insulin administration. Yet, plasma free fatty acids were not as elevated in the diabetic insulin-treated group as they were in the diabetic untreated group (1933 μM) during the control period. In all three treatment groups, plasma arterial free fatty acid concentrations were significantly ($P<.05$) depressed by β-hydroxybutyrate infusion. Again, the venoarterial differences were, for the most part, different ($P<.01$) from zero, indicating significant tissue uptake or release.

Plasma free fatty acid net fluxes are given in Table 7. There were no significant changes in portal-drained visceral free fatty acid net flux in either of the three groups. This is to be expected, since the majority of dietary fat is absorbed via the lymph system, and the splanchnic tissues are not generally considered to be a significant tissue of fat storage. A significant ($P<.05$) decrease in free fatty acid uptake by the liver upon β-hydroxybutyrate infusion was noted in all three treatment groups. However, the diabetic untreated sheep did not demostrate as dramatic a decrease as was observed in the normal and diabetic insulin-treated groups. As a consequence, total splanchnic free fatty acid uptake was significantly decreased in the normal and diabetic insulin-treated sheep, but not in the diabetic untreated animals. Diabetic untreated animals demonstrated significantly ($P<.05$) greater net free fatty acid uptake by the hepatic and total splanchnic tissues during both the control and β-hydroxybutyrate infusion periods than that observed in the normal animals, indicating extensive hepatic metabolism of this ketogenic substrate.

TABLE 7. FREE FATTY ACID NET FLUX, (μM/MIN).

	PDV[1]	HEP[2]	TSP[3]
Normal (n=5)			
Control	97 ± 33[4]	-284 ± 31	-187 ± 50
BOHB[5] Infusion	76 ± 19	-108 ± 19*	-33 ± 31*
DIT[6] (n=3)			
Control	3 ± 9†	-280 ± 49	-276 ± 50
BOHB Infusion	32 ± 10	-94 ± 22*	-62 ± 16*
DUT[7] (n=3)			
Control	43 ± 27	-485 ± 82†	-442 ± 81†
BOHB Infusion	-10 ± 43†	-319 ± 66*†	-329 ± 38†

*Significantly different from control, P<.05.

†Significantly different from normal, P<.05.

[1]Portal-drained viscera

[2]Hepatic

[3]Total splanchnic

[4]Means ± SEM

[5]β-Hydroxybutyrate

[6]Diabetic insulin-treated

[7]Diabetic untreated

Insulin

Control and β-hydroxybutyrate infusion period arterial plasma insulin concentrations and venoarterial differences (μU/ml) were determined for all three treatment groups (Table 8). Preinfusion plasma insulin levels in arterial blood in normal sheep averaged approximately 38 μU/ml, which is a good standard value in ruminant animals (Ferguson and Cox, 1975; Brockman and Bergman, 1975b). Infusion of the ketone body significantly ($P<.10$) increased arterial plasma insulin levels (49.3 μU/ml) in these animals. Diabetic insulin-treated sheep had identical control and β-hydroxybutyrate infusion period arterial plasma insulin levels of 22.1 μU/ml. This was not surprising considering that their insulin-secreting cells were pharmacologically destroyed, and thus, they were maintained on fixed subcutaneously-administered insulin doses as reported above in Materials and Methods. The diabetic untreated ewes had similar control and β-hydroxybutyrate infusion period plasma insulin concentrations of less than 3.0 μU/ml. Significant venoarterial differences are seen for the most part only on normal sheep, indicating that pancreatic production (P-A, in this case) of insulin is completely shut down in alloxanized sheep.

Plasma insulin net fluxes are given in Table 9. Portal-drained visceral net flux of insulin is a direct measurement of pancreatic β-cell insulin secretion, since it is the only vein draining the gland. Preinfusion and β-hydroxybutyrate infusion period portal-drained visceral insulin net flux were calculated to be 24.8 mU/min and 35.7 mU/min, respectively, in the normal experimental group. This

TABLE 8. INSULIN CONCENTRATIONS AND VENOARTERIAL DIFFERENCES, (μU/ML).

	A[1]	H[2]-A	H-P[3]	P-A
Normal (n=5)				
Control	38.2 ± 5.4[4]	4.2 ± 1.9	-16.2 ± 3.9*	20.3 ± 3.9*
BOHB[5] Infusion	49.3 ± 4.3†	4.2 ± 2.0	-26.8 ± 3.9*	30.9 ± 3.6*
DIT[6] (n=3)				
Control	22.1 ± 1.9††	-3.0 ± 0.8*	-1.3 ± 0.8	-1.7 ± 0.6
BOHB Infusion	22.1 ± 1.5††	-2.9 ± 0.5*	-1.5 ± 0.7	-1.4 ± 0.7
DUT[7] (n=3)				
Control	2.8 ± 0.4††	-0.5 ± 0.4	-1.3 ± 0.5	0.8 ± 0.7
BOHB Infusion	2.9 ± 0.5††	-0.2 ± 0.5	-0.1 ± 0.5	-0.1 ± 0.5

*Significantly different from 0, P<.01.

†Significantly different from control, P<.10.

††Significantly different from normal, P<.01.

[1]Caudal aorta

[2]Hepatic vein

[3]Portal vein

[4]Means ± SEM

[5]β-Hydroxybutyrate

[6]Diabetic insulin-treated

[7]Diabetic untreated

TABLE 9. INSULIN NET FLUX, (mU/MIN)

	PDV[1]	HEP[2]	TSP[3]
Normal (n=5)			
Control	24.8 ± 5.4[4]	-16.7 ± 5.2	8.2 ± 4.4
BOHB[5] Infusion	35.7 ± 5.2*	-26.6 ± 4.0*	9.2 ± 4.4
DIT[6] (n=3)			
Control	-0.9 ± 0.4†	-1.8 ± 1.0†	-2.7 ± 0.8†
BOHB Infusion	-0.6 ± 0.3†	-2.8 ± 0.7†	-3.4 ± 0.7†
DUT[7] (n=3)			
Control	0.7 ± 0.7†	-1.5 ± 0.7†	-0.8 ± 0.6
BOHB Infusion	0.1 ± 0.6†	-0.2 ± 0.6†	-0.1 ± 0.6†

*Significantly different from control, P<.05.

†Significantly different from normal, P<.05.

[1]Portal-drained viscera

[2]Hepatic

[3]Total splanchnic

[4]Means ± SEM

[5]β-Hydroxybutyrate

[6]Diabetic insulin-treated

[7]Diabetic untreated

demonstrates a significant ($P<.05$) 44.0% increase in insulin secretion by the β-cells upon ketone body infusion. Concomitantly, hepatic uptake of plasma insulin was significantly ($P<.05$) increased by 62.8% upon β-hydroxybutyrate infusion in these same animals. Therefore, there was no difference in net total splanchnic insulin release (8.2 versus 9.2 mU/min) during the control and β-hydroxybutyrate infusion periods, respectively, in the normal ewes. Diabetic insulin-treated and untreated animals had no significant rates of flux across any of the tissues measured, a function of their diabetic condition.

CHAPTER V

DISCUSSION

This study examines the possibility of ketone body auto-regulation in anesthetized sheep. Three experimental categories were considered in order to investigate the role of insulin in the regulation of ketogenesis. These included normal, diabetic insulin-treated, and diabetic 72-hour untreated animals.

Ketone Bodies

The mean total ketone body concentration of arterial blood in normal animals was 600 μM (Tables 2 and 4, pages 52 and 56). This value is below that considered to be ketotic in ruminants (Baird, 1982), and corresponds with that obtained by Kaufman and Bergman (1974). The mean arterial acetoacetate and β-hydroxybutyrate concentrations in the normal sheep were 35.6 and 565 μM, respectively. According to Lindsay and Leat (1975) acetoacetate concentration in sheep whole blood ranges between 10 and 86 μM, while β-hydroxybutyrate falls within 180 and 420 μM. Hence, β-hydroxybutyrate was slightly elevated in the normal animals, while acetoacetate remained at a normal physiological level. The elevated arterial β-hydroxybutyrate observed in these animals could be due to the fact that the sheep had undergone surgery and were still under anesthesia (sodium pentobarbital) when the experiments were conducted.

Infusion of β-hydroxybutyrate at a rate of 0.4 g/kg$^{0.75}$/hr enchanced acetoacetate release from 24.7 to 67.9 μM/min (Table 3, page 54) and stimulated β-hydroxybutyrate uptake from 137 to -394 μM/min (Table 5, page 57) by the portal-drained viscera in normal animals. Portal-drained visceral values include ketone bodies produced by the rumen epithelium (Seto et al., 1955; Roe et al., 1966). Roe et al. (1966) reported a portal-drained visceral ketone body production rate of approximately 333 μM/min in normal 60 kg sheep. Preinfusion ketone body production by the portal-drained viscera is due to volatile fatty acid (more specifically, butyrate) metabolism by the rumen epithelium (Seto et al., 1955). In addition, normal animals exhibited an increased hepatic acetoacetate uptake from -35.8 to -112.0 μM/min (Table 3, page 54) along with an increased hepatic β-hydroxybutyrate output from 395 to 679 μM/min (Table 5, page 57) upon β-hydroxybutyrate infusion. Baird et al. (1975) have reported net acetoacetate uptake and net β-hydroxybutyrate release by livers of healthy cows. The result was a 53% decrease in net total splanchnic ketone body output upon β-hydroxybutyrate infusion.

Diabetic insulin-teated sheep had a mean preinfusion arterial ketone body concentration of 1069 μM (Tables 2 and 4, pages 52 and 56). Even though the animals were considered to be non-ketotic (Baird, 1982) β-hydroxybutyrate concentration was abnormally high (1023 μM). Acetoacetate concentrations of approximately 46 μM resemble those obtained by other researchers (Lindsay and Leat, 1975). Upon infusion of β-hydroxybutyrate portal-drained visceral release of acetoacetate increased from 4.0 to 62.7 μM/min (Table 3, page 54)

while there was a turnabout in portal-drained visceral β-hydroxybutyrate metabolism from a release of 9 μM/min to an uptake of -278 μM/min (Table 5, page 57). β-Hydroxybutyrate infusion caused a switch in hepatic acetoacetate metabolism from a release of 12.5 μM/min to an uptake of -177 μM/min, while β-hydroxybutyrate fluxes remained stable at approximately 815 μM/min. The result was a 37% net decrease in total splanchnic ketone body release (774.5 to 487.5 μM/min) upon β-hydroxybutyrate infusion.

Alloxan-diabetic sheep that had daily insulin injections withheld for 72 hours comprised the third and final experimental category. These animals had mean preinfusion arterial acetoacetate and β-hydroxybutyrate concentrations of 411 and 13771 μM (Tables 2 and 4, pages 52 and 56), respectively, to yield to total ketone body concentration of over 14 mM. According to the guidelines set by Baird (1982) these animals were experiencing a severe case of clinical ketosis. There was little change in net portal-drained visceral turnover of acetoacetate upon infusion of β-hydroxybutyrate (-9.4 versus 11.9 μM/min) (Table 3, page 54). However, β-hydroxybutyrate net flux across portal-drained visceral tissue exhibited a 50% decrease in output upon ketone body infusion (Table 5, page 57). This decrease was not considered significant due to the high error involved in the assay procedure. It is of interest to note the high preinfusion β-hydroxybutyrate release by the portal-drained viscera (450 μM/min) in these diabetic 72-hour untreated sheep. Hepatic acetoacetate release tended to decrease (71.7 to 13.0 μM/min), while β-hydroxybutyrate release by the liver tended to increase upon infusion of

β-hydroxybutyrate. This yielded a slight, but non-significant decrease in total splanchnic acetoacetate net flux (62.3 to 24.9 μM/min) and no significant change in total splanchnic β-hydroxybutyrate net flux was observed as a result of β-hydroxybutyrate infusion in the diabetic 72-hour untreated group (1588 to 1672 μM/min). Therefore, unlike that observed for normal and diabetic insulin-treated sheep, there was no decrease in total ketone body release by the total splanchnic bed.

Baird et al. (1968) reported β-hydroxybutyrate/acetoacetate concentration ratios of approximately 24, 18, and 5 in non-lactating, lactating, and ketotic cows, respectively. In the rat the normal β-hydroxybutyrate/acetoacetate concentration ratio averages 2.0 (Krebs et al., 1969). Preinfusion arterial β-hydroxybutyrate/acetoacetate concentration ratios in the present study were approximately 15.9, 22.1, and 33.5 for the normal, diabetic insulin-treated, and diabetic untreated sheep, respectively. It is apparent that ruminants have a greater basal β-hydroxybutyrate/acetoacetate concentration ratio than do non-ruminants.

Free Fatty Acids

In fed, nonpregnant sheep plasma free fatty acid concentrations range between 100 to 600 mmol/l (Lindsay and Leat, 1975). It has been documented that free fatty acid levels are elevated in the blood of ketotic ruminants (Baird et al., 1972; Baird, 1977). Remesy and Demigne (1983) demonstrated that the major precursors of hepatic ketogenesis are free fatty acids. In addition, the rate of hepatic

free fatty acid uptake and subsequent oxidation is directly proportional to circulating levels of free fatty acids (Van Harken et al., 1969; Boyd et al., 1982). Therefore, it must follow that regulating the amount of free fatty acid reaching the liver will consequently alter hepatic ketone body production in ketosis-prone individuals. The present study measured free fatty acid net fluxes as a result of ketone body infusion in order to determine the mode of action of ketone body auto-regulation.

Preinfusion arterial plasma free fatty acid concentration in the normal sheep averaged 872 μM (Table 6, page 59). The elevated free fatty acid concentration may have been due to the anesthetic agent (sodium pentobarbital) used or to the fact that the experiments were conducted immediately following surgery, and thus, after a 24-hour fasting (see Materials and Methods). The latter seems more appropriate in this study since the preinfusion plasma free fatty acid level of the normal sheep resemble those observed in 24-hour fasted sheep (Trenkle and Kuhlemeier, 1966; Heitmann and Metzler, 1983). Upon ketone body infusion, arterial free fatty acid concentration decreased 39% to 528 μM. This may be due to the inhibition of adipose tissue hormone-sensitive lipase by β-hydroxybutyrate (Björntorp, 1966) or by increased pancreatic insulin secretion due to an elevated β-hydroxybutyrate level (Björntorp and Schersten, 1967), both of which would result in a lower plasma free fatty acid concentration. There was no net change in free fatty acid flux across the portal-drained viscera of normal animals upon β-hydroxybutyrate infusion (97 to 76 μM/min) (Table 7, page 61), but there was a 62% decrease in free fatty acid

uptake by the liver observed during infusion of the ketone body. The result was a significant 82% decrease in net total splanchnic free fatty acid uptake from -187 to -33 μM/min.

As observed in normal animals, ketone body infusion caused a 42% decrease in circulating levels of free fatty acids from 1186 μM to 684 μM in diabetic insulin-treated sheep (Table 6, page 59). The abnormally high preinfusion arterial free fatty acid concentration (1186 μM) may be due to both the 24-hour fasting period preceeding surgical cannulation of the blood vessels and inadequate doses of subcutaneous insulin injections. Preinfusion arterial free fatty acid concentration in the diabetic insulin-treated group was significantly ($P<.01$) greater than that observed in the normal group. Plasma free fatty acid concentrations ranging between 1000 and 1500 μM have been reported in spontaneously ketotic cows (Baird, 1977), and 2-5 day fasted sheep (Trenkle and Kuhlemeier, 1966; Heitmann and Metzler, 1983). Apparently, the diabetes was not under control in these animals. As was observed with normal sheep, diabetic insulin-treated sheep demonstrated no significant change in portal-drained visceral free fatty acid net flux (3 to 32 μM/min) (Table 7, page 61), while a significant ($P<.05$) 66% decrease in net hepatic free fatty acid uptake was observed during β-hydroxybutyrate infusion. This yielded a 77% decrease in net total splanchnic free fatty acid uptake (-276 to 62 μM/min), which is similar to that of the normal animals.

Diabetic untreated sheep experienced high arterial preinfusion free fatty acid concentrations (1933 μM) (Table 6, page 59) along with elevated ketone body levels (see above). It is of interest

that reports confirming such elevated free fatty acid levels are related only to food-deprived ketotic lactating cows (Baird, 1977), which are in a negative energy state. Moreover, mature wethers deprived of feed for 5 days still had lower circulating free fatty acid levels (approximately 1350 μM) than the diabetic untreated sheep in these experiments (Heitmann and Metzler, 1983). It is obvious that these animals were experiencing an extreme negative energy balance. Although there was a significant ($P<.05$) 11% decrease in arterial free fatty acid concentration, it was not as dramatic as that observed in normal and diabetic insulin-treated animals (39 and 42%, respectively). Portal-drained visceral free fatty acid turnover switched from net release of free fatty acids (43 μM/min) to net uptake (-10 μM/min) upon β-hydroxybutyrate infusion (Table 7, page 61). A significant 34% decrease in hepatic free fatty acid uptake was also observed as compared to 62 and 66% decreases observed in the other treatments. This yielded a net, but non-significant, 25% decrease in net total splanchnic free fatty acid uptake from -442 to -329 μM/min. This is much lower than the 82 and 77% decreases observed in normal and diabetic insulin-treated sheep.

Uptake of free fatty acids by the liver of normal rats has been shown to be proportional to circulating levels of free fatty acids (Van Harken et al., 1969). Keller et al. (1977) reported similar findings in dogs. Rat liver has been shown to take up 50% of circulating free fatty acids (Thompson and Darling, 1975). In this study, hepatic extraction of free fatty acids in the normal, diabetic insulin-treated, and diabetic untreated animals were 13.86%, 15.24%,

and 12.59%, respectively, and upon ketone body infusion decreased to 8.0%, 7.9%, and 9.3%, accordingly. It appears that elevated ketone body levels not only reduce peripheral tissue lipolysis (Björntorp, 1966; Björntorp and Schersten, 1971) but also impede free fatty acid uptake by the liver.

Insulin

Insulin is an anabolic hormone secreted by the β-cells of the islets of Langerhans in the pancreas. It promotes the synthesis of glycogen, lipids, and amino acids while it inhibits the degradation of these substances (for reviews see Czech, 1977, 1980; Prior and Smith, 1982). The role of insulin in the regulation of ketogenesis appears to be preventive rather than stimulatory. According to Prior and Smith (1982) the concensus that insulin is a lipogenic hormone may hold true for non-ruminants and not ruminants. Yet, West and Passey (1967) demonstrated in sheep that insulin administration lowers plasma free fatty acid concentrations. Since free fatty acids are the major precursors in hepatic ketone body production (McGarry and Foster, 1980; Robinson and Williamson, 1980) and the rate of hepatic free fatty acid uptake (Van Harken et al., 1969) and oxidation (Boyd et al., 1982) have been shown to be proportional to circulating levels of free fatty acids, it follows that an increase in lipogenesis and/or a decrease in peripheral tissue lipolysis in the presence of insulin would result in decreased free fatty acid uptake by the liver, and consequently, a lower rate of ketogenesis. Moreover, it has been reported that elevated blood levels of ketone bodies may induce

insulin secretion by the pancreatic β-cells (Madison et al, 1964), which could be a major auto-regulatory mechanism of ketogenesis. Keller et al. (1977) showed that an elevated plasma free fatty acid concentration will not influence circulating insulin levels.

Plasma insulin in ruminants varies between 5 to 40 μU/ml (Bassett et al., 1971; Trenkle, 1972; Ferguson and Cox, 1975). An arterial insulin concentration of 38.2 μU/ml was observed in the normal sheep during the preinfusion period (Table 8, page 63). Infusion of β-hydroxybutyrate significantly ($P<.10$) increased arterial plasma insulin levels by 29%, which agree with the findings of Madison et al. (1964). β-Hydroxybutyrate significantly ($P<.05$) increased portal-drained visceral insulin appearance from 24.8 to 35.7 mU/min, which translates to a 44% increase in insulin secretion (Table 9, page 64). Insulin uptake by the liver increased 60% (-16.7 to -26.6 mU/min) with the infusion of β-hydroxybutyrate. Consequently, no change in net total splanchnic insulin flux was observed. Hepatic removal of portal blood insulin was 67 and 74.5% during the control and β-hydroxybutyrate infusion periods, respectively. This is slightly higher than the 45% hepatic insulin uptake reported by Brockman and Bergman (1975). In the normal sheep the liver took up 13.4 and 16.8% of the total circulating insulin during the preinfusion and β-hydroxybutyrate infusion periods, respectively. Again, this was slightly higher than the 8% reported by Brockman and Bergman (1975). It is of interest to note that hepatic extraction of 50% of portal blood insulin did not differ significantly in consicious and sodium pentobarbital anesthetized dogs (Ishida et al., 1983).

Diabetes was induced pharmacologically with intravenous doses of alloxan (50 mg/kg) as suggested by Rerup (1970). Alloxan is considered a beta-cytotoxin because of its specific degradative properties on the insulin-secreting cells of the pacreas (Rerup, 1970). The β-cells of both the diabetic insulin-treated and diabetic untreated animals were non-functional as demonstrated by the non significant portal-arterial differences observed Table 8, page 63, and by the resulting portal-drained visceral (i.e., pancreatic) insulin procuction rates (Table 9, page 64).

Preinfusion arterial insulin concentration in the diabetic insulin-treated sheep was 22.1 μU/ml and remained unchanged with the infusion of β-hydroxybutyrate (Table 8, page 63). Unlike the increased insulin secretion observed with β-hydroxybutyrate infusion in the normal animals, diabetic insulin-treated sheep had lost their insulin-secreting capabilities due to the administration of alloxan and were solely dependent on daily subcutaneous insulin injections as described in the Materials and Methods section. Hence, they were not able to respond to the elevated ketone body levels as proposed by Madison et al. (1964) and as observed in the normal, intact animals. Diabetic untreated sheep had arterial insulin concentrations below 3.0 μU/ml in both the preinfusion and ketone body infusion phases of the experiment. Hepatic uptake of total circulating insulin averaged 7% in the diabetic insulin-treated individuals and 18% in the diabetic untreated animals during both phases of the experiment (Table 9, page 64) with no significant difference between the preinfusion and β-hydroxybutyrate infusion periods. Hepatic extraction of insulin in

diabetic insulin-treated sheep resembled that observed by Brockman and Bergman (1975) in normal sheep, while diabetic untreated sheep had a slightly higher hepatic uptake of insulin.

Summary

Ketosis is a metabolic disorder in which large amounts of ketone bodies are produced due to an impaired carbohydrate and/or lipid metabolism. It has been shown that an overproduction, rather than an underutilization of ketone bodies is the major cause of ketosis in ruminants (Bergman and Kon, 1964a). Factors considered to provoke ketosis include starvation, excessive fetal nutrient demands, and high milk production (Brockman, 1979; Hibbitt, 1979; Baird, 1982). Note that in each of these cases, the ketosis-susceptible individual would be in a negative energy balance, which could be attributed to the fact that in all cases, the nutrient demands far exceed nutrient intake (Hibbitt, 1979). To meet this challenge a homeorhetic transformation would increase peripheral tissue lipolysis, and consequently, free fatty acid transport to the liver (Bauman and Currie, 1980). As a result, there would be an increased hepatic intramitochondrial fatty acid oxidation (Boyd et al., 1982) and subsequent enhancement of hepatic ketogenesis (Remesy and Demigne, 1983). An elevated glucagon/insulin concentration ratio is essential in the etiology of ketosis (Brockman, 1976, 1979; Fain and Shepherd, 1979; McGarry and Foster, 1979; Woodside, 1979).

The ketone body β-hydroxybutyrate may prove to have autoregulating capabilities. There is evidence that an elevated

circulating ketone body concentration, and more precisely an elevated β-hydroxybutyrate concentration, may have antilipolytic qualities (Madison et al., 1964; Björntorp, 1966; Björntorp and Schersten, 1967). If so, β-hydroxybutyrate would decrease plasma free fatty acids, which in turn would decrease free fatty acid oxidation and subsequent hepatic production of ketone bodies. Hence, the phrase ketone body auto-regulation.

The present study investigated the possibility of ketone body auto-regulation in ruminants. Using mature crossbred ewes in either the normal, diabetic insulin-treated, or diabetic 72-hour untreated experimental groups permitted the study of insulin's role in the regulation of ketone bodies in ruminants.

The normal animals exhibited insulin, free fatty acid, acetoacetate, and β-hydroxybutyrate concentrations of approximately 38 μU/ml, 872 μM, 36 μM, and 565 μM, respectively, during the control period. Upon β-hydroxybutyrate infusion arterial concentrations of insulin increased by 29% while free fatty acids decreased 39%. This agrees with evidence already presented by Madison et al. (1964), Björntorp (1966), and Björntorp and Schersten (1967). Insulin production and secretion by the β-cells of the pancreas was enhanced 44% during the infusion phase, as evidenced by portal-drained visceral net flux of insulin, but hepatic extraction of the hormone also increased 60% indicating that the net increase in arterial insulin concentration was due to an increased pancreatic insulin production rather than a decreased insulin uptake. In reference to the decrease in arterial free fatty acid observed during the simulated elevation

of ketone bodies, hepatic uptake of the fatty acids decreased almost 62%. Hence, the decrease in plasma free fatty acid must have been due to decreased peripheral tissue lipolysis rather than increased free fatty acid uptake. In addition, net total splanchnic release of total ketone bodies tended to decrease with β-hydroxybutyrate infusion. Even though there was a marked increase (~72%) in β-hydroxybutyrate release by the liver, acetoacetate uptake by the liver decreased 2-fold resulting in the decreased total splanchnic total ketone body release.

It is of interest to note that alloxan-diabetic sheep given daily subcutaneous doses of insulin as determined by blood glucose levels exhibited similar results upon β-hydroxybutyrate infusion as those observed in the normal sheep. Diabetic insulin-treated sheep had preinfusion arterial insulin, free fatty acid, acetoacetate, and β-hydroxybutyrate concentrations of 22.1 μU/ml, 1186 μM, 46.3 μM, and 1023 μM, respectively. Elevated β-hydroxybutyrate and free fatty acid levels may be due to improper subcutaneously-administered insulin dosages. The same trends observed with the normal animals upon β-hydroxybutyrate infusion were noted in the diabetic insulin-treated animals, with the obvious exception of increased pancreatic insulin release. Arterial free fatty acid concentration showed a 42% decrease during ketone body infusion. Again, it must have been due to decreased peripheral tissue lipolysis in response to elevated ketone body levels, as suggested by Björntorp (1966) and Björntorp and Schersten (1967), because there was a 62% decrease in hepatic free fatty acid uptake. Moreover, net total splanchnic output of

ketone bodies decreased, as was observed in the normal sheep. From this particular set of data it appears that an increased pancreatic insulin release in response to an elevated blood ketone body level is not a vital factor in the regulation of hepatic ketogenesis in terms of free fatty acid availability to the liver.

Alloxan-diabetic sheep which were not given their daily insulin doses for three days were unable to respond to elevated ketone body levels due to β-hydroxybutyrate infusion as was observed in the normal and diabetic insulin-treated sheep. A slight but significant 11% decrease in arterial free fatty acid concentration and a 34% decrease in hepatic free fatty acid extraction upon β-hydroxybutyrate infusion was observed. No net changes were noted in total splanchnic ketone body and free fatty acid net fluxes.

Normal and diabetic insulin-treated sheep responded similarly to β-hydroxybutyrate infusion. Considering that the latter group had non-functioning pancreatic β-cells, and thus, unable to respond to elevated ketone bodies as suggested by Madison et al. (1964), and yet, were still able to decrease peripheral tissue lipolysis and total splanchnic ketogenesis, it appears that an increased pancreatic insulin release is not vital for ketone body regulation. Moreover, the lack of response demonstrated by the diabetic untreated sheep indicates that even though an increased insulin secretion does not appear to be required, insulin itself is still essential in the regulation of ketogenesis. Therefore, adipocyte insulin receptor adaptations may play a major role in the regulation of lipolysis and hepatic ketogenesis by insulin.

CHAPTER VI

CONCLUSION

Elevated ketone body levels due to β-hydroxybutyrate infusion depressed plasma free fatty acid levels and impeded hepatic uptake of free fatty acids in normal, diabetic insulin-treated, and diabetic untreated sheep. An increase in pancreatic insulin release was observed in the normal sheep, but, of course, was absent in the diabetic animals. Yet, in both normal and diabetic insulin-treated, but not in diabetic untreated sheep, a net increase in total splanchnic acetoacetate uptake and a concomitant decrease in net total splanchnic β-hydroxybutyrate release during infusion of the ketone body was observed. Therefore, it was concluded that the effects of β-hydroxybutyrate infusion on ketone body and free fatty acid concentrations and fluxes may be mediated at the insulin receptor site and not by increased pancreatic insulin production, and/or at the site of hepatic free fatty acid uptake.

LIST OF REFERENCES

LIST OF REFERENCES

Alberti, K. G. M. M., D. G. Johnston, A. Gill, A. J. Barnes, and H. Orskou. (1978). Hormonal regulation of ketone-body metabolism in man. Biochem. Soc. Symp. 43:163-182.

Baird, G. D., K. G. Hibbit, G. D. Hunter, P. Lund, M. Stubbs, and H. A. Krebs. (1968). Biochemical aspects of bovine ketosis. Biochem. J. 107:683-689.

Baird, G. D., R. J. Heitzman and K. G. Hibbit. (1972). Effects of starvation on intermediary metabolism in the lactating cow. A comparison with metabolic changes occurring during bovine ketosis. Biochem. J. 128:1311-1318.

Baird, G. D., H. W. Symonds, and R. Ash. (1975). Some observations on metabolite production and utilization in vivo by the gut and liver of adult dairy cows. J. Agric. Sci., Camb. 85:281-296.

Baird, G. D. (1977). Aspects of ruminant intermediary metabolism in relation to ketosis. Biochem. Rev. 5:819-827.

Baird, G. D. (1982). Primary ketosis in the high-producing dairy cow: Clinical and subclinical disorders, treatment, prevention, and outlook. J. Dairy Sci. 65:1-10.

Balasse, E. O. and M. A. Neef. (1975). Inhibition of ketogenesis by ketone bodies in fasting humans. Metabolism 24:999-1007.

Bassett, J. M. (1971). The effects of glucagon on plasma concentrations of insulin, growth hormone, glucose, and free fatty acids in sheep: Comparison with the effects of catecholamines. Aust. J. Biol. Sci. 24:311-320.

Bassett, J. M., R. H. Weston, and J. P. Hogan. (1971). Dietary regulation of plasma insulin and growth hormone concentrations in sheep. Aust. J. Biol. Sci. 24:321-330.

Bassett, J. M. (1972). Plasma glucagon concentrations in sheep: Their regulation and relation to concentrations of insulin and growth hormone. Aust. J. Biol. Sci. 25:1277-1287.

Bassett, J. M. (1974). Diurnal patterns of plasma insulin, growth hormone, corticosteroid and metabolite concentrations in fed and fasted sheep. Aust. J. Biol. Sci. 27:167-181.

Bassett, J. M. (1975). Dietary and gastrointestinal control of hormones regulating carbohydrate metabolism in ruminants. I. W. McDonald and A. C. I. Warner, eds. Digestion and Metabolism in the Ruminant. Armidale, New South Wales, Australia: University of New England Publishing. Unit; 383-398.

Bates, M. W., H. A. Krebs, and D. H. Williamson. (1968). Turnover rates of ketone bodies in normal, starved and alloxan-diabetic rats. Biochem. J. 110:655-661.

Bauman, D. E. (1976). Intermediary metabolism of adipose tissue. Federation Proc. 35:2308-2313.

Bauman, D. E. and W. B. Currie. (1980). Partitioning of nutrients during pregnancy and lactation: A review of mechanisms involving homeostasis and homeorhesis. J. Dairy Sci. 63:1514-1529.

Beatty, C. H., R. D. Peterson, R. M. Bocek, and E. S. West. (1959). Acetoacetate and glucose uptake by diaphragm and skeletal muscle from control and diabetic rats. J. Biol. Chem. 234(1):011-015.

Beatty, C. H., A. Marco, R. D. Peterson, R. M. Bocek, and E. S. West. (1960). Acetoacetic acid metabolism by skeletal muscle fibers from control and diabetic rats. J. Biol. Chem. 235(10):2774-2777.

Bensadoun, A. and J. T. Reid. (1962). Estimation of rate of portal blood flow in ruminants: Effect of feeding, fasting, and anesthezia. J. Dairy Sci. 45:540-543.

Bergman, E. N. and K. Kon. (1964a). Acetoacetate turnover and oxidation rates in ovine pregnancy ketosis. Am. J. Physiol. 206(2):449-452.

Bergman, E. N. and K. Kon. (1964b). Factors affecting acetoacetate production rates by normal and ketotic pregnant sheep. Am. J. Physiol. 206(2):453-457.

Bergman, E. N., R. S. Reid, M. G. Murray, J. M. Brockway, and F. G. Whitelaw. (1965). Interconversions and production of volatile fatty acids in the sheep rumen. Biochem. J. 97:53-58.

Bergman, E. N. and S. J. Roberts. (1967). Urinary glucose excretion and blood concentrations in ketotic cattle after treatment with glucose. Cornell Vet. 67(4):624-630.

Bergman, E. N. (1971). Hyperketonemia--ketogenesis and ketone body metabolism. J. Dairy Sci. 54:936-948.

Bergman, E. N., R. P. Brockman, and C. F. Kaufman. (1974). Glucose metabolism in ruminants: Comparison of whole-blood turnover with production by gut, liver, and kidneys. Federation Proc. 33:1849-1854.

Bergman, E. N. (1975). Production and utilization of metabolities by the alimentary tract as measured in portal and hepatic blood. pp. 292-305 in Digestion and Metabolism in the Ruminant. I. W. McDonald and A. C. I. Warner, eds. The University of New England Publishing Unit, Australia.

Bergstrom, J. D., K. A. Rabbins, and J. Edmond. (1982). Acetoacetyl-coenzyme A synthetase activity in rat liver cytosol: A regulated enzyme in lipogenesis. Biochem. Biophys. Res. Commun. 106(3):856-862.

Berzins, R. and J. G. Manns. (1979). How concentrate feeding affects glucoregulatory hormones in ruminants: Implications in bovine ketosis. J. Dairy Sci. 62:1739-1745.

Björntorp, P. (1966). Effect of ketone bodies on lipolysis in adipose tissue in vitro. J. Lipid. Res. 7:621-626.

Björntorp, P. and T. Schersten. (1967). Effect of β-hydroxybutryate on lipid mobilization. Am. J. Physiol. 212(3):683-687.

Blackard, W. G., E. W. Hull, and A. Lopez-S. (1971). Effects of lipid on growth hormone in humans. J. Clin. Invest. 50(7):1439-1443.

Blum, J. W., D. Froehli, and P. Kunz. (1982). Effects of catecholamines on plasma free fatty acids in fed and fasted cattle. Endocrinology. 110:452-456.

Bossi, E., K. Zuppinger, H. P. Siegrist, U. Wiesmann, and N. Herschkowitz. (1982). Age-dependent utilization of D-β-OH-butyrate and oleic acid as glucose substitutes by neonatal mouse brain cell cultures. Pediatr. Res. 16:579-582.

Bowen, J. M. (1964). Peripheral, hepatic, and nonhepatic splanchnic effects of insulin in sheep. Cornell Vet. 54:58-65.

Boyd, R. D., R. A. Britton, H. Knoche, B. D. Moser, E. R. Peo, Jr., and R. K. Johnson. (1982). Oxidation rates of major fatty acids in fasting neonatal pigs. J. Anim. Sci. 55(1):95-100.

Brady, P. S., R. F. Scofield, S. Ohgaku, W. C. Schumann, G. E. Bartsch, J. M. Margolis, K. Kumaran, A. Horvat, S. Mann, and B. R. Landau. (1982). Pathways of acetoacetate's formation in liver and kidney. J. Biol. Chem. 257(16):9290-9293.

Brass, E. P., and C. L. Hoppel. (1978). Disassociation between acid-insoluble acylcarnitines and ketogenesis following carnitine administration in vivo. J. Biol. Chem. 253(15):5274-5276.

Bremer, J. (1981). The effect of fasting on the activity of liver carnitine palmitoyltransferase and it's inhibition by malonyl-CoA. Biochim. Biophys. Acta. 665:628-631.

Brockman, R. P., and J. G. Manns. (1974). Effects of glucagon on activities of hepatic enzymes in sheep. Cornell Vet. 64(2):217-224.

Brockman, R. P., and E. N. Bergman. (1975a). Effect of glucagon on plasma alanine and glutamine metabolism and hepatic gluconeogenesis in sheep. Am. J. Physiol. 228(6):1627-1633.

Brockman, R. P., and E. N. Bergman. (1975b). Quantitative aspects of insulin secretion and its hepatic and renal removal in sheep. Am. J. Physiol. 229(5):1338-1343.

Brockman, R. P. (1976). Effects of glucagon and insulin on lipolysis and ketogenesis in sheep. Can. J. Comp. Med. 40:166-170.

Brockman, R. P., J. G. Manns, and E. N. Bergman. (1976). Quantitative aspects of secretion and hepatic removal of glucagon in sheep. Can. J. Physiol. Pharmacol. 54:666-670.

Brockman, R. P. (1978). Roles of glucagon and insulin in the regulation of metabolism in ruminants-A review. Can. Vet. J. 19:55-62.

Brockman, R. P. (1979). Roles for insulin and glucagon in the development of ruminant ketosis-A review. Can. Vet. J. 20:121-126.

Brunengraber, H., M. Boutry, and J. M. Lowenstein. (1973). Fatty acids and 3-β-hydroxysterol synthesis in the perfused rat liver. J. Biol. Chem. 248(8):2656-2669.

Buckley, B. M., and D. H. Williamson. (1975). Acetoacetyl-CoA synthetase; a lipogenic enzyme in rat tissues. FEBS Lett. 60:7-10.

Byers, F. M. (1982). Nutritional factors affecting growth of muscle and adipose tissue in ruminants. Federation Proc. 41:2562-2566.

Carrol, E. J., and R. E. Hungate. (1954). The magnitude of microbial fermentation in the bovine rumen. Appl. Microbiol. 2:205-214.

Chan, C. P., and J. S. Stern. (1982). Adipose lipoprotein lipase in insulin-treated diabetic lean and obese Zucker rats. Am. J. Physiol.

Christiansen, R. Z. (1977). Regulation of palmitate metabolism by carnitine and glucagon in hepatocytes isolated from fasted and carbohydrate refed rats. Biochim. Biophys. Acta. 488:249-262.

Cole, T. G., H. G. Wilcox, and M. Heimberg. (1982). Effects of adrenalectomy and dexamethasone on hepatic lipid metabolism. J. Lipid Res. 23:81-91.

Cook, G. A., M. T. King, and R. L. Veech. (1978). Ketogenesis and malonyl coenzyme A content of isolated rat hepatocytes. J. Biol. Chem. 253(8):2529-2531.

Cook, G. A., D. A. Otto, and N. W. Cornell. (1980). Differential inhibition of ketogenesis by malonyl-CoA in mitochondria from fed and starved rats. Biochem. J. 192:955-958.

Czech, M. P. (1977). Molecular basis of insulin action. Ann. Rev. Biochem. 46:359-384.

Czech, M. P. (1980). Insulin action and the regulation of hexose transport. Diabetes. 29(5):399-409.

de Bruijne, J. J., N. Altszuler, J. Hampshire, T. J. Visser, and W. H. L. Hackeng. (1981). Fat mobilization and plasma hormone levels in fasted dogs. Metabolism. 30(2):190-194.

Declercq, P. E., L. J. Debeer, and G. P. Mannaerts. (1982). The inhibition by glucagon of triacylglycerol synthesis in isolated rat hepatocytes. Societe Belge de Biochime, Reunion de Louvain-en-Wolueve (Bruxelles) 20, Fevrier. B13-B14.

Demaugre, F., H. Buc, J. Girard, and J. P. Leroux. (1982). Role of the mitochondrial metabolism of pyruvate on the regulation of ketogenesis in rat hepatocytes. Metabolism. 32(1):40-48.

DiMarco, J. P., and C. Hoppel. (1975). Hepatic mitochondrial function in ketogenic states: Diabetes, starvation, and after growth hormone administration. J. Clin. Invest. 55:1237-1244.

Dobbing, J., and J. Sands. (1971). Vulnerability of developing brain. IX. The effect of nutritional growth retardation on the timing of the brain growth spurt. Biol. Neonate. 19:363-378.

Dougherty, R. W. (1981). Cannulation of arteries and veins. Experimental Surgery in Farm Animals. The Iowa State University Press, Ames, Iowa. 56-68.

Etherton, T. D. (1982). The role of insulin-receptor interactions in regulation of nutrient utilization by skeletal muscle and adipose tissue: A review. J. Anim. Sci. 54(1):58-67.

Evringham, A., E. Brenneman, and S. M. Horvath. (1959). Influence of sodium pentobarbital on splanchnic blood flow and related functions. Am. J. Physiol. 197(3):624-626.

Fain, J. N., and R. E. Shepherd. (1979). Hormonal regulation of lipolysis: Role of cyclic nucleotides, adenosine, and free fatty acids. Advances in Experimental Medicine and Biology vol. 111. Hormones and Energy Metabolism, edited by D. M. Klachko, R. R. Anderson and M. Heimberg. Plenum Press. New York and London.

Ferguson, K. A., and R. I. Cox. (1975). Hormones. The Blood of Sheep: Composition and Function. M. H. Blunt, ed. Springer-Verlag Berlin, Heidelberg, New York. 101-121.

Ford, E. J. H., and J. W. Boyd. (1960). Some observations on bovine acetonaemia. Res. Vet. Sci. 1:232-241.

Garfinkel, A. S., N. Baker, and M. C. Schotz. (1967). Relationship of lipoprotein lipase activity to triglyceride uptake in adipose tissue. J. Lipid Res. 8:274-280.

Gill, R. D., and I. C. Hart. (1980). Properties of insulin and glucagon receptors on sheep hepatocytes: A comparison of hormone binding and plasma hormones and metabolites in lactating and non-lactating ewes. J. Endocr. 84:237-247.

Gill, R. D., and I. C. Hart. (1981). Insulin and glucagon binding to hepatocytes in relation to circulating hormones and metabolites in goats maintained on different diets. Horm. Metab. Res. 13:603-609.

Gilmore, J. P. (1958). Effect of anesthesia and hepatic sampling site upon hepatic blood flow. Am. J. Physiol. 195(2):465-468.

Graham, A. D., and G. D. Phillips. (1981). Plasma glucose, lactate and free fatty acid responses to adrenaline in chronically warm- and cold-exposed sheep. Can. J. Anim. Sci. 61:919-924.

Guynn, R. W., D. Veloso, and R. L. Veech. (1972). The concentration of malonyl-coenzyme A and the control of fatty acid synthesis *in vivo*. J. Biol. Chem. 247(22):7325-7331.

Hagenfeldt, L. (1975). Turnover of individual free fatty acids in man. Federation Proc. 34:2246-2249.

Hales, C. N., J. P. Luzio, and K. Siddle. (1978). Hormonal control of adipose-tissue lipolysis. Biochem. Soc. Symp. 43:97-135.

Hall, Susan E. H., and T. M. Bolton. (1981). A problem associated with the measurement of ketone body kinetics *in vivo*. Can. J. Physiol. Pharmacol. 59(11):1178-1180.

Hart, I. C., J. A. Bines, S. V. Morant, and J. L. Ridley. (1978). Endocrine control of energy metabolism in the cow: Comparison of the levels of hormones (prolactin, growth hormone, insulin and thyroxine) and metabolites in the plasma of high- and low-yielding cattle at various stages of lactation. J. Endocr. 77:333-345.

Hatefi, Y., and T. Fakouhi. (1968). Control of β-hydroxybutyrate and acetoacetate oxidation by inorganic phosphate and adenosine 5'-diphosphate in heart mitochondria. Arch. Biochem. Biophys. 125(1):114-125.

Heimberg, M., I. Weinstein, and M. Kohout. (1969). The effects of glucagon, dibutyryl cyclic adenosine-3', 5'-monophosphate, and concentration of free fatty acid on hepatic lipid metabolism. J. Biol. Chem. 244(19):5131-5139.

Heitmann, R. N., and D. R. Metzler. (1983). Effects of β-hydroxy-butyrate on plasma free fatty acids in mature wethers. Fed. Proc. 42:532. Abstr.

Henderson, G. D., L. C. Read, and A. M. Snoswell. (1982). Studies of liver lipids in normal, alloxan-diabetic and pregnancy-toxaemic sheep. Biochim. Biophys. Acta. 710:236-241.

Herdt, T. H., J. B. Stevens, W. G. Olson, and V. Larson. (1981a). Blood concentrations of β-hydroxybutyrate in clinically normal Holstein-Friesian herds and in those with a high prevalence of clinical ketosis. Am. J. Vet. Res. 42(3):503-506.

Herdt, T. H., J. B. Stevens, J. Linn, and V. Larson. (1981b). Influence of ration composition and energy balance on blood β-hydroxybutyrate (ketone) and plasma glucose concentrations of dairy cows in early lactation. Am. J. Vet. Res. 42(7):1177-1180.

Hermansen, K. (1982). Stimulatory effect of beta-hydroxybutyrate on the release of somatostatin from the isolated pancreas of normal and streptozotocin-diabetic dogs. Diabetes. 31:270-274.

Hertelendy, F., and D. M. Kipnis. (1973). Studies on growth hormone secretion: V. Influence of plasma free fatty acid levels. Endocrinol. 92(2):402-410.

Hibbitt, K. G. (1979). Bovine ketosis and it's prevention. Veterinary Record. 105:13-15.

Hiebert, J. M., J. M. McCormick, and R. H. Egdahl. (1972). Direct measurement of insulin secretory rates: Studies in shocked primates and postoperative patients. Ann. Surg. 176:296-304.

Hochachka, P. W., J. R. Neely, and W. R. Driedzic. (1977). Integration of lipid utilization with Krebs cycle activity in muscle. Federation Proc. 36:2009-2014.

Honey, R. N., A. Arimura, and G. C. Weir. (1981). Somatostatin neutralization stimulates glucagon and insulin secretion from the avian pancreas. Endocrinol. 109:1971-1974.

Hood, V. L., E. Danforth, Jr., E. S. Horton, and R. L. Tannen. (1982). Impact of hydrogen ion on fasting ketogenesis: Feedback regulation of acid production. Am. J. Physiol. 242:F238-F245.

Howard, B. V., J. S. Reitman, B. Vasquez, and L. Zech. (1983). Very-low-density lipoprotein triglyceride metabolism in non-insulin-dependent diabetes mellitus: Relationship to plasma insulin and free fatty acids. Diabetes. 32(3):271-276.

Ide, T., and J. A. Ontko. (1981). Increased secretion of very low density lipoprotein triglyceride following inhibition of long chain fatty acid oxidation in isolated rat liver. J. Biol. Chem. 256(20):10247-10255.

Ide, T., H. Oku, and M. Sugano. (1982). Reciprocal responses clofibrate in ketogenesis and triglyceride and cholesterol secretion in isolated rat liver. Metabolism. 31(10):1065-1072.

Ingle, D. L., D. E. Bauman, and U. S. Garrigus. (1972a). Lipogenesis in the ruminant: in vitro study of tissue sites, carbon source and reducing equivalent generation for fatty acid synthesis. J. Nutr. 102:609-616.

Ingle, D. E., D. E. Bauman, and U. S. Garrigus. (1972b). Lipogenesis in the ruminant: in vivo site of fatty acid synthesis in sheep. J. Nutr. 102:617-624.

Ishida, T., R. M. Lewis, C. J. Hartley, M. L. Entman, and J. B. Field. (1983). Comparison of hepatic extraction of insulin and glucagon in conscious and anesthetized dogs. Endocrinol. 112(3):1098-1109.

Kark, R. A. P., J. P. Blass, J. Avigan, and W. K. Engel. (1971). The oxidation of β-hydroxybutyric acid by small quantities of type-pure red and white skeletal muscle. J. Biol. Chem. 246(14):4560-4566.

Katz, M. L., and E. N. Bergman. (1969a). Simultaneous measurements of hepatic and portal venous blood flow in the sheep and dog. Am. J. Physiol. 216(4):946:952.

Katz, M. L., and E. N. Bergman. (1969b). Hepatic and portal metabolism of glucose, free fatty acids, and ketone bodies in the sheep. Am. J. Physiol. 216:953-960.

Katz, M. L., and E. N. Bergman. (1969c). A method for simultaneous cannulation of the major splanchnic blood vessels of the sheep. Am. J. Vet. Res. 30(4):655-661.

Kaufman, C. F., and E. N. Bergman. (1971). Renal glucose, free fatty acid, and ketone body metabolism in the unanesthetized sheep. Am. J. Physiol. 221(4):967-972.

Kaufman, C. F., and E. N. Bergman. (1974). Renal ketone body metabolism and gluconeogenesis in normal and hypoglycemic sheep. Am. J. Physiol. 226(4):827-832.

Kawai, K., and R. H. Unger. (1982). Inhibition of glucagon secretion by exogenous glucagon in the isolated, perfused dog pancreas. Diabetes. 31(6):512-515.

Kawai, K., and R. H. Unger. (1983). Opposing actions of glucagon and insulin on sphlanchnic D cell function. J. Clin. Invest. 71:721-725.

Keller, U., J.-L. Chiasson, J. E. Liljenquist, A. D. Cherrington, A. S. Jennings, and O. B. Crofford. (1977). The roles of insulin, glucagon, and free fatty acids in the regulation of ketogenesis in dogs. Diabetes. 26(11):1040:1051.

Koch, A. (1982). Partition of energy for growth in the steer. Growth. 46:60-73.

Kopec, B., and I. B. Fritz. (1973). Comparison of properties of carnitine palmitoyltransferase I with those of carnitine palmitoyltransferase II and preparation of antibodies to carnitine palmitoyltransferases. J. Biol. Chem. 248(11):4069-4074.

Koper, J. W., M. Lopes-Cardozo, and L. M. G. Van Golde. (1981). Preferential utilization of ketone bodies for the synthesis of myelin cholesterol in vivo. Biochim. Biophys. Acta. 666:411-417.

Krebs, H. A., P. G. Wallace, R. Hems, and R. A. Freedland. (1969). Rates of ketone-body formation in the perfused rat liver. Biochem. J. 112:595-600.

Kronfeld, D. S., M. G. Simesen, and D. L. Dungworth. (1960). Liver glycogen in normal and ketotic cows. Res. Vet. Sci. 1:242-247.

Kronfeld, D. S., F. Raggi, and C. F. Ramberg, Jr. (1968). Mammary blood flow and ketone body metabolism in normal, fasted, and ketotic cows. Am. J. Physiol. 215(1):218-227.

Kronfeld, D. S. (1971). Hypoglycemia in ketotic cows. J. Dairy Sci. 54:949-961.

Kronfeld, D. S. (1972). Ketosis in pregnant sheep and lactating cows. A review. Austr. Vet. J. 48:680-687.

Lehninger, A. L. (1975). Biochemistry, 2nd ed. Worth Publishers, Inc. New York.

Liljenquist, J. E., J. D. Bomboy, S. B. Lewis, B. C. Sinclair-Smith, P. W. Felts, W. W. Lacy, O. B. Crofford, and G. W. Liddle. (1974). Effects of glucagon on lipolysis and ketogenesis in normal and diabetic men. J. Clin. Invest. 53:190-197.

Lindsay, D. B., and W. M. F. Leat. (1975). Carbohydrate and lipid metabolism. The Blood of Sheep: Composition and Function. M. H. Blunt ed. Springer-Verlag Berlin, Heidelberg, New York. 45-62.

Lloyd, L. E., B. E. McDonald, and E. W. Crampton. (1978). Fundamentals of Nutrition, 2nd ed. W. H. Freeman and Company, San Francisco.

Lopez-Cardozo, M., and S. G. Van den Bergh. (1972). Ketogenesis in isolated rat liver mitochondria. I. Relationships with citric cycle and with the mitochondrial energy state. Biochim. Biophys. Acta. 283:1-15.

Lowenstein, J. M. (1971). Effect of (-)-hydroxycitrate on fatty acid synthesis by rat liver in vivo. J. Biol. Chem. 246(3):629-632.

Madison, L. L., D. Mebane, R. H. Unger, and A. Lochner. (1964). The hypoglycemic action of ketones. II. Evidence for a stimulating feedback of ketones on the pancreatic beta cells. J. Clin. Invest. 43:408-415.

Maynard, L. A., J. K. Loosli, H. F. Hintz, and R. G. Warner. (1979). Animal Nutrition, 7th ed. McGraw-Hill Book Company, New York.

McCarthy, R. D., J. C. Shaw, J. L. McCarthy, S. Lakshanan, and J. B. Holter. (1958). Production and absorption of organic acids by the perfused goat rumen. Proc. Soc. Exp. Biol. Med. 99:556-559.

McGarry, J. D., and D. W. Foster. (1969). Ketogenesis and cholesterol synthesis in normal and neoplastic tissues of the rat. J. Biol. Chem. 244(15):4251-4256.

McGarry, J. D., M. J. Guest, and D. W. Foster. (1970). Ketone body metabolism in the ketosis of starvation and alloxan diabetes. J. Biol. Chem. 245(17):4382-4390.

McGarry, J. D., and D. W. Foster. (1971). The regulation of ketogenesis from octanoic acid: The role of the tricarboxylic acid cycle and fatty acid synthesis. J. Biol. Chem. 246(4):1149-1159.

McGarry, J. D., J. M. Meier, and D. W. Foster. (1973). The effects of starvation and refeeding on carbohydrate and lipid metabolism in vivo and in the perfused rat liver: The relationship between fatty acid oxidation and esterification in the regulation of ketogenesis. J. Biol. Chem. 248(1):270-278.

McGarry, J. D., and D. W. Foster. (1974a). Studies with (+)-octanoylcarnitine in experimental diabetic ketoacidosis. Diabetes. 23:485-493.

McGarry, J. D., and D. W. Foster. (1974b). The metabolism of (-)-octanoylcarnitine in perfused livers from fed and fasted rats: Evidence for a possible regulatory role of carnitine acyltransferase in the control of ketogenesis. J. Biol. Chem. 249(24):7984-7990.

McGarry, J. D., P. H. Wright, and D. W. Foster. (1975). Hormonal control of ketogenesis. Rapid activation of hepatic ketogenic capacity in fed rats by anti-insulin serum and glucagon. J. Clin. Invest. 55:1202-1209.

McGarry, J. D., and D. W. Foster. (1977). Hormonal control of ketogenesis. Arch. Intern. Med. 137:494-501.

McGarry, J. D., G. P. Mannaerts, and D. W. Foster. (1977). A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis. J. Clin. Invest. 60:265-270.

McGarry, J. D., G. F. Leatherman, D. W. Foster. (1978a). Carnitine palmitoyltransferase I. The site of inhibition of hepatic fatty acid oxidation by malonyl-CoA. J. Biol. Chem. 253(12): 4128-4136.

McGarry, J. D., G. P. Mannaerts, and D. W. Foster. (1978b). Characteristics of fatty acid oxidation in rat liver homogenates and the inhibitory effect of malonyl-CoA. Biochim. Biophys. Acta. 530:305-313.

McGarry, J. D., M. J. Stark, and D. W. Foster. (1978c). Hepatic malonyl-CoA levels of fed, fasted and diabetic rats as measured using a simple radioisotopic assay. J. Biol. Chem. 253(22): 8291-8293.

McGarry, J. D., Y. Takabayashi, and D. W. Foster. (1978d). The role of malonyl-CoA in the coordination of fatty acid synthesis and oxidation in isolated rat hepatocytes. J. Biol. Chem. 253(22):8294-8300.

McGarry, J. D., and D. W. Foster. (1979a). In support of the roles of malonyl-CoA and carnitine acyltransferase I in the regulation of hepatic fatty acid oxidation and ketogenesis. J. Biol. Chem. 254(17):8163-8168.

McGarry, J. D., and D. W. Foster. (1979b). Hormonal control of ketogenesis. Advances in Experimental Medicine and Biology, vol. 111. D. M. Klachko, R. R. Anderson, and M. Heimberg eds. Plenum Press. New York and London.

McGarry, J. D., and D. W. Foster. (1980a). Regulation of hepatic fatty acid oxidation and ketone body production. Ann. Rev. Biochem. 49:395-420.

McGarry, J. D., and D. W. Foster. (1980b). Effects of exogenous fatty acid concentration on glucagon-induced changes in hepatic fatty acid metabolism. Diabetes. 29(3):236-240.

McNeill, D. A., J. H. Herbein, and S. J. Ritchey. (1982). Hepatic gluconeogenic enzymes, plasma insulin and glucagon response to magnesium deficiency and fasting. J. Nutr. 112:736-743.

Meier, J. M., J. D. McGarry, G. R. Faloona, R. H. Unger, and D. W. Foster. (1972). Studies of the development of diabetic ketosis in the rat. J. Lipid Res. 13:228-233.

Menahan, L. A., W. T. Hron, D. G. Hinkelman, and H. M. Miziorko. (1981). Interrelationships between 3-hydroxy-3-methyglutaryl-CoA synthase, acetoacetyl-CoA and ketogenesis. Eur. J. Biochem. 119:287-294.

Menahan, L. A., and W. T. Hron. (1981). Regulation of acetoacetyl-CoA in isolated perfused rat hearts. Eur. J. Biochem. 119:295-299.

Metzler, D. E. (1977). Biochemistry: The Chemical Reactions of Living Cells. Academic Press, New York, San Francisco, London.

Miles, J. M., S. L. Nissen, R. A. Rizza, J. E. Gerich, and M. W. Haymond. (1983). Failure of infused β-hydroxybutyrate to decrease proteolysis in man. Diabetes. 32(3):197-205.

Müller, J. J., H. Köster, and H. J. Seitz. (1981). Effect of thyroid state on ketogenic capacity of the isolated perfused liver of starved rats. Biochim. Biophys. Acta. 666:475-481.

O'Donnell III, J. A., and R. A. Freedland. (1980). Ketogenesis from oleate and octanoate in isolated rat hepatocytes. J. Nutr. 110(12):2365-2373.

Ogata, F., and Y. Hirasawa. (1982). Free fatty acids alter the electrophoretic mobility of serum lipoproteins. Biochem. Biophys. Res. Comm. 107(2):417-421.

Ohgaku, S., P. S. Brady, W. C. Schumann, G. E. Bartsch, J. M. Margolis, K. Kumaran, S. B. Landau, and B. R. Landau. (1982). A method for quantitating the contributions of the pathways of acetoacetate formation and its application to diabetic ketosis in vivo. J. Biol. Chem. 257(16):9283-9289.

Olefsky, J. M., S. Marshall, P. Berhanu, M. Saekow, K. Heidenreich, and A. Green. (1982). Internalization and intracellular processing of insulin and insulin receptors in adipocytes. Metabolism. 31(7):670-690.

Olsen, M. E., O. K. Faber, and C. Binder. (1983). Hepatic extraction of insulin after carbohydrate hyperalimentation. Acta Endocrinologica. 102:416-419.

Ontko, J. A. (1972). Metabolism of free fatty acids in isolated liver cells. Factors affecting the partition between esterification and oxidation. J. Biol. Chem. 247(6):1788-1800.

Ontko, J. A. (1973). Effects of ethanol on the metabolism of free fatty acids in isolated liver cells. J. Lipid Res. 14:78-86.

Ott, D. B., and P. A. Lachance. (1981). Biochemical controls of liver cholesterol biosynthesis. Amer. J. Clin. Nutr. 34:2295-2306.

Owen, O. E., V. E. Trapp, C. L. Skutches, M. A. Mozzoli, R. D. Hoeldtke, G. Boden, and G. A. Reichard, Jr. (1982). Acetone metabolism during diabetic ketoacidosis. Diabetes. 31(3):242-248.

Pace, C. S., and J. T. Tarvin. (1982). Somatostatin: Mechanism of action in pancreatic islet β-cells. Diabetes. 30(10):836-842.

Pande, S. V. (1971). On rate-controlling factors of long chain fatty acid oxidation. J. Biol. Chem. 246(17):5384-5390.

Parvin, R., and S. V. Pande. (1979). Enhancement of mitochondrial carnitine and carnitine acylcarnitine translocase-mediated transport of fatty acids into liver mitochondria under ketogenic conditions. J. Biol. Chem. 254(12):5423-5429.

Pethick, D. W., D. B. Lindsay, P. J. Barker, and A. J. Northrop. (1981). Acetate supply and utilization by the tissues of sheep in vivo. Br. J. Nutr. 46:97-100.

Prior, R. L., and S. B. Smith. (1982). Hormonal effects on partitioning of nutrients for tissue growth: Role of insulin. Federation Proc. 41:2545-2549.

Radloff, H. D., and L. H. Schultz. (1966). Hormonal regulation of plasma free fatty acid concentration in ruminants. J. Dairy Sci. 49:971-975.

Reilly, P. E. B. and E. J. H. Ford. (1966). The effects of different dietary contents of protein on amino acid and glucose production and on the contribution of amino acids to gluconeogenesis in sheep. Br. J. Nutr. 26(2):249-263.

Remesey, C., and C. Demigne. (1983). Changes in availability of glucogenic and ketogenic substrates and liver metabolism in fed or starved rats. Ann. Nutr. Metab. 27:57-70.

Rerup, C. C. (1970). Drugs producing diabetes through damage of the insulin secreting cells. Pharmacol. Rev. 22(4):485-518.

Robertson, A., H. Paver, P. Barden, and T. G. Marr. (1960). Fasting metabolism of the lactating cow. Res. Vet. Sci. 1:117-124.

Robinson, A. M., and D. H. Williamson. (1978). Utilization of D-3-hydroxy-[3-^{14}C]-butyrate for lipogenesis *in vivo* in lactating rat mammary gland. Biochem. J. 176:635-638.

Robinson, A. M., and D. H. Williamson. (1980). Physiological roles of ketone bodies as substrates and signals in mammalian tissues. Physiol. Rev. 60:143-187.

Roe, W. E., E. N. Bergman, and K. Kon. (1966). Absorption of ketone bodies and other metabolites via the portal blood of sheep. Am. J. Vet. Res. 27(118):729-736.

Roeder, L. M., S. E. Poduslo, and J. T. Tildon. (1982a). Utilization of ketone bodies and glucose by established neural cell lines. J. Neuroscience Res. 8:671-682.

Roeder, L. M., J. T. Tilden, and J. H. Stevenson. (1982b). Antiketonemic effect of glycerol during development of the rat. J. Nutr. 112(7):1273-1280.

Rous, S. (1977). Fasting and insulin regulation of the utilization of acetoacetate for fatty acid synthesis. Arch. Biochem. Biophys. 179:328-333.

Sabel, K.-G., R. Olegard, M. Mellander, and K. Hildingsson. (1982). Interrelation between fatty acid oxidation and control of gluconeogenic substrates in small-for-gestational-age (SGA) infants with hypoglycemia and with hypoglycemia and with normoglycemia. Acta Paediatr. Scand. 71:53-61.

Sauer, F., and J. D. Erfle. (1966). On the mechanism of acetoacetate synthesis by guinea pig liver fractions. J. Biol. Chem. 241(1):30-37.

Schade, D. S., and R. P. Eaton. (1975). Glucagon regulation of plasma ketone body concentration in human diabetes. J. Clin. Invest. 56:1340-1344.

Schade, D. S., W. Woodside, and R. P. Eaton. (1979). The role of glucagon in the regulation of plasma lipids. Metabolism 28:874-886.

Schwalm, J. W., and L. H. Schultz. (1976a). Relationship of insulin concentration to blood metabolites in the dairy cow. J. Dairy Sci. 59:255-261.

Schwalm, J. W., and L. H. Schultz. (1976b). Blood and liver metabolites in fed and fasted diabetic goats. J. Dairy Sci. 59:262-269.

Seto, K., T. Tsuda, and M. Umezu. (1955). The mechanism of the ketone bodies production with rumen epithelium. I. The ketone bodies production from volatile fatty acids with rumen epithelium and the influence of substances concerned with the citric acid cycle on their production. Tohoku J. Agr. Res. 6(2):91-98.

Sherwin, R. S., R. G. Hendler, and P. Felig. (1975). Effect of ketone infusions on amino acid and nitrogen metabolism in man. J. Clin. Invest. 55:1382-1390.

Siess, E. A., R. I. Kientsch-Engel, and O. H. Wieland. (1982). Role of free oxaloacetate in ketogenesis: Derviation from the direct measurement of mitochondrial [3-hydroxybutyrate]/[acetoacetate] ratio in hepatocytes. Eur. J. Biochem. 121:493-499.

Singh, B., H. Osmundsen, and B. Borreback. (1982). The time course of glucagon action on the utilization of [U-^{14}C] palmitate by isolated hepatocytes. Arch. Biochem. Biophys. 217(1):244-250.

Snoswell, A. M., and P. P. Koundakjian. (1972). Relationships between carnitine and coenzyme A esters in tissues of normal and alloxan-diabetic sheep. Biochem. J. 127:133-141.

Snoswell, A. M., R. P. Trimble, R. C. Fishlock, G. B. Storer, and D. L. Topping. (1982). Metabolic effects of acetate in perfused rat liver studies on ketogenesis, glucose output, lactate uptake and lipogenesis. Biochim. Biophys. Acta. 716:290-297.

Spitzer, J. J. (1975). Application of tracers in studying free fatty acid metabolism of various organs *in vivo*. Federation Proc. 34:2242-2245.

Stamm, G. W. (1975). Veterinary Guide for Farmers. R. C. Klussendorf, ed. pp 120-122. Book Division, The Hearst Corporation, New York, N.Y.

Stern, J. R., M. J. Coon, and A. Del Campillo. (1953). Enzymatic breakdown and synthesis of acetoacetate. Nature London 171:28-30.

Stern, J. R., and G. E. Miller. (1959). On the enzymatic mechanism of acetoacetate synthesis. Biochim. Biophys. Acta. 35:576-577.

Stern, J. R. (1971). A role of acetoacetyl-CoA synthetase in acetoacetate utilization by rat liver cell fractions. Biochem. Biophys. Res. Commun. 44:1001-1007.

Thompson, G. E., and K. F. Darling. (1975). The hepatic uptake of individual free fatty acids in sheep during noradrenaline infusion. Res. Vet. Sci. 18:325-327.

Trenkle, A., and K. V. Kuhlemeier. (1966). Relationship of rumen volatile fatty acids, blood glucose and plasma nonesterified fatty acids in sheep. J. Anim. Sci. 25:1111-1115.

Trenkle. A. (1972). Radioimmunoassay of plasma hormones: Review of plasma insulin in ruminants. J. Dairy Sci. 5(8):1200-1211.

Trout, D. L., E. H. Estes, and S. J. Friedberg. (1960). Titration of free fatty acids of plasma: A study of current methods and a new modification. J. Lipid Res. 1:199-202.

Tutwiler, G. F., and H. J. Brentzel. (1982). Relation of oxidation of long-chain fatty acids to gluconeogenesis in the perfused liver of the guinea pig: Effect of 2-tetradecylglycidic acid (McN-3802). Eur. J. Biochem. 124:465-470.

Unger, R. H., and L. Orci. (1981). Glucagon and the A-cell: Physiology and pathophysiology. New Eng. J. Med. 304:1518-1524 and 1575-1580.

Van Harken, D. R., C. W. Dixon, and M. Heimberg. (1969). Hepatic lipid metabolism in experimental diabetes. V. The effect of concentration of oleate on metabolism of triglycerides and on ketogenesis. J. Biol. Chem. 244(9):2278-2285.

Varnam, G. C. E., M. K. Jeacock, and D. A. L. Shepherd. (1978). Hepatic ketone-body metabolism in developing sheep and pregnant ewes. Br. J. Nutr. 40:359-367.

Veerkamp, J. H., and H. T. B. Van Moerkerk. (1982). The effect of malonyl-CoA on fatty acid oxidation in rat muscle and liver mitochondria. Biochim. Biophys. Acta. 710:252-255.

Weidmann, M. J., and H. A. Krebs. (1969). The fuel of respiration of rat kidney cortex. Biochem. J. 112:149-166.

West, C. E., and R. F. Passey. (1967). Effect of glucose load and of insulin on the metabolism of glucose and of palmitate in sheep. Biochem. J. 102:58-64.

Williamson, D. H., and J. Mellanby. (1965). Acetoacetate and D-(-)-β-hydroxybutyrate. Methods of Enzymatic Analysis 2nd ed. pp. 454-461. H. V. Bergemeyer, ed. Academic Press, New York.

Williamson, D. H., M. W. Bates, and H. A. Krebs. (1968). Activity and intracellular distribution of enzymes of ketone-body metabolism in rat liver. Biochem. J. 108:353-361.

Williamson, D. H., and E. Whitelaw. (1978). Physiological aspects of the regulation of ketogenesis. Biochem. Soc. Symp. 43:137-161.

Williamson, D. H., and R. Hems. (1978). Metabolism and function of ketone bodies. In: Essays in Cell Metabolism. W. Bartley, H. L. Kronberg, and J. R. Quayle, eds. New York: Wiley; 257-281.

Williamson, J. R., R. Scholz, and E. T. Browning. (1969). Control mechanisms of gluconeogenesis and ketogenesis. II. Interactions between fatty acid oxidation and the citric acid cycle in perfused rat liver. J. Biol. Chem. 244(17):4617-4627.

Winder, W. W., K. M. Baldwin, and J. O. Holloszy. (1975). Exercise-induced increase in the capacity of rat skeletal muscle to oxidize ketones. Can. J. Physiol. Pharmacol. 53:86-91.

Windmueller, H. G., and A. E. Spaeth. (1978). Identification of ketone bodies and glutamine as the major respiratory fuels in vivo for postabsorptive rat small intestine. J. Biol. Chem. 253(1):69-76.

Woodside, W. F. (1979). Influence of insulin and glucagon on ketogenesis by isolated rat hepatocytes. Advances in Experimental Medicine and Biology vol. 111. D. M. Klachko, R. R. Anderson, and M. Heimberg, eds. Plenum Press, New York and London.

Yeh, Y.-Y., V. L. Streuli, and P. Zee. (1977). Ketone bodies serve as important precursors of brain lipids in the developing rat. Lipids. 12:957-964.

Yeh, Y.-Y., P. Zee, G. L. Owen, and E. Walker. (1982). Effect of postnatal undernutrition on ketone metabolism and its relation to metabolic changes in brain and liver of rats. J. Nutr. 112(6):1162-1168.

Zammit, V. A. (1981). Intrahepatic regulation of ketogenesis. Trends Biochem. Sci. 6:46-49.

APPENDIXES

APPENDIX A

SURGICAL PACK AND SUPPLIES FOR THE CATHETERIZATION OF THE FEMORAL ARTERY, AND FEMORAL, HEPATIC, PORTAL, AND MESENTERIC VEINS.

8 Towel clamps

5 Female Tuohy borsts

2 Bulldog clamps

3 Atraumatic surgical needles, #4

3 Traumatic surgical needles, #4

4 13 gauge stainless steel hypodermic needles

1 Rat-toothed thumb forcep, 6"

1 Thumb forcep, 6"

6 Straight-edge mosquito hemostats

8 Curved-edge mosquito hemostats

4 Allis tissue forceps

1 Eye-dressing forcep, 3"

1 Curve-edge eye-dressing forcep, 3"

1 Iris scissors

1 Mixter scissors

1 Blunt/Blunt scissors

1 Blunt/Sharp scissors

1 #4 Scalpel handle (uses #21 blade)

1 #7 Scalpel handle (uses #11 blade)

2 6" Needle holders

1 4" Needle holder

1 Finochietto, 12-18"

1 Blunt probe

1 Straight-edge hemostat with protected jaws

1 Skin needle, 18"

2 Saline bowls

1 Glass syringe, 5cc

1+ Heparin 10cc vial

1+ 2 x 2 gauze sponges package

1+ 4 x 4 gauze sponges package

3 Gauze lap packs

Silk suture material, size 0

*If catheters are to be autoclaved along with the surgical instruments use Silastic medical grade tubing. If using Tygon-type of material, sterilize the catheters with ethylene oxide or a comparable cold-sterilization agent.

Catheters

1.) FEMORAL ARTERIAL CATHETER:

80 cm in length

Silastic: 0.044 x 0.065 // Tygon: 0.050 x 0.090 (inches)

Double cuffs 20 cm from the end.

2.) FEMORAL VENOUS CATHETER:

80 cm in length

Silastic: 0.044 x 0.065 // Tygon: 0.050 x 0.090 (inches)

Double cuffs 20 cm from the end.

3.) HEPATIC VEIN CATHETER:

80 cm in length

Silastic: 0.044 x 0.065 // Tygon: 0.050 x 0.090 (inches)

Single cuffs at 2.5, 6.5, and 10.5 cm from the end.

4.) PORTAL VEIN CATHETER:

A. Direct cannulation via purse-string suture...

80 cm in length

Silastic: 0.044 x 0.065 // Tygon: 0.050 x 0.090 (inches)

Double cuffs 2.5 - 3.0 cm from the end.

B. Cannulation via a mesenteric vein...

80 - 100 cm in length

Silastic: 0.044 x 0.065 // Tygon: 0.050 x 0.090 (inches)

Double cuffs 25 - 40 cm from the end as determined by palpation to be approximately 2.5 cm from the portal - hepatic junction

5.) MESENTERIC VIEW CATHETER:

80 cm in length

Silastic: 0.044 x 0.065 // Tygon: 0.040 x 0.070 (inches)

Single or Double cuff(s) 10 cm from the end.

APPENDIX B

PARA-AMINOHIPPURIC ACID ANALYSIS REAGENTS

Para-aminohippuric Acid Assay Reagents:

A. 20% Trichloroacetic acid (TCA)

B. 1.2 N HCl

C. $NaNO_2$ (100 mg/100 ml H_2O)

Make up on day of use

D. Ammonium sulfamate (500 mg/100 ml H_2O)

Make up within a month, and store in amber container

E. Coupling reagent (N-1 Naphthylethylenediamine dihydrochloride, 100 mg/100 ml H_2O)

Stored in amber bottle and refrigerated — indefinitely

PAH Stock - 10% Solution for Infusion:

A. PAH Stock Solution:

1. 44.9 g PAH acid powder
2. 10.5 g NaOH
3. Bring upto volume with commercial physiological saline solution (PSS).
4. Final volume = 500 ml

Dissolve each reagent in PSS separately. Add NaOH solution to PAH solution and stir. To finish dissolving, set in 40°C water bath and stir until completly dissolved. Filter through a Buchner funnel with #42 Whatman filter paper. Titrate filter to pH 7.4 with 1 N

HCl or 4N NaOH. Stored frozen (-20°C).

B. 1.5% PAH Infusion Solution:

Mix 75 cc of stock solution with 425 ml PSS. Final volume = 500 ml. Store frozen (-20°C).

APPENDIX C

KETONE BODY ANALYSIS REAGENTS

Ketone Body Analysis Reagents.

A. Treatment of whole blood.

1. 1 M $HClO_4$
2. KOH

B. Acetoacetate assay:

1. Phosphate buffer. (pH 6.8, 0.1 M)

   a. 1.36 g KH_2PO_4 (Potassium phosphate monobasic) ⟶ 100 ml

   b. 1.74 g K_2HPO_4 (Potassium phosphate) ⟶ 100 ml

   Mix equal volumes of a and b, check pH, and neutralize with either a or b, so that the final pH is 6.8

2. NADH (approximately 1 mM)

   Boehringer, Grade II, disodium salt, 97% pure

   0.005 g ⟶ 6 ml

   Make up on day of use and keep cold

3. 3-β-Hydroxybutyrate dehydrogenase.

   Boehringer. 5 mg/ml

4. Acetoacetate standards. (Anhydrous M. wt. 108.0)

   Sigma. Lithium salt, 90-95% pure.

   1.5 mM stock solution = 0.01620 g ⟶ 100 ml

   Make up on day of use

   a. 1.5 mM = 20 parts stock solution + 0 parts H_2O

   b. 0.15 mM = 2 parts (a) + 18 parts H_2O

c. 0.10 mM = 12 parts (b) + 8 parts H_2O

d. 0.075 mM = 4 parts (b) + 4 parts H_2O

e. 0.050 mM = 10 parts (c) + 10 parts H_2O

f. 0.025 mM = 10 parts (e) + 10 parts H_2O

g. 0.000 mM = 0 parts std + 20 parts H_2O

C. β-Hydroxybutyrate assay:

1. Tris-HCl buffer (pH 8.5, 0.1M)

   2.42 g tris ⟶ 50 ml

   Bring to pH 8.5 with HCl if needed

   Bring to final volume up to 200 ml

   Can be stored on the shelf indefinately

2. Hydrazine-Tris buffer (pH 8.5)

   Make up on day of use

   2.5 ml of hydrazine hydrate

   0.05 g EDTA

   12.5 ml 1 M HCl

   Bring final volume up to 50 ml with Tris-HCl buffer

3. NAD^+ (approximately 14 mM)

   Boehringer. Grade II. Free acid. 98% pure

   0.03 ⟶ 3 ml

   Make up on day of use and keep cold

4. 3-β-Hydroxybutyrate dehydrogenase.

   Boehringer. 5 mg/ml

5. β-Hydroxybutyrate standards

   Sigma. Sodium salt. Enzymatic purity approximately 96%

Stock soln. 2mM = 0.0656 g ⟶ 250 ml

Make working standards of 2.0 mM, 1.6 mM, 1.2 mM, 0.8 mM, 0.4 mM and 0 mM.

Make up on day of use

APPENDIX D

PLASMA FREE FATTY ACID ANALYSIS REAGENTS

1. Titration mixture.

   Thymol blue (0.01% in 90% ethanol)

2. Extraction mixture.

   40 Isopropanol: 10 Petroleum ether: 1 1N H_2SO_4

   Heptane may be substituted for petroleum ether

3. Petroleum ether

4. 0.05% H_2SO_4

5. 0.0018 N NaOH

   Make up on day of use

   (Stored as 0.18 N NaOH, $\longrightarrow$ 100 ml)

6. Nitrogen gas

APPENDIX E.

INSULIN RADIOIMMUNOASSAY MATERIALS AND REAGENT PREPARATIONS

Insulin Radioimmunoassay Materials:

1. $NaH_2PO_4 \cdot H_2O$
2. NaCl
3. Disodium ethylene dinitro-tetra-acetate
4. Methiolate (Thimerosol)
5. 5 M NaOH
6. Bovine serum albumin

   RIA Grade — Fraction 5
7. Guinea pig serum

   Miles Laboratories, Inc.

   or

   Research Products International
8. Bovine pancreatic crystalline insulin (24-25 IU/mg)

   Sigma Chemical Co.
9. Guinea pig anti-bovine insulin (1^{st} Ab)

   Miles Laboratories, Inc.
10. ^{125}I-Insulin

    Amersham Corp.
11. Anti-guinea pig IgG

    Miles Laboratory, Inc.

    or

    Research Products International
12. Borosilicate culture tubes (12 x 75 mm)

Insulin Radioimmunoassay Reagent Preparations:

1. Phosphate Buffered Saline (PBS-EDTA)

   Dissolve in 800 ml DD H_2O;

   1.38 g $NaH_2PO_4 \cdot H_2O$

   8.77 g NaCl

   9.305 g EDTA$\cdot 2H_2O$ Disodium Salt

   Adjust to pH 7.6 with 5M NaOH

   Add 100 mg Thimersol and bring to 1000 ml in volumetric flask

   Store at 2^{o}-4^{o}C.

2. 1% BSA-PBS-EDTA

   12-24 hours prior to each assay add 1% BSA to cold PBS-EDTA in a beaker

   Dissolve using a stirring magnet

   Readjust pH to 7.6 using 5M NaOH.

3. 0.25% GPS-1% BSA-PBS-EDTA

   Add 250 µl guinea pig serum to 100 ml 1% BSA-PBS-EDTA

4. 1^{st} Antibody

   Reconstitute lyophilized guinea pig anti-bovine insulin antibody according to manufacturer's directions.

   For Miles Ab use 1 ml cold DD H_2O and then add 9 ml 1% BSA-PBS-EDTA to give a 1/10 dilution.

   1^{st} antibody should be diluted so that buffer control tubes contain 50% of the total tubes' CPM.

   (This lab has been using $\frac{1}{7000}$ or $\frac{1}{8000}$ dilution: 0.05 ml of 1/10 Ab diluted to 35 or 40 ml using 0.25% GPS-1% BSA-PBS-EDTA.

5. Insulin Standard

   A. 78.43 mg bovine insulin (Sigma I5500) diluted in 500 ml 1% BSA-PBS-EDTA (since lot contained 25.5 U/mg, therefore 78.43 mg = 2000)

      Stir continuously for 1-2 hours at room temperature

      Final solution contains 4 U/ml

   B. 1 ml of 4 U/ml solution is diluted to 100 ml in 1% BSA-PBS-EDTA to give a 40 mU/ml solution.

      Freeze solution in 0.5 ml aliquots using liquid nitrogen

   C. To prepare standard solution, 0.2 ml of the 40 mU/ml solution is diluted to 4 ml in 1% BSA-PBS-EDTA to give a 2 mU/ml solution. The following dilutions of the 2 mU/ml solution are then used to give solutions used in the standard curve:

Standard (μU/ml)	2mU/ml Solution Volume (ml)	1% BSA-PBS-EDTA Volume (ml)
200	0.5	4.5
120	0.3	4.7
80	0.2	4.8
40	0.1	4.9
20	0.05	4.95
12	0.06	9.94
8	0.04	9.96
4	0.02	9.98
1	0.005	9.995

200 μl of these solutions are then used in corresponding standard tubes.

6. Labelled Insulin

   ^{125}I-Insulin is diluted with 1% BSA-PBS-EDTA to give a solution containing enough ^{125}I-Insulin so that approximately 10,000 CPM is in the 100 µl sample.

7. 2nd Antibody

   A. When using Miles Ab:

      Lyophilized Ab is reconstituted using 2 ml cold DD H_2O

      Dilute the solution to 20 ml using 1% BSA-PBS-EDTA

   B. When using Research Products Inc. Ab:

      Antibody is thawed and diluted $\frac{1}{80}$ using 1% BSA-PBS-EDTA

8. Guinea Pig Serum

   Guinea pig serum should be frozen in 500 µl aliquots

APPENDIX F

INSULIN RADIOIMMUNOASSAY PROTOCOL

Insulin Assay Protocol Using 400 µl Samples

Tube no.		Plasma or standard	PBS no GPS	PBS w̄ GPS	1st Ab in PBS w̄ GPS	Insulin	2nd Ab in PBS no GPS
1- 3	Total	-	-	-	-	100 µl	-
4- 6	N.S.	-	500 µl	100 µl	-		200 µl
7- 9	0	-	400 µl		100 µl		
10-12	1	200 µl	200 µl				
13-15	4						
16-18	8						
19-21	12						
22-24	20						
25-27	40						
28-30	80						
31-33	120						
34-36	200						
37-39	0	-	400 µl				
40-42	Pooled cow	400 µl	-				
43-45	Pooled calf	400 µl	-				
46-47	Unknown samples	400 µl	-	100 µl	100 µl	100 µl	100 µl

VITA

Juan Marcos Fernandez was born in Havana, Cuba on November 2, 1958. He emigrated to the United States of America on December 30, 1963, and was immediately granted permanent residence status. The author attended elementary schools in Miami, Florida and Maywood, Illinois, and received his high school diploma in June, 1976 from Porviso East High School in Maywood.

He enrolled at Illinois State University the following August, and majored in Agricultural Science with minors in Biology and Chemistry. He graduated in December 1980 and received his Bachelor of Science in Agriculture. The author stayed at Illinois State University during the Spring semester of 1981 as a non-degree student.

Mr. Fernandez accepted a research assistantship at the University of Tennessee, Knoxville, and began study toward a Master's degree in the area of ruminant nutrition in September 1981. The Master of Science degree in Animal Science was awarded in August, 1983.

The author will commence study toward the Doctor of Philosophy degree in August of 1983 at North Carolina State University, Raleigh, where he has accepted a research assistantship.

During his stay at The University of Tennessee the author met and married the former Sheila Ogle Watt, and now has a step-daughter, Julia Christan Watt.