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AUTOREGULATION OF KETOGENESIS IN FASTED SHEEP

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Dianne J. Dawes

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ABSTRACT

The effects of beta-hydroxybutyrate at rates simulating maximum utilization on portal-drained viscera (PDV), hepatic (HEP), and hindquarter (RUMP) net fluxes of acetoacetate (AcAc), beta-hydroxybutyrate (BOHB), free fatty acids (FFA), insulin (IN) and glucagon (GN) were measured in normal (NOR), diabetic insulin treated (DIT) and diabetic untreated (DUT) 3-day fasted sheep. The sheep were equipped with chronic indwelling catheters in the femoral artery and portal, hepatic, mesenteric, and femoral veins. Beta-hydroxybutyrate was infused into a jugular vein. Para-aminohippuric acid (PAH) was infused into a mesenteric vein (1.5% at 0.764 ml/min) in order to measure blood flow rates across the downstream tissues. Hindquarter blood flow was determined by infusing PAH into the femoral artery and measuring femoral artery-jugular vein PAH differences. Three pre- and post-BOHB samples were taken simultaneously from the artery and portal, hepatic, and femoral veins. Net fluxes were calculated by multiplying venoarterial differences by whole blood flow rates. Briefly, BOHB infusion decreased FFA levels in the NOR and DIT and to a lesser extent in DUT animals. Hepatic uptake of FFA decreased in NOR and DIT, but not DUT. Portal-drained visceral and RUMP release of FFA decreased in NOR and DIT, but not DUT ewes. Production of both AcAc and BOHB decreased during BOHB infusion in all three groups. Betahydroxybutyrate infusion increased pancreatic production of insulin in the DIT and NOR sheep but had no effect on IN levels in DUT sheep, while exerting no effect on GN production.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	LITERATURE REVIEW	4
	Ketone Body Synthesis	4
	Ketone Body Utilization	7
	Hyperketonemia	9
	Regulation of Ketogenesis	10
	Extrahepatic Regulation of Ketogenesis	11
	Intrahepatic Regulation of Ketogenesis	17
	Autoregulation of Ketogenesis	21
III.	MATERIALS AND METHODS	23
	Catheter Preparation	23
	Pre-operative Care and Anesthesia	24
	Leg Surgery	24
	Abdominal Surgery	25
	Post-operative Care of the Animals	25
	Experimental Protocol	26
	Chemical Analysis	27
	Statistical Analysis	30
IV.	RESULTS	31
	Whole Blood Flow Rates	32
	Free Fatty Acids	32
	Acetoacetate	35
	Beta-hydroxybutyrate	37
	Insulin	39
	Glucagon	41
V.	DISCUSSION	43
	Free Fatty Acids	44
	Ketone Bodies	46
	Insulin	49
	Glucagon	51
	Conclusion	53
LIST OF REFERENCES		55
APPENDIXES		65
APPENDIX 1		66
APPENDIX 2		69
APPENDIX 3		72
APPENDIX 4		75
APPENDIX 5		78

CHAPTER PAGE

APPENDIXES (continued)
APPENDIX 6. 83
APPENDIX 7. 89
APPENDIX 8. 93
APPENDIX 9. 95
APPENDIX 10 97
APPENDIX 11 99
APPENDIX 12 101
APPENDIX 13 103
APPENDIX 14 105
APPENDIX 15 107
APPENDIX 16 109
APPENDIX 17 111
APPENDIX 18 113
APPENDIX 19 115
APPENDIX 20 117

VITA. 119

LIST OF TABLES

TABLE		PAGE
1.	Physiological roles of ketone bodies in peripheral tissues of man and rat.	8
2.	Whole blood flow rates in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted ewes	33
3.	Effects of beta-hydroxybutyrate on free fatty acid arterial concentrations and net fluxes in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted ewes	34
4.	Effects of beta-hydroxybutyrate on acetoacetate arterial concentrations and net fluxes in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted ewes.	36
5.	Effects of beta-hydroxybutyrate on beta-hydroxybutyrate arterial concentrations and net fluxes in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted ewes	38
6.	Effect of beta-hydroxybutyrate on insulin arterial concentrations and net fluxes in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted ewes.	40
7.	Effects of beta-hydroxybutyrate on glucagon arterial concentrations and net fluxes in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted ewes.	42
A-1.	Venoarterial differences of glucose in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted ewes.	94
A-2.	Venoarterial differences of free fatty acids in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted ewes pre and post beta-hydroxybutyrate infusion. . .	96
A-3.	Venoarterial differences of acetoacetate in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted ewes pre and post beta-hydroxybutyrate infusion.	98

TABLE PAGE

A-4. Venoarterial differences of beta-hydroxybutyrate in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted ewes pre and post beta-hydroxybutyrate infusion. 100

A-5. Venoarterial differences of insulin in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted ewes pre and post beta-hydroxybutyrate infusion 102

A-6. Venoarterial differences of glucagon in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted ewes pre and post beta-hydroxybutyrate infusion. . . 104

A-7. Effects of beta-hydroxybutyrate infusion on extraction ratios of glucose in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted sheep 106

A-8. Effects of beta-hydroxybutyrate infusion on extraction ratios of free fatty acids in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted sheep. 108

A-9. Effects of beta-hydroxybutyrate infusion on extraction ratios of acetoacetate in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted sheep 110

A-10. Effects of beta-hydroxybutyrate infusion on extraction ratios of beta-hydroxybutyrate in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted sheep 112

A-11. Effects of beta-hydroxybutyrate infusion on extraction ratios of insulin in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted sheep 114

A-12. Effects of beta-hydroxybutyrate infusion on extraction ratios of glucagon in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted sheep 116

A-13. Effects of beta-hydroxybutyrate infusion on arterial concentrations and net fluxes of glucose in normal, diabetic insulin-treated, and diabetic untreated 3-day fasted ewes 118

CHAPTER I

INTRODUCTION

In the late 1800's ketone bodies were discovered in the urine of diabetics. This led to the conclusion that they were byproducts of metabolism which were unable to be utilized by the body. In addition, ketone bodies were once considered to be intermediates in the oxidation of fatty acids. However, it has been reported that the normal intermediates in the oxidation of fatty acids are the CoA derivatives of ketone bodies, not the ketone bodies themselves. In addition, Lehninger and Greville (1953) showed that one of the intermediates formed through fatty acid oxidation was L (+)- beta-hydroxybutyrate-CoA while beta-hydroxybutyrate in the blood exists in the (D)-form. Since then many investigators as reviewed by Campbell and Best (1953) have found that ketone bodies can serve a physiological function by supplying tissues with an alternate fuel of respiration during energy deficient states.

More recent investigations have 1) determined the various sites of ketone body production, 2) identified the precursors of ketone body production, 3) described the biochemical pathways of ketone body synthesis, and 4) elucidated possible regulatory mechanisms involved in ketone metabolism.

The three ketone bodies which exist in the blood are aceto-acetate, beta-hydroxybutyrate, and acetone. However, it has been clearly established that acetoacetate and beta-hydroxybutyrate are

the only physiologically significant ketone bodies. Until recently acetone has been regarded an nonmetabolizable and physiologically insignificant, since large quantities were consistently found in the urine. However, Hetenyi and Farrarotto (1985) have shown that a small fraction (1.37%) of the carbon atoms of glucose were derived from acetone via its conversion to lactate. Nonetheless, future reference to "ketone bodies" in this thesis will include only acetoacetate and beta-hydroxybutyrate.

The production of ketone bodies is known as ketogenesis. Ketogenesis occurs primarily in the liver of nonruminants (Williamson and Whitelaw, 1978) and to an equal extent in the rumen epithelium and liver of normal fed ruminants (Katz and Bergman, 1969; Annison and Lewis, 1969; Baird, 1977). The major precursors of ketogenesis are free fatty acids in the liver (Fritz, 1961; Leng and West, 1969; Mayes and Felts, 1967; Havel et al., 1962) and volatile fatty acids, particularly butyrate, in the alimentary tract (Seto et al., 1955; Bergman, 1970). Control of ketone body production can occur extra-hepaticly, through control of the rate of release of free fatty acids from the adipocyte, and intrahepaticly, via control of the fate of the free fatty acids in the liver, i.e. beta-oxidation or re-esterification (McGarry and Foster, 1978). In addition, McGarry and Foster (1977, 1978) suggest that two hormones (insulin and glucagon) are involved in the regulation of ketogenesis. Finally, it has been shown that ketone bodies themselves have a regulatory role in their own production (Madison et al., 1964; Bjorntorp and Shersten, 1967; Balasse et al., 1971). There is no doubt that glucagon and especially

insulin have definite roles in the autoregulation of ketogenesis. It is possible that ketone bodies can regulate their own production, independent of the influence of insulin.

CHAPTER II

LITERATURE REVIEW

Ketone Body Synthesis

Both ruminants and nonruminants depend on glucose as a primary source of energy. While nonruminants absorb glucose across the alimentary tract into the portal blood, the amount of glucose absorbed across the alimentary tract of the ruminant is negligible (Roe et al., 1966). Therefore, the ruminant relies on hepatic gluconeogenesis as a source of glucose. Bergman et al. (1965) reported that 80% of the total digestible energy was provided by volatile fatty acids and that proprionate is the primary precursor for hepatic gluconeogenesis. However, in times of energy restriction, ketone bodies become an alternate source of energy for both ruminants and nonruminants. Alimentary and hepatic ketogenesis contribute equally to total ketone body concentration in the ruminant but it is primarily hepatic ketogenesis which supplies ketone bodies in the starved ruminant (Brockman, 1976; Bergman, 1970). Alimentary ketogenesis is negligible in the nonruminant.

The major precursors of hepatic ketogenesis are free fatty acids (Fritz, 1961; Leng and West, 1969; Mays and Felts, 1967; Havel et al., 1962), derived from the mobilization of free fatty acids from fat stores, particularly adipocytes (Hengfeldt and Warren, 1968; Mayes, 1962). It has been estimated that free fatty acids contribute between 30-55% of the oxidizable energy in energy deficient states

(Baxter, 1962; Lindsay, 1975; Pethic, 1982; Wilson, 1984). The major free fatty acids that exist in the blood of ruminants are palmitic acid, oleic acid, and stearic acid (Thompson and Darling, 1975). The uptake of free fatty acids by the liver is proportional to the concentration of free fatty acids in the blood (Fritz and Yul, 1963; Fine and Williams, 1960; Evan et al., 1963). Once the free fatty acids are taken up by the liver they must be converted to long chain acyl carnitine, transported across the mitochondrial membrane, and undergo beta-oxidation and the formation of acetyl-CoA in order to form ketone bodies.

The first step in the production of ketone bodies is the conversion of acetyl CoA to acetoacetyl CoA. There are then two major pathways for the conversion of acetoacetyl CoA to acetoacetate. The first is the beta-hydrox,beta-methylglutaryl CoA pathway (Figure 1) consisting of a stepwise conversion of acetoacetyl-CoA to beta-hydroxy, beta-methylglutaryl CoA and finally to acetoacetate. The enzymes involved are beta-hydroxy,beta-methylglutaryl CoA synthetase and lyase. Both are rate limiting enzymes. The second and less predominant pathway involves the direct deacylation of acetoacetyl CoA to acetoacetate. This reaction is catalyzed by acetyl CoA deacylase, also a rate limiting enzyme. A great deal of controversy has arisen as to which pathway is predominant. The majority of the available evidence indicates that most, if not all, of the acetoacetate production in the liver occurs via the beta-hydroxy-beta-methylglutaryl CoA pathway (Ohgaku et al., 1981; Williamson et al., 1968) since the acetyl CoA deacylase activity is only 20-33%

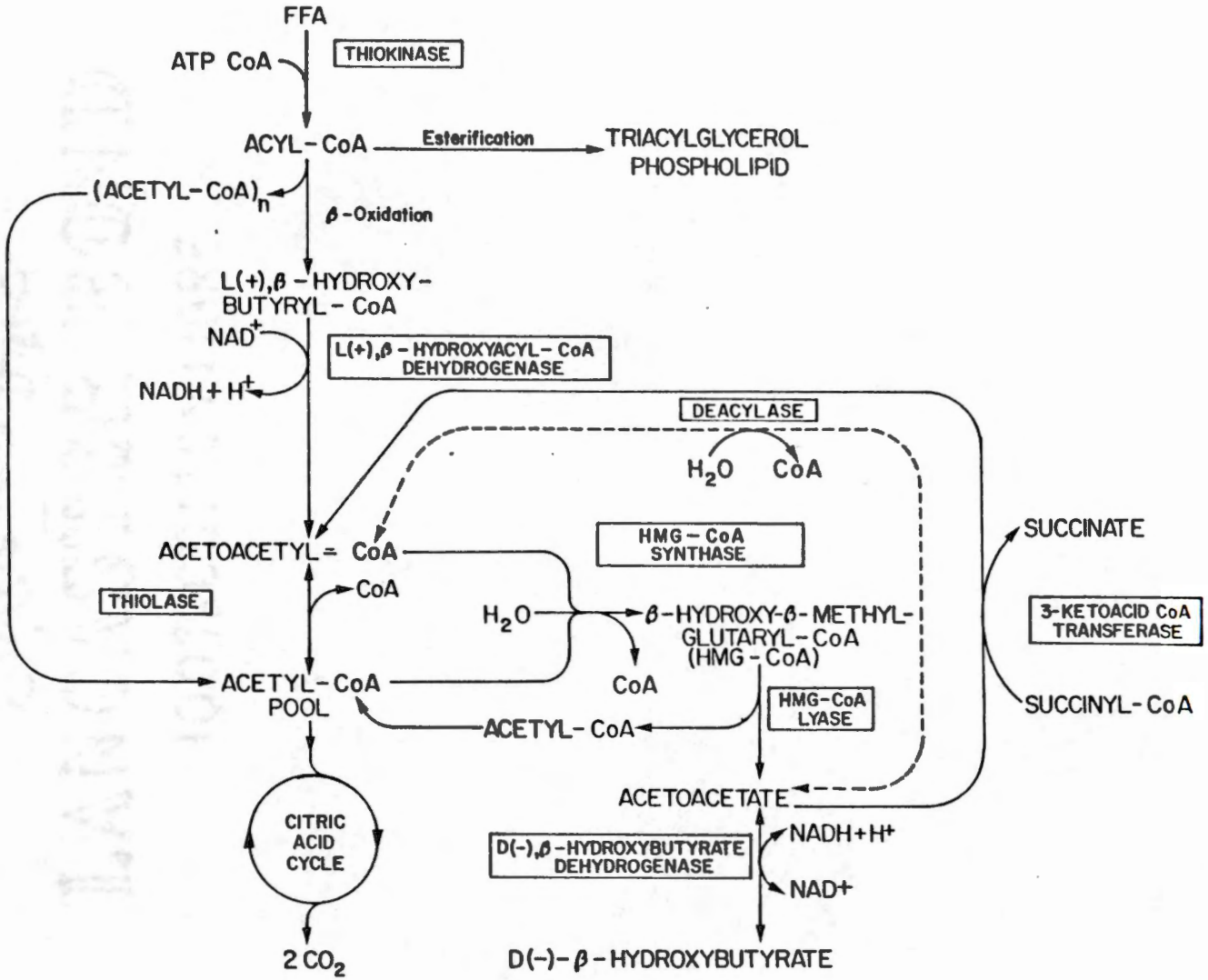

Figure 1. Ketone body metabolism in the liver.

that of the beta-hydroxy-beta-methylglutaryl synthetase and lyase. Once acetoacetate has been produced it can be converted to beta-hydroxybutyrate in the presence of 3-beta-hydroxybutyrate-dehydrogenease and NADH, as demonstrated in various tissues by Kulka et al. (1961) and Bergman et al. (1965). According to Katz and Bergman (1969), the rate of interconversion is dependent upon the redox state of the cellular compartment.

Ketone Body Utilization

Ketone bodies can be utilized as a source of energy in several tissues (McCann, 1975; Krebs, 1961; Williamson and Krebs, 1961; Kaufmann and Bergman, 1971; Katz and Bergman, 1976). Despite the tendency of ketone bodies to favor oxidation, they also serve as lipogenic precursors in the lactating mammary gland (Robinson and Williams, 1978). Extensive information of ketone body turnover rates has been reported in sheep (Bergman and Kon, 1964); in rats (McGarry and Foster, 1970, Bates et al., 1968; Wahren et al., 1984) and in neonatal rats (Shambaugh, 1985). A summary of the utilization of ketone bodies by several tissues has been tabulated by Williamson and Whitelaw (1978) (Table 1).

The first step in the utilization of ketone bodies is the conversion of acetoacetate to acetoacetyl CoA. This reaction is catalyzed by 3-keto-oxoacid transferase. This enzyme is present in most tissues throughout the body except for the liver; therefore, the hepatic tissues are unable to utilize ketone bodies (Koundakjian and Snoswell, 1970). Acetoacetyl CoA is then converted back to acetyl

Table 1. Physiological roles of ketone bodies in peripheral tissues of man and rat.

Tissue	Metabolic effects	Reference	Species
Brain	Oxidizable substrate	Hawkins et al. (1971)	Rat
	Lipid precursor	Ruderman et al. (1974)	Rat
	Inhibits pyruvate oxidation	Edmond (1974)	Rat
		Patel and Owen (1976)	Rat
		Owen et al. (1967)	Man
		Gottstein et al. (1971)	Man
Heart	Oxidizable substrate	Williamson and Krebs (1961)	Rat
	Inhibits glucose utilization and pyruvate oxidation	Randle et al. (1964)	Rat
		Lassers et al. (1971)	Man
Kidney	Oxidizable substrate	Krebs et al. (1965)	Rat
	Inhibits glucose utilization	Underwood and Newsholme (1967)	Rat
		Owen et al. (1969)	Man
Skeletal muscle	Oxidizable substrate	Ruderman and Goodman (1973)	Rat
	Inhibits pyruvate oxidation	Ruderman et al. (1977)	Rat
		Owen and Reichard (1971)	Man
Lactating mammary gland	Oxidizable substrate	Hawkins and Williamson (1972)	Rat
	Lipid precursor	Williamson et al. (1975)	Rat
	Inhibits glucose utilization and pyruvate oxidation; relieved by insulin	Robinson and Williamson (1977a,b)	Rat

Source: Williamson and Whitelaw (1978).

CoA through a thiolase reaction. The acetyl CoA can then enter the TCA cycle where it can be fully oxidized to CO_2 and H_2O.

Hyperketonemia

The whole blood concentration of ketone bodies represents the balance between net hepatic and alimentary ketogenesis and net utilization of ketone bodies by the peripheral tissues (Williamson and Whitelaw, 1978). Concentrations typically range between 100-390 μM in the nonruminant (Bates et al., 1968; Garbel and Minzel 1974); Robinson and Williamson, 1980) and 100-500 μM in the ruminant (Baird et al., 1972; Bergman and Kon, 1964). Lindsay and Leat (1975) reported concentrations of 10-80 uM for acetoacetate and 120-480 uM for beta-hydroxybutyrate in the ruminant. An increase of ketone bodies above normal levels may result during energy deficient states. Williamson and Whitelaw (1978) suggest that elevated concentrations are due not only to overproduction but a defect in the ability of the tissues to utilize ketone bodies. However, Bergman and Kon (1964) suggest that overproduction rather than underutilization is responsible for the elevated ketone body concentration, since utilization of ketone bodies is concentration dependent up to a maximum of 10 mg/ml. Prolonged hyperketonemia may result in a pathological condition known as ketosis. Since Baird (1982) reported an incidence of bovine ketosis between 2-15% in the U.S. and Western Europe and Kronfield (1971) found that one of the physiological manifestations of ketosis is a decrease in milk production, it is obvious that this metabolic

disorder may have economical implications and therefore a better understanding of the regulation of ketone body production is important.

Regulation of Ketogenesis

The regulation of ketogenesis is a multifaceted and highly intricate process and has been reviewed extensively (Alberti et al., 1978; Williamson and Whitelaw, 1978; Brockman, 1979; Fain and Shepherd, 1979; McGarry and Foster, 1977, 1980; Zammit, 1981). It has been suggested by McGarry and Foster (1977) that regulation occurs both extra-hepaticly, at the level of the release of free fatty acids from the adipocyte, and intra-hepaticly by control of the fate of the free fatty acids once taken up by the liver. In addition, McGarry and Foster (1975) have suggested that two hormones, insulin and glucagon, play major roles in the regulation of ketogenesis. Several other hormones including growth hormone, glucocorticoids, epinephrine, and adrenocorticotropic hormone also exert regulatory effects. However, insulin and glucagon play the more important homeostatic roles (Radloff and Shultz, 1966). A negative correlation between insulin concentration and rate of ketogenesis has been demonstrated by McGarry et al. (1973), while glucagon has been shown to have ketogenic activity (Menahan and Wieland, 1969; Williamson, 1967; McGarry et al., 1975). It appears to be the ratio of the two hormones which dictates the incidence of increase or decrease of ketogenesis. For instance, a high insulin/glucagon ratio would not favor ketogenesis while a low insulin/glucagon ratio would. Moreover, Brockman (1976) demonstrated that insulin has a more potent effect on the regulation

of ketone body production based on the fact that low levels of insulin are capable of blocking the ketogenic effects of glucagon.

Insulin exerts an antiketogenic effect at the adipocyte (Figure 2) by facilitating the uptake and conversion of glucose to glycerol-1-phosphate in the adipocyte, thereby enhancing fatty acid re-esterification, and by inhibiting the hydrolysis of triacylglycerides to free fatty acids resulting in a net decrease in free fatty release from the adipocyte (Annison, 1960). Insulin also plays a role in determining whether free fatty acids will be re-esterified or oxidized once they reach the liver. Ample evidence exists which shows that insulin enhances re-esterification in the liver while inhibiting the transport of free fatty acids into the mitochondria.

Glucagon also exerts its effects both at the adipocyte and liver (Figure 3). It is well known that glucagon stimulates adipose tissue lipolysis and that glucagon favors the transport of fatty acids into the mitochondria where they are oxidized while inhibiting re-esterification of fatty acids within the liver (McGarry and Foster, 1980).

Extrahepatic Regulation of Ketogenesis

The hydrolysis of triacylglycerides in the adipocyte is of significant importance in the availability of free fatty acids for the production of ketone bodies. In general, the release of free fatty acids depends on an overall anabolic/catabolic state within the adipocyte.

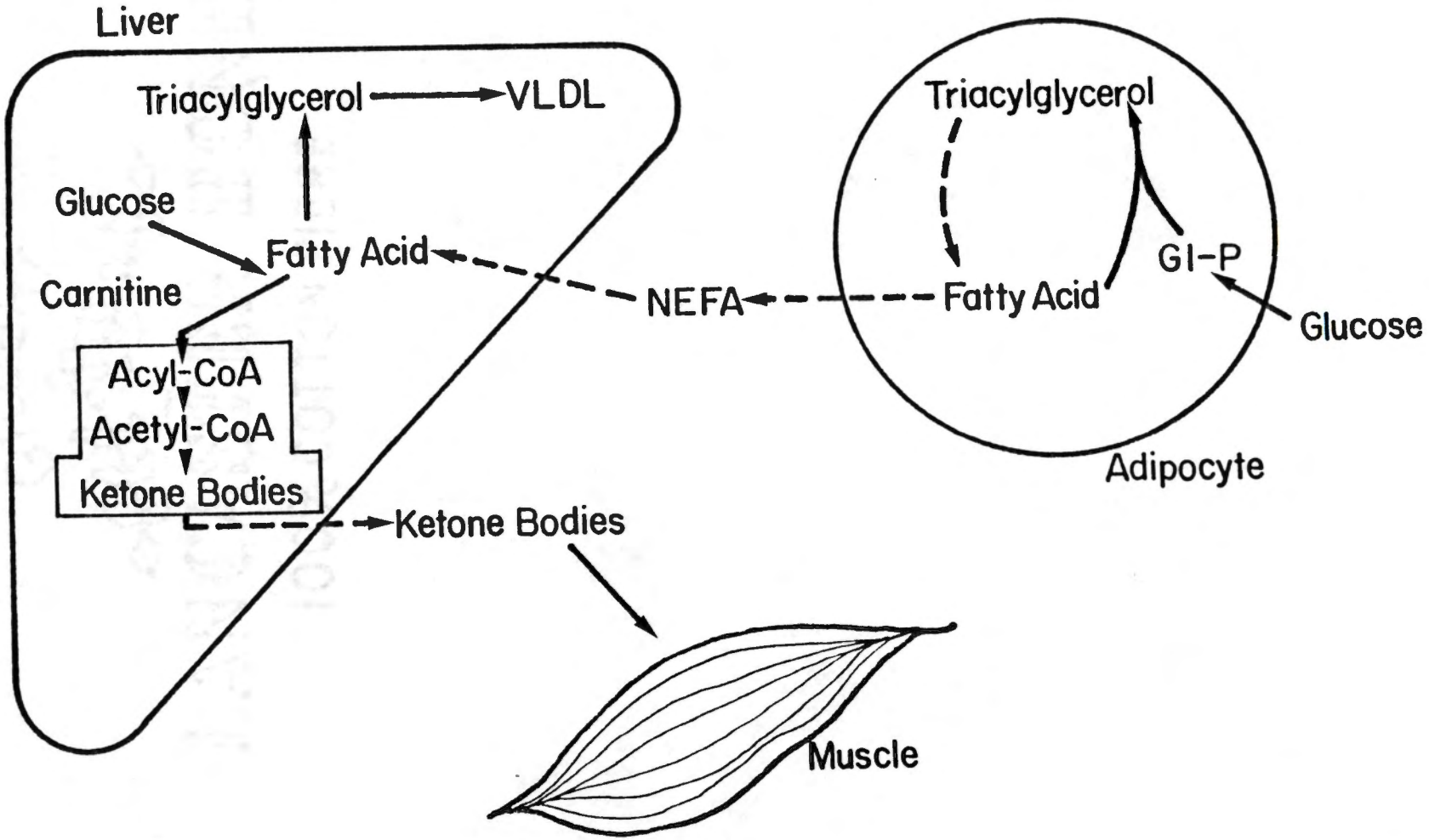

Figure 2. Effects of insulin on ketone body metabolism (Alberti et al., 1978).

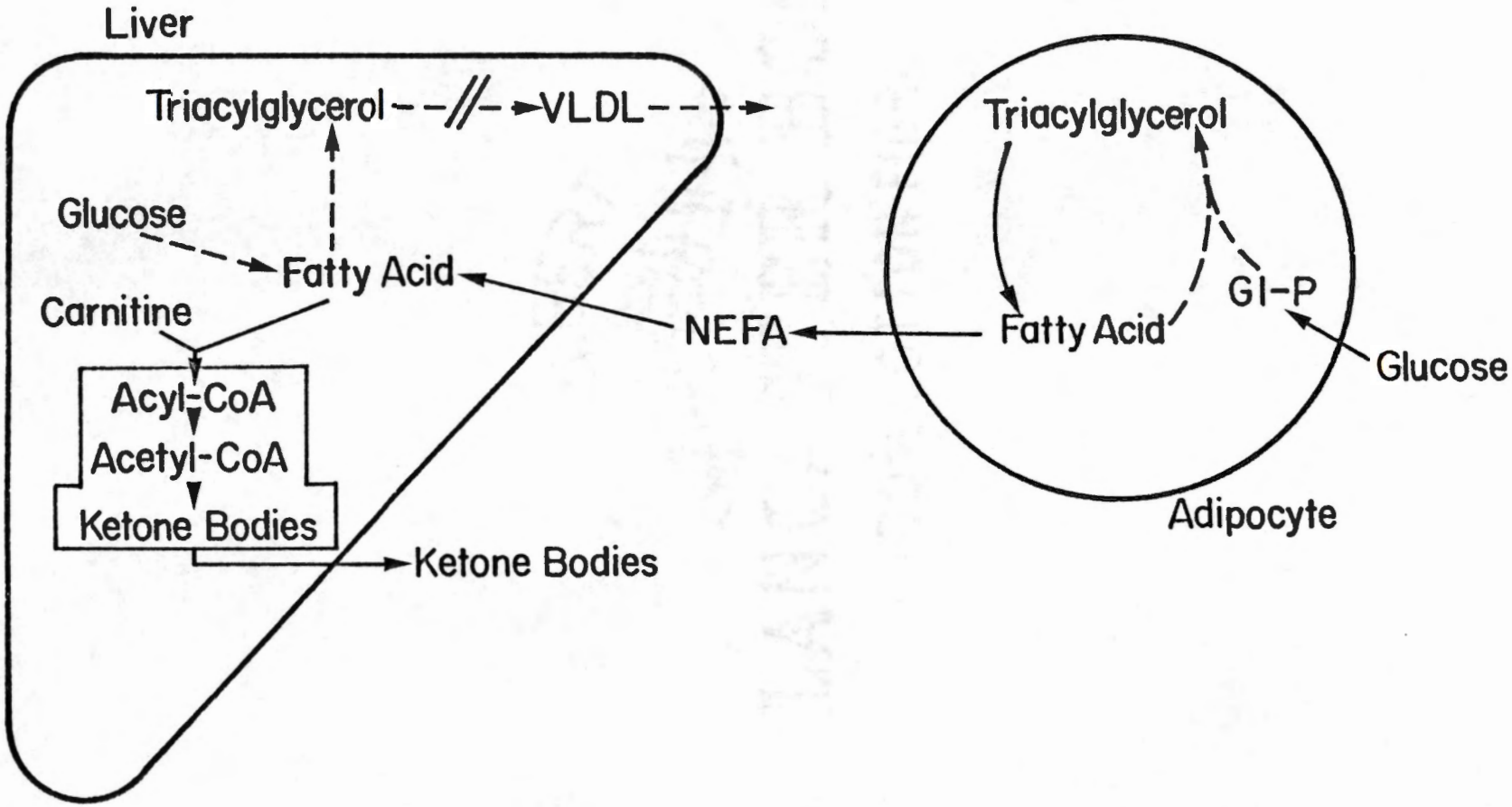

Figure 3. Effects of glucagon on ketogenesis with insulin absent (Alberti et al., 1978).

Insulin and glucagon are the primary homeostatic hormones for this mechanism.

The enzymes involved in fatty acid metabolism within the adipocyte are hormone sensitive lipase (Rizack, 1961) and lipoprotein lipase (Sadur and Eckel, 1982). The rate of lipolysis is determined to a large degree by hormone sensitive lipase, since its primary effect is the hydrolysis of triacylglycerides to free fatty acids (Rizack, 1961; Hollenberg et al., 1961; Bjorntorp, 1966). Lipoprotein lipase catalyses the hydrolysis of triacylglyceride rich lipoprotein to fatty acids in the blood, which then enter the adipocyte and are stored as triacylglycerides (Sadur, 1984). These two enzyme systems are primarily under the control of insulin and glucagon. Both insulin and glucagon exert their effect through interaction with hormone specific receptor systems on the plasma membrane of the adipocyte.

Glucagon carries out its lipolytic effects through a secondary messenger mediated phosphorylation cascade series of reactions beginning with the binding of the hormone to its receptor (Figure 4). The glucagon receptor acts in the same manner as the beta-adrenergic receptors beginning with the activation of adenylate cyclase. Until recently it was thought that the receptor ligand complex bound directly to adenylate cyclase (Shramm, 1977). However, Gilman (1984) has reported that the hormone-receptor complex activates adenylate cyclase through a guanine nucleotide regulatory protein which itself binds to and activates the adenylate cyclase. The activation of adenylate cyclase results in the conversion of ATP, in the matrix of the cell,

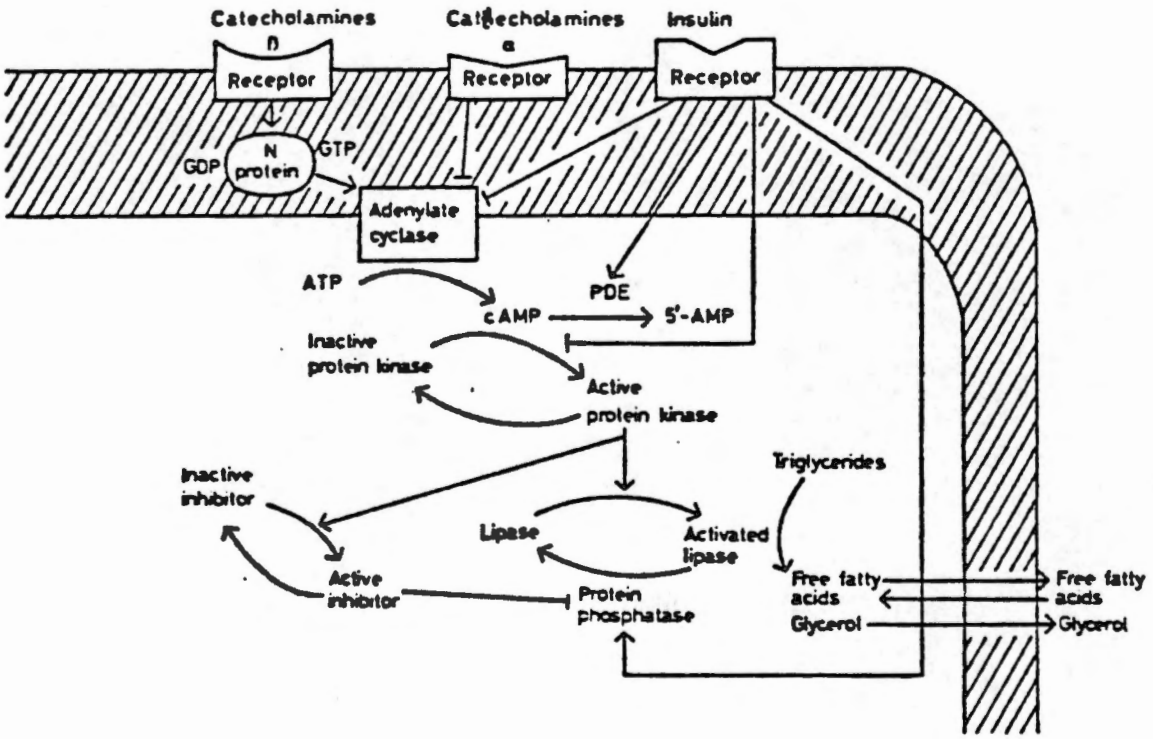

Figure 4. Hypothetical model for the regulation of lipolysis in the fat cell by catecholamines and insulin, through the cyclic AMP system (Engfeldt, 1982).

to cAMP. The effects of cAMP are mediated by the action of cAMP-dependent protein kinase. Protein kinase is a general phosphorylating enzyme which catalyzes the activation of hormone sensitive lipase (Hales et al., 1968; Corbin et al., 1972). Corbin et al. (1972) have suggested that protein kinase consists of both a regulatory and a catalytic subunit and that the holoenzyme displays no activity. The binding of cAMP to the regulatory subunit allows its disassociation of the catalytic subunit resulting in activation of the catalytic subunit. It is the active catalytic subunit which stimulates the activity of the hormone-sensitive lipase, followed by fatty acid mobilization and release from the adipocyte.

The antilipolytic effects of insulin also involve a secondary messenger mediated system (Figure 4). The initial step of the effects of insulin begins with the binding of insulin with the surface protein receptors on the adipocyte plasma membrane (Czech, 1976). Binding studies conducted by Gammeltoft and Gliemann (1973) have shown that only a small amount of insulin is required to cause metabolic effects in the fat cell. Insulin carries out its effects in several ways (Figure 4). It has been well established that insulin inhibts adenylate cyclase activity. Also, Engfeld (1982) and Correze et al. (1976) have reported that insulin stimulates phosphodiesterase activity. The inhibition of adenylate cyclase activity subsequently decreases cAMP production while phosphodiesterase converts the existing cAMP in the cell back to AMP. It appears that the presence of intracellular Ca^{++} is required for the effect of insulin. In support of this evidence,

Kissenban et al. (1974) demonstrated an influx of Ca^{++} into the cell from the plasma membrane and from the endoreticulum (Jarret et al., 1972).

Insulin can also directly inhibit hormone sensitive lipase independently of the cAMP mediated system through activation of protein phosphatase, a nonspecific dephosphorylating agent (Jungas and Ball, 1983). Finally, concomitant with the inhibition of free fatty acid release from the adipocyte, is the enhancement of fatty acid re-esterification by insulin. This is accomplished through stimulation of lipoprotein lipase (Sadur et al., 1984) and facilitation of glucose uptake and subsequent conversion to glycerol-1-P. Studies on adipose tissue from fed and fasted animals showed a positive correlation between triacylglyceride uptake and lipoprotein lipase activity (Cherkes and Gordon, 1959). Therefore, high insulin concentrations would favor the re-esterification of free fatty acids and inhibit fatty acid mobilization resulting in an overall decrease in free fatty acid release from the adipocyte.

Intrahepatic Regulation of Ketogenesis

Elevated free fatty acid concentration, although necessary, is not sufficient in itself to promote ketogenesis. Several studies have demonstrated only very small increases in ketogenesis in fed as compared with fasted animals at the same level of substrate availability (Madison et al., 1964; Mayes and Felts, 1967).

Two primary routes of metabolism are available to long chain fatty acids subsequent to entry into the liver (Figure 1, page 6).

They can either be re-esterified in the presence of glycerol-1-phosphate to phospholipids or triacylglycerides, or be converted to long chain acyl CoA, transported in the mitochondria, and undergo subsequent oxidation (McGarry and Foster, 1977; Williamson and Whitelaw, 1978). The partitioning of free fatty acids between these two metabolic pathways is of major regulatory significance in the production of ketone bodies (Williamson, 1967; Williamson and Whitelaw, 1978; Fritz, 1961; Ontko and Zilversmit, 1966). The final result of beta-oxidation, in the mitochondria, is the formation of acetyl CoA, which can be oxidized via the TCA cycle, translocation into cytosal and utilized for lipogenesis, or utilized for ketone body production. The partitioning of acetyl CoA between these metabolic pathways represents a second regulatory site for ketogenesis (McGarry et al., 1974). The first branchpoint depends on the preference towards re-esterification of the free fatty acids or their transport across the mitochondrial membrane and into the mitochondria.

Fritz (1961) reported that the mitochondrial membrane acts as a barrier limiting the passage of long-chain fatty acyl CoA into the mitochondria. McGarry and Foster (1978) elucidated the roles of carnitine acyl transferase I and II, located on the outer and inner surface of the inner membrane of the mitochondria, in facilitating the transport of the long-chain acyl CoA across the membrane. Additionally, McGarry and Foster (1974) showed that long-chain, but not short-chain, fatty acids are dependent on the carnitine acyltransferases for translocation into the mitochondria. The activity of

carnitine acyl transferase may be influenced by many factors. The combined results of studies conducted by McGarry and Foster (1975), Lund et al. (1980), Zammit (1981), and Parvin and Pande (1979) have suggested that the level of carnitine acyltransferase activity may be influenced by carnitine concentration in the blood. Additional studies (McGarry et al., 1974; Robinson and Zammit, 1982; Zammit, 1981; Paulson, 1984) have demonstrated the influence of malonyl CoA, an intermediate in the lipogenic pathway, on carnitine acyltransferase activity. In short, these investigations found that malonyl CoA inhibits the activity of the carnitine acyltransferase I, thus ensuring that transport of fatty acids and subsequent oxidation does not occur.

Insulin and glucagon have major roles in the intra-hepatic regulation of ketogenesis, particularly at the branchpoint between fatty acid re-esterification and transport into the mitochondria. Madison et al. (1964) proposed that insulin enhances fatty acid re-esterification by activating acetyl CoA carboxylase, an enzyme involved in the conversion of acetyl CoA to malonyl CoA in the lipogenic pathway. In addition, Holland and Hardies (1985) reported that the mechanism of insulin activation of acetyl CoA carboxylase was a direct phosphorylation of the enzyme.

The effects of glucagon on intra-hepatic fatty acid metabolism have been widely investigated. Keller et al. (1977), Alberti (1978), and McGarry et al. (1974) suggested that fatty acid transport into the mitochondria is enhanced by the stimulation of the activity of carnitine acetyltransferase I by glucagon. Concomitant with the

increased fatty acid oxidation was an inhibition of malonyl CoA synthesis by glucagon (McGarry, 1979). The source of inhibition of malonyl CoA production occurs directly through the inhibition of acetyl CoA carboxylase by glucagon. Swenson and Porter (1985) reported that the inactivation of acetyl CoA carboxylase is accomplished through its phosphorylation by glucagon.

The second branchpoint in fatty acid metabolism involves the partition of acetyl CoA between lipogenesis, oxidation and ketone production. This branchpoint is controlled more by the energy state of the animal, the flow of carbons towards gluconeogenesis, and the mitochondrial NADH/NAD ratio than by the direct effects of glucagon or insulin. The availability of mitochondrial oxaloacetate is necessary for acetyl CoA to enter the TCA cycle. If the flow of carbons favors gluconeogenesis, acetyl CoA condenses with oxaloacetate and enters the TCA cycle instead of being used for ketone body production. However, in energy deficient states, the gluconeogenic intermediates at some point become exhausted, particularly oxaloacetate, and acetyl CoA becomes available for use in ketone body production (Lehninger and Krebs, 1966). Therefore the availability of oxaloacetate is the link between the two pathways. The factor which affects the availabililty of oxaloacetate in the mitochondria is the NADH/NAD ratio which is influenced directly by the level of fatty acid oxidation and, therefore, indirectly by the insulin/glucagon ratio. A high NADH/NAD ratio results in the conversion of oxaloacetate to malate which may be transported into the cytoplasm from the mitochondria. The net result is an increase in acetyl CoA availability for ketone

body production. In addition, Tubbs and Garland (1964) have shown that fatty acid acyl CoA derivatives inhibit citrate synthase, the enzyme which catalyzes the condensation of oxaloacetate and acetyl CoA to form citrate, thereby enhancing the conversion of oxaloacetate to malate.

Autoregulation of Ketogenesis

Several investigators have demonstrated the ability of ketone bodies to regulate their own production by inhibiting free fatty acid release from the adipocyte (Madison et al., 1964; Bjorntorp and Shersten, 1967; Balasse and Ooms, 1969; Senoir and Loridan, 1960; Jenkins, 1967). Madison et al. (1964) demonstrated an increased pancreatic production of insulin in the presence of high beta-hydroxybutyrate concentration and suggested that it is through this mechanism that control of beta-hydroxybutyrate production occurs. Additional studies have shown that beta-hydroxybutyrate decreases free fatty acid release from the adipocyte independently of insulin (Bjorntorp and Shersten, 1967; Hotta et al., 1971). Beta-hydroxybutyrate has also been shown to have the ability to inhibit ketone body production intra-hepaticly in the presence of insulin. Heitmann and Fernandez (1986) have proposed that intra-hepatic autoregulation of ketogenesis may occur in the absence of insulin.

Kronfeld (1971) found that milk production is dependent upon glucose uptake by the mammary gland. Laarveld and Brockman (1983) suggested that the tissues of the mammary gland, along with brain tissues, are able to utilize glucose independent of insulin.

Therefore, mammary tissues must compete with those tissues which involve insulin facilitated uptake of glucose for glucose sources. For these reasons it is obvious that an insulin independent mechanism autoregulatory mechanism of hepatic ketogenesis would be desirable to allow mammary tissues to compete more favorably with other tissues for the uptake of glucose and prevent decreases in milk production during ketosis. The effects of BOHB infusion on the net flux of free fatty acids, acetoacetate, beta-hydroxybutyrate, insulin and glucagaon were measured in physiologically ketogenic diabetic insulin treated and diabetic untreated ewes in order to determine if insulin is required for the autoregulation of ketogenesis.

CHAPTER III

MATERIALS AND METHODS

Nonpregnant nonlactating suffolk crossbred ewes weighing 55-65 Kg and ranging from 1 to 3 years of age were used in the experimental protocol. The ewes were housed in individual indoor 1.8 x 3.0 meter pens at 19-25°C and with natural lighting. They were fed 400 grams of commercially prepared alfalfa pellets twice daily at 0800 and 1700 hours. Water and trace mineral salts were available ad libitum.

Five normal, five diabetic insulin-treated, and five diabetic untreated unanesthetized ewes were studied. The ewes were made diabetic pharmacologically with a single 50 mg/Kg intravenous dose of alloxan. Blood glucose levels were measured daily and appropriate subcutaneous injections (approximately 50 IU) of insulin (Iletin-100) were administered to maintain euglycemia.

Catheter Preparation

Catheters for the femoral artery and the hepatic, portal, and femoral veins were made from tygon tubing (1.27 x 2.29 mm). An additional catheter was prepared for the mesenteric vein using 1.02 x 1.78 mm tygon tubing. All catheters were 80 cm in length. Silastic cuffs were added to allow the catheter to be anchored into position, thus restricting movement of the catheter tip. The catheters were coated with a 7% heperanized TDMAC, gas (ethylene oxide) sterilized

and allowed to aerate without heat for at least 72 hours prior to surgery.

Pre-operative Care and Anesthesia

Ewes were fasted at least 24 hours prior to surgeries. On the morning of the surgery a polyvinyl catheter (1.12 x 1.65 mm) was placed into a jugular vein of the ewe. Sodium Pentobarbital (65 mg/ml) was administered as a pre-general anesthesia into the jugular vein. Once the animal was surgically anesthetized, an endotracheal tube was inserted into the trachea to allow for the passage of oxygen and halothane, the general anesthesia, and to prevent inspiration of saliva or rumen contents. Surgical anesthesia was maintained at 2.0-3.5% halothane and approximately 2.5 l/min. oxygen. Vital signs were monitored throughout the surgery.

Leg Surgery

Ewes were placed in dorsal recumbency and surgically prepared by clipping and scrubbing the inguinal region on the medial side of the leg with iodine solution. Alcohol was applied to the area and allowed to dry. Catheters were placed in the femoral artery and femoral veins as described by Dougherty (1981). An incision was made just medial to the M. sartorius. The M. sartorius and M. gracilis were separated by blunt dissection, allowing the exposure of the femoral vein and femoral artery. An incision was made into both the femoral artery and femoral vein for the passage of the tygon catheters fitted with silastic cuffs. Approximately 20 cm of the catheters were inserted so that their tips resided in the caudal aorta and caudal

vena cava. The silastic cuffs were ligated to the blood vessel using non-degradable 000 silk. The catheters were filled with deparin (1000 U/ml), exteriorized with a sterilized skin needle, and secured to the rump using branding cement.

Abdominal Surgery

Immediately following the completion of the leg surgery the ewe was placed in left lateral recumbancy. The area from the loin area to the linea alba and from the lumbar vertebrae to the thirteenth rib was clipped, scrubbed with iodine, and, finally, rinsed with alcohol. The portal and hepatic veins were catheterized as described by Katz and Bergman (1969) with the exception of the portal vein which was introduced into the porta-hepatis via a branch located on the caudal surface of the left lateral lobe of the liver. Confirmation of location was made through palpation of the porta-hepatis. The silastic cuffs of the catheters were anchored directly to hepatic parenchyma using 000 cardiovascular silk. Finally, a 1.02 x 1.78 mm tygon catheter was placed in a branch of the common mesenteric vein and anchored into position with 000 silk. Catheters were flushed with physiological saline and filled with heparin (1000 U/ml) and exteriorized through a sterilized skin needle. The catheters were secured to the lumbar region of the ewe's back with surgical tape and branding cement.

Post-operative Care of the Animals

Following the surgery, the ewe was placed on its sternum in a clean stall and closely supervised until it was able to stand without assistance. Combiotic (penicillin G and streptomycin) was administered

intramuscularly in decreasing daily doses (5, 3, 3, and 3 ml) for 4 days. All animals were usually consuming full rations within 24 hours. Animals were allowed 10 days for recovery before experiments. The catheters were flushed daily with a physiological saline solution (0.9% NaCl) and refilled with heparin. One day prior to each experiment heparin was replaced with NaEDTA (6%) to prevent activation of lipoprotein lipase.

Experimental Protocol

Normal, diabetic insulin-treated, and diabetic untreated sheep represented the three treatment groups. Feed was withdrawn for 3 days prior to experiments in order to induce a physiologically ketogenic state. Similarly, insulin injections were withheld for 3 days from the diabetic untreated ewes.

On the morning of the experiment, para-aminohippuric acid (1.5%), a nonmetabolizable dye, was infused at 0.764 ml/min. into the mesenteric vein and allowed to equilibrate with the body fluids for 1 hour. Three serial blood samples were taken at 30-minute intervals simultaneously from the femoral artery and the hepatic, portal, and femoral veins. These samples represented the control period. Immediately following the control period, beta-hydroxybutyrate was infused at rates simulating maximum utilization ($0.4 g/Kg^{0.75/hour}$) of ketone bodies (Bergman and Kon, 1969) into the jugular vein. Following a 1-hour equilibration period, three sets of beta-hydroxybutyrate infusion samples were taken as described for the control period. Finally, at the end of the last set of infusion samples, para-aminohippuric

acid was infused into the femoral artery (1.5% at 0.764 ml/min.) and allowed to equilibrate with the body fluids for 30 minutes. Three serial samples were obtained simultaneously from the femoral vein and a jugular vein and were used to calculate blood flow across the hindquarters.

At the end of each experiment all animals immediately received 400 g of alfalfa pellets. The diabetic untreated ewes also received a subcutaneous injection of insulin.

Chemical Analysis

All samples were analyzed for plasma free fatty acids, insulin, and glucagon and whole blood concentration of acetoacetate beta-hydroxybutyrate, para-aminohippuric acid, and glucose.

Para-aminohippuric acid was determined as described by Katz and Bergman (1969) (see Appendix 1).

Free fatty acid levels were determined using a NEFA-C kit (WAKO, Dallas, Texas) based on the following principles: 1) free fatty acids are converted to their CoA esters by acyl-CoA synthetase (ACS):

$$\text{R-COOH} + \text{ATP} + \text{CoA} \xrightarrow{\text{ACS}} \text{Acyl-CoA} = \text{AMP} + \text{ppi}$$

The acyl-CoA is oxidized by acyl-CoA oxidase (ACOD) to yield hydrogen peroxide:

$$\text{Acyl-CoA} + O_2 \xrightarrow{\text{(ACOD)}} \text{2.3-trans-enoyl CoA} + H_2O_2$$

The hydrogen peroxide is acted on by peroxidase (POD) in the presence of 3-methyl,N-ethyl,N-beta-hydroxyethyl-anilin (MEHA) and

4-aminoantipyrine (AAP) to form a product with purple color which can be read at 550 nm.

$$H_2O_2 + \text{MEHA} + \text{AAP} \xrightarrow{\text{POD}} \text{purple quinone} + H_2O$$

A description of free fatty acid analysis can be found in Appendix 2.

Whole blood acetoacetate and beta-hydroxybutyrate were determined using an enzymatic assay (Williamson and Mellanby, 1965) described fully in Appendixes 3 and 4.

Insulin and glucagon concentrations were determined by double antibody radioimmunoassay techniques developed by Aiello as acknowledged by McNeill et al. (1982) and Faloona and Unger (1972) for insulin and glucagon, respectively, and modified by Reynolds and Miller (personal communication). The antibodies used were guinea-pig antibovine insulin antibody and goat antiguinea-pig IgG for insulin assays, and 30K rabbit antiglucagon antibody and goat anti-rabbit IgG (2nd antibody) for the glucagon assays. The procedures for the assays can be found in Appendixes 5 and 6.

Whole blood glucose levels were measured using a Sigma 510 blood glucose kit as detailed in Appendix 7.

Whole blood flow rates were calculated using the Fick Principle:

$$\frac{I}{C_v - C_a} = F$$

where F is blood flow rate in ml/min. and C_v and C_a represent concentrations of para-amino hippuric acid (O.D./ml) in the vein and artery, respectively, and I is infusion rate of para-amino hippuric acid (O.D. units/min.).

Blood flow rates of hepatic and portal veins were measured directly and the hepatic arterial flow was derived indirectly by calculating the difference between the two. Plasma flows were determined by subtracting that portion of the flow represented by the packed cell volume.

Net fluxes of each metabolite were calculated across portal-drained viscera by using the following equation:

$$PDV = F_{pv} \ (C_{pv} - C_a)$$

where PDV = portal-drained viscera

F_{pv} = portal vein blood flow (l/min.)

C_{pv} = concentration of the metabolite in the portal vein

C_a = concentration of the metabolite in the femoral artery

Net fluxes of metabolites across the hepatic tissues were calculated as follows:

$$HEP = F_{pv} \ (C_{hv} - C_{pv}) + F_a \ (C_{hv} - C_a)$$

where HEP = hepatic net flux

F_{pv} = blood flow across the portal vein (l/min.)

F_a = arterial blood flow (l/min.)

C_{hv} = concentration of metabolite in the hepatic vein

C_{pv} = concentration of metabolite in the portal vein

C_a = concentration of metabolite in the femoral artery

Total splanchnic flux was calculated by using the following equation:

$$TSP = F_{hv} \ (C_{hv} - C_a)$$

where TSP = total splanchnic net flux (μM/min.)

F_{hv} = hepatic blood flow

C_{hv} = concentration of metabolite in the hepatic vein

C_a = concentration of metabolite in the femoral artery

Lower hindquarter flux was calculated as follows:

$$Rump = F_{jv} (D_{jv} - C_a)$$

where Rump = lower hindquarter

F_{jv} = blood flow across the jugular vein

F_a = concentration

C_{jv} = concentration of metabolite in the jugular vein

C_a = concentration of metabolite in the femoral artery.

Statistical Analysis

Data was analyzed by a two-way analysis of variance to determine the significance of differences due to beta-hydroxybutyrate infusion. Duncan's New Multiple Range Test was used to determine the significance of differences among the three treatment groups.

CHAPTER IV

RESULTS

Venoarterial differences of glucose, free fatty acids, acetoacetate, beta-hydroxybutyrate, insulin, and glucagon across portal-drained viscera, liver, and lower hindquarter were determined for normal, diabetic insulin-treated, and diabetic untreated 3-day fasted sheep. Values were used as an aid to determine whether net uptake or release of each metabolite by each tissue was significant from zero and can be found in Appendixes 8-13. They will be referred to within the text only when pertinent. Extraction ratios were calculated for each metabolite across each tissue. Extraction ratios represent that portion of a metabolite available to a tissue (concentration x blood flow) that is removed by the tissue (net uptake). Significant differences among extraction ratios indicate that changes in rates of net flux were not passive or solely due to changes in concentration and that some physiological mechanism must be involved. Extraction ratios for glucose, free fatty acid, AcAc, BOHB, insulin and glucagon can be found in Appendixes 14-19. Extraction ratios are included in tables within the text where pertinent to physiological explanation of the data. Glucose arterial concentrations and net fluxes were determined but were erratic and not instrumental in the interpretation of the other data. Therefore they can be found in Appendix 20.

Whole Blood Flow Rates

Whole blood flow rates for normal, diabetic insulin-treated, and diabetic untreated ewes can be found in Table 2. No differences were found between control or beta-hydroxybutyrate infusion period blood flows. Consequently, these data were grouped by treatment. Hepatic blood flow ranged between 2.0 and 2.8 l/min. Portal flows were between 1.5 and 2.2 l/min. and represented approximately 80% that of the hepatic values, which is in close agreement with results reported in fed and fasted sheep (Brockman and Bergman, 1975; Heitmann and Fernandez, 1986; Katz and Bergman, 1969). Blood flow across the caudal vena cava ranged between 1.0 and 1.5 l/min.

Free Fatty Acids

Plasma arterial concentrations and net flux of free fatty acids are shown in Table 3. Arterial concentrations were 912 μM and 1060 μM during the control period in the normal and diabetic insulin-treated ewes and increased two-fold in untreated diabetics. The elevated arterial concentrations, relative to normal fed animals (Heitmann et al., 1986), reflect significant omental and peripheral lipolysis in normal fasted sheep and peripheral release in diabetic insulin-treated (but not diabetic untreated) ewes and despite significant hepatic utilization in all groups. Hepatic utilization in the diabetic untreated group was 153 μmol/min. and was two- and three-fold lower than the levels of 275 and 406 μmol/min. observed in the diabetic insulin treated and normal sheep. Upon infusion of beta-hydroxybutyrate,

Table 2. Whole blood flow rates in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted ewes.[1]

	Portal vein (l/min.)	Hepatic vein (l/min.)	Caudal vena cava (l/min.)	Portal ÷ hepatic
NOR (5)	2.251	2.814	0.985	0.80
	±0.428	±0.536	±0.164	±0.01
DIT (5)	2.012	2.400	0.824	0.84
	±0.516	±0.595	±0.194	±0.02
DUT (5)	1.526	2.071	1.504	0.74
	±0.175	±0.224	±0.236	±0.05

Values are means ± SEM; (n) is the number of animals studied.

Table 3. Effects of beta-hydroxybutyrate (BOHB) on free fatty acid arterial concentrations and net fluxes in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted ewes.[1,2,3]

	Artery (μM)	Portal-drained viscera (μmol/min)	Liver (μmol/min)	Hepatic extraction ratio	Lower hindquarters (μmol/min)
NOR (5)					
Control	912 ± 122	247 ± 62	-406 ± 93	0.16 ± 0.02	92 ± 21
BOHB infusion	270 ± 49**	69 ± 43*	-83 ± 44**	0.10 ± 0.02*	21 ± 9**
DIT (5)					
Control	1060 ± 116	-5 ± 65†	-275 ± 56	0.16 ± 0.02	71 ± 23
BOHB infusion	455 ± 35**	-2 ± 28	-44 ± 41**	0.04 ± 0.03**	25 ± 14*
DUT (5)					
Control	1985 ± 148†	11 ± 13†	-153 ± 69†	0.05 ± 0.03†	13 ± 9†
BOHB infusion	1244 ± 147*	-25 ± 25	-145 ± 29	0.13 ± 0.03*	24 ± 18

[1]Values are means ± SEM; (n) is the number of animals studied.

[2]Statistically different by paired T test from Control: *P < .05; **P < .01.

[3]Statistically different by unpaired T test from Normal: †P < .01.

the arterial concentration of free fatty acids decreased by 605-742 μM in all groups. The decrease in arterial concentration was due to a decreased omental and peripheral lipolysis in normal and decreased peripheral release in diabetic insulin-treated sheep. No changes were observed in diabetic untreated sheep. In addition, hepatic utilization decreased by 80 and 85% in the normal and diabetic insulin-treated sheep. Hepatic extraction ratios of free fatty acids decreased in normal and diabetic insulin-treated ewes upon beta-hydroxybutyrate infusion, indicating the existence of a physiological control mechanism. The lowered hepatic extraction ratio observed in diabetic untreated sheep suggests that this mechanism may be functioning prior to beta-hydroxybutyrate infusion.

Acetoacetate

Arterial concentration and net fluxes of acetoacetate are given in Table 4. Acetoacetate arterial concentration was 98 μM for the normal sheep but increased two-fold in diabetic insulin-treated sheep and ten-fold in diabetic untreated sheep. The arterial concentration of acetoacetate increased significantly upon infusion of beta-hydroxybutyrate in all three treatment groups. However, this increase was an artifact of the infusion since beta-hydroxybutyrate can be converted to acetoacetate (Watson and Lindsay, 1972). For the most part, net flux rates of acetoacetate across the portal-drained viscera were not significant from zero (Appendix 10). Since alimentary ketogenesis is dependent upon exogenous substrates and since these animals had been fasted for 3 days, the observed rates were as expected.

Table 4. Effects of beta-hydroxybutyrate (BOHB) on acetoacetate arterial concentrations and net fluxes in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted ewes.[1,2,3]

	Artery (μM)	Portal-drained viscera (μMol/min.)	Liver (μMol/min.)	Lower hindquarters (μMol/min.)
NOR (5)				
Control	98 ± 9	-2.2 ± 10.9	90.0 ± 23.0	-15.6 ± 2.1
BOHB infusion	153 ± 17*	61.6 ± 14.6**	-117.8 ± 25.5**	-17.6 ± 4.7
DIT (5)				
Control	176 ± 25†	24.1 ± 39.8	99.9 ± 62.8	-23.9 ± 6.4
BOHB infusion	392 ± 71**	7.5 ± 8.8	-49.9 ± 21.1*	-50.6 ± 20.4
DUT (5)				
Control	1244 ± 190††	-27.9 ± 51.3	562.5 ± 112.0	-113.3 ± 31.1††
BOHB infusion	1658 ± 155*	-159.2 ± 138.1	-20.2 ± 205.0**	-655.0 ± 235.1**

[1]Values are means ± SEM; (n) is the number of animals studied.

[2]Statistically different by paired T test from Control: *$P < .05$; **$P < .01$.

[3]Statistically different by unpaired T test from Normal: †$P < .05$; ††$P < .01$.

Hepatic release of acetoacetate, during the control period, was similar in the normal and diabetic insulin-treated groups (90-100 μmol/min.), but was five-fold higher in diabetic untreated sheep. Of particular interest relative to autoregulation was the switch from a state of hepatic production of acetoacetate to utilization when beta-hydroxybutyrate was infused. Utilization of acetoacetate by the hindquarters increased during untreated diabetes. However, this was due to increased acetoacetate concentrations since extraction ratios by the rump tissues were consistently in the range of 0.15-0.20 for all three treatments groups (Appendix 16).

Beta-hydroxybutyrate

Arterial concentrations and net fluxes of beta-hydroxybutyrate are given in Table 5. Arterial concentrations were 1303 and 2017 μM during the control period in normal and diabetic insulin-treated ewes, respectively. A ten-fold increase to 13,302 μM was noted in diabetic untreated sheep. The elevated arterial concentrations, relative to normal fed animals (Heitmann et al., 1986), might be explained by elevated ketone body production rates by the livers of all three groups, particularly that of diabetic untreated sheep which was two-fold that of normal and diabetic insulin-treated ewes. The infusion of beta-hydroxybutyrate elicited an increase in arterial concentration of beta-hydroxybutyrate in all three groups which was an artifact of the infusion. Of particular importance, as with acetoacetate, was the supression of hepatic beta-hydroxybutyrate production to nearly 25-35% of the original preinfusion rates.

Table 5. Effects of beta-hydroxybutyrate (BOHB) on beta-hydroxybutyrate arterial concentrations and net fluxes in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted ewes.[1,2,3]

	Artery (μM)	Portal-drained viscera (μMol/min.)	Liver (μMol/min.)	Lower hindquarters (μMol/min.)
NOR (5)				
Control	1303 ± 58	-122 ± 61	1304 ± 125	-174 ± 24
BOHB infusion	2873 ± 221**	-220 ± 85	461 ± 91**	-385 ± 52*
DIT (5)				
Control	2017 ± 258†	-386 ± 205	1032 ± 337	-196 ± 80
BOHB infusion	4554 ± 528**	423 ± 191*	235 ± 201**	-266 ± 141
DUT (5)				
Control	13302 ± 788	-29 ± 231	2904 ± 458†	-604 ± 336†
BOHB infusion	22066 ± 1501**	297 ± 567	798 ± 563**	-1189 ± 334*

[1]Values are means ± SEM; (n) is the number of animals studied.

[2]Statistically different by paired T test from Control: *$P < .05$, **$P < .01$.

[3]Statistically different by unpaired T test from Normal: †$P < .05$.

Similarly to the results observed for acetoacetate, flux rates across the portal-drained viscera for beta-hydroxybutyrate were, for the most part, not significant from zero (Appendix 11). Utilization of beta-hydroxybutyrate by the lower hindquarters in the normal group was 174 μMol/min. and approximately 13% of the available substrate (concentration x blood flow). Utilization rates increased with beta-hydroxybutyrate infusion and untreated diabetes in a concentration dependent manner.

Insulin

Insulin arterial concentration and net fluxes can be found in Table 6. Insulin arterial concentration was 18 μU/ml during the control period for the normal group and pancreatic production was 8.4 μU/min. Since the beta-cells of the pancreas were nonfunctional in the diabetic insulin-treated group, the arterial concentration of 42 μU/ml is due solely to an exogenous subcutaneous injection of insulin. Finally, the arterial concentration of insulin in the diabetic untreated group was not significantly different from zero due to the absence of a functioning pancreas and the exogenous source. The portal-drained viscera rates observed were reflective of the pancreatic production of insulin. A net production of 8.4 μU/min. during the control period was observed for the normal grouop while the diabetic insulin-treated and diabetic untreated groups demonstrated no significant rates of flux, indicating that beta-cell function had ceased. During beta-hydroxybutyrate infusion a two-fold increase in arterial concentration was observed along with, and possibly due

Table 6. Effect of beta-hydroxybutyrate (BOHB) on insulin arterial concentrations and net fluxes in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted ewes.[1,2,3]

	Artery (μU/ml)	Portal-drained viscera (μU/min.)	Liver (μU/min.)	Hepatic extraction ratio
NOR (5)				
Control	18 ± 3	8.4 ± 1.3	-3.0 ± 1.0	0.06 ± 0.01
BOHB infusion	32 ± 5*	17.0 ± 3.0*	-10.0 ± 2.6*	0.12 ± 0.03*
DIT (5)				
Control	42 ± 7†	-2.5 ± 2.6†	3.3 ± 5.2	-0.08 ± 0.09
BOHB infusion	45 ± 10	-8.9 ± 5.2	4.0 ± 7.1	-0.20 ± 0.10
DUT (5)				
Control	3 ± 0†	0.6 ± 0.9†	0.5 ± 0.3	-0.19 ± 0.10
BOHB infusion	3 ± 0	1.5 ± 0.8	0.4 ± 0.7	-0.04 ± 0.09

[1]Values are means ± SEM; (n) is the number of animals studied.

[2]Statistically different by paired T test from Control: *$P < .05$.

[3]Statistically different by unpaired T test from Normal: †$P < .01$.

to, a concomitant two-fold increase in pancreatic production and despite an increase in hepatic utilization. Hindquarter net fluxes of insulin were small (less than 1.0 μU/min.) and not significantly different from zero (Appendix 12). Consequently, these data were omitted for clarity. However, the hindquarter venoarterial data can be found in Appendix 11. Hepatic insulin extraction ratios were 0.06 and 0.12 in normal control and beta-hydroxybutyrate infusion periods and similar to values reported by Brockman and Bergman (1975).

Glucagon

Glucagon arterial concentration and net fluxes are shown in Table 7. Arterial glucagon was higher than that of fed sheep (Heitmann et al., 1986) for all three groups, but represented good standard values for 3-day fasted animals. Glucagon concentration increased nearly three-fold in diabetic untreated sheep. A two-fold increase in pancreatic production from 5.2 to 11.8 and 9.8 μg/hour was observed between normal and diabetic insulin-treated and diabetic untreated ewes. No changes were observed in hepatic uptake of glucagon. Insulin/glucagon ratios increased in diabetic insulin-treated sheep, but decreased in diabetic untreated sheep, and these changes were mainly due to changes in insulin concentration. The infusion of beta-hydroxybutyrate had no significant effects on the arterial concentrations, net fluxes, or extraction ratios of glucagon. Hindquarter net fluxes of glucagon were low (less than 1.0 μg/h) and not significantly different from zero. Consequently, these data were omitted for clarity. However, the hindquarter venoarterial data can be found in Appendix 13.

Table 7. Effects of beta-hydroxybutyrate (BOHB) on glucagon arterial concentrations and net fluxes in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted ewes.[1,2,3]

	Artery (pg/ml)	Portal-drained viscera (μg/h)	Liver (μg/h)	Insulin ÷ glucagon
NOR (5)				
Control	239 ± 57	5.2 ± 2.0	-2.6 ± 1.2	3.13 ± 0.80
BOHB infusion	249 ± 41	4.3 ± 0.7	-1.9 ± 0.4	3.84 ± 0.89
DIT (5)				
Control	244 ± 51	11.9 ± 3.1	-3.9 ± 1.8	5.87 ± 1.44*
BOHB infusion	196 ± 32	10.2 ± 2.7	-1.9 ± 1.8	7.55 ± 1.99
DUT (5)				
Control	673 ± 106**	9.8 ± 1.7*	-1.2 ± 1.6	0.29 ± 0.08*
BOHB infusion	584 ± 95	6.2 ± 1.1	0.0 ± 1.6	0.25 ± 0.05

[1]Values are means ± SEM; (n) is the number of animals studied.

[2]Statistically different by unpaired T test from Normal: *P < .05, **P < .01.

[3]Insulin converted from μU/m to pg/m by multiplying by 23.3.

CHAPTER V

DISCUSSION

The regulation of ketogenesis has been investigated extensively, mainly in nonruminants. McGarry and Foster (1977) have proposed a bihormonal theory which describes the role of both insulin and glucagon in regulating hepatic ketogenesis intra-hepaticly and extra-hepaticly. In addition, the work of Madison et al. (1964) and Bjorntorp (1966) has shown that ketone bodies are capable of inhibiting their own production via inhibition of free fatty acid release from the adipocyte. Bjorntorp and Shersten (1967) demonstrated the same results in a pancreactomized dog and thus independent of insulin. The present study was conducted to measure the effects of beta-hydroxybutyrate infusion on physioketogenic ewes both in the presence and in the absence of insulin. To establish the conditions required to achieve the research goals, three groups of animals were used: normal, diabetic insulin-treated, and diabetic untreated ewes. All three groups were made ketogenic by 3 days of fasting. Net fluxes of free fatty acids, acetoacetate, beta-hydroxybutyrate, insulin, and glucagon were measured across portal-drained viscera, lower hindquarters, and, most importantly, the liver during control and beta-hydroxybutyrate infusion periods. Relationships were studied between fluxes among the three treatment groups.

Free Fatty Acids

Plasma free fatty acid concentration in normal animals was approximately 912 μM and similar to values reported by Lindsay and Leat (1975). Slightly higher values of 1500 μM/l were reported by Heitmann and Metzler (1983) for 5-day fasted sheep. Elevated plasma free fatty acids may be attributed to significant omental lipolysis (portal-drained visceral release of 247 μmol/min.) and peripheral lipolysis (lower hindquarter release of 92 μmol/min.) Hepatic uptake of free fatty acids was eleven- to twelve-fold higher than normal fed sheep (Heitmann et al., 1986). These values are in close agreement with previous reports (Katz and Bergman, 1969; Thompson and Darling, 1975). Infusion of beta-hydroxybutyrate resulted in a nearly 70% decrease in plasma concentration of free fatty acids concomitant with a 72% and 77% decrease in portal-drained visceral and lower hindquarter release and despite decreased hepatic utilization from 406 μmol/min. to 83 μmol/min. in normal, intact, fasted sheep.

Pre-infusion plasma concentration of free fatty acids in the diabetic insulin-treated animals was 1060 μM/l. Significant peripheral lipolysis may, in part, explain the elevated free fatty acid concentration. However, no significant omental lipolysis occurred. As was observed in the normal 3-day fasted animals, hepatic utilization was elevated above normal fed values (Heitmann et al., 1986). Again, plasma free fatty acid concentration decreased upon infusion of beta-hydroxybutyrate as did lower hindquarter release and hepatic utilization. However, no effect was observed in portal-drained visceral

flux of free fatty acids. These results indicate that a functioning beta cell is not required for the observed change in free fatty acid fluxes.

Perhaps of most importance was a decrease in the hepatic extraction ratio upon infusion of beta-hydroxybutyrate in the normal and diabetic insulin-treated animals. Many investigators have shown that hepatic uptake of free fatty acids is concentration-dependent and that the transport kinetics cannot be saturated even at very high concentrations of free fatty acids. Therefore, any change in the extraction ratio would indicate the existence of a physiological mechanism in the control of free fatty acid uptake by the liver.

Diabetic untreated pre-infusion plasma free fatty acid concentration was approximately 1985 μM/l and double that observed in normal and diabetic insulin-treated animals. A possible explanation could be that diabetes was completely out of control. Infusion of beta-hydroxybutyrate again decreased plasma free fatty acids from 1985 to 1244 μM/l.

Of particular interest was the significantly lower free fatty acid uptake by the liver when compared with the rates of normal and diabetic insultin-treated animals. In addition, portal-drained visceral and lower hindquarter release of free fatty acids were no longer significant. These data, plus the observed decrease in hepatic extraction ratio during the control period, suggest that some autoregulation of ketogenesis may be already occurring.

Ketone Bodies

Since free fatty acids are the major precursors of hepatic ketogenesis (McGarry and Foster, 1980; Robinson and Williams, 1980), it would follow that changes in hepatic free fatty acid fluxes due to infusion of beta-hydroxybutyrate should, in turn, cause a change in hepatic production of ketone bodies.

Pre-infusion arterial concentrations of both acetoacetate and beta-hydroxybutyrate in normal 3-day fasted sheep were 98 and 912 μM and about two-fold higher than values reported in fed sheep (Lindsay and Leat, 1975; Heitmann et al., 1986). In normal fed animals, alimentary ketogenesis contributes 60% of the total net release of acetoacetate and beta-hydroxybutyrate by total splanchnic tissues (Baird, 1977; Bergman, 1970). However, in this study, the animals were fasted for 3 days and, therefore, instead of alimentary release of acetoacetate and beta-hydroxybutyrate, a net utilization of 2.2 μmol/min. and 122.2 μmol/min., respectively, was observed. This may be due to rumen epithium utilization of ketone bodies as a source of energy.

Net hepatic production of both acetoacetate and beta-hydroxybutyrate (90 and 1304 μmol/min.) was higher than in fed sheep (-43 and 188 μmol/min.) as reviewed by Heitmann et al. (1986), probably due to higher uptake of free fatty acids by the liver. Hindquarter utilization of ketone bodies was 15.6 and 175 μmol/min. for acetoacetate and beta-hydroxybutyrate, respectively, and represented approximately 14% of available circulating substrates. Bergman and Kon (1964) demonstrated similar values of 10-20% and suggested

that utilization of ketone bodies is concentration-dependent up to a maximum 10 mg/100 ml.

Infusion of beta-hydroxybutyrate in normal 3-day fasted sheep resulted in a two-fold increase of both acetoacetate and beta-hydroxybutyrate arterial concentrations. In both cases the increase was an artifact of the infusion. The increase in acetoacetate can be explained by an interconversion of the infused beta-hydroxybutyrate to acetoacetate by 3-beta-hydroxybutyrate dehydrogenase.

An increase in beta-hydroxybutyrate uptake in normal 3-day fasted sheep by portal-drained viscera from 122 to 220 μmol/min. was accompanied by an increased release of acetoacetate (-2.0 to 62 μmol/min.). Since Watson and Lindsay (1972) reported appreciable activity of 3-beta-hydroxybutyrate dehydrogenase in the rumen epithelium, it is likely that the increased uptake of beta-hydroxybutyrate by portal-drained viscera resulted in its conversion to acetoacetate, thereby increasing the quantity of acetoacetate released.

Beta-hydroxybutyrate infusion in normal 3-day fasted sheep decreased hepatic production of beta-hydroxybutyrate 64% concomitant with an 80% decrease in hepatic uptake of free fatty acids. In addition, hepatic production of acetoacetate (90 μmol/min.) switched to a net uptake of 117.8 μmol/min. It is unlikely that the liver actually utilized acetoacetate since hepatic tissues lack the rate limiting enzyme of acetoacetate utilization, 3-keto oxoacid CoA transferase. A more likely explanation for the hepatic uptake of acetoacetate is its conversion to beta-hydroxybutyrate. Lower hindquarter

uptake of both ketone bodies was still approximately 14% of the available circulating metabolite during beta-hydroxybutyrate infusion.

Pre-infusion arterial concentrations of both acetoacetate and beta-hydroxybutyrate in diabetic insulin-treated sheep were two-fold higher than those of the normal animals. However, no changes were observed in net fluxes of acetoacetate or beta-hydroxybutyrate across the portal-drained viscera, liver, or lower hindquarters. As observed in the normal animals, hepatic acetoacetate flux switched from net production to utilization and hepatic release decreased 77% during beta-hydroxybutyrate infusion concomitant with an 80% decrease in hepatic fatty acid uptake. Therefore, a functional beta cell is not required for ketogenic regulation. However, insulin may still be involved in ketone autoregulation since beta-hydroxybutyrate, in concentrations similar to those observed in diabetic keto-acidosis restored insulin receptor binding in human lymphocytes (Misbin et al., 1978).

Arterial concentrations of acetoacetate and beta-hydroxybutyrate in diabetic untreated animals were greater than ten-fold higher than in normal sheep and fully indicative of uncontrolled diabetic ketogenesis. The high arterial concentrations were probably due to six- and two-fold increases in the rates of hepatic production of acetoacetate and beta-hydroxybutyrate (562.5 and 2904 μmol/min., respectively). Infusion of beta-hydroxybutyrate resulted in the same changes in ketone body flux rates observed for the normal and diabetic insulin-treated sheep. Lower hindquarter uptake increased

in a concentration-dependent fashion, but, most importantly, hepatic release of acetoacetate switched to uptake and hepatic production of beta-hydroxybutyrate decreased 73%. Since these ewes were untreated functional diabetics, it is obvious that the autoregulation of ketogenesis can occur in the absence of insulin.

Insulin

Insulin's effects of ketone body production have been investigated by several researchers. Annison et al. (1960) demonstrated the antilipolytic properties of insulin. In addition, Alberti et al. (1968) reported that insulin enhances hepatic fatty acid re-esterification over oxidation. Since free fatty acids are the major precursors of hepatic ketogenesis, and it is clear that insulin inhibits free fatty acid release from the adipocyte, it follows that elevated concentrations of insulin would result in the suppression of ketogenesis. Madison et al. (1964) have demonstrated the ability of beta-hydroxybutyrate to stimulate pancreatic production of insulin in rats. Since the normal intact sheep in this current study also demonstrated increased insulin concentrations and pancreatic production rates during beta-hydroxybutyrate infusion, an insulin-dependent negative feedback mechanism of ketogenesis functions similarly to the circulating glucose control mechanism described by Williamson and Whitelaw (1978). However, since ketogenesis is also slowed in diabetic untreated sheep, an insulin-dependent mechanism appears to function as well. The relative physiological importance of these two mechanisms was not measured in this study.

Pre-infusion arterial insulin concentration in normal 3-day fasted sheep was 18 μU/ml and well within the normal range of 5-40 μU/ml reported by Basset et al. (1971). Pancreatic production as represented by portal-drained visceral release, was 8.4 μU/min. Net hepatic uptake of insulin was 3 μU/min. and 36% that of the pancreatic production. Total hepatic insulin extraction was 6%. These values are similar to those reported by Brockman and Bergman (1975). Upon infusion of beta-hydroxybutyrate, insulin arterial concentration increased two-fold concomitant with a two-fold increase in pancreatic production and despite a three-fold increase in hepatic utilization and an increased hepatic extraction ratio.

Pre-infusion arterial insulin concentration in diabetic insulin-treated sheep was 42 μU/ml. This was due solely to exogenous insulin source since portal-drained visceral flux of insulin was not different from zero, indicative of a nonfunctional beta cell. Hepatic fluxes of insulin were not different from zero. Obviously, infusion of beta-hydroxybutyrate did not increase arterial concentration or portal-drained visceral release of insulin, thus confirming the nonfunctional state of the pancreas. Insulin arterial concentration of diabetic untreated animals was not significantly different from zero since there was no endogenous nor exogenous source of insulin. Infusion of beta-hydroxybutyrate obviously had no effect on arterial concentration or portal-drained visceral flux of insulin.

Glucagon

McGarry and Foster (1977) suggested that glucagon also has regulatory influences on ketogenesis. Glucagon exerts a catabolic effect on lipid metabolism at the adipocyte, and thereby increases circulating free fatty acids. In addition to this extrahepatic control, evidence has been presented to confirm intrahepatic regulation of ketogenesis by glucagon (McGarry and Foster, 1980). This regulation may occur through the effects that glucagon has on both malonyl CoA and acetyl CoA carboxylase. McGarry and Foster (1980) reported that glucagon inhibits the production of malonyl CoA and Swenson and Porter (1985) suggested that this inhibition was accomplished through the inactivation of acetyl CoA carboxylase, the enzyme which converts acetyl CoA to malonyl CoA. The inhibition of this reaction results in a reversal of the inhibition of carnitine acyltransferase I by malonyl CoA, thereby allowing fatty acyl-CoA to be transported into the mitochondrial membrane where it undergoes subsequent beta-oxidation and ketone body production.

Brockman (1976) suggested that the action of glucagon may be muted by insulin. Similarly, Witters (1981) and Brownsey and Denton (1982) have reported that insulin activates hepatic acetyl CoA carboxylase in the presence of glucagon. Consequently, insulin/glucagon ratios may have more physiological significance than the actual hormonal concentration.

Glucagon arterial concentrations in the normal 3-day fasted ewes was 239 pg/ml. This value was higher than that observed in

fed sheep (Heitmann et al., 1986) but was similar to that reported by Sartin et al. (1985) in lactating dairy cows. Pancreatic production of glucagon, as represented by portal-drained visceral release, was 5.2 pg/hour and hepatic uptake was 2.6 pg/hour. These values are similar to those reported by Brockman et al. (1976) and Basset (1972). The insulin/glucagon ratio was 3.1 which was 65% lower than in fed sheep (Heitmann et al., 1986), but similar to that of normal lactating cows (Sartin et al., 1985).

Arterial concentrations and net fluxes of glucagon in diabetic insuliin-treated sheep were not significantly different from the normal fed ewes. However, insulin/glucagon ratio was two-fold higher than observed in normal animals due to a two-fold increase in arterial insulin concentration through an exogenous source.

Pre-infusion arterial concentration of glucagon in the diabetic untreated animals was almost three-fold higher than in normal sheep due to a significant two-fold increase in portal-drained visceral release. The insulin/glucagon ratio was significantly lower than observed for either normal or diabetic insulin-treated animals, as would be expected since the pancreatic production and exogenous source of insulin had been removed. The infusion of beta-hydroxybutyrate had no effects on glucagon concentration, net fluxes, or insulin/glucagon ratios in any of the treatments. It would appear that glucagon would be capable of exerting its extrahepatic ketogenic effects by increasing omental and peripheral lipolysis and increasing hepatic uptake of free fatty acids. However, this was not the

case in diabetic untreated sheep. Portal-drained visceral and hind-quarter release of free fatty acids were lower than normal or diabetic insulin-treated animals and the insulin/glucagon ratios were higher. In addition, the infusion of beta-hydroxybutyrate, again, had no influence on the flux of glucagon across portal-drained viscera or liver. Since beta-hydroxybutyrate infusion elicited a decrease in free fatty acid release by portal-drained viscera and uptake by the liver in addition to decreased hepatic ketogenesis in all three treatment groups while exerting no effect on glucagon, it may be concluded that glucagon's role in the regulation of ketogenesis is passive and only secondary to that of insulin.

Conclusion

It appears from the data compiled from this study that insulin does play an important part in ketogenic autoregulation since infusion of beta-hydroxybutyrate did result in a two-fold increase in arterial concentrations and pancreatic production of insulin in normal animals and was accompanied by decreased omental and peripheral lipolysis and decreased hepatic production of ketone bodies. However, the same results were observed in diabetic insulin-treated animals, indicating that an increase in pancreatic production of insulin is not necessary to elicit the autoregulatory mechanism. Moreover, since beta-hydroxybutyrate infusion continued to exert autoregulatory properties in the complete absence of insulin, it may be concluded that an insulin-independent autoregulatory mechanism also exists. The exact mechanism remains undefined. However, several possibilities based on previous reports may be suggested. McGarry et al. (1978)

have demonstrated the role of carnitine acyltransferase I for the transport of long chain fatty acids into the mitochondria. Horano et al. (1985) reported that glucagon was responsible for the activation of carnitine acyltransferase I by phosphorylating it. It may be suggested from this evidence that beta-hydroxybutyrate directly exerts an inhibitory effect on pancreatic glucagon release, thereby indirectly controlling the activity of carnitine acyltransferase I. However, both this study and the work of Sensenig et al. (1986) reported no change in glucagon flux due to infusion of beta-hydroxybutyrate. An alternative suggestion might be that beta-hydroxybutyrate itself may allostearically inhibit the activity of carnitine acyltransferase I. Since McGarry et al. (1977, 1978, 1980) have suggested that malonyl CoA inhibits carnitine acyltransferase I activity and that acetyl CoA carboxlase activity is essential for the formation of malonyl CoA, it is possible that ketone bodies may regulate their own production through allostearically stimulating acetyl CoA carboxylase.

This study established the existence of an insulin-independent intra-hepatic autoregulatory mechanism of ketogenesis. Further investigation is required to elucidate the exact mechanism or mechanisms.

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APPENDIXES

APPENDIX 1

PAH - ANALYSIS

I. Reagents for Filtrate

A. 20% Trichloracetic Acid (TCA)

B. Distilled H_2O

II. Method for Filtrate

A. Pipette 1 ml of blood or standard into 15 ml conical centrifuge containing 5 ml distilled H_2O. Add drop by drop, swirling the flask.

B. Pipette 5 ml blood mixture into second 15 ml conical centrifuge tube containing 20% TCA - (5 ml). Add drop by drop, mixing well.

C. Let stand 60 minutes or overnight (can be left at this stage).

D. Filter through Whatman #42 filter paper into large test tube.

E. Store in refrigerator until ready for analysis.

F. Standards from 1.5% PAH infusion solution made along with this using same method as for blood filtrate.

1. Make up a 1:500 dilution for sheep from 1.5% infusion solution; 1:100 for dog from 0.25% infusion solution.

2. From this dilution make up standard dilution 20, 40, 60, 80, 100% (stock dilution). (Be sure to run this through the blood filtrate procedure.)

3. Blank mixed with 1/2 distilled H_2O and 1/2 TCA (20%).

III. Reagents for Analysis

A. 1.2 N Hcl (1:10 dil. of 12.4 N Hcl close enough).

B. $NaNO_2$ (100 mg/100 ml H_2O) freshly made up day of analysis.

C. Ammonium sulfamate (500 mg/100 ml H_2O) made up within a month.

D. Coupling reagent (N-1 Naphthylethylenediamine Dihydrochloride) - 100 mg/100 ml H_2O (stored in brown bottle in refrigerator indefinitely).

IV. Analysis

A. Pipette 1 ml of filtrate into test tubes (in duplicate).

Pipette 1 ml of standards into test tubes (single).

Pipette 1 ml of blank into test tubes (duplicate).

B. Add boiling chip to each tube and place marble on top.

C. Boil for 30 min. after fine bubbles appear on each tube. Flame should be low. Boil slowly for the 30 min.

D. Cool at room temperature. Remove marbles when cool.

E. Add .02 ml of 1.2 N NcL to all tubes with blowout pipette. Mix well.

Start time clock and add:

a) .1 ml $NaNO_2$ with blowout pipette. Mix well.

b) .1 ml ammonium sulfamate - within 3-5 min. Mix well.

c) .1 ml coupling reagent - within 3-5 min. Mix Well.

Wait 10 min. for color development.

Read on Evelyn Colorimeter at 540 mu.

V. PAH Stock - 10% Solution for infusion.

A. 44.9 grams PAH acid powder (Eastman Chemical Corp., Pequannock, N.J.). (Yields 1.5% solution of sodium salt.)

10.5 grams NaOH

500 ml PSS Commercial - (sterile)

Dissolve each reagent in PSS separately. Then add NaOH solution to PAH and stir. To finish dissolving, set in 40°C water bath and stir until completely dissolved. Filter through Buchner funnel with #42 Whatman filter paper. Titrate filtrate to pH 7.4 with 1N Hcl or 4 N NaOH. Bring final volume b 500 ml.

B. For 1.5% infusion solution take 75 cc of stock and bring to 500 ml. volume with PSS.

APPENDIX 2

ENZYMATIC ANALYSIS OF FREE FATTY AICDS

(NEFA-C Kit from WAKO)

Procedure:

A. Preparation of Standards

Dilute the standard provided (1000 μeq/1) with distilled H_2O to provide 125, 250, and 500 μeq/1 standards. Validation tests show distilled H_2O is an acceptable diluent.

B. Preparation of Reagents

1. Mix color reagent A by adding 10 ml of the specific diluent to each vial. Gently invert the vial until contents are completely dissolved and then combine with 13.3 ml dH_2O. Mix again and record preparation date.*

   *Solutions should be stored between 2°C and 8°C and are stable for 5 days. Do not freeze solutions or expose them to direct sunlight. Color reagents may be combined only if they have the same lot number.

2. Mix color reagent B by adding 20 ml of specific "B" diluent per vial. Invert until contents are dissolved, then combine with 33.3 ml dH_2O. Record preparation date and store as for "A" solution above.

C. Assay

1. Assay each sample and standard in duplicate.

2. Add 25 µl of serum, plasma or standard to each tube, followed by 350 µl of the combined Reagent A + H_2O mix (these steps can be performed simultaneously using the Brinkman). Vortex and (if necessary) refrigerate for up to 60 minutes.

3. Incubate tubes in a waterbath at 37°C for exactly 20 minutes.

4. Following incubation add 0.8 ml of the Reagent B + H_2O mix to each tube. Vortex and incubate a second time at 37°C for exactly 20 min.

5. Remove tubes from incubator, allow to equilibrate at room temperature for 5 minutes, then record OD at 550 nm using a blank prepared with reagents as zero.

6. Process results through TI-59 NEFA program to generate concentrations and CoV between duplicates.

Tube I.D.	Standard	Plasma	Reagent $A+H_2O$	Reagent $B+H_2O$
Blank	--	--	0.35	0.80
Std - 125 µeq/l	25	--		
- 250 "		--		
- 500 "		--		
-1000 "		--		
-2000 "	50*	--		
Sample	--	25		

*50 µl of the 1000 µeq/l standard provided in the kit. This standard is not required for routine work in our laboratory.

APPENDIX 3

ACETOACETATE ASSAY

Must be done on the same day as sampling.

Reagents

1. Phosphate buffer (pH 6.8, 0.1 M)
   a. 1.36 g KH_2PO_4 → 100 ml
   b. 1.74 g K_2HPO_4 → 100 ml
   Mix equal volumes of a) and b)
   Check pH, add a) or b) if necessary.

2. NADH (approx. 1 mM)
   Boehringer. Grade II, disodium salt, 97% pure. Cat. no. 128023.
   0.005 g → 6 ml.
   Make up on day of use. Bergmeyer recommends 6 mM NADH but as we use the fluorimeter which is very sensitive, this is off the scale. If you are using a spectrophotometer, you can calculate the minimum amount of NADH you need from the molor extinction coefficient ($6.22 \times 10^3\ M^{-1}cm^{-1}$), or you can "back it off."

3. 3-hydroxybutyrate dehydrogenase. Boehringer. 5 mg/ml
   Cat. no. 127841.

4. Acetoacetate standards
   Sigma. Lithium salt. 90-95% pure.
   Stock solution 1.5 mM 0.01751g → 100 ml.
   Make up on day of use.
   Dilute to the following concentrations for the working standards: 0.15, 0.10, 0.075, 0.05, 0.025 mM. I usually do two zero conc. standards as blanks.

   The standards must be taken through the entire procedure as small amounts of perchlorate do affect enzyme activity. Pipette into a graduated conical centrifuge tube:
   1 ml standard and 1 ml 1 M $HClO_4$.
   Neutralize to pH 6-8
   Make volume up to 2.5 ml with dH_2O.
   Centrifuge 1,000 g for 10-15 min.

Assay Procedure

Pipette into cuvettes:

Buffer	0.5 ml
Sample	1.0 ml for fed sheep or 1.0 ml standard (0.5 ml + 0.5 ml dH_2O for fasted sheep).
NADH	0.05 ml

Read E_1 at (340 nm)

Add 0.005 ml 3-HB dehydrogenase
Incubate at room temperature for approximately 20 min.
Read E_2 (340 nm)

APPENDIX 4

3-HYDROXYBUTYRATE ASSAY

Samples may be stored at -20°C for at least 1 week.

Reagents

1. tris-HCl buffer (pH 8.5, 0.1 M)
   2.42 g tris → approx. 50 ml.
   pH → 8.5 with 1 M HCl
   volume → 200 ml.

2. Hydrazine-tris buffer
   Make up on day of use.
   2.5 ml hydrazine hydrate
   0.05 g EDTA
   12.5 ml 1 M HCl
   volume → 50 ml <u>with tris-HCl buffer</u>
   check pH = 8.5

3. NAD^+ (approx. 14 mM)
   Boehringer. Grade II. Free acid. 98% pure. Cat. no. 127990
   0.03 g → 3 ml.

4. 3-HB standards
   Calbiochem. Sodium salt. Enzymic purity approx. 96%.
   Stock 2 mM 0.0656 g → 250 ml.
   Working standards: 2.0, 1.6, 1.2, 0.8, 0.4, and 0 mM. The standards must go through the whole procedure and are treated as described in the acetoacetate assay.

Assay Procedure

Pipette into cuvettes:

Sample or standard	0.25 ml - fed	0.1 ml sample - fasted
H_2O	0.75 ml - fed	0.9 ml H_2O - fasted
Buffer	0.5 ml	
NAD^+	0.05 ml	

Read E_1 (340 nm)
Add 0.005 ml 3-HB dehydrogenase
Incubate at room temperature for about 45 min.
Read E_2.

Important

NAD and hydrazine form a complex which absorbs at 340 nm. Therefore, a slow constant increase in absorbance occurs.

It is like the acetoacetate assay but in reverse. So pipette the NAD and enzyme and take the absorbance readings at timed intervals as described for the acetoacetate assay.

Calculations

As for acetoacetate, except ΔE is calculated from $E_2 - E_1$.

APPENDIX 5

RADIOIMMUNOASSAY OF INSULIN

Reagent Preparation:

1. Phosphate Buffered Saline (PBS-EDTA)

   Dissolve in 800 ml DD H_2O;
   1.38 grams $HaH_2PO_4 \cdot H_2O$
   8.77 grams NaCl
   9.305 grams EDTA$\cdot 2H_2O$ Disodium Salt
   Adjust pH to 7.6 with 5 M NaOH
   Add 100 mg Thimersol and bring to 1000 ml in volumetric flask
   Store at 2° - 4°C

2. I% BSA-PBS

   Before each assay (usually 12-24 hours prior to) add 1% BSA to cold PBS in a beaker and dissolve using stirring magnet. After BSA is dissolved, readjust pH to 7.6 using 5 M NaOH.

   If I am running 3 assays of 123 tubes I will generally mix 2.5 g BSA with 250 ml PBS. This is usually enough BSA for setting up the assays the first day and diluting labelled insulin. Sometimes I have to mix 100 ml on day 3 to dilute the second Ab.

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

   - add 250 µl Guinea Pig Serum to 100 ml 1% BSA-PBS
   This is enough to mix the first Ab and add to culture tubes as a diluent and carrier for 3 assays.

4. 1st Ab

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

   For Miles Ab we used 1 ml cold DD H_2O and then added 9 ml 1% BSA-PBS-EDTA to give a 1/10 dilution. We then froze this solution in 0.15 ml aliquots (in polystyrene tubes using liquid nitrogen).

   These aliquots can then be thawed and diluted for each assay. 1st Ab should be diluted so that Bo (Buffer control) tubes contain about 50% of the total tubes CPM.

   We have been using a 1/7000 or 1/8000 dilution. To obtain these dilutions, 0.05 ml of the 1/10 dilution is diluted to 35 or 40 ml using 1% BSA-0.25% GPS-PBS-EDTA.

   I use a Gibson P-5000 to measure the 34.95 or 39.95 ml PBS.

5. Insulin Standards

78.43 mg Bovine Insulin (Sigma I5500) was diluted in 1% BSA-PBS-EDTA (at room temperature with 1-2 hours stirring). Our insulin lot contained 25.5 U/mg, therefore 78.43 mg = 2000 U.

1 ml of this 4 U/ml solution was then diluted to 100 ml in 1% BSA-PBS-EDTA to give a 40 mU/ml solution. We then froze this (in 0.5 ml aliquots) using liquid nitrogen.

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 our standard curve.

Standard	2 mU/ml Solution Volume	BSA-PBS-EDTA Volume
200 µU/ml	0.5 ml	4.5 ml
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 is then used in each standard tube.

6. Labelled Insulin

Labelled 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 a 100 µl sample.

7. Second Ab

When using Miles Ab:

Lyophilized Ab is reconstituted using 2 ml cold DD H_2O, this solution is then diluted to 20 ml using 1% BSA-PBS.

When using RPI Ab:

RPI Ab is thawed and diluted 1/80 using 1% PBA-PBS. Currently RPI Ab is frozen in 1 ml aliquots which could be diluted to 80 ml. This would provide enough 2nd Ab for 3 assays plus some left over which could be quick frozen and thawed prior to using again. When using either 2nd Ab there will always be some that is not

used. I generally quick freeze it in polystyrene culture tubes and thaw it (only once) when I need it. When I do use it I add it to other Ab from other "pools" that I have mixed that day or thawed before I use any. This assures we have a more uniform Ab solution in each assay.

8. Guinea Pig Serum

Guinea pig serum should be frozen in 500 µl aliquots.

Procedures:

Day 1 - add plasma or standards, diluent and 1st antibody
Day 2 - add radio iodinated insulin
Day 3 - add 2nd Ab
Day 5 - centrifuge, pour off and count precipitate

Day 1 -

Label culture tubes. We usually run 3 assays containing 123 tubes each. Our centrifuge can hold 120 tubes (3 total tubes are not centrifuged) and we prepare a standard curve for each centrifuge load.

Mix 1% BSA with PBS-EDTA before use (usually the night before) and have its pH at 7.6.

Thaw an aliquot each of 1st Ab, insulin standard solution and Guinea pig serum. Prepare insulin standard solutions, GPS-BSA-PBS-EDTA (100 ml is adequate for 3 assays) and 1st Ab as described.

Dispense BSA-PBS-EDTA (200 500 µl when using 400 µl samples) and GPS-BSA-PBS (100 µl) into tubes as needed, standard solutions (200 µl), plasma samples (400 µl or 200 µl) and 1st Ab (100 µl) to all but total and non-specific binding tubes. Vortex tubes and incubate 24 hours at 2-4°C.

Make arrangements for gamma counter use on days 2 and 5.

Day 2 -

Prepare labelled insulin and add 100 µl to all tubes, vortex and incubate 24 hours at 2-4°C.

Day 3 -

Prepare 2nd Ab and add 200 µl to all but total tubes, vortex and incubate 48 hours at 2-4°C.

Day 5 -

Centrifuge at 3000 RPM for 30-35 minutes (do not centrifuge or decant total tubes).

Pour off supernatant into waste bottle (quickly/gently) and place upside down on Kimwipes in a foil-lined box for at least 15 minutes.

Wipe out the top half of each tube with a Kimwipe on a wooden applicator stick and count for 1 to 1.5 minutes.

Dispose of culture tubes ASAP.

APPENDIX 6

RADIOIMMUNOASSAY OF GLUCAGON

Reagent Preparation

1. 0.2 M Glycine-Merthiolate

   Dissolve in 900 ml DD H_2O;
   15 g Glycine
   0.1 g Thimerosol
   Adjust pH to 8.8 with 3 M NaOH
   Bring to 1000 ml in volumetric flask
   Store at 2-4°C

2. 0.05 M ETDA-Glycine-Merthiolate;

   Dissolve in 900 ml 0.2 M Glycine-Merthiolate;
   18.6 g Na_2-EDTA
   Adjust pH to 8.8 with 10 M NaOH
   Bring to 1000 ml with 0.2 M Glycine-Merthiolate in volumetric flask
   Store at 2-4°C

   I now dissolve 15 g Glycine and 0.1 g Thimerosol in 850 ml DD H_2O, adjust pH to 8.8 with 3 M NaOH, then add 18.6 g Na_2-EDTA, adjust pH to 8.8 with 10 M NaOH, and bring to 1000 ml with DD H_2O in volumetric flask.

3. 1% BSA-Glycine-Merthiolate

   Dissolve 1 g BSA in 100 ml 0.2 M Glycine-Merthiolate, check pH (should not change)

   - use cold buffer in a beaker with a stirring magnet.

   - 100 ml should be enough for 3 assays of 123 tubes, with some left over.

4. 0.1 M Benzamidine - 1% BSA-0.2 M Glycine Merthiolate

   - prepare 1% BSA-Glycine-Merthiolate as in #3 and add 1.566 g Benzamidine Hydrochloride

   - check pH, adjust to 8.8 with ≈75 µl 3 M NaOH.

5. 0.25% Normal Rabbit Serum (NRS) - EDTA-Glycine-Merthiolate

   Dilute 250 µl Normal Rabbit Serum to 100 ml in 0.05 M EDTA-Glycine-Merthiolate.

74 ml are needed for diluting the first Ab for 3 assays of 123 tubes.

- mix immediately before use and keep at 2-4°C

6. Rabbit Ani Glucagon Antibody (First Ab)

Dr. Unger has been shipping 25 µl of Ab (lyophillized) with a recommended final culture tube dilution of 1:100,000;

to reconstitute Ab with this volume and titer;

- Remove stopper (after tapping tube lightly to dislodge any Ab on the stopper) and add 5 ml of 1% BSA-Glycine-Merthiolate, replace the stopper, mix by gentle inversion. Let the solution sit (at 2-4°C) for 1 hour, inverting it every 10-15 minutes. This gives a 1:200 dilution. Transfer this solution to a cold 15 ml centrifuge tube (on ice), wash the original tube with 5 ml 1% BSA-Glycine-Merthiolate and add to the original 5 ml to give a 1:400 dilution.

- This solution should be frozen and stored in 500 µl aliquots, using liquid nitrogen and 12 x 75 mm polypropylene culture tubes.

- We have been using a 1/50 dilution of this 1/400 solution for our working solution; thaw 1 500 µl aliquot/123 tube assay and dilute to 25 ml using 0.25% NRS-EDTA-Glycine-Merthiolate (we use 200 µl/tube).

- For 3 assays dilute 1.5 ml to 75 ml.

- I have been measuring the NRS-EDTA-Glycine-Merthiolate in a graduated cylinder and rinsing the 1st Ab storage tubes with 2-3 ml of buffer 4-5 times.

- Mix immediately before use and keep at 2-4°C.

7. Glucagon Standard Solution

- Weigh out 10 mg Crystalline Glucagon (Jim Bacon's balance is excellent for this); dissolve in 20 ml 0.02 N HCl to give a solution of 0.5 mg/ml, the optical density of this solution should be 1.055 @ 278 nm. (Harris et al., 1979). If the OD is not 1.055, then correct the dilution to give an equivalent final concentration assuming that:

$$\frac{0.5 \text{ ml/ml}}{1.055 \text{ OD}} = \frac{X \text{ mg/ml}}{\text{actual OD}}$$

- Dilute 1 ml of the 0.5 mg/ml solution to 100 ml in 1% BSA-Glycine-Merthiolate to give a 5 ng/µl solution.

- Dilute 0.5 ml of this 5 mg/µl solution to 500 ml using 1% BSA-Glycine-Merthiolate to give a 0.005 ng/µl solution.

- I corrected our dilution scheme for the OD obtained here; if we need 1500 ng/500 ml, 2500 ng/5 ng/µl = 500 µl needed if OD ok
  2500 ng/ X ng/µl = $\frac{2500}{X}$ µl needed to correct for OD

- Quick freeze this solution in 2.1 ml aliquots. (Since we are using 1.917 ml/standard curve/assay, use liquid nitrogen and polypropylene tubes.)

- I originally froze our standard solution in 3.5 ml aliquots and am thawing 2 aliquots for 3 assays, which is wasting >1 ml each time.

- Standard solution is used at this dilution, thawed immediately before use and kept at 2-4°C.

8. Labelled Glucagon

- Reconstitute (10 µCi Cambridge ^{125}I-Glucagon) using 5.0 ml Benzamidine-BSA-Glycine-Merthiolate and dilute such that 100 µl contains 8,000 CPM.

- Instructions sent with the Cambridge RIA Glucagon Kit recommend letting the vial of labelled glucagon sit at room temperature for 30 minutes after adding the buffer, then inverting gently a few times before diluting/using.

- At least 12.3 ml are needed for each 123 tube assay (100 µl/tube) so I try to mix 38 ml for 3 assays.

- The remaining ^{125}I-Glucagon solution (10 µCi/5 ml) should be quick frozen (using liquid nitrogen and polyproplyene 12 x 75 mm culture tubes), usually in 500 µl aliquots.

9. Goat Anti Rabbit IgG (Second Ab)

- Thaw the 10 ml received from RPI and quick freeze in 1 ml aliquots.

- A 1 ml aliquot is then diluted to 60 ml using 0.2 M Glycine-Merthiolate.

- 300 µl is needed per tube, so I mix 120 ml for 3 assays, and quick freeze the remaining 12 ml in 3 ml aliquots in polypropylene tubes.

- This 1/60 dilution should only be thawed once.

10. Normal Rabbit Serum

- Should be frozen in 1 ml aliquots.

Procedure

Day 1 - Add buffer, standard, plasma and 1st antibody, incubate 12 hours
Day 1 + 12 hr - add labelled glucagon, incubate 48 hr
Day 3 - Add 2nd Ab, incubate 48 hr
Day 5 - Centrifuge, pour off, count precipitant

- Make sure there are adequate quantities of all reagents before beginning.

- Mix buffers needed before beginning, usually 12-24 hours beforehand.

- Label culture tubes. I usually run 3 assays of 123 tubes each. Our centrifuge (Beckman table top-refrigerated) holds 120 tubes (the 3 total tubes are not centrifuged) and we prepare a standard curve for each centrifuge load.

Day 1:

Thaw: 3 500 µl aliquots 1st Ab
3 2.1 ml aliquots standard solution
1 aliquot NRS (at least 250 µl)
3 1 ml aliquots of pooled plasma needed

- samples (39/assay = 117)

Prepare 100 ml 0.25% NRS-EDTA-Glycine-Merthiolate and use to dilute 1st Ab as previously described (1.5 ml 1st Ab 75 ml NRS-Buffer).

Dispense 1% BSA-Glycine-Merthiolate, standard solution, plasma and 1st antibody as needed. Vortex tubes and incubate 12 hours at 2-4°C.

Make arrangements for gamma counter use.

Day 1 (+ 12 hr):

Prepare labelled glucagon, add 100 µl to all tubes, vortex, incubate 48 hr at 2-4°C.

Day 3:

Prepare 2nd Ab and add 300 µl to all but total tubes, vortex; incubate 48 hr at 2-4°C.

Day 5:

Centrifuge at 3000 RPM (at least 1500 g @ 2-4°C) for 40 minutes (do not centrifuge or decant total tubes).

Pour off supernatant into a waste bottle (quickly/gently) and place upside down on Kimwipe in a foil-lined box for 15 minutes.

Wipe out the top half of each tube with a Kimwipe on a wooden applicator stick and count 1.5 minutes.

Dispose of tubes ASAP.

APPENDIX 7

GLUCOSE

There are two fundamental procedures which may be employed for the determination of glucose in biologic fluids using the Sigma test system:

a) Direct - applicable to serum or plasma.

b) Deproteinized filtrates - applicable to whole blood and colored or turbid serum or plasma.

The choice of procedure depends primarily on the condition of the specimen. If samples are highly Ictaric or markedly hemolyzed, protein-free filtrates should be prepared.

Use of Plasma or Serum Directly

1. Label three or more tubes or cuvets as follows:

   Blank, Standard, Test 1, Test 2, etc.

2. To Blank, add: 0.5 ml water.

   To Standard, add: 0.5 ml of a 20-fold dilution of glucose standard solution, stock no. 635-100 (1 part solution plus 19 parts water); or: 25 µl (0.025 ml) of standard plus 0.5 ml water.

   To each Test add: 0.5 ml of a 20-fold dilution of sample (1 part serum or plasma plus 19 parts water); or: 25 µl (0.025 ml) of saple plus 0.5 ml water.

3. To each tube, add: 5.0 ml of combined enzyme-color reagent solution (Reagent A").

   Mix each tube thoroughly.

4. Incubate all tubes at 37°C for 30 ± 5 minutes or at room temperature for 45 minutes.

   NOTE: Avoid exposure to direct sunlight or bright daylight.

5. At the end of incubation period, remove all tubes from water bath. Read A of Standard and Test, using Blank as reference at 425 - 475 nm.

   Readings should be completed within 30 minutes.

6. Calculate your Test values as follows:

$$\text{Serum Glucose (mg/100 ml)} = \frac{A_{Test}}{A_{Standard}} \times 100$$

NOTE: If Test result is greater than 300 mg/100 ml, repeat assay using a 40-fold rather than a 20-fold dilution of sample in Step 2 above. Multiply new result by 2.

Use of Deproteinized Blood, Plasma or Serum

NOTE: The following procedure is used on whole blood, plasma or serum samples which are markedly colored or turbid.

1. Label three or more tubes or flasks as follows: Blank, Standard, Test 1, Test 2, etc.

2. To each add: 1.8 ml water

3. To Blank add: 0.2 ml water.

   To Standard add: 0.2 ml glucose standard solution, stock no. 635-100

   To each Test, add: 0.2 ml blood, plasma, or serum. Swirl to mix and hemolyze blood.

4. a) Add to each tube: 1.0 ml barium hydroxide solution, stock no. 14-3.

   b) Add to each tube: 1.0 ml zinc sulfate solution, stock no. 14.4.

      Stopper tightly.

      Mix well by shaking.

   c) Filter or centrifuge to obtain clear filtrate or supernatant.

5. Transfer 0.5 ml of clear filtrate or supernatant from each tube to another series of clean dry tubes or cuvets, correspondingly labeled Blank, Standard, Test 1, Test 2, etc.

6. To each tube, add: 6.0 ml combined enzyme-color reagent solution (Reagent A").

   Mix each tube well.

7. Incubate all tubes at 37°C for 30 ± 5 minutes or at room temperature for 45 minutes.

   NOTE: Avoid exposure to direct sunlight or bright daylight.

8. At the end of incubation period, remove all tubes from water bath. Read A of Standard and Test, using Blank as reference at 425 - 475 nm.

   Readings should be completed within an additional 30 minutes.

9. Calculate your Test values as follows:

$$\text{Serum glucose (mg/100 ml)} = \frac{A_{Test}}{A_{Standard}} \times 100$$

   NOTE: If Test result is greater than 300 mg/100 ml, repeat the assay starting with Step 5 by using 0.25 ml of Test filtrate (from Step 4, c) plus 0.25 ml H_2O. Multiply new result by 2.

APPENDIX 8

Table A-1. Venoarterial differences of glucose in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted ewes.

	H-A	H-P	P-A	V-A
NOR (5)				
Control	3 ± 0.5 †	4 ± 0.5 †	-0.7 ± 0.7	0.2 ± 0.6
BOHB infusion	1 ± 0.4 †	2 ± 0.4 †	-0.5 ± 0.5	0.4 ± 0.5
DIT (5)				
Control	6 ± 3 †	5 ± 4	1 ± 4	-1 ± 4
BOHB infusion	8 ± 3 †	4 ± 3	4 ± 4	5 ± 4
DUT (5)				
Control	8 ± 4	11 ± 4 †	-3 ± 4	-2 ± 4
BOHB infusion	5 ± 2 †	7 ± 2 †	-2 ± 2	-6 ± 3

Values are means ± SEM; (n) is the number of animals studied.

†Statistically different from normal PR |T|; $p < .05$.

APPENDIX 9

Table A-2. Venoarterial differences of free fatty acids in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted ewes pre and post beta-hydroxybutyrate (BOHB) infusion.

	H-A	H-P	P-A	V-A
NOR (5)				
Control	-71 ± 26 †	201 ± 41 †	130 ± 28 †	123 ± 31 †
BOHB infusion	-16 ± 26	-41 ± 16 †	24 ± 18	23 ± 16
DIT (5)				
Control	-127 ± 26 †	-159 ± 22 †	32 ± 31	97 ± 20 †
BOHB infusion	-28 ± 16	-33 ± 23	5 ± 15	26 ± 15
DUT (5)				
Control	-98 ± 41 †	-109 ± 38 †	10.5 ± 14	15 ± 11
BOHB infusion	-150 ± 29 †	-126 ± 27 †	-25 ± 21	25 ± 16

Values are means ± SEM; (n) is the number of animals studied.

†Statistically different from normal PR |T|; $p < .05$.

APPENDIX 10

Table A-3. Venoarterial differences of acetoacetate in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted ewes pre and post beta-hydroxybutyrate (BOHB) infusion.

	H-A	H-P	P-A	V-A
NOR (5)				
Control	32 ± 9 †	33 ± 8 †	-1.4 ± 6	-16 ± 2 †
BOHB infusion	-20 ± 7 †	-44 ± 9 †	24 ± 4 †	-20 ± 6 †
DIT (5)				
Control	25 ± 17	24 ± 15	2 ± 12	-28 ± 7 †
BOHB infusion	-25 ± 9 †	28 ± 8 †	3 ± 4	-47 ± 14 †
DUT (5)				
Control	246 ± 67 †	267 ± 47 †	-21 ± 33	-93 ± 19 †
BOHB infusion	-110 ± 136	-12 ± 106	-98 ± 92	-378 ± 101 †

Values are means ± SEM; (n) is the number of animals studied.

†Statistically different from normal PR |T|; $p < .05$.

APPENDIX 11

Table A-4. Venoarterial differences of beta-hydroxybutyrate in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted ewes pre and post beta-hydroxybutyrate (BOHB) infusion.

	H-A	H-P	P-A	V-A
NOR (5)				
Control	520 ± 95†	559 ± 83†	-39 ± 25	-177 ± 22†
BOHB infusion	73 ± 37†	175 ± 29†	-102 ± 48†	-379 ± 31†
DIT (5)				
Control	315 ± 83†	405 ± 80†	-92 ± 70	-186 ± 68†
BOHB infusion	230 ± 57†	75 ± 59	155 ± 58†	-191 ± 110
DUT (5)				
Control	1305 ± 268†	-1374 ± 222†	-69 ± 163	-355 ± 153†
BOHB infusion	529 ± 375	254 ± 268	275 ± 668	-858 ± 238†

Values are means ± SEM; (n) is the number of animals studied.

†Statistically different from normal PR $|T|$; $p < .05$.

APPENDIX 12

Table A-5. Venoarterial differences of insulin in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted ewes pre and post beta-hydroxybutyrate infusion.

	H-A	H-P	P-A	V-A
NOR (5)				
Control	3 ± .9†	-2 ± .6†	6 ± 1.3†	-0.1 ± .3†
BOHB infusion	3 ± .8†	-6 ± 1.5†	9 ± 1.7†	0.5 ± .6
DIT (5)				
Control	1 ± 3	3 ± 3	-2 ± 2	-0.1 ± 3
BOHB infusion	-1 ± 5	4 ± 4	-5 ± 3	-4 ± 4
DUT (5)				
Control	1 ± .6	0.6 ± .3	0.6 ± .7	0.2 ± .8
BOHB infusion	2 ± .8	0.1 ± .4	1 ± .7	0.5 ± .8

Values are means ± SEM; (n) is the number of animals studied.

†Statistically different from normal PR |T|; $p < .05$.

APPENDIX 13

Table A-6. Venoarterial differences of glucagon in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted ewes pre and post beta-hydroxybutyrate (BOHB) infusion.

	H-A	H-P	P-A	V-A
NOR (5)				
Control	18 ± 7†	-24 ± 8†	43 ± 13†	-26 ± 10†
BOHB infusion	26 ± 9†	-22 ± 4†	48 ± 11†	-26 ± 10†
DIT (5)				
Control	60 ± 13†	-50 ± 16†	110 ± 19†	-10 ± 21
BOHB infusion	78 ± 15†	-28 ± 12†	106 ± 22†	7 ± 11
DUT (5)				
Control	106 ± 25†	-59 ± 20†	166 ± 29†	-4 ± 15
BOHB infusion	75 ± 21†	-28 ± 19	104 ± 18†	-26 ± 9†

Values are means ± SEM; (n) is the number of animals studied.

†Statistically different from normal PR |T|; $p < .05$.

APPENDIX 14

Table A-7. Effects of beta-hydroxybutyrate (BOHB) infusion on extraction ratios of glucose in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted sheep.

	Portal-drained viscera	Hepatic	Total splanchnic	Lower hindquarter
NOR (5)				
Control	-0.01 ± .02	0.08 ± .01	0.07 ± .01	0.00 ± .01
BOHB infusion	-0.01 ± .01	0.04 ± .01	0.03 ± .08	0.01 ± .01
DIT (5)				
Control	0.01 ± .04	0.05 ± .03	0.07 ± .03	-0.03 ± .03
BOHB infusion	0.07 ± .05	0.02 ± .02	0.08 ± .02	0.01 ± .03
DUT (5)				
Control	-0.01 ± .03	0.01 ± .02	0.06 ± .02	-0.00 ± .02
BOHB infusion	-0.01 ± .01	0.04 ± .01	0.03 ± .01	-0.01 ± .02

Values are means ± SEM; (n) is the number of animals studied.

APPENDIX 15

Table A-8. Effects of beta-hydroxybutyrate (BOHB) infusion on extraction ratios of free fatty acids in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted sheep.

	Portal-drained viscera	Hepatic	Total splanchnic	Lower hindquarter
NOR (5)				
Control	0.1 ± .03	-0.2 ± .02	0.2 ± .04	-0.1 ± .02
BOHB infusion	0.1 ± .05	-0.1 ± .04	0.2 ± .08	0.01 ± .08
DIT (5)				
Control	0.04 ± 03†	-0.2 ± .03	0.1 ± .02	-0.1 ± .02
BOHB infusion	0.01 ± .03	0.04 ± .05*	0.05 ± .03	0.04 ± .04
DUT (5)				
Control	0.0 ± .01†	0.5 ± .01†	.00 ± 0.0	-.04 ± 0.2 †
BOHB infusion	-.03 ± .02	-.13 ± .04	.02 ± .01	-.14 ± .04

Values are means ± SEM; (n) is the number of animals studied.

†Statistically different from normal PR $|T|$; $p < .05$.

APPENDIX 16

Table A-9. Effects of beta-hydroxybutyrate (BOHB) infusion on extraction ratios of acetoacetate in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted sheep.

	Portal-drained viscera	Hepatic	Total splanchnic	Lower hindquarter
NOR (5)				
Control	-0.0 ± .08	0.3 ± .08	-0.8 ± .03	0.3 ± .12
BOHB infusion	.02 ± .04 †	-0.2 ± .04 †	-0.11 ± .03 †	-0.11 ± .03 †
DIT (5)				
Control	-0.01 ± .05	0.18 ± .09	-0.18 ± .81	0.16 ± .03
BOHB infusion	0.0 ± .01	-0.09 ± .03	-.12 ± .03	-0.09 ± .03*
DUT (5)				
Control	0.0 ± .03	0.3 ± .05	-0.1 ± .03	0.28 ± .07
BOHB infusion	-0.03 ± .07	-0.01 ± .06 †	-0.22 ± .05	-0.04 ± .07*

Values are means ± SEM; (n) is the number of animals studied.

†Statistically different from normal PR |T|; $p < .05$.

*Statistically different from control PR |T|; $p < .05$.

APPENDIX 17

Table A-10. Effects of beta-hydroxybutyrate (BOHB) infusion on extraction ratios of beta-hydroxybutyrate in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted sheep.

	Portal-drained viscera	Hepatic	Total splanchnic	Lower hindquarter
NOR (5)				
Control	-0.04 ± .02	0.42 ± .06	-0.14 ± .02	0.38 ± .07
BOHB infusion	0.04 ± .02	0.06 ± .01†	-0.15 ± .02	0.02 ± .01†
DIT (5)				
Control	0.07 ± .05	0.23 ± .04†	-0.11 ± .03	0.15 ± .05†
BOHB infusion	0.03 ± .01†	0.02 ± .01*	-0.03 ± .02*	0.05 ± .02
DUT (5)				
Control	0.0 ± .01	0.10 ± .02†	-0.02 ± .01†	0.12 ± .02†
BOHB infusion	0.0 ± .02	0.02 ± .01*	-0.04 ± .12	0.02 ± .18

Values are means ± SEM; (n) is the number of animals studied.

†Statistically different from normal PR $|T|$; $p < .05$.

*Statistically different from control PR $|T|$; $p < .05$

APPENDIX 18

Table A-11. Effects of beta-hydroxybutyrate (BOHB) infusion on extraction ratios of insulin in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted sheep.

	Portal-drained viscera	Hepatic	Total splanchnic	Lower hindquarter
NOR (5)				
Control	0.30 ± .05	0.06 ± .02	-0.03 ± .02	0.17 ± .04
BOHB infusion	0.33 ± .05	-0.11 ± .03	0.04 ± .03	0.10 ± .02
DIT (5)				
Control	0.02 ± .06	0.08 ± .08	0.08 ± .05	0.08 ± .07
BOHB infusion	-0.06 ± .05	0.20 ± .10	0.03 ± .05	0.14 ± .10
DUT (5)				
Control	0.30 ± .22	0.20 ± .15†	0.06 ± .12	0.50 ± .22
BOHB infusion	0.61 ± .36	-0.04 ± .10	0.04 ± .13	0.66 ± .45

Values are means ± SEM; (n) is the number of animals studied.

†Statistically different from normal PR $|T|$; $p < .05$

APPENDIX 19

Table A-12. Effects of beta-hydroxybutyrate (BOHB) infusion on extraction ratios of glucagon in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted sheep.

	Portal-drained viscera	Hepatic	Total splanchnic	Lower hindquarter
NOR (5)				
Control	0.19 ± .03	-0.05 ± .12	-0.09 ± .01	0.08 ± .02
BOHB infusion	0.22 ± .03	-0.05 ± .01	-0.09 ± .02	0.11 ± .03
DIT (5)				
Control	0.58 ± .12 †	-0.12 ± .03	-0.00 ± .05	0.30 ± .09†
BOHB infusion	0.59 ± .12	-0.02 ± .04*	0.04 ± .06	0.42 ± .07
DUT (5)				
Control	0.27 ± .04	-0.00 ± .02	-0.00 ± .03	0.20 ± .05
BOHB infusion	0.21 ± .04	-0.00 ± .03	-0.05 ± .04	0.14 ± .01

Values are means ± SEM; (n) is the number of animals studied.

†Statistically different from normal PR |T|; $p < .05$.

*Statistically different from control PR |T|; $p < .05$.

APPENDIX 20

Table A-13. Effects of beta-hydroxybutyrate infusion on arterial concentrations and net fluxes of glucose in normal (NOR), diabetic insulin-treated (DIT), and diabetic untreated (DUT) 3-day fasted ewes.

	Arterial concentration	Portal-drained viscera	Liver	Lower hindquarter
NOR (5)				
Control	48 ± 2†	-45 ± 5	35 ± 5	2 ± 2
BOHB infusion	47 ± 49	-3 ± 3	16 ± 3*	1 ± 1
DIT (5)				
Control	35 ± 16	13 ± 36†	15 ± 37	12 ± 16
BOHB infusion	29 ± 14	40 ± 28	49 ± 22	18 ± 14
DUT (5)				
Control	171 ± 12	-17 ± 25	78 ± 28	-21 ± 25
BOHB infusion	182 ± 12	-11 ± 10	44 ± 11	-38 ± 22

Values are means ± SEM; (n) is the number of animals studied.

†Statistically different from normal PR $|T|$; $p < .05$.

*Statistically different from control $|T|$; $p < .05$.

VITA

Dianne Jean Dawes-Torre was born in Pittsburgh, Pennsylvania August 3, 1962. She attended elementary school and high school in Pittsburgh. Dianne attended The Pennsylvania State University where she received her Bachelor of Science in Animal Science in 1984.

Dianne Dawes accepted a research assistantship at The University of Tennessee, Knoxville and studied for two years with Dr. R. N. Heitmann in the area of ruminant nutrition.

Dianne Dawes was married to Patrick Torre on August 9, 1986, and received the Master of Science degree August 29, 1986.

Dianne plans to apply to veterinary school in January 1987.