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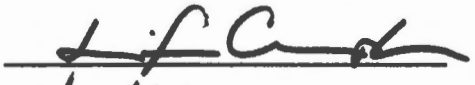
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GAMMA-BUTYROBETAINE DOES NOT RESTORE BETA-HYDROXYBUTYRATE INHIBITION OF HEPATIC KETOGENESIS IN DIABETIC, UNTREATED EWES

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee

Jennifer Lynn Campbell
August 1993

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DEDICATION

This thesis is dedicated to my parents

Richard Allen Campbell

and

Joan Martha Campbell

who have always encouraged me to be my best.

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ABSTRACT

A possible insulin-independent regulatory mechanism of hepatic ketogenesis was studied in eight experiments on five ewes surgically catheterized in hepatic, portal, and mesenteric veins, and femoral artery and femoral vein. Ewes were made diabetic with alloxan (50 mg/kg bw, iv). Euglycemia was maintained with daily insulin injections (Iletin-100 ~35 IU/d) until three days prior to each experiment, when injections were withheld to induce ketosis. Ewes were housed in 1.8 x 3.0 m indoor pens under natural lighting in a thermoneutral environment. Experiments consisted of three infusion periods: (1) para-aminohippurate (1.5% @ 0.764 ml/min) infusion into the common mesenteric vein (control); (2) para-aminohippurate as in (1) and beta-hydroxybutyrate (1.18 mmol/min) into the common mesenteric vein; and (3) para-aminohippurate and beta-hydroxybutyrate as in (2) and gamma-butyrobetaine (1.18 mmol/min) into the common mesenteric vein. For each period, each infusate was allowed to equilibrate for one hour and then three blood samples were taken simultaneously from the hepatic, portal, and femoral veins and femoral artery at thirty minute intervals and analyzed for beta-hydroxybutyrate, acetoacetate, non-esterified fatty acids, glucose, and para-aminohippurate. Blood flows were calculated by measuring downstream dilution of para-aminohippurate and net fluxes were calculated by multiplying tissue veno-arterial concentrations by tissue

blood flow. Blood glucose (≈10 mM) was unaffected by infusion. Beta-hydroxybutyrate decreased hepatic flow (3.6 to 2.5 L/min, P<.01), but did not change portal flow. Consequently, portal flow contribution to the liver increased from 73 to 83% (P<.01) during beta-hydroxybutyrate infusion. Relative to control, beta-hydroxybutyrate infusion decreased (P<.01) non-esterified fatty acid arterial concentrations (2.1 to 1.1 mM) and hepatic (0.7 to 0.2 mmol/min) and total splanchnic (0.6 to 0.2 mmol/min) net uptakes. Beta-hydroxybutyrate infusion increased acetoacetate arterial concentrations (1.2 to 1.5 mM, P<.05) and decreased (P<.05) hepatic release (0.5 to 0.1 mmol/min)(i.e., decreased ketogenesis) and total splanchnic release (0.6 to 0.1 mmol/min). Similar to its effects on acetoacetate, beta-hydroxybutyrate infusion increased beta-hydroxybutyrate arterial concentrations (7.6 to 12.3 mM, P<.01) but decreased net hepatic release (1.6 to 0.7 mmol/min, P<.01) and total splanchnic release (2.1 to 1.3 mmol/min, P<.1). Gamma-butyrobetaine infusion did not reverse any beta-hydroxybutyrate effects. According to this data beta-hydroxybutyrate may regulate ketogenesis by decreasing hepatic non-esterified fatty acid uptake and subsequent conversion to acetoacetate and beta-hydroxybutyrate. Gamma-butyrobetaine, in contrast, had no effect on hepatic ketogenesis nor non-esterified fatty acid uptake, suggesting that beta-hydroxybutyrate inhibition was not at the carnitine

acyltransferase level or that gamma-butyrobetaine may not be extracted by the liver in quantities sufficient to counteract observed effects of beta-hydroxybutyrate.

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CHAPTER I

INTRODUCTION

Hyperketonemia can be a result of starvation, diabetes mellitus, late gestation or early lactation (18). It is described by elevated circulating NEFA accompanied by increased fatty acid utilization and decreased carbohydrate utilization (20,22). The stress of late pregnancy or early lactation induces increased lipolysis, making more NEFA available to the liver as substrate (19). Increased hepatic uptake of NEFA may result in elevated production of ketone bodies, i.e. beta-hydroxybutyrate and acetoacetate, causing ketosis (34). Hepatic production of ketone bodies beyond extrahepatic utilization results in a buildup of ketone bodies in the blood (34), marked by lowered blood pH known as ketoacidosis. This condition can progress to coma and death if untreated.

Under fasting conditions, alimentary ketogenesis ceases and hepatic ketogenesis increases (24). The ruminant liver switches from fatty acid reesterification to ketone body production and fatty acid oxidation during a matter of hours (34,36,55). Ketone body concentrations rise more markedly than any other fuel source during starvation (38). Ketone body production accompanied by decreased hepatic glucose output can result in a 30% reduction in peripheral tissue uptake of glucose. In this way protein precursors of glucose are spared, and glucose is conserved for the brain

and other obligatory tissues (30).

Ketones are a very efficient fuel source during periods of high metabolic demand. Unlike NEFA, ketones can be transported in the blood at high concentrations in the free state. Due to their toxicity in the free state, NEFA require an albumin transporter which lowers their diffusion rate into the cell and limits their availability. Ketones can be transported at concentrations high enough to replace glucose as an energy source (30,38).

Control of ketogenesis occurs at the prehepatic and hepatic level (34). Triglycerides in adipose tissue are broken down by hormone-sensitive lipase, releasing free fatty acids and glycerol. The hormones insulin and glucagon exert a strong influence on hormone-sensitive lipase; insulin inhibiting its action by dephosphorylation, thereby limiting the amount of precursor available for hepatic ketogenesis (34), and glucagon stimulating it via cAMP-dependent phosphorylation (6,16). Bjorntorp (7) proposed that ketones may suppress hormone-sensitive lipase directly. Drackley (15) proposed that ketone bodies may regulate NEFA oxidation at a site within the liver. Ketone bodies may have an autoregulatory effect on their own production by stimulating insulin release, which inhibits the release of free fatty acids from adipose tissue and therefore limits the amount of substrate available for ketogenesis (15,19,34,55).

CHAPTER II

LITERATURE REVIEW

Ketone Body Production

Free fatty acids are released from adipose tissue and transported in the blood bound to albumin (38) (Figure 1). Inside the liver, the acyl unit is activated to an acyl-CoA by acyl-CoA synthase and ATP. The acyl-CoA may be esterified to an acylglycerol and undergo triacylglycerol synthesis (21), or enter the hepatic mitochondria to undergo beta-oxidation (34). Transport across the inner mitochondrial membrane involves the carnitine palmitoyltransferase (CPT) mechanism. Within the matrix, the acyl-CoA undergoes beta-oxidation to acetyl-CoA, which is either fully oxidized to CO_2 via the tricarboxylic acid (TCA) cycle, or converted to ketone bodies.

Zammit (55) proposed that the shift from triacylglyceride synthesis to oxidation during ketogenesis can be ascribed to a decrease in long-chain fatty acyl-CoA entering the esterification pathway due to an increase in acyl-carnitine production. In fasted rats, mitochondrial glycerol-3-phosphate acyl-transferase (GPAT) activity decreased by 40% (55). This enzyme is responsible for the union of acyl-CoA and glycerol-3-phosphate in the formation of triacylglyceride. Its decreased activity implies a lowered rate of esterification during starvation. Starved

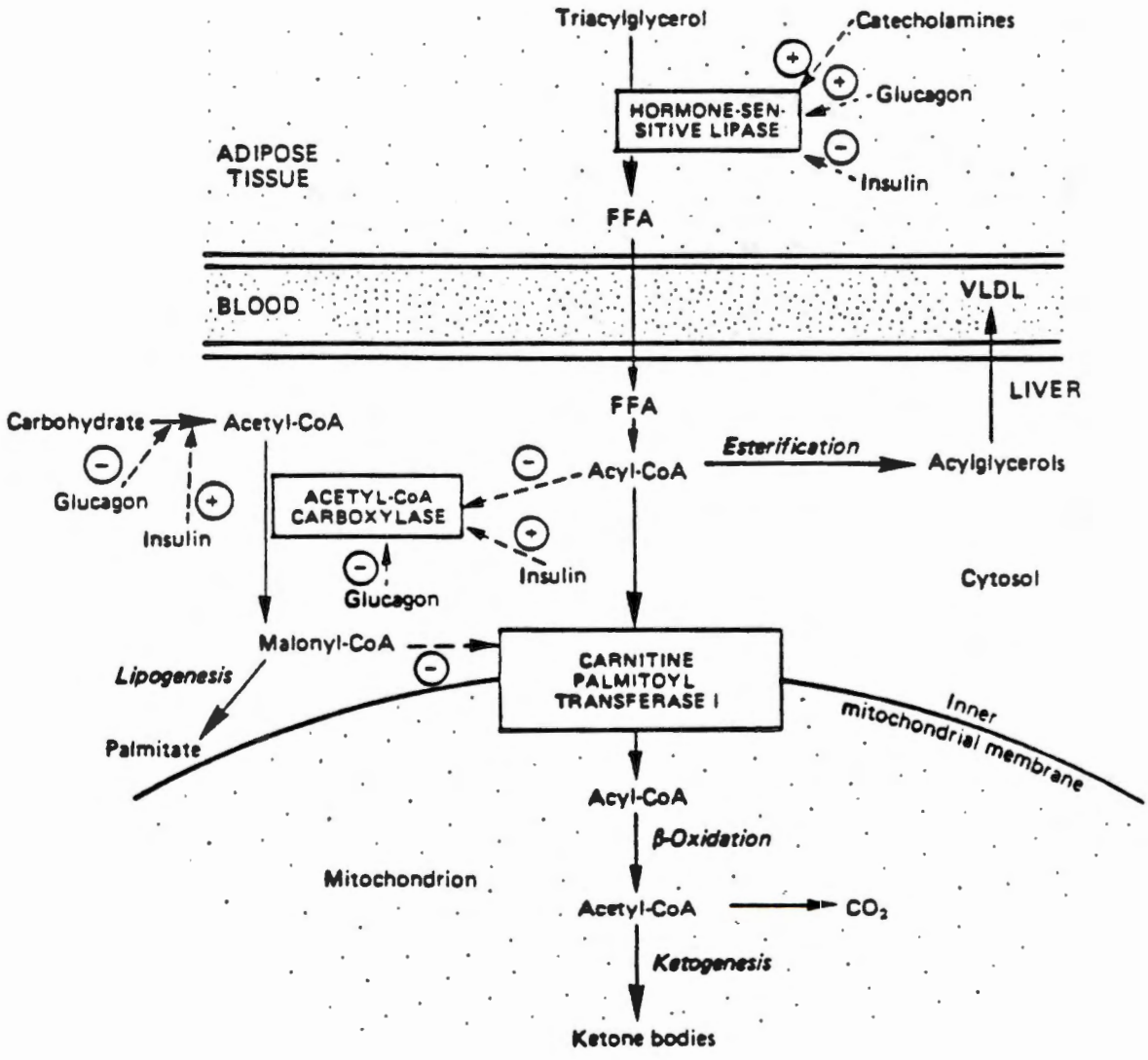


Figure 1: Regulation of long-chain fatty acid oxidation in the liver. (FFA, free fatty acids; VLDL, very low density lipoproteins.) (Harper's, 1985).

rats also exhibited a two to three-fold increase in CPT-I activity as opposed to fed rats (55), implying an increase in long-chain fatty acid transport into the mitochondria.

Upon entering the oxidation pathway, the acyl-CoA is bound reversibly with carnitine by CPT-I, which is loosely bound to the outer side of the inner mitochondrial membrane, to become acyl-carnitine (13,34,48) (Figure 2). Acyl-carnitine exchanges with free carnitine across the mitochondrial membrane via carnitine acyl-carnitine translocase. Within the matrix, the acylcarnitine is split to the original acyl-CoA and free carnitine by CPT-II on the inner side of the mitochondrial membrane (13). The acyl-CoA is then available to undergo beta-oxidation.

Any inhibition of the CPT transport mechanism effects the flow of NEFA into ketone bodies (34), and it has been suggested that this is the rate-limiting step in fatty acid oxidation (37). Malonyl-CoA is a strong competitive inhibitor of CPT-I, but not CPT-II, because of the impermeability of the inner mitochondrial membrane. By binding to CPT-I, malonyl-CoA inhibits acyl-CoA transport into the matrix. Declercq et al. (13) proposed that CPT-I and CPT-II are separate entities, CPT-II being smaller than CPT-I and showing different sensitivity to inhibitors, such as malonyl-CoA.

The acyl-CoA unit itself has an inhibitory effect on acetyl-CoA carboxylase, the enzyme responsible for the

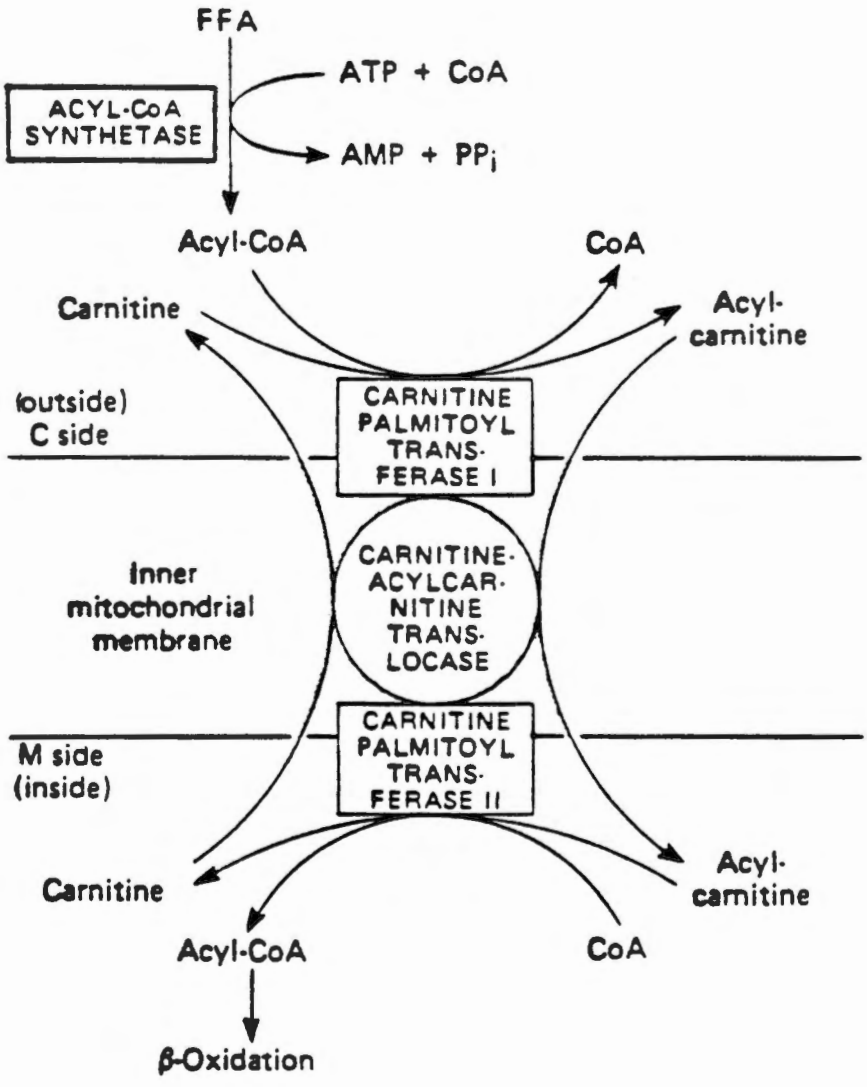


Figure 2: Role of carnitine in the transport of long-chain fatty acids through the inner mitochondrial membrane (Harper's, 1985).

conversion of acetyl-CoA to malonyl-CoA. Increased concentrations of acyl-CoA decrease malonyl-CoA inhibition of the CPT-I transport mechanism (35), thereby relieving the inhibition which may have prevented entrance to the mitochondria and incorporation of the acyl-CoA into ketone bodies instead of acylglycerols.

Inside the matrix, the acetyl-CoA required for the Krebs cycle joins with oxaloacetate to form citrate via citrate synthase, whereas the excess intramitochondrial acetyl-CoA combines to form acetoacetyl-CoA (48). This is the first committed step in the hydroxymethylglutaryl-CoA (HMG-CoA) pathway (56). The reaction is catalyzed by acetyl-CoA acetyltransferase, which competes with citrate synthase for acetyl-CoA (56). Studies suggest that citrate synthase has a lower K_m for acetyl-CoA within the hepatic mitochondria than does acetyl-CoA acetyltransferase (56). If so, rates of ketogenesis are dependent largely upon the amount of oxaloacetate available to draw acetyl-CoA into the TCA cycle and away from the ketogenic pathway (2,3).

A close relationship exists between ketogenic rates and free oxaloacetate concentrations in isolated hepatocyte mitochondria (56). Oxaloacetate concentrations depend upon equilibrium with malate, which is determined by the mitochondrial $NAD^+/NADH$ ratio; and conversion of oxaloacetate to phosphoenolpyruvate (PEP) within the cytosol by PEP carboxykinase (PEPCK) (3,56). Baird (2) found

significant differences between oxaloacetate concentrations in livers of normal lactating cows and livers of ketotic cows. Excess NADH from beta-oxidation converts oxaloacetate to malate, shunting malate out of the mitochondria and into the cytosol for gluconeogenesis (3). PEPCK activity is increased in ketotic sheep, resulting in increased conversion of oxaloacetate to PEP (3). These two factors enhance gluconeogenesis with concomitant depletion of the mitochondrial oxaloacetate supply, resulting in reduced entrance of acetyl-CoA into the TCA cycle and subsequent flow of acetyl-CoA through the ketogenic pathway.

The elevated glucose requirement of pregnancy and lactation increases the conversion of propionate, lactate, and amino acids to glucose by the liver and kidney (3). Since these substrates may be used for oxaloacetate production, this contributes to the oxaloacetate deficiency, and enhances ketogenesis.

Under the influence of the above factors, acetyl-CoA is directed into the ketogenic pathway and groups of two acetyl-CoA molecules condense to form acetoacetyl-CoA. Acetoacetyl-CoA is then joined with acetyl-CoA by HMG-CoA synthase to form HMG-CoA (Figure 3). Acetoacetyl-CoA is a negative feedback inhibitor of HMG-CoA synthase. HMG-CoA is split by HMG-CoA lyase to form acetoacetate and a different acetyl-CoA from the original molecule. Acetoacetate can be spontaneously decarboxylated to produce acetone, or it can

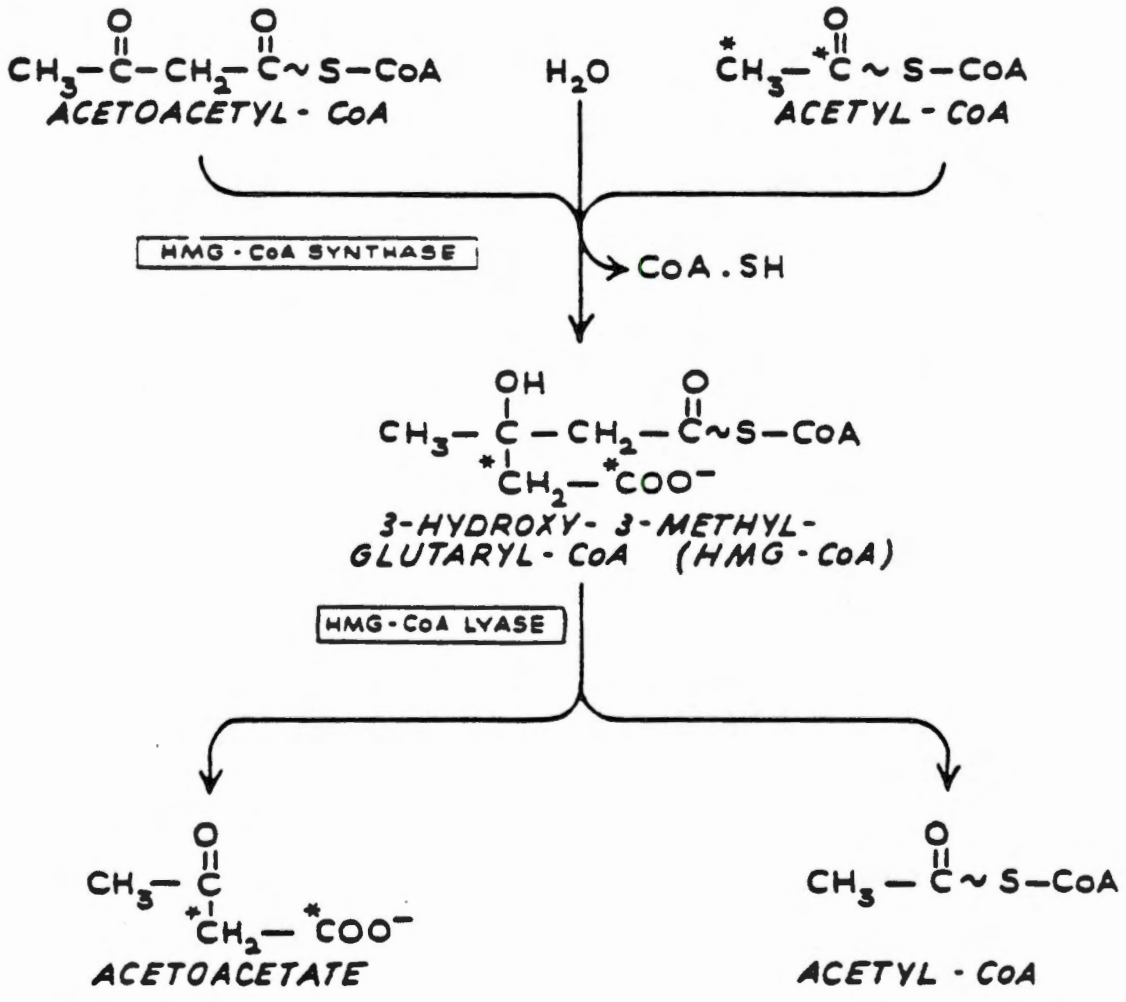


Figure 3: Formation of acetoacetate through intermediate production of HMG-CoA (Harper's, 1985).

be converted to beta-hydroxybutyrate by the addition of two hydrogens (Figure 4). This reaction relies upon beta-hydroxybutyrate dehydrogenase, located in the cytosol of sheep liver and kidney cortex (27), which is influenced by the cytosolic NADH/NAD^+ ratio. During periods of increased fatty acid oxidation, NADH production in the mitochondria results in a lowered cytosolic NADH/NAD^+ ratio, and very little acetoacetate is converted to beta-hydroxybutyrate by beta-hydroxybutyrate dehydrogenase (56). Beta-hydroxybutyrate dehydrogenase is not present in sheep liver mitochondria, prohibiting acetoacetate conversion to beta-hydroxybutyrate in sheep liver mitochondria even under reducing conditions (27). Beta-hydroxybutyrate dehydrogenase activity in cow liver is $1/30^{th}$ that in rat liver (2).

HMG-CoA synthase is considered the rate-limiting enzyme of the ketogenic pathway. Although the other ketogenic enzymes have been found in the heart, kidney and intestine, HMG-CoA synthase has been isolated only in the liver and in very small quantities in rumen epithelium (28). The hindlimb of fasted and diabetic dogs appears to be a negligible source, and HMG-CoA synthase has not been found in the extrahepatic tissues of the dog (1). Its near restriction to hepatic tissue accounts for the liver being the primary site of ketogenesis in non-ruminants and fasted ruminants (36).

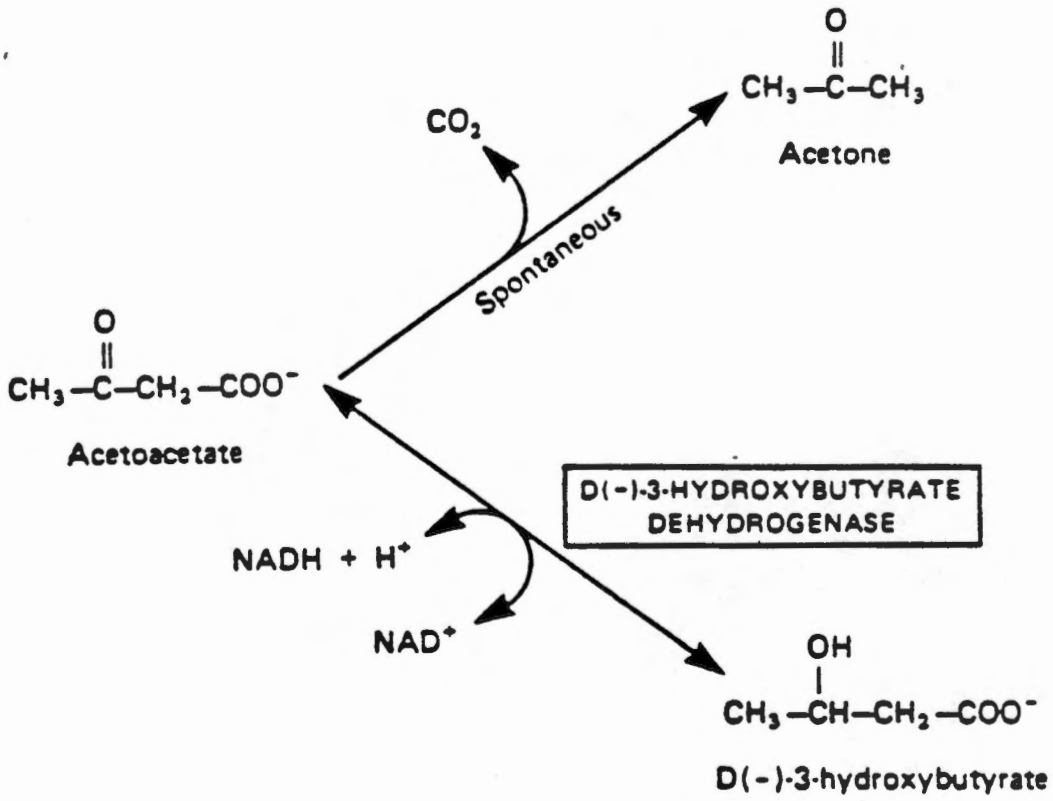


Figure 4: Interrelationships of the ketone bodies (Harper's, 1985).

Although alimentary ketogenesis provides approximately 7% of the fed ruminant's energy requirements through the conversion of butyrate and some acetate to ketone bodies (18), the liver is the main source of ketone bodies during energy restriction, pregnancy, and lactation, relying on NEFA released from adipose tissue (2,19).

Ketone Body Utilization

Ketone bodies produced in the liver are transported in the blood to extrahepatic tissues. Beta-hydroxybutyrate is oxidized to acetoacetate, which is then activated by joining with succinyl-CoA to form acetoacetyl-CoA and succinate, as catalyzed by 3-oxoacid-CoA-transferase (38). This enzyme is abundant in most tissues except the liver, explaining why the liver does not utilize the ketone bodies it produces. Acetoacetyl-CoA is cleaved in the presence of a free CoA to form two acetyl-CoA units. The first and last steps of this pathway are catalyzed by beta-hydroxybutyrate dehydrogenase and acetoacetyl-CoA thiolase, which appear to be abundant in all mammalian tissues (36).

Under normal, fed conditions the ruminant liver experienced a net acetoacetate uptake, and a net beta-hydroxybutyrate production (18). After three days of fasting, acetoacetate uptake reversed to production, and beta-hydroxybutyrate production increased more than ten-fold (20). This condition was similar in one-day fasted,

alloxan-diabetic sheep maintained at euglycemia with insulin injections. However, one-day fasted, untreated diabetic animals displayed elevated acetoacetate production rates, and beta-hydroxybutyrate production levels twenty-fold above normal. This accelerated ketogenesis following a lack of insulin concurs with insulin inhibition of adipose lipolysis at hormone-sensitive lipase (19).

When three day fasted animals were infused with a ketone, acetoacetate production reversed to net uptake, and beta-hydroxybutyrate production fell to 461 umol/min. This data represents the reversal of the fasting condition by the ketone to the fed state (11).

The mitochondrial location of beta-hydroxybutyrate dehydrogenase in rumen epithelium (28), and an increased $NADH/NAD^+$ ratio in the fed ruminant, favors a high beta-hydroxybutyrate:acetoacetate ratio. During progressive fasting, alimentary ketogenesis ceases and peripheral free fatty acid release increases to provide substrate for hepatic ketogenesis. Hypoinsulinemia accompanied by hyperglucagonemia induces free fatty acid release from adipose and subsequent hepatic oxidation. Elevated hepatic oxidation of free fatty acid contributes to an increased mitochondrial $NADH/NAD^+$ ratio, which, coupled with the cytosolic location of beta-hydroxybutyrate dehydrogenase in the hepatocyte, results in an increased acetoacetate:beta-hydroxybutyrate ratio (18,27).

Acetoacetate may be responsible for the central nervous system depression associated with ketosis (27). The lack of beta-hydroxybutyrate dehydrogenase in ruminant hepatocyte mitochondria may explain the high susceptibility of ruminants to ketosis. Under fasting conditions, the ruminant liver can not reduce the acetoacetate:beta-hydroxybutyrate ratio. This contrasts with non-ruminants such as the rat, where the enzyme is mitochondrial, and an increased mitochondrial $NADH/NAD^+$ ratio results in accelerated conversion of acetoacetate to beta-hydroxybutyrate, thereby having a detoxifying effect (27).

Acetoacetate utilization was concentration dependent up to about 1 mM, at which point maximal utilization was constant at $0.4 g/hr/kg^{3/4}$ (4,5). Acetoacetate contribution to total exhaled CO_2 increased from 2% under normal conditions to 20% during pregnancy ketosis (5), implying that utilization of acetoacetate during ketosis increased ten-fold. This suggests that overproduction rather than underutilization was responsible for the toxic effects of acetoacetate during ketosis.

Insulin and Glucagon

During periods of carbohydrate deprivation, fatty acids are oxidized in place of glucose, providing a glucose sparing effect. This is known as the Randle glucose-fatty acid cycle. Diabetics, however, experience abnormal fatty

acid metabolism, with elevated NEFA levels (Yasuhiro,90)(47). Insulin is required for the uptake of glucose by most mammalian tissues, and its deficit results in hyperglycemia with concomitant hyperlipidemia, as the body attempts to utilize fatty acids during glucose unavailability.

Ketone bodies are an important energy source for diabetics, and concentrations elevated greater than ten-fold normal levels are well documented (19). This suggests that insulin has an important role in the regulation of ketogenesis (34). Because insulin did not stimulate beta-hydroxybutyrate uptake in the perfused hindquarter of diabetic or starved rats, it can be proposed that the diabetic state displayed not only uncontrolled ketogenesis, but a lack of ketone body uptake as well, resulting in ketosis (22).

Keller et al. (25) stated that insulin exhibits two methods of ketogenesis inhibition: the suppression of hormone-sensitive lipase and a direct hepatic effect.

Somatostatin, an inhibitor of insulin and glucagon secretion, produced a significant decrease in hepatic glucose production. The results were similar when glucagon deficiency was achieved by somatostatin infusion accompanied by intraportal insulin infusion. This suggested that glucagon was necessary for the stimulation of hepatic glucose production. Indeed, insulin deficiency accompanied

by glucagon infusion resulted in a two-fold increase in glucogenesis (25).

Insulin deficiency in the presence of basal glucagon levels resulted in a 185% increase in hepatic ketogenesis within one hour. NEFA infusion induced a further increase. Hypoglucagonemia with exogenous insulin did not present a significant difference from control or bihormonal deficiency (25). Based on the elevated rates of hepatic ketogenesis observed when insulin deficiency was coupled with basal glucagon levels, it appeared that insulin had a modulating effect on ketone body production in three-day fasted dogs. The lack of an increase with selective glucagon deficiency suggested that insulin displayed a stronger suppressive role than glucagon did a stimulatory one.

Insulin infusion produced an increase in mitochondrial GPAT activity in isolated rat liver, as reported by Zammit (56), substantiating insulin's role in stimulating triglyceride synthesis while inhibiting oxidative pathways such as ketogenesis. Insulin lowered acetoacetate formation in ketotic twin-pregnant ewes within one-half hour of infusion (4). Insulin decreases hepatic NEFA uptake, which reverses ketosis in normal subjects. However, endogenous NEFA are readily available in subjects with fatty liver, and only administration of agents blocking the carnitine transport system can reverse ketosis in these subjects (34).

Insulin stimulates malonyl-CoA production by increasing

acetyl-CoA carboxylase activity. Malonyl-CoA inhibits fatty acid transport into the mitochondria via CPT-I. Therefore, insulin has an indirect inhibition on hepatic ketogenesis.

The contribution of NEFA to ketogenesis increased in diabetic humans displaying low insulin levels accompanied by elevated NEFA and ketone body concentrations (6). The elevated NEFA levels were possibly due to an insulin deficiency, which removed inhibition of hormone-sensitive lipase at the adipocyte. However, when NEFA was supplied by triacylglyceride emulsion infusion, mild hyperinsulinemia did not affect the production of ketone bodies from NEFA, which suggested that insulin had a stronger regulatory role at the adipocyte versus the hepatic site (6).

Beylot (6) proposed that insulin had no direct hepatic effect toward inhibiting ketogenesis. In human patients, the proportion of NEFA used for ketogenesis was unaltered by increasing insulin levels. It should be noted that insulin levels were physiological, and insulin may have had an effect at pharmacological levels (6).

Insulin secretion <u>in vivo</u> may be a factor of nutrient oxidation rates, including fatty acid oxidation rates. Short- term NEFA infusion in dogs resulted in insulin release, and stimulated insulin release in isolated pancreatic islets and in perfused pancreas (54). Yasuhiro and Grill (54) reported that fatty acid oxidation in the pancreatic islets inhibited insulin secretion. Intravenous

infusion of a 10% lipid emulsion in isolated perfused rat pancreas produced a marked increase in insulin secretion after 3 hours of administration, with the effect lessening over time until insulin secretion was inhibited after 48 hours. Inhibition occurred with normal and elevated glucose levels (54). If increased fatty acid oxidation stimulated insulin release, insulin had a negative feedback on peripheral lipolysis and acyl-CoA transport into the mitochondrial matrix.

Glucagon infusion caused a decrease in hepatic removal of ketone bodies in both normal and diabetic sheep (8). Glucagon was proposed to have a stimulatory effect on hepatic ketogenesis; possibly through the CPT-I mechanism (26). Glucagon diminishes the production of malonyl-CoA by inhibiting acetyl-CoA carboxylase activity. A decrease in glucagon removes the inhibition on acetyl-CoA carboxylase, allowing malonyl-CoA levels to rise in the presence of insulin. This interferes with the CPT-I system, and limits ketogenic capacity. Glucagon also stimulates hormone-sensitive lipase via cAMP-PK, elevating circulating NEFA concentrations, which makes more substrate available for ketone production.

Glucagon release was studied in an experiment involving infusion of a ketone following a fatty acid emulsion. The fat emulsion produced slight but not significant increases in glucagon release, which were not altered by subsequent

infusion of the ketone. Glucagon did not appear to be a regulatory mechanism in the inhibition of hepatic ketogenesis brought on by beta-hydroxybutyrate (45).

Glucagon infusion induced an increase in hepatic acetoacetate production but no change in beta-hydroxybutyrate production in two-day fasted dogs. Keller and Shulman (26) proposed that glucagon stimulated mitochondrial respiration; thereby lowering the beta-hydroxybutyrate:acetoacetate ratio. This suggested that the hyperglucagonemia and insulin deficiency of diabetes enhanced fatty acid oxidation and ketogenesis. Basal glucagon levels may not affect hepatic ketogenesis unless insulin deficiency is present (25). Given these conditions, the stimulation of ketogenesis can be related to mechanisms observed in acute diabetes. Glucagon injection in rats resulted in hepatic metabolism resembling that observed in starved animals (55).

Glucagon acted at the hepatic level of regulation to increase the proportion of NEFA converted to ketone bodies in human subjects (6). However, insulin was able to reverse glucagon's effect. A deficiency of insulin accompanied by hyperglucagonemia may contribute to ketosis through unchecked lipolysis and ketogenesis (8).

Malonyl-CoA

Malonyl-CoA is a strong reversible inhibitor of CPT-I (13,15), the mechanism responsible for initiating the transport of free fatty acids across the inner mitochondrial membrane so that oxidation may occur. Both malonyl-CoA concentrations and acetyl-CoA carboxylase activity are decreased during ketosis (56), which removes direct inhibition of CPT-I. However, Cook et al. (10) suggested that reduced sensitivity of CPT-I to malonyl-CoA inhibition, rather than malonyl-CoA concentrations, may be a more important regulator in starved rats. Malonyl-CoA concentrations were inversely related to ketogenic rates in fed rats, but not in starved rats. Starved rats required much higher levels of malonyl-CoA in order to produce inhibition of ketogenesis (10). Cook (10) proposed that this was due to increased rates of malonyl-CoA deacylates during starvation, but cultures showed equal rates of malonyl-CoA loss in fed and starved states. It appears that the liver is less sensitive to malonyl-CoA inhibition in the starved state. Zammit (56) proposed that mitochondrial CPT-I may respond to malonyl-CoA and "remember" hepatic malonyl-CoA concentrations either by covalent modification (ie. phosphorylation of the CPT-I) or by binding of an effector molecule to the CPT-I.

Malonyl-CoA inhibition of CPT-I is readily reversible, and can be overcome by high concentrations of palmitoyl-CoA.

Muscle mitochondria are more sensitive to malonyl-CoA inhibition than are liver mitochondria (13).

Malonyl-CoA concentrations varied with diet, exhibiting an increase during carbohydrate feeding when liver metabolism was directed toward fatty acid synthesis (36). As hepatic malonyl-CoA concentrations rise, fatty acid oxidation is inhibited (21). Insulin release is stimulated, promoting the breakdown of carbohydrate to acetyl-CoA and subsequent conversion of acetyl-CoA to malonyl-CoA (35). Acetyl-CoA carboxylase, the enzyme responsible for this step, is stimulated by insulin and inhibited by glucagon (21). High levels of malonyl-CoA inhibit CPT-I, in essence shutting down fatty acid oxidation (36). In contrast, starvation is characterized by elevated glucagon release, which lowers malonyl-CoA concentrations and suppresses fatty acid synthesis. Increased flux of acyl groups into the hepatic mitochondria stimulates fatty acid oxidation and ketogenesis (36).

McGarry and Foster (35) treated male rats with glucagon and 5-(tetradecyloxy)-2-furoic acid (RMI 14,514); both inhibitors of hepatic malonyl-CoA synthesis; and reported that malonyl-CoA suppression accompanied by elevated carnitine concentrations in the liver contributed to increased ketogenesis in animals fed glucose, lactate and pyruvate in order to maximize fatty acid synthesis. Enhancement of fatty acid oxidation by glucagon and RMI

14,514 was not as prevalent when rats were fed high concentrations of oleic acid. It is possible that oleate had an inhibitory effect on acetyl-CoA carboxylase, thereby lowering malonyl-CoA production. Therefore, the long chain fatty acid relieved malonyl-CoA inhibition on fatty acid oxidation.

Methyl Palmoxirate

Methyl palmoxirate is a fatty acid analog of palmitate which irreversibly blocks CPT-I (32), inhibiting the oxidation of long chain fatty acids, but not short chain fatty acids or palmitoyl-carnitine (48). Because fatty acid oxidation results in decreased hepatic glucose utilization and increased gluconeogenesis in species with cytosolic PEPCK, methyl palmoxirate has an insulin-independent effect of lowering glucose concentrations by stimulating glucose utilization while decreasing glucose production. This results in lowered blood glucose and ketone concentrations (48). The liver appears to be more sensitive to methyl palmoxirate than is muscle (48).

A human diabetic patient in severe hyperglycemia and insulin resistance responded to oral methyl palmoxirate with reduced fasting plasma glucose concentrations from 463 to 306 mg/dl. The respiratory quotient increased from .71 to .84, implying that carbohydrate oxidation increased during treatment. Glucose production was lowered, and plasma NEFA

concentrations fell (32).

Oral methyl palmoxirate reduced plasma ketone concentrations 83 to 93% in fasted rats 4 to 6 hours after administration. Ketones were significantly lowered with oral doses ten-fold less than those required to significantly reduce blood glucose, suggesting that methyl palmoxirate is beneficial in the treatment of ketosis (48).

Carnitine

Carnitine is essential to fatty acid translocation into the mitochondria (34). Cardiac and skeletal muscle rely heavily on fatty acids as an energy source, and therefore require carnitine (40). Palmitic acid and other long chain fatty acids require carnitine as a transporter across the mitochondrial membrane (39). Species lacking in CPT-I, such as the blowfly, are incapable of oxidizing long chain fatty acids (36).

Carnitine enhances fatty acid oxidation in vitro (16). Isolated rat mitochondria with a carnitine deficiency presented inhibited ketogenesis (10). Addition of carnitine to bovine liver slices and ovine hepatocytes increased NEFA oxidation (15). However, in hepatocytes from fasted sheep, additional carnitine did not increase NEFA oxidation where endogenous carnitine levels were already elevated (15). Carnitine infused into the cranial mesenteric did not increase hepatic ketogenesis in diabetic ewes (52).

Carnitine concentrations are increased in the liver of lactating dairy cows as opposed to non-lactating cows, and fasted sheep versus fed sheep.

Carnitine may be obtained from the diet or synthesized by the body (42). It is excreted in the blood, urine and milk (16). During periods of stress or starvation, proteolysis results in an increase in circulating amino acids (3). Trimethyllysine is released from skeletal muscle and taken up by the kidney, where it is converted to gamma-butyrobetaine (15) (Figure 5). Gamma-butyrobetaine is then rapidly converted to carnitine in the liver and transported to extrahepatic tissues (40). It has been proposed that acetyl-carnitine is transported via the blood between the muscle and liver (14).

Stress increased carnitine synthesis by the liver (16). Plasma free carnitine levels in human subjects decreased during exercise, as free carnitine was incorporated into acetyl-carnitine due to fat oxidation (14). An increase in protein turnover produces a subsequent increase in trimethyllysine from protein, possibly stimulating carnitine biosynthesis (40).

Perfused rat intestine and liver absorbed gamma-butyrobetaine, but kidney did not (40). Male rats supplemented with 1% dietary gamma-butyrobetaine displayed elevated L-carnitine levels, suggesting that gamma-butyrobetaine was absorbed by the intestine, taken up by the

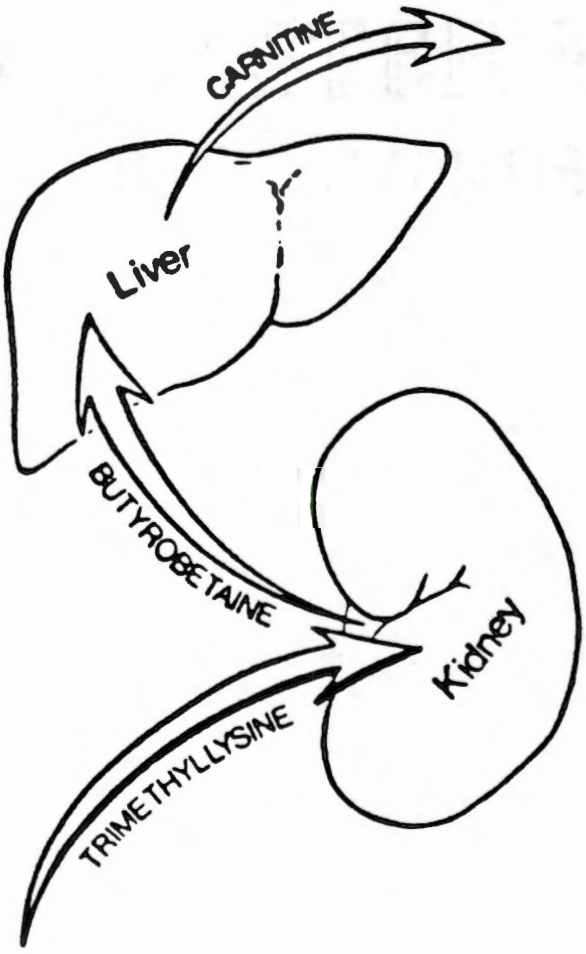


Figure 5: Synthesis of carnitine (Frenkel, 1980).

liver, and converted to L-carnitine.

When hepatocytes of fed rats were exposed to glucagon and RMI 14,514, fatty acid oxidation and ketogenesis were increased. Subsequent addition of carnitine caused ketogenesis to increase to rates observed in fasted animals (35). Carnitine concentrations as low as 10^{-5} M increased long chain fatty acid oxidation in cardiac muscle (9). This data suggested that carnitine accelerated ketogenesis through enhancement of the CPT-I transport mechanism. Although carnitine acyl-transferases have been identified in most mammalian tissues (36), CPT-I is specific to liver, heart, kidney and skeletal muscle mitochondria (21).

Beta-hydroxybutyrate Infusion

Beta-hydroxybutyrate infusion decreased NEFA concentrations in fasted and diabetic goats and sheep (16), and increased insulin levels in normal sheep (19). Elevated insulin may inhibit adipose lipolysis, thereby lowering the level of ketone bodies produced from NEFA (16).

In diabetic, insulin-treated sheep, ketone infusion had a significant effect in returning accelerated ketogenesis to rates observed in the fed state. Even in a diabetic, untreated ewe, ketone infusion significantly reduced beta-hydroxybutyrate production and reversed acetoacetate production to net uptake (11). Beta-hydroxybutyrate infusion may reduce hepatic ketogenesis by inhibiting CPT-I.

Three mechanisms are possible: a decrease in pancreatic glucagon production, the stimulation of acetyl-CoA carboxylase, and the direct inhibition of CPT-I by binding (11).

Infusion of beta-hydroxybutyrate resulted in a significant decrease in arterial plasma NEFA concentrations in alloxanized sheep and dogs (30). Heitmann and Fernandez (19) reported lowered NEFA levels and decreased hepatic uptake of NEFA following beta-hydroxybutyrate infusion in normal, diabetic insulin-treated, and diabetic untreated sheep. Ketone infusion lowered hepatic extraction ratios in normal and diabetic insulin-treated sheep, but not in diabetic untreated sheep (19), which suggested that insulin had an inhibitory effect on NEFA uptake by the liver.

Hepatic NEFA uptake increased 70% in diabetic untreated sheep due to elevated free fatty acid levels, resulting in constant hepatic extraction ratios (19). Madison (30) reported arterial NEFA concentration decreases of over 50% following femoral vein ketone infusion, which suggested that due to the similarity of the ketone infusion and insulin infusion, the ketone may have produced a decrease in free fatty acid by stimulating insulin secretion.

Beta-hydroxybutyrate infusion in partially diabetic alloxanized dogs resulted in lowered arterial glucose and NEFA concentrations. A 196% rise in pancreatic-venous insulin concentration was measured after 80 minutes of beta-

hydroxybutyrate or acetoacetate infusion at 2.46 mmol/min via the femoral vein (30).

Pancreatic-arterial infusion of ketone bodies at 0.125 mmol/min resulted in rapid release of insulin from the pancreas. Pancreatic-venous insulin levels rose 142% within 15 minutes and 800% by 80 minutes (30). The immediate rise in insulin concentrations following ketone infusion may have been responsible for the hypoglycemia observed with the hyperketonemia (30). The decrease in blood glucose levels in partially diabetic alloxanized dogs with elevated blood glucose levels due to induced hyperketonemia, coupled with no change in depancreatized dogs with near normal blood glucose levels, led Madison et al. (30) to propose that the release of insulin due to hyperketonemia is not similar to glucose action.

Lipid Infusion

Lipid and heparin infusion resulted in an increase in both arterial NEFA concentrations and hepatic free fatty acid uptake, which was not affected by the presence or absence of both pancreatic hormones. This increase in substrate availability induced a 149% increase in hepatic ketogenesis, mostly attributable to increased beta-hydroxybutyrate production (25). Lipid infusion also stimulated lipoprotein lipase activity (39).

Infusion of fatty acid emulsion and heparin caused no

significant change in plasma glucagon or insulin levels, but resulted in a four to five-fold increase in glycerol concentration due to lipoprotein lipase stimulation and precursor supply. Keller et al. (25) used glycerol concentrations to monitor lipolysis, and reported no significant change in arterial glycerol concentration with hormone fluxes. Glycerol uptake by the liver followed first order kinetics despite hormone level alterations.

Oleic acid infusion in fed, diabetic untreated sheep caused a significant increase in ketogenesis, whereas octonoate infusion did not (50). This suggests that CPT-I is involved in the regulation of ketogenesis, since the long chain fatty acid is transported by CPT-I, but the medium chain fatty acid is not (33).

In addition, beta-hydroxybutyrate infusion on top of the fat emulsion reduced ketogenesis only with the long chain fatty acid, suggesting that the C 18 was regulated at CPT-I (50). The purpose of this study was to establish if gamma-butyrobetaine would be taken up by the liver and converted to carnitine, and if the exogenous carnitine would reverse the inhibition of hepatic ketogenesis brought on by beta-hydroxybutyrate infusion in alloxan-diabetic ewes.

CHAPTER III

MATERIALS AND METHODS

Animals

A possible insulin-independent regulatory mechanism of hepatic ketogenesis was studied in eight experiments conducted on five non-pregnant, non-lactating, alloxan-diabetic crossbred ewes weighing 46 to 103 kg. Animals were housed individually in 1.8 x 3.0 m indoor pens under natural lighting with ambient temperature maintained between 17 and 25 °C. Commercially prepared alfalfa pellets were fed according to the National Research Council recommendation in two daily aliquots of 450 g at 0800 and 1700 h. Water and trace mineralized salt were provided ad libitum. Animals were accustomed to laboratory personnel prior to any experimentation and only mild restraint was required for sample collection.

Surgery

Catheters were surgically positioned in hepatic, portal (23) and femoral veins, a femoral artery (57), and two mesenteric veins of each animal, so that the tips resided in the hepatic vein, portal vein, caudal vena cava, caudal aorta, and the common mesenteric vein (see Appendix 1). Blood samples from the caudal aorta and caudal vena cava were used in arterial and venous determinations of glucose,

NEFA, acetoacetate and beta-hydroxybutyrate. Hepatic and portal vein blood samples were used to measure liver and portal-drained visceral fluxes of glucose, NEFA, acetoacetate, and beta-hydroxybutyrate. The common mesenteric vein served for infusion of para-aminohippuric acid (PAH, Eastman-Kodak Co., Rochester, N.Y.) for whole blood flow rate determinations.

Diabetes

Animals were allowed at least ten days to recover from surgery, at which time ewes were made diabetic with a single intravenous dose of alloxan (Sigma Chemical Co.) at 50 mg/kg body weight at 1700 h. Alloxan specifically destroys the beta cells of the pancreas, inducing diabetes (43). This allowed the study of insulin-independent effects. Animals were closely monitored over the subsequent 24 hour period to ensure a diabetic state, confirmed by arterial glucose concentrations. Daily insulin injections of Iletin-100 were administered subcutaneously at 1300 h to reestablish and maintain euglycemia (2.5 to 3.5 mM). Insulin was withheld 3 days prior to each experiment, inducing a ketotic state for the study of insulin-independent effects. Feed intake was noticeably decreased by the third day of insulin withdrawal.

Experiments

On the experimental day, infusion consisted of three superimposed periods, with all infusates into the common mesenteric vein (see Appendix 2). Each period was two hours in length, with the initial hour for equilibration of each additional infusate and one hour for sample collection. The equivalence of a 30 minute infusion for each new infusate was pulsed into the infusion site at the commencement of each period. The control period began at 0700 h with PAH infusion (1.5% Eastman-Kodak Co.) into the first of the two mesenteric veins at 0.764 ml/min (Model 940, Harvard Apparatus). During the second period, PAH was infused as in the first period, while dl-beta-hydroxybutyrate was infused into the same mesenteric vein at 1.18 mM/min (1.5445 M solution, Sigma Chemical Co.). Finally, the last period consisted of PAH and dl-beta-hydroxybutyrate infused into the first mesenteric vein, while gamma-butyrobetaine (1.5445 M, Sigma Chemical Co.) was introduced into the second mesenteric vein. Gamma-butyrobetaine was infused at a rate equimolar to the BOHB infusion.

Following the 1 hour equilibration period, 12 ml blood samples were drawn simultaneously from each of the four remaining catheter sites at 30 minute intervals. This procedure was performed as each new infusate was administered. Samples were drawn into sterile syringes containing 0.4 ml 6% NaEDTA, gently mixed, and immediately

chilled on ice. Throughout the experiment catheter patency was maintained with 6% NaEDTA solution. NaEDTA was used as an anti-coagulant instead of heparin, because heparin activates lipoprotein lipase and results in false high NEFA values. All analyses were completed on the day of the experiment, except for insulin. Plasma was separated and frozen for future analysis of insulin.

Analyses

Whole blood was then analyzed for acetoacetate and beta-hydroxybutyrate on all samples, and PAH on all but femoral venous samples. Plasma was analyzed for NEFA on all samples. Packed cell volume, blood glucose and plasma insulin values were determined only on femoral arterial samples. If arterial insulin concentrations had been significant, portal insulin samples would have been analyzed to determine portal minus arterial values describing pancreatic insulin release. Plasma percent was determined by subtraction of the hematocrit value from 1.

Analysis for ketone bodies, i.e. beta-hydroxybutyrate and acetoacetate, was performed on 2 ml whole blood in cold 1 M perchloric acid (53). Glucose was determined by spectrophotometer using a commercial kit (Sigma Chemical Co.) and 200 ul whole blood. One ml of whole blood was analyzed for PAH (24). A Wako NEFA-C kit (Wako Chemical Co.) was used for the enzymatic assay of non-esterified

fatty acids. Arterial insulin concentrations were determined from 200 ul of serum stored frozen, and a human insulin kit (DPC #TKIN1). NaEDTA has a tendency to cause false high insulin values with this kit. An equivalent volume of NaEDTA was added to a second standard curve to account for NaEDTA presence in the samples. The regression equation for the second standard curve was: $y = 1.6206 + .6580\ x$, $r = .9934$.

Calculations

The rate of PAH infusion (optical density units/min) was divided by the venoarterial PAH concentration difference (optical density units/L) across each tissue to determine the blood flow rates. Blood flow rates for hepatic and portal veins were measured directly and the difference between these two values was the hepatic arterial flow. Multiplying blood flow rates by plasma percent determined the plasma flow rates.

The venoarterial concentration differences across the tissue multiplied by the respective blood flow rate equaled the net flux. A net release is indicated by positive values, whereas a net uptake is indicated by negative values. Hepatic extraction ratios were calculated as the fraction of the metabolite taken up by the organ relative to the total concentration presented to the organ per unit time. Significant differences between treatments would

indicate intraorgan effects independent of blood flow and concentration.

Statistical Analysis

Data was analyzed by comparing subsequent means of consecutive treatments without interference to any other treatments. Means were separated by an unpaired t-test within and among sheep and significance was considered at the levels of ($p<.05$) and ($p<.01$), to determine the significance of the infusates, dl-beta-hydroxybutyrate and gamma-butyrobetaine, on energy metabolite concentrations and fluxes.

CHAPTER IV

RESULTS

Whole Blood Flow

Whole blood flow rates for the portal and hepatic veins as well as the portal to hepatic vein blood flow ratio are shown in Table 1. Sequential infusions had no significant effect on portal blood flow rates, which ranged from 2.1 to 2.5 L/min. Hepatic blood flow was decreased from 3.6 to 2.5 L/min ($P<.01$) as a result of beta-hydroxybutyrate infusion. Gamma-butyrobetaine infusion had no effect. The portal to hepatic ratio was low at 73% in the control period (20), probably due to decreased portal circulation in the fasted state and increased hepatic artery contribution bringing peripheral NEFA to the liver. This ratio increased from 73% to 83% ($p<.01$) after beta-hydroxybutyrate infusion, explained by increased portal vein contribution as more blood was drained from splanchnic tissues as opposed to peripheral tissues. This could be due to exogenous beta-hydroxybutyrate from the mesenteric vein reversing the control. Gamma-butyrobetaine had no effect on the portal to hepatic ratio or blood flow rates.

Glucose and Plasma Percent

Arterial glucose was not significantly altered during the sampling period, remaining at 10 mM (Table 2); basically

TABLE 1

EFFECTS OF SEQUENTIAL BETA-HYDROXYBUTYRATE AND GAMMA-BUTYROBETAINE INFUSIONS ON BLOOD FLOW RATES AND PORTAL HEPATIC FLOW RATIOS IN UNTREATED, ALLOXAN-DIABETIC EWES[1]

TREATMENT	PORTAL VEIN BLOOD FLOW	HEPATIC VEIN BLOOD FLOW	PORTAL/ HEPATIC RATIO
	----------(L/min)----------		--(%)--
CONTROL	2.5 ±.2	3.6 ±.2	73 ±3
BOHB	2.1 ±.2	2.5** ±.2	83** ±2
pSTD[2]	.2	.3	4
GBB	2.2 ±.1	2.9 ±.2	79 ±3
pSTD[3]	.2	.3	3

1. means ± standard error of means
2. pooled standard deviation of control and BOHB
3. pooled standard deviation of BOHB and GBB
* Significantly different from the preceding infusion period, $p < .05$.
** Significantly different from the preceding infusion period, $p < .01$.

TABLE 2

EFFECTS OF SEQUENTIAL INFUSION OF BETA-HYDROXYBUTYRATE AND GAMMA-BUTYROBETAINE ON MEANS OF ARTERIAL CONCENTRATIONS FOR GLUCOSE AND PLASMA PERCENT IN UNTREATED, ALLOXAN-DIABETIC EWES[1]

TREATMENT	GLUCOSE (mM)	PLASMA PERCENT
CONTROL	10.01 ±.32	.74 ±.01
BOHB	9.84 ±.30	.76 ±.01
pSTD[2]	.43	.01
GBB	9.98 ±.26	.76 ±.01
pSTD[3]	.39	.01

1. means ± standard error of means
2. pooled standard deviation of control and BOHB
3. pooled standard deviation of BOHB and GBB
* Significantly different from the preceding infusion period, $p < .05$.
** Significantly different from the preceding infusion period, $p < .01$.

a three fold elevation of normal levels in non-diabetic sheep (20). Sequential infusions of beta-hydroxybutyrate and gamma-butyrobetaine had no effect on plasma glucose.

Plasma percent (Table 2) ranged between 74 and 76; slightly high. Blood withdrawal did not result in a significant change in plasma percent, meaning that blood sampling did not stress the animals.

Non-esterified Fatty Acids

Infusion of beta-hydroxybutyrate resulted in a 50% reduction in arterial NEFA concentrations from 2144 to 1067 μmol ($p<.01$, Table 3). Hepatic net uptake, indicating removal of NEFA from the blood, decreased from 658 to 240 μmol/min ($p<.01$) upon beta-hydroxybutyrate infusion. Total splanchnic uptake of NEFA also decreased 71% during beta-hydroxybutyrate infusion ($p<.01$). Portal-drained-visceral release of NEFA was unaltered. Calculated hepatic extraction ratios ranged from 9% to 6%, with no significant change throughout the study. Sequential infusion of gamma-butyrobetaine had no effect on NEFA concentrations or net fluxes. Beta-hydroxybutyrate and gamma-butyrobetaine had no significant effect ($p>.05$) on NEFA femoral venoarterial differences.

TABLE 3

EFFECTS OF SEQUENTIAL BETA-HYDROXYBUTYRATE AND GAMMA-BUTYROBETAINE INFUSION ON BLOOD PLASMA NEFA ARTERIAL CONCENTRATIONS, NET FLUX RATES, AND HINDQUARTER VENOARTERIAL DIFFERENCES IN UNTREATED, ALLOXAN-DIABETIC EWES[1]

TREATMENT	ARTERIAL	PORTAL DRAINED VISCERAL	HEPATIC	HEPATIC EXTRACTION RATIO	TOTAL SPLANCHNIC	FEMORAL VEIN - ARTERY
	-(μmol)-	---(μmol/min)----		---(%)---	(μmol/min)	-(μmol)-
CONTROL	2144	59	-658	9	-599	110
	±97	±61	±117		±129	±29
BOHB	1067**	66	-240**	6	-174**	116
	±137	±28	±30		±39	±28
pSTD[2]	164	69	123		137	41
GBB	1175	8	-217	7	-209	95
	±130	±25	±23		±29	±20
pSTD[3]	184	39	39		50	35

1. means ± standard error of means
2. pooled standard deviation of control and BOHB
3. pooled standard deviation of BOHB and GBB
* Significantly different from the preceding infusion period, $p < .05$.
** Significantly different from the preceding infusion period, $p < .01$.

Acetoacetate

Arterial acetoacetate concentrations were 1167 μmol during the control period and increased to 1503 μmol ($p<.05$, Table 4) following beta-hydroxybutyrate infusion. Portal drained visceral net release of acetoacetate was 98 μmol/min during the control period, indicative of alimentary ketogenesis. Portal drained visceral release was unaltered by infusion. Infusion of beta-hydroxybutyrate decreased hepatic net release of acetoacetate from 508 to 102 μmol/min ($p<.01$). Because of the significant decrease in hepatic release of acetoacetate following beta-hydroxybutyrate infusion, total splanchnic release of acetoacetate also decreased from 605 to 70 μmol/min ($p<.01$). Femoral venoarterial differences always showed a net uptake of acetoacetate by the hindquarter with no significant change during any infusion period. Blood flow was not measured across the rump. However, because portal vein contribution to blood flow increased during ketone infusion, peripheral blood flow rates could not have increased concomitant to a splanchnic increase, assuming a constant cardiac output. Therefore, it was assumed that hindquarter blood flow was unaltered. Subsequent gamma-butyrobetaine infusion had no effect on any acetoacetate measurements.

TABLE 4

EFFECTS OF SEQUENTIAL BETA-HYDROXYBUTYRATE AND GAMMA-BUTYROBETAINE INFUSION ON ACETOACETATE ARTERIAL CONCENTRATIONS, NET FLUX RATES, AND HINDQUARTER VENOARTERIAL DIFFERENCES IN UNTREATED, ALLOXAN-DIABETIC EWES[1]

TREATMENT	ARTERIAL	PORTAL DRAINED VISCERAL	HEPATIC	TOTAL SPLANCHNIC	FEMORAL VEIN ARTERY
	---(μmol)---	----------(μmol/min)----------			-(μmol)-
CONTROL	1167	98	508	605	-36
	±108	±52	±95	±113	±28
BOHB	1503*	-33	102**	70**	-64
	±126	±42	±54	±44	±25
pSTD[2]	163	68	112	124	38
GBB	1468	35	96	132	-70
	±133	±28	±26	±44	±19
pSTD[3]	180	52	61	64	32

1. means ± standard error of means
2. pooled standard deviation of control and BOHB
3. pooled standard deviation of BOHB and GBB
* Significantly different from the preceding infusion period, $p < .05$.
** Significantly different from the preceding infusion period, $p < .01$.

Beta-hydroxybutyrate

Arterial beta-hydroxybutyrate concentrations were 7582 μmol during the control period and increased to 12,349 μmol ($p<.01$, Table 5) due to beta-hydroxybutyrate infusion. Portal drained visceral net release of beta-hydroxybutyrate indicated alimentary ketogenesis of beta-hydroxybutyrate and was not affected by any infusion. Conversely, hepatic net release of beta-hydroxybutyrate was decreased by more than 50% from 1586 to 700 μmol/min with beta-hydroxybutyrate infusion ($p<.01$). Total splanchnic net beta-hydroxybutyrate flux was not affected by any infusion period. Femoral venoarterial differences always showed a net uptake of beta-hydroxybutyrate by the hindquarter. Beta-hydroxybutyrate venoarterial difference increased from 31 to 439 μmol ($p<.01$) following ketone infusion. Subsequent infusion of gamma-butyrobetaine had no effect on net hepatic beta-hydroxybutyrate production.

Insulin

Arterial insulin concentrations are shown in Table 6. Concentrations were small but different from zero, ranging from 0.46 to 3.80 μIU/ml. This was similar to Fernandez (19) and may represent cross reaction of the technique. The mean of all concentrations throughout the control period was 1.63 ± .48 μIU/ml, and did not change significantly during either infusion, so that all insulin means were combined,

TABLE 5

EFFECTS OF SEQUENTIAL BETA-HYDROXYBUTYRATE AND GAMMA-BUTYROBETAINE INFUSION ON BETA-HYDROXYBUTYRATE ARTERIAL CONCENTRATIONS, NET FLUX RATES, AND HINDQUARTER VENOARTERIAL DIFFERENCES IN UNTREATED, ALLOXAN-DIABETIC EWES[1]

TREATMENT	ARTERIAL	PORTAL DRAINED VISCERAL	HEPATIC	TOTAL SPLANCHNIC	FEMORAL VEIN ARTERY
	---(μmol)---	----------(μmol/min)----------			-(μmol)-
CONTROL	7582	532	1586	2119	31
	±330	±240	±227	±361	±71
BOHB	12349**	597	700**	1297	-439**
	±564	±241	±158	±306	±106
pSTD[2]	639	348	283	484	131
GBB	13051	466	1260	1726	-268
	±544	±257	±337	±353	±179
pSTD[3]	767	360	381	477	213

1. means ± standard error of means
2. pooled standard deviation of control and BOHB
3. pooled standard deviation of BOHB and GBB
* Significantly different fromn the preceding infusion period, $p < .05$.
** Significantly different from the preceding infusion period, $p < .01$.

TABLE 6

EFFECTS OF SEQUENTIAL INFUSION OF BETA-HYDROXYBUTYRATE AND GAMMA-BUTYROBETAINE ON MEANS OF ARTERIAL CONCENTRATIONS OF INSULIN IN UNTREATED, ALLOXAN DIABETIC EWES[1]

TREATMENT	INSULIN (μIU/ml)
Control	1.63
	±.48
BOHB	1.04
	±.46
pSTD[2]	.68
GBB	1.26
	±.36
pSTD[3]	.60

1. means ± standard error of means
2. pooled standard deviation of control and BOHB
3. pooled standard deviation of BOHB and GBB
* Significantly different from the preceding infusion period, $p < .05$.
** Significantly different from the preceding infusion period, $p < .01$.

resulting in a concentration of 1.25 ± .17 μIU/ml. Since all other metabolites indicated successful alloxan diabetes, portal insulin was not measured.

CHAPTER V

DISCUSSION

This study was designed to evaluate the role of gamma-butyrobetaine in restoring hepatic ketogenesis following beta-hydroxybutyrate inhibition in insulin withdrawn alloxan-diabetic ewes. Arterial concentrations and net portal drained visceral and hepatic fluxes and femoral venoarterial differences of NEFA, acetoacetate and beta-hydroxybutyrate were measured in untreated, alloxan-diabetic ewes, as well as arterial concentrations of glucose and insulin.

Infusion of beta-hydroxybutyrate into insulin-withdrawn, diabetic ewes resulted in inhibition of hepatic beta-hydroxybutyrate production ($p<.01$) and reversal of acetoacetate production to hepatic uptake ($p<.01$)(11). This suggested that neither a functioning pancreas nor exogenous insulin were required for the autoregulation of ketogenesis. Infusion of oleic acid increased hepatic release of both acetoacetate and beta-hydroxybutyrate ($p<.05$), whereas octanoate infusion altered neither ketone production level. Infusion of beta-hydroxybutyrate following the oleic acid infusion decreased hepatic production ($p<.05$), suggesting that CPT-I is a possible regulatory mechanism (45).

Wells (50) proposed that carnitine, because it contributes to the CPT-I transport mechanism, may reverse the inhibition of ketogenesis brought on by beta-

hydroxybutyrate infusion. Carnitine did not restore hepatic ketogenesis when infused at a rate equimolar to beta-hydroxybutyrate infusion; however, assays revealed that infused carnitine was not removed by the liver.

This study proposed that gamma-butyrobetaine is taken up by the liver upon infusion, and converted to carnitine; thereby stimulating the CPT-I transport mechanism and reversing any inhibition of hepatic ketogenesis brought on by beta-hydroxybutyrate infusion.

Alloxan Diabetes

Mean arterial insulin concentrations ranged from 0.46 to 3.80 μIU/ml over the eight experiments (Table 6), with the average of the means being 1.25 ± .17 μIU/ml, and significantly different from zero, suggesting that either some Ilentin-100 insulin remained or some cross reactivity to the technique occurred.

The mean insulin concentration for the eighth experiment was 3.80 ± .98 μIU/ml, significantly higher than the other experiments, indicating perhaps that this ewe retained some Ilentin-100 from her last injection prior to the withdrawal period. However, NEFA, acetoacetate, and beta-hydroxybutyrate results were not different from the other seven experiments.

Arterial glucose concentrations were 10 mM, well above normal levels (20), indicating that the animals were indeed

diabetic. Arterial concentrations of acetoacetate, beta-hydroxybutyrate and NEFA were elevated relative to normal sheep (18,20), also suggesting that these animals were in an insulin-deficient state.

A diabetic state ensures that acetyl-CoA carboxylase will not be stimulated by endogenous insulin, which would promote conversion of acetyl-CoA to malonyl-CoA, producing an inhibitory effect on CPT-I (16), i.e., fat transport across the inner mitochondrial membrane would decrease. An insulin-withdrawn diabetic state removes all effects of exogenous insulin, and forces the animal to rely heavily on ketones as an energy source.

In a previous study, a decrease in glucagon release was eliminated as a possible mechanism contributing to the beta-hydroxybutyrate inhibition of CPT-I (46). If glucagon levels decrease, lowered stimulation of hormone-sensitive lipase results in lower concentrations of circulating NEFA, making less NEFA available for ketogenesis. Also, a decrease in glucagon levels results in lowered inhibition of acetyl-CoA carboxylase, resulting in increased malonyl-CoA production, which inhibits CPT-I. However, Sensenig (45) observed a slight increase in portal drained visceral release of glucagon, concomitant to beta-hydroxybutyrate inhibition of hepatic ketogenesis, which suggested that glucagon was not a contributing factor to regulation.

Plasma Percent

Plasma percent was not altered during the experimental period, suggesting that the volume of blood sampled presented no stress to the ewes.

Portal and Hepatic Blood Flow

Portal vein blood flow showed no change following addition of either infusates, whereas hepatic vein blood flow decreased ($p<.01$) following beta-hydroxybutyrate infusion. This resulted in an increased ($p<.01$) portal to hepatic ratio (Table 1). An increased portal to hepatic vein ratio implies that contribution from the hepatic artery was lessened. This could be explained by the exogenous supply of nutrients infused into the mesenteric vein during the second period, causing the portal vein contribution to be increased relative to the hepatic artery since the portal vein is carrying nutrients due to the ketone infusion. Concomitant to the increase in nutrients from the portal vein, the decrease in arterial NEFA concentration ($p<.01$) may account for lowered hepatic vein blood flow, as less nutrients come from the systemic circulation as opposed to alimentary circulation.

Acetoacetate

Arterial acetoacetate concentrations were elevated relative to normal sheep (18) and increased ($p<.05$)

following beta-hydroxybutyrate infusion. This increase in arterial levels represented conversion of beta-hydroxybutyrate to acetoacetate within the animal.

Portal drained visceral release of acetoacetate during the control period was indicative of alimentary ketogenesis from rumen fermentation of organic matter and volatile fatty acids, especially butyrate. However, variation was high between sheep, since some of the ewes decreased feed intake by the third day of insulin withdrawal. Consequently, none of the portal-drained visceral acetoacetate flux rates were significantly different with infusate treatments.

Hepatic release of acetoacetate during the control period was 508 μmol/min (Table 4) and similar to values reported in diabetic, untreated ewes (12,19,46,51,52). Infusion of beta-hydroxybutyrate decreased hepatic ketogenesis as observed by Dawes (12), Heitmann (19), and Sensenig (46), but not Wells (51,52). However, Wells reported arterial insulin concentrations of 13.4 μIU/ml, suggesting that the ewes were not in complete insulin-withdrawal.

Total splanchnic release of acetoacetate dropped from 605 to 70 μmol/min ($p<.01$), depicting a net decrease in acetoacetate release by the gut and liver following ketone infusion, due to lowered production of acetoacetate by the liver.

Femoral venoarterial differences for acetoacetate were

not significantly different from zero, but did not differ between infusion periods, suggesting that the rump did take up acetoacetate. Uptake by the hindquarter was not altered by any infusion.

Infusion of gamma-butyrobetaine did not alter acetoacetate concentrations or flux rates to a significant level.

Beta-hydroxybutyrate

Arterial beta-hydroxybutyrate concentrations were elevated relative to normal sheep (18) and increased ($p<.01$, Table 5) following beta-hydroxybutyrate infusion. This increase represents the ketone being infused. All other values were corrected for ketone infusion.

Beta-hydroxybutyrate portal drained visceral fluxes were positive, indicative of alimentary ketogenesis from rumen fermentation of organic matter, and were unaltered by infusions, suggesting that infusion did not decrease beta-hydroxybutyrate production by the gut.

Hepatic release of beta-hydroxybutyrate during the control period was 1586 μmol/min and similar to values reported on diabetic untreated ewes (12,19,46,51,52). Infusion of beta-hydroxybutyrate decreased hepatic ketogenesis as observed by Dawes (12), and Heitmann (19), but not Wells (51,52). However, Wells has arterial insulin concentrations of 13.4 μIU/ml, suggesting that the ewes were

not in complete insulin-withdrawal.

Total splanchnic release of beta-hydroxybutyrate was not altered significantly, despite the reduction in hepatic release, although there was a numerical decline.

Hindquarter uptake of beta-hydroxybutyrate was negative. It increased with infusion, suggesting that exogenous beta-hydroxybutyrate provided a significant energy source for the hindquarter.

Gamma-butyrobetaine infusion had no significant effects on beta-hydroxybutyrate concentrations or flux rates.

Non-esterified Fatty Acids

Arterial NEFA concentrations were significantly decreased ($p<.01$, Table 3) by beta-hydroxybutyrate infusion. It has been proposed that beta-hydroxybutyrate may have an autoregulatory effect on hepatic ketogenesis by inactivating hormone-sensitive lipase and thus lowering circulating NEFA concentrations (15). However, venoarterial differences across the rump in this study showed no significant changes in NEFA concentrations after beta-hydroxybutyrate infusion, suggesting that beta-hydroxybutyrate had no effect on peripheral lipolysis. Dawes (11) demonstrated that beta-hydroxybutyrate infusion decreased NEFA flux across the rump in normal and diabetic insulin-treated ewes but not in diabetic insulin-withdrawn ewes, suggesting that the mechanism of beta-hydroxybutyrate inhibition at the

adipocyte may work through insulin. Blood flow rates were not measured across the hindquarter, because PAH was infused into the common mesenteric vein and would have been cleared by the kidney, but portal vein contribution to blood flow increased during beta-hydroxybutyrate infusion ($p<.01$, Table 1), suggesting that with a constant cardiac output, peripheral flow rates would not increase concomitant to a splanchnic increase. Assuming no increase in rump flow, no change in venoarterial difference of NEFA represents no change in peripheral lipolysis following beta-hydroxybutyrate infusion. Ketone infusion did not appear to alter hormone-sensitive-lipase activity.

Wells (50) reported no decrease in net hepatic release of beta-hydroxybutyrate following beta-hydroxybutyrate infusion in insulin-withdrawn, alloxan-diabetic ewes. Heitmann and Fernandez (19) reported no change in ketogenesis due to beta-hydroxybutyrate infusion in diabetic, untreated sheep. In contrast, Dawes (11) reported significant decreases in net hepatic release of beta-hydroxybutyrate following beta-hydroxybutyrate infusion in normal, insulin-treated, and insulin-withdrawn ewes. Hepatic production of both acetoacetate and beta-hydroxybutyrate decreased significantly in this study following beta-hydroxybutyrate infusion ($p<.01$). This concurs with a lowered hepatic uptake of NEFA following beta-hydroxybutyrate infusion ($p<.01$). Wells (50) also

reported a decreased hepatic uptake of NEFA, but cited a significantly decreased hepatic extraction ratio as well. In contrast, the hepatic extraction ratio in this study was slightly but not significantly lowered. Katz and Bergman (24) reported constant hepatic extraction ratios between 9 and 13% in pregnant and fasting sheep. A constant hepatic extraction ratio concomitant with decreased circulating NEFA levels agrees with decreased ketogenic production, since hepatic extraction obeys first order kinetics (15,56).

Portal drained visceral release of NEFA was not significantly altered by infusions. Total splanchnic uptake decreased following beta-hydroxybutyrate infusion ($p<.01$), which concurred with the decrease in hepatic uptake of NEFA ($p<.01$) with a constant portal drained visceral release. Gamma-butyrobetaine infusion had no effect on NEFA concentrations or flux rates.

The average plasma NEFA of the ruminant is approximately 17 carbons in length (49). Using this figure, each NEFA arriving at the liver is capable of producing 4.25 ketone bodies (24). Table 3 shows hepatic uptake of NEFA in the control period was 658 μmol/min. Multiplied by a factor of 4.25, this represents a potential production of 2797 ketone bodies. Hepatic production rates of beta-hydroxybutyrate and acetoacetate for this period were 1586 and 508 μmol/min, representing a total of 2094 four-carbon units. This suggests that 75% of the NEFA taken up by the

liver in an insulin-withdrawn diabetic state is converted to ketone bodies. In the second period, hepatic uptake of NEFA was 240 μmol/min, and ketone production by the liver totalled 802 μmol/min, representing 79% of hepatic NEFA uptake. These percentages concurred with Katz and Bergman (24).

It appears that although the infusion of beta-hydroxybutyrate lowered both hepatic uptake of NEFA ($p<.01$) and production of beta-hydroxybutyrate and acetoacetate ($p<.01$), it did not alter the proportion of NEFA taken up by the liver which was converted to ketone bodies. Infusion of gamma-butyrobetaine resulted in no change in hepatic NEFA uptake, which was 217 μmol/min, or a potential 922 μmol/min ketone bodies. Although acetoacetate production did not change significantly at 96 μmol/min, beta-hydroxybutyrate production measured 1260 μmol/min, bringing total ketone production to 1356 μmol/min. It is not possible that 1356 μmol/min were produced from a potential NEFA contribution of 922 μmol/min. One explanation is that other sources contributed to ketone production, such as butyrate and amino acids, but this is unlikely considering that feed intake had decreased one to two days prior to the experiments in some ewes. Hepatic uptake of butyrate in fed ewes was only 21 μmol/min, resulting in a potentially small contribution of 21 μmol/min to ketone bodies, and this decreased by 71% during energy restriction (29). A more likely explanation

is that the rate of beta-hydroxybutyrate production measured in the third infusion period was numerically but not significantly elevated due to tremendous variation among and between sheep associated with determinations at high concentrations. Since values of NEFA contribution to ketone production in the first two periods agree with Katz and Bergman (24), and because gamma-butyrobetaine infusion caused no significant changes, it is likely that NEFA contribution to hepatic ketogenesis in the third period was not significantly different from those values in the first and second periods.

Since the rate of re-esterification of NEFA is passive and totally dependent upon the rate of beta-oxidation, it would appear that the rate of hepatic ketogenesis is dependent upon NEFA uptake and the rate of beta-oxidation. Because the percent NEFA converted to ketones did change, beta-hydroxybutyrate does not appear to have a regulatory effect associated with insulin or CPT-I. Control of the beta-hydroxybutyrate mechanism may be within NEFA uptake. Gamma-butyrobetaine had no effects on beta-hydroxybutyrate or acetoacetate concentrations or flux rates, suggesting that either it was not taken up by the liver, was not converted to carnitine, or was not a regulatory mechanism.

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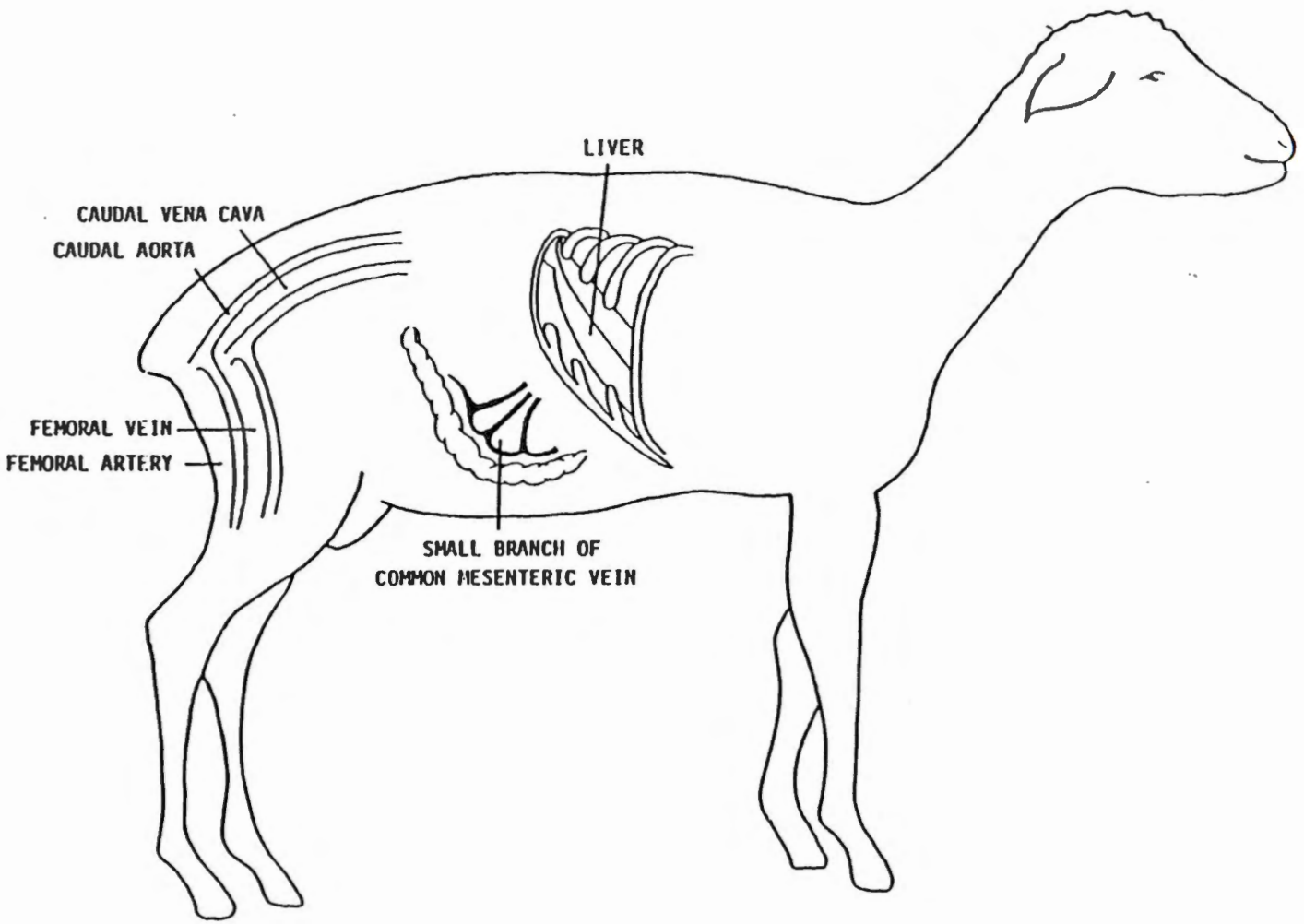
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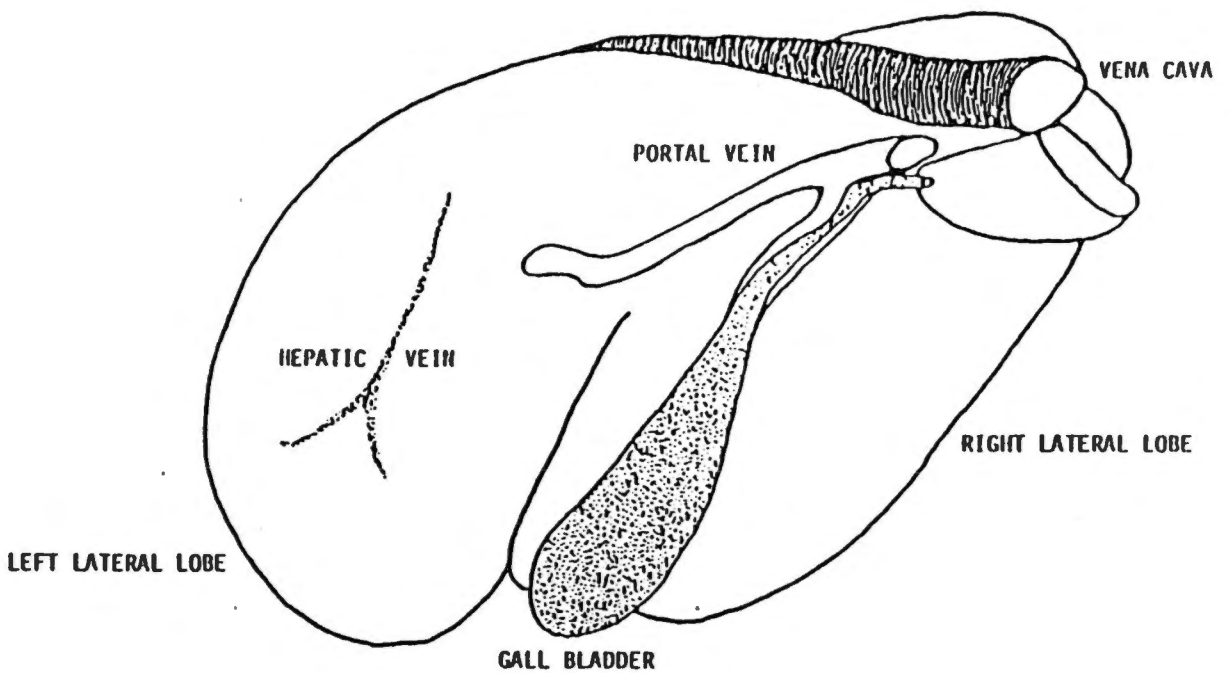
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APPENDICES

APPENDIX A



APPENDIX A.1 Figure showing location of catheters within sheep.



CAUDAL SURFACE OF LIVER

APPENDIX A.2 Figure showing location of catheters within liver.

APPENDIX B

PROTOCOL

TIME (min)	------INFUSATES----- PAH	BOHB	GBB	COMMENT
0	|			INFUSED 1.5% PAH @ 0.764 ml/min
60	|			SAMPLE 1A, 1H, 1P, 1V
90	|			SAMPLE 2A, 2H, 2P, 2V
120	|			SAMPLE 3A, 3H, 3P, 3V
121	|	|		INFUSED BOHB 1.18 mmol/min
180	|	|		SAMPLE 4A, 4H, 4P, 4V
210	|	|		SAMPLE 5A, 5H, 4P, 5V
240	|	|		SAMPLE 6A, 6H, 6P, 6V
241	|	|	|	INFUSED GBB 1.18 mmol/min
270	|	|	|	SAMPLE 7A, 7H, 7P, 7V
300	|	|	|	SAMPLE 8A, 8H, 8P, 8V
330	|	|	|	SAMPLE 9A, 9H, 9P, 9V

APPENDIX B. Experimental protocol.

APPENDIX C

GLUCOSE ANALYSIS

Whole blood glucose determination utilizing the Sigma Chemical Company Glucose Procedure No. 510 Kit is based upon the following coupled enzymatic reactions:

$$\text{Glucose} + 2H_2O + O_2 \xrightarrow{\text{glucose oxidase}} \text{Gluconic acid} + 2H_2O_2$$

$$H_2O_2 + \text{o-Dianisidine (colorless)} \xrightarrow{\text{peroxidase}} \text{oxidized o-Dianisidine (brown color)}$$

Reagents (included with kit):

A) PGO Enzymes (500 units glucose oxidase, 100 Purpurogalin units peroxidase and buffer salts), (cat. # 510-6).

One capsule is diluted with dH_2O to 100 ml and stored in an amber bottle at 0 to 5 °C.

B) o-Dianisidine Dihydrochloride, (cat. # 510-50).

Pre-weighed vial is diluted with dH_2O to 20 ml and stored at 0 to 5 °C.

1.6 ml of this solution is added to 100 ml of PGO enzyme solution.

C) Glucose Standard Solution (100mg/dl), (cat. # 635100) Consists of a solution of beta-D-glucose, 100 mg/dl in benzoic acid, 0.1%, and stored at 0 to 5 °C.

Other Reagents

A) 0.3 N Barium Hydroxide Solution, Sigma (cat. # 14-3)

B) 0.3 N Zinc Sulfate Solution, Sigma (cat. # 14-4)

Sample Collection and Preparation

Blood samples should be collected in a container with a suitable anticoagulant. Immediately after sample is collected, pipette 0.2 ml blood into test tube containing 1.8 ml dH_2O, then mix. To each tube add 1.0 ml barium hydroxide, vortex, then add 1.0 ml of zinc sulfate and vortex again.

Blank and standard are prepared in a similar manner except 0.2 ml dH_2O and 0.2 ml of glucose standard are used, respectively, in place of blood.

Tubes are then centrifuged at 3g for 15 min. at 0 to 5 °C.

Analysis

Prepare unknown, blank, and standard tubes in duplicate as follows:

To each tube add 0.2 ml of supernatant from the respective centrifuged unknown, blank and standard tubes followed by 2.0 ml of PGO/color solution and vortex.

Incubate all tubes at 37 °C for 30 min. in a waterbath or at room temperature for 45 min.

Avoid direct exposure to sunlight or bright light.

Following incubation, remove all tubes from the waterbath.

Read at 450 nm.

Readings should be completed within 30 min.

Calculations

$$\text{Blood Glucose (mg/dl)} = \frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times 100$$

APPENDIX D

NON-ESTERIFIED FATTY ACID ANALYSIS

Principle

Plasma non-esterified fatty acid concentrations can be determined using a WAKO NEFA C kit which is based upon the following reactions:

$$R\text{-}COOH + ATP + CoA \xrightarrow[\text{acyl-CoA synthase}]{} Acyl\text{-}CoA + AMP + PP_i$$

$$Acyl\text{-}CoA + O_2 \xrightarrow[\text{acyl-CoA oxidase}]{} 2,3\text{-trans-enoyl-CoA} + H_2O_2$$

$$2\ H_2O_2 + MEHA + 4\text{-}AAP \xrightarrow[\text{peroxidase}]{} \text{Purple Quinone Product} + 4H_2O$$

MEHA = 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline
4-AAP = 4-aminoantipyrine

Reagents (included with kit):

A) Color Reagent "A":
   - Acyl-CoA synthase
   - Ascorbate oxidase
   - CoA
   - ATP
   - 4-aminoantipyrine

B) Diluent for Color Reagent "A":
   - 0.05 M Phosphate Buffer, pH 6.9
   - Magnesium chloride
   - Surfactant
   - Stabilizers

C) Color Reagent "B":
   - Acyl-CoA oxidase
   - Peroxidase

D) Diluent for Color Reagent "B":
   - MEHA
   - Surfactant

E) NEFA Standard Solution:
   - Oleic acid (1000 μeq/L)
   - Surfactant
   - Stabilizers

Procedure

1) Preparation of standards:

Dilute the standard provided (1000 µeq/l) with dH_2O to provide 125, 250, and 500 µeq/liter standards. Validation tests show that dH_2O is an acceptable dilutent.

2) Preparation of reagents:

Mix color reagent "A" by adding 10 ml of the specific dilutent "A" to each vial. Gently invert the vial until contents are completely dissolved and then combine with 13.3 ml dH_2O. Store at 2°C to 10°C for up to 5 days.

Mix color reagent "B" by adding 20 ml of the specific diluent "B" to each vial. Gently invert until contents are dissolved and then combine with 33.3 ml dH_2O. Store at 2°C to 10°C for up to 5 days.

Analysis

1) Assay each sample and standard in duplicate.

2) Add 0.4 ml dH_2O to each tube.

3) Add 0.01 ml of serum, plasma or standard to each tube, followed by 0.35 ml of the combined reagent "A" plus dH_2O mixture. Mix and refrigerate for up to 60 min if necessary.

4) Incubate tubes in a waterbath at 37°C for exactly 20 min.

5) Following incubation, add 0.8 ml of the combined reagent "B" plus dH_2O mixture to each tube, mix and incubate a second time at 37°C for exactly 20 min.

6) Remove tubes from waterbath, allow to equilibrate at room temperature for 5 min, then record the OD at 550 nm using a blank prepared with reagents as zero.

Important

Do not mix reagents and supplies from test kits bearing different lot numbers.

The assay is sensitive to heparin and plasma samples using heparin as an anticoagulant are unsuitable.

Samples which are hemolyzed or overtly icteric may yield inaccurate results.

Ascorbic acid interferes with assay so ascorbate oxidase is automatically included in reagent "A" mixture in WAKO kit.

APPENDIX E

ACETOACETATE ANALYSIS

Principle

The acetoacetate analysis is a spectrophotometric determination based upon a compound's (nicotinamide adenine dinucleotide, NAD) unique property to absorb a specific quantity of light of specific wavelength. Acetoacetate is indirectly determined from the concentration of NAD following the chemical reaction:

$$\underset{\text{acetoacetate}}{HOOC\text{-}CH_2\text{-}CO\text{-}CH_3} + NADH + H^+ \longrightarrow \underset{\text{beta-hydroxybutyrate}}{HOOC\text{-}CH_2\text{-}CHOH\text{-}CH_3} + NAD^+$$

Acetoacetate is reduced, in a 1:1 stoichiometry, using reduced nicotinamide adenine dinucleotide (NADH) in the presence of the enzyme beta-hydroxybutyrate dehydrogenase resulting in the formation of beta-hydroxybutyrate and oxidized nicotinamide dinucleotide (NAD^+). The change in absorption between the reduced and the oxidized forms of nicotinamide adenine dinucleotide results in the calculation of acetoacetate.

Treatment of Blood

1) Mix equal volumes of whole blood and 1 M $HCLO_4$ immediately after sampling.

2) Centrifuge at 3g for approximately 15 minutes.

3) Neutralize excess $HCLO_4$ with KOH so that the final pH is between 6.0 and 8.0.

4) Centrifuge off the potassium perchlorate formed at approximately 3g for 15 minutes and then pipette from the supernatant.

5) Samples must be analyzed the day of collection.

Reagents

1) 0.1 M Phosphate Buffer (pH 6.8)

   1.36 g KH_2PO_4 ---> 100 ml dH_2O

   1.74 g K_2HPO_4 ---> 100 ml dH_2O

Mix equal volumes of each solution. Check pH and add one or the other to balance pH.

2) NADH (approx. 1 mM) (Disodium Salt) (Grade II, 98% Pure) (Boehringer Mannheim, cat. # 128023)

0.010 g NADH ---> 6.0 ml dH_2O

Make up on day of use.

3) 3-Hydroxybutyrate Dehydrogenase (5.0 mg/ml) (Grade II) (Boehringer Mannheim, cat. # 127841)

4) 1.5 mM Acetoacetic Acid (ACAC) (Lithium Salt) (90 to 95% Pure) (Sigma Chemical Co., cat. # A-8509)

0.01620 g CH_3COCH_2COOH ---> 100 ml dH_2O

Standards

Dilute to the following concentrations for the working standards: 0.15, 0.09, 0.075, 0.045, and 0.0225 mM.

Use two zero concentration standards as blanks.

The standards must be taken through the entire procedure as small amounts of perchlorate will affect enzyme activity. Pipette into conical centrifuge tubes:

1.0 ml standard and 1.0 ml of 1 M $HCLO_4$.

Neutralize to pH 6.0 to 8.0. (Remember to record the volume of buffer added to adjust pH !!!).

Centrifuge at 3g for 15 minutes.

Standard dilutions:

A) 0.15 ----> 2.0 ml of 1.5 mM ACAC plus 18.0 ml dH_2O

B) 0.09 ----> 12.0 ml of A plus 8.0 ml dH_2O

C) 0.075 ---> 4.0 ml of A plus 4.0 ml dH_2O

D) 0.045 ---> 10.0 ml of B plus 10.0 ml dH_2O

E) 0.025 ---> 10.0 ml of D plus 10.0 ml dH_2O

Analysis

Pipette into 12 X 75 mm culture tubes:

0.5 ml buffer
Sample - 0.25 ml plus 0.75 ml dH_2O
Standard - 1.0 ml
0.05 ml NADH

Read E_1 at 340 nm.

Add 0.005 ml 3-Hydroxybutyrate Dehydrogenase, vortex, and incubate at room temperature for approximately 22 minutes.

Read E_2 at 340 nm.

Important

NADH will "react" slowly at room temperature but at about the same rate in all samples. The zero concentration ACAC standards can be used as a control for this. Pipette NADH into all of the cuvettes at known time intervals, i.e., 15 to 60 s. Then read E_1 in all cuvettes at same timed intervals. Incubate and read E_2 at same timed intervals.

Calculations

Calculate E_1 minus E_2 for all standards, samples and blanks:

Change in E of standard minus change in E of blank.

Change in E of sample minus change in E of blank.

Plot a standard curve of change in E of standard versus concentration.

Read the concentration of the samples from the curve.

Multiply by appropriate dilution factors.

APPENDIX F

BETA-HYDROXYBUTYRATE ANALYSIS

Principle

The beta-hydroxybutyrate analysis is a spectrophotometric determination based upon a compound's (nicotinamide adenine dinucleotide, NAD) unique property to absorb a specific quantity of light of specific wavelength. Beta-hydroxybutyrate is indirectly determined from the concentration of NAD following the chemical reaction:

$$\underset{\text{beta-hydroxybutyrate}}{HOOC\text{-}CH_2\text{-}CHOH\text{-}CH_3} + NAD^+ \longrightarrow \underset{\text{acetoacetate}}{COOH\text{-}CH_2\text{-}CO\text{-}CH_3} + NADH + H^+$$

Beta-hydroxybutyrate is oxidized, in a 1:1 stoichiometry, using oxidized nicotinamide adenine dinucleotide (NAD^+) in the presence of the enzyme beta-hydroxybutyrate dehydrogenase resulting in the formation of acetoacetate and reduced nicotinamide dinucleotide (NADH). The change in absorption between the reduced and the oxidized forms of nicotinamide adenine dinucleotide results in the calculation of beta-hydroxybutyrate.

Treatment of Blood

1) Mix equal volumes of whole blood and 1 M $HCLO_4$ immediately after sampling.

2) Centrifuge at 3g for approximately 15 minutes.

3) Neutralize excess $HCLO_4$ with KOH so that the final pH is between 6.0 and 8.0.

4) Centrifuge off the potassium perchlorate formed at approximately 3g for 15 minutes and then pipette from the supernatant.

5) Samples must be analyzed the day of collection.

Reagents

1) 0.1 M Tris-HCL Buffer (pH 8.5)

   2.42 g Tris ---> 50 ml dH_2O

   pH ---> 8.5 with 1 M HCL

   Final volume ---> 200 ml

2) Hydrazine-Tris Buffer (Burnt Bacon Buffer)

2.5 ml Hydrazine Hydrate

0.05 g EDTA

12.5 ml 1 M HCL

Volume ---> 50 ml with Tris-HCL Buffer

Check pH, should be 8.5

Make up on day of use.

3) 14 mM NAD^+ (Free Acid) (Grade II, 98% Pure) (Boehringer Mannheim, cat. # 127990)

0.06 g MAD^+ ---> 3.0 ml dH_2O

3) 3-Hydroxybutyrate Dehydrogenase (5.0 mg/ml) (Grade II) (Boehringer Mannheim, cat. # 127841)

4) 2.0 mM 3-Hydroxybutyric Acid (Sodium Salt) (98% Pure) (Sigma Chemical Co., cat. # H-6501)

0.0656 g $CH_3CHOHCH_2COOH$ ---> 250 ml dH_2O

Standards

Dilute to the following concentrations for the working standards: 2.0, 1.6, 1.2, 0.8, and 0.4 mM.

Use two zero concentration standards as blanks.

The standards must be taken through the entire procedure as small amounts of perchlorate will affect enzyme activity. Pipette into conical centrifuge tubes:

1.0 ml standard and 1.0 ml of 1 M $HCLO_4$.

Neutralize to pH 6.0 to 8.0. (Remember to record the volume of buffer added to adjust pH !!!).

Centrifuge at 3g for 15 minutes.

Standard dilutions:

A) 2.0 ----> 20.0 ml of 2.0 mM BOHB plus 0.0 ml dH_2O

B) 1.6 ----> 16.0 ml of 2.0 mM BOHB plus 4.0 ml dH_2O

C) 1.2 ----> 12.0 ml of 2.0 mM BOHB plus 8.0 ml dH_2O

D) 0.8 ----> 8.0 ml of 2.0 mM BOHB plus 12.0 ml dH_2O

E) 0.4 ----> 4.0 ml of 2.0 mM BOHB plus 16.0 ml dH_2O

Analysis

Pipette into 12 X 75 mm culture tubes:

0.5 ml buffer
Sample - 0.10 ml plus 0.90 ml dH_2O
Standard - 0.25 ml plus 0.75 ml dH_2O
0.05 ml NAD^+

Read E_1 at 340 nm.

Add 0.005 ml 3-Hydroxybutyrate Dehydrogenase, vortex, and incubate at room temperature for approximately 45 minutes.

Read E_2 at 340 nm.

Important

NAD^+ and hydrazine form a complex which absorbs at 340 nm. Therefore, a slow constant increase in absorbance occurs. This is similar to 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

Same as for acetoacetate, except change in E is calculated from E_2 minus E_1.

APPENDIX G

INSULIN ANALYSIS

Principle

^{125}I-labeled insulin competes with insulin in the patient sample for sites on insulin-specific antibody immobilized to the wall of a polypropylene tube. After incubation, isolation of the antibody-bound fraction is acheived by decanting the supernatant. The tube is then counted in a gamma counter, the counts being inversely related to the amount of insulin present in the patient sample. The quantity of insulin in the sample is then determined by comparing the counts to a standard curve.

Collection

Collect blood and separate serum from cells. Avoid hemolysis, since it has been shown to interfere with insulin immunoassays involving standard separation methods. Samples may be stored at 2 to 8°C for 7 days, or up to 3 months at -20°C. Avoid repeated thawing and freezing. Allow samples to come to room temperature before assay; do not thaw frozen samples by heating in a water bath. Mix by *gentle* swirling or inversion.

Reagents

1) ^{125}I Insulin

   Add 100 ml dH_2O and mix by gentle inversion. Store at 2 to 8°C up to 30 days.

2) Standards

   Reconstitute calibrator "A" with 6.0 ml dH_2O. Reconstitute calibrators "B" through "G" with 3.0 ml dH_2O.

Procedure

1) Label four uncoated 12 X 75 mm polypropylene tubes "T" (total counts) and "NSB" (nonspecific binding). Label fourteen coated tubes (provided in kit) "A" (maximum binding) and "B" through "G" in duplicate.

2) Pipette 200 µl of calibrator "A" into the "NSB" and "A" tubes. Pipette 200 µl of each remaining calibrator, control, and patient sample into the tubes prepared. Pipette directly to the bottom.

3) Add 1.0 ml of ^{125}I Insulin to every tube and vortex. Set the "T" tubes aside. Insulin addition should be

completed in no more than forty minutes after step 2.

4) Incubate for 18 to 24 hours at room temperature.

5) Decant all tubes except the "T" tubes. Allow tubes to drain for 2 to 3 minutes, then strike sharply on absorbent paper to shake off residual droplets.

6) Count for 1 minute in a gamma counter.

Calculation

1) Calculate for each tube the average "NSB"-corrected counts per minute:

$$\text{Net Counts} = \text{Average CPM} - \text{Average "NSB" CPM}$$

2) Determine the binding oe each tube as a percent of maximum binding "MB", with the "NSB"-corrected counts of the "A" tubes taken as 100%:

$$\text{Percent Bound} = \frac{\text{Net Counts}}{\text{Net "MB" Counts}} \times 100 .$$

3) Plot Percent Bound on the vertical axis against Concentration on the horizontal axis for each of the calibrators "B" through "G", and draw a straight line through these six points. Insulin concentrations for the unknowns may then be estimated from the line by interpolation.

Important

All components must be at room temperature before use.

An automatic pipetter is not recommended for this assay.

Crossreactivity with pro-insulin at mid-curve is approximately 40%.

The procedure can detect as little as 1.2 μIU/ml insulin.

EDTA plasma yields falsely elevated insulin levels in this assay. Therefore, if EDTA has been used as an anti-coagulant, a second set of standards should be used with an equivalent amount of EDTA added as present in the samples.

APPENDIX H

PARA-AMINOHIPPURIC ACID ANALYSIS

Reagents for Filtrate

1) Double Distilled H_2O

2) 20% Trichloroacetic Acid (TCA) (CCl_3COOH)
(Sigma Chemical Co., cat. # T-6399)

Method for Filtrate

1) Pipette 1.0 ml of blood or standard into 15 ml centrifuge tube containing 5.0 ml dH_2O.

2) Pipette 5.0 ml of blood or standard mixture into second 15 ml centrifuge tube containing 5.0 ml 20% TCA.

3) Let stand 60 min or overnight (can be left at this stage if refrigerated.

4) Filter through Whatman #4 filter paper into 16 x 150 mm glass culture tubes.

5) Add boiling chip to each culture tube and place a marble on top of each tube.

6) Boil over low heat in water bath for 30 min after fine bubbles appear in each tube.

7) Cool at room temperature and remove marbles when cool.

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

Make up a 1:500 dilution for sheep from 1.5% infusion solution; 1:100 for dog from 0.25% infusion solution (considered 10:10).

Be sure to run this dilution through the filtrate procedure (do not have to boil).

Blank mixed with half dH_2O and half TCA (20%).

Reagents for Analysis

1) 1.2 N Hydrochloric Acid (HCl)
(Fisher Scientific Co., cat. # A-144-S)

(1:10 dilution of 12.4 N HCl close enough)

2) Sodium Nitrite ($NaNO_2$)
(Sigma Chemical Co., cat. # S-2252)

100 mg $NaNO_2$ ---> 100 ml H_2O

3) Ammonium Sulfamate ($NH_4OSO_2NH_2$)
(Sigma Chemical Co., cat. # A-8670)

500 mg Ammonium Sulfamate ---> 100 ml H_2O

4) Coupling Reagent N(-1-Naphthyl)Ethylenediamine
(Sigma Chemical Co., cat. # N-5889)

500 mg coupling reagent ---> 100 ml H_2O

All reagents should be refrigerated in brown bottles and can be stored indefinitely.

Analysis

1) Pipette 1.0 ml of filtrate, standard or blank into test tube (in duplicate).

2) Add 0.2 ml of 1.2 N HCl to all tubes and vortex.

The following steps should be done with no more than 5 min between additions and tubes should be vortexed after each addition.

Start time clock and add to all tubes:

0.1 ml sodium nitrite

0.1 ml ammonium sulfamate

0.1 ml coupling reagent

3) Wait 10 min (minimum) for color development.

4) Read absorbance on spectrophotometer at 540 nm.

5) Calculations are made as a percentage of 100 standard.

PAH Stock Solution

1) Para-Aminohippuric Acid (PAH)
(Eastman Kodak Corp., cat. # 5704)

2) Sodium Hydroxide (NaOH)
(Fisher Scientific Co., cat # S-318)

Dissolve each reagent separately in Physiological Sterile Saline (PSS):

44.8 g PAH

10.5 g NaOH

Then add NaOH solution to PAH solution and stir. May have to heat gently to fully dissolve. Filter through Whatman #4 filter paper. Titrate filtrate to pH 7.4 with 1 N HCl or 4 M NaOH. Bring final volume to 500 ml. Yields a 1.5% solution of sodium salt.

VITA

Jennifer Lynn Campbell was born in Salem, Massachusetts on January 5, 1968 to Mr. and Mrs. Richard A. Campbell. She was raised and educated in Topsfield, Massachusetts and attended Steward Elementary School. She moved to Boxford, Massachusetts at age ten and attended Masconomet Regional Junior and Senior High School. She graduated from Masconomet Regional Junior and Senior High School in June, 1985. She worked for two years full-time at the Nancy Z. Bender Insurance Agency in Boston, Massachusetts, before attending the University of New Hampshire in September, 1987. She received her Bachelor of Science degree in Life Science and Agriculture , majoring in Animal Science, from the University of New Hampshire in May 1991. Following her Bachelor's degree, she accepted the position of Graduate Research Assistant with Dr. R. N. Heitmann at the University of Tennessee, Knoxville, to begin studies toward a Master of Science degree in Animal Science, specializing in ruminant nutrition. She graduated August 13, 1993, with a Master of Science degree in Animal Science. The author is a member of the American Society of Animal Science.