

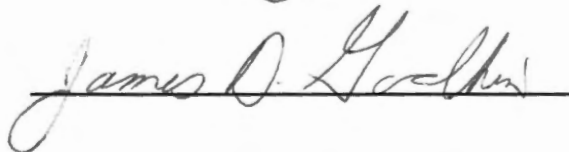
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Energy Restriction Effects on Splanchnic and
Peripheral Tissue Metabolite Fluxes
with or without the Addition
of Lasalocid

A Thesis
Presented for the
Master of Science Degree

The University of Tennessee
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Louis N. Lembo
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ABSTRACT

Concentration and net fluxes of glucose (GLU), nonesterified fatty acids (NEFA), acetoacetate (ACAC), β -hydroxybutyrate (BOHB) and volatile fatty acids (VFA) were studied in mature, non-pregnant, non-lactating ewes with or without the addition of the ionophore Lasalocid. Chronic indwelling catheters were placed in portal, hepatic and mesenteric veins, and the caudal vena cava and aorta at least 14 days prior to experiments. Ewes were fed alfalfa pellets at 100% or 50% of the NRC metabolizable energy requirement during the first study. During the second study, the feeding regimen was identical except for the addition of Lasalocid (44 mg/kg) to the feed. On experiment days, paraaminohippurate (PAH, 1.5% @ .764 ml/min.) was infused via the mesenteric catheter in order to measure blood flow across downstream tissues. After a one hour equilibration period, a series of 6 samples (12 ml) were drawn simultaneously at 30 min. intervals from the portal and hepatic veins and the caudal vena cava and aorta. Flux rates were calculated by multiplying venoarterial (V-A) differences by blood flow rates across the respective tissue. Rump V-A differences were calculated instead of flux rates because a simultaneous blood flow could not be

measured. Whole blood was analyzed for PAH, GLU, ACAC and BOHB. Plasma was analyzed for NEFA and VFA's.

During the first study, energy restriction (ER) reduced splanchnic blood flow specifically at the expense of the portal vein contribution to the liver (HEP) indicating that a larger percentage of peripherally released metabolites reached the liver. Energy restriction also decreased ($P < .05$) arterial glucose from 2.68 to 2.41 mM due to increased ($P < .05$) glucose uptake by portal-drained viscera (PDV). Liver GLU release (30 mmol/h) was unchanged. NEFA arterial concentrations increased ($P < .01$) 2 fold due to a 3 fold increase ($P < .01$) in rump release and despite a 2.5 fold increase ($P < .01$) in hepatic uptake. Hepatic extraction of NEFA also increased ($P < .01$) by 50%. Because HEP NEFA uptake increased during ER, HEP release of ACAC decreased ($P < .01$), while release of BOHB increased ($P < .05$) 2 fold. However, PDV release of both ketones decreased ~60% ($P < .05$, ACAC; $P < .05$, BOHB) due to the energy restriction. Arterial acetate concentration decreased ($P < .01$) during ER by 36% due to a decrease ($P < .01$) in PDV release of ~50% (927 to 439 $\mu\text{mol/min}$). There was no significant HEP flux of acetate, but rump uptake decreased ($P < .01$) 43%, dependent upon concentration. Arterial propionate concentration was unaffected by ER despite a decrease ($P < .01$) of 60% in PDV release due to a concomitant

decrease ($P < .01$) of 60% in HEP uptake. Rump uptake of propionate was not affected by ER, remaining constant at ~25-35% of circulating concentrations. Arterial butyrate concentrations statistically decreased ($P < .01$) from 8 to 5 μM with ER, however, this may not be physiologically significant because both PDV release and HEP uptake decreased ($P < .01$) resulting in no change in total splanchnic output. This work demonstrates that decreasing ME intake by 50% increases the animals reliance on endogenous fuels (i.e. hepatic ketogenesis and peripheral lypolysis) because potential ME from VFA PDV release decreased probably due to decreased organic matter fermentation.

During the second study, energy restriction did not change splanchnic blood flow. Arterial glucose concentration actually increased ($P < .01$) from 2.36 to 2.56 mM despite a decrease ($P < .05$) in total splanchnic release of 40%. Liver release of GLU was not statistically different, however, it was approx. 33% lower than what was expected (20 vs. 30 mmol/h). NEFA arterial concentrations increased ($P < .01$) 2 fold despite a 2 fold increase ($P < .01$) in hepatic uptake and a switch from rump release to utilization ($P < .01$). Arterial ACAC concentrations did not change despite a decrease ($P < .01$) of 60% in portal vein viscera (PDV) release. BOHB arterial concentrations increased

($P < .01$) due to an increase ($P < .01$) in HEP production of 45%. Acetate arterial concentrations decreased ($P < .01$), as did portal concentrations. PDV acetate release decreased ($P < .01$) almost 65%, resulting in decreased ($P < .01$) TSP release. Propionate arterial concentrations decreased ($P < .01$) almost 50%, while portal concentrations decreased ($P < .01$) almost 65%. PDV release of propionate decreased almost 75%. HEP propionate uptake decreased nearly 70%. However, TSP release still saw a decrease ($P < .01$) from 31 to 2 $\mu\text{mol}/\text{min}$. Butyrate arterial concentrations decreased ($P < .01$), as did portal concentrations. There was no change in TSP butyrate release, despite a decrease ($P < .01$) of 65% in PDV release. HEP butyrate uptake also decreased ($P < .01$) 66%. Valerate portal concentrations decreased ($P < .05$) 40%. PDV release of valerate decreased ($P < .05$) 40%, as did HEP uptake. There were no significant net flux changes with either of the branched chain volatile fatty acids, isobutyrate and isovalerate.

"There is no subject more captivating, more worthy of study than nature. To understand this great mechanism, to discover the forces which are active, and the laws which govern them, is the highest aim of the intellect of man"

Nikola Tesla, 1891

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CHAPTER 1

INTRODUCTION

The body remains in a dynamic state, ever changing, in order to react to a wide variety of factors, ranging from changes in the environment to changes in the diet. An animal must be able to adapt to these changes in order to survive and reproduce to insure the survival of the species. An animal, or species, which can not successfully adapt to an ever changing environment will die off and become extinct.

Most of the classic mechanistic work was performed on animals in a post-absorptive state. In the ruminant, a three day fast is required before the animal is considered to be in a post-absorptive state. However, in practice, fasting probably only occurs in response to an other, more serious condition, such as a disease state. A more realistic scenario that would be encountered in the field is a partial energy restriction, perhaps due to inadequate trough space, or environmental conditions, such as flooding or snow cover. Therefore, the first study was conducted to quantify the metabolic responses which occur in ewes adapting to restrictions in energy intake.

Ionophores have been shown to decrease feed-to-gain ratios, thus making the animal more efficient. The second part of this study was conducted to determine whether this

increased efficiency could be demonstrated in animals in a negative energy balance.

CHAPTER 2

REVIEW OF LITERATURE ENERGY METABOLISM

Glucose Production:

There are three primary sources which provide glucose to the ruminant animal: the gastrointestinal tract (via absorption), liver and kidneys. Depending upon the diet, very little glucose is provided via the GI tract, in fact, portal utilization may account for up to 20% of glucose turnover (Bergman, et al., 1970). This may appear paradoxical since most of what is fed to a ruminant is rich in glucose, such as cellulose and starches. These compounds are broken down in the rumen to their basic monosaccharide constituents, however, rumen microorganisms utilize these monosaccharides as substrates to produce various end-products, among them: acetate, butyrate and propionate (Katz and Bergman, 1969a). The end result is that little or no glucose will by-pass the rumen to be absorbed directly by the small intestines. (Roe, et al., 1966)

Minimal glucose absorption directly from the diet places the ruminant animal in a peculiar situation. Since glucose derived directly from the diet is inadequate to meet the animal's needs, ruminants must rely entirely upon gluconeogenesis for their glucose supply (Baird, 1977;

Heitmann, et al., 1986). Bergman (1973) estimated that approximately 98% of a ruminant's glucose demand was met through liver and kidney glucose production, the majority of which being derived from the liver (Katz and Bergman, 1969b).

Substrates for gluconeogenesis generally fall into four categories: 1) propionate, 2) amino acids, 3) glycerol and 4) lactate and pyruvate. The rate of gluconeogenesis is dependent upon the rate at which these precursors are supplied to glucogenic tissues. Other substrates, such as valerate and isobutyrate, can contribute to net glucose production, however they are present in too small a quantity to be of major physiological importance (Bergman, 1973).

The relative contributions of each of the major substrates varies according to the animal's energy balance. For example, in the fed state, propionate and lactate will be readily available from rumen fermentation, and may account for up to 80% of net glucose production (Baird, 1977). In the fasted state, propionate, which is derived exclusively from ruminal fermentation, will be low (Bergman et al., 1966). However, there will be an increased glycerol release from the breakdown of triglycerides in adipose tissue which can partially relieve the shortage of gluconeogenic substrates (Heitmann, et al., 1986).

Propionate is the only major volatile fatty acid (VFA) that can contribute directly to gluconeogenesis. Net glucose production from propionate can occur because propionate enters the TCA cycle at succinate and therefore none of the molecule is lost as CO₂ (Figure 1).

The majority of glucose production which occurs directly from propionate occurs in the liver simply because circulating levels of propionate are low (Cook and Miller, 1966). The liver, which is the first major tissue downstream of the digestive tract, extracts about 90% of the propionate which is presented to it and is quite efficient at converting propionate into glucose. Leng (1970) estimated that 50% to 60% of the propionate taken up by the liver is converted directly to glucose.

Propionate may also contribute indirectly to glucose production via conversion to lactate in rumen epithelium, which may account for 1.0 to 4.6% of the propionate absorbed by the epithelium (Weigand, et al., 1972). Since circulating levels of lactate are generally higher than circulating levels of propionate, glucose production in the kidneys may be stimulated by an increase in ruminal propionate production. However, glucose production from propionate, either directly or indirectly, would not exceed two-thirds of the total glucose production from gluconeogenic substrates (Bergman, 1973).

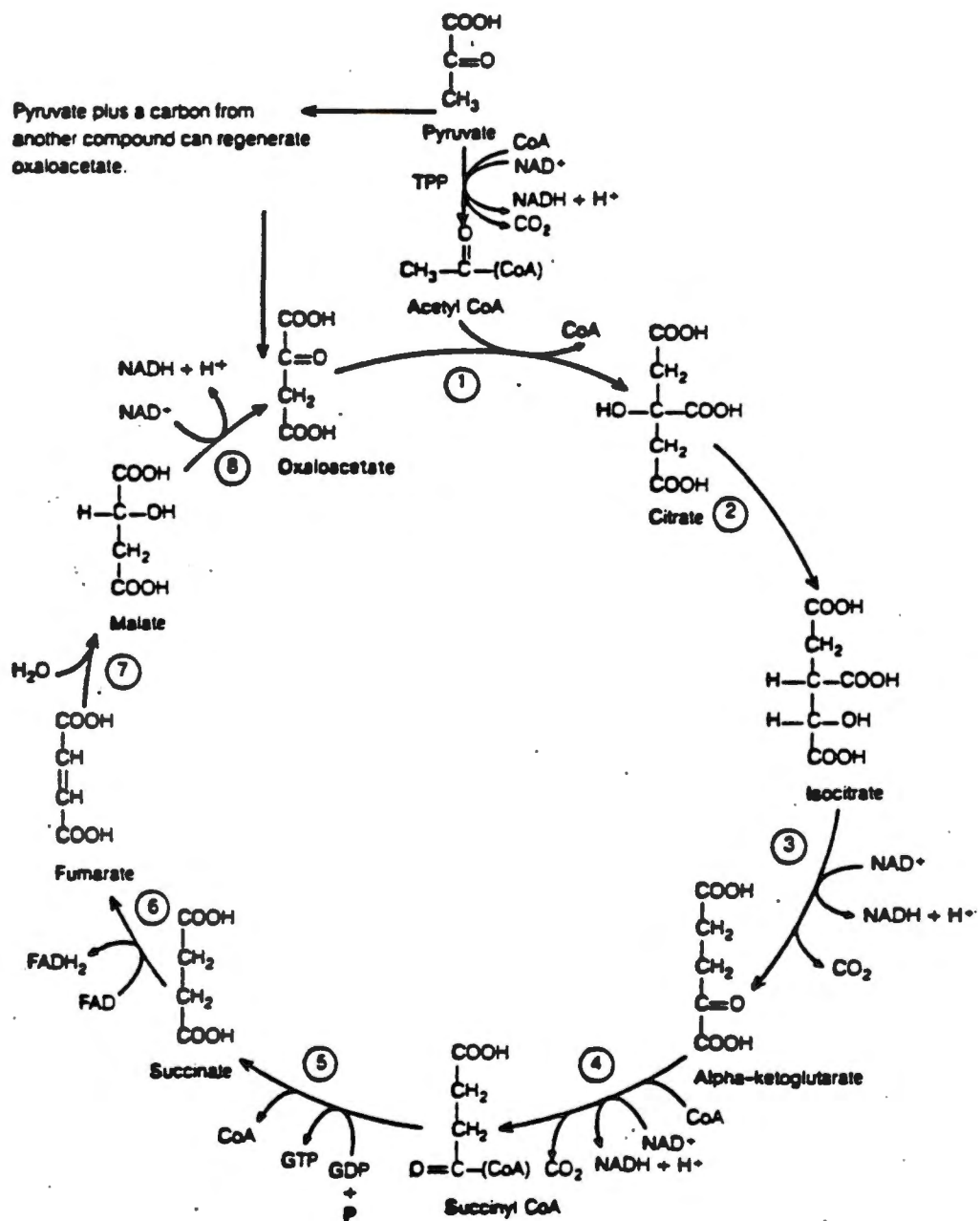


Figure 1
The TCA Cycle

Amino acids are a second major source of gluconeogenic substrates. Heitmann and Bergman (1978) estimated that almost 30% of glucose in whole blood may be derived from six amino acids. Over 25% of the total glucose came from just three: glutamate, glutamine and alanine.

The first step towards gluconeogenesis for an amino acid involves the removal of the amino group, via transamination or oxidative deamination. Transamination is used in muscle cells to transport the toxic amino groups to the liver for urea formation. Transamination is accomplished by transferal of an amino group from an amino acid to an α keto-acid, such as α ketoglutarate or oxaloacetate to alanine or glutamine, respectively. The new amino acid is then transferred via the blood stream to the liver for deamination and use as a substrate for gluconeogenesis.

Other cells, such as those of the gut, which release little or no glutamate or glutamine, rid amino acids of their amino groups via oxidative deamination. This pathway results in the formation of an α keto-acid and a free ammonium ion. The ammonium ion can then either be metabolized in the liver to form urea or can be shuttled back to the rumen for incorporation into amino acids by rumen microorganisms.

Glycerol is probably not a very important gluconeogenic precursor in the fed ruminant. The main function of glycerol is to serve as the backbone for triglycerides in adipose tissue. However, since adipose tissue possesses insufficient glycerokinase activity to produce the glycerol-3-phosphate from glycerol needed for both gluconeogenesis and triglyceride synthesis (Bauman, 1976), triglyceride breakdown results in an unusable endproduct: glycerol. The glycerol molecule can be transported to the liver, which possesses glycerolkinase activity, for subsequent metabolism to glucose via triose-phosphate. Conversion of glycerol into glucose does not involve the TCA cycle (Figure 1).

During times of metabolic stress, there is mobilization of body fat stores and an associated concomitant rise in glycerol release from adipose tissue. This added glycerol may be used to replace some of the glucogenic substrates that are limited in the diet or provide supplementary substrates when glucose turnover rate increases. Thus while glycerol may be relatively unimportant in the fed animal, it can play a major role in times of stress, possibly accounting for up to 40% of the ruminant animals glucose production (Bergman, 1973).

The final major pathway for gluconeogenesis involves lactate, pyruvate and the Cori cycle. Circulating lactate

must be separated into two components: that derived from the Cori cycle and that derived from ruminal fermentation. Cori cycle derived lactate conversion to glucose can not be considered true net glucose production, while ruminally derived lactate can contribute to net glucose production. The Cori cycle involves conversion of glucose to lactate, usually under periods of oxygen shortage or intense work (e.g. muscle during exercise). Lactate can then be transported to the liver or kidneys for subsequent conversion to glucose. This cycle allows for the use of lactate by tissues which do not possess the ability to metabolize lactate directly.

Lactate can be converted to pyruvate by the enzyme lactate dehydrogenase. Pyruvate may also be released from working tissue during anaerobic glycolysis and the gut via ruminal fermentation. Conversion of lactate to pyruvate is very important because lactate can not enter the TCA cycle directly, while pyruvate can (Figure 1).

Glucose Utilization:

In both ruminants and non-ruminants, blood glucose levels must be adequately maintained. Bergman (1973) estimated that only about 10% of respired CO₂ was derived from direct oxidation of glucose, however, there are at least 6 major systems for which glucose is an obligate

requirement: the nervous system, adipose tissue (triacylglycerol synthesis), muscle tissue, mammary tissue (milk synthesis), the fetus and red blood cells (Bergman, et al., 1970). Therefore, even though glucose plays a minor role in meeting a ruminants total energy requirements, an adequate supply of glucose is none the less essential.

The derivation of energy from glucose occurs in two stages. The first stage is referred to as the Embden-Meyerof or glycolytic pathway and involves utilization of 2 moles of ATP to convert 1 mole of glucose into 2 moles of glyceraldehyde-3-phosphate (Figure 2). The 2 moles of glyceraldehyde-3-phosphate can then be oxidized to form 2 moles pyruvate, 4 moles ATP and 2 moles NADH. If we assume 1 mole of NADH can yield 3 moles ATP via oxidative phosphorylation, then the conversion of 2 moles glyceraldehyde-3-phosphate to 2 moles pyruvate could yield a maximum of 10 moles ATP. Subtracting the 2 moles ATP needed to start the glycolytic pathway, there is a net yield of 8 moles ATP per mole of glucose converted to pyruvate.

During periods of oxygen deprivation, such as during exercise, or relatively anaerobic conditions, such as in the rumen, pyruvate will be converted to lactate in order to provide NAD^+ for the conversion of glyceraldehyde-3-

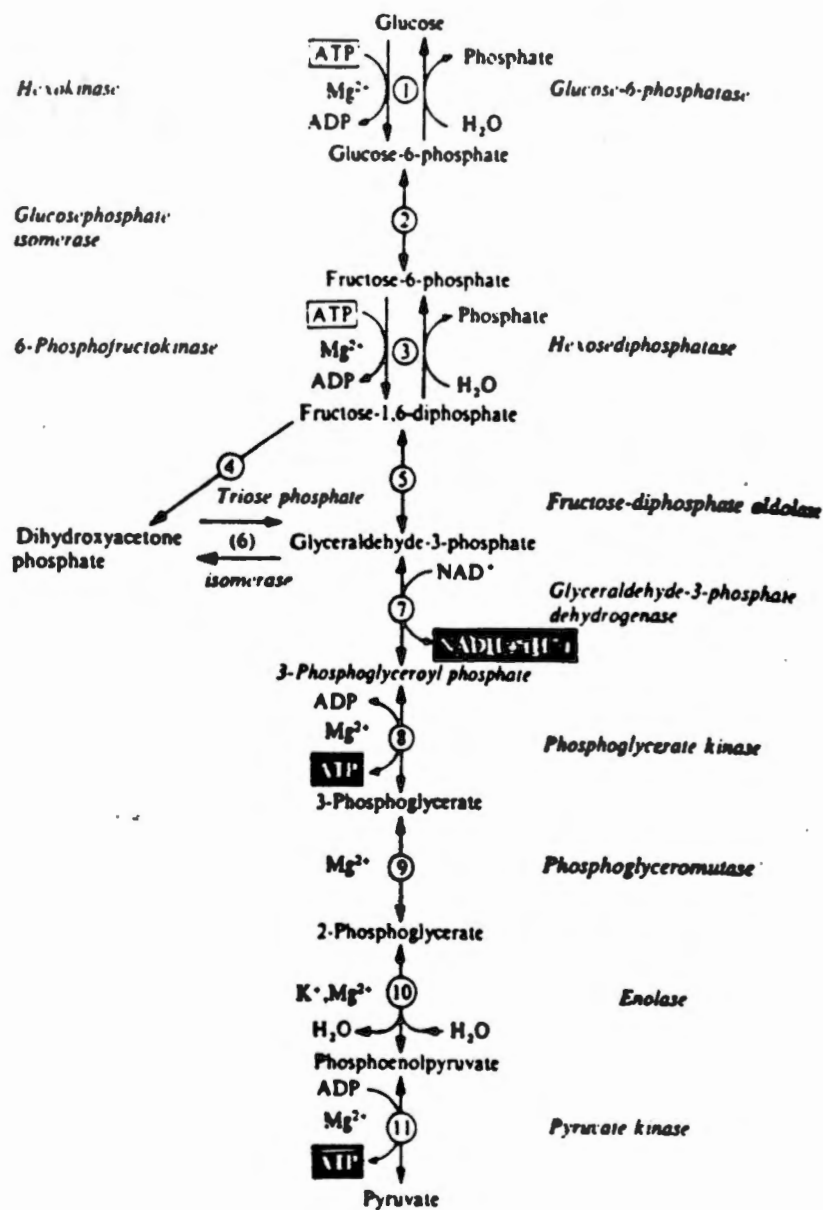


Figure 2
Glycolytic Pathway

phosphate to 3-phosphoglycerol phosphate in order for the glycolytic pathway to continue. A net production of 2 moles of ATP is generated, therefore, by oxidation of glucose to lactate.

The lactate generated from the breakdown of glucose can be oxidized if it can once again be converted back to pyruvate. Pyruvate is converted to acetyl CoA by the enzyme pyruvate dehydrogenase which begins the second stage of glycolysis, the TCA cycle. Oxidation of acetyl CoA via the TCA cycle can yield a maximum of 15 moles of ATP equivalents per mole of acetyl CoA oxidized. Since one mole of glucose can yield 2 moles acetyl CoA, a maximum of 38 moles of ATP equivalents can be generated via oxidative degradation of glucose to CO₂ and water.

Glucose and Triglyceride Synthesis:

The two major functions of glucose in adipose tissue are the synthesis of NADPH and generation of α glycerol-phosphate for triglyceride formation. Ruminants can not produce fatty acids directly from glucose due to extremely low adipose tissue activity of two key enzymes: ATP-citrate lyase and NADP-malate dehydrogenase (Bauman, 1976). Without ATP-citrate lyase and NADP-malate dehydrogenase the adipocyte is unable to transfer mitochondrial acetyl CoA to the cytosol for fatty acid synthesis.

In the ruminant, synthesis of NADPH for fat synthesis can occur via two metabolic pathways: the pentose monophosphate shunt and the isocitrate cycle. The pentose phosphate pathway can produce 2 moles of NADPH for each mole of glucose entering the cycle and is the major source of NADPH for fat synthesis (Bauman, 1976). The two regulatory reactions in the monophosphate pathway involve the conversion of glucose to 6-phosphoglucon- δ -lactone and 6-phosphogluconate to ketogluconate-6-phosphate, which are regulated by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, respectively (McDonald, 1988) (Figure 3).

The isocitrate cycle produces NADPH by the conversion of isocitrate to α ketoglutarate via isocitrate dehydrogenase. This cycle possesses a relatively high activity, when compared to non-ruminants, allowing ruminants to produce a large amount of NADPH from this cycle. The major advantage of NADPH production via isocitrate dehydrogenase is the glucose sparing effect. Ruminants can produce an equal amount of NADPH for fat synthesis utilizing less glucose than would a non-ruminant.

Nonesterified Fatty Acid Metabolism:

In the non-ruminant animal, Nonesterified fatty acids (NEFA), or free fatty acids, provide a buffer between the

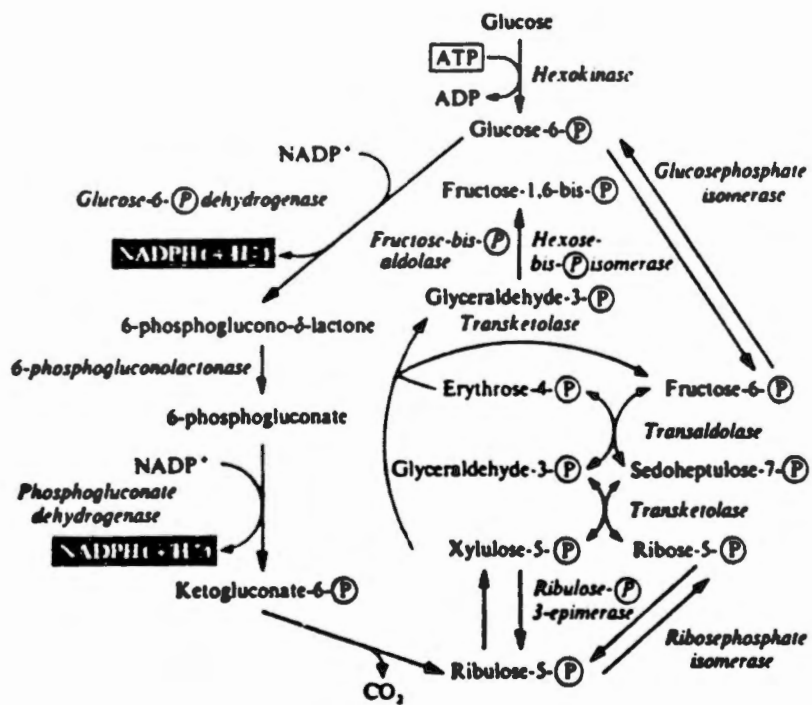


Figure 3
The Pentose Monophosphate Shunt

fed and the fasted state. Ruminant animals generally remain in a fed state as evidenced by the amount of time which a ruminant must be fasted before changes are seen in blood metabolite concentrations and fluxes (Heitmann et al., 1986). However, NEFA's play a very important role in the fed ruminant and an even more important role in times of metabolic stress, such as energy restriction or lactation (Leat, 1974).

Steinberg (1963) stated that adipose tissue mobilization occurs primarily and almost exclusively in the form of free fatty acids. Net release (or uptake) of NEFA's from adipose tissue is the result of a balance between two opposing pathways: esterification (to form triglycerides for storage) and hydrolysis (of stored triglycerides) (Van Soest, 1982).

Control over NEFA mobilization is still unclear. Bauman (1976) suggested that lipolysis is regulated via the cyclic AMP second messenger system and hormone sensitive lipase stimulation, however, he notes that the initial hormone activators are still under debate. Steinberg (1963) listed 11 different hormonal factors which have been shown to increase NEFA release from non-ruminant adipose tissue. Most of the hormones appear to increase NEFA release by increasing triglyceride breakdown and decreasing

triglyceride synthesis, but not shutting synthesis down completely.

Plasma NEFA concentrations in ruminants are lower than in non-ruminants. This should not be surprising given that 60-80% of ruminant energy expenditure in the fed state is provided by VFA oxidation (Blaxter, 1962). The liver, which is a major NEFA consumer in the ruminant will only manufacture about 5% of NEFA's produced in the whole animal (Van Soest, 1982). Therefore the majority of NEFA's reaching the liver for processing must be plasma fatty acids derived from either the diet or from adipose tissue breakdown.

NEFA uptake by the liver is concentration dependent and has been shown to remain relatively constant (10-20%) over a variety of conditions (Katz and Bergman, 1969b; Heitmann, et al., 1986). There are four major pathways with which the liver may dispose of fatty acids: Biliary excretion as lipoproteins, storage and secretion as acylglycerols, complete oxidation to CO₂, or incomplete oxidation to ketone bodies. The extent to which plasma derived NEFA is incorporated into lipoproteins for biliary excretion is not known. However, Moore and Christie (1984) estimated that 18-38% of the lipids that enter the small intestine of a ruminant are lipids released in bile indicating that biliary excretion and absorption could be a

very important method of returning NEFA back to adipose tissue.

Formation of acylglycerols can occur in the liver for either export in association with apo-proteins or for storage. Emery et al. (1992) speculated that excretion could not be an important means of removing fat from the ruminant liver, basing their conclusions on hepatocyte culture data. Acylglycerol storage by the ruminant liver is minimal and control over storage is not yet completely understood. For example, fatty liver, which is an accumulation of fat in a liver beyond the level which is normally encountered, may be the result of a normal physiological response (Blood and Studdert, 1988).

While NEFA biliary excretion, storage, and acylglycerol secretion may be important means of hepatic NEFA metabolism, the most important pathway for NEFA disposal is oxidation. The first step in oxidation of fatty acids (called β -oxidation) is activation via acyl CoA synthetase. This occurs in the cytosol and requires two ATP equivalents. Carnitine is then added to the resulting acyl-CoA by the enzyme carnitine acyl transferase I (CAT I) producing an acyl carnitine which can be transported into the mitochondria, where β -oxidation occurs. CAT I is the rate limiting step in the oxidation of fatty acids. Once inside the mitochondria, the acyl carnitine molecule

interacts with carnitine acyl transferase II resulting in separation of the fatty acid CoA and the carnitine molecules.

Oxidation of the resulting acyl CoA involves sequential removal of two carbon units from the parent fatty acid molecule. Removal of the two carbon acetyl CoA fragments results in the production of an FADH_2 and NADH or five ATP equivalents. The acetyl CoA moiety can then be oxidized via the TCA cycle which will yield another 24 ATP equivalents, for a total of 29 ATP equivalents for each acetyl CoA derived from β -oxidation of a fatty acid.

Ketone Body Metabolism:

The two major sources of ketone bodies in the ruminant are the liver, via incomplete oxidation of plasma NEFA, and ruminal epithelium, via oxidation of dietary substrates, such as the volatile fatty acids acetate and, especially, butyrate (Bergman, 1971). In the fed animal, the alimentary tract is the primary source of ketone bodies (Katz and Bergman, 1969b). The liver is capable of ketone body production in the fed state, however, Katz and Bergman (1969a) showed that only 0 to 30% of fatty acids taken up by the liver, a major source of ketone body substrate, was converted to ketone bodies.

Hepatic uptake of free fatty acids and subsequent β -oxidation within the mitochondria results in acetyl CoA which can then be converted to acetoacetate via hydroxymethylglutaryl (HMG) CoA (McGarry and Foster, 1980). Acetoacetate is then converted via β -hydroxybutyrate dehydrogenase (BHBD) to β -hydroxybutyrate (BOHB), which is released exclusively by the liver in the fed state (Baird, 1977; Katz and Bergman, 1969). However, BHBD in the ruminant hepatocyte is mostly cytosolic, meaning that acetoacetate is the only ketone body to leave the mitochondria (Emery, et al., 1992) and subsequently, BOHB production is subject to cytosolic NAD/NADH ratios not mitochondrial (Koundakjian and Snoswell, 1970).

Alimentary ketogenesis occurs mainly via catabolism of butyrate and some acetate from ruminal fermentation of the diet (Bergman, 1971). Since the diet provides the substrate, portal drained viscera ketone body release may be slowed or even stopped through fasting (Roe, et al., 1966). However, during fasting, the liver will increase ketone body output which can equal or even surpass the amount produced by the gut (Katz and Bergman, 1969b; Heitmann, et al., 1986).

Alimentary production of ketone bodies involves conversion of acetate and butyrate through the same pathway, the HMG-CoA cycle. Acetate is converted to

acetoacetate through the addition of CoA and subsequent metabolism via the HMG-CoA cycle of which acetoacetate is a direct endproduct (Conn, et al., 1987). Butyrate is converted to Acetoacetate in rumen wall mucosa via activation to butyryl CoA, after being absorbed from the rumen. Rumen mucosa possesses both butyryl CoA synthetase and HMG-CoA synthase, the key enzymes for ketogenesis (Baird, 1977). As in the liver, portal production of ketone bodies occurs almost entirely as BOHB (Katz and Bergman, 1969).

As discussed earlier, acetoacetate and BOHB are interconvertible via BHBD. However, acetoacetate is not as stable as BOHB and can spontaneously decarboxylate to form acetone which is not utilized well by ruminant tissues (Bergman, 1971). Acetone itself is also unstable and quite volatile giving rise to the odor which is sometimes observed on the breath of untreated diabetic animals. The relative stability of BOHB may account for the ratio of BOHB/acetoactetate release observed in the ruminant even though production of BOHB from acetoacetate involves utilization of NADH.

However, it must be noted that ketone body utilization is mediated through metabolism of acetoacetate via succinyl Co A transferase. Therefore, tissues must possess BHBD in order to utilize BOHB. Most tissues, including skeletal

muscle (Ruderman and Goodman, 1973), the kidneys (Baird, 1977) and the heart, can use ketone bodies and may even "prefer" them under certain conditions, which can contribute to the sparing of glucose. (Heitmann, et al., 1987)

Ketone body utilization occurs via conversion of acetoacetate to acetoacetyl Co A, and subsequent splitting by thiokinase to 2 acetyl CoA's, which can then be readily metabolized through the TCA cycle. Bergman (1971) estimated that maximal whole body ketone utilization in sheep occurs at about 20 mg/100 ml, expressed as acetone, and also concluded that at maximal utilization, such as in a ketotic state, a sheep could derive approximately 20-30% of respiratory CO₂ from ketone body oxidation.

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LASALOCID

Introduction:

Lasalocid belongs to a class of compounds called carboxylic ionophores, so named for the terminal carboxyl group which they all possess (Painter and Pressman, 1985). These compounds are chemically inert, except for their ability to transport ions across biological membranes. This feature has allowed new insight into the metabolism of certain organs and various organelles by allowing the researcher to perturb the delicate concentration and electrical gradients needed by many biological systems to function effectively. Most recently, ionophores, especially monensin and lasalocid, have been studied as agricultural feed additives to improve feed efficiency in ruminants.

Ionophore Basic Properties:

Simply, ionophores facilitate movement of cations across biological membranes and down their concentration gradients. In order to accomplish this feat, ionophores must possess four basic properties (Painter and Pressman, 1985): (1) The molecule must contain both polar and non-polar groups. The non-polar fraction of the molecule allows the entity to move about freely in a lipid bi-layer. The polar fraction of the molecule allows the ionophore to

"trap" the cation and stabilize the cation's positive charge. (2) The molecule must be able to assume a stable configuration which directs its polar portions toward a central cavity suitable for surrounding a cation. This conformation is usually stabilized by the formation of intra-molecular hydrogen bonds. The conformation must also result in the ionophore's non-polar portion facing outward in order for the molecule to remain lipid soluble. The size and electrical characteristics of the cavity formed will determine cation preference. (3) Ion trapping and releasing must occur at rapid rates. A molecule which complexes or decomplexes slowly will allow the cell time to reestablish a concentration gradient and therefore be ineffective. (4) The overall size and shape of the ionophore-cation complex must allow for unrestricted movement through the lipid bi-layer.

Lasalocid Mode of Action:

Lasalocid's ability to transport ions across biological membranes is a function of its two structural configurations. The equilibrium that exists between the two arrangements can be shifted by altering the polarity of the solvent in which the molecule resides. Figure 4 represents the acyclic, quasi-linear conformation of lasalocid which is dominant in a high-polarity medium.

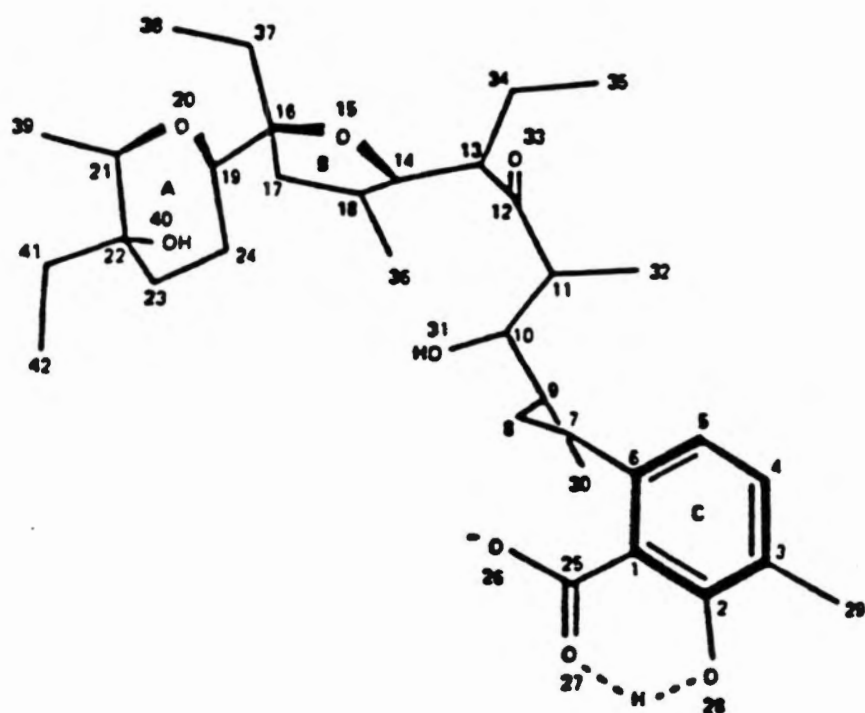


Figure 4
Lasalocid: Acyclic Conformation

Figure 5 represents lasalocid in a cyclic configuration which is held in place by intramolecular, head-to-tail hydrogen bond interactions. This is the predominant form under low polarity conditions and also allows the ionophore-cation complex to form a stable zwitterion which readily diffuses through biological membranes. The arrows in Figure 5 indicate the bonds which rotate allowing lasalocid to quickly change conformations.

Lasalocid's mode of transport has been identified as an electrically neutral, exchange-diffusion process, because while cation concentration gradients are dissipated, electrical gradients are not. The sequence of events occurring during ion transport by lasalocid are shown in Figure 6.

Cation transport is initiated by a protonated ionophore molecule diffusing toward a membrane interface. At the interface, the proton is released, changing the molecule's polarity and thereby trapping the molecule at the membrane-medium interface. The ionophore is now able to complex with a cation. Upon encountering a suitable cation, the ionophore will shift to its cyclic conformation, displacing the cation's associated water molecule and essentially trapping the ion, forming a stable zwitterionic complex. This neutral compound is now able to leave the membrane interface and diffuse to the opposite

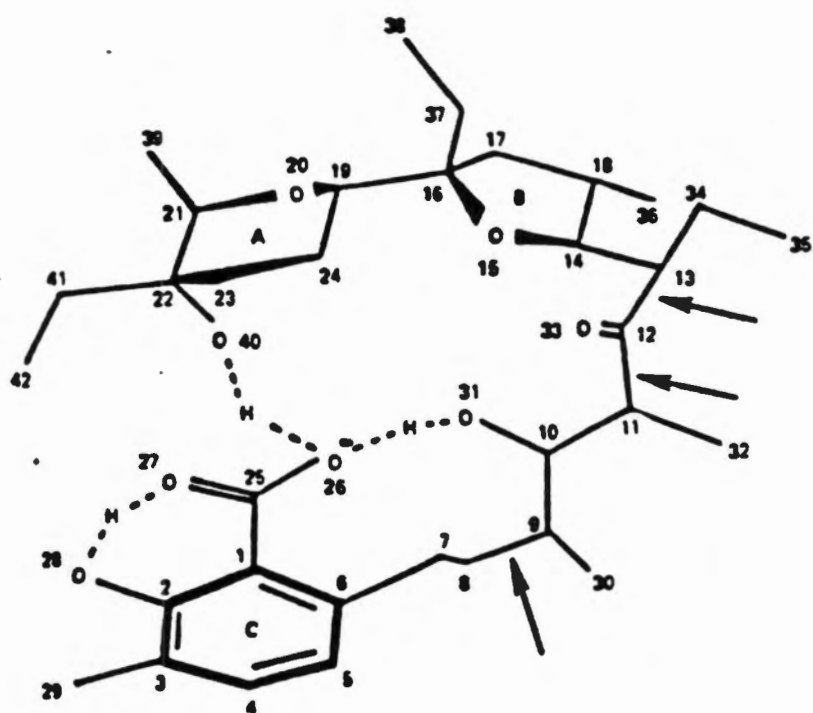
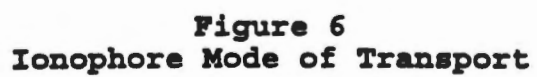


Figure 5
Lasalocid: Cyclic Conformation



interface. Once at the opposite interface, if the situation is energetically favorable, the cation will decomplex with the ionophore, complex with a water molecule and enter into solution. At this time, one of three events can occur. The ionophore may complex with a like cation in what would be termed self-exchange. This exchange does not involve a change in the concentration gradient of the cation. Secondly, the ionophore may complex with a different cation. This event is termed exchange-diffusion and will affect the concentration gradients of both cations. By this mechanism, an ionophore may dissipate two concentration gradients simultaneously. Finally, the ionophore may assimilate a proton. This option may or may not affect the pH of either side of the membrane. The transport cycle may only continue if one of the above events occurs, maintaining an electrically neutral transport process. Upon assimilation of a cation or proton, the neutral ionophore may once again diffuse through the membrane and complete the cycle.

The scheme presented above is generally accepted as the mode of transport for lasalocid in a 1:1 complex with a monovalent cation. Lasalocid also possesses the ability to transport divalent cations, such as Ca^{+2} and Mg^{+2} , as well. A stable configuration is achieved by lasalocid forming a 2:1 complex with the divalent cation. In this 2:1 complex,

there are no new, intermolecular bonds formed between the two ionophore molecules to hold them in position, the configuration is held together primarily by the enclosed cation. Electrically neutral transport is maintained by transport of another divalent cation or by each molecule transporting a monovalent cation back.

The ability of lasalocid to transport both monovalent and divalent cations must be considered in biological situations. Research concerning the ability of lasalocid to transport various cations of physiological importance *in vitro* has shown that the ionophore possesses a stronger affinity for K^+ than Na^+ , and Ca^{+2} over Mg^{+2} (Painter and Pressman, 1985). No definitive data has been published detailing lasalocid's cation preference *in vivo*. However, given that an ionophore's preference is based on the size and electrical characteristics of the cavity formed upon encountering a cation, one may assume that *in vitro* results will translate well *in vivo*.

Changes in Ruminal Dynamics:

The majority of lasalocid's effects may be traced back to the ability of the ionophore to transport cations and dissipate concentration gradients. Russell and Strobel (1989) concluded that the effects of ionophore supplementation observed in the rumen are primarily due to

increased ion flux through microbial membranes the cation selectivity of the ionophore, the ion gradient and microbial sensitivity. As previously discussed, lasalocid may transport both monovalent (e.g. K^+ , Na^+) and divalent (e.g. Ca^{+2} , Mg^{+2}) cations, which are sufficiently abundant in rumen fluid (Russell and Strobel, 1989). Concentration gradients of ions which the ionophore will transport are also a factor and may be more important than ionophore selectivity. Differing concentration gradients, along with different pH levels, associated with different feeding regimens may account for some of the variable responses associated with ionophore supplementation observed in the literature. The mechanisms by which lasalocid alters ruminal fermentation characteristics are due to shifts in rumen microbial populations because of microbial sensitivity to the ionophore (Nagaraja et al., 1987). Initial reports on rumen microbial ionophore sensitivity *in vitro* indicated that, in general, organisms that produced succinate and fumarate were resistant to the ionophore's antibiotic actions. This lead Bergen and Bates (1984) to conclude that ionophore resistance was somehow related to the enzyme fumarate reductase. This theory was soon challenged by the fact that *Ruminococcus flavefaciens*, an organism known to produce succinate, was also sensitive to ionophores.

Current research suggests ionophore resistance is related closely to cell wall structure (Chen and Wolin, 1979). Gram-negative organisms, which tend to be ionophore-resistant, generally possess an outer cell wall. This outer cell wall is impermeable to many macromolecules and solute movement is mediated by structures embedded within the wall (Russell and Strobel, 1989). These structures form hydrophilic channels in the hydrophobic outer wall. Due to the ionophore's relatively hydrophobic structure, passage of the molecule is inhibited. Gram-positive organisms which lack an outer cell wall are more likely to be sensitive to ionophore action. Ruminant protozoa, which also lack an outer cell wall, have also been shown to be ionophore-sensitive (Dennis et al., 1986). Gram-positive organisms are the primary producers of acetate, butyrate, H_2 , formate and ammonium. Selection against these organisms should result in decreased lactate and methane production, with an increase in the molar proportion of propionate. However, improvements in animal production will be related to the ability of the resistant organisms to fill the void left by the resistant organisms (e.g. cellulose digestion) (Russell and Strobel, 1989).

Modifications of Ruminant Metabolism:

A great number of biological responses have been attributed to ionophore feeding. The most widely recognized response being the modification of ruminal volatile fatty acid (VFA) production. Numerous researchers have documented a change in the acetate:propionate ratio in the rumen of ionophore-fed animals (Nagaraja et al., 1987; Ricke et al., 1984; Van Maanen et al., 1978). Propionate production appears to be increased at the expense of acetate, while total volatile fatty acid concentrations remain constant (Armmentano and Young, 1983). Initially, researchers believed that increased ruminal propionate would lead to an increased production of glucose from propionate in the animal. This was suggested because Judson and Leng (1973) had demonstrated an increase in gluconeogenesis with an intra-mesenteric propionate infusion. However, even though many studies demonstrate an increase in ruminal propionate production, Harmon et al. (1989) found no increase in portal drained viscera (PDV) propionate release in ionophore-supplemented animals. This has led many to speculate as to the benefits of increased ruminal propionate production.

Van Maanen (1978) speculated that the excess propionate could either be oxidized directly to CO_2 , or could contribute carbon to the synthesis of other compounds, which would otherwise require glucose as a

substrate. Harmon (1989) suggested that an increase in ruminal propionate production may have a glucose sparing effect on ruminal epithelium. Decreased utilization of glucose as an energy source may decrease the amount of gluconeogenic substances need to maintain euglycaemia. This phenomenon may decrease the need for amino acid use in gluconeogenesis, which could also produce a protein-sparing effect (Schelling, 1984).

Schelling (1984) discussed three more ways in which increased ruminal production of propionate may be more beneficial. First, propionate production appears to be more energy efficient than acetate production. Chalupa (1977) reported the efficiencies of fermenting hexoses to acetate, propionate and butyrate to be 62, 109 and 78%, respectively. Therefore, increasing ruminal production of propionate should increase energy retention by minimizing fermentative losses. Second, propionate may be more efficiently utilized by tissues than acetate (Orskov et al., 1979). Finally, propionate may be oxidized directly or may be used as a substrate for gluconeogenesis, allowing for some flexibility to respond to varying metabolic situations.

Animals fed ionophores have also been shown to exhibit modified feed intake and digestibility, depending on the type of diet fed. Feed intake depression appears to be

greater with high grain diets than with roughage based diets. High grain diet ionophore supplementation generally decreased feed intake, while body weight gains remained steady (Delfino et al., 1988). Therefore, feed/gain ratios were improved. With high roughage diets, ionophore supplementation generally did not affect feed intake, or increased it slightly, but resulted in an increase in body weight gain, which also improved feed/gain ratios (Schelling, 1984).

In general, overall digestibility of a feed is unchanged with ionophore supplementation (Spears, 1990). Digestibility of high concentrate diets, which possess highly fermentable carbohydrates, is not affected by supplementation. High fiber diets tend to show a moderate increase in digestibility, perhaps due to an increase in ruminal retention time (Rogers and Davis, 1982). Spears (1990) demonstrated that lasalocid could increase apparent digestible energy by 2% in cattle, however, neither monensin nor lasalocid appeared to affect digestibility in sheep. Delfino et al. (1988) found no change in the digestibility of an ionophore-supplemented, high concentrate diet fed to cattle, but reported a 10-21% increase in the net energy for maintenance portion of the diet. They attributed the increased efficiency to a decrease in fecal energy and methane losses. However, part

of the increase in efficiency may be due to the lower heat increment associated with propionate metabolism (Smith, 1971).

While it is still unclear whether or not ionophores affect true digestibility, several researchers have noted lasalocid's ability to alter the primary site of starch digestion (Spears, 1990; Delfino et al., 1982). Muntifering (1981) showed a 19% decrease in apparent ruminal starch fermentation in monensin-fed animals, with no significant change in overall digestibility. He concluded that more starch was by-passing the rumen and was being degraded in the small intestine, leading to an increase in the proportion of energy being absorbed as glucose. Considered along with Orskov's (1977) observation that absorbed glucose is used more efficiently than absorbed propionate in the ruminant and Smith's (1971) observation that VFA utilization may be a primary source of wasteful heat production, increased starch digestion in the lower GI tract could account for some of the increased efficiency of ionophore supplementation. However, not all researchers have been able to demonstrate an increased PDV glucose flux in ionophore supplemented animals (Harmon, 1989).

Methane production and loss can represent as much as 12% of feed energy intake (Russell and Strobel, 1989).

Ionophores may decrease methanogenic loss by as much as 30% (Schelling, 1984). The mechanism for this increased efficiency is still unclear. Depression of certain microorganisms (e.g. *Ruminococcus albus*) could be responsible for a decrease in methane production. However, Chen and Wolin (1979) have suggested that methanogens may not be the primary target of ionophores in the rumen. Currently, it is believed that the decrease in methane production is a result of decreased H₂ availability, possibly as a result of increased incorporation into propionate (Chalupa, 1977). Bergen and Yokoyama (1977) also speculated that decreasing acetate production may lead to a decrease in methanogenesis. Even though this depression in methane production does contribute to conserving feed energy, the overall result is only a slight improvement in efficiency of animal production (Schelling, 1984).

Conclusions:

Ionophores contribute a great deal to improving ruminant animal efficiency. They accomplish this by producing a myriad of biological effects ranging from increased protein digestibility to earlier puberty in heifers (Schelling, 1984). However, at this time, all the effects seen with ionophore-supplementation can be

attributed to the ionophore's ability to interact with microorganisms in the rumen.

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CHAPTER 3

EFFECTS OF ENERGY RESTRICTION ON NET METABOLITE FLUX

ABSTRACT

Concentration and net fluxes of glucose (GLU), nonesterified fatty acids (NEFA), acetoacetate (ACAC), β -hydroxybutyrate (BOHB) and volatile fatty acids (VFA) were studied in mature, non-pregnant, non-lactating ewes. Chronic indwelling catheters were placed in portal, hepatic and mesenteric veins, and the caudal vena cava and aorta at least 14 days prior to experiments. Ewes were fed alfalfa pellets at 100% or 50% of the NRC metabolizable energy requirement. On experiment days, paraaminohippurate (PAH, 1.5% @ .764 ml/min.) was infused via the mesenteric catheter in order to measure blood flow across down stream tissues. After a one hour equilibration period, a series of 6 samples (12 ml) were drawn simultaneously at 30 min. intervals from the portal and hepatic veins and the caudal vena cava and aorta. Flux rates were calculated by multiplying venoarterial (V-A) differences by blood flow rates across the respective tissue. Rump V-A differences were calculated instead of flux rates because a simultaneous blood flow could not be measured. Whole blood was analyzed for PAH, GLU, ACAC and BOHB. Plasma was analyzed for NEFA and VFA's. Energy restriction (ER)

reduced splanchnic blood flow specifically at the expense of the portal vein contribution to the liver (HEP) indicating that a larger percentage of peripherally released metabolites reached the liver. Energy restriction also decreased ($P<.05$) arterial glucose from 2.68 to 2.41 mM due to increased ($P<.05$) glucose uptake by portal-drained viscera (PDV). Liver GLU release (30 mmol/h) was unchanged. NEFA arterial concentrations increased ($P<.01$) 2 fold due to a 3 fold increase ($P<.01$) in rump release and despite a 2.5 fold increase ($P<.01$) in hepatic uptake. Hepatic extraction of NEFA also increased ($P<.01$) by 50%. Because HEP NEFA uptake increased during ER, HEP release of ACAC decreased ($P<.01$), while release of BOHB increased ($P<.05$) 2 fold. However, PDV release of both ketones decreased ~60% ($P<.05$, ACAC; $P<.05$, BOHB) due to the energy restriction. Arterial acetate concentration decreased ($P<.01$) during ER by 36% due to a decrease ($P<.01$) in PDV release of ~50% (927 to 439 $\mu\text{mol}/\text{min}$). There was no significant HEP flux of acetate, but rump uptake decreased ($P<.01$) 43%, dependent upon concentration. Arterial propionate concentration was unaffected by ER despite a decrease ($P<.01$) of 60% in PDV release due to a concomitant decrease ($P<.01$) of 60% in HEP uptake. Rump uptake of propionate was not affected by ER, remaining constant at ~25-35% of circulating concentrations. Arterial butyrate

concentrations statistically decreased ($P < .01$) from 8 to 5 μM with ER, however, this may not be physiologically significant because both PDV release and HEP uptake decreased ($P < .01$) resulting in no change in total splanchnic output. This work demonstrates that decreasing ME intake by 50% increases the animals reliance on endogenous fuels (i.e. hepatic ketogenesis and peripheral lypolysis) because potential ME from VFA PDV release decreased probably due to decreased organic matter fermentation.

INTRODUCTION

An adaptive state is a stable state that is maintained as long as the stimulus that evoked it is maintained (Waterlow, 1986). During periods of energy restriction, a new plane of energy balance is attained through a reduction in energy output and changes in intermediary metabolism (Shetty, 1990). The changes in body composition can be monitored by measuring venoarterial differences and flux rates across various tissue beds. The laboratories of Bergman (for review, see Bergman, 1973) and Heitmann (Heitmann, et al., 1987; Heitmann, et al., 1986; Heitmann and Fernandez, 1986) have used flux data to study splanchnic tissues in fasting ewes under a variety of conditions.

Flux data has shown that in ewes fasted at least 3 days, glucose arterial concentrations will fall from approximately 50 mg/100 ml to approximately 35 mg/100 ml due to decreased hepatic production (Bergman, et al., 1970; Katz and Bergman, 1969). This change is not seen in ewes fasted only 24 hours (Heitmann et al, 1986) indicating that ewes must be feed deprived for at least 72 hours before they can be considered to be in a post-absorptive state. Fasting will also increase circulating levels of Nonesterified fatty acids (Bergman et al., 1970; Baird, 1977; Leat and Ford, 1966), resulting in an increase in alterative energy sources for the liver to act upon. Oxidation of fatty acids can result in CO₂, from complete oxidation, or the ketone bodies, Acetoacetate (ACAC) and β -hydroxybutyrate (BOHB), from incomplete oxidation. During fasting, total ketone body production will increase (Leat and Ford, 1966). However, this increase is due solely to a change in liver metabolism. Portal drained viscera release of ketone bodies will slow or even stop, due to lack of volatile fatty acid substrate in the rumen (Bergman and Wolff, 1971; Roe, et al., 1966). Liver production of both ketones bodies will increase dramatically, however. BOHB release from the liver can increase as much as 5-7 fold, while liver uptake of ACAC is changed to a net release (Katz and Bergman, 1969; Heitmann et al., 1986).

Fasting ruminants has helped to elicit some of the mechanisms behind some metabolic disorders, such as ketosis. However, under current farming systems, it is unlikely that a ruminant will receive no dietary substrate for a period of 72 hours or more. A more likely event, short term energy restriction can result from poor management, i.e. inadequate trough space, or from varying environmental conditions, i.e. snow cover or low dry matter spring pastures (Donohue and Kronfield, 1990). Short term energy restriction, when added to an other stressors, such as pregnancy or lactation, can result in severe metabolic disorders (Herdt and Emery, 1992). The objective of this study was to quantify the changes in metabolite concentrations and fluxes which occur during short term energy restriction.

MATERIALS AND METHODS

Animals and Housing:

Effects of energy restriction were studied in mature non-pregnant, non-lactating ewes of various breeds and crosses, weighting between 55 and 75 kg. The animals were housed in individual 1.8 x 3.0 m pens at approximately 17-22 degrees Celsius. The ewes were fed commercially prepared alfalfa pellets daily at approximately 0800 hours. All sheep were accustomed to being handled, therefore

restraint was not necessary during the experimental periods. Water was available at all times.

Surgical Protocol:

Animals were surgically implanted with chronic indwelling catheters placed so that the tips would reside in the following locations: 1) caudal aorta (A), 2) caudal vena cava (V), 3) common mesenteric vein (M), 4) hepatic vein (H) and 5) portal vein (P), as described by Katz and Bergman (1969) and modified by Zanzalari et al. (1989)

Briefly, the caudal aorta and vena cava catheters were implanted by first making a 9 cm incision in the inguinal region, just medial to the M. satorius. The muscle layers were then separated to expose the femoral vein and aorta.

Hepatic and portal catheters were implanted by first making a 22 cm incision starting 4 cm caudal to the 13th rib and 5 cm ventral to the first lumbar vertebra, following the costal arch. The left later lobe of the liver was exposed, palpated, an incision was made and catheters were inserted. Hepatic veins were indentified though movement of a tiny air bubble inserted into the catheter. Portal vein catheters were identified by palpation of the porta hepatis.

Mesenteric vein catheters were implanted by isolating a branch of the common mesenteric vein and inserting a

catheter so that the tip resided in the common mesenteric vein.

Experimental Protocol:

Animals were fed alfalfa pellets (17% Crude protein) at either 100% or 50% of their metabolizable energy (ME) requirement as determined by the National Research Council (NRC) (1984) for at least ten days following surgery. Animals which were fed 100% ME, and whose catheters remained functional after a first experiment, were then fed 50% ME for at least ten days and a second experiment was performed. The ewes were weighted before being placed on their respective diets. Animals in the 100% ME group maintained their body weight. No measurement was made at the end of the 50% ME trials. A total of 5 ewes were used (three ewes had two experiments performed). Two ewes only had one experiment performed due to a loss of catheter patency.

On experiment days, Para-aminohippurate (PAH) (Eastman Kodak, Rochester, NY) was infused (1.5% solution @ .764 ml/min.) and allowed to equilibrate for one hour before samples were taken. Six serial blood samples were then taken simultaneously from the A, H, P and V vessels at consecutive thirty minute intervals. Whole blood was analyzed for glucose, PAH, acetoacetate and β -

hydroxybutyrate. Plasma was analyzed for nonesterified fatty acids (NEFA) on the day of the experiment. Aliquots of plasma were set aside and frozen for subsequent analysis for volatile fatty acid (VFA) concentrations.

Analysis:

Glucose concentrations were determined utilizing a glucose determination kit, No 510 (Sigma Chemical Co, St. Louis, Mo.). The exact procedure is described in Appendix 1.

Plasma NEFA concentrations were analyzed utilizing a WAKO NEFA-C kit (WAKO Pure Chemical Industries, Osaka, Japan). The exact procedure is described in Appendix 2.

The ketone bodies, acetoacetate and β -hydroxybutyrate, were analyzed enzymatically based on the method of Williamson et. al. (1962). The exact procedure is described in Appendix 3 and 4, respectfully.

Volatile fatty acid concentrations were determined based on the method of Reynolds et. al. (1986), with modifications by Quigley et al (1991). The exact procedure is described in Appendix 5.

For whole blood flow, PAH concentrations were determined utilizing the method of Katz and Bergman (1969). The exact procedure is described in Appendix 6.

Calculations:

Blood flow was determined by the indicator-dilution method, using the following equation:

$$BF(l/min) = PAH_p / (V-A)$$

PAH_p represents the infusion rate of PAH in OD/min. $V-A$ represents the venoarterial difference in PAH concentration for the tissue. Portal and hepatic vein flows were measured directly and hepatic artery contributions were measured by hepatic-portal difference.

Flux data was determined by the following equations:

$$\begin{aligned} PDV \text{ (mM/min)} &= PF \text{ (L/min)} \times (P-A) \text{ (mM)} \\ HEP \text{ (mM/min)} &= HF \text{ (L/min)} \times (H-P) \text{ (mM)} + \\ &\quad AF \text{ (L/min)} \times (H-A) \text{ (mM)} \\ TSP \text{ (mM/min)} &= HF \text{ (L/min)} \times (H-P) \text{ (mM)} \end{aligned}$$

PDV, HEP, and TSP represent portal drained viscera, hepatic and total splanchnic flux, respectively. Total splanchnic flux is equal to $PDV + HEP$, however, TSP was calculated separately. PF, HF and AF, represent portal vein, hepatic vein and arterial blood flow, respectively. P, H and A represent portal vein, hepatic vein and hepatic artery metabolite concentrations, respectively. Rump flux data was not obtained because simultaneous blood flow measurements could not be obtained.

Extraction ratios were only calculated when net fluxes were determined to be negative and statistically different from zero ($P < .05$) by the following equation:

$$\text{Extraction ratio} = (\text{net uptake} / (\text{BF} \times \text{A})) \times 100\%$$

Extraction ratios represent the amount of a metabolite presented to a tissue that is taken up by that tissue.

Statistics:

Differences between groups was tested for significance by t-test. Venoarterial concentration and net flux rate differences also were determined to be different from zero by t-test.

RESULTS

Splanchnic blood flow is shown in table 1. Portal vein flow decreased ($P < .05$) from 1.88 to 1.60 L/min during energy restriction. Hepatic vein blood flow tended to decrease ($P < .06$) (2.35 vs. 2.08 L/min) with energy restriction, however, the ratio of portal blood flow to hepatic blood flow remained constant at approximately 80%. Hepatic artery blood flow was unaffected by energy restriction, remaining just under .50 L/min.

Glucose arterial concentrations decreased ($P < .05$) with energy restriction from 2.68 to 2.41 mM (Table 2). Portal drained viscera flux decreased ($P < .05$) from a release that was not significantly different from zero (1.57 mmol/h) at 100% ME to utilization (-8.70 mmol/h) at 50% ME. Hepatic release of glucose remained constant at approximately 30 mmol/h and there was no significant change in total

Table 1
Splanchnic Blood Flow (L/min) in ewes fed at
100% and 50% of NRC requirements for ME

	Portal Vein	Hepatic Vein	Hepatic Artery	<u>Portal</u> <u>Hepatic</u>
100%	1.88	2.35	0.47	0.80
50%	1.60	2.08	0.48	0.78
pSD ¹	0.12	0.14	0.09	0.03
P<	.05	.06	NS	NS

¹pSD - Pooled Standard Deviation

Table 2
Glucose arterial concentration (A), portal drained viscera (PDV), hepatic (HEP) and total splanchnic (TSP) flux rates, and rump venoarterial differences (V-A) and extraction (RUMP Ex) in ewes fed at 100% and 50% of NRC requirements for ME

	A	PDV	HEP	TSP	V-A	RUMP Ex
	mM	-----mmol/h-----			mM	%
100%	2.68	1.57	30.71	32.28	-.07	2.5
50%	2.41	-8.70	29.65	20.65	-.11	5.0
pSD ¹	.1	4.0	5.5	5.9	.03	1.0
P<	.05	.05	NS	NS	NS	.05

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

splanchnic output. Glucose venoarterial difference across the rump did not change and remained at approximately .10 mM.

Nonesterified fatty acid arterial concentrations increased ($P < .01$) almost 2 fold with energy restriction from 233 to 431 μM (Table 3). Portal drained viscera release was not significantly different, however, hepatic uptake increased ($P < .01$) almost 2.5 fold from 47 to 113 $\mu\text{mol/h}$. Hepatic extraction increased ($P < .01$) 50% from 12 to 18% with energy restriction. The increase in hepatic extraction resulted in an increase ($P < .01$) in total splanchnic uptake from 32 to 84 $\mu\text{mol/h}$. Rump venoarterial difference may have compensated for the increased hepatic uptake because of increased ($P < .01$) fatty acid release (23 vs 77 μM) with energy restriction.

Acetoacetate flux and concentrations are shown in table 4. Arterial acetoacetate concentrations increased ($P < .05$) with energy restriction from 20.7 to 31.5 μM . Portal drained viscera release of acetoacetate decreased ($P < .05$) from 30.2 to 19.3 $\mu\text{mol/min}$ with energy restriction, however, hepatic uptake decreased ($P < .01$) from 32.6 to 2.0 $\mu\text{mol/min}$ resulting in an increase ($P < .01$) in total splanchnic release from -2.4 to 17.3 $\mu\text{mol/min}$. Rump venoarterial difference of acetoacetate was unaffected by energy restriction.

Table 3
Nonesterified fatty acid arterial concentration (A), portal drained viscera (PDV), hepatic (HEP) and total splanchnic (TSP) flux rates, hepatic extraction (HEP Ex), and rump venoarterial differences (V-A) in ewes fed at 100% and 50% of NRC requirements for ME

	A	PDV	HEP	HEP Ex	TSP	V-A
	μM	-- $\mu\text{mol/min}$ --		%	$\mu\text{mol/min}$	μM
100%	233	16	-47	12	-32	23
50%	431	29	-113	18	-84	77
pSD ¹	37	8	13	2	13	18
P<	.01	NS	.01	.01	.01	.01

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

Table 4
Acetoacetate arterial concentration (A), portal
drained viscera (PDV), hepatic (HEP) and total
splanchnic (TSP) flux rates, and rump venoarterial
differences (V-A) and extraction (RUMP Ex) in ewes fed
at 100% and 50% of NRC requirements for ME

	A	PDV	HEP	TSP	V-A	RUMP Ex
	μM	----- $\mu\text{mol}/\text{min}$ -----			μM	%
100%	20.7	30.2	-32.6	-2.4	-5.1	12.5
50%	31.5	19.3	-2.0	17.3	-8.3	25.4
pSD ¹	4.2	4.2	6.3	6.1	2.7	9.2
P<	.05	.05	.01	.01	NS	NS

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

Arterial β -hydroxybutyrate concentrations and total splanchnic output were not significantly different between the 100% and the 50% groups (Table 5). However, there was a switch in the origin of the ketone. Portal drained viscera release decreased ($P<.05$) from 269 to 170 $\mu\text{mol}/\text{min}$, while hepatic release increased ($P<.05$) almost 2 fold from 189 to 365 $\mu\text{mol}/\text{min}$ with energy restriction. Rump utilization was not changed with energy restriction and remained constant at approximately 50 μM .

Acetate arterial concentrations (Table 6) decreased ($P<.01$) from 978 to 622 μM with energy restriction, as did portal vein concentrations (1689 vs. 1076 μM). Portal drained viscera released decreased ($P<.01$) from 927 to 439 $\mu\text{mol}/\text{h}$ and hepatic acetate flux was not significantly different from zero. However, the change in portal flux was enough to cause a decrease ($P<.01$) in total splanchnic output (834 vs. 456 $\mu\text{mol}/\text{h}$). Rump venoarterial difference of acetate decreased ($P<.01$) with energy restriction from 317 to 181 μM , but this was concentration dependent, because rump extraction ratios were not significantly different.

Propionate arterial concentrations were not different, despite changes in splanchnic tissue flux rates (Table 7). Portal concentrations were lower ($P<.01$) (215 vs. 128 μM). Portal drained viscera release decreased ($P<.01$) from 263 to 104 $\mu\text{mol}/\text{min}$, as did hepatic uptake (245 vs. 96

Table 5
 β -Hydroxybutyrate arterial concentration (A),
portal drained viscera (PDV), hepatic (HEP) and
total splanchnic (TSP) flux rates, and rump
venoarterial differences (V-A) and extraction (RUMP
Ex) in ewes fed at 100% and 50% of NRC requirements
for ME

	A	PDV	HEP	TSP	V-A	RUMP Ex
	μM	----- $\mu\text{mol/min}$ -----			μM	%
100%	475	269	189	458	-46	9.7
50%	555	170	365	535	-50	10.8
pSD ¹	54	41	69	88	14	2.9
P<	NS	.05	.05	NS	NS	NS

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

Table 6
Acetate arterial (A) and portal (P) concentrations, portal drained viscera (PDV), hepatic (HEP) and total splanchnic (TSP) flux rates, and rump venoarterial differences (V-A) and extraction (RUMP EX) in ewes fed at 100% and 50% of NRC requirements for ME

	A	P	PDV	HEP	TSP	V-A	Rump Ex
	----- μ M-----		--- μ mol/min-----			μ M	%
100%	978	1689	927	-93	834	-317	33
50%	622	1076	439	16	456	-181	25
pSD ¹	78	113	79	67	84	37	4
P<	.01	.01	.01	NS	.01	.01	NS

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

Table 7
 Propionate arterial (A) and portal (P) concentrations,
 portal drained viscera (PDV), hepatic (HEP) and total
 splanchnic (TSP) flux rates, hepatic extraction
 (HEP EX) and rump venoarterial differences (V-A) in
 ewes fed at 100% and 50% of NRC requirements for ME

	A	P	PDV	HEP	HEP Ex	TSP	V-A
	---- μ M-----		--- μ mol/min--	%	μ mol/min	μ M	
100%	19	215	263	-245	82	18	-7
50%	17	128	104	-96	72	8	-5
pSD ¹	2	19	26	24	2	4	1
P<	NS	.01	.01	.01	.01	.05	NS

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

$\mu\text{mol}/\text{min}$). Hepatic extraction also decreased ($P<.01$) from 82 to 72% indicating that the extraction of propionate was not simply concentration dependent. However, the decreases in hepatic uptake and extraction were not enough to off-set the decrease in portal release, resulting in a decrease ($P<.05$) in total splanchnic release (18 vs. 8 $\mu\text{mol}/\text{min}$). Rump venoarterial difference of propionate was not changed.

Butyrate arterial (8 vs. 5 μM) and portal (27 vs. 15 μM) concentrations both decreased ($P<.01$) with energy restriction (Table 8). Portal drained viscera release decreased ($P<.01$) almost 66% from 25 to 9 $\mu\text{mol}/\text{min}$. However, hepatic uptake also decreased ($P<.01$) almost 66% from 21 to 6 $\mu\text{mol}/\text{min}$, resulting in no change in total splanchnic output. Hepatic extraction ratios were not significantly different. Rump venoarterial difference was small and not significantly different from zero.

Valerate portal concentrations decreased ($P<.01$) from 9 to 4 μM with energy restriction (Table 9). Portal drained viscera release was also lower ($P<.01$), decreasing from 12 to 4 $\mu\text{mol}/\text{min}$. Hepatic uptake was 100% of portal drain viscera release, and consequently decreased ($P<.01$) from 12 to 4 $\mu\text{mol}/\text{min}$.

The branched volatile fatty acids, isobutyrate (Table 10) and isovalerate (Table 11), showed no significant

Table 8
Butyrate arterial (A) and portal concentrations,
portal drained viscera (PDV), hepatic (HEP) and total
splanchnic (TSP) flux rates, hepatic extraction (HEP
EX) and rump venoarterial differences (V-A) in ewes
fed at 100% and 50% of NRC requirements for ME

	A	P	PDV	HEP	HEP Ex	TSP	V-A
	---- μ M----		- μ mol/min-		%	μ mol/min	μ M
100%	8	27	25	-21	47	4	-2
50%	5	15	9	-6	26	3	0
pSD ¹	1	3	4	4	12	2	1
P<	.01	.01	.01	.01	NS	NS	NS

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

Table 9
Valerate portal concentration (P), and portal
drained viscera (PDV) and hepatic (HEP) flux
rates in ewes fed at 100% and 50% of NRC
requirements for ME

	P	PDV	HEP
	μM	----- $\mu\text{mol/min}$ -----	
100%	9	12	-12
50%	4	4	-4
pSD ¹	1	2	2
P<	.01	.01	.01

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

Table 10
Isobutyrate arterial and portal (P) concentrations, portal drained viscera (PDV), hepatic (HEP) and total splanchnic (TSP) flux rates, and rump venoarterial differences (V-A) in ewes fed at 100% and 50% of NRC requirements for ME

	A	P	PDV	HEP	TSP	V-A
	--- μM ---		----- $\mu\text{mol}/\text{min}$ -----			μM
100%	4	13	12	-13	-1	-2
50%	2	13	10	-9	1	0
pSD ¹	1	1	2	2	2	1
P<	NS	NS	NS	NS	NS	NS

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

Table 11
Isovalerate portal concentration (P), and
portal drained viscera (PDV) and hepatic (HEP)
flux rates in ewes fed at 100% and 50% of NRC
requirements for ME

	P	PDV	HEP
	μM	----- $\mu\text{mol/min}$ -----	
100%	11	12	-13
50%	12	11	-9
pSD ¹	1	1	2
P<	NS	NS	NS

¹pSD - Pooled Standard Deviation
Note: Negative numbers indicate metabolite uptake

change in any metabolite measured as a result of energy restriction.

DISCUSSION

Blood Flow:

Many researchers have demonstrated that splanchnic blood flow correlates positively with the amount of energy consumed by the animal (Burrin, et al., 1989; Bergman and Wolff, 1971; Mineo, et al., 1991). The decrease in portal and hepatic blood flows observed in this study agrees with data from other laboratories. While the change in hepatic blood flow was not highly significant ($P < .06$), there is previous data (Katz and Bergman, 1969) suggesting that this decrease was real. The fact that hepatic artery blood flow remained the same during energy restriction, a period when there is an increased reliance on endogenous fuels, shows that there must be a mechanism for ensuring a constant supply of nutrients to the liver. This mechanism may be controlled at the level of portal blood flow, since hepatic artery contribution does not change. The portal/hepatic ratio of approximately 80% observed in this study is in close agreement with data produced in this and other laboratories which have demonstrated that this ratio will remain constant under a variety of conditions, including

pregnancy (Bergman, et al., 1970) and fasting (Bergman and Wolff, 1971; Heitmann et al., 1986).

Glucose Metabolism:

The decrease in glucose arterial concentration observed in this study is consistent with decreases observed in other laboratories in feed restricted animals (for review, see Baird, 1977). Because hepatic production of glucose did not decrease, increased portal drained visceral glucose consumption could have explained some of the decrease in arterial concentration. However, some other tissues may have changed their individual glucose metabolism as well. One organ in particular, the kidney, which may be responsible for up to 15% of glucose production in the ruminant (Bergman, 1973), may have decreased glucose production. The majority of gluconeogenic substrates absorbed by the gut must first pass through the liver. Since liver production of glucose was not affected by energy restriction, a time when there is less exogenous fuel available to the liver, an increased reliance on endogenous substrates may have limited gluconeogenic precursor availability to the kidneys.

Free Fatty Acid and Ketone Body Metabolism:

Increased arterial concentrations are a generally recognized response to restrictions in energy intake (Emery et al., 1992; Sheety, 1990; Leat and Ford, 1966). Therefore, the 2 fold increase observed in this study would be considered a normal response. This increased arterial concentration appears to be a result of increased rump release, since portal drained viscera release did not change. It should be remember that portal drained viscera nonesterified fatty acid release represents omental lypolysis because absorbed fat is released via the lymph system to the venous blood at the thoracic duct.

Increased hepatic uptake was not due solely to the increase in circulating concentrations, as evidenced by the increase in the hepatic extraction ratio. The 50% increase in the extraction ratio indicates that a mechanism may be involved which attempts to maintain an adequate supply of energy to the liver, in spite of decreased exogenous substrate and blood flow. Whatever the mechanism, the liver did increase free fatty acid uptake during energy restriction greater than 2 fold, which accounted for all the of the increase in total splanchnic uptake.

Increased free fatty acid uptake by the liver has been associated with an increase in ketone body concentrations

(Emery et al., 1992). Bergman (1971) showed that ketone body release during energy restriction ketosis was a result of over production by the liver, not under utilization by extra-hepatic tissues. In the present study, arterial concentrations of acetoacetate did increase. The main reason for this increase appears to be a lack of uptake by the liver. Total splanchnic output increase 33%, in spite of a significant decrease in portal drained viscera release, because of lack of acetoacetate uptake by the liver

This laboratory and others have demonstrated a change in liver acetoacetate metabolism during energy restriction. Most researchers report a change from liver uptake to liver release after a three day fast (Roe et al, 1966; Katz and Bergman, 1969; Heitmann, et al., 1989). This change was not seen in the current experiment. The assumption can then be made that animals fed at 50% of their requirements would not fall into a ketotic state. However, since arterial concentrations were elevated, it appears that the animal's ability to utilize ketone bodies had already reached a maximum. While these animals appeared healthy, even during the energy restriction periods, it appears that ewes which remain at 50% ME for extended periods may develop signs of ketosis.

β -hydroxybutyrate arterial concentrations did not rise with energy restriction, probably because total splanchnic release did not change. There was a change in the source of the ketone body. Portal drained viscera release decreased probably due to the decrease in available substrate from the rumen. Hepatic production increased, possibly due to the increased fatty acid uptake.

Volatile Fatty Acid Metabolism:

All of the major volatile fatty acids - acetate, propionate and butyrate - demonstrated a decrease in portal concentration with energy restriction. However, splanchnic metabolism of each fatty acid varied. Acetate arterial concentration decreased 36% despite a 42% decrease in rumen utilization. The rumen venoarterial difference was passive and concentration dependent, since extraction ratios did not change. The majority of the arterial concentration change was probably due to a 45% decrease in total splanchnic release. The decrease in splanchnic release was a result of decreased portal drained viscera release, as hepatic flux was not significantly different between treatments. These results are consistent with previously published data (Bergman and Wolff, 1971).

Portal drained viscera release of propionate decreased approximately 60% with energy restriction. Hepatic uptake

also decreased by the same percentage, but not enough to maintain total splanchnic output, which decreased significantly. This is similar to data produced by Bergman et al. (1966), also in 50 % feed restricted animals. The decrease in hepatic propionate uptake was not simply concentration dependent, as there was also a significant drop in the hepatic extraction ratio. Arterial propionate concentrations did not decrease, however, possibly due to an increase in ketone body utilization by tissues which possess the capability to use both ketone bodies and short chain fatty acids.

There was no change in total splanchnic butyrate release. This occurred because the decrease in hepatic uptake paralleled the decrease in portal drained viscera release, which has been demonstrated earlier by Bergman and Wolff (1971). However, there was an unexpected decrease in arterial concentration. Since rump venoarterial differences and total splanchnic release were not different from zero, it is possible that some other tissues, such as the kidney cortex (Pennington, 1951), began using butyrate due to the decrease in substrates which occurred with the change in energy balance.

Valerate portal concentrations decreased significantly, as did portal flux. Hepatic uptake also decreased, however, this may have been a direct result of

the decrease in portal release. Since little or no arterial valerate was present, it is difficult to state whether or not the liver possesses the ability to utilize greater quantities of valerate.

The branched-chain volatile fatty acids, isobutyrate and isovalerate, showed no significant change in flux rates or concentrations. However, since the concentrations of these acids are so low, they were not considered to be of major physiological importance.

CONCLUSION

As expected, a 50% restriction in energy intake decreased the energy released by the portal drained viscera almost 50% (Table 12). However, only a fraction of the energy fed to the animals appeared in the portal vein as metabolites measured in this study. Other sources of energy, such as nonesterified fatty acids and amino acids, released from the gut were not measured and may have counted for a large portion of the ingested energy. Also not measured were losses due to methane production and the undigestible portion of the feed.

Table 12 illustrates that while a 60% decrease in the major volatile fatty acids occurred, ketone body release only decreased approximately 35%. Therefore, rumen epithelium maintained its ketogenic capacity, converting

approximately 90% of the available butyrate to ketone bodies.

Table 12
Energy release as VFA or ketone bodies from portal
drained viscera of sheep fed at 50 or 100% of
NRC requirements for ME

	100%	50%	%Decrease
Acetate	1167	553	53
Propionate	578	229	60
Butyrate	79	28	65
Isobutyrate	38	31	18
Valerate	49	16	67
Isovalerate	49	45	8
β -Hydroxybutyrate	790	499	37
Acetoacetate	78	50	36
Total	2828	1451	48

Literature Cited

Literature Cited

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CHAPTER 4

EFFECTS OF ENERGY RESTRICTION AND LASALOCID ON NET METABOLITE FLUX

ABSTRACT

Concentration and net fluxes of glucose (GLU), nonesterified fatty acids (NEFA), acetoacetate (ACAC), β -hydroxybutyrate (BOHB) and volatile fatty acids (VFA) were studied in mature, non-pregnant, non-lactating ewes. Chronic indwelling catheters were placed in portal, hepatic and mesenteric veins, and the caudal vena cava and aorta at least 14 days prior to experiments. Ewes were fed alfalfa pellets at 100% or 50% of the NRC metabolizable energy requirement with the addition of the ionophore lasalocid. On experiment days, paraaminohippurate (PAH, 1.5% @ .764 ml/min.) was infused via the mesenteric catheter in order to measure blood flow across down stream tissues. After a one hour equilibration period, a series of 6 samples (12 ml) were drawn simultaneously at 30 min. intervals from the portal and hepatic veins and the caudal vena cava and aorta. Flux rates were calculated by multiplying venoarterial (V-A) differences by blood flow rates across the respective tissue. Rump V-A differences were calculated instead of flux rates because a simultaneous blood flow could not be measured. Whole blood was analyzed for PAH, GLU, ACAC and BOHB. Plasma was analyzed for NEFA

and VFA's. Energy restriction (ER) did not change splanchnic blood flow. Arterial glucose concentration actually increased ($P < .01$) from 2.36 to 2.56 mM despite a decrease ($P < .05$) in total splanchnic (TSP) release of 40%. Liver (HEP) release of GLU was not statistically different, however, it was approx. 33% lower than what was expected (20 vs. 30 mmol/h). NEFA arterial concentrations increased ($P < .01$) 2 fold despite a 2 fold increase ($P < .01$) in hepatic uptake and a switch from rump release to utilization ($P < .01$). Arterial ACAC concentrations did not change despite a decrease ($P < .01$) of 60% in portal vein viscera (PDV) release. BOHB arterial concentrations increased ($P < .01$) due to an increase ($P < .01$) in HEP production of 45%. Acetate arterial concentrations decreased ($P < .01$), as did portal concentrations. PDV acetate release decreased ($P < .01$) almost 65%, resulting in decreased ($P < .01$) TSP release. Propionate arterial concentrations decreased ($P < .01$) almost 50%, while portal concentrations decreased ($P < .01$) almost 65%. PDV release of propionate decreased almost 4 fold. HEP propionate uptake decreased nearly 3.5 fold. However, TSP release still saw a decrease ($P < .01$) from 31 to 2 $\mu\text{mol}/\text{min}$. Butyrate arterial concentrations decreased ($P < .01$), as did portal concentrations. There was no change in TSP butyrate release, despite a decrease ($P < .01$) of 65% in PDV release. HEP butyrate uptake also

decreased ($P < .01$) 3 fold. Valerate portal concentrations decreased ($P < .05$) 40%. PDV release of valerate decreased ($P < .05$) 40%, as did HEP uptake. There were no significant net flux changes with either of the branched chain volatile fatty acids, isobutyrate and isovalerate.

INTRODUCTION

Lasalocid belongs to a class of compounds called carboxylic ionophores, so named for the terminal carboxyl group which they all possess (Painter and Pressman, 1985). These compounds are chemically inert, except for their ability to transport ions across biological membranes. This feature has allowed new insight into the metabolism of certain organs and various organelles by allowing the researcher to perturb the delicate concentration and electrical gradients needed by many biological systems to function effectively. Most recently, ionophores, especially monensin and lasalocid, have been studied as agricultural feed additives to improve feed efficiency in ruminants.

The majority of lasalocid's effects may be traced back to the ability of the ionophore to transport cations and dissipate concentration gradients. Russell and Strobel (1989) concluded that the effects of ionophore supplementation observed in the rumen are primarily due to

increased ion flux through microbial membranes, the cation selectivity of the ionophore, ion gradients and microbial sensitivity. Lasalocid may transport both monovalent (e.g. K^+ , Na^+) and divalent (e.g. Ca^{+2} , Mg^{+2}) cations, which are sufficiently abundant in rumen fluid (Russell and Strobel, 1989). Concentration gradients of ions which the ionophore will transport are also a factor and may be more important than ionophore selectivity. Differing concentration gradients, along with different pH levels, associated with different feeding regimens may account for some of the variable responses associated with ionophore supplementation observed in the literature. The mechanisms by which lasalocid alters ruminal fermentation characteristics are due to shifts in rumen microbial populations because of microbial sensitivity to the ionophore (Nagaraja et al., 1987).

Current research suggests ionophore resistance is related closely to cell wall structure (Chen and Wolin, 1979). Gram-negative organisms, which tend to be ionophore-resistant, generally possess an outer cell wall. Gram-positive organisms which lack an outer cell wall are more likely to be sensitive to ionophore action. Ruminal protozoa, which also lack an outer cell wall, have also been shown to be ionophore-sensitive (Dennis et al., 1986). Gram-positive organisms are the primary producers of

acetate, butyrate, H_2 , formate and ammonium. Selection against these organisms should result in decreased lactate and methane production, with an increase in the molar proportion of propionate. However, improvements in animal production will be related to the ability of the resistant organisms to fill the void left by the resistant organisms (e.g. cellulose digestion) (Russell and Strobel, 1989).

A great number of biological responses have been attributed to ionophore feeding. The most widely recognized response being the modification of ruminal volatile fatty acid (VFA) production. Numerous researchers have documented a change in the acetate:propionate ratio in the rumen of ionophore-fed animals (Nagaraja et al., 1987; Ricke et al, 1984; Van Maanen et al, 1978). Propionate production appears to be increased at the expense of acetate, while total volatile fatty acid concentrations remain constant (Armstrong and Young, 1983). Initially, researchers believed that increased ruminal propionate would lead to an increased production of glucose from propionate in the animal. This was suggested because Judson and Leng (1973) had demonstrated an increase in gluconeogenesis with an intra-mesenteric propionate infusion. However, even though many studies demonstrate an increase in ruminal propionate production, Harmon et. al.(1989) found no increase in portal drained viscera (PDV)

propionate release in ionophore-supplemented animals. Animals fed ionophores have also been shown to exhibit modified feed intake and digestibility, depending on the type of diet fed. While it is still unclear whether ionophores affect true digestibility, several researchers have noted lasalocid's ability to alter the primary site of starch digestion (Spears, 1990; Delfino et al., 1982). Ionophores may also decrease methanogenic loss by as much as 30% (Schelling, 1984).

All the effects seen with ionophore supplementation can be attributed to the ionophore's ability to interact with microorganisms in the rumen. These interactions in the rumen will cause changes in metabolite release from the portal drained viscera. However, in all the experimental data generated to date rumen, microbes have been in a state of positive energy balance. The objective of the study was to determine the effects of ionophore supplementation on metabolite concentrations and fluxes in splanchnic tissues during short term energy restriction.

MATERIALS AND METHODS

Animals and Housing:

Effects of energy restriction were studied in mature non-pregnant, non-lactating ewes of various breeds and crosses, weighing between 55 and 75 kg. The animals were

housed in individual 1.8 x 3.0 m pens at approximately 17-22 degrees Celsius. The ewes were fed commercially prepared alfalfa pellets daily at approximately 0800 hours. All sheep were accustomed to being handled, therefore restraint was not necessary during the experimental periods. Water was available at all times.

Surgical Protocol:

Animals were surgically implanted with chronic indwelling catheters placed so that the tips would reside in the following locations: 1) caudal aorta (A), 2) caudal vena cava (V), 3) common mesenteric vein (M), 4) hepatic vein (H) and 5) portal vein (P), as described by Katz and Bergman (1969) and modified by Zanzalari et al. (1989)

Briefly, the caudal aorta and vena cava catheters were implanted by making a 9 cm incision in the inguinal region, just medial to the M. satorius. The muscle layers were then separated to expose the femoral vein and aorta.

Hepatic and portal catheters were implanted by first making a 22 cm incision starting 4 cm caudal to the 13th rib and 5 cm ventral to the first lumbar vertebra, following the costal arch. The left lateral lobe of the liver was exposed, palpated, an incision was made and catheters were inserted. Hepatic veins were indentified though movement of a tiny air bubble inserted into the

catheter. Portal vein catheters were identified by palpation of the porta hepatis.

Mesenteric vein catheters were implanted by isolating a branch of the common mesenteric vein and inserting a catheter so that the tip resided in the common mesenteric vein.

Experimental Protocol:

Animals were fed alfalfa pellets (17% protein) at either 100% or 50% of their metabolizable energy (ME) requirement as determined by the National Research Council (NRC) (1984) plus Lasalocid (Bovatec 20 Liquid, Hoffman-LaRoche, Inc., Nutley, NJ) for at least ten days following surgery. Animals which were fed 100% ME, and whose catheters remained functional after a first experiment, were then fed 50% ME for at least ten days and a second experiment was performed. The ewes were weighted before being placed on their respective diets. Animals in the 100% ME group maintained their body weight. No measurement was made at the end of the 50% ME trials. A total of 5 ewes were used (three ewes had two experiments performed). Two ewes only had one experiment performed due to a loss of catheter patency.

On experiment days, Para-aminohippurate (PAH) (Eastman Kodak, Rochester, NY) was infused (1.5% solution @ .764

ml/min.) and allowed to equilibrate for one hour before samples were taken. Six serial blood samples were then taken simultaneously from the A, H, P and V vessels at consecutive thirty minute intervals. Whole blood was analyzed for glucose, PAH, acetoacetate and β -hydroxybutyrate. Plasma was analyzed for nonesterified fatty acids (NEFA) on the day of the experiment. Aliquots of plasma were set aside and frozen for subsequent analysis for volatile fatty acid (VFA) concentrations.

Analysis:

Glucose concentrations were determined utilizing a glucose determination kit, No 510 (Sigma Chemical Co, St. Louis, Mo.). The exact procedure is described in Appendix 1.

Plasma NEFA concentrations were analyzed utilizing a WAKO NEFA-C kit (WAKO Pure Chemical Industries, Osaka, Japan). The exact procedure is described in Appendix 2.

The ketone bodies, acetoacetate and β -hydroxybutyrate, were analyzed enzymatically based on the method of Williamson et. al. (1962). The exact procedure is described in Appendix 3 and 4, respectfully.

Volatile fatty acid concentrations were determined based on the method of Reynolds et. al. (1986), with modifications by Quigley et al (1991). The exact procedure

is described in Appendix 5. VFA determination was only performed on the 100% ME group due to loss of plasma samples.

For whole blood flow, PAH concentrations were determined utilizing the method of Katz and Bergman (1969). The exact procedure is described in Appendix 6.

Due to a loss of catheter patency, rump data was obtained on only two animals in the 50% ME group.

Calculations:

Blood flow was determined by the indicator-dilution method, using the following equation:

$$BF(l/min) = PAH_p / (V-A)$$

PAH_p represents the infusion rate of PAH in OD/min. $V-A$ represents the venoarterial difference in PAH concentration for the tissue. Portal and hepatic vein flows were measured directly and hepatic artery contributions were measured by hepatic-portal difference.

Flux data was determined by the following equations:

$$\begin{aligned} PDV \text{ (mM/min)} &= PF \text{ (L/min)} \times (P-A) \text{ (mM)} \\ HEP \text{ (mM/min)} &= HF \text{ (L/min)} \times (H-P) \text{ (mM)} + \\ &\quad AF \text{ (L/min)} \times (H-A) \text{ (mM)} \\ TSP \text{ (mM/min)} &= HF \text{ (L/min)} \times (H-P) \text{ (mM)} \end{aligned}$$

PDV, HEP, and TSP represent portal drained viscera, hepatic and total splanchnic flux, respectively. Total splanchnic flux is equal to PDV + HEP, however, TSP was calculated separately. PF, HF and AF, represent portal vein, hepatic

vein and arterial blood flow, respectively. P, H and A represent portal vein, hepatic vein and hepatic artery metabolite concentrations, respectively. Rump flux data was not obtained because simultaneous blood flow measurements could not be obtained.

Extraction ratios were only calculated when net fluxes were determined to be negative and statistically different from zero ($P < .05$) by the following equation:

$$\text{Extraction ratio} = (\text{net uptake} / (\text{BF} \times \text{A})) \times 100\%$$

Extraction ratios represent the amount of a metabolite presented to a tissue that is taken up by that tissue.

Statistics:

Differences between groups was tested for significance by t-test. Venoarterial concentration and net flux rate differences also were determined to be different from zero by t-test.

RESULTS

Splanchnic blood flow is shown in table 1. Portal vein flow did not decrease during energy restriction, remaining constant at approximately 1.80 L/min. Hepatic vein blood flow was also unchanged. The ratio of portal blood flow to hepatic blood flow remained constant at

Table 1
Splanchnic Blood Flow (L/min) in ewes fed at 100%
and 50% of NRC requirements for ME plus ionophore

	Portal Vein	Hepatic Vein	Hepatic Artery	<u>Portal</u> <u>Hepatic</u>
100%	1.91	2.41	0.50	0.81
50%	1.78	2.17	0.39	0.83
pSD ¹	0.12	0.18	0.09	0.03
P<	NS	NS	NS	NS

¹pSD - Pooled Standard Deviation

approximately 82%. Hepatic artery blood flow did not change significantly with energy restriction.

Paradoxically, glucose arterial concentrations increased ($P < .05$) with energy restriction from 2.36 to 2.54 mM (Table 2). Portal drained viscera and hepatic glucose fluxes were not significantly different, however total splanchnic output was statistically lower ($P < .05$) in the energy restricted animals (22.27 vs. 17.63 mmol/h). Rump glucose venoarterial difference did not change and was approximately 1.0 mM.

Nonesterified fatty acid arterial concentrations increased ($P < .01$) almost 2 fold with energy restriction from 179 to 366 μ M (Table 3). Portal drained viscera release was not significantly different, however, hepatic uptake increased ($P < .01$) almost 2 fold from 42 to 95 μ mol/h. The increase in hepatic uptake resulted in an increase ($P < .01$) in total splanchnic uptake from 22 to 54 μ mol/h. Rump fatty acid release decreased ($P < .01$), changing from a net release (9 μ M) in 100% animals, to a net uptake (10 μ M) in fed restricted animals.

Acetoacetate flux and concentrations are shown in table 4. Arterial acetoacetate concentrations were not changed with energy restriction remaining around 30 μ M. Portal drained viscera release of acetoacetate decreased

Table 2
Glucose arterial concentration (A), portal drained viscera (PDV), hepatic (HEP) and total splanchnic (TSP) flux rates, and rump venoarterial differences (V-A) and extraction (RUMP Ex) in ewes fed 100% and 50% of NRC requirements for ME plus ionophore

	A	PDV	HEP	TSP	V-A	RUMP Ex
	mM	-----mmol/h-----			mM	%
100%	2.36	-1.44	23.71	22.27	-0.07	2.92
50%	2.54	-4.44	17.63	13.20	-0.03	1.34
pSD ¹	.09	2.38	3.84	3.41	.84	2.09
P<	.05	NS	NS	.05	NS	NS

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

Table 3
Nonesterified fatty acid arterial concentration (A),
portal drained viscera (PDV), hepatic (HEP) and total
splanchnic (TSP) flux rates, hepatic (HEP Ex)
extraction, and rump venoarterial differences (V-A)
in ewes fed 100% and 50% of NRC requirements
for ME plus ionophore

	A	PDV	HEP	HEP Ex	TSP	V-A
	μM	-- $\mu\text{mol/h}$ ---		%	$\mu\text{mol/h}$	μM
100%	179	19	-42	-11.9	-22	9
50%	366	41	-95	-14.5	-54	-10
pSD ¹	34	12	17	2.1	10	6
P<	.01	NS	.01	NS	.01	.01

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

Table 4
 Acetoacetate arterial concentration (A), portal
 drained viscera (PDV), hepatic (HEP) and total
 splanchnic (TSP) flux rates, and rump venoarterial
 differences (V-A) and extraction (RUMP Ex) in ewes
 fed at 100% and 50% of NRC requirements for ME plus
 ionophore

	A	PDV	HEP	TSP	V-A	RUMP Ex
	μM	----- $\mu\text{mol/min}$ -----			μM	%
100%	35.8	64.5	-25.1	39.3	-10.4	20.2
50%	28.3	23.9	-12.7	11.2	-17.0	43.7
pSD ¹	4.6	10.4	17.1	19.0	8.5	25.7
P<	NS	.01	NS	NS	NS	NS

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

($P < .01$) from 64.5 to 23.9 $\mu\text{mol/min}$ with energy restriction, however, hepatic uptake and total splanchnic output were unaffected by energy restriction. Rump uptake of acetoacetate was not changed by energy restriction.

Arterial β -hydroxybutyrate concentrations increased ($P < .01$) during energy restriction from 391 to 581 μM (Table 5). Portal drained viscera release was unchanged, while hepatic release increased ($P < .01$) almost 2 fold from 218 to 399 $\mu\text{mol/min}$ with energy restriction. As a result, total splanchnic output increased ($P < .05$) from 421 to 564 $\mu\text{mol/min}$. Rump utilization was not changed with energy restriction and remained constant at approximately 60 μM .

Acetate arterial concentrations (Table 6) decreased ($P < .01$) from 1067 to 515 μM with energy restriction, as did portal vein concentrations (2115 vs. 989 μM). Portal drained viscera release decreased ($P < .01$) from 1410 to 515 $\mu\text{mol/min}$, but hepatic acetate flux was highly variable and not significantly different from zero. However, the change in portal flux was enough to cause a decrease ($P < .01$) in total splanchnic output (1428 vs. 362 $\mu\text{mol/min}$).

Propionate arterial concentrations decreased ($P < .01$) from 28 to 16 μM (Table 7). Portal concentrations were also lower ($P < .01$) (321 vs. 113 μM). Portal drained viscera release decreased ($P < .01$) from 394 to 102 $\mu\text{mol/min}$,

Table 5
 β -Hydroxybutyrate arterial concentration (A), portal
drained viscera (PDV), hepatic (HEP) and total
splanchnic (TSP) flux rates, and rump venoarterial
differences (V-A) and extraction (RUMP Ex) in ewes fed
at 100% and 50% of NRC requirements for ME plus
ionophore

	A	PDV	HEP	TSP	V-A	RUMP Ex
	μM	----- $\mu\text{mol/min}$ -----			μM	%
100%	391	203	218	421	-73	20.3
50%	581	165	399	564	-53	11.0
pSD ¹	42	41	60	63	26	7.0
P<	.01	NS	.01	.05	NS	NS

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

Table 6
Acetate arterial (A) and portal (P) concentrations, portal drained viscera (PDV), hepatic (HEP) and total splanchnic (TSP) flux rates, and rump venoarterial differences (V-A) and extraction (RUMP EX) in ewes fed 100% and 50% of NRC requirements for ME plus ionophore

	A	P	PDV	HEP	TSP	V-A	Rump Ex
	----- μ M-----		----- μ mol/min-----			μ M	%
100%	1067	2115	1410	19	1428	-391	37.9
50%	515	989	515	-153	362	—	—
pSD ¹	140	347	301	134	347	—	—
P<	.01	.01	.01	NS	.01	—	—

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

Table 7
Propionate arterial (A) and portal (P) concentrations, portal drained viscera (PDV), hepatic (HEP) and total splanchnic (TSP) flux rates, hepatic extraction (HEP Ex) and rump venoarterial differences (V-A) in ewes fed 100% and 50% of NRC requirements for ME plus ionophore

	A	P	PDV	HEP	HEP Ex	TSP	V-A
	---- μ M----		-- μ mol/min--		%	μ mol/min	μ M
100%	28	321	394	-363	80	31	-7
50%	16	113	102	-100	76	2	—
pSD ¹	4	58	71	66	3	8	—
P<	.01	.01	.01	.01	NS	.01	—

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

as did hepatic uptake (363 vs. 100 $\mu\text{mol}/\text{min}$). However decrease in hepatic uptake was not enough to off-set the decrease in portal release, resulting in a decrease ($P<.01$) in total splanchnic release (31 vs. 2 $\mu\text{mol}/\text{min}$).

Butyrate arterial (7 vs. 2 μM) and portal (32 vs. 11 μM) concentrations both decreased ($P<.01$) with energy restriction (Table 8). Portal drained viscera release decreased ($P<.01$) 65% from 34 to 12 $\mu\text{mol}/\text{min}$. However, hepatic uptake also decreased ($P<.01$) 70% from 27 to 8 $\mu\text{mol}/\text{min}$, resulting in no change in total splanchnic output.

Isobutyrate arterial and portal concentrations were both unchanged by energy restriction (Table 9). Portal drained viscera release of isobutyrate was lowered ($P<.01$) from 23 to 10 $\mu\text{mol}/\text{min}$. Hepatic uptake was not significantly different.

Valerate portal concentrations decreased ($P<.05$) from 10 to 6 μM with energy restriction (Table 10). Portal drained viscera release was also lower ($P<.05$), decreasing from 14 to 8 $\mu\text{mol}/\text{min}$. However, hepatic uptake mirrored portal drain viscera release, as uptake decreased ($P<.01$) from 14 to 8 $\mu\text{mol}/\text{min}$.

Isovalerate portal concentrations were not significantly different, however portal drained viscera flux were lower ($P<.01$), decreasing from 22 to 11 $\mu\text{mol}/\text{min}$

Table 8

Butyrate arterial (A) and portal (P) concentrations, portal drained viscera (PDV), hepatic (HEP) and total splanchnic (TSP) flux rates, hepatic extraction (HEP Ex) and rump venoarterial differences (V-A) in ewes fed 100% and 50% of NRC requirements for ME plus ionophore

	A	P	PDV	HEP	HEP Ex	TSP	V-A
	----- μ M-----		- μ mol/min-		%	μ mol/min	μ M
100%	7	32	34	-27	54	7	-2
50%	2	11	12	-8	56	3	—
pSD ¹	1	5	7	6	9	2	—
P<	.01	.01	.01	.01	NS	NS	—

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

Table 9
Isobutyrate arterial (A) and portal (P)
concentrations, portal drained viscera (PDV), hepatic
(HEP) and total splanchnic (TSP) flux rates, and rump
venoarterial differences (V-A) in ewes fed 100% and
50% of NRC requirements for ME plus ionophore

	A	P	PDV	HEP	TSP	V-A
	--- μ M----		----- μ mol/min-----			μ M
100%	5	22	23	-21	2	-1
50%	6	15	10	-13	-3	—
pSD ¹	1	4	4	4	2	—
P<	NS	NS	.01	NS	.05	—

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

Table 10
Valerate portal concentration (P), and portal
drained viscera (PDV) and hepatic (HEP) flux
rates in ewes fed at 100% and 50% of NRC
requirements for ME plus ionophore

	P	PDV	HEP
	μM	----- $\mu\text{mol/min}$ -----	
100%	10	14	-14
50%	6	8	-8
pSD ¹	2	2	2
P<	.05	.05	.05

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

Table 11
Isovalerate portal concentration (P), and
portal drained viscera (PDV) and hepatic (HEP)
flux rates in ewes fed at 100% and 50% of NRC
requirements for ME plus ionophore

	P	PDV	HEP
	μM	----- $\mu\text{mol/min}$ -----	
100%	19	22	-20
50%	14	11	-11
pSD ¹	3	3	4
P<	NS	.01	.05

¹pSD - Pooled Standard Deviation

Note: Negative numbers indicate metabolite uptake

during energy restriction (Table 11). However, hepatic uptake mirrored portal drain viscera release, as uptake decreased ($P<.01$) from 20 to 11 $\mu\text{mol}/\text{min}$.

DISCUSSION

Blood Flow:

Many researchers have demonstrated that splanchnic blood flow varies with the amount of energy consumed by the animal (Burrin, et al., 1989; Bergman and Wolff, 1971; Mineo, et al., 1991). The study immediately previous and presented above also showed decreased portal and hepatic vein blood flow. However, in this study although the actual rates were extremely similar to the study above, there were no significant changes in splanchnic blood flow between the energy restricted and full-fed animals due to slightly higher variability. The portal/hepatic ratio of approximately 80% observed in this study is in close agreement with data produced in this and other laboratories which have demonstrated that this ratio will remain constant under a variety of conditions, including pregnancy (Bergman, et al., 1970) and fasting (Bergman and Wolff, 1971; Heitmann et al., 1986).

Glucose Metabolism:

Glucose arterial concentrations actually rose in the energy restricted animals. This result conflicts with the decrease in arterial concentrations seen in non-supplemented energy restricted animals (see Baird, 1977 and the above study). In previous work with energy restricted animals reported above, hepatic glucose production remained similar in both 100% and 50% fed animals. Hepatic production in this study also remained similar between groups, but, it was approximately two thirds of the rate of the non-supplemented animals (20 vs. 30 mmol/h). However, Harmon et al. (1993) reported no change in hepatic glucose release in steers supplemented with monensin. Therefore, hepatic production could not explain the rise in arterial glucose concentrations. Portal drained viscera uptake was not significantly different from zero. This was different from the significant net uptake which occurred in non-supplemented energy restricted animals in the previous study, and data generated from ionophore treated animals in studies by Harmon and co-workers (Harmon et al., 1993; Harmon et al. 1989; Harmon and Avery, 1987). The previous energy restriction study showed an increase in portal drained viscera glucose uptake concomitant with the decrease in arterial concentrations. Paradoxically, total

splanchnic release of glucose decreased ($P < .05$) making interpretation of the observed increase in glucose difficult, unless there was a concomitant and greater decrease in glucose utilization by other tissues. Rump venoarterial difference decreased numerically, but not significantly.

Free Fatty Acid and Ketone Body Metabolism:

Increased concentrations of circulating free fatty acids are a generally recognized response to restrictions in energy intake (Emery et al., 1992; Sheety, 1990; Leat and Ford, 1966). Lasalocid supplementation did not change this response. There was no change in portal drained viscera release, but hepatic uptake increased, resulting in a decrease in total splanchnic release. Since peripheral lypolysis as measured by rump venoarterial difference in this study is considered to be the major supplier of free fatty acids during energy restriction, the reason for the increase in arterial free fatty acid is unclear.

Increased free fatty acid uptake by the liver has been associated with an increase in ketone body concentrations (Emery et al., 1992). Bergman (1971) showed that ketone body release during energy restriction ketosis was a result of over production by the liver, not under utilization by extra-hepatic tissues. However, with lasalocid

supplementation, there was no significant change in hepatic acetoacetate uptake. Total splanchnic acetoacetate release was not statistically different, with energy restriction, despite a 66% decrease in portal drained viscera release due to variability. Energy restricted non-supplemented animals, in the above study, did have a decrease in both total splanchnic and hepatic release, which probably caused the decrease in arterial concentrations observed in that study. Lasalocid supplemented animals did not have a decreased arterial acetoacetate concentration during energy restriction. One possible explanation for this may have been that lasalocid fed animals had numerically higher portal drained viscera acetoacetate release rates than the non-supplemented animals, although no statical analyses were performed between the two.

β -hydroxybutyrate arterial concentrations increased, a trend seen in fasted animals, but one which was observed numerically but not significantly during energy restriction in the previous non-lasalocid supplemented work on 50% energy restriction. In the previous study, portal drained viscera β -hydroxybutyrate release decreased and there was a concomitant increase in hepatic output, resulting in no change in total splanchnic release and increased arterial concentrations. However, in lasalocid fed animals portal drained viscera release did not change significantly,

resulting in an increase in total splanchnic release. Therefor, the rise in arterial concentrations may very well have been a result of the increased hepatic release.

Total portal drained viscera ketone body release rates were also numerically greater than the previous energy restriction study. When total ketone body production was expressed as a percentage of total butyrate production, both studies revealed that approximately 90% of ruminal butyrate produced was converted to ketone bodies. A butyrate conversion rate of 90% has been demonstrated by other researchers as well (Weigand, et al., 1975). However, it appears that lasalocid supplementation did increase the amount of acetate produced, while β -hydroxybutyrate production remained about the same. This coincides with data produced from rumen epithelium studies from lasalocid supplemented animals (B. Housewright, unpublished data), indicating that lasalocid may be changing redox potentials within rumen epithelium cells.

Volatile Fatty Acid Metabolism:

All of the major volatile fatty acids - acetate, propionate and butyrate - demonstrated a decrease in portal concentration with energy restriction. At 100% ME, all major volatile fatty acid portal concentrations were numerically higher in lasalocid fed animals verses data

obtained in the previous non-lasalocid supplemented energy restriction study, however, no statistical analyses were performed between the two. At 50% ME, the portal volatile fatty acid concentrations were very similar between the two studies, indicating that lasalocid may not prove beneficial to animals in a feed restriction induced negative energy balance.

Acetate arterial concentrations decreased almost 50% with energy restriction. The majority of the decrease was probably due to a nearly 75% decrease in total splanchnic release. Hepatic flux was not significantly different, however, portal drained viscera release decreased approximately 60%. These changes were also seen in the previous study, indicating that ionophore supplementation did not affect the trends associated with energy restriction. Rump data in 50% animals was not available due to losses in catheter patency (see Materials and Methods).

Much attention has been given to propionate metabolism in ionophore supplemented animals. However, most of the work has been performed in animals in a positive energy balance, and there has been little change in propionate metabolism reported except for changes in ruminal concentrations. In this study, there was a significant decrease in arterial propionate concentrations, which was

not seen in the previous non-lasalocid supplemented, energy restricted study. This may have been due to an increased level of circulating propionate which resulted from a total splanchnic release which was almost 50% greater than in the previous study, although no statistical analyses were performed between the two. Harmon's laboratory has published contrasting data in this area. Harmon and Avery (1987) showed an increase in arterial propionate, but no change in total splanchnic release. However, Harmon et al. (1993) showed no change in arterial propionate concentrations, but stated that differences in diets may account for some of the variability associated with ionophore data. Both studies were performed in cattle utilizing monensin and the diets contained variable amounts of concentrates. Portal drained viscera and hepatic fluxes of propionate both decreased with energy restriction, which was similar to trend observed in the previous study, although there was no change in hepatic extraction. Perhaps, intact lasalocid released from the gut (Spears, 1990; Donoho, 1984) may have altered this aspect of liver metabolism.

There was no change in total splanchnic butyrate release, which was not different from zero. This occurred because the decrease in hepatic uptake paralleled the decrease in portal drained viscera release, which has been

previously demonstrated earlier by Bergman and Wolff (1971). However, there was an unexpected decrease in arterial concentration, but because the values were so low this is not believed to be of great physiological importance.

The changes in both butyrate and valerate seen in this study were very similar to those seen in the previous study indicating that lasalocid supplementation had very little effect on butyrate or valerate metabolism.

Both isobutyrate and isovalerate portal drain viscera flux decreased significantly, as did hepatic uptake of isovalerate. However since concentrations of these acids are so low, it is difficult to interpret the physiological significance of these events.

CONCLUSION

The amount of energy released as ketone bodies and volatile fatty acids is shown in Table 12. Lasalocid caused an increase in the molar quantities of the major volatile fatty acids - acetate, propionate and butyrate - appearing in the portal vein and, consequently, increased the amount of energy released by portal drained viscera in the ewes fed 100% ME only, when compared to results obtained in the previous study. The ionophore did not appear to affect the amount of energy released by the

portal drained viscera in ewes fed 50% ME, in fact, the amount of energy released was very similar to the previous energy restriction study, which suggest that Lasalocid may not alter rumen metabolism similarly in both positive and negative energy balances. Therefore, from the data obtained in this experiment, it appears that Lasalocid may not be beneficial to animals fed only 50% of National Research Council (1984) recommendations for metabolizable energy.

Table 12
 Energy release as VFA or ketone bodies from portal
 drained viscera of sheep fed at 50% or 100% of NRC
 requirements for ME plus ionophore

	100%	50%	%Decrease
Acetate	1776	649	63
Propionate	866	224	74
Butyrate	106	38	64
Isobutyrate	72	31	57
Valerate	57	33	42
Isovalerate	90	45	50
β -Hydroxybutyrate	596	484	19
Acetoacetate	166	62	62
Total	3729	1566	58

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Literature Cited

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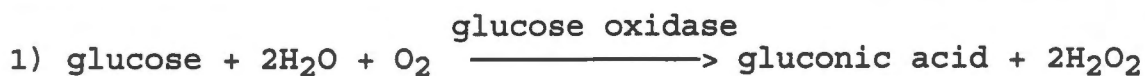
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APPENDICES

APPENDIX 1

GLUCOSE ANALYSIS

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



Reagents (included with kit):

- A) PGO enzymes (500 units glucose oxidase, 100 Purpurogalin units peroxidase and buffer salts), Catalog No. 510-6.
- B) o-Dianisidine Dihydrochloride, Catalog No. 510-50.
- C) Glucose Standard Solution (100 mg/dl), Catalog No. 635-100.

Other Reagents:

- A) 0.3N Barium Hydroxide Solution, Sigma Catalog No. 14-3.
- B) 0.3N Zinc Sulfate Solution, Sigma Catalog No. 14-4.

Reagent Preparation:

- A) Color Reagent: one capsule o-Dianisidine Dihydrochloride diluted with 20 ml H₂O.
- B) Enzyme Solution: one capsule PGO enzymes diluted in 100 ml H₂O.
- C) Enzyme-Color Reagent Solution: 1.6 ml of color reagent is added to 100 ml enzyme solution.
- D) All reagents should be stored in amber bottles at 0-5°C.

Procedure:

- 1) Tubes are labeled STD, BLANK, sample 1, etc. and 1.8 ml H₂O is added to all tubes.
- 2) The blank receives an additional .2 ml H₂O, the STD receives .2 ml glucose and each sample tube receives .2 ml of the respective whole blood sample
- 3) 1.0 ml of barium hydroxide is added to all tubes; vortex.

- 4) 1.0 ml of zinc sulfate is added to all tubes; vortex.
- 5) Tubes are centrifuged at 3 g for 15 min.
- 6) .2 ml of the resulting clear supernatant is then transferred to a new set of tubes labeled as above.
- 7) To each tube add 2.0 ml of the enzyme-color reagent solution.
- 8) Tubes are then incubated for in a 37°C water bath for 30 min. or allowed to equilibrate at room temperature for 45 min.
- 9) Absorbance is measured at 450 nm. All readings should be completed within 30 min.

Calculations:

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

APPENDIX 2

NEFA ANALYSIS

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



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

4-AAP = 4-aminoantipyrine

Reagents (included with kit):

- A) Color Reagent A:
 - Acyl-coenzyme A synthetase
 - Ascorbate oxidase
 - Coenzyme A
 - ATP
 - 4-AAP
- B) Diluent for Color Reagent A:
 - Phosphate buffer
 - Magnesium chloride
 - Surfactant
 - Stabilizers
- C) Color Reagent B:
 - Acyl-coenzyme A oxidase
 - Peroxidase
- D) Diluent for Color Reagent B:
 - MEHA
 - Surfactant
- E) NEFA Standard Solution:
 - Oleic acid (1000 μeq/L)
 - Surfactant
 - Stabilizers

Reagent Preparation:

- A) Color Reagent A Solution: Mix 10 ml of Diluent for Color Reagent A with one vial of color reagent A. Gently invert bottle until powder is completely dissolved, then add 13.3 ml H₂O. Store at 2°C to 10°C until ready to use.
- B) Color Reagent B Solution: Mix 20 ml of Diluent for Color Reagent B with one vial of color reagent A. Gently invert bottle until powder is completely dissolved, then add 33.3 ml H₂O. Store at 2°C to 10°C until ready to use.
- C) Standards: dilute NEFA standard solution provided with H₂O to provide standards of 125, 250, and 500 µeq/L.

Sample Collection and Preparation:

Blood should be collected using a suitable anticoagulant, such as EDTA. Heparin use may yield inaccurate results due to activation of hormone sensitive lipase. After adding whole blood and anticoagulant, tubes should be spun at 3 g for 15 mins. to provide the plasma fraction necessary. This test may also be run on serum by adding whole blood to a clotting tube and harvesting the serum after centrifugation. Samples which are hemolyzed or overtly icteric may yield inaccurate results.

Procedure:

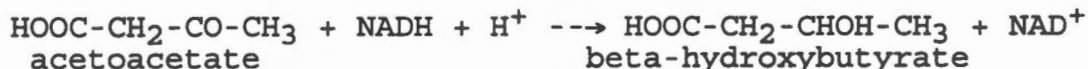
- 1) Tubes are labeled as standards (e.g. 0, 125, 250, etc.) and as samples in duplicate.
- 2) Add .4 ml H₂O to all tubes.
- 3) Add 25 µm of serum, plasma or standard to the appropriate tube, followed by 350 µL of Color Reagent A Solution. Vortex.
- 4) Incubate in a 37° waterbath for exactly 20 min.
- 5) After removing from waterbath, add 800 µL of Color Reagent B Solution to all tubes. Vortex.
- 6) Incubate in a 37° waterbath for exactly 20 min.
- 7) Remove tubes from bath and allow to equilibrate at room temperature for 5 mins.
- 8) Record OD at 550 nm.
- 9) Calculation of sample values should be made against a new standard curve each time the assay is run.

APPENDIX 3

ACETOACETATE ANALYSIS

Principle

The acetoacetate analysis is a spectrophotometric determination based upon a compound's (nicotinamide adenine dinucleotide, NAD, in this analysis) 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:



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 HClO₄ immediately after sampling.
- 2) Centrifuge at 3 g for approximately 15 minutes.
- 3) Neutralize excess HClO₄ with KOH so that the final pH is between 6.0 and 8.0.
- 4) Centrifuge off the potassium perchlorate formed at approximately 3 g 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)
 $1.36 \text{ g KH}_2\text{PO}_4 \longrightarrow 100 \text{ ml dH}_2\text{O}$
 $1.74 \text{ g K}_2\text{HPO}_4 \longrightarrow 100 \text{ ml dH}_2\text{O}$

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 dH₂O

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 CH₃COCH₂COOH ----> 100 ml dH₂O

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 HClO₄.

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

Centrifuge at 3 g for 15 minutes.

Standard dilutions:

- A) 0.15 ----> 2.0 ml of 1.5 mM ACAC plus 18.0 ml dH₂O
- B) 0.09 ----> 12.0 ml of A plus 8.0 ml dH₂O
- C) 0.075 ----> 4.0 ml of A plus 4.0 ml dH₂O
- D) 0.045 ----> 10.0 ml of B plus 10.0 ml dH₂O
- E) 0.025 ----> 10.0 ml of D plus 10.0 ml dH₂O

Analysis Pipette into 12 X 75 mm culture tubes:

0.5 ml buffer
Sample - 0.25 ml plus 0.75 ml dH₂O
Standard - 1.0 ml
0.05 ml NADH

Read E₁ at 340 nm.

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

Read E₂ 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 E₁ in all cuvettes at same
timed intervals. Incubate and read E₂ at same timed
intervals.

Calculations

Calculate E₁ minus E₂ 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.

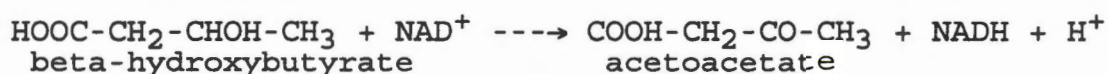
Multiply by appropriate dilution factors.

APPENDIX 4

BETA-HYDROXYBUTYRATE ANALYSIS

Principle

The beta-hydroxybutyrate analysis is a spectrophotometric determination based upon a compound's (nicotinamide adenine dinucleotide, NAD, in this analysis) 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:



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 3 g 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 3 g 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 NAD^+ ----> 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 $\text{CH}_3\text{CHOHCH}_2\text{COOH}$ ----> 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 3 g for 15 minutes.

Standard dilutions:

- A) 2.0 -----> 20.0 ml of 2.0 mM BOHB plus 0.0 ml dH₂O
- B) 1.6 -----> 16.0 ml of 2.0 mM BOHB plus 4.0 ml dH₂O
- C) 1.2 -----> 12.0 ml of 2.0 mM BOHB plus 8.0 ml dH₂O
- D) 0.8 -----> 8.0 ml of 2.0 mM BOHB plus 12.0 ml dH₂O
- E) 0.4 -----> 4.0 ml of 2.0 mM BOHB plus 16.0 ml dH₂O

Analysis

Pipette into 12 X 75 mm culture tubes:

0.5 ml buffer
Sample - 0.10 ml plus 0.90 ml dH₂O
Standard - 0.25 ml plus 0.75 ml dH₂O
0.05 ml NAD⁺

Read E₁ at 340 nm.

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

Read E₂ 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 E₂ minus E₁.

APPENDIX 5

VOLATILE FATTY ACID ANALYSIS

Volatile fatty acid concentrations were determined in plasma based on the method of Reynolds et. al. (1986), with modifications by Quigley et al (1991). The exact procedure is described below.

Reagents:

- A) H₂O/Triton-X mixture (20 μ l Triton-X100/ 250 ml H₂O)
- B) 1M Sodium Hydroxide (NaOH)
- C) 10mM Sodium Hydroxide (NaOH)
- D) 1N Hydrochloric acid (HCl)
- E) 0.3N Zinc Sulfate (ZnSO₄)
- F) 0.3N Barium Hydroxide (BaOH)
- G) Internal Standard (1.31mM 2-ethylbutyric acid)
- H) 0.01M Oxalic acid

Note: The concentrations of zinc sulfate and barium hydroxide must completely neutralize each other.

Column Preparation:

A) Packing:

- 1) Anion columns are prepared using BIO-REX 5 resin (100-200 Mesh, Bio-Rad Cat. No. 140-7841)
- 2) Cation columns are prepared using AG 50W-8 resin (100-200 Mesh, Bio-Rad Cat. No. 142-1441)
- 3) Resins are first prepared by making a slurry of each and pouring off fines. Stir resins twice more and allow to settle, removing fines each time.
- 4) Stir slurry once again and transfer resins into columns (Poly-Prep Columns, Bio-Rad Cat. No. 731-1550).
- 5) Each column receives:
 - a) Cation column receives 1.8 ml resin
 - b) Anion column receives 0.6 ml resin

B) Regeneration:

1) Cation

- a) Fill columns with 1M NaOH and stir resin
- b) Rinse twice with water
- c) Fill columns with 1N HCl twice
- d) Rinse twice with H₂O

2) Anion

- a) Fill columns twice with 1M NaOH and stir resin with first filling
- b) Rinse twice with H₂O

Notes:

- 1) Columns must be stores generated and resin must not be allowed to dry out
- 2) Stirring columns more often will minimize establishment of channels through resin

Assay:

A) Regenerate columns, as above, and fill with H₂O

B) In 50 ml centrifuge tubes:

- 1) add 6 ml H₂O/Triton-X mixture
- 2) add 2 ml plasma; vortex
- 3) let stand 5 minutes
- 4) add 4 ml BaOH; vortex ; let stand 5 minutes
- 5) add 4 ml ZnSO₄ vortex ; let stand 5 minutes
- 6) centrifuge for 15 min. at 13,000 rpm (15,000g)

C) After centrifugation, pour supernatant into 50 ml tube

D) Add 0.2 ml internal standard to all tubes

E) Allow H₂O to drain from columns, then place cation columns on top of anion columns.

F) Pour supernatant/internal standard mixture onto the cation columns. Allow to drip through both columns completely

G) Rinse tube twice with 2 ml H₂O and pour over columns, allowing each rinse to drip through completely

H) Rinse top column once with 2 ml H₂O, allow to drip through

- I) Remove top columns. Fill columns with H₂O
- J) Rinse bottom column once with 2 ml H₂O, allow to drip through
- K) Place a vial beneath each bottom column; add 10 ml of 10 mM NaOH and allow to drip through completely; pH of collected liquid should be above 11, check at least one sample in each rack
- L) Place vials in convection oven until dry (Temperature should not exceed 50°C)
- M) Reconstitute dry sample with 1 ml 0.01M oxalic acid
- N) Add 0.1 ml of regenerated cation resin to solution
- O) Allow sample to stand for one hour
- P) Solution is filtered using a 0.45 µm syringe filter (Aerodisc* No. 4184, Gelman Science) and transferred to a gas chromatograph vial and capped
- Q) Samples were analysed for volatile fatty acids by gas chromatography (30 m x 0.53 mm x 1.0 µm capillary column coated with stabilwax-DA, flame ionization detector, helium carrier at 6 ml/min.) using a Hewlett Packard model 5890 gas chromatograph.
- R) Sample acid concentrations were corrected for recovery of internal standard

APPENDIX 6

PARA-AMINOHIPPURIC ACID ANALYSIS

Reagents for Filtrate:

- 1) Double Distilled (DD) H₂O
- 2) 20% Trichloroacetic Acid (TCA) (CCL₃COOH)
(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 DD H₂O.
- 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).

Notes:

- 1) Be sure to run this dilution through the filtrate procedure (do not boil).
- 2) Blank mixed with half DD H₂O 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 is adequate)
- 2) Sodium Nitrite (NaNO_2)
(Sigma Chemical Co., cat. # S-2252)
100 mg NaNO_2 ---> 100 ml H_2O
- 3) Ammonium Sulfamate ($\text{NH}_4\text{OSO}_2\text{NH}_2$)
(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.
Start time clock and add to all tubes:
 - a) 0.1 ml sodium nitrite
 - b) 0.1 ml ammonium sulfamate
 - c) 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.

Note: The reagents in step 2 should be added with no more than 5 min between additions and tubes should be vortexed after each addition.

PAH Stock Solution:

Reagents:

- 1) Para-Aminohippuric Acid (PAH) (Eastman Kodak Corp., cat. # 5704)
- 2) Sodium Hydroxide (NaOH) (Fisher Scientific Co., cat # S-318)

Procedure:

- 1) Dissolve each reagent separately in Physiological Sterile Saline (PSS):
 - a) 44.8 g PAH
 - b) 10.5 g NaOH
- 2) Add NaOH solution to PAH solution and stir. (May heat gently to fully dissolve.
- 3) Filter through Whatman #4 filter paper.
- 4) Titrate filtrate to pH 7.4 with 1 N HCl or 4 M NaOH.
- 5) Bring final volume to 500 ml.

Note: Yields a 1.5% solution of sodium salt.

VITA

Louis Nicholas Lembo was born in Providence, Rhode Island, on August 4, 1969. He received his high school diploma from Classical High School in May 1987. In September of that year, he entered the University of Rhode Island and received his Bachelor of Science in Animal Science from URI in May, 1991. He then entered The University of Tennessee and received his Master of Science degree in Animal Science in August, 1993.

Louis is currently residing in Knoxville, Tennessee working for a local veterinarian.