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Studies of rumen epithelial cell metabolism

Matthew Reid Waldron

To the Graduate Council:

I am submitting herewith a thesis written by Matthew Reid Waldron entitled "Studies of rumen epithelial cell metabolism." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Richard N. Heitmann, Major Professor

We have read this thesis and recommend its acceptance:

James D. Quigley III, F. Neal Schrick

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

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Associate Vice Chancellor and
Dean of The Graduate School

STUDIES OF RUMEN EPITHELIAL CELL METABOLISM

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Matthew Reid Waldron

August 1996

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Abstract

The metabolism of rumen epithelial cells was studied in two experiments. The first experiment compared the metabolism of cells harvested from steers and ewes to determine the existence of a species effect. The second experiment compared the metabolism of cells harvested from four locations within the ewe rumen to determine if cellular origin has an effect on metabolism. In the first study, rumen epithelial cells were harvested from the anterior cranial pillar (ACP) of 4 ewes and 4 steers fed fescue hay. Whole tissue from ewes (~150 cm^2) was excised following death by captive bolt and exsanguination. Rumen epithelial cells were obtained from steers from clipped papillae harvested after exteriorizing the rumen through a rumen fistula. In the second study, tissue (~150 cm^2) was excised from the ACP, ventral sac floor (VSF), caudal pillar surface (CPS), and dorsal sac ceiling (DSC) of each ewe rumen. Cells were isolated using a serial tryptic digestion procedure in both experiments. Thereafter, 1 mL of cell isolate was incubated for 2 h in 6 mL of media containing 25 m*M* propionate and 10 m*M* butyrate. Incubations were terminated at 0, 30, 60, 90, and 120 min and analyzed for B-hydroxybutyrate (BOHB) and acetoacetate (ACAC) as mitochondrial, and lactate (LACT) and pyruvate (PYR) as

cytosolic redox estimators. All metabolite concentrations and ratios increased over the incubations indicating continuous cellular activity. Cell yield averaged 21 and 5 x 10^6 cells/mL for ewes and steers respectively, and mean cell viability for both was 92% in the first study. Final 2 h concentrations (*n*mol/10^6 cells) of BOHB, ACAC, LACT, and PYR were 123 ± 20, 36 ± 17, 25 ± 7, and 2.9 ± 1.1 for ewes, and 177 ± 20, 90 ± 18, 74 ± 7, and 5.6 ± 1.1 for steers. Ratios of BOHB to ACAC and LACT to PYR were 3.4 and 8.8 for ewes, and 1.8 and 13.2 for steers ($P > .10$). Steers produced BOHB ($P < .06$), ACAC ($P < .01$), LACT ($P < .01$), and PYR ($P < .10$) at higher levels than did ewes indicating that a species difference existed in the metabolism of rumen epithelial cells. Cell yield was 22, 22, 24, and 14 ± 6 x 10^6cells/mL and viability was 92, 92, 94, and 87% for ACP, VSF, CPS, and DSC, respectively in the second study. Final 2 h concentrations (nmoles/10^6 cells) were 123, 113, 163, and 158 ± 35 for BOHB; 38, 42, 24, and 45 ± 10 for ACAC; 25.3, 20.6, 10.1, and 20.4 ± 5.6 for LACT; and 2.54, .98, 1.06, and 1.31 ± .61 for PYR in the ACP, VSF, CPS, and DSC incubations, respectively. The amount of metabolite produced did not differ ($P > .05$) between tissues indicating that cellular location was not a significant factor in the metabolism of rumen epithelial cells. These experiments

were designed to study various aspects of rumen epithelial cell metabolism. Cells from steers produced more BOHB, ACAC, LACT, and PYR than those from ewes. However, metabolism of cells isolated from different areas of the ewe rumen did not differ.

"On the plains of hesitation bleach the bones of countless millions, who at the dawn of victory laid down to rest, and resting died."

-Unknown

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Introduction

According to Wardrop and Coombe (1961), the development of rumen function and physical development in the lamb can be divided into three phases: 1) 0-3 weeks of age - Non-ruminant phase, 2) 3-8 weeks of age - Transition phase, 3) >8 weeks of age - Adult ruminant. In early life, the ruminant is functionally a simple-stomached animal. At birth, the rumen is small, undeveloped, and unused. Suckled milk bypasses the rumen, via the esophogeal groove through the undeveloped reticulum and omasum, and enters directly into the abomasum. From this point, digestion and absorption progress as in the non-ruminant. As the ruminant grows and its natural dietary progression from milk to solid feed occurs, the pregastric chambers of the stomach grow and develop. Wardrop (1961a) reported that in the fetus, the rumen mucosal surface is smooth, consisting of a stratified cuboidal type epithelium, while post-natal specimens bear small conical papillae of stratified squamous epithelium. Following birth, in normally fed animals (with access to concentrate and forage), papillae increase rapidly in size and become "tongue-shaped". Within 24 hours of birth, feed and the environment inoculate the rumen with microflora that rapidly proliferate in this ideal milieu. Rumen microflora exist in a symbiotic relationship with the host animal. The

rumen provides a warm (39°C), moist environment and substrate for the microbes which in turn digest complex carbohydrates (many of which are undigestable to the animal) to volatile fatty acids (VFA). The VFA are almost completely absorbed across the rumen epithelium and can be used as a source of energy by the host. During their absorption, however, some VFA are metabolized by the rumen epithelial cells to other products such as ketone bodies, lactate, or CO_2 and are available as an immediate source of energy for the tissue. The microbes also incorporate non-protein nitrogen into their cellular proteins and cytoskeletons. Ultimately, microbes pass into the abomasum where they are digested and serve as a source of high quality protein to be absorbed along the remainder of the gastro-intestinal tract. Thus, the ruminants' existence largely depends on microbial protein and its fermentation of complex carbohydrates.

Discussion

Rumen Epithelial Development

The rumen epithelium is an interface between the animal and its environment. Like other epithelial tissue, that of the rumen consists of several differentiated cellular layers. Henriksson and Habel (1961) suggested the following terminology for the rumen epithelium based on histological study: stratum basale, stratum spinosum, stratum transitionale, and stratum corneum. They suggested that the stratum granulosum is included within the stratum transitionale, but popular nomenclature has persisted in placing the stratum granulosum in place of the stratum transitionale. Of these layers, the basale and spinosum are the most metabolically active, with cells of the granulosum progressing towards keratinization. Galfi et al. (1982) proposed a histochemical method for identifying the degree of differentiation of isolated rumen epithelial cells using cellular enzymatic activities. They reported lactate dehydrogenase activity to be high only in basale and spinosum, while alkaline phosphatase activity was high only in granulosum and corneum. Thus, rumen epithelial cells can be differentiated according to metabolic activity by assessing certain enzyme activities.

Gross morphological study of the rumen epithelium reveals a diverse mucosal surface. Characteristically, the anterior surface of the cranial pillar and the dorsal surface of the caudal pillar show extensive papillation, while the ceiling of the dorsal sac is void of papillary development (personal observation). More variable is the papillation of the ventral surface of the dorsal sac and that of the lateral pillars. Under some conditions and in some animals, these regions also display extensive papillation. Wardrop (1961a) reported the formation of papillae occurs as the basal epithelium (containing the capillary network, and thus the absorptive site) folds. This folding of the basal layers results in an increase in absorptive area, not just in mucosal surface area. The stimulus for this folding has been the focus of much research. Brownlee (1956) reported that calves fed a milk-only diet exhibited no papillary development, whereas milk + hay, milk + grass, and milk + concentrate diets resulted in increased papillary development. Phillipson and McAnally (1942) concluded that rumen bacteria ferment carbohydrates and produce volatile fatty acids as a by-product. These acids are stable in the rumen, do not pass to the abomasum in large quantities under normal rumen conditions, and appear to be absorbed almost entirely in the rumen or

omasum. These VFA, their absorption, or metabolism are thought to be the cause for rumen papillary development. Flatt et al. (1958) reported that papillary development was the result of chemical stimuli from the end-products of rumen fermentation since the physical stimulus of intraruminal plastic sponges alone did not elicit development of rumen papillae. Sander et al. (1959) reported that administration of solutions of Na-butyrate or Na-propionate caused marked development of the rumen mucosa, whereas Na-acetate, NaCl, or glucose, showed little effect. Other researchers have also hypothesized that end-products of fermentation were the stimulus for papillary development (Harrison et al., 1960; Wardrop, 1961b; and Tamate et al., 1962). Tamate et al. (1962) proposed that the epithelial cells are deficient in 3- or 4-carbon VFA. When a sufficient supply becomes available, the cells begin multiplying and migrate to form papillae. However, this hypothesis fails to explain the internal folding of the epithelial tissue that Wardrop (1961a) proposed. Although it is generally believed that VFA are the effectors of papillary development, the exact mechanism of stimulation has yet to be determined.

Rumen development can also regress when the end-products of a fermentative diet are not provided. Tamate et

al. (1962) reported that older milk-fed calves showed less papillary development than did calves 0 to 3 days of age, and thus suggested an early post-natal regression of papillae may have occurred. Wilson (1963) and Harrison et al. (1960) reported that rumen papillae disappeared in calves switched from a forage and concentrate diet to a milk-only diet. Other researchers have also reported papillae that appeared less developed and more conical in shape following exposure to a milk-only diet (Walker and Simmonds, 1962; Wardrop, 1961b). The latter reported normal papillary histology, but noted a less extensive development of stratum granulosum and stratum corneum cell layers. Henriksson and Habel (1961) reported that the thickness of the stratum basale and stratum spinosum were less in milk-fed calves than in hay and grain-fed calves.

As stated by Brownlee (1956), the development of the rumen musculature is not *pari passu* with papillary development. This was concluded after rumen weight (reflecting the musculature) increased six-fold with no corresponding increase in extent of papillation. Tamate et al. (1962) and Harrison et al. (1960) reported that the presence of inert bulk in the rumen increased the development of rumen muscle, but had no effect on papillary

development. Thus, development of rumen muscle is independent of mucosal development.

Rumen Epithelial Absorption

Metabolic activity and mucosal development of the ruminal epithelium are highly correlated. In fact, Sander et al. (1959) concluded that growth of the rumen papillae is probably a result of the metabolism of certain compounds by the rumen wall, and/or their effect on blood flow to the rumen. As stated previously, the stratum basale and stratum spinosum of rumen epithelium are metabolically active. When combined with other cells of the gastro-intestinal tract, overall oxygen consumption by the gut comprises about 25% of the total oxygen consumption (Huntington, 1990). This percentage is very significant as the gut contributes only 6-8% to total body weight. Kelly et al. (1993) studied some of the factors that contribute to the oxygen consumption of rumen epithelial cells. Using ouabain as an inhibitor of Na^+,K^+-ATPase respiration and cyclohexamide as an inhibitor of protein synthesis, it was determined that each of these metabolic activities account for 20% of cellular oxygen consumption. It is understandable that the Na^+,K^+-ATPase would consume a considerable portion of cellular energy because of its role in ion balance and absorption.

Rumen epithelial cells directly receive the end-products of rumen fermentation and absorption is a high priority in this tissue. Danielli et al. (1945) reported that most substances absorbed across the rumen epithelium move from higher to lower concentrations. This is true with the exception of ion fluxes to maintain an electrochemical balance. Under normal rumen conditions, VFA, NH_3, Cl^-, and Na^+ are absorbed from the mucosal to the serosal cell surface, while HCO_3^- and urea are the most common secretogogues of the rumen epithelium. Sodium ions, some Cl^-, and small amounts of K^+ are actively transported (Stevens, 1973) at the serosal surface, while HCO_3^- travels via an anti-port mechanism with Cl^-.

The absorption of Na^+ and Cl^- appear to be linked via HCO_3^-. That is, cellular H^+ concentration is affected by Na^+ flux as it participates in electroneutral ion exchange with Na^+ on the mucosal cell surface. The change in H^+ concentration elevates cellular pH, and thereby increases cellular bicarbonate concentrations. This activates the Cl^-/HCO_3^- exchange, as it is dependent on the availability of intracellular HCO_3^- (Gäbel and Martens, 1991). Thus, under some conditions, as Na^+ transport is increased, so is the transport of Cl^-.

The flux of water across rumen epithelium has been controversial. Many *in vivo* studies indicate little movement of water across this tissue under normal physiological conditions. However, some *in vitro* studies have shown moderate to large net fluxes of water are possible. This may be resultant to the methods used. von Engelhardt (1970) suggested that a net flux of water is prevented, or intensely inhibited, by a zone of high osmotic pressure in the deep layers of the epithelium. Dobson et al. (1976), however, reported significant flux of H_2O across the rumen of conscious cows due to the existence of osmotic gradients. Further, it was concluded that these gradients could be created or enhanced by factors that affect blood flow. Recently, López et al. (1994) also reported the net movement of water across the rumen wall of sheep. Movement occurred according to the osmotic pressure gradient, where either a net efflux or influx of rumen water was observed corresponding to osmotic pressures that were lower or higher than 341 mosmol/kg, respectively.

At normal rumen pH (6.0-6.8), most rumen VFA are in the dissociated form and must co-transport across the mucosal surface with Na^+. This co-transport was initially supported by Danielli et al. (1945) who reported that the fatty acid anion is absorbed only with an equivalent amount of sodium

passage. The main driving forces of VFA transport are intracellular pH and pCO_2 as water and CO_2 form carbonic acid (H_2CO_3) within the cell. Carbonic acid is split by carbonic anhydrase to form bicarbonate and free H^+. This free hydrogen ion combines with the VFA anion to form the undissociated form of the VFA. The absorption rate of the undissociated acids increases with increasing carbon chain length (butyrate > propionate > acetate) (Danielli et al., 1945). Perhaps this is due to the fact that rumen epithelium metabolizes individual VFA in the same order, thereby increasing the concentration gradient between the lumen and the cells (Merchen, 1988). The undissociated acid moves across the serosa into the capillary lumen. Once in the blood (pH ~ 7.4), the VFA quickly returns to the dissociated form of the acid and thus, is trapped in the capillary lumen. The rate of VFA transport increases with decreasing rumen pH due to increased proportions of each acid in its undissociated form (Stevens and Stettler, 1966).

Both D-(-)- and L-(+)-lactate are organic acids commonly present in rumen digesta. They may result from carbohydrate fermentation by rumen microbes, or can be ingested in fermented feeds such as silage. In either case, rumen concentrations can be substantial and, if too high, can contribute to acid-base disturbances in the rumen.

Absorption of lactate depends on substrate concentration and rumen pH. At decreasing pH and increasing substrate concentrations, lactate absorption increases (Jones et al., 1969). Absorptive mechanisms appear to be different for the two forms of the acid. Harmon et al. (1985) reported that at low rumen pH (~4.8), D-(-)-lactate absorption increased six-fold, whereas L-(+)-lactate absorption was increased by only 70%.

The absorption of ammonia occurs simply due to concentration gradients, as changes in rumen concentration are closely paralleled by changes in the portal vein ammonia concentration (Lewis et al., 1957). Rémond et al. (1993b) noted that ammonia absorption is positively correlated to blood flow through the rumen capillaries. Factors such as osmotic gradients, butyrate, and CO_2 which stimulate blood flow, also increase the absorption of ammonia.

Urea recycling is of great importance to the ruminant. Rumen fermentation results in the absorption of large quantities of ammonia across the rumen wall. If all absorbed ammonia were to remain in the animals' blood, ammonia toxicity would quickly occur. Thus, ammonia is removed from the blood by the liver and is released into the blood as urea. In addition to secretion in urine and saliva, much urea is cleared from the blood by transfer

across the rumen mucosa into the rumen. This transfer appears to be passive in nature (Houpt, 1970), but can be modified by parameters of the rumen environment. Rémond et al. (1993b) reported that the net transfer of urea was stimulated by CO_2, negatively related to ammonia absorption, and was independent of ruminal blood flow. Also, a decrease in net rumen urease activity decreased the transfer of urea into the rumen. This decreased transfer could occur due to an accumulation of urea in the lumen as a result of reduced urea hydrolysis to ammonia in the rumen lumen and epithelium (Rémond et al., 1993a).

Rumen Epithelial Metabolism

As stated previously, rumen epithelial cells are active metabolically. Pennington and Sutherland (1956) reported that these cells contain the necessary components for a functional citric acid cycle, since the addition of most acid intermediates resulted in increased rates of tissue oxygen consumption. Rumen epithelial cells also metabolize a quantity of VFA during absorption to ketone bodies, lactate, CO_2, and other metabolites in addition to normal cellular metabolism. Barcroft et al. (1944) reported that the proportion of higher molecular weight acids were greater in rumen contents than in the blood. This finding was supported by Annison et al. (1957) who noted a much lower

concentration of butyrate under normal feeding conditions, relative to the levels of acetate and propionate, in the portal system than in rumen contents. Bergman and Wolff (1971) and Britton and Krehbiel (1993) concur that, during absorption, the rumen epithelium metabolizes 90, 50, and 30% of butyrate, propionate, and acetate, respectively.

The major by-products of VFA metabolism in the rumen epithelium are the ketone bodies, acetoacetate (ACAC) and D(-)ß-hydroxybutyrate (BOHB). Sutton et al. (1963) reported that ketone bodies were produced from both acetate and butyrate. In a finding that has been widely accepted, Pennington (1952) reported that a large proportion of butyrate and a smaller amount of acetate are converted to ketone bodies by rumen epithelial tissue. As a reason for ketogenesis of VFA, Galfi et al. (1991) reported that butyrate inhibits DNA synthesis and the entire mitotic process of the rumen epithelial cell. They postulate that ketogenesis of butyrate may occur as a mechanism to remove butyrate from the cell. Yamdagni et al. (1968) investigated the ketogenic properties of longer chain fatty acids in the goat rumen. Some direct ruminal absorption of C_4-C_{10} fatty acids and a resultant ketogenesis, either in the rumen wall or liver, occurred.

Activation to the corresponding Co-A ester must occur prior to the metabolism of any VFA in the rumen epithelium or liver. The presence of one VFA often affects the rate at which another is used. Baldwin and Jesse (1991), working with isolated rumen epithelial cells, reported that additions of acetate reduced, whereas added propionate stimulated ketone body production from butyrate. VFA activation has been proposed to be involved in the autoregulation of VFA uptake and metabolism. Ash (1972) noted that butyryl-CoA synthetase had the highest single CoA-synthetase activity in rumen epithelium. In addition, Ash and Baird (1973) and Harmon et al. (1991) reported that butyrate inhibited both acetyl-CoA and propionyl-CoA synthetase activities in rumen epithelium. Thus butyrate is the VFA primarily metabolized in rumen epithelium. Beck et al. (1984) demonstrated an enhanced ketogenic effect of glucose on butyrate. Perhaps glucose provides added reducing equivalents to the tissue enhancing the activation potential of the tissue.

Butyrate is the major ketogenic substrate in rumen epithelium (Pennington, 1952). Several metabolic pathways have been proposed for the conversion of VFA to ketone bodies in this tissue. Most of these pathways involve the conversion of butyryl-CoA to acetoacetyl-CoA (ACAC-CoA) as

common initial steps. In this conversion, butyryl-CoA is transformed into crotonyl-CoA by butyryl-CoA dehydrogenase. Crotonyl-CoA is converted into L(+)-3-hydroxybutyryl-CoA in a reaction catalyzed by crotonase. In the final step, NAD^+ is reduced to form NADH + H+, during the reaction that produces ACAC-CoA from L(+)-3-hydroxybutyryl-CoA. This reaction is catalyzed by L(+)-3-hydroxybutyryl-CoA dehydrogenase.

The first and most widely accepted pathway of ruminal ketogenesis is the 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) pathway (Figure 1), proposed by Lynen et al. (1958). This first involves the conversion of butyrate to acetoacetyl-CoA. ACAC-CoA is then condensed with an acetyl-CoA moiety in a reaction catalyzed by HMG-CoA synthase to yield HMG-CoA. HMG-CoA lyase catalyzes the cleavage of HMG-CoA yielding acetyl-CoA and acetoacetate (ACAC).

A second pathway which incorporates the steps of the HMG-CoA pathway into it, is the thiolase pathway (Figure 1). Here, ACAC-CoA can be cleaved via a thiolase-catalyzed reaction into two acetyl-CoA moieties. These molecules enter the acetyl-CoA pool. Under conditions favoring ketogenesis, two acetyl-CoA moieties can be re-linked via another thiolase-catalyzed reaction to yield one ACAC-CoA molecule. This ACAC-CoA can then be metabolized using the

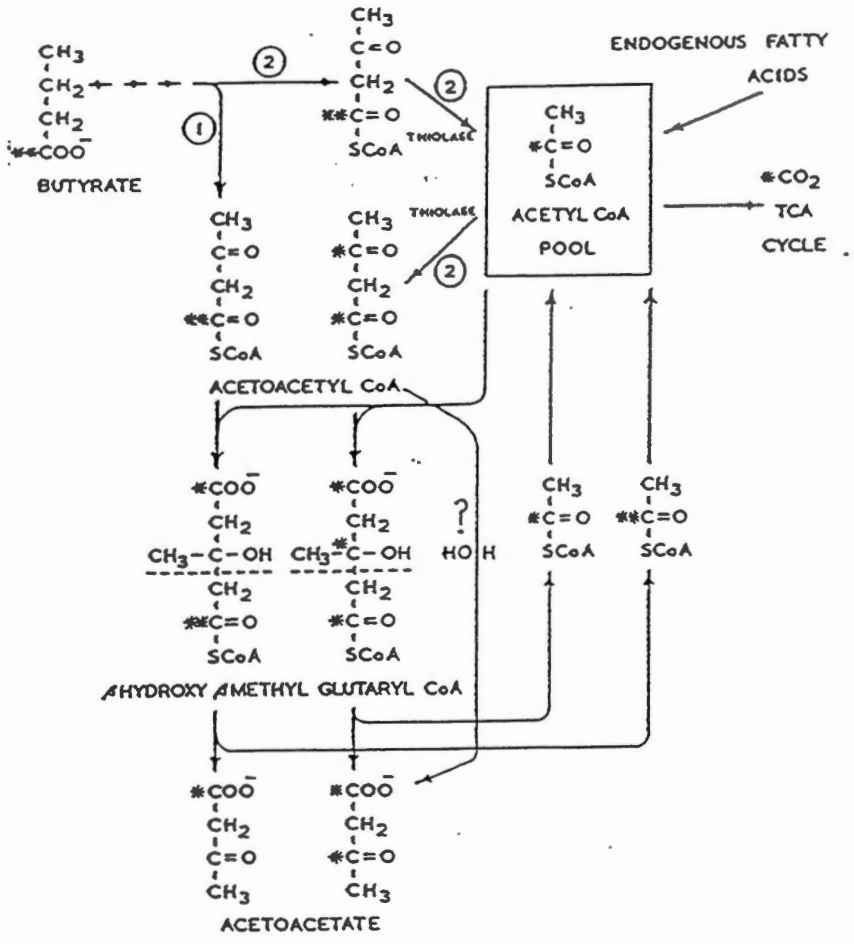

Figure 1. The conversion of butyrate to acetoacetate via the HMG-CoA (1) and thiolase (2) pathways. (Hird and Symons, 1961)

remainder of the HMG-CoA pathway to produce ACAC (Hird and Symons, 1961). This can be classified as its own pathway, but in reality it is just a method by which acetyl-CoA can be incorporated into ketogenesis.

A third pathway that exists in bovine liver and may be present in rumen epithelium was proposed by Drummond and Stern (1960). According to this route, the direct deacylation of ACAC-CoA to form ACAC, occurs via a specific acetoacetyl-CoA deacylase enzyme.

A fourth pathway that was reported to be active in crude extracts of rumen papillae was proposed by Bush and Milligan (1971). This pathway involves the transfer of the CoA group from ACAC-CoA to succinate, thereby producing ACAC and succinyl-CoA. This reaction is catalyzed by the enzyme 3-oxo acid CoA transferase (commonly called succinyl-CoA transferase in this reaction).

The fifth, and most recent pathway was reported to be present in bovine and ovine rumen epithelium by Emmanuel et al. (1982). In this pathway (Figure 2), butyryl-CoA is converted to crotonyl-CoA in the reaction catalyzed by butyryl-CoA dehydrogenase. Vinylacetyl-CoA isomerase then aids in the conversion of crotonyl-CoA to vinylacetyl-CoA. Next, a yet to be isolated enzyme (a hydratase) converts vinylacetyl-CoA into D(-)-3-hydroxybutyryl-CoA (BOHB-CoA).

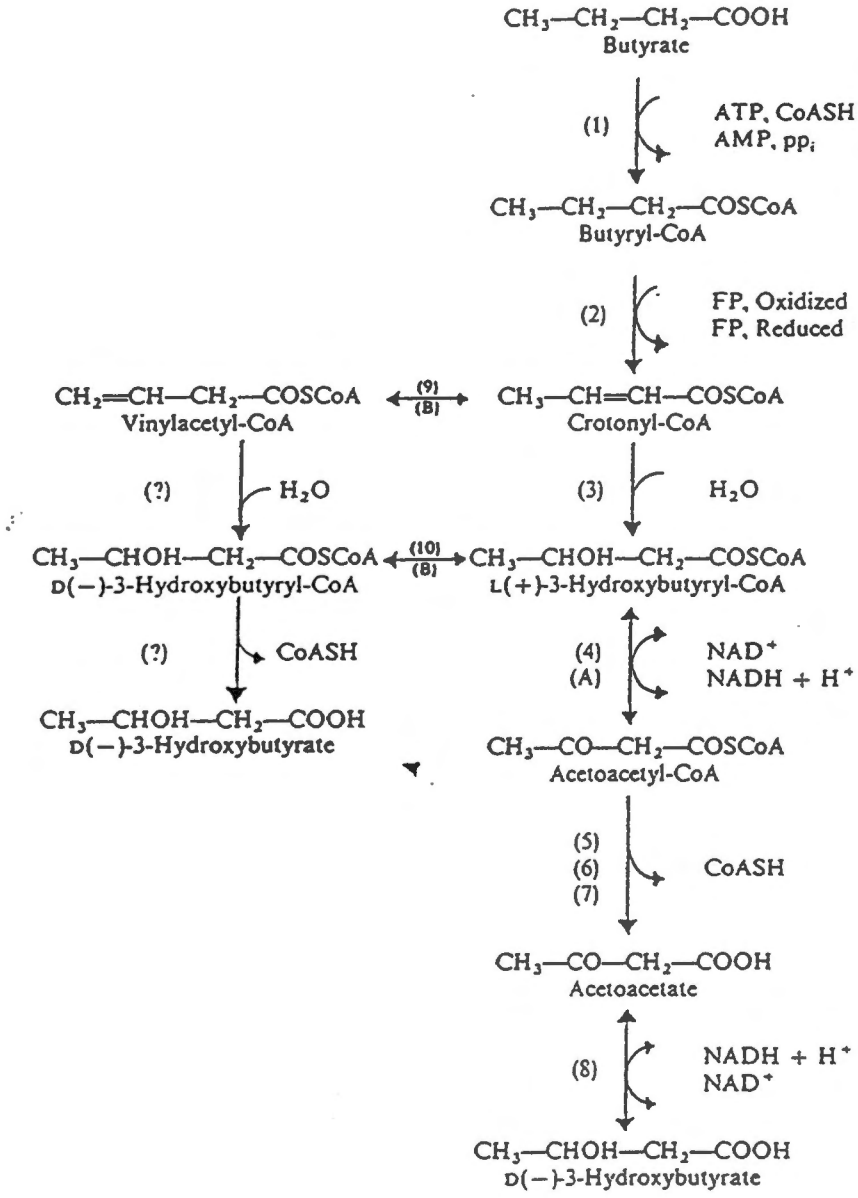

Figure 2. Metabolic routes of D(-)-3-hydroxybutyrate synthesis in rumen epithelium. Numbers in brackets refer to: 1 = Butyryl-CoA synthetase (EC 6.2.1.2); 2 = Butyryl-CoA dehydrogenase (EC 1.3.99.2); 3 = Crotonase (EC 4.2.1.17); 4 = L(+)-3-Hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.35); 5 = Deacylase (EC 3.1.2.11); 6 = 3-Oxo acid CoA transferase (EC 2.8.3.5); 7 = 3-hydroxy-3-methylglutaryl-CoA synthase (EC 4.1.3.5) plus 3-hydroxy-3-methylglutaryl-CoA lyase (EC 4.1.3.4); 8 = D(-)-3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30); 9 = Vinylacetyl-CoA isomerase (EC 5.3.3.3); and 10 = 3-Hydroxybutyryl-CoA racemase (EC 5.1.2.3). (Emmanuel et al., 1982)

This molecule can then have either one of two fates: 1) BOHB-CoA can be converted to BOHB with the aid of an unknown enzyme, or 2) BOHB-CoA can be converted to L(+)-3-hydroxybutyryl-CoA in a reaction catalyzed by 3-hydroxybutyryl-CoA racemase. When this latter path is followed, the L(+)-3-hydroxybutyryl-CoA can be converted to ACAC-CoA and any of the previously described pathways can be utilized for ketogenesis.

All of these reactions have been shown to exist in at least the crude extracts of rumen epithelium. Controversy exists as to which pathway is the most quantitatively important. The HMG-CoA pathway is the most widely accepted, although this was also the first pathway described. In comparing two of the described pathways, Hird and Symons (1961) reported that if only the HMG-CoA pathway and its analog, the thiolase pathway, were operating in the rumen epithelium of sheep, the thiolase pathway would account for only 26% of ketone body formation. In support of the succinyl transferase pathway, Bush and Milligan (1971) reported that the enzyme activities for the HMG-CoA and ACAC-CoA deacylase pathways accounted for 16 and 14%, respectively, of the total capacity of the tissue to remove ACAC-CoA. Meanwhile, the activity of succinyl-CoA transferase was sufficient to account for 70% of the ACAC-

CoA removal. The authors also noted that these data could help to explain the lack of inhibition of ketogenesis by propionate in the rumen epithelium. Baldwin and Jesse (1991) supported this theory in their report of stimulation of ketogenesis by propionate. However, recently Baldwin and Jesse (personal communication) reported that added succinate increased lactate formation and decreased BOHB formation by rumen epithelial cells. Therefore, propionate-induced stimulation of BOHB formation from butyrate is not mediated via succinate, but is more likely a result of the shift in the mitochondrial NADH/NAD^+ ratio. Perhaps all of the pathways exist and are of some importance under different conditions.

Once ACAC is produced, it can be converted to BOHB in a reaction catalyzed by BOHB dehydrogenase. In contrast to ruminant liver where the enzyme exists in the cytosol (Nielsen and Fleischer, 1969; Koundakjian and Snoswell, 1970), in rumen epithelium cellular BOHB dehydrogenase is mitochondrial. During the reaction, NADH + H^+ is oxidized to NAD^+ with the release of BOHB. This reaction proceeds freely in the presence of NADH, and is thus often used as an indicator of the mitochondrial redox potential (NADH:NAD).

Metabolism of VFA to ketone bodies is quantitatively the most important function of rumen epithelium. Hird and

Weidemann (1964) reported that ketone body synthesis could account for as much as 70% of the rumen epithelial oxygen uptake in mature lambs. This estimate is probably high according to a report by Kelly et al. (1993) that approximately 40% of the oxygen uptake by these cells is used for Na^+,K^+-ATPase and protein synthesis. However, the metabolic conversion of VFA into ketone bodies is of significant quantity, and thus uses a significant portion of cellular oxygen consumption.

Acetate is present in the largest molar proportion of VFA in the rumen. Most of the acetate produced is absorbed across the rumen. Peters et al. (1992) reported that at moderate levels in the rumen, 87% of acetate is absorbed by rumen epithelium. They also noted that increased amounts of acetate are absorbed with increased acetate production, but the fraction of acetate absorbed is lower. Bergman and Wolff (1971) reported that only about 30% of the absorbed acetate is metabolized by the rumen epithelium. Activated acetate (acetyl-CoA) enters the cellular acetyl-CoA pool, where it can be oxidized in the citric acid cycle. Pennington (1952) reported that some acetate can also be converted to ketone bodies in this tissue.

Other than butyrate and acetate, propionate is the other major VFA absorbed across the rumen epithelium. Once

absorbed, a considerable portion (32%) can be used for glucose synthesis in the liver (Leng et al., 1967). Britton and Krehbiel (1993) reported that about 50% of propionate is metabolized by the rumen epithelium during absorption. Much of this may be oxidized to CO_2 by the tissue (Pennington and Sutherland, 1956). Some propionate is converted to lactate by the rumen epithelium, but according to Weekes and Webster (1975) lactate is probably not a major end-product. In fact, Weekes (1972) reported that the calculated conversion of propionate to lactate and pyruvate averaged only 3%. Although not of quantitative importance, lactate production from propionate enhances the cellular lactate pool.

Summary

The ruminant animal has developed a unique digestive and absorptive strategy. Living in symbiosis with rumen microbes, the ruminant is able to gain nutritive value from otherwise unavailable sources. Ammonia and microbial protein serve as a source of nitrogen for the animal. Volatile fatty acids, the main products of carbohydrate fermentation, are absorbed across the rumen wall and serve as a source of energy for the animal. During their absorption, VFA are metabolized extensively by the rumen epithelium. Most butyrate never reaches the portal blood, as it is metabolized rapidly to ketone bodies. A large quantity of propionate is oxidized by the epithelial cells for energy, as is a portion of acetate. Thus, the rumen and its microbes combine to allow the ruminant to survive and adapt to poor quality nutritive environments.

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Part 1.

Rumen Epithelial Cell Metabolism of Ewes and Steers

Abstract

Metabolism of epithelial cells isolated from rumens of ewes and steers was compared to determine species differences. Cells were harvested from the anterior cranial pillar (ACP) of the rumen from 4 ewes and 4 steers fed fescue hay. For ewes, whole tissue (~150 cm^2) was excised following death by captive bolt and exsanguination. In steers, cells were obtained from clipped papillae harvested after exteriorizing the rumen through a rumen fistula. Cells were then isolated using a serial tryptic digestion procedure. One mL of isolate was incubated for 2 h in 6 mL of media containing 25 m*M* propionate and 10 m*M* butyrate. Incubations were terminated at 0, 30, 60, 90 and 120 min and analyzed for ß-hydroxybutyrate (BOHB) and acetoacetate (ACAC) as mitochondrial and lactate (LACT) and pyruvate (PYR) as cytosolic redox estimators. Cell yield averaged 21 and 5 x 10^6 cells/mL for ewes and steers, respectively, and mean cell viability for both was 92 ± 3%. Metabolite concentrations and hydroxy to ketone ratios increased over the 2 h incubations indicating continuous cell activity. Final 2 h concentrations (*n*mol/10^6 cells) of BOHB, ACAC, LACT, and PYR were 123 ± 20, 36 ± 17, 25 ± 7, and 2.9 ± 1.1 for ewes, and 177 ± 20, 90 ± 18, 74 ± 7, and 5.6 ± 1.1 for

steers. Ratios of BOHB to ACAC and LACT to PYR were 3.4 and 8.8 for ewes, and 1.8 and 13.2 for steers ($P > .10$). Steers produced BOHB ($P < .06$), ACAC ($P < .01$), LACT ($P < .01$), and PYR ($P < .10$) at higher levels than did ewes indicating that a species difference existed in the metabolism of rumen epithelial cells.

Key Words: Steers, Ewes, Cells, Epithelium, Rumen, Metabolism.

Introduction

Sheep are often used as a biological model for cattle because they are smaller, easier to house, and less costly. Calves are sometimes used for similar reasons, but are not used as a model of mature cattle in nutrition research because of their lack of ruminal development until about eight weeks of age (Wardrop and Coombe, 1961; Baldwin and Jesse, 1992). Rumen epithelial metabolism has been studied using tissue from calves (Bush, 1988), cattle (Weigand et al., 1975), lambs (Wardrop and Coombe, 1961), and sheep (Pennington and Sutherland, 1956; Jesse et al., 1992). Beck et al. (1984) compared rumen epithelial metabolism of sheep and cattle using a perfused epithelial sheet and reported that tissue from both species utilized butyrate at similar rates, but that sheep tissue produced a higher ß-hydroxybutyrate to acetoacetate ratio than did cattle tissue. The objective of this study was to compare the production of ß-hydroxybutyrate, acetoacetate, L-(+)-lactate, and pyruvate by rumen epithelial cells in an isolated cell system to determine if a species effect exists between ewes and steers.

Materials and Methods

Materials

Hepes (N-2-Hydroxyethylpiperazine-N'-2-Ethane Sulfonic Acid) and trypsin (1:250) were from Gibco BRL (Life Technologies, Inc., Grand Island, NY). Bovine serum albumin (BSA) was fatty acid free, #A-6003 (Sigma Chemical Co., St. Louis, MO). Sodium salts of propionate and butyrate used for incubation substrates were from Sigma Chemical. All other chemicals were reagent grade compounds from either Sigma Chemical or Fisher Scientific (Fair Lawn, NJ).

Animals

Animals used in this study were four 2 year-old Angus steers with rumen cannulas and four mature, hysterectomized, crossbred ewes. One steer or one ewe was used on each experiment day. All had access to trace-mineralized salt blocks and were fed fescue hay and offered water for ad libitum intake for at least 14 d. Feed samples were analyzed for DM, ash, CP, and macro-mineral content by proximate analysis using AOAC (1975) methods.

Cell Isolation - Steers

Steers were brought to the holding facility and fed for ad libitum intake until 18 h prior to sampling, at which time feed was withdrawn. At approximately 0800, a steer was placed in a head-gate and its rumen cannula was removed.

Approximately 30 mL of ruminal fluid was collected by straining rumen digesta through several layers of cheesecloth. These samples were immediately taken to the laboratory, centrifuged (15000 x g; 4°C), and frozen for later analysis (Appendix 5) of VFA concentrations. The reticulo-rumen was then evacuated with the aid of a large canister vacuum cleaner, and the anterior cranial pillar was retracted to the rumen fistula such that the tissue of the anterior surface was exposed. Rumen papillae were excised from this surface using a pair of sharp, curved surgical scissors. Papillae were harvested for a duration of approximately 12 min and placed into 37°C Krb-Hepes (Table 1) buffer. Rumen contents were replaced and the tissue was immediately transported to the laboratory (< 2 min). Papillae were rinsed with Krb-Hepes buffer on a 1 mm nylon mesh to remove adherent blood and feed particles. Papillae were placed in a digestion flask and 100 mL of a 3% trypsin solution (Krb-Hepes buffer used as the solvent) containing .016 g $CaCl_2$ dihydrate was added. The flask was placed in a slow shaking 37°C orbital hot air incubator (New Brunswick Scientific Co., Edison, NJ) for 15 min; thereafter the contents were filtered through a 1 mm nylon mesh into a 250 mL Erlenmeyer flask. Tissue remaining on the mesh was rinsed with 37°C Krb-Hepes buffer to release any adhering

Table 1. Krb-Hepes buffer[a]

Compound	Amount
KH_2PO_4	.3239 g
$MgSO_4$	.5866 g
KCl	.706 g
Hepes[b]	13.0 g
NaCl	14.4 g

[a]Compounds in solution with 2 L distilled water and bubbled with O_2/CO_2 (95%:5%) for 20 minutes (pH 7.4).

[b]N-2-Hydroxyethylpiperazine-N'-2-Ethane Sulfonic Acid.

cells into the collection flask. Recovered cells were discarded from digestion. The tissue on the mesh was placed back into the digestion flask and subjected to another 15 minute 3% trypsin digestion. The same recovery procedure was used and cells from this digestion were also discarded. The third digestion was a 15 minute digestion in a 2% trypsin solution. After filtering and rinsing the cells through the 1 mm nylon mesh, recovered cells were filtered through a 300 µm nylon mesh into 50 mL centrifuge tubes. The filtrate was spun in a Sorvall RC2-B centrifuge (Ivan Sorvall Inc., Newtown, CT) at 70 x g (764 rpm) for 6 minutes at 4° C. If the pellet contained a dark ring of keratinized cells it was discarded. If keratinized cells were not predominant the supernatant liquid was decanted and cells were resuspended (washed) in 37°C Krb-Hepes buffer. The suspension was recentrifuged and the cells were washed twice more. Pelleted cells remained in KRB-Hepes buffer at room temperature until they were combined with cells from later digestions. Subsequent digestions (4-8) were in 2% trypsin solution for 12 min. Cells from digestions four through seven usually had little keratinization and were combined to yield a cell pool. These cells were recentrifuged and resuspended in 18 mL of Krb-Hepes buffer. Cell numbers and viability were then assessed using a hemacytometer and

trypan blue exclusion. Cells that did not contain trypan blue dye were considered to be viable.

Cell Isolation - Ewes

Ewes were brought to the surgical facility and killed according to USDA specifications using captive bolt followed by exsanguination. The abdomen was opened immediately and the rumen was excised. Approximately 30 mL of ruminal fluid was collected by straining rumen digesta through several layers of cheesecloth. These samples were immediately taken to the laboratory, centrifuged (15000 x g; 4°C), and frozen for later analysis (Appendix 5) of VFA concentrations. About 150 cm^2 of rumen tissue was removed from the anterior side of the cranial pillar. The tissue was rinsed with warm tap water to remove adherent feed particles and placed in 37°C Krb-Hepes buffer. Tissues were immediately transported to the laboratory (< 2 min). The exterior muscle and connective tissue layers were then peeled from the epithelial sheet and discarded. The epithelial sheet was minced to approximately 1 cm^2 pieces and subjected to serial tryptic digestion procedure similar to that outlined for steers except that digestions four through seven were a 2% trypsin solution for 10 min. Also, digestions three through five were usually combined to yield the working cell pool.

Cell Incubations

Incubation flasks (25 mL Erlenmeyer) were prepared by adding 5 mL of a premix solution containing 5X Krb (84 mL) (5x Krb was prepared by adding 10.5 mL H_2O to a flask containing 2, .5, 50, .5, and 1.5 mL of .77 *M* KCl, .77 *M* KH_2PO_4, .77 *M* NaCl, .77 *M* $MgSO_4$, and .77 *M* $NaHCO_3$, respectively.), 1 *M* Hepes (10.5 mL), 100 m*M* butyrate (42 mL), 250 m*M* propionate (42 mL), BSA (84 mL), and H_2O (87.5 mL). Flasks were placed in a 37°C water bath and assigned incubation periods of 0, 30, 60, 90, or 120 minutes. Cell suspensions (1 mL) were added to each flask at 30 second intervals, yielding a total of 6 mL per flask. The final concentration of reagents in these flasks was Krb (1X), Hepes (.25 mM), butyrate (10 mM), propionate (25 mM), and BSA (.25 mM). At the end of incubation, cells in each flask were killed by adding .4 mL of concentrated $HClO_4$. Flasks were placed on ice for 10 min, then .8 mL of saturated potassium carbonate was added as a buffering agent. Flasks were left on ice for at least 10 min after buffering, and were decanted into 15 mL centrifuge tubes. Tubes were centrifuged at 1500 x g for 20 min in a Beckman TJ-6 centrifuge (Beckman Instruments, Palo Alto, CA).

Analyses

Supernatant liquid was analyzed for acetoacetate (ACAC) and beta-hydroxybutyrate (BOHB; Williamson and Mellanby, 1965), and L-(+)-lactate (LACT) and pyruvate (PYR) using procedures modified from the Sigma kits. Procedures are described fully in Appendices 1 through 4.

Statistics

Data were analyzed using a completely randomized experimental design with species being the classification variable. Production of each metabolite across the range of cell numbers for steers (2-7 x 10^6cells/mL) and ewes (9-39 x 10^6cells/mL) was analyzed using Proc Reg (SAS, 1989). Linear regressions and subsequent *F* tests of the slopes for each metabolite indicated that the slopes across cell numbers were not different, thereby satisfying concerns that differences in cell numbers may have influenced cellular metabolite production.

Results

Results of feed analysis (Table 2) indicated poor quality forage was utilized in this study. A predicted intake of 1200 g/d DM (NRC, 1984b) met approximately 107% of ME and 80% of CP requirements for mature ewes, and a predicted intake of 3550 g/d DM (NRC, 1984a) met approximately 121% of ME and 54% of CP requirements for steers. The estimates for CP intake were calculated by multiplying the analyzed CP level of the feed (Table 2) by the NRC predicted DM intake for each species. The estimates for ME intake were calculated by multiplying the ME value of mature fescue hay from the NRC recommendations by the predicted intake for each species. The predicted ME intakes are overestimated because according to CP levels the NRC assumes better quality forage than was utilized in this study. Due to calorimeter problems, no measurements of gross energy content of the hay were available.

Total rumen VFA concentrations for ewes and steers were 54 and 57 (SE = 15.1) m*M*, respectively. Concentrations did not differ between species when compared by T-test. Molar percentages of individual VFA did not differ between sheep and steers with the exception of butyrate, which was higher in steers than in ewes ($P < .01$; Table 3).

Table 2. Fescue hay composition

Component	Value
DM (%)	94.3
CP[a] (%)	7.1
Ash[a] (%)	7.0
Calcium[a] (g/mg)	4.52
Phosphorus[a] (g/mg)	2.49

[a] = Composition on a dry matter basis.

Table 3. Rumen VFA Concentrations[a]

VFA	Ewes	Steers	pSTD[b]	*P* value
Total VFA (mM)	54	57	15.1	NS[c]
Individual Acids -	(mol/100mol)			
Acetate	75	73	2.68	NS
Propionate	18	16	1.71	NS
Butyrate	4	9	1.26	.01
Isobutyrate	1.06	.74	.30	NS
Isovalerate	1.20	.62	.36	NS
Valerate	.47	.49	.14	NS

[a]Total VFA concentration and molar percentages of individual rumen acids immediately prior to tissue collection.

[b]Pooled standard deviation.

[c]$P > .10$

Mean ruminal cell harvest from steers and ewes ranged from 2.5 to 41.3 x 10^6cells/mL and cell viability ranged from 87 to 96%. The number of viable cells used for incubation ranged from 2.3 to 38.5 x 10^6cells/mL (Table 4). Harvested and live cell numbers both tended to be different between species ($P < .10$).

Graphs of metabolite production and ratios during the 2 h incubation period are shown in Figures 1 through 6. All metabolite concentrations increased over time with the exception of ACAC in ewes, which reached an asymptote at 2 h. This sustained metabolite production indicated continuous cellular activity during the 2 h incubation. Cells from steers produced more BOHB ($P < .06$), ACAC ($P < .05$), and LACT ($P < .01$) during the 2 h incubation than did those from ewes (Table 5). Steer cells tended to produce more PYR than ewe cells during the 2 h incubation ($P = .10$; Table 5). Variation of PYR in several flasks during the 2 h incubation was responsible for the lack of significance during this incubation; however, steer cells produced significantly more pyruvate ($P < .01$) than did ewe cells during all other incubation times (Figure 5). Metabolite ratios were calculated using reported means (ratios of the means) instead of averaging individual ratios from each experimental unit (means of the ratios). The means of the

Table 4. Cell Yield and Viability[a]

	Ewes	Steers	pSTD	*P*
Harvested Cells[b]	21.50	4.41	7.57	.1
Range	(9.90-41.30)	(2.51-7.71)	...	...
Viability (%)	92	92	3	NS[c]
Range	(87-96)	(90-94)	...	...
Live Cells[b]	19.68	4.05	6.94	.1
Range	(9.45-38.55)	(2.33-6.94)		

[a]Numbers and viabilities of cells recovered from the serial tryptic digestion procedure.

[b]10^6cells/mL.

[c]$P > .10$

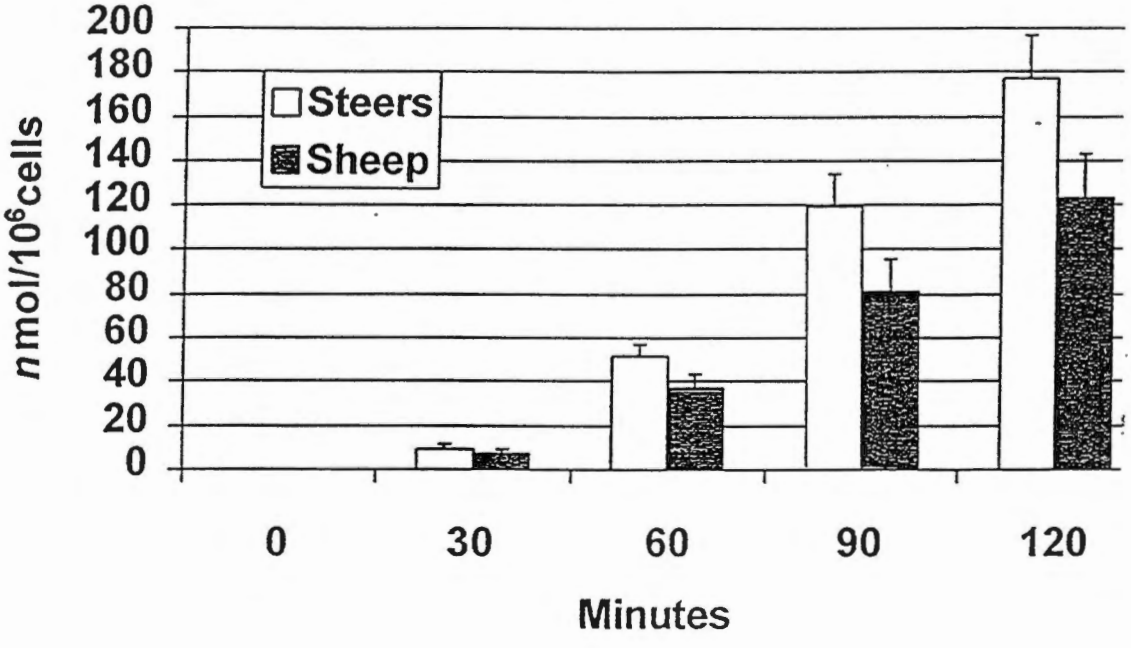

Figure 1. Production of BOHB during the 2 h incubation.

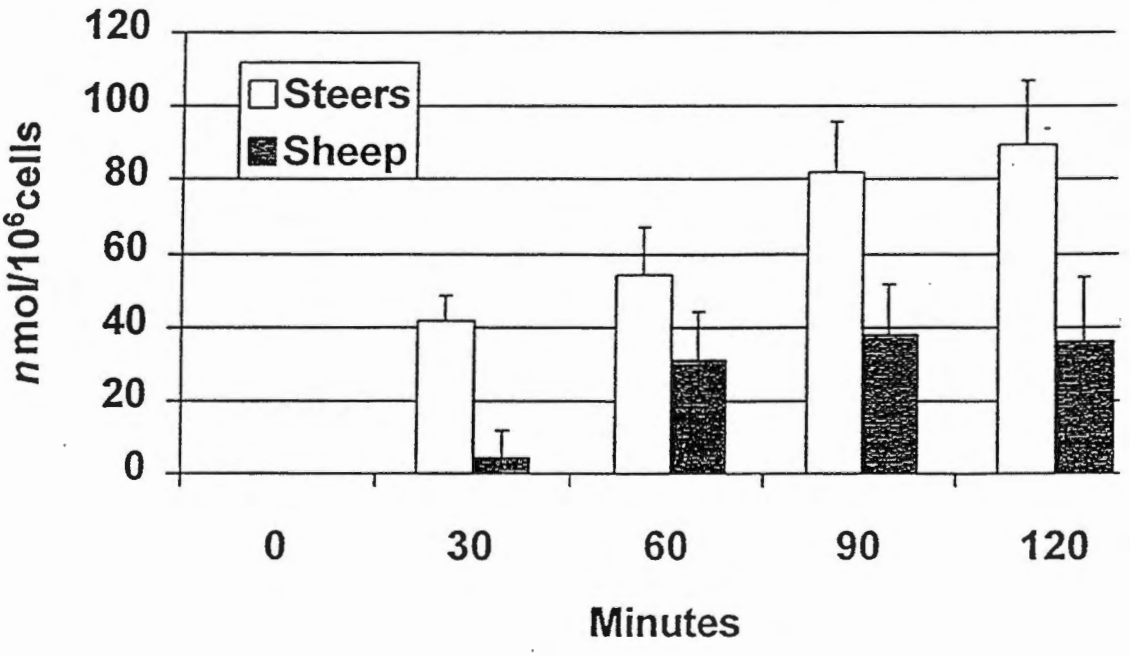

Figure 2. Production of ACAC during the 2 h incubation.

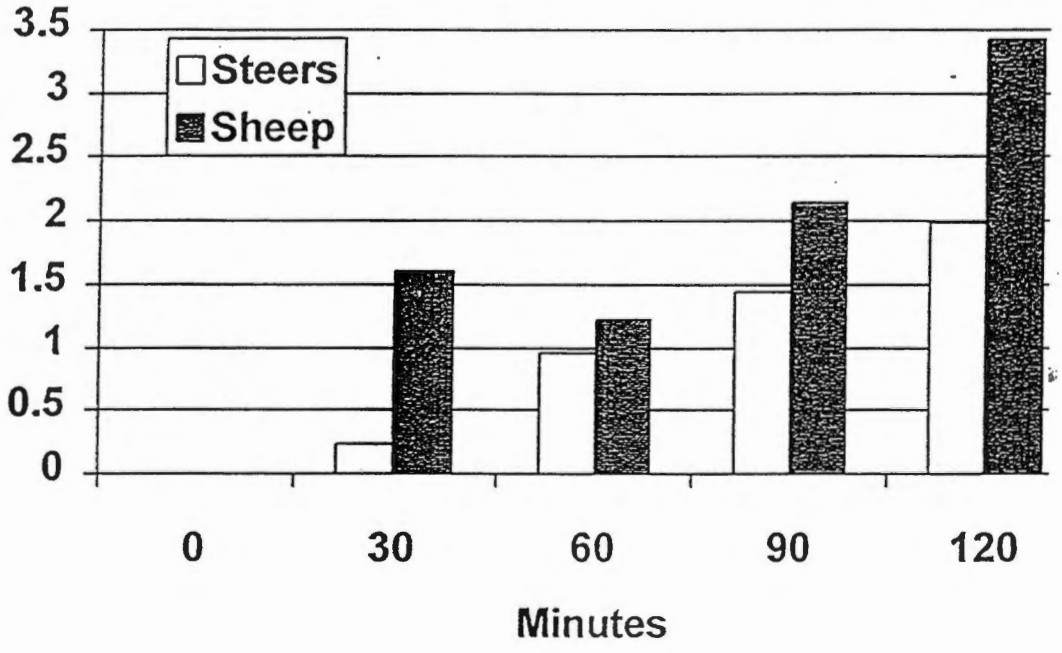

Figure 3. Ratio of BOHB to ACAC during the 2 h incubation.

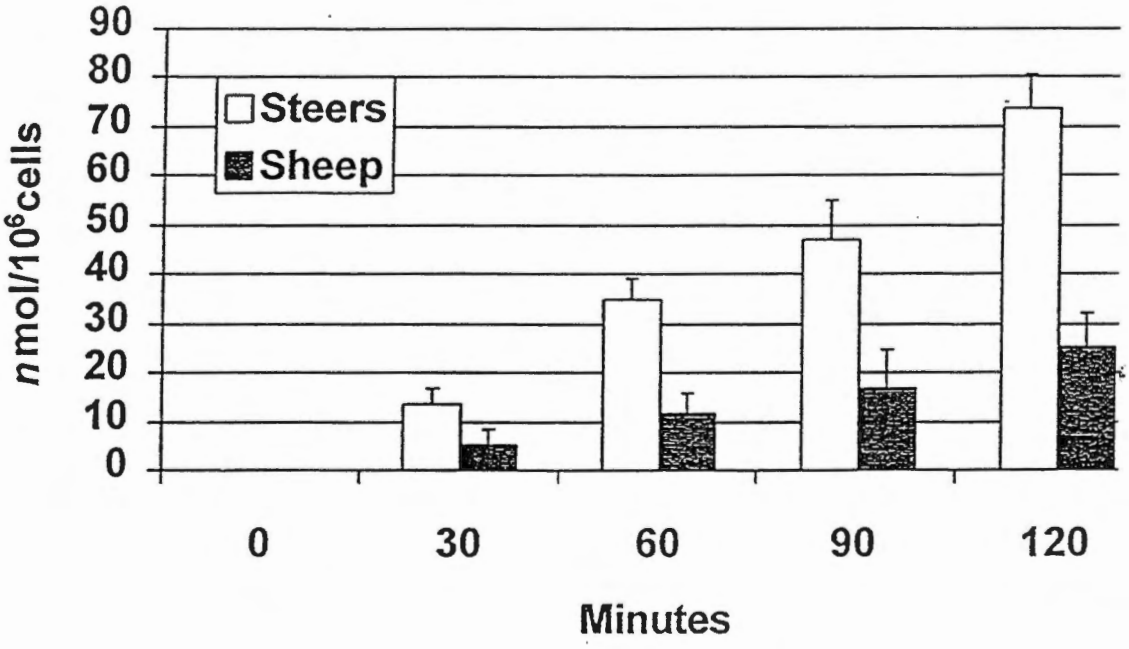

Figure 4. Production of LACT during the 2 h incubation.

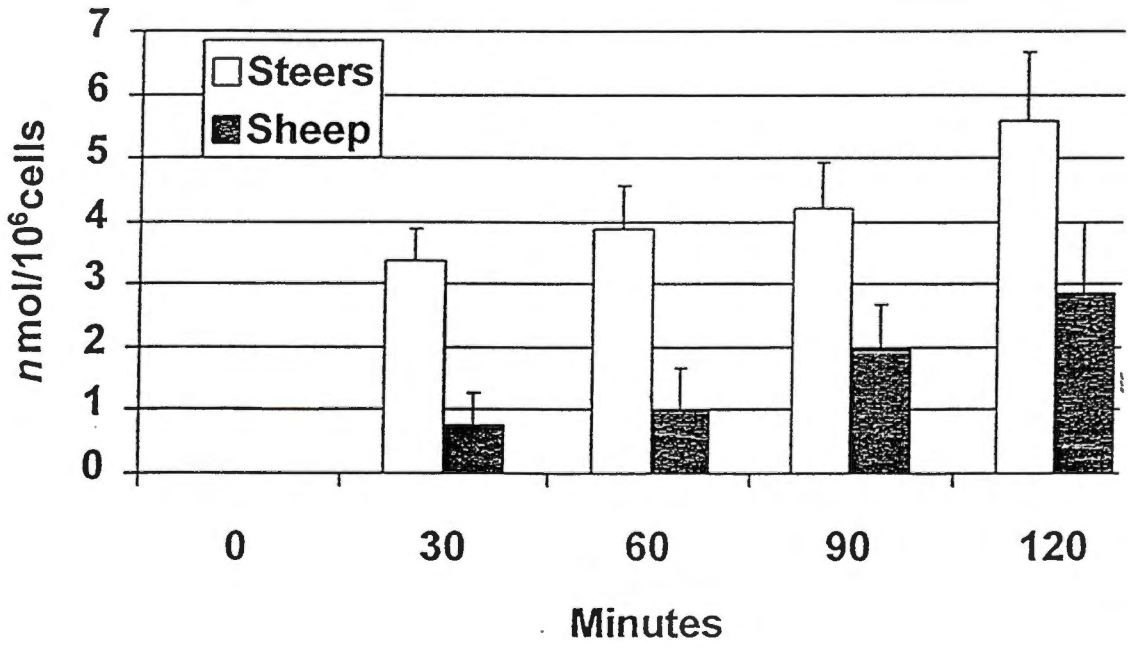

Figure 5. Production of PYR during the 2 h incubation.

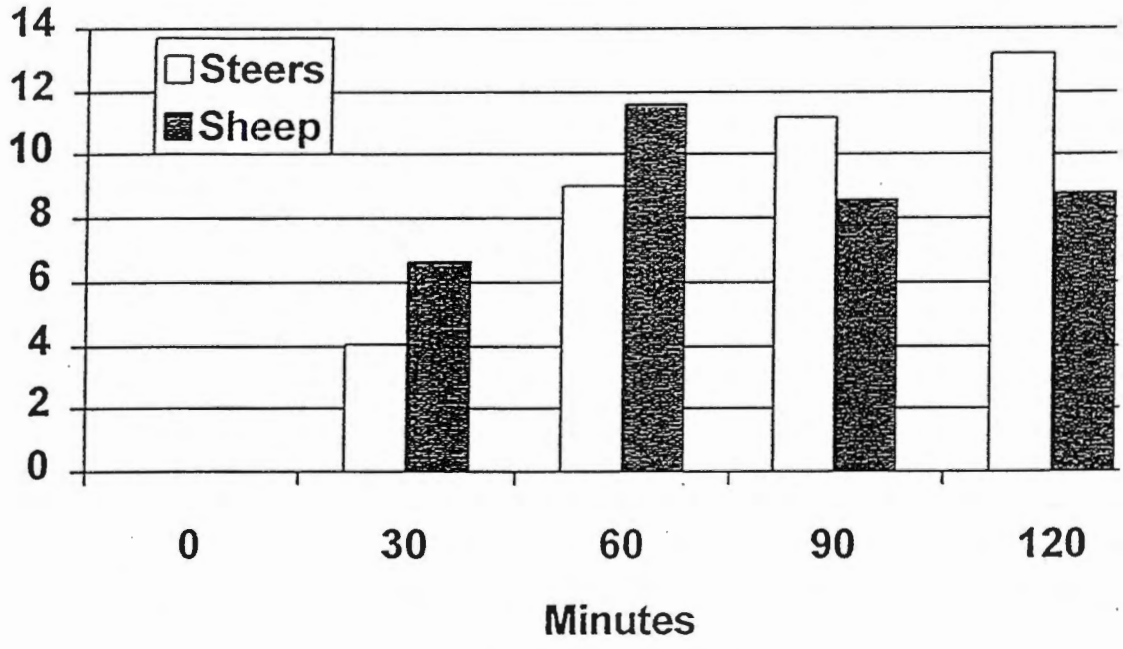

Figure 6. Ratio of LACT to PYR during the 2 h incubation.

Table 5. Metabolite production[a] by steers and ewes

Metabolite	Steers	Ewes	SEM	*P*
BOHB	177	123	20	.06
ACAC	90	36	18	.05
BOHB/ACAC	1.98	3.43	...	...
LACT	74	25	7.3	.01
PYR	5.6	2.9	1.1	.10
LACT/PYR	13.20	8.85	...	...

[a] *n*moles/10^6 cells after 120 minutes of incubation.

Metabolites were: BOHB = ß-hydroxybutyrate, ACAC = acetoacetate, LACT = L-(+)-lactate, and PYR = pyruvate.

ratios were calculated, but variation in the data caused some means to become exaggerated. Therefore the most conservative method of analysis (ratios of the means) was reported in an attempt to minimize Type I errors.

Discussion

This study comparing rumen epithelial metabolism between ewes and steers hinges on several assumptions that could have influenced the data. First, it is assumed that no differences in metabolism occurred due to animal sex. Weekes (1972) reported that breeding state did not affect the activity of four enzymes involved in conversion of propionate to lactate and pyruvate when activities were expressed per unit weight of rumen mucosa and suggested that sex hormone levels did not influence rumen epithelial metabolism. However, to minimize sex hormone differences, animals used in the current study were hysterectomized ewes and non-implanted steers. Thus, neither group of animals possessed actively secreting gonads.

The second assumption is that animal age did not affect metabolic activity of epithelial tissue. The exact age of the ewes was not established; however, all were greater than 3 yr. Steers were approximately 2 yr of age. Since there are numerous reports that epithelial tissue from 8 wk old ruminants metabolize VFA at rates similar to tissue from adult ruminants (Wardrop and Coombe, 1961; Bush, 1988; and Baldwin and Jesse, 1992), it was important to have the epithelium, rather than the whole animal, at the same stage of development. Thus, all animals were placed on the fescue

hay diet 21 d prior to tissue harvest so that epithelial tissue was at the same stage of maturity.

Apart from age, stage of growth was also of concern in the current study. It is assumed that the mature ewes were undergoing little growth. Weights were not recorded for the steers during this experiment, but a review of animal records indicates that the steers gained approximately 100 lbs over an 18 mo period surrounding the study. This indicates that these 2 yr old animals were not in a rapid state of growth. High plasma concentrations of GH are associated with animals exhibiting rapid growth (McDowell and Annison, 1991). GH stimulates cell division and protein synthesis via its main effector, insulin-like growth factor I (IGF-I) (Etherton and Kensinger, 1984). Baldwin (1995) reported increased proliferation of rumen epithelial cells when cells were incubated with IGF-I *in vitro*. The fact that these animals were not growing rapidly suggests that growth hormone (GH) levels were not elevated sufficiently to increase rumen epithelial proliferation.

The low CP and ME of the fescue hay (Table 2) was not sufficient to meet the maintenance requirements of mature ewes or growing steers. This poor quality forage may have resulted in insufficient N for microbial growth and subsequent low numbers of rumen microbes (Dearth et al.,

1974). Low numbers of rumen microbes would further lower the amount of protein liberated from the forage (Wallace and Cotta, 1988), as much of the N in this poor quality hay would be nonsoluble in the lignified fraction. It also follows that the reduced number of rumen microbes would result in lower production of VFA that could be absorbed and metabolized across the rumen epithelium. Low levels of VFA (especially butyrate) would cause little stimulatory effect for systemic insulin release (Trenkle, 1978). Insulin stimulates rumen epithelial cell mitosis and negates the inhibitory effect of butyrate on epithelial proliferation (Galfi et al., 1991). A lack of insulin would thereby cause lower metabolic activity of the epithelial cells by not initiating mitosis.

All metabolites measured in this experiment were higher or tended to be higher in cells from steers than those from ewes. Differing levels of individual VFA in the rumen cause changes in the morphological development (Sander et al., 1959; Gaebel et al., 1987) and metabolic processes of the rumen epithelium (Galfi et al., 1991). Fell and Weekes (1975) and Weigand et al. (1967) reported enzyme activities per unit tissue weight were not greatly affected by diet in growing lambs or cattle, respectively. However, it is not known if exposure to individual VFA immediately prior to

metabolic study predisposes epithelial cells to preferentially metabolize that substrate. If these cells were able to adapt to existing levels of VFA in the rumen, higher concentrations of ruminal butyrate in steers may have contributed to increased production of ketone bodies. However, this fails to explain the higher levels of lactate and pyruvate produced by steers. Since ruminal propionate concentrations did not differ between species (Table 3), no enzyme adaptation would have occurred for the production of lactate or pyruvate. Higher butyrate in the rumen of steers may have increased the mitogenic rate of the rumen epithelium *in vivo* (Sakata and Tamate, 1976). Increased mitogenesis would stimulate cellular metabolic activity and result in higher metabolite production by steer cells. However, the presence of butyrate in the incubation media *in vitro* may have resulted in the suspension of cellular mitogenesis (Kruh, 1982) and a progression towards cellular cornification (Galfi et al., 1983). This mitotic inhibition by butyrate *in vitro* would delete any increased metabolic activity inherent in steer cells.

Beck et al. (1984) reported no differences in metabolite production from butyrate by rumen epithelial tissue of cattle and sheep. The lack of differences reported in their study may be due to the use of epithelial

tissue sheets, whereas our study utilized epithelial cells resultant from serial tryptic digestion. Steer tissue was more heavily keratinized than ewe tissue in this study, and it took more digestions to remove this keratinized cell layer (3 or 4 digestions for steers, and 2 digestions for ewes). This higher degree of keratinization would cause slower movement of substrate to the cells of an undigested epithelial sheet and result in lower metabolite production by bovine tissue relative to ovine.

The ratio of BOHB to ACAC (an indicator of mitochondrial redox potential) is similar to *in vivo* portal vein data for ruminants, ranging from 2-4:1 (Heitmann et al, 1987). The ratios reported in this study are encouraging in light of other *in vitro* data which has had difficulty in mimicking *in vivo* reports. Weigand et al. (1975) reported a BOHB/ACAC of about 1:2 from rumen papillae incubated with butyrate. Beck et al. (1984) reported ratios ranging from 4-7:1 (cattle) and 12-17:1 (sheep) when incubating epithelial sheets in the presence of butyrate. The ratios reported in the present study are similar to those reported by Baldwin (1994) and suggest that conditions achieved in this study may reflect actual *in vivo* physiological conditions.

The ratios of LACT to PYR tended to be large and variable due to the small amount and variability of pyruvate produced by cells in this study. The ratio reported in this study for ewes (8.82) is within the range (3 - 9.1) reported by Weekes (1972).

The ratios of BOHB to ACAC and LACT to PYR were similar between species during the incubation periods. These ratios represent the redox status of the mitochondria and the cytosol, eliminating cellular energy status as a potential cause for species differences.

Species differences in metabolite production could be due to differing enzymatic activities between species. However, no differences in enzymatic activities were reported in the literature and some key enzymes appear to have similar activities in both species. Emmanuel and Milligan (1983) and Bush and Milligan (1971) reported that the activity of 3-oxoacid CoA transferase (a proposed key enzyme in ruminal ketogenesis) in bovine rumen epithelium was 26 ± 4 and 15 ± 6 μmol/h/g of tissue. Leighton et al. (1983) reported the activity of this enzyme to be 24 ± 7 μmol/h/g of tissue in ovine epithelium. Also, Bush and Milligan (1971) and Leighton et al. (1983) reported the activity of 3-hydroxy-3-methylglutaryl-CoA synthase (a key enzyme in ketogenesis via the 3-hydroxy-3-methylglutaryl-CoA

pathway) to be 3.6 ± 2.6 and 13.2 ± 2.4 µmol/h/g of tissue for bovine and ovine, respectively. Emmanuel and Milligan (1983) reported that the activity of bovine rumen epithelial lactate dehydrogenase was 3338 ± 167 µmol/h/g of tissue, which is similar to the reported value for ovine of 3402 ± 418 µmol/h/g of epithelium (Leighton et al., 1983). Similar activities of 3-oxoacid CoA transferase and lactate dehydrogenase between species and a higher activity of HMG-CoA synthase in sheep do not support the higher metabolite production by steers. However, other enzyme activities in these pathways could differ and regulate metabolite production.

Cellular mass action ratios may affect metabolite production within species. Differences in substrate supply of PYR or ACAC from endogenous anaerobic glucose oxidation or aerobic oxidation of non-esterified fatty acids (Jesse et al., 1992) may result in higher metabolite production by steers. However, these substrates would have to be present from residual systemic supply because glucose and non-esterified fatty acids were not present in the incubation media. Therefore, this explanation is not likely because cells were harvested several hours before incubation, during which time they utilized any endogenous substrates.

The higher production of metabolites by steer cells could be a reflection of the higher metabolic rate present in bovine than in ovine. When expressed according to metabolic body weight ($BW^{.75}$), bovine (>75 kcal/$kg^{.75}$) possess a higher rate of metabolism than do ovine (<60 kcal/$kg^{.75}$; Kleiber, 1961). Britton and Krehbiel (1993) attributed the higher portal-drained visceral (PDV) oxygen consumption present in cattle (23.2 - 26.7% of whole body O_2 consumption vs. 19% for sheep) to be a result of the smaller size of the stomach and small intestine in sheep. The higher metabolite production reported in this study would also be consistent with the higher PDV O_2 consumption reported for cattle.

Conclusion

The objective of this study was to determine if a species effect exists between sheep and steers in the basal metabolism of rumen epithelial cells. Cells from steers produced more of the measured metabolites than did cells from ewes. Although similar enzyme systems and metabolic pathways are present in the rumen epithelium of sheep and cattle, caution is needed when using sheep tissue as a model for bovine rumen epithelial metabolism.

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Part 2.

Metabolism of Epithelial Cells from Different Areas of the Ewe Rumen

Abstract

Cells were harvested from 4 rumen locations in 4 ewes fed fescue hay to determine if cell origin has an effect on cellular metabolism. Tissue (~ 150 cm^2) was excised from the anterior cranial pillar (ACP), ventral sac floor (VSF), caudal pillar surface (CPS) and dorsal sac ceiling (DSC). Cells were isolated using serial tryptic digestion. One mL of isolate was incubated for 2 h in 6 mL of media containing 25 m*M* propionate and 10 m*M* butyrate. Incubations were terminated at 0, 30, 60, 90 and 120 min and analyzed for ß-hydroxybutyrate (BOHB) and acetoacetate (ACAC) as mitochondrial and lactate (LACT) and pyruvate (PYR) as cytosolic redox estimators. Cell yield was 22, 22, 24 and 14 ± 6 x 10^6 cells/mL and viability was 92, 92, 94 and 87% for ACP, VSF, CPS and DSC. All metabolite concentrations and ratios of redox pairs increased over the incubations indicating continuous cellular activity. Final 2 h concentrations (*n*moles/10^6 cells) were 123, 113, 163, and 158 ± 35 for BOHB; 38, 42, 24, and 45 ± 10 for ACAC; 25.3, 20.6, 10.1, and 20.4 ± 5.6 for LACT; and 2.54, .98, 1.06, and 1.31 ± .61 for PYR in the ACP, VSF, CPS, and DSC incubations, respectively. No differences existed between tissues in the amount of metabolite produced ($P > .05$) indicating that cellular location was not a critical factor

in the metabolism of rumen epithelial cells.

Key Words: Sheep, Cells, Rumen, Epithelium, Metabolism.

Introduction

Tissue has been harvested from a number of locations within the rumen for the study of rumen epithelial metabolism. Baldwin and Jesse (1992) used tissue from the whole rumen of young lambs and tissue from the cranial ventral sac in older ruminants. Hird and Weidemann (1964) harvested tissue from the dorsal sac, while others did not specify the location epithelial tissue was collected from (Bush, 1988; Sakata and Tamate, 1979). The exposure of different areas of the rumen to certain substrates (e.g., presence or absence of ruminal liquor) and cells harvested from various anatomical and morphological structures of the rumen could affect epithelial cell metabolism. The objective of this study was to compare the *in vitro* metabolism of cells harvested from different locations within the ewe rumen to determine if cell location, tissue morphology, and proximal substrates affect the rate that these cells metabolize rumen substrates.

Materials and Methods

Materials

Hepes (N-2-Hydroxyethylpiperazine-N'-2-Ethane Sulfonic Acid) and trypsin (1:250) were from Gibco BRL (Life Technologies, Inc., Grand Island, NY). Bovine serum albumin (BSA) was fatty acid free, #A-6003 (Sigma Chemical Co., St. Louis, MO). Sodium salts of propionate and butyrate used for incubation substrates were also from Sigma Chemical. All other chemicals were reagent grade compounds from either Sigma Chemical or Fisher Scientific (Fair Lawn, NJ).

Animals

Four crossbred, hysterectomized, mature ewes had access to trace-mineralized salt blocks and were fed a poor quality fescue hay and offered water for ad libitum intake for at least 14 d prior to initiation of the experiment. Forage samples were analyzed for DM, ash, CP, and macro-mineral content by proximate analysis (AOAC).

Cell Isolation

Ewes were brought to the surgical facility and killed according to USDA guidelines using captive bolt followed by exsanguination. The abdomen was opened immediately and the rumen was excised. Ruminal fluid samples were collected for analysis (Appendix 5) of ruminal VFA concentrations. Portions (~ 150 cm^2)of rumen tissue were removed from the

anterior cranial pillar (ACP), ventral sac floor (VSF), dorsal caudal pillar (CPS), and dorsal sac ceiling (DSC). Each tissue was rinsed with warm tap water to remove adherent feed particles and placed in 37°C Krb-Hepes buffer (Table 1). Tissues were immediately transported to the laboratory (< 2 min). The exterior muscle and connective tissue layers were then peeled from the back of the epithelial sheets. The muscular and connective tissues were discarded and each epithelial sheet was minced to pieces about 1 cm^2 in size. Epithelium from each tissue was placed into one of four labeled digestion flasks. One-hundred mL of a 3% trypsin solution containing .016 g $CaCl_2$ dihydrate was added to each flask. Flasks were placed in a slow shaking 37°C orbital hot air incubator (New Brunswick Scientific Co., Edison, NJ) for 15 minutes. Flasks were then removed from the incubator and contents were filtered through a 1 mm nylon mesh into 250 mL Erlenmeyer flasks. Tissue remaining on mesh was rinsed with 37°C Krb-Hepes buffer to release any adhering cells into the collection flasks. Recovered cells were discarded from this digestion. The tissue on the mesh was placed back into digestion flasks and subjected to another 15 min 3% trypsin digestion. The same recovery procedure was used and cells from this

Table 1. Krb-Hepes buffer[a]

Compound	Amount
KH_2PO_4	.3239 g
$MgSO_4$	.5866 g
KCl	.706 g
Hepes[b]	13.0 g
NaCl	14.4 g

[a]Compounds in solution with 2 L distilled water and bubbled with O_2/CO_2 (95%:5%) for 20 minutes (pH 7.4).

[b]N-2-Hydroxyethylpiperazine-N'-2-Ethane Sulfonic Acid.

digestion were also discarded. The third digestion was a 15 min digestion in a 2% trypsin solution. After filtering and rinsing the cells through the 1 mm nylon mesh, recovered cells were filtered through a 300 µm nylon mesh into 50 mL centrifuge tubes. The filtrate was centrifuged in a Sorvall RC2-B centrifuge (Ivan Sorvall Inc., Newtown, CT) at 70 x g (764 rpm) for 6 min at 4° C. If the pellet contained a dark ring of keratinized cells, it was discarded. If keratinized cells were not predominant, the supernatant liquid was decanted and cells were resuspended (washed) in 37°C Krb-Hepes buffer. The suspension was recentrifuged and cells were washed twice more. Pelleted cells remained in KRB-Hepes buffer at room temperature until they were combined with cells from later digestions. Subsequent digestions (4-7) were in a 2% trypsin solution for 10 min. Cells from digestions three through five usually had little keratinization and were combined to yield a cell pool. Digestions served to harvest cells from mainly the stratum basale and stratum spinosum layers, and most cells from the stratum granulosum and stratum corneum were discarded. The progression of cells from the basale outward to the corneum is concurrent with cellular degeneration. Cells of the granulosum exhibit signs of degeneration (Steven and Marshall, 1970) and cells of the corneum display extensive

keratinization (Henriksson and Habel, 1961). Therefore, the metabolically active cells from the stratum basale and stratum spinosum were collected. These cells were recentrifuged and resuspended in 18 mL of Krb-Hepes buffer. Cell numbers and viability were then assessed using a hemacytometer and trypan blue exclusion. Cells that did not contain trypan blue dye were considered to be viable. This isolation procedure was performed simultaneously for the epithelium from each of the four tissue sites.

Cell Incubations

Incubation flasks (25 mL Erlenmeyer) were prepared with 5 mL of a premix solution containing 5X Krb (84 mL) (5x Krb was prepared by adding 10.5 mL H_2O to a flask containing 2, .5, 50, .5, and 1.5 mL of .77 *M* KCl, .77 *M* KH_2PO_4, .77 *M* NaCl, .77 *M* $MgSO_4$, and .77 *M* $NaHCO_3$, respectively.), 1 *M* Hepes (10.5 mL), 100 m*M* butyrate (42 mL), 250 m*M* propionate (42 mL), BSA (84 mL), and H_2O (87.5 mL). Flasks were placed in a 37°C water bath and assigned incubation periods of 0, 30, 60, 90, or 120 min. Cell suspensions (1 mL) were added to each flask at 30 second intervals, yielding a total of 6 mL per flask. The final concentration of reagents in these flasks was Krb (1X), Hepes (.25 m*M*), butyrate (10 m*M*), propionate (25 m*M*), and BSA (.25 m*M*). At the end of incubation, cells in each flask were killed by adding .4 mL

of concentrated perchloric acid. Flasks were placed on ice for 10 min, then .8 mL of saturated potassium carbonate was added as a buffering agent. Flasks were left on ice for at least 10 min after buffering, and were decanted into 15 mL centrifuge tubes. Tubes were centrifuged at 1500 x g for 20 min in a Beckman TJ-6 centrifuge (Beckman Instruments, Palo Alto, CA).

Analyses

Supernatant liquid was analyzed for acetoacetate (ACAC) and beta-hydroxybutyrate (BOHB; Williamson and Mellanby, 1965), and L-(+)-lactate (LACT) and pyruvate (PYR) using procedures modified from the Sigma kits. Procedures are described fully in Appendices 1 through 4.

Statistics

Data were analyzed as a completely randomized design using animal and rumen site as classification variables. Orthogonal contrasts were used to assess differences between rumen locations.

Results

Results of feed analysis (Table 2) indicated poor quality forage was utilized in this study. A predicted intake of 1200 g/d DM met approximately 107% of ME and 80% of CP requirements for mature ewes (NRC, 1984). The estimates for CP intake were calculated by multiplying the analyzed CP level of the feed (Table 2) by the NRC predicted DM intake for mature ewes. The estimates for ME intake were calculated by multiplying the ME value of mature fescue hay from the NRC recommendations by the ewes' predicted intake. The predicted ME intakes are overestimated because according to CP levels the NRC assumes better quality forage than was utilized in this study.

Total rumen VFA concentration for ewes immediately following exsanguination was 54 (SE = 15.1) m*M*. Acetate (75%) was present in the largest molar percentage (mol/100mol), followed by propionate (18%), and butyrate (4%). Isobutyrate, isovalerate, and valerate were 1.06, 1.2, and .47% of total ruminal VFA, respectively.

The mean number of harvested cells from the four tissues ranged from 14 to 24 x 10^6cells/mL and cell viability ranged from 87 to 94%. The number of viable cells used for incubation ranged from 12.5 to 22.5 x 10^6cells/mL (Table 3) and did not differ ($P > .10$) among tissue sites.

Table 2. Fescue Hay Composition

Component	Value
DM (%)	94.3
CP[a] (%)	7.1
Ash[a] (%)	7.0
Calcium[a] (g/mg)	4.52
Phosphorus[a] (g/mg)	2.49

[a] Composition on a dry matter basis.

Table 3. Cell yields and viabilities

Tissue[a]	Harvested cells		Viability		Live cells	
	mean[b]	SEM	mean[c]	SEM	mean[b]	SEM
ACP	21.50	7.48	.92	.02	19.68	6.87
VSF	22.02	6.92	.92	.01	20.02	6.14
CPS	24.00	7.70	.94	.01	22.50	7.20
DSC	14.32	2.76	.87	.05	12.53	2.67

[a]ACP = anterior cranial pillar; VSF = ventral sac floor; CPS = caudal pillar surface; and DSC = dorsal sac ceiling.

[b]10^6cells/mL

[c] x 100 = %

Graphs of metabolite production and ratios during the 2 h incubation period are shown in Figures 1 through 6. Metabolite production generally increased during the incubation for metabolites at all tissue sites. Two-hour metabolite production by each tissue is reported in Table 4. The reported ratios were calculated using the reported means (ratios of the means) instead of averaging individual ratios from each experimental unit (means of the ratios). The means of the ratios were calculated, but variation in data caused some trends to become exaggerated. Therefore, the most conservative method of analysis (ratios of the means) was reported in an attempt to minimize Type I errors.

Statistical evaluation of metabolite production at 2 h of incubation was based on three orthogonal contrasts between the four tissues (Table 4). No differences were observed in the production of BOHB, ACAC, LACT, or PYR between tissues for the comparisons made, except that the ACP tended to produce more LACT than the CPS ($P = .07$).

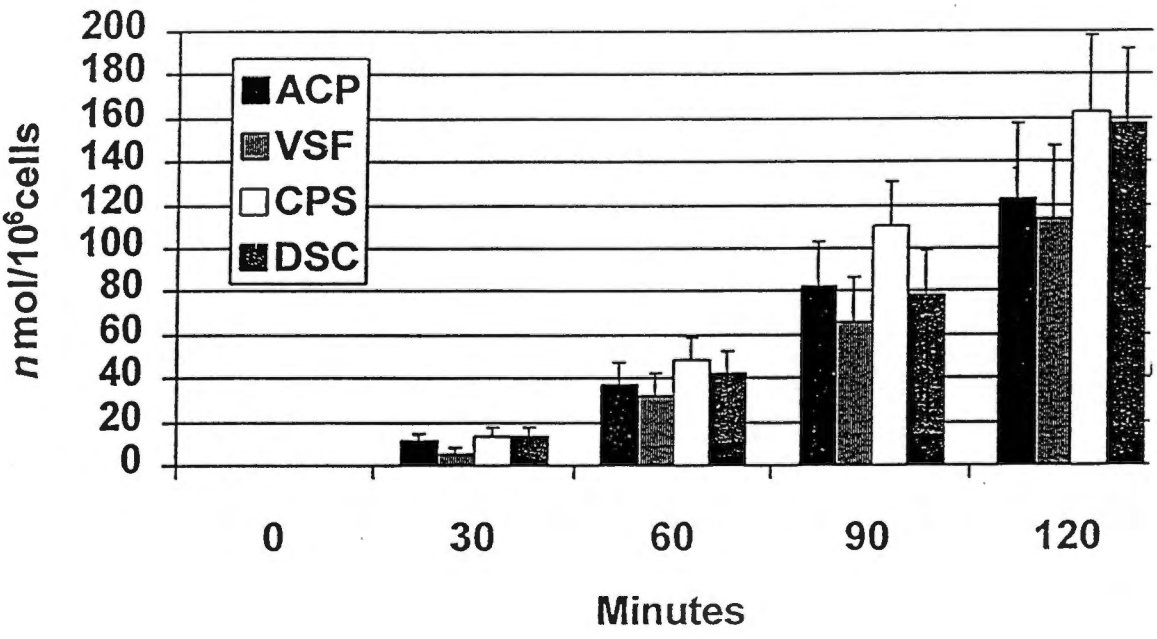

Figure 1. Production of BOHB during the 2 h incubation.

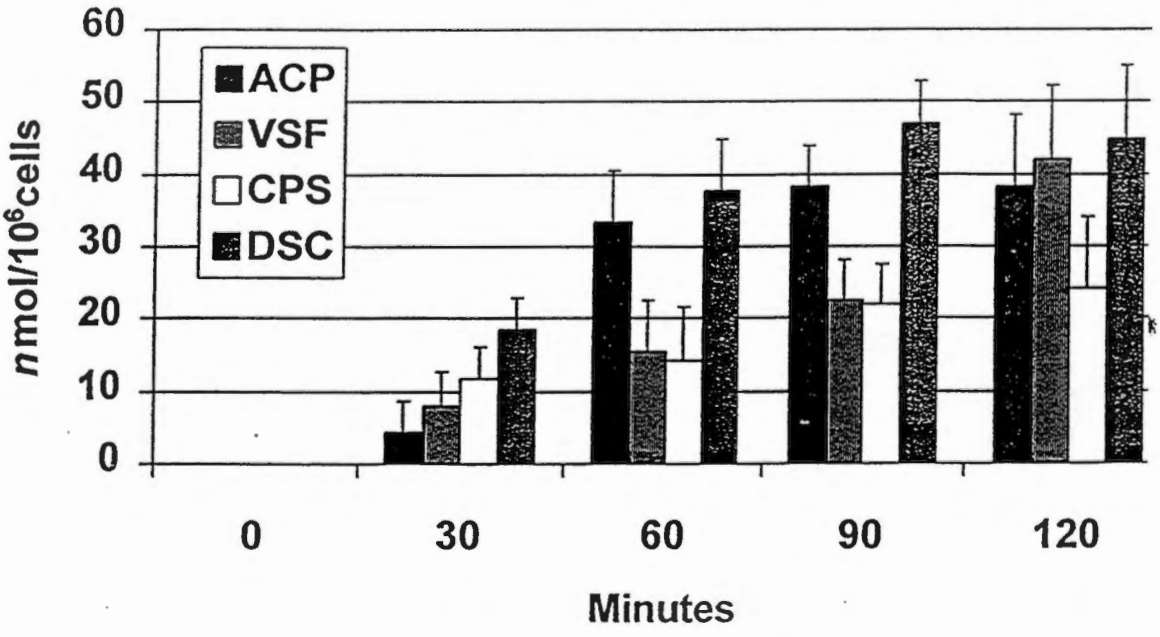

Figure 2. Production of ACAC during the 2 h incubation.

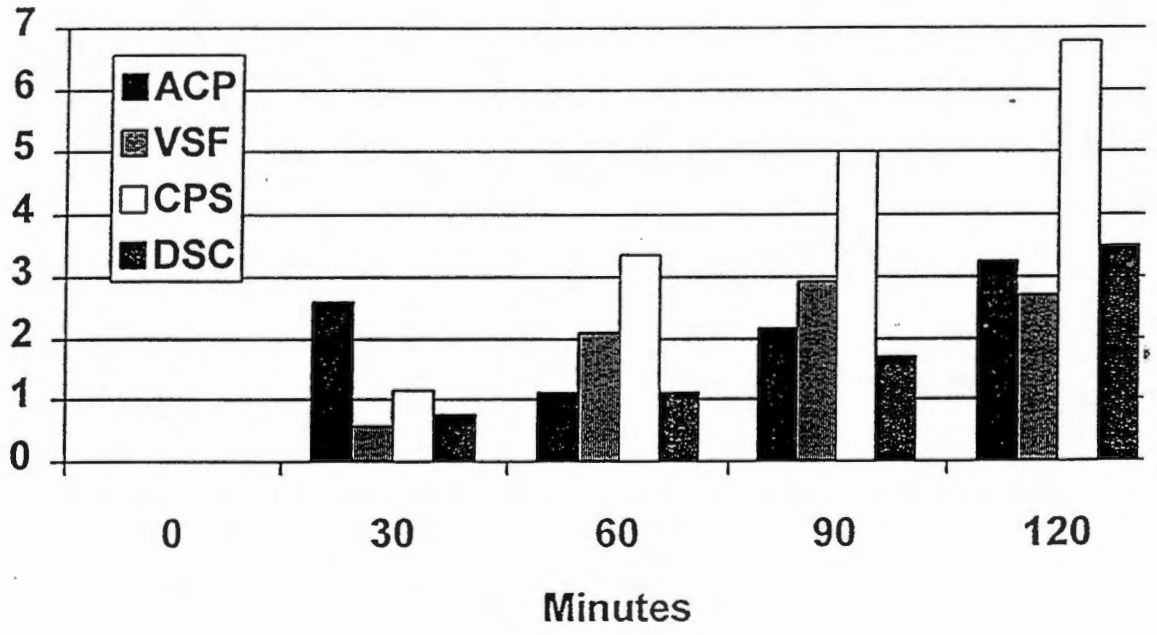

Figure 3. Ratio of BOHB to ACAC during the 2 h incubation.

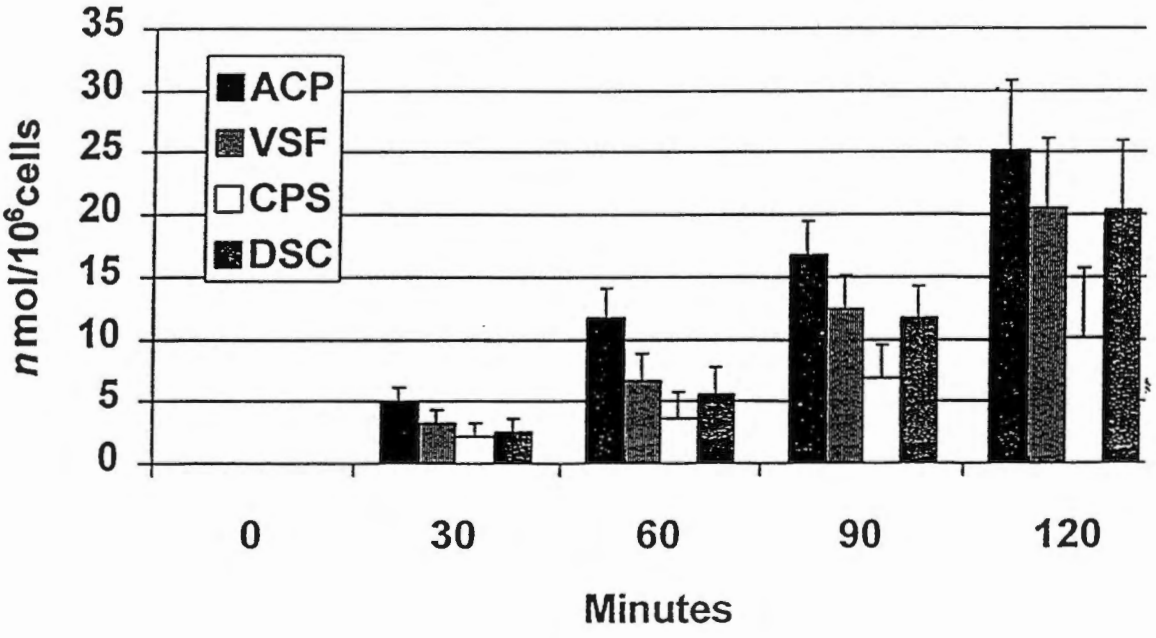

Figure 4. Production of LACT during the 2 h incubation.

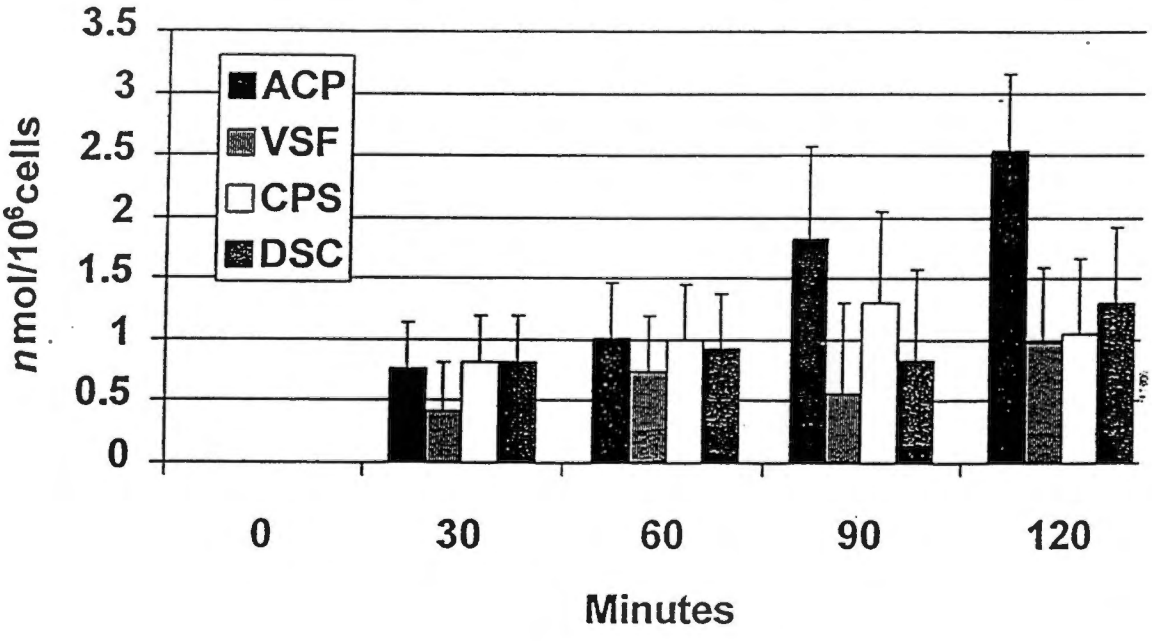

Figure 5. Production of PYR during the 2 h incubation.

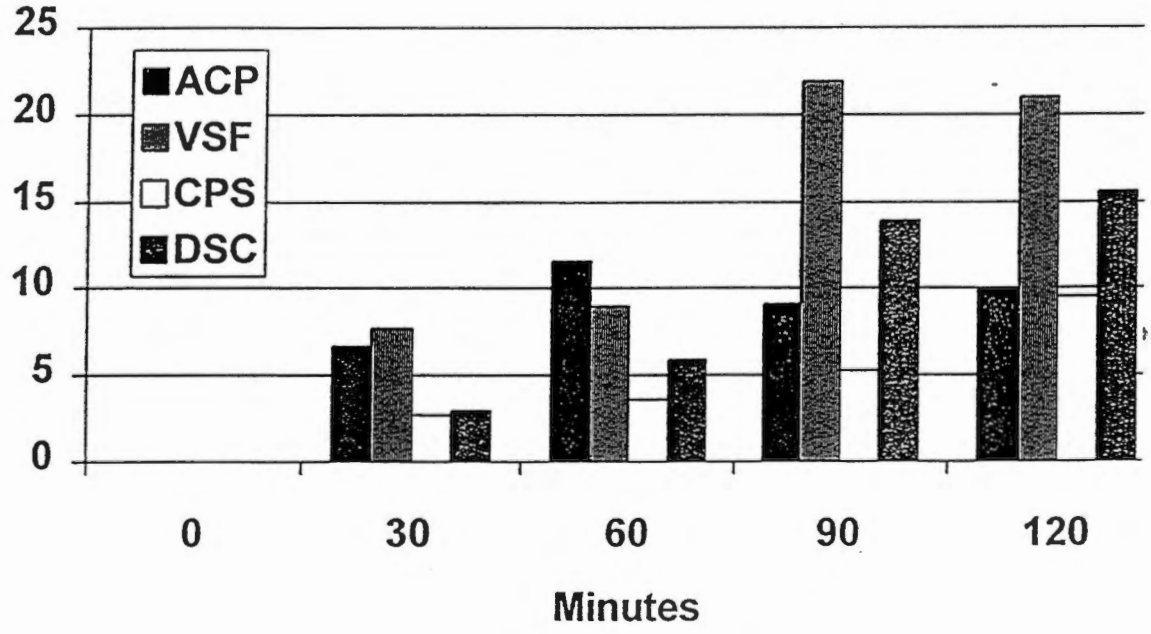

Figure 6. Ratio of LACT to PYR during the 2 h incubation.

Table 4. Metabolite production by tissue[a]

	ACP	VSF	CPS	DSC	pSEM[b]	Contrasts[b] 1	2	3
BOHB	123	113	163	158	34.5	NS[d]	NS	NS
ACAC	38	42	24	45	10.0	NS	NS	NS
BOHB/ACAC	3.24	2.69	6.79	3.51	...	..	..	..
LACT	25.3	20.6	10.1	20.4	5.56	NS	NS	.07
PYR	2.54	0.98	1.06	1.31	.61	NS	NS	NS
LACT/PYR	9.96	21.02	9.52	15.57	...	..	..	..

[a] *n*moles/10^6 cells after 120 minutes of incubation. Tissues were: ACP = anterior cranial pillar; VSF = ventral sac floor; CPS = caudal pillar surface; DSC = dorsal sac ceiling. Metabolites were: BOHB = ß-hydroxybutyrate; ACAC = acetoacetate; LACT = L-(+)-lactate; and PYR = pyruvate.

[b] contrasts: 1 = (ACP + CPS) / (VSF + DSC); 2 = VSF / DSC; 3 = ACP / CPS

[c] pooled standard error of the mean

[d] $P > .10$

Discussion

The low CP of the fescue hay (7.1%, Table 2) was not sufficient to meet the maintenance requirements of mature ewes. This poor quality forage may have resulted in insufficient N for microbial growth and subsequent low numbers of rumen microbes (Dearth et al., 1974). Low numbers of rumen microbes would further lower the amount of protein liberated from the forage (Wallace and Cotta, 1988), as much of the N in this poor quality hay would be nonsoluble in the lignified fraction. It also follows that the reduced number of rumen microbes would result in lower production of VFA that could be absorbed and metabolized across the rumen epithelium. Low levels of VFA (especially butyrate) would cause little stimulatory effect for systemic insulin release (Trenkle, 1978). Insulin stimulates rumen epithelial cell mitosis and negates the inhibitory effect of butyrate on epithelial proliferation (Galfi et al., 1991). A lack of insulin would thereby cause lower metabolic activity of the epithelial cells by not initiating mitosis.

The rumen is composed of four non-discreet physiological compartments, each defined by specific microbial populations (Czerkawski and Cheng, 1988). Microflora are: 1)adherent to the ruminal particulate matter, 2)in solution in the ruminal liquid, 3)loosely

adherent to the particulate matter (thereby associated with both the particulate and liquid fractions), and 4)associated with the rumen epithelial wall. Each of these microbial compartments consists of populations that metabolize components of the proximal substrate (Williams and Strachan, 1984). Microbes tightly bound to the particulate fraction digest mostly structural carbohydrates. Microbes in the liquid fraction metabolize soluble carbohydrates and nitrogen components, while the population that is associated with both the particulate and liquid fractions metabolizes substrates from both compartments. Microbes associated with the rumen epithelium are largely facultative anaerobes that metabolize substrates that are transferred from the host across the epithelium such as urea, oxygen, and sloughed epithelial tissue (Wallace et al., 1979). This compartmentation within the rumen results in compartmentation of microbial metabolite production. Czerkawski and Breckenridge (1982) reported that the concentrations of total VFA and of NH_3 were greater in the particulate-containing compartments than in the liquid compartment. Owens and Goetsch (1988) reported higher acetate levels associated with the solid-phase or "mat" of the rumen digesta. By-product compartmentation did not affect cellular metabolism in this study. This finding

suggests that rumen epithelial cells do not adapt to the presence of proximal metabolites and subsequently do not metabolize these substrates preferentially.

Conflicting data exists concerning the effect of VFA on the rumen epithelium *in vivo* and *in vitro*. VFA, especially butyrate and propionate, are known to stimulate rumen epithelial proliferation *in vivo* (Sakata and Tamate, 1978, 1979). However, butyrate, and to a lesser extent propionate, inhibit rumen epithelial cell mitosis *in vitro* (Galfi et al., 1991). The increased mitosis of this tissue *in vivo* is likely stimulated by hormonal influences which relieve the inhibitory effects of VFA (Sakata et al., 1980) and cannot be produced naturally *in vitro*. Addition of hormones such as insulin (Neogrady et al., 1989 as quoted by Galfi et al., 1991) and other growth factors (IGF-I, IGF-II, and EGF) to *in vitro* studies removed the inhibitory effect of VFA on rumen epithelial cell proliferation (Baldwin, 1995), indicating that the *in vivo* response to VFA may stem from these paracrine and endocrine influences. The stimulation of mitosis *in vivo* increases the absorptive and metabolic activity of the tissue. Increased absorptive capacity occurs due to greater surface area for absorption and heightened metabolic activity (Galfi et al., 1991). The increased metabolic activity of the epithelial cells

increases the concentration gradient of VFA from the lumen to the intracellular spaces. Sutton et al. (1963) reported that ruminal VFA increased the absorptive ability of rumen epithelium. Measurements of VFA uptake or absorption were not taken in the present study; only metabolite production by the cells was measured.

Rumen epithelium is reported to adapt to levels of VFA in the rumen (Fell and Weekes, 1975). This adaptation occurs as metabolic activity of the cells fluctuates with changes in the mitogenic state (Goodlad, 1981). Research *in vivo* supports this adaptation (Gaebel et al., 1987); however, little evidence supports adaptation *in vitro* (Weekes, 1972 as quoted by Fell and Weekes, 1975; Young et al., 1965). This suggests messenger mechanisms other than VFA, which change the metabolic activity and absorptive capacity of the tissue, may participate in rumen epithelial adaptation.

The *in vitro* study of cells does not include all parameters present *in vivo*. For example, hormonal and growth factor responses present *in vivo* do not occur *in vitro*. Other factors in this study, such as the removal of keratinized cell layers, create conditions that are not physiologically normal. In spite of these shortcomings, *in vitro* research allows aspects of cell metabolism to be

examined that would otherwise be masked by normal physiological responses, such as hormone release, *in vivo*.

The current study indicates that rumen epithelial cells do not individually adapt to proximal stimuli present during ruminal absorption of VFA. Instead, this study confirms reports of other messenger mechanism involvement. It indicates that adaptation does not occur at the cellular level, but that the rumen adapts to ruminal substrate at the tissue level by responding to systemic influences.

Conclusion

Tissue was harvested from a number of locations within the rumen to study metabolism of epithelial cells. This study indicated that, with the exception of the ACP tending ($P = .07$) to produce more LACT than the CPS, cells from different sites within the rumen did not differ in production of BOHB, ACAC, LACT, or PYR. Hence, it appears that cell origin within the rumen is not of significant concern in the experimental design of rumen epithelial research.

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Literature Cited

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APPENDICES

Appendix 1.

ß-Hydroxybutyrate Assay for Rumen Cells

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 (340nm). ß-hydroxybutyrate concentration is indirectly determined from the concentration of NAD^+ following the chemical reaction:

$$HOOC\text{-}CH_2\text{-}CHOH\text{-}CH_3 + NAD^+ \text{----} > COOH\text{-}CH_2\text{-}CO\text{-}CH_3 + NADH + H^+$$

ß-hydroxybutyrate acetoacetate

ß-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 allows for the calculation of ß-hydroxybutyrate concentration.

Reagents

1) .1 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

4) ß-Hydroxybutyrate Dehydrogenase (5.0 mg/ml)

(Grade II) (Boehringer Mannheim, cat. # 127841)

5) 2.0 nM ß-Hydroxybutyric Acid (Sodium Salt)

(98% Pure) (Sigma Chemical Co., cat. # H-6501)

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

Standards

Dilute to the following concentrations for the working standards: 2.0, 1.6, 1.2, .8, and .4 mM.

Standard dilutions:

A) 2.0 ----> 20.0 mL of 2.0 mM BOHB plus .0 mL dH_2O

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

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

D) .8 ----> 8.0 mL of 2.0 mM BOHB plus 12.0 mL dH_2O

E) .4 ----> 4.0 mL of 2.0 mM BOHB plus 16.0 mL dH_2O

Use two zero concentration standards as blanks.

The standards and blanks must be taken through the same procedure as the cell supenatant because small amounts of perchlorate will affect enzyme activity. Pipette into conical centrifuge tubes:

6.0 mL standard (or blank) + .4 mL perchlorate

Place tubes on ice for ten minutes, then buffer with .8 mL potassium carbonate. Centrifuge at 1500 X g for 15 minutes.

Analysis

Pipette into 12 x 75 mm culture tubes:

0.5 mL buffer

1.0 mL sample or standard

50 µL NAD^+ solution

Mix using a Vortex mixer and read E_1 at 340 nm.

Add 10 µL ß-Hydroxybutyrate Dehydrogenase, mix using a vortex, and incubate at room temperature for approximately 45 minutes. Read E_2 at 340 nm.

Important

NAD^+ and hydrazine form a complex which absorbs at 340 nm. Therefore, a slow constant increase in absorbance occurs. This is similar to the acetoacetate assay but in reverse. So pipette the NAD^+ and enzyme and take the absorbance readings at timed intervals so that all changes are relative.

Calculations

Calculate E_2 minus E_1 for all standards, samples, and blanks. Plot a standard curve of delta E of standard versus the "known concentration. Read the concentration of the samples from the curve.

Appendix 2.

Acetoacetate Assay for Rumen Cells

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 (340 nm). Acetoacetate concentration is indirectly determined from the concentration of NAD following the chemical reaction:

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

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

Reagents

1) .1 M Phosphate Buffer (pH 6.8)

- 1.36 g KH_2PO_4 ----> 100 mL dH_2O

- 1.74 g K_2HPO_4 ----> 100 mL dH_2O

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

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

0.010 g NADH ----> 6.0 mL dH_2O

Make up on day of use.

3) ß-hydroxybutyrate Dehydrogenase (5.0 mg/mL) (Grade II)(Boehringer Mannheim, cat. # 127841)

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

0.01620 g CH_3COCH_2COOH ----> 100 mL dH_2O

Standards

Dilute to the following concentrations for the working standards: .15, .09, .075, .045, and .0225 mM.

Standard dilutions:

A) .15 ----> 2.0 mL of 1.5 mM ACAC plus 18.0 mL dH_2O

B) .08 ----> 12.0 mL of A plus 8.0 mL dH_2O

C) .075 ----> 4.0 mL of A plus 4.0 mL dH_2O

D) .045 ----> 10.0 mL of B plus 10.0 mL dH_2O

E) .025 ----> 10.0 mL of D plus 10.0 mL dH_2O

Use two zero concentrations standards as blanks.

The standards and blanks must be taken through the same procedure as the cell supernatant because small amounts of perchlorate will affect enzyme activity. Pipette into conical centrifuge tubes:

6.0 mL standard (or blank) + .4 mL perchlorate

Place tubes on ice for ten minutes, then buffer with .8 mL potassium carbonate. Centrifuge at 1500 X g for 15 minutes.

Analysis

Pipette into 12 x 75 mm culture tubes:

0.5 mL buffer

1.0 mL sample or standard

50 µL NADH solution

Mix using a Vortex mixer and read E_1 at 340 nm.

Add 10 µL ß-Hydroxybutyrate Dehydrogenanse, mix using a Vortex, and incubate at room temperature for approximately 22 minutes. Read E_2 at 340 nm.

Important

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

Calculations

Calculate E_1 minus E_2 for all standards, samples and blanks. Plot a standard curve of delta E of standard versus the "known" concentration. Read the concentration of the samples from the curve.

Appendix 3.

Lactate Assay for Rumen Cells

This procedure is based on the Sigma Lactate Kit #826-UV. The standard calibrations and the procedures have been modified for the quantification of lactate produced by isolated rumen epithelial cells.

Principle

This procedure is based on the conversion of lactate to pyruvate by lactate dehydrogenase in the presence of NAD. When excess NAD is present, all lactate will be converted to pyruvate. Measurement of NADH before and after the addition of lactate dehydrogenase will show the amount of lactate originally present because the absorbance of NAD (280 nm) differs from that of its reduced analog NADH (absorbance 340 nm).

Reagents

The following reagents can be purchased from Sigma Chemical Co.:

Lactate Dehydrogenase, Catalog No. 826-6.

NAD, Catalog No. 260-110.

Lactic Acid, Catalog No. 826-10.

-Concentration 4.44mmol/liter

Glycine Buffer, Catalog No. 826-3. Contains glycine (.6 mol/liter), and hydrazine (.5 mol/liter). pH = 9.2 Store at 2 - 6 degrees C. Discard if turbidity is present.

Glycine buffer may be made by adding 23 g glycine to 20 mL hydrazine hydrate (54% hydrazine, Sigma #H9507) and bringing up to 500 mL with water. The pH of the solution should be approximately 9.

Perchloric Acid 70%

Potassium Carbonate, saturated

KRB-Hepes Buffer Solution (obtain from cell isolation procedure)

Premix Solution (obtain from cell isolation procedure)

Other Materials Required

Spectrophotometer capable of measuring absorbance at 340 nm.

Pipeting Devices.

Erlenmeyer Flasks (3), 25 mL.

Centrifuge.

Centrifuge Tubes.

Test Tubes, 12 x 75.

Vortex Mixer.

Preparation of Standards

The following standards will be used to generate a standard curve and regression equation from which measured absorbances can be assigned appropriate lactate concentrations.

Prepare an "initial standard" by combining the following into a small beaker:

2 mL lactic acid standard solution

2 mL KRB-Hepes buffer solution

Mix well, then add the following to each of three 25 mL erlenmeyer flasks:

1 mL "initial standard" (from above)

5 mL rumen cell premix solution

.4 mL perchloric acid (70%)

Chill the flasks on ice for ten minutes, then add .8 mL of saturated potassium carbonate solution. Chill the flasks on ice for ten more minutes, then pour the contents of flasks into centrifuge tubes. Spin the tubes at high speed for 30 minutes. Combine the supernatents to yield the lactate "working standard" of concentration .308 umol/ml. Mix well. Next, prepare the calibrated standards for the standard curve by adding the following to five labelled test tubes:

Test Tube #	Working Standard	Water (ddH2O)	Calibrated Standard Conc. (umol/ml)
1	1.0 mL	0 mL	.308
2	.75 mL	.25 mL	.231
3	.50 mL	.50 mL	.154
4	.25 mL	.75 mL	.077
5	0 mL	1.0 mL(buffered water)	.00

Mix each tube with a Vortex. Cover and refrigerate these calibrated standards until their addition to the microtiter plates.

Assay Procedure

Add 10 mg NAD to a tared vial. (One vial will be sufficient to measure 30 samples or standards.) To each NAD vial needed, add 2.0 mL glycine buffer and 4.0 mL water. Cap and invert the vials several times to dissolve NAD. Combine contents of vials if more than one is to be used. Solution is stable for four hours at room temperature, or 24 hours at 2 - 6 degrees C.

Pipet 200 ul of NAD/glycine solution into each well of a 96-well microtiter plate. Add 20 ul sample or standard. Mix gently.

Read absorbance using KinetiCalc software (see below) at 340 nm. This is E_1.

Add 5 ul of LDH to each well. Incubate plates for 15 minutes at 37 degrees C (or 30 min at 25 C).

Read absorbance using KinetiCalc software at 340 nm. This is E_2.

Appendix 4.

Pyruvate Assay for Rumen Cells

This assay is based on the Sigma Pyruvate Kit #726. The standard calibrations and the procedures have been modified for the quantification of pyruvate produced by isolated rumen epithelial cells.

Principle

This procedure is based on the conversion of pyruvate to lactate by lactate dehydrogenase in the presence of NADH. When excess NADH is present, all pyruvate will be converted to lactate. Measurement of NADH before and after the addition of lactate dehydrogenase will show the amount of pyruvate originally present because the absorbance of NADH (340 nm) differs from that of its oxidized analog NAD (absorbance 280 nm).

Reagents

The following reagents can be purchased individually through Sigma Diagnostics or can be purchased in the kit form as Sigma Kit #726.

Trizma Base Solution, Catalog No. 726-4

NADH, Catalog No.340-101

Pyruvic Acid Standard Solution, Catalog No. 726-10

-Pyruvic acid, 4 mg/dl (0.45 mmol/L)

Lactate Dehydrogenase, Catalog No. 826-6

Perchloric Acid, 70% - NOT PROVIDED IN KIT

Potassium Carbonate, saturated - NOT PROVIDED IN KIT

Premix Solution - NOT PROVIDED IN KIT (obtain from cell isolation procedure)

Materials Required But NOT Provided

Spectrophotometer capable of measuring absorbance at 340 nm.

Pipeting devices.

Erlenmeyer Flasks(3), 25 mL.

Centrifuge.

Centrifuge Tubes.

Test tubes, 12 x 75.

Vortex Mixer.

Preparation of Standards

The following standards will be used to generate a standard curve and regression equation from which measured

absorbances can be assigned appropriate pyruvate concentrations.

Prepare a "working standard" by adding the following to each of three 25 mL erlenmeyer flasks:

1 mL pyruvic acid standard solution

5 mL rumen cell "premix" solution

.4 mL perchloric acid (70 %)

Chill the flasks on ice for ten minutes, then add .8 mL of saturated potassium carbonate. Chill flasks on ice for ten more minutes, then pour contents of flasks into centrifuge tubes. Spin the tubes at high speed for 30 minutes. Combine the supernatents to yield the pyruvate "working standard" of concentration .063131 umol/ml.

Pipet 4.3 mL of Trizma Base Solution into each of two NADH vials. Invert the vials several times to dissolve the NADH. Combine the solutions into 1 vial and mix well. Prepare the calibrated standards by adding the following to five labelled test tubes:

Test Tube #	Trizma/ NADH Solution	Water (ddH2O)	Working Standard	Calibrated Standard Conc.(umol/ml)
1	1.0 mL	0 mL	2.0 mL	.042087
2	1.0 mL	.5 mL	1.5 mL	.031566
3	1.0 mL	1.0 mL	1.0 mL	.021044
4	1.0 mL	1.5 mL	.5 mL	.010522
5	1.0 mL	2.0 mL (buffered H2O for tube 5)	0 mL	.00

Mix the tubes with a Vortex, and wait two minutes. Read and record the absorbance of each of these standards at 340 nm. This is the E_1 reading. Then add 10 ul lactate dehydrogenase, and mix with a Vortex. Allow the standards to sit for five minutes, then read and record the absorbance of the standards again. This is the E_2 reading. $E_1 - E_2$ = the change in absorbance for each standard calibration.

Procedure for Measurement of Samples

1) Pipet 2.2 mL Trizma Base Solution into each NADH vial to be used. (Each vial will be sufficient for 8 samples).

Combine the solutions from each vial into a beaker and mix well.

2) Pipet the following into 12 x 75 test tubes:

- 1.0 mL sample
- .25 mL Trizma Base Solution

MIX using Vortex. -(Mixing is essential. Solution must be brought to proper pH by thorough mixing before adding NADH.)

- .25 mL NADH solution from Step 1. Mix using Vortex.

3) Read and record absorbance at 340 nm using buffered water as the zero reference. This measurement is E1.

4) Quickly add 10 ul lactate dehydrogenase to each tube. Mix using a Vortex.

5) After 2 - 5 minutes, again read and record absorbance. This measurement is E_2.

Appendix 5.

Ruminal Fluid VFA Analysis

1) Centrifuge raw ruminal fluid at 15000 X g for 15 minutes in 15 mL polypropylene tubes.

2) Place two 3 mL aliquots from the supernatant into 5 mL polypropylene tubes. Stopper tubes and store in freezer.

3) Thaw above tubes when ready to complete the analysis.

4) Place 2.5 mL from each thawed tube into separate 15 mL polypropylene centrifuge tubes.

5) Add .5 mL 25% H_3PO_4 solution (with 30 µM/mL 2-ethylbutyric acid as internal standard) to each centrifuge tube.

6) Allow acidified sample to stand 30 minutes on ice.

7) Centrifuge at 15000 X g for 15 minutes.

8) Place 1 mL into the vial for gas chromatograph analysis.

VITA

Matthew Reid Waldron was born in Batavia, New York on April 10, 1971. He grew up in Oakfield, NY and received his high school diploma from Oakfield-Alabama Central High School in June, 1989. In August of that year he entered SUNY New Paltz from which he transferred to Cornell University in August 1991. He received a Bachelor of Science, in Animal Science, from Cornell in May, 1993. He then entered The University of Tennessee and received his Master of Science degree in Animal Science in August, 1996.