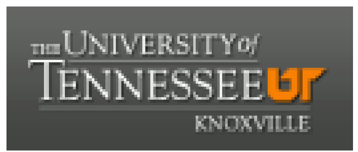

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To the Graduate Council:

I am submitting herewith a thesis written by Robert Clements Gillis entitled "Evaluation of primary rumen epithelial cell culture techniques in sheep." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Richard N. Heitmann, Major Professor

We have read this thesis and recommend its acceptance:

Michael Smith, John Waller

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

To the Graduate Council:

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We have read this thesis and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of the Graduate School

EVALUATION OF PRIMARY RUMEN EPITHELIAL CELL CULTURE TECHNIQUES IN SHEEP

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

Robert Clements Gillis
May 2000

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Abstract

Objectives of this study were to determine if the number of cells incubated in primary rumen epithelial cell cultures affects production rates of metabolites and to standardize reporting criteria by obtaining an optimum mode of data expression. Epithelial tissue was excised from five Suffolk x Dorset crossbred sheep and subjected to serial tryptic digestion to isolate cells. Isolated cells were incubated for 90-minutes in 25 m*M* propionate and 10 m*M* butyrate at concentrations of 0.5, 1, 5, 10, 20 and 40-million cells per flask (total vol. = 3mL). Production of acetoacetate (AcAc), β-hydroxybutyrate (β-HBA), lactate (LAC) and pyruvate (PYR) were measured. Data were expressed as either cell number, cell dry matter or cell total protein alone or per epithelial wet tissue weight, body weight (BW) or metabolic BW to generate twelve different forms of data expression. Coefficients of variation were calculated for all 12 modes of expression. Expressing data per cell number resulted in the lowest variation ($P < .01$) and data adjusted for metabolic BW had less variation than BW. Acetoacetate concentrations were largest at 0.5-million cells/flask ($P < .05$) and there were no differences between 1, 5, 10 and 20 and only 40 differed from 0.5 and 5-million cells/flask. β-HBA concentrations were largest at 1 and 5-million cells/flask. However, 1 and 5 only differed significantly ($P < .05$) from 20 and 40-million cells/flask. Lactate and PYR concentrations were largest at 1-million cells/flask, but no significant differences were found. Ratios of β-HBA:AcAc were below one for the 0.5-million cells/flask indicating low mitochondrial redox potentials ($P < .05$). A suggested range of rumen epithelial cells to include in incubations is 5 to 20-million/flask. This range will minimize the potential for altered metabolite production caused by incubating large cell quantities as well as the

experimental error associated with using low cell numbers. When rumen tissue is taken from animals of the same species, size and stage of development, data adjusted by cell number is preferred. However, it is recommended that metabolic BW, cell protein and cell dry matter be included to facilitate future comparison between laboratories and species.

Table of Contents

Section *Page*

1. Review of Literature 1
    - Ruminant Foregut Anatomy 1
    - Volatile Fatty Acid Metabolism 2
    - Techniques Used to Study Rumen Metabolism 3
    - *In Vivo* Study of Rumen Epithelium Metabolism 5
    - *In Vitro* Study of Rumen Epithelium Metabolism 10
2. Materials and Methods 19
    - Materials 19
    - Animals 19
    - Cell Isolation 19
    - Cell Incubations 22
    - Analyses 23
3. Results and Discussion 25
4. Conclusions 40

Literature Cited 41

Appendices 48
- Appendix 1: Beta-hydroxybutyrate Assay for Microtiter Plates 49
- Appendix 2: Acetoacetate Assay for Microtiter Plates 53
- Appendix 3: Lactate Assay for Microtiter Plates 56
- Appendix 4: Pyruvate Assay for Microtiter Plates 59
- Appendix 5: List of Abbreviations 62

Vita 64

List of Tables

Table *Page*

1. Krb-Hepes buffer, Premix and 5X Krb solutions.................................. 20

2. Characteristics measured for individual sheep.................................... 26

3. Cell dry matter and total protein for various cell dilutions........................ 27

4. Metabolite production over 90-minute incubation period......................... 28

5. Acetoacetate (ACAC) and β-hydroxybutyrate (β-HBA) concentrations following 90-minute incubation with rumen epithelial cells at various cellular concentrations... 30

6. Lactate (LAC) and pyruvate (PYR) concentrations following 90-minute incubation with rumen epithelial cells at various cellular concentrations...... 33

7. Average coefficients of variation (CV) for each metabolite at each dilution.. 34

8. Comparison of coefficients of variation (CV) between million cells, mg cell dry matter and cell total protein modes of expression......................... 36

9. Comparison of coefficients of variation (CV) between cellular expressions and their respective adjustments by wet tissue weight (g), body weight (kg) and metabolic body weight ($BW^{.75}$).. 37

1. Review of Literature

Ruminant Foregut Anatomy

A foregut fermenting animal has a nonsecretory fore stomach and a secretory hind stomach. In "true" ruminant foregut fermentors, the fore stomach consists of three compartments, the rumen, reticulum and omasum. The hind stomach consists of the abomasum (Leek, 1993). The main site of fermentation occurs in the rumen. The rumen consists of a dorsal and ventral sac, which are partially separated by the cranial and caudal pillars and the right and left longitudinal pillars (Leek, 1993). The rumen mucosal surface is covered with papillae (Hofmann, 1988). Which are unevenly distributed throughout the rumen. The size and number of papillae as well as their distribution is affected by diet (Hofmann, 1988). The change in papillae, as a result of the diet, is most prevalent on the mucosa of the dorsal wall of the dorsal sac (Hofmann, 1988). An increase in butyric and propionic acids stimulates the growth of papillae while acetic acid, which is associated with a diet of fibrous forage, causes the papillae to regress (Hofmann, 1988). Papillae are most abundant in the cranial sac and least abundant on the ruminal pillars and the dorsal rumen wall (Hofmann, 1988).

The rumen epithelium consists of four cell layers: *stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum* (Steven and Marshall, 1970). The cells of the *stratum basale* are columnar in shape with many mitochondria and ovoid nuclei (Steven and Marshall, 1970). These cells lie adjacent to the basal lamina. The *stratum spinosum* cells are oval in shape with few mitochondria and irregularly shaped nuclei (Steven and Marshall, 1970). The *stratum granulosum* cells are situated parallel to the luminal surface and have pyknotic nuclei. There are also many more fibrils in the

stratum granulosum than in previous cell layers (Steven and Marshall, 1970). The *stratum corneum* cells are in direct contact with the lumen of the rumen. There are few nuclei present and the cells consist of a granular material (Steven and Marshall, 1970). These cells are keratinized and are most likely used as a defense barrier against the harsh conditions of the rumen (Gálfi et al., 1983; Baldwin, 1998). Leighton et. al., 1983, reported that the process of ketogenesis in the rumen epithelium is performed only in the mitochondria. Therefore, *stratum basale* and *stratum spinosum* cell layers contribute most to the metabolic properties of the tissue, because they have the greatest numbers mitochondria (de Lahunta, 1965; Baldwin, 1998).

Volatile Fatty Acid Metabolism

The major metabolites produced by bacterial fermentation in the rumen are volatile fatty acids (VFA), specifically acetic, propionic and butyric acids (Pennington, 1952). Volatile fatty acids represent a crucially important source of energy for the ruminant animal (Annison and Armstrong, 1970). Volatile fatty acids are absorbed through the epithelial lining of the rumen, which acts as a barrier to nutrient diffusion and maintains metabolite concentration gradients (Annison and Armstrong, 1970; Baldwin, 1998).

Acetate is not used by the liver, but is either directly oxidized by the tricarboxylic acid cycle as acetyl-CoA in the muscles or forms ketone bodies in the rumen epithelium (Bergman, 1993). Acetate is converted to acetyl-CoA in the cytoplasm via acetyl-CoA synthetase. Acetyl-CoA synthetase is widely distributed throughout most non-hepatic animal tissues (Annison and Armstrong, 1970).

The rumen epithelium or liver removes all of the absorbed butyrate before it reaches the general blood circulation (Bergman, 1993). The rumen epithelial cells metabolize butyrate to acetoacetate (AcAc) and β-hydroxybutyrate (β-HBA) (Annison and Armstrong, 1970; Bush and Milligan, 1971) so called ketone bodies. Acetoacetate is converted and reversible to β-HBA by β-hydroxybutyrate dehydrogenase (Koundakjian and Snoswell, 1970; Emmanuel et al., 1982; Emmanuel, 1980). Acetoacetate is the parent ketone body formed first during synthesis by β-hydroxy-β-methylglutaryl-CoA lyase (HMG-CoA lyase), but it must be reduced to β-HBA for transport in the blood since AcAc is unstable (Bergman, 1993). Non-hepatic tissues utilize the ketone bodies converting them to acetyl-CoA which enter the TCA cycle for oxidation to CO_2 (Bergman, 1993).

Propionate is the only one of the three VFA's that is glucogenic and is the most important precursor of glucose (Annison and Armstrong, 1970). Propionate may be directly absorbed into portal blood, removed by the liver and be converted to glucose. Propionate also may be converted to lactate and pyruvate in the rumen epithelium which, in turn, go through hepatic gluconeogenesis (Annison and Armstrong, 1970). As much as 70 percent of glucose formation can come from propionate if the animal is under heavy grain feeding and as little as none if the animal is being starved (Bergman, 1993).

Techniques Used to Study Rumen Metabolism

There are basically two types of experiments that can be conducted to study the metabolism of the rumen epithelium. They are *in vivo* which means in a living cell or organism and *in vitro* which is an experimental situation outside the organism and

literally means "in glass" (Griffiths et al., 1993). Each type of experiment has its own advantages and limitations.

The limitations of the *in vivo* method are the difficulty in measuring the tissue content of the substance and its metabolite. Measuring the amount of a substance or its metabolites transported in the blood requires knowledge of blood flow and control of the transepithelial electrochemical potential difference (Stevens, 1970). The main advantage of the *in vivo*'s method is that one knows the physiological conditions within the body should be close to the physiological conditions of an average animal on an average day.

The major limitation of *in vitro* experiments is the ability to prove that the tissue is under normal physiological conditions and that it is viable (Stevens, 1970). Another limitation is the possibility of eliminating by purification certain components that may be essential to the biological function of the tissue (Lehninger et al., 1993). The main advantages of *in vitro* techniques are that one can have independent control over most parameters and one can achieve complete recovery of metabolites (Stevens, 1970).

Because *in vivo* and *in vitro* experiments each have roughly the opposite sets of advantages and limitations, it would be ideal to conduct at least one experiment of each type to get a true idea of the physiology of a specific tissue. *In vivo* studies can determine the range and magnitude of a tissue's absorptive function while *in vitro* studies can conclude the mechanisms of the absorption (Stevens, 1970). The *in vivo* methods used to study the metabolism of the rumen epithelium include temporary isolated rumen, miniature rumen, venoarterial (V-A) concentration difference and V-A difference in conjunction with isotope dilution. *In vitro* methods include sheets of isolated rumen

epithelium, Ussing chamber, perfused rumen, isolated rumen papillae and isolated rumen epithelial cells.

In Vivo Study of Rumen Epithelium Metabolism

The temporary isolated rumen technique allows for the isolation of the rumen in the live animal. It is similar to the *in vitro* technique of the perfused rumen. For this technique, a fistulated animal is used. Typically the rumen contents are removed and kept in a 39 °C water bath. The rumen is then washed with a buffer solution until clean. A saliva collector developed by Hydén (1958) is inserted into the esophagus. The saliva collector continuously recycles the saliva by bypassing the rumen to the omasum via a catheter. The rumen outflow is prevented by placing a piece of foam rubber into a balloon which, in turn, is placed into the reticulo-omasal orifice (Martens, 1983; Gaebel et al., 1987; Dahlborn and Holtenius, 1989). From this point, a buffer of known volume and concentration with the appropriate VFA is placed into the isolated rumen. This is gassed with CO_2 in order to keep the buffer mixed. Samples are taken at zero time and thereafter and analyzed for VFA concentration. A water soluble, non-absorbable marker is placed in the buffer at the beginning of the experiment. The recovery of this marker at the end of the experiment estimates leakage during the experiment (Martens, 1983).

This technique has several disadvantages. First, there has to be someone with surgical expertise to implant the fistula. As far as the technique itself is concerned, there a several possible sources of error. A sheep can produce between 0.5 and 1.34 liters of saliva per hour (6 to 16 $L \cdot day^{-1}$ in sheep and 60 to 160 $L \cdot day^{-1}$ in cattle) and the removal of this saliva before it reaches the rumen can have adverse affect on the VFA's since saliva is an important mechanism for neutralizing roughly half the VFA's in the

forestomach (Leek, 1993). Because of unneutralized VFA, the pH may fall and it is possible that a low pH will affect the metabolism of the epithelial cells, thereby reducing the active transport from the rumen to the blood (Gaebel et al., 1987). Dahlborn and Holtenius (1989) reported that isolation of the rumen caused an increase in plasma renin activity as well as the release of aldosterone and cortisol. More importantly, they reported high levels of vasopressin which were maintained throughout the experiment. These high levels of vasopressin could adversely affect the absorption of VFA due to the change in the physiological blood flow. Other potential problems are the accuracy of the fluid volume measurements due to possible leakage of saliva into the rumen or possible leakage of buffer into the omasum (Martens, 1983). Dahlborn and Holtenius (1989) concluded that the technique of the isolated rumen affected the fluid homeostasis, but how this affected absorption, they do not know.

A technique similar to the isolated rumen is the rumen pouch or miniature rumen. This technique was developed by Tsuda (1956). It is based on the classical Pavlov (1910) experiment where he formed a pouch out of a dog's stomach to demonstrate gastric secretion. An animal is anaesthetized and an incision is made transversely on the skin inferior to the last rib to access the rumen. A surgical procedure developed by Pavlov (1910) is utilized to create the pouch. The pouch is created from the dorsal sac of the rumen. It takes an average of three hours of surgery to create the pouch. The conical pouch is completely separate from the rest of the rumen (except for the blood supply and the nerve) and has a volume of 20-40 cc. The height and depth of the pouch are roughly 5 cm and 8 cm respectively. To run an experiment, an animal must first heal for two weeks. If the animal heals properly, one inserts a catheter into the bottom of the pouch or

miniature rumen. A syringe is connected to the catheter, and a solution with known concentrations is injected into the miniature rumen. At one hour intervals, samples are taken from the miniature rumen to determine the change in concentration (Tsuda, 1956a and 1956b).

The main advantage to the rumen pouch is that it allows for controlled studies in conscious animals (Stevens, 1970). There are, however, several sources of error. First, the miniature rumen is created from the dorsal sac where the papillae are shorter and fewer in number than the remainder of the rumen. Tsuda (1956a) concludes that this could result in less active absorption. In contrast, Waldron (1996) showed that metabolism was the same in the dorsal sac as in the rest of the rumen and that the origin of the cells from the rumen should not be a concern when performing rumen epithelial research. It was reported by Tsuda (1956a) that a few weeks post operation the epithelium in the rumen pouch had begun to be replaced by new epithelium. The new epithelium had a decreased height of papillae, thickening of the epithelium and muscle layer as well as a deepening of the notches in the secondary papillae. Tsuda (1956b) also reported that a fistula would sometimes spontaneously form between the miniature rumen and the rumen proper. In such cases, attempts to close the fistula by surgery were unsuccessful.

The technique of venoarterial concentration difference (V-A difference) is measured by the implantation of catheters into the arterial input and venous output of an organ or tissue (Heitmann, 1989). Net flux of a metabolite can be calculated when one multiplies the V-A difference with the blood flow (Huntington and Reynolds, 1987). The resulting net flux is a measurement of the net rates of production or utilization of a

metabolite in a tissue or organ (Heitmann, 1989). A negative flux indicates an uptake of the metabolite by the tissue and a positive flux indicates the tissue is releasing the metabolite (Heitmann, 1989).

An extraction ratio can also be calculated. It is the ratio of the metabolite presented to the tissue to the amount of the metabolite utilized by the tissue (Heitmann, 1989). If one measures V-A difference over the liver, one must take special care because the liver has two sources of blood supply. Katz and Bergman (1969a) developed a way to take these two sources into account, as well as distinguish between the portal metabolism and liver metabolism. It should be noted that simultaneous measurements of the total hepatic and portal blood flow are needed to distinguish between the portal and liver metabolism (Katz and Bergman, 1969b). They utilized chronic indwelling multiple catheters: one in the portal vein, one in the hepatic artery and one in the hepatic vein.

The main advantage to the V-A difference technique is that it can be performed on conscious animals that are under near normal conditions (Huntington and Reynolds, 1987). The main disadvantage to V-A difference is that one needs to be trained surgically in order to properly catheterize the animals. There are several possible sources of error associated with this technique. First, metabolites produced *de novo* in the organ could be utilized locally by the organ, thereby never making it into the blood stream and never being measured (Bergman, 1975). It was also reported by Bergman (1975) that simple V-A difference along with blood flow rates can seriously underestimate absorption. Samples must also be withdrawn slowly, so as not to disturb the laminar blood flow (Bergman, 1975). The sampling of multicathetered animals can also produce

a large error if the samples from each site are not removed simultaneously (Heitmann, 1989).

Veno-arterial difference combined with isotope dilution measurements refine single net flux rates of metabolites. Isotope dilution is used to measure the turnover rates in the whole body as well as irreversible utilization, *de novo* production, conversion and interconversion (Bergman, 1975; Heitmann, 1987). Radioactive labeled metabolites are used, typically ^{14}C-labeled. From this, one can distinguish between the specific organ utilization and the net rate of production and utilization which could be occurring simultaneously over a specific organ (Katz et al., 1970; Bergman, 1975). The idea behind the calculations is that the labeled metabolite is infused into the arterial or inflowing blood and any newly formed compound will be unlabeled if it is produced de novo in the organ (Bergman, 1975). This also helps eliminate the error associated with interconversion. Reducing the error associated with interconversion allows measurement of the amount of a metabolite being converted to another metabolite (Heitmann, 1987).

A key disadvantages of V-A difference coupled with isotope dilution, with regards to turnover rates, is that they can only be made on a whole animal basis and not on specific organs (Katz and Bergman, 1969b). Isotope dilution also has several possible sources of error, such as the method measures gross production rates (of VFA) which may exceed the net production rates (Warner, 1964). Rates can also be underestimated when looking at rates of production and utilization of individual tissues due to the reconversions of labeled breakdown products back into the original compound (Bergman, 1975). Other sources of error are associated with the measurement of blood flow (this error is also valid with V-A difference alone). Many researchers measure blood flow rate

one time and assume it will remain constant, or at least show only minimal variation (Warner, 1964). Blood flow rates can also be mismeasured due to inadequate mixing of the dye (para-amino hippuric acid (PAH) or sulfobromophthalein (BSP)) in the blood. The dye is used to measure the rate of blood flow (Katz and Bergman, 1969c). Rates of blood flow can also be miscalculated due to incorrect cannulation of the veins. If the cannula is introduced too far into the vein some postcaval blood could be collected, thereby increasing the concentrations of the dyes (Katz and Bergman, 1969c). Overall, the main limitations to V-A difference coupled with isotope dilution are the researchers time, money and energy (Heitmann, 1987).

In Vitro Study of Rumen Epithelial Metabolism

Hird and Weidemann (1964) conducted an experiment that utilized sheets of isolated rumen epithelium that had been mounted as diaphragms to study the transport and metabolism of butyrate. The apparatus used consisted of two thick cylindrically hollowed Perspex plates. The hollowed compartments had capacities of 25 mL and 35 mL respectively. The holes on the top of the plates allowed solutions to be added or withdrawn from the compartments. The tissue was mounted between the two plates. After the tissue was mounted, a Krebs-Ringer phosphate buffer (pH 7.4) solution was simultaneously added to each of the chambers (20 mL to one and 30 mL to the other). The substrate (VFA) was then added to the 30-mL side of the apparatus. The apparatus was then placed in a water bath up to the level of the fluid surrounding the tissue and was held at a constant temperature of 38 °C. An initial sample was taken at five minutes and subsequent samples of two mL were taken every 15 minutes for two hours (Hird and Weidemann, 1964). Since this system does not have a blood supply, ketone bodies

accumulate in the papillae and have a probability of leaking into the medium before diffusing towards the muscle side. Therefore, ketone data obtained by this technique would differ from that of data found by *in vivo* techniques. Another potential problem is that this technique cannot control the transepithelial electrical potential difference which may affect the driving force and transport of the VFA (Stevens, 1970; Rechkemmer et al., 1995).

The Ussing technique, which was developed by Ussing and Zerahn (1951), permits the measurement of the transepithelial potential difference and the elimination of the transepithelial electrical potential by a mechanism of short-circuiting this potential. This allows for control of the differences between opposing bathing solutions as well as a way to determine viability by controlling the tissue current and resistance (Stevens, 1970). This was demonstrated in a classical experiment using frog skin epithelium (Ussing and Zerahn, 1951). Frog skin epithelium, like rumen epithelium, has an outer keratinized cell layer with a *stratum spinosum* and *stratum basale* layers underneath (Ussing and Windhager, 1964). Because of these similarities, the rumen epithelium works well in the Ussing chamber. For this procedure, the rumen epithelium was placed between two chambers containing Ringer's solution. The electrical potential was measured using agar-Ringer bridges connecting the two solutions to a potentiometer. Another pair of bridges, connected to a battery, was used to short-circuit the tissue potential. The current used to short-circuit the tissue was equal to the current generated by the tissue. With identical solutions in the chambers, the ion transport can be studied since the electrochemical gradients are eliminated (Stevens, 1964). Using this procedure in conjunction with flux measurements and isotope dilutions allows one to detect whether

the ions are actively or passively transported across the tissue (Stevens, 1964; Rechkemmer et al., 1995). A source of error that may arise using the Ussing technique in conjunction with isotope dilutions is the interconversion of the VFA (Warner, 1964; Rechkemmer et al., 1995).

Other sources of error can arise in the use of the Ussing chamber. If the VFA are converted to ketone bodies, they would not be detected by the usual Ussing chamber technique (Rechkemmer et al., 1995). Also potentials are somewhat smaller than those observed *in vivo* (Keynes & Harrison, 1970). Finally lower flux rates are observed at high VFA concentrations. This may be a result of unstirred layer effects. Unstirred water layer effects may be the rate-limiting step of VFA transport at high concentrations (Rechkemmer et al., 1995).

The perfused rumen technique utilizes an intact rumen. This technique allows for estimates of VFA absorption with control over the rumen acid concentration, rumen pH and the rate of blood flow through the rumen vascular system (Brown et al., 1960). McCarthy et al. (1958) concluded that the perfused rumen technique superbly measured the absorption and production of VFA's from an animals rumen.

The animal is anesthetized and exsanguinated via a cannula inserted into the carotid artery. The blood is collected and treated with heparin to prevent clotting. Once the blood stops flowing, the researcher opens the abdominal cavity and ties ligatures around the esophagus and duodenum to isolate the stomach. The stomach is then removed from the body and placed in a 0.9% saline bath at 39 °C. The right ruminal artery and vein then receive glass cannulas. The cannula in the artery is connected via tygon tubing to a gravity bottle that is roughly five feet above the saline bath. A tygon

tube is also connected to the venous cannula and ran into a 500 mL graduated cylinder. The rumen vascular system is then flushed with approximately 500 mL of physiological saline. Samples of the saline perfusate are saved for VFA analysis. Following the rinse, the filtered heparinized blood (filtered through either a glass wool and cotton plug or through an isolated goat liver (Brown et al., 1960; McCarthy et al., 1958)) begins the perfusion. Arterial blood samples were taken at the beginning and at the end of the experiment and venous blood samples were taken at intervals during the perfusion from the graduated cylinder. Blood flow rates were measured as the volume of venous blood was collected over a specified period of time. Rumen fluid samples were taken through a tygon tube inserted in the rumen through the esophagus (Brown et al., 1960).

One of the largest sources of error is the inability to obtain a physiological blood flow rate. Rates of the perfused rumen were reported to be roughly 25 – 50% of the estimated *in vivo* rate (McCarthy et al., 1958). Brown et al. (1960) concluded that, "since the flow rates are unphysiological, one must conclude that the absorption rates are also abnormal." It has also been reported by McCarthy et al., (1958) that low butyric acid levels and few to no ketone bodies were detected. This most likely was due to the conversion of butyric acid to β-hydroxybutyrate by the rumen epithelium (Brown et al., 1960). One also has to be aware of the interconversion of the VFA's, which skew one's estimates (McCarthy et al., 1958). McCarthy et al. (1958) extrapolated their data out to 24 hours and found that their production results accounted for only 53% of the total energy needed by the animal in a 24 hour period. This clearly reflects the unphysiological environment created by this technique. It is also difficult to get a true estimate because one cannot emulate the rate of flow of saliva into the rumen and the rate

of flow of material out of the rumen (Warner, 1964). Stevens (1970) concludes that the perfused rumen technique has numerous sources of error. Preparation of the perfused rumen is very difficult and one cannot ensure the viability of the rumen epithelium. Because of this the results do not always agree with other techniques.

The technique of isolating rumen epithelial cells began with a study performed by Pennington (1952). In that study it was stated that rumen epithelium is an perfect tissue to be used to study metabolism of an animal in an *in vitro* type experiment due to its large surface area and being thin and tough. Pennington (1952) isolated and incubated whole papillae in order to measure the amount of metabolite produced. He reported that the rate of disappearance of butyrate in the epithelium was much greater than the rate of disappearance in the muscle. This indicated that the epithelium was the predominantly metabolically active tissue, not the underlying layer of muscle tissue. It was also reported finding mostly acetoacetate and very little β-hydroxybutyrate. This does not correspond with *in vivo* data. Typical *in vivo* data shows a ratio of β-HBA to AcAc of 2-4:1 (Heitmann, 1987). One of the probable causes for this discrepancy is that Pennington removed the tissue from the animal and immediately placed it into a partly frozen Krebs-Ringer solution. Baldwin and Jesse (1991) reported that transporting tissue in ice-cold buffers dramatically inhibits the production of β-HBA by the cells. Because of this discrepancy and ground breaking concept, other techniques evolved from Pennington's original work in 1952.

Weigand et al. (1975) also isolated rumen papillae. They set out to investigate the metabolic activity of the rumen mucosa on cattle fed either an all grain diet or an all hay diet. Weigand et al. (1975) reported an inverse β-HBA to AcAc ratio that ranged

between 1:1.8-2.5. Hodson et al. (1967) and Sutton et al. (1963) also reported an inverse ratio in that roughly one-third of the ketone bodies produced were β-HBA. The inverse ratios reported by Sutton et al. (1963) are probably due to the fact that they, like Pennington (1952), transported the tissue in ice-cold buffer. On the other hand, Weigand et al. (1975) collected tissue according to Cook et al. (1968) which transported the tissue in a 39° C buffer. The inverse ratios reported by Weigand et al. (1975) were most likely caused by the heterogeneity of the tissue being measured. The heterogeneity is compounded by the diet. The animals that were fed a high-concentrate diet exhibited hyperkeratosis and a thickened *stratum corneum* cell layer. Both the thickened cell layer and the hyperkeratosis lower the portion of metabolically active tissue per unit weight of papillae and thereby lower the VFA utilization per unit. Because of this, a need arose to eliminate the extra tissue components, i.e. the keratinized cell layers, and isolate the metabolically active cells. This allows for accurate measurement of the metabolic activity of the rumen mucosa and comparison of two different diets.

The first relatively successful rumen epithelial cell isolation was performed by Weekes in 1974. He treated the tissue with papain and reported achieving a relatively pure prep of epithelial cells obtained though centrifugation. He reported that the papain resulted in an advancing inhibition of oxygen uptake by the isolated cells. The lack of oxygen being consumed implies that the cells are not metabolizing to their normal degree. Weekes concluded that the papain inactivated some of the respiratory enzymes. Weekes also reported that the papain critically impaired the isolated cells' ability to form lactate and pyruvate from the added propionate in a 30-minute incubation. It was further reported that papain denatured all four enzymes being measured: lactate dehydrogenase,

NADP-malate dehydrogenase, glutamate dehydrogenase and β-hydroxybutyrate dehydrogenase. Lactate dehydrogenase is the enzyme least affected by the papain (Weekes, 1974). He also compounded the error by transporting the tissue in ice-cold buffer.

Gálfi et al. (1980) isolated and cultured the stratum basale and stratum spinosum layers of rumen epithelial tissue. Gálfi et al. (1980) isolated these cell layers via a serial trypsinisation procedure. They changed the trypsin solution every 30 minutes until non-epithelial cells appeared to be in the suspension. Gálfi et al. (1980) did, however, transport the tissue in ice-cold buffer. It also showed that sodium n-butyrate inhibits the growth of ruminal epithelial cell cultures *in vitro* (Gálfi et al., 1981 and 1983; Neogrády et al., 1989). The main limitation to Gálfi's experiments is that it took roughly eight hours to isolate the cells of the stratum basale and stratum spinosum cell layers. With this amount of time required for isolation, it is very difficult to obtain a physiologically accurate measurement of metabolism.

Inooka et al. (1984) also utilized a serial trypsinisation technique as did Gálfi et al., 1980, except Inooka was able to obtain isolated cells in approximately two hours. Inooka et al. (1984) were also able to culture two different types of cells: epithelial cells and fibroblast-like cells. Both epithelial cell cultures and fibroblast-like cell cultures were able to be carried out over 30 days and they concluded that colonies grew from the initial colonies (Inooka et al., 1984 and 1986).

The most recent rumen epithelial cell isolation was preformed by Baldwin and Jesse (1991). They also utilized a serial trypsinisation, as did Inooka et al. (1984). Most

importantly, Baldwin and Jesse (1991), demonstrated that transporting rumen tissue in cold buffer dramatically affects the tissue's ability to produce β-HBA. They isolated the stratum basale cell layer in approximately two hours. They reported that after the first 15-minute trypsin digest most of the stratum corneum layer had been removed and by the 3rd digest only stratum basale cell remained. They also conducted short-term (two-hour) incubation beyond the isolation period. The technique obtained cell viability between 90 and 95% (Baldwin and Jesse, 1991). The data attained from the experiments was representative of *in vivo* data previously reported. Baldwin and Jesse (1991) selected VFA concentrations for the experiment that were approximations of expected *in vivo* concentration found in the rumen lumen. They concluded, as did Weigand et al. (1975), that the presence of the keratinized cells in the intact rumen may cause a concentration gradient between the rumen liquor and intercellular fluid, which would be observed *in vivo* and would be unaccounted for *in vitro* (Baldwin and Jesse, 1991).

Presently, the technique developed by Baldwin and Jesse (1991) is the best technique for measuring the metabolism of rumen epithelium *in vitro*. However, one of the limitations of the procedure is that the animal must be sacrificed. Waldron (1996) was able to obtain physiological data by gathering the papillae of a steer through a fistula. By using fistulated animals, studies can be run on a single animal that extend over time and test the changing of diets on the rumen metabolism. Recently (Baldwin and McLeod, 2000), multiple animals in different experimental groups were used to study the various effects of diet. The rumen metabolism of an individual animal can also be measured through different physiological stages and growth.

The most important facet of the technique that is not yet known is whether the dissociation of the cell layers has an adverse effect on the metabolism of those cells. Do the outer cell layers help in controlling the VFA concentrations and production? Furthermore, the process of cell counts needs to be calibrated in order to get them in true ratios that would be found *in vivo*. Does the number of cells incubated in a flask affect the rates of production of the metabolites being examined and what would be the best mode of expression to accurately report the data obtained? Thus, the objectives of the present study are to determine the optimum cell concentration for incubations and the ideal mode of expression of data in order to standardize the reporting criteria for primary cell incubations, thus making it easier to compare data among laboratories and species. Once these concerns have been addressed the present technique of isolating rumen epithelial cells should be that much closer to identifying what is truly going on inside the animal.

2. Materials and Methods

Materials

Bovine Serum Albumin (BSA) #A-6003 and, sodium salts of butyrate and propionate used for incubation substrates, were from Sigma Chemical Company, St. Louis, Mo. Trypsin (1:250) and Hepes (N-2-Hydroxyethylpiperazine-N'-2-Ethane Sulfonic Acid) were from Gibco BRL (Life Technologies, Inc., Grand Island, NY). All other chemicals were reagent grade compounds either from Fisher Scientific (Fair Lawn, NJ), Mallinckrodt Chemical (Paris, KY), or Sigma Chemical.

Animals

Animals used in this study were one-year old Suffolk x Dorset crossbred sheep with an average weight of 74 kg (ranged between 50 and 91 kg). All ewes were fed the same rations: CO-OP lamb grower/finisher hi-energy-bov (Tennessee Farmers Cooperative, Lavergne, TN) prior to slaughter.

Cell Isolation

Sheep were brought to The University of Tennessee and slaughtered according to USDA specifications, stunning with a captive bolt followed by exsanguination. The abdomen was opened and the rumen was exposed. A piece of rumen tissue approximately 15 centimeters square was excised anterior and posterior to the cranial pillar. Size and location of removed tissue varied, depending on papillae density. Tissue was rinsed with warm tap water to remove any feed particles and was immediately placed into 37 °C Krb-Hepes buffer (Table 1) and transported to the laboratory. Muscle and connective tissue were peeled away from the epithelium and the epithelial sheet was weighed. Using a standard scalpel, the epithelial sheet was minced to approximately 1

Table 1. Krb-Hepes buffer, Premix and 5X Krb solutions

	Compound	Amount
Krb-Hepes buffer[a]	NaCl	14.4 (g)
	Hepes[c]	13.0 (g)
	KCl	0.706 (g)
	$MgSO_4$	0.5866 (g)
	KH_2PO_4	0.3239 (g)
Premix[b]	Deionized H_2O	63.5 (mL)
	5X Krb	60 (mL)
	1 *M* Hepes[c]	7.5 (mL)
5X Krb	0.77 *M* NaCl	50 (mL)
	Deionized H_2O	10.5 (mL)
	0.77 *M* KCl	2 (mL)
	0.77 *M* $NaHCO_3$	1.5 (mL)
	0.77 *M* $MgSO_4$	0.5 (mL)
	0.77 *M* KH_2PO_4	0.5 (mL)

[a]Compounds in solution with 2 L deionized H_2O and bubbled with O_2:CO_2 (95%:5%) for 20 minutes at pH = 7.4

[b]Solution was continuously bubbled with O_2:CO_2 (95%:5%) at pH = 7.4

[c]N-2-Hydroxyethlpiperazine-N'-2-Ethane Sulfonic Acid

cm^2 and was placed in a 5% trypsin solution (5g trypsin and 0.016g $CaCl_2$ in 100 mL DiH_2O) in a 500-mL Erlenmeyer flask. The flask was incubated in a 37 °C slow shaking orbital hot air incubator (New Brunswick Scientific Co., Inc., Edison, NJ, Model G24 Environmental Incubator Shaker) for 15 minutes. Flask contents were poured across a 1-mm nylon mesh (Spectra Mesh, Spectrum Laboratory Products, Los Angeles, CA) into another 500-mL Erlenmeyer flask. Tissue on the mesh was rinsed with 37 °C Krb-Hepes buffer to wash any adhering cells into the collection flask. The remaining tissue was placed back into the 500-mL digestion flask and was subjected to a second 15-minute incubation in a 5% trypsin solution. The second digest was poured across a 1-mm mesh into a collection flask. The resulting cell containing solution was poured across a 300-µm mesh (Spectra Mesh) into 50-mL centrifuge tubes. The 300-µm mesh was rinsed with 37 °C Krb-Hepes buffer to remove any adhering cells. The cells were centrifuged (Beckman Model TJ-6 Centrifuge, Beckman Instruments, Palo Alto, CA) at 70 x *g* (580 rpm) for eight minutes at 4 °C. The supernatant was decanted and cells were resuspended (washed) in two 50-mL centrifuge tubes with 37 °C Krb-Hepes buffer, which were spun at 70 x *g* for eight minutes at 4 °C. The supernatant was decanted and the cells were resuspended in one 50-mL centrifuge tube with 37 °C Krb-Hepes buffer and were spun following the previous procedure. Pelleted cells remained in Krb-Hepes buffer at room temperature. The two subsequent digestions (3 and 4) were in a 4% trypsin solution and were incubated for 15 minutes. The same recovery procedure (described for 2nd digestion) was used for all remaining digestions. All digestions thereafter were in 3% trypsin solution and incubated for 10 minutes. Digests five through eight were usually combined to form a cell pool. Digests were not included in the cell pool if the digestion

exhibited a dark ring of keratinized cells surrounding the pellet. Cell pool was formed by decanting the supernatant from the desired digests and combining the pellets in one 50mL centrifuge tube with approximately 30-40 mL of 37 °C Krb-Hepes buffer. Cell numbers and viability were determined using a hemacytometer and trypan blue dye exclusion technique (Sigma, 1998). Cells that did not absorb the dye after a 4-minute incubation were considered to be viable. The cell pool was separated into six 25-mL beakers with cell concentrations of 80, 40, 20, 10, 2 and 1 million cells/mL. A 500-million cell aliquot was taken from the fluid cell pool and was frozen for later analysis.

Cell Incubations

Incubations were carried out in 25-mL Erlenmeyer flasks. Incubation flasks were prepared by adding 1.3 mL of premix (Table 1), 0.3 mL of 100 m*M* butyrate, 0.3 mL 250 m*M* propionate, 0.6 mL 1.25 *M* bovine serum albumin and 0.5 mL of cells of various concentrations. Flasks were assigned a cell concentration (40, 20, 10, 5, 1 and 0.5 million cells/mL) and an incubation time (0, 30, 60 or 90 minutes). All six concentrations were incubated at each time interval in triplicate. Cell concentrations were suspended and 0.5 mL of the appropriate cell concentration was added to each incubation flask. The flasks were gassed for 20 seconds with a 95:5% O_2:CO_2 mixture. Flasks were sealed with a rubber serum cap and placed in slow shaking water bath at 37 °C (Yamato Shaking Water Bath Model BT-47, Yamato Scientific Co., LTD and Precision Reciprocal Shaking Water Bath Model 25, Precision Scientific Chicago, IL). At the end of an incubation cells were killed by adding 0.2 mL of concentrated perchloric acid to the flask. The flasks were placed on ice for 10 minutes. At the end of the 10 minutes, 0.4 mL of saturated potassium carbonate was added to neutralize the contents.

Flasks were left on ice for an additional 10 minutes and were decanted into 16x100mm culture tubes. Culture tubes were centrifuged at 1500 x *g* (2684 rpm) for 20 minutes. After the spin the supernatant was decanted into clean 16x100mm culture tubes.

Analyses

Supernatants were analyzed for acetoacetate (AcAc), β-hydroxybutyrate (β-HBA), lactate (LAC) and pyruvate (PYR) (Williamson and Mellanby, 1965; Mellanby and Williamson, 1965; Sigma procedure # 726-uv and procedure #826-uv respectively). All four assays were modified for use on a microtiter plate reader (Bio Kinetics Reader model EL340, Bio-Tek Instruments, Winooski, VT). Assay procedures are outlined in appendices 1-4. Zero time flasks were used to account for endogenous metabolite production prior to the incubation period. Dry mater (DM) and total protein (TP) measurements were taken from the 500 million cell aliquots. Dry matter was measured by freeze drying (Freeze Mobile model 24, The Virtis Company, Gardiner, NY). Total protein was determined from dried samples using the Kjeldahl method (Bradstreet, 1965). The metabolite concentration data were expressed as nmoles per million cells, mg cell DM or mg cell TP. Each of these three forms of expression were then compared further per wet tissue weight (g), body weight (kg) (BW) or metabolic BW ($kg^{.75}$). This generated twelve forms of data expression for metabolite concentration.

Statistical analysis was performed using incubation flasks as the experimental units and the number of cells added to the flask as the treatment. Data reported are means of five different cell preparations (animals) and triplicate incubations were conducted on each cell preparation at each dilution. The effects of metabolite and cell dilutions were tested with the interaction of cell dilution x metabolite production. Statistical analysis

was conducted using the Mixed model procedure of SAS (1999) to test for significant interactions between metabolite production and cell dilution. Individual coefficients of variation (CV) were determined for each mode of data expression at each cell dilution. A paired t-test (Steele and Torrie, 1960) was used to analyze the CV's determined from the twelve modes of data expression. The paired t-test allowed comparison of all possible combinations of the twelve different modes of data expression by canceling out dilution to dilution variation.

3. Results and Discussion

The average cell viability for the sheep was 92.83% (ranged between 91.22% and 94.83%). This average cell viability is very similar to that of Waldron (1996) and slightly higher than that reported by Baldwin and Jesse (1996) and Klotz (1999). Characteristics of individual sheep, including epithelial tissue weight, body weight, metabolic body weight, % cell viability and viable cell yield are presented in Table 2. Measurements of cell DM and cell TP taken from the 500-million cell aliquots are shown in Table 3, and also included are the respective amounts present in 0.5, 1, 5, 10, 20 and 40-million cell dilutions. Cell DM weights were found to be less than what Baldwin and Jesse (1996) reported using lambs, but comparable with values reported by Klotz (1999) using heifers. This difference in cell DM is postulated to result from different sampling sizes, animal-to-animal variation, and methodological differences (Klotz, 1999). Cell DM indicates that animals had roughly the same size cells, helping to exclude the possibility of a difference in metabolism resulting from one animal having larger epithelial cells than another. Cell TP indicates that the animals had similar amounts of cellular protein. It is unlikely that interanimal variation resulted from differences in metabolism because of varying protein levels.

Production of AcAc, β-HBA, LAC, and PYR increased throughout the 90-minute incubation period, indicating continuous cellular activity (Table 4). For β-HBA and AcAc, the slope decreased as the cell dilution increased. For LAC and PYR, the 0.5-million cell dilution slope was larger than the 1-million cell dilution; however, from the 1-million cell dilution to 40-million cell dilution, the slope decreased as seen in the prior two metabolites.

Table 2. Characteristics measured for individual sheep

Animal	Epithelial Tissue Wt.	Body Wt.	Metabolic Wt.	Cell Viability	Viable Cell Yield
	g	kg	$kg^{.75}$	%	million cells/mL
1	193.09	91	29.46	94.83	100.99
2	114.92	80	26.75	92.17	82.49
3	154.62	91	29.46	92.53	111.49
4	88.94	50	18.80	91.22	119.50
5	129.68	57	20.74	93.25	83.00

Table 3. Cell dry matter and total protein for various cell dilutions[a]

Animal	Cell Dry Matter[b] (mg)	Cell Dilution (million cells) 0.5	1	5	10	20	40
		mg cell dry matter					
1	368.2	0.37	0.74	3.68	7.36	14.73	29.46
2	402.8	0.40	0.80	4.03	8.06	16.11	32.22
3	383.6	0.38	0.77	3.84	7.67	15.34	30.69
4	261.8	0.26	0.52	2.62	5.24	10.47	20.94
5	345.5	0.34	0.69	3.45	6.91	13.82	27.64
	Cell Crude Protein[b] (%)	mg cell total protein					
1	66.24	0.24	0.49	2.44	4.88	9.75	19.51
2	70.45	0.28	0.57	2.84	5.67	11.35	22.70
3	70.70	0.27	0.54	2.71	5.42	10.85	21.70
4	69.82	0.17	0.35	1.75	3.50	7.00	14.00
5	70.58	0.24	0.49	2.44	4.88	9.75	19.51

[a]Total protein was calculated by multiplying the cell DM of the different dilutions by percentage of crude protein.

[b]Values are measurements taken from 500-million cell aliquots.

Table 4. Metabolite production over 90-minute incubation period

Cell Dilutions (million cells)		Intercept	Slope[a]	SE[b]	r^2	n
Acetoacetate	0.5	-7.68	3.40	0.60	0.64 **	20
	1	100.62	0.76	1.64	0.01	20
	5	-16.02	1.82	0.40	0.55 **	20
	10	-12.31	1.06	0.27	0.46 **	20
	20	3.70	0.63	0.14	0.53 **	20
	40	2.64	0.27	0.06	0.51 **	20
β-hydroxybutyrate	0.5	-31.83	2.31	0.47	0.58 **	20
	1	-26.56	2.77	0.45	0.68 **	20
	5	-27.17	2.91	0.41	0.74 **	20
	10	-13.98	2.00	0.31	0.70 **	20
	20	-7.33	1.37	0.17	0.78 **	20
	40	-3.93	1.00	0.10	0.83 **	20
Lactate	0.5	-5.79	0.27	0.11	0.26 *	19
	1	-2.06	0.47	0.11	0.49 **	20
	5	-1.14	0.21	0.04	0.61 **	20
	10	-1.14	0.17	0.02	0.76 **	20
	20	-.34	0.13	0.01	0.90 **	20
	40	.09	0.09	0.01	0.75 **	20
Pyruvate	0.5	-5.07	0.23	0.28	0.03	20
	1	-6.82	0.53	0.22	0.25 *	20
	5	1.81	0.11	0.07	0.11	20
	10	.46	0.04	0.03	0.07	20
	20	.22	0.02	0.02	0.05	20
	40	.35	0.009	0.01	0.02	20

[a]Slope expressed as nmoles of metabolite produced
[b]SE of slope
*Indicates a significance of $P < .05$
**Indicates a significance of $P < .01$

The concentration of endogenous metabolite measured at zero time was subtracted from the 90-minute metabolite incubation resulting in a measurement of a production concentration for 90 minutes. This correction can produce negative production concentrations if the zero time concentration being subtracted is larger than the amount of metabolite produced during the 90-minute incubation.

Production ratios, concentration ratios, and rates of production for AcAc and β-HBA are presented in Table 5. Acetoacetate production concentrations were greatest at the 0.5-million cell dilution ($P < .05$). Concentrations increased between 1 and 5-million and there was no difference between 1, 5, 10, 20-million cell dilution. The 40-million cell dilution differed from 0.5 and 5-million cell dilutions ($P < .05$). β-hydroxybutyrate production concentrations were largest at 1 and 5-million cell dilutions. However, 1 and 5 only differed significantly from 20 and 40-million cell dilutions. For both AcAc and β-HBA the 40-million cell dilution produced significantly less ($P < .05$) amounts of the metabolites than the amounts produced at the 0.5-million cell dilution. This could be a result of the available substrate. Both 0.5 and 40-million cell dilutions had the same amount of substrate available to them, but the 40-million cell dilution had 39.5 million more cells competing for the available substrate than did the 0.5-million cell dilution. Because of this, simple physical exposure to the substrate may have resulted in a lower rate of production for the 40-million cell dilution. However, Baldwin and McLeod (2000) reported that a concentration of 10 m*M* butyrate per liter was enough to saturate the enzyme systems. In the present experiment 100 m*M* butyrate per liter was used so there should have been ample substrate available for the 40-million cell dilution.

Table 5. Acetoacetate (AcAc) and β-hydroxybutyrate (β-HBA) concentrations following 90-minute incubation with rumen epithelial cells at various cellular concentrations[a]

Cell Dilution (million cells)	Flask concentrations, nmoles/million cells			
			β-HBA/AcAc[b]	
	AcAc[c]	β-HBA[d]	Conc-ratio[e]	Prod-ratio
0.5	282.61^{x} ± 37.97	205.99^{xy} ± 26.06	0.98^{wz} ± 0.40	0.73
1	77.30^{yz} ± 81.59	256.86^{x} ± 25.39	0.64^{z} ± 0.11	3.32
5	143.88^{z} ± 33.19	260.99^{x} ± 26.80	1.61^{wy} ± 0.21	1.81
10	91.38^{yz} ± 18.52	177.66^{xyz} ± 21.30	3.43^{xyz} ± 1.75	1.94
20	57.38^{yz} ± 11.21	126.72^{yz} ± 14.72	2.05^{xy} ± 0.31	2.21
40	23.87^{y} ± 4.30	88.13^{z} ± 9.89	3.33^{x} ± o.69	3.69

[a]nanomoles produced·$1x10^{6}$ $cells^{-1}$·$90\text{-}minutes^{-1}$.

[b]β-HBA/AcAc concentrations-ratios were calculated by taking the mean of ratios resulting from concentrations uncorrected for endogenous metabolite and the production-ratio were calculated by taking the ratio of the means corrected for endogenous metabolite.

[c]AcAc means and SEM calculated using n=13 for 0.5-, n=14 for 1- and n=15 for 5-, 10-, 20- and 40-million cell dilutions.

[d]β-HBA means and SEM calculated using n=15 for all cell dilutions.

[e]β-HBA/AcAc concentration ratio means and SEM calculated using n=9 for 0.5-, n=6 for 1-, n=14 for 40- and n-15 for 5-, 10- and 20-million cell dilutions.

[wxyz]Means within a column not sharing like superscripts are different ($P < .05$).

Concentration ratios are used as an indication of the redox state of cells in incubation flasks. Concentration ratios are not corrected for endogenous metabolites. Emmanuel (1981) demonstrated that changes in the redox state (NADH:NAD) of the cell will affect the ratio of ketone production of the epithelial cells. Therefore, redox state can be used to determine if the cell is functioning in a physiological manner. Conversely, production ratios are corrected for zero time concentrations and are calculated by taking the ratio of each redox pair for their respective cell dilution. Production ratios are used to identify changes in the mitochondrial (β-HBA and AcAc) or cytosolic (LAC and PYR) redox pairs. The β-HBA:AcAc production ratio (Table 5) was below one for the 0.5-million cell dilution and was an indicator of abnormal metabolism. Ratios of β-HBA:AcAc from 1 to 40-million cell dilution greater than one indicating cells were physiologically normal. Both AcAc and β-HBA concentrations decreased as the per-million cell dilutions increased. The concentration ratios (Table 5) for 0.5 and 1-million cell dilutions were 0.98:1 and 0.64:1. This is similar to 1:1 reported by Klotz (1999) for 0.5 and 1-million cell dilutions. However, both the present study and that of Klotz (1999) are lower than any other previously published *in vivo* or *in vitro* data. Concentration ratios for 5, 10, 20 and 40 million cell dilutions were 1.6 to 3.4:1 and are similar to the ratio 2 to 4:1 reported by Baldwin and Jesse (1996) and Waldron (1996) using sheep and steers respectively. Beck et al. (1984) reported ratios of 11-16:1 in slices of sheep epithelium, which are considerably higher than the ratios in the current study. Klotz (1999) also reported ratios that were slightly higher (4.7 to 9.3:1). The ratios of 1.6-3.4:1 are lower than those obtained *in vivo* from the bovine portal vein by Weigand et al.

(1972) who reported ratios of 6.6-8.2:1. The fact that the ratios for the 0.5 and 1-million cell dilutions were below 1.0 is an indicator that the cells were exposed to non-physiological conditions, causing a reduced production of β-HBA. Klotz (1999) reported the same findings for cell dilutions of .5 and 1-million.

Production ratios, concentration ratios, and production concentrations for LAC and PYR are presented in Table 6. Concentration LAC and PYR were largest at the1-million cell dilution; however, no significant differences were found. Similar to AcAc and β-HBA, production of both LAC and PYR decreased as the number of cells incubated increased. Production ratios of LAC:PYR were below one for the 0.5 and 1-million cell dilutions, further suggesting that cell metabolism in these dilutions was not representative of cells found in the animal. Concentration ratios were similar, except that the 5-million cell dilution was less than the 10-million cell dilution ($P < .1$). The concentration ratios from 5 to 40-million cell dilutions ranged from 1.54 to 2.29:1. These ratios are lower than 4.82 to 7.13:1 reported by Klotz (1999) and 3 to 9.1:1 reported by Weigand et al. (1972).

Coefficients of variation (CV) were calculated for each metabolite expressed in 12 different units of conversion for each cell dilution. This generated a mean (n = 12 unit conversions) CV for each metabolite at each dilution (Table 7). Coefficients of variation for AcAc were lowest at the 40-million cell dilution ($P < .05$). Coefficients of variation for β-HBA were lowest at the 0.5-million cell dilution ($P < .05$), but was not statistically different from the 1 and 40-million cell dilutions ($P < .05$). For LAC, the CV for the 40-million cell dilution was the lowest ($P < .05$). Coefficients of variation for 5, 10, and 20-million cell dilutions were lower than the 0.5 and 1-million cell dilutions ($P < .05$). For

Table 6. Lactate (LAC) and pyruvate (PYR) concentrations following 90-minute incubation with rumen epithelial cells at various cellular concentrations[a]

Cell Dilution (million cells)	Flask concentrations, nmoles/million cells			
			LAC/PYR[b]	
	LAC[c]	PYR[d]	Conc-ratio[e]	Prod-ratio
0.5	23.17[x] ± 5.90	31.68[x] ± 14.50	2.19[xy] ± 1.83	0.73
1	37.58[x] ± 7.02	48.58[x] ± 14.84	4.19[xy] ± 2.38	0.77
5	19.44[x] ± 2.06	13.43[x] ± 3.54	1.54[y] ± 0.14	1.45
10	15.40[x] ± 1.32	3.10[x] ± 1.55	2.29[x] ± 0.35	4.97
20	11.62[x] ± 0.70	2.40[x] ± 1.05	1.88[xy] ± 0.22	4.84
40	8.58[x] ± 0.79	0.83[x] ± 0.63	1.92[xy] ± 0.23	10.34

[a]nanomoles produced $\cdot 1\times10^6$ cells$^{-1}\cdot$90-minutes^{-1}.

[b]Lac/Pyr concentrations-ratio were calculated by taking the mean of ratios resulting from concentrations uncorrected for endogenous metabolite and the production-ratios were calculated by taking the ratio of the means corrected for endogenous metabolite.

[c]Lactate means and SEM calculated using n=12 for 0.5- and n=15 for 1-, 5-, 10-, 20- and 40-million cell dilutions.

[d]Pyruvate means and SEM calculated using n=14 for 0.5- and n=15 for 1-, 5-, 10-, 20- and 40-million cell dilutions.

[e]Lac/Pyr concentration ratio means and SEM calculated using n=4 for 0.5-, n=11 for 1- and n=15 for 5-, 10-, 20- and 40-million cell dilutions.

[xy]Means within a column not sharing like superscripts are different ($P < .1$).

Table 7. Average coefficient of variation (CV) for each metabolite at each dilution[a]

Cell Dilution (million cells)	Acetoacetate	β-hydroxybutyrate	Lactate	Pyruvate
0.5	64.62^{y} ± 2.62	59.79^{z} ± 2.83	88.44^{x} ± 1.01	209.18^{v} ± 1.21
1	349.13^{v} ± 32.72	53.09^{zy} ± 3.36	84.00^{x} ± 2.85	110.33^{y} ± 1.55
5	83.57^{w} ± 0.99	46.60^{y} ± 2.47	51.83^{y} ± 2.92	98.01^{z} ± 1.29
10	79.60^{x} ± 0.88	75.67^{x} ± 4.58	52.89^{y} ± 3.89	197.44^{w} ± 2.11
20	77.02^{x} ± 0.89	74.66^{x} ± 4.68	44.49^{y} ± 4.00	146.38^{x} ± 3.02
40	56.17^{z} ± 1.29	53.03^{zy} ± 2.55	32.20^{z} ± 1.74	337.48^{u} ± 7.55

[a]Means and SEM for all CV's used n=12 except the 1-million cell dilution of acetoacetate which used n=6 and the 0.5-million cell dilution of lactate which used n=9.

[uvwxyz]Mean CV's within a column not sharing like superscripts are different ($P < .05$).

PYR, the 5-million cell dilution had the lowest CV and all six cell dilutions were different ($P < .05$).

The purpose of evaluating the CV's of the different data conversions was to standardize reporting criteria so that data can be compared from different labs and hopefully different species. Data were compared based on either cell number, cell DM or cell TP alone (Table 8) or per epithelial wet tissue weight, body weight (BW) or metabolic BW (Table 9). Since a pared t-test was used, values in Tables 8 and 9 are not actual CV's, rather the difference between two CV's. Therefore a negative number implies that the CV for the first conversion is a lower than the second conversion being compared. The million cell conversion had the least amount of variation for LAC and β-HBA measurements when compared to cellular DM and TP ($P < .01$). For PYR, cell TP had a significantly lower CV than cell number and cellular DM. For β-HBA and LAC, cell DM had a lower CV when compared to TP ($P < .01$). In the present experiment expressing data on a per million cell basis appears to be the ideal data conversion compared to cell DM or cell TP, because cell DM and TP measurements remained unchanged between cell isolated from animals used in this study. Coefficients of variation for cell number, cell DM, and cell TP were lower than their respective CV's further corrected by wet tissue weight, BW and metabolic BW for all four metabolites ($P < .05$) (Table 9). Correction for wet tissue weight was done, because the density of the tissue may have an overall affect on the rate of metabolite production. Specifically a greater density may require a longer digestion time to equally disassociate the desired cell layers from the tissue. Thus possibly subjecting the desirable cells to more stress than

Table 8. Comparison of coefficients of variation (CV) between million cells, cell dry matter and cell total protein modes of expression[a][b]

Comparison	Acetoacetate	β-hydroxybutyrate	Lactate	Pyruvate
Million cells vs. Cell DM	-1.06	-12.81**	-11.33**	-0.61
	± 1.30	± 1.41	± 1.57	± 2.13
Million cells vs. Cell TP	-0.97	-14.58**	-12.94**	1.20
	± 1.49	± 1.56	± 1.74	± 1.97
Cell DM vs. Cell TP	0.76	-1.78**	-1.62**	1.83**
	± 0.60	± 0.15	± 0.15	± 0.32

[a]Values are not actual CV's, rather the difference between the two as compared by a paired t-test.
[b]Mean and SEM for acetoacetate, lactate and pyruvate CV's used n=20 and β-hydroxybutyrate used n=24.
** Indicates a significant difference between two conversions being compared ($P < .01$).

Table 9. Comparison of coefficients of variation (CV) between cellular expressions and their respective adjustments by wet tissue weight (g), body weight (kg) and metabolic body weight ($BW^{.75}$)[ab]

Comparison	Acetoacetate	β-hydroxybutyrate	Lactate	Pyruvate
Cell conversions vs. Wet tissue wt., g	-5.71 * ± 2.51	-24.21 ** ± 2.37	-21.44 ** ± 2.70	-17.09 * ± 6.53
Cell conversions vs. Body wt., kg	-2.73 ± 2.16	-16.36 ** ± 1.80	-13.42 ** ± 2.19	-10.34 † ± 5.55
Cells conversions vs. Metabolic $BW^{.75}$	-1.07 ± 1.64	-11.19 ** ± 1.45	-8.50 ** ± 1.73	-7.05 † ± 3.83
Wet tissue wt. vs. Body wt.	2.97 ** ± 0.65	7.85 ** ± 0.59	8.01 ** ± 0.58	6.75 ** ± 2.09
Wet tissue wt. vs. Metabolic $BW^{.75}$	4.63 ** ± 1.00	12.72 ** ± 0.92	12.64 ** ± 1.00	10.47 ** ± 3.09
Body wt. vs. Metabolic $BW^{.75}$	1.66 ** ± 0.65	4.97 ** ± 0.34	4.75 ** ± 0.46	3.66 * ± 1.65

[a]Values are not actual CV's, rather the difference between the two as compared by a paired t-test.
[b]Mean and SEM for acetoacetate, lactate and pyruvate CV's used n=15 and β-hydroxybutyrate used n=18.
†Indicates a significant difference between two conversions being compared ($P < .1$).
*Indicates a significant difference between two conversions being compared ($P < .05$).
** Indicates a significant difference between two conversions being compared ($P < .01$).

cells from a less dense tissue. Digestion of tissues with different wet weights had no apparent effect on the overall metabolism of the cells in culture.

Correcting data for body weight was a correction for variation in rumen size. The size of the rumen directly correlates with the size of the animal. Total gastrointestinal alimentary tract weight makes up 3.6% of the BW of mature sheep and the ruminoreticulum consists of 14.9 grams of organ weight per kilogram of BW (Lyford, 1988). Percentage of BW contributed by the rumen changes dramatically from birth until maturity. For example, a lamb's ruminoreticulum at birth makes up of 3.3 g for each kg of BW and at 16 weeks 17.9 g for each kg of BW (Lyford, 1988). However, similar rates of VFA metabolism have been seen between mature ruminants and ruminants as young as 60 days of age (Bush, 1988). The present study concluded in that presenting data on a cell number basis is preferred over data converted by BW (Table 9).

Coefficients of variation for metabolic BW were significantly lower than body weight for AcAc, β-HBA, LAC, and PYR ($P < .01$). This is important when comparing different species such as sheep, cattle or deer since each of these species are different sizes and basal rates of metabolism.

There are however, several limitations associated with primary cell culture technique. First, it is difficult to obtain a 100% homogenous pool of cells from the desired cell layers, specifically cells from the *stratum basale* and *stratum spinosum* cell layers. This is important to do because cells from the luminal *stratum granulosum* layer have cytosolic lactate dehydrogenase (Gálfi et al., 1982), but contain few mitochondria, therefore these cells will have low levels β-hydroxybutyrate dehydrogenase (Steven and Marshall, 1970). Increased ratios of cytosolic lactate dehydrogenase to mitochondrial β-

hydroxybutyrate dehydrogenase (in cell pools that are not 100% homogenous for the *stratum basale* and *stratum spinosum* cell layers) could theoretically affect production rates and the ratios of compartmental redox pairs. Also, the inclusion of unwanted cell layers could adversely affect substrate availability. The second limitation is the ability to get an "actual" count of how many cells are being incubated. Counts are made by taking a fraction of the total cell suspension and counting the number of cells with a hemacytometer. It is difficult to get a true representative fraction from the cell pool to count, because the cells are continuously settling out of solution and/or clumping. Because of this it is difficult to get a second, third, etc. representative fraction to actually incubate once the count has been made. This error is intensified with the lower numbered cell dilutions. An additional error associated with the viable cell count is in the estimation of cell viability by trypan blue exclusion. It is a subjective measurement that varies from person to person due to differences as to when one considers the cell to have absorbed the dye (dead cells absorb dye).

Regardless of these concerns, the technique of isolating rumen epithelial cells in this present study is still an ideal technique to optimally measure rumen epithelial metabolism *in vitro*. This technique eliminates confoundment by morphological differences in rumen epithelial tissue when investigating the effects of diet/energy intake on tissue metabolism and absorption.

4. Conclusions

In conclusion, it would be best not to incubate any cell dilution under 5-million because the production ratios for AcAc and β-HBA were consistently below one for the 0.5 and 1-million cell dilutions which indicates an abnormal cellular metabolism during the 90-minute incubation. Conversely, the incubation of 40-million cells or greater is not recommended due to the difficulty obtaining that many viable cells. A suggested ideal range of cells to include in an incubation flask is 5 to 20-million. This range minimizes the potential for altered metabolite production possibly caused by incubating large cell quantities as well as the experimental error associated with using low cell numbers.

It is also recommended that data be expressed on per cell number and, if necessary, data adjusted for metabolic BW is preferable over that of BW. This should be used in conjunction with animals of roughly the same age. When comparing different species, animals of varying weights or stages of development, it is recommended that data also be converted to cell DM and cell TP to facilitate comparison by other laboratories and to simplify the future incorporation of data with other experiments.

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Literature Cited

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Appendices

Appendix 1.

Beta-hydroxybutyrate Assay for Microtiter Plates

Beta-hydroxybutyrate Assay for Microtiter Plates

The procedure for the measurement of D-β-hydroxybutyrate is based on the procedure described by Williamson and Mellanby (1965). The standard calibrations and the procedures have been modified for quantification of β-HBA produced by isolated rumen epithelial cells and for measurement on a microtiter plates.

Principle

The β-HBA analysis is a spectrophotometric determination based upon a compound's (nicotinamide adenine dinucleotide, NAD^+) unique property to absorb a specific quantity of light of specific wavelength (340 nm). β-hydroxybutyrate concentration of NAD^+ following the chemical reaction:

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

β-hydroxybutyrate acetoacetate

β-hydroxybutyrate is oxidized, in a 1:1 stoichiometry, using oxidized nicotinamide adenine dinucleotide (NAD^+) in the presence of the enzyme β-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 β-HBA concentration.

1. **Prepare dilutions of standards.**

   Use 0.1261g/ 100 ml β-HBA for 10.0 mM (D-βHBA, in dessicator in refrigerator) This stock standard can be made in large amounts and frozen for future use.

   Serial dilution can be prepared from stock β-HBA: 0.01 mM, 0.025 mM, 0.05 mM, 0.1 mM, 0.5 mM and 0.75 mM.

From 10 mM stock β-HBA:

0.1 mM ——→	*0.01 mM*	
40 μL **10 mM**	400 μL **.1 mM**	
3960 μL d.d. H_2O	3600 μL d.d. H_2O	
0.5 mM ——→	*0.05 mM* ——→	*0.025 mM*
200 μL **10 mM**	450 μL **.5 mM**	1500 μL **.05 mM**
3800 μL d.d. H_2O	3500 μL d.d. H_2O	1500 μL d.d. H_2O
0.75 mM		
225 μL **10 mM**		
2775 μL d.d. H_2O		

A. After dilutions are prepared, bring the volume down to 3 ml
B. Add 0.2 ml perchloric acid concentrated
C. Add 0.4 ml K_2CO_3 5.8 mM
D. Vortex each tube
E. Spin at 1500 x *g* (2684 rpm) for 10 min

Note: Standards are treated the same as samples.

2. **Tris – EDTA – Hydrazine hydrate buffer (50 ml)**

25 ml 0.2 mM Tris (Sigma T 1378)
0.75 ml 0.1 M EDTA
1 ml Hydrazine hydrate (Sigma H0883)

pH to 8.5 with HCL and raise to 50 mL

Note: This buffer is only good for day of use. Also initial pH of this buffer is a little high about pH 10. Six mM HCl is needed to bring to proper pH.

3. **Working buffer**

Tris – EDTA – Hydrazine hydrate buffer (50 mL)
5.55 ml d.d. H_2O
0.0555 g NAD (stored in dessicator in freezer)

4. **Into 96–well plates:**

Sample/std: 125 μL
Buffer: 125 μL

5. **Procedure:**

1. Prepare standards
2. Add sample/stds, buffer. (plate in increasing conc. Of std., i.e. A1, A2 0.01 mM to A11, A12 0.75 mM; also H12 plate water.)
3. Take initial reading at 340 nm
4. Add 1 μL of **β-hydroxybutyrate dehydrogenase** to each well (Sigma H6126)
5. Incubate at 37 °C for 45 minutes*
6. Read final at 340 nm

** Hr is required when more than one plate is assayed at a time*

Appendix 2.

Acetoacetate Assay for Microtiter Plates

Acetoacetate Assay for Microtiter Plates

The measurement of AcAc is based on a procedure described by Mellanby and Williamson (1965). The standard calibration procedures have been modified for quantification of acetoacetate produced by isolated rumen epithelial cells for measurement on a microtiter plate.

Principle

The AcAc 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 of NAD following the chemical reaction:

$$HOOC{-}CH_2{-}CO{-}CH_3 + NADH + H^+ \longrightarrow HOOC{-}CH_2{-}CHOH{-}CH_3 + NAD^+$$

Acetoacetate → β-hydroxybutyrate

Acetoacetate is reduced, in a 1:1 stoichiometry, using reduced nicotinamide adenine dinucleotide (NADH) in the presence of the enzyme β-hydroxybutyrate dehydrogenase resulting in the formation of β-HBA and oxidized nicotinamide adenine dinucleotide (NAD^+). The change in nicotinamide adenine dinucleotide results allows for the calculation of the AcAc concentration.

1. **Prepare dilutions of standards.**

   Use 0.0108 g/ 10 mL AcAc for 10.0 mM (stored in dessicator in freezer, must be made fresh on day of use.)

   Serial dilutions are made to include standards: 0.001 mM, 0.003 mM, 0.01 mM, 0.1 mM, 0.3 mM and 1.0 mM.

   From 1.0 mM AcAc

0.1 mM ⟶	0.01 mM ⟶	0.001 mM
400 μL **1.0 mM**	400 μL **0.1 mM**	400 μL **0.01 mM**

3600 μL d.d. H_2O 3600 μL d.d. H_2O 3600 μL d.d. H_2O

0.3 mM ———→ 0.003 mM
1200 μL **1.0 mM** 40 μL **0.3 mM**
2800 μL d.d. H_2O 3960 μL d.d. H_2O

A. After dilutions are prepared bring down the volume of each standard to 3 mL (i.e. 0.1 M and 0.01 mM remove 600 μL, 0.003 mM and 0.001 mM remove 1 mL)
B. Add 0.2 mL of concentrated perchloric acid
C. Add 0.4 mL K_2CO_3 5.8 mM (stored in refrigerator)
Note: a white vapor will result and some bubbling in the tube. This procedure should be preformed on ice.
D. Vortex each tube
E. Centrifuge at 1500 x *g* (2684 rpm) for 10 min.
F. Use cleared supernatant for the standard curve

2. **Phosphate buffer and NADH**

13.6g/L for 0.1 M KH_2PO_4
17.4g/L for 0.1 M K_2HPO_4
Buffer is ½ 0.1 M KH_2PO_4 and ½ K_2HPO_4, pH to 6.8

Note: this buffer comes pretty close to 6.8. Not much acid is needed to bring to proper pH. 0 .00426 g of NADH (dry powder in dessicator in refrigerator) added to 10 mL of phosphate buffer. This is enough buffer for one 96 well tray.

3. **Into 96–well plates:**

Sample/std: 150 μL
Buffer: 100 μL

4. **Procedure:**

1. Prepare standards (this should be made last since AcAc degrades fairly rapidly).
2. Add samples/stds, buffer. (Plate in increasing conc. Of stds, i.e. A1, A2 0.001 mM to A11, A12 1.0 mM) Leave well H12 to plate water.
3. Take initial reading at 340 nm
4. Add 1 μL of **β–hydroxybutyrate dehydrogenase** to each well. (Sigma H6126) **SHAKE**
5. Incubate at 37 °C for 45 minutes*
6. Read final at 340 nm

** 1 hr incubation if more than one tray is running at a time*

Appendix 3.

Lactate Assay for Microtiter Plates

Lactate Assay for Microtiter Plates

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 LAC to PYR by lactate dehydrogenase in the presence of NAD^+. When excess NAD^+ is present, all LAC will be converted to PYR. Measurement of NADH before and after the addition of lactate dehydrogenase will show the amount of lactate originally present because the absorbency of NAD^+ (280 nm) differs from that of its reduced analog NADH (absorbency of 340 nm).

1. **Prepare dilutions of standards**

Use Lactic Acid (Sigma L1750 in dessicator in freezer).

Prepare a **10 mM** stock solution (this solution can be prepared in large quantities of aliquots and stored in freezer for future use (lactate MW 90.08). For 50 mL of 10 mM stock mix 0.04504/50 mLs.

Serial dilutions can be prepared from 10 mM stock Lactate: 0.01 mM, 0.02 mM, 0.03 mM, 0.04 mM, 0.08 mM and 0.1 mM.

From **10 mM** stock Lactate:

1.0 mM ——→	0.1 mM ——→	0.01 mM
1.0 mL **10 mM**	400 µL **1.0 mM**	400 µL **0.1 mM**
9.0 mL d.d. H_2O	600 µL d.d. H_2O	400 µL d.d. H_2O

	Amount **1.0 mM**	Amount of d.d. H_2O
0.02 mM	60 µL	2940 µL
0.03 mM	90 µL	2910 µL
0.04 mM	120 µL	2880 µL
0.08 mM	240 µL	2750 µL

A. After dilutions are prepared, bring down volume to 3 mL. (Remove 600 μL from 0.1 mM and 1.0 mL from 0.01 mM. The rest of the standards are at 3 mL)
B. Add 0.2 mL concentrated perchloric acid
C. Add 0.4 mL K_2CO_3 5.8 mM
D. Vortex each tube
E. Spin at 1500 x *g* (2684 rpm) for 10 min

Note: Standards are treated the same as samples.

2. **Stock buffer solution**

Glycine Buffer		**Example: (50 mL)**
Glycine, 0.6 mol/L	(MW 75.07)	2.25 glycine
Hydrazine, 0.5 mol/L	(MW 50.06)	1.25 ml hydrazine hydrate

pH to 9.2 at 25 °C, store in refrigerator

Note: This initial pH comes pretty close to 9.2

3. **Working buffer solution**

22 mL H_2O
15 mL Glycine buffer
0.080 g NAD

4. **Into 96–well plate**

Sample/std: 125 μL
Buffer: 125 μL

5. **Procedure:**

1. Prepare standards
2. Add sample/stds, buffer to plate. (Plate in increasing conc. of std., i.e. A1, A2 0.01 mM to A11, A12 0.1 mM. Also plate water in H12 to use as a blank.)
3. Read plate with appropriate blank well identified at 340 nm.
4. Add 2 μL of **Lactate dehydrogenase** to all wells
5. Incubate, covered for 90 minutes*
6. Read a second time, again using appropriate blank

Note: A reading at 45 min is also required to make assay complete.

Appendix 4.

Pyruvate Assay for Microtiter Plates

Pyruvate Assay for Microtiter Plates

This procedure is based on the Sigma Pyruvate Kit (726–UV). The standard calibrations and the procedure have been modified for the quantification of PYR produced by isolated rumen epithelial cells and for measurement on a microtiter plate.

Principle

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

1. **Prepare dilutions of standards**

Use Pyruvic Acid (Sigma P2256 in dessicator in refrigerator)

Prepare a **10 mM** stock solution (this solution can be prepared in large quantities of aliquots and stored in the freezer for future use, mix 0.1100 g/100 mL.

Serial dilutions can be prepared from stock pyruvate: 0.005 mM, 0.010 mM, 0.05 mM, 0.1 mM, 0.3 mM and 0.5 mM.

From 10 mM stock pyruvate:

0.1 mM ⟶	0.010 mM	
40 µL **10 mM**	400 µL **.1 mM**	
3960 µL d.d. H_2O	3600 µL d.d. H_2O	
0.5 mM ⟶	0.05 mM ⟶	0.005 mM
200 µL **10 mM**	400 µL **0.5 mM**	400 µL **0.05 mM**
3800 µL d.d. H_2O	3600 µL d.d. H_2O	3600 µL d.d. H_2O
0.3 mM		
90 µL **10 mM**		
2910 µL d.d. H_2O		

A. After dilutions are prepared, bring down the volume to 3 mL
B. Add 0.2 mL of concentrated perchloric acid
C. Add 0.4 mL K_2CO_3 5.8 mM
D. Vortex each tube
E. Spin at 1500 x *g* (2684 rpm) for 10 min.

Note: *Standards are treated the same as samples.*

2. **Trizma base**

Trizma, 1.5 mol/L	(MW 121.1)	**Example: (100 mL)** 18.18g TRIZMA BASE
pH to 8.5		

Note: *This is the stock buffer.*

3. **Working buffer**

3 mL water
22 mL Trizma base (stock buffer)
0.0128 g NADH (in dessicator in refrigerator)

4. **Into 96–well plates:**

Sample/std: 150 µL
Buffer: 100 µL

5. **Procedure:**

1. Prepare standards
2. Add sample/stds and buffer to plate. (Plate in increasing concentration standard, i.e. A1, A2 0.005 mM to A11, A12 0.5 mM; also H12 plate water.)
3. Read plate with the appropriate blank well identified at 340 nm.
4. Add 2 µL of **lactate dehydrogenase** to all wells
5. Incubate, covered, at 37 °C for 30 minutes*
6. Read a second time at 340 nm, again using the appropriate blank

Note: *If multiple trays are running, extra time is not required to run this assay.*

Appendix 5.

List of Abbreviations

List of Abbreviations

VFA	Volatile fatty acid
AcAc	Acetoacetate
β-HBA	Beta-hydroxybutyrate
LAC	Lactate
PYR	Pyruvate
CV	Coefficient of variation
BW	Body weight
DM	Dry matter
TP	Total protein
V-A	Veno-arterial
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (REDUCED)

Vita

Robert Clements Gillis was born in Baltimore, Maryland on March 28, 1975, and is the son of David J. and Katherine M. Gillis. He received his high school diploma from The St. Paul's School, in Brooklandville, Maryland, in June, 1993. He then entered Gettysburg College in Gettysburg, Pennsylvania, majoring in Biology with a minor in Chemistry. He received a Bachelor of Science degree in Biology in May, 1997. He then entered The University of Tennessee, Knoxville in August, 1997, and began his graduate work in Animal Science on a teaching assistantship. Under the tutelage of Dr. R. N. Heitmann, he focused on the study of ruminant nutrition. He became a member of Gamma Sigma Delta and Phi Kappa Phi honor societies. He received his Master of Science degree in Animal Science in May, 2000.

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