ABSTRACT

Title of Dissertation: ESTIMATION OF RUMEN MICROBIAL PROTEIN PRODUCTION AND RUMINAL PROTEIN DEGRADATION.

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Animal agricultural production systems are a major source of nitrogen (**N**) which may contribute to potential environmental pollution and one way to reduce losses of N to the environment is through feeding protein closer to requirements without overfeeding. This experiment was conducted to evaluate the effect of two rumen degraded protein (**RDP**) sources (non-protein N in the form of urea and amino acid-N in the form of casein) on microbial N (**MN**) flow, digestibility and production in lactating dairy cows. Eight ruminally and duodenally cannulated Holstein cows were fed one of four dietary treatments in a repeated 4x4 Latin square. The first diet (**BASE**) served as the negative control and contained 12.2% crude protein (**CP**). The remaining diets contained either urea (**UREA**), casein (**CAS**), or a combination of both (**U+C**) on an equal N basis and contained 15.0% CP. Cows were infused with Co-EDTA, Cr-mordanted NDF and ^{15}N which were used as markers for liquid, solid and bacteria flow, respectively. Intake, duodenal MN flow, milk production, and

digestibility were lower when cows were fed the BASE diet and there were differences in MN flow between the UREA, CAS or U+C diets. Ruminal starch digestibility was highest when cows were fed the U+C diet and NDF digestibility was higher when cows were fed the CAS and U+C diets. Therefore a source of RDP with amino acids was required to maximize both fiber and starch digestibility.

In the same study flow rates of various particle sizes from reticulum and duodenal samples were compared as well as the bacterial attachment to these particles as they flow through the digestive tract. Digesta collected from both the reticulum and the duodenum were poured over a set of sieves to allow for particle size separation. Flow rates of DM, NDF and N differed depending on particle size and the composition of the various sieve fractions differed but was still similar between reticulum and duodenal samples. Bacterial attachment differed depending on particle size and location in the digestive tract. These results indicate the importance of particle size passage from the rumen and the usefulness of flow markers to adjust for unrepresentative sampling from both the rumen and the duodenum.

A better understanding of the responses of MN flow due to RDP source can lead to improved diet formulation models which can be used to balance dairy cattle rations for optimum production yet minimize losses of N from the cow and therefore to the environment.

ESTIMATION OF RUMEN MICROBIAL PROTEIN PRODUCTION AND RUMINAL PROTEIN DEGRADATION

by

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AA ……..…….amino acid

AAN ……...……..amino acid nitrogen

ADF ……..……acid detergent fiber

ADF-N …...…….acid detergent fiber nitrogen

APE ……..……atom percent excess

AUC ……...……..area under the curve

BCVFA ……...……..branched chain volatile fatty acids

CP ……..…….crude protein

DIM …….days in milk

DM ……...……dry matter

DMF …….dimethylformamide

DMI ……...……dry matter intake

FCM ……..……fat corrected milk

FSG ……..……..functional specific gravity

IRS …… infrared spectroscopy

LAB ……..……liquid-associated bacteria

MN ……...……..microbial nitrogen

MTBSTFA ……..……..*N*-(*tert*-butyl-dimethylsilyl)-*N*-methyltriflouroacetamide

MUN ……..……..milk urea nitrogen

N …….……nitrogen

NB …...……..natural background

NE_L …….……..net energy of lactation

NDF ……..……neutral detergent fiber

NDF-N ……neutral detergent fiber nitrogen

NH_3-N …….……ammonia nitrogen

NPN ……..nonprotein nitrogen

NRC …….……National Research Council

RDP ……...……ruminally degraded protein

RUP ……..……ruminally undegraded protein

SAB …….……..solid-associated bacteria

SAS ……...……Statistical Analysis System

SBM …….……..soybean meal

SCC ……...……..somatic cell count

SD …….……..standard deviation

SEM ……..…….standard error of the mean

SSA ……..……sulfosalicylic acid

TBDMS ……..……. tert-butyldimethylsilyl

TT ……...……total tract

VFA ……...…….volatile fatty acids

Chapter 1

REVIEW OF LITERATURE

INTRODUCTION

Animal agricultural production systems are major sources of nonpoint pollution affecting quality of water sources (Williams, 1995). The major nutrients that are considered pollutants from agricultural systems are nitrogen (**N**), phosphorus, and methane (Kohn et al., 1997). Nitrogen has been identified as the foremost source of nonpoint water pollution (Thomann et al., 1994) and the potential negative impacts of N have become an area of public concern. Substantial efforts have gone into managing nutrients on dairy farms to maximize profit while reducing the risk of pollution to protect water resources (Lanyon, 1994). Nitrogen losses from dairy farming can be reduced through improvements in diet formulation (Tamminga, 1992; Kohn et al., 1997). Reducing dietary protein and increasing the efficiency of protein use within the cow can lead to reductions in N loss from dairy cows (Tamminga, 1992). Therefore, the goal of this dissertation is to provide new information to aid in reducing N excretion from dairy cows while maintaining a high level of production that is economically efficient.

Much research has gone into understanding the requirement for rumen degraded protein (**RDP**) of dairy cows, and the effects of various sources of RDP on digestion and lactation performance have been evaluated. Various groups of rumen bacteria respond differently to the type of protein they receive. However, limited research has evaluated the effects of non-protein nitrogen (**NPN**) and/or amino acid-N (**AA-N**) on microbial protein yield, lactation performance and N metabolism in lactating dairy cows. Additionally, since 1965, there has been considerable research investigating the use of microbial-derived purines to predict microbial protein flow out

of the rumen as microbial protein is essential to dairy cows; however, the effect of NPN and/or AA-N on purine excretion has not been evaluated.

Contribution of Nitrogen from Dairy Farming

The environmental impact of farming is of great public concern. As urban expansion continues to increase, farm land is turned into housing developments and shopping malls. Additionally, the human population continues to increase in size, in consequence, increasing the demand for agricultural commodities such as meat, milk, fruits and vegetables. However, as urban sprawl increases developments on fertile farm land, producers are left to generate high-quality products in less space. In the animal industry, this trend has lead to confined animal feeding operations where a large number of animals are housed in a relatively small area. A large amount of waste is generated from these feeding operations which must be handled in a safe and effective way as nutrients such as phosphorus and N contained in the waste may contribute to potential air and water pollution.

Reactive N lost to the environment from agriculture contributes to eutrophication of streams and estuaries, ground water contamination, smog and acidity of soils and water (Galloway, 2002). Only 21 to 38% of the N that was brought on to farms as feed, fertilizer and via N fixation by legumes was exported off farms as meat and milk (Klausner, 1993). The remaining N (farm inputs minus exported products) is eventually lost from the farm to air and water resources (Kohn et al., 1997). Dairy cows contribute a minimum of 750,000 metric tons of N per year into the environment of the United States (St-Pierre and Thraen, 1999). Nitrogen can enter the environment through volatilization of ammonia to the air, nitrate leaching into ground water and

run-off to surface water from manure or other fertilizers. Even when manure and other fertilizers are properly managed, much N is lost to the environment. Therefore, reducing N losses requires better feeding and herd management to reduce the need for crop production and manure application (Kohn et al., 1997).

In 2003, the National Research Council (**NRC**) published a report on predicting excretion of N from animal feeding operations. In this mass balance approach, the importance of including animal nutrition into the whole-system analysis was indicated. However, the Environmental Protection Agency's (**EPA**) current method of determine release of ammonia from animal feeding operations is based on emissions per animal unit. This calculation does not include manure management practices and more importantly, animal nutrition, which is deemed to be important in predicting N excretion from animal feeding operations.

N Metabolism in the Rumen

Dietary protein is used by ruminants for maintenance, reproduction, milk production and growth. This protein is obtained from dietary protein that escapes rumen degradation and from microbial protein synthesized in the rumen. Both of these sources of protein are subsequently broken down in the true stomach and absorbed in the small intestine. There are two types of dietary crude protein: rumen undegraded protein (**RUP**) and rumen degraded protein (**RDP**). Ruminal microbes require RDP to meet their N needs. Since microbial protein alone cannot meet the animal's requirements to support high milk production, a source of RUP is also needed the diet. There are also two types of RDP: non-protein nitrogen (**NPN**) and

true protein. The NPN is comprised of ammonia and urea, while true protein is comprised of chains of amino acids.

The first step in ruminal protein degradation involves the attachment of bacteria to feed particles, followed by microbial protease activity (Brock et al., 1982). From 70 to 80% of ruminal microorganisms attach to undigested feed particles (Craig et al., 1987) and 30 to 50% of these bacteria possess proteolytic activity (Prins et al., 1983). The proteolytic activity of the rumen microbes and the type of dietary protein are the primary factors determining the rate and extent to which protein degradation occurs (Bach et al., 2005). Different proteases are necessary for complete protein degradation due to the large number of different bonds within a protein (Wallace et al., 1997) and the end products resulting from this process are peptides and AA.

The peptides and AA resulting from microbial proteolyic activity are then transported into the microbial cell. Inside the cell, peptides can be degraded into AA by peptidases and AA can then be incorporated into microbial protein or further deaminated to volatile fatty acids (**VFA**) , CO_2 or ammonia (Tamminga, 1979). If the bacteria are in need of energy, the peptide or AA will be deaminated and the carbon (**C**) chains will be fermented into VFA; however, if adequate energy is available, the AA will be transaminated or used for microbial protein synthesis (Bach et al., 2005).

Compared to non-ruminants, ruminant animals are relatively inefficient at converting dietary crude protein (**CP**) into a usable N source because of the extensive fermentative activity of the rumen microbial population (Broderick et al., 1991). A significant portion of the N fed to high producing dairy cows is not incorporated into

microbial protein which results in an elevated rumen NH_3 pool that is inevitably lost through urinary urea-N excretion (Sannes et al., 2002). Rumen microbial protein synthesis is critical for high producing dairy cows as it makes up 60 to 85% of the metabolizable protein requirements for maintenance, growth, gestation, and lactation in dairy cattle (Stern et al., 1994). Understanding of the requirements of rumen microbes will aid in formulating rations to maximize microbial nitrogen (**MN**) yield while avoiding over-feeding N which could potentially lead to environmental pollution.

Preferred N Source of Rumen Microbes

Rumen microbes are able to convert NPN to high-quality protein for use by dairy cows, but they also degrade high-quality dietary protein to ammonia (Van Soest, 1994). Ammonia is the main source of N for microbial protein synthesis (Nolan, 1975) and 82% of the bacterial strains isolated from one animal grew with NH_3 as the sole N source (Bryant and Robinson, 1962). Cows can thrive with urea as the only source of dietary N (Virtanen, 1966); however feeding true protein to cattle typically improves performance (Stock et al., 1986; Rooke and Armstrong, 1989). Both *in vitro* and *in vivo*, the addition of protein or AA-N has also been shown to also increase fiber digestion and microbial protein yield (Maeng and Baldwin, 1976; Cotta and Russell, 1982; Rooke and Armstrong, 1989). Growth of most rumen bacterial strains has been shown to improve when preformed AA are present (Hungate, 1966). Rumen microbes take up amino acids in the form of peptides more rapidly than free amino acids (Wright, 1967).

The AA that make up microbial protein may be synthesized *de novo* using ammonia-N (**NH_3-N)** and C-chains which are derived from a variety of pathways (Wallace et al., 1997). The C-chains result from carbohydrate or amino acid catabolism while NH_3-N is derived from AA, NPN, or urea recycling back to the rumen from the blood, deamination of AA and other sources. Amino acids are produced during proteolysis of feed protein, proteolysis of bacterial and protozoal protein released after cells are lysed (intra-ruminal recycling), release of AA from bacteria and protozoa, and from degradation of sloughed rumen epithelial cells (Demeyer and Fievez, 2004). Additionally, peptides and AA may be taken up intact by the rumen microbes and incorporated directly into microbial protein or transaminated prior to incorporation into protein (Bach et al., 2005).

In the rumen, cellulolytic bacteria primarily use NH_3-N while amyolytic bacteria prefer to use AA-N and they are more proteolytic than cellulolytic bacteria (Siddons and Paradine, 1981; Wallace et al., 1997). Several species of rumen bacteria have been shown to require specific AA for growth (Forsberg, 1978; Jones and Pickard, 1980). In mixed cultures, microbial growth was stimulated with certain AA or groups of AA (Maeng et al., 1976) while certain bacterial strains require AA in the peptide form (Pittman and Bryant, 1964). Several studies have evaluated the effects of feeding NPN versus an AAN source both *in vitro* and *in vivo* on MN yield, N metabolism and production with various results.

Effects of AAN versus NPN

In pure bacterial cultures, both AA and peptide supplementation increased the maximum specific growth rate of several cellulolytic and amyolytic bacterial strains as

compared to supplementation of $(NH_4)_2SO_4$ (Cruz Soto et al., 1994). However, the degradation of cellulose by three cellulolytic bacterial species (*F. succinogenes, R. albus, R. flavefaciens*) was enhanced when the pure cultures were incubated with $(NH_4)_2SO_4$ and AA, but not peptides.

In an initial *in vivo* study evaluating the effects of AAN or NPN in cattle, Armentano et al. (1993) fed mid-lactation Holstein cows either a degradable true protein source in the form of soybean meal (**SBM**) or urea. Ruminal NH_3-N tended to be higher and ruminal branched-chain volatile fatty acids (**BCVFA**) were lower when cows were fed urea compared to a degradable true protein source. No differences in dry matter intake (**DMI**), milk production or milk components were noted.

In a similar study, Broderick et al. (1993) reported no differences in DMI, milk yield or milk urea N (**MUN**) when multiparous Holstein cows were fed diets containing urea or soybean meal (**SBM**). However, plasma urea N was increased when cows were fed the urea diet. Additionally, DMI increased by 2 kg/d and milk yield increased by 3 kg/d when cows were fed the low-DM alfalfa silage supplemented with an AA-N source compared to urea, but the difference was not detected for the same supplements to the high-DM alfalfa silage (Broderick et al., 1993). Regardless of silage DM, plasma urea N, MUN, and ruminal ammonia were higher when cows were fed urea compared to an AA-N source (Broderick et al., 1993).

To further evaluate responses to N sources, using urinary purine excretion to predict the flow of MN from the rumen, Cruz Soto et al. (1994) found no differences between infusing peptides, AA or ammonia into the rumen of adult sheep. No

difference in N retention, digestibility, or N excretion in urine or feces was noted. However there was an increase in ruminal BCVFA when sheep were infused with AA and peptides as compared to the urea infusion.

As a follow-up study, when sheep were supplemented with either casein or urea, N source had no effect on MN yield with hay-based diets (Chikunya et al., 1996). However, casein supplementation resulted in an increased bacterial count and MN yield when added to a beet pulp-based diet possibly because of the rapidly degradable fiber. Additionally, ruminal NH_3-N concentration was higher when urea was fed but no difference in ruminal digestibility was noted across dietary treatments (Chikunya et al., 1996).

More recently, Sannes et al. (2002) compared feeding urea or SBM as the primary protein source to lactating Holstein cows. Unfortunately, the diets were not iso-nitrogenous as the diet supplemented with SBM contained over one percentage unit more CP (DM basis) than the urea-supplemented diet. This difference may have resulted in the observed increase in ruminal NH_3, urinary N excretion, and MUN when cows were fed the SBM-supplemented diet as compared to the urea-supplemented diet where only an increase in ruminal BCVFA was reported. However, no changes in DMI, milk yield, microbial protein, or purine excretion were noted between dietary treatments.

Estimating Microbial Protein Production – Use of Purine Derivatives

External markers such as ^{15}N or ^{35}S as well as internal markers such as nucleic acids have been used to determine ruminal microbial protein production (Broderick and Merchen, 1992). However, determining digesta flow is necessary when using

these markers which require cannulated animals (Broderick and Merchen, 1992). As a result, finding a non-invasive method to estimate microbial protein production in the rumen of cattle would be beneficial in ration formulation.

In ruminants, purines are excreted as purine derivatives (**PD**) in urine and milk as allantoin, uric acid, xanthine and hypoxanthine. Because of the high xanthine oxidase activity found in the blood of cattle, xanthine and hypoxanthine are converted to uric acid in blood and tissues prior to urinary excretion (Chen et al., 1990). Through the use of nucleic acid infusion, PD were found to originate from the catabolism of purines of both endogenous and exogenous origin (Verbic et al., 1990). Concentrations of nucleic acids in the rumen are used to estimate MN production because nucleic acids from the diet were shown to be degraded in the rumen (Smith and McAllen, 1970). As a result, most of the purines and pyrimidines found in the duodenum are assumed to originate from microbial protein production.

Topps and Elliott (1965) originally suggested that urinary excretion of PD such as allantoin could be a useful indicator of rumen microbial protein flow from the rumen. Since then, many researchers have used urinary and milk excretion of PD including allantoin and uric acid as a non-invasive method to predict rumen microbial protein production with varying results.

Purine Derivatives in Urine

Balcells et al. (1991) concluded that urinary allantoin may be a useful index to estimate duodenal input of purines when animals are fed close to or above their maintenance requirements. Measuring all PD excreted in urine may provide a more accurate estimate of microbial protein production than allantoin alone (Giesecke et al.,

1984; Lindberg et al., 1989). However, urinary allantoin excretion more precisely estimated microbial protein synthesis than all PD excreted in urine because allantoin is excreted in greater concentration compared to the other PD and therefore less error is associated with its measurement (Puchala and Kulasek, 1992).

Several studies have correlated excretion of allantoin, uric acid and total purines to MN flow with variable results. In sheep, a correlation ($R^2 = 0.49$; $P < 0.05$) between urinary excretion of allantoin and duodenal MN flow was observed (Lindberg et al., 1989; Puchala and Kulasek, 1992). A linear relationship ($R^2 = 0.64$) was reported between urinary allantoin excretion and the flow of nucleic acids to the duodenum of sheep (Offer et al., 1978; Antoniewicz et al., 1980). Estimates of MN based on urinary PD excretion by heifers were consistently lower (more that 50 g/d MN) than direct measurements of purine base flow through the intestine (Martín-Orúe et al., 2000). Lindberg and Jacobsson (1990) concluded that urinary purine excretion in ruminants was unaffected by moderate changes in energy intake and by large changes in protein intake.

Various feeding régimes have influenced PD excretion in ruminants with little effect on uric acid but a more pronounced effect on allantoin excretion. In beef cows, increasing SBM content of the diet resulted in increased urinary allantoin and urea excretion but no changes were noted for uric acid (Susmel et al., 1993; Susmel et al., 1994; Susmel et al., 1995). Feeding a high concentrate diet was shown to increase excretion of urinary allantoin and uric acid in multiparous Holstein cows (Gonda et al., 1996; Valadares et al., 1999). A linear relationship was determined by Vercoe (1976) between digestible DMI and urinary allantoin excretion in beef steers and buffalos. In

multiparous Holstein cows, increasing dietary protein resulted in increased urinary allantoin excretion (Moorby et al., 1996; Broderick, 2003). A 36% neutral detergent fiber (**NDF**) diet resulted in lower milk allantoin concentration and urinary PD excretion than a 32 or 28% NDF diet (Broderick, 2003). Ruminal infusion of casein increased the daily yield of MN and PD as compared to duodenal casein infusion (Khalili and Huhtanen, 2002). Reynal and Broderick (2005) reported increased MN yield and urinary allantoin excretion when cows were fed 13.2% RDP as compared to 12.3, 11.7 or 10.6% RDP but no dietary effect on urinary uric acid excretion was noted.

Purine Derivatives in Milk

Only a few studies have evaluated the relationship between MN at the duodenum and allantoin excretion in milk. Allantoin excretion in milk was positively correlated with MN flow ($R^2 = 0.28$; $P < 0.0001$) in lactating multiparous Holstein cows; however, this was determined averaging responses across ten different experiments (Timmermans et al., 2000). Shingfield and Offer (1998) and Giesecke et al. (1994) reported a high correlation between milk allantoin excretion and concentration with milk yield in Holstein cows. However, individual cow milk allantoin concentration and excretion were poorly correlated with urinary purine excretion or calculated microbial protein supply (Shingfield and Offer, 1998). Kirchgessner and Kreuzer (1985) reported that though milk urea increased as dietary crude protein increased, milk allantoin concentration was not altered. As DMI increased, milk yield increased causing a subsequent increase in the overall yield of milk allantoin though there was no change in milk allantoin concentration when

lactating cows were energy and protein depleted followed by normal or excessive nutrient supply (Kirchgessner and Windisch, 1989).

The dietary effects of PD secretion in milk have been reported with variable results. Feeding diets high in energy was shown to increase excretion of milk allantoin in lactating cows (Kirchgessner and Kaufmann, 1987; Lebzien et al., 1993; Valadares et al., 1999). Dry matter intake was also found to be positively correlated with allantoin excretion in milk (Gonda and Lindberg, 1997). When lactating beef cows were fed increasing amounts of urea, no changes in milk allantoin or uric acid were noted (Susmel et al., 1995). Feed restriction has also been shown to influence milk and urine allantoin and uric acid concentrations (González-Ronquillo et al., 2004). Broderick (2003) fed 15.1, 16.7 or 18.4% CP and noted no effect on milk allantoin concentration. Giesecke et al. (1994) showed that energy intake was correlated with the concentration of allantoin in milk in Holstein cows ($R^2 = 0.80$; $P < 0.001$).

Correlation of Urine and Milk PD Excretion

Gonda and Lindberg (1997) reported that urinary excretion of allantoin was positively correlated with its excretion in milk in lactating dairy cows. Allantoin concentrations in milk were correlated with urinary excretion of allantoin in Holstein cows (Vagnoni et al., 1997). Additionally, milk allantoin excretion was highly correlated with urinary PD excretion when milk yield was included as a covariate in the model (Shingfield and Offer, 1998). In ewes, allantoin excretion in milk was not correlated with its excretion in urine although its relationship with urinary purine excretion tended towards significance (Martín-Orúe et al., 1996).

Purine Derivatives in Plasma

Little research has been conducted on the circulating concentrations of PD in plasma as results on the correlation of plasma PD concentration to PD excretion have been variable (Giesecke et al., 1994; Gonda and Lindberg, 1994; Chen et al., 1995). Plasma allantoin was found to be correlated with urinary allantoin in lambs ($R^2 = 0.88$; Fukihara et al., 2003) and to milk and urinary allantoin in cows ($R^2 = 0.84$; Giesecke et al., 1994). Additionally, plasma allantoin is correlated with energy intake and milk yield (Giesecke et al., 1994). It would be beneficial; however, to develop a technique utilizing spot samples of plasma since collection of total urine in the field is difficult (Chen et al., 1997).

Digesta Flow

In order to directly measure passage of microbial protein or undigested feed, it is necessary to determine the passage rate of digesta.. Passage rate from the rumen plays an important role in controlling appetite (Welch, 1982), ruminal fill (Jung and Allen, 1995), extent of ruminal degradation (Ørskov and McDonald, 1979), and efficiency of microbial protein synthesis (Harrison and McAllan, 1980). The rate-limiting step in digestion in the rumen is the physical breakdown of feed particles (Mosely and Jones, 1984). Flow of digesta out of the rumen is influenced primarily by the size and density of the feed particle. Particles above a certain critical size are thought to be retained in the rumen and are rarely found further down the digestive tract which led to the concept of critical size (Ulyatt et al., 1986). It has been suggested that 1.18 mm is the critical size for both sheep and cattle (Poppi et al., 1980, 1985) though cattle tend to selectively retain a higher proportion of particles than do

sheep (Lechner-Doll et al., 1990). Studies have shown, however, that digesta can leave the rumen of dairy cows at particle sizes above 4.75 mm and are excreted in feces at 2.36 mm (Huhtanen et al., 1997). Even in sheep, digesta particles above 2.0 mm can flow out of the rumen (Troelsen and Campbell, 1968).

Not only has it been reported that critical size plays a role in particle passage, but the idea of functional specific gravity (**FSG**) also affects retention of particles in the rumen (Lechner-Doll et al., 1990). The idea of FSG pertains to the solid, liquid and gaseous makeup of a feed particle. A particle that has been digested and has little bacterial attachment and/or is undergoing minimal fermentation would have a high FSG and have a greater probability of flowing out of the rumen than one with a lower FSG. Since bacterial fermentation occurring on feed particles causes the entrapment of gas, the FSG would be lower and these particles would selectively be retained in the rumen.

Rumen bacteria contribute a large proportion of N flowing through the duodenum of dairy cattle (Clark et al., 1992). Therefore understanding the flow of ruminal bacteria attached to various particle sizes will contribute to understanding the digestive process and may facilitate determination or prediction of microbial protein flow rates. Particle-associated bacteria range from 50 to 70% of the total bacteria in the rumen (Cheng et al., 1977; Merry and McAllan, 1983; Craig et al., 1987) with the remainder in the liquid fraction of rumen contents. Few studies have compared the flow of particle-associated bacteria from the rumen to other segments of the digestive tract (Ørskov et al., 1986; Ahvenjärvi et al., 2000) or between various particle sizes (Yang et al., 2001). As a result, since rumen bacteria are an integral part of nutrition

and digestion in the dairy cow, understanding of the attachment of bacteria to various particle sizes flowing through the digestive tract is required to accurately quantify microbial protein flow.

Omasal and Rumen Sampling

Cannulation has made the study of the intricacies of digestion and nutrient utilization through the gastrointestinal tract of ruminants possible. Cannulation permits access to the digestive tract for digesta sampling, infusions, digestibility determinations and various other determinations that may aid in understanding digestive processes in ruminants. However, duodenal cannulation has been linked to decreased feed intake and decreased milk yield (McRae and Wilson, 1977; Wenham, 1979). For some nutrients not absorbed in the omasum, the quantity leaving the rumen through the reticulo-rumen orifice should equal the quantity flowing through the duodenum. Therefore, sampling digesta at the reticulo-rumen orifice should enable prediction of what is flowing through the duodenum, avoiding duodenal cannulations. Samples at the reticulo-rumen orifice would contain less endogenous N than in the duodenum (Ørskov et al., 1986) and better represent the particle- and liquid-associated bacterial fractions (Ahvenjärvi et al., 2000). Digestion and absorption may occur as digesta flows to the duodenum (Faichney et al., 1997) and the acidity and enzyme activity encountered as particles flow through of the abomasum (Firkins et al., 2006) may influence digesta make-up and flow rate.

When samples were collected from the omasum, Huhtanen et al. (1997) inserted the sampling device through the rumen cannula and reported minimal effects on digestion and production. A decrease in DMI was observed and the digesta that

was collected seemed to underestimate the particulate phase though the double-marker method (Faichney, 1994) was used to calculate true digesta flow. Other procedures require omasal cannulation (Rupp et al., 1994) or consist of inserting a tube through the reticulo-omasal orifice via the ruminal cannula to collect digesta using a vacuum (Punia et al., 1988). The tube must be reinserted each time of sampling for the latter.

Ahvenjärvi et al. (2000) determined digesta flow at the omasum and at the duodenum of cows and found that less organic matter was flowing through the omasum than through the duodenum. However, more NDF and acid-detergent fiber (**ADF**) was flowing out of the omasum than through the duodenum. Digestibility of organic matter was higher when based off of omasal canal flow than when calculated using the duodenal flow but the opposite relationship was found for NDF and ADF. There was, however a strong correlation between flows of organic matter, NDF and ADF between the two sampling sites. Differences were also found between microbial N flows as sampled from the rumen, the omasum or the duodenum though correlations between duodenal and omasal canal flows existed.

Therefore, there is the possibility to use rumen, reticulum or omasal samples to predict flows of various digestible fractions through the duodenum. This could result in fewer cannulations of research animals as well as a better understanding of digesta flow, especially MN flow, through the dairy cow.

Conclusion

As the public concern regarding the environment increases, animal agriculture must be prepared to deal with regulations regarding how waste, consisting of various forms of N, is managed on the farm. The contribution of N to the environment can be

decreased through dietary protein manipulation and through feeding cows closer to their protein requirements without overfeeding. Rumen microflora are of great importance to the overall nutrition of the dairy cow, but are especially important to meeting her protein requirements. Through understanding these rumen bacteria and what substrates increase their growth rate, their flow through the digestive tract may increase, therefore increasing the supply of high-quality protein to the small intestine for absorption. Additionally, being able to predict how much MN is flowing through the digestive system is also of great importance in ration formulation. Through the use of purine bases, MN flow may be accurately predicted which would be a novel on-farm technique to assist in ration formulation, as well as decreasing the use of cannulated animals. Cannulations could also be minimized by using rumen or reticulum samples to determine flows of various digesta fractions out of the rumen and through the duodenum.

Therefore, there are several objectives to this research. The first objective of this research was to evaluate the effect of two RDP sources (urea vs. casein) on microbial protein flow out of the rumen and through the duodenum, production, purine excretion and nitrogen metabolism. The second objective of this research was to determine the flow rates of various particle sizes out of the rumen and through the duodenum as well as determine the composition and microbial attachment for the different particle sizes. The final objective of this research was to determine differences in measurements of DM, NDF, starch, N and MN flow, and DM, NDF and starch digestibility when measured from the reticulum compared to measurements from the duodenum.

Chapter 2

COMPARISON OF ANALYTICAL METHODS AND THE INFLUENCE OF MILK COMPONENTS ON MILK UREA NITROGEN RECOVERY

ABSTRACT

The objectives of this study were to compare analytical instruments used in independent laboratories to measure milk urea nitrogen **(MUN)** and determine if any components in milk affect the recovery of MUN. Milk samples were collected from 100 Holstein cows fed one ration in a commercial dairy herd with a rolling herd average of 9500 kg. Half of each sample was spiked with 4 mg/dl of urea N while the other half was not, to determine recovery. Both milk samples were sent to 14 independent laboratories involved in the MUN Quality Control Program through National Dairy Herd Improvement Association and analyzed for MUN, fat, protein, lactose, somatic cell count **(SCC)**, and total solids. The laboratories analyzed MUN using CL-10 (n=3), Skalar (n=2), Bentley (n=3), Foss 4000 (n=3) or Foss 6000 (n=3) systems. When recovery of MUN was evaluated among the 5 analytical methods, the mean recoveries for the Bentley, Foss 6000 and Skalar systems were 92.1 (SE = 2.76%), 95.4 (SE = 10.1%), and 95.1% (SE = 7.61%), respectively, and did not differ from each other. However, MUN recovery was 85.0% (SE = 2.8%) for the CL-10 system and 47.1% (SE = 9.9%) for the Foss 4000 system, both of which differed from the other three systems. Recoveries from Foss 4000, Foss 6000 and Skalar varied among laboratories using the same instrument. As initial MUN concentration increased, recovery decreased using the Bentley and CL-10 systems. Increasing milk fat resulted in a decrease in recovery using the Foss 6000 system. For four of the five methods, recovery of MUN was not associated with specific milk components. Recovery of MUN was inconsistent for laboratories using the Foss 4000 and the Foss

6000 method and using these systems may result in an overestimation or underestimation of MUN.

INTRODUCTION

Milk urea nitrogen (**MUN**) can be a practical indicator of the protein utilization of lactating cows in dairy cattle nutrition programs (Jonker et al., 1999). Monitoring MUN offers the potential to evaluate the protein concentration in lactating cow rations, reduce farm expenses, and reduce environmental nitrogen loading (Jonker et al., 1998). However, this potential depends largely on the accuracy of MUN values from the laboratory.

Each month, National DHIA sends 12 bulk tank samples in duplicate, selected for a range in milk components, to each DHIA laboratory and to reference testing laboratories to characterize accuracy among laboratories (National DHIA, 2003). This procedure may improve accuracy among laboratories, but does not reveal sources of variation and error in MUN measurement. Also, the use of bulk-tank milk may underestimate variation associated with changes in milk composition because of the tighter range in milk components as compared to individual cow milk.

Most laboratories that test for MUN use one of five automated methods: CL-10™ (Eurochem, 00040 Ardea (Roma) Via Pontina Km.34), Skalar™ (Skalar, 56000 Oakbrook Pkwy, Norcross, GA 30093), Bentley Chemspec™ (Bentley Instruments Inc., 4004 Peavey Rd, Chaska, MN 55318) , Foss 4000™ (Foss Inc., Eden Prairie, MN 55344), or Foss 6000™ (Foss Inc., Eden Prairie, MN 55344). The CL-10, Skalar, and Bentley systems calculate MUN by measuring the ammonia formed from urea after treating the sample with urease. The CL-10 measures the change in pH caused

by the release of ammonia. The Skalar and Bentley use a modified Berthelot reaction for colorimetric determination of the ammonia formed from urease hydrolysis of urea. The Foss 4000 system and the newer Foss 6000 system measure MUN indirectly using infrared spectroscopy **(IRS)**.

The first objective of this study was to compare the accuracy of analytical instruments used to determine MUN and identify differences in initial MUN and recovery of added urea. The second objective was to determine if milk fat, protein, lactose, or somatic cell count **(SCC)** influence the recovery of added urea.

MATERIALS AND METHODS

Sample Preparation

In November of 2002, a commercial Holstein dairy herd in Maryland was sampled. Cows averaged 164 days in milk and average milk production was 30 kg/d. Individual milk samples (1.5 L) were collected from 100 Holstein cows and were immediately placed on ice and processed within 4 hours of collection.

Milk from each cow was mixed and then divided into two 750-ml sub-samples using volumetric flasks. Each sub-sample was then transferred to an Erlenmeyer flask. To one flask, 1 ml of water was added as a control. The other flask was spiked with 1 ml of urea solution to result in a final concentration of 4 mg/dl higher than the control. Each flask was inverted 10 times to mix and then divided into 14 randomly-numbered 50-ml milk vials containing a preservative (Broad Spectrum Microtabs II, D&F Control Systems Inc., 3401 Crow Canyon Road, Son Ramon, CA 94583).

Sample Analysis

Fourteen independent laboratories were selected based on their participation in the National DHIA MUN Quality Control Program. All milk samples were sent overnight in Refrigerated Styro-Shippers (Lincoln Suppliers Inc., Owatonna, MN). Each set of samples was analyzed on a CL-10 (3 laboratories), Bentley (3 laboratories), Skalar (2 laboratories), Foss 4000 (3 laboratories), or Foss 6000 (3 laboratories). Milk samples sent to each laboratory included 100 spiked and 100 control samples.

Recovery Calculation

Recovery of added urea N was calculated by the difference in the analyzed MUN concentration between the control and treated milk samples. This difference was then divided by 4 mg/dl (amount of urea added) resulting in the fraction recovered where 1 indicates perfect recovery.

Statistics

Statistical analyses were performed using PROC MIXED of SAS Version 8 (1999). Contrast statements were used to compare methods and laboratories within methods. The model used to analyze differences in initial MUN concentrations and MUN recovery among laboratories and methods included the random effects of cow and laboratory nested within method and the fixed effect of method as follows:

$$Y_{ijk} = \mu + C_i + m_j + L(m)_{jk} + e_{ijk}$$

where

Y_{ijk} = observations for dependent variables;

μ = overall mean;

C_i = random effect of cow i;

m_j = fixed effect of method j;

$L(m)_{jk}$ = random effect of laboratory k nested within method j;

e_{ijk} = residual error.

The nested effects of $L(m)_{jk}$ were allowed to have variances that differed among methods. The hypothesis of heterogeneous variances was tested using a likelihood ratio statistic (Littell et al., 1996). Multiple regression models were used to evaluate the effects of milk components on MUN recovery for each analytical method. A full model including the fixed effect of laboratory, milk fat, milk protein, initial MUN concentration, log SCC and interactions of each component within laboratory was evaluated. Insignificant ($P > 0.10$) variables and interactions were removed one at a time and the reduced model resulted for each analytical method. Results are presented as least-square means. Significance was noted at $P < 0.05$ and trends at $0.05 < P < 0.10$.

RESULTS AND DISCUSSION

Recovery Among Methods

Initial MUN concentration across all methods and laboratories averaged 11.7 mg/dl (SE = 1.6 mg/dl) and did not differ among methods ($P > 0.10$). Even though the average initial MUN concentrations were comparable, there were significant differences in the variance of initial MUN among laboratories within the same method (likelihood ratio statistic χ^2=132.0, df=4; $P < 0.001$). The lowest variation occurred within the CL-10 method while the highest variation occurred within the Foss 4000 method. Additionally, milk fat averaged 4.1% (SE = 0.50%) and milk protein averaged 3.2% (SE = 0.34%).

Recoveries of added urea N for each analytical method are reported in Table 2-1. Recoveries for the Bentley, Foss 6000 and Skalar methods were 92.1, 95.4, and 95.1%, respectively, and did not differ from each other ($P > 0.10$). The recovery for the Foss 6000 method may be misleading, however, because two laboratories had average recoveries greater than 105% and the SE for that method was 10.1%. Recovery for the CL-10 method averaged 85.0% (SE = 2.76%) and was significantly lower than the Bentley, Foss 6000 and Skalar methods ($P < 0.05$). However, recovery for the CL-10 method was higher than that of the Foss 4000 method which averaged 47.1% (SE = 9.88%; $P < 0.05$). Recovery for the Foss 4000 method which is an IRS instrument was significantly lower than for all the other methods ($P < 0.05$).

After interviewing several laboratory managers, it was found that the majority of laboratories on this study were using a set of 12 randomly selected bulk tank samples to calibrate the machines on a weekly basis. These samples are first analyzed on a CL-10 instrument as a reference point and are sent out to laboratories to be used for calibration. The range in MUN values varies from week to week and may result in a range as small as 12 to 22 mg/dl with most samples in the middle of the range. Such a set of calibration samples would place little weight on high or low MUN samples possibly explaining the inaccuracy of high and low measurements.

There were significant differences in the variance of MUN recovery among laboratories within the same method (likelihood ratio statistic χ^2=115.6, df=4; $P <$ 0.001). The highest variation in recovery occurred within the Foss 4000 and 6000 methods while the remaining three methods had similar low variability.

Recovery Among Laboratories

To determine if the recovery for the Foss 4000 and the other methods was consistent across laboratories, each laboratory within a method was evaluated and results are presented in Table 2-2. Since the identity of individual laboratories could not be revealed, each laboratory within a method was identified by a letter (eg., A, B, or C). There was little variation among recoveries for the Bentley and CL-10 analytical methods and there were no differences among laboratories for each method ($P > 0.10$). However, overall recovery using the Bentley method was higher than recovery for the CL-10 method ($P < 0.05$). Therefore the Bentley method is both more repeatable and may be the most reliable way to analyze MUN. Since only two laboratories using the Skalar method were evaluated in this study, no assumptions about the repeatability and accuracy of MUN analysis will be made. Two laboratories utilizing the Foss 6000 method had recoveries of over 105% while the third laboratory had a recovery of only 75.3% ($P < 0.0001$). As a result, while an average recovery of 95.4% may appear to be adequate, this method may not be repeatable and may result in an overestimation or underestimation of MUN depending on the laboratory used. Finally, the Foss 4000 method had recoveries ranging from 30.4 to 64.2% and the recoveries for all three laboratories were different from each other ($P < 0.0001$). As a result, not only is the recovery far below what is desirable resulting in an underestimation of MUN, but there is also too much variation between laboratories to result in an accurate MUN value.

Effect of Milk Components on Recovery

Because MUN recovery was incomplete and variable among methods and laboratories, milk components were analyzed to determine if they interfered with

MUN recovery. Samples were analyzed for fat, protein, lactose, SCC and initial MUN. A full multiple regression model including laboratory, milk fat, milk protein, initial MUN concentration, log SCC, total solids and interactions of each component with laboratory was evaluated for each method individually. Insignificant ($P > 0.10$) variables and interactions were removed one at a time resulting in a reduced model for each analytical method (i.e. a backward variable selection procedure; Kleinbaum et al., 1998). Results from each reduced model are presented in Table 2-3. Initial MUN concentration had an inverse negative effect on recovery for the Bentley and CL-10 methods whereby as initial MUN increased, recovery decreased. This effect may be due to the narrow range of standards used by each lab to calibrate the instrument. Though significant, the effect on recovery was small at 1.8% or 1.6% of recovery per 1 mg/dl of MUN for the Bentley and CL-10 methods, respectively ($P < 0.001$). It has been previously shown that milk fat percentage is positively correlated with MUN in high-producing herds (Rajala-Schultz and Saville, 2003), and that high milk fat may result in misleading MUN values (Carlsson and Bergström, 1994). Milk fat decreased MUN recovery among laboratories using the Foss 6000 method ($P < 0.05$). In this case, there was a decrease in recovery of 5.8 percentage units for every 1 unit increase in fat percentage. Effects associated with laboratory and interactions with laboratory, especially with the Foss 6000 method, may be due to variation in analyzing these components between laboratories.

CONCLUSIONS

Milk urea nitrogen recovery for the Foss 4000 method was significantly lower than the recovery for the other methods including CL-10, Skalar, Bentley, and Foss

6000. Additionally, recovery for laboratories using this method was quite variable ranging from 30.4 to 64.2%. Therefore, analyzing MUN using the Foss 4000 may result in an underestimation for the higher values of MUN. Since recoveries were greater or less than 100% for the Foss 6000 method depending on the laboratory, using this system may result in an overestimation or underestimation of MUN. The Bentley instrument was the most repeatable among laboratories and resulted in higher recoveries as compared with the CL-10. This study suggests that improved calibration among laboratories using the same method may improve the reproducibility and accuracy of MUN values. Consistent and accurate MUN values are important if they are to be used to better formulate dairy rations or estimate urinary N excretion.

Table 2-1. Percent Recovery of Urea Nitrogen Among Analytical Methods.

Method	Recovery (%)[1]	SE (%)
Bentley	92.1[a]	2.76
CL-10	85.0[b]	2.76
Foss 4000	47.1[c]	9.88
Foss 6000	95.4[a]	10.1
Skalar	95.1[a]	7.61

[a,b,c]Means within a column with unlike superscripts differ ($P < 0.05$).

[1]Recovery = (Treated MUN - Control MUN)/4 mg/dl.

Table 2-2. Percent Recovery of Milk Urea Nitrogen Among Laboratories

Lab	Recovery (%)[1]	SE (%)	*P* value[2]
Bentley A[3]	97.6	1.41	NS[4]
Bentley B	88.9	1.48	
Bentley C	89.9	1.57	
CL-10 A	86.7	1.33	NS
CL-10 B	88.7	1.44	
CL-10 C	79.6	1.07	
Foss 4000 A	46.7	5.90	< 0.0001
Foss 4000 B	30.4	1.50	
Foss 4000 C	64.2	1.58	
Foss 6000 A	105.3	3.87	< 0.0001
Foss 6000 B	105.8	2.39	
Foss 6000 C	75.3	1.39	
Skalar A	88.3	0.84	0.0022
Skalar B	101.8	7.72	

[1]Recovery = (Treated MUN - Control MUN)/4 mg/dl.

[2]*P* value = significance of difference among laboratories within each method.

[3]A, B, C = denotes different laboratories within a method.

[4]NS = not significant.

Table 2-3. Effect of Milk Components on Milk Urea Nitrogen Recovery

Method	Variable[1]	*P*-value
Bentley	Lab	< 0.05
	Initial MUN (slope = -1.8% per mg/dl)	< 0.001
	Laboratory*Lactose	< 0.05
CL-10	Laboratory	< 0.0001
	Initial MUN (slope = -1.6% per mg/dl)	< 0.001
Foss 4000	Laboratory	< 0.0001
Foss 6000	Fat (slope = -5.8% per % fat)	< 0.05
	Laboratory*Fat	< 0.01
	Laboratory*Protein	< 0.01
Skalar	Laboratory	= 0.10

[1]Significant variables left in the reduced model.

Chapter 3

EFFECT OF RUMINALLY DEGRADED PROTEIN SOURCE ON RUMINAL DIGESTION AND MICROBIAL YIELD IN HOLSTEIN COWS

ABSTRACT

To evaluate the effect of two ruminally degraded protein sources (protein vs. non-protein N) on ruminal digestion and microbial protein flow, eight early lactation Holstein cows were arranged in a repeated 4x4 Latin square design balanced for carryover effects with 21-d periods. All diets were isoenergetic (1.64 Mcal/kg) and had the same rumen undegraded protein (**RUP**) content (5.6%). Cows were fed either a base diet containing 12.2% CP (**BASE**) or one of three treatment diets containing 15% CP supplemented with urea (**UREA**), casein (**CAS**) or both (**U+C**). Dry matter intake was lowest for cows fed the BASE diet (20.3 kg/d), which could be attributed to lower DM and NDF digestibility, while cows fed the other three diets averaged 22.5 kg/d and were more digestible. Ruminal starch digestibility was highest when cows were fed the U+C diet and NDF digestibility was higher when cows were fed the CAS and U+C diets. Total tract digestibility of NDF and starch was lower for cows fed the BASE diet but did not differ between the UREA, CAS and U+C diets. Microbial N flow through the duodenum in cows fed the BASE diet was 237 g/d compared with 292 g/d for cows fed the other three diets. Milk yield averaged 28.0 kg/d (SEM=2.6) for cows fed the BASE diet compared 33.7 kg/d for cows fed the U+C diet. Cows fed the UREA and CAS diets yielded 33.2 and 32.6 kg/d of milk, respectively, which were not different from each other but were higher than the BASE diet. However, when milk yield was expressed as 4% fat-corrected milk yield, cows fed the UREA, CAS and U+C diets responded similarly. Milk fat and protein percentages did not differ among treatments. Milk urea nitrogen was lowest for cows fed the BASE diet averaging 6.6 mg/dl (SEM = 0.86) while MUN from cows fed the other diets averaged

12.5 mg/dl and did not differ from each other. Though the energy content of the diets was the same, the addition of casein to the diet may have provided more available amino acids to the rumen microbes which may have shifted the microbial population in the rumen. Digestibility was improved when casein and the combination of urea and casein were fed compared to urea alone as the main source of RDP. Therefore a source of RDP with amino acids was required to maximize both fiber and starch digestibility.

Keywords: rumen degraded protein, amino acids, non-protein nitrogen urea, microbial nitrogen

INTRODUCTION

Reactive N lost to the environment from agriculture contributes to eutrophication of streams and estuaries, ground water contamination, smog and acidity of soils and water (Galloway, 2003). Only 21 to 38% of the N that was brought on to farms as feed, fertilizer and via N fixation by legumes was exported off the farm as meat and milk (Klausner, 1993). The remaining N (farm inputs minus exported products) is eventually lost from the farm to air and water resources (Kohn et al., 1997). Dairy cows contribute a minimum of 750,000 metric tons of N per year into the environment of the United States (St-Pierre and Thraen, 1999). Nitrogen can enter the environment through volatilization of ammonia to the air, nitrate leaching into ground water and run-off to surface water from manure or other fertilizers. Even when manure and other fertilizers are properly managed, much N is lost to the environment. Therefore, reducing N losses requires better feeding and herd

management to reduce the need for crop production and manure application (Kohn et al., 1997).

Compared to non-ruminants, ruminant animals are relatively inefficient at converting dietary crude protein (**CP**) into a usable N source because of the extensive fermentative activity of the rumen microbial population (Broderick et al., 1991). A significant portion of the N fed to high producing dairy cows is not incorporated into microbial protein which results in an elevated rumen NH_3 pool that is inevitably lost through urinary N excretion (Sannes et al., 2002). Rumen microbial protein synthesis is critical for high producing dairy cows as it makes up 60 to 85% of the metabolizable protein requirements for maintenance, growth, gestation, and lactation in dairy cattle (Stern et al., 1994). Understanding of the requirements of rumen microbes will aid in formulating rations to maximize microbial nitrogen (**MN**) yield while avoiding over-feeding N which could potentially lead to environmental pollution.

In the rumen, cellulolytic bacteria primarily use ammonia-N (**NH_3-N)** while amyolytic bacteria prefer to use amino acid N (**AAN**) as they are more proteolytic than cellulolytic bacteria (Siddons and Paradine, 1981; Wallace et al., 1997). Cows can thrive with urea as the only source of dietary N (Virtanen, 1966); however feeding true protein to cattle typically improves performance (Stock et al., 1986; Rooke and Armstrong, 1989). Several studies have evaluated the effects of feeding urea versus an AAN source both in vitro and in vivo on MN yield, N metabolism and production with various results. In pure bacterial cultures, both amino acid and peptide supplementation increased the maximum specific growth rate for several cellulolytic and amyolytic bacteria as compared to the addition of $(NH_4)_2SO_4$ (Cruz Soto et al.,

1994). In 1993, Armentano et al. fed mid-lactation Holstein cows either a degradable true protein source or urea and reported no differences in intake, milk production or milk components. Using urinary purine excretion to predict the flow of MN from the rumen, Cruz Soto et al. (1994) found no differences between infusing peptides, amino acids or ammonia into the rumen of adult sheep. Broderick et al. (1993) reported no differences in DMI, milk yield or MUN when cows were fed urea or soybean meal. However, when an AAN source was added to a low DM alfalfa silage, DMI increased by 2 kg/d and milk yield increased by 3 kg/d as compared to adding urea though MN yield was not quantified. When sheep were fed either a hay-based or beet pulp-based diet supplemented with either casein or urea, nitrogen source had no effect on MN yield with the hay-based diet (Chikunya et al., 1996). However, casein supplementation resulted in an increased bacterial count and MN yield when added to a beet pulp-based diet possibly because of the rapidly degradable fiber (Chikunya et al., 1996). Sannes et al. (2002) compared feeding urea or SBM as the primary protein source to lactating cows and no changes in DMI, milk yield, microbial protein or purine excretion was noted.

To date, however, there has been no study evaluating the effects of non-protein nitrogen (**NPN**) and/or AAN on MN yield, production and N metabolism in lactating dairy cows. Therefore, the first objective of this study was to evaluate the effect of two RDP sources (urea vs. casein) on MN flow through the duodenum using ^{15}N as a microbial protein marker. The second objective was to determine differences, if any, on production performance, ruminal and duodenal digestibility and nitrogen metabolism due to source of RDP.

MATERIALS AND METHODS

Cows and Treatments

Animal experiments were conducted at the Beltsville Agricultural Research Center in accordance with the USDA and University of Maryland animal care and use committees. Eight multiparous Holstein cows were ruminally and duodenally cannulated approximately 4 wk prior to their expected calving date. After parturition, cows were divided into to two groups based on days in milk (**DIM**). Cows in the first group averaged 36.3 DIM (SEM = 9.5) while cows in the second group averaged 35 DIM (SEM = 2.5) at the start of the trial. Treatments were applied to cows arranged in a repeated 4x4 Latin square design balanced for carryover effects with 21-d periods. Cows were housed in tie-stalls in a climate-controlled building and were exercised twice daily.

Cows were fed once daily at 1400 h targeting 10% orts. Treatment diets were based on corn silage and all ingredients were kept constant across treatments except wheat straw, corn starch, urea and casein (Table 3-1). Treatment diets were built upon the base diet (**BASE**) which contained 0.33% urea (DM basis) to increase the N content. The urea diet (**UREA**) contained less wheat straw and more corn starch to make up for the addition of the urea which contained no energy. The casein diet (**CAS**) contained the same amount of wheat straw and urea as the base diet but had less corn starch than any other dietary treatment because of the addition of casein which is both an energy and N source. The urea and casein diet (**U+C**) contained urea and casein to supply equal amounts of nitrogen to the diet.

Marker Dosing Timeline

Each of the four treatment periods lasted 21 d. The first 14 d were for adaptation, d 15 through 21 were for marker infusions and the last 3 d were for sample collections. For microbial protein yield determination, labeled ammonium sulfate [$(^{15}NH_4)_2SO_4$] was continuously infused into the rumen. To determine flow rates out of the rumen and through the duodenum, Co-EDTA and Cr-mordanted NDF were intraruminally administered as liquid and solid passage markers, respectively (Uden et al., 1980). Cows were continuously infused with $(^{15}NH_4)_2SO_4$ and Co-EDTA at the rate of 1 g/d and 54 mg Co/d, respectively, from 1200 on d 15 through 1200 on d 21. Intraruminal dosing of Cr-mordanted NDF occurred every 8 h from 1000 h on d 16 for 24 h and the dosing rate changed to every 4 h starting at 1000 h on d 17 through 0800 on d 21.

Sample Collection Timeline

Total collection of urine and feces began at 1200 h on d 19 and ended at 1200 h on d 21. Rumen, reticulum and duodenal sampling occurred every 4 h at 1200, 1600, 2000 and 2400 h on d 19, at 0400, 0800, 1400, 1800 and 2200 h on d 20 and at 0200, 0600 and 1000 h on d 21. Spot urine, fecal and blood samples were collected every 4 h at 1200, 1600, 2000 and 2400 h on d 19 and at 0400 and 0800 h on d 20. Milk samples were collected during the PM milking on d 19, both AM and PM milkings on d 20 and the AM milking on d 21.

Milking and Milk Sampling

Cows were milked twice daily in tie-stalls at 0700 h and 1800 h. Milk yield was recorded for both the am and pm samples. Milk samples obtained during the collection period (p.m. d 19, a.m. and p.m. d 20 and a.m. d 21) were composited in

proportion to total milk yield. One composite sample was sent to Lancaster DHIA (Manheim, PA) for component analysis including fat, protein, solids, SCC and MUN (Bentley Chemspec; Chaska, MN), and 4% FCM (kg/d) was calculated as 0.4 x milk yield (kg/d) + 15 x fat yield (kg/d) (NRC, 2001). Another composite sample (40 ml) was freeze-dried and analyzed for ^{15}N enrichment (UC Davis Stable Isotope Facility, Davis, CA). A final composite sample (40 ml) was analyzed for NH_3-N and ^{15}N enrichment of NH_3-N.

Urine Collection

Indwelling Folley bladder catheters were inserted at 1200 on d 19 of each collection period for total urine collection. Catheters were promptly removed at 1200 on d 21 of each period. Urine was collected in plastic jugs containing 50% HCl to prevent ammonia volatilization by keeping the pH below 3. At the end of the collection, urine was mixed thoroughly, sub-sampled and frozen for later analysis of N and NH_3-N.

Rumen Sampling

Rumen liquid was sampled using a PVC pipe (12.7 mm i.d.) with a mesh affixed to the end to keep particles out of the sample. The opposite end of the PVC pipe was attached to a vacuum. Approximately 250 ml of fluid was collected at each sampling time point from the caudal, cranial, dorsal and ventral areas of the rumen. The pH of this sample was immediately determined and recorded. For rumen NH_3-N and ^{15}N enrichment of NH_3-N determination, 10 ml of rumen fluid was added to 0.2 ml of 10% H_2SO_4 to prevent volatilization. Another 10 ml portion of rumen fluid was

added to 10 ml formic acid for later analysis of VFA. Both samples for NH_3-N and VFA analysis were composited over each 48 h period and frozen until later analysis.

Reticulum Sampling

A sample from the reticulum was collected using PVC pipe (12.7 mm i.d.) attached to a vacuum with additional holes drilled no farther than 2 inches from the end to allow for both fluid and particle collection. Approximately 250 ml of sample was collected every 4 h. This sample was poured over a set of sieves to allow for particle separation and liquid collection. The sieves were 2 mm, 1 mm, 0.5 mm and 0.25 mm from top to bottom. The liquid fraction was differentially centrifuged for microbial pellet formation. The liquid was centrifuged at 500 x g for 15 min at 4 °C for solid associated bacteria (**SAB**) pellet formation. The SAB pellet was immediately frozen and later freeze-dried and composited over each period for analysis of N and ^{15}N. The supernatant was decanted and recentrifuged at 4640 x g for 30 min at 4 °C for liquid associated bacteria (**LAB**) pellet formation. The LAB pellet was immediately frozen and later freeze-dried and composited over each period for analysis of N and ^{15}N.

Duodenal Sampling

A 250 ml sample was collected from the duodenum every 4 hours. This sample was poured over the same set of sieves previously described. The particles were gently rinsed and composited over each period. Particle fractions were later analyzed for total N, ^{15}N to determine SAB and Cr to determine the solid flow rate. The liquid fraction that remained in the collection pan was composited over each

period and frozen. This liquid fraction was then freeze dried for analysis of total N, ^{15}N to determine LAB and Co to determine the liquid flow rate.

Diet and Particle Analysis

The DM percentage of corn silage samples was determined bi-weekly for diet adjustment to ensure consistent forage to concentrate ratio (DM basis) throughout each period. Daily samples of corn silage, wheat straw and concentrate mix were collected and composited from d 15 through 21 of each period. These samples were oven-dried at 60°C, ground through a Wiley mill (1-mm screen; Arthur H. Thomas, Philadelphia, PA) and analyzed for NDF, ADF and lignin sequentially (Mertens, 2002). Starch was also measured by an enzymatic method (Karkalas, 1985) and glucose was measured with a glucose oxidase method (Glucose kit #510; Sigma Chemical Co., St. Louis, MO). Additionally, the samples were analyzed for DM (100°C overnight), ash (500°C overnight) and total N by Dumas combustion (Leco FP428, Leco Corp., St. Joseph, MI). Particles collected from the duodenum were analyzed for NDF (Van Soest and Wine, 1967), starch and total N.

Ammonia Analysis

Ammonia was analyzed in rumen fluid and milk. At each sampling time point, 10 ml of rumen fluid was added to 0.2 ml of 10% H_2SO_4 to prevent volatilization of NH_3. These samples were composited over the 48 h collection period and frozen. For analysis of NH_3-N, each sample was diluted 20x. On a 96-well plate, 25 _l of sample, 100 _l of phenol-nitroprusside-tartrate and 100 _l of alkaline hypochlorite were added in each well in duplicate. The plate was allowed to sit for 30 min for the reaction to occur and then was run on a plate reader at an absorbance of 570 nm.

To quantify ^{15}N-NH_3 in rumen fluid, the NH_3 had to be extracted from solution. To do this, each sample was thawed and mixed thoroughly. In a 1 L volumetric flask, 100 ml aliquot of the rumen fluid and 80 ml of NaOH was added to release the NH_3 from solution. This flask was immediately placed on a burner with the top of the flask attached to a condenser to trap the NH_3. The tube on the end of the condenser was placed in and Erlenmeyer flask containing 100 ml of 4% boric acid containing a color indicator which changed from red to blue once NH_3 bubbled into the solution. The rumen fluid plus NaOH was allowed to boil until the 100 ml volume of boric acid increased to approximately 150 ml. The NH_3 trapped in the boric acid was brought to a volume of 200 ml in a volumetric flask and transferred to a storage container and frozen. To quantify ^{15}N enrichment of NH_3-N, this sample was freeze dried, placed into tin capsules and analyzed (UC Davis Stable Isotope Facility, Davis, CA). For quantification of ^{15}N-NH_3 in milk, NH_3 was extracted using the same procedure as used for rumen fluid except 30 ml of milk and 2 ml of boric acid were used per sample.

VFA Analysis

Analysis of VFAs in rumen fluid using gas chromatography was preformed based on procedures described by Richardson et al. (1989) and López et al. (1999). An internal standard of 20 mM 2-ethylbutyric acid in a 20% o-phosphoric acid solution was used. A 0.8 ml aliquot of rumen fluid was added to 0.20 ml of the internal standard and mixed. This was centrifuged at 7,000 x g for 30 min for removal of solids from the aqueous phase. The supernatant (0.5 ml) was filtered through a 0.2 μm filter. To this filtered supernatant, 0.7 ml of diethyl ether was added for the

extraction and mixed for 1 min. This was centrifuged at 3,000 x g for 10 min to ensure separation of ether and aqueous phase. An aliquot of the ether interphase was removed and put into a gas chromatography vial with glass insert making sure no water was associated with the ether. Samples and standards were run on the gas chromatogram using conditions reported by Koenig et al (2003). A capillary Supelco Nukol column (30 m x 0.25 mm i.d., 1 μ phase thickness, bonded PEG) and a flame ionization detector were used. Helium was used as the carrier gas. Oven temperature settings are as follows: 100°C for 1 min, ramped at 20°C/min to 140 °C, and then at 8°C/min to 200°C, hold for 5 min. The injector and detector temperatures were 200°C and 250°C, respectively. A standard was run on the gas chromatogram 8-10 times before samples were run and all standards and samples injected at a volume of 1 μl.

Urea Analysis

Reagents for urea analysis. Dimethylformamide (**DMF**) and *N*-(*tert*-butyl-dimethylsilyl)-*N*-methyltriflouroacetamide (**MTBSTFA**) were purchased from Pierce (Rockford, IL). [$^{15}N_2$]Urea was obtained from Cambridge Isotope Laboratories (Andover, MA). Ammonia solution and urea were obtained from Sigma (St. Louis, MO). The AG50W-X8 resin (100-200 mesh, H form) was obtained from Bio-Rad Laboratories (Hercules, CA).

Sample Preparation. Previously frozen composite urine samples not containing an internal standard were thawed. Since an inadequate amount of labeled urea was added at the time of sample collection, duplicate samples (100 _l) were mixed with 100 _l of a new internal standard containing $5.13x10^{-3}$ mg of [$^{15}N_2$]urea/g

of distilled water. These samples were then ready for isolation and derivatization as described below.

Isolation and Derivatization. After samples were prepared as previously described, samples were mixed with 200 _l of 0.1 N HCl to ensure acidity and applied to packed column containing 0.5 ml of the cation-exchange resin (AG50W-X8, 100-200 mesh, H form). The column was rinsed twice with 2ml ddH_20. Urea was eluted with 2ml of 2M NH_3OH followed by 1 ml of ddH_2O. A 50 _l volume of the elutate was transferred to a V-vial and dried at 40 ºC under nitrogen. The TBDMS derivatives of urea were formed by reacting with 100 _l of DMF-MTBSTFA (1:1) at 90 ºC for 20 min. The reaction mixture was then analyzed by the GC/MS.

GC/MS Instrumentation. The samples were analyzed on a HP 6890 gas chromatograph coupled with a HP 5973N quadrupole mass selective detector (Hewlett-Packard, Wilmington, DE). The GC was fitted with a 30 m x 0.25 mm i.d. (0.25 _m film thickness) HP-5 capillary column (Agilent, Wilmington, DE). Injections (1 _l) were made in the split mode using a 40:1 split ratio. Helium was used as the carrier gas at a flow of 0.9 ml/min. The injector port temperature was 250 ºC and the column temperature program was from 160 to 190 ºC at 10 ºC/min and then from 190 to 300 ºC at 30 ºC/min with a 1 min hold at the end of the run. The GC/MS auxiliary temperature was 280 ºC.

The mass spectrometer was operated under electron impact ionization conditions with the following source parameters: electron energy, 70 eV; detector current, 2600 EMVolts; source temperature, 230 ºC; and quadrapole temperature, 150 ºC. The M+0 and M+2 ions were *m/z* 231 and 233 for urea.

Cobalt and Chromium Analysis

All fractions that were flowing through the duodenum including particle and liquid fractions as well as the Cr mordanted NDF used for dosing in the rumen were digested using a nitric/percholoric acid digestion (Perkin-Elmer, 1982). Approximately 0.2 g of sample was weighed out into a 40 ml beaker in duplicate. To this, 10 ml of 70% nitric acid was added in the ventilation hood for organic matter digestion, covered with a watch glass and left over night. Samples were heated on a hot plate and once all samples were boiling, the hot plate was turned off samples were cooled. Concentrated perchloric acid (4 ml) was added to the cooled samples. The hot plate was turned on again and once all the samples were boiling the hot plate was turned off and samples were cooled. The watch glasses were removed from each beaker and rinsed with distilled water above the beaker to ensure no sample condensation was lost. The content of each beaker was transferred to a 100 ml volumetric flask and brought to volume using distilled water. The beaker was covered with parafilm and mixed well by inverting the flask several times. Samples were then transferred into labeled conical vials for analysis of Cr and Co via atomic absorption spectrophotometry (Perkin-Elmer 5100PC Atomic Absorption Spectrophotometer).

Flow Rate and ^{15}N enrichment calculations

Assuming that negligible breakdown and absorption of Cr-mordanted NDF and Co-EDTA occurs as digesta flows out of the rumen and to the duodenum, the intraruminal infusions of both Cr-mordanted NDF and Co-EDTA should equal the flow rate through the duodenum. In order to determine the flow rate of both the solid and liquid fractions the following calculation was used (Faichney, 1993):

Digesta flow = (Co or Cr dose rate)/(Co or Cr concentration in digesta)

Each particle size in the solid fraction was analyzed separately providing four particle flows and one liquid flow. The total N in SAB, LAB and particle fractions collected in the duodenum as well as urine and milk was determined by Dumas combustion (Leco FP428, Leco Corp., St. Joseph, MI). Additionally, these samples (except urine) were analyzed for ^{15}N enrichment (UC Davis Stable Isotope Facility, Davis, CA). Background samples of LAB from the reticulum were collected prior to continuous infusions of $(^{15}NH_4)_2SO_4$ for each period to determine the natural background (**NB**). The following calculation was used to determine the ^{15}N enrichment above the NB (**^{15}N-APE**) in each sample:

$$^{15}\text{N-APE} = {}^{15}\text{N-atom \%} - {}^{15}\text{NB}$$

Assuming that the LAB pellet collected from the reticulum consisted purely of microbial protein and that both the LAB and SAB represent what is flowing with the liquid and solid fractions, the following calculation was used to determine the %N in from microbial origin using SAB as an example:

%N from Microbial Origin in SAB = ^{15}N-APE in SAB/^{15}N-APE in LAB

To then calculate the yield (g/d) of MN flowing in the solid phase through the duodenum, the following calculation was used:

SAB flow = (solid flow x % N in solid fraction)

x (%N from Microbial Origin)

Apparent rumen digestibility (%) and amount digested (g/d) was calculated using flows of nutrients through the duodenum. The apparent digestibility of starch, for example, was calculated using the following equation:

Apparent rumen digestibility =

(1 – duodenal starch flow/starch intake) x 100

The amount of starch apparently digested (g/d) in the rumen calculated using the following equation:

Apparently digested in rumen =

starch intake x apparent rumen digestibility

Apparent total tract (**TT**) digestibility (%) was calculated using the following equation:

Apparent TT digestibility =

(1-fecal starch output/starch intake) x 100

The amount of starch apparently digested (g/d) in the TT was calculated as follows:

Apparently digested in the TT =

starch intake x apparent TT digestibility

Statistics

Data were analyzed using JMP Version 4 (2000). The model included the random effect of cow and the fixed effect of treatment and period.

$Y_{ijk} = \_ + T_i + P_j + A_k + \_{ijk}$

Where Y_{ijk} is the response variable, _ is the overall mean, T_i is the fixed effect of treatment, P_j = fixed effect of period, A_k is the random effect of cow and $\_{ijk}$ is the error term. Results are presented as least square means and significance was declared at $P < 0.05$ and trends at $0.05 < P < 0.10$.

For rumen pH, the area under the curve (**AUC**) was calculated using the trapezoidal rule (Jones, 1997).

RESULTS AND DISCUSSION

Diet Ingredients and Chemical Composition

All treatment diets were corn silage based and varying amounts of wheat straw, corn starch, urea and casein were added to keep all diets isoenergetic and the UREA, CAS and U+C diets isonitrogenous (Table 3-1). The chemical composition of each treatment diet (Table 3-2) was the same for all components except CP which was 12.2% for the base diet and 14.5, 15.0 and 14.7 for the UREA, CAS, and U+C diets, respectively, which did not differ. Values for RDP%, RUP%, NPN intake and NE_L (Mcal/kg) were predicted using NRC (2001).

Intake

Dry matter intake was lowest for cows fed the BASE diet at 20.3 kg/d as compared to cows fed the other three treatment diets averaging 22.5 kg/d which could be directly linked to the decrease in MN yield ($P < 0.0001$; Table 3-4). When cows were fed diets varying in RDP from 10.6 to 13.2%, DMI averaged 1.5 kg/d less for cows fed 10.6% RDP (Reynal and Broderick, 2005). Additionally there was a tendency for cows fed a diet in high RDP to consume 0.3 kg/d more DM than cows fed an adequate RDP diet (Hirstov et al., 2004). There was no difference in DMI across the UREA, CAS and U+C diets (Table 3-4) which are similar to results when cows were fed diets supplemented with a true protein source compared with urea (Broderick et al., 1993; Armentanto et al., 1993; Sannes et al., 2002). Additionally, N intake was significantly lower when cows were fed the BASE diet which is a function

of both the CP % of the diet and the DMI (Table 3-6). Neither NDF nor starch intakes differed across dietary treatments (Table 3-4).

Total N and Microbial Protein Flow

Total flow of N through the duodenum was not different across dietary treatments though there was less N in the liquid fraction when cows were fed the BASE diet as compared to the other three diets ($P = 0.006$; Table 3-3). Duodenal total CP flow was lower when cows were fed diets supplemented with animal proteins versus plant proteins (Mabjeesh et al., 1996) but not when cows were fed high and low amounts of rumen-available protein supplemented to either a high or low rumen-available non structural carbohydrate diet (Aldrich et al., 1993). In the duodenum, LAB flow represented about 35-40% of the total MN flow and was significantly lower when cows were fed the BASE diet ($P = 0.0003$). The lower total MN flow through the duodenum is probably related to both the decrease in DMI as well as the low CP % of the diet in which N could be limited for microbial growth. Total MN flow through the duodenum was not different among the UREA, CAS and U+C diets indicating that both NPN and AAN result in similar microbial growth. These results are similar to previously reported MN yields in sheep fed a hay-based diet (Chikunya et al., 1996) and in lactating cows (Sannes et al., 2002).

Digestibility

Apparent total tract DM digestibility was lower when cows were fed the BASE diet as compared to the other three treatment diets ($P = 0.03$; Table 3-4). However, previous work showed that heifers fed diets varying in CP from 11.9 to 20.1 % showed no differences in apparent total tract DM digestibility (Gabler and Heinrichs,

2003) and no differences were observed when cows were fed adequate or high RDP diets (Hirstov et al., 2004). There was a tendency for apparent ruminal DM digestibility (%) to be suppressed when cows were fed the BASE diet and the UREA diet which could be a function of the lower CP % compared to the other two treatments or the inability of ruminal bacteria to use urea as efficiently as an AAN source.

Apparent ruminal digestibility of NDF (%) was lowest for cows fed the BASE diet while cows fed the CAS and U+C diets had the highest digestibilities ($P < 0.0001$; Table 3-4) though no differences were found in NDF intake. Previously, ruminal NDF digestibility was not altered when cows were fed diets varying in RDP % (Hirstov et al., 2004; Flis and Wattiaux, 2005; Reynal and Broderick, 2005). In addition, apparent total tract NDF digestibility was over 8% lower for cows on the BASE diet with no differences among the other three treatments. Rumen microbes seems to be more efficient digesting NDF when supplied with AAN or a combination of AAN and NPN as opposed to strictly urea which is typically (in the form of NH_3) used by fiber-digesting bacteria.

Though starch intake was not different across dietary treatments, apparent ruminal starch digestion (%) was highest when cows were fed the U+C diet ($P = 0.007$; Table 3-4). It is speculated that though microbial protein yield was not different, the combination of AAN and NPN (U+C diet) may have favored the amylolytic bacteria population resulting in the higher starch digestion. Both apparent ruminal and total tract digestibilities (%) were lower for cows fed the BASE diet than

the other three diets indicating that the amyolytic bacteria were in inadequate supply in the rumen for complete digestion of starch on this treatment.

Rumen Metabolism

Ruminal pH averaged across sampling times did not differ across treatment diets (Table 3-5). Ruminal pH did not differ when cows were fed urea or SBM (Sannes et al., 2002) or when sheep were fed urea or casein (Chikunya et al., 1996). However, when the AUC (Jones, 1997) was calculated across time for each dietary treatment, cows fed the BASE diet had lower areas than cows fed the other three dietary treatments indicating that the rumen was at a lower pH for a greater amount of time.

Ruminal NH_3-N was numerically higher for cows fed the UREA diet than cows fed the other three treatment diets which are similar to results found by Armentano et al. (1993) and Chikunya et al. (1996) but different from those found by Sannes et al. (2002). Since the degradation of urea is instantaneous in the rumen, it is not surprising that ruminal NH_3-N concentrations were numerically higher when cows were fed a diet where the main source of N was urea. The BASE diet also contained 0.33% urea, but since the CP % was much lower, the rumen microbial population was essentially starved for N. Presumably, the microbes rapidly used the available N in the form of NH_3 resulting in the numerically lower value for NH_3-N. The amount of MN from NH_3-N was calculated using ^{15}N enrichment of the NH_3 pool. Though not significant, numerically more MN came from NH_3-N when cows were fed the BASE diet. However, the amount of N flowing through the NH_3 pool in the rumen (g/d) was significantly lower when cows were fed the BASE diet as compared to the other three

treatments due to the lower protein degradation. There were no differences, however, in N flow, MN from NH_3-N or N flowing through the NH_3 pool between the UREA, CAS and U+C diets**.** The question arises about the increase in ruminal NH_3-N when cows were fed the UREA diet when the parameters previously mentioned were not different. It is possible that more N was recycled back into the rumen increasing ruminal NH_3-N when cows were fed the BASE diet. In fact, since numerically less N absorption occurred when cows were on this treatment this theory is further supported (Table 3-6). When cows were fed the CAS diet it is likely that rumen bacteria rapidly used the available NH_3-N resulting in a lower ruminal NH_3-N concentration. Rumen NH_3-N may have equilibrated with AAN from casein, thus resulting in equal incorporation into rumen NH_3-N, and similar flow rates through the NH_3-N pool without causing an increase in rumen NH_3-N concentration.

Total VFA concentration (mM) was not different between the UREA, CAS and U+C diets, similar to results previously reported from sheep (Cruz Soto et al., 1994; Chikunya et al., 1996) or cows (Sannes et al., 2002) fed either an AAN source or urea. Since microbial protein yield was lower when cows were fed the BASE diet it is not surprising that total VFA concentration was also lower. For individual VFA, only acetate and isobutyrate lower on the BASE diet (Table 3-5). No differences were observed for acetate production when cows (Armentano et al., 1993; Sannes et al., 2002) or sheep (Cruz Soto et al., 1994; Chikunya et al., 1996) were fed either an AAN source or urea. Fiber-digesting bacteria, known producers of acetate (Van Soest, 1994), could have been inhibited as NDF digestibility was also lower for cows fed the BASE diet (Table 3-4). Isobutyrate, a branched chain VFA (**BCVFA**), tended to be

lower for cows fed the BASE diet but did not differ between the UREA, CAS and U+C diets. Armentano et al. (1993) reported a tendency for cows to have greater rumen isobutyrate concentrations when fed SBM as compared to urea, and total BCVFA were lower when cows were fed urea as compared to SBM (Sannes et al., 2002). Since ruminal BCVFA arise from the deamination and decarboxylation of branch-chain AA (Allison, 1970), it is not surprising that the BCVFA should be higher when cows were fed an AAN source as compared MPM. However, in this study, isobutyrate was only lowered by the BASE diet which may have been a function of the limited supply of AA in the rumen.

Urine yield was lowest for cows fed the BASE diet and highest for cows fed the U+C diets ($P = 0.02$; Table 3-5). The increase in dietary CP % resulted in an increase in urinary excretion in Holstein cows (Sannes et al., 2002; Broderick, 2003). Additionally, increasing the RDP concentration 10.6 to 13.2% resulted in an increase in urine volume (Reynal and Broderick, 2005). Since urine is the primary excretory route for N, it is not surprising that urine yield would be higher for the UREA, CAS and U+C diets. Moreover, urine volume was highest for cows fed the U+C diet though Sannes et al. (2002) reported that the source of CP did not alter urinary excretion.

Urinary NH_3-N was not different between dietary treatments. Urea concentration in urine was lowest for cows fed the base diet but did not differ between the UREA, CAS and U+C diets ($P = 0.007$; Table 3-5). Urea yield (g/d) was lowest for cows fed the base diet and highest for cows fed the U+C diet. As RDP increased from 10.6 to 13.2 % so did urinary urea excretion (Reynal and Broderick, 2005).

Cows fed the UREA diet excreted over 25 g/d less of urea than cows fed the U+C diet which is similar to previously published results where cows fed urea had lower urinary urea output than cows fed SBM (Sannes et al., 2002).

Nitrogen Balance

Nitrogen retention was calculated as N intake minus milk N, urinary N and fecal N. Nitrogen intake was over 100 g/d less between the BASE diets and the remaining three treatment diets which averaged over 520 g/d ($P < 0.0001$; Table 3-6). Absorption of N from the rumen was not different across treatments though numerically less absorption occurred when cows were fed the U+C diet. Nitrogen absorption was unaffected by dietary treatment as digesta flowed from the reticulum through the remainder of the digestive tract. Milk and urinary N output was lower for cows fed the BASE diet as compared to the other three dietary treatments as N was used to a greater extent in the cow because of the low CP and RDP diet. Similar results were found by Kalscheur et al. (2006) when cows were fed 6.8% RDP. Source of RDP seemed to have little affect on retained N as a percentage of N intake which was also found when sheep were intraruminally infused with urea, AA or peptides (Cruz Soto et al., 1994). However, cows fed the U+C diet had higher urinary N excretion (g/d) which is of a concern as increased N excretion can lead to increased environmental pollution. Additionally, urinary N excretion was lower when cows were fed the UREA and CAS diets as compared to the U+C diet though it is not clear as to why this occurred. When cows were fed diet supplemented with either urea of SBM more N was excreted in the urine and milk when cows were fed SBM which may have been due to the higher CP % in the SBM diet (Sannes et al., 2002)

Milk Yield and Composition

Milk yield was significantly altered by dietary treatment as cows fed the BASE diet had the lowest yield compared to the other treatments ($P < 0.0001$; Table 3-7) which could be due to the lower DMI. Cows fed the UREA and U+C diets had similar yields, however, when 4% FCM yield was calculated, no differences among the UREA, CAS and U+C existed. No differences in milk yield were reported with cows were fed AAN versus urea (Armentano et al., 1993; Sannes et al., 2002) Fat and protein percentages did not differ across treatments, however yields (kg/d) of both were lower for cows fed the BASE diet compared to the other three treatments. This could be a result of the increased DMI and MN flow as well as apparent fiber digestibility when cows were fed the UREA, CAS or U+C diets. These results are similar to those of Armentano et al. (1993) and Sannes et al. (2002) who noted no differences in fat and protein yield when cows were fed urea or an AAN source. Fat yield tended to be lower for cows fed the BASE diet which could be a function of ruminal acetate production ($P = 0.0809$; Table 3-7). As expected, since the BASE diet contained significantly less protein, MUN concentrations were also lower but there were no differences in the remaining three dietary treatments due to source of RDP.

The N content of the milk was lowest for cows fed the BASE diet and higher for cows fed the remaining three diets. When the ^{15}N enrichment of milk protein was calculated, more of the N in the milk was from bacterial origin for cows fed the UREA, CAS and U+C diets as compared to the BASE diet. Hristov et al. (2004) calculated the amount of milk N from bacterial N and found that approximately 60% of the N in milk was from bacteria regardless of whether a high- or low RDP-diet was

fed, which is considerably greater than the 30 to 40 % found in this study. Milk NH_3 concentration was numerically higher when cows were fed the UREA diet which follows a similar trend to ruminal NH_3. However, the relationship between ruminal and milk NH_3 the two was determined, the relationship was slight.

Conclusions

Feeding a diet containing only 12% CP to lactating dairy cows decreases DMI, milk yield and duodenal MN yield. This decrease in MN yield resulted in a decrease in the apparent digestibilities of DM, NDF and starch in both the rumen and total tract. Either the low protein or the low RDP value of this diet inhibited MN yield resulting in the observed decreases.

Ruminally degraded protein source did not affect MN flow through the duodenum when cows were fed adequate CP. It is possible that the microbial profile in the rumen changed depending on the N source though the microbial populations were not quantified. Though MN yield was not different between the two RDP sources, apparent digestibility of NDF was highest when cows were fed an AAN source or a combination of AAN and NPN. Starch digestibility was highest when cows were fed a combination of AAN and NPN indicating that the microbial population in the rumen requires both sources to maximum starch digestibility.

Table 3-1. Ingredient and ingredient composition of treatment diets (% of DM).

	Treatments			
	BASE	UREA	CAS	U+C
Ingredient				
Corn Silage[1]	46.2	46.2	46.2	46.2
Wheat Straw[2]	7.24	5.18	7.24	6.21
Concentrate Mix[3, 4]				
Corn Starch	3.67	4.62	0.23	2.43
Fine Grd Corn	30.2	30.2	30.2	30.2
48% SBM	1.71	1.71	1.71	1.71
Soy Pass	6.57	6.57	6.57	6.57
Mineral/Vitamin Mix[5]	0.88	0.88	0.88	0.88
Dicalcium Phosphate	0.61	0.61	0.61	0.61
Limestone	0.83	0.83	0.83	0.83
Trace mineral salt	0.63	0.63	0.63	0.63
Megalac	1.19	1.19	1.19	1.19
Urea	0.33	1.44	0.33	0.89
Casein	0.00	0.00	3.44	1.72

[1]Contained 29.0% DM, 8.65% CP, 43.1% NDF, 25.6% ADF, 1.97% NDF-N, 1.43% ADF-N, 3.2% Lignin and 4.2% Ash.

[2]Contained 95.1% DM, 4.0% CP, 76.7% NDF, 47.9% ADF, 2.3% NDF-N, 1.36% ADF-N, 8.1% Lignin and 7.3% Ash.

[3]Contained 93.9% DM, 12.0% NDF, 4.0% ADF, 15.1% NDF-N, 3.0% ADF-N, 1.3% Lignin and 8.3% Ash.

[4]Concentrate mix CP content: Base – 17.09%, Urea - 23.65%, Casein - 23.08%, Urea+Casein - 22.43%.

[5]Contained 33.0% Mg, 9.0% S, 6.15% K, 84.0 ppm Se, 960,000 IU/lb vitamin A, 240,000 IU/lb vitamin D and 3,200 IU/lb vitamin E.

Table 3-2. Chemical composition of treatment diets (% of DM).

	Treatments			
	BASE	UREA	CAS	U+C
Nutrient				
DM, %	46.5	46.1	46.0	46.1
CP, %	12.2	14.5	15.0	14.7
Starch, %	40.9	39.8	37.6	41.3
NDF, %	30.3	28.5	29.9	28.9
NDF-N, %	6.11	7.74	7.45	6.28
ADF, %	16.8	15.7	16.8	16.2
ADF-N, %	1.50	1.69	1.55	1.51
Lignin, %	2.34	2.17	2.41	2.36
Ash, %	7.59	7.73	7.79	7.81
Predicted values[1]				
RDP, %	6.0	8.3	8.9	8.5
RUP, %	6.2	6.2	6.1	6.2
NPN intake, g	56	269	60	166
NE_L, Mcal/kg	1.63	1.64	1.63	1.64

[1]Values predicted from NRC (2001).

Table 3-3. Least square means of total and microbial nitrogen flow through the duodenum of cows fed diets varying in RDP source.

	Treatments					
	BASE	UREA	CAS	U+C	SE	$P \leq$
Duodenal total N flow						
Liquid fraction, g/d	155[a]	197[b]	185[b]	183[b]	9.2	0.004
Solid fraction, g/d	627	622	559	514	81	NS
Total N flow, g/d	782	819	744	697	84	NS
Duodenal microbial N flow						
LAB[1], g/d	82.5[a]	120[b]	111[b]	111[b]	7.3	0.0003
LAB, % of microbial-N	34.9	39.8	36.8	36.8		
SAB[2], g/d	154	182	191	193	26	NS
SAB, % of microbial-N	65.1	60.2	63.2	63.2		
Total Microbial N, g/d	237[a]	300[b]	288[b]	304[b]	27	0.04

[a,b]Means in the same row with unlike letters differ (P <0.05).

[1]LAB = liquid associated bacteria.

[2]SAB = solid associated bacteria.

Table 3-4. Least square means of intakes and digestibility of DM, NDF and starch from cows fed diets varying in RDP source.

	Treatments					
	BASE	UREA	CAS	U+C	SE	$P \leq$
DMI, kg/d	20.3[a]	22.8[b]	22.6[b]	22.0[b]	0.8	< 0.05
DM apparently digested in rumen, kg/d	13.3[a]	16.2[b]	16.7[b]	16.5[b]	1.01	0.03
%	65.6[a]	71.0[a]	73.6[b]	75.3[b]	3.33	0.08
DM flow from the duodenum, kg/d	6.4	5.7	5.3	4.9	0.8	NS
DM apparently digested in the total tract, kg/d	14.6[a]	16.8[b]	17.1[b]	16.7[b]	0.62	0.02
%	72.5	76.1	76.0	76.5	1.6	0.03
NDF intake, kg/d	6.14	6.29	6.77	6.36	0.32	NS
NDF apparently digested in rumen, kg/d[1]	2.2[a]	2.8[b]	3.3[b]	3.0[b]	0.2	0.001
%	43.5[a]	52.7[b]	59.6[c]	57.4[bc]	1.9	< 0.0001
NDF flow from the duodenum, kg/d	2.8[a]	2.5[a]	2.2[b]	2.3[b]	0.2	0.02
NDF apparently digested in the total tract, kg/d	1.8[a]	2.3[b]	2.0[a]	2.3[b]	0.14	0.09
%	34.9	43.7	41.6	44.0	2.8	NS
Starch intake, kg/d	8.33	8.84	8.54	9.06	0.6	NS
Starch apparently digested in rumen, kg/d[1]	4.9[a]	5.8[a]	5.5[a]	7.0[c]	0.63	0.06
%	76.5[a]	83.3[b]	81.3[b]	89.7[c]	2.2	0.007
Starch flow from the duodenum, kg/d	1.5[a]	1.2[ab]	1.1[b]	0.74[c]	0.2	0.008
Starch apparently digested in the total tract, kg/d	6.8	7.4	6.9	7.6	0.6	NS
%	99.3[a]	99.7[b]	99.7[b]	99.7[b]	0.10	0.01

[a-c]Means in the same row with unlike letters differ ($P < 0.05$).

Table 3-5. Least square means of ruminal metabolism and urinary excretion from cows fed diets varying in RDP source.

	Treatments					
	BASE	UREA	CAS	U+C	SE	$P \leq$
Ruminal metabolism						
Rumen pH	6.24	6.17	6.17	6.16	0.07	NS
pH, AUC[1]	277[a]	287[b]	290[b]	287[b]	3.54	0.04
Rumen NH_3-N, mg/dl	6.98	10.4	8.17	8.08	1.01	NS
Rumen bacteria from NH_3-N, %[2]	58.6	50.7	51.8	53.7	6.41	NS
N flow through NH_3 pool, g/d[3]	133[a]	178[b]	183[b]	185[b]	22.3	0.05
Total VFA, mM	52.1[a]	66.2[b]	64.8[b]	59.2[ab]	3.54	0.03
Acetate, mM	31.1[a]	40.4[b]	40.7[b]	35.9[ab]	2.34	0.02
Propionate, mM	12.2	17.9	15.8	14.8	1.76	NS
A:P ratio	2.70	2.89	2.71	2.55	0.30	NS
Butyrate, mM	6.68	5.87	5.74	5.89	0.48	NS
Isobutyrate, mM	0.66[a]	0.89[b]	0.82[b]	0.84[b]	0.06	0.08
Valarate, mM	0.97	0.96	1.03	0.90	0.14	NS
Isovalarate, mM	0.48	0.62	0.56	0.56	0.04	NS
Urine yield, kg/d	11.6[a]	13.0[a]	13.4[a]	15.8[b]	1.61	0.02
NH_3-N, mg/dl	0.37	0.50	0.48	0.38	0.10	NS
Urea, g/L	3.99[a]	6.20[b]	7.65[b]	7.35[b]	0.73	0.007
Urea, g/d	44.9[a]	85.5[b]	97.0[bc]	112[c]	9.20	0.0003

[a-c]Means in the same row with unlike letters differ ($P < 0.05$).
[1]AUC = Area under the curve calculation (Jones, 1997).
[2]Calculated using ^{15}N enrichment of the NH_3-N fraction in rumen fluid.
[3]N flow through NH_3 pool = (^{15}N dose rate/^{15}N enrichment of rumen NH_3 pool)*100.

Table 3-6. Least square means of intake, partitioning and utilization, of N from cows fed diets varying in RDP source.

	Treatments					
	BASE	UREA	CAS	U+C	SE	$P \leq$
N intake, g/d	394[a]	535[b]	501[b]	529[b]	21.5	0.0002
N flow from rumen, g/d	782	819	744	697	84	NS
N absorption from rumen,[1] g/d	-388	-287	-243	-168	82	NS
N absorption lower tract,[2] g/d	659	702	635	581	78.9	NS
Milk N, g/d	101[a]	125[b]	125[b]	137[b]	9.96	0.0005
Milk N, % of intake	25.4	23.7	25.0	26.2	1.95	NS
Urinary N, g/d	60.6[a]	107[b]	119[bc]	140[c]	10.9	0.0002
Urinary N, % of intake	15.6[a]	19.4[ab]	24.3[b]	26.7[b]	2.06	0.003
Fecal N, g/d	123	117.1	109	116	10.8	NS
Fecal N, % of intake	30.8	22.0[b]	22.3[b]	22.1[b]	2.15	0.01
Retained N,[3] g/d	109	183	148	121	24.1	NS
Retained N, % of intake	13.1	18.9	19.4	11.0	2.92	NS
N efficiency,[4] %	28.1	34.7	28.3	22.8	4.48	NS

[a-c]Means in the same row with unlike letters differ ($P < 0.05$).

[1]N absorption from the rumen = N intake – N flow from the rumen.

[2]N absorption from lower tract = N flow from rumen – fecal N.

[3]Retained N = N intake - (milk N + urinary N + fecal N).

[4]N efficiency (%) = 100 x Milk N (g/d) / Intake N (g/d).

Table 3-7. Least square means for milk yield and composition from cows fed diets varying in RDP source.

	Treatments					
	BASE	UREA	CAS	U+C	SE	$P \leq$
Milk yield, kg/d	28.0[a]	33.2[bc]	32.6[b]	33.7[c]	2.62	< 0.0001
4% FCM, kg/d[1]	23.1[a]	27.9[b]	27.1[b]	28.5[b]	2.29	0.01
Protein, %	2.41	2.51	2.54	2.53	0.08	NS
Fat, %	2.87	2.93	2.93	3.05	0.23	NS
Solids, %	5.48[a]	5.61[b]	5.61[b]	5.62[b]	0.10	0.03
SCC (x 1000)	122	190	330	176	158	NS
Protein, kg/d	0.67[a]	0.83[b]	0.82[b]	0.84[b]	0.07	0.001
Fat, kg/d	0.79[a]	0.97[b]	0.94[b]	1.00[b]	0.10	0.08
Solids, kg/d	1.52[a]	1.86[b]	1.82[b]	1.88[b]	0.14	0.003
MUN, mg/dl	6.61[a]	12.64[b]	11.74[b]	13.26[b]	0.86	< 0.0001
Milk N, g/d	101[a]	125[b]	125[b]	137[b]	9.96	0.0005
Milk N from bacterial origin, %[2]	29.3[a]	36.2[b]	37.0[b]	34.5[b]	1.50	0.007
Milk N from bacteria, g/d	30.3[a]	46.1[b]	46.6[b]	48.2[b]	4.36	0.002
Milk NH_3-N, mg/dl	0.08[a]	0.13[b]	0.10[a]	0.12[a]	0.02	0.10

[a-c]Means in the same row with unlike letters differ ($P < 0.05$).

[1]4% FCM (kg/d) = 0.4 x milk yield (kg/d) + 15 x fat yield (kg/d) (NRC, 2001).

[2]Calculated using ^{15}N enrichment of the N fraction in milk.

Chapter 4

EFFECT OF PARTICLE SIZE ON FLOW RATE FROM THE RUMEN AND THROUGH THE DUODENUM OF LACTATING DAIRY COWS

ABSTRACT

The objective of this study was to compare flow rates of various particle sizes from reticulum and duodenal samples as well as obtain a better understanding of bacterial attachment to these particles as they flow through the digestive tract. Cows were infused with Co-EDTA, Cr-mordanted NDF and ^{15}N which were used as markers for liquid, solid and bacteria flow, respectively. Samples were collected from 8 ruminally and duodenally cannulated lactating Holstein cows every 4 h for 48 h over 4 periods (n=32). Samples were poured sequentially through sieves measuring 2.0, 1.0, 0.5, and 0.25 mm. Fractions were composited over 48 h for each cow. The flow of DM was greatest for the 0.5 mm fraction both out of the rumen and through the duodenum and was not different between these two locations. Additionally, there was no difference in flow rate of the 1.0 mm fraction between the two sampling sites and this particle size accounted for approximately 25% of total DM flow. Though total NDF flow was 0.4 kg/d greater out of the rumen as compared to the duodenum, flow rates were similar regarding the 0.25, 0.5 and 1.0 mm fractions between the two sampling sites. Starch flow through the duodenum was much greater and inversely related to samples obtained from the reticulum most likely due to under-sampling of larger starch particles in the reticulum. Particle-associated N flow was not different for the 0.5, 1.0 and ≥ 2.0 mm sieve fractions between both locations, and N flow was greatest in the 0.5 mm fraction. In the rumen, the percentage of particle-associated N from bacterial origin increased as particle size increased indicating that bacterial attachment was greater with the larger particles possibly due to continued degradation. The opposite was found in the duodenum as more bacteria were in the liquid fraction possibly due

to detachment from particles in the abomasum because of enzymes and acidity. In conclusion, flow rates of DM, NDF and N differed depending on particle size. Additionally, the composition of the various sieve fractions differed but was similar between reticulum and duodenal samples. Bacterial attachment differed depending in particle size and location in the digestive tract. The distribution of particles as sampled differed from the distribution of particles as flowed from both sampling locations. These results indicate the importance of particle size passage from the rumen and the usefulness of flow markers to adjust for unrepresentative sampling from both the rumen and the duodenum.

Keywords: Dairy Cows, Digesta Flow, Cannulas, Microbial Protein, Particle Size

INTRODUCTION

Passage rate from the rumen plays an important role in controlling appetite (Welch, 1982), ruminal fill (Jung and Allen, 1995), extent of ruminal protein degradation (Ørskov and McDonald, 1979), and efficiency of microbial protein synthesis (Harrison and McAllan, 1980). The rate-limiting step in digestion in the rumen is the physical breakdown of feed particles (Mosely and Jones, 1984). Flow of digesta out of the rumen is influenced primarily by the size and density of the feed particle. Particles above 1.18 mm, or the critical size for passage out of the rumen for both sheep and cattle (Poppi et al., 1980, 1985), are thought to be retained in the rumen and are rarely found further down the digestive tract (Ulyatt et al., 1986). Studies have shown, however, that digesta can leave the rumen of dairy cows at particle sizes above 4.75 mm and are excreted in feces at 2.36 mm (Huhtanen et al.,

1997). Even in sheep, digesta particles above 2.0 mm can flow out of the rumen (Troelsen and Campbell, 1968).

Not only has it been reported that critical size plays a role in particle passage, but the idea of functional specific gravity (**FSG**) also affects retention of particles in the rumen (Lechner-Doll et al., 1990). The idea of FSG pertains to the solid, liquid and gaseous makeup of a feed particle. A particle that has been digested and has little bacterial attachment and/or is undergoing minimal fermentation would have a high FSG and have a greater probability of flowing out of the rumen than one with a lower FSG. Since bacterial fermentation occurring on feed particles causes the entrapment of gas, the FSG would be lower and these particles would selectively be retained in the rumen.

Rumen bacteria contribute a large proportion of N flowing through the duodenum of dairy cattle (Clark et al., 1992). Therefore understanding the flow of ruminal bacteria attached to various particle sizes will contribute to understanding the digestive process and may facilitate determination or prediction of microbial protein flow rates. Particle-associated bacteria range from 50 to 70% of the total bacteria in the rumen (Cheng et al., 1977; Merry and McAllan, 1983; Craig et al., 1987) with the remainder in the liquid fraction of rumen contents. Few studies have compared the flow of particle-associated bacteria from the rumen to other segments of the digestive tract (Ørskov et al., 1986; Ahvenjärvi et al., 2000) or between various particle sizes (Yang et al., 2001). As a result, since rumen bacteria are such an integral part of nutrition and digestion in the dairy cow, understanding of the attachment of bacteria to

various particle sizes flowing through the digestive tract may help more accurately quantify microbial protein flow.

Therefore, the first objective of this study was to determine the flow rates of various particle sizes out of the rumen and through the duodenum. The second objective was to determine the composition and microbial attachment for different particle sizes passing from the rumen to the duodenum. The third objective was to quantify the apparent changes in flow of feed fractions and changes in particle size distribution as digesta flows from the rumen to the duodenum.

MATERIALS AND METHODS

Cows and Treatments

Animal experiments were conducted at the Beltsville Agricultural Research Center in accordance with the USDA and University of Maryland animal care and use committees. Eight multiparous duodenally and ruminally cannulated Holstein cows were arranged in a repeated 4x4 Latin square design balanced for carryover effects with 21 d periods. Cows were fed one of four dietary treatments. Details regarding cows and treatments have previously been reported (Peterson et al. 200#).

Flow Markers

Each of the four treatment periods lasted 21 d. The first 14 d were for adaptation, the last 7 days were for marker infusions and the last 3 d were for sample collections. To determine microbial nitrogen (**MN**) yield and microbial attachment to various particle sizes, labeled ammonium sulfate [$(^{15}NH_4)_2SO_4$] was continuously infused into the rumen. To determine flow rates out of the rumen and through the duodenum, Co-EDTA and Cr-mordanted NDF were intraruminally administered as

liquid and solid passage markers, respectively (Uden et al., 1980). Cows were continuously infused with $(^{15}NH_4)_2SO_4$ at the rate of 1 g/d and with Co-EDTA at the rate of 54 mg Co/d from 1200 on d 15 through 1200 on d 21. Intraruminal dosing of Cr-mordanted NDF occurred every 8 h from 1000 h on d 16 for 24 h and the dosing rate changed to every 4 h starting at 1000 h on d 17 through 0800 on d 21.

The Cr-mordanted NDF was ground using a Wiley Mill (2-mm screen; Arthur H. Thomas, Philadelphia, PA) to ensure particles small enough that would be candidates to pass out of the reticulum. It was important that the Cr-mordanted NDF be ground so that it consisted of particles of < 0.25, 0.25, 0.5, 1 and 2 mm so that the flow of each particle size could be determined. A subsample of each batch of Cr-mordanated NDF was wet-sieved over screens measuring 0.25, 0.5, 1 and 2 mm to calculate the distribution of particles entering the rumen.

Reticulum and Duodenal Sampling

Samples were collected from the reticulum through a 4 in. rumen cannula using a PVC pipe (12 mm i.d.) attached to a vacuum with additional holes drilled no farther than 2 inches from the end to allow for both fluid and particle collection. Duodenal samples were collected using a t-shaped duodenal cannula that had been surgically placed 6 wk prior to parturition. Samples (250 ml each) were collected from reticulum and duodenum every 4 h at 1200, 1600, 2000 and 2400 h on d 19, at 0400, 0800, 1400, 1800 and 2200 h on d 20 and at 0200, 0600 and 1000 h on d 21. Both the reticulum and duodenal samples were poured over a set of five sieves to allow for particle separation and liquid collection. The sieve sizes were, from largest to smallest, 2.0, 1.0, 0.5 and 0.25 mm. At the bottom of the sieves was a collecting

pan to collect particles smaller than 0.25 as well as the liquid fraction. Once the liquid in the collecting pan was removed, all sieves remained stacked and each sieve was sequentially rinsed with a gentle stream of water. This rinse allowed for movement of particles from the larger screens to the smaller ones that may have been attached to other larger particles or the sides of the sieves. After rinsing, the remaining water was gently pressed out of the particles and each sieve was composited over the collection period and frozen. These sieve samples were later dried and analyzed for Co, Cr, N and ^{15}N.

The liquid fraction from the reticulum samples was differentially centrifuged for microbial pellet formation. After the high-speed centrifugation was complete, the supernatant was decanted and immediately frozen. The supernatant samples were later thawed, composited over each period and freeze-dried for analysis of Co (liquid passage marker). The liquid fraction that remained in the collecting pan from the duodenal samples was composited over each period and frozen. This fraction was then freeze-dried and later analyzed for Co, Cr, N and ^{15}N.

Sieve Fraction Analysis

The frozen reticulum and duodenal particle composites were weighed and dried in a 55° C oven. Dry matter of each sieve was recorded. Each sample was then ground Wiley Mill (1-mm screen; Arthur H. Thomas, Philadelphia, PA) prior to analysis. Particles were analyzed for NDF (Van Soest and Wine, 1967) and starch which was measured by an enzymatic method (Karkalas, 1985) and glucose was measured with a glucose oxidase method (Glucose kit #510; Sigma Chemical Co., St. Louis, MO). Total N and NDF-N were quantified by Dumas combustion (Leco

FP428, Leco Corp., St. Joseph, MI). To determine the attachment of microbes to each particle fraction, sieve samples were analyzed for ^{15}N enrichment (UC Davis Stable Isotope Facility, Davis, CA). All fractions that were flowing out of the reticulum and into the duodenum including microbial pellets, supernatants, sieve fractions, as well as the Cr mordanted NDF used for dosing in the rumen were digested using a nitric/percholoric acid digestion (Perkin-Elmer, 1982). Details on the Cr and Co analysis have been published (Peterson et al. 200#).

Flow rate and ^{15}N enrichment calculations

In steady state, the rate of intraruminal infusion of both Cr-mordanted NDF and Co-EDTA should equal the flow rate out of the rumen. And, assuming that negligible breakdown and absorption occurs as digesta flows out of the rumen and to the duodenum, the flow of Cr and Co out of the rumen should equal the flow rate through the duodenum. In order to determine the flow rate of both the solid and liquid fractions the following calculation was used for each particle size fraction or liquid sample (Faichney, 1993):

Digesta flow = (Co or Cr dose rate)/(Co or Cr concentration in digesta)

To quantify ^{15}N enrichment of sieve fractions, background samples were collected prior to continuous infusions of $(^{15}NH_4)_2SO_4$ for each period to determine the natural background (**NB**). The following calculation was used to determine the ^{15}N enrichment above the NB (**^{15}N-APE**) in each sample:

$$^{15}N\text{-APE} = {}^{15}N\text{-atom \%} - {}^{15}NB$$

Assuming that the liquid-associated bacteria (**LAB**), obtained from the high-speed centrifugation of the reticulum liquid, consisted purely of MN, the following calculation was used to determine the %N in from microbial origin:

%N from Microbial Origin in Sieve Fraction =

^{15}N-APE in Sieve Fraction/^{15}N-APE in LAB

To then calculate the yield (g/d) of MN flowing in the sieve fraction out of the rumen, the following calculation was used:

MN flow in Sieve Fraction = (sieve fraction flow x % N in sieve fraction)

x (%N from Microbial Origin in Sieve Fraction)

Statistics

Data was analyzed using JMP Version 4 (SAS, 2000). To determine sampling location and sieve fraction differences, the model included the random effect of cow and the fixed effect of sampling location, sieve fraction size and period:

$Y_{ijkl} = \_ + L_i + S_j + P_k + A_l + L_i*S_j + \_{ijkl}$

Where Y_{ijkl} is the response variable, _ is the overall mean, L_i is the fixed effect of sampling location, S_j is the fixed effect of sieve fraction size, P_k is the fixed effect of period, A_l is the random effect of cow, L_i*S_j is the interaction between sampling location and sieve fraction size, and $\_{ijkl}$ is the error term. Data is presented as least square means and significance was declared at $P < 0.05$ and trends at $0.05 < P < 0.10$.

RESULTS AND DISCUSSION

Dry Matter Flow between Sampling Sites

Total water flow was approximately 6 L/d greater through the duodenum than out of the rumen (Table 4-1; $P < 0.0001$). The liquid collected in the pan after pouring

the samples over the sieves was also analyzed for Cr to determine if any detachment had occurred from the particles to which they were affixed. The Cr in the liquid flowing out of the rumen was negligible and accounted for only 2-6% of the total Cr flow. For the duodenum, only 5-14% of the total Cr flow was in the liquid fraction. The liquid flow marker, Co-EDTA, was not found in the particle fractions. The reason for the observed low levels of detachment or reattachment of markers probably relates to the thorough separation of particles using wet sieving. As a result, the double-marker method to determine digesta flow was not used (Faichney, 1993), but instead, each particle fraction received a separate flow with the mordanted particles of each particle size serving as a separate marker. Therefore, there were four particle flow markers (≥ 2.0, 1.0, 0.5, and 0.25 mm) and one liquid flow marker.

Total DM flow (kg/d) was approximately 1 kg/d greater out of the rumen as compared to the duodenum which is a function of more particles flowing in the 0.25 and ≥ 2.0 mm fractions ($P = 0.009$; Table 4-1). Flow rates of all sieve fractions were different between the two sampling sites except for the 0.5 mm fraction which represented over 40% of the total DM flow. More DM was flowing through the duodenum than out of the rumen in the < 0.25 mm fraction which could be due to particle size reduction as samples flowed through the omasum and abomasum. However, more DM was flowing in the 0.25, 1.0 and 2.0 mm fractions out of the rumen than through the duodenum.

When DM was expressed as a percentage of total flow, almost 10% of the total DM flow through the duodenum was found in the < 0.25 mm fraction which was significantly greater than the 3.3% flowing out of the rumen ($P < 0.0001$, Table 4-1).

Particle distribution between the reticulum and duodenum was similar among the 0.25, 0.5 and 1.0 mm fractions. Mean particle size flowing out of the rumen was 710 _m compared to 659 _m flowing through the duodenum (SEM = 27.3; $P = 0.10$). Though mean particle size tended to be smaller flowing through the duodenum, when the mean was expressed as $\log_{10}$mean (Olaisen et al., 2001), the values were not different between the two sampling locations (reticulum = 2.84 and duodenum = 2.81; SEM = 0.02). There was a tendency, however, for a higher proportion of DM flowing out of the rumen in the 2.0 mm fraction as compared to flowing through the duodenum. This can be explained due to digestion and absorption occurring in the omasum and abomasum (Faichney et al., 1997). Proportionally it seems that DM flow (% of total) was similar for the majority of particles regardless of sampling sight though total flow (kg/d) was greater out of the rumen.

Dry Matter Flow between Sieve Fractions

When DM flow out of the rumen and through the duodenum was evaluated across the various sieve fractions, it was found that flow rates differed significantly ($P < 0.0001$; Table 4-1). Most of the DM flowed with particles measuring 0.5 mm which represented slightly over 40% of the total DM flow both out of the rumen and through the duodenum. The least amount of DM flowed with the < 0.25 and 2.0 mm fractions and combined represented less than 15% of DM flowing out of the rumen and almost 20% of the DM flowing through the duodenum. Troelsen and Campbell (1968) reported that most of the particles flowing out of the rumen, omasum and abomasum were retained on the 0.25 and 0.5 mm sieves though this data came from sheep which may have resulted in the different distributions. When samples were collected from

the omasum, approximately 35% of the DM collected was greater than 2.36 mm while the same percentage was found to be < 0.15 mm (Huhtanen et al., 1997). In this study, over 60% of the DM that flow out of the rumen was 0.5 mm or less. Since microbial attachment on larger particles (1 mm and above) is expected for continued digestion (Table 4-6), these particles should have a lower FSG making their retention in the rumen longer (Lechner-Doll et al., 1990).

Comparison of DM distribution using flow markers or as sampled

The distribution of DM flowing out of the rumen and through the duodenum was calculated using the various particle sizes of Cr-mordanted NDF as flow markers. Additionally, when samples were collected from both sites, the distribution of DM in the digesta was also quantified (Table 4-2). The distribution of DM differed greatly as calculated using flow markers as compared to the sample collected. Approximately 70% of the DM as sampled was flowing in the < 0.25 and ≥ 2.0 mm fractions both out of the rumen and through the duodenum. However, as calculated using the flow marker, over 70% of the DM was flowing in the 0.25, 0.5 and 1.0 mm fractions both out of the rumen and through the duodenum. This suggests that samples collected from both locations were not representative of what was actually flowing. The unrepresentative sampling is not an issue as long as an adequate range of accurate flow markers are used, but would result in inaccurate flow estimates otherwise. For the reticulum, more of the < 0.25 mm fraction (DM in the liquid phase) was flowing than the other fractions relative to the calculated flow. The same holds true for the duodenum, but there was less of a difference between the as sampled and as flowed distribution for the < 0.25 mm fraction. Dry matter flow as calculated divided by the

DM flow as sampled, provides an index of how representative the sample was as collected from either sampling site. Numbers greater than one suggest under-sampling while numbers less than one suggest over-sampling. In the < 0.25 mm fraction, DM was over-sampled from both the reticulum and duodenum and DM was under-sampled in the 0.25, 0.5 and 1.0 mm fractions. As a result, it is apparent that both liquid and solid markers are necessary in order to accurately quantify flows out of the rumen. Additionally, because duodenal samples were significantly different from the actual flow of digesta, markers would be useful for more accurate digesta flow values.

NDF between Sampling Sites and Sieve Fractions

Total NDF flow was different between sampling locations though the difference was only 0.4 kg/d (Table 4-3; $P = 0.0008$). Flow of NDF was not different between reticulum and duodenal samples for the 0.25, 0.5 and 1.0 mm sieve fractions. The only difference, though numerically small, was for the ≥ 2.0 mm fraction. Moreover it seems that NDF flow is essentially the same between both sampling locations regardless of particle size. When expressed as a percentage of total NDF flow, there was no difference between the distribution of NDF between the sampling sites for the 0.25, 0.5 and 1 mm fractions. The only difference existed with the ≥ 2.0 mm fraction where a greater percentage of NDF was flowing out of the rumen than through the duodenum. In total, most of the NDF (70%) was flowing in the 0.5 and 1.0 mm fractions regardless of sampling location. As bacteria continue to digest the larger NDF particles, the size of these particles decrease and they become a part of the smaller sieve fractions. Since DM flow as greatest in the 0.5 mm fraction, NDF flow is also greatest for that sieve size.

Starch Flow

Total starch flow out of the rumen was 1 kg/d less than that flowing through the duodenum ($P < 0.0001$; Table 4-4). Starch flow out of the rumen and through the duodenum was the same for the 0.25 mm fraction. However, starch flow was significantly greater through the duodenum for particle sizes above 0.5 mm ($P < 0.0001$). Sampling error from the reticulum is the most likely explanation for this occurrence. It is speculated that the larger particles of starch were under-sampled because of their location in the rumen and that the particle size measuring 0.25 mm was more ubiquitous throughout the digesta. Again, since DM flow was highest with the 0.5 mm fraction, starch flow is also greater in that fraction while starch flow was lowest in the 2.0 mm fraction due to the numerically lower DM flow in this fraction. Additionally, it may be possible that starch in the 2.0 mm fraction was still being fermented which tends to decrease the FSG (Faichney, 1993) making the retention in the rumen longer. As a percentage of total starch flow through the duodenum, 80% of the flow was divided equally between the 0.5 and 1.0 mm fractions. Upon visual evaluation of these sieves, cracked corn particles were apparent and may have escaped complete ruminal digestion because of their density and tendency to migrate towards the bottom of the rumen increasing the possibility for passage.

Nitrogen Flow

Total N flowing out of the rumen or through the duodenum was not significantly different (Table 4-5). The N flowing with the 0.5 mm fraction was the largest and contributed to 40% of the total N flow both out of the rumen and through the duodenum. Total N flow was the same between the 0.5, 1.0 and 2.0 mm fractions.

There was a greater flow of N associated with the 0.25 mm fraction out of the rumen than that flowing through the duodenum though this relationship was the opposite for the ≤ 0.25 mm fraction. As a percentage of total N flow, there was no difference between the distribution through the 0.5, 1.0 and 2.0 mm fractions which represent approximately 65% of the total N flow.

Microbial Nitrogen Flow

When N from bacterial origin (%) was determined using ^{15}N enrichment of digesta particles, it was found that 47% of the N flowing out of the rumen in the 2.0 mm sieve fraction was of bacterial origin (Table 4-6). As the sieve size decreased so did the contribution of N from bacteria which was opposite of previously published results (Yang et al., 2001). However, one explanation of the higher percentage of bacteria attached to the larger particles in the current study is that these bacteria are still in the process of digesting the larger feed particles. Additionally, Craig et al. (1987) reported that anywhere from 70-80% of ruminal microorganisms are attached to undigested feed particles and 50-65% of the particle-associated N was from microbial origin. A larger proportion of MN was associated with the smaller sieve fractions as compared to the larger fractions (1.0 and 2.0 mm). Though these results are slightly lower than previously published results (Clark et al., 1992), the trend is similar (Legay-Carmier and Bauchart, 1989; Yang et al., 1989; Yang et al., 2001).

When flow of MN was quantified, most of the MN flowing through the duodenum was in the ≤ 0.25 fraction (Table 4-7). However, most of the MN flowing out of the rumen was associated with the 0.5 mm fraction. This is probably due to the pH change and enzymatic secretions as digesta flows through the abomasum which

may result in bacterial detachment from particles and lysing of bacterial cells (Van Soest, 1994; Firkins et al., 2006). There were no differences in MN flow between sampling location for the 0.25, 0.5 and 1.0 mm fractions. Additionally, the distribution of MN flowing out of the rumen was similar to that flowing through the duodenum for the 0.25, 0.5 and 1.0 mm fraction.

CONCLUSIONS

Flow of water through the duodenum appeared to be much higher than that out of the rumen which suggests water secretion into the omasum. DM flow from the rumen was 1 kg/d higher than to the duodenum. Particle size distribution (% of total DM flow) was similar between reticulum and duodenal samples. Dry matter flowed mostly in the 0.5-mm fraction followed by the 1.0-mm size.

Total flow of NDF was only 0.4 kg/d higher out of the rumen than through the duodenum and there were no differences in the amount of NDF flowing in the 0.25, 0.5 and 1.0 mm sieve fractions between sampling sites. Possibly due to the higher DM flow through the 0.5 mm fraction, NDF flow was also highest for the 0.5 mm fraction both out of the rumen and through the duodenum. Additionally, the distribution of NDF (kg/d) across the sieves was quite similar between sampling sites. Due to sampling error of larger starch particles from the rumen, starch flow out of the rumen was under-estimated though flow rates in the 0.25 mm fraction were similar between sampling sites. Particle-associated N and MN flow across the sieves were similar between the two sampling sites. Bacteria seem to detach from larger particles as they flow through the omasum, abomasum and through the duodenum resulting in more MN flowing with the smaller particles.

Overall, the flow rates DM, NDF and N across the 0.25, 0.5, 1.0 and 2.0 mm sieves were similar between both sampling locations. Flow rates differ, however, depending on particle size and most of the digesta that is flowing seems to be about 0.5 mm. However, the distribution of flow across the sieves was also similar between samples collected in the reticulum and those collected from the duodenum. Additionally, the composition of the various sieve fractions differed but was still similar between reticulum and duodenal samples. As a result, it can be concluded that using reticulum sampling to accurately estimate the flow of DM, NDF and N through the duodenum is possible.

Table 4-1. Differences in particle size flow of DM as sampled from the reticulum and the duodenum.

	Sampling Location			
	Reticulum	Duodenum	SEM	$P \leq$[1]
Total water flow, L/d	14.3	20.9	0.67	< 0.0001
Total DM flow, kg/d	7.21	6.14	0.64	0.009
Flow by particle size, kg/d				
< 0.25 mm	0.24[a]	0.54[a]	0.02	< 0.0001
0.25 mm	1.21[b]	0.94[a]	0.13	0.009
0.5 mm	3.06[c]	2.69[b]	0.40	NS
1.0 mm	1.93[d]	1.42[c]	0.20	0.08
≥ 2.0 mm	0.77[a]	0.55[a]	0.14	0.006
SEM	0.19	0.19		
$P \leq$[2]	< 0.0001	< 0.0001		
Flow, % of total DM flow				
< 0.25 mm	3.3[a]	8.8[a]	0.57	< 0.0001
0.25 mm	16.8[b]	15.3[b]	1.00	NS
0.5 mm	42.4[c]	43.8[c]	2.25	NS
1.0 mm	26.8[d]	23.1[d]	2.56	NS
≥ 2.0 mm	10.7[e]	9.0[a]	1.38	0.06
SEM	1.73	1.73		
$P \leq$[2]	< 0.0001	< 0.0001		

[a-e]Refers to significant differences ($P < 0.05$) between sieve fractions in vertical columns.

[1]Indicates significance between sampling location (reticulum and duodenum).

[2]Indicates significance between sieve sizes (0.25, 0.5, 1.0 and 2.0mm).

Table 4-2. Comparison of DM distribution (%) between sampling locations.

	Measurement				
	As sampled[1]	As flowed[2]	SEM	$P \leq$[3]	Flowed/Sampled[4]
Reticulum					
< 0.25 mm	63.8	3.3	0.73	< 0.0001	0.06
0.25 mm	7.49	16.8	0.86	< 0.0001	2.46
0.5 mm	9.93	42.4	1.66	< 0.0001	4.33
1.0 mm	7.39	26.8	1.96	< 0.0001	3.73
2.0 mm	13.2	10.7	0.97	0.07	1.12
Duodenum					
< 0.25 mm	38.1	8.8	1.09	< 0.0001	0.3
0.25 mm	6.8	15.3	0.71	< 0.0001	2.56
0.5 mm	13.2	43.8	1.68	< 0.0001	3.38
1.0 mm	16.2	23.1	1.73	0.003	1.47
2.0 mm	29.8	9.0	1.22	< 0.0001	0.3

[1]Dry matter distribution (%) as sampled from the reticulum or duodenum.
[2]Dry matter distribution (%) as calculated using flow markers from the reticulum or duodenum.

[3]Indicates significance between as sampled and as calculated values.
[4]Dry matter distribution of flow as predicted using flow markers/dry matter distribution determined from actual reticulum sample.

Table 4-3. Differences in particle size flow of NDF as sampled from the reticulum and the duodenum.

	Sampling Location			
	Reticulum	Duodenum	SEM	$P \leq$[1]
Total flow, kg/d	2.88	2.47	0.14	0.0008
Flow by particle size, kg/d				
0.25 mm	0.50[a]	0.47[a]	0.04	NS
0.5 mm	1.24[b]	1.15[b]	0.10	NS
1.0 mm	0.79[c]	0.61[a]	0.09	NS
≥ 2.0 mm	0.35[a]	0.24[c]	0.04	0.0002
SEM	0.07	0.07		
$P \leq$[2]	< 0.0001	< 0.0001		
Flow, % of total NDF flow				
0.25 mm	17.4[a]	19.0[a]	1.10	NS
0.5 mm	43.1[b]	46.6[b]	2.23	NS
1.0 mm	27.4[c]	24.7[a]	2.70	NS
≥ 2.0 mm	12.2[a]	9.7[c]	1.25	0.02
SEM	2.08	2.08		
$P \leq$[2]	< 0.0001	< 0.0001		

[a-c]Refers to significant differences ($P < 0.05$) between sieve fractions in vertical columns.

[1]Indicates significance between sampling location (reticulum and duodenum).

[2]Indicates significance between sieve sizes (0.25, 0.5, 1.0 and 2.0mm).

Table 4-4. Differences in particle size flow of starch as sampled from the reticulum and the duodenum.

	Sampling Location			
	Reticulum	Duodenum	SEM	$P \leq$[1]
Total flow, kg/d	0.27	1.2	0.07	< 0.0001
Flow by particle size, g/d				
0.25 mm	71.3[a]	73.5[a]	7.63	NS
0.5 mm	131[b]	500[b]	38.6	< 0.0001
1.0 mm	53.3[a]	487[b]	60.8	< 0.0001
≥ 2.0 mm	15.1[c]	187[a]	19.7	< 0.0001
SEM	9.08	52.6		
$P \leq$[2]	< 0.0001	< 0.0001		
Flow, % of total starch flow				
0.25 mm	26.3[a]	5.9[a]	1.18	< 0.0001
0.5 mm	48.4[b]	40.1[b]	2.02	NS
1.0 mm	19.7[a]	39.0[b]	3.29	0.003
≥ 2.0 mm	5.6[c]	15.0[c]	1.42	< 0.0001
SEM	2.00	2.84		
$P \leq$[2]	< 0.0001	< 0.0001		

[a-c]Refers to significant differences ($P < 0.05$) between sieve fractions in vertical columns.

[1]Indicates significance between sampling location (reticulum and duodenum).

[2]Indicates significance between sieve sizes (0.25, 0.5, 1.0 and 2.0mm).

Table 4-5. Difference in association of N to different particle sizes as flowed from the reticulum and duodenum.

	Sampling Location			
	Reticulum	Duodenum	SEM	$P \leq$[1]
Total flow, g/d	796	763	43.7	NS
Flow by particle size, g/d				
< 0.25 mm	88.5[a]	180[a]	12.3	< 0.0001
0.25 mm	169[b]	114[b]	15.5	< 0.0001
0.5 mm	326[c]	288[c]	42.2	NS
1.0 mm	164[b]	138[b]	19.7	NS
≥ 2.0 mm	48.4[d]	42.8[d]	7.49	NS
SEM	22.49	22.49		
$P \leq$[2]	< 0.0001	< 0.0001		
Flow, % of total N flow				
< 0.25 mm	11.1[a]	23.6[a]	1.93	0.01
0.25 mm	21.2[b]	14.9[a]	1.28	0.02
0.5 mm	41.0[c]	37.8[b]	2.31	NS
1.0 mm	20.6[b]	18.1[a]	2.72	NS
≥ 2.0 mm	6.1[a]	5.6[d]	0.76	NS
SEM	2.02	2.06		
$P \leq$[2]	< 0.0001	< 0.0001		

[a-d]Refers to significant differences ($P < 0.05$) between sieve fractions in vertical columns.

[1]Indicates significance between sampling location (reticulum and duodenum).

[2]Indicates significance between sieve sizes (0.25, 0.5, 1.0 and 2.0mm).

Table 4-6. Difference in the percentage of N flowing from microbial origin as sampled from the reticulum and the duodenum.

	Sampling Location			
	Reticulum	Duodenum	SEM	$P \leq$[1]
N flow, % from bacterial origin				
< 0.25 mm	75.4[a]	58.9[a]	4.17	0.005
0.25 mm	27.0[b]	30.4[b]	2.06	NS
0.5 mm	33.3[b]	28.7[b]	3.36	NS
1.0 mm	38.3[b]	21.0[c]	2.68	0.01
2.0 mm	47.0[c]	24.8[d]	2.62	0.003
SEM	3.36	2.31		
$P \leq$[2]	< 0.0001	< 0.0001		

[a-c]Refers to significant differences ($P < 0.05$) between sieve fractions in vertical columns.

[1]Indicates significance between sampling location (reticulum and duodenum).

[2]Indicates significance between sieve sizes (0.25, 0.5, 1.0 and 2.0mm).

Table 4-7. Difference in particle size flow of MN as sampled from the reticulum and the duodenum.

	Sampling Location			
	Reticulum	Duodenum	SEM	$P \leq$[1]
Total flow, g/d	286	266	22.4	NS
Flow by particle size, g/d				
< 0.25 mm	66.7[ab]	106[a]	8.97	< 0.0001
0.25 mm	43.9[a]	34.4[bd]	7.12	0.08
0.5 mm	91.8[b]	77.8[c]	10.9	NS
1.0 mm	58.0[ab]	37.1[b]	20.1	NS
≥ 2.0 mm	25.2[a]	10.2[d]	5.52	0.03
SEM	13.2	7.61		
$P \leq$[2]	0.02	0.0002		
Flow, % of total MN flow				
< 0.25 mm	23.4[a]	39.9[a]	1.66	0.0007
0.25 mm	15.4[ab]	13.0[bd]	3.97	NS
0.5 mm	32.1[c]	29.3[c]	5.31	NS
1.0 mm	20.3[ab]	14.0[b]	6.27	NS
≥ 2.0 mm	8.8[b]	3.8[d]	1.60	0.04
SEM	5.04	3.25		
$P \leq$[2]	0.003	< 0.0001		

[a-d]Refers to significant differences ($P < 0.05$) between sieve fractions in vertical columns.

[1]Indicates significance between sampling location (reticulum and duodenum).

[2]Indicates significance between sieve sizes (0.25, 0.5, 1.0 and 2.0mm).

CHAPTER 5

THE USE OF RETICULUM SAMPLES TO PREDICT FLOWS OF VARIOUS DIGESTIBLE FRACTIONS THROUGH THE DUODENUM

ABSTRACT

The objective of this study was to compare measurements of digesta flow using samples from the reticulum to measurements using duodenal samples. Eight multiparous duodenally and ruminally cannulated Holstein cows were arranged in a repeated 4x4 Latin square design balanced for carryover effects with 21-d periods. Solid- and liquid-flow markers of Cr-mordanted NDF and Co-EDTA were continuously infused for steady state calculations. Reticulum and duodenal samples were collected every 4 h for 48 h and composited over each period. Total DM flow was 1 kg/d higher out of the rumen than through the duodenum though the majority of samples were close to their corresponding duodenal samples. Flow of NDF was 0.4 kg/d higher when estimated using reticulum samples compared to duodenal samples. Starch flow out of the rumen was estimated at 0.27 kg/d using reticulum samples compared to 1.2 kg/d flowing through the duodenum, indicating that starch was under-sampled in the reticulum, over-sampled in the duodenum, or both. Total N and MN flow was similar out of the rumen and through the duodenum. There was a strong correlation between duodenal-predicted MN flow and reticulum-predicted MN flow ($R^2 = 0.61$) and 95% of reticulum samples fell within 94 g/d of their corresponding duodenal samples. The variability attributed to various parameters in the model was similar for all digestible fractions between the two sampling sites. Though flow rates of the various digestible fractions were slightly different, many similarities existed between the two sampling sites. Additionally, there was little variability and most of the samples fell within an acceptable range from their corresponding duodenal samples. The digestibility of both DM and NDF were also similar whether calculated

using duodenal or reticulum flow. Starch was quite different between sampling sites probably due to sampling error. Overall, the flows of most digesta fractions out of the rumen can be used to predict what is flowing through the duodenum; however, the flow of starch needs to be determined directly from the duodenum.

Keywords: cannulation, digestibility, flow

INTRODUCTION

Cannulation has made the study of the intricacies of digestion and nutrient utilization through the gastrointestinal tract of ruminants possible. Cannulation permits access to the digestive tract for digesta sampling, infusions, digestibility determinations and various other determinations that may aid in understanding digestive processes in ruminants. However, duodenal cannulation has been linked to decreased feed intake and decreased milk yield (McRae and Wilson, 1977; Wenham, 1979). For some nutrients not absorbed in the omasum, the quantity leaving the rumen through the reticulo-rumen orifice should equal the quantity flowing through the duodenum. Therefore, sampling digesta at the reticulo-rumen orifice should enable prediction of what is flowing through the duodenum, avoiding duodenal cannulations. Samples at the reticulo-rumen orifice would contain less endogenous N than in the duodenum (Ørskov et al., 1986) and better represent the particle- and liquid-associated bacterial fractions (Ahvenjärvi et al., 2000). Digestion and absorption may occur as digesta flows to the duodenum (Faichney et al., 1997) and the acidity and enzyme activity encountered as particles flow through of the abomasum (Firkins et al., 2006) may influence digesta make-up and flow rate.

In order to assess whether digesta samples collected from the reticulum are representative of digesta flowing through the duodenum, the flow rates of various digestible fractions must be statistically compared. In 1986, Bland and Altman suggested a series of steps that could be used to evaluate agreement or disagreement between two methods. They noted that the common use of correlation coefficients to establish agreement between two methods is incorrect as correlation coefficients give an idea of the relationship between two analyses, but not the conformity between them.

Therefore the objective of this paper was to determine differences in measurements of DM, NDF, starch, N and microbial N (**MN**) flow, and DM, NDF and starch digestibility when measured from the reticulum compared to measurements from the duodenum

MATERIALS AND METHODS

Animal experiments were conducted at the Beltsville Agricultural Research Center in accordance with the USDA and University of Maryland animal care and use committees. Eight multiparous duodenally and ruminally cannulated Holstein cows were arranged in a repeated 4x4 Latin square design balanced for carryover effects with 21 d periods. Cows were fed one of four dietary treatments. Details regarding cows and treatments have previously been reported (Peterson et al. 200#). Chromium-mordanted NDF, Co-EDTA and $(^{15}NH_4)_2SO_4$ were intraruminally infused to determine solid, liquid and MN flow out of the rumen and through the duodenum. Reticulum and duodenal samples were collected every 4 h over the last 48-h of each period. Samples were poured over a set of sieves to allow for particle separation and

liquid collection. The sieve mesh sizes were ≥ 2 mm, 1 mm, 0.5 mm and 0.25 mm. The bottom pan was used for liquid collection and the DM in the liquid represented a separate particle fraction (< 0.25 mm) The particles were gently rinsed, composited over each period and analyzed as previously described (Peterson et al., 200#). Flow of DM (F_{DM}) out of the rumen or into the duodenum was calculated according to the formula:

$$F_{DM} = \sum_{i=1}^{n} (F_i / C_i)$$

where F_i is the infusion rate of each particle size or liquid flow marker (i), and C_i is the concentration of each marker as a fraction of the total DM in that particle fraction.

Statistics

Data were analyzed using JMP Version 4 (SAS, 2000). To determine differences in flow rates as calculated using reticulum or duodenal samples, the model included the random effect of cow and the fixed effects of sampling location, treatment and period:

$Y_{ijkl} = \_ + L_i + P_j + T_k + L_i*T_k + A_l + \_{ijkl}$

Where Y_{ijkl} is the response variable, _ is the overall mean, L_i is the fixed effect of sampling location, P_j is the fixed effect of period, T_k is the fixed effect of dietary treatment, L_i*T_k is the interaction between sampling location and treatment, A_l is the random effect of cow, and $\_{ijkl}$ is the error term. Data is presented as least square means and significance was declared at $P < 0.05$ and trends at $0.05 < P < 0.10$.

To determine agreement between estimating DM, NDF, starch, N and MN flow from reticulum and duodenal samples and digestibilities of DM, NDF and starch

using reticulum or duodenum flows, a statistical procedure developed by Bland and Altman (1986) was used. Through this procedure, the difference between the two sampling sites is regressed on the mean of the two. Using the generated scatter plot with the regression line and determining mean and/or slope biases, agreement or disagreement between the two sampling locations can be determined. Additional information regarding the statistical methodology can be found in Appendix A.

Additionally, the percentage of variation among reticulum or duodenal samples attributed to the variance from cow, treatment and period was determined by dividing the variance component estimate for each component by the total variance component (Kohn et al., 2004). If similarities exist between the two sampling sites, the distribution of the variation should be similar.

The 95% limits of agreement were calculated as the mean difference ±1.96 time the standard deviation of the differences. Therefore, it is estimated that for 95% of the reticulum samples, the observed value would be greater than the duodenal value minus the lower limit and less that the duodenal value plus the upper limit (Bland and Altman, 1995).

RESULTS AND DISCUSSION

Dry Matter Flow

Water flow rates through the duodenum were found to be higher than out of the rumen ($P < 0.0001$; Table 5-1). Liquid flow rates were reported to be higher through the abomasum than out of the rumen (Ørskov et al., 1986). DM flow was measured to be slightly over 1 kg/d greater leaving the rumen than through the duodenum. Results from Ahvenjarvi et al (2000) indicate that more organic matter

was flowing through the duodenum than the omasal canal. This may be because the flow rates of particles sampled in the rumen were slower than flow rates of markers of those particles or due to absorption in the omasum and abomasum.

Though DM flow was found to be higher out of the rumen than through the duodenum, when total DM flow from the rumen was regressed on DM flow through the duodenum a strong relationship existed ($P < 0.0001$; $r^2 = 0.83$; Peterson et al., 200#). As suggested by Bland and Altman (1986), the correlation coefficient establishes a relationship between the two sampling sites but not the agreement between them. When the average DM flow between the two sampling sites was plotted against the difference between the two, no slope or mean biases were detected (Figure 5-2). The RMSE was 0.69 kg/d which is an estimate of the total error of the model. The slope of the regression line was not different from 0, indicating no apparent linear bias. The variance associated with the individual parameters in the model was distributed similarly between the two sampling sites (Figure 5-3). Over half of the variability is associated with the random effect of cow averaging 51.1% between the two sampling sites. For the limits of agreement, if DM from the duodenum is flowing at 5 kg/d, there is a 95% probability that the DM flow through the reticulum would be >4.25 and <6.83 kg/d. Therefore, the range is quite small.

Typically, duodenal flows of nutrients are used to determine apparent ruminal digestibility, however, if rumen data can be used with the same results then duodenal cannulation may be avoided. The same procedure was used to evaluate the differences in calculating apparent ruminal digestibility either using flows of nutrients out of the rumen or through the duodenum (Table 5-5). Apparent ruminal DM digestibility was

8.1% higher using duodenal samples as compared to reticulum samples. Evaluating the amount of DM digested in the rumen using the duodenal flow resulted in 1.4 kg/d higher value than using flow out of the rumen. This indicates that using reticulum samples to determine ruminal apparent DM digestibility may provide underestimated values. When the difference in apparent ruminal digestibility between the two sampling sites was regressed on the mean of the two, there was no significant mean or linear bias. As a result, calculating apparent ruminal DM digestibility was similar whether using reticulum or duodenal samples.

Neutral Detergent Fiber Flow

The flow of NDF (kg/d) was 0.4 kg/d higher through the rumen compared to the duodenum (Table 5-1; $P = 0.0008$), possibly due to continued digestion in the omasum. Ahvenjärvi et al. (2000) found 3.2 kg of NDF flowing through the omasum while 2.9 kg/d were found flowing through the duodenum. Though duodenal NDF flow was lower through the duodenum than out of the rumen, when NDF flow as calculated from reticulum samples was regressed on NDF flow as calculated from duodenal samples, a significant relationship existed ($P < 0.0001$; $r^2 = 0.82$; Peterson et al., 200#). The average NDF flow between the two sampling sites was plotted against the difference between the two (Table 5-2; Bland and Altman, 1986). The RMSE for this regression line was 0.35 kg/d and the slope was not significant. The distribution of the variation associated with the model for each sampling site indicated that about 30% of the variability was attributed to cow effects and a large portion of the variation was associated with the error term (Table 5-3). The effects of treatment and period attributed to about 40% of the variation when NDF flow was calculated using

duodenal samples compared to under 20% for the reticulum. However, when the 95% limits of agreement were established, there is a small range of difference between reticulum and duodenal samples (Table 5-4). As a result, NDF flow through the reticulum may be a good predictor of NDF flow through the duodenum as long as the disappearance of digesta due to digestion and absorption in the omasum and abomasum (Faichney et al., 1997) is accounted for.

Digestibility of NDF using rumen flow data averaged 47% but was 53% when using duodenal flow data (Table 5-5). The results obtained using the duodenal flow data was closer to previously published values (Hirstov et al., 2004). On average, NDF apparently digested in the rumen was 0.4 kg/d greater when using duodenal digesta flows as compared to rumen digesta flows. The variability associated with the digestibility calculations was small. As a result, calculating apparent ruminal NDF digestibility was similar whether using reticulum or duodenal samples.

Starch Flow

Starch flow was approximately 1 kg/d less out of the rumen than through the duodenum (Table 5-1). Starch may have been under-sampled in the reticulum compared to those samples obtained from the duodenum or perhaps starch flowing through the duodenum was over-sampled. Since starch and small corn particles are extremely dense, they should be situated close to the rumen floor or at the reticulo-rumen orifice waiting to flow. If situated out of reach of the sampling device, it is possible that the sample obtained from the rumen did not accurately represent what was flowing out of the rumen. Starch flow through the duodenum, however, was similar to results found by (Theurer et al., 1999) who fed dry-rolled sorghum to

crossbred steers. In a review article on starch digestion by Huntington (1997), ground corn should be approximately 50% digestible in the rumen. So if a cow is consuming 18.0 kg DM/d consisting of 40% starch with 50% digestibility then approximately 3.6 kg of starch should be leaving the rumen. However, sampling from the reticulum, in this case, resulted in only 0.27 kg/d which is about 20% of what was flowing through the duodenum. Along with sampling error, the fact that the reticulo-omasal orifice is the main area for obstruction for digesta passage may provide some insight on the differences in flow (Balch and Campling, 1962, 1965).

Starch digestibility was different using flow out of the rumen at 96% compared to using duodenal flow (average = 83%). It is speculated that sampling in the reticulum did not result in a representative sample and that using duodenal flow data to calculate the digestibility of starch will result in a more accurate estimate.

Nitrogen Flow

Total N flow averaged 796 g/d out of the rumen and 763 g/d through the duodenum which were not different from each other (Table 5-1). When total N flow out of the rumen was regressed upon total N flow through the duodenum, a significant relationship exists ($P < 0.0001$; $r^2 = 0.82$; Peterson et al., 200#). When the average N flow between the two sampling sites was plotted against the difference between reticulum and duodenal flow, the regression line was not significant (Table 5-2; Bland and Altman, 1986). The RMSE was 140 g/d, and 95% of the reticulum N flows will fall within -239 and 203 g/d of their corresponding duodenal samples (Table 5-4). Additionally, the distribution of variation between the reticulum and duodenum was similar indicating that each parameter in the model was attributing to the same amount

of variation regardless of sampling site. Therefore, N flow between the two sampling sites was similar.

Microbial N flow

Determination of MN flow through the digestive tract has typically been predicted using duodenal samples. Total MN flow was only 20 g/d higher out of the rumen than through the duodenum which was not statistically different (Table 5-1). This suggests that sampling from the reticulum may provide an accurate estimate of the various N fractions flowing through the duodenum. A significant relationship existed when rumen-predicted MN flow was plotted against duodenal-predicted MN flow ($P < 0.0001$; $r^2 = 0.6195$; Figure 5-1). A scatter plot of the differences of estimated MN yield as determined from either the reticulum or duodenum was regressed on the mean of the two estimates (Figure 5-1). There was no mean or slope bias associated with this data and the RMSE was 58.6 g/d (Table 5-2). If MN flow through the duodenum was estimated at 200 g/d than there is a 95% probability that the reticulum sample would be >154.7 and <282.1 g/d (Table 5-4). The distribution of the variation (Table 5-3) was different with lower random variance and more of the variation attributed to treatment effects for the reticulum samples. Treatment effects were significant for both the reticulum and duodenal samples, indicating that both methods were similar though the distribution of the variance was different. The lower random variance for reticulum samples may result from reduced impact of digestion of microbial protein on measurement in the rumen compared to measurement in the duodenum.

Conclusion

Though flow rates of the various digestible fractions were slightly different, many similarities existed between the two sampling sites. Additionally, there was little variability and most of the samples fell within an acceptable range from their corresponding duodenal samples. The digestibility of both DM and NDF were also similar whether calculated using duodenal or reticulum flow. Starch was quite different between sampling sites probably due to sampling error. Overall, the flows of most digesta fractions out of the rumen can be used to predict what is flowing through the duodenum; however, the flow of starch needs to be determined directly from the duodenum.

Table 5-1. Effect of sampling location on flow rates of various digestible fractions.

	Sampling Location			
	Reticulum	Duodenum	SEM	$P \leq$[1]
Water flow, L/d	14.3	20.9	0.67	< 0.0001
DM flow, kg/d	7.21	6.14	0.64	0.009
NDF flow, kg/d	2.88	2.47	0.14	0.0008
Starch flow, kg/d	0.27	1.2	0.07	< 0.0001
Total N flow, g/d	796	763	43.7	NS
Total MN flow, g/d	286	266	22.4	NS

[1]Indicates significance between sampling location (reticulum and duodenum).

Table 5-2. Regression of the difference between the two sampling sites on the mean of the two sampling sites.[1]

			95% Limits of agreement[1]	
	Mean Difference	SD	Lower limit	Upper limit
DM flow, kg/d	0.54	0.66	-0.75	1.83
NDF flow, kg/d	0.37	0.32	-0.26	1.00
Starch flow, kg/d	0.96	0.12	0.72	1.20
Total N flow, g/d	31.7	138	-239	302
Total MN flow, g/d	18.4	32.5	-45.3	82.1

[1]For 95% of samples, the measurement of the indicated digesta fraction as measured from the reticulum will be greater than the lower limit (reference value minus indicated) and less than the higher limit (reference value plus indicated) relative to a measurement from the duodenum; Limits = 1.96(SD) ± mean difference (Bland and Altman, 1995).

Table 5-3. Variance of flows of various digestible fractions determined by reticulum or duodenal samples.

			Variance attributed to source (%)			
	Mean	SD	Cow	Treatment	Period	Error
DM flow, kg/d						
Reticulum	7.21	2.37	51.6*	4.2	9.3	34.9
Duodenum	6.14	2.20	50.5*	5.4	17.2*	26.9
NDF flow, kg/d						
Reticulum	2.88	0.55	31.7	6.6	11.0	50.7
Duodenum	2.47	0.53	30.6	18.6*	20.2*	30.6
Starch flow, kg/d						
Reticulum	0.27	0.10	44.7*	28.3	2.7*	24.3
Duodenum	1.2	0.53	19.1	7.1	20.9	52.9
Total N flow, g/d						
Reticulum	796	227	37.9	3.8	15.2	43.1
Duodenum	763	240	45.7*	4.0	17.7	32.6
Total MN flow, g/d						
Reticulum	286	92.4	49.9**	22.3**	14.3**	13.5
Duodenum	266	82.6	45.7**	10.4*	16.8*	27.1

*Indicates significance ($P < 0.05$) in the model.

**Indicates significance ($P < 0.01$) in the model.

Table 5-4. The 95% limits of agreement for flows of digestible fractions measured from different locations.[1]

	Model Parameters			
	Regression Equation	R^2	$P \leq$[2]	RMSE
DM flow, kg/d	0.04x + 261	0.02	NS	0.69
NDF flow, kg/d	0.04x + 275	0.03	NS	0.35
Starch flow, kg/d	1.61x - 252	0.90	< 0.0001	0.16
Total N flow, g/d	77.4 - 0.06x	0.03	NS	140
Total MN flow, g/d	0.13x – 18.0	0.03	NS	58.6

[1]Two sampling sites: reticulum versus duodenum.

[2]Indicates significance of the regression line.

Table 5-5. Effect of using reticulum or duodenal samples to calculate apparent ruminal digestibility of various digestible fractions.

	Sampling Location			
	Reticulum	Duodenum	SEM	$P \leq$[1]
Apparent ruminal digestibility[2]				
DM, %	60.7	68.8	3.37	0.002
NDF, %	46.6	53.4	1.01	< 0.0001
Starch, %	96.2	82.3	1.10	< 0.0001
Apparently digested in rumen[3]				
DM, kg/d	11.1	12.5	0.85	0.01
NDF, kg/d	2.47	2.85	0.14	0.0002
Starch, kg/d	6.94	5.86	0.42	0.03

[1]Indicates significance between sampling location (reticulum and duodenum).
[2]Apparent ruminal digestibility using DM as example = (1 - reticulum or duodenal DM flow/DM intake) x 100.
[3]Apparently digested in rumen using DM as example = DM intake x apparent ruminal digestibility.

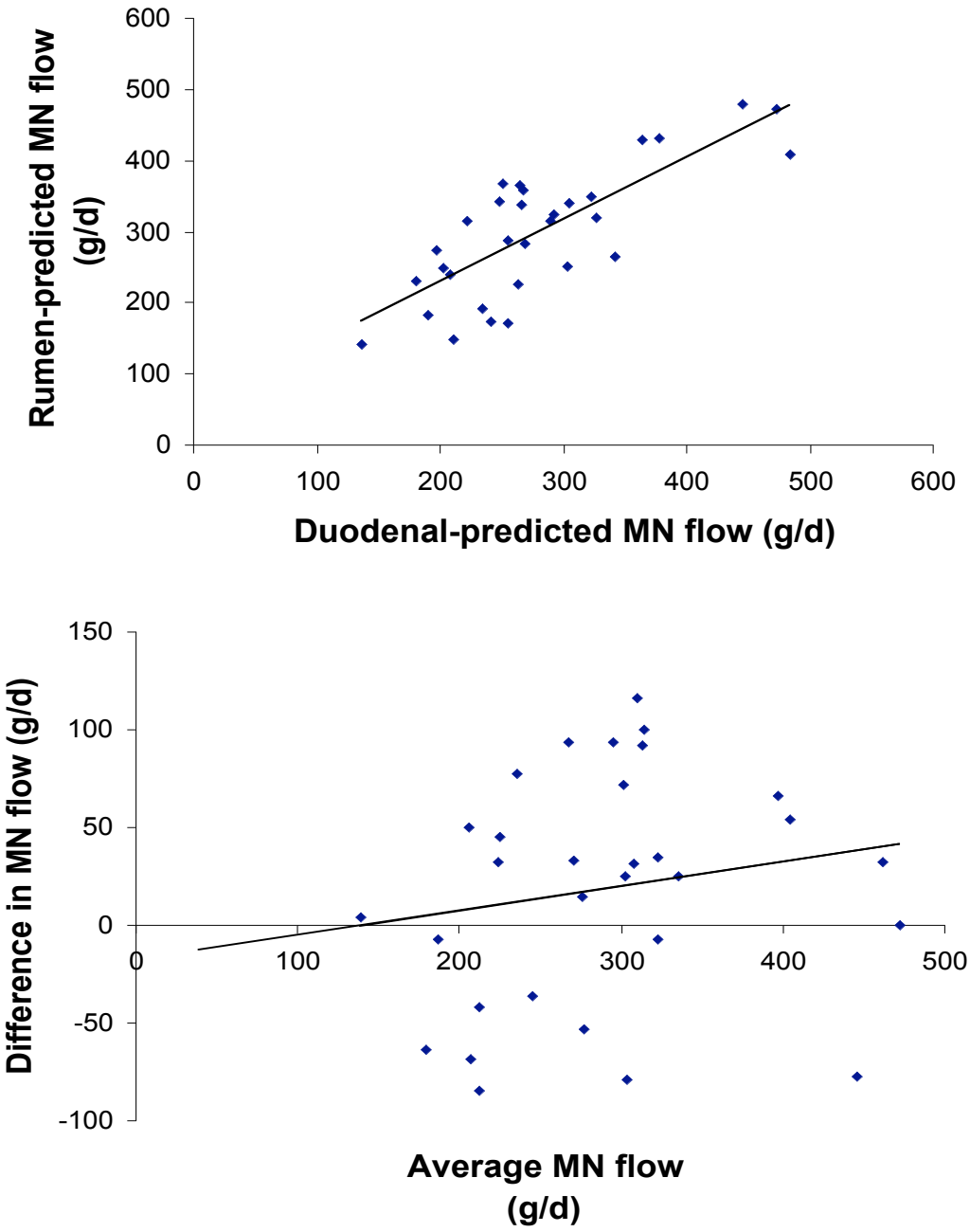

Figure 5-1. Relationship between duodenal-predicted MN flow (g/d) and rumen-predicted MN flow (g/d). The equation is Y = 53.61 + 0.875X with an R^2 of 0.61 and *P* < 0.0001 (TOP). Relationship between the average MN flow (g/d) out of the rumen and through the duodenum and the difference in total MN flow (g/d) between them. The equation is Y = -17.98 + 0.126X with an R^2 of 0.03 and *P* = NS (BOTTOM).

CHAPTER 6

USE OF ALLANTOIN AND URIC ACID IN MILK, URINE AND PLASMA TO PREDICT RUMEN MICROBIAL PROTEIN PRODUCTION

ABSTRACT

The objective of this paper was to evaluate the flow of PD through digestion and metabolism. The second objective was to determine if allantoin and uric acid concentration in milk and urine and circulating in plasma could be used to predict MN flow through the duodenum. Eight early lactation Holstein cows were arranged in a repeated 4x4 Latin square design balanced for carryover effects with 21 d periods. Cows were fed either a base diet containing 12.8% CP (**BASE**) or one of three treatment diets containing 16% CP supplemented with urea (**UREA**), casein (**CAS**) or both (**U+C**). Urinary allantoin and uric acid concentrations were unaffected by dietary treatment. Urinary allantoin excretion was lower when cows were fed the BASE diet as compared to the other three dietary treatments which was a function of the lower urine yield or the lower MN flow through the duodenum. There was no affect of sampling time on plasma concentrations of uric acid or allantoin. Plasma allantoin concentration was lowest when cows were fed the BASE diet and did not differ among the other three dietary treatments. Milk concentration and excretion of allantoin was lowest for cows fed the BASE diet which may have been a function of milk yield. Milk uric acid concentration followed the same trend as allantoin, however, no differences in daily uric acid output were noted. The correlations between allantoin and uric acid in milk, urine and plasma with MN flow through the duodenum were small (r^2 = 0.2 to 0.17). However, cows fed the BASE diet had lower MN flows through the duodenum compared to the other three dietary treatments which corresponds to the lower allantoin concentration in urine, plasma and milk. In conclusion, the overall effect of dietary treatments on concentrations of allantoin and

uric acid in urine, plasma and milk were similar. The lower CP content of the BASE diet and the lower DMI from cows on this diet may have resulted in the significantly lower concentration of PB. Trends observed between duodenal MN flow and allantoin in urine, plasma and milk were similar. However, only small relationships existed between the different PB and MN flow through the duodenum indicating that the excretion of these PB may not be a good index of MN flow.
Keywords: purine bases, allantoin, uric acid, microbial nitrogen

INTRODUCTION

External markers such as ^{15}N or ^{35}S as well as internal markers such as nucleic acids have been used to predict ruminal microbial protein production (Broderick and Merchen, 1992). However, determining digesta flow is necessary when using these markers which require cannulated animals (Broderick and Merchen, 1992). As a result, finding a non-invasive method to estimate microbial protein production in the rumen of cattle would be beneficial in ration formulation.

In ruminants, purine derivatives (**PD**) are excreted in urine and milk as allantoin, uric acid, xanthine and hypoxanthine. Because of the high xanthine oxidase activity found in blood of cattle, xanthine and hypoxanthine are converted to uric acid in blood and tissues prior to urinary excretion (Chen et al., 1990). Through the use of nucleic acid infusion, purines were found to originate from the catabolism of purines of both endogenous and exogenous origin (Verbic et al., 1990). Concentrations of nucleic acids in the rumen are used to estimate microbial nitrogen (**MN**) production because nucleic acids from the diet are thought to be degraded in the rumen (Smith

and McAllen, 1970). Therefore, the majority of purines found in the duodenum are assumed to originate from microbial protein production.

Topps and Elliott (1965) originally suggested that urinary excretion of PD such as allantoin could be a useful indicator of rumen microbial protein synthesis. Since then, many researchers have used urinary and milk excretion of purine derivatives including allantoin and uric acid with variable results as a non-invasive method to predict rumen microbial protein production.

In sheep, a correlation ($R^2 = 0.49$; $P < 0.05$) between urinary excretion allantoin and duodenal MN flow was observed (Lindberg et al., 1989; Puchala and Kulasek, 1992). A linear relationship ($R^2 = 0.64$) was reported between urinary allantoin excretion and the flow of nucleic acids to the duodenum of sheep (Offer et al., 1978; Antoniewicz et al., 1980). Estimates of MN based on urinary PD excretion in heifers were consistently lower (more that 50 g/d MN) than direct measurements of purine bases through the intestine (Martín-Orúe et al., 2000). Lindberg and Jacobsson (1990) concluded that urinary purine excretion in ruminants was unaffected by moderate changes in energy intake and by large changes in protein intake. Possibly due to variability with milk samples, a poor relationship between milk allantoin excretion with calculated microbial protein flow has been reported in Holstein cows (Shingfield and Offer, 1998). Additionally, results on the correlation of plasma PD concentration to PD excretion have been variable (Giesecke et al., 1994; Gonda and Lindberg, 1994; Chen et al., 1995). It would be beneficial; however, to develop a technique utilizing spot samples of plasma since collection of total urine in the field is difficult (Chen et al., 1997).

The effect of various diet compositions on allantoin and uric acid excretion has also been evaluated in dairy cattle. Broderick (2003) fed 15.1, 16.7 or 18.4% CP and noted no effect on milk allantoin concentration but less urinary PD were excreted when cows were fed the 15.1% CP diet. Ruminal infusion of casein increased the daily yield of MN and PD as compared to duodenal casein infusion (Khalili and Huhtanen, 2002). Reynal and Broderick (2005) reported increased MN yield and urinary allantoin excretion when cows were fed 13.2% RDP as compared to 12.3, 11.7 or 10.6% RDP but no dietary effect on urinary uric acid excretion was noted. In 1994, Giesecke et al. reported a correlation of plasma allantoin concentration to milk allantoin concentration ($R^2 = 0.78$; $P < 0.001$) indicating that milk allantoin might be used as a good indicator of blood allantoin. The effects of feeding urea versus SBM (non-protein N versus amino acid N) on microbial protein yield and total PD excretion has been reported and no differences were noted for either (Sannes et al., 2002).

Overall, the use of allantoin and uric acid as a marker of MN flow has not been adequately evaluated. Therefore the objective of this paper was to evaluate the model the flow of PD flow through digestion and metabolism. The second objective was to determine if allantoin and uric acid concentration in milk and urine and circulating in plasma could be used to predict MN flow through the duodenum.

MATERIALS AND METHODS

Cows and Treatments

Animal experiments were conducted at the Beltsville Agricultural Research Center in accordance with the USDA and University of Maryland animal care and use committees. Eight multiparous duodenally and ruminally cannulated Holstein cows

were arranged in a repeated 4x4 Latin square design balanced for carryover effects with 21 d periods. Cows were fed one of four dietary treatments. Details regarding cows and treatments have previously been reported (Peterson et al. 200#).

Sample Collection

A total urine collection was preformed from 1200 on d 19 through 1200 on d 21 using indwelling Foley catheters that were connected to 25L containers containing 50% HCl (v/v). The urine was well mixed and a 500 _l volume of the composited urine was spiked with 250 _l of internal standard (containing [5-^{13}C, 1-^{15}N]allantoin and [1,3-$^{15}N_2$]uric acid and frozen until later analysis. Two additional aliquots (500 _l) of unspiked urine composites were also collected in eppendorf tubes and frozen.

Milk (PM milking on d 19, AM and PM milking on d 20 and the AM milking on d 21) was composited for each cow over each period (n=32). A 500 _l aliquot of the composited raw milk was spiked with 250 _l of same internal standard previously mentioned and frozen for later analysis. Two additional aliquots (500 _l) of unspiked milk composites were also collected in eppendorf tubes and frozen.

Blood samples (10 ml) were collected at 1200, 1600, 2000 and 2400 on d 19 and at 0400 and 0800 on d 20 into hepranized tubes. These samples were immediately placed on ice and centrifuged at 3000 x *g* for 10 min for separation of plasma. Plasma (500 _l) was removed and to that, 250 _l of the internal standard was added in duplicate. These samples were frozen for later analysis. Two additional aliquots (500 _l) of unspiked plasma were also collected in eppendorf tubes and frozen.

Allantoin and Uric Acid Procedure

The allantoin and uric acid procedure used to analyze the urine, milk and plasma samples was an adaptation of the gas chromatography/mass spectrometry (**GC/MS**) method by Chen et al. (1998).

Reagents for Allantoin and Uric Acid analysis. Dimethylformamide (**DMF**) and *N*-(*tert*-butyl-dimethylsilyl)-*N*-methyltriflouroacetamide (**MTBSTFA**) were purchased from Pierce (Rockford, IL). [5-^{13}C, 1-^{15}N]Allantoin and [1,3-$^{15}N_2$]uric acid were obtained from Isotec (Miamisburg, OH). Ammonia solution, allantoin, and uric acid were obtained from Sigma (St. Louis, MO). The AG1-X8 resin (100-200 mesh, Cl form) was obtained from Bio-Rad Laboratories (Hercules, CA).

Standards and Sample Preparation. To 500 _l of each milk composites, urine composites, and plasma samples, 250 _l of an internal standard containing [5-^{13}C, 1-^{15}N]allantoin, [1,3-$^{15}N_2$]uric acid was added at the time of collection and prior to freezing. However, since an insufficient amount of [5-^{13}C, 1-^{15}N]allantoin was added to composite urine samples, an aliquot of unspiked sample (100 _l) was spiked with a new internal standard (200 _l) and mixed. The new internal standard contained 0.13 mg of [1,3-$^{15}N_2$]uric acid/g of distilled water and 0.26 mg of [5-^{13}C, 1-^{15}N]allantoin/g of distilled water. Urine samples were then ready for isolation and derivitization.

An insufficient amount [5-^{13}C, 1-^{15}N]allantoin was added to the milk samples. Therefore an aliquot of unspiked composited milk (500 _l) was spiked with a new internal standard (200 _l) and vortexed. To precipitate proteins from milk samples, 100 _l of 64% sulfosalicylic acid (**SSA**) was added. These samples were immediately vortexed and centrifuged at 3000 x *g* for 5 minutes. The lipid layer on the top of the sample was carefully removed and the supernatant was passed through a 0.2 um filter

to remove any remaining proteins and lipids. These samples were then ready for isolation and derivatization.

Spot plasma samples containing internal standard were thawed at room temperature. To each tube, 100 _l of 64% SSA was added for protein precipitation. These samples were immediately vortexed and centrifuged at 3000 x *g* for 5 minutes. The supernatant was decanted and these samples were then ready for isolation and derivatization. A unique standard curve had to be developed for each sample type which was used in the quantification of each compound.

Isolation and Derivatization. After samples were prepared, samples were mixed with 500 _l of 6 M NH_3OH solution and applied to packed column containing 1 ml of the anion-exchange resin (AG1-X8, 100-200 mesh, Cl form). To wash the column, 4ml ddH_20 was added. The allantoin and uric acid were eluted with 4ml of 0.1 N HCl. A 500 _l volume of the eluate was transferred to a V-vial and dried at 90 ºC under nitrogen. The tert-butyldimethylsilyl derivatives of allantoin and uric acid were formed by reacting with 50 _l of DMF-MTBSTFA (1:1) at 130 ºC for 20 min. The reaction mixture was then analyzed by the GC/MS.

GC/MS Instrumentation. The samples were analyzed on a HP 6890 gas chromatograph coupled with a HP 5973N quadrupole mass selective detector (Hewlett-Packard, Wilmington, DE). The GC was fitted with a 30 m x 0.25 mm i.d. (0.25 _m film thickness) HP-5 capillary column (Agilent, Wilmington, DE). Injections (1 _l) were made in the split mode using a 40:1 split ratio for urine and milk composite samples and a 5:1 split ratio for plasma samples. Helium was used as the carrier gas at a flow of 0.9 ml/min. The injector port temperature was 250 ºC and the

column temperature program was from 150 to 300 ºC at 20 ºC/min with a 2 min hold. The GC/MS auxiliary temperature was 280 ºC.

The mass spectrometer was operated under electron impact ionization conditions with the following source parameters: electron energy, 70 eV; detector current, 2600 EMVolts; source temperature, 230 ºC; and quadrapole temperature, 150 ºC. The M+0 and M+2 ions were *m/z* 398 and 400, respectively for allantoin and were *m/z* 567 and 569, respectively for uric acid.

Statistics

Data were analyzed using JMP Version 4 (SAS, 2000). To determine dietary treatment differences, the model included the random effect of cow and the fixed effect of treatment and period.

$Y_{ijk} = \_ + T_i + P_j + A_k + \_{ijk}$

Where Y_{ijk} is the response variable, _ is the overall mean, T_i is the fixed effect of treatment, P_j is the fixed effect of period, A_k is the random effect of cow and $\_{ijk}$ is the error term. To determine the effect of time on plasma allantoin concentrations, the model included the random effects of cow, time nested within cow, and treatment by time nested within cow as well as the fixed effects of treatment and period.

$Y_{ijk} = \_ + T_i + P_j + A_k + R(A_k) + T_i*R(A_k) + \_{ijk}$

Where Y_{ijk} is the response variable, _ is the overall mean, T_i is the fixed effect of treatment, P_j is the fixed effect of period, A_k is the random effect of cow, $R(A_k)$ is time nested within the random effect of cow, $T_i*R(A_k)$ is the interaction of treatment, T_i, with time nested within the random effect of cow, $R(A_k)$, and $\_{ijk}$ is the error term.

Data is presented as least square means and significance was declared at $P < 0.05$ and trends at $0.05 < P < 0.10$.

RESULTS AND DISCUSSION

Urinary Purine Excretion

Urinary allantoin concentration was not different among dietary treatments but, on average, was 30% lower than some previously reported values (Table 6-1; Valadares et al., 1999; Reynal and Broderick, 2005). When allantoin excretion was calculated, cows fed the BASE diet had the lowest allantoin excretion and there was no difference between the UREA, CAS and U+C diets. Allantoin excretion in urine increased as heifers were fed increasing amounts of CP from 11.9 to 20.1% (Gabler and Heinrichs, 2003). Sannes et al. (2002) reported no differences in total PD excretion (urine+milk) when cows were fed urea or SBM. In this study, allantoin averaged 94% of the total PD excretion in urine which is similar to previous results (Vagnoni et al., 1997; Valadares et al., 1999). Neither urinary uric acid concentration nor excretion was altered by dietary treatment. Similar results were found when cows were fed diets varying in RDP % (Reynal et al., 2005; Reynal and Broderick, 2005).

Cows fed the BASE diet had the lowest MN flow through the duodenum while cows fed the other three dietary treatments were not different from each other (Peterson et al., 200#). When urinary allantoin concentration and yield used to predict MN flow through the duodenum (Peterson et al., 200#), no significant relationship was noted (Table 6-2). However, when duodenal MN flow was predicted using urinary uric acid concentration and yield, the slope was significant, but the r^2 values for both

of these relationships were still small. As a result, this data indicates a poor relationship between MN flow through the duodenum and urinary PD excretion.

Plasma Purine Concentrations

The effect of time on allantoin and uric acid concentrations in plasma was evaluated prior to determining dietary treatment effects (Figure 6-1). No time effect was found for plasma allantoin concentration which averaged 210 _mol/L across the six time points. Additionally, there was no difference in uric acid concentration between plasma samples averaging 41.0 _mol/L over the 24-h sampling period. This indicates that using spot plasma samples to determine circulating allantoin and uric acid is possible as there are no significant differences over time. However, the use of spot sampling techniques tend to increase the variability associated with the measurement (Valadares et al., 1999)

Plasma allantoin was affected by dietary treatment where cows fed the BASE diet had the lowest circulating allantoin concentration and cows fed the U+C diets had the highest concentrations (Table 6-1). When sheep were abomasally infused with purines at the rate of 5, 10 or 20 mmol/d, sheep receiving 5 mmol/d exhibited the lowest plasma PD concentration (Chen et al., 1997). In cattle, plasma allantoin concentrations averaged 184.5 _mol/L (SD = 47.8) when measured by Giesecke et al. (1994) which are very close to the values from this study. However, Martín-Orúe et al. (1996) indicated that the average allantoin concentration was 113.2 _mol/L (SEM = 5.55) in ewes.

When duodenal MN flow was regressed against plasma allantoin concentration, no relationship existed. However, when plasma uric acid concentration

was used to predict MN flow through the duodenum, the slope of the line was significant ($P = 0.03$; Table 6-2). The r^2 for this regression line was only 0.16 which, though significant, still indicates a poor relationship between plasma uric acid concentration and MN flow through the duodenum.

Milk Purine Excretion

Milk allantoin concentration was lowest when cows were fed the base diet and highest when cows were fed the U+C diet ($P = 0.03$; Table 6-1). Milk allantoin concentration has been positively correlated with energy intake (Kirchgessner and Kaufmann, 1986; Lebzien et al., 1993). However, when cows were limit-fed (González-Ronquillo et al., 2004) or fed diets with various protein content and degradability (Gonda and Lindberg, 1997) no changes in milk allantoin concentration were noted. In this study, cows fed the BASE diet had lower MN flow through the duodenum than cows on the other three dietary treatments (Peterson et al., 200#) which may explain why milk allantoin concentration was lower on this diet.

Total daily excretion of milk allantoin was lowest when cows were fed the BASE diet but did not differ among the other three dietary treatments ($P = 0.04$, Table 6-1) which may be a function of the lower milk production when cows were fed the BASE diet (Peterson et al., 200#). The influence of milk yield on allantoin excretion has been documented (Gonda and Lindberg, 1997; Shingfield and Offer, 1998). Neither energy source (Shingfield and Offer, 1998) nor intake (González-Ronquillo et al., 2004) altered milk allantoin excretion. Additionally, no relationship was observed between CP intake and milk allantoin concentration (Kirchgessner and Kreuzer, 1985).

Uric acid concentration in milk was lowest for cows fed the BASE diet and highest for cows fed the U+C diet ($P < 0.05$; Table 6-1). However, no effect on uric acid excretion was noted. No effect of intake (González-Ronquillo et al., 2004) or ruminally protected amino acid supplementation (Timmermans et al., 2000) has been reported. Uric acid represented, on average, 37% of the total PD excreted from the mammary gland across all four dietary treatments. This is slightly higher than results reported by others (Giesecke et al., 1994; Gonda and Lindberg, 1997) however these studies used HPLC to quantify the uric acid content in milk whereas GC/MS was used to evaluate milk samples in this study.

Duodenal flow of MN tended to be correlated to milk allantoin concentration and excretion though the r^2 values are quite low ($P = 0.08$; Table 6-2). Previous studies have also indicated a lack of a relationship between microbial flow and milk allantoin in dairy cattle (Shingfield and Offer, 1998) and in sheep (Martín-Orúe et al., 1996). No relationship was found between duodenal MN flow and uric acid concentration or excretion in milk which is similar to previously reported results (Timmermans et al., 2000; González-Ronquillo et al., 2004).

CONCLUSION

The overall effect of dietary treatments on concentrations of allantoin and uric acid in urine, plasma and milk were similar. The lower CP content of the BASE diet and the lower MN flow through the duodenum of cows on this diet may have resulted in the significantly lower concentration of PD. Excretion of allantoin in urine and milk was a function of the different urine and milk yields across dietary treatments. There was no effect of time on plasma concentrations of uric acid or allantoin

indicating that spot samples may be used to measure the concentration of PD. Only small relationships existed between the different PD and MN flow through the duodenum though the trends among treatments for the PD and MN flows were similar. This indicates that the excretion or concentration of allantoin and uric acid may not be a good index of MN flow.

Table 6-1. Concentration and excretion of uric acid and allantoin in urine, plasma, and milk from cows fed diets varying in RDP source.

	Treatments					
	BASE	UREA	CAS	U+C	SE	$P \leq$
Urine						
Allantoin, mmol/L	7.94	7.40	8.48	7.55	0.78	NS
Allantoin, mmol/d	81.3[a]	97.1[ab]	107[b]	116[b]	8.45	0.03
Uric acid, mmol/L	0.60	0.51	0.49	0.42	0.11	NS
Uric acid, mmol/d	6.51	6.79	5.92	6.97	1.28	NS
Plasma						
Allantoin, _mol/L	191[a]	201[ab]	216[ab]	231[b]	26.1	< 0.05
Uric acid, _mol/L	44.2	37.3	40.7	41.8	4.08	NS
Milk						
Allantoin, _mol/L	130[a]	220[ab]	199[ab]	267[b]	41	0.03
Allantoin, mmol/d	3.78[a]	7.25[b]	6.48[ab]	7.71[b]	1.2	0.04
Uric acid, _mol/L	105[a]	109[ab]	118[ab]	120[b]	6.5	< 0.05
Uric acid, mmol/d	3.16	3.64	3.82	3.61	0.4	NS

[a-c]Numbers with different letters differ ($P < 0.05$).

Table 6-2. Prediction of duodenal MN flow (g/d)[1] from allantoin and uric acid concentration and excretion in urine, plasma and milk.

	Equation[2]	r^2	RMSE	$P \leq$[3]
Urine				
Allantoin, mmol/L	221 + 7.85x	0.05	84.2	NS
Allantoin, mmol/d	249 + 0.34x	0.01	85.7	NS
Uric acid, mmol/L	340 – 111x	0.14	79.8	0.04
Uric acid, mmol/d	349 – 10.1x	0.17	78.4	0.03
Plasma				
Allantoin, _mol/L	205 + 0.34x	0.16	79.2	0.03
Uric acid, _mol/L	241 + 0.92x	0.03	85.2	NS
Milk				
Allantoin, _mol/L	211 + 0.38x	0.11	80.8	0.08
Allantoin, mmol/d	225 + 9.62x	0.10	80.9	0.08
Uric acid, _mol/L	244 + 0.33x	0.01	86.7	NS
Uric acid, mmol/d	243 + 10.4x	0.02	85.9	NS

[1]Duodenal MN flow (g/d) has previously been reported (Peterson et al., 200#).

[2]Equation of the regression line where MN flow (g/d) is on the Y axis.

[3]Indicates significance of the slope.

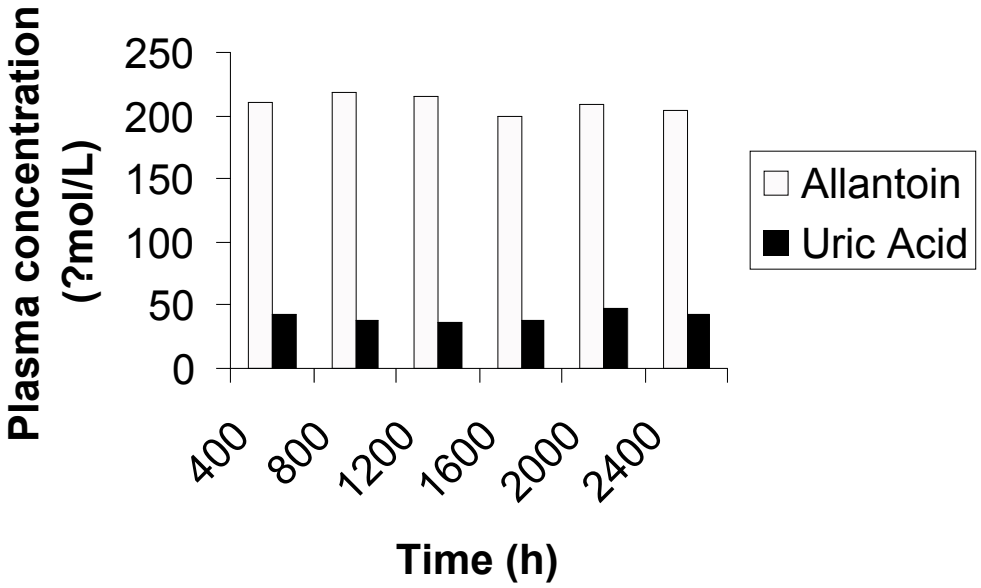

Figure 6-1. Effect of time on allantoin and uric acid concentrations (_mol/L) in plasma from cows fed diets varying in RDP source (*P* = NS).

APPENDIX A

Original Literature Review from Original Research Proposal

INTRODUCTION

Animal agriculture has been identified as a major source of nonpoint nitrogen **(N)** pollution of water resources (Thomann et al., 1994) and the potential negative impacts of N have become an area of public concern. Substantial efforts have gone into managing nutrients on dairy farms to maximize profit while reducing the risk of pollution to protect water resources (Lanyon, 1994).

Nitrogen is released into the environment through volatilization of ammonia to the air, nitrate leaching into ground water and run-off to surface water. In the air, volatile nitrogen oxides have been blamed for ozone loss and global warming, and volatile ammonia contributes to acid rain (Johnson et al., 1992; Tamminga, 1996; Trefil, 1997). Even when N is properly managed, there is a potential for run-off, leaching and volatilization of N from manure or other fertilizers applied to crops. Therefore, reducing nutrient losses requires better feeding and herd management to reduce the need for crops and manure application (Kohn et al., 1997). In order to decrease manure N output, it is necessary that N utilization by the animal is improved and that cows are fed at requirements without overfeeding N. Reductions in ruminal N loss are possible by reducing the dietary N concentration or by improving the efficiency of retaining rumen degraded N through microbial protein synthesis (Tamminga, 1992).

Microbial protein synthesis in the rumen makes up 60 to 85% of the crude protein (**CP**) requirements for maintenance, growth, gestation, and lactation in dairy cattle (Stern et al., 1994). Microbial protein is a high quality protein, similar in amino acid (**AA**) profile to milk protein and is highly digestible (O'Conner et al., 1990). Prediction of rumen microbial protein production would enable diets to be formulated more accurately to meet the protein requirement of the dairy cow without overfeeding. Routine estimation of rumen microbial protein production in the field could be used to fine-tune and evaluate rations. Not only would this reduce the cost of the ration for the producer but it would also decrease N excretion which could eventually lead to environmental pollution. However, to date, there is no proven noninvasive method to estimate rumen microbial protein production.

BACKGROUND

Purine Derivatives. In ruminants, purines derivatives are excreted in urine and milk as allantoin, uric acid, xanthine and hypoxanthine. They originate from the catabolism of purines of both endogenous and exogenous origin (Verbic et al., 1990). Concentrations of nucleic acids in the rumen are used to estimate microbial nitrogen (**MN**) production because nucleic acids from the diet are thought to be degraded in the rumen (Smith and McAllen, 1970). As a result, most of the purines and pyrimidines found in the duodenum are assumed to originate from microbial protein production.

It was originally suggested that urinary excretion of purine derivatives such as allantoin could be a useful indicator of rumen microbial protein synthesis (Topps and Elliott, 1965). Since then, many researchers have used urinary and milk excretion of

purine derivatives including allantoin and uric acid as a non-invasive method to predict rumen microbial protein production with varying results.

Purine Derivatives in Urine. Feeding a high concentrate diet was shown to increase excretion of urinary allantoin and uric acid in multiparous Holstein cows (Valadares et al., 1999). Additionally, they demonstrated that spot urine sampling yielded satisfactory estimates of purine derivative excretion versus total urine collection. Gonda et al. (1996) reported that though feeding a high concentrate diet resulted in an increase in urinary allantoin excretion, a high fat diet (5.8% vs 2.8% fat) had no effect in lactating cows. A linear relationship was determined by Vercoe (1976) between digestible dry matter intake and urinary allantoin excretion in beef steers and buffalos. In multiparous Holstein cows, increasing dietary protein resulted in increased urinary allantoin excretion (Moorby et al., 1996).

In beef cows, increasing soybean meal or urea content of the diet resulted in increased urinary allantoin and urea excretion while no changes were noted for uric acid (Susmel et al., 1993; Susmel et al., 1994; Susmel et al., 1995). Urinary excretion of allantoin has been shown to be correlated with the amount of nucleic acids infused post ruminally to sheep (Antoniewicz et al., 1980; Giesecke et al., 1984; Fujihara et al., 1987; Chen et al., 1990; Balcells et al., 1991) and steers (Verbic et al., 1990). It was concluded that urinary allantoin may be a useful index to estimate duodenal input of purines when animals are fed close to or above their maintenance requirements (Balcells et al., 1991).

Several studies have correlated excretion of allantoin, uric acid and total purines to microbial N flow (Lindberg et al., 1989; Puchala and Kalasek, 1992;

Johnson et al., 1998). In sheep, Lindberg et al. (1989) observed a high correlation between urinary excretion of purines and allantoin and duodenal microbial N flow. Similar results were found by Puchala and Kulasek (1992) in ewes. Johnson et al. (1998) concluded that uric acid excretion in urine can be used to predict microbial N production in lactating Holstein cows.

Lindberg and Jacobsson (1990) concluded that urinary purine excretion in ruminants is unaffected by moderate changes in energy intake and by large changes in protein intake. However, only a 3 d adaptation period was allowed in between changing dietary protein concentrations and/or energy content fed to sheep. This 3 d period was immediately followed by a 3 d data collection which was used to reach their results. It has also been reported that urinary allantoin excretion cannot be used to predict microbial N production accurately among cows at different stages of lactation (Johnson et al., 1998). However, in the six experiments used to reach these conclusions, only 6 Holstein cows were followed through an entire lactation. As a result, the conclusions from these two studies may be misleading.

Purine Derivatives in Milk. Feeding diets high in energy was shown to increase excretion of milk allantoin in lactating cows (Kirchgessner and Kaufmann, 1987; Lebzien et al., 1993; Valadares et al., 1999). Dry matter intake was also found to be positively correlated with allantoin excretion in milk (Gonda and Lindberg, 1997). Several studies have found a correlation between microbial protein N at the duodenum and allantoin excretion in milk (Lebzien et al., 1993; Giesecke et al., 1994; Timmermans et al., 2000). Giesecke et al. (1994) showed that the daily amount of microbial protein entering the duodenum was correlated with the concentration of

allantoin in milk in Holstein cows. Allantoin excretion in milk was positively correlated with microbial nitrogen flow in lactating multiparous Holstein cows (Timmermans et al., 2000). Additionally they used milk allantoin output to develop a prediction equation to estimate microbial N flow to the duodenum, including milk yield in their model (milk allantoin output (mmol/d) = -0.58 + 0.0089 microbial nitrogen (g/d) + 0.099 milk (kg/d)).

Shingfield and Offer (1998) and Giesecke et al. (1994) reported a high correlation between milk allantoin excretion and concentration with milk yield in Holstein cows. Additionally, auto-correlation with milk yield accounted for milk allantoin excretion and concentration being highly correlated with calculated microbial protein supply (Shingfield and Offer, 1998). However, individual cow milk allantoin or excretion was poorly correlated with urinary purine excretion or calculated microbial protein supply (Shingfield and Offer, 1998).

Kirchgessner and Kreuzer (1985) reported that though milk urea increased as dietary crude protein increased, milk allantoin concentration was not altered. As dry matter intake increased, milk yield increased causing a subsequent increase in the overall yield of milk allantoin. There was no change in milk allantoin concentration when lactating cows were energy and protein depleted followed by normal or excessive nutrient supply (Kirchgessner and Windisch, 1989). When lactating beef cows were fed urea in order to increase rumen microbial growth, no changes in milk allantoin or uric acid were noted (Susmel et al., 1995). Martin-Orue et al. (1996) concluded that milk purine excretion in ewes was not a reliable alternative to urinary purine excretion though statistical inferences in this paper are questionable.

Correlation of Milk and Urine Purine Excretion. Gonda and Lindberg (1997) reported that urinary excretion of allantoin was positively correlated with its excretion in milk in lactating dairy cows. Allantoin concentrations in milk were correlated with urinary excretion of allantoin in Holstein cows (Vagnoni et al., 1997). Additionally, milk allantoin excretion was highly correlated with urinary purine derivative excretion when milk yield was included as a covariate in the model (Shingfield and Offer, 1998). In ewes, allantoin excretion in milk was not correlated with its excretion in urine although its relationship with urinary purine excretion tended towards significance (Martin-Orue et al., 1996). However, in this study, milk yield was correlated with milk allantoin concentrations though this information was not used as a covariate in the previous statement.

D-Amino Acids in Milk. The D-stereoisomer of alanine and glutamic acid are found in constant amounts in both gram-positive and gram-negative bacterial cell walls (Schleifer and Kandler, 1972). It was proposed by Garrett et al. (1982) that D-amino acids **(DAA)** may be possible markers for estimation of rumen microbial protein production. However, for DAA to be a useful marker they must not be present in feedstuffs or converted to the L-form during digestion. It was reported that DAA in cow's milk originate from the digestion of proteins containing DAA from the peptidoglycan layer in the cell walls of rumen microbes (Rooke et al., 1984; Schleifer and Kandler, 1972).

There are three possible sources of DAA in milk: rumen microflora, lysis of cell walls of lactic bacteria, or by racemases in milk (Gandolfi et al., 1992). The D-forms of Asp, Gly and Ala are the only three DAA detected in fresh raw milk samples

(Csapo et al., 1995). In total, DAA make up only 3.4% of the total free D- and L-amino acids (Csapo et al., 1995). Since DAA are often products of bacterial metabolism, Csapo et al, (1995) evaluated the effect of mastitis of DAA concentration. It was found that as somatic cell count **(SCC)** increased (determined with the California Mastitis Test), the concentration of DAA, especially D-Asp, D-Glu and D-Ala, also increased. Additionally, it was determined that foremilk had higher concentrations of D-Asp, D-Glu and D-Ala than milk sampled later in the milking process. This could be due to the fact that foremilk has a higher SCC (Forster et al., 1967).

If an accurate method to quantification DAA can be developed, perhaps a prediction equation can be developed to correlate rumen microbial protein yield to DAA concentration in milk. However, it is necessary that SCC be analyzed in all milk samples and put in the model as a covariate to account for any variation associated with SCC.

Estimation of Ruminal Protein Degradation. In order to develop a prediction equation to estimate protein degradation in the rumen, it is necessary to quantify the amount of protein entering the rumen through the diet, leaving the rumen as undigested protein and microbial protein and flowing to the duodenum. In 1997, Lykos et al. published a method to evaluate microbial protein flow out of the rumen. They collected approximately 1.3 L of ruminal digesta from the reticuloormasal orifice every 3 h to quantify the amount of microbial protein exiting the rumen. A portion of this was homogenized in a blender at low speed and filtered through cheesecloth. The liquid fraction was later used to quantify the amount of bacteria

associated with the liquid fraction leaving the rumen. The solid fraction was soaked in chilled saline and homogenized at high speed and filtered through cheesecloth. This fraction was later used to quantify the amount of bacteria associated with the solid fraction leaving the rumen.

APPENDIX B

Adopting New Techniques: Statistical Procedures to Determine the Agreement between two Laboratory Methods

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ABSTRACT

As laboratory technologies advance, it is inevitable that new procedures will be adopted to replace older, perhaps more tedious techniques. These new techniques are more automated or more precise than the out-of-date counterpart. Prior to adoption of a new technique, it is necessary to ensure that the new technique agrees adequately with the old one. As suggested by Bland and Altman (1986), the use of a correlation coefficient is inadequate to determine agreement, therefore, a set of steps to evaluate agreement was proposed. This paper will illustrate the use of SAS and JMP to evaluate agreement. The example used will include the evaluation of agreement on data collected under different conditions (e.g. treatment, genetic strains, tissues, etc.). To evaluate agreement, an acceptable difference must first be established. This is the maximum amount that observations from two techniques can differ and still be considered in agreement. The statistical techniques used will include graphics, descriptive statistics and linear models to draw appropriate conclusions regarding agreement between the two techniques.

INTRODUCTION

As scientific analyses advance, new techniques are introduced to analyze the same parameter, or so it is assumed. Too often these new technologies are simply brought into a lab with little or no evaluation of how they match-up with what they are soon to replace. This can present a problem when the new technology, though it may be easier, cheaper or less tedious, gives results that are more variable, less consistent or even magnitudes different than any result ever given from the old technique. As a result, it is imperative that new technologies are compared to their old counterparts and adequately assessed prior to adoption into the laboratory.

In 1986, Bland and Altman suggested a series of steps that could be used to evaluate agreement or disagreement between two technologies. They noted that the common use of correlation coefficients to establish agreement between two methods is incorrect as correlation coefficients give us an idea of the relationship between two analyses, but not the conformity between them. Therefore the objective of this paper is to detail the correct statistical approach to establish agreement and disagreement between two techniques.

STATISTICAL ANALYSIS

Datasets. Although experimental data were available, they did not illustrate all of the problems we wished to demonstrate. Therefore, two simulated datasets (n=20 per group) were created using SAS® (Version 9.1, 2003) to demonstrate both agreement and disagreement between two techniques. In these datasets not only are two techniques evaluated, but the techniques are also evaluated on two tissue types (liver vs. mammary) from two genotypes (dairy cattle and goats), in this case two different

species. Each technique within the two datasets was independently analyzed for the effects of genotype and tissue type. These analyses are not a formal comparison of methods, but illustrate the typical results that might be reported if a researcher was using one of the two techniques. The data were then combined for an evaluation of the differences between the two techniques for each dataset.

Individual technique analysis. Statistical analyses for each analytical technique (1 and 2) were performed using JMP® (Version 4, 2002) and code is provided using regression and mixed model procedures of SAS® (Version 9.1, 2003).

The model used for these analyses was as follows:

$$Y_{ijk} = \_ + G_i + A_{k(i)} + T_j + (GT)_{ij} + \_{ijk}$$

Where Y_{ijk} is the observed value for either Technique 1 or Technique 2 for the i^{th} genotype, j^{th} tissue type and k^{th} animal; _ is the overall mean; G_i is the fixed effect of i^{th} genotype; $A_{l(i)}$ is the random effect of k^{th} animal nested within the i^{th} genotype; T_j is the fixed effect of j^{th} tissue type; $(GT)_{ij}$ is the interaction between the i^{th} genotype and the j^{th} tissue type; and $\_{ijk}$ is random error. Least square means (±SEM) are presented to indicate average difference between genotypes and tissue types. Significant effects were declared at $P < 0.05$.

Difference between techniques. Bland and Altman's paper suggest, but did not include an approach for evaluation of agreement, when there is a slope bias (non constant bias) in the data. In addition, their example is for one sample or group of n observations. Their methods could then be applied separately to each group or treatment when multiple groups or treatments are required. The mean of these two techniques as well as the difference between the two techniques were calculated.

Statistical analyses were performed using JMP as well as the mixed model procedure of SAS. The difference between the two techniques was regressed on the mean of the two techniques to examine the effect of the magnitude of the response. The model used for analysis of the difference between the two analytical techniques was as follows:

$D_{ijk} = \_ + G_i + A_{k(i)} + T_j + (GT)_{ij} + b_1M_{ijk} + b_2M_{ijk}(G_i) + b_3M_{ijk}(T_j) + b_4M_{ijk}(GT)_{ij} + \_{ijk}$

Where D_{ijk} is the observed difference between the two techniques for the i^{th} genotype, j^{th} tissue type and k^{th} animal; _ is the overall mean; G_i is the fixed effect of i^{th} genotype; $A_{l(i)}$ is the random effect of k^{th} animal nested within the i^{th} genotype; T_j is the fixed effect of j^{th} tissue type; $(GT)_{ij}$ is the interaction between the i^{th} genotype and the j^{th} tissue type; b_1M_{ijk} is the linear regression of the differences between the two techniques on the means of the two techniques; $b_2M_{ijk}(G_i)$, $b_3M_{ijk}(T_j)$ and $b_4M_{ijk}(GT)_{ij}$ are the interactions between the linear regression and fixed effects; and $\_{ijk}$ is random error.

Scatter plots of differences with regression lines are used to illustrate the agreement of analytical techniques over the range of values observed for the sample results. Residual variances are reported to assess the precision of each analytical technique for combinations of genotype and tissue type. The variability associated with the analytical techniques, tissues and genotypes were evaluated and compared using Akaike's Information Corrected Criterion (**AICC**) as a 'goodness of fit' indicator. Estimates of the residual variances for each combination of genotype and tissue type, and for each analytical technique are reported.

RESULTS USING JMP

Individual technique analysis. Both techniques within the agreement and disagreement datasets were first independently evaluated (Table 1). For the agreement dataset, both the dairy cow and goat genotypes did not differ from each other within each technique. There was, however, a difference in tissue as liver tissue had significantly higher values than that of mammary tissue within both techniques. This holds true within the genotype*tissue interaction where liver tissue from both dairy cows and goats had higher values than mammary tissue from both genotypes.

Table 1. Independent evaluation of techniques 1 and 2 in the agreement and disagreement datasets using JMP.						
Agreement Dataset	Technique 1			Technique 2		
	LS Means	SE	*P* value	LS Means	SE	*P* value
Genotype						
Dairy cow	2.32	0.13	0.79	2.32	0.13	0.91
Goat	2.27	0.13		2.30	0.13	
Tissue						
Liver	2.69	0.10	<0.0001	2.70	0.10	<0.0001
Mammary	1.90	0.10		1.91	0.10	
Genotype*Tissue						
Dairy cow/Liver	2.86	0.14	<0.0001	2.87	0.14	<0.0001
Goat/Liver	2.51	0.14		2.53	0.14	
Dairy cow/Mammary	1.78	0.14		1.77	0.14	
Goat/Mammary	2.03	0.14		2.06	0.14	
Disagreement Dataset	Technique 1			Technique 2		
	LS Means	SE	*P* value	LS Means	SE	*P* value
Genotype						
Dairy cow	2.36	0.15	0.67	4.74	0.16	0.0007
Goat	2.28	0.15		3.94	0.16	
Tissue						
Liver	2.74	0.15	<0.0001	4.73	0.16	0.001
Mammary	1.90	0.15		3.96	0.16	
Genotype*Tissue						
Dairy cow/Liver	2.95	0.21	0.12	5.87	0.23	<0.0001
Goat/Liver	2.53	0.21		3.59	0.23	
Dairy cow/Mammary	1.78	0.21		3.62	0.23	
Goat/Mammary	2.03	0.21		4.30	0.23	

For the disagreement dataset, there was no difference between genotype for Technique 1 however dairy cows had significantly higher values using Technique 2 than did goats (Table 1). This is the first indication that these two techniques may not be giving the same result. However, when tissue type was evaluated, liver tissue within both techniques was significantly higher than mammary tissue but Technique 1 was giving values much lower than Technique 2. This is another clue that these two techniques may be different. Finally, when the interaction between genotype*tissue

was evaluated, there was a significant interaction with Technique 2 and not Technique 1.

These results indicate that in the case of the disagreement dataset, the relationship between the two techniques is not just a simple rescaling of the data. The differences between the two techniques are more complex in the case of the disagreement data set. As a result it is necessary to evaluate this dataset (as well as the agreement dataset to provide an example) further using the methods presented in Bland and Altman (1986), with some modifications and additions, to assess the nature of the differences between the two techniques.

Difference between techniques. The first step to evaluate difference between techniques is to plot the difference of the two techniques (Technique 1 minus Technique 2) versus the mean of the two techniques. In Figure 1 and 2 we can see that, with a sample mean of 2.69 and 1.91 across both genotypes for liver and mammary tissue, respectively, all samples are within ±0.3 of the mean. Typically a maximum acceptable difference (MAD) needs to be established *a priori* by the researcher for evaluation of the difference between the two techniques. In other words, what is the maximum difference between techniques that the researcher would consider acceptable, if the new technique is to be adopted into the laboratory. This MAD could be an absolute difference (e.g. ±0.4) or could be expressed as a percentage of the mean. In this case, if we accept data, which falls within ±0.4 of the mean, then these two techniques are similar enough to each other in order to be considered interchangeable. However, since the data represents samples from the population of differences, Bland and Altman suggest that the MAD be evaluated allowing some

probability that differences might fall out side the MAD value. For example, one might simply estimate the cutoffs for the center 95% of the population of differences. That is, estimate the 2.5 percentile and 97.5 percentile for the distribution of differences. Additionally, it is important to note that there is neither a mean or slope bias (mean difference close to zero, slope close to zero) associated with these data which means that samples, whether high or low, are all falling in an acceptable range. These data can be plotted by genotype and tissue type for further evaluation, which is what we will do for the disagreement dataset.

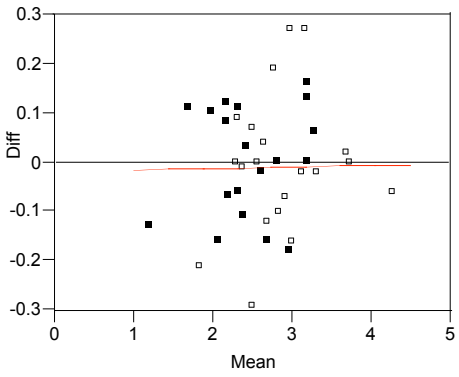

Figure 1. Plot of the difference between Technique 1 and Technique 2 vs. the mean for liver tissue from both dairy cows (_) and goats (_) from the agreement dataset.

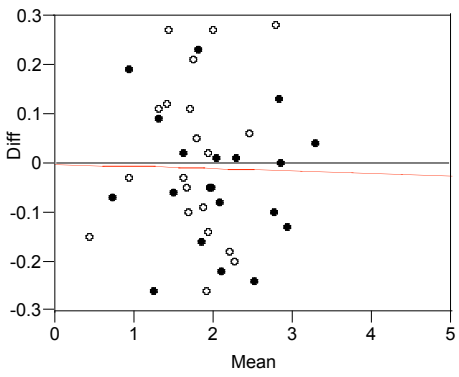

Figure 2. Plot of the difference between Technique 1 and Technique 2 vs. the mean for mammary tissue from both dairy cows (_) and goats (_) from the agreement dataset.

As indicated by the significant interaction in Table 1, it is clear that the two techniques are resulting in differences due to both genotype and tissue type. As a result, we will look at all four graphs of technique differences vs. the means for dairy cow/liver, dairy cow/mammary, goat/liver and goat/mammary combinations. Figure 3 shows a serious slope bias: as mean sample values increase to 7, they approach an agreement between

the two techniques (near 0 difference). However, since most of the sample means are between 3 and 6, Technique 2 is reading highest at lower values (mean=3) and the difference decreases as the mean increases. As a result, these two techniques obviously do not agree with dairy cow liver tissue, except for a very small range of values around a mean of 7.

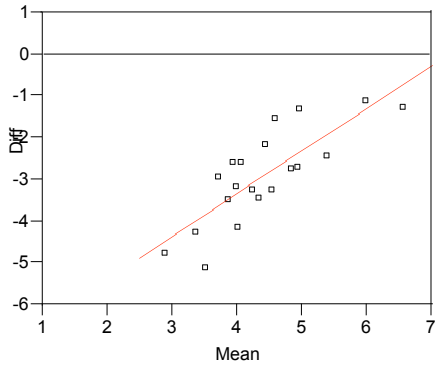

Figure 3. Plot of the difference between Technique 1 and Technique 3 vs. the mean for liver tissue from dairy cows (_) from the disagreement dataset.

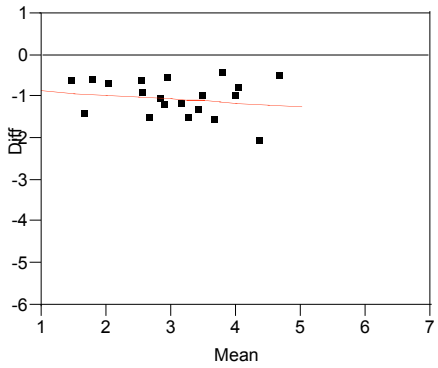

Figure 4. Plot of the difference between Technique 1 and Technique 2 vs. the mean for liver tissue from goats (_) from the disagreement dataset.

In Figure 4, a mean bias is present as most of the samples analyzed using Technique 1 are running consistently one unit lower than Technique 2. With an average of about 3 units for both techniques and a difference between them of 1 unit, it should be obvious that these techniques are providing quite different answers, but unlike Figure 3 the disagreement between the techniques is not associated with the magnitude of the mean.

For mammary tissue from both dairy cows and goats (Figure 5 and 6, respectively), we can see that

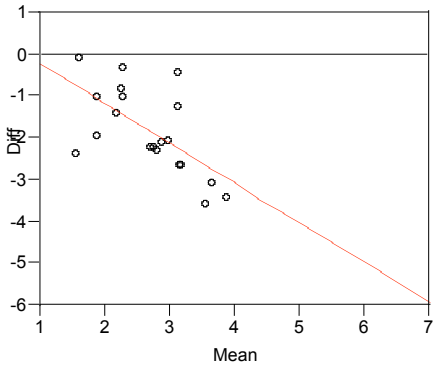

Figure 5. Plot of the difference between Technique 1 and Technique 2 vs. the mean for mammary tissue from dairy cows (_) from the disagreement dataset

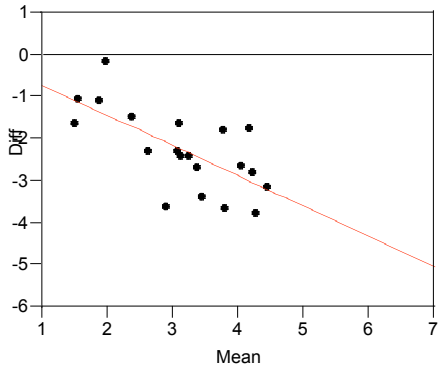

Figure 6. Plot of the difference between Technique 1 and Technique 2 vs. the mean for mammary tissue from goats (_) from the disagreement dataset

there is a slope bias associated with both genotypes. In this case, as sample means increase, samples deviate more and more away from an agreement between the two techniques. Again, ideally most of the data points should fall along the reference line (at 0 difference) and the slope should be near zero either towards or away from this line. As a result, from Figures 3-6, we can see that the two techniques are not similar regardless of genotype or tissue type for the disagreement data set.

RESULTS USING SAS

Individual technique analysis. For the agreement data set one would expect the results using SAS would not differ from those JMP. The only reason for including this section is to present the SAS code that one would use to fit the model described in the method section. The code below is for the mixed procedure in SAS. The information present here is for the analysis of Technique 1. The results for Technique 2 were analyzed using the same procedures, but are not presented.

```
proc mixed data=agree;
class Genotype Tissue;
model Tech1 = Genotype Tissue Genotype*Tissue;
```

```
random AnimalID(Genotype);
repeated / group=Genotype*Tissue;
lsmeans Genotype Tissue Genotype*Tissue;
```

The repeated statement is included to provide an evaluation of homogeneity of variances (HOV). A group option on the repeated statement is used to request that a separate residual variance be calculated for each combination of genotype and tissue. The mixed procedure generates a number of goodness of fit (GOF) statistics that can be used to evaluate how well the random portion is fit. For this example the AICC statistics are compared to evaluate if the data are better fit using a single pooled residual variance or if the data are better fit using the four separate residual variances. The above analysis fits four separate residual variances and resulted in the following AICC statistic.

AICC (smaller is better) 117.2

When the repeated statement was removed from the mixed procedure the AICC statistic was obtained for an analysis with a single pooled residual variance. The single pooled variance model resulted in a smaller AICC indicating a better fit of these data than fitting four separate residual variances. Thus the assumption of HOV is supported.

AICC (smaller is better) 112.1

Based on this result for the final analysis the repeated statement was dropped and another option to the model statement was added. The 'outp' option creates the data set named **resids** containing the model residual and predicted values, which are useful in evaluating the normality assumption for the analysis. It is the results of this analysis that is exactly the same as those found in Table 1.

```
proc mixed data=agree;
class Genotype Tissue;
model Tech1 = Genotype Tissue Genotype*Tissue / outp=resids;
random AnimalID(Genotype);
lsmeans Genotype Tissue Genotype*Tissue;
```

For the disagreement data set we would use the first mixed code above, however the resulting GOF statistics leads to a different conclusion. Again only the analysis for Technique 1 will be presented because it adequately illustrates the differences in the analysis for the disagreement data as compared to the agreement data. Following the approach above, the mixed model needs to be fit with and without the repeated statement to obtain the GOF statistics for the two potential models.

When the repeated statement was included the following AICC statistics were obtained for each residual variance.

AICC (smaller is better) 207.7

Covariance Parameter Estimates

Cov Parm	Group	Estimate
Residual	Genotype*Tissue Dairy Liver	1.8769
Residual	Genotype*Tissue Dairy Mammary	0.2844
Residual	Genotype*Tissue Goat Liver	0.7684
Residual	Genotype*Tissue Goat Mammary	0.4752

Omitting the repeated statement resulted in the following output:

AICC (smaller is better) 217.7

Covariance Parameter Estimates

Cov Parm	Estimate
Residual	0.8535

Based on the AICC statistics, the four residual variances model is a better fit of the data. That is, the GOF statistics indicate that these data should not be considered to

have HOV. The following mixed code will provide an appropriate analysis of variance of these data without pooling the residual variances. It will also conduct tests using the separate variances by the same approach that are commonly presented for the two sample t test with unequal variances with the Satterthwaite estimation of the degrees of freedom (df).

```
proc mixed data=disagree;
class Genotype Tissue;
model Tech1 = Genotype Tissue Genotype*Tissue / ddfm=sat outp=resids;
random AnimalID(Genotype);
repeated / group=Genotype*Tissue;
lsmeans Genotype Tissue Genotype*Tissue;
```

The resulting ANOVA and means output follows:

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Genotype	1	49.9	0.17	0.6857
Tissue	1	49.9	16.43	0.0002
Genotype*Tissue	1	49.9	2.56	0.1162

Least Squares Means

Effect	Genotype	Tissue	Estimate	Standard Error	DF
Genotype	Dairy		2.3633	0.1644	24.6
Genotype	Goat		2.2792	0.1251	36
Tissue		Liver	2.7398	0.1821	32.4
Tissue		Mammary	1.9026	0.0976	35.7
Genotype*Tissue	Dairy	Liver	2.9470	0.3064	19
Genotype*Tissue	Dairy	Mammary	1.7795	0.1191	19
Genotype*Tissue	Goat	Liver	2.5327	0.1967	19
Genotype*Tissue	Goat	Mammary	2.0257	0.1547	19

The results for Technique 2 also indicated heterogeneous variances and assuming normal distributions of residuals within genotype and tissue combinations, the above approach would provide and appropriate analysis.

Difference between techniques.

The following SAS code was used to analyze the differences between the two techniques.

```
proc reg data=agree;
by Genotype Tissue;
model Diff  = Mean;
plot Diff*Mean / pred vaxis=-.6 to .6 by .2 vref=-.4 .4;

proc mixed data=agree covtest;
class Genotype animalid Tissue;
model Diff = Genotype Tissue Genotype*Tissue
        Mean
        Mean*Genotype
        Mean*Tissue
        Mean*Genotype*Tissue;
random animalid(Genotype);
lsmeans Genotype Tissue Genotype*Tissue;
```

The regression procedure was primarily used to generate the graphs of the relationship between the technique differences and the mean. The plot statement option 'pred' request that observed differences, the regression line, and the 95% individual prediction belts be included in the plot. The option 'vref=-.4 .4' request that horizontal reference lines be drawn at the difference values -.4 and .4. These correspond to our MAD values for this example. The BY statement in the code would generate a plot for each level of genotype and tissue. Figure 7 is an example of the regression plot from the agreement data from the above code.

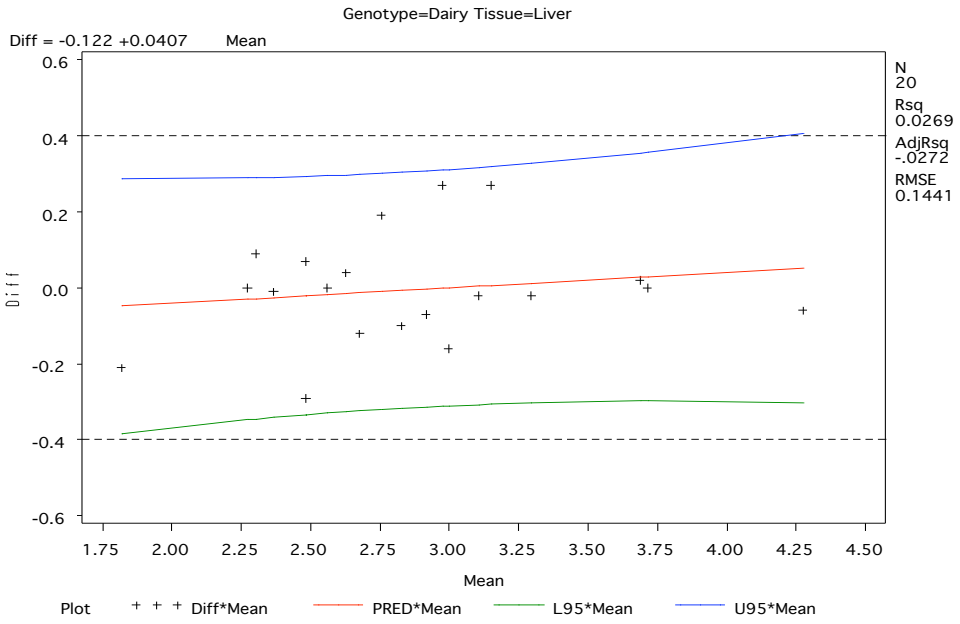

Figure 7. Plot of the difference between Technique 1 and Technique 2 vs. the mean for dairy cattle liver tissue for the agreement data set. The curved lines are the 95% individual prediction intervals and the horizontal dashed lines are the MADs.

As pointed out earlier in the paper observed differences are all within ±.3. However, note that the individual prediction intervals are beyond ±.3. In this case, the 95% individual prediction belts are within the MAD in the range of observed values. It is obvious that mean values below approximately 1.7 and above 4.3 would not be in agreement given that one of the two prediction belts will be outside either the upper or lower MAD. The other difference plots are similar for the agreement data and would be interpreted in the same manner.

The analysis using the mixed procedure was run on centered data for the mean (continuous independent variable) to simplify the interpretation of the SAS hypotheses. The last three lines are tests of hypotheses about the differences between slopes, while the first three lines are tests of hypotheses about mean differences at the grand mean of the X values. In this example there is no evidence that slopes are

different by genotype or tissue type or that the slopes differ from zero. That is, statistically the four regression lines could be represented as a single over all pooled regression with a non-significant slope. Likewise there is no evidence that the mean differences are a function of genotype and tissue type. The ANOVA output from the mixed procedure follows:

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Genotype	1	38	0.42	0.5205
Tissue	1	34	0.15	0.6970
Genotype*Tissue	1	34	1.26	0.2686
Mean	1	34	0.08	0.7812
Mean*Genotype	1	34	1.28	0.2659
Mean*Tissue	1	34	0.02	0.8902
Mean*Genotype*Tissue	1	34	0.03	0.8708

Examination of the mean differences and the tests of hypotheses that the mean differences are different from zero, completes the analysis of agreement for these data. The least squares means output from this analysis follows. Note that the mean differences are not different from zero for any mean.

Least Squares Means

Effect	Genotype	Tissue	Estimate	Standard Error	DF	t Value	Pr > |t|
Genotype	Dairy		0.002891	0.03314	38	0.09	0.9309
Genotype	Goat		-0.02389	0.02463	38	-0.97	0.3382
Tissue		Liver	-0.01860	0.02898	34	-0.64	0.5252
Tissue		Mammary	-0.00239	0.02941	34	-0.08	0.9357
Genotype*Tissue	Dairy	Liver	-0.02843	0.04640	34	-0.61	0.5441
Genotype*Tissue	Dairy	Mammary	0.03422	0.04732	34	0.72	0.4746
Genotype*Tissue	Goat	Liver	-0.00878	0.03473	34	-0.25	0.8020
Genotype*Tissue	Goat	Mammary	-0.03900	0.03493	34	-1.12	0.2721

The analysis of the disagreement data set is based on the same code as above except that due to the lack of HOV it was necessary to add a 'repeated / group=Genotype*Tissue' statement to the analysis along with the option 'ddfm=sat' to the model statement.

The following is the output from the analysis of the disagreement data set. For this genotype and tissue type none of the observed values fall within the MAD. Only for a very short range of values is the upper individual prediction belt within the MAD. To be considered in agreement both individual prediction belts must be contained within the MAD for the relevant range of values. Although the slopes and widths of the 95% prediction intervals changed, the methods failed to be in agreement for any of the genotype and tissue type combinations.

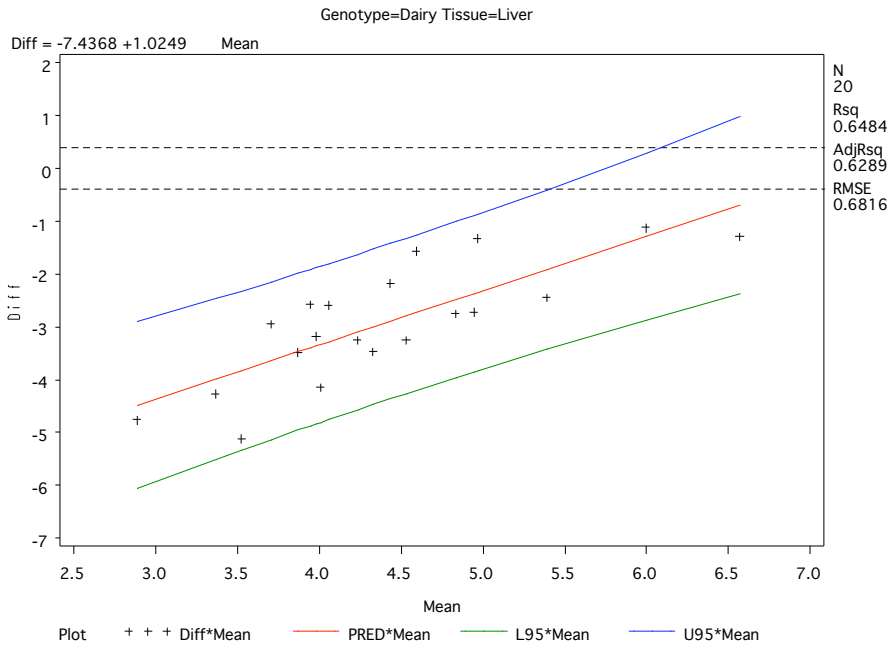

Figure 8. Plot of the difference between Technique 1 and Technique 2 vs. the mean for dairy cattle liver tissue for the disagreement data set. The curved lines are the 95% individual prediction intervals and the horizontal dashed lines are the MADs.

Examination of the regression ANOVAs (not shown) indicated that there was a significant slope ($P < 0.01$) bias for three of the four genotype and tissue type combinations. Only for liver tissue from goats was the slope not significantly different from zero ($P > 0.4$).

The results of the mixed model analysis are presented below. From the analysis of variance it is clear that for these data the magnitude of the slope bias and the mean bias is dependent on genotype and tissue type.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Genotype	1	55.5	54.85	<.0001
Tissue	1	55.5	0.45	0.5054
Genotype*Tissue	1	55.5	51.73	<.0001
Mean	1	52.9	3.52	0.0661
Mean*Genotype	1	52.9	5.11	0.0279
Mean*Tissue	1	52.9	43.83	<.0001
Mean*Genotype*Tissue	1	52.9	11.86	0.0011

Examination of the output from the least squares means indicate that the mean bias is significantly different from zero for every combination of genotype and tissue type.

Least Squares Means

Effect	Genotype	Tissue	Estimate	Standard Error	DF	t Value	Pr > |t|
Genotype	Dairy		-3.2300	0.1758	36	-18.37	<.0001
Genotype	Goat		-1.7380	0.09839	30	-17.66	<.0001
Tissue		Liver	-2.5516	0.1328	24.2	-19.22	<.0001
Tissue		Mammary	-2.4165	0.1515	31.3	-15.95	<.0001

Genotype*Tissue	Dairy	Liver	-4.0220	0.2446	18	-16.44	<.0001
Genotype*Tissue	Dairy	Mammary	-2.4380	0.2526	18	-9.65	<.0001
Genotype*Tissue	Goat	Liver	-1.0811	0.1035	18	-10.45	<.0001
Genotype*Tissue	Goat	Mammary	-2.3950	0.1674	18	-14.31	<.0001

CONCLUSIONS

The Bland and Altman paper outlines methods for assessing the agreement between two methods for a single sample of n observation. This paper adds two important tools to their approach. Using JMP and/or SAS we have illustrated how to assess mean and slope bias for data sets containing multiple groups or treatments. Slope bias was assessed using the regression procedure. Individual prediction intervals are use to compare results to the researchers maximum acceptable difference. The mixed model procedure was used to examine the differences in mean and slope bias among different groups. In addition, a default test in the least squares means output is useful to determine if the mean difference between groups is different from zero.

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