EFFECTS OF AMMONIA LOADING ON LYSINE UTILIZATION BY GROWING CATTLE

by

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ABSTRACT

Six ruminally-cannulated Holstein steers (202 ± 15 kg) were used to study the effects of ruminal ammonia loading on whole-body lysine utilization. Steers were housed in metabolism crates and used in a 6 × 6 Latin square design. All steers received 2.52 kg of DM/d of a diet (10.1% CP) containing 82% soybean hulls, 8% wheat straw, 5% cane molasses, and 5% vitamins and minerals. Ten g/d of urea was infused continuously into the rumen of all steers to ensure adequate ruminal ammonia concentrations; concurrently, steers were ruminally infused continuously with 200 g/d acetic acid, 200 g/d propionic acid, and 50 g/d of butyric acid and abomasally infused with 300 g/d of glucose continuously to increase energy supply without increasing microbial protein supply. Steers were also abomasally infused continuously with an excess of all essential amino acids except lysine to ensure that lysine was the only limiting amino acid. Treatments were arranged as a 3 × 2 factorial with 3 additional levels of urea (0, 40, or 80 g/d) continuously infused ruminally to induce ammonia loading and 2 levels of lysine (0 or 6 g/d) continuously infused abomasally. Treatments did not affect fecal N output (P = 0.37). Lysine supplementation decreased (P < 0.01) urinary N excretion from 51.9 g/d to 44.3 g/d, increased (P < 0.01) retained N from 24.4 to 33.3 g/d, and tended (P = 0.09) to reduce plasma urea-N. Urea infusions linearly increased retained N (26.7, 28.8, and 31.1 g/d; P = 0.05) and also linearly increased (P < 0.01) urinary N excretion (31.8, 48.1, and 64.4 g/d), urinary urea (21.9, 37.7, and 54.3 g/d), urinary ammonia (1.1, 1.4, and 1.9 g/d), and plasma urea (2.7, 4.0, and 5.1 mM) for 0, 40, and 80 g urea/d, respectively. Assuming that retained protein is 6.25 × retained N and contains 6.4% lysine, the incremental efficiencies of infused lysine utilization were 51, 59, and 69% for steers

receiving 0, 40, and 80 g/d of urea, respectively, suggesting that the ruminal ammonia loads might improve the efficiency of lysine utilization; this is supported by the observed increases in whole-body protein deposition in response to ammonia loading of our steers that were, by design, lysine deficient.

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Chapter 1 – A Review of Literature

INTRODUCTION

The efficiency of amino acid (AA) utilization in growing cattle has remained an enigma in beef nutrition. Although the latest NRC (1996) lists specific AA requirements, the accuracy of those requirements is questionable. This is because NRC assumes a uniform efficiency of utilization for each essential AA. If efficiency of use was the same for whole protein and all AA, one could suggest that the AA profile of protein flowing to the small intestine is ideal. In practical situations, this suggestion would not be true. Additionally, the AA requirements recommended by the NRC (1996) were based on estimates of absorbable AA, body weight, and AA composition of tissue. Depending on the diet, microbial protein may contribute the highest percentage of AA reaching the duodenum. For sheep, Storm and Ørskov (1984) suggested that nitrogen (N) retention would be limited by methionine and lysine if microbial protein was the only source of metabolizable protein. To avoid these limitations, dietary escape protein should be included in growing cattle rations. Appropriate supplementation of essential AA would require knowledge about individual AA efficiencies and predictions of microbial protein synthesis. This review will focus on efficiency of lysine utilization in cattle.

Among the essential AA limiting in cattle diets, lysine is a key AA of interest. In contrast to pigs who utilize up to 90% of lysine hepatic uptake for protein synthesis (Stoll et al., 1998), ruminants are relatively inefficient in lysine utilization (Batista et al., 2015). Although few studies have calculated lysine efficiency in cattle from growing animal response curves, recent data indicated that the incremental efficiency was 40% (Batista et al., 2015), indicating that at least half of all supplemental lysine was wasted by growing

cattle. By altering the animal's metabolism, the efficiency of lysine utilization could increase, resulting in lower feed costs for dietary lysine supplementation. Increasing ammonia (NH_3) loads to cattle may serve as a possible method for increasing lysine's efficiency for protein accretion. If excess NH_3 were supplied in the form of feed grade urea, the liver may spare lysine from catabolism and remove toxic NH_3 from blood as a high priority (Awawdeh et al., 2005). Whether or not this method is effective, one should consider the environmental consequences of increasing N excretion by cattle.

LYSINE REQUIREMENTS OF GROWING CATTLE

Under some situations, average daily gain (ADG) and feed efficiency improve when bioavailable lysine is supplied to growing cattle fed high-corn diets (Abe et al., 1997). Conversely, estimates of lysine requirements for growing animals have been variable because published studies involved different body weights and different diet compositions. For example, body weights differed by almost 300 kg between Tzeng and Davis (1980) who used 45 kg steers and Titgemeyer et al. (1988) who used 313 kg steers. In general, an increase in body weight increases AA requirements by the animal (NRC, 1996). Additionally, the 75% corn diet used by Titgemeyer et al. (1988) was first-limiting in lysine, whereas the milk diet supplied by Tzeng and Davis (1980) was not lysine deficient due to the presence of casein in milk. Thus, the influence of initial body weight and diet composition led to a wide variation in estimated lysine requirements, ranging from 13 g/d in Tzeng and Davis (1980) and 30 g/d in Titgemeyer et al. (1988).

The method used for determining lysine requirements is also important for obtaining accurate estimates. For example, the lysine requirements determined by plasma lysine concentrations in Tzeng and Davis (1980) exceeded the requirements

calculated from N balance by approximately 2 g/d. By considering energy supply as a variable, Ludden and Kerley (1998) estimated a requirement of 8 g/d of lysine for 280 kg steers fed a 56% corn diet. Abe et al. (1997, 1999) also published lysine requirements for newly-weaned steers adapted to corn-based diets. In those studies, lysine supplementation improved ADG for the first week after weaning. When steers reached body weights between 100 to 200 kg, lysine supplementation no longer improved ADG or feed efficiency. Replacing whole corn with soybean meal in the diet also decreased the benefits of lysine supplementation on growth (Abe et al., 1998). In that study, steers were less responsive to lysine and more responsive to methionine because dietary soybean meal was most liming in methionine (Abe et al., 1998).

For 240-kg steers fed a mixture of corn and corn gluten meal, supplementation of ruminally-protected lysine (50% lysine + 15% methionine) increased ($P < 0.05$) ADG and feed efficiency after 56 d of treatment (Klemesrud et al., 2000a). A follow-up study by the same authors (Klemesrud et al., 2000b) determined that the daily requirement for intestinal lysine was 40.5 g/d, which was similar to estimates of Zinn (1988). In Zinn (1988), steers fed a urea supplement were estimated to have a daily lysine requirement of 45 g/d. Given this difference, the limiting AA supply from urea-supplemented diets either changed the lysine requirement of steers, or the true lysine requirement was greater than what was predicted by Klemesrud et al. (2000b).

To ensure that growing steers were not limited by other AA, Wessels et al. (1997) abomasally infused 5.3 g/d of lysine and a mixture of other AA continuously to steers fed 85% rolled corn diets. Steers receiving AA infusions had a greater response in N retention than control steers that did not receive supplemental AA. The disadvantage of

Wessels et al. (1997) was the absence of response curves for lysine and other AA. If a diet was deficient in lysine, supplying additional lysine would increase N retention by the animal (Batista et al., 2015). If excessive amounts of lysine were supplemented, N retention would not show further improvements. Beyond the lysine requirement, unutilized or other AA would be catabolized by the liver and used to synthesize urea (Abdoun et al., 2010). Depending on dietary protein intake, the urea may be recycled back to the GI tract or excreted in urine. These urinary N losses result in higher feed costs and increased environmental pollution (Awawdeh et al., 2005).

LYSINE REQUIREMENTS OF LACTATING CATTLE

Although specific requirements for lysine are listed in the dairy NRC (2001), these requirements are based on estimated requirements for total MP. Another problem is that the efficiency of AA utilization for milk protein production has a fixed value of 67% for all AA. When Vyas and Erdman (2009) tested for differences in efficiency between lysine and methionine using lactation response curves, they found that the efficiencies for lysine and methionine were different; moreover, the efficiency of lysine utilization was 39% when 80 g/d of lysine was provided and 25% when 200 g/d of lysine was provided. These lysine efficiencies were 42 to 63% lower than the NRC (2001) estimate of 67%. Haque et al. (2012) observed an increase ($P < 0.05$) in milk production, milk protein synthesis, and efficiency when lactating cows were provided with 2 levels of CP (12.9% CP from corn silage alone or 14.5% CP from corn silage + soybean meal) and duodenally infused with a mixture of lysine, methionine, leucine, and histidine. Although Apelo et al. (2014) stated that 10% CP was considered adequate in most dairy diets, the accuracy of that statement requires further validation. If 10% CP was truly adequate, the observed

responses to AA infusion in Haque et al. (2012) may suggest that at least one of the infused AA limited production independent of CP supply.

When lactating cows were fed a 16% CP corn-based diet and infused with 0, 22.5, 45, 90, or 180 g/d of L-lysine-HCl, incremental infusions beyond 22.5 g/d led to numerical decreases ($P > 0.05$) in milk production efficiency (King et al., 1991). The only response in milk production to lysine infusions was observed between 0 and 22.5 g/d L-lysine-HCl (37% incremental efficiency). The lack of response beyond 22.5 g/d L-lysine-HCl may suggest that the lysine requirement for maximal milk yield was somewhere between 0 and 22.5 g/d L-lysine-HCl. This range is too large for estimating the exact lysine requirement. For milk protein synthesis, Wang et al. (2010) determined that a lysine to methionine ratio of 3:1 in MP would provide the greatest milk protein yield when supplemental lysine and methionine were provided in the diet. According to Guinard and Rulquin (1994), lysine supplementation stimulates AA extraction by the mammary gland, resulting in more substrate for milk protein synthesis in secretory cells. In that study, the amount of milk protein was increased from 670 to 702 g/d when lactating cows were duodenally infused with 0 or 9 g/d of lysine, respectively. In contrast, no differences in milk yield were detected in response to lysine infusions. When infusions of lysine exceeded 9 g/d, milk protein also remained constant. Based on the research model used by Guinard and Rulquin (1994), part of the increase in milk protein output between 0 and 9 g/d may be attributed to possible changes in microbial protein supply because N containing feedstuffs, such as corn silage, were supplied orally. Although AA were infused directly into the duodenum, no information on microbial protein contribution was available in that study. Any change in microbial protein supply

will change the overall composition and quantity of MP available for productive processes, resulting in confounded results. As an alternative to Guinard and Rulquin (1994), a semi-purified diet low in N would be recommended to prevent changes in microbial protein supply.

LYSINE SUPPLEMENTATION IN SEMI-PURIFIED DIETS

The experimental diet used by Campbell et al. (1997) was semi-purified, containing 83% soybean hulls and 8% wheat straw. This diet was designed to supply adequate amounts of ruminally-available N but limited amounts of AA to the small intestine. In addition, controlled amounts of AA were delivered to the small intestine by abomasal infusion. This design eliminated confounding factors that affect AA supply to cattle by preventing changes in ruminal fermentation among treatments and allowing direct provision of all supplemental AA to the small intestine (Awawdeh et al., 2004). If the AA were provided to the rumen, they would be deaminated to NH_3 by the ruminal microbes (Abdoun et al., 2006). To ensure that only the AA of interest was limiting, the other essential AA were abomasally infused in excess of the animals suspected requirements. Under this design, the animal could have responded to the AA of interest without limitation of other nutrients (Awawdeh et al., 2004). If the animal was deficient, the animal would have responded to higher levels of the AA. If the requirement was satisfied, N retention would have plateaued.

The semi-purified diet of Campbell et al. (1997) was energy deficient; therefore, energy supply to experimental animals was increased by ruminal and abomasal infusions. Glucose was supplied as an energy source in the abomasal infusate to prevent its utilization by ruminal microbes. Once in the abomasum, glucose passed to the small

intestine and could be absorbed. Volatile fatty acids, including acetate, propionate, and butyrate, were also supplied as an energy source in ruminal infusate. Volatile fatty acids should have been readily absorbed across the ruminal wall; moreover, ruminal microbes would have been unable to utilize them as an energy source, because these acids are microbial end products of fermentation. According to Lambert et al. (2004), semi-purified diets containing 83% soyhulls and 8% wheat straw were deficient in vitamins such as pyridoxine, folic acid, and cyanocobalamin. For this reason, the authors recommend supplementation of 10 mg/d pyridoxine, 10 mg/d folic acid, and 100 μg of cyanocobalamin to meet these vitamin requirements of growing cattle.

EFFICIENCY OF LYSINE UTILIZATION

The latest beef cattle NRC (1996) predicted that the efficiency of lysine utilization was the same as MP and all other essential AA. Although this prediction would make diet formulation easier than having different estimates of efficiency for each AA, later research demonstrated that each essential AA had different efficiencies of utilization when growing steers were limited by a single AA (Campbell et al., 1997; Loest et al., 2001; Awawdeh et al., 2004; McCuistion et al., 2004; Awawdeh et al., 2005). The incorrect prediction by the NRC (1996) was based on a Cornell Net Carbohydrate and Protein System equation where protein efficiency = 0.834 - 0.00114 × equivalent shrunk weight (Ainslie et al., 1993). This equation would only be applicable to intact protein and failed to consider the different metabolic pathways and uses for individual AA. Conversely, it does consider that AA efficiency for protein accretion decreases at greater body weights (Titgemeyer, 2012).

When lysine efficiencies were estimated using different diets, efficiency values were highly variable. For example, Burris et al. (1976) recorded an incremental efficiency of 13% when N retention was used as a response variable with growing steers (258 kg) fed a corn-based diet. In contrast, van Weerden and Huisman (1985) recorded an efficiency of 35% when calves (62 kg) were fed a milk replacer limiting in lysine. One reason for this difference could be younger cattle used by van Weerden and Huisman (1985). Slabbert et al. (1988) found an incremental efficiency of 30% when growing steers (230 kg) were fed whole-maize grain diets with molasses and urea. Interestingly, this efficiency was close to the value determined by van Weerden and Huisman (1985) who used steers with similar body weights.

Efficiency values greater than NRC (1996) estimates have also been calculated for lysine utilization. When growing steers were fed whole corn and corn gluten feed, the incremental efficiency of ruminally-protected lysine (50% lysine + 15% methionine) was near 100% (Klemesrud et al., 2000a). Because ADG was used as the response variable, it was difficult to distinguish how much of the gain was muscle or fat. Thus, the amount of lysine utilized for protein deposition could not be accurately determined. When a response curve for lysine infusion was used with growing steers fed a semi-purified diet, the incremental efficiency of lysine utilization was 40% (Batista et al., 2015). These efficiency values could indicate that 60% of all supplemental lysine was catabolized by growing cattle.

CATABOLISM OF LYSINE

Fate of Carbon Skeleton

There are two common pathways for lysine catabolism. The main pathway synthesizes a saccharopine intermediate from lysine and α-ketoglutarate in the liver. This pathway has a total of 11 reactions (Voet et al., 2013). Two molecules of carbon dioxide are produced through the initial formation of the saccharopine intermediate, which is an 11 carbon compound carrying a net negative charge. Although the amine group on the 5th carbon of lysine (ε-amino group) is transferred to α-ketoglutarate, this reaction does not use pyridoxal phosphate as a cofactor as typical transaminase enzymes do (Florence et al., 1973). Instead, saccharopine formation is catalyzed by the bifunctional enzyme α-aminoadipic semialdehyde synthase. Once formed, the same enzyme hydrolyzes the saccharopine to α-aminoadipic-6-semialdehyde, producing glutamate as a byproduct (Hallen et al., 2013). The irreversible nature of this reaction is a primary reason why lysine is an essential AA in mammals (Hallen et al., 2013). The dual function of α-aminoadipic semialdehyde synthase is attributed to its two catalytic subunits named lysine-α-ketoglutarate reductase and saccharopine dehydrogenase (Hallen et al., 2013). The catalytic activity of lysine-α-ketoglutarate reductase occurs at the ε-amino group end of lysine to produce the saccharopine while the catalytic activity of saccharopine dehydrogenase occurs at the carboxyl group end to produce α-aminoadipic-6-semialdehyde (Hallen et al., 2013). The subsequent oxidation of α-aminoadipic-6-semialdehyde and production of NADH by aminoadipate semialdehyde dehydrogenase produces α-aminoadipate. Aminoadipate aminotransferase enzyme, which uses pyridoxal phosphate, transfers the α-amino group of α-aminoadipate to α-ketoglutarate, producing α-ketoadipate and glutamate. One FAD+ and two NAD+ are also utilized as electron acceptors further down the pathway (Voet et al., 2013). The final product of lysine

catabolism is acetoacetyl-CoA. This acetoacetyl-CoA is further converted to the ketone body acetoacetate by acetoacetyl-CoA hydrolase (Voet et al., 2013). Thus, lysine is a ketogenic AA.

The second pathway of lysine catabolism is the pipecolic acid pathway (Florence et al., 1973). The pipecolic pathway involves synthesis of α-keto-ε-amino caproic acid from lysine and reduction of α-keto-ε-amino caproic acid to pipecolic acid (Hallen et al., 2013). Pipecolic acid is further converted to α-aminoadipic-6-semialdehyde and this reaction is catalyzed by α-aminoadipic semialdehyde dehydrogenase (Struys and Jakobs, 2010). The remainder of the pathway proceeds as it would with last steps of the saccharopine pathway.

Lysine catabolism is also important for synthesis of carnitine. In fatty acid catabolism, carnitine is used to transport fatty acids into the mitochondria for oxidation (Melegh et al., 1993). Various enzymes within cellular lysosomes convert lysine to trimethyllysine using histone methyltransferases as methyl group donors (Wang et al., 2003). This trimethyllysine serves as a necessary precursor for carnitine biosynthesis through 4 metabolic reactions (Melegh et al., 1993).

Fate of Ammonia

The ε-amino group of lysine has several potential fates. Once lysine is catabolized by the liver, the two glutamate molecules produced in the saccharopine pathway or one glutamate molecule produced in the pipecolic acid pathway can pass into the bloodstream or be excreted in the form of urea or NH_3. To synthesize urea, glutamate dehydrogenase enzyme in the liver splits glutamate into α-ketoglutarate and free NH_3, which can be incorporated by carbamoyl phosphate synthase-I (CPS I) in urea cycle.

Glutamate can also be transaminated to aspartate, which supplies the second N atom for urea synthesis.

Urea may be excreted in the urine or be recycled back to the rumen (Reynolds et al., 1992). The two modes of recycling occur via saliva or diffusion from the bloodstream across the ruminal epithelium (Lapierre and Lobley, 2001). This recycled urea can be catabolized by bacterial urease and incorporated into microbial protein. Free glutamate produced from lysine catabolism may also be used to synthesize glutamine in perivenous hepatocytes (Haussinger et al., 1992). If glutamine is synthesized by hepatocytes, the kidney may extract it from the blood and catabolize it to urinary NH_3 (Weiner and Verlander, 2014).

METABOLIC COSTS OF CATABOLISM

Although NH_3 is incorporated into urea, researchers were uncertain if both N atoms could originate from free NH_3 molecules. Krebs (1973) determined that NH_3 was a substrate for CPS I in the urea cycle and that the second N atom of urea was donated by aspartate; however, the origin of this aspartate-N remained a controversy. Lobley et al. (1995) and Reynolds et al. (1991) suggested that essential AA are catabolized as a secondary source of N during urea synthesis, whereas Lobley et al. (1996) and Mutsvangwa et al. (1999) suggested that the aspartate was synthesized from free NH_3. One reason for controversy was that the results changed depending on the conditions of each study. For example, animals in Lobley et al. (1995) were infused with NH_4Cl whereas Lobley et al. (1996) used NH_4CO_3 as an NH_3 source. Studies may also differ in energy supply, diet composition, and feeding frequency. Thus, the results of one study should not be extrapolated beyond the conditions from which they were developed.

Others may argue that the results of in vitro studies may not be representative of what happens in vivo.

Reynolds et al. (1991) proposed that mitochondrial NH_3 may not contribute both N atoms for urea synthesis when the liver receives large amounts of NH_3. To compensate, the liver would catabolize AA to generate aspartate for urea cycle. In contrast, Milano et al. (2000) suggested that AA catabolism was not a required source of urea N. Instead, AA catabolism may serve as a source of energy for driving the urea cycle forward (Milano et al., 2000). For each cycle, an equivalent of 4 ATP are consumed. Two molecules of ATP are required for carbamoyl phosphate synthesis and one ATP is hydrolyzed to AMP to form arginosuccinate from citrulline (Voet et al., 2013). If AA are catabolized for the urea cycle, there may be limitations in growth and other productive functions (Parker et al., 1995). Studies comparing the effects of high-concentrate and high-forage diets on NH_3 detoxification concluded that a high forage-to-concentrate ratio increased NH_3 absorption and urea production by the liver (Reynolds et al., 1991). These increases were explained by the higher protein content in some forages relative to grains. In Reynolds et al. (1991), the ratio of urea production to NH_3 absorption was used as an indicator of how much urea N was supplied by NH_3. If the ratio was greater than 1:1, N was assumed to be provided by AA. When a 75% alfalfa diet was fed to growing beef heifers, Reynolds et al. (1991) reported an average urea-N production to NH_3-N absorption ratio of 1.7:1.0, suggesting AA-N input to urea cycle. If urea production from AA was avoided, N retention and muscle growth may be enhanced (Sadiq et al., 2008). This strategy would be most useful in conserving limiting AA such as lysine.

LYSINE BIOSYNTHESIS

Lysine is considered an essential AA because mammals are unable to synthesize it within the body. In contrast, plants and bacteria have the ability to synthesize lysine from the AA aspartate (Sun and Huang, 2011). Lysine is a component of the cell wall of gram-positive bacteria in the rumen, whereas the intermediate of lysine biosynthesis (meso-DAP) is a cell wall component of gram-negative bacteria (Dogovski et al., 2012). The pathway used to synthesize meso-DAP and lysine is known as the DAP pathway (Dogovski et al., 2009). The initial step involves phosphorylation of aspartate to aspartyl phosphate. This intermediate is reduced to aspartate semialdehyde by aspartate-semialdehyde dehydrogenase (Dogovski et al., 2009). In the presence of dihydrodipicolinate (DHDP) synthase enzyme, aspartate semialdehyde and pyruvate react to form DHDP, which is subsequently reduced to tetrahydrodipicolinate (THDP) in the rumen (Hutton et al., 2007). This product serves as a branch point for two separate pathways. Some rumen bacteria convert the THDP to N-succinyl-2-amino-6-ketopimelate, whereas other bacteria convert the THDP to meso-DAP directly (Hutton et al., 2007). The bacterial species that synthesize N-succinyl-2-amino-6-ketopimelate use the succinyl pathway to convert this compound to meso-DAP under three additional reactions (Hutton et al., 2007). Thus, both pathways yield the same intermediate (meso-DAP). In the final step, meso-DAP is converted to lysine by DAP decarboxylase (Dogovski et al., 2012). Although these reactions exist in the rumen, the lysine produced may not be enough to satisfy the needs of ruminant animals for growth or other productive functions (Storm and Ørskov, 1984). The antagonistic effects of other AA

such as arginine may also limit lysine availability for protein synthesis in peripheral tissues if adequate amounts of lysine were available postruminally (Ball et al., 2007).

LYSINE-ARGININE INTERACTIONS

In the small intestine and kidney, lysine and arginine utilize the same transporters (Closs et al., 2004). As a result of this competition, too much of one AA may lead to saturation of transporters. For example, an excess of supplemental lysine may limit the absorption of arginine into blood (Jones et al., 1967). These antagonistic effects have been observed in both chicks (Austic and Scott, 1975) and rats (Jones et al., 1966); however, it was uncertain whether the arginine deficiency was due to transporter competition. Austic and Scott (1975) observed an increase in feed intake when excess lysine was removed from the diet. Thus, feed intake may have been a confounding factor for their response. In dogs, Czarnecki et al. (1985) suggested that competition between lysine and arginine occurred for kidney reabsorption when lysine was supplemented. Unlike rats and dogs, chicks lack CPS-I enzyme because uric acid serves as the excretory product for NH_3 in birds (Ball et al., 2007). Without some urea cycle enzymes, arginine cannot be synthesized.

Lysine-Arginine antagonism has not been observed in species other than pigs and chickens. When cat diets contained excess lysine, there were no differences ($P > 0.05$) in plasma arginine concentrations or dry matter intake (Fascetti et al. 2004). Similar results have also been observed in swine fed high amounts of lysine (Edmonds and Baker 1987). In cattle, Abe et al. (1998) reported that antagonism between lysine and arginine did not occur; excess lysine administration through the reticular groove of calves led to increases ($P < 0.05$) in plasma arginine concentrations. Although the mechanisms for observed

plasma arginine increases were not fully understood, the authors found no dietary imbalances with other limiting AA.

SUPPLEMENTATION OF CO-LIMITING AMINO ACIDS

In many feeding regimens for cattle, lysine is often co-limiting with methionine. When the diets were high in corn and corn byproducts, lysine was the most limiting AA (Burris et al., 1976; Abe et al., 1997). In contrast, soybean meal-supplemented diets were most limiting in methionine (Abe et al., 1998). Regardless, the responses to supplemental lysine and methionine diminished when cattle achieved greater body weights (Abe et al., 1999). These decreases could be explained by the greater feed intake in older steers relative to younger steers (Greenwood and Titgemeyer, 2000). To estimate the first and second limiting AA in ruminants when microbial protein was the sole source of metabolizable protein, Richardson and Hatfield (1978) combined numerical responses in urinary N excretion with estimates of fecal N from the last period of their study. Methionine was first limiting and lysine was second limiting. Greenwood and Titgemeyer (2000) argued that the rankings were not entirely justified because the N retention values were highly dependent on fecal N and several fecal N values were excluded from the calculations. Additionally, there were no significant differences in N retention between the methionine-lysine infusate and the methionine-tryptophan infusate. Despite these rankings, lysine and methionine have improved performance of growing cattle when supplemented together (Deetz et al., 1985; Viera et al., 1991).

The amount of CP in the diet should also be considered. Ludden and Kerley (1998) found that supplemental lysine in corn diets containing 13.2% CP did not improve performance, whereas Veira et al. (1991) observed increases ($P < 0.03$) in ADG (0.92 to

1.07 kg/d) and improvements in feed efficiency (7.88 to 6.81 F:G; $P < 0.01$) when steers were fed a grass silage diet with 18% CP and supplemented with 8.2 g/d of ruminally protected lysine and 2.6 g/d of ruminally protected methionine. Veira et al. (1991) suggested that both supplemental methionine and lysine were beneficial because the grass component of the diet was low in ruminally-undegradable protein. Other studies found that grain diets containing soybean meal improved both ADG and feed efficiency in growing cattle when supplemental lysine and methionine were provided in the ration (Van Amburgh et al., 1993; Oke et al., 1986); however, the lysine provided by Oke et al. (1986) was five-fold less than what was provided by Van Amburgh et al. (1993). The similarity in response between those studies indicated that methionine was primarily responsible for the observed treatment effects (Greenwood and Titgemeyer, 2000). If this suggestion were true, the ranking proposed by Richardson and Hatfield (1978) would be supported. To ensure that lysine and methionine are utilized for productive purposes, it is important to determine if essential AA utilization is negatively affected by feeding situations that result in elevated NH_3 absorption from the rumen.

AMMONIA LOADING

Ammonia loading is the process of adding NH_3 to the digestive tract of animals. If each AA has a different metabolic pathway, one could assume that NH_3 loading might have different effects when different AA are limiting. If NH_3 detoxification required input of AA, greater levels of NH_3 infusion would lead to greater catabolism of AA. To determine if AA catabolism was correlated with hepatic NH_3 loading, Lobley et al. (1995) infused NH_4Cl into the mesenteric vein of sheep. After 5 d of infusion, significant increases ($P < 0.05$) in leucine oxidation were observed as infused NH_4Cl increased.

Despite this increase in leucine catabolism, the low blood pH reported by the authors (pH = 7.36) may have been a confounding variable (Reaich et al., 1992; McCuistion et al., 2004). According to Pitts (1964), the increase in protein breakdown is a homeostatic response to acidotic conditions known as renal ammoniagenesis (Pitts, 1964). Plasma AA such as leucine can be transaminated to glutamate, aminated to glutamine in the liver, and later catabolized to α-ketoglutarate and NH_4^+ by phosphate-dependent glutaminase enzyme (Curthoys and Watford, 1995). The α-ketoglutarate is catabolized, yielding two bicarbonate molecules that can help counterbalance acidosis in the animal (Pitts, 1964). The catabolism of glutamine to bicarbonate also increases excretion of H^+ ions because NH_4^+ is a product of renal ammoniagenesis (Mutsvangwa et al., 2004). Alternatively, urea production consumes bicarbonate during the generation of carbamoyl phosphate. Thus, during acidotic conditions, less N-acetylglutamate is produced by N-acetylglutamate synthase, which is an activator of CPS-I enzyme in urea cycle. Without the stimulatory effects of N-acetylglutamate, CPS-I activity is inhibited, thereby increasing NH_3 detoxification via glutamine synthesis as an alternative to urea synthesis. This switch in NH_3 detoxification pathways leads to increased NH_4^+ excretion at the expense of urinary urea during metabolic acidosis. For these reasons, the acidosis induced by NH_4Cl in Lobley et al. (1995) would likely explain the increase in hepatic AA catabolism they observed.

Mutsvangwa et al. (1997) used ^{15}N labeled alanine to track the metabolism of dietary AA in ovine hepatocytes in vitro. If AA N was catabolized to support urea cycle activity, the labeled AA would yield greater concentrations of urea containing at least one atom of ^{15}N. In that study, NH_3 loading on the liver was induced by adding graded

amounts of urea to the diet. When the sheep hepatocytes were isolated, Mutsvangwa et al. (1997) observed greater production of ^{15}N labeled urea relative to unlabeled urea when greater NH_3 loads were supplemented to the sheep from which the hepatocytes were isolated.

To investigate changes in essential AA metabolism in response to NH_3 loading, Mutsvangwa et al. (1999) isolated hepatocytes from sheep and incubated them with 1 of 4labeled AA (methionine, alanine, phenylalanine, and leucine). The ^{15}N alanine incubation had the greatest increase ($P < 0.05$) in labeled urea production in response to increased NH_3 concentrations, whereas the ^{15}N methionine incubations had the lowest concentration of labeled urea ($N^{14}N^{15}$ urea) produced by hepatocytes at the highest level of NH_4Cl (Mutsvangwa et al., 1999). The hepatocyte incubations containing phenylalanine and leucine were not affected ($P > 0.05$) by NH_3 loads. The observed increase ($P < 0.05$) in labeled urea in the alanine incubation was attributed to transamination reactions of alanine to glutamate (Mutsvangwa et al., 1999). In the cytosol, glutamate and oxaloacetate react to form α-ketoglutarate and aspartate (Voet et al., 2013). This aspartate can subsequently be used in the urea cycle as a N source for arginosuccinate synthesis. The glutamate produced by transamination can be deaminated by glutamate dehydrogenase to yield ^{15}N labeled NH_3 for carbamoyl phosphate synthesis (Voet et al., 2013). Incorporating NH_3 into carbamoyl phosphate would explain why some urea molecules from hepatocytes contained 2 atoms of labeled N instead of 1 in the studies of Mutsvangwa et al. (1999) and Lobley et al. (1995).

ALTERNATIVE THEORY FOR AMMONIA DETOXIFICATION

Several researchers suggested that the urea cycle does not have an obligate need for AA N (Lobley et al., 1996; Milano et al., 2000). Critics of Lobley et al. (1995) suggested that the observed increase in AA catabolism was confounded by the acidotic state of their experimental sheep. To avoid development of acidosis and increases in renal ammoniagenesis, a follow-up study by Lobley et al. (1996) induced NH_3 loading in sheep using NH_4CO_3 as a substitute for NH_4Cl. The NH_4CO_3 eliminated acid base balance as a confounding variable and allowed unbiased comparisons between treatments (Lobley and Milano, 1997). By using the ratio of urea production to NH_3 absorption as an indicator of NH_3 N input, the authors found no significant ($P > 0.05$) changes in ^{15}N labeled leucine oxidation when NH_3 loading was increased. Hence, the hepatic detoxification of NH_3 did not require AA breakdown (Lobley et al., 1996). These results were consistent with sheep hepatocytes receiving both NH_4Cl and AA loads (Lou et al., 1995) and rat livers that were incubated with NH_4Cl (Brosnan et al., 1996). The lack of response to NH_4Cl may be attributed to the in vitro settings of those studies. If individual organs are isolated from an animal, the metabolic pathways involved in maintaining whole-body homeostasis may not occur. Hence, the isolated liver may not catabolize AA to counteract acidotic conditions in the culture.

Energy supply is another confounding factor in NH_3 detoxification studies. For example, Mutsvangwa et al. (1996) found that NH_4Cl supplementation in vitro decreased ^{14}C labeled alanine and glutamate release by hepatocytes. The authors suggested that high-N diets increased NH_3 absorption and subsequently reduced AA availability for protein synthesis. If the hepatocytes were energy deficient, AA may have been catabolized as an energy source (Mutsvangwa et al., 1996). To verify this assumption,

Mutsvangwa et al. (1997) supplied additional energy in the form of propionate. This propionate supplementation created a net decrease in AA catabolism in vitro compared to Mutsvangwa et al. (1996). Thus, NH_3 N sources may supply both N atoms for urea synthesis if energy supply remains adequate.

In general, the use of hepatocytes to study liver metabolism has yielded inconsistent results (Zhang et al., 2012). One reason is that in vitro studies lack representative amounts of specific hepatocytes (Haussinger et al., 1992). Results may also differ when NH_3 input exceeds liver processing capacity (Reynolds and Huntington., 1988). Symonds et al. (1981) previously determined that liver's maximum tolerance was 1.84 mmol/min per kg of liver weight. To induce changes in urea synthesis, Milano and Lobley (2001) infused NH_4CO_3 into the mesenteric vein of sheep in excess of the maximum NH_3 tolerance level. When liver capacity was exceeded, NH_3 removal remained constant ($P > 0.05$) while urea concentrations in culture increased. This observation could be explained by the heterogeneous population of hepatocytes in the liver (Katz, 1992).

HEPATOCYTE DIVERSITY

There are two types of hepatocytes occupying the liver. The first type is called periportal cells. When portal blood reaches the liver, periportal cells are first to receive metabolites such as NH_3 (Haussinger, 1990). These are the hepatocytes responsible for urea cycle activity. Although urea cycle enzymes have a low affinity for NH_3, this inconvenience is outweighed by their high capacity for NH_3 molecules (Katz, 1992). Any unused NH_3 is passed to the perivenous cells further inside the liver. The perivenous cells contain glutamine synthetase which incorporates unused NH_3 into glutamine

(Milano and Lobley, 2000). This system has low capacity, but high affinity, for NH_3 (Katz, 1992; Haussinger et al., 1992). By working in cohort, the urea cycle in periportal cells and glutamine synthetase in perivenous cells successfully detoxifies hazardous NH_3 from the portal blood. Regardless of NH_3 concentration, periportal cells convert the majority of NH_3 into urea, whereas perivenous cells extract the small remainder for glutamine synthesis (Katz, 1992). Some glutamine may be extracted by the kidney with glutaminase, yielding NH_3 for excretion in urine. Periportal hepatocytes may also extract glutamine and use glutaminase to liberate NH_3 for utilization by CPS-I enzyme, which synthesizes carbamoyl phosphate from ornithine, NH_3, bicarbonate, and two ATP. Excess glutamine can also be transaminated to glutamate, which is enzymatically converted to glutamate semialdehyde (GSA) by pyroline-5-carboxylate synthetase (Arentson et al., 2012). The GSA can serve as a precursor for ornithine or for proline, depending on the metabolic pathway and AA needs of the body (Arentson et al., 2012).

UREA CYCLE

Ruminal bacteria produce large amounts of NH_3 from deamination of AA (Rychlik et al., 2002). The primary source of NH_3 in the rumen is microbial breakdown of dietary true protein and hydrolysis of dietary non-protein N (NPN). Other sources of NH_3 include urea from recycling (Abdoun et al., 2006). Ruminal NH_3 can be absorbed across the ruminal wall and either assimilated for microbial protein production or passed into the lower gut (Lapierre and Lobley, 2001). Absorbed NH_3 enters the portal blood and travels to the liver for urea synthesis (Meijer et al., 1990). In the urea cycle, NH_3 and bicarbonate combine to form carbamoyl phosphate. When ornithine is available to the liver, ornithine transcarbamoylase catalyzes the condensation of carbamoyl phosphate

with ornithine to produce citrulline (Morris, 2002). Arginosuccinate synthetase incorporates aspartate into citruilline to yield arginosuccinate, which is further cleaved by arginosuccinate lyase into one molecule of arginine and one molecule of fumarate (Morris, 2002). The fumarate can enter the Krebs cycle (Voet et al., 2013), whereas the hepatic arginine is broken down by arginase. Arginase produces 1 molecule of urea and 1 molecule of ornithine that can re-enter the cycle.

Urea synthesis benefits mammals by removing toxic NH_3, disposing of excess AA-N, and regulating acid-base balance (Haussinger, 1990). This acid-base regulation results from assimilation of basic bicarbonate by carbamoyl phosphate synthase I in urea cycle (Haussinger, 1990). For every molecule of urea synthesized, one bicarbonate is used to make carbamoyl phosphate (Kothe and Powers-Lee, 2004). As more urea is synthesized, more bicarbonate is eliminated by the body. If the animal is suffering from alkalosis, it will increase urea cycle activity to maintain homeostasis (Haussinger, 1990). This change in urea cycle activity is regulated by the activities of glutaminase and N-acetylglutamate synthase enzymes in response to alkaline pH. During alkaline conditions, glutaminase activity increases, which increases glutamate synthesis from glutamine. In response to increases in glutamate and alkaline pH, the activity of N-acetylglutamate synthase increases, resulting in greater production of N-acetylglutamate from glutamate and acetyl-coA (Nissim, 1999). Once produced, N-acetylglutamate directly stimulates CPS-I, the rate-limiting enzyme of urea cycle, by allosteric regulation (Nissim, 1999). Conversely, if the animal is experiencing acidosis, N-acetylglutamate production decreases, thereby decreasing urea cycle activity to conserve bicarbonate in the body.

For urea synthesis to occur, two N atoms are required for each cycle. One atom from free NH_3 is used for synthesis of carbamoyl phosphate, while the other is donated from aspartate (Lobley et al., 1995). As mentioned earlier, carbamoyl phosphate synthase I forms carbamoyl phosphate from assimilation of bicarbonate and NH_3. The N atom from aspartate may originate from different AA (Lobley et al., 1995). Transamination reactions and glutamate dehydrogenase activity can supply the aspartate for urea cycle by combining α-ketoglutarate with NH_3 and transferring glutamate's amino group to oxaloacetate (Lobley and Milano, 1997). Thus, NH_3 may supply either one or two N atoms for urea synthesis.

HEPATIC GLUTAMINE PRODUCTION

Any NH_3 that escapes urea synthesis in periportal cells can be used for glutamine synthesis in perivenous cells. Although the amount of NH_3 exceeded urea cycle capacity in the study of Lobley et al. (1996), the synthesis of glutamine by the liver cells did not increase. This observation may be explained by the low capacity for NH_3 by perivenous cells (Katz, 1992). Plasma concentrations of glutamine were numerically increased from 0.28 mM to 0.44 mM when NH_4CO_3 infusion increased from 25 to 150 μmol/min. If glutamine synthesis remained constant in the liver, glutamine synthesis in muscle was likely stimulated (Lobley et al., 1996). Leweling et al. (1996) observed similar increases in muscle glutamine concentrations when excess ammonium salt was intravenously infused into rats. This increase was associated with increases ($P < 0.05$) in intracellular glutamate and decreases ($P < 0.05$) in intracellular branched chain AA. The authors suggested that the branched chain AA were transaminated to replace the glutamate lost during glutamine synthesis. Another possibility was that ammonium salt supplementation

increased branched chain AA catabolism to supply more aspartate for NH_3 detoxification (Lobley et al., 1995). If more urea cycle enzymes were present, this inefficient utilization of branch chain AA may have been avoided. According to Meijer et al. (1990), there are more urea cycle enzymes present in animals fed high-protein diets than in animals fed low-protein diets. This increase in active enzymes would increase the liver's capacity for NH_3. If NH_3 levels are low, the liver may remove plasma AA as its next priority (Lobley et al., 1996).

EFFICIENCY OF AA USE

Over half of all absorbed AA are catabolized by the liver (Lobley et al., 1996). Any increase in AA catabolism would reflect an inefficiency by the animal. The efficiency of AA use for protein synthesis is based on several factors such as bioavailability of the diet, energy supply, and animal AA requirements (Millward et al., 2008). If the requirements are satisfied, AA will be used more efficiently. Unfortunately, small proportions are still catabolized when animals are fed below their requirement (Ball et al., 2007). If the AA pools exceed the requirements, efficiency of use decreases. Alternative endpoints of absorbed AA include urinary urea, urinary NH_3, oxidation to carbon dioxide, retention in blood, and synthesis of creatinine (Wright, 1995).

Most liver metabolism research in ruminants has used continuous feeding frequencies and infusions to establish a balance between protein supply and tissue requirements (Reynolds et al., 1994). When plasma N supply is elevated, the liver removes surplus AA using transamination, deamination, and the urea cycle (Munro, 1978). Once the amine group is removed, the liberated carbon skeletons are used as building blocks for both lipid biosynthesis and gluconeogenesis (Voet et al., 2013). To

determine liver capacity for AA loading, Wray-Cahen et al. (1997) infused incremental amounts of AA into the mesenteric vein. In that study, the proportion of non-essential AA removed by the liver decreased ($P < 0.05$) when AA were infused into the mesenteric vein. Interestingly, greater AA infusions led to greater ($P < 0.05$) absorption of AA across the PDV and decreased ($P < 0.05$) the percentage of AA extracted by the liver. Despite this observation, the concentrations removed by the liver increased ($P < 0.05$) at greater rates of AA infusion. The authors suggested that this increase in urea synthesis was due to inadequate energy supply to support increased protein deposition. As a consequence, essential AA such as lysine were catabolized as a source of energy (Wray-Cahen et al., 1997).

Wray-Cahen et al. (1997) also found that nonessential AA output from the liver decreased with AA infusions, thereby decreasing AA availability to peripheral tissues for protein deposition; however, plasma phenylalanine and histidine concentrations were depressed in the sheep receiving AA infusions. The authors attributed this to an imbalance in the AA infusate. By using an AA infusate based on the AA profile of microbial protein determined by Storm and Øskov (1984), Lobley et al. (1998) found that hepatic removal of NH_3 remained constant as intravenous AA infusions increased. This observation may have indicated that excess AA were catabolized for the urea cycle (Milano and Lobley, 2000). Because the highest levels of AA infusions were considered to be above the maximum capacity established by Symonds et al. (1981), the conclusions of Lobley et al. (1998) may not be valid under practical situations. Regardless, changes in metabolism from excess N is worth further investigation.

EFFECTS OF AMMONIA LOADING ON DIFFERENT AMINO ACIDS

When N retention was used as a response variable in early NH_3 loading studies with sheep (Norton et al., 1982) and cattle (Moorby and Theobald, 1999), authors reported no changes in N retention when greater ruminal NH_3 (Norton et al., 1982) or duodenal NH_3 loads were provided (Moorby and Theobald, 1999). The main weakness of those studies was that experimental animals were not limited by individual AA (Awawdeh et al., 2005). If adequate amounts of each AA were available to the animal, changes in N retention would not be expected with greater NH_3 loads. When growing cattle were limited by methionine, NH_3 loading via ruminal urea infusion did not improve ($P > 0.05$) N retention (Awawdeh et al., 2004). In that study, average N retention changed from 28.0 to 26.6 and 27.9 when 0, 40 g/d or 80 g/d urea was infused with 5 g/d methionine. Efficiency of methionine utilization increased from 23 to 27% between 0 and 40 g/d of urea and decreased from 27 to 18% between 40 and 80 g/d of urea. In contrast, McCuistion (2004) found numerical improvements in N retention from 35.1 to 37.1 g/d that were not significant ($P = 0.16$) when growing steers were limited by histidine and infused with 0 or 80 g/d urea, respectively. In that study, the incremental efficiency of histidine utilization increased from 50 to 81% for 0 and 80 g/d of supplemental urea. Interestingly, Awawdeh et al. (2005) found significant improvements in whole-body N retention when steers were limited by leucine. This was demonstrated by the increase in N retention from 24.7 to 29.2 g/d ($P < 0.05$) when urea infusion changed from 0 to 80 g/d at the highest level of supplemental leucine (6 g/d). The incremental efficiency of leucine utilization increased from 24 to 43% for 0 and 80 g/d of urea, respectively, when leucine supplementation increased from 0 to 4 g/d. These findings may be attributed to differences in metabolism throughout the body. Lysine, methionine, and histidine are

primarily catabolized by the liver, whereas branch chain AA such as leucine are metabolized in tissues throughout the body (Harper et al., 1984). These results indicated that NH_3 loading may have different effects when different AA are limiting (Awawdeh et al., 2005). The increase in N retention in Awawdeh et al. (2005) was unrelated to changes in ruminal fermentation because there were no significant differences in digestibility in response to urea infusions. Awawdeh et al. (2005) suggested that NH_3 supply to the liver may have reduced whole-body leucine catabolism by increasing the availability of glutamine and alanine to peripheral tissues. Transamination of branched chain AA such as leucine to alpha-keto acids is a reversible process that can transfer an amino group to glutamate and pyruvate, forming glutamine and alanine, respectively (Voet et al., 2013). If the rate of leucine transamination decreases in response to NH_3 loading, more leucine would be available to increase whole body N retention (Awawdeh et al., 2005; Harper et al., 1984). Additionally, increased transamination of α-ketoisocaproate to leucine would create a similar response in N retention by increasing leucine production (Awawdeh et al., 2005). These ideas were supported by Lobley et al. (1996) where NH_3 loading increased plasma glutamine and decreased plasma glutamate and aspartate in sheep. Because aspartate is a N donor in urea cycle, more aspartate was likely used to detoxify NH_3 loads (Awawdeh et al., 2005). The decreases in plasma glutamate could be explained by greater glutamine synthetase activity in perivenous cells, resulting from greater bypass of NH_3 though periportal cells (McCuistion et al., 2004; Awawdeh et al., 2005). Although transamination would explain the improvements in leucine utilization, the exact mechanism was still uncertain. Awawdeh et al. (2004; 2005) and McCuistion et al. (2004) also observed decreases in plasma alanine with urea

infusions from 150 to 132 μM ($P < 0.05$) during methionine restriction, 305 to 278 μM ($P < 0.05$) during leucine deficiency, and from 255 to 213 μM during histidine deficiency ($P = 0.18$). These decreases may have resulted from transamination reactions that converted alanine to aspartate for the urea cycle. The lack of decreases in N retention observed by Awawdeh et al. (2004), McCuistion et al. (2004), and Awawdeh et al. (2005) could be interpreted to suggest that NH_3 detoxification to urea did not require input of AA N when ruminal NH_3 loads were elevated.

REGULATION OF LYSINE CATABOLISM

Hepatic lysine degradation predominantly uses the saccharopine pathway, located within the mitochondrial matrix (Voet et al., 2013). According to Blemings et al. (1998), transport of lysine into the mitochondria is the rate-limiting factor for lysine oxidation. In that study, the activity of key lysine catabolic enzymes in the mitochondria (lysine α-ketoglutarate reductase and saccharopine dehydrogenase) and total lysine oxidation were assessed in the liver of rats fed diets containing 5, 20, and 60% casein. Although lysine oxidation increased ($P < 0.05$) with greater casein intake, the overall activity of both lysine α-ketoglutarate reductase and saccharopine dehydrogenase enzymes were 6 to 106-fold greater than lysine oxidation. If more lysine had entered the mitochondria, the difference between lysine oxidation and catabolic enzyme activity may have been minimized.

Lysine catabolism is also regulated by hormonal mechanisms. Based on the findings of Scislowski et al. (1994), the production of ^{14}C labeled CO_2 from saccharopine pathway was controlled by the intracellular concentrations of lysine, α-ketoglutarate, and NADPH in hepatocytes. In that study, hepatocytes were isolated from rats receiving

glucagon injections. The rats treated with glucagon had greater ($P < 0.05$) activities of lysine-α-ketoglutarate reductase and saccharopine dehydrogenase enzymes, which catalyze the initial steps of the catabolic pathway (Voet et al., 2013). This observation was consistent with Snodgrass et al. (1978) who reported an increased secretion of glucagon stimulated AA catabolism by the liver. Snodgrass et al. (1978) suggested that glucagon increased mitochondrial calcium concentrations which activated isocitrate dehydrogenase and α-ketoglutarate dehydrogenase in the Krebs cycle. This theory was supported by the 30% increase in mitochondrial calcium observed by Scislowski et al. (1994) when glucagon was injected into the peritoneal cavity of live rats. The glucagon treatment used in their experiment was designed to mimic the increase in glucagon secretion that occurs when high-protein meals are consumed (Scislowski et al., 1994). The mechanism by which glucagon increased lysine oxidation involved increased utilization of α-ketoglutarate by α-ketoglutarate dehydrogenase and increased activity of lysine-α-ketoglutarate reductase and saccharopine dehydrogenase (Scislowski et al., 1994).

Catabolic enzymes for lysine are present in the liver (Voet et al., 2013); however, an *in vivo* study with piglets fed high-protein diets (250 g protein/d) reported that intestinal oxidation of lysine accounted for a 30% of whole-body lysine catabolism (van Goudoever et al., 2000). These researchers also reported that most lysine taken up by the portal drained vicera (PDV) escaped oxidation. These observations were interpreted to suggest that lysine catabolism may also occur in the intestine (Burrin et al., 2002) via several proposed mechanisms. The first proposed mechanism suggested that the dorsal villi of the small intestine are most active in dietary AA breakdown and lysine oxidation

(van Goudoever et al., 2000). This idea was supported by a study that supplemented leucine for protein synthesis in different sections of intestinal villi in rats (Alpers, 1972). The second proposed mechanism suggested that intracellular transport of AA differs when lysine enters the apical membrane of enterocytes versus the basolateral membrane (van Goudoever et al., 2000). This was supported by the presence of different AA transporters on the apical and basolateral membrane (Bröer, 2008). The third proposed mechanism had the least amount of evidence and suggested that intestinal microbes oxidize dietary lysine. In Shulman et al. (1995), an AA mixture containing lysine was directly infused into the duodenum and absorbed in the jejunum at a greater rate than bacterial assimilation of lysine.

In van Goudoever et al. (2000), low protein diets were fed at a level sufficient to maintain N homeostasis. Low-protein diets (milk replacer containing 100 g protein) were also associated with greater ($P < 0.05$) utilization of all of the essential AA by the PDV relative to high-protein diets (milk replacer containing 250 g protein), indicating that PDV tissues required large amounts of essential AA during protein restriction (Burrin et al., 2002). van Goudoever et al. (2000) also suggested that the large quantity of AA required by the PDV were needed to maintain the structure and function of the gastrointestinal tract to serve as a barrier against pathogens, produce large amounts of immunoglobulins (Brandtzaeg et al., 1987), break down macromolecules, and absorb nutrients.

CONCLUSION

Lysine is an often-limiting AA and cattle utilize supplemental lysine rather inefficiently. To reduce supplementation costs, additional methods are needed to increase

efficiency of lysine use for protein deposition. Ammonia loading is a possible approach that has demonstrated variable results for different essential AA. It is unknown how lysine responds to NH_3 loads, even though lysine is a substrate for hepatocyte catabolism. Recent studies indicated that AA would not be catabolized as a source of urea N when energy supply was adequate and acidotic conditions were avoided. If this is true, NH_3 loading may have minimal effects on lysine utilization; however, the unique catabolic pathway of lysine may affect the response to incremental NH_3 infusions.

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CHAPTER 2 - EFFECT OF RUMINAL AMMONIA SUPPLY ON LYSINE UTILIZATION BY GROWING STEERS

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ABSTRACT

Six ruminally cannulated Holstein steers (202 ± 15 kg) were used to study the effects of ruminal ammonia loading on whole-body lysine utilization. Steers were housed in metabolism crates and used in a 6 × 6 Latin square design. All steers received 2.52 kg of DM/d of a diet (10.1% CP) containing 82% soybean hulls, 8% wheat straw, 5% cane molasses, and 5% vitamins and minerals, and 10 g/d of urea was ruminally infused continuously to ensure adequate ruminal ammonia concentrations. All steers were ruminally infused continuously with 200 g/d acetic acid, 200 g/d propionic acid, and 50 g/d of butyric acid and abomasally infused with 300 g/d of glucose continuously to increase energy supply without increasing microbial protein supply. Steers were also abomasally infused continuously with an excess of all essential amino acids except lysine to ensure that lysine was the only limiting amino acid. Treatments were arranged as a 3 × 2 factorial with 3 levels of urea (0, 40, or 80 g/d) continuously infused ruminally to induce ammonia loading and 2 levels of lysine (0 or 6 g/d) continuously infused abomasally. Treatments did not affect fecal N output ($P = 0.37$). Lysine supplementation decreased ($P < 0.01$) urinary N excretion from 51.9 g/d to 44.3 g/d, increased ($P < 0.01$) retained N from 24.4 to 33.3 g/d, increased ($P < 0.01$) plasma lysine, and decreased ($P \leq 0.05$) plasma serine, tyrosine, valine, leucine, and phenylalanine. Lysine supplementation also tended ($P = 0.09$) to reduce plasma urea-N. Urea infusions linearly increased retained N (26.7, 28.8, and 31.1 g/d; $P = 0.05$) and also linearly increased ($P < 0.01$) urinary N excretion (31.8, 48.1, and 64.4 g/d), urinary urea (21.9, 37.7, and 54.3 g/d), urinary ammonia (1.1, 1.4, and 1.9 g/d), plasma urea (2.7, 4.0, and 5.1 mM), and linearly decreased plasma alanine ($P = 0.04$) and plasma glycine ($P < 0.01$). Assuming that

retained protein is 6.25 × retained N and contains 6.4% lysine, the incremental efficiencies of infused lysine utilization were 51, 59, and 69% for steers receiving 0, 40, and 80 g/d of urea, respectively, indicating that the ruminal ammonia loads may improve the efficiency of lysine utilization. This is supported by observed increases in whole body-protein deposition in response to ammonia loading of our steers that were, by design, lysine deficient.

INTRODUCTION

Nitrogen (N) retention studies identified lysine (Lys) as the second limiting amino acid (AA) for growing cattle (Richardson and Hatfield, 1978). Despite its status as a limiting AA, plasma Lys is subject to catabolism by the liver (Ball et al., 2007). To increase Lys availability for protein synthesis in peripheral tissues, hepatic Lys catabolism needs to be suppressed. By feeding high-protein diets, subsequent increases in ammonia absorption may be a potential mechanism to limit catabolism of essential AA by increasing ammonia detoxification by hepatocytes. In contrast, Wilton et al. (1988) observed that urea N produced by the liver exceeded the input of supplemental ammonia when ammonium salt was infused into the mesenteric vein of cattle. To account for this excess, Lobley et al. (1995) observed that AA-N was utilized for ammonia detoxification by the liver; however, this did not fit with the findings of Luo et al. (1995) where plasma ammonia supplied both N atoms required for urea synthesis in sheep. Additionally, Lobley et al. (1996) found no effects of supplemental ammonia load on catabolism of Leu, suggesting that urea synthesis was not dependent on AA-N (Milano et al., 2000). Thus, ammonia loading had no negative impacts on protein deposition in ruminants.

In a research setting, ammonia loading can be induced by feeding non protein N (NPN) sources such as urea. When growing cattle were limited by Met, ammonia loading did not improve N retention (Awawdeh et al. 2004). Conversely, McCuistion (2004) found numerical improvements in N retention that were not significant when growing steers were limited by His. Interestingly, Awawdeh et al. (2005) reported significant improvements in N retention when steers were limited by Leu. These results may have indicated that ammonia loading can have different effects when different AA are limiting.

Our objective was to determine the effects of ruminal ammonia loading on protein deposition when growing steers were limited by Lys.

MATERIALS AND METHODS

Animals and Treatments

All animal procedures were approved by the Kansas State University Institutional Animal Care and Use Committee.

Six ruminally cannulated Holstein steers (202 ± 15 kg) were used in a 6 × 6 Latin square design and housed in metabolism crates within a temperature-controlled room at 22°C. A seventh steer was also used in the study and given the same treatment sequence as one of the other steers. Some observations were excluded from the data set because feed refusals were large, and notably all observations from one steer were excluded. Infusion lines of flexible Tygon polyvinylchloride tubing (2 mm i.d.) were placed in the rumen and abomasum through the ruminal cannula. A perforated vial was attached to the end of the ruminal infusion lines to prevent direct infusion of VFA onto the ruminal wall. Rubber flanges (7-cm diameter) were attached to the end of the abomasal lines to anchor them in the abomasum. Ruminal and abomasal infusates (each containing 4 kg/d of solution) were continuously infused using a peristaltic pump (Model CP-78002-10; Cole-Parmer Instrument Company, Vernon Hills, IL).

Preceding the study, steers were adapted to the experimental diet (Table 1) and to similar infusions for a similar experiment. All steers had ad libitum access to water and received the same diet at 2.52 kg DM/d in 2 equal portions at 12-h intervals. The experimental diet (along with the basal ruminal infusion of 10 g urea/d) was formulated to provide adequate amounts of ruminally degradable protein but limited metabolizable

AA (Campbell et al., 1997). All steers were continuously infused with 200 g of acetic acid/d, 200 g of propionic acid/d, and 50 g of butyric acid/d into the rumen and 300 g of dextrose/d into the abomasum as energy sources for the animal; these infusions served as energy sources for the animal without increasing ruminal microbial protein production.

All steers received continuous infusions of an AA-containing solution into the abomasum (Table 2). This solution provided monosodium glutamate as a source of Glu, Gly, and all essential AA except Lys to ensure that only Lys was limiting (Greenwood and Titgemeyer, 2000).

Treatments were arranged in 3 × 2 factorial with 3 levels of ruminally infused urea (0, 40, and 80 g/d) and 2 levels of abomasally infused Lys (0 and 6 g/d). Experimental periods lasted 6 d (2 d of adaptation and 4 d for total collection of feces and urine). Two days of adaptations were adequate (Moloney et al., 1998; Schroeder et al., 2007) because the treatments were not expected to alter ruminal fermentation.

Daily abomasal infusates (Table 2) were prepared by dissolving branched chain AA in 833 g of water containing 72 g of 6 *M* HCl. After L-Leu, L-Ile, and L-Val were dissolved, L-Met, L-His-H_2O-HCl, L-Thr, L-Phe, L-Arg-HCl, L-Trp, and Gly were added to the mixture. Once dissolved, 330 g of dextrose was added to the AA mixture. Glutamate (as monosodium glutamate) was dissolved separately in 448 g of water. The Lys-HCl also was dissolved separately as a 10% (wt/wt) solution. The 3 solutions (AA/dextrose mixture, Glu, and Lys) were mixed together in individual bottles and water was added to bring the daily abomasal infusate weight to 4 kg. Pyridoxine HCl (10 mg/d), folic acid (10 mg/d), and cyanocobalamin (0.1 mg/d) were dissolved and added to

the abomasal infusate because steers under these experimental conditions may become deficient in at least one of those vitamins (Lambert et al., 2004).

Ruminal infusates were prepared by mixing solutions containing acetic acid, propionic acid, and butyric acid with solutions providing the basal 10 g/d of urea plus each urea treatment (0, 40, or 80 g/d). Water was added to bring the daily ruminal infusate weight to 4 kg.

Sample Collection and Analysis

Representative samples of the experimental diet were collected from d 2 to 5 of each period, stored at −20°C, and ground in a Wiley Mill through a 1-mm screen (Thomas Scientific, Swedesboro, NJ) before analysis. Orts, if any, were collected from d 3 to 6, combined, stored at −20°C, dried at 55°C in a forced-air oven, and ground through a 1-mm screen before analysis. Total urine and feces were collected daily from d 3 to 6 of each period and weighed to determine total output. Urine was collected in buckets containing 900 mL of 10% (wt/wt) H_2SO_4 to prevent loss of ammonia. Representative samples of feces (10% of total) and urine (1% of total) from individual steers were pooled within period and stored at −20°C for later analyses.

Feed samples, orts, and feces were analyzed for DM in a forced-air oven for 24 h (105°C). These samples were subsequently combusted at 450°C for 8 h to determine OM. Feed samples, orts, wet feces, and urine were analyzed for N using a TruMac N Analyzer (LECO Corporation, St. Joseph, MI).

Jugular blood samples were collected 10 h after the morning feeding on the last day of each period. Blood was collected into vacuum tubes (Becton Dickinson, Franklin Lakes, NJ) containing sodium heparin. The tubes were immediately placed on ice, then

centrifuged for 15 min at 1,000 × *g* at 4°C to obtain plasma. Plasma samples were stored at −20°C for later analysis of glucose, urea, and AA. Plasma glucose was measured using methods of Gochman and Schmitz (1972). Plasma urea and urinary urea were measured using the method of Marsh et al. (1965). Urinary ammonia was measured using the method of Broderick and Kang (1980). For AA analysis, an equal volume of 10% (wt/vol) sulfosalicylic acid, containing 1 m*M* norleucine as an internal standard, was added to plasma to deproteinize the samples. Plasma AA were measured by cation exchange chromatography with post-column derivitization with *o*-phthalaldehyde. A 4 × 100 mm lithium ion-exchange column (Pickering Laboratories, Mountain View, CA) and lithium eluents (Pickering Laboratories) were used. Flow rate was 0.3 mL/min and the total run time was 130 min. The initial eluant contained 0.7% lithium citrate, 0.6% lithium chloride, and 0.2% sulfolane and was pumped for 39.1 min. The second eluant contained 2.7% lithium citrate and <0.1% lithium chloride and was pumped for 18 min. The third eluant contained 3.4% lithium chloride and 1.5% lithium citrate and was pumped for 21 min. The fourth eluant contained 2.56% lithium chloride, 1.05% lithium citrate, and 0.18% lithium hydroxide and was pumped for 30 min, after which the initial eluant was used to re-equilibrate the column for 21.9 min. The column temperature was 33°C for the initial 25 min, then increased to 70°C for 85 min before being returned to 33°C for the final 20 min. The *o*-phthalaldehyde reagent (Pickering Laboratories) was mixed with column effluent at a rate of 0.3 mL/min and allowed to react for 10 s at 21°C prior to fluorescence detection with excitation at 330 nm and emission at 465 nm (HP 1046A Fluorescence Detector; Agilent Technologies, Santa Clara, CA).

Statistical Analysis

Data were analyzed using the Mixed procedure of SAS System 9.3 for Windows (SAS Inst., Inc., Cary, NC). Fixed effects in the model included Lys, urea, Lys × urea, and period; steer was included as a random effect. Linear and quadratic effects of urea and their interactions with Lys were evaluated using orthogonal polynomial contrasts. Treatment means were calculated using the LSMEANS option.

Efficiencies of Lys utilization were calculated by regressing Lys deposition (N retention, g N/d x 6.25 g protein/g N x 0.064 g Lys/g protein; Ainslie et al., 1993) against supplemental Lys within urea treatment using the Mixed procedure of SAS. The model included the random effect of steer, fixed effects of period and urea treatment, and Lys within urea treatment as a regression variable. Slopes were compared with contrasts that evaluated the linear and quadratic effects of urea level.

RESULTS

Nitrogen Retention and Excretion

There were no significant interactions between Lys and urea for diet digestibility or N retention data (Table 3).

Effects of Lys. Abomasal Lys supplementation increased N intake ($P < 0.01$) from 98.2 to 99.4 g/d and increased ($P < 0.01$) N retention from 24.8 to 33.8 g/d. This increase was linked to decreased ($P < 0.01$) urinary N excretion from 51.9 to 44.3g/d. Lysine infusion decreased ($P < 0.01$) urinary urea-N from 42.4 to 33.5 g/d, whereas urinary ammonia was not affected by Lys supply ($P = 0.17$). Lysine supplementation had no effect on fecal N ($P = 0.75$), dry matter digestibility ($P = 0.96$), nor organic matter digestibility ($P = 0.84$).

Effects of Urea. Nitrogen intake increased linearly ($P < 0.01$) with urea infusions, whereas fecal N output did not differ among treatments ($P = 0.37$). Between 0 to 80 g/d of urea infusion, total urinary N increased linearly ($P < 0.01$) from 31.8 to 64.4 g/d, urinary urea-N increased linearly ($P < 0.01$) from 21.9 to 54.3 g/d, and urinary ammonia increased linearly ($P < 0.01$) from 1.1 to 1.9 g/d. With incremental urea infusions, N retention increased linearly ($P = 0.05$). At 0, 40, and 80 g/d of ruminally infused urea, steers retained 27.1, 29.3, and 31.5 g N/d, respectively.

The efficiencies of Lys utilization were 51, 59, and 69% for steers receiving 0, 40, and 80 g/d of urea, respectively. Neither linear ($P = 0.52$) nor quadratic ($P = 0.96$) effects of urea were significant for these efficiencies.

Plasma Metabolites

There was only 1 interaction (quadratic) between Lys and urea for plasma metabolites (arginine), and this interaction had no clear explanation.

Effects of Lys. Lysine supplementation tended to decrease ($P = 0.09$) plasma urea. Additionally, Lys supplementation had no effect on plasma glucose ($P = 0.27$).

Lysine supplementation increased ($P < 0.01$) plasma Lys concentrations and decreased ($P < 0.05$) plasma concentrations of Val, Leu, Ser, Tyr, Phe, and taurine (Table 4). Plasma Arg increased ($P < 0.01$) with Lys supplementation.

Effects of Urea. Supplemental urea induced ammonia loading as demonstrated by linear increases ($P < 0.01$) in plasma urea (Table 4). Urea infusions did not affect plasma glucose concentrations. Plasma Gly, Ala, taurine, and total AA linearly decreased ($P < 0.05$) with urea infusions. Plasma Ser also tended to decrease linearly ($P = 0.06$) with urea infusions.

DISCUSSION

Significance of Research Model

In ruminants fed corn-based diets, Lys is considered the first-limiting AA (Burris et al., 1976; Abe et al., 1997). Greenwood and Titgemeyer (2000) determined that the experimental soybean hull-based diet used in this study provided deficient amounts of Lys as well as of other essential AA, suggesting that this diet would be useful for studying Lys utilization in growing cattle. In our model, Lys was designed to be the sole limiting AA in growing cattle. The amount of intestinally absorbable Lys provided by our soybean-hull diet was 7.1 g/kg DM intake, so based on intakes of 2.52 kg DM/d, the experimental diet provided 17.9 g/d of absorbable Lys, which was not adequate for maximal N retention. By supplementing essential and nonessential AA (Table 2) above their suspected requirements, the confounding effects of other AA limiting protein deposition were avoided. The Lys treatment was also designed to be below the linear response surface for N retention, which ranged from 0 to 9 g/d of supplemental Lys (Batista et al., 2015). By increasing the supply of Lys, whole-body protein deposition (N retention) was enhanced and catabolism of AA other than Lys was decreased. Another important aspect of the research model was that 10 g/d urea was ruminally infused to all animals regardless of treatments. This 10 g/d of urea was designed to provide ruminal ammonia concentrations necessary for optimal microbial growth and normal rumen function. In essence, this urea infusion replaced dietary urea that was provided in previous work with this research model (Campbell et al., 1997, Schroeder et al., 2006, 2007); the substitution from diet to ruminal infusion was made to reduce variation in dietary N content and to provide a more constant input of N to the rumen. The fact that

diet digestibility and fecal N did not change with greater urea infusions would suggest that the basal amount of urea infused to steers was enough to support microbial protein synthesis. Thus, our model likely eliminated ruminal fermentation as a confounding factor.

Nitrogen Retention

Effects of Lys. The increases in N retention with Lys infusions indicated that steers were deficient in Lys in our research model. By making a limiting AA available for protein synthesis, less of the non-limiting AA are diverted towards catabolism. Such decreases in AA catabolism would appear as decreases is N excretion, which was also observed when Lys was supplemented. If appropriate amounts of each AA are not available to tissues, initiation of protein synthesis may be inhibited. This decrease in protein synthesis can result in decreases in protein deposition because deposition is a balance between synthesis and degradation. Both processes occur simultaneously throughout the body as a result of continuous protein turnover. Thus, our increases in whole-body N retention reflected greater protein synthesis relative to protein degradation when Lys, the sole limiting AA in our model, was supplemented to growing cattle. Our highest level of Lys (6 g/d) was within the linear response surface to Lys established by Batista et al. (2015).

Effects of Urea. The increase in N retention with urea infusions indicates that less Lys, the limiting AA, was catabolized by steers when greater ruminal ammonia loads were provided. According to van Goudoever et al. (2000), Lys catabolism can occur in both the liver and small intestine. Lysine sparing may have taken place in either the liver, the small intestine, or at both sites. Previous work with pigs (Ball et al., 2007) indicated

that the liver catabolizes a small proportion of Lys, even when the animal is fed below its Lys requirement. If this wasteful catabolism occurs in growing steers, the ruminal ammonia loads may have reduced hepatic Lys breakdown through some alteration of transport or metabolism. When faced with increased ammonia supply, the liver would increase ammonia detoxification, perhaps foregoing metabolism of non-toxic compounds such as Lys.

Some Lys may also be oxidized in the small intestine; van Goudoever et al. (2000) found that 30% of whole-body Lys oxidation occurred in the small intestine when piglets were fed a high-protein diet. If this phenomenon is true in cattle, the sparing of Lys by greater ammonia loads may have occurred in the intestine. This mechanism would presumably be dependent on some ruminal ammonia reaching the intestinal lumen and decreasing AA deamination by an unknown mechanism. By decreasing AA deamination in the intestine, less ammonia would be absorbed across the GI tract.

Lys Efficiency

Although there were no treatment differences when we regressed deposited Lys against Lys intake, the calculated values for incremental efficiency (51, 59, and 69% for 0, 40, and 80 g/d of urea) were interpreted to suggest that the efficiency of Lys utilization for protein deposition may increase with greater ruminal ammonia loads. This suggestion was supported by the overall increases in N retention in response to urea supplementation in our Lys-limiting model, because the ammonia loading could affect utilization of the basal dietary Lys as well as the supplemental Lys.

Our measures of incremental efficiency of Lys utilization (51 to 69%) were substantially less than what was calculated by Klemesrud et al. (2000). One difference

may be the use of ADG as a response variable by Klemesrud et al. (2000), whereas we based our calculations on N retention. Our calculated efficiency of Lys utilization at the lowest ammonia load (51%) was also less than the NRC (1996) prediction of 65%, which is calculated based on BW of the cattle. Our average efficiency (60%) as well as the efficiency value when 80 g/d of urea was provided (69%) were close to the NRC (1996) prediction. In contrast, Burris et al. (1976) calculated an efficiency of 13% for growing steers fed a corn-based diet. This lower value by Burris et al. (1976) may have resulted from the experimental conditions in that study. Unlike the current study, Burris et al. (1976) did not infuse a mixture of other AA, making it uncertain that their cattle were only limited by Lys. When N retention was used as a response variable in Batista et al. (2015), the incremental efficiency of Lys utilization was 40%. In that study, experimental conditions were essentially equivalent to our lowest urea treatment, which yielded an incremental efficiency of 51%. Given that the steers used by Batista et al. (2015) were 1 mo younger and that AA efficiency is expected to decrease with greater BW, the reason for the greater Lys efficiency in the current study is unclear. Regardless, the difference between studies is not extremely large and both values can be interpreted to suggest an inefficient utilization of Lys by growing cattle.

Using a growth model based on data from 11 growth trials (a total of 543 individually-fed beef steers), Wilkerson et al. (1993) estimated metabolizable Lys requirements at 31.2 g/d, which represented 8% of metabolizable protein requirements. Thus, if body protein contains 6.4% Lys (Ainslie et al. 1993), the efficiency of Lys utilization for growth would be estimated to be 80% (6.4%/8%) of the efficiency of metabolizable protein. Assuming that body weight gain contained 15% protein, the

efficiency of metabolizable protein utilization for growth in the model of Wilkerson et al. (1993) would be 49%. Thus, the efficiency of Lys utilization would be calculated to be 39%, which is reasonably close to the efficiency of Lys utilization at our lowest level of urea infusion (51%).

Urinary N Excretion

The increase in urinary urea with greater ruminal ammonia loads can be explained by greater urea cycle activity when more ammonia reached the liver for detoxification. The rise in urinary ammonia can be attributed to increased conversion of ammonia to Gln by perivenous hepatocytes (Katz, 1992). If more ammonia becomes available in the liver, more ammonia may reach perivenous hepatocytes which scavenge residual ammonia that is not incorporated into urea (Katz, 1992). In contrast to periportal cells, which carry out the urea cycle, perivenous hepatocytes use ammonia as a substrate for glutamine synthetase, which catalyzes the amidation of Glu to Gln. A variable percentage of this newly synthesized Gln will travel to the kidney to be catabolized to urinary ammonia. Our observed increases in urinary ammonia excretion in response to urea supplementation were consistent with those of McCuistion et al. (2004) when growing steers were limited by His and those of Awawdeh et al. (2005) when growing steers were limited by Leu. In the current study, as well as in the work of McCuistion et al. (2004) and Awawdeh et al. (2005), the increases in urinary ammonia were not large. In the current study, urinary ammonia represented only 3% of the total urinary N, whereas urinary urea represented 84% of total urinary N at our highest level of urea infusion, suggesting that increases in total urinary N were mainly attributed to increases in urinary urea.

Urea infusions linearly increased (P = 0.04) total output of urine (acidified urine weights were 10.3. 11.0, and 12.3 kg/d for 0, 40, and 80 g/d urea; SEM = 1.2 kg/d). This increase may be attributable to the observed increases in urinary urea, which by increasing osmotic load would increase urinary water excretion. Lysine supplementation also increased (P = 0.03) total urine output (acidified urine weights were 10.3 and 12.0 kg/d for 0 and 6 g/d Lys; SEM = 1.1 kg/d). This response to Lys was unexpected in that urinary urea excretion was decreased by Lys, and this would decrease the urinary osmotic load. We used Lys-HCl as our Lys source, and the provision of Cl may have increased urinary excretion, although the amount of Cl was relatively small. Unknown factors, such as changes in hormonal secretion by the intestine in response to AA supply, also may have contributed to the response. Increases in urinary excretion in response to ammonia loading or Lys supplementation could have biological or economic implications for cattle producers.

Implications of Ammonia Loading

The goal of ammonia loading in our study was to simulate conditions in which cattle consume high-protein diets, which consequently results in increased ammonia absorption from the rumen and potentially the intestine. Once inside the rumen, urea is hydrolyzed by bacteria, predominantly absorbed as ammonia across the rumen epithelium, transported to the liver for detoxification as urea, and subsequently excreted in urine (Abdoun et al., 2006). The implications of this process on AA utilization and protein deposition have been uncertain as prior research has yielded contradicting results. For example, Lobley et al. (1995) and Mutsvangwa et al. (1996) suggested that ammonia detoxification in hepatocytes contributed to inefficient use of AA. In those studies, AA

extracted by the liver were presumably catabolized as a N source to support urea synthesis. Conversely, Luo et al. (1995) and Milano et al. (2000) suggested that AA use for ureagenesis was not biologically important if acidotic conditions were avoided.

Previous studies in sheep (Norton et al., 1982) and cattle (Moorby and Theobald, 1999) found no changes in N balance when ammonia load was increased. For example, N balance remained constant when ammonium chloride was intraruminally infused into sheep fed a pelleted-grass diet (Norton et al., 1982). In Moorby and Theobald (1999), N retention numerically became less negative, from -31 to -20 g/d, in lactating dairy cows fed perennial ryegrass silage when they were duodenally infused with ammonium acetate; variation was large, as might be expected for lactation dairy cattle, which may have limited the ability to detect differences. Although the authors detected no significant changes in N balance, those experimental animals were not limiting in supply of a specific individual AA. For this reason, ammonia loading may have had limited observable effects on AA metabolism.

The N retention responses in our study demonstrated that ammonia loading improved protein deposition in a model where Lys was designed to be limiting. The increases in N retention in response to urea infusion were consistent with McCuistion et al. (2004) who reported that N retention increased from 35.1 to 37.1 g/d when supplemental urea was increased from 0 to 80 g/d in growing steers limited by His. A lack of response would have been consistent with Awawdeh et al. (2004) where N retention of steers was limited by Met availability. In contrast, Awawdeh et al. (2005) observed significant improvements in N retention from 24.7 to 29.2 g/d when ammonia loading was induced in Leu-deficient steers. This finding may be attributed to differences

in metabolism throughout the body. Lysine, Met, and His are primarily catabolized by the liver, whereas branch chain AA such as Leu are metabolized throughout the body. Additionally, the initial catabolic step for Leu is a reversible transamination reaction, whereas this is not the case for Met, His, and Lys. Taken as a whole, the results of studies with cattle limited by Met, His, Leu, or Lys support the concept that increased ammonia detoxification does not require AA N inputs.

Plasma Metabolites

Effects of Lys. The numerical decreases in plasma urea with Lys supplementation were interpreted to suggest that less urea was produced from AA-N when a limiting AA was available for protein synthesis. Considering that steers in our model were Lys deficient, the increases in plasma Lys with supplementation of 6 g/d Lys would contradict the prediction of Bergen (1979) that plasma AA should not increase until the supply of that specific AA exceeds the requirement for protein deposition, which Batista et al. (2015) determined to be 9 g/d for maximal N retention. However, that same study reported increases in plasma Lys when 6 or 9 g/d of Lys was supplemented to steers under experimental conditions similar to our study. These increases in plasma Lys accompanied with increases in protein deposition before the requirement was exceeded in Batista et al. (2015) suggests that the increases in plasma Lys in response to Lys supplementation was a reasonable expectation and that the prediction by Bergen (1979) may not be applicable to Lys in growing cattle. Although plasma Lys increased in response to Lys supplementation in our study, it should also be noted that the Lys concentrations in plasma were not particularly high for any of the treatments. The observed decreases in plasma Val, Leu, Ser, Tyr, and Phe concentrations with Lys

infusions may have resulted from increased uptake and use for protein deposition when Lys, the sole limiting AA in our study, was provided. If appropriate concentrations or amounts of each AA are not available to peripheral tissues, protein deposition can be limited. Decreases in plasma concentrations of Val and Leu in response to supplementation of a limiting AA were also observed in growing steers limited by Met (Awawdeh et al., 2004) or His (McCuistion et al., 2004). Additionally, Awawdeh et al. (2004) observed decreases in Tyr when Met-deficient steers were supplemented with Met. In the current study, Lys infusions also led to decreases in plasma taurine, suggesting that less taurine was produced as a product of Met metabolism subsequent to increased Met utilization for protein deposition (Stipanuk and Ueki, 2011).

It would be tempting to suggest that the observed increases ($P < 0.01$) in plasma Glu in response to Lys supplementation were a result of changes in ureagenesis that affected Glu production or utilization; however, the lack of a plasma Glu response to urea infusion does not support this concept. The instability of Gln in plasma samples, which results in conversion of Gln to Glu, also makes interpretation of plasma Glu concentrations difficult, and it should be noted that concentrations of Gln plus Glu were not affected by treatments. The increases in plasma Arg with Lys infusion were also difficult to interpret. It may be possible that Lys supplementation altered Arg synthesis, Arg catabolism, or Arg excretion via changes in either enzymatically-mediated processes or in Arg transport.

Effects of Urea. The linear increase in plasma urea with urea infusions can be explained by increases in hepatic ureagenesis from ammonia absorbed across the rumen

wall (Reynolds and Kristensen, 2008). Once released into venous blood, urea can either be excreted in urine by the kidney or be recycled to the gastrointestinal tract.

The lack of change in plasma Lys with urea infusion might be interpreted as a result of offsetting effects on Lys concentrations. Improvements in Lys efficiency would reflect decreases in Lys catabolism, which could lead to increases in plasma Lys concentration. At the same time, the observed increases in protein deposition with greater ammonia loads would increase removal of Lys from plasma and potentially decrease its concentration. If both processes occurred to similar extents, there would be little or no net change in Lys concentration.

Significant decreases in plasma Gly with urea infusions were consistent with McCuistion et al. (2004) when ammonia loading was induced in growing steers limited by His. McCuistion et al. (2004) attributed the decreases in Gly to increased utilization of Gly for synthesis of Asp which can enter urea cycle when ammonia detoxification is upregulated in periportal hepatocytes. By using excess Gly as an Asp-N source for urea cycle, essential AA such as Lys may be spared from wasteful catabolism. This concept is supported by numerical decreases in Ser with greater urea infusions because Gly can be a precursor for Ser synthesis via serine hydroxymethyltransferase (Fu et al., 2001); once produced, Ser can be deaminated to pyruvate and carboxylated to oxaloacetate, which is the direct precursor to Asp (Jitrapakdee and Wallace, 1999). Similar to plasma Gly, Ala concentrations were linearly decreased in response to ruminal ammonia loads, perhaps reflecting an increase in Ala utilization. Decreases in Ala were also reported when ammonia loading was induced in growing steers limited by Met (Awawdeh et al., 2004), His (McCuistion et al., 2004), and Leu (Awawdeh et al., 2005). Via transamination

reactions, Ala may be used as a source of Asp-N for ammonia detoxification (Harper et al., 1984). Decreases in Ala in response to ammonia loading were consistent with an in vitro study by Mutsvangwa et al. (1996) where hepatocytes isolated from sheep supplemented with 20 g urea/kg DM had greater utilization of Ala for gluconeogenesis than hepatocytes from sheep that did not receive supplemental urea.

Conclusions

Overall, Lys supplementation improved whole-body protein deposition when growing steers were deficient in dietary Lys. No metabolic cost for protein deposition was demonstrated when ammonia loading was induced in Lys-deficient steers. Instead, daily N retention increased with greater urea infusions, suggesting that ruminal ammonia loading may improve the efficiency of Lys utilization. Despite potential improvements in cattle performance, the environmental and economic pitfalls may outweigh the benefits of providing excess N supplies.

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Table 1. Composition of experimental diet

Item	% of DM
Ingredient	
Pelleted soybean hulls	81.70
Wheat straw	8.11
Cane molasses	4.80
Calcium phosphate	2.07
Sodium bicarbonate	1.31
Calcium carbonate	1.09
Magnesium oxide	0.44
Trace mineral salt[1]	0.22
Vitamin premix[2]	0.14
Sulfur	0.11
Se premix[3]	0.011
Bovatec-91[4]	0.018
Nutrient	
Crude Protein	10.1
OM	89.2

[1] Composition > 95.5% NaCl, 0.24% Mn, 0.24% Fe, 0.05% Mg, 0.032% Cu, 0.032% Zn, 0.007% I, and 0.004% Co.
[2] Provided 5,300 IU vitamin A/kg diet DM, 3,593 IU vitamin D/kg diet DM, and 48 IU vitamin E/kg diet DM.
[3] Provided 0.065 mg Se/kg diet DM from sodium selenite.
[4] Supplied 36 mg lasalocid/kg diet DM.

Table 2. Abomasal infusate mixture

Ingredient	Amount, g/d
L-Lysine-HCl[1]	0 or 7.64
L-Methionine	10
L-Threonine	15
L-Histidine-HCl-H_2O[2]	8
L-Phenylalanine	20
L-Arginine-HCl[3]	15
L-Tryptophan[4]	5
L-Leucine	20
L-Isoleucine	15
L-Valine	15
Monosodium glutamate	150
Glycine	40
Dextrose	330
	mg/d
Pyridoxine-HCl	10
Folic acid	10
Cyanocobalamin	0.1

[1] Feed grade (78.8%); provided 6 g Lys/d.
[2] Provided 5.9 g histidine/d.
[3] Provided 12.4 g of arginine/d.
[4] Feed grade (98%); provided 4.9 g of tryptophan/d.

Table 3. Effects of abomasal Lys supplementation and ammonia loading via ruminal urea infusion on N balance and diet digestibilities in growing steers

	0 g/d of Lys			6 g/d of Lys				*P*-value[1]				
	Urea, g/d											
Item	0	40	80	0	40	80	SEM[2]	Lys	U-L	U-Q	Lys × U-L	Lys × U-Q
n	5	6	4	4	5	7						
Nitrogen, g/d												
Infused	39.4	58.0	76.5	40.6	59.1	77.7	-	-	-	-	-	-
Dietary	40.3	40.1	40.3	40.3	40.3	40.3	0.38	0.88	0.90	0.64	0.94	0.61
Total Intake	79.7	98.1	116.8	80.9	99.4	118.0	0.38	<0.01	<0.01	0.64	0.94	0.61
Fecal	21.7	21.5	21.3	20.7	21.5	21.7	1.3	0.75	0.72	0.78	0.37	0.84
Urinary	34.7	51.8	69.2	28.9	44.4	59.6	2.2	<0.01	<0.01	0.99	0.33	0.91
Ammonia	0.9	1.2	2.1	1.4	1.6	1.7	0.18	0.17	<0.01	0.41	0.03	0.19
Urea	25.2	41.4	60.8	18.7	33.9	47.9	2.2	<0.01	<0.01	0.77	0.12	0.49
Retained	23.3	24.8	26.3	31.0	33.7	36.6	2.3	<0.01	0.05	0.98	0.52	0.96
Digestibility, %												
DM	74.6	74.4	76.6	75.6	75.3	74.6	1.7	0.96	0.57	0.53	0.12	0.34
OM	76.2	76.1	78.3	76.9	77.0	76.2	1.6	0.84	0.44	0.63	0.14	0.30

[1] U-L = Linear effect of urea; U-Q = Quadratic effect of urea.

[2] For n = 4.

Table 4. Effects of abomasal Lys supplementation and ammonia loading via ruminal urea infusion on plasma urea, glucose, and AA in growing steers

	0 g/d of Lys			6 g/d of Lys								
	Urea, g/d							*P*-value[1]				
Item	0	40	80	0	40	80	SEM[2]	Lys	U-L	U-Q	Lys × U-L	Lys × U-Q
n	5	6	4	4	5	7						
Urea, m*M*	2.9	4.5	5.1	2.4	3.4	5.1	0.63	0.09	<0.01	0.80	0.54	0.27
Glucose, m*M*	5.9	6.4	6.3	6.3	6.5	6.3	0.33	0.45	0.60	0.27	0.56	0.86
Amino acid, μ*M*												
Lysine	21	23	22	39	37	38	5.4	<0.01	0.97	0.93	0.81	0.60
Alanine	168	182	158	192	182	160	12.8	0.24	0.04	0.11	0.26	0.42
Arginine	65	74	67	79	71	87	7.9	<0.01	0.33	0.61	0.47	0.02
Aspartic acid	10.5	13.0	9.6	13.8	13.4	11.9	1.7	0.12	0.39	0.19	0.77	0.35
Citrulline	73	69	76	66	65	71	7.5	0.16	0.35	0.27	0.87	0.76
Glutamate	74	77	77	102	90	88	7.4	<0.01	0.39	0.75	0.23	0.56
Glutamine	289	315	286	294	285	280	29.4	0.52	0.65	0.45	0.78	0.39
Glu + Gln	366	392	365	401	378	369	32.9	0.68	0.50	0.64	0.54	0.43
Glycine	632	600	530	643	530	453	54.6	0.27	<0.01	0.99	0.38	0.66
Isoleucine	66	67	70	65	68	64	6.3	0.68	0.83	0.76	0.66	0.57
Leucine	64	66	69	56	60	54	5.9	0.02	0.78	0.61	0.44	0.48
Methionine	34.7	33.5	32.2	31.3	34.5	31.2	3.9	0.61	0.65	0.50	0.68	0.53
Ornithine	54	55	56	61	53	50	8.7	0.93	0.56	0.86	0.37	0.85
Phenylalanine	73	70	71	68	69	58	3.9	0.05	0.12	0.49	0.24	0.18
Serine	120	127	109	94	95	80	7.2	<0.01	0.06	0.07	0.77	0.69
Taurine	56	52	47	49	36	41	5.4	<0.01	0.05	0.24	0.99	0.22
Threonine	157	148	154	153	168	135	19.5	0.94	0.47	0.50	0.64	0.21
Tryptophan	53	61	60	56	57	53	5.5	0.40	0.59	0.22	0.17	0.84
Tyrosine	50	54	53	38	40	44	6.4	<0.01	0.38	0.84	0.84	0.70
Valine	258	272	274	243	235	226	21.1	0.02	0.99	0.83	0.32	0.87

Total AA	2430	2459	2328	2447	2298	2116	138.6	0.17	0.05	0.60	0.30	0.73

[1] U-L = Linear effect of urea; U-Q = Quadratic effect of urea.
[2] For n = 4.