Balancing for amino acids in support of lactation.

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Protein is an Essential Nutrient

Protein is one of six essential nutrients needed to survive. It is composed of carbon, hydrogen, oxygen, nitrogen (**N**), and sulfur. The latter two elements are what differentiate protein from carbohydrates, which contain only carbon, hydrogen, and oxygen. Generalizing protein as one comprehensive category allows simple categorization of a substrate that humans and animals must consume in their diets. However, this mindset does not accurately depict what they need to synthesize body tissue (skin, muscle, organs, etc.), gestational tissue, milk protein for young, and to carry out countless other processes required for basic life functions.

Protein is actually composed of smaller molecules called amino acids (**AA**), which are strung together to form a long chain. The AA can be envisioned as links in the chain. However, this chain is unconventional, as its links come in 20 different shapes (the 20 different AA). Because the length of the chain and the order of AA in each chain vary, and both dictate the characteristics of the protein, an essentially infinite number of proteins can be created. For example, if a protein is composed of 200 AA (a smaller sized protein), there a 20^{200} ($1.6 * 10^{260}$) possible combinations of the AA and thus 20^{200} different proteins.

Animal cells make more than 20,000 different proteins. These proteins provide the structural framework of the cell and conduct much of the work that allows the cell to metabolize nutrients, grow, divide, and maintain itself. Examples of unique proteins include:

- hair and wool fibers, which are composed of a number of long protein chains wound together by the hair follicle cell and secreted as a hair or wool fiber;
- a mesh of translucent proteins forming the retina of the eye;
- a series of fibrin proteins bound together to form a muscle fiber;
- actin protein, which acts as the motor to cause muscle fiber contraction;
- keratin sheets created by skin cells before dying to create the outer skin layer;
- and the caseins, beta-lactoglobulin, and alpha-lactalbumin proteins secreted with milk.

Metabolizable Protein and Amino Acids

Metabolizable protein (**MP**) represents the true protein available to the cow absorbed from the intestine. Metabolizable protein includes digested microbial protein (**MCP**) and protein escaping degradation in the rumen (**RUP**). The Dairy NRC (2001) includes endogenous protein in this term, but this is not a correct representation as it is derived

from previously absorbed microbial and ruminally undegraded feed protein, and thus does not represent "new" protein.

Although animal N requirements are commonly stated in terms of MP, the true requirements are for the specific AA resident in that protein. The animal actually does not have a protein requirement, per se. Because there is a diversity of AA composition in the absorbed protein, stating animal requirements in terms of MP inherently causes requirement over-prediction to compensate for variation in AA composition. This is perhaps most apparent when feeding diets constructed largely from maize products which are inherently low in lysine (Polan et al., 1991). Such a diet could be created to meet MP requirements, but animals typically still respond to the addition of a protected lysine source or more protein that also provides lysine to the ration (Vyas and Erdman, 2009).

When one or more AA are deficient, production will generally decline relative to a sufficient diet. When such diets and their corresponding milk production are used to generate MP requirement prediction equations, the AA deficient diets will categorize as MP deficient resulting in a solution that calls for more MP than if all of the diets had a good mix of AA. This results in higher MP requirements which serve to ensure the most limiting AA in the diet always exceed requirements. Thus, we are often overfeeding many AA when basing requirements on MP alone.

Animals can be successfully fed a lower MP diet if the AA composition of that diet is better matched to AA requirements. This was demonstrated by Haque et al. (2012) using diets with less than 13% crude protein (**CP**). The idea of having a perfect mix of AA is generally referred to as the "ideal protein" concept. Basing requirements on the individual AA, rather than on MP alone, clearly identifies which AA are being wasted and those that are insufficient. This allows construction of diets which achieve the "ideal" profile. Feeding an ideal protein profile at 100% of the animal's AA requirements will yield maximum N efficiency (percentage of dietary N that is converted to a marketable product). This is beneficial from an environmental standpoint, as dairy cattle excrete any unused dietary N in their urine and feces.

Though some of the N in stored manure may be captured by growing plants and microbes in the soil after land application, much of it is lost as ammonia. This is evident by the smell of ammonia associated with barn floors and manure storage facilities. The ammonia is primarily derived from catabolism of urea in urine. It is rapidly converted to ammonia by action of the enzyme urease which is present in feces. As soon as the urine and feces mix on the barn floor, the conversion of urea into ammonia begins, and ammonia then vaporizes from the manure. The loss of ammonia continues during manure storage, resulting in half or more of the N in manure being lost to the air.

Ammonia emissions are a form of environmental pollution (Uuml et al., 2001, Agle et al., 2008). Ammonia in the air combines with sulfur and nitrogen oxides from car and power plant exhaust to form small particles less than 2.5 micrometers. These particles cause the haze visible in the air and evidence is growing that they contribute to lung problems including asthma.

Economic and Environmental Benefits of Precision N Feeding

In addition to the environmental impact of ammonia emissions, there is also a large financial cost. Protein is an expensive dietary nutrient representing approximately 42% of the cost of a lactating cow ration in the United States (St-Pierre, 2012). In a survey carried out on 103 large-scale dairies across the US (613 ± 46 cows; 34.5 ± 0.3 kg of milk per cow per day), nutritionists reported feeding diets with 17.8 ± 0.1 % crude protein (Caraviello et al., 2006). A meta-analysis of 846 experimental diets found a similar mean diet CP content and identified that conversion efficiencies for dietary and metabolizable N (based on NRC, 2001) to milk protein averaged 24.6 % and 42.6 %, respectively (Hristov et al., 2004). Assuming the same intake and diet composition (22.1 kg/d DMI and 17.8 % CP), over a 10-month lactation, the 9 million dairy cattle in the US excrete an estimated 1.3 million metric tons (mmt) of N per year (Livestock, Dairy, and Poultry Outlook: August 2012, LDPM-218, Dairy Economic Research Service, USDA). It is likely a survey today would observe slightly lower protein diets than what the aforementioned researchers observed given the significant increase in protein cost since 2006. However, the efficiency of conversion of dietary protein to milk protein does not generally exceed 30% until dietary CP levels fall below 15.5%. It is unlikely that the current average dietary CP% on US dairy farms is this low.

The dairy extension group at Ohio State determines the cost of a pound of MP in dairy rations on a bi-monthly basis (Buckeye Dairy News (www.dairy.osu.edu/newsletter/buckeye-dairy-news). Over the past 5 years, this value has remained relatively constant at $0.43/lb, although the most recent analyses shows a much lower value, presumably due to the impact of Chinese tarrifs on imported soybean products. Presumably this value will return to the historic trend line when the trade war is resolved. Using the historic trend, a ration for a cow producing 80 lbs of milk should contain approximately 5.44 lbs of MP which can be readily met with a diet containing 16.1% CP resulting in a N efficiency of 28.7%.

Successful removal of 1 lb of MP from the ration would result in a reduction in ration cost of $0.43/cow/d, a dietary CP level of 13.1%, and a N efficiency of 35.1%. Thus, there are both economic and environmental incentives to feed less dietary protein; the challenge is doing so without sacrificing production. To accomplish this, accurate and precise nutrition models that are based on AA rather than on MP must be implemented in ration balancing software. This would allow nutritionists to reliably match the supply of AA to the animal's needs, along with the desired level of production.

Ruminal Outflow of Microbial and Undegraded Feed Protein

Regardless of whether the requirement system is based on MP or on AA, predictions of the supply of MP must be unbiased. Estimates of the supplied AA are derived from predictions of the true feed protein escaping ruminal degradation, and the true microbial protein synthesized in the rumen. If either are biased, then the estimates of the supply of AA associated with each will be biased. Although the 2001 Dairy NRC represented a significant improvement in accuracy and precision over earlier systems, predictions of microbial and undegraded feed protein (RUP) were apparently biased (Roman-Garcia et al., 2016, White et al., 2016, White et al., 2017a, White et al., 2017b). Undegraded feed protein flow was found to be overpredicted on average by 40 g N/d for a typical

animal, and the error increased as RUP flow increased. This signals fundamental problems in model structure, which consequently contributes to similar bias in predicting milk production. Bateman et al. (2005) and Broderick et al. (2010) observed similar problems.

Correlation analyses indicated the RUP bias problem was associated with passage rate (K_p) estimates. The K_p equations used by the Dairy NRC (2001) were biased compared to K_p measurements from studies that used indigestible NDF as a marker (Krizsan et al., 2010). A recent study of K_p on forage-based diets also supports bias in prediction of particulate K_p (Gregorini et al., 2015). Recent work by our group has demonstrated that the derivation of new estimates for K_p from observed RUP flows resulted in very modest gains in precision but substantial reductions in bias.

The revised estimates for RUP led to similar adjustments in RDP (calculated by difference from total N flows). The latter were used by Moraes et al. (unpublished) along with new estimates of ruminal starch and fiber degradation to derive an updated prediction of microbial protein outflow. The approach taken provides an integrated representation of ruminally degraded N and carbohydrate sources that substantially improved precision and accuracy relative to the previous equation. However, the potential contribution of recycled blood urea in microbial protein (Reynolds and Kristensen, 2008) was not represented. This likely is only important when attempting to feed very low protein diets (less than 13% CP), and consequently may be ignored for now.

Ruminal Outflow of AA and Absorption from the Intestine

A number of updates and revisions to the NRC 2001 system for predicting AA flows were undertaken (Fleming et al., 2019). These included corrections for hydration and incomplete recovery of AA from acid hydrolysis of proteins (Lapierre et al., 2016), as well as revised estimates of the composition of AA in microbial protein (Sok et al., 2017). Past efforts have largely ignored the difference in mass of AA when bound in protein versus when in free form. In the former, a molecule of water is removed across each peptide bound holding the AA together to form the protein resulting in a mean mass loss of 15%. This was reflected in research models such as Molly (Baldwin et al., 1987), but had been overlooked by the prior NRC committee (2001). The AA composition of proteins is almost always reported in a hydrated form given that the measurements are made on free AA after acid hydrolysis. Correction for the mass of hydration, incomplete recovery of AA from acid hydrolysis, and updated microbial AA composition resulted in removal of much of the mean bias observed by NRC (2001). These corrections mostly aligned predicted AA flows with observed total AA outflows from the rumen. The model tended to overpredict several AA, including methionine, which was felt to possibly reflect technical challenges including incomplete recovery from hydrolysis due to inadequate temperature control during digestion or introduction of oxygen in the hydrolysis tubes leading to some oxidative losses.

Although this work improved estimates of RUP AA flows from the rumen, predictions of absorbed AA are likely still biased and less precise than desired due to the assumption that all AA are digested to the same extent as the protein contained in the microbes and

RUP. We have recently adapted an approach used by Maxin et al. (2013) to assess the absorbed supply of each AA from individual dietary ingredients (Estes et al., 2018, Huang et al., 2019). The method makes use of an infusion of a ^{13}C labeled AA mixture derived from enriched algae to assess the entry rate of each AA. The labeled AA are introduced into the jugular vein, consequently, measurements reflect appearance in blood thereby accounting for all losses before blood appearance, i.e. ruminal and intestinal.

We have assessed AA entry as a fraction of the AA in the source ingredient for 9 ingredients: corn silage, grass hay, alfalfa hay, corn grain, soyhulls, distiller's grains, brewer's grains (Huang et al., 2019), blood meal, and feather meal (Estes et al., 2018). In all cases except feather meal, the proportions of AA in the source ingredient that entered the blood pool varied considerably across the essential AA. Thus, either the composition of the RUP deviates significantly from the ingredient or the digestibility of individual AA in the RUP vary considerably, or both. In the case of the feather and blood meals, the modified 3-step and Ross assays failed to reflect the observed digestibility of those ingredients indicating those methods are not reliable for evaluation of AA availability (Estes et al., in preparation). Additional work is required to establish methods of AA availability assessments that can be conducted in real or near-real time for field application.

Fixed AA Use Efficiencies and the Ideal Protein Concept

A portion of the problem in predicting milk or milk protein responses to MP supply noted above is the model assumption that the conversion of MP to milk protein, after subtracting maintenance use, is a constant 65%. In a summary of literature data, Lapierre et al. (2007) found that the highest MP conversion efficiency was 43%. Efficiency decreased as milk protein output (and MP supply) increased. In a summary of publications that reported responses to post-ruminally infused casein, Hanigan et al. (1998) found a similar maximum efficiency of conversion of about 45% with an average conversion efficiency of 22%. Whitelaw et al. (1986) abomasally infused casein at 3 different levels and observed responses at each level with efficiencies of conversion ranging from 40 for the first increment to 15% for the last increment. Each of these demonstrates that the maximum efficiency is less than the assumed 65% and varies depending on the overall supply and other factors. These problems are not resolved if MP is replaced with individual AA. In fact, adherence to the same scheme for the multiple essential AA (EAA) maintains the slope bias associated with too high of an assumed efficiency and introduces significant mean bias.

Historically, we have used the concept of a first-limiting AA as the basis for determination of requirements. The concept is based on a hypothesis which has become known as the Law of the Minimum. Sprengel (1828) formulated this concept based on plant growth responses to soil minerals. The original thesis stated that a nutrient can limit plant growth, and when limiting, additional growth will be proportional to additional supply. This is a logical approach rooted in the concept of conservation of mass. Clearly, nutrient output cannot exceed nutrient input if it cannot be synthesized, and output will be less than input if the transfer is less than 100% efficient, which is often the case.

This concept is strongly supported by volumes of data over the past 175 years. Von Liebig (see Paris, 1992 for a translation) subsequently restated and expanded the hypothesis indicating that if a nutrient was limiting growth, responses to other nutrients could not occur (von Liebig, 1862). Mitchell and Block (1946) used von Liebig's extension of Sprengel's thesis to develop the concept of the order of limiting AA, which was described using an analogy of a water barrel with broken staves. Based on this formulation, if any nutrient is limiting milk production, then only the addition of that nutrient to the diet will result in a positive milk yield response, e.g. the single-limiting nutrient paradigm. The ideal protein concept loosely aligns with this framework in that it is assumed there is an ideal AA profile that should be provided to an animal and that profile will remain largely fixed as production levels change.

Whereas the observations of Sprengel are well supported, that of von Liebig and the subsequent derivations have not fared as well. The single-limiting AA theory and the ideal protein concept hold true provided the efficiencies of use of absorbed AA remain constant regardless of supply. For example, if the supply of methionine is doubled, the allowable protein production from methionine must also double (and the reciprocal for halving supply). Additionally, doubling the supply and use of methionine should exactly double the need for histidine. If this is true, then one can easily determine which nutrient is most limiting by calculation of the allowable protein yield from each AA based on the composition of the protein and the known efficiencies, and this can be extended to energy and other required inputs. If the result of that calculation indicates that if inadequate histidine is being provided, then one would predict a response to the addition of histidine, and the same for any other nutrient that is apparently deficient.

However, the transfer efficiency of absorbed AA to milk protein is not fixed. Because AA removal from blood is regulated in concert with needs for milk protein synthesis (Bequette et al., 2000), the efficiency of AA transfer from the gut to milk protein is variable. This complicates application of the ideal protein calculations and undermines the concept of a single-limiting nutrient. If there are interactions among nutrients or among nutrients and the environment that affect efficiency, the predictions of which nutrient is first-limiting will be faulty as will predictions of allowable protein production.

Work at the cellular level over the past 30 years has clearly demonstrated that protein synthesis and AA transport in support of such synthesis are highly regulated by the mix of AA available in the cell, the energy status of the cell, and hormonal signals denoting overall energy status in the animal (Bequette et al., 2000, Appuhamy et al., 2009, Appuhamy et al., 2011, Appuhamy et al., 2012). These regulatory mechanisms control protein synthesis, and therefore tie rates of protein synthesis to substrate supply and energy state in the animal. One can think of them as the accelerator pedal in a car. Pressing on the pedal does not, in and of itself, make the car go faster. It controls fuel entry to the engine, and more fuel does make the car go faster. Thus the pedal controls car speed. A shortage of one or more AA causes a reduction in the regulatory system which results in a slowing of the rate of protein synthesis and secretion, i.e. less milk protein. The cells sense the deficiency and respond by increasing the activity of transporters for those AA to minimize the deficiency and limit the response to a very modest decline in protein synthesis.

High insulin concentrations outside of the cell denote adequate energy supply in the animal, which stimulates protein synthesis and consequently stimulates AA transport into the cell. Inadequate supplies of ATP due to a shortage of acetate and other energetic precursors deriving from ruminal fermentation and absorbed nutrients causes a reduction in protein synthesis and in AA transport, although the effects of this latter signal are modest perhaps becoming more potent under severe shortages (Arriola Apelo et al., 2014a, Castro et al., 2016a) (Bequette et al., 2000). The signals arising from each of these factors are integrated in an additive manner via a set of regulatory mechanisms within the cells resulting in the ability to respond to several factors at a time. As a result, the cells have some latitude in adjusting their activity in their attempts to maintain production. Returning to our analogy it is equivalent to multiple gas pedals and the average of them being used to set the rate of fuel delivery to the engine.

Such variation in AA transport and use causes variable efficiency of transfer from absorbed AA to protein production. This undermines the ideal protein concept by creating a range of inputs that can achieve similar efficiency. This also contributes to muted responses to AA as the efficiency declines as the supply increases. The additivity of the responses across factors also is inconsistent with the single-limiting nutrient concept. If provision of more than one nutrient or hormone can partially or completely offset the loss or deficiency of another, there is almost an infinite number of combinations of AA, energy substrates, and hormonal concentrations that will result in the very same amount of milk. This concept is demonstrated *in vitro* by Clark et al. (1978) and (Arriola Apelo et al., 2014b), and *in vivo* by Rius et al. (2010a), Hanigan et al. (2000), Liu et al. (2017), and Yoder et al. (in press, JDS) clearly demonstrating that the response surface is complex and not well represented by the "Law of the Minimum" when applied to lactational responses to AA. Therefore, current protein and AA requirement models for lactation inappropriately represent the underlying biology, which leads to inflated prediction errors and large bias in predicted responses.

Milk Protein Output from Energy and AA Supplies

Work to define the mechanisms controlling mammary AA uptake and subsequent use for milk protein production has progressed considerably over the past 15 years. Fairly robust mechanistic models of mammary metabolism capture the independent and additive effects of key essential AA, energy supply, and insulin (Hanigan et al., 2000, Hanigan et al., 2001, Hanigan et al., 2002, Castro et al., 2016b).

These concepts are at least partially captured if one represents milk protein production as an additive function of several key EAA and energy supply. In such an equation, the partial effect of each EAA and energy can be captured in the model. Evaluation of over 950 treatment means from the literature that included protein infusions, individual AA infusions, and rumen-protected amino acids **(RPAA)** feeding studies resulted in a very robust set of equations that contained 7 EAA plus digestible energy, animal body weight, and digestible NDF. The AA were well defined by the data and the estimates were very stable during cross-evaluation. They included arginine, histidine, isoleucine, leucine, lysine, methionine, and threonine. Phenylalanine estimates were not stable on cross-evaluation likely indicating inadequate, independent observations of responses to that AA. Tryptophan **(Trp)** generally solved for negative responses, but it was unclear if

that reflected a true inhibition or if it was an artifact of little independent manipulation of Trp. Valine responses were poorly supported by the data.

A key component to the prediction equation is the inclusion of digestible energy. It is a very strong driver of milk protein output and reflects the linkage between energy and protein supplies to the animal. Milk and body proteins cannot be synthesized at maximal rates if the energy supply to the animal is inadequate. Inclusion of energy in the milk protein equation reflects this and mirrors the inclusion of ruminally digestible carbohydrate in the microbial growth equation.

Evaluation of the new equations shows a near halving of the prediction errors and complete removal of the slope and mean bias present in the prior system. Although milk protein is not directly predicted by the NRC (2001), one can generate a prediction using the observed milk protein concentration from each treatment and the predicted milk volume from the model. Using this approach yielded an error of prediction of 24.9% for MP plus NEL with 18% of the error due to mean bias and 21% due to slope bias. When each of the EAA were also considered using the first-limiting nutrient approach, the overall error became 29.0%, and the mean and slope bias proportions were 46 and 5%, respectively. This contrasts with an error of 13.9% for the new equation based on supplies of the EAA and energy with no mean or slope bias.

Initial testing of this system in a ration balancer environment has shown that given a range of RPAA at prices similar to those available today, diets can be balanced for cows producing 100 lbs of milk per day with less than 14% CP and at costs less than the NRC (2001) MP based costs. We are in the process of setting up an animal study to test these predictions.

Conclusion

Rations can be balanced at levels well below 15% CP, probably even below 13%, if we are able to reliably match AA supply with true animal needs. Current models of AA requirements used in field application programs have significant accuracy and precision problems due to less robust parameter estimates and a post-absorptive system framework that is inconsistent with the known biology. We have addressed both issues resulting in a new system that better represents the biology and exhibits much greater accuracy and precision, allowing us to achieve N efficiencies of 35% or greater in lactating cattle.

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