

Evaluation of ruminal nitrogen availability in liquid feeds

Report to the Liquid Feed Committee of the American Feed Industry Association

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Background

Under many conditions, protein supplements provided to ruminants are of optimal value when the protein (nitrogen) is completely available for use by the ruminal microbes. Various approaches are available to assess the ruminal availability of proteins in feedstuffs. These include measures conducted in live animals, in situ disappearance of nitrogen from Dacron bags, and in vitro ammonia release (and similar assays where other end-products of protein degradation are measured).

Live animal evaluations are ideal, but are too slow and costly for routine evaluation of a wide range of feeds. *In situ* incubation of substrates in Dacron bags provides a good measure for many feedstuffs, but it measures the insoluble proteins that remain following fermentation and, as such, is of little value in measuring availability of soluble nitrogenous compounds in liquid feeds. Moreover, in situ methodologies are based on the premise that protein solubility is synonymous with degradability, but this is not the case. Many soluble nitrogen compounds are not ruminally degraded, and, conversely, nitrogen in some insoluble compounds can be degraded by ruminal microbes. Test tube assays measuring ammonia release work well for some feeds. However, feeds that have a low protein concentration or a highly fermentable carbohydrate component are difficult to evaluate because microbes will take up much of the ammonia that is produced from the feed. Although microbial inhibitors can be added to in vitro assays to prevent utilization of ammonia or amino acids by microbes, those assays are only valid over short incubation periods and may not work well for sources of non-protein nitrogen because not all of the end-products may contribute to the ammonia pool directly.

For these experiments, we used an in vitro assay where we do not measure the feedstuff nitrogen directly, but rather the microbial mass that accumulates as a result of assimilation of dietary nitrogen. The microbial mass is proportional to the amount of available nitrogen in the sample because microbial growth is most limited by the availability of protein/nitrogen. In our assay, buffered rumen fluid is incubated with an

excess amount of an energy substrate (starch) and the nitrogen source to be tested. Following 12 h of incubation, the fermentation is stopped, and the amount of cytosine (a marker of microbial mass) present in the tube is measured.

General methods

Fermentations were conducted in 50 mL centrifuge tubes. Each tube contained 20 mL of anaerobic McDougall's buffer, 10 mL of strained rumen fluid, 0.75 g of starch to serve as an energy source, and a sample supplying 20 mg N. Standards were prepared to contain 0, 10, 20, or 30 mg N (from an equal mixture of urea and casein on a N basis), which leads to linear increases in the amount of cytosine (marker of microbial cell mass) produced. Tubes were allowed to ferment for 12 hours at 39°C with occasional mixing. After 12 hours, 0.1 mL of a formalin solution (100 mL of 37% formaldehyde plus 9 g NaCl in 1 L of solution) was added, and samples were frozen. After thawing, samples were centrifuged at $30,000 \times g$ for 15 minutes, the supernatant was removed, and the resulting pellet containing the bacteria was lyophilized, transferred to a screw-cap tube, hydrolyzed in perchloric acid, and analyzed for cytosine content by HPLC. Standard curves were analyzed by linear regression of cytosine production on amount of N added to the tubes. In some of our work, we observed that addition of sugar to the tubes led to small increases in cytosine independent of the N added. Therefore, standards were prepared with and without added sugars (0.25 g) and the amount of cytosine resulting from sugars (or other readily fermentable carbohydrates) in samples was subtracted before the available N was calculated from the standard curve.

Initially, samples were fermented in quadruplicate and individually analyzed for cytosine. In the final experiment, each sample or standard was fermented in six replicates, and these were prepared individually for analysis of cytosine and then aliquots from three tubes were mixed together prior to HPLC analysis. Variation was predominantly related to differences between tubes, so this strategy allowed us to account for this variation with a large number of replicates (6) but not unduly increase the cost associated with the HPLC analysis of the samples.

We previously demonstrated linear relationships between the amount of casein (a purified protein that is considered completely available to ruminal microbes) added to fermentation tubes and the amount of microbial cytosine that was produced. Similar responses were achieved when non-protein nitrogen sources, such as urea, were tested. We tested graded levels of casein, urea, and an equal mix of casein and urea, and responses were similar regardless of whether the available nitrogen was provided as a true protein source or as a non-protein nitrogen source. Thus, the assay is robust with regard to the type of substrates that could be evaluated.

Carbohydrates other than the starch (which we purposely added as an energy source) impact the relationship between available nitrogen and microbial cell mass. The practical concern is that some test ingredients might provide enough sugar to impact the results. We tested two levels of a mixture of sucrose, glucose, and fructose added to the fermentation tubes and measured the response in microbial production of cytosine *in vitro*. The lower level of sugar (0.25 g/tube) led to small increases in cytosine production, but the higher level (0.5 g/tube) did not change the amount of cytosine produced. Therefore, we developed standard curves with and without 0.25 g sugars to

correct for the amount of cytosine that was produced from carbohydrate rather than nitrogen supply. The response to the added sugar is consistently present, but it does not greatly impact the predicted ruminal N availabilities.

Effects of processing on ruminal nitrogen availability

We tested the effects of base ingredient, heating, addition of minerals, and storage on the ruminal availability of nitrogen in liquid feeds. For preparing the test products, we used the following base ingredients: cane molasses, steep liquor, distiller's solubles, and concentrated separator byproduct. We also made products using purified components to mimic the base ingredients but with "contaminants" removed. The goal of using the purified components was to model the effects of individual components that might be present in the various base ingredients. The purified components included 1) 55% sucrose, 2) 33% sucrose plus 11% glucose plus 11% fructose, 3) 30% starch, partially hydrolyzed by amylase, plus 4.5% lactic acid, and 4) 5% soluble starch. Products were made by adding either casein or urea as the nitrogen source to mimic true protein and non-protein nitrogen in feeds, respectively. Most of the crude protein contained in the products was supplied by the casein or the urea. For assessing mineral additions, salt (NaCl) was added at 2% of the product weight and phosphoric acid was added at 4% of the product weight (with a pH of at least 3 being maintained). Heating of the products was accomplished by placing the samples in a boiling water bath for 15 minutes, whereas unheated products were maintained at room temperature throughout the process. For our evaluation of the effects of heating of the liquid feeds, we tested the products that contained 2% added NaCl.

In this study (Table 1), we observed that the base ingredient used to manufacture the product impacted the availability of the protein. Notably, products made with concentrated separator byproduct had lower ruminal availability of nitrogen than the other products. In general, products made with typical feed ingredients had lower availability than those made with the purified components. The specific base ingredients used to manufacture products were evaluated in a later assay. The cane molasses contained 6.4% crude protein with 14% ruminally available, the CSB contained 13.7% CP with 4% available, the steep contained 16.8% CP with 50% available, and the distiller's solubles contained 8.3% CP with 17% availability. The differences among these four base ingredients can explain some of the differences in the products, particularly the lower availability of nitrogen from products manufactured from CSB. However, some of the differences among products must still be attributed to interactions between the base ingredients and the protein sources (urea and casein) that were added to those products.

The decrease in ruminal nitrogen availability in response to heating was rather dramatic, and this response was dependent upon whether the primary source of nitrogen was casein or urea (Table 2). There was a much larger depression in nitrogen availability in response to heating for products containing casein than for those containing urea. This suggests that true proteins are more able to enter into heat-dependent reactions that impact availability. Interestingly, the base ingredient used to make the product did not greatly affect the response to heating (Table 3). We expected that products with more reducing sugars would be more impacted by heating, but this was not observed. For

example, heating decreased the availability of nitrogen in products made with sucrose by 42%, but ruminal nitrogen availability was only decreased 33% by heating in products made with a mixture of sucrose, glucose, and fructose.

Mineral additions to unheated products (as salt or phosphoric acid) also had a large impact on nitrogen availability. Addition of salt to the products decreased nitrogen availability by an average of 21%, whereas addition of 4% phosphoric acid decreased nitrogen availability by 50%. However, the responses to the mineral additions were somewhat dependent upon the source of protein (casein vs urea, Table 4) as well as the base ingredient used to make the product (Table 5). For example, the negative effect of NaCl was greater for those products made with urea than for those made with casein. In contrast, the negative effect of phosphoric acid additions was rather dramatic for products made with either casein or urea.

For evaluating effects of storage, we utilized products containing the phosphoric acid and stored them at room temperature for 0, 2, or 8 weeks prior to analysis. Mold growth was prevalent in some samples, particularly when stored for 8 weeks. Across all samples (Table 6), storage for 2 weeks reduced ruminal nitrogen availability from 99 to 91%, whereas after storage for 8 weeks the availability was greater than in unstored samples (104%). It is likely that the increases in nitrogen availability over the longer period of storage are due to the mold growth in these samples leading to a more available product. There were some differences among the samples with regard to their response to storage, but these effects seem to be driven mostly by two starch/urea products that had very low availabilities when stored for 2 weeks (Table 7).

In summary, processing characteristics can impact the availability of nitrogen from liquid feeds. Important variables include the base ingredient, the source of nitrogen, mineral additions, and heating. Additionally, significant interactions between some of these variable were also present. It is clear that future research will be needed to fully characterize these effects so that negative impacts of manufacturing on protein availability can be prevented, thereby allowing the feed industry to exploit the full value of the feed ingredients that are employed.

Evaluation of nitrogen availability in base ingredients used in liquid feeds

For this experiment, we were able to obtain seven samples of cane molasses, two samples of beet molasses, five samples of concentrated separator byproduct, four samples of steep liquor, four samples of whey, three samples of condensed corn distillers solubles, and single samples of soy solubles and of hemicellulose extract (wood molasses). The samples were analyzed for total crude protein content ($N \times 6.25$) and for ruminal availability of the nitrogen.

Table 8 provides the crude protein contents of the samples. The large range in crude protein concentrations for some of the products should be noted, and much of the range in protein concentrations can be attributed to differences in moisture among the samples. For example, whey and condensed whey were grouped together.

There were clear differences among the ingredients with regard to the ruminal N availability (Table 9). Molasses products (including cane, beet, and CSB) all contained less available nitrogen than steep, condensed distillers solubles, and whey. Also, we observed large differences within the different sources of some of these products.

Notably, we observed a cane molasses sample that had availability as low as -33% as well as a cane molasses sample as high as 31%. Large ranges among samples were observed for all of the ingredients except beet molasses, where we were limited to two samples.

Among the different samples of the cane molasses, it appeared that the protein concentration in the samples was related to the availability. Samples with higher crude protein contents also had greater ruminal nitrogen availabilities (Figure 1). It is unknown if there is a cause-effect relationship or if this relationship would hold across a greater number of samples. However, it may indicate that certain factors that impact the protein concentration (e.g., the initial materials utilized or the manufacturing process) also impact the nitrogen availability.

One issue with this data set is the negative values that were obtained for a number of the samples. These resulted from fermentations that yielded less microbial growth when the samples were added than when no nitrogen source was added. If none of the sample nitrogen was available, we would expect a value of 0. Thus, it is possible that some of the samples contained compounds that inhibited microbial growth and led to underestimates in N availability. In some cases, this might invalidate our conclusions, whereas in others we could still correctly differentiate among samples.

The importance of the ruminal N availabilities for these samples is, in part, dependent upon the concentration of protein that they contain. For example, it is most likely of little importance that the nitrogen in wood molasses was unavailable because the product contained less than 1% crude protein. In contrast, the CSB and steep both contained a fair amount of crude protein and, thus, the differences in availability between these two products would be of practical importance.

Table 1. Ruminally available nitrogen in liquid feed products containing casein or urea as the primary nitrogen source and manufactured from different base ingredients

Base ingredient ¹	Ruminally available nitrogen
	--- % ---
Cane molasses	73
Concentrated separator byproduct	47
Distiller's solubles	67
Steep liquor	72
55% sucrose	81
33% sucrose, 11% glucose, 11% fructose	87
30% hydrolyzed starch, 4.5% lactate	69
5% soluble starch	100
SEM	6.4

¹Products contained 2% added NaCl and represent averages of unheated products and products heated in a boiling water bath for 15 minutes.

Table 2. Effects of heating and nitrogen source on ruminally available nitrogen in liquid feeds¹

Nitrogen source	Unheated	Heated ²
	--- % available nitrogen ---	
Casein	106	45
Urea	80	67
SEM	4.5	

¹Values represent averages across products manufactured with a range of base ingredients.

²Products were heated in a boiling water bath for 15 minutes.

Table 3. Effects of heating and base ingredient on ruminally available nitrogen in liquid feeds containing casein or urea as the primary nitrogen source

Base ingredient ¹	Unheated	Heated ²
	--- % available nitrogen ---	
Cane molasses	87	59
Concentrated separator byproduct	69	26
Distiller's solubles	81	52
Steep liquor	91	53
55% sucrose	103	59
33% sucrose, 11% glucose, 11% fructose	105	70
30% hydrolyzed starch, 4.5% lactate	86	53
5% soluble starch	123	76
SEM	9.0	

¹ Products contained 2% added NaCl and represent averages of products containing casein or urea as the primary nitrogen source.

² Products were heated in a boiling water bath for 15 minutes.

Table 4. Effect of mineral additions and nitrogen source on ruminal nitrogen availability in liquid feeds¹

Mineral addition	Nitrogen source	
	Casein	Urea
	--- % available nitrogen ---	
None	110	124
2% NaCl	106	80
4% H ₃ PO ₄	61	55
SEM	4.8	

¹ Values represent averages across products manufactured with a range of base ingredients.

Table 5. Effect of mineral additions and base ingredient on ruminal nitrogen availability in liquid feeds¹

Base ingredient	Mineral addition		
	None	2% NaCl	4% H ₃ PO ₄
	--- % available nitrogen ---		
Cane molasses	89	87	49
Concentrated separator byproduct	84	69	70
Distiller's solubles	115	81	60
Steep liquor	134	91	52
55% sucrose	131	103	64
33% sucrose, 11% glucose, 11% fructose	124	105	51
30% hydrolyzed starch, 4.5% lactate	126	86	57
5% soluble starch	136	123	62
SEM	9.6		

¹Values represent averages across products containing casein or urea as the primary nitrogen source.

Table 6. Ruminally available nitrogen in liquid feed products stored at room temperature for 0, 2, or 8 weeks

Storage time	Ruminally available nitrogen
	--- % ---
None	99
2 weeks	91
8 weeks	104
SEM	1.6

¹Products contained 4% added phosphoric acid and represented products manufactured with a range of base ingredients.

Table 7. Effect of storage on ruminal N availability of various products

Base ingredient	Casein-based products			Urea-based products		
	no storage	2 weeks storage	8 weeks storage	no storage	2 weeks storage	8 weeks storage
	--- % available nitrogen ---					
Cane molasses	75	79	86	102	89	98
CSB	85	61	79	76	89	94
Distiller's solubles	75	73	92	81	98	107
Steep liquor	74	89	89	106	86	101
55% sucrose	117	120	135	116	118	122
Sucrose/glucose/fructose	131	112	123	110	117	94
Hydrolyzed starch	130	126	122	118	36	83
5% soluble starch	111	111	118	70	51	122

SEM = 6.4. CSB = Concentrated separator byproduct; Sucrose/glucose/fructose = 33% sucrose, 11% glucose, 11% fructose; Hydrolyzed starch product contained 4.5% lactic acid.

Table 8. Crude protein content of ingredients

Ingredient	n	Crude protein	
		Average	Range
		--- %, as is basis ---	
Cane molasses	7	5.5	4.0 to 6.8
Beet molasses	2	12.3	10.9 to 13.6
Concentrated separator byproduct	5	12.3	10.1 to 16.1
Steep	4	18.5	16.8 to 19.2
Condensed corn distillers solubles	3	9.5	6.2 to 14.1
Whey	4	3.2	0.9 to 5.1
Soy solubles	1	8.8	
Wood molasses	1	0.8	

Table 9. Ruminal nitrogen availability content of ingredients

Ingredient	n	Crude protein availability	
		Average	Range
		--- % available nitrogen ---	
Cane molasses	7	1	-33 to 31
Beet molasses	2	-1	0 to 2
Concentrated separator byproduct	5	-10	-34 to 11
Steep	4	38	19 to 56
Condensed corn distillers solubles	3	24	17 to 29
Whey	4	37	23 to 49
Soy solubles	1	11	
Wood molasses	1	-30	

Figure 1. The relationship between crude protein content and ruminal nitrogen availability in sources of cane molasses.

