Technical Note: Effect of Removal of Microbial Cells by Centrifugation on Peptide and α -Amino Nitrogen Concentrations in Ruminal Fluid¹

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ABSTRACT

We evaluated the effect of centrifuging rumen fluid prior to analysis on concentrations of α -amino N (AAN) and peptides. Rumen fluid was collected from steers fed grain-based diets at either various times after feeding or after dosing the rumens with solubilized casein. Fluid was either directly processed for peptide analysis by acidifying 10 ml of rumen fluid with 0.5 ml of 70% (wt/wt) perchloric acid, or first centrifuged at $500 \times g$ for 20 min to remove protozoa and then at $30,000 \times g$ for 15 min to remove bacterial cells prior to further processing. By removing microbial cells, intracellular AAN and peptides were not included in subsequent analyses. Concentrations of AAN were determined using an automated trinitrobenzene sulfonic acid assay, and peptides were determined as the increase in AAN following acid hydrolysis of the samples. When casein was not dosed, removal of microbial cells prior to analysis decreased concentrations of both AAN and peptides, and the decrease was greater for AAN (2.2 mM) than for peptides (1.2 mM). Dosing with casein led to much higher concentrations of ruminal peptides and AAN. After casein dosing, decreases in AAN and peptide concentrations due to prior centrifugation (2.1 mM and 1.0 mM for AAN and peptides, respectively) were similar to the decreases observed before the casein dosing. Results suggest that the contribution of intracellular AAN and peptides to the concentrations in ruminal fluid are relatively constant across broad ranges of dietary protein supply for cattle fed corn-based diets.

(**Key words:** amino acid, peptide, rumen fluid, centrifugation)

Abbreviation key: AAN = α -amino nitrogen.

INTRODUCTION

Peptides are an important intermediate in ruminal protein metabolism, but despite their importance, they are not routinely measured in nutrition experiments. Moreover, peptides can be measured using various techniques (Wallace et al., 1990), so there is not a robust database available for assessing typical ruminal concentrations. One issue relevant to the measurement of peptides and α -amino N (AAN) is the contribution of microbial cells. Intracellular concentrations of AAN and peptides would be expected to cause a larger percentage change in measured amounts when overall concentrations are low, but this has not been studied. Some researchers have prepared ruminal samples for peptide and AAN analysis by first removing microbes with a centrifugation step (Chen et al., 1987), but this step also has been omitted (Wessels et al., 1996). This paper describes the impact of microbial cell removal by centrifugation on ruminal fluid peptide and AAN concentrations over a broad range of peptide and AAN concentrations.

For our study, five steers (345 kg) were maintained with ad libitum access to five dietary treatments. One diet contained 72.1% dry-rolled corn, 12.0% soybean meal, and 12% alfalfa (dietary CP = 14.2%), and this diet was supplemented with no antibiotic, with 175 mg/d virginiamycin, or with a combination of 250 mg/d monensin and 100 mg/d tylosin. The other diet contained 62.9% dry-rolled corn, 30.0% wet corn gluten feed, and 5.0% alfalfa (dietary CP = 14.4%), and this diet was supplemented with either no antibiotic or with 175 mg/d virginiamycin. DMI averaged 7.1 kg/d.

The samples were collected only during the first period of a Latin square experiment (Ives et al., 2002), so there was no replication over the diets or the steers. On 2 consecutive d, samples were collected from each steer before feeding and then 2, 4, 6, 8, and 10 h after feeding. On the following day, steers were fed then dosed intraruminally 2 h later with 350 g of solubilized CN to increase substrate concentrations such that the capacity of the ruminal microbes for proteolysis and deamination could be measured. Samples were then collected 1, 2, 3, 4, and 6 h after the CN dosing.

Received April 3, 2002.

Accepted May 24, 2002.

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¹Contribution no. 02-394-J, Kansas Agric. Exp. Sta., Manhattan.

Ruminal samples were processed with two methods prior to analysis of AAN and peptides. One portion of the sample (10 ml) was immediately mixed with 0.5 ml of 70% (wt/wt) HClO_4 and then frozen until later analysis. Another portion of the sample (30 ml) was first centrifuged at $500 \times g$ for 20 min (4°C) to sediment feed particles and protozoa, and the supernatant was then centrifuged at $30,000 \times g$ for 15 min (4°C) to remove bacterial cells. The cell-free supernatant (10 ml) was then mixed with 0.5 ml of 70% (wt/wt) HClO_4 and frozen until later analysis. Samples prepared with each of these methods were then similarly handled and analyzed for AAN and peptides. The precipitation of proteins with HClO_4 is similar to that described by Chen et al. (1987).

Thawed ruminal samples were prepared for analysis by centrifuging at $30,000 \times g$ for 15 min at 4°C to remove precipitated proteins and collecting the supernatant. Samples were analyzed for AAN by use of an automated trinitrobenzene sulfonic acid assay (Palmer and Peters, 1969, as described by Wessels et al., 1996), and peptides were determined as the increase in AAN following acid hydrolysis (samples were mixed with equal volumes of 12 N HCl and hydrolyzed under N_2 at 105°C for 24

h). The trinitrobenzene sulfonic acid assay measures primary amines, so the estimates of AAN include the N present on the termini of peptides, whereas the estimates of peptide N would not include the terminal amino groups on peptides. Thus, it is impossible to definitively determine the average size of the peptides in samples.

Data were analyzed using the mixed procedure of SAS (SAS System for Windows, Release 8.01, SAS Inst. Inc., Cary, NC) as a split-plot, with terms describing the sample collection (animal and hour) in the main-plot and with the method of analysis (with or without centrifugation) and its interactions with main-plot terms in the sub-plot. Separate analyses were conducted for samples collected after CN dosing.

Figures 1 and 2 show the concentrations of peptides and AAN on days without (Figure 1) and with (Figure 2) CN dosing. For comparison, ruminal ammonia concentrations, measured in samples deproteinized with meta-phosphoric acid, were 8.4, 17.5, 11.8, 6.5, 6.2, and 5.5 mM at 0, 2, 4, 6, 8, and 10 h after feeding and 17.7, 23.6, 26.4, 26.9, and 17.1 mM at 1, 2, 3, 4, and 6 h after CN dosing.

Figure 1. Concentrations of peptides and α -amino N in ruminal fluid of steers as influenced by time after feeding and method of sample preparation. Centrifuged samples were centrifuged at $500 \times g$ and $30,000 \times g$ to remove microbial cells prior to mixing with perchloric acid. Uncentrifuged ruminal fluid was mixed directly with perchloric acid. SEM = 0.68 for α -amino N and 0.36 for peptide N.

Figure 2. Concentrations of peptides and α -amino N in ruminal fluid of steers as influenced by time after ruminal dosing of CN and method of sample preparation. Centrifuged samples were centrifuged at $500 \times g$ and $30,000 \times g$ to remove microbial cells prior to mixing with perchloric acid. Uncentrifuged ruminal fluid was mixed directly with perchloric acid. SEM = 0.43 for α -amino N and 1.39 for peptide N.

For normally-fed animals (Figure 1), peptide and AAN concentrations peaked at 2 h after feeding, with the peak being greater for AAN than for peptides. For both peptides and AAN, the samples that had been centrifuged to remove microbial cells had lower concentrations, reflecting the removal of the intracellular pools of those compounds. The average decrease in AAN due to microbial cell removal was 2.2 mM, whereas the average decrease in peptide concentration due to microbial cell removal was 1.2 mM. The greater decrease in AAN than peptides is not surprising, as cells would be expected to have greater concentrations of AAN.

As expected, concentrations of peptides and AAN were increased by the ruminal CN dosing (Fig. 2). Although samples were not collected directly prior to the CN dosing, concentrations of peptides and AAN at dosing would be expected to be similar to those observed at 2 h after feeding on the previous days. Concentrations of both peptides and AAN decreased from 1 h after dosing until the final collection, demonstrating further degradation as well as passage of these compounds. Averaged over all time points and both methods of sample preparation, peptide concentrations after CN dosing were 4.4 times those observed when CN was not dosed. Similarly, AAN was increased to a value three times as large.

For the samples collected after CN dosing, centrifugation to remove microbial cells decreased both peptide (1.0 mM) and AAN (2.1 mM) concentrations. These decreases were very similar to those observed when CN

was not dosed, suggesting that the contribution of intracellular AAN and peptides to the concentrations in ruminal fluid are relatively constant across a broad range of dietary CP supply for cattle fed corn-based diets. Thus, although absolute concentrations of both peptides and AAN were impacted by removal of microbial cells prior to analysis, the use of this step may not be critical for evaluating treatment differences. However, removal of microbial cells changed the concentrations of peptide and AAN, suggesting that removal of microbial cells is important if the size of the extracellular pools of these compounds is of primary interest.

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