

Effect of Inhibitor Concentration and End-Product Accumulation on Estimates of Ruminal *In Vitro* Protein Degradation*

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ABSTRACT

Effects of varying the concentrations of hydrazine sulfate (HS) and chloramphenicol (CAP), inhibitors of microbial-N uptake and protein synthesis, on rates of protein degradation estimated from net appearance of NH₃ and total amino acids (TAA) were studied in a ruminal *in vitro* fermentation system. Without inhibitors, recoveries of N added as NH₃ and TAA were 4 and 6% after 4-h incubations, and apparent degradation rates estimated from release of NH₃ and TAA for casein, solvent soybean meal (SSBM), and expeller soybean meal (ESBM) approached 0. Increasing inhibitor concentrations from the standard amounts of 1 mM HS plus 30 mg of CAP/L to 2 mM HS plus 90 mg of CAP/L gave rise to numerically greater N recoveries and degradation rates, but these differences were not statistically significant. Compared with the standard inhibitor concentrations, use of 2 mM HS, without CAP, yielded similar recoveries and rates, but 30 or 90 mg of CAP/L, without HS, was not satisfactory. Versus that with 1 mM HS plus 30 mg of CAP/L, media containing 2 mM HS plus 90 mg of CAP/L gave increased TAA recoveries and higher rates for casein, but not SSBM, in the presence of added starch. Faster degradation rates were obtained for casein, but slower rates for SSBM and ESBM, in Sweden versus Wisconsin using inocula from cows fed different diets but with similar CP and energy contents. Differences in microbial catabolism of peptides may account for differences in degradation rates observed between Sweden and Wisconsin. Adding NH₃ plus free and peptide-bound amino acids

to the inoculum reduced apparent degradation rates, possibly via end-product inhibition. Analysis of data from multiple time-point incubations indicated that casein degradation followed simple, first-order kinetics, while a biexponential model fitted degradation patterns for both SSBM and ESBM.

(Key words): protein degradation, rumen, inhibitor *in vitro*)

Abbreviation key: CAP = chloramphenicol, ESBM = expeller soybean meal, HS = hydrazine sulfate, IIV = inhibitor *in vitro*, SSBM = solvent soybean meal, TAA = total AA.

INTRODUCTION

In the inhibitor *in vitro* (IIV) method, rates of protein degradation are quantified from the time-course of N appearance as NH₃ and total free AA (TAA) in the presence of hydrazine sulfate (HS) and chloramphenicol (CAP; Broderick, 1987a). Adding 1 mM HS and 30 mg of CAP/L to the medium did not alter proteolytic activity of mixed ruminal microorganisms (Broderick, 1987b) and yielded quantitative recovery of NH₃ and TAA. However, protein degradation rates obtained with the IIV method for the tannin-containing forages sainfoin and lespedeza were very slow or approximated to zero (Broderick and Albrecht, 1997). Moreover, rates of about 0.01/h were determined by IIV for blood meal and corn gluten meal, while rates estimated *in vivo* for the same proteins were 0.05 and 0.10/h (Reynal and Broderick, 2003). It is possible that some uptake of degradation end-products may still occur in the IIV system; this “leakage” would be most problematic for estimating rates of slowly degraded proteins. More than 50% of the total CP in alfalfa silage was present as NPN (Broderick, 1995); degradation rates obtained by IIV for the residual (insoluble) protein in these silages were slower than those obtained using uninhibited ruminal inoculum in which ¹⁵N was used to account for microbial uptake of degraded-N (Peltekova and Broder-

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ick, 1996). This suggested that rates may have been underestimated with the IIV method due to the presence of NPN compounds of alfalfa silage. The addition of HS and CAP results in accumulation of NH_3 , free AA, and small peptides, which may also contribute to end-product inhibition in the IIV system. Veresegyhizy et al. (1993) reported that adding more than 19 mM NH_3 or 12 mM total free AA inhibited the rate of casein degradation by ruminal microbes *in vitro*.

The objectives of these studies on the IIV system were to: 1) reevaluate effects of inhibitor concentrations on NH_3 and TAA recovery and observed degradation rates; 2) assess the importance of accumulation of NH_3 , free AA, and small peptides as end-product inhibitors; and 3) evaluate use of mono- and biexponential kinetic models for quantifying protein degradation.

MATERIALS AND METHODS

Locations and Donor Animals

Experiments were conducted at Uppsala, Sweden, and Madison, Wisconsin. Inocula were obtained from donor cows (Swedish Red or Holstein Friesian) surgically fitted with 10-cm ruminal cannulas (Bar Diamond, Parma, ID); surgical care and general maintenance of the animals followed IACUC requirements at both locations. Donor cows were fed one of 2 different lactation diets based on grass silage plus concentrate (a mixture of barley, oats, sugarbeet pulp, rapeseed meal, soybean meal, and vitamins and minerals) in Sweden or on alfalfa silage and corn silage plus concentrate (a mixture of rolled high moisture corn, soybean meal, and vitamins and minerals) in Wisconsin. Diets averaged (DM basis) 17% CP and 1.60 Mcal/kg of NE_L in Sweden and 16.5% CP and 1.55 Mcal/kg of NE_L in Wisconsin (NE_L computed at 3 \times maintenance; NRC, 2001). Donor cows were fed *ad libitum* but intake was not measured.

Protein Sources

The *in vitro* studies reported here were conducted with 3 protein sources: casein (no. C-5890, Sigma Chemicals, St. Louis, MO), one sample of solvent-extracted soybean meal (SSBM), and one sample of expeller-extracted soybean meal (ESBM; West Central Coop., Ralston, IA). The designation ESBM.1 was used in this paper to distinguish this sample from a second expeller soybean meal that was used in our other work. Protein sources were ground with a cyclone mill (Udy Corp., Fort Collins, CO) fitted with a 1-mm screen. More complete composition data are reported on these proteins in the companion paper (Broderick et al., 2004).

Standard *In Vitro* Protocol

In studies conducted in both Sweden and Wisconsin, inocula were prepared using only preincubated strained ruminal fluid. Whole ruminal contents were collected from beneath the fibrous mat in the rumen and filtered at the barn through 2 layers of cheesecloth into a warmed thermos bottle that had been flushed with CO_2 . This filtrate was transported to the laboratory and then filtered again through 4 layers of cheesecloth. In Wisconsin, four 10-mL aliquots were centrifuged (10,000 \times g, 30 min, 4°C); pellets were dried (48 h in 60°C forced draft oven) and analyzed later for total N. Duplicate aliquots were taken in Sweden. Because of the proximity of animal facilities at both locations, <10 min elapsed from initial collection of whole ruminal contents until the preincubations were begun under CO_2 . Per liter of strained ruminal fluid, 8 g of maltose (Sigma no. M-2250), 4 g of xylose (Sigma no. X-1500), 4 g of soluble starch (Sigma no. S-2004), and 2.5 g of NaHCO_3 were weighed into an Erlenmeyer flask and suspended with slow stirring in 100 mL of McDougall's buffer (McDougall, 1948) that had been freshly gassed with CO_2 . Four grams of citrus pectin (Sigma no. P-9135) was dispersed in 100 mL of water with heating and stirring and then added to the flask before flushing with CO_2 . Filtered strained ruminal fluid (1000 mL) and 0.2 mL of surfactant (Antifoam 204; Sigma no. A-6426) were added and the inoculum preincubated for 3 or 4-h using slow stirring in a 39°C bath. The flask headspace was continually flushed with a CO_2 stream during preincubation to maintain anaerobiosis. At 0 h and every hour samples were taken for analysis of NH_3 , and TAA and pH was measured; if pH was <6.2, pH was adjusted to 6.4 by slow addition of 3 N NaOH. Two solutions for inhibiting NH_3 and TAA uptake were prepared by dissolving 72 mM of HS (Sigma no. H-7394) and 2250 mg/L of CAP (Sigma no. C-0378) in 39°C McDougall's buffer. Twenty-five milliliters of each, plus 0.293 g of mercaptoethanol (Sigma no. M-6250), were added to give an inoculum with 1.5 mM HS, 45 mg of CAP/L, and 3 mM mercaptoethanol. Concentrations of HS and CAP were varied in some experiments. This inoculum was held at 39°C under a CO_2 stream until dispensed at the start of the incubation (typically about 20 min).

Incubations were conducted in either 50-mL polypropylene centrifuge tubes (Wisconsin) or 50-mL round-bottom glass tubes (Sweden). In Wisconsin experiments, protein substrate equal to 1.9 to 2.0 mg of N (equivalent to 14.0 to 70 mg of the protein sources studied, weighed to the nearest 0.1 mg) was added to each tube. Then 5 mL of warm (39°C) McDougall's buffer was added to each tube to wet the proteins, tube headspace flushed with CO_2 , and tubes were stoppered, and held

at 39°C for about 1 h prior to inoculation. Incubations were begun by dispensing 10 mL of inoculum/tube using either a repipet or a Cornwall repeating syringe. Two times these amounts were used in incubations conducted in Sweden, and inoculum was dispensed using a Cornwall syringe, but otherwise the same protocols were used. Four blank tubes containing all ingredients except protein substrate were used for each time point in each incubation. Final concentrations in the standard medium were 1.0 mM HS, 30 mg of CAP/L, 2 mM mercaptoethanol (to maintain reducing conditions), and 0.13 mg/mL of protein-N from each source; however, HS and CAP concentrations were an experimental variable in some trials. Immediately after inoculating, tube headspace was flushed with CO₂, tubes were capped with Bunsen valves, swirled, and incubated at 39°C in a warm room (Wisconsin) or a water bath (Sweden). Mixing was accomplished using a wrist-arm shaker at 150 cycles/min. (Wisconsin) or by hand swirling at 1-h intervals (Sweden). A preliminary study in Sweden showed that mixing tube contents every hour yielded the same degradation rates as continuous shaking. Incubations were run from 0 to 4 h with hourly sampling in most studies. In Sweden, incubations were killed by transferring 1-mL aliquots of medium to 1.5-mL centrifuge tubes containing 0.1 mL of 55%, wt/vol, TCA (final sample concentration of 5%, wt/vol). Killed samples were immediately capped and mixed by inversion, then placed on ice for 30 min before centrifuging (10 min at 17,700 × g). In Wisconsin, incubations were killed by adding 1.25 mL of 65%, wt/vol, TCA to the entire 15 mL of medium in each tube. Tube contents were mixed immediately by swirling, placed on ice for 30 min, and then about 4 mL of sample was transferred to 12- × 75-mm plastic tubes and centrifuged (15 min at 15,300 × g). Supernatants were either held at 5°C until analyzed for degraded-N later the same day or stored at -18°C for later analysis. All incubation experiments were replicated twice.

Computation of Rate and Extent of Degradation

Rate and extent of protein degradation were computed from net release of NH₃ and TAA as described in the companion paper (Broderick et al., 2004). Reiterating, fractions degraded (FD) and fractions undegraded (FUD) at each time-point were computed:

$$\begin{aligned} \text{FD} &= \text{degraded-N/protein-N} \\ \text{FUD} &= 1 - \text{FD}, \end{aligned}$$

where degraded-N was the net release of N in mg/tube detected as NH₃ and TAA and protein-N was the total N in mg/tube added from each protein source. Degraded-N

released as NH₃ and TAA in TCA supernatants was computed using the equation:

$$\begin{aligned} \text{degraded-N, mg} &= \{ ([\text{NH}_3]_{\text{prot}} - [\text{NH}_3]_{\text{blank}}) \\ &\times 0.0140067 + ([\text{TAA}]_{\text{prot}} - [\text{TAA}]_{\text{blank}})/(\text{TAA/N}) \} \times \text{Vol}, \end{aligned}$$

where [NH₃]_{prot} and [NH₃]_{blank} and [TAA]_{prot} and [TAA]_{blank} were NH₃ and TAA concentrations in mM (μmol/mL) in, respectively, protein-containing and blank tubes, 0.0140067 was the NH₃-N constant (mg N/μmol NH₃), TAA/N was 50 μmol/mg N, which was the mean ratio of TAA (after HCl hydrolysis) to total N in the proteins studied, and Vol was the total tube volume in milliliters. The TAA released by HCl-hydrolysis were assumed to represent all of the degradable N in each protein.

Rates of ruminal protein degradation (k_d) were estimated in most experiments by fitting FUD data to a single exponential model: 1) by nonlinear regression of FUD on time at each time-point using the nonlinear procedure of SAS (1999-2000); or 2) using ln FUD only at times = 0 h (FUD₀) and 4 h (FUD₄) from the equation:

$$\text{degradation rate (k}_d\text{), /h} = [\ln(\text{FUD}_4) - \ln(\text{FUD}_0)]/4.$$

The potentially degradable fraction of total CP (fraction B) was computed using the equation:

$$\text{fraction B, \%} = (\text{FUD}_0) \times 100.$$

As discussed earlier (Broderick et al., 2004), FUD₀ was not corrected for undegradable-N. Extents of ruminal protein escape were computed using the equation (Waldo et al., 1972):

$$\text{ruminal protein escape, \%} = \text{B} \times [k_p/(k_d + k_p)],$$

where k_p, the ruminal passage rate, was set equal to 0.06/h. In one study, the nonlinear procedure of SAS (1999-2000) was used to fit FUD data from SSBM and ESBM.1 to the biexponential model:

$$\begin{aligned} \text{ruminal protein escape, \%} &= \\ &\text{B}_1 \times [k_p/(k_1 + k_p)] + \text{B}_2 \times [k_p/(k_2 + k_p)], \end{aligned}$$

where B₁ and B₂ were the protein fractions degraded, respectively, at rates k₁ and k₂, and passage rate, k_p, was set equal to 0.06/h for both fractions. Fractions B₁ and B₂ were set to sum to FUD₀.

Chemical Analyses

Proteins were analyzed for total N, DM, acid detergent insoluble-N, and acid-hydrolyzed to TAA as de-

scribed earlier (Broderick et al., 2004). Protein hydrolysates and TCA-supernatants from in vitro incubations in all Swedish studies were analyzed for NH_3 and TAA using phenol-hypochlorite and ninhydrin assays adapted to continuous-flow analysis as described earlier (Broderick and Kang, 1980; Broderick, 1987a) except with an analysis rate of 60/h. In Wisconsin, NH_3 was determined by phenol-hypochlorite assay using flow-injection analysis (Lachat method 18-107-06-1-A; Lachat Quick-Chem 8000 FIA; Zellweger Analytical, Milwaukee, WI) and TAA were determined using a fluorimetric procedure based on o-phthalaldehyde (Roth, 1971) that also was adapted to flow-injection analysis. Other details on sample analyses, including the rationale for using the mean value of 50 μmol of TAA/mg total N to compute FD and mean composition data on the proteins studied are in the companion paper (Broderick et al., 2004).

Experimental Studies

Effect of inhibitor concentration. Two trials were conducted in Sweden to study varying the concentrations of the inhibitors HS and CAP in ruminal inocula on estimated protein degradation rates. In the first experiment, solutions containing 36 mM HS and 1080 mg of CAP/L were dissolved in McDougall's buffer. To nine 500-mL Erlenmeyer flasks, the following respective volumes (mL) of McDougall's buffer, 36 mM HS, and 1080 mg of CAP/L were added: A) 50, 0, and 0; B) 40, 10, and 0; C) 30, 20, and 0; D) 40, 0, and 10; E) 20, 0, and 30; F) 30, 10, and 10; G) 20, 20, and 10; H) 10, 10, and 30; and I) 0, 20, and 30. A magnetic stir bar was placed in each flask, and flasks were flushed with CO_2 , stoppered, and warmed to 39°C in a water bath. After completing 3 h of preincubation using the standard protocol, the inoculum was made 3 mM mercaptoethanol, and 190 mL was added to each flask (total 240 mL). Flasks were swirled, flushed again with CO_2 , stoppered, and replaced in the water bath. After 10 min had elapsed, 20 mL of each inocula was dispensed while stirring to duplicate tubes prepared (using the standard Swedish protocol) to contain: no additive (blanks), 4.0 mg of substrate N added as casein, SSBM, or ESBM.1, or 66.7 μmol of NH_3 or 66.7 μmol of TAA (added as acid-hydrolyzed casein; Sigma no. C-9386) in 10 mL of McDougall's buffer (previously warmed to 39°C). As inocula were dispensed, 0-h samples were withdrawn and killed with TCA, then tubes were flushed with CO_2 , capped with Bunsen valves and incubated at 39°C. The sequence of inoculum addition was A) through I); an average of 2.5 min was required to inoculate each set of tubes (total elapsed time 20 min). In this experiment, tubes were sampled again and killed after 4 h only.

Analysis of TCA supernatants for NH_3 and TAA was done using continuous-flow analysis, and degradation parameters were computed using the 2-point equation; NH_3 and TAA recoveries were computed based on the concentrations present at 0 h for each of the 9 inocula.

A second trial compared the effect of energy added to inocula F (containing the standard inhibitor concentrations of 1.5 mM HS and 45 mg of CAP/L) versus inocula I (containing inhibitor concentrations of 3 mM HS and 135 mg of CAP/L). The 2 inocula were prepared as in the 9-inocula experiment, except 720 mL was made of each. Twenty tubes were prepared for both inocula: duplicate blanks, 10 with 4.0 mg of substrate N added as casein, and 8 with 4.0 mg of substrate N added as SSBM. Soluble starch was added to duplicate casein tubes at 0, 34, 86, 215, and 430 mg/tube; soluble starch was added to duplicate SSBM tubes at 0, 65, 172, and 344 mg/tube. McDougall's buffer (10 mL/tube) was added and tubes were warmed to 39°C. Inoculation of tubes, swirling, sampling at 0 and 4 h, analyses and computations of degradation parameters were done as in the 9-inocula experiment.

End-product concentration. Two trials were conducted in Sweden to assess whether the end-products from protein degradation inhibit observed rates. In the first experiment, 1.44 L of inoculum I containing 3 mM HS, 135 mg of CAP/L, and 3 mM mercaptoethanol, and 1.44 L of McDougall's buffer containing 2 mM HS, 90 mg of CAP/L, and 2 mM mercaptoethanol, were prepared as described and held at 39°C under CO_2 . Ten glass tubes and ten 100-mL Erlenmeyer flasks were prepared in duplicate: blank, 4.0 mg of substrate N added as casein, SSBM, or ESBM.1, or 66.7 μmol of NH_3 or 66.7 μmol of TAA (added as acid-hydrolyzed casein). McDougall's buffer (10 mL/vessel) was added to tubes and flasks, and they were then flushed with CO_2 , stoppered, and replaced in the water bath. Just before inoculation, 30 mL of the McDougall's buffer containing the HS, CAP, and mercaptoethanol was added to each flask. Tubes and flasks were then inoculated with 20 mL of inoculum, swirled, sampled for 0-h analyses, then flushed with CO_2 , stoppered with Bunsen valves, and replaced in the 39°C water bath. All vessels contained 2 mM HS, 90 mg of CAP/L and 2 mM mercaptoethanol, and the typical amounts of protein and inoculum. However, flasks were diluted to twice the typical volume. Vessels were swirled and sampled every hour through the 4-h incubation. Analyses and computations of degradation parameters were done using the standard protocol described above.

In the second experiment, preincubation for 4-h was used in preparation of 1.44 L of inoculum I (containing 3 mM HS, 135 mg of CAP/L, and 3 mM mercaptoethanol) that was then held for 30 min at 39°C under CO_2 .

Sixteen tubes were prepared in quadruplicate with the standard Swedish protocol: blank or plus 4.0 mg of substrate N added as casein, SSBM, or ESBM.1. McDougall's buffer (10 mL/tube) was added, and tubes were stoppered and placed in the 39°C water bath. Just before starting the incubation, the inoculum was split: half was control and to the other half was added 5.0 mM NH₃ as (NH₄)₂SO₄, 333 mg/L of acid-hydrolyzed casein, and 83 mg/L of enzymatically hydrolyzed casein (Sigma no. C-1026). Tubes were then inoculated with 20 mL of either control inoculum or inoculum with added N, then swirled, sampled at 0 h, flushed with CO₂, stoppered with Bunsen valves, and replaced in the 39°C water bath. Tubes were swirled every hour, sampled again at 4 h only and then analyzed, and degradation parameters were computed using the 2-point protocol described above.

Time-course of protein degradation. A study conducted in Sweden examined the effect of background concentration of N end-products in conjunction with more frequent sampling over a 6- rather than 4-h incubation to estimate degradation parameters. The same methods used in the second end-product inhibition experiment were employed to prepare 2.40 L of inoculum I (containing 3 mM HS, 135 mg of CAP/L, and 3 mM mercaptoethanol) that was held for 30 min at 39°C under CO₂. Sixteen 250-mL Erlenmeyer flasks were prepared in quadruplicate: blank or 20.0 mg of substrate N added as casein, SSBM, or ESBM.1. McDougall's buffer (50 mL/flask) was added, and flasks were stoppered and placed in the 39°C water bath. Just before the incubation was started, the inoculum was split; half was control and to the other half was added 5.0 mM NH₃ as (NH₄)₂SO₄, 333 mg/L of acid-hydrolyzed casein, and 83 mg/L of enzymatically hydrolyzed casein. Flasks were inoculated with 100 mL of either control inoculum or inoculum with added N, then swirled, sampled for 0-h analyses, flushed with CO₂, stoppered with Bunsen valves, and replaced in the 39°C water bath. Flasks were swirled and sampled every 20 min throughout the 6-h incubation. Analyses and computation of FD and FUD from degraded-N at each time-point were conducted with the standard protocol. These data were used with nonlinear analysis of the mono-exponential model for all 3 proteins and with the biexponential model for SSBM and ESBM.1 as described above.

Statistical Analysis

Quadruplicate blank and duplicate substrate-containing tubes were assayed in duplicate for NH₃ and TAA at each time-point in each incubation. Means from these assays were used to compute net N release, and FD and FUD values, for each protein replicate tube

at each time-point by the methods described. Protein degradation parameters determined in this way were statistically analyzed at Wisconsin with the general linear model of SAS (1999-2000). Effect of inhibitor concentrations was assessed using a model including incubation run, replicate(run), and inoculum source (9 inhibitor concentrations). Recoveries of added N and degradation rates of individual proteins were analyzed separately. Effect of inhibitor concentration and starch addition was assessed using a model including incubation run, replicate(run), inoculum (F vs. I), starch level, and inoculum × starch interactions. Degradation rates of casein and SSBM were analyzed separately. Effect of location was assessed using a model including protein substrate, location (Wisconsin vs. Sweden), and protein × location interaction. Effect of end-product inhibition was assessed using a model including incubation run, replicate(run), protein substrate, medium (control or diluted inoculum; with or without added N), and protein × run and protein × medium interactions. Effect of method for estimating degradation parameters was assessed using a model including incubation run, replicate(run), protein substrate, method (nonlinear regression vs. two-point assay), and protein × run and protein × method interactions. Replicate(run) was used as the error term in testing for significance of treatments and 2-way interactions in all models except in the test of location effect. Interaction terms were removed from models when $P \geq 0.25$. Differences were declared significant at $\alpha < 0.05$. When the F -test for a treatment was significant ($P \leq 0.05$), mean separation was conducted by least significant difference at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Effect of Inhibitor Concentration

Inhibitor concentrations of 1 mM HS and 30 mg/L of CAP were identified in earlier studies as "optimal" based on maximal N recoveries from added NH₃ and TAA and on maximal degradation rates for both casein and SSBM (Broderick, 1987a). We were interested in confirming the effectiveness of these concentrations under Swedish conditions and, because of the toxic nature of hydrazine (Lenga, 1988), in determining whether elevated levels of CAP alone would be as effective as the mixture of these compounds. The effect of 9 different combinations of inhibitors on recovery of added N and rate of protein degradation was evaluated (Table 1). As observed previously, microbial uptake invalidated rates estimated from appearance of protein degradation products without inhibitors in the medium. Recoveries of NH₃ and TAA (added as acid-hydrolyzed casein) were very low, only 4 and 6%, and apparent degradation rates approached 0 for all three proteins, on control

Table 1. Effect of inhibitor concentrations on N recoveries and observed rates of protein degradation.¹

Inoculum	[HS], mM	[CAP], mg/L	Recovery, ² %		Degradation rate, /h		
			NH ₃ -N	CHA-N	Casein	SSBM	ESBM.1
A	0	0	4.2 ^c	6.2 ^d	0.032 ^e	0.010 ^c	-0.001 ^c
B	1	0	92.1 ^a	85.3 ^a	0.259 ^{bc}	0.096 ^a	0.022 ^b
C	2	0	100.3 ^a	95.6 ^a	0.286 ^{ab}	0.093 ^a	0.036 ^a
D	0	30	20.6 ^c	26.0 ^c	0.108 ^d	0.018 ^c	-0.002 ^c
E	0	90	52.7 ^b	55.7 ^b	0.232 ^c	0.044 ^{bc}	0.001 ^c
F	1	30	95.2 ^a	92.0 ^a	0.292 ^{ab}	0.100 ^a	0.027 ^{ab}
G	2	30	97.5 ^a	94.9 ^a	0.291 ^{ab}	0.087 ^{ab}	0.033 ^a
H	1	90	98.9 ^a	93.2 ^a	0.309 ^a	0.091 ^a	0.027 ^{ab}
I	2	90	100.4 ^a	95.7 ^a	0.318 ^a	0.121 ^a	0.033 ^a
SE			5.6	4.4	0.010	0.014	0.003
Probability ³							
Inoculum			<0.001	<0.001	<0.001	0.005	<0.001

^{a,b,c,d,e}LS means in the same column with differing superscripts were different ($P < 0.05$).

¹CAP = Chloramphenicol; CHA-N = N added as acid-hydrolyzed casein; HS = hydrazine sulfate; SSBM = solvent soybean meal; ESBM.1 = expeller soybean meal. In vitro incubations conducted in Sweden.

²Recovery of N as NH₃ + TAA, added to the media as NH₃ or CHA-N, after 4-h incubations.

³Probability of significant effects of inoculum source using replicate-within-incubation run as the error term.

inoculum A. Adding HS only, at 1 or 2 mM, yielded N recoveries that were not different from those with standard inoculum F (1 mM HS and 30 mg of CAP/L). Adding 30 or even 90 mg/L of CAP without HS gave lower recoveries and rates. More recent work in which up to 300 mg of CAP/L was added did not yield N recoveries or degradation rates that were equivalent to those with 1 mM HS plus 30 mg/L of CAP (data not shown). The 3 inocula with elevated HS and CAP (G, H, and I) were not different from standard inoculum F, indicating that there was no apparent advantage of increasing HS or CAP when the other inhibitor was also present. With this combination of compounds, it was clear that HS could not be deleted from the medium. Hydrazine functions as an inhibitor of a large number of microbial pyridoxine enzymes, including those involved in transamination and other amino transfer reactions (Sauberlich, 1968). Chloramphenicol is a classical antibiotic that inhibits protein synthesis (Gale and Folkes, 1953; Mahler and Cordes, 1966). The addition of HS inhibited incorporation of N from both NH₃ and exogenous free AA. However, the finding that casein rate was increased when 30 mg/L of CAP was also added with HS (Broderick, 1987a), versus 1 mM HS alone (our original inhibitor concentration; Broderick, 1978), suggested that some N originating from protein degradation was still used for protein synthesis. Peptides produced during extracellular proteolysis are transported into microbial cells (Broderick et al., 1988; Wallace et al., 1999). We speculated that peptides derived from extracellular proteolysis may have contributed to an intracellular free AA pool that was used directly for protein synthesis even in the presence of HS. This may explain why add-

ing CAP with HS was required for complete recovery of protein degradation products. Earlier work showed that methylhydrazine, phenylhydrazine, and *p*-nitrophenylhydrazine also were effective for inhibiting NH₃ and free AA uptake by mixed ruminal organisms (Broderick and Balthrop, 1979). However, these compounds have LD50 that indicate greater toxicity than HS (Lenga, 1988). Of course, this work did not preclude other, as yet unidentified inhibitors that may be as effective but less toxic.

Although not significantly different, NH₃ and TAA recoveries and casein and SSBM degradation rates were numerically greater using 2 mM HS plus 90 mg of CAP/L (inoculum I, Table 1). Based on this observation, a study compared degradation rates obtained with the standard 1/30 medium versus the 2/90 medium, with or without addition of soluble starch, that could have stimulated microbial-N uptake and underestimated rates. In this experiment, conducted in Sweden, there was a significant effect of starch addition, and overall degradation rates were slower for both casein and SSBM using the standard 1/30 versus 2/90 (media F and I; Table 2). Expressing starch addition as if the protein were present in a concentrate feed with increasing carbohydrate dilution indicated that there was no decrease in apparent degradation rate until casein and SSBM were diluted to, respectively, less than 3.5 and 1.8% N. The 2 proteins behaved differently in the 2 media: SSBM degradation rates were not different when starch addition diluted total N to 7.2 (no starch) and 3.3% N in medium F or 7.2, 3.3, and 1.8% N in medium I. For casein, all rates determined with medium I were more rapid than those with medium F.

Table 2. Effect of inhibitor concentration and soluble starch addition on degradation rates observed for casein and SSBM.¹

Inoculum	[HS], mM	[CAP], mg/L	Starch, mg/mL	N-dilution, ² % of DM	Casein rate, /h	Starch, mg/mL	N-dilution, ² % of DM	SSBM rate, /h
F	1	30			0.252			0.092
I	2	90			0.333			0.102
SE					0.004			0.002
	Across inocula		0.0	14.05	0.295 ^{ab}	0.0	7.17	0.101 ^a
			1.1	6.37	0.303 ^a	2.2	3.32	0.100 ^a
			2.9	3.47	0.301 ^a	5.7	1.77	0.097 ^{ab}
			7.2	1.64	0.283 ^{bc}	11.5	1.01	0.090 ^b
			14.3	0.86	0.279 ^c			
SE					0.005			0.003
Probability ³								
Inoculum					<0.001			0.030
Starch					0.001			0.001
Inoculum*Starch					0.586			0.371

^{a,b,c}LS mean degradation rates in the same column across inocula among the five starch additions with differing superscripts are different ($P < 0.05$).

¹CAP = Chloramphenicol; HS = hydrazine sulfate; SSBM = solvent soybean meal. In vitro incubations conducted in Sweden.

²Effective N concentration resulting from diluting protein source by addition of soluble starch to the medium.

³Probability of significant effects of inoculum source, starch addition, and their two-way interaction, using replicate-within-incubation run as the error term.

These results were taken to mean that, at least under Swedish conditions, the 2/90 medium would give rise to more reliable estimates of degradation rate for casein (and perhaps other rapidly degraded proteins) because microbial uptake of degradation end-products was prevented.

Effect of Location

Differences in rates by location were found when results from these same samples of protein, determined at both locations using inocula from lactating cows (i.e., at high feed intakes), were compared (Table 3): casein degradation rates were more rapid, and rates for the 2 soybean meals slower, in Sweden versus Wisconsin. Although overall mean rates for all 3 proteins were not different ($P = 0.49$), there was a clear protein \times location interaction. We speculated that, despite apparently

similar relative intakes of NE_L and CP, different microbial populations and degradative enzyme activities were found in the rumens at these locations. There are substantial differences among ruminal microbes in their ability to catabolize small oligopeptides and inocula rich in *Prevotella ruminicola* and other members of the genus *Prevotella*, which are active peptide degraders, would be expected to clear peptides more rapidly (Wallace et al., 1999). As reported in our companion paper (Broderick et al., 2004), Wisconsin work showed that adding N in peptides to that released as NH₃ and TAA substantially increased estimated degradation rates for casein but not SSBM or ESBM. Based on these findings, it appeared that peptide hydrolysis activity may have been relatively low in ruminal inocula used in Wisconsin studies. Other Wisconsin work indicated that fluid-phase microbes were most effective in degrading SSBM and that both SSBM and ESBM were less

Table 3. Effect of location of IIV incubation on protein degradation rates observed for casein, solvent soybean meal (SSBM), and expeller soybean meal (ESBM.1).

Protein	Location (n) ¹	LS Mean/h	SE	$P > F^2$
Casein	Sweden (36)	0.258	0.008	<0.001
	Wisconsin (102)	0.207	0.005	
SSBM	Sweden (32)	0.067	0.008	<0.001
	Wisconsin (92)	0.119	0.005	
ESBM.1	Sweden (18)	0.017	0.011	0.355
	Wisconsin (73)	0.029	0.006	
Location (all 3 proteins)				0.492
Location \times Protein				<0.001

¹Number of IIV incubations conducted at each location used in computing LS mean degradation rate.

²Probability of significant effect of location and location-by-protein interaction using replicate-within-incubation run as the error term.

Table 4. Effects of medium dilution or end-product addition on initial NH₃ and total AA (TAA) concentrations and on observed rates of protein degradation.¹

Item	Medium dilution ²		SE	P > F ³
	1X	2X		
NH ₃ , mM	2.48	1.50	0.24	<0.051
TAA, mM	2.46	1.35	0.15	<0.013
Casein rate, /h	0.238	0.232	0.023	0.857
SSBM rate, /h	0.055	0.057	0.002	0.699
ESBM.1 rate, /h	0.017	0.021	0.001	0.106
Overall mean rate, /h	0.104	0.103	0.008	0.970
Item	End-product addition ⁴		SE	P > F ²
	0	+		
NH ₃ , mM	1.05	5.09	0.01	<0.001
TAA, mM	1.38	2.01	0.01	<0.001
Casein rate, /h	0.327	0.283	0.013	<0.001
SSBM rate, /h	0.059	0.046	0.006	0.013
ESBM.1 rate, /h	0.018	0.020	0.002	0.641
Overall mean rate, /h	0.135	0.117	0.002	<0.001

¹In vitro incubations conducted in Sweden.

²Extent of dilution of incubation medium; 1X = undiluted and 2X = dilution of medium to twice its original volume by addition of McDougall's buffer.

³Probability of significant effects of medium dilution (top panel) or addition of degradation end-products (bottom panel) on initial NH₃ and of TAA concentrations and observed degradation rates.

⁴Nitrogen added to "+" vessels as NH₄SO₄ plus acid- and enzymatically-hydrolyzed casein to increase background concentrations of protein breakdown products.

rapidly degraded using inocula obtained after feeding (Broderick et al., 2004). We speculated that introduction of small particulates into the rumen with feeding may have reduced numbers of fluid-phase organisms and that, perhaps because of lower initial populations, proteolytic activity was reduced in Swedish inocula such that apparent degradation rates for the 2 soybean meals were depressed. Although there was substantial variation among individual experiments, increased degradation rates after feeding were observed consistently for all 3 proteins in Wisconsin. None of these speculations have been tested experimentally.

Effect of Degradation End-Product Concentration

Earlier, we observed that preincubating inocula with added peptides, but not with added NH₃, increased background NH₃ and TAA concentrations in the medium and was associated with reduced degradation rates (Broderick et al., 2004). Thus, under conditions in which NH₃ and/or TAA accumulated or where background concentrations were elevated, end-product inhibition may have reduced estimates of protein degradation in the IIV system. The magnitude of this possible effect was assessed in 2 experiments. In the first, the standard incubation medium was compared to the same medium diluted to twice the original volume using McDougall's buffer. Doubling medium volume reduced background concentrations of both NH₃ and TAA by 40

and 45%, respectively, but had no effect on apparent rates of degradation of casein, SSBM, or ESBM (top panel, Table 4). These concentrations were not unusually high and initial NH₃ levels often approached 10 mM in other incubations (Broderick et al., 2004). In the second experiment, a mixture of NH₃, acid-hydrolyzed casein plus enzymatically hydrolyzed casein was added to a series of time-course incubations. Mean background concentrations of NH₃ were increased from 1.0 to 5.1 mM and of TAA were increased from 1.4 to 2.0 mM. Addition of the N metabolites reduced the apparent degradation rates for casein and SSBM rates by 13 and 22%, respectively, but did not affect degradation of ESBM (bottom panel, Table 4). This latter result suggested that NH₃ concentration exceeding 5 mM may result in end-product inhibition, at least for more rapidly degraded proteins. Vereseghyzy et al. (1993) observed inhibition of protein degradation in vitro when NH₃ exceeded 19 mM. Thus, it appeared that methods such as preincubation that reduced background NH₃ were valuable. These findings also suggested that free AA were not inhibitory to protein degradation in these studies because background TAA did not exceed 2 mM in most incubations. It is possible that peptides, important intermediates in protein catabolism (Broderick et al., 1988; Wallace et al., 1999) but not measured in this study, might act as end-product inhibitors. We observed peptide accumulation only in casein IIV incubations (Broderick et al., 2004) and peptides may accu-

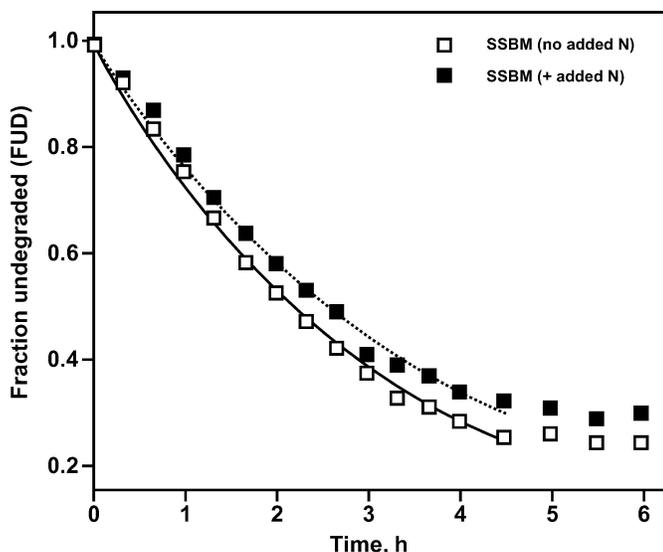


Figure 1. Protein degradation curves obtained in Sweden for casein over 6 h using the IIV system, with and without N added at 0 h as a mixture of NH_4SO_4 plus acid- and enzymatic-hydrolyzed casein to increase background concentrations of protein breakdown products. Equations obtained using nonlinear regression (SAS, 1999-2000) to fit a mono-exponential model to data from 0 to 4.5 h were: $\text{FUD} = 0.993 * e^{-0.266t}$ (plus added N); and $\text{FUD} = 0.993 * e^{-0.313t}$ (no added N). Error bars are not visible because they fall within the plot symbols.

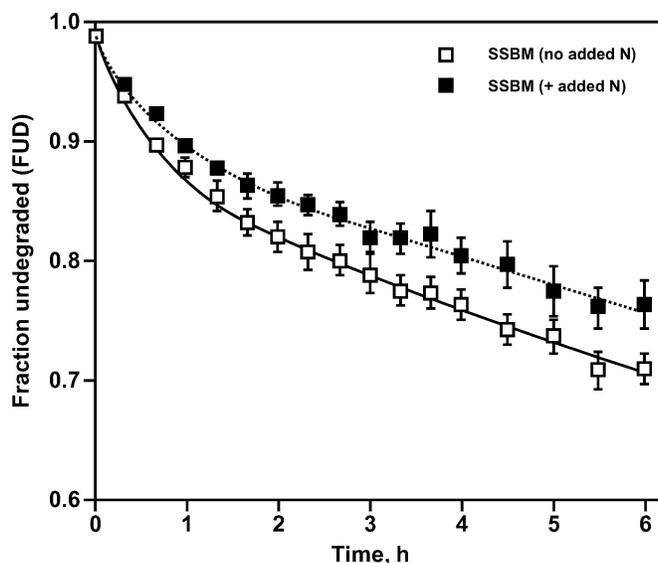


Figure 2. Protein degradation curves obtained in Sweden for solvent soybean meal (SSBM) over 6 h using the IIV system, with and without N added at 0-h as a mixture of NH_4SO_4 plus acid- and enzymatic-hydrolyzed casein to increase background concentrations of protein breakdown products. Equations obtained from non-linear regressions (SAS, 1999-2000) to fit a bi-exponential model to data were: $\text{FUD} = 0.088 * e^{-1.447t} + 0.900 * e^{-0.029t}$ (plus added N); and $\text{FUD} = 0.114 * e^{-1.628t} + 0.875 * e^{-0.036t}$ (no added N). Error bars are ± 1 SE.

mulate during breakdown of rapidly degraded proteins (Broderick and Craig, 1989).

Time-Course of Protein Degradation

We expected that degradation of casein would be a mono-exponential process but that degradation of both SSBM and ESBM might require a 2-compartment model with 2 fractions degraded at 2 different rates. The time-course of degradation of these 3 proteins over 6 h, with and without added background N-metabolites, is shown in Figures 1 to 3. Casein degradation was adequately described by a mono-exponential model over 4.5 h of the 6-h incubation (Figure 1). Without added background N, 74% of the casein was degraded by 4.5 h. Degradation may have deviated from first-order beyond this time because most of the substrate was degraded or because of accumulation of N end-products in the IIV system. Degradation of casein in the presence of added N also was linear over 4.5 h, but only 68% of the original casein was degraded; degradation in this case did not proceed linearly until 74% of the casein was consumed. This suggested that end-product accumulation in the IIV system was more important than substrate limitation in slowing casein degradation below the initial first-order rate.

Degradation of both SSBM (Figure 2) and ESBM (Figure 3) were well described by biexponential models. This was expected because soybean meals (Broderick and Clayton, 1992) and most protein concentrates (Broderick and Cochran, 2000) may be considered to be mixtures of soluble and insoluble protein fractions that are degraded at 2 different rates. Breakdown of SSBM, but not ESBM, was slowed by addition of N metabolites to the medium. Degradation parameters obtained using the nonlinear model of SAS (1999-2000) for both the mono- and biexponential kinetic fits of the data are in Table 5. Rates estimated for rapidly degraded fractions B_1 were very fast, ranging from 1.35 to 1.63/h for 4.3 to 11.4% of the total CP in SSBM and ESBM.1. Degradation rates ranging from 0.87 to 1.08/h were estimated for the rapidly degraded fractions in commercial samples of solvent and screw-press extracted cottonseed meal using a biexponential model (Broderick and Craig, 1980). Degradation rates for casein, estimated using a Michaelis-Menten variant of the present method, ranged from 0.68 to 0.99/h over a large number of IIV incubations (Broderick and Clayton, 1992). The addition of degradation end-products reduced the rates obtained with the mono-exponential model for casein and SSBM, but not for ESBM. Significant effects of protein source, N addition, and their 2-way interactions, were found using biexponential fitting of the soy-

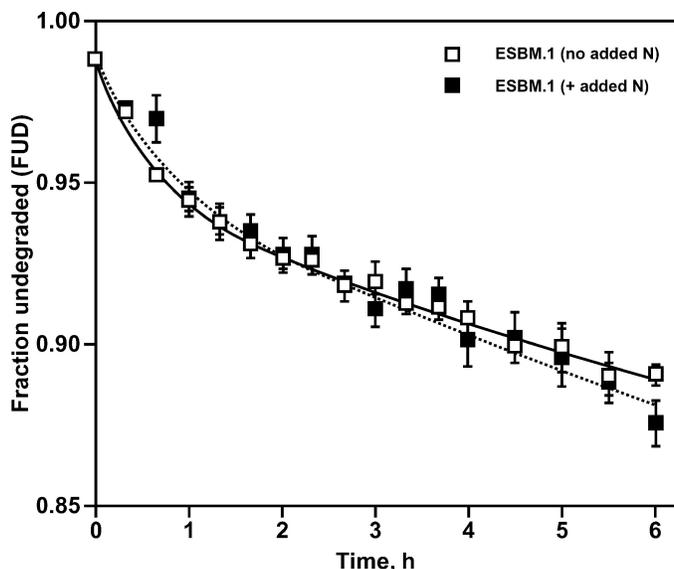


Figure 3. Protein degradation curves obtained in Sweden for expeller soybean meal (ESBM.1) over 6 h using the IIV system, with and without N added at 0-h as a mixture of NH_4SO_4 plus acid- and enzymatic-hydrolyzed casein to increase background concentrations of protein breakdown products. Equations obtained from non-linear regressions (SAS, 1999-2000) to fit a biexponential model to data were: $\text{FUD} = 0.043 * e^{-1.353t} + 0.946 * e^{-0.012t}$ (plus added N); and $\text{FUD} = 0.046 * e^{-1.617t} + 0.943 * e^{-0.010t}$ (no added N). Error bars are ± 1 SE.

bean meals. These occurred because N addition had no effect on ESBM degradation parameters but reduced the apparent size of rapidly degraded fraction (B_1) and increased the size of more slowly degraded fraction (B_2) for SSBM, without altering either degradation rate. Heating cottonseed meal reduced the size and rate of

degradation of the rapidly degraded fraction and increased the size and reduced the rate for the slowly degraded fraction (Broderick and Craig, 1980). However, N addition did not influence the total protein escape estimated using the biexponential model. Similar interpretations of results were obtained using either mono- or biexponential models. Note that the mono-exponential model predicted about 9% less protein escape for both SSBM and ESBM.

Many of the incubations conducted during these studies (e.g., where several inocula or inhibitor concentrations were tested in one experiment as shown in Tables 1 to 3) involved using only 0- and 4-h time-points with casein, SSBM, and ESBM. For all 3 proteins, there was no difference in rate or protein escape estimated with the mono-exponential model using the nonlinear model (nlin; Table 6) in SAS (1999-2000) versus using only the two 0- and 4-h time points (4-h; Table 6). Overall, mean rates were 0.124/h with the nonlinear model in SAS and 0.123/h using only 0- and 4-h data ($P = 0.902$), and escapes averaged 47% by both methods ($P = 0.844$). We concluded that, although the biexponential model fits better the data from the soybean meals, using the abbreviated approach, without multiple time-point measurements or nonlinear regression, did not compromise results from our inocula and inhibitor studies.

SUMMARY AND CONCLUSIONS

Effects of varying the concentrations of the metabolic inhibitors HS and CAP inhibitors on rates of protein degradation estimated from net appearance of NH_3 and TAA were studied in ruminal in vitro experiments con-

Table 5. Results obtained using mono- or biexponential models, and effect of adding N-compounds, on parameters of protein degradation observed for casein, solvent soybean meal (SSBM), and expeller soybean meal (ESBM.1).¹

Protein	Addition ²	Mon-exponential			Bi-exponential				
		B_1 , %	k, /h	Escape, %	B_1 , %	k1, /h	B_2 , %	k2, /h	Escape, %
Casein	0	99.3	0.313	16.0
	+	99.3	0.266	18.3
SSBM	0	98.8	0.068	46.6	11.4	1.628	87.5	0.036	55.2
	+	98.8	0.053	52.8	8.8	1.447	90.0	0.029	61.6
ESBM.1	0	98.9	0.021	72.8	4.6	1.617	94.3	0.010	81.2
	+	98.9	0.022	72.3	4.3	1.353	94.6	0.012	79.4
SE		<0.1	0.002	0.7	0.6	0.099	0.5	0.002	1.1
Probability ³									
Protein		0.003	<0.001	0.001	0.002	0.797	0.001	0.007	0.006
Added N		0.953	0.016	0.114	0.026	0.341	0.014	0.288	0.310
Protein*added N		0.758	0.034	0.201	0.037	0.839	0.024	0.127	0.138

¹LS means of protein degradation parameters. In vitro incubations conducted in Sweden.

²Nitrogen added to "+" vessels as NH_4SO_4 plus acid- and enzymatic-hydrolyzed casein to increase background concentrations of protein breakdown products. Mean concentrations (mM) were: NH_3 , 1.1 (0) and 5.1 (+) and TAA, 1.4 (0) and 2.0 (+).

³Probability of significant effects of protein source, added N, and their 2-way interaction, using replicate-within-incubation run as the error term.

Table 6. Effect of method of estimating degradation parameters on observed rate and estimated ruminal escape of casein, solvent soybean meal (SSBM), and expeller soybean meal (ESBM).¹

Protein	Method ²	Mono-exponential		Bi-exp. escape, %
		Rate, /h	Escape, %	
Casein	nlin	0.289	17.1	...
	4-h	0.290	17.1	...
SSBM	nlin	0.060	49.7	58.4
	4-h	0.058	50.5	...
ESBM.1	nlin	0.022	72.5	80.3
	4-h	0.022	72.4	...
SE		0.003	0.5	0.6
Probability ³				
Protein		<0.001	0.001	...
Method		0.902	0.844	...
Protein × method		0.903	0.922	...

¹LS means of observed degradation rate and estimated escape. Ruminal escape estimated using a passage rate of 0.06/h. In vitro incubations conducted in Sweden.

²The nlin = nonlinear regression fit of data using SAS (1999-2000) model nlin; 4-h = only the ln fractions undergraded at 2 time-points (t = 0 and 4-h) used to compute rate and escape. Bi-exp. escape = mean protein escapes for the two soybean meals estimated using SAS (1999-2000) to fit the bi-exponential model (see Table 5).

³Probability of significant effects of protein source, method of data analysis, and their 2-way interaction, using replicate-within-incubation run as the error term.

ducted in Sweden and Wisconsin. Without adding inhibitors to ruminal inocula, recoveries of NH₃ and TAA were only 4 and 6% after 4-h incubations, and degradation rates estimated for casein, SSBM, and ESBM were very low. Versus the standard inhibitor concentrations of 1 mM HS plus 30 mg of CAP/L, 2 mM HS without CAP yielded similar recoveries and rates but 30 or 90 mg of CAP/L, without HS, was not effective. In Sweden, 2 mM HS plus 90 mg of CAP/L appeared to give rise to increased TAA recoveries and higher rates for casein, but not for SSBM, in the presence of added starch. For the same protein samples, faster degradation rates were obtained for casein, but slower rates for SSBM and ESBM, in Sweden versus Wisconsin, using ruminal inocula from lactating cows fed diets with similar CP and energy content. Location differences may have resulted from differences in peptide catabolism between microbial populations in the rumens of donors in Sweden and Wisconsin. Adding NH₃, free AA, and peptides to inocula reduced apparent degradation rates, possibly via end-product inhibition. Casein degradation followed simple, first-order kinetics, while a biexponential model was required to fit the degradation patterns for both SSBM and ESBM.

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