

In Vitro Determination of Ruminal Protein Degradability Using [¹⁵N]Ammonia to Correct for Microbial Nitrogen Uptake^{1,2}

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ABSTRACT: An in vitro procedure was developed to estimate rate and extent of ruminal protein degradation using [¹⁵N]H₃ to quantify uptake of protein degradation products for microbial protein synthesis. Incubations were conducted for 6 h in stirrer flasks with ruminal inoculum plus buffer, reducing solution, pectin, soluble carbohydrates, and added (¹⁵NH₄)₂SO₄. Seven protein concentrates were tested in the system. Samples of media were analyzed for ¹⁵N enrichment of NH₃, microbial N, and total solids N. Degradation rate was computed from net (i.e., protein-added minus blank) release of NH₃ N plus net synthesis of microbial protein N; escape was estimated assuming ruminal passage rate = .06/h. Over the course of the incubations, pH was stable at 6.6, protozoal numbers increased slightly, and

microbial protein content increased by more than 200%. Free AA had not accumulated at the end of the incubations. Microbial protein synthesis was a linear function ($P < .001$; $r^2 = .780$) of extent of degradation. Mean degradation rates and ruminal escapes determined were, respectively, .569/h and 10% (casein), .148/h and 29% (solvent soybean meal), .036/h and 63% (expeller soybean meal), .026/h and 70% (low solubles fish meal), .063/h and 49% (high solubles fish meal), .034/h and 64% (corn gluten meal), and .050/h and 55% (roasted soybeans). Overall, degradation rates averaged 28% greater than those previously estimated using an inhibitor in vitro system; however, rates obtained for the fish meals using the ¹⁵N method were slower.

Key Words: Rumen Digestion, Protein Degradation, Nitrogen-15, Protein Quality, Protein Concentrates

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Introduction

The undegraded intake protein content of feeds is recognized by the NRC (1989) as a major factor limiting the productivity of high-yielding dairy cows. In vitro methods based on N solubility (Crooker et al., 1978; Vérité et al., 1979), commercial proteases (Krishnamoorthy et al., 1983; Poos-Floyd et al.,

1985), or both (Aufreere et al., 1991) have been proposed to estimate the rate and extent of ruminal protein degradation. The first approach has been criticized because solubility does not distinguish degraded from undegraded protein (Owens and Zinn, 1982); solubility may be applicable only for testing specific protective treatments on individual proteins. Systems using ruminal inoculum have advantages over those based on commercial proteases in terms of simulating the dynamics and specificity of in vivo microbial protein degradation. Mahadevan et al. (1987) used proteases isolated from mixed ruminal microbes to estimate protein degradability. Accumulation of NH₃ and AA in ruminal in vitro incubations may be misleading indicators of protein degradation because protein breakdown and microbial protein synthesis occur simultaneously. Attempts to overcome this problem have involved ruminal in vitro incubations using 1) gas production to quantify microbial N incorporation (Raab et al., 1983) and 2) inhibitors of NH₃ and AA anabolism to obtain quantitative recovery of protein degradation products (Broderick, 1987).

Microbial incorporation of N compounds derived during protein degradation may be determined by means of tracer substances such as ¹⁵N. Al-Rabbat et

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Table 1. Composition of the incubation mixture

Component	Amount per vessel ^a
Buffer ^b	70 mL
Reducing solution ^b	12 mL
Soluble carbohydrates ^c	737 mg
Pectin ^d	990 mg
Protein N ^e	45.0 mg
(¹⁵ NH ₄) ₂ SO ₄ ^f	5.77 mg
Inoculum ^g	240 mL

^aIncubation vessels were spinner flasks (Broderick, 1987).

^bMacromineral buffer and reducing solution were prepared as described by Goering and Van Soest (1970) except NH₄HCO₃ was replaced by an equimolar amount of KHCO₃ and reducing agent was added at 5 mM rather than 6 mM.

^cSoluble carbohydrates contained 121 mg of maltose, 66 mg of glucose, 66 mg of sucrose, and 484 mg of soluble potato starch and were dissolved in 4 mL of buffer^b and added to vessels at 0 h and again after 3 h of incubation. Two milliliters of hydroxide solution (1.0 N KOH and .5 N NaOH) also was added after 3 h of incubation.

^dCitrus fruit pectin added to incubation vessels at 0 h dissolved in 33 mL of buffer^b.

^eProtein (12.5 mg of N/100 mL) was added to vessels (except blanks) and soaked for 1 h at 39°C in buffer^b, reducing solution, and soluble carbohydrate and pectin solutions, before addition of (¹⁵NH₄)₂SO₄ and inoculum.

^f(¹⁵NH₄)₂SO₄ enrichment was 73% atom percentage excess of ¹⁵N (equivalent to .93 mg of ¹⁵N per flask). (¹⁵NH₄)₂SO₄ was added at 0 h to each vessel dissolved in 1 mL of water.

^gInoculum prepared from equal volumes of strained ruminal fluid and buffer^b extract of ruminal solids (see Materials and Methods). Total volume at the start of incubations was 360 mL/vessel.

al. (1971a) and Harmeyer et al. (1976) proposed using ¹⁵NH₃ in vitro for estimating NH₃ utilization for microbial protein synthesis. The objective of the present study was to develop and evaluate an in vitro method for quantifying ruminal degradation of feed protein concentrates using ¹⁵NH₃ to correct for incorporation of protein degradation products for microbial protein synthesis.

Materials and Methods

Digesta were obtained from two ruminally cannulated cows in mid to late lactation fed a total mixed diet containing (DM basis) 40% alfalfa silage (55% DM), 20% corn silage (40% DM), 28.2% cracked shelled corn, 10% soybean meal, 1.1% dicalcium phosphate, and .7% trace mineral salt, plus vitamins A, D, and E. Equal amounts of whole ruminal contents were collected 1.5 h after morning feeding from the ventral rumina of both cows, mixed, and squeezed through two layers of cheesecloth. To enrich the inoculum with microbes loosely attached to feed particles (Craig et al., 1984), the remaining solids were washed four times through two layers of cheesecloth with a volume of warm (39°C) buffer (Goering and Van Soest, 1970) equal to the volume of strained ruminal fluid (Table 1). The buffer wash was mixed with the strained ruminal fluid and filtered

through eight layers of cheesecloth into 4-L graduated cylinders. Cylinders were held under CO₂ at 39°C for 30 min, during which time gas-entrapped, small feed particles rose to the top of the cylinder. This band of buoyant feed particles was removed by vacuum aspiration; typically approximately 15% of inoculum volume was removed. Remaining inoculum was mixed to resuspend protozoa and pH was determined (pH Meter 130, Corning, Medfield, MA); protozoal counts (Ogimoto and Imai, 1981) also were made in two of the four incubations. Just before inoculating the flasks, the NH₃ concentration of the inoculum was measured. If NH₃ was less than 20 mg of N/100 mL, then sufficient buffered (NH₄)₂SO₄ solution was added to the inoculum to give an NH₃ concentration of 24 mg of N/100 mL (equivalent to 16 mg of NH₃ N/100 mL during the incubation).

Incubation vessels were spinner flasks with nominal volumes of 250 mL (Bellco, Vineland, NJ) and actual capacities of 450 mL (Broderick, 1987). Before inoculation, vessels were dosed with (Table 1) protein source (45 mg of N; 12.5 mg of N/100 mL), 70 mL of buffer, and 12 mL of reducing solution (Goering and Van Soest, 1970), 737 mg of soluble carbohydrate mixture (dissolved in 4 mL of buffer) and 990 mg of citrus fruit pectin (No. P-9135, Sigma, St. Louis, MO; dissolved with heating in 33 mL of buffer). The carbohydrate source was a mixture of substrates that were readily and slowly fermented and designed to support normal growth and fermentation. Protein sources were soaked in these solutions at 39°C for 1 h before inoculum was added. Just before incubation, 5.77 mg of (¹⁵NH₄)₂SO₄ enriched with 73 atom % excess ¹⁵N (dissolved in 1 mL of water) was added to each vessel; blank vessels contained all components except the protein source. Incubations began with addition, using a peristaltic pump, of 240 mL of well-mixed inoculum to each spinner flask (total volume 360 mL). Incubations were 6 h long; vessels were stirred continually. At 3 h, to each vessel were added 2 mL of hydroxide solution (1.0 N KOH and .5 N NaOH) plus an additional 737 mg of soluble carbohydrate mixture (dissolved in 4 mL of buffer). All manipulations were carried out under O₂-free CO₂ gas. All blanks and protein sources were incubated in triplicate; incubations were repeated four times. The pH of each vessel was recorded at 6 h. Protozoal counts (Ogimoto and Imai, 1981) also were made at 6 h in each vessel in two of the four incubations.

Duplicate 25-mL samples were taken from each vessel at 0 h (immediately after inoculating and mixing) and at 6 h by means of a vacuum pipet with a large opening; both samples were cooled in an ice bath and processed immediately. One sample was treated with 1% vol/vol formalin and bacterial solids isolated by centrifuging to remove protozoa and feed residues (551 × g, 4°C for 10 min), decanting the supernatant, then recentrifuging at high speed (31,000 × g, 4°C for 25 min). Total solids were isolated without formalin

by high-speed centrifugation ($31,000 \times g$, 4°C for 25 min). Supernatants from total solid isolation were removed, deproteinized by adding trichloroacetic acid (TCA) to final concentration of 5% wt/vol, holding on ice for 30 min, and recentrifuging ($31,000 \times g$, 4°C for 25 min); TCA supernatants were stored (-20°C) for later analysis. Pellets from both bacterial and total solids were resuspended and washed twice with .9% wt/vol NaCl containing 1% vol/vol formalin solution then recentrifuged ($31,000 \times g$, 4°C for 25 min), followed by freeze-drying.

Deproteinized TCA supernatants from 0 and 6 h were thawed and analyzed for NH_3 and total AA concentrations (Broderick and Kang, 1980). In two incubations, peptides were determined only in blank and casein vessels at 6 h from the increase in free AA upon hydrolysis of TCA supernatants (6 N HCl for 20 h at 105°C). The ^{15}N enrichments of the NH_3 pools at 0 and 6 h were determined in TCA supernatants as follows: samples were made basic ($\text{pH} > 10$) with 2 M NaOH and NH_3 was steam-distilled and trapped in .1% H_2SO_4 . The distillate was treated with 1 mL of 40% wt/vol aqueous KMnO_4 to eliminate interfering methylamines from ^{15}N mass spectrometry; distillates were evaporated down to about 1-mL volume. The N_2 was released in evacuated Rittenberg flasks by reacting samples with NaBrO. Flasks then were attached to a specially designed entry port of an isotope ratio mass spectrometer (Varian MAT Mass Spectrometer, Flozham Park, NJ) and ^{15}N enrichment determined using O_2 -free air as background. Non- NH_3 N (NAN) was determined in freeze-dried pellets from bacterial and total solids by removing NH_3 with K_2CO_3 treatment (Nagel and Broderick, 1992) then analyzing for total N by Kjeldahl digestion (AOAC, 1980) using a copper digestion catalyst (Kjeltabs, Tecator, Herndon, VA). Kjeldahl digestates (15 mL) were treated with 15 mL of 13 N NaOH then steam-distilled, concentrated, and analyzed for ^{15}N -enrichment as described for TCA supernatants.

Seven proteins, previously studied in in vitro and in vivo experiments, were tested (% N, DM basis): casein (14.43% N; Sigma, St. Louis, MO), solvent soybean meal (7.49% N; **SSBM**), expeller soybean meal (7.71% N; **ESBM**), low solubles fish meal (10.85% N; **LSFM**), high solubles fish meal (10.93% N; **HSFM**), corn gluten meal (11.39% N; **CGM**), and roasted whole soybeans (5.87% N; **RSB**).

Figure 1 depicts the N transactions that were assumed to occur during the ruminal in vitro incubations. Microbial (bacterial) protein N (NAN) became enriched over the course of 6 h from $^{15}\text{NH}_3$ incorporation. The ^{15}N content of "pure" bacterial NAN was estimated in bacteria isolated by differential centrifugation. The ^{15}N content of total solid NAN, which was assumed to include residual feed NAN (carried over from the inoculum), microbial NAN, and undegraded test protein NAN, was estimated in total solids isolated as the pellet from high-speed centrifugation.

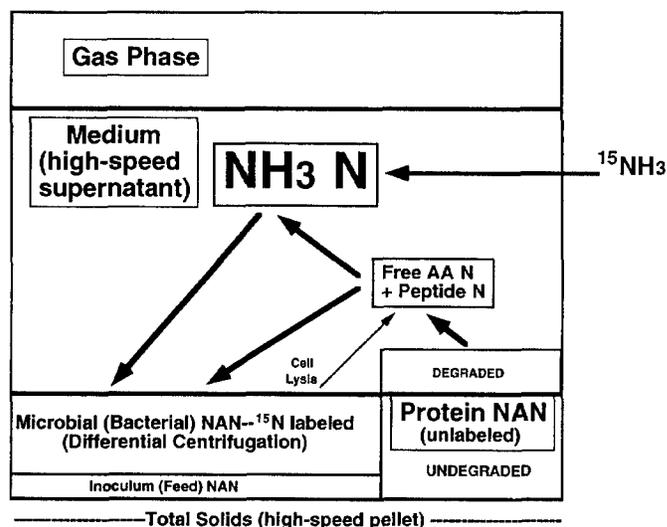


Figure 1. Diagram showing the N transactions in the ruminal in vitro incubation flasks and the sample separation scheme. The NH_3 pool was labeled with ^{15}N ; microbial (bacterial) protein N became enriched over the 6-h incubations from $^{15}\text{NH}_3$ incorporation. Enrichment of ^{15}N in "pure" bacterial non-ammonia N (NAN) was estimated in samples isolated by differential centrifugation. Enrichment of ^{15}N in total solid NAN, which was assumed to include feed residual NAN (from the inoculum), microbial NAN, and undegraded test protein NAN, was isolated as the pellet from high-speed centrifugation. Because there was no net accumulation of peptides and free AA, degraded protein N will appear as the net increase in N in the NH_3 and microbial NAN pools.

Several additional assumptions were made: 1) equal amounts of microbial plus residual feed N from the inoculum were dosed into all vessels at the beginning of the incubation; 2) by 6 h, bacterial and protozoal NAN equilibrated to the same ^{15}N enrichment; 3) differences at 6 h in microbial NAN between blank and protein-added vessels originated from incorporation of peptides, AA, and NH_3 released from added protein, and, because there was no net accumulation at 6 h of peptides and free AA from degradation of test or microbial proteins, 4) the extent of protein degradation at 6 h could be computed from net release of NH_3 N plus net formation of microbial protein N.

Extent of protein degradation (PD) within each set of eight vessels was computed using the following equation:

$$\text{PD, \%} = [(\text{NAR}_6 + \text{NMN}_6) / \text{Prot. N}] \times 100 \quad [1]$$

where NAR_6 is the net (i.e., blank corrected) NH_3 N released ($\text{mg}/100 \text{ mL}$) at 6 h, NMN_6 is the net (i.e.,

blank corrected) microbial NAN synthesized (mg/100 mL) at 6 h, and Prot. N is the amount of protein N added at 0 h (12.5 mg of N/100 mL). The NAR_6 observed for each protein was computed:

$$NAR_6 = [NH_3]_{prot.} - [NH_3]_{blank} \quad [2]$$

where $[NH_3]_{prot.}$ and $[NH_3]_{blank}$ are NH_3 N concentrations at 6 h for protein and blank vessels, respectively. The NMN_6 was computed for each protein using the following equations:

$$MN_6 = (TS^{15}N_6/B^{15}N_6) \times TSN_6 \quad [3]$$

$$NMN_6 = (MN_6)_{prot.} - (MN_6)_{blank} \quad [4]$$

where MN_6 is microbial NAN concentration (mg/100 mL) at 6 h, $TS^{15}N_6$ and $B^{15}N_6$ are atom percentage excess of ^{15}N enrichments of total solids NAN and bacterial NAN at 6 h, TSN_6 is total solids NAN (mg/100 mL) at 6 h, and $(MN_6)_{prot.}$ and $(MN_6)_{blank}$ are microbial NAN concentrations (mg/100 mL) at 6 h in protein-added and blank vessels.

Twenty-four vessels (three sets of eight) were used in each of four separate incubations; degradability data were computed within each set of eight (one blank and seven protein-added vessels). Three separate estimates of each variable were obtained in three of the four incubations. However, $^{15}NH_3$ recoveries approaching 100% were observed in one set in one incubation (suggesting that carbohydrates inadvertently were omitted from those eight vessels) and degradation estimates were possible with only two sets in that incubation. Four individual estimates of extent of degradation at 6 h for casein were greater than 100% (102 to 109%); thus, the mean extent of degradation (Equation [1]) from each incubation was used to compute single estimates ($n = 4$) of degradation rate (k_d) and estimated ruminal escape for each protein with the following equations:

$$k_d, /h = \{ \ln[(100 - PD, \%)/100] \} / 6 \text{ h} \quad [5]$$

$$\text{Estimated escape, \%} = [k_p / (k_d + k_p)] \times 100 \quad [6]$$

where k_p is the rate of ruminal DM passage (assumed = .06/h). The mean extent of degradation from one incubation for casein was 100.6%, so no rate or escape could be estimated from that run; rate and escape for casein are means from three incubations only. Therefore, an adjusted degradation rate and ruminal escape was computed, relative to casein, for each protein in each set ($n = 11$) by first calculating an adjusted protein degradation (**APD**):

$$APD, \% = (96.08/PD_{cas}) \times PD_{prot.} \quad [7]$$

where PD_{cas} and $PD_{prot.}$ are the individual extents of degradation (Equation [1]) observed at 6 h for, respectively, casein and each of the other six proteins, and 96.08 is the percentage casein degradation required in 6 h to yield a $k_d = .540/h$ and a ruminal escape of 10% (McDonald and Hall, 1957) at $k_p = .06/h$. Adjusted degradation rates and estimated ruminal escapes were computed by substituting APD for PD in Equation [5] and using the resulting k_d in Equation [6].

Statistical analysis was conducted using the GLM procedures of SAS (1985). The model used to test for differences among treatments for most variables included treatment ($n = 8$ for blank and protein-added vessels, or $n = 7$ for net differences due to protein source), incubation run ($n = 4$ for all variables except $n = 2$ for protozoal counts), replicate ($n = 3$ for three runs and $n = 2$ for one run), and treatment \times run interaction. When the interaction was not significant ($P > .05$), it was pooled with error. Analysis of extent of degradation at 6 h, degradation rate, and estimated escape were done using a single mean computed for each protein from each incubation run ($n = 4$ or $n = 3$ for casein), weighted for number of replicates ($n = 3$ or 2 per run); the model included only treatment ($n = 7$) and run ($n = 4$). Analysis of adjusted extents of degradation at 6 h, degradation rates, and estimated escapes (computed relative to casein) were done using the same model as for net differences, except $n = 6$ for treatments. When significant effects ($P < .01$) due to treatment were detected, separation of means was by LSD at $P = .05$ (Steel and Torrie, 1980).

Results and Discussion

Mean pH of the medium was 6.67 at 0 h; pH at 6 h did not differ among treatments ($P = .17$), averaging 6.60 (Table 2). The concentration of bacterial N, estimated by differential centrifugation, increased from 3.5 mg/100 mL at 0 h to an average 11.1 mg/100 mL of medium at 6 h, an increase of 217%. Other work (D. B. Vagnoni, unpublished data) with this same carbohydrate mixture under the same conditions showed no accumulation of lactate, yielded rates of VFA formation that were linear over 6 h and similar to rates obtained using the carbohydrate mixture of Van Nevel and Demeyer (1977), and confirmed the value of adding base at 3 h. The stable pH indicated that the fermentation in the present work was not directed toward overgrowth of *Streptococcus bovis* and excessive production of lactate (Russell and Hino, 1985), despite extensive bacterial growth. Use of pectin, a more slowly degraded carbohydrate whose fermentation does not yield lactate (Strobel and Russell, 1986), also may have prevented overgrowth by *S. bovis*. Protozoal numbers averaged 1.44×10^5 at 0 h and actually increased over the course of the

Table 2. Protein metabolism data from in vitro incubations^a

Item	Time, h	Blank	Casein	SDBM	ESBM	LSFM	HSFM	CGM	RSB	SEM	P > F ^b
NH ₃ ¹⁵ N, atom % excess	0	1.411	1.412	1.411	1.410	1.441	1.399	1.405	1.411	.012	.421
NH ₃ N, mg/100 mL	0	16.28 ^{tuw}	16.19 ^{uw}	16.11 ^w	16.28 ^{uw}	16.18 ^{uw}	16.37 ^{tu}	16.35 ^{tu}	16.46 ^t	.08	.037
pH	6	6.68	6.61	6.58	6.55	6.59	6.61	6.58	6.60	.03	.174
Protozoa × 10 ⁵ c	6	1.88 ^t	1.63 ^w	1.63 ^w	1.91 ^t	1.84 ^t	1.65 ^w	1.75 ^u	1.90 ^t	.03	.045
Total AA, mM	6	.352 ^{tu}	.397 ^t	.317 ^u	.330 ^u	.367 ^{tu}	.392 ^t	.350 ^{tu}	.318 ^u	.021	.038
Apparent total AA release, mM	6	—	.045	-.035	-.022	.015	.040	-.002	-.034	.025	.097
NH ₃ ¹⁵ N, atom % excess	6	1.048 ^t	.865 ^y	.838 ^z	.963 ^w	1.002 ^u	.960 ^w	.972 ^w	.918 ^x	.006	<.001
NH ₃ N, mg/100 mL	6	8.70 ^y	15.35 ^t	11.42 ^u	8.76 ^y	10.08 ^x	11.03 ^w	8.89 ^y	8.99 ^y	.11	<.001
Apparent NH ₃ N release (A), mg/100 mL	6	—	6.65 ^t	2.72 ^u	.06 ^y	1.39 ^x	2.33 ^w	.19 ^y	.29 ^y	.11	<.001
Total solid N, mg/100 mL	6	24.73 ^z	28.50 ^y	33.05 ^x	35.78 ^u	34.22 ^w	33.86 ^{wx}	36.86 ^t	36.40 ^{tu}	.34	<.001
Total solid ¹⁵ N, atom % excess	6	.455 ^t	.278 ^z	.336 ^u	.333 ^{uw}	.315 ^x	.303 ^y	.326 ^w	.335 ^{uw}	.003	<.001
Bacterial ¹⁵ N, atom % excess	6	.567 ^t	.312 ^y	.456 ^x	.540 ^u	.537 ^u	.481 ^w	.551 ^{tu}	.540 ^u	.006	<.001
Microbial N, mg/100 mL	6	19.84 ^z	25.34 ^t	24.45 ^u	22.20 ^{wx}	20.28 ^z	21.44 ^y	21.94 ^{xy}	22.75 ^w	.22	<.001
Net microbial N (B), mg/100 mL	6	—	5.50 ^t	4.61 ^u	2.36 ^{wx}	.44 ^z	1.60 ^y	2.10 ^{xy}	2.91 ^w	.26	<.001
Degraded protein N (A + B), mg/100 mL	6	—	12.16 ^t	7.33 ^u	2.42 ^y	1.83 ^y	3.93 ^w	2.29 ^y	3.20 ^x	.23	<.001
Degraded protein N, %	6	—	97.0 ^t	58.6 ^u	19.3 ^y	14.6 ^y	31.4 ^w	18.3 ^y	25.6 ^x	1.9	<.001

^aSSBM = solvent soybean meal; ESBM = expeller soybean meal; LSFM = low solubles fish meal; HSFM = high solubles fish meal; CGM = corn gluten meal; RSB = roasted soybeans.

^bProbability of a significant treatment effect.

^cProtozoal numbers determined in only two of the four incubations.

^{t,u,w,x,y,z}Means within rows with different superscripts differ ($P < .05$).

Table 3. Protein degradation estimates

Method and variable	Protein source ^a							SEM	P > F ^b
	Casein	SSBM	ESBM	LSFM	HSFM	CGM	RSB		
Weighted means ^c									
Degraded protein N (6 h), %	97.0 ^u	58.6 ^w	19.3 ^{yz}	14.6 ^z	31.4 ^x	18.3 ^{yz}	25.6 ^{xy}	3.2	<.001
Degradation rate (k _d), /h	.569 ^u	.148 ^w	.036 ^x	.026 ^x	.063 ^{wx}	.034 ^x	.050 ^{wx}	.037	<.001
Estimated escape, %	10.0 ^z	29.1 ^y	62.9 ^{uw}	70.1 ^u	49.2 ^x	64.4 ^{uw}	55.3 ^{wx}	4.3	<.001
Computed relative to casein ^d									
Degraded protein N (6 h), %	96.1	57.9 ^u	19.2 ^y	14.4 ^z	31.1 ^w	18.2 ^{yz}	25.5 ^x	1.5	<.001
Degradation rate (k _d), /h	.540	.145 ^u	.036 ^y	.026 ^y	.063 ^w	.034 ^y	.050 ^x	.004	<.001
Estimated escape, %	10.0	29.5 ^z	63.6 ^w	71.0 ^u	49.6 ^y	64.8 ^w	55.7 ^x	2.0	<.001
Inhibitor in vitro ^e									
Degradation rate (k _d), /h	.307	.137	.030	.034	.066	.017	.045	—	—
Estimated escape, %	17.0	30.2	65.2	62.8	46.1	74.5	56.1	—	—

^aSSBM = solvent soybean meal; ESBM = expeller soybean meal; LSFM = low solubles fish meal; HSFM = high solubles fish meal; CGM = corn gluten meal; RSB = roasted soybeans.

^bProbability of a significant effect due to protein source.

^cDegradation computed using ¹⁵NH₃ metabolism data (see Table 2 and Materials and Methods). Degradation rates and estimated escapes are weighted means from four values for all proteins except casein, which are from three values.

^dProtein degradation computed by adjusting 6-h degradations relative to casein, assuming a casein degradation of 96.1%, corresponding to a degradation rate (k_d) = .54/h and an escape of 10% at a passage rate (k_p) = .06/h.

^eDegradation rates and estimated escapes obtained previously for the same proteins using the inhibitor in vitro method (Broderick, 1987). Data are from Broderick (1992) (LSFM and HSFM), Broderick et al. (1990) (CGM), and Faldet and Satter (1991) (RSB). Values for casein, SSBM and ESBM are means from incubations conducted in all three experiments.

^{u,w,x,y,z}Means within rows with different superscripts differ (P < .05).

incubations, averaging 1.77×10^5 at 6 h; numbers differed somewhat among treatments at 6 h (Table 2; P = .045). Protozoa are difficult to cultivate in vitro (Bonhomme, 1990) but are important to normal ruminal protein degradation. The role of protozoa in the breakdown of soluble (Hino and Russell, 1987) and insoluble proteins (Ushida and Jouany, 1985; Ushida et al., 1991) has been demonstrated. Wallace et al. (1990) showed that protozoa were involved in ruminal hydrolysis of dipeptides in vitro. Our data indicate that a stable fermentation with active microbial growth was maintained throughout the incubations.

Table 2 summarizes means of the protein metabolism data used in Equations [1] to [7] to compute rates and extents of protein degradation. Although there were small differences in NH₃ concentrations among protein-added vessels at 0 h, none was different from the blank; ¹⁵N-enrichment of NH₃ at 0 h was not different among treatments. Also, there were no differences in total AA at 6 h among blank and protein treatments; apparent release of total AA were not different from zero (P = .097). Analyses conducted in two incubations indicated that peptide levels were not different between blank and casein vessels at 6 h (data not shown). Thus, there was no significant accumulation of free AA or peptides as end products of protein degradation in the incubations, and these pools may be ignored in degradation computations. High speed centrifugation, followed by washing with saline plus formalin, was used in isolation of total solids NAN. This approach probably was satisfactory because free AA and peptides did not accumulate.

However, it may be possible to isolate total solids NAN and determine net microbial NAN more conveniently simply by adding acid (to stop enzymic activity in total solid samples) then lyophilizing. If adding alkali would stop deamination, then NH₃ removal for NAN determination could be accomplished more easily during the drying step.

As expected, NH₃ concentrations at the end of the incubation were greater in protein-added vessels than in blanks due to NH₃ release from protein breakdown. Recoveries of added ¹⁵NH₃ ranged from 35.4 (RSB) to 58.1% (casein). The ¹⁵N-enrichment of NH₃ at 6 h generally was inversely related to NH₃ concentration, due to dilution of the essentially equal 0-h ¹⁵NH₃ pools by ¹⁴NH₃ produced from protein breakdown. However, ¹⁵NH₃-enrichment at 6 h for SSBM was slightly lower than that for casein despite lower NH₃ concentration. Total NH₃ production over the incubations, computed by subtracting 0-h NH₃ from the 6-h NH₃ concentration corrected for ¹⁵NH₃ recovery, was 10.3 and 10.9 mg of N/100 mL for casein and SSBM, respectively. This suggested that, although approximately equal amounts of NH₃ were formed in the casein and SSBM incubations, relatively more NH₃ was incorporated into microbial protein with SSBM because NH₃ concentration at 6 h was greater with casein (Table 2). We estimated microbial N formation from ¹⁵N enrichment of isolated bacterial N and total solid N (Equation [3]). Diaminopimelic acid may be an unsatisfactory marker because greater degradation of microbial protoplasm than of cell wall material would lead to overestimation of microbial N (Masson et al., 1991).

Table 3 reports mean extents of degradation at 6 h, as well as the corresponding rates of degradation and estimated escapes, computed for a ruminal passage rate of .06/h. Mean extents of protein degradation at 6 h ranged from 14.6 (LSFM) to 97.0% (casein). Estimated degradation rates ranged from .026 to .569/h, which yielded estimated ruminal escapes ranging from 10 to 70%. Extents of protein degradation at 6 h also were adjusted relative to casein by assuming that casein had a 6-h degradation of 96.1% (which is equivalent to a ruminal escape of 10%; McDonald and Hall, 1957). This adjustment allowed a relative degradation rate and estimated ruminal escape to be computed for each replicate within run ($n = 11$); thus, SEM for both traits were much smaller than without adjustment. However, this computation had little effect on overall means for rates and escapes. Also in Table 3 are ruminal degradabilities obtained with the same feedstuffs by the inhibitor *in vitro* (IIV) method (Broderick, 1987). Degradation rates averaged across all proteins were 28% more rapid than those estimated by the IIV method; degradabilities were greater for five of the seven proteins studied. However, rates obtained for LSFM and HSFM were 23 and 4% slower, respectively, than those estimated by IIV. Possible inhibition by fish oil of microbial growth (Galbraith et al., 1971) may have had a greater effect on degradation obtained using the ^{15}N rather than the IIV method because microbial growth already is suppressed by inhibitors (hydrazine and chloramphenicol) in the IIV technique.

Mean ruminal escapes from *in vivo* determinations reported by the NRC (1989) for three of these proteins were similar to those obtained in our study: 35 (SSBM), 60 (fish meal), and 55% (CGM). A degradation rate observed *in vivo* for the casein (.462/h; Broderick, 1978) may be compared to that obtained by the ^{15}N procedure (.569/h). Mean ruminal escape of casein estimated by the ^{15}N procedure (10%) was identical to that observed *in vivo* in sheep (McDonald and Hall, 1957). In an *in vitro* study, Raab et al. (1983) found degradation rates, computed from 12 and 24 h incubations, of .164 and .192/h for casein and .115 and .094/h for soybean meal; the rate obtained for CGM (24 h only) was .043/h. There can be substantial variation in protein degradability among samples of the same feedstuff due to processing or other factors. However, these comparisons indicate that the ^{15}N method we developed yielded satisfactory estimates of ruminal degradation rates and escapes.

Because of microbial uptake of NH_3 for growth, apparent NH_3 release (protein-added minus blank) substantially underestimated protein degradation; apparent NH_3 release for ESBM, CGM, and RSB was almost nil (Table 2). Originally, we planned to correct NH_3 release for $^{15}\text{NH}_3$ recovery to account for microbial uptake of protein degradation products. However, this proved unsatisfactory for computing

either absolute or relative estimates of protein degradation because direct incorporation of NAN for microbial growth varied with protein source. The average of ^{15}N enrichment at 0 h and 6 h for NH_3 (precursor) and the ^{15}N enrichment at 6 h for microbial N (product) may be used to estimate proportions of microbial N coming from the NH_3 and NAN pools (IAEA, 1985). Variation among incubations in origins of microbial N are shown in Figure 2. Except for casein, proportions of microbial N coming from NH_3 and NAN were similar among incubations. The more resistant a protein was to microbial degradation the greater was the proportion of the microbial N coming from NH_3 . More rapidly degraded proteins such as casein would be expected to have greater rates of release of peptides and free AA (Broderick and Wallace, 1988), thus favoring greater NAN and lower NH_3 incorporation.

A strong preference of the microbes to use NAN rather than N from the abundant NH_3 pool is apparent despite NH_3 concentrations well in excess of "optima" of 5 (Satter and Slyter, 1974) and 8.5 mg of N/100 mL (Kang-Meznarich and Broderick, 1981) reported for *in vitro* and *in vivo* studies, respectively. Proportions of microbial N incorporated from $^{15}\text{NH}_3$ in this experiment ranged from 28% (casein) to 46% (blank, CGM and RSB). To make these estimates, it was necessary to assume that all the bacterial N isolated at 6 h was newly formed. This is unlikely, and the actual ^{15}N enrichment of newly formed microbial protein N probably was greater than the observed enrichment due to dilution from unlabeled microbial N carried over from the inoculum. Mean bacterial concentrations (isolated by differential centrifugation) were 3.5 and 11.1 mg of N/100 mL at 0 h and 6 h, respectively. Mean microbial N at 6 h, estimated from ^{15}N enrichment, was 22.3 mg/100 mL, indicating that differential centrifugation isolated approximately half the microbial N. Although this isolation was not quantitative, an estimated maximum of 3.5/11.1, or 32%, of the microbial N at 6 h could have been carried over from the inoculum. Because only newly synthesized protein would be labeled, enrichment from $^{15}\text{NH}_3$ of bacterial N isolated at 6 h may have been diluted to as little as 68% of actual. Thus, the maxima for microbial N from NH_3 could fall in the range of 40% (28/.68) to 68% (46/.68). Some microbial N in the inoculum would be degraded; therefore, these latter estimates represent upper limits.

Our uncorrected estimates were low compared with *in vitro* results of 62 to 78% for bacteria and 61% for mixed ruminal microbes (Al-Rabbat et al., 1971a) and *in vivo* estimates of 50 to 65% for bacteria and 31 to 55% for protozoa (Mathison and Milligan, 1971) and 42% for mixed ruminal microbes (Al-Rabbat et al., 1971b). However, Russell et al. (1983) found that, when carbohydrate availability was increased up to 200 mg/L, most of the N incorporated into microbial

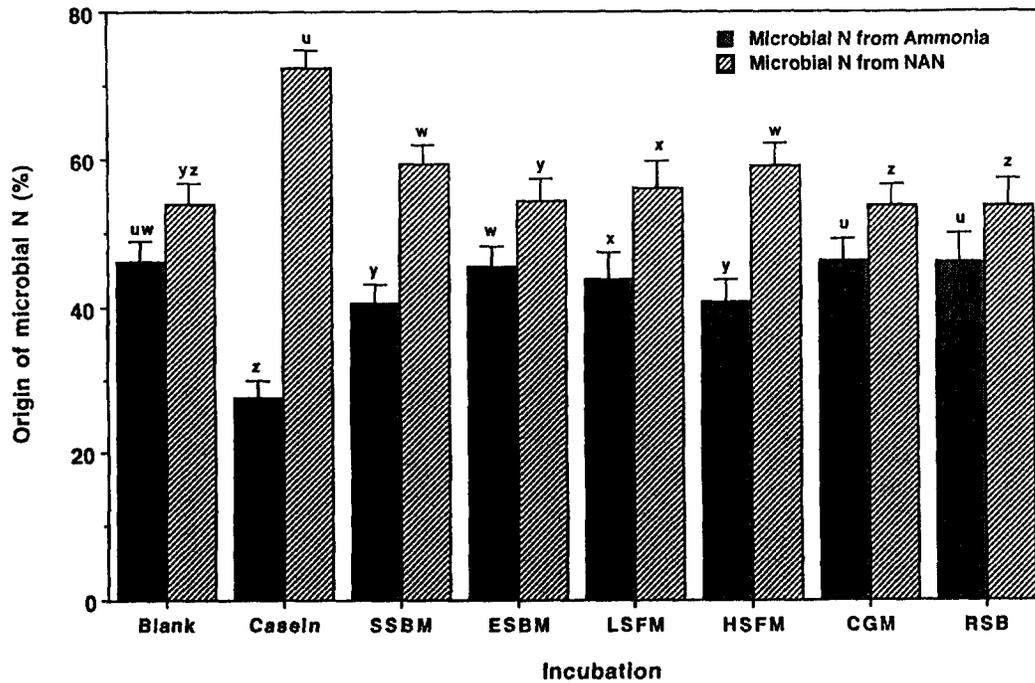


Figure 2. Proportion of N incorporated into microbial protein N originating from NH_3 and non-ammonia N (NAN) at 6 h (the end of the incubation) estimated from average ^{15}N enrichment of NH_3 N at 0 and 6 h and ^{15}N enrichment of bacterial NAN at 6 h (means \pm SD). Letters represent mean separations, within microbial N source from NH_3 N or NAN, determined by LSD ($P < .05$). SSBM = solvent soybean meal; ESBM = expeller soybean meal; LSFM = low solubles fish meal; HSFM = high solubles fish meal; CGM = corn gluten meal; RSB = roasted soybeans.

protein came directly from AA without going through the NH_3 pool. Although the present experiment was designed to provide adequate energy, excess fermentable carbohydrate may have stimulated direct incorporation of AA into cellular protein. In our study, some of the microbial protein that apparently originated from NAN probably was derived from NH_3 produced from intracellular deamination of AA that did not equilibrate with extracellular $^{15}\text{NH}_3$.

Several groups (Bryant and Robinson, 1962; Maeng et al., 1976; Cotta and Russell, 1982; Russell et al., 1983) found that free AA and acid or enzymatic hydrolysates of casein stimulated microbial growth even when NH_3 and VFA were not limiting. Argyle and Baldwin (1989) reported up to a fourfold increase in microbial growth over the NH_3 control when AA mixtures or tryptic digests of casein were added to in vitro media. Means for net microbial N from each of our incubations were regressed on extent of degradation at 6 h for the seven proteins (Figure 3). These were highly correlated ($P < .001$; $r^2 = .780$), suggesting a stimulatory effect of peptides and free AA on microbial protein synthesis. A similar response in bacterial N formation was reported by Stokes et al. (1991) in continuous culture studies with mixed ruminal organisms when degradable protein was added as peanut meal. Net microbial N formation for the fish meals tended to be lower than that for the

other five proteins: all eight values for LSFM and HSFM fell below the regression line (Figure 3). Oil contents of fish meals in our study were (DM basis; Broderick, 1992) 6.4 (LSFM) and 9.9% (HSFM). Fish oil has large amounts of fatty acids longer than 18 carbons (Opstvedt, 1984); these may inhibit microbial growth (Galbraith et al., 1971). An inhibitory effect of fish oil may account for the suppression of net microbial N that we observed. Hussein et al. (1991) found that, compared with soybean meal, feeding fish meal to provide oil concentrations equivalent to five to six times those used in our study decreased bacterial N flow from continuous culture fermentors.

The relationship of net microbial N synthesis to degradation rate is shown in Figure 4. Microbial protein formation seemed to be a linear function of degradation rate when data were regressed from all proteins except casein ($P < .001$; $r^2 = .676$). Mean net microbial protein only increased from 4.6 (SSBM) to 5.5 (casein) mg of N/100 mL as degradation rate increased from .148 to .569/h. The regression of net protein synthesis on degradation rate using only the seven data points from casein and SSBM for which rates were available also was significant ($P = .093$; $r^2 = .468$). Intersection of the two regression lines at .14/h suggested that dietary proteins with degradabilities greater than this rate would give rise to little increase

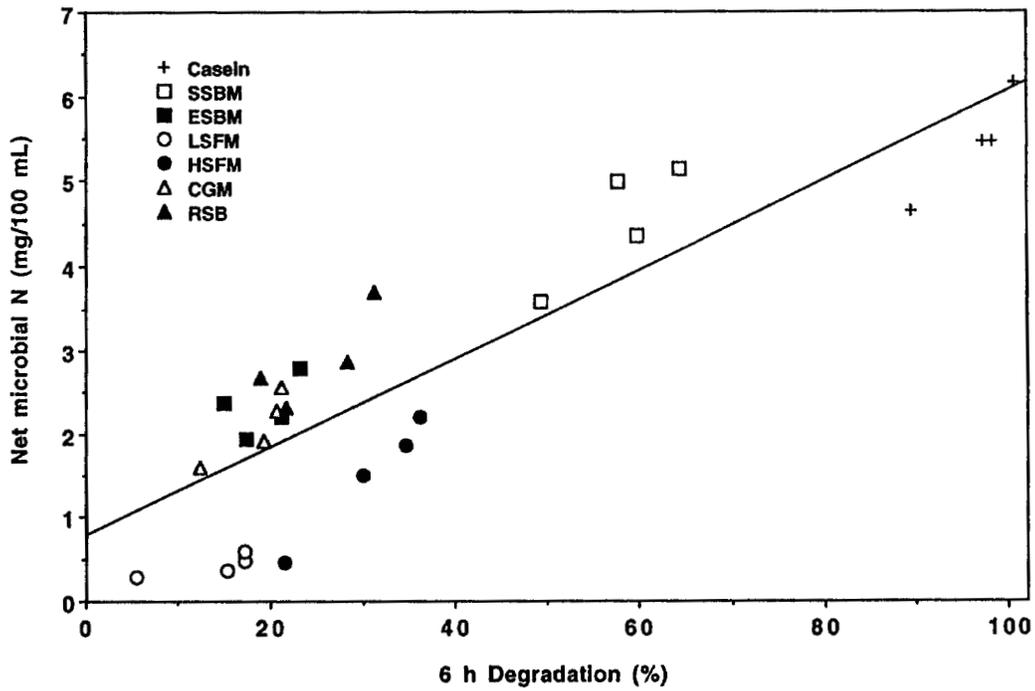


Figure 3. Regression of net synthesis of microbial protein N on extent of degradation at 6 h (the end of the incubation). $Y = .053 X + .778$ ($P < .001$; $r^2 = .780$). SSBM = solvent soybean meal; ESBM = expeller soybean meal; LSFM = low solubles fish meal; HSFM = high solubles fish meal; CGM = corn gluten meal; RSB = roasted soybeans.

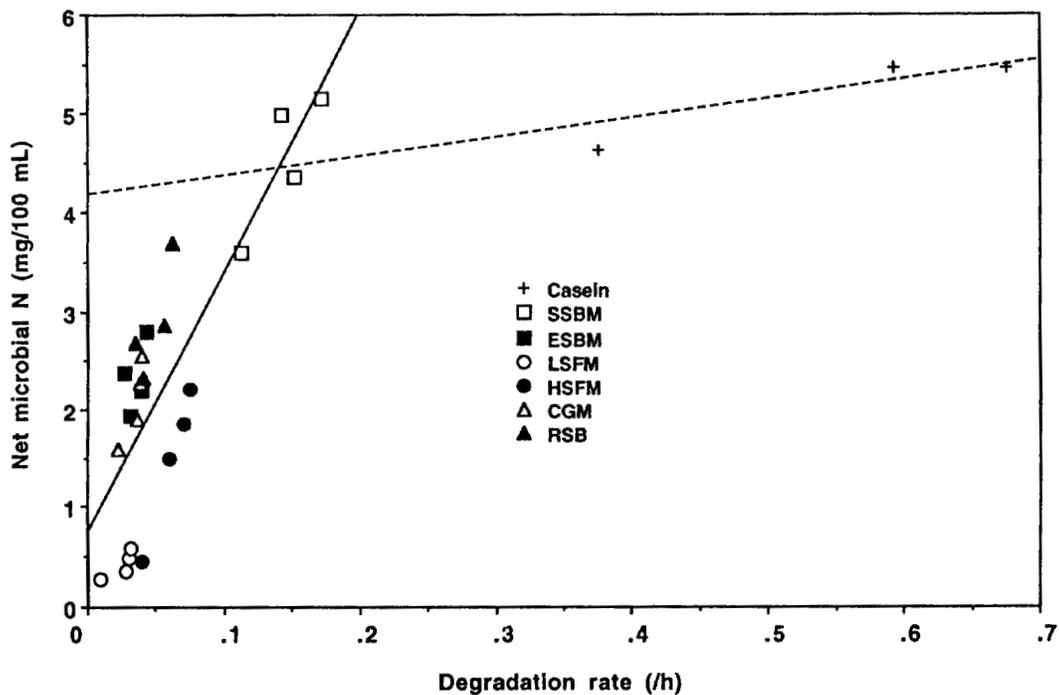


Figure 4. Regressions of net synthesis of microbial protein N on rates of protein degradation. Regressions are for all proteins except casein [$-$; $Y = 25.98 X + .779$ ($P < .001$; $r^2 = .676$)] and for SSBM and casein only [$- - -$; $Y = 1.97 X + 4.18$ ($P = .093$; $r^2 = .468$)]. SSBM = solvent soybean meal; ESBM = expeller soybean meal; LSFM = low solubles fish meal; HSFM = high solubles fish meal; CGM = corn gluten meal; RSB = roasted soybeans.

in microbial protein synthesis. Assuming a ruminal passage rate, $k_p = .06/h$, $k_d = .14/h$ corresponded to a mean ruminal escape of 30%. Tabular data of the NRC (table 6-4; 1989) indicated that the optimal undegraded intake protein, as a proportion of total dietary protein, for lactating dairy cows at a number of levels of production was approximately 37%.

Implications

Estimation of ruminal protein degradability from NH_3 release only, whether or not corrected for recovery of added $^{15}\text{NH}_3$, did not yield useful data because direct microbial incorporation of non- NH_3 N differed among proteins with different degradation rates. However, net release of NH_3 N plus net formation of microbial N, computed by subtracting blank concentrations, yielded satisfactory estimates of rate and extent of protein degradation for seven standard proteins. An in vitro method based on use of $^{15}\text{NH}_3$ to estimate microbial N may serve as a more theoretically sound procedure with which to compare other in vitro tests for protein degradability.

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