

# Preservation of Ruminal Microorganisms for In Vitro Determination of Ruminal Protein Degradation<sup>1,2</sup>

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**ABSTRACT:** Ruminal microorganisms, preserved either lyophilized or frozen, were compared with freshly strained ruminal fluid for proteolytic activity and as inoculum source for determination of ruminal protein degradation rates by the inhibitor in vitro method. Dialysis and glycerol addition had no effect on the proteolytic activity of preserved microorganisms. Net release of NH<sub>3</sub> and total amino acids from protein using the fluid plus particle-associated microorganisms was higher than that found using the fluid-associated microorganisms alone. Method of inoculum preservation altered total proteolytic activity, but harvesting bacteria using centrifugal force greater than 5,000 × *g* did not increase proteolytic activity of the pellet. The proposed method for harvesting and preserving microorganisms consisted of centrifuging strained ruminal fluid at 5,000 × *g* (30 min at 4°C),

stirring the pellet in a 50:50 (vol/vol) solution of glycerol-McDougall's buffer for 15 min, and then storing at -20°C. Protein degradation rates in incubations with preserved microorganisms were four to eight times slower than when using fresh ruminal fluid; however, feed proteins were ranked similarly for degradation rate. Preincubating the preserved microorganisms reduced blank concentrations of NH<sub>3</sub> and total amino acid and increased protein degradative activity of the preserved inoculum. Degradation rates with preincubated, preserved inocula were similar to those obtained using fresh ruminal fluid. These results indicated that mixed ruminal microorganisms can be preserved by freezing and, after a preincubation period of 6 h, used as the inoculum source for in vitro estimation of ruminal protein degradation.

Key Words: In Vitro, Ruminal Digestion, Protein Degradation, Preserved Microorganisms

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## Introduction

The NRC (1989) recommends in vivo estimation of ruminal protein degradation; however, that method is cumbersome and not appropriate for routine use (Stern et al., 1994). An enzymatic procedure would be rapid, easy to use and economical, but the degradative activities of several commercial proteases commonly used to assess ruminal protein degradation were found not to mimic that of mixed ruminal microbes (Luchini et al., 1996). Thus, results with commercial enzymes may be misleading. Broderick (1987) developed an

inhibitor in vitro (IIV) method that used strained ruminal fluid (SRF); however, use of SRF requires continued availability of cannulated animals. This may be inconvenient for commercial laboratories.

The objectives of Exp. 1 and 2 were to evaluate the effects of dialysis, addition of glycerol, and centrifugal force on the proteolytic activity of harvested, mixed ruminal microorganisms (MRM) and to compare two methods of preservation of MRM. The objectives of Exp. 3 were to reduce the NH<sub>3</sub> and total amino acid (TAA) concentrations in blanks and to increase ruminal proteolytic activity of preserved MRM. Experiment 4 was conducted to test the hypothesis that preincubation was required for preserved MRM to achieve protein degradative activity similar to that of fresh SRF.

## Materials and Methods

Whole ruminal contents were obtained from a ruminally cannulated lactating dairy cow approximately 2 h after being fed a diet of (g/kg DM) 600

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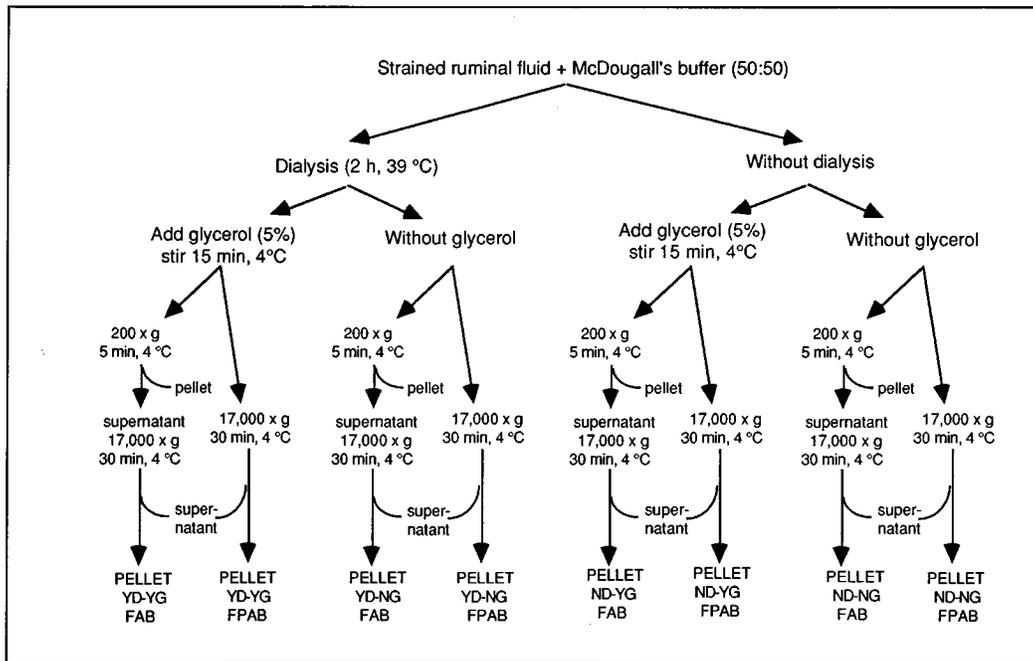


Figure 1. Ruminal fluid processing for Exp. 1; YD = dialyzed 2 h; ND = not dialyzed; YG = 5 mL glycerol per 100 mL SRF; NG = no addition of glycerol; FAB = fluid associated bacteria; FPAB = fluid plus particle associated bacteria. All pellets were lyophilized and stored at  $-20^{\circ}\text{C}$ .

alfalfa silage, 282 shelled cracked corn, 100 soybean meal, 11 dicalcium phosphate, and 7 trace mineral salt. Ruminal contents were squeezed through two layers of cheesecloth to obtain a given volume of SRF. To extract some of the particle-associated microorganisms, the remaining solids residue was washed four times with a total volume of pre-warmed ( $39^{\circ}\text{C}$ ) McDougall's buffer (McDougall, 1948) equal to the original volume of SRF. The SRF plus the buffer extract were mixed and filtered through eight layers of cheesecloth.

**Experiment 1.** The SRF plus buffer extract prepared as described above was divided into two portions (Figure 1): one was dialyzed against .9% (wt/vol) NaCl for 2 h in a  $39^{\circ}\text{C}$  warm room and the other was held under  $\text{O}_2$ -free  $\text{CO}_2$  (Hungate, 1969) at room temperature and processed immediately. The dialyzed and undialyzed portions were further divided into two equal aliquots. One aliquot was mixed with 5 mL of reagent-grade glycerol per 100 mL of SRF and stirred for 15 min in an ice water bath; the other aliquot was untreated. The glycerol-treated and untreated aliquots were allocated to two different centrifugation procedures to harvest the fluid plus particle-associated bacteria (**FPAB**) or only the fluid-associated bacteria (**FAB**). The FPAB were harvested as the pellet from centrifugation at  $17,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ . The FAB were harvested by differential centrifugation at  $200 \times g$  for 5 min at  $4^{\circ}\text{C}$ , discarding the sediment, then centrifuging the supernatant at  $17,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ . Microbial pellets from the FAB and FPAB

were frozen and lyophilized (model 24DX48, Virtis Co., Gardener, NY), and the resulting powders were stored at  $-20^{\circ}\text{C}$ . On the day of the incubation, lyophilized powder from each of the eight treatments was reconstituted to a volume equal to that of the original SRF using warm ( $39^{\circ}\text{C}$ ) McDougall's buffer containing 5 g of maltose/L. Reconstituted inocula were incubated with blanks or expeller soybean meal (**ESBM**), solvent soybean meal (**SSBM**), high-solubles fish meal (**HSFM**) and low-solubles fish meal (**LSFM**).

**Experiment 2.** Fresh SRF plus buffer extract was prepared as described in Exp. 1 and then separated into three portions that were centrifuged at 5,000, 17,000, or  $30,000 \times g$  for 30 min at  $4^{\circ}\text{C}$  (Figure 2). Hsu and Fahey (1990) suggested using centrifugation at  $4,640 \times g$  for 30 min to harvest ruminal microorganisms. The  $30,000 \times g$  was the highest force possible with the rotor used;  $17,000 \times g$  was selected as control because it was used in Exp. 1 and was a midpoint between the other two forces. Pellets obtained after each centrifugation were divided into two aliquots. A 50:50 (vol/vol) solution of glycerol-McDougall's buffer was added to one aliquot at 5% of the original volume (2.5% [vol/vol] glycerol final concentration), and the mixture was stirred for 20 min in an ice bath under  $\text{O}_2$ -free  $\text{CO}_2$ . The stirred pellet was transferred to a glass bottle and stored at  $-20^{\circ}\text{C}$ . The other aliquot was freeze-dried as in Exp. 1 and stored at  $-20^{\circ}\text{C}$ . On the day of the incubation, lyophilized powder or frozen pellet from each of the six treatments was

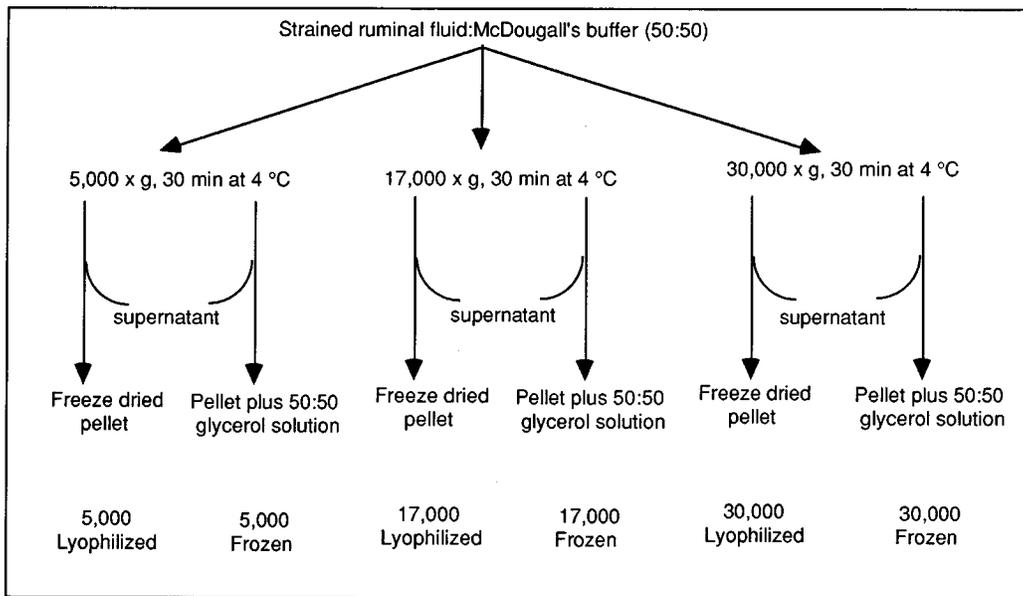


Figure 2. Ruminal fluid processing for Exp. 2; 5,000, 17,000, and 30,000  $\times g$  were the centrifugal forces used for collection of microbial pellets, which were either frozen or lyophilized.

reconstituted to a volume equal to that of the original SRF using warm (39°C) McDougall's buffer containing 5 g/L of maltose. Reconstituted inocula were used in incubations with blanks or ESBM, SSBM, HSBM, and LSM.

*Experiment 3.* The SRF plus buffer extract was prepared as described in Exp. 1 followed by centrifugation at 5,000  $\times g$  for 30 min at 4°C (Figure 3). The supernatant was discarded, and a 50:50 (vol/vol) solution of glycerol-McDougall's buffer was added to

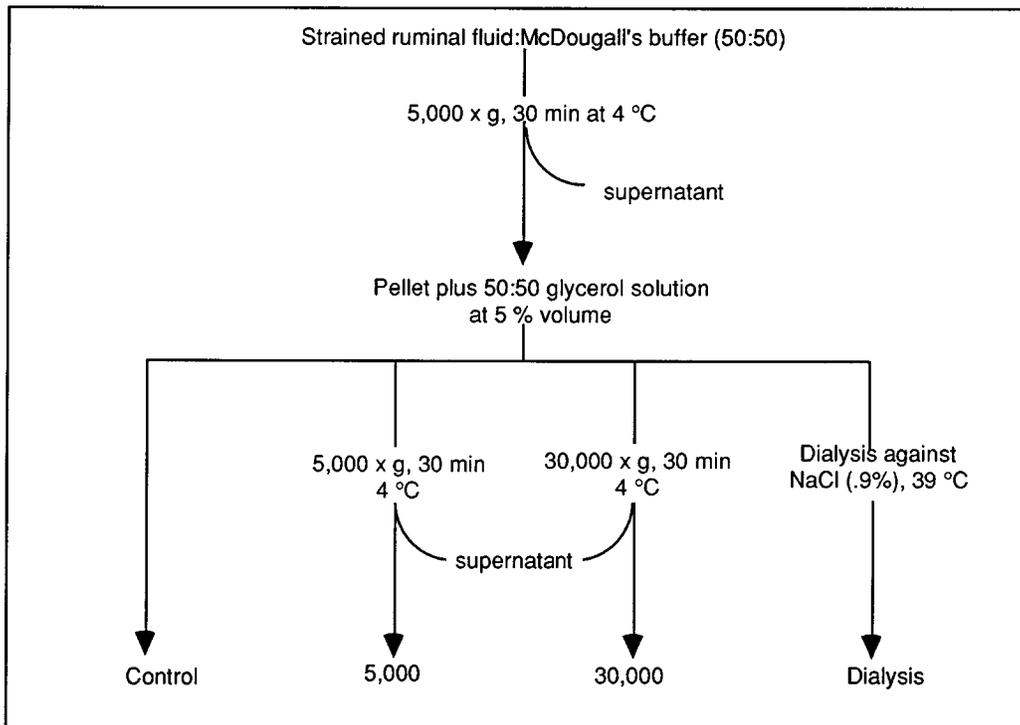


Figure 3. Ruminal fluid processing for Exp. 3; thawed microbial pellets were centrifuged at 5,000 or 30,000  $\times g$  or dialyzed against a .9% NaCl solution for 2 h.

Table 1. Components of the defined medium

Component	Amount, mL/L
Rumen buffer: <sup>a</sup> macromineral and micromineral solutions <sup>b</sup>	739
Pectin solution <sup>c</sup>	100
Soluble carbohydrates solution <sup>d</sup>	50
Vitamin solution <sup>e</sup>	100
VFA solution <sup>f</sup>	10
Hemin solution <sup>g</sup>	1
Mercaptoethanol <sup>h</sup>	.16

<sup>a</sup>From Goering and Van Soest (1970), except that  $\text{NH}_4\text{HCO}_3$  was replaced by an equimolar amount of  $\text{KHCO}_3$ .

<sup>b</sup>From Goering and Van Soest (1970); solution contained the following (g/L):  $\text{KHCO}_3$ , 1.28;  $\text{NaHCO}_3$ , 7.86;  $\text{Na}_2\text{HPO}_4$  anhydrous, 1.42;  $\text{KH}_2\text{PO}_4$  anhydrous, 1.54;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , .15;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , .0182;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , .0138;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , .0014;  $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$ , .011.

<sup>c</sup>Solution contained 2.65 g pectin diluted in 100 mL of heated (70°C) buffer-mineral solution and stirred vigorously for 1 h (Hristov and Broderick, 1994).

<sup>d</sup>Solution contained the following (g/50 mL buffer-mineral solution): maltose, .675; glucose, .337; sucrose, .337; starch, 2.5 (Hristov and Broderick, 1994).

<sup>e</sup>Vitamins were mixed as follows (mg/L): thiamine HCl, 20 mg; Ca-D-panthotenate, 20; nicotinamide, 20; riboflavin, 20; pyridoxine HCl, 20; *p*-aminobenzoic acid, 1; biotin, .5; folic acid, .125; vitamin B<sub>12</sub>, .2; tetrahydrofolic acid, .125 (Schaefer et al., 1980).

<sup>f</sup>The VFA were mixed as follows (mL/100 mL) and adjusted to pH 7 with NaOH: acetic acid, 17; propionic acid, 6; *n*-butyric acid, 4; isobutyric acid, 1; *n*-valeric acid, 1; iso-valeric acid, 1; DL- $\alpha$ -methylbutyric acid, 1 (Hespell and Bryant, 1981).

<sup>g</sup>Hemin, 100 mg, was diluted to 100 mL in a solution of 50 mL of 50% (vol/vol) ethanol and 50 mL of .05 N NaOH (Hespell and Bryant, 1981).

<sup>h</sup>Added as reducing agent (Broderick, 1987).

the MRM pellet at 5% of the original volume of the SRF plus buffer extract from which it was obtained. The mixture was stirred for 15 min in bottles placed in an ice bath under O<sub>2</sub>-free CO<sub>2</sub>. Stirred pellets were transferred to glass bottles and stored at -20°C. On the day of the incubation, the microbial pellets were thawed and left untreated (control), centrifuged at 5,000 × *g* or 30,000 × *g* for 30 min at 4°C, or dialyzed for 2 h at 39°C against a .9% NaCl solution (Figure 3). Pellets were reconstituted as for Exp. 1 and 2 and used as inocula in incubations with blanks or ESBM and SSBM.

**Experiment 4.** On the day of incubation, the MRM pellet prepared as the control from Exp. 3 was thawed at room temperature and reconstituted to a volume equivalent to the discarded supernatant using a warm (39°C) defined medium (Table 1) that contained the following: 1) macro- and micro-mineral buffer solution similar to that used by Goering and Van Soest (1981), except that  $\text{NH}_4\text{HCO}_3$  was replaced by an equimolar amount of  $\text{KHCO}_3$  (Hristov and Broderick, 1994); 2) a mixture of carbohydrates designed to support normal growth and fermentation (Hristov and Broderick, 1994); 3) a vitamin solution (Schaefer et al., 1980); 4) VFA and hemin solutions (Hespell and Bryant, 1981); and 5) mercaptoethanol as a reducing agent (Broderick, 1987). The reconstituted MRM was preincubated for 6 h in a warm (39°C) room in a flask flushed with O<sub>2</sub>-free CO<sub>2</sub>. During this time, the pH

was monitored hourly. Hourly aliquots from each flask were transferred to 12 × 75 mm disposable centrifuge tubes, trichloroacetic acid added to a final concentration of 5% (wt/vol), and tubes held for 30 min in an ice water bath (Broderick, 1987). Tubes then were centrifuged for 20 min at 10,000 × *g* at 4°C and supernatants analyzed for NH<sub>3</sub> and TAA (Broderick and Kang, 1980). After the 6-h preincubation period, a second MRM pellet was thawed and reconstituted with warm (39°C) McDougall's buffer to which maltose (5 g/L) also was added. The 0 h and 6 h preincubated MRM inocula were used in incubations with blanks, ESBM, and SSBM. These protein sources were selected because they are routinely used in our laboratory as standard proteins for the IIV incubations with fresh SRF.

All experiments were repeated three times, and incubations were performed in duplicate 50-mL plastic centrifuge tubes to which proteins were added at .125 mg N/mL incubation media (Broderick, 1987). Proteins were soaked with warm (39°C) McDougall's buffer or the defined media for 45 min before the addition of the inoculum (Broderick, 1987). Chloramphenicol (**CAP**) and hydrazine sulfate (**HS**) were added to the inoculum at 30 μg/mL and 1 mM final medium, respectively, 10 min before incubation (Broderick, 1987). Tubes were dosed with inoculum, flushed with O<sub>2</sub>-free CO<sub>2</sub>, and capped with rubber stoppers with Bunsen valves. Incubations were performed in a 39°C warm room for 0 and 6 h for Exp. 1 and 2, for 0 and 4 h for Exp. 3, and 0 and 4 h or 0, 1, 2, 4 and 6 h for fresh SRF or preserved MRM, respectively, in Exp. 4. Incubations were stopped by adding trichloroacetic acid at a final concentration of 5% (wt/vol) and placing tubes in an ice bath for approximately 30 min. Aliquots from each tube were transferred to 15 × 75 mm disposable test tubes and centrifuged at 15,000 × *g* for 20 min at 4°C. Supernatants were analyzed for NH<sub>3</sub> and TAA (Broderick and Kang, 1980). Degradation rates were computed as for the IIV method of Broderick (1987).

Data from Exp. 1, 2 and 3 were analyzed using ANOVA by the GLM procedure of SAS (1985). Data from Exp. 1 were analyzed as a split-plot in a randomized complete block design, with dialysis as the whole plot and glycerol and differential centrifugation in a 2 × 2 factorial arrangement of treatments as the subplot (Steel and Torrie, 1980). Data from Exp. 2 were analyzed as a split-plot design, with centrifugal force (5,000, 17,000, or 30,000 × *g*) as the whole plot and storage method (frozen or lyophilized) as the split-plot (Steel and Torrie, 1980). Experiments 3 and 4 were analyzed as randomized complete block designs, and the model included block and treatment; treatment means were separated using a protected least significant difference test (Steel and Torrie, 1980). For Exp. 4, a two-tail, unequal variance *t*-test (Steel and Torrie, 1980) was performed to compare NH<sub>3</sub> and TAA concentration in blanks after 0 and 6 h

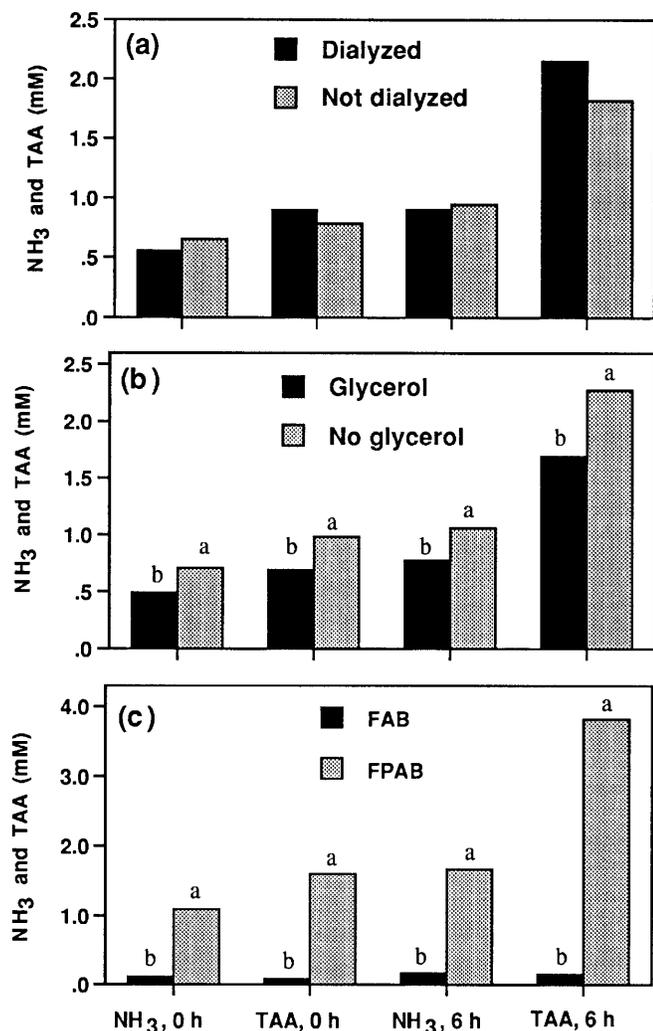


Figure 4. Effect of (a) dialysis, (b) glycerol addition, and (c) use of fluid associated bacteria (FAB) or fluid plus particle associated bacteria (FPAB) on concentrations of NH<sub>3</sub> and total amino acids (TAA) in blanks after 0 and 6 h of incubation. Dialysis was performed for 2 h; 5 mL of glycerol was added per 100 mL of SRF. The FAB and FPAB were harvested by differential centrifugation. The SEM for panels (a) and (b) were .03, .06, .05, and .13 for NH<sub>3</sub> (0 h), TAA (0 h), NH<sub>3</sub> (6 h), and TAA (6 h), respectively; SEM for panel (c) were .04, .03, .05, and .08 for NH<sub>3</sub> (0 h), TAA (0 h), NH<sub>3</sub> (6 h), and TAA (6 h), respectively. Means with different superscripts differ ( $P < .05$ ) (Exp. 1).

of preincubation. Unless otherwise noted, values were considered significant when  $P < .05$ .

## Results and Discussion

There were no significant interactions for any of the variables tested in Exp. 1; therefore, only the main effects will be discussed. Dialysis had no effect on the

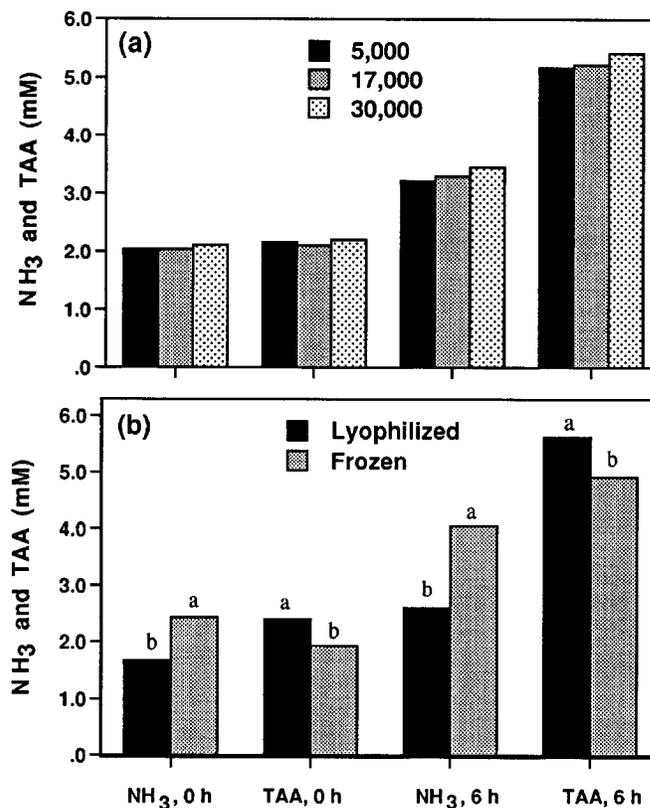


Figure 5. Effect of (a) centrifugation speed and (b) lyophilization or freezing on concentrations of NH<sub>3</sub> and total amino acids (TAA) in blanks after 0 or 6 h of incubation with preserved ruminal microorganisms. Strained rumen fluid was centrifuged at 5,000, 17,000, or 30,000  $\times g$  for 30 min, and pellets were either lyophilized or mixed with a 50:50 (vol/vol) solution of glycerol-McDougall's buffer at 5% its original volume and stored at  $-20^{\circ}\text{C}$ . The SEM for panel (a) were .07, .09, .07, and .13 for NH<sub>3</sub> (0 h), TAA (0 h), NH<sub>3</sub> (6 h), and TAA (6 h), respectively; SEM for panel (b) were .04, .07, .05, and .04 for NH<sub>3</sub> (0 h), TAA (0 h), NH<sub>3</sub> (6 h), and TAA (6 h), respectively. Bars with different superscripts differ ( $P < .05$ ) (Exp. 2).

NH<sub>3</sub> and TAA concentrations in the blanks at 0 h or 6 h of incubation (Figure 4a). The increase in TAA concentrations after the 6-h incubations indicated there had been proteolysis of nitrogenous substrates from the pellet and confirmed the inhibitory effect of CAP and HS on the uptake of end products of protein degradation by the ruminal microbes (Broderick, 1987). Glycerol, when added to the microbial pellet, was an effective cryoprotectant, reducing the concentration of both NH<sub>3</sub> and TAA at 0 and 6 h (Figure 4b). Microbial cells can be protected from freeze-thaw injury by using compounds that are soluble, nontoxic, and able to penetrate the cells, thereby acting to prevent increased electrolyte concentrations during freezing (Fennema, 1973). There are many

Table 2. Effect of dialysis, glycerol addition, and centrifugal force on net release<sup>a</sup> (mM) of NH<sub>3</sub> and total amino acids (TAA) from expeller soybean meal (ESBM), solvent soybean meal (SSBM), high solubles fish meal (HSFM), and low solubles fish meal (LSFM) after 0 and 6 h of incubation (Exp. 1)

Feed sample	Dialysis <sup>b</sup>			Glycerol addition <sup>c</sup>		Centrifugal force		
	Yes	No	SEM	Yes	No	FAB <sup>d</sup>	FPAB <sup>e</sup>	SEM
<b>ESBM</b>								
NH <sub>3</sub> , 0 h	.006	.002	.003	.004	.004	.007	<.001	.006
TAA, 0 h	.015	.011	.004	.022	.005	.021	.006	.005
NH <sub>3</sub> , 6 h	.067	.058	.006	.064	.063	.024 <sup>g</sup>	.102 <sup>f</sup>	.005
TAA, 6 h	.182	.168	.021	.187	.163	.045 <sup>g</sup>	.305 <sup>f</sup>	.019
<b>SSBM</b>								
NH <sub>3</sub> , 0 h	-.004	-.005	.004	-.003	-.006	.007 <sup>g</sup>	-.016 <sup>f</sup>	.007
TAA, 0 h	.038 <sup>f</sup>	-.018 <sup>g</sup>	.002	.036	.020	.044	.012	.011
NH <sub>3</sub> , 6 h	.076	.075	.007	.077	.075	.030 <sup>g</sup>	.120 <sup>f</sup>	.012
TAA, 6 h	.230	.197	.033	.224	.204	.074 <sup>g</sup>	.350 <sup>f</sup>	.048
<b>HSFM</b>								
NH <sub>3</sub> , 0 h	.057	.050	.002	.054	.052	.060	.047	.005
TAA, 0 h	.115	.110	.002	.117	.108	.115	.109	.005
NH <sub>3</sub> , 6 h	.186	.140	.039	.190	.136	.098 <sup>g</sup>	.229 <sup>f</sup>	.034
TAA, 6 h	.329	.320	.038	.339	.313	.160 <sup>g</sup>	.492 <sup>f</sup>	.032
<b>LSFM</b>								
NH <sub>3</sub> , 0 h	.037	.031	.004	.035	.033	.043	.025	.008
TAA, 0 h	.064	.057	.002	.067	.055	.067	.054	.010
NH <sub>3</sub> , 6 h	.115	.106	.003	.115	.106	.071 <sup>g</sup>	.150 <sup>f</sup>	.008
TAA, 6 h	.240	.210	.017	.230	.230	.107 <sup>g</sup>	.355 <sup>f</sup>	.017

<sup>a</sup>Blank corrected.  
<sup>b</sup>Dialyzed for 2 h at 39°C against .9% (wt/vol) NaCl.  
<sup>c</sup>Glycerol-McDougall's solution (50:50) added at 5% (vol/vol) of original volume.  
<sup>d</sup>Fluid associated bacteria harvested by differential centrifugation (Figure 1).  
<sup>e</sup>Fluid plus particle associated bacteria harvested after a 17,000 × g, 30-min centrifugation.  
<sup>f,g</sup>Means in paired columns with different superscripts differ (P < .05).

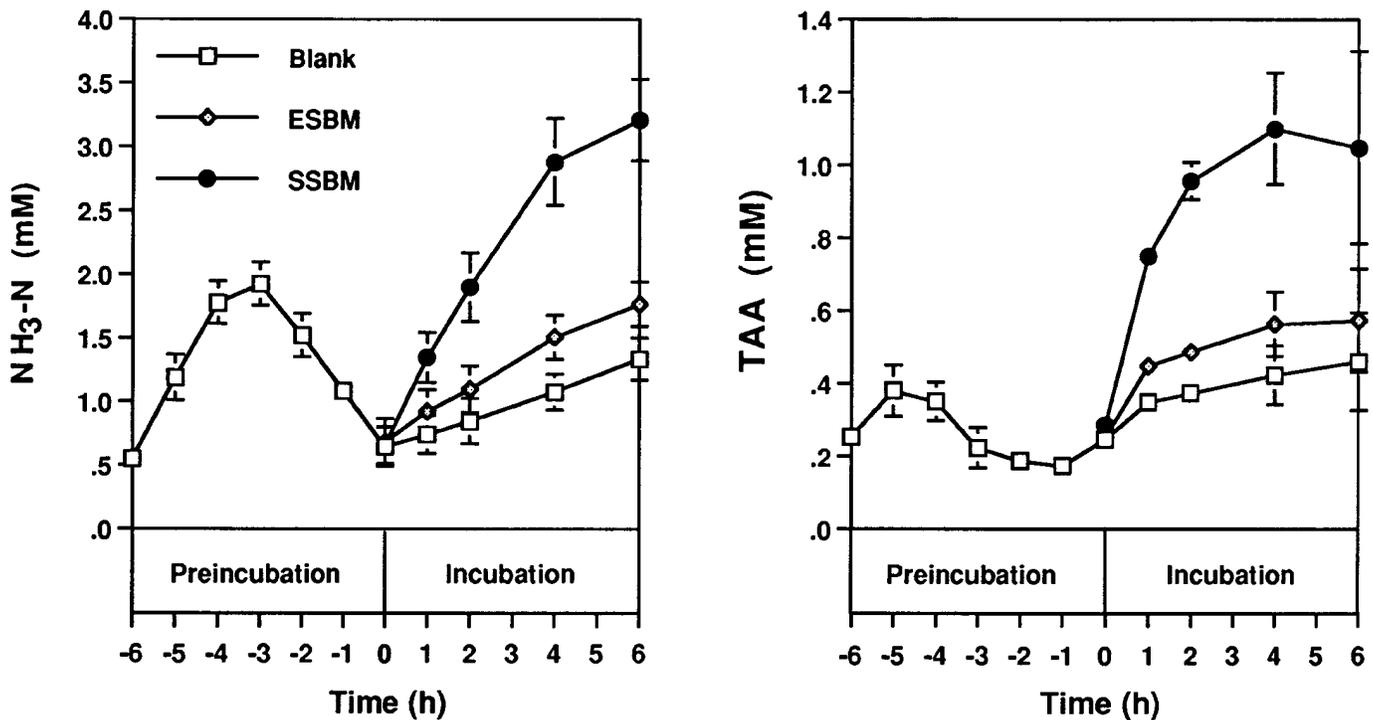


Figure 6. Concentrations of NH<sub>3</sub> and total amino acids (TAA) during the preincubation and incubation periods with blank, expeller soybean meal (ESBM), and solvent soybean meal (SSBM) using freeze-stored mixed ruminal microorganisms. Error bars correspond to ± 1 SD (Exp. 4).

Table 3. Effect of centrifugal force and storage method on net release<sup>a</sup> (mM) of ammonia (NH<sub>3</sub>) and total amino acids (TAA) from expeller soybean meal (ESBM), solvent soybean meal (SSBM), high solubles fish meal (HSFM), and low solubles fish meal (LSFM) after 0 and 6 h incubations (Exp. 2)

Feed sample	Centrifugal force (g) <sup>b</sup>			SEM	Storage method <sup>cd</sup>		SEM
	5,000	17,000	30,000		Lyophilized	Frozen	
<b>ESBM</b>							
NH <sub>3</sub> , 0 h	.016	.014	.013	.004	.010	.019	.004
TAA, 0 h	.015	.025	.031	.008	.016 <sup>f</sup>	.031 <sup>e</sup>	.003
NH <sub>3</sub> , 6 h	.236	.201	.205	.029	.159 <sup>f</sup>	.270 <sup>e</sup>	.032
TAA, 6 h	.337	.310	.317	.028	.348	.294	.025
<b>SSBM</b>							
NH <sub>3</sub> , 0 h	.022	.025	.016	.003	.017	.024	.006
TAA, 0 h	.049	.062	.060	.008	.066 <sup>e</sup>	.048 <sup>f</sup>	.004
NH <sub>3</sub> , 6 h	.321	.225	.257	.021	.229	.306	.052
TAA, 6 h	.656	.582	.618	.036	.582	.656	.032
<b>HSFM</b>							
NH <sub>3</sub> , 0 h	.089	.076	.078	.009	.076	.086	.006
TAA, 0 h	.109	.121	.122	.009	.113	.122	.008
NH <sub>3</sub> , 6 h	.272	.248	.272	.028	.238	.290	.026
TAA, 6 h	.788	.751	.747	.061	.629 <sup>f</sup>	.895 <sup>e</sup>	.040
<b>LSFM</b>							
NH <sub>3</sub> , 0 h	.073	.066	.064	.004	.060	.076	.007
TAA, 0 h	.062	.077	.079	.008	.083	.063	.006
NH <sub>3</sub> , 6 h	.250 <sup>e</sup>	.177 <sup>f</sup>	.157 <sup>f</sup>	.016	.179	.210	.026
TAA, 6 h	.584	.470	.489	.060	.416 <sup>f</sup>	.613 <sup>e</sup>	.048

<sup>a</sup>Blank corrected.

<sup>b</sup>Samples centrifuged at 5,000, 17,000, or 30,000 × g for 30 min at 4°C.

<sup>c</sup>Lyophilized bacterial pellets stored at -20°C.

<sup>d</sup>Frozen bacterial pellets stored at -20°C after addition of a 50:50 glycerol-McDougall's solution at 5% (vol/vol) of original volume.

<sup>e,f</sup>Means within centrifugal force or storage method with different superscripts differ ( $P < .05$ ).

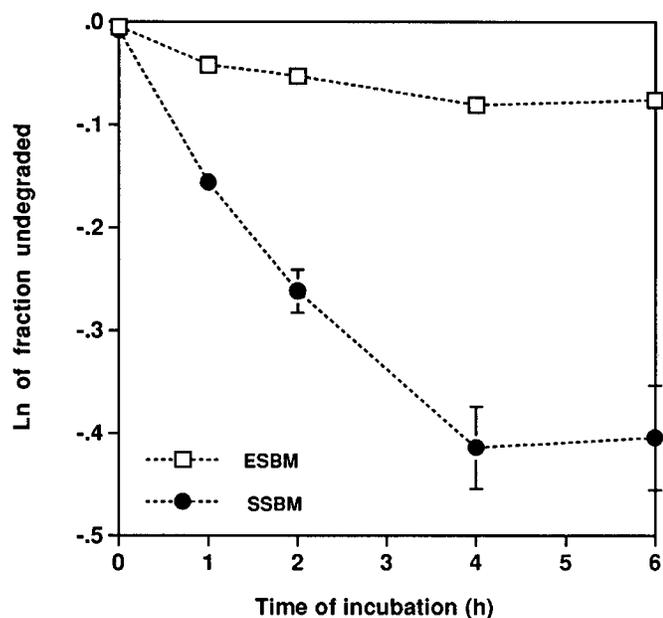


Figure 7. Degradation curves of expeller soybean meal (ESBM) and solvent soybean meal (SSBM) in incubations using freeze-stored mixed ruminal microorganisms (MRM). Error bars correspond to  $\pm 1$  SD (Exp. 4).

cryoprotectant compounds in use (e.g., egg white, corn syrup, rice flour, sucrose); however, the most commonly used is glycerol (Fennema, 1973). Microbial fractionation through differential centrifugation had a marked effect on NH<sub>3</sub> and TAA concentrations in the blanks (Figure 4c). The FAB pellet had lower NH<sub>3</sub> and TAA concentrations than FPAB at both 0 and 6 h. Moreover, from 0 to 6 h, blank NH<sub>3</sub> and TAA concentrations increased from .11 and .08 mM, respectively, to .17 and .16 mM in the FAB inocula, and from 1.1 and 1.6 mM to 1.7 and 3.8 mM in the FPAB inocula. These results suggest that the FPAB pellet contained greater quantities of nitrogenous substances that were degraded by the ruminal microorganisms.

Net release of NH<sub>3</sub> and TAA from degradation of feed proteins after 0 and 6 h of incubation is shown in Table 2. Net release of NH<sub>3</sub> and TAA from the feeds at 0 h, which represents fraction A (protein degraded at 0 h), was not affected by dialysis, glycerol addition or centrifugation force. Dialysis and glycerol addition did not affect net release of NH<sub>3</sub> or TAA after 6 h of incubation from any of the feeds. However, greater degradative activity was found in FPAB pellets than in FAB pellets. Greater net release of both NH<sub>3</sub> and TAA from all tested feeds with FPAB suggested that microbial proteolytic activity was associated mainly with the feed particles. However, concentrations of

Table 4. Concentrations of NH<sub>3</sub> and total amino acids (TAA) at 0 or 6 h in blank, expeller soybean meal (ESBM) and solvent soybean meal (SSBM) incubations using preserved ruminal microorganisms (Exp. 4)

Feed sample	Control <sup>a</sup>	Dialysis <sup>b</sup>	Centrifugal force ( <i>g</i> ) <sup>c</sup>		SEM
			5,000	30,000	
Total concentration (mM)					
Blanks					
NH <sub>3</sub> , 0 hr	1.01	.780	.790	.760	.076
TAA, 0 hr	.780 <sup>f</sup>	1.27 <sup>e</sup>	.642 <sup>f</sup>	.663 <sup>f</sup>	.085
NH <sub>3</sub> , 6 hr	2.77 <sup>e</sup>	1.52 <sup>f</sup>	2.08 <sup>f</sup>	1.93 <sup>f</sup>	.193
TAA, 6 hr	4.02 <sup>e</sup>	2.82 <sup>f</sup>	3.07 <sup>f</sup>	3.00 <sup>f</sup>	.148
Net concentration (mM) <sup>d</sup>					
ESBM					
NH <sub>3</sub> , 0 hr	.020	.013	.005	.003	.006
TAA, 0 hr	.023	.018	.022	.018	.005
NH <sub>3</sub> , 6 hr	.303	.137	.168	.222	.063
TAA, 6 hr	.267	.205	.168	.225	.063
SSBM					
NH <sub>3</sub> , 0 hr	.017	.012	.003	.008	.006
TAA, 0 hr	.050	.050	.050	.042	.006
NH <sub>3</sub> , 6 hr	.275	.221	.325	.272	.082
TAA, 6 hr	.507	.393	.475	.420	.060

<sup>a</sup>Microbial pellet was reconstituted as in the text and used as inoculum.

<sup>b</sup>Microbial pellet was dialyzed for 2 h against a .9% (wt/vol) NaCl solution, then reconstituted and used as inoculum.

<sup>c</sup>Microbial pellets were centrifuged at 5,000 or 30,000 × *g* for 30 min at 4°C, and pellets were reconstituted and used as inocula.

<sup>d</sup>Blank corrected.

<sup>e,f</sup>Means in rows with different superscripts differ (*P* < .05).

NH<sub>3</sub> and TAA in the 0 and 6 h blanks in the FPAB incubations were between three to nine times greater than the net release of these metabolites from the feeds (Figure 4, Table 2). These relatively high blank concentrations are undesirable because ruminal protein degradation of feed proteins is computed from net release of NH<sub>3</sub> and TAA.

There were no significant interactions for any of the variables tested in Exp. 2; therefore, only the main effects will be discussed. The NH<sub>3</sub> and TAA concentrations in the blanks were not affected by centrifugal force (Figure 5a); NH<sub>3</sub> concentrations, however, were lower at 0 and 6 h, and TAA concentrations were higher at 6 h, for lyophilized than for frozen bacterial pellet (Figure 5b). Higher NH<sub>3</sub> concentration in the frozen pellets may be expected because centrifugation would not remove all the NH<sub>3</sub> and some NH<sub>3</sub> probably

was volatilized during lyophilization. Higher TAA concentration in the lyophilized pellet at 0 h indicated that some microbial lysis occurred. As in Exp. 1, NH<sub>3</sub> and TAA concentrations in the blanks were high relative to their net release from feeds (Figure 5a and b; Table 3). The increase in concentration of both NH<sub>3</sub> and TAA in the blanks during the 6-h incubation period indicated that proteolysis of nitrogenous compounds in the pellet took place during that time. As in the blanks, centrifugal force had no effect on net release of NH<sub>3</sub> or TAA at 0 or 6 h from any of the feeds tested (Table 3). For some feeds, however, there was greater net release of these metabolites after the 6-h incubation period using inoculum prepared from the frozen pellet. These results indicated that ruminal microorganisms may be harvested at the lower centrifugal force of 5,000 × *g* and can be stored frozen instead of being lyophilized.

Table 5. Concentrations of NH<sub>3</sub> and total amino acid (TAA) in blanks from incubations with preserved, mixed ruminal microorganisms preincubated for 0 or 6 h

Preincubation time	Blanks incubated for			
	0 h		4 h	
	NH <sub>3</sub>	TAA	NH <sub>3</sub>	TAA
0 h	1.00	.90	2.06	2.87
6 h	.64	.24	1.07	.42
<i>P</i> <	.005	.001	.001	.001

Table 6. Fraction A<sup>a</sup> and rate and extent of degradation of fraction B<sup>a</sup> from expeller soybean meal and solvent soybean meal using the inhibitor in vitro method in incubations with preserved mixed ruminal microorganisms (MRM), preincubated for 0 or 6 h, or with fresh strained ruminal fluid (SRF)

Feed	Preserved MRM		SRF	SEM
	Preincubation time			
	0 h	6 h		
	Fraction A (%)			
Expeller soybean meal	.61	.50	.70	.49
Solvent soybean meal	.75	.80	.87	.23
	Degradation rate (h <sup>-1</sup> )			
Expeller soybean meal	.010 <sup>e</sup>	.019 <sup>d</sup>	.038 <sup>c</sup>	.001
Solvent soybean meal	.023 <sup>e</sup>	.101 <sup>d</sup>	.166 <sup>c</sup>	.006
	Extent of degradation (%)			
Expeller soybean meal	16.5 <sup>e</sup>	24.3 <sup>d</sup>	39.0 <sup>c</sup>	1.6
Solvent soybean meal	27.8 <sup>d</sup>	63.0 <sup>c</sup>	71.0 <sup>c</sup>	2.2

<sup>a</sup>Computed as described by Broderick (1987).

<sup>c,d,e</sup>Means in rows with different superscripts differ ( $P < .05$ ).

Results from Exp. 3 are in Table 4. There were no differences in NH<sub>3</sub> concentrations among treatments, but the dialysis treatment gave rise to higher TAA concentrations in the blanks at 0 h. This may indicate that during the 2-h period, high-molecular-weight polypeptides were generated that were not removed by dialysis. However, after the 6-h incubation, all three treatments were equally effective in reducing the NH<sub>3</sub> and TAA concentrations in the blanks compared with the control. Net release of NH<sub>3</sub> and TAA from SSBM was greater than from ESBM; however, blank concentrations were relatively high in all treatments. Net release of NH<sub>3</sub> and TAA from ESBM and SSBM from all three treatments after 0 or 6 h was not different from that of the control. Thus, total proteolytic activity was not increased by any of these treatments.

As depicted in Figure 6, the NH<sub>3</sub> and TAA concentrations of the inoculum changed during preincubation; both increased during the first 2 to 3 h, then decreased to about the original concentrations after 5 and 6 h of preincubation in Exp. 4. Increased concentrations after 2 and 3 h of preincubation could be due to degradation of protein present in the MRM pellet or to bacterial lysis. Decreased concentration of both NH<sub>3</sub> and TAA from 3 to 6 h of preincubation suggested net bacterial N uptake. The medium (Table 1) provided all the nutrients thought to be required for bacterial growth except N; thus, N requirements for the microbial population would have been supplied from components present in the MRM pellet or from autolysis of the microbial biomass. Results obtained after 6-h incubations of blank, ESBM and SSBM samples also are shown in Figure 6. Increased NH<sub>3</sub> and TAA concentrations in blanks likely reflected the inhibition by CAP and HS of bacterial uptake of NH<sub>3</sub> and TAA released from cell lysis and proteolysis. As expected, the SSBM had higher release of TAA and

NH<sub>3</sub> than ESBM, reflecting its greater degradability.

Plots of the natural log of the fraction undegraded (FUD) from 0 to 6 h obtained from incubations with ESBM and SSBM indicated that the degradative activity of the inoculum diminished after 4 h (Figure 7). Reduced activity with time may be due to end product inhibition or to microbial lysis in the absence of microbial growth because of the presence of the inhibitors (Broderick, 1987). However, decreased activity also may have been a function of time after CAP and HS addition. Despite substantial differences in end product accumulation between ESBM and SSBM, degradation activity toward both proteins was diminished after 4 h of incubation.

Blank NH<sub>3</sub> and TAA concentrations after 0 and 4 h of incubation were lower for preincubated MRM (Table 5;  $P < .005$ ). This suggested that, during the preincubation period, N substrates were utilized for microbial growth. When a microbial population is inoculated into fresh medium, a lag phase usually occurs before rapid growth begins because the cells may be depleted of essential coenzymes or other required constituents (Brock, 1979). Also, a lag phase may ensue when a population is transferred from a richer to a poorer culture medium, or because the inoculum contains cells that have been damaged and time is required for cellular repair (Brock, 1979). Comparisons of fraction A and rate and extent of degradation obtained with preserved MRM, preincubated for 0 or 6 h, and fresh SRF are shown in Table 6. As expected, there were no differences in fraction A; however, degradation rates and extents were different among treatments. Degradation rates of ESBM and SSBM obtained in incubations with fresh SRF were four and eight times more rapid, respectively, than for MRM preincubated for 0 h, and two and 1.6 times more rapid than for MRM preincubated for 6 h. Thus,

preincubation of the reconstituted MRM had two beneficial effects: 1) reduced blank concentrations, which would improve precision of measurement of degradation products; and 2) increased rates and extents of protein degradation, which indicated that microbial activity was more similar to that of fresh SRF.

### Implications

These results indicated that ruminal microbes can be harvested by centrifugation at relatively slow speed ( $5,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ ) and the sedimented mixed ruminal microorganisms preserved at  $-20^{\circ}\text{C}$  until thawed, reconstituted, and used as the inoculum for determining protein degradability. After reconstitution, frozen mixed ruminal microorganisms required preincubation to enhance degradative activity. Preincubation both reduced blank  $\text{NH}_3$  and total amino acid concentrations and increased rate and extent of microbial degradation of expeller soybean meal and solvent soybean meal to values comparable to those of fresh strained ruminal fluid. Preserved, preincubated mixed ruminal microorganisms may be useful as the *in vitro* inoculum for determining the rank-ordering of feeds with respect to ruminal protein degradability.

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