In Vitro Determination of Ruminal Protein Degradation Using Freeze-Stored Ruminal Microorganisms[1,2]

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ABSTRACT: Mixed ruminal microorganisms were harvested from a lactating dairy cow and preserved frozen or lyophilized. Fermentation characteristics of freshly strained ruminal fluid, frozen microorganisms, or lyophilized microorganisms were evaluated during a 24-h pre-incubation and a 4-h incubation with test proteins. Differences observed during the first 4 to 6 h in total amino acid concentration, optical density, pH and VFA concentrations, acetate:propionate ratio, and lactate concentration largely disappeared later in the pre-incubation. Protein degradation rates determined for expeller and solvent meals were .015 and .092 h^{-1}, .015 and .101 h^{-1}, and .005 and .019 h^{-1}, with fresh ruminal fluid, frozen microorganisms, and lyophilized microorganisms, respectively. Regression of degradation rates obtained with fresh ruminal fluid on those obtained with pre-incubated, frozen microorganisms indicated the two methods were well correlated (r^2 = .98 and .94 in two experiments). Mean in vitro degradability obtained for 17 feeds using pre-incubated, frozen microbes was 89% of that obtained using the in situ method; however, in situ degradation rates for these same feeds averaged only 67% of those obtained using frozen microorganisms. Ruminal undegraded protein values for nine samples of heated soybeans and soybean meal, determined using frozen microbes, were overestimated relative to in vivo values (in vivo = 1.1 + .8 in vitro; r^2 = .77). These results indicated that ruminal microorganisms can be preserved by freezing and used as the inoculum for in vitro determination of ruminal protein degradation after overnight pre-incubation.

Key Words: In Vitro, In Situ, Rumen, Protein Degradation, Preserved Microbes

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Introduction

A reliable in vitro method for determining ruminal protein degradation would be valuable because of greater speed and lower expense than in vivo and in situ methods. We developed a method for preservation of mixed ruminal microorganisms (**MRM**; Luchini et al., 1996) to reduce day-to-day variation in the degradative activity of the inoculum and to minimize the need for ruminally cannulated donor cows. A pre-incubation period for the reconstituted MRM inoculum was used to increase its proteolytic activity; however, the optimum length of pre-incubation time, and changes in the fermentation characteristics, were not studied. The objectives of Exp. 1 were to compare inocula prepared from MRM preserved using two different methods with inocula prepared from fresh strained ruminal fluid (**SRF**). These inocula were compared for fermentation activity over a 24-h pre-incubation period and for protein degradation activity after an 18-h pre-incubation period. Experiments 2, 3, and 4 were conducted to compare the rate and extent of ruminal degradation for a number of feed proteins, determined in vitro using pre-incubated frozen MRM with those determined in vitro, using fresh SRF, and determined by in situ and in vivo methods.

Materials and Methods

Frozen MRM preparation

Whole ruminal contents were obtained from a ruminally cannulated lactating dairy cow about 2 h

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after it was fed a diet of (g/kg DM) 600 alfalfa silage, 282 shelled cracked corn, 100 soybean meal, 11 dicalcium phosphate, and 7 trace mineralized salt. The SRF was obtained by squeezing the ruminal contents through two layers of cheesecloth to yield a given volume of SRF. To extract some of the particle-associated microorganisms, the remaining solids residue was washed four times with four equal volumes of pre-warmed (39°C) McDougall's buffer (1948); the total volume of buffer used to wash the solids was equal to the original volume of SRF. The SRF and buffer extract were mixed then filtered through eight layers of cheesecloth. The MRM were harvested by transferring 400-mL aliquots of the filtrate to 500-mL plastic centrifuge bottles, capping, and centrifuging at 5,000 × *g* for 30 min at 4°C. The supernatants were discarded, the pellets were re-suspended in 10 mL of reagent-grade glycerol, added as a cryoprotectant (Fennema, 1973), and stored at –80°C (frozen MRM).

Experiment 1

Frozen and lyophilized MRM were compared to fresh SRF in this experiment. Typically, 4.8 L of fresh SRF plus buffer extract was prepared. Half (2.4 L) was processed and stored as frozen MRM as described and half (2.4 L) was processed as lyophilized MRM as follows: reagent-grade glycerol was added at 5% of the volume of SRF plus buffer extract, the mixture was stirred for 30 min, then transferred to 500-mL bottles and centrifuged as described above. Supernatants were discarded and pellets were frozen and lyophilized (lyophilized MRM) using a Virtis model 24DX48 freeze drier (The Virtis Co., Gardener, NY), and stored at –20°C. This experiment had two phases: Phase 1 evaluated fermentation of the inocula during a 24-h pre-incubation period and Phase 2 evaluated protein degradative activity of the inocula.

Phase 1. On the day of the experiment, frozen MRM pellets and lyophilized MRM powder were reconstituted in defined media as described by Luchini et al. (1996). Whole ruminal contents were processed and fresh SRF plus buffer extract (fresh SRF) was prepared as described except that extraction was done using the defined medium rather than McDougall's buffer. Concentration of carbohydrates (**CHO**) added to the medium used to wash the ruminal contents was increased two-fold to equalize fermentable energy among the three inocula. Concentrations of VFA, vitamins, macro- and microminerals were not increased because it was assumed that the SRF would contain these nutrients in concentrations similar to the defined medium.

Fresh SRF, frozen MRM, and lyophilized MRM inocula were pre-incubated in a 39°C room for 24 h in duplicate 250-mL spinner flasks equipped with screw-capped side-arms (Bellco, Vineland, NJ) and Bunsen valves. Samples were taken from one side-arm of the spinner flask as O_2-free CO_2 (Hungate, 1969) was flushed through the other side-arm. Samples were analyzed for pH, bacterial mass, total amino acids (**TAA**), and NH_3 every hour from 0 to 8 h and every 2 h from 8 to 24 h, and for fermentation products every 2 h from 0 to 24 h. Gas production was measured at the same time points directly from plunger displacement by placing 20 mL of each inoculum in duplicate, graduated 100-mL glass syringes with 5 to 10 mL of dead space filled with O_2-free CO_2, in a 39°C water bath (Menke et al., 1979). Inoculum DM content was determined after 24 h by transferring contents remaining in each syringe into centrifuge tubes, centrifuging at 15,000 × *g* for 15 min, discarding the supernatant, and then weighing the pellets after drying for 48 h at 65°C. Rate of gas production was expressed per gram of inoculum DM.

Aliquots of each inoculum were transferred to 12- × 75-mm disposable tubes and processed for analysis of fermentation products by the method of Siegfried et al. (1984), modified as follows: 1 mL of a 3.57 *M* $Ca(OH)_2$ suspension was added to 1 mL of sample and, after vortexing, .5 mL of .4 *M* $CuSO_4$ reagent containing 46.5 m*M* of crotonic acid as internal standard was added, the tube was capped and vortexed. Samples were refrigerated for at least 30 min then centrifuged at 10,000 × *g* for 20 min at 4°C. Supernatants were decanted into 12- × 75-mm plastic tubes and 25 μL of reagent-grade concentrated H_2SO_4 were added; tubes were capped, vortexed, frozen once at –20°C, then thawed and stored frozen at –20°C. On the day of analysis, samples were thawed and centrifuged (10,000 × *g* for 20 min at 4°C), and supernatants were transferred to HPLC auto sampler vials. Analysis was performed using a Varian HPLC model VISTA 5500 instrument (Varian, Walnut Creek, CA) equipped with a variable wavelength UV detector set at 210 nm and a refractive index detector, and an Aminex HPX87H (BioRad, Richmond, CA) cation exchange column. The column was maintained at 42°C; the mobile phase contained .015 *N* H_2SO_4 and .25 m*M* EDTA and was pumped at a flow rate of .6 mL/h. Data were acquired and chromatogram peaks integrated from both channels using a Shimadzu EZ-Chrom data system (Shimadzu Scientific Instruments, Columbia, MD). Lactic acid and glycerol co-eluted from the Aminex HPX87H column; however, glycerol is not detected at 210 nm (Masson et al., 1991). Bacterial mass was estimated from optical density (**OD**) by transferring 1-mL aliquots of each inoculum to 12- × 75-mm disposable plastic tubes containing 3 mL of macro- plus micromineral buffer (Goering and Van Soest, 1970). Tubes were centrifuged at 2,700 × *g* for 3 min at room temperature, then OD was read at 600 nm (Newbold and Rust, 1991) using a Spectronic 70 spectrophotometer (Baush & Lomb, Rochester, NY). Inoculum pH was monitored with a Corning model 360i pH meter (Corning Inc., Corning, NY). The TAA and NH_3 were

determined by transferring 2-mL aliquots of inoculum to 12- × 75-mm disposable plastic tubes containing .167 mL of 65% (wt/vol) trichloroacetic acid, placing tubes in an ice bath for 30 min, centrifuging (10,000 × *g* for 20 min at 4°C), then analyzing supernatants (Broderick and Kang, 1980). Phase 1 was replicated three times.

Phase 2. Between 1600 and 1700, frozen MRM pellets and lyophilized MRM were reconstituted with defined media (Luchini et al., 1996) in flasks gassed with O_2-free CO_2. Flasks were capped with rubber stoppers with Bunsen valves and stirred overnight in a 39°C room. Between 0800 and 0900 on the following morning, whole ruminal contents were obtained from the ruminally cannulated cow and fresh SRF was prepared as described for Phase 1, except that no extra CHO were added to the medium when washing the solids. The fresh SRF was dialyzed against aqueous .9% (wt/vol) NaCl for 2 h in a 39°C room to reduce background NH_3 and TAA. Between 1000 and 1100, chloramphenicol and hydrazine sulfate, inhibitors of bacterial utilization of TAA and NH_3 (Broderick, 1987), were added to the three inocula 15 min before they were used in the incubations. Incubations were run for 4 h in a 39°C room in spinner flasks (Luchini et al., 1996) containing a total volume of 180 mL: 60 mL of defined medium plus 120 mL of inoculum. Duplicate flasks were run without added protein (blanks), or with expeller soybean meal (**ESBM**) or solvent soybean meal (**SSBM**), added at .125 mg N/mL incubation (Broderick, 1987); ESBM and SSBM were hydrated in defined medium for 45 min before adding inoculum. Incubations were replicated three times. Using the methods described previously, gas production, pH, OD, and VFA were determined hourly only in blank flasks; concentrations of NH_3 and TAA were determined hourly in blank, ESBM, and SSBM flasks.

Experiment 2

The frozen MRM pellet was thawed at room temperature and reconstituted to a volume equal to that of the original SRF plus buffer wash from which it was obtained using warm (39°C) defined medium (Luchini et al., 1996). Flasks were gassed with O_2-free CO_2, capped with rubber stoppers with Bunsen valves, and pre-incubated overnight (16 to 18 h) in a warm (39°C) room. The following morning, this inoculum was used in the inhibitor in vitro method (**IIV**; Broderick, 1987). Seven proteins previously tested in the IIV method with fresh SRF (Hristov and Broderick, 1994) were used as substrates in the inoculum prepared from pre-incubated, frozen MRM: casein, SSBM, ESBM, high solubles fish meal, low solubles fish meal, roasted soybeans, and corn gluten meal. These samples were milled (1 mm, Wiley Mill; Arthur H. Thomas Co., Philadelphia, PA) and stored at 20 to 24°C until used in this experiment. Incubations were run in spinner flasks in a 39°C room for 4 h (Luchini et al., 1996) with a total volume of 150 mL: 50 mL of defined medium plus 100 mL of inoculum. Duplicate flasks were run without added protein (blanks) or with one of the seven proteins added at .125 mg N/mL final incubation. Proteins were hydrated in the 50 mL of defined medium for 45 min prior to adding inoculum. Inhibitors (chloramphenicol at 30 μg/mL and hydrazine at 1 m*M* in the final incubation) were added to the defined medium used to soak the proteins before addition of the inoculum. Samples were taken at 0 h then every 30 min, acidified with trichloroacetic acid, and analyzed for NH_3 and TAA as described in Exp. 1. Incubations were replicated three times.

Experiment 3

Rates and extents of ruminal protein degradation of 17 feeds were estimated using the in situ method by Calsamiglia et al. (1992). Rates of ruminal protein degradation of the same 17 feeds were determined with the IIV method using inoculum prepared from fresh SRF and incubated for 0 and 4 h (Broderick, 1987), or using inoculum prepared from frozen MRM pre-incubated for 18 h and, based on results from Exp. 2, incubated for 0 and 2 h as described in Exp. 2. Extents of protein escape were computed assuming a ruminal passage rate of .06/h (Broderick, 1987). Incubations were replicated three times.

Experiment 4

In vivo ruminal protein degradations of four lots of whole soybeans, either raw or heated to an exit temperature of 141, 149, or 157°C, were determined using four steers (BW 373 kg) cannulated at the rumen, duodenum, and ileum and were reported by Aldrich et al. (1995). In vivo ruminal protein degradations of five lots of SSBM, roasted at 165°C for 0, 75, 150, 180, or 210 min, were determined using six mature wethers (BW 72 kg) cannulated at the rumen, duodenum, and ileum and were reported by Demjanec et al. (1995). The IIV degradation of these same protein sources was estimated with the IIV method using inoculum prepared from fresh SRF and incubated for 0 and 4 h as described earlier (Broderick, 1987), or using inoculum prepared from frozen MRM pre-incubated for 18 h and incubated for 0 and 2 h as described in Exp. 2.

Statistical Analyses

Data from Exp. 1 were analyzed using ANOVA by the GLM procedures of SAS (1989). Experiments were analyzed as a randomized complete block design using ruminal fluid collection date as block (Steel and Torrie, 1980). The model included block, treatment, and block × treatment interactions. Differences between treatment means were determined by protected

Table 1. Rates of gas production of fresh strained ruminal fluid (SRF), frozen mixed ruminal microorganisms (MRM), and lyophilized MRM during the 24-hour pre-incubation period (Exp. 1, Phase 1)

	Rate of gas production, mL/h·g DM[a]				
Period, h	Fresh SRF	Frozen MRM	Lyophilized	RMSE[b]	*P*
0–1	26.4	14.5	6.5	16.6	.120
1–2	28.5[c]	32.2[c]	7.9[d]	12.2	.044
2–3	32.9[d]	38.1[c]	21.9[e]	2.9	<.001
3–4	22.3[e]	45.5[d]	54.2[c]	4.0	<.001
4–5	18.9[d]	35.9[c]	35.9[c]	4.6	<.001
5–6	14.3[e]	26.9[c]	23.1[d]	2.0	<.001
6–7	11.5[d]	20.7[c]	14.1[d]	2.3	<.001
7–8	9.5[d]	19.7[c]	17.5[c]	1.9	<.001
8–10	7.7[e]	16.7[c]	13.4[d]	1.5	<.001
10–12	6.0[d]	13.9[c]	13.7[c]	2.0	<.001
12–14	2.6[d]	10.5[c]	8.7[c]	1.7	<.001
14–16	3.6[e]	12.4[c]	7.5[d]	1.2	<.001
16–18	3.7[e]	11.8[c]	7.0[d]	1.7	<.001
18–20	2.4[e]	12.1[c]	5.9[d]	.9	<.001
20–22	1.3[e]	11.4[c]	4.4[d]	1.5	<.001
22–24	2.1[e]	10.9[c]	3.8[d]	1.0	<.001

[a]Means of six rates for fresh SRF and frozen MRM and four rates for lyophilized MRM.
[b]Square root of the mean square error.
[c,d,e]Means within row with different superscripts differ.

LSD; significance was declared at $P < .05$ unless otherwise noted. Regression analysis by the GLM procedures of SAS (1989) was used to assess the relationship between ruminal protein degradabilities estimated by the different methods tested in Exp. 2, 3, and 4.

Results and Discussion

Experiment 1

Phase 1. Rates of gas production (mL/[h·g DM]) of each inoculum from Phase 1 are shown in Table 1. Gas production of lyophilized MRM was lower than in the other two inocula for the first 4 h. By 4 h, gas production rates had peaked for all three media and then declined. Gas production rates of the frozen MRM tended to be higher than for the other two inocula. The lag time observed with lyophilized MRM suggested that at least 2 h was required for rehydration of the microorganisms prior to initiation of fermentation. Inoculum prepared from fresh SRF began to produce gas immediately; there was a short, nonsignificant time lag before the beginning of gas production with the frozen MRM. Although the concentrations of fermentable CHO in the inocula were comparable, differences in gas production may be explained by the amount of added glycerol because it is fermented by ruminal organisms (Stewart and Bryant, 1988). The amount of glycerol added to frozen MRM and lyophilized MRM was similar. However, glycerol was added to the frozen MRM inoculum pellet after centrifugation, whereas most of the glycerol probably was discarded with the supernatant after centrifugation of the lyophilized MRM. Fresh SRF had no added glycerol.

Concentrations of VFA during the 24 h pre-incubation period with fresh SRF suggested a normal fermentation (Figure 1a). At 0 h, concentrations of acetic, propionic, and butyric acids were 73, 21, and 13 m*M*, respectively. Although acetic acid increased from 73 to 120 after 24 h, concentrations of propionic and butyric acids were stable. Moreover, lactic acid production was not detected, suggesting that there was no overgrowth of lactic acid-producing bacteria. As expected, the two preserved MRM inocula had initial VFA concentration reflecting that added to the medium; however, their fermentation patterns differed from each other (Figures 1b and 1c). The increase of acetic acid in frozen MRM was similar to that in fresh SRF, but frozen MRM had a much larger increase in propionic acid. These increases in acetic and propionic acids may be due to fermentation of glycerol. Ruminal microorganisms ferment glycerol to propionate, lactate, succinate, and acetate (Stewart and Bryant, 1988). During the first 4 h of incubation there was some production of lactate. When ruminant animals are fed rapidly fermentable CHO, lactate concentration may increase due to an overgrowth of *Streptococcus bovis*. However, lactate concentration was negligible after 12 h in frozen MRM. Lactate concentration may have decreased because of growth of lactic acid fermenting bacteria (Stewart and Bryant, 1988) or because numbers of lactic acid-producing bacteria may have diminished with reduced amounts of rapidly fermentable CHO as fermentation progressed. Although acetic and propionic acids also increased in

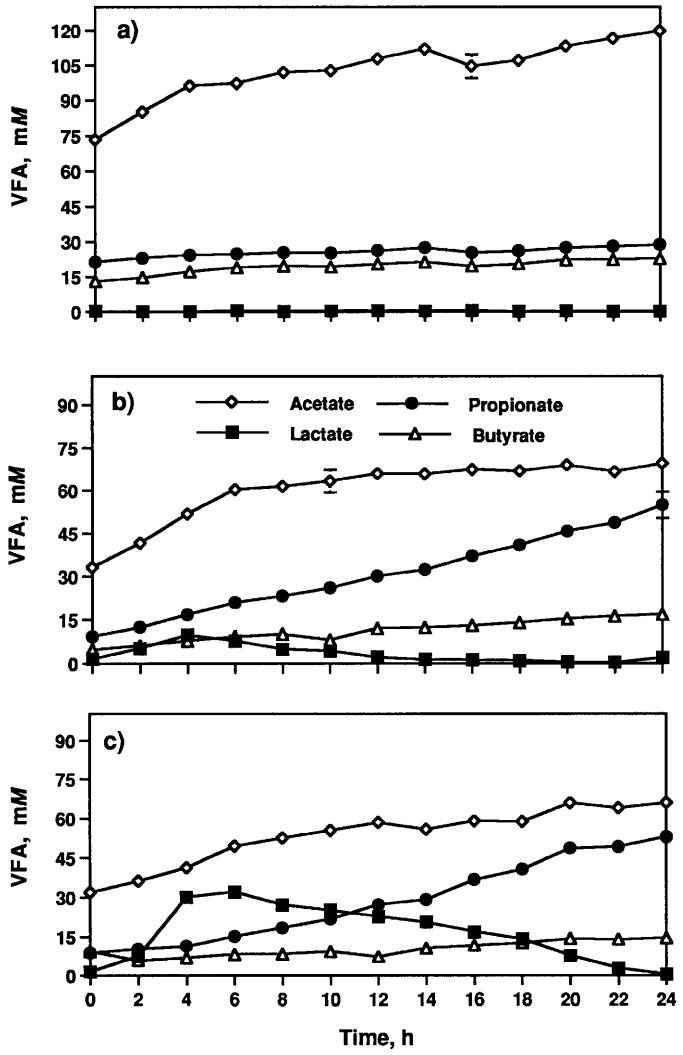

Figure 1. Concentrations of VFA (m*M*) during the 24 h pre-incubation period with (a) fresh strained ruminal fluid (SRF), (b) frozen mixed ruminal microorganisms (MRM), and (c) lyophilized MRM. Error bars correspond to ± 1 SE (n = 3) (Exp. 1, Phase 1).

lyophilized MRM, there were some differences from frozen MRM in the time course of the fermentation: acetic acid increased linearly and there was a large increase of lactic acid (to 32 m*M*). This suggested that there was an overgrowth of lactic acid-producing bacteria. The decline in lactic acid concentration after 6 h again indicated depletion of rapidly fermentable CHO and subsequent depression of the lactic acid-producing bacteria. Thus, VFA patterns suggested there may have been shifts in the microbial populations in the two preserved MRM inocula during pre-incubation; however, after 6 to 8 h, the only difference from fresh SRF was the increasing concentration of propionate. Despite these differences in VFA concentrations, acetate to propionate ratio was within the normal range of in vivo values (Van Soest, 1994).

Because measurement of bacterial cells using culture OD is reliable only at low culture densities (Ingraham et al., 1983), it was necessary to dilute samples to give OD values in the linear range and to multiply observed OD by the appropriate dilution factor. Changes in OD during the 24 h pre-incubation period are depicted in Figure 2. Both fresh SRF and lyophilized MRM showed a lag time, exponential growth, then a stationary phase from about 6 to 24 h. Lag times are characterized by slow growth, during which the cells reestablish the chemical composition necessary for exponential growth; exponential growth is the normal consequence of the constantly increasing catalytic capacity of a culture of multiplying bacteria (Ingraham et al., 1983). When some nutrient becomes exhausted, or some toxic waste product accumulates, balanced growth can no longer continue, and the bacteria enter stationary phase. For both the fresh SRF and lyophilized MRM, a limiting nutrient triggering stationary phase apparently was depleted about the same time. The frozen MRM showed slow, linear growth throughout the 24 h; the greater gas production of inocula prepared from frozen MRM (Table 1) reflected its greater OD. A source of N was not added to the medium; although ruminal microorganisms are effective N scavengers (Schaefer et al., 1980), it is possible that N was limiting in both the frozen and lyophilized inocula. Depletion of readily fermentable energy also may have limited bacterial growth, particularly in fresh SRF inoculum where NH_3 concentration was high and not limiting (Figure 3). Lower pH in this inoculum (data not shown) also may have contributed to its stationary phase.

There was a decrease in the pH of all three inocula sources during the first 4 to 5 h of pre-incubation: At 0 h, the pH was 6.4, 6.9, and 6.9, for fresh SRF, frozen MRM, and lyophilized MRM, respectively, declining to pH 5.9, 6.4, and 6.4 after 5 h. The pH may have stabilized after 5 h because of depletion of readily fermentable CHO, thus slowing subsequent rates of VFA production. Pectin and starch composed a large proportion of the fermentable CHO in the medium (Luchini et al., 1996). Pectin has a half-life in the rumen of approximately 4 h (Hatfield and Weimer, 1995). Thus, it is unlikely that all the CHO in the medium would have been depleted in less than 1 h. The defined medium contained 95 m*M* HCO_3^{-1} (pK_a = 6.37) from $KHCO_3$ and $NaHCO_3$ plus 10 m*M* of monohydrogen phosphate (HPO_4^{-2}; pK_a = 12.4) from Na_2HPO_4 and 11 m*M* of dihydrogen phosphate ($H_2PO_4^{-1}$; pK_a = 6.8) from KH_2PO_4. In vivo concentrations of these buffers in ruminal liquor ranged from 34 to 80 m*M* of HCO_3^{-1} and 10 to 17 m*M* of HPO_4^{-2} (Counotte et al., 1979). Thus, the high buffering capacity of the medium, compared with ruminal liquor, also may have contributed to pH stability. Lower buffering capacity of its medium and higher total VFA concentrations help explain the lower pH of the fresh SRF inoculum.

Figure 3a shows the changes in NH_3 concentrations in the three inocula. The NH_3 concentration of fresh

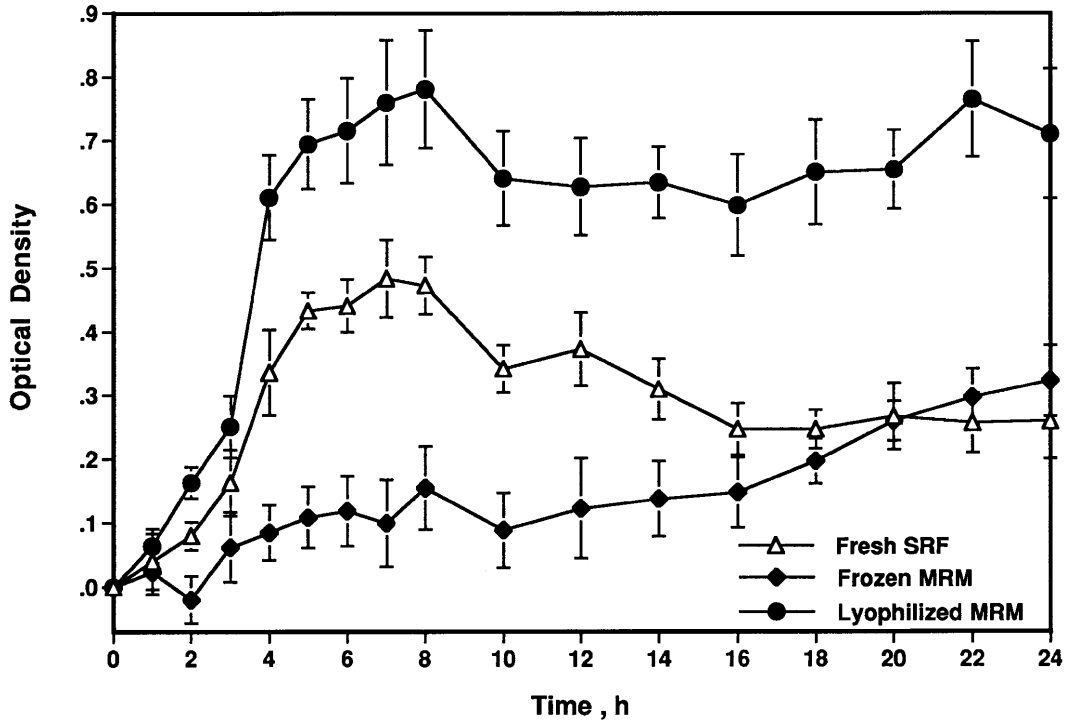

Figure 2. Optical density (OD) of fresh strained ruminal fluid (SRF), frozen mixed ruminal microorganisms (MRM), and lyophilized MRM inocula during the 24-h pre-incubation period. Error bars correspond to ± 1 SE (n = 3) (Exp. 1, Phase 1).

SRF was higher than that of frozen and lyophilized MRM throughout the 24-h pre-incubation period. The initial drop in NH_3 concentration in fresh SRF inoculum probably reflected net microbial N uptake during the first 4 h. However, after this initial drop, there was an increase to levels more than double those at 0 h. This may have resulted from NH_3 production from catabolism of lysed cells. Net release of NH_3 and TAA at any time point is estimated most accurately when blank concentrations are relatively low. The

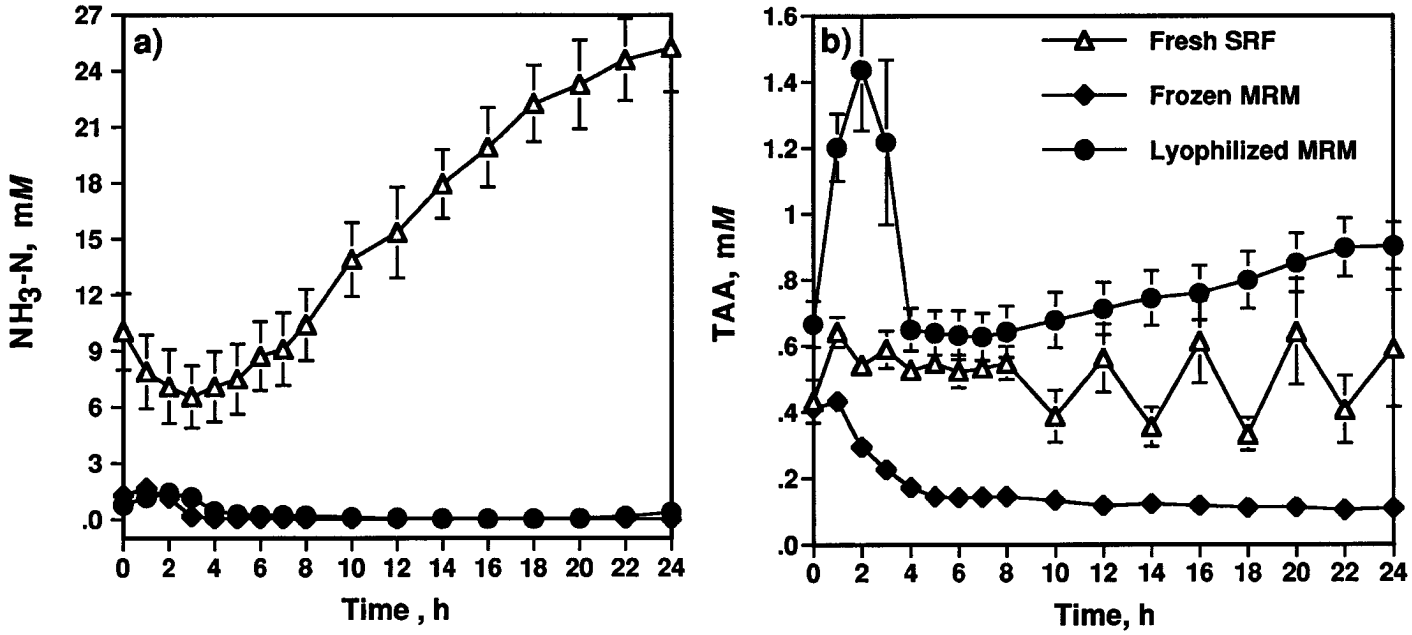

Figure 3. Ammonia (NH_3) and total amino acid (TAA) release from fresh strained ruminal fluid (SRF), frozen mixed ruminal microorganisms (MRM), and lyophilized MRM during the 24-h pre-incubation period. Error bars correspond to ± 1 SE (n = 3) (Exp. 1, Phase 1).

Table 2. Rates of gas production from blank samples of fresh strained ruminal fluid (SRF), frozen mixed ruminal microorganisms (MRM), and lyophilized MRM during the incubation period (Exp. 1, Phase 2)

	Rate of gas production, mL/h·g DM				
Period, h	Fresh SRF	Frozen MRM	Lyophilized MRM	SEM	*P*
0–1	−3.9	−13.0	−11.5	6.44	.58
1–2	6.3[a]	4.4[ab]	3.7[b]	.75	.09
2–3	5.3	5.1	4.0	.78	.45
3–4	4.8[a]	2.1[b]	2.1[b]	.64	.02

[a,b]Means in the same row with different superscripts differ.

NH_3 concentrations during pre-incubation of both frozen MRM and lyophilized MRM were opposite that of fresh SRF. At 0 h, NH_3 concentration was very low; NH_3 increased during the first 1 to 2 h, then decreased after 4 h to levels lower than at 0 h. This suggested that the initial NH_3 formation from proteolysis of lysed cells or other nitrogenous compounds present in the MRM pellet was depleted by 4 to 5 h of pre-incubation. Concentrations of NH_3 from 5 to 24 h in inocula containing lyophilized MRM and frozen MRM ranged from .04 to .28 and .03 to .05 m*M*, respectively. The very low NH_3 concentrations in frozen MRM may have limited bacterial growth. The TAA concentration in fresh SRF was stable during the first 8 h; the apparent fluctuation from 8 to 24 h was nonsignificant (Figure 3b). Lyophilized MRM had increased TAA concentrations during the first 2 h, suggesting some microbial lysis; by 4 h, however, TAA had dropped to concentrations similar to those at 0 h, and remained relatively stable until the end of the pre-incubation at 24 h. After 1 h, TAA concentrations in frozen MRM declined, reaching .15 m*M* by 5 h, and remaining there until 24 h.

Phase 2. The VFA concentrations during the 4-h incubation with fresh SRF and the two pre-incubated inocula, prepared from frozen MRM and lyophilized MRM, are shown in Figures 4a, 4b, and 4c, respectively. Acetic, propionic, and butyric acid concentrations increased from 39.7 to 49.2, 13.1 to 14.7, and 8.0 to 10.7 m*M* in fresh SRF, from 58.3 to 65.5, 36.3 to 41.3, and 10.8 to 12.6 m*M* in frozen MRM, and from 53.5 to 58.7, 24.5 to 29.9, and 7.3 to 8.4 m*M* in lyophilized MRM. This indicated that microbial fermentation occurred during incubation in the presence of the inhibitors. The three inocula had similar VFA pattern; however, the lactate accumulation of the lyophilized MRM suggested either the presence of lactic acid-producing bacteria or the absence of lactic acid-fermenting bacteria (Figure 4c).

Rates of gas production during Phase 2 for the 4-h incubations with the three inocula are shown in Table 2. Although the frozen and lyophilized MRM inocula had been pre-incubated for 18 h, all three inocula had similar patterns of gas production. During

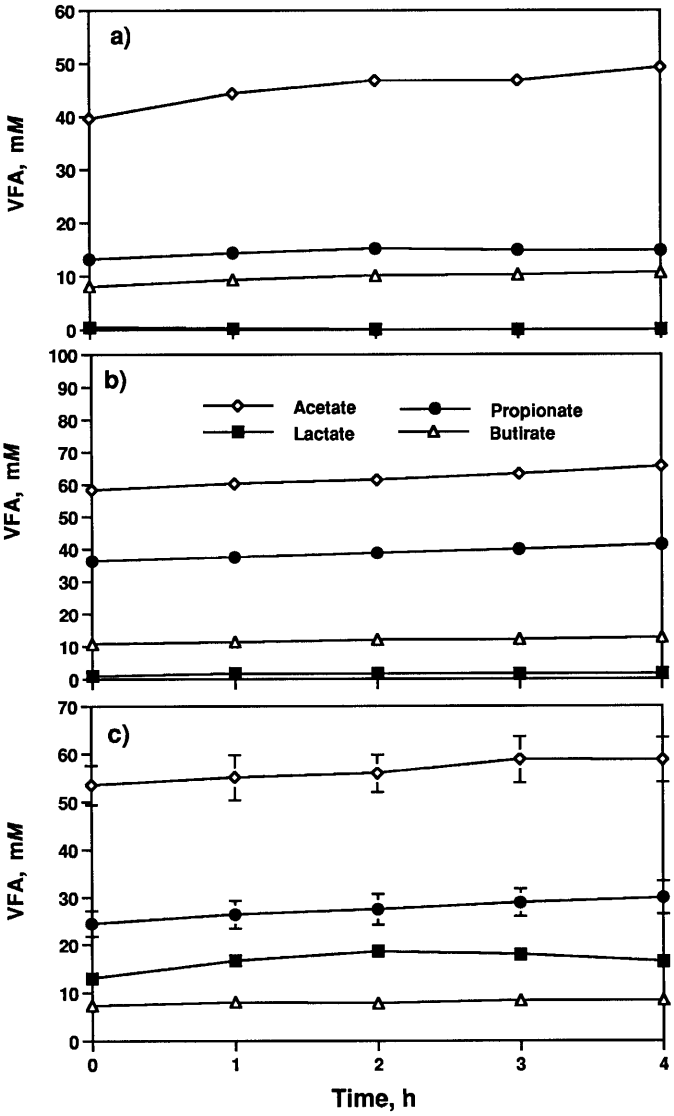

Figure 4. Concentrations of VFA (m*M*) during the 4-h incubation period with (a) fresh strained ruminal fluid (SRF), (b) frozen mixed ruminal microorganisms (MRM), and (c) lyophilized MRM. Error bars correspond to ± 1 SE (n = 3) (Exp. 1, Phase 2).

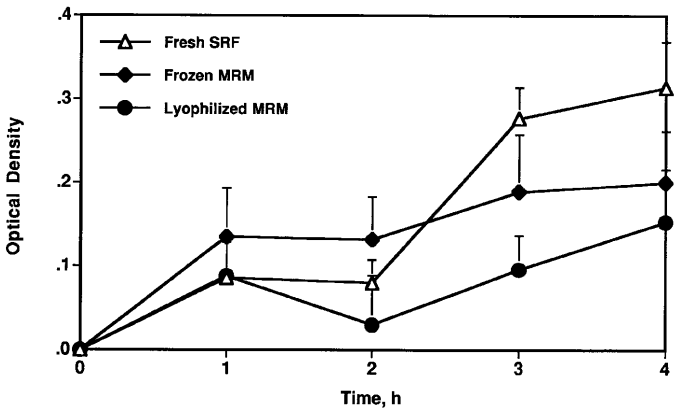

Figure 5. Optical density (OD) of fresh strained ruminal fluid (SRF), frozen mixed ruminal microorganisms (MRM), and lyophilized MRM during the 4-h incubation period. Error bars correspond to ± 1 SE (n = 3) (Exp. 1, Phase 2).

the 1st h, the apparent negative gas productions probably resulted from CO_2 going into solution because of a disequilibrium between the gaseous and dissolved phases of CO_2. After equilibrium, however, there was net gas production until the end of the incubations at 4 h. Fresh SRF and frozen MRM had higher rates of gas production than lyophilized MRM; gas production tended to decrease with time. The slower rates of gas production with these three inocula than with the uninhibited inocula during pre-incubation in Phase 1, may be explained by the presence of the inhibitors in Phase 2.

Net changes in OD during the 4-h incubation suggested that there was a small amount of microbial growth, despite the presence of chloramphenicol and hydrazine (Figure 5); however, net OD was greater than zero ($P \leq .03$) only for fresh SRF at 3 h and 4 h. Net OD was not greater than zero ($P \geq .09$) at any time point for the other two inocula. The significant increase in OD for fresh SRF corresponded to the greater gas production for that inoculum. This growth may be due to utilization of nutrients added to the spinner flasks prior to the incubation. The increase in OD in the presence of the inhibitors was unexpected. Previously, we obtained quantitative recovery of NH_3 and TAA (Broderick, 1987) and virtually zero uptake of $^{15}NH_3$ (Hristov, unpublished data, 1991) during in vitro incubations containing the same concentrations of these inhibitors. Net microbial growth would not be expected if there were quantitative recovery of NH_3 and TAA.

The pH of the three inocula during the 4-h incubation dropped from 6.64, 6.70, and 6.68, to 6.29, 6.44, and 6.42, respectively, for fresh SRF, frozen MRM, and lyophilized MRM. This pH decline may be explained by fermentation of the nutrients in the inocula added to the spinner flasks prior to the incubation. Throughout the 4-h period, the fresh SRF

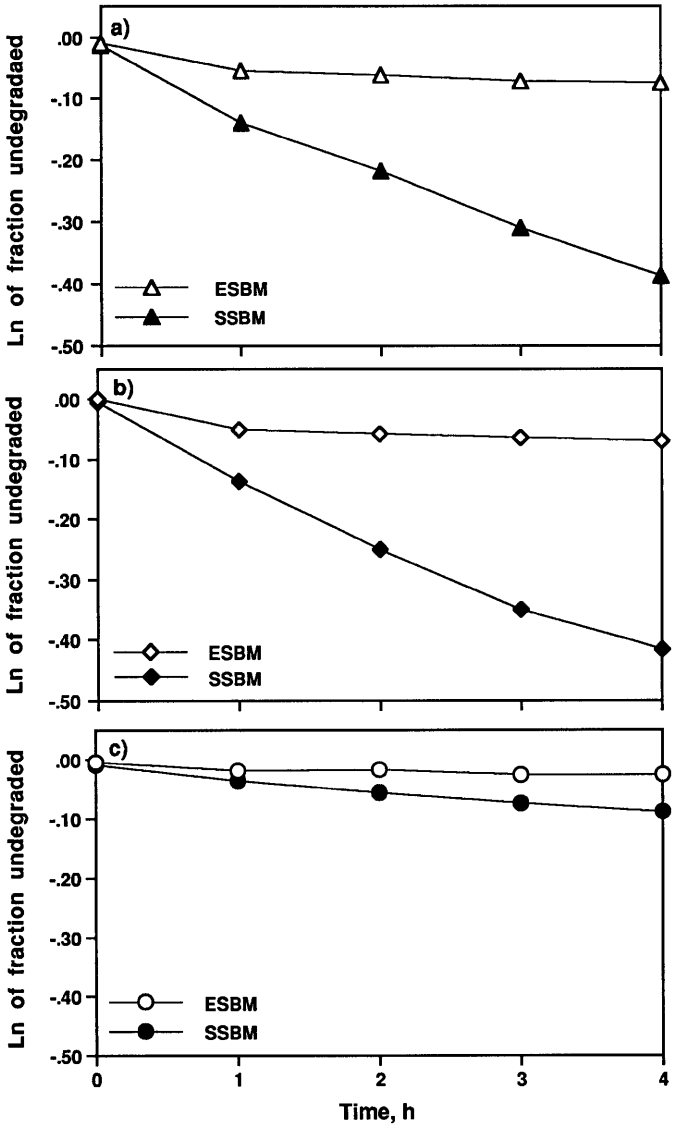

Figure 6. Protein degradation curves of expeller soybean meal (ESBM) and solvent soybean meal (SSBM) in incubations with (a) fresh strained ruminal fluid (SRF): ESBM = –.015 h^{-1} – .025 (r^2 = .78) and SSBM = –.092 h^{-1} – .030 (r^2 = .99); (b) frozen mixed ruminal microorganisms (MRM): ESBM = –.015 h^{-1} – .018 (r^2 = .75), and SSBM = –.104 h^{-1} – .024 r^2 = .99); and (c) lyophilized MRM: ESBM = –.005 h^{-1} – .009 (r^2 = .99), and SSBM = –.019 h^{-1} – .014 (r^2 = .99). Degradation rates estimated from the slopes of degradation curves though 4 h (Exp. 1, Phase 2).

inoculum had lower pH than the frozen and lyophilized MRM. The fresh SRF inoculum contained some ruminal liquor, but the other two inocula were reconstituted only with the defined medium.

Coefficients of determination (r^2) for single exponential fits (Figures 6a, 6b, and 6c) for ESBM were .78, .75, and .79, and for SSBM were .99, .99, and .99 for, respectively, fresh SRF, frozen MRM, and lyophilized MRM. The degradation rate for ESBM obtained

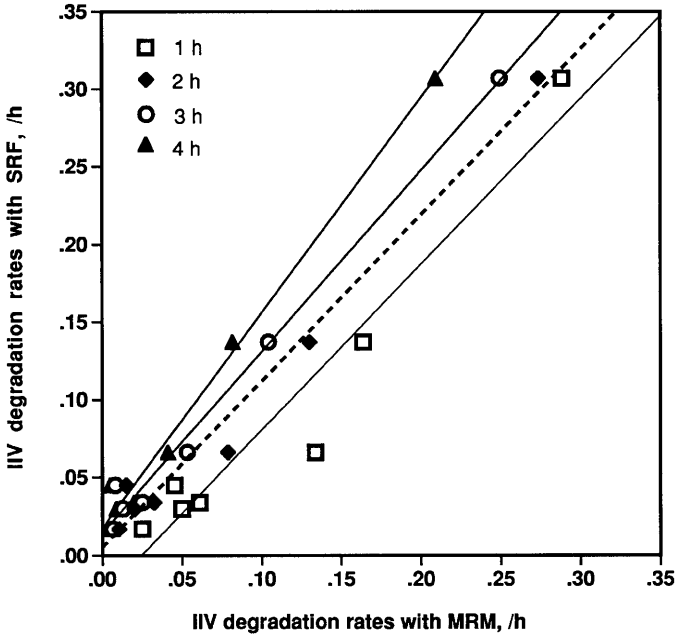

Figure 7. Protein degradation rates (k_d) of casein, solvent soybean meal, high solubles fish meal, low solubles fish meal, expeller soybean meal, roasted soybean meal, and corn gluten meal by the inhibitor in vitro (IIV) method. Data with fresh strained ruminal fluid (SRF) from Hristov and Broderick (1994). Equations (± SE) from regression of k_d from SRF on k_d from frozen mixed ruminal microorganisms (MRM), incubated for 1, 2, 3, and 4 h, were: SRF = 1.07 (± .13) $MRM_{1\ h}$ – .026 (± .02) (r^2 = .94); SRF = 1.07 (± .07) $MRM_{2\ h}$ + .005 (± .008) (r^2 = .98); SRF = 1.17 (± .05) $MRM_{3\ h}$ + .014 (± .006) (r^2 = .99); and SRF = 1.39 (± .07) $MRM_{4\ h}$ + .017 (± .006) (r^2 = .99) (Exp. 2).

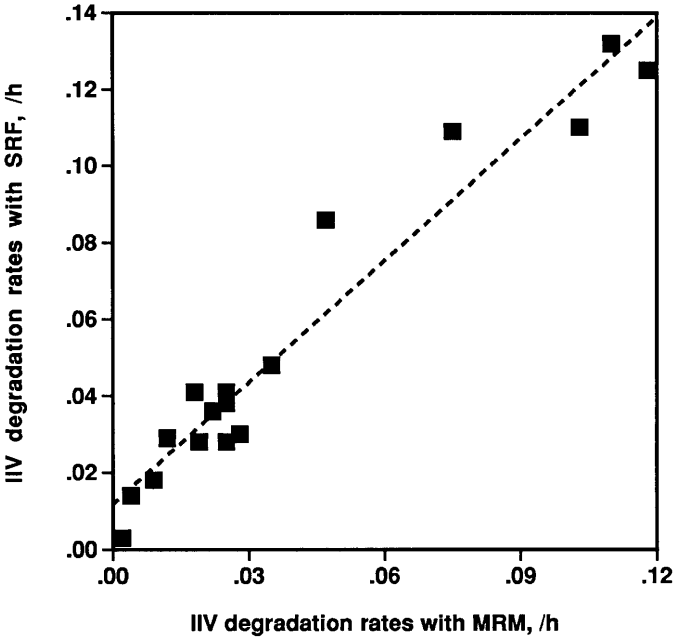

Figure 8. Equation (± SE) from regressing protein degradation rates (k_d) estimated with the inhibitor in vitro (IIV) method using fresh strained ruminal fluid (SRF) as inocula on k_d obtained using frozen mixed ruminal microorganisms (MRM) as inocula was: SRF = 1.06 (± .07) MRM + .012 (± .004) (r^2 = .94). Proteins were the 17 feeds also tested in situ (Calsamiglia et al., 1994) (Exp. 3).

with lyophilized MRM was not different (P = .12) from those obtained with the other two inocula; however, the rate for SSBM was slower (P < .001) with lyophilized MRM. These results indicated that lyophilized MRM, even after pre-incubation for 18 h, did not have protein degradative activity comparable to inocula prepared from either fresh SRF or frozen MRM. The lyophilization methods used here may have killed such a large number of important proteolytic organisms that pre-incubation could not restore their activity to normal levels.

Experiment 2

Results from the IIV incubations with fresh SRF and frozen MRM as inocula are shown in Figure 7. Regressions of protein degradation rates (k_d) obtained with fresh SRF incubated for 4 h on the k_d obtained using frozen MRM incubated for 1, 2, 3, and 4 h yielded, respectively, r^2 of .94, .98, .99, and .99; slopes of 1.07, 1.07, 1.17, and 1.39, and intercepts of –.026, .005, .014, and .017. Slopes were not different from one for regressions using frozen MRM incubated for 1 h (P = .144) and 2 h (P = .556); however, slopes were greater than one for regressions using frozen MRM incubated for 3 h (P = .036) and 4 h (P = .015). Likewise, intercepts were not different from zero for regressions from frozen MRM incubated for 1 h (P = .600) and 2 h (P = .320), but intercepts were greater than zero for regressions using frozen MRM incubated for 3 h (P = .008) and 4 h (P < .001). The increased slopes and intercepts at 3 h and 4 h suggested that there was a relative decrease in degradation rate with frozen MRM as incubation time increased. Thus, rates obtained in either 1- or 2-h incubations with frozen MRM would be more reliable because they were more similar to those from incubations with fresh SRF. The 2-h incubations yielded greater net release of NH_3 and TAA and, hence, greater accuracy in estimating degradation rates. Therefore, results obtained using the 2-h incubation time were considered more reliable.

Experiment 3

Degradation rates obtained for 17 protein sources using the IIV method with fresh SRF (4-h incubations) or frozen MRM (2-h incubations) as inocula are shown in Figure 8. Similar to Exp. 2, regression of the k_d obtained with fresh SRF on the k_d from using frozen MRM yielded a slope that was not different

Table 3. Protein degradation parameters obtained in situ[a] and with the inhibitor in vitro (IIV)[b] method using frozen mixed ruminal microorganisms (MRM) as the inoculum (Exp. 4)

	Intercept[c]		Degradation rate[d]		Degradability[e]	
Protein source	In situ	IIV-MRM	In situ	IIV-MRM	In situ	IIV-MRM
Soybean meal	75.8	99.1	.113	.118	73.7	66.6
Meat and bone meal	73.9	98.5	.020	.110	44.2	65.2
Meat and bone meal	67.7	98.8	.020	.103	49.2	63.6
Solvent-extracted cottonseed meal	79.8	98.7	.044	.075	54.0	56.1
Expeller soybean meal (Soy-plus)	81.6	99.1	.049	.047	54.9	44.4
Menhaden fish meal	75.3	98.6	.008	.035	33.6	37.7
Hydrolyzed feather meal	93.0	99.4	.004	.028	12.1	32.2
Hydrolyzed feather meal	82.2	99.3	.006	.025	24.7	29.9
Dried distillers grains	76.1	97.2	.038	.025	53.2	31.4
Brewer's grain	78.3	98.5	.034	.025	49.8	30.5
Hydrolyzed feather meal	67.5	99.0	.016	.022	46.4	27.6
Dried distillers grains	80.1	98.7	.016	.019	36.4	25.0
Brewer's grain	88.2	99.1	.028	.018	39.9	23.8
Soybean meal, lignosulfonate	91.5	99.1	.020	.012	30.9	17.4
Corn gluten meal	91.3	99.2	.007	.009	18.2	13.7
Ring dried blood meal	90.9	100.0	.002	.004	12.1	6.3
Conventionally dried blood meal	93.8	99.9	.002	.002	9.2	3.3

[a]Calsamiglia et al. (1992).
[b]Broderick (1987).
[c]Fraction remaining undegraded at 0 h.
[d]Protein degradation rate (k_d) estimated by the IIV and in situ methods.
[e]Extent of protein degradation, % = (100 − intercept) + {intercept × [k_d/(k_d + .06)]}.

from one (P = .383) and was well correlated (r^2 = .94). However, the intercept was greater than zero (P = .008), indicating that degradation rate was relatively slower with frozen MRM for more slowly degraded proteins. Comparison of the ruminal degradation of the same proteins, estimated by the in situ method and the IIV technique using frozen MRM as inoculum, are given in Table 3. Although the mean IIV protein degradability obtained with frozen MRM was only 89% of that estimated using the in situ method, the mean in situ k_d was only 62% of that using frozen MRM. Differences in the k_d between the two methods were reported previously (Broderick et al., 1988). In that study, the k_d measured by the IIV method for seven proteins averaged three times the mean in situ k_d. However, extent of protein degradation estimated in situ averaged 83% of that by the IIV technique. These researchers suggested that slower in situ degradation rates were compensated for by greater estimates of protein degraded at 0 time, causing the extents of ruminal degradation estimated by the two methods to converge.

Experiment 4

Rates of ruminal degradation (k_d) of heat-treated soybeans and SSBM, estimated after 2 h (frozen MRM) or 4 h (fresh SRF) IIV incubations are shown in Figure 9. Regression of the k_d obtained with fresh SRF on the k_d from frozen MRM yielded a slope of .91 that was not different from one (P = .383) and a strong correlation (r^2 = .88). However, the intercept of .020 was greater than zero (P = .008), suggesting that, as in Exp. 3, degradation rates with frozen MRM were disproportionately slower for more slowly degraded proteins. The differences in the error bars between the two methods reflected the smaller variation with frozen MRM: CV with frozen MRM ranged from 3.6 to 16% compared with CV of 5.3 to 171% with fresh SRF. Ruminal undegraded protein values for the same nine soy proteins, estimated in vivo and in vitro using frozen MRM, are shown in Figure 10. Whole soybeans were either raw or heat-treated in a commercial roaster using three different exit temperatures plus steeping for 30 min (Aldrich et al., 1995). In vivo ruminal undegraded protein increased with the first heat treatment but declined at the two highest exit temperatures (Figure 10). Aldrich et al. (1995) suggested that this unexpected result may have occurred because roasted soybeans became more brittle with increased heating, rendering the soybean protein more degradable in the rumen. Roasted SSBM was prepared by heating at constant temperature (165°C) for 0 to 210 min (Demjanec et al., 1995). As expected, increasing roasting time increased in vivo ruminal protein escape, from 34.9% (unheated SSBM) to 92.9% (SSBM roasted at 165°C for 210 min). Regression of ruminal undegraded protein, reported from the two in vivo studies with heat-treated SSBM and soybeans, on the ruminal protein escape estimated in vitro with the frozen MRM yielded

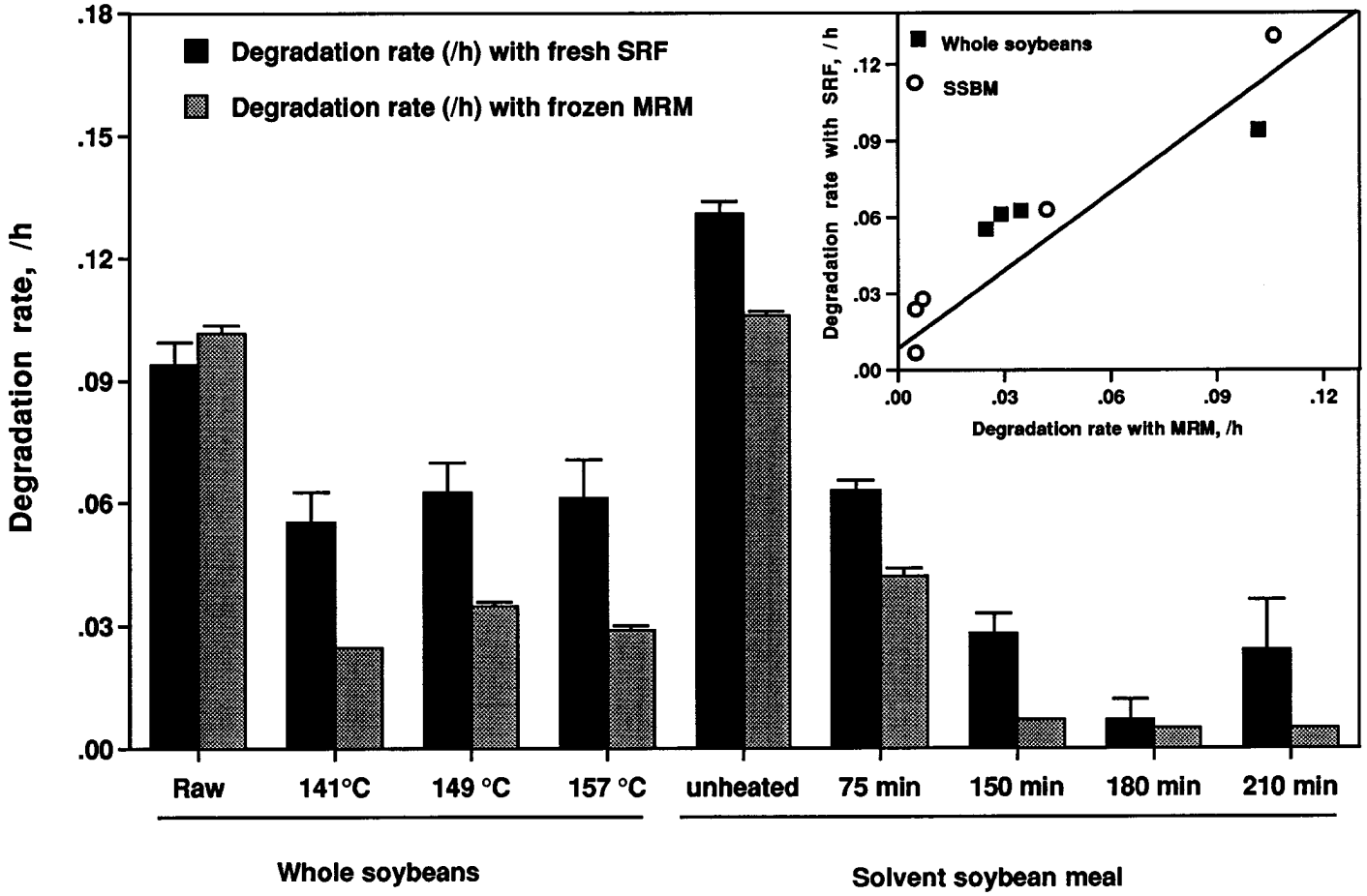

Figure 9. Protein degradation rates (k_d) estimated by the inhibitor in vitro method (IIV) using fresh strained ruminal fluid (SRF) or frozen mixed ruminal microorganisms (MRM) as inocula. Whole soybeans were either raw or heated at 141, 149, or 157°C (Aldrich et al., 1995), and solvent soybean meal (SSBM) was either unheated or heated at 165°C for 75, 150, 180 and 210 min (Demjanec et al., 1995). Equation (± SE) from regressing degradation rates from SRF on the degradation rates from MRM was: SRF = .91 (± .13) MRM + .02 (± .007) (r^2 = .88). Error bars correspond to ± 1 SE (n = 3) (Exp. 4).

slope of .80 (not different from one; P = .256) and intercept of 1.1% (not different from zero; P = .925). Ruminal escape obtained with frozen MRM was not as well correlated with in vivo estimates (r^2 = .77) as were degradation rates determined using frozen MRM and fresh SRF (Figure 9). In vitro estimates of ruminal escape were computed from the observed protein degradation rate using an assumed ruminal passage rate (.06 h^{-1}). Neither degradation rate nor passage rate was determined in the two in vivo studies. Moreover, an effect of heating on particle breakdown, which may have increased in vivo protein degradation (Aldrich et al., 1995), would not have influenced in vitro results because all samples were ground through a 1-mm Udy mill screen. Overall, these results indicated that frozen MRM, after pre-incubation, can be used as the IIV inoculum for estimating rate and extent of ruminal protein degradation.

Implications

Protein degradation rates obtained after 2-h incubations with frozen, mixed ruminal microorganisms agreed closely with those obtained after 4-h incubations with fresh ruminal fluid. Ruminal protein degradation determined by in situ and in vitro methods tended to converge, despite differences in degradation rates and fractions degraded at 0 h. Protein escapes estimated in vitro using pre-incubated, frozen mixed ruminal microorganisms were well correlated with in vivo escapes determined for nine samples of heat-treated soybeans and SSBM. In these experiments, freeze-drying did not prove to be a satisfactory method for preserving mixed ruminal microorganisms. These results indicated that mixed ruminal microorganisms, preserved frozen, may be used for the in vitro determination of ruminal protein degradation.

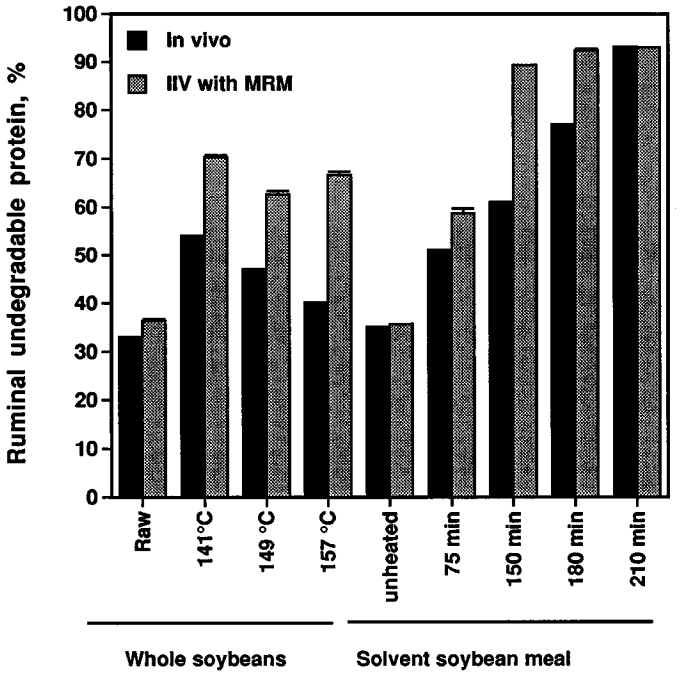

Figure 10. In vivo and in vitro estimated ruminal undegraded protein. In vivo estimated values for whole soybean samples from Aldrich et al. (1995) and for solvent soybean meal samples from Demjanec et al. (1995). Soybeans were either raw or heated in a commercial roaster to an exit temperature of 141, 149, or 157°C. Solvent soybean meal samples were either raw or heated to 165°C in a forced-air oven for 75, 150, 180, and 210 min. In vitro estimates were made using frozen mixed ruminal microorganisms (MRM) in the inhibitor in vitro (IIV) method. Error bars correspond to ± 1 SE (n = 3) (Exp. 4).

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