Quantitation of Microorganisms Associated with the Particulate Phase of Ruminal Ingesta

W. MICHAEL CRAIG,2 GLENA. BRODERICK AND D. BRADFORD RICKER

U.S. Dairy Forage Research Center, U.S. Department of Agriculture, Agricultural Research Service, University of Wisconsin, Madison, WI 53706

ABSTRACT Microbial organic matter associated with rumen particulate and fluid phases was quantified using 15N as the microbial marker in two rumen fistulated cows fed a 65% alfalfa haylage diet. During two collection periods, feed was removed 1 h after initiation of feeding, and cows were dosed with (15NH₄)₂SO₄. Whole rumen contents were sampled before feeding and at various times up to 12 h after feeding. Fluid microorganisms were those that passed through eight layers of cheesecloth. Particle-associated microorganisms were obtained by chilling squeezed particles prior to seven successive extractions with saline solution. The amount of microorganisms removed from particles ranged from 32.1 to 59.9% as measured by 15N. Organic matter (mg/mL strained rumen fluid equivalent) of fluid and particle-associated microorganisms was respectively: 10.7 and 47.5; 12.5 and 35.5; 12.2 and 30.1; 10.7 and 26.1; 10.9 and 26.7; and 8.9 and 20.6, at 2, 3, 4, 7, 9 and 11 h after initiation of feeding. These ratios indicated that 70—80% of microbial organic matter in whole rumen contents was associated with the particulate phase and that particle-associated microbial organic matter was greatest soon after feeding. Analysis of 15N in extracted rumen particles indicated that 50—65% of particle nitrogen and 17—27% of particle dry matter was of microbial origin. These results provide evidence that particle-associated microorganisms make up a major proportion of the total microorganisms in ruminal ingesta and that a large proportion of ingesta particle N can be of microbial origin. J. Nutr. 117: 56—62, 1987.

INDEXING KEY WORDS:
• particulate microorganisms • fluid microorganisms • 15N • rumen microorganisms

Results from electron microscopy work indicate that a large number of bacteria [1], protozoa [2] and fungi [3] are attached to undigested feed particles in the rumen. All of these attached microorganisms seem to play a role in digestion of plant cell walls [4]. Orpin [5] found that in vitro bacterial attachment to hay particles was rapid, and maximum attachment occurred within 15 min. Also, in vitro digestion rates [6] and volatile fatty acid (VFA) production [7, 8] have been enhanced by including particulate microorganisms in the in vitro medium.

Quantitative information, although limited, indicates that microorganisms associated with particles may constitute a large proportion of the rumen microbial population. Forsberg and Lam [9] found that 75% of microbial ATP was associated with the rumen particulate fraction of whole rumen contents obtained from a cow consuming a hay diet. When the cow was fed a grain diet, less ATP was associated with the particulate fraction. McFadyen and McAllan [10], using diaminopimelic acid (DAPA) as a microbial marker, estimated that 50% of bacteria were associated with undigested particles in the rumen approximately 16 h after steers consumed a barley and hay diet.

If a significant proportion of the total rumen microorganisms are associated with undigested particles, measurements pertaining to rumen microbes should be obtained from particulate as well as fluid populations. The goal of this study was to quantify rumen microorganisms associated with the particulate and fluid phase at various time points after feeding cows a forage-based diet.


2To whom correspondence should be addressed. Current address is: Department of Animal Science, Louisiana State University, Baton Rouge, LA 70803.
MATERIALS AND METHODS

Two lactating Holstein cows equipped with rumen fistulae were fed every 12 h. Diet dry matter (DM) consisted of 65% alfalfa haylage [International Feed Number (IFN) 3-00-217], 30% high moisture corn [IFN 4-20-770] and 5% soybean meal [IFN 5-20-637]. The alfalfa haylage contained 60% DM. Feed was available for 1 h after which any uneaten feed was removed. Cows were fed in this manner for 10 d before initiation of the first collection period. There were two collection periods, 14 d apart. During each collection period, DM consumption averaged 2.3% of body weight per 24-h period. Whole rumen contents (WRC) were sampled before feeding, immediately after feed removal and 1, 2, 3, 4, 6, 8, 10 and 12 h after feed removal. Immediately after feed was removed, cows were dosed with 200 mL distilled water containing 2 g of 15N enriched ammonium sulfate [73% enriched, Monsanto Company, Mound Facility, Miamisburg, OH] and 500 mL of chromium ethylenediaminetetracetic acid (EDTA) containing 20,000 ppm chromium. To obtain a representative sample of rumen contents, WRC subsamples were taken from 10 different locations using a 60-mL container. One subsample was from the reticulum and the other nine were from caudal, medial and ventral locations in the rumen.

Subsamples were composited, weighed and squeezed through eight layers of cheesecloth. The amount of strained rumen fluid (SRF) obtained was measured in a graduated cylinder and recorded. In order to remove residual fluid microorganisms not removed by the squeezing process, the particles were washed once with 0.85% wt/vol saline solution (equivalent to 20% of the volume of SRF obtained). The wash solution was added to the strained rumen fluid. This washing process may have also removed some of the particle-associated microorganisms. However, the fluid population was defined as microorganisms obtained in SRF plus one particle wash. A 40 mL aliquot was transferred to a vial containing 1 mL saturated mercuric chloride. The sample was chilled and centrifuged (30,000 x g, 30 min, 4°C). Resulting supernatants were analyzed directly for chromium by atomic absorption spectrometry and for ammonia and total free amino acids [11]. Mean dilution rate of chromium EDTA and rumen liquid volume were 0.138/h and 81.4 L, respectively.

To prevent microbial lysis, a final concentration of 0.5% wt/vol formaldehyde was included in the SRF plus saline wash solution. This concentration was lower than the 7.4% wt/vol formaldehyde level used by Isaacsen et al. [12]. Preliminary data indicated that 0.5 to 1% wt/vol formaldehyde was adequate for recovering maximum microbial organic matter and nitrogen. After addition of formaldehyde, the solution was chilled at 4°C before centrifugation.

Particle-associated microorganisms were defined as those remaining on particles after obtaining SRF plus one particle wash. Microorganisms were removed by the following procedure. Approximately 100 g of squeezed particles (wet basis) were weighed into a vessel containing 600 mL of extraction solution (0.85% wt/vol saline containing 0.5% wt/vol formaldehyde and 0.1% wt/vol Tween 80). After chilling for approximately 24 h, contents were strained through eight layers of cheesecloth. Particles were then resuspended and washed seven times with 250-mL volumes of the extraction solution. Hence, a total of 2350 mL of solution was used in removing microorganisms from each 100 g sample of squeezed particles.

Microbial deposits were obtained for both populations by centrifugation (30,000 x g, 30 min, 4°C). Deposits were washed once with 0.85% wt/vol saline solution and recentrifuged. All microbial deposits and particle samples were lyophilized and allowed to equilibrate at room temperature for 3 d before determination of organic matter (OM) and DM [13]. Data for fluid microorganisms were expressed as mg OM per milliliter SRF. The amount of SRF collected during the straining procedure ranged from 230 to 435 mL, and 250 mL were centrifuged when possible. Microbial deposits ranged from 1.5 to 4.1 g OM. Total nitrogen (N) was determined by Kjeldahl digestion followed with NH₃ assay by colorimetry [13].

Microbial enrichment of 15N was determined by mass spectrometry [14]. The enrichment in each population was measured to investigate changes after a pulse dose of 15N and to determine if fluid microbial 15N enrichment was indicative of that of particle-associated microorganisms. During the first 4 h after dosing, fluid microorganisms had a higher 15N enrichment than the particle-associated population (Fig. 1). From 6 to 12 h after dosing, 15N enrichment was similar for both populations. Therefore, for several hours after dosing, 15N enrichment of fluid microbes did not reflect that of particle-associated microbes. In this study, 15N enrichment of particle-associated microorganisms obtained at specific times was used as a marker to quantify microbes removed from particles. To estimate total amounts of microbes associated with particles, non-ammonia nitrogen (NAN) and NAN-15N were determined on nonextracted particles. Ammonia was removed from particles by adding 5 mL 1 N KOH to approximately 0.4 g of particles contained in a 250-mL Kjeldahl digestion tube. Samples were placed in a 50°C oven for 1 h to volatilize ammonia. Preliminary trials using [NH₄]₂SO₄ and asparagine indicated that 99% of ammonia and 3% of asparagine N were removed using this procedure.

The particle-associated microbial mass was expressed as mg OM/mL SRF equivalent and computed using the equation:

\[
\text{particle-associated microorganisms} = \frac{[\text{mg OM in microbial deposit} + \frac{[\text{PDME/PDMC} \times 0.64 \times 15N\text{ rec}}{\text{ml SRF col}}]}}
\]
FIGURE 1 Enrichment of $^{15}$N in fluid (•) and particle-associated (▲) microorganism after pulse dose with $^{15}$N. Pooled SEM for fluid and particle-associated microorganisms were 0.011 and 0.010 $\mu$g $^{15}$N, respectively.

where mg deposit OM is obtained after centrifuging 1500 mL of extraction solution; PDME/PDMC is the fraction of total particle dry matter collected which was extracted; the constant 0.64 is fraction of volume extracted from which particle-associated microorganisms were harvested [1500 mL/2350 mL]; $^{15}$N rec is the proportion of particle-associated NAN-$^{15}$N extracted and recovered in the microbial deposit; and SRF col is the original volume of SRF collected. The $^{15}$N rec was calculated by dividing total $^{15}$N in particle-associated microbial deposit by total NAN-$^{15}$N added to extraction jar. Using the volume of SRF as the final denominator allows for comparison of both populations on an equivalent ruminal basis.

Using this approach, it is not necessary to assume that the composition of $^{15}$N enrichment of the two microbial fractions is the same or that every organism incorporates $^{15}$N ammonia. It is assumed that the composition and $^{15}$N enrichment of microbial fractions isolated by these techniques are representative of the fluid and particle-associated total microbial pools in the in vivo rumen. Also, the assumption is made that nonmicrobial contamination is not sufficient to invalidate measurements of microbial fractions. Since microscopic feed residues can contaminate microbial deposits, many researchers use differential centrifugation, discarding the protozoa and particulate fractions sedimenting at slow speeds and retaining the bacterial fraction subsequently pelleted at high speeds. Differential centrifugation was not used in this study since the goal was to measure total microorganisms in both populations. Protozoa and fungi that sediment at slow speeds can play an important role in rumen function, and bacteria are also present in the form of clumps that sediment with protozoa and small particles (Olubobokun, J. A. & Craig, W. M., unpublished data and ref. 15). A preliminary experiment in which cows consumed a diet similar to that fed in this study, and in which SRF was filtered through eight layers of cheesecloth, indicated that the protozoal fraction ($500 \times g$ for 5 min) contained approximately twofold more dry matter than the bacterial fraction ($30,000 \times g$ for 30 min). The N content of the protozoal fraction was only slightly lower than that of the bacterial fraction (7.2 vs. 7.8% of DM), indicating feed particle contamination was not much greater in the protozoal fraction. Therefore, total microbial mass was more accurately measured by centrifugation of strained fluid at $30,000 \times g$ for 30 min.

Chemical changes in the two populations over the feeding period are reported in another publication (16). The N concentration in both fluid and particulate microbial extracts ranged from 6.4 to 9.7% of organic matter and was within the range reported for rumen bacteria (17—19). Lower N concentrations for both populations were obtained soon after feeding and were due to a dilution effect caused by accumulation of high levels of storage polysaccharide (16). Others have shown that N levels of rumen bacteria decrease due to large increases in nonstructural carbohydrates soon after feeding (18, 19).

Data were analyzed by least-squares procedures as outlined by Steel and Torrie (20). A completely randomized one-way analysis of variance was used to test if differences were observed over time. When differences were detected ($P<0.05$), means were compared by Fisher's protected LSD (20). Differences between SRF and particle-associated microbial organic matter across time points were tested with paired t-tests.
RESULTS

Recovery of particle-associated NAN-15N with the extraction procedure is presented in Table 1. As indicated by NAN-15N extraction, 32 to 52% of particle-associated microorganisms were recovered from particles taken 1, 2, 3, 6, 8 and 10 h after feed was removed. The proportion of particle-associated microorganisms recovered tended to increase with time after feeding. Recovery of 15N at 1 h was lower (P<0.05) than that recovered from 6 to 10 h after feed removal. Recoveries at 4 and 12 h are not reported since these samples were lost during handling.

Proportions of microbial organic matter contained in the two populations are presented in Table 2. Fluid microbial mass ranged from 8.9 to 12.5 mg OM/mL SRF with no differences (P>0.10) observed due to time after feeding. Particle-associated microbial mass was 2.3 to 4.4 times greater (P<0.01) than that in the fluid fraction (Table 2). Also, differences (P<0.05) with time after feeding were observed in the particulate fraction. The largest amount of microbial organic matter was observed at 1 h (47.5 mg) and lowest at 10 h after feed removal (20.6 mg). The sum of fluid and particulate microbial mass at each time reflects total microbial mass of rumen contents. Total microbial organic matter/mL SRF equivalent ranged from 58.3 mg at 1 h to 29.5 mg at 10 h after feed removal. This decrease was similar to that observed in the particulate phase, since 70 to 80% of the total mass was attributed to the particulate phase (Table 2).

Changes in rumen levels of ammonia and total free amino acids correspond to changes in particle-associated microorganisms (Fig. 2). Ammonia concentrations peaked 1 h after feed removal (P<0.01) and returned to levels similar to prefeeding levels by 6 h (P>0.10). Total free amino acid concentrations were highest when feed was removed (P<0.01) and returned to prefeeding levels by 3 h after feed was removed. Rumen free amino acids are normally low (21), similar to the levels observed from 3 to 12 h after feeding in this study. The high concentration (15 mM) obtained at time of feed removal (1 h after initiation of feeding) was due to high levels of free amino acids contained in the haylage (approximately 0.7% of dry matter).

Nitrogen content of squeezed particles ranged from

---

**TABLE 1**

<table>
<thead>
<tr>
<th>Nonammonia 15N</th>
<th>Recov. microbes</th>
<th>Recovery</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time after feed removed</td>
<td>Preextracted particles</td>
<td>Recovered microbes</td>
<td>Recovery</td>
</tr>
<tr>
<td>h</td>
<td>µg 15N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>219.2</td>
<td>70.5</td>
<td>32.1b</td>
</tr>
<tr>
<td>2</td>
<td>252.0</td>
<td>109.6</td>
<td>40.0ab</td>
</tr>
<tr>
<td>3</td>
<td>282.8</td>
<td>120.5</td>
<td>40.5ab</td>
</tr>
<tr>
<td>6</td>
<td>237.6</td>
<td>127.2</td>
<td>52.4a</td>
</tr>
<tr>
<td>8</td>
<td>261.6</td>
<td>129.4</td>
<td>49.9a</td>
</tr>
<tr>
<td>10</td>
<td>233.7</td>
<td>107.4</td>
<td>46.2a</td>
</tr>
<tr>
<td>Pooled SEM</td>
<td>40.3</td>
<td>24.2</td>
<td>4.43</td>
</tr>
</tbody>
</table>

1Extraction procedure described in Materials and Methods section.
2The 2 cows were allowed to consume feed for 1 h.
3Mean particulate nonammonia 15N added to extraction jars; DM added to jars ranged from 20 to 25 g.
4Mean particle-associated microbial 15N recovered in 30,000 x g centrifuged deposit.
5Means in column not sharing a common superscript are different (P<0.05).

---

**TABLE 2**

<table>
<thead>
<tr>
<th>Changes in microbial organic matter after feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time after feed removed</td>
</tr>
<tr>
<td>h</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>Pooled SEM</td>
</tr>
</tbody>
</table>

1The 2 cows were allowed to consume feed for 1 h.
2The quantity of particle-associated microorganisms was calculated using respective recovery values in Table 1 and the equation described in the Materials and Methods section.
3Means in columns not sharing a common superscript are different (P<0.05).
4Sum of fluid and particle-associated microorganisms.
5Values in parentheses represent percent of the total microbial OM associated with each fraction.

---

**FIGURE 2** Change in rumen fluid ammonia (•) and total free amino acids (■) before feeding (BF), 1 h after initiation of feeding (AF) and various time points after feeding. Pooled SEM for ammonia and amino acids were 2.32 and 1.03 µmol/mL, respectively.
2.9% (1 h) to 2.3% (6 h) during the collection period (Table 3). The portion of particle N which was plant N or microbial N was calculated using $^{15}$N. The microbial portion was computed by dividing $^{15}$N enrichment of particle N by that of the deposit from microbes extracted from the same sample. The assumption was made that deposit N represented microbial N, whereas particle N represented plant plus microbial N. Using this approach, microbial N was estimated to be 50 to 65% of total particle N. Microbial N (product of g particle N and % microbial N) ranged from 1.3 to 1.8 g/100 g particle DM, with no detectable changes ($P = 0.10$) due to time of collection (Table 3). Plant N ranged from 1.40 (2 h) to 0.86 g/100 g DM (10 h), and tended to decrease linearly ($P<0.08$) with time after feeding. The actual amount of plant protein degradation cannot be computed in this study since DM disappearance from the rumen, due to passage or digestion, was not measured.

The ratio of microbial N associated with particles (Table 3) to microbial N in the deposit prepared from extracted microorganism provides an estimate of the amount of particle DM that is microbial (Table 4). During the collection period, microbial DM ranged from 17 to 30% of the rumen particle dry matter. The percentage microbial DM obtained at 1 h was higher ($P<0.05$) than those obtained from 2 h to 10 h after feed was removed.

### DISCUSSION

The procedure used to remove particulate microorganisms consisted of chilling, use of a surfactant and multiple extractions. Each of these steps has been shown to contribute to removal of particulate bacteria and protozoa [7, 15, 22]. However, little is known about the proportion of total microbial OM removed using these procedures. Results from this study indicate that, although an extensive extraction procedure was used, only 32–52% of particle-associated microbes was removed. Also, the percentage of microbes removed tended to be lowest soon after feeding. These findings agree with the suggestion of Leedle et al. [17] that microbes attach tenaciously to forages soon after feeding. One possible reason for the lower amount of removal in this study is that many more microbes were attached at the earlier times (Table 2). The absolute amount of OM removed was not lower at earlier times. Approximately 15 mg OM/mL SRF equivalent was removed 1 h after feed was removed, decreasing to 10 mg OM/mL SRF, 10 h after feed removal [data not shown].

Fluid microbial OM did not change ($P>0.10$) during the 10 h after feed removal (Table 2). Others have reported that microbial mass or numbers, obtained from SRF or blended WRC, either did not change or decreased soon after feeding [17, 23]. Observations such as these and other measurements (e.g., RNA-to-protein ratios) have led researchers to conclude that microbial growth is slow (near stationary phase) in the rumen [24, 25]. In this study, changes in fluid microbial OM did not reflect the changes observed with particle-associated microbial OM. In addition to greater OM (mg/mL SRF equivalent) at all times, particle-associated microbial mass declined with time after feeding. Particle-associated microbial OM was highest 1 h after feed removal and lowest at 10 h after feed removal. This change in microbial OM suggests that microbial attachment to feed is rapid and indicates that microbial mass associated with particles increases in response to feeding. Microbial mass, as measured in this study, cannot be used to quantify number or type of viable microorganisms present. However, it is probable that the increase in microbial mass reflects, in part, an increase in microbial numbers. In vitro doubling times of 2 h or less
have been reported for some strains of ruminal bacteria (26, 27). Chemical analyses of the microbial samples in this study indicated there was a rapid change in cell composition after feeding (16). Nonstructural polysaccharide (expressed as glycogen equivalent) of particulate fraction increased from 11 to 34% of OM while crude protein decreased from 57 to 40% of OM. Although results from this study do not provide information on specific causes for changes in microbial mass due to feeding, it is apparent that particle-associated microorganisms, as well as fluid microorganisms, should be quantitated when rumen microbial measurements are made.

Particle-associated and total microbial OM were maximal at approximately the same time as when maximal rumen concentrations of ammonia and total free amino acids were observed (Fig. 2). The relationship between these metabolites to attachment and growth is not known. Maeng and Baldwin (28) observed that addition of free amino acids to rumen contents in vitro increased microbial cell yield by 87–156%, 1 h after dosing. The high levels of free amino acids observed in this study were due to feeding large amounts of high protein silage. It is possible that high concentrations of free amino acids occurring ruminally may increase cell yield and/or attachment.

Since a large proportion of particulate N was microbial (50 to 65%, Table 3), losses of plant N due to digestion were masked. Therefore, techniques used to measure N disappearance from solids must consider microbial N contamination. This may account for apparent increases in N of certain feedstuffs after short-term in situ incubations (29).

In this study, 15N ratios were used to estimate the proportion of particle N that was microbial. In using these ratios, the assumption is made that there is little or no plant contamination in microbial deposits. The amount of error is dependent on plant N contamination rather than plant DM contamination. Using data from this study, a 10% contamination from plant material containing 2% N would reduce the actual proportions of microbial N in total particulate N only 3 to 4% (Table 3). When plant N contamination is minimal, 15N enrichment ratios may be used to estimate microbial N contamination of particles. These ratios may also be used to measure microbial DM, as conducted in this study; however, the validity of this approach will depend on the extent of plant DM contamination in the microbial deposits.

ACKNOWLEDGMENTS

We wish to thank Len Strozinski, Leland Danz and Merilyn Marti for their excellent assistance in animal handling and care. The assistance of R. H. Burris in mass spectrometry of 15N is gratefully acknowledged.

LITERATURE CITED


