APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1993, p. 3250–3254
0099-2240/93/103250-05$02.00/0

Vol. 59, No. 10

Effect of Monensin on the Specific Activity of Ammonia Production by Ruminal Bacteria and Disappearance of Amino Nitrogen from the Rumen

C.-M. J. YANG[1] AND J. B. RUSSELL[2,3]*

Department of Animal Science[1] and Section of Microbiology,[2] Division of Biological Sciences, Cornell University, and USDA Agricultural Research Service,[3] Ithaca, New York 14853-8101

Received 2 February 1993/Accepted 2 August 1993

When unadapted mixed ruminal bacteria (312 mg of protein per liter) were treated with monensin (5 mM) in vitro, the rates of ammonia production from enzymatic digests of casein, gelatin, and soy protein (0.5 g of N per liter) were decreased from 46 ± 2 to 24 ± 1, 20 ± 1 to 7 ± 1, and 40 ± 2 to 18 ± 2 nmol/mg of protein per min, respectively. Monensin also caused a decrease in ammonia production in vivo. Nonlactating dairy cows which were fed 0.56 kg of timothy hay 12 times per day had a steady-state ruminal ammonia concentration of 2.7 ± 0.1 mM, and the ammonia concentration decreased to 1.2 ± 0.2 mM when monensin (350 mg/day) was added to the diet. The decrease in ammonia production was associated with a 10-fold reduction (4.1×10^6 versus 4.2×10^5/ml) in the most probable number of ammonia-producing ruminal bacteria that could use protein hydrolysate as an energy source. Monensin had little effect on the most probable number of carbohydrate-utilizing ruminal bacteria (6.5 versus 7.0×10^8/ml). The addition of protein hydrolysates (560 g) to the rumen caused a rapid increase in the ammonia concentration, but this increase was at least 30% lower when the animals were fed monensin. Since the specific rates of microbial utilization for soy, casein, and gelatin hydrolysates were lower in monensin-fed animals (0.80 ± 0.04 versus 0.33 ± 0.03, 0.55 ± 0.01 versus 0.18 ± 0.02, and 0.25 ± 0.01 versus 0.06 ± 0.02 h^{-1}, respectively), it appeared that monensin was increasing the rate of passage of amino acid nitrogen from the rumen.

Amino acid degradation in the rumen is a nutritionally wasteful process that often produces more ammonia than the microorganisms can utilize, and this excess ammonia represents a loss of dietary nitrogen (1). Because ruminal bacteria can take up amino acids at a rapid rate (6) and there is little free amino acid nitrogen in ruminal fluid (35), it had generally been assumed that proteolysis was the rate-limiting step in ruminal protein degradation (29). Winter et al. (34) noted that ruminal fluid sometimes had large amounts of nonammonia, nonprotein nitrogen, but the role of peptides as intermediates in ruminal protein degradation was largely ignored. In vitro studies indicated that peptides were resistant to ruminal degradation (4, 12, 26, 32, 36), and recent studies showed that peptides could accumulate in vivo (10, 11).

The ionophore monensin is commonly fed to beef cattle to improve the efficiency of feed utilization (17). Monensin was originally marketed as a methane inhibitor, but early work indicated that it also caused a decrease in ruminal ammonia (16). Since monensin has little effect on proteolysis (30), it appeared that peptide or amino acid escape might be responsible for the "protein sparing."

Recently isolated, monensin-sensitive ruminal bacteria (8, 27) produced ammonia at a much faster rate than monensin-resistant ruminal bacteria (3, 18), but their contribution to ammonia production in vivo had not been elucidated. The results presented here indicate that monensin (i) decreases the production rate of ammonia in vitro and in vivo and (ii) increases the flow of amino nitrogen from the rumen. These changes may be explained by a decreased number of monensin-sensitive, ammonia-producing bacteria which do not require carbohydrates as an energy source.

* Corresponding author.

MATERIALS AND METHODS

In vitro experiments. Rumen contents were obtained from two ruminally fistulated, nonlactating Holstein cows (approximately 700 kg each). The cows were fed 2.5 kg of timothy hay and 2.5 kg of a commercial concentrate mix (16% crude protein) twice daily. At 1.5 h after feeding, ruminal contents were squeezed through four layers of cheesecloth into a glass flask and allowed to stand for 1 h (39°C) anaerobically. After gas production had buoyed small feed particles to the top and protozoa had sedimented to the bottom, bacteria were collected from the middle section of the flask. The bacteria were harvested by centrifugation (10,300 × *g* for 15 min at 15°C in a CO_2 atmosphere) and transferred anaerobically to a basal medium containing 292 mg of $K_2HPO_4 \cdot 3H_2O$, 240 mg of KH_2PO_4, 480 mg of Na_2SO_4, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot H_2O$, 4,000 mg of Na_2CO_3, and 600 mg of cysteine per liter and either casein (Trypticase [0.5 g of N per liter]; BBL Laboratories, Cockeysville, Md.), gelatin, or soy hydrolysate (0.5 g of N per liter) (U.S. Biochemical Corp., Cleveland, Ohio). The final cell concentration was one optical density (measured by a Gilford 260 spectrophotometer at 600 nm with a 10-mm light path) or 312 mg of protein per liter. Triplicate bottles (80 ml) were sealed with butyl rubber stoppers and aluminum seals and incubated at 39°C. Monensin (5 mM final concentration) was dissolved in ethanol, and the final concentration of ethanol was 2% (vol/vol). Samples (taken after 0, 2, 4, 6, 12, 24, 48, 72, and 96 h) were centrifuged (13,000 × *g* at 22°C for 5 min), and the cells were washed once with 0.9% NaCl. The cell-free supernatants and cells were frozen (−20°C).

In vivo study. The same ruminally fistulated Holstein cows were fed 0.56 kg (dry matter basis) of chopped timothy hay (9% crude protein, 41% acid detergent fiber, 65% neutral

detergent fiber, and 17% nonstructural carbohydrates) every 2 h with a carousel feeder for 4 weeks. Casein, gelatin, or soy hydrolysate (560 g) (U.S. Biochemical Corp.) was dissolved in distilled water (2 liters) and added into the rumens through the fistulas on different days. Rumen fluid (300 ml) was obtained from the ventral and anterior sections of the rumens by suction at 0, 1, 2, 3, 4, 5, 6, 7, 8, 11, 12, and 24 h after administration of the hydrolysates. The rumen fluid was strained through four layers of cheesecloth and centrifuged (10,300 × *g* at 4°C for 20 min). The extracted ruminal fluid was stored at −20°C. Rumen fluid dilution rate was estimated from the disappearance of cobalt EDTA (Co-EDTA). The animals were dosed with 6 g of Co-EDTA, and samples were taken after 2, 4, 6, 8, 11, 13, 15, and 24 h. Cell-free samples were analyzed for Co with an inductively coupled plasma spectrometer (model 3400; Applied Research Laboratories, Sunland, Calif.). The animals were fed monensin for 4 weeks, and there was at least 1 week between the additions of protein hydrolysate.

Specific activity and enumeration. Particle-free ruminal fluid from the cows fed timothy hay 12 times per day was incubated with or without casein hydrolysate (0.5 g of N per liter), and the initial rate of ammonia production (specific activity from 0 to 6 h) was determined. The feed particle-free ruminal fluid was serially diluted (10-fold increments) in the medium described by Caldwell and Bryant (5) and either mixed carbohydrates (2 g of glucose and maltose and 1.5 of cellobiose per liter) or Casamino Acids (15 g/liter) (Difco Laboratories, Detroit, Mich.) were provided as an energy source. Triplicate tubes were incubated at 39°C for 72 h, and growth was scored by the increase in optical density (20).

Peptides and amino acids. Amino nitrogen utilization was estimated by a ninhydrin procedure (36). Cell-free supernatant was deproteinized (10), and samples (100 ml) were dried in an oven (110°C) to remove ammonia and hydrolyzed with HCl (6 N) (at 110°C for 24 h under N_2 gas). The samples were adjusted to pH 5.4 with NaOH and assayed by ninhydrin with glycine as a standard.

The amino acid composition of the residual amino nitrogen was estimated by high-pressure liquid chromatography (HPLC). Samples (25 μl) were hydrolyzed with HCl and derivatized with phenylisothiocyanate prior to being added to the HPLC column (14). This procedure gave the total amino acid composition of the samples. Peptides were then estimated by difference from samples which had been run through a C-18 Sep-Pak to remove peptides (Millipore Corporation, Milford, Mass.).

Other analyses. Ammonia was measured by the colorimetric method of Chaney and Marbach (7). Cell samples were boiled in 0.2 N NaOH for 15 min, and protein was assayed by the method of Lowry et al. (21).

Statistics. The in vivo experiments were conducted with two fistulated cows, and the standard deviations were calculated (see Fig. 1 to 3, error bars).

RESULTS

In vitro batch cultures. When mixed rumen bacteria were incubated in the basal medium which did not contain protein hydrolysate (controls), there was little increase in ammonia concentration (Fig. 1). However, when the bacteria were provided with protein hydrolysate, a rapid increase in ammonia concentration was noted. The ammonia production rates for soy and casein were faster than those for gelatin hydrolysate. When monensin (5 μM) was added, there was a decrease in ammonia production, which was associated with a decreased utilization of nonammonia, nonprotein, ninhydrin-reactive nitrogen (data not shown).

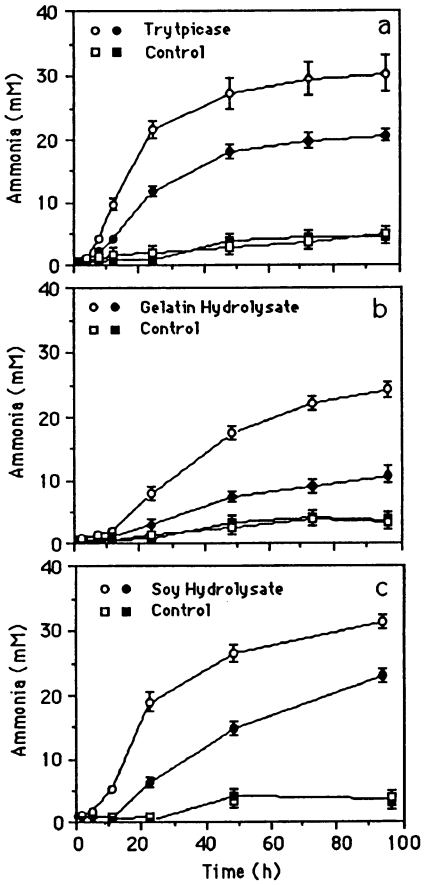

FIG. 1. Effect of monensin (5 mM) (closed symbols) on ammonia production in vitro from Trypticase (a), gelatin hydrolysate (b), or soy hydrolysate (c). Controls lacking monensin (open symbols) or protein hydrolysate are also shown. The standard deviations are indicated by error bars.

Ammonia concentrations in vivo. When the ruminally fistulated cows were fed the timothy hay diet 12 times a day at 80% of the National Research Council recommendation (22), the ruminal ammonia concentrations (2.7 ± 0.1 mM) achieved a steady state, which is characteristic of continuous culture. On the basis of the disappearance of Co-EDTA, the rumen fluid dilution rate was 0.07 ± 0.002 h^{-1} and the rumen volume was 80 ± 3 liters. The day-to-day variation in ruminal ammonia was less than 5%. Monensin had little effect on food intake, fluid dilution rate (0.066 ± 0.001 h^{-1}), or rumen volume (84 ± 1 liters), but there was a 50% decrease in the concentration of ammonia (1.2 ± 0.2 mM). This decrease in ruminal ammonia concentration was evident within 3 to 5 days after monensin supplementation.

Specific activity and MPN. When mixed ruminal bacteria from the cows were incubated in vitro with Trypticase (0.5 g of N per liter) on different days, ammonia was produced at a linear rate of 27.4 ± 2.2 nmol/mg of protein per min for 6 h. The most probable number (MPN) of bacteria in ruminal fluid which could utilize mixed carbohydrates (glucose, maltose, and cellobiose) as an energy source was 6.5 × 10^8/ml, but only 4.1 × 10^6/ml could use Casamino Acids as an energy source for growth. When the cows were fed 350

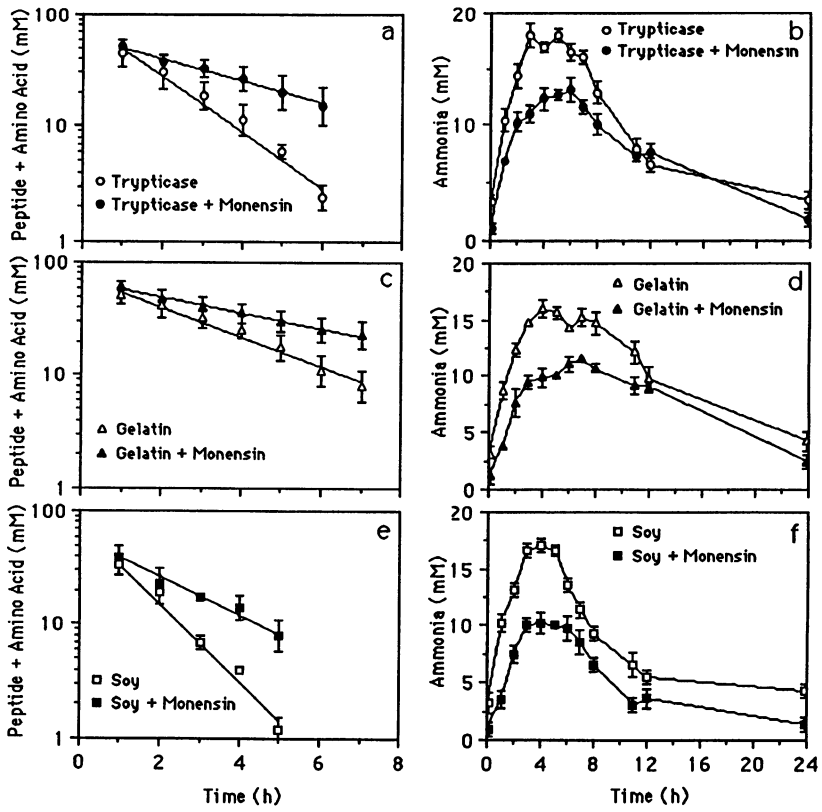

FIG. 2. Effect of monensin (closed symbols) on disappearance of peptide and amino acid nitrogen from the rumen and ruminal ammonia accumulation after Trypticase (a and b), gelatin hydrolysate (c and d), or soy hydrolysate (e and f) was added to the rumen. Controls without monensin (open symbols) are shown. The standard deviations are indicated by error bars.

mg of monensin per day, the specific activity decreased to 17.2 ± 1.5 nmol/mg of protein per min. Monensin had little influence on the MPN of carbohydrate-utilizing bacteria (7.0×10^8/ml), but the MPN of the bacteria utilizing Casamino Acids decreased 10-fold to 4.2×10^5/ml.

Peptide and amino acid disappearance in vivo. When protein hydrolysates were added directly to the rumens, the nonammonia, nonprotein, ninhydrin-reactive nitrogen (peptides and amino acids) decreased at a logarithmic rate (Fig. 2a, c, and e), and this disappearance caused an increase in ruminal ammonia concentrations (Fig. 2b, d, and f). The disappearance rate of soy hydrolysate was greater than that of Trypticase, and the disappearance rate of Trypticase was greater than that of gelatin hydrolysate. The disappearance rate was highly correlated ($r^2 = -0.94$) with the proline contents of the protein hydrolysate (Fig. 3). When the cows were fed 350 mg of monensin per day, the rates of nonammonia, nonprotein, ninhydrin-reactive nitrogen disappearance decreased (Fig. 2a, c, and e) and the ammonia concentrations were not as high (Fig. 2b, d, and f).

When the ruminal fluid samples were reanalyzed by HPLC, 60 to 90% of the nonammonia, nonprotein, ninhydrin-reactive nitrogen was recovered as amino acid nitrogen. The composition of the residual amino acid nitrogen varied with the protein hydrolysate, but the composition of the residue was remarkably similar to the original material at all times (data not shown). Most of the amino acids in the casein, gelatin, and soy hydrolysates were found in peptides (75, 90, and 98%, respectively). When soy hydrolysate was added to the rumen, free amino acids never accumulated. With gelatin hydrolysate and Trypticase, there was some increase in the concentration of free amino acids but most of the residual nitrogen was always present as peptide nitrogen. Monensin had little effect on the proportions of amino acid and peptide nitrogen.

DISCUSSION

Previous studies showed that monensin decreased ammonia production in vitro (9, 25, 30, 33) and in vivo (16, 24), but

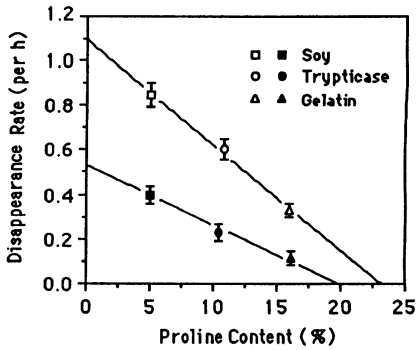

FIG. 3. Relationship between proline content of protein hydrolysates and their rates of disappearance from the rumen with (closed symbols) and without (open symbols) monensin. The standard deviations are indicated by error bars.

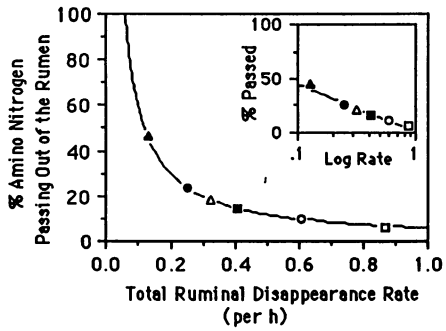

FIG. 4. Relationship between percent ruminal disappearance rate and the amounts of peptide and amino acid nitrogen passing out of the rumen. Results for soy (□, ■), Trypticase (○, ●), and gelatin hydrolysates (△, ▲) without (open symbols) and with (closed symbols) monensin are shown.

there has been much less documentation that monensin did indeed increase amino acid nitrogen flow from the rumen. Goodrich et al. (17) reviewed the effect of monensin on protein utilization and noted that animal growth responses were usually greatest when diets contained true protein. Poos et al. (24) reported that monensin caused an increased flow of amino acid nitrogen to the lower gut, but in other studies the flow of nonammonia nitrogen was only marginally increased (2). In some cases, the increased flow of plant amino acids was countered by a decreased flow of microbial protein (24).

For many years, it had been assumed that the most active ammonia-producing ruminal bacteria were gram-negative, monensin-resistant, carbohydrate-fermenting species (3, 13, 15) and the effect of monensin on ammonia production in vitro and in vivo could not be readily explained (16, 25, 30). Recent work showed that previously unidentified monensin-sensitive ruminal bacteria had a much higher specific activity of ammonia production (8, 27). Since the MPN of carbohydrate-utilizing bacteria did not decline, it appeared that the amino acid-sparing effect of monensin was due to a decreased number of the gram-positive, monensin-sensitive bacteria which could utilize amino acids and peptides as an energy source for growth. The latter bacteria were recently identified as *Peptostreptococcus anaerobius*, *Clostridium sticklandii*, and *Clostridium aminophilum* sp. nov. (23).

Since there is little amino nitrogen absorption in the rumen (28) and ruminal disappearance is a first order function of the passage and microbial utilization rates (19, 31), it was possible to estimate the amounts of peptides and amino acids which passed out of the rumen. As the total disappearance rate increased, there was a logarithmic decrease in the fraction of nonammonia, nonprotein, ninhydrin-reactive material passing out of the rumen (a ruminal degradation much faster than the passage rate [Fig. 4]). When the total disappearance rate decreased, passage accounted for a much greater proportion of the disappearance. With soy hydrolysate, only 6% of the peptides and amino acids escaped ruminal degradation, but the fraction was two to four times greater for Trypticase and gelatin hydrolysates.

The effect of monensin on amino acid nitrogen passage from the rumen varied with the protein hydrolysate. Monensin caused a 60% decrease in the disappearance rate of soy hydrolysate, but this decrease had only a small effect on the amounts of peptides and amino acids passing out of the rumen (Fig. 4). The passage rate was still much less than the microbial utilization rate. Monensin had a much greater impact on the amount of gelatin hydrolysate which passed. In the control animals (no monensin), only 19% of the gelatin hydrolysate would have passed, but nearly 50% passed when monensin was added.

In vitro experiments indicated that ruminal bacteria utilized proline-containing peptides at a slow rate (36) and that the proline content of the protein hydrolysate was inversely correlated with the ruminal disappearance rate. Monensin decreased the ruminal disappearance rate of all three protein hydrolysates irrespective of their proline content. Since proline-containing peptides were utilized slowly, one might have expected an increased passage of proline from the rumen. No such trend was noted. Because the amino acid composition of residual amino acid nitrogen was similar to that of the original material, it appeared that proline was protecting a variety of other amino acids from ruminal degradation.

The amino acid status of ruminant animals is dependent on the kinetics of ruminal fermentation (proteolysis, peptide utilization, microbial growth, and amino acid deamination). If the diet has enough ruminally degradable carbohydrate, ammonia can be used as a nitrogen source for microbial growth, and under these conditions, ammonia does not represent a significant loss of feed nitrogen. When the supply of ruminally degradable carbohydrate is low, much of the ruminally degradable protein can be deaminated and ammonia will accumulate. The loss of protein as excess ruminal ammonia can be reduced by the selection of feed proteins which are resistant to ruminal proteinases and peptidases. Ammonia losses may also be decreased by inhibiting ruminal bacteria which have a high specific activity of amino acid uptake and ammonia production.

ACKNOWLEDGMENTS

This research was supported by the U.S. Dairy Forage Research Center, Madison, Wis.

We thank Earl Cary for cobalt analyses and Lilly Research Laboratories for providing monensin.

REFERENCES

1. **Allison, M. J.** 1969. Biosynthesis of amino acids by ruminal microorganisms. J. Anim. Sci. **29**:797–807.
2. **Beever, D. E., H. R. Losada, D. L. Gale, M. C. Spooner, and M. S. Dhanoa.** 1987. The use of monensin or formaldehyde to control the digestion of the nitrogenous constituents of perennial ryegrass (Lolium perenne cv. Melle) and white clover (Trifolium repens cv. Blanca) in the rumen of cattle. Br. J. Nutr. **57**:57–67.
3. **Bladen, H. A., M. P. Bryant, and R. N. Doetsch.** 1961. A study of bacterial species from the rumen which produce ammonia from protein hydrolyzate. Appl. Microbiol. **9**:175–180.
4. **Brodrick, G. A., R. J. Wallace, and N. J. McKain.** 1988. Uptake of small neutral peptides by mixed rumen microorganisms in vitro. J. Sci. Food Agric. **42**:109–118.
5. **Caldwell, D. R., and M. P. Bryant.** 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. Appl. Microbiol. **14**:794–801.
6. **Chalupa, W.** 1976. Degradation of amino acids by the mixed rumen microbial population. J. Anim. Sci. **43**:828–834.
7. **Chaney, A. L., and E. P. Marbach.** 1962. Modified reagents for determination of urea and ammonia. Clin. Chem. **8**:130–132.
8. **Chen, G., and J. B. Russell.** 1989. More monensin-sensitive, ammonia-producing bacteria from the rumen. Appl. Environ. Microbiol. **55**:1052–1057.
9. **Chen, G., and J. B. Russell.** 1991. Effect of monensin and a protonophore on protein degradation, peptide accumulation, and deamination by mixed ruminal microorganisms in vitro. J.

Anim. Sci. **69**:2196–2203.
10. **Chen, G., J. B. Russell, and C. J. Sniffen.** 1987. A procedure for measuring peptides in rumen fluid and data suggesting that peptide uptake is the rate-limiting step in ruminal protein degradation. J. Dairy Sci. **70**:1211–1219.
11. **Chen, G., C. J. Sniffen, and J. B. Russell.** 1987. Concentration and estimated flow of peptides from the rumen of dairy cattle: effects of protein quality, protein solubility and feeding frequency. J. Dairy Sci. **70**:983–992.
12. **Chen, G., H. J. Strobel, J. B. Russell, and C. J. Sniffen.** 1987. Effect of hydrophobicity on utilization of peptides by ruminal bacteria in vitro. Appl. Environ. Microbiol. **53**:2021–2025.
13. **Chen, M., and M. J. Wolin.** 1979. Effect of monensin and lasalocid-sodium on the growth of methanogenic and rumen saccharolytic bacteria. Appl. Environ. Microbiol. **38**:72–77.
14. **Cohen, S. A., T. L. Tarvin, and B. A. Bidlingmeyer.** 1984. Analysis of amino acid using pre-column derivatization with phenylisothiocyanate. Am. Lab. **28**(8):48–59.
15. **Dennis, S. M., T. G. Nagaraja, and E. E. Bartley.** 1981. Effects of lasalocid or monensin on lactate-producing or -using bacteria. J. Anim. Sci. **52**:418–426.
16. **Dinius, D. A., M. E. Simpson, and P. B. Marsh.** 1976. Effect of monensin fed with forage on digestion and the ruminal ecosystem of steers. J. Anim. Sci. **42**:229–234.
17. **Goodrich, R. D., J. E. Garrett, D. R. Gast, M. A. Kirick, D. A. Larson, and J. C. Meiske.** 1984. Influence of monensin on the performance of cattle. J. Anim. Sci. **58**:1484–1498.
18. **Hino, T., and J. B. Russell.** 1986. Relative contributions of ruminal bacteria and protozoa to the degradation of protein in vitro. J. Anim. Sci. **64**:261–270.
19. **Hungate, R. E.** 1966. The rumen and its microbes, p. 206–240. Academic Press, New York.
20. **Ingraham, J. L., O. Maaloe, and F. C. Neidhardt.** 1983. Growth of the bacterial cell, p. 227–265. Sinauer Associates, Inc., Sunderland, Mass.
21. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
22. **National Research Council.** 1989. Nutrient requirements of dairy cattle. National Academy Press, Washington, D.C.
23. **Paster, B. J., J. B. Russell, C.-M. J. Yang, J. M. Chow, C. R. Woese, and R. Tanner.** 1993. Phylogeny of the ammonia-producing ruminal bacteria *Peptostreptococcus anaerobius*, *Clostridium sticklandii*, and *Clostridium aminophilum* sp. nov. Int. J. Syst. Bacteriol. **43**:107–110.
24. **Poos, M. I., T. L. Hanson, and T. J. Klopfenstein.** 1979. Monensin effects on diet digestibility, ruminal protein bypass and microbial protein synthesis. J. Anim. Sci. **48**:1516–1524.
25. **Russell, J. B., and S. A. Martin.** 1984. Effects of various methane inhibitors on the fermentation of amino acids by mixed rumen microorganisms in vitro. J. Anim. Sci. **59**:1329–1338.
26. **Russell, J. B., C. J. Sniffen, and P. J. Van Soest.** 1983. Effect of carbohydrate limitation on degradation and utilization of casein by mixed rumen bacteria. J. Dairy Sci. **66**:763–775.
27. **Russell, J. B., H. J. Strobel, and G. Chen.** 1988. Enrichment and isolation of a ruminal bacterium with a very high specific activity of ammonia production. Appl. Environ. Microbiol. **54**:872–877.
28. **Smith, R. H.** 1975. Digestion and metabolism in the ruminant, p. 399–415. University of New England Publishing Unit, Sydney, Australia.
29. **Tamminga, S.** 1979. Protein degradation in the forestomachs of ruminants. J. Anim. Sci. **49**:1615–1627.
30. **Van Nevel, C. J., and D. I. Demeyer.** 1977. Effect of monensin on rumen metabolism in vitro. Appl. Environ. Microbiol. **34**: 251–257.
31. **Waldo, D. R., L. W. Smith, and E. L. Cox.** 1972. Model of cellulose disappearance from the rumen. J. Dairy Sci. **55**:125–134.
32. **Wallace, R. J., and N. McKain.** 1989. Some observations on the susceptibility of peptides to degradation by rumen microorganisms. Asian-Australasian J. Anim. Sci. **2**:333–335.
33. **Whetstone, H. D., C. L. Davis, and M. P. Bryant.** 1981. Effect of monensin on breakdown of protein by ruminal microorganisms in vitro. J. Anim. Sci. **53**:803–809.
34. **Winter, K. A., R. R. Johnson, and B. A. Dehority.** 1964. Metabolism of urea nitrogen by mixed cultures of rumen bacteria grown on cellulose. J. Dairy Sci. **47**:793–797.
35. **Wright, D. E., and R. E. Hungate.** 1967. Amino acid concentrations in rumen fluid. Appl. Microbiol. **15**:148–151.
36. **Yang, C.-M. J., and J. B. Russell.** 1992. Resistance of proline-containing peptides to ruminal degradation in vitro. Appl. Environ. Microbiol. **58**:3954–3958.