Why Do Many Ruminal Bacteria Die and Lyse So Quickly?

ABSTRACT

Studies using $^{15}$N have indicated that as much as 50% of the microbial mass turns over before N passes to the lower gut, and this N recycling significantly decreases the availability of microbial protein. Protozoa digest bacteria and smaller protozoa, but bacterial protein can turn over even if protozoa are not present. Fibrobacter succinogenes cultures lyse even when they are growing, and the lysis rate is independent of growth rate. When extracellular sugar is depleted, F. succinogenes secretes an extracellular proteinase that inactivates the autolysins. This method of autolytic regulation decreases the turnover of stationary cells. Bacteriophage and anaeroplasma can cause lysogeny, but, as yet, there is little proof that these processes are important determinants of bacterial turnover in vivo. Dietary manipulations (e.g., salt feeding and particle size reduction) that increase liquid and solid dilution rates can increase bacterial flow by decreasing bacterial residence time and turnover. Some dead ruminal bacteria are able to maintain their cellular integrity, and the ratio of dead to live cells in ruminal fluid may be as great as 10:1. Bacterial survival appears to be at least partially explained by the method of sugar transport. When bacteria rely solely on mechanisms of ion-coupled sugar symport, an energized membrane is necessary for the reinitiation of growth. If group translocation (phosphotransferase system) is the mechanisms of transport, uptake can be driven by phosphoenolpyruvate, and an energized membrane and the storage of intracellular reserve materials are not an absolute criteria for survival. In some cases, N deprivation accelerates death. When Prevotella ruminicola was limited for N under conditions of excess energy, methylglyoxal production caused a rapid decrease in viability. The impact of bacterial death in the rumen is not clear-cut. If the rate of fermentation is zero-order with respect to cell concentration (substrate-limited), cell death would have little impact on digestion.

(Key words: rumen, bacteria, death, lysis)

Abbreviation key: MPN = most probable number, PTS = phosphotransferase system.

INTRODUCTION

Protein was first recognized as an essential nutrient nearly 200 yr ago. Most mammals with simple stomachs require at least eight essential AA (65). In the 1940s, Loosli et al. (54) demonstrated that ruminal microorganisms could synthesize most of these AA. In later studies, Virtanen (88) maintained lactating cows on protein-free diets with urea as the only N source and concluded that ruminal microorganisms provided ruminants with a de novo method of synthesizing essential AA. In conventional diets, microbial protein provides 40 to 90% of AA entering the small intestine (78).

Recommendations of the NRC (62) used a constant microbial yield, employed more or less fixed coefficients of digestion, and ignored microbial protein turnover. The Cornell Net Carbohydrate Protein System uses first-order rate constants to predict digestion and passage (78). This system also has a microbial turnover equation that discounts the theoretical (maintenance corrected) growth yield, but this empirical adjustment is always set at 20%. Microbial turnover constants as great as 90% have been reported (27), but, until recently, little information was available regarding the regulation of microbial turnover in the rumen.

MICROBIAL TURNOVER STUDIES

Radioactive isotopes of N (e.g., $^{13}$N) have extremely short half-lives, but mass spectrometry can differentiate $^{15}$N from $^{14}$N. Nitrogen turnover prolongs the dilution of $^{15}$N label from the rumen, and Nolan and Leng (63) noted that ammonia N...
dilution showed biphasic kinetics (Figure 1). Based on the observation that the initial rate of $^{15}$N dilution was 14 times greater than the final dilution rate, it appeared that 35% of the ammonia N was turning over. Some of this turnover was caused by ammonia absorption from the rumen and its return as $^{15}$N urea, but Nolan and Leng (63) indicated that only one-third of the total recycling could be explained in this fashion. The remainder was attributed to the turnover of microbial protein.

Firkins et al. (27) concluded that microbial protein turnover should produce peptides, AA, and nucleic acids, and they estimated microbial turnover from the flux of N through this NAN pool. Based on their calculations, the turnover of microbial protein was as high as 90%, but the NAN pool was very small, and the enrichment of $^{15}$N was low. Aharoni et al. (2) fractionated the NAN pool into nucleic acids and amide N and concluded that only a small fraction (approximately one-third) of the total NAN turnover could be attributed to microbial recycling. The additional turnover was not precisely determined, but Aharoni et al. (2) suspected that it might be amide N (e.g., glutamine).

The quotient of total flux (grams per hour) and pool size (grams) should be an index of growth rate (per hour), and Cottle et al. [as cited by Leng and Nolan (52)] reported growth rates of 0.05 and 0.08/h for bacteria and protozoa, respectively. Firkins et al. (27) indicated that the ruminal bacteria were growing at 0.08/h, but this value did not address turnover. If microbial turnover was 90%, the true growth rate (growth rate/(1 - turnover)) would have been 0.8/h (Figure 2). Given the observation that the maximum specific growth rate of most ruminal bacteria is <0.5/h (77), turnover >50% would be unrealistic. Overestimation of turnover would cause an underestimation of microbial yield and microbial N flow.

**Protozoal Predation**

Protozoal predation has often been cited as the key factor causing bacterial turnover in the rumen (64, 95). This conclusion has been based on a variety of observations: 1) protozoal mass is in some cases nearly as great as the bacterial mass (95), 2) protozoa can engulf bacteria rapidly (21), 3) little
solubilization of labeled bacterial protein occurs unless protozoa are present (90), 4) the ruminal ammonia concentration is generally greater when protozoa are present (87), and 5) defaunation can increase the performance of ruminants on low protein diets (7). However, many of these observations have been based on in vitro studies that did not truly mimic the ruminal environment. In vivo, protozoa take up small feed particles as well as bacteria (95), and most of the bacteria are present in biofilms rather than as free cells (58). In vitro studies usually have few competing feed particles, and this difference would also cause an overestimation of protozoal predation. Ruminal ammonia concentrations are almost always lower when protozoa are absent (87), but even this observation can be misleading. Because protozoa lyse easily in vitro (3, 20), the additional ammonia could have been derived from protozoa protein rather than from bacteria. Based on the observation that little protozoal protein ever seems to leave the rumen (1, 5, 51, 92), an alternative explanation is feasible. The protozoa lyse, and protozoal protein is then degraded by the bacteria.

**Bacterial Autolysis**

Krebs et al. (47) used $^{15}$N to estimate microbial protein turnover in defaunated sheep and concluded that ruminal bacteria were turning over even though protozoa were not present. This conclusion was supported by the observation that pure cultures of ruminal bacteria can lyse (8, 10, 11, 13, 24, 37, 47, 80). Lysis is generally described as a feature of starving cultures, but the mechanisms of cell lysis have not been studied in great detail. Aerobic bacilli lyse easily, and they frequently have been used as a model to study autolysis (73).

Bacterial growth is a balance of cell-wall synthesis and degradation, and, in the surface stress model of Koch (44), Gram-positive rods first deposit peptidoglycan at the inner surface. The older, outer layers are cut by autolytic enzymes, and stress is gradually transferred to more recently synthesized portions of the peptidoglycan (44, 45). Because each new layer of peptidoglycan is slightly longer than the preceding one, the wall is continually expanded.

Doyle et al. (25) noted that compounds that destroyed membrane potential promoted the lysis of bacilli, and a model of autolysis was introduced that was based on the localization of protons near the cell membrane (38). Rapidly growing bacteria would have high membrane potential and would accumulate protons at the cell surface. This low pH, in turn, would inhibit autolysin. Conversely, starvation could dissipate the membrane potential, increase pH, and activate the autolysins.

Compounds that decreased membrane potential accelerated the lysis of the ruminal bacterium, *Fibrobacter succinogenes*, but even growing cells with a high membrane potential were lysing and turning over (94). In *F. succinogenes*, lysis appears to be a property of growing cells that is independent of growth rate rather than a starvation or death response. When cells enter the stationary phase, the absolute lysis rate decreases, and this transition was inhibited by phenylmethylsulfonyl fluoride, an inhibitor of serine proteinases. Because this inhibitor only promoted the lysis of cells going into stationary phase, *F. succinogenes* appeared to be regulating its autolysis via a mechanism involving the proteolytic degradation of autolysins.

**Anaeroplasma**

Hungate (36) observed bacteriolytic microorganisms in the rumen similar to mycoplasma, and several anaeroplasma were subsequently isolated from the rumen. Anaeroplasma would promote bacterial lysis, but the anaeroplasma isolated by Robinson and Hungate (72) only attacked heat-killed, Gram-negative bacteria (70, 71). Anaerobic mycoplasma that were capable of infecting live, Gram-negative bacteria were present at less than $10^4$/ml of ruminal fluid [Prins and van Den Vortenbosch, as cited by Robinson (69)]. Ruminal anaeroplasma require lipopolysaccharide and cholesterol, but it is not clear whether anaeroplasma are truly parasitic (69).

**Bacteriophages**

Bacteriophages have been found at $1 \times 10^7$ to $1.6 \times 10^{10}$/ml of ruminal fluid (40, 42, 68). Lytic bacteriophages promote immediate bacterial lysis, but lysogenic phages can be retained in the bacterial DNA until a later time. Klieve et al. (41) concluded that lytic ruminal phages were of little importance, but 25% of the ruminal bacteria contained chromosomally stable lysogenic prophages. Large numbers of phages have been observed in the period shortly after feeding (43), but phage numbers alone are not indicative of bacterial turnover. One bacterium can give rise to as many as 47 phages (82), but the polysaccharide coating of ruminal bacteria may protect them from phage infection (58).

Figure 3. The relationship between dilution rate from the rumen and magnitude of microbial turnover at three different lysis rates: 0.1 (●), 0.01 (○), and 0.001 (▲).

Ruminal Dilution Rates

Harrison et al. (32) noted that the infusion of mineral salts increased the fluid dilution rate and flow of AA N from the rumen and concluded that the efficiency of microbial synthesis was being improved. Salts should have no direct effect on bacterial growth rate or maintenance, but would increase the ruminal fluid dilution rate. When the fluid dilution rate increases, greater opportunity exists for bacteria to pass out of the rumen prior to lysis and turnover (Figure 3).

VIABILITY

Direct Count Versus Viable Count

Animal viability can be assessed by respiration, heart function, brain waves, and other means, but such criteria are not so easily adapted to bacteria. Bacterial viability is usually assessed by reproduction or growth. Even early work (36, 91), indicated that the total count of ruminal bacteria was often 10-fold greater than the viable count, and this observation indicated that a large fraction of the ruminal bacteria might be dead. Bryant and Robinson (9, 12) noted the greatest ratio of viable to total cells just prior to feeding. Leedle et al. (49) reported that the difference between the total and viable counts was 10-fold just after feeding and only 2-fold 12 h after feeding. Similar trends were noted with diets of forage and concentrate, and losses in viability did not seem to be highly specific to diet.

In an effort to see whether ruminal bacteria differed in their ability to survive, Leedle et al. (40) plated samples on semi-selective media. Glucose- and starch-fermenting bacteria showed little variation during the feeding cycle, but cellulolytic and xylanolytic bacteria decreased dramatically 2 h postfeeding. The ruminal contents were homogenized, but it was not clear whether the decreases in cellulolytic and xylanolytic bacteria were due to death or merely to attachment. After 16 h postfeeding, viability declined slowly, and this decrease was almost certainly caused by starvation and death.

When mixed ruminal bacteria were anaerobically washed and starved for as little as 2 h, viable cell number declined 60%, but the stress of washing could not be excluded as a cause of death (50). If the cells were really starving, a decrease in polysaccharide, protein, or nucleotides would be expected, but no such responses were observed. Subsequent increases in viable cell count were attributed to cryptic growth, but only 8 bacterial cells disappeared for each new cell synthesized. The cryptic growth of Klebsiella (Aerobacter) aerogenes involved the turnover of 50 dead cells for the synthesis of 1 new cell (66).

Postgate and Hunter (66) postulated that the proportion of live cells would be dependent on growth rate in natural systems, such as the rumen. Selenomonas ruminantium (59, 61) and Ruminococcus flavefaciens (89) had higher viable cell counts at faster growth rates in continuous culture, but the survival of Megasphaera elsdenii (60), Sel. ruminantium (59, 61), and R. flavefaciens (89) was greater at slower dilution rates. These latter results indicate that substrate availability and growth rate are not reliable indices of survival.

Bacterial growth in a recovery medium is not always predictable. Postgate and Hunter (66) reported that rich media could actually decrease viable cell counts, and Kaprelyants and Kell (39) observed a greatly improved cell count when Micrococcus luteus was resuscitated in a minimal nutrient medium prior to plating. The substrate-accelerated death of K. aerogenes was counteracted by cyclic AMP, a regulator of catabolite repression (14), but most ruminal bacteria seem to have little cyclic AMP (22). Selenomonas ruminantium survived better in spent medium than in minimal medium, but differences in oxygen or redox potential could not be excluded (61).
When the ruminal bacterium, *Prevotella ruminicola*, was starved for N, intracellular ATP, intracellular potassium, membrane potential, and viable cell number declined rapidly (75). Because death-resistant mutants had lower rates of glucose transport (75), it appeared that glucose was causing the toxicity. Subsequent work indicated that *P. ruminicola* was unable to regulate its glucose metabolism, and the excess glucose was converted to methylglyoxal (76). Methylglyoxal is an alternative end product of glucose fermentation (85) that can kill cells and inhibit protein synthesis (31).

The idea that ruminal bacteria are very sensitive to starvation is supported by the observation that pure cultures of ruminal bacteria often require frequent transfer intervals (59, 60, 89, 93), but even this conclusion is based entirely on the assumption that growth and viability are synonymous. The extrapolation of in vitro viable counts to the rumen is also confounded by the fact that some ruminal bacteria have never been cultured in the laboratory. Quin's oval is a very large, commonly observed, and easily identified bacterium that has never been isolated (48).

**Most Probable Number Versus Plate Count**

Since the time of Koch and his treatise on the germ theory of disease (46), microbiologists have relied heavily on agar media and colony-forming units as an assay of viable cells. However, many bacteria do not grow well on agar surfaces (57). Most probable number (MPN) dilution methods allow the bacteria to grow in liquid medium, and MPN are often 10- or even 100-fold greater than plate counts. Determinations of MPN require a large number of replicates for an accurate estimate.

**Adenylate Charge, Membrane Potential, and Vital Stains**

Because ATP is a ubiquitous energy carrier in all living cells, it has been used as an indicator of viability (34). In theory, ATP would be needed to energize cell membranes, maintain transport activities, and reinitiate growth. Because metabolic processes are often more responsive to adenylate ratios than the ATP concentration per se, adenylate energy charge \((\frac{[\text{ATP}]+0.5[\text{ADP}]}{[\text{ATP}]+[\text{ADP}]+[\text{AMP}]})\) has been used as an index of viability (4, 23). Viability of *Escherichia coli* did not decline until the energy charge was <0.8 (15).

When mixed ruminal bacteria were separated by low speed centrifugation into free and particle-bound fractions, the bound bacteria had 10-fold more ATP than the fluid phase bacteria (29), but no attempt was made to correlate ATP and viability. Based on ATP alone, fluid phase ruminal bacteria would be classified as nonviable (96, 97), but Erfle et al. (26) indicated that the energy charge of free-living ruminal bacteria was not particularly low (approximately 0.7 to 0.9).

Assuming that an energized membrane is an absolute criterion of viability, some bacteriologists have used the uptake or exclusion of vital stains to assess viability (39, 57). The viability of *F. succinogenes* was highly correlated with membrane potential (93), but viable cultures of *Streptococcus bovis* did not have a membrane potential (74). *Clostridium aminophilum* (strain F) never has a high membrane potential (16), and high membrane potentials prevented *Peptostreptococcus anaerobius* (strain C) from transporting glutamine (6). Based on these comparisons, membrane potential is not an absolute criterion of viability.

**Mathematical Approaches**

Hungate (36) incubated ruminal microorganisms with a large excess of glucose and used the initial rate of gas production to estimate the fraction of ruminal bacteria that were in the fluid phase versus those that were attached to feed particles. This method was based on first-order kinetics and the assumption that rate of glucose fermentation would be proportional to the cell concentration. Feedstuffs in the rumen are usually substrate-limiting, and the fermentation rate is normally zero-order with respect to cells. Bacteria often exhibit a lag before they enter the growth phase, but this lag phase is not well understood. The lag time of *F. succinogenes* was highly correlated with a decline in viable cell numbers (93), and the proportion of viable cells could be extrapolated from the intercept of the growth curve (Figure 4). Cultures that had a greater proportion of viable cells had shorter lag times.

**ENDOGENOUS METABOLISM**

When exogenous energy sources are no longer available, endogenous metabolism must be fueled by the turnover of intracellular components. Once the cell mass decreases, there is less protoplasm to energize. Ribonucleic acid, DNA, and protein are costly to synthesize (33), but those components yield relatively little energy per unit of mass (23) and are not always utilized (59, 60, 61, 89). Polyphosphate and glycogen store energy more efficiently and are more readily utilized (28).
Escherichia coli stores glycogen when the growth medium has an abundance of sugar and other nutrients are limiting (67), but maximal glycogen deposition occurs just before sugar depletion. Glycogen synthesis appears to be triggered by a decrease in growth rate and an increase in cyclic AMP (35, 67). Escherichia coli only degrades glycogen when it is starving, and this careful utilization allows E. coli to remain energized and viable for days (81).

Many ruminal bacteria store glycogen-like materials (17, 18), and glycogen reserves decrease during nutrient deprivation (59, 60, 61, 89, 93). The glycogen depletion of F. succinogenes was a simple first-order function (Figure 5), and the initial rate of glycogen degradation was 10-fold greater than the endogenous rate needed to maintain cell viability (93). Because the glycogen was prematurely degraded, F. succinogenes had a short half-life. After 100 h of starvation, the viable cell count was <10^2/ml.

The rapid death rate of F. succinogenes could be explained by its method of sugar transport. Fibrobacter succinogenes does not have a phosphoenolpyruvate phosphotransferase system (PTS) to take up sugar and must use sodium symport mechanisms for sugar transport (19, 30, 55). When the endogenous metabolic rate of F. succinogenes was <0.02 mg of glycogen/mg of protein per h (93), the membrane potential declined, sodium accumulated, sugar transport was no longer possible, and the viable cell count decreased.

Streptococcus bovis does not store glycogen (79), but streptococci such as S. bovis can use phosphoenolpyruvate reserves and the PTS to drive sugar transport and reinitiate growth (86). Selenomonas
ruminantium has a PTS for glucose (56) and stores large amounts of glycogen (83). The rapid death rate of Sel. ruminantium (59, 61) may be related to lysis rather than to starvation per se. Starved mycoplasma accumulate sodium, swell, and lyse (53), and Sel. ruminantium also seems to swell when it enters stationary phase (83, 84).

**CONCLUSIONS**

Many ruminal microorganisms may not survive for long periods of time, but the impact of microbial death on ruminant performance is questionable. The rumen normally operates as a substrate-limited, continuous culture device that is first-order with respect to substrate, and, by this definition, the cells would be in excess. Differential rates of death could have an impact on microbial ecology, but, as yet, there has been little information to suggest that the death of individual species would affect fermentation end products. Lysis and turnover are phenomena that appear to have a greater impact on animal performance. One might envision several approaches for decreasing microbial turnover: 1) the elimination of protozoa, 2) genetic modification of the ruminal bacteria to decrease autolysis, and 3) increase in the ruminal dilution rates to remove microbial protein from the rumen before lysis can occur. Defaunation is not yet a practical approach, and bacterial autolysis and growth may be highly integrated processes. Given these constraints, modification of ruminal dilution rates via salt or particle size reduction is the most attractive solution.

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**REFERENCES**