ASPECTS OF METABOLIC REGULATION IN RUMEN BACTERIA

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ABSTRACT

Aspects of Metabolic Regulation

In Rumen Bacteria

By

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Aspects of metabolic regulation in rumen bacteria were studied using in vitro culture techniques. Growth rate of ruminal bacteria was varied by changing energy source and substrate limitation (glucose and nitrogen). Ribonucleic acid:protein ratios of rumen bacteria were highly correlated with specific growth rate, μ. As μ increased, RNA/protein and RNA/DNA values increased. The regression of RNA/protein on μ (six microorganisms) was $Y = .62\mu + .23$. Glucose and nitrogen limitation affected ($P<.01$) RNA:protein ratios of seven predominant ruminal strains. Nitrogen limited cultures had the highest RNA:protein ratio (.52), followed by nutritional sufficiency (.28) and glucose limitation (.12). The DNA:protein ratio of ruminal bacteria was not affected by variation in growth rate. Macromolecular composition of rumen bacteria may be used as an indicator of microbial activity in the rumen.

Proteolytic activity was assayed using azocasein as substrate. An interaction ($P<.001$) was observed between growth substrate and stage of growth as factors that affect exoprotease activity of Bacteroides ruminicola GA33. A similar interaction ($P<.01$) was observed between

nutritional status and stage of growth. It is concluded that energy limitation interferes with the mechanism that normally depresses ($P<.01$) exoprotease activity of stationary phase cultures of B. ruminicola GA33. Several other factors including rate of growth ($P<.01$) and nutritional status ($P<.01$) have been identified that influence the proteolytic activity of this organism. A plot of exoprotease activity vs dilution rate for a glucose limited continuous culture indicates that a complex, interacting set of controls may be involved in regulating the proteolytic activity of B. ruminicola GA33.

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INTRODUCTION

A truly symbiotic relationship exists between the host ruminant and its accompanying microbes. Structural polysaccharides of plants cannot be degraded by animal digestive processes and the herbivore relies on the microbes in the rumen to convert these to products which it can utilize for energy and growth.

Rumen Conditions Defined

A salient feature of the rumen is the rather constant set of conditions which support a complex microbial ecosystem (Hungate, 1966). Rumen bacteria are anaerobic and require rather exacting conditions to flourish. The presence of extremely dense microbial populations in the rumen (Gall et al., 1947; Bryant and Burkey, 1953; Bryant and Robinson, 1961; Caldwell and Bryant, 1966) indicate that these conditions are being met.

There are several factors that influence microbial growth in the rumen. Rumen temperature is maintained at approximately 39 degrees centigrade (Hungate, 1966) and an influx of feed and water insures that the bacterial population in the rumen is a dynamic one. The pH of the rumen is normally buffered between pH 6.0 and 7.0 (Bryant, 1977) by bicarbonate and phosphate present in saliva (McDougall, 1948). Ruminal absorption of volatile fatty acids (Pennington, 1952; Pennington, 1954) and ammonia (Hungate, 1966) also influences rumen pH. Osmotic pressure is generally hypotonic with respect to blood (Clarke and Bauchop, 1977). The

gas phase above rumen contents is highly anaerobic and contains gases which arise from the active fermentation in the rumen. The Eh of rumen contents ranges from a -250 to -450 mV. Thus, the rumen consists of a highly reduced environment capable of supporting the growth of anaerobic microorganisms comprising the rumen ecosystem (Bryant, 1977).

Rumen Ecosystem Defined

The complex interactions between rumen microorganisms foster a striking diversity within the microbial population. Approximately 200 species of bacteria and 20 species of protozoa have been isolated from the rumen (Russell and Hespell, 1981). Several explanations have been proposed to account for the abundance of niches which exist within the ruminal ecosystem.

The feed of ruminants is very complex and contains a variety of carbohydrates (both complex and simple), proteins, fats and numerous other organic compounds such as alkaloids and minerals (Hungate, 1966). Those organisms capable of vigorous growth on the available nutrients will survive. Bacterial species can compete for a given niche by optimizing one or more of several factors (Russell and Hespell, 1981). These include maximum growth rate, substrate affinity, tolerance to low pH, growth efficiency, the ability to metabolize a variety of substrates, the ability to metabolize recalcitrant substrates or resistance to predation.

An important determinant of relative species success is the maximum growth rate which a given species can achieve. Streptococcus bovis can achieve a doubling time of around 20 minutes (Russell et al., 1979), almost triple that of other ruminal strains. When substrate is plentiful, S. bovis can dominate the ruminal ecosystem (Bartley et al., 1975). This condition occasionally exists when cattle enter a feedlot or are shifted

from a primarily roughage diet to one high in concentrates (Bartley et al., 1975) and the pH of the rumen declines dramatically. During this period the predominate bacteria are Megasphaera elsdenii, Streptococcus bovis, and eventually a variety of lactobacilli. Under these rather specific conditions tolerance to pH appears to be an important factor regulating the population mix within the rumen.

Ruminal soluble substrate concentrations are usually very low (Takahashi and Nakamura, 1969). At low substrate concentrations the relationship between substrate concentration and growth rate in a batch culture can be described by the Monod equation:

$$\mu = \mu \text{ max } x \ (S/[K_S + S])$$

where: S = concentration of the growth limiting nutrient, μ = specific growth rate and μ max = maximum specific growth rate obtainable in the absence of any substrate limitation. The term K_S is a constant and is defined as that growth limiting substrate concentration which allows the organism to grow at one half the maximum specific growth rate. It is a measure of the affinity of an organism for the growth limiting nutrient and as such is also a major ecologial determinant in the rumen.

In the early 1960's Bauchop and Elsden (1960) proposed that yield (Y_{ATP}) of bacterial cells (g/mole ATP) was a constant at around 10.5. In the late 1960's and early 1970's many contradictions to this constant appeared in the literature (Hobsen and Summers, 1967; Hobsen and Summers, 1972; Howlett et al., 1976; Jenkinson and Woodbine, 1979). Theoretical calculations by Stouthammer (1973) indicated that Y_{ATP} could be as high as 32.5 for growth of an organism in a complex medium. Stouthammer and Bettenhausen (1973) extrapolated from experimental results and suggested that much of the discrepancy could be explained by the utilization of

increasingly greater proportions of energy for maintenance as the bacterial culture grows at slower growth rates. This relationship has been defined by Pirt (1965) and is expressed as:

$$[1/y^{obs}_{ATP}] + [(\mu/m) + (1/y^{max}_{ATP})]$$

where m = maintenance coefficient (mole/hr/g dry wt); μ = specific growth rate (hr^{-1}) and y^{max}_{ATP} extrapolated yield (g/mole) at $\mu = \infty$. Maintenance for individual species can vary greatly (Russell and Baldwin, 1979), even though the maintenance energy of the total rumen population is low compared to other bacteria (Issacson, 1973). The relative amount of energy required for maintenance can also be a determinant which affects the final population characteristics of the rumen ecosystem.

Predation by protozoa may be involved in the selection process as well. The rate of bacterial engulfment in the rumen can be quite substantial and under certain conditions approaches 20,000 bacteria/protozoan/hr (Coleman, 1975). Coleman and Sandford (1979) have elegantly demonstrated that Entodinium and other ruminal protozoal species show a selective response in their engulfment of mixed bacterial populations. For instance, Entodina species preferentially engulfed cellulytic bacteria as opposed to other ruminal strains.

The Rumen As A Continue Culture

Hungate (1966) proposed that the mechanics of the anaerobic fermentation in the rumen are comparable to those of a vat with a constant supply of fresh nutrient in which the culture constituents (bacterial cells, fluid and fermentation end products) are removed at a rate equalling the supply of fresh medium. Such a system has been defined as a continuous feed system akin to the continuous culture commonly utilized under laboratory conditions.

Hungate (1966) has outlined the mathematical design used to define the dilution of a pulsed marker added to a continuous fermentation system with a given turnover rate. The average time that rumen contents remain in the rumen must equal the time required for an equivalent amount of food to enter the rumen. Otherwise, the volume of the rumen would change. Many researchers have used these mechanics to study rumen function through the use of appropriate markers that flow with the various ruminal fractions (Faichney, 1975). This information has been used to calculate digestion of different components of the diet (Schneider and Flatt, 1975), microbial protein flowing to the lower gastrointestinal tract (Smith, 1975) and total non-ammonia nitrogen contribution to the nitrogen status of the host animal (Orskov, 1982).

Three different types of markers have been used to correspond to the three phases commonly associated with the rumen:

1) particulate markers - markers of the flow of undigested feed particles which remain insoluble within the rumen;

2) liquid markers - markers of the flow of liquid from the rumen;

3) microbial markers - markers which specifically measure the flow of microorganisms irrespective of their phasic distribution.

Faichney (1975) defined the ideal marker in the following manner:

1) It must be non-absorbable

2) It must not effect or be affected by the gastrointestinal tract or its microbial population

3) It must be physically similar to or intimately associated with the material it is to mark; and

4) The analytical procedure used to estimate it must be specific and sensitive and must not interfere with other analysis.

Soluble markers commonly used are PEG and Cr-EDTA while Cr_2O_3, ruthenium and a variety of rare earths (i.e., Lanthanium and Yutterbium) have frequently been used as particulate markers (Faichney, 1975). Microbial markers that have been used include RNA (Smith, 1975), DNA (Wolstrup and Jenson, 1979), Diaminopimelic acid (Theurer, 1982), and D-alanine (Garrett et al., 1982). The latter two are components of the bacterial cell wall.

Review of Growth Kinetics in the Rumen

The analogy of the rumen to a continuous fermenter is acceptable when used to define rumen turnover. On the other hand, direct application of the continuous culture model to define microbial growth in the rumen has some serious limitations. The major assumption inherent in continuous culture design is that a defined growth medium is used which contains a single growth limiting nutrient (Pirt, 1975). Because a direct relationship exists between the dilution rate and the concentration of growth limiting nutrient reaching the cells, an equivalence between dilution rate and growth rate of the bacterial culture exists at steady state. This relationship is defined by the following equation:

$$D = ([\mu \text{ max} \times S]/K_s])$$

where: D = dilution rate and S = steady state concentration of the growth limiting nutrient in the culture vessel. At steady state $\mu = D$ such that an increase in D will result in a comparable increase in μ.

This condition does not exist universally in the rumen. Intake is sporadic, especially when animals are fed only one or two times daily; rumen volume is not constant; rate of salivary flow is not constant (Hungate, 1966); and the rate at which material leaves the rumen is not constant, regardless if attributable to digestion (VanSoest, 1982)

fermentation (Hungate, 1966), absorption (Annison et al., 1957) or spillover to the lower gastrointestinal tract (Faichney, 1975). Transient feed supply would result in a burst of microbial growth followed by periods during which growth is stationary (Koch, 1971). Gas and VFA production (Hungate, 1966), P^{33} labeling (Bucholtz and Bergen, 1973) and simulated cell production with time after feeding (Baldwin and Denham, 1979) all indicate periods of growth transition in the rumen. A bacterial culture undergoing growth transients spends much time in periods of unbalanced growth, undergoing both shifts up and down during which it "gears up" for growth then destroys or alters much of its metabolic machinery in an effort to economize its maintenance expenditures (Koch, 1971). The continuous culture, on the other hand, exists in a state of balanced growth where every component, both structural and metabolic, doubles at exactly the same rate (Mandelstam and McQuillen, 1973).

El Shazley and Hungate (1965) extrapolated ruminal bacterial growth rate from increase in maximal rates of gas production between initial rumen samples and samples that had been incubated for one hour. These authors assume that fermentative activity is coupled to growth of microorganisms. Because uncoupled growth has been reported in rumen microorganisms (Jenkinson and Woodbine, 1979), this procedure may overestimate microbial growth. Using this technique, Hungate (1966) proposed that the average growth constant of rumen microbes in vivo was .06-.08 per hour. He states "The rumen resembles a continuous fermentation system with a growth rate similar to a late stage of a batch culture . . . where specific growth rate is .066 populations per hour" and further points out that substrate is limiting and products inhibitory during much of the feeding cycle (Hungate, 1966). Oxford (1964) takes

this one step further when he suggests "The fact that rumen bacteria seem to be acting so well within their theoretical potentialities, apparently dividing on the average only once or twice a day instead of several times per hour as many bacteria are capable of doing at 39° C, surely means that as biochemical agents they are more often acting in the resting state or even the stage of decline than in the logrithmic phase of growth . . . it seems likely that their logorithmic phase lasts for only very brief periods immediately after fresh fodder reaches the rumen. But their enzymic makeup will be very different according to stage of growth."

The attachment of bacteria to feed particles during the digestion process is another confounding factor. Hungate (1966) noticed a large decline in gas production when samples containing little or no particulate matter were used as innocula. Direct microscopic examination shows that much of the bacterial population is bound to feed particles (Akin, 1979; Cheng and Costerton, 1980). Cheng and Hungate (1976) found higher counts of bacteria in media containing solid preparations from alfalfa fiber than in media containing dissolved fiber oligosaccharides. Minato and Suto (1978) used Tween^{80} to detach bacteria from digesta particles and showed that approximately equal proportions of bacteria were associated with feed particles as with rumen fluid. Viable counts of adherent and free bacteria separated by homogenization also indicate that approximately one half of the bacteria present in the rumen are bound (Leedle and Hespell, 1980; Gillett, 1982). Furthermore, Forsberg and Lam (1977) found that 75% of the ATP measured in rumen samples was associated with feed particles.

The residence time of larger fibrous feedstuffs in the rumen is longer than the liquid residence times (on the order of 25 vs 10 hours) (Ellis, 1982). If a direct correlation exists between dilution rate of rumen contents and growth rate of rumen bacteria, then a two pool model must be invoked to describe microbial growth on feed particles which turn over at one rate and microbial growth in the rumen fluid which would tend to turn over with the liquid fraction.

If one assumes that the feed particles passing through the rumen are maximally colonized, or at least colonized to the same degree, than an exponential rate constant would by applicable. However, time required for colonization is greater than that required for turnover of the initial particulate fraction after its ingestion (Akin, 1979; Van Soest, 1982). A model describing linear growth of bacterial colonies on agar has been proprosed (Pirt, 1975). Application of this model to microbial growth on feed particles introduces a variable that renders unacceptable direct application of a continuous culture model to rumen microbial growth.

The Rumen As An Anaerobic Digestion Chamber

The nutrition of the ruminant depends heavily on the complex microbial interactions and metabolism that constitute rumen function. Up to 85% of the digestible dry matter of the normal production diet is digested by the rumen microbes (Bryant, 1977) with ensuing accumulation of volalite fatty acids, CO_2, methane, NH_3 and ultimately, microbial cells. The volatile fatty acids serve to meet the energy requirement of the host animal while microbial cells (which flow down the digestive tract) contribute significantly to the protein nutriture of the animal (Hungate, 1966). Because many of the rumen microorganisms can synthesize essential B vitamins, the ruminant can be maintained on diets devoid of otherwise essential vitamins (Hungate, 1966).

The major carbohydrate constituents of the ruminant diet frequently include fibrous materials such as cellulose and hemicellulose which are not susceptible to direct enzymatic attack by mammalian enzymes produced by either nonruminants or ruminants. There are no vertebrates that synthesize the enzymes capable of hydrolyzing the β linkages associated

with the chemical structure of these materials (Prins, 1977). The rumen ecosystem, however, possesses the ability to cleave β glycosidic linkages and, in the process, provide a substantial contribution to the nutritional status of the host animal.

A diagram indicating the points of rumen function that might best be manipulated to the host's advantage is shown in Figure 1. The diet of a ruminant contains both carbohydrates and proteins which are subject to hydrolysis and utilization by the rumen microbes. Proteins are subjected to enzymatic processes which first degrade them to amino acids and peptides and finally to ammonia and carbon skeletons. Energy is conserved as ATP during fermentation of sugars arizing from the digestion of complex polysaccharides. Anabolic reactions which utilize ATP as an energy currency then construct cellular materials from the end products of these digestive processes. Manipulation of these events to maximize the rumen's contribution to host physiology can take the following forms:

1) Changes in the fermentation to either improve dry matter digestibility or alter the stoichiometry of the end products (Chalupa, 1980).

2) Decreases in proteolysis of dietary protein to increase the amount which "bypasses" rumen degradation and reaches the lower gastrointestinal tract (GIT) intact (Chalupa, 1975).

3) Increases in both efficiency and yield in microbial growth (Bergen and Yokoyama, 1982).

The following chapters address various aspects of manipulation and/or assessment of rumen function.

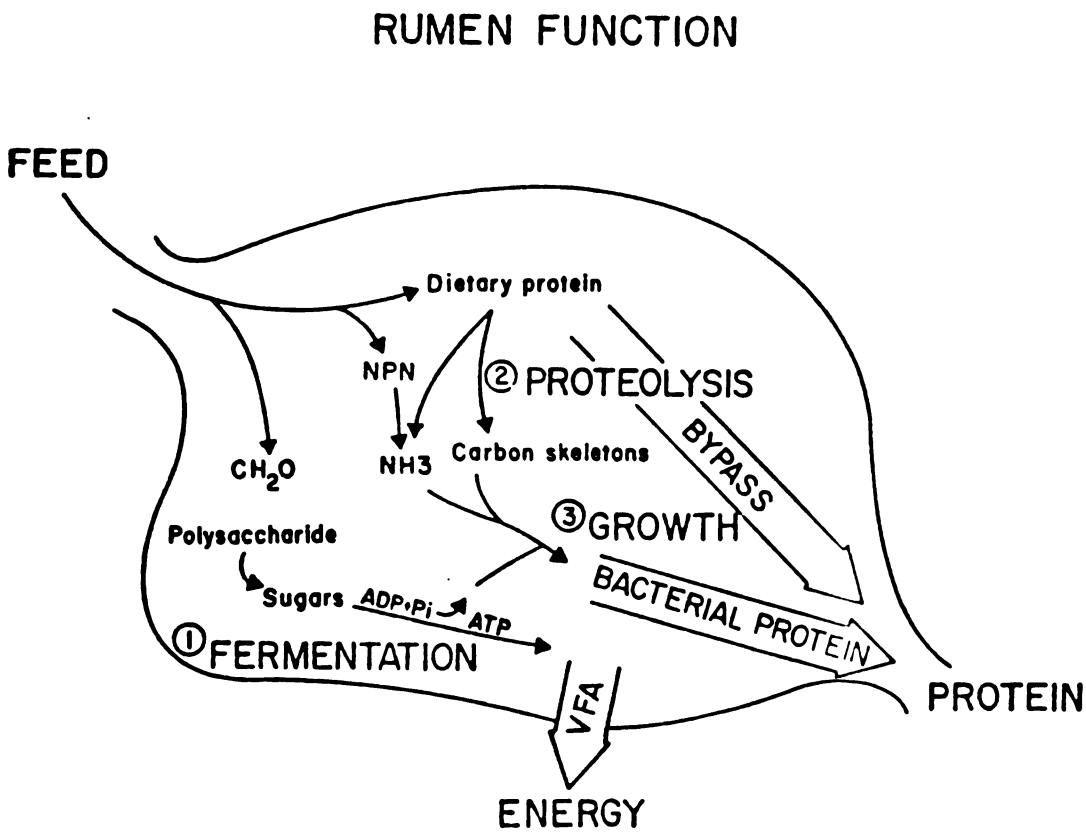

FIGURE 1. DIAGRAM OF RUMEN FUNCTION.

CHAPTER I

NUCLEIC ACID - PROTEIN RATIOS OF PURE CULTURES AND MIXED RUMINAL BACTERIA

Review of Literature

Effective nitrogen utilization by the ruminant requires a supply of protein of sufficient quantity and quality to satisfy the requirements of the animal for both maintenance and production. An adequate description of nitrogen reaching the small intestine requires measurement of the contributions made by both ruminally undergraded dietary protein and microbial protein synthesized during the fermentation of the feedstuff. One common approach has been to gauge the microbial contribution by using an internal marker of microbial protein such as ribonucleic acid (RNA). This approach assumes that most RNA reaching the lower gut is of microbial origin (Smith and McAllan, 1970).

The proportion of microbial nitrogen (N) in abomasal or duodenal N can be calculated by multiplying the ratio [(total N/bacterial dry matter) : (RNA-N/bacterial dry matter)] by {(RNA-N/abomasal dry matter) : (NonAmmonia Nitrogen (NAN)/abomasal dry matter)}. Microbial flow is then determined by multiplying this figure by total NAN passage to the lower GIT. Obviously, a prerequisite of this marker technique is the maintenance of a constant ratio of RNA-N:total-N in the bacterial population leaving the rumen.

Ellis and Pfander (1965) and Smith (1969) suggested that this relationship was constant in rumen bacteria under a variety of dietary

conditions and that it might be useful as a marker of microbial protein synthesis. Analyses of rumen microorganisms taken from sheep fed diets devoid of nucleic acids indicated that a relatively constant (10.4 - 14.8%) of the total microbial nitrogen could be attributed to ribonucleic acid N. These estimates were calculated assuming a constant Nitrogen: Phosphorus ratio of 15:3.8. Smith (1969) reported that approximately 19% of the total microbial N was nucleic acid. Smith (1969) also conducted an analysis of five species of rumen bacteria and concluded that RNA-N:total-N ratios were less variable than DNA-N/total-N.

Since these early studies, many workers have reported digesta passage studies where RNA-N/total-N has been utilized as a marker for microbial protein synthesis (Theurer, 1980). Unfortunately, a considerable range exists in the estimates of microbial yield using this technique (Table 1). This variation may be due to species variation, a physiological response of the rumen microoganisms or to difficulties with sample analysis.

Table 1. Summary of Experiments Reporting Microbial Protein Yield

FLOW MARKER	MICROBIAL MARKER	MICROBIAL PROTEIN YIELD[a] MEAN	RANGE
Chromium	RNA	12	6-20
Lignin	RNA	19	12-23

[a]Theurer, 1979
[b]Grams Microbial N/Kg DOM

Several problems have been identified with the use of this technique. These include contribution of dietary nucleic acid to the pool of nucleic acid measured postruminally (McAllan and Smith, 1973) and fluctuations

over time in the RNA-N:total-N ratio of mixed rumen bacteria (Smith, 1975). Recently, a correction factor of .85 has been proposed to account for the contribution of exogenous dietary RNA to nucleic acid measurements made postruminally (McAllan and Smith, 1973).

Mixed rumen bacteria sampled just before feeding showed significantly lower RNA-N/total-N then bacteria taken 4-6 hrs after feeding (Smith and McAllan, 1974). Czerakawski (1976) reported that the relative composition of microbial preparations varied more with diet than with time after feeding. RNA-N/total-N varied from .09 for a hay diet to .18 for diets containing high levels of grain. Considerable variation in the RNA-N:total-N ratio between individual animals and different diets was observed by Ling and Buttery (1978). Workers at Kansas State (Arambel et al., 1982) found extensive variation in RNA-N/total-N and DNA-N/total-N of 17 pure ruminal strains harvested in the stationary phase. Exact growth conditions were not reported.

Most in vivo RNA-N/total-N values vary from .16 - .20 for samples taken at several times after feeding and pooled prior to analysis (Poos et al., 1979 and Isichei, 1980). DNA-N/total-N values in the literature vary from .03 (Ellis and Pfander, 1965) to .07 (Smith, 1969). By using appropriate conversion factors (6.25 x N for protein, and 6.76 x N for RNA) (Ling and Buttery, 1978) the following calculation can be made:

$$(6.76 \times .18)\ [(1 - .18 - .05) \times 6.25] = .25$$

This value represents a conversion from RNA-N/total-N to RNA/protein and is representative of values reported by Gillett (1982) and Barao (1983).

Work with enteric bacteria has indicated that microbial RNA content is not constant per unit of cell mass and hence may be unsuitable as a

microbial biomass marker (Maaloe and Kjeldgaard, 1966). RNA content varies with the physiological state or specific growth rate of bacteria (Maaloe and Kjeldgaard, 1966; Rosset et al., 1966; Neirlich, 1978; and Ingrahm et al., 1983). RNA/protein values of .1-.2 are usually obtained for enteric organisms growing near stationary phase (Koch, 1971).

Bacteria in the rumen can be broadly divided into free floating organisms in the small particle-liquid phase and attached (adherent) or bound organisms in the large particule phase (Cheng and Costerton, 1980). Hungate (1966) and Oxford (1964) have indicated that rumen bacteria are present at 10^{10} to 10^{11} per g rumen contents and grow at an average rate of .06 to .07 doublings/hr. If all ruminal bacteria were growing at this rate, the RNA:protein ratios would be expected to be low and constant (Nierlich, 1978; Bergen et al., 1982). Variations in overall RNA/protein ratios of rumen bacteria would only occur if a sizeable fraction of the population were growing more rapidly. This is likely for free bacteria after feeding, especially when large quantities of readily fermetable carbohydrate are present in the diet.

The objectives of the work reported in this chapter were 1) To determine DNA/protein and RNA/protein values of several predominant ruminal bacteria when cultured at different specific growth rates and harvested at mid log and stationary phase 2) To apply this information to interpretation of existing data on the variation of RNA/protein that has been observed in the rumen with time after feeding and 3) To determine the effect of nutrient limitation on the RNA/protein of predominant ruminal bacteria.

Materials and Methods

Organisms

Butyrvibrio fibrisolvens D1, Selenomonas ruminantium GA192, Ruminococcus albus 7, and Bacteroides succinogenes S85 were obtained from the culture collection of the Department of Dairy Science, University of Illinois. Butyrivibrio fibrisolvens H106 and Ruminococcus flavefaciens C94 were a kind gift from Dr. B. A. Dehority, Department of Animal Science, Ohio Agricultural Research and Development Center. Bacteroides ruminicola subsp brevis (ATCC, 19188) and subsp ruminicola (ATCC, 19199) and Selanomonus ruminantium subsp lactilytica (ATCC 19205) were purchased from the American Type Culture Collection.

Streptococcus bovis was isolated from the rumen of a steer fed alfalfa hay. A bright orange colony was isolated from a 10^8 dilution into a roll tube containing medium 98-5 (Bryant and Robinson, 1961). The colony was one of the first to appear during a 24 hour incubation. The isolated cells are gram positive cocci that exist as singlets or pairs. This species ferments glucose, sucorse, maltose, mannose, cellobiose, lactose, fructose, galactose and soluble starch; but not xylose or arabinose. It is a homofermentative lactate producer and grows with a specific growth rate over 2.0 on most substrates.

Routine transfers of stock cultures were performed at monthly intervals. Gram stains and wet mounts of the respective microorganisms were examined bimonthly as a check for culture purity.

Cultivation

The Hungate technique was used in the preparation of media and cultivation of microorganisms. Inoculation and sampling were performed while continuously gassing with O_2 free CO_2 which had been passed

through a heated quartz column containing elemental copper. Cultures were grown at a constant 39°C.

Media

Bacteria were maintained on slants of Medium 10 (Caldwell and Bryant, 1966) supplemented with 10% (v/v) clarified rumen fluid and modified to contain .1% (v/v) of the volatile fatty acid mix. The defined experimental medium is shown in Table 2.

Variation in Growth Rate

Specific growth rate of a bacteria species is a function of the rate at which a given substrate is metabolized and the genetic potential of that strain to synthesize cellular material and divide. Growth rate of ruminal bacteria can be varied by changing energy source (Russell et al., 1979) or limiting sugar concentration in the growth medium (Russell and Baldwin, 1979). Both of these techniques were used in this work to grow ruminal strains at a diversity of growth rates. S. ruminantium was grown in media containing as the primary energy substrate .5% (w/v) fructose, glucose, sucorse, galactose, maltose and cellobiose, respectively (listed in the order reflecting ability to support rapid growth); B. ruminicola in media containing glucose, galactose, sucrose, fructose, lactose, maltose and cellobiose; B. fibrisolvens in media containing sucrose, fructose, cellobiose, galactose, glucose, xylose, maltose; B. succinogenes in media containing glucose and cellobiose; R. albus in media containing cellobiose and glucose; and R. flavefaciens in media containing cellobiose and glucose. In addition, each of these strains was grown in a medium containing .1% (w/v) glucose and the celluloylytics (B. succinogenes, R. albus and R. flavefaciens) were grown in a cellobiose medium from which the amino acid solution was deleted. Five separate growth trials and

Table 2. Experimental Medium for Pure Culture of Ruminal Bacteria[a]

Medium Components	Percentage in Medium
Carbohydrate source[b]	1 to .5 (w/v)
Yeast extract	.01 (w/v)
Vitamin solution[c]	1.0 (v/v)
Amino acid solution[de]	1.0 (v/v)
Mineral solution I[f]	7.5 (v/v)
Mineral solution II[f]	7.5 (v/v)
Hemin solution[f]	.5 (v/v)
VFA solution[g]	.33 (v/v)
Na_2CO_3 solution (8%)	5.0 (v/v)
Cysteine-HCl Na_2S $9H_2O$ solution[f]	2.0 (v/v)
Deionized water[2]	76.0 (v/v)

[a]Prepared under CO_2; final pH 6.8.
[b]Carbohydrate sources varied to achieve varying growth rates; glucose at various concentrations, maltose, cellobiose, lactose, galactose, xylose, fructose and sucrose used at .5%.
[c]Contained in mg/liter: thiamine HCl, 20; Calcium pantothenate, 20; nicotiamide, 20; riboflavin, 20; pyridoxine-HCl, 20; para-aminobenzoic acid, 1; biotin and B_{12}, .2.
[d]Contained .5% of aspartic acid, glutamic acid, threonine, serine, proline, glycine, alanine, valine, methinonine, isoleucine, leucine, tyrosine, phenylalanine, lysine, tyrptophan, histidine, arginine, asparagine, glutamine and cystine.
[e].1% Trypticase was substituted for amino acid solution during cultivation of B. ruminicola.
[f]Holdeman et at. (1977).
[g]Contained in ml/liter: acetic acid, 548.4; propionic acid, 193.5; n-butyric acid, 129.0; n-valeric acid, 32.3; isovaleric acid, 32.3; isobutyric acid 32.3; DL 2-methylbutyric acid, 32.3.

sampling periods were employed. The growth rate observed with a given bacterium on a particular substrate was not always constant. Russell et al. (1979) reported a similar observation in their study of maximum growth rates of rumen bacteria and suggested that genetic variation in the inoculum could affect maximum growth rate observed under a given set of conditions.

Determination of Growth Limiting Conditions

The K_s value of an organism for a given substrate is defined as the concentration of growth limiting substrate that will support one half maximal growth rate (Lynch and Poole, 1979). An approximate K_s that could be used to limit growth of the bacteria used in experiment one was determined for glucose, NH_3, amino acids and peptides. Varying concentrations of each of these nutrients were utilized to achieve a stepwise increase in both growth rate and yield up to a maximum value for both parameters. The appropriate modifications of the experimental medium included the following:

1. Use of .01, .05, .1, .2, .3, .4, .5 and 1.0% (w/v) glucose in the experimental medium to test for limitation by glucose concentration.
2. Use of .005, .01, .025, .05, .1, .15, .25, .5 and 1.0% (v/v) of an acidified (pH 4.0) amino acid mix (.5% (w/v) glycine, alanine, valine, leucine, isoleucine, serine, threonine, methionine, aspartic acid, asparagine, glutamic acid, glutamine, arginine, lysine, histidine, phenylalinine, tyrosine, tryptophan and proline) to test for amino acid limitation.
3. Use of .01, .05, .1, .2, .3, .4, .5, 1.0, 2.0, 3.0 and 7.5% (v/v) Mineral 2 solution in the experimental medium modified by removal

of the amino acid mix. This set of media were used to test for NH_3 limitation. Other minerals remained constant through appropriate mineral supplementation.

4. Substitution of .005, .01, .025, .05, .1 and .2% Trypticase™ (BBL Microbiology systems) for the amino acid mix to test for limitation by peptide.

Experiment Protocol - Experiment 1

Bacteria were grown in 500 ml round bottom flasks containing 300 ml of the experimental medium. Medium composition was designed to contain only trace Tricloracetic acid (TCA) precipitable protein or nucleotide sources.

Cultures were taken through several serial transfers in a medium identical to the experimental medium before they were used to inoculate experimental flasks. Only cultures in mid-log phase were used a inocula. Inocula represented 3% of the final volume of the experimental cultures.

Growth of bacteria was monitored by measurements of optical denisty (600 nm) of 5-ml samples taken from experimental flasks at various time intervals. The specific growth constant (μ) was computed by taking the slope of the ln optical density vs time curve. Preliminary growth curves for the various bacteria were prepared and final cell dry matter yield and the start of the retardation phase determined. Thirty five ml samples for RNA, DNA and protein analysis were initially taken at approximately mid-log phase of growth, which was defined as batch growth to an optical density of .5 $\pm$ .1. Each culture was then grown for a total of 48 h and a final sample representing stationary phase bacteria was taken. All culture growth had plateaued at final sampling.

Fifty percent TCA was added to the sample to yield a final concentration of 5% and the sample was placed on ice for at lease 30 min.

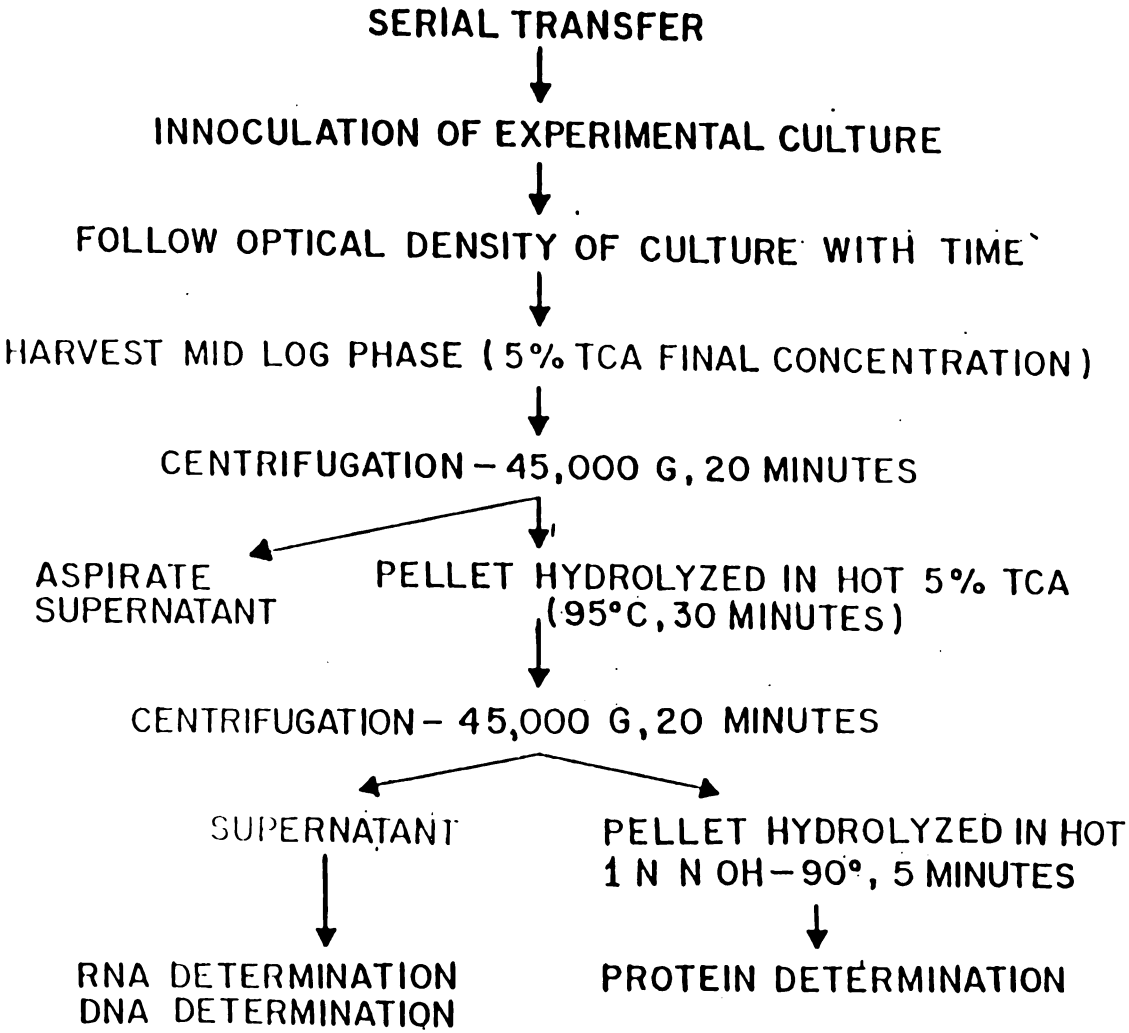

FIGURE 2. FLOW CHART OF THE METHODS USED TO DETERMINE THE RELATIONSHIP BETWEEN SPECIFIC GROWTH RATE AND RNA/PROTEIN RATIO OF RUMEN BACTERIA.

The sample was centrifuged for 20 min. at 45,000 x g and the supernatant aspirated leaving the precipitate as an intact pellet. This pellet was washed with cold 5% TCA and the centrifugation process repeated. The final pellet was hydrolyzed in 4 ml 5% TCA at 95C for 30 min. After cooling, the hydrolyzate was centrifuged at 45,000 x g for 20 min and the supernatant carefully removed with a pasteur pipette. The RNA and DNA were determined in this supernatant by the orcinol and diphenylamine procedure, respectively, using hydrolyzed yeast RNA and DNA as standards (Munro and Fleck, 1969).

The remaining pellets were solublized in 1 N NaOH for 5 min at 90C and analyzed for protein by the Hartree modification of Lowry (Folin-phenol) procedure. Bovine serum albumin served as protein standard (Hartree, 1972).

Experimental Protocol - Experiment 2

Bacteria were grown in 16 mM test tubes containing 10 ml of experimental medium prepared to provide the following nutritional states:

1. glucose, nitrogen sufficiency
2. glucose limitation, nitrogen sufficiency
3. glucose sufficiency, nitrogen limitation

In order to establish these conditions the experimental medium was prepared either as listed in Table 2, or modified to contain either glucose NH_3 or amino acids/peptide at the concentration representing the K_s value for each species tested (Table 3).

Experimental cultures were inoculated with inoculum grown in experimental medium through two successive generations. Inocula represented 3% of the final volume of the experimental culture. Growth was monitored at 600 nM with a Spectronic 70 (B & L) spectrophotometer and

Table 3. Adjustments to Experimental Medium to Impose Nutritional Limitations During the Stationary Phase.

	Limitation	
Species	Glucose	Nitrogen[a]
S. bovis	.1% (w/v)	.01% amino acid mix (v/v)
S. ruminantium	.05%	.01% amino acid mix
B. fibrisolvens	.1%	.01% amino acid mix
R albus	.05%	.15 mg% NH_3 (w/v)
R. flavefaciens	.1%	.15 mg% NH_3
B. succinogenes	.1%	.05 mg% NH_3
B. ruminicola	.08%	.01% Trypticase

[a].01% (w/v) dithiothreitol and 2% (v/v) Na_2S solution [2.5% (w/v)] were used as reducing agents.

bacterial samples were obtained 36 hours after growth had ceased. A second sample was obtained at 60 hours for S. bovis. Samples were processed and analyzed as in experiment one.

Statistical Methods

The in vitro bacterial studies (Experiment 1) were assessed by regression analysis (Gill, 1978) and were analyzed using the Genstat statistical package (Lawes Agricultural Trust, 1980). Bacterial RNA/protein vs μ were also specifically evaluated at μ from .1 to .5 by regression analysis and confidence intervals calculated for the y intercept and the slope of the regression (Gill, 1978). Experiment 2 was analyzed as a replicated 3 x 7 factorial design (Gill, 1978) using the general linear models procedure of the Statistical Analysis System (Freund and Littell, 1981) for main effects, interactions and overall means.

Results

Experiment 1

A total of seven strains of pure culture ruminal bacteria were grown at various μ and harvested for DNA, RNA and protein analysis at mid-log and stationary phase (μ=0). The combined RNA/protein (Figure 3) and RNA/DNA values (Figure 4) for six bacteria (except S. bovis) were plotted as nucleic acid/protein value (Y) against specific growth rate (μ). Clearly, as μ increased from .1 to 1.2 RNA/protein and RNA/DNA values increased for all organisms studied. The overall regression for RNA/protein vs μ was $Y=.62\ \mu + .23$, $r = .86$. The effect of μ on RNA/protein over μ from .1 to 1.2 was significant at $P<.005$. Based on the R^2, 74% of the RNA-protein variation was due to μ.

Typically, rumen liquid phase turnover varies from .03 to .12 h^{-1} or more (Bergen et al., 1982) and, hence, rumen organisms most likely do not

exhibit a μ greater than this range except during unrestricted growth of unattached bacteria immediately after feeding. To relate RNA/protein to over a more physiological range, RNA/protein was regressed against μ from .1 to .5. The regression was $Y=.9\mu + .14$, $r = .78$ ($R^2 = .61$). The effect of μ on RNA/protein over μ from .1 to .5 was significant at $P<.005$. The confidence intervals for the Y intercept and slope were $\pm$ .06 and $\pm$ .08 respectively. The overall regression for RNA/DNA vs μ was $Y=7.0\mu + 1.8$, $r = .89$ and based on the R^2, 79% of the variation in RNA/DNA was due to μ.

From the overall regression in Figure 1, the Y intercept was .23 with a CID of $\pm$ .1. This value represents the RNA/protein when $\mu = 0$. When preparations of mixed ruminal bacteria, isolated at various times after feeding, are assayed for RNA/protein, the values range from .2 to .3 (Isichei, 1980). The RNA/protein during stationary phase (48 h incubation) was also determined for each organism. Each culture that was sampled during exponential growth was also sampled during stationary phase. The data are presented in Table 4. The values obtained range from .18 to .47 (mean of .25) for the seven bacteria. The correlation between μ and RNA/protein (Table 4) ranged from .7 to .97 for the individual strains. The Y intercept for each regression then represents the RNA/ protein at $\mu = 0$. These RNA/protein obtained from the regression equations at $\mu = 0$ ranged from .08 to .29, with a mean of .21. The mean of the individual Y intercept ($\mu = 0$) based RNA/protein values and RNA/protein (stationary phase, $\mu = 0$) observed experimentally after 48 h incubation and from mixed ruminal bacteria preparations (Isichei, 1980) are comparable.

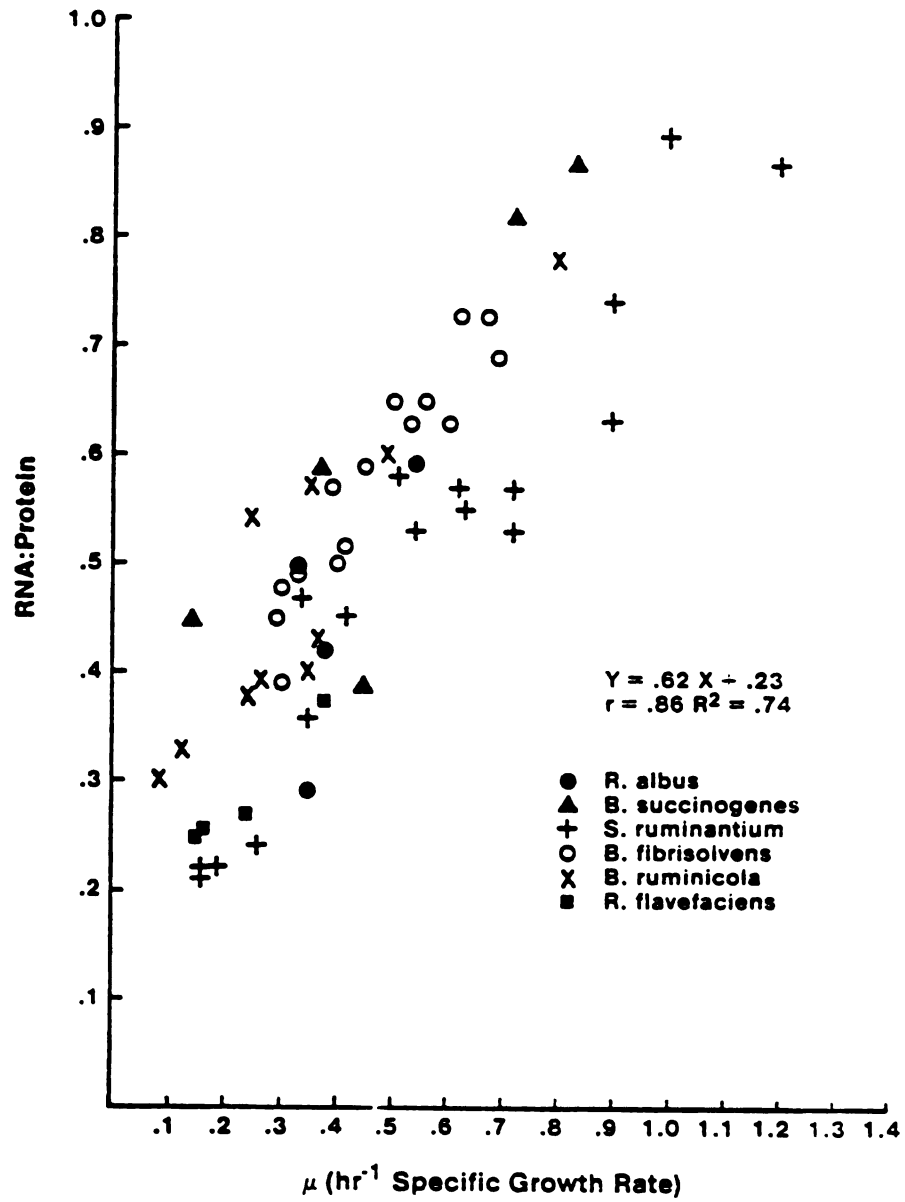

FIGURE 3. RNA:PROTEIN RATIOS AT SPECIFIC GROWTH RATES FROM .1 TO 1.2 DOUBLINGS PER HOUR FOR SIX RUMINAL BACTERIA

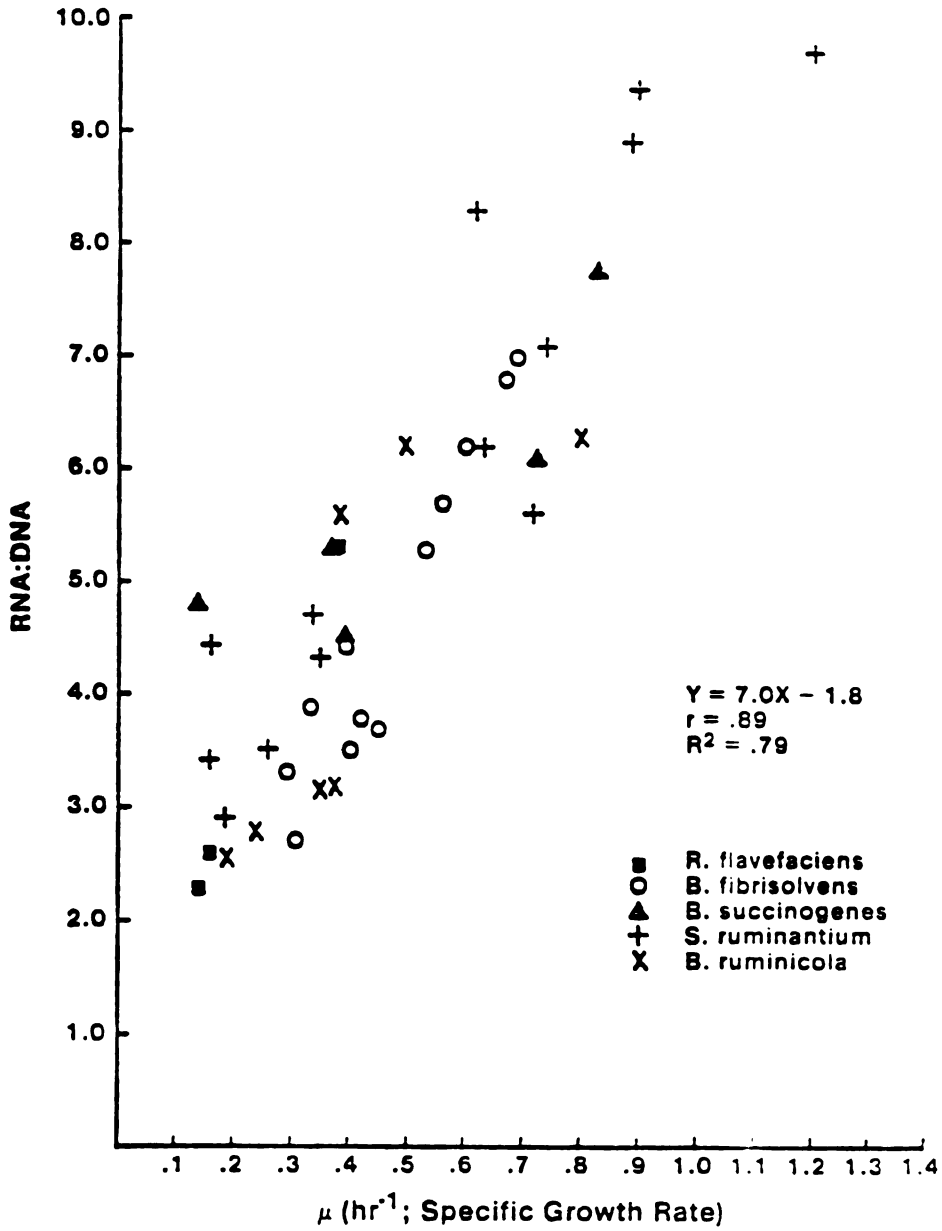

FIGURE 4. RNA:DNA RATIOS AT SPECIFIC GROWTH RATES FROM .1 TO 1.4 DOUBLINGS PER HOUR FOR FIVE RUMINAL BACTERIA.

Table 4. Observed and Predicted RNA/Protein Values for Zero Growth in Rumen Bacteria

Bacteria	RNA-protein at stationary phase	RNA-protein predicted when $\mu = 0$	Correlation between μ and RNA/protein
B. fibrisolvens	.24 ± .07[a]	.27	.93
R. albus	.18 ± .01[b]	.08	.70
B. succinogenes	.18 ± .05[b]	.29	.85
B. ruminicola	.23 ± .04[a]	.26	.90
R. flavefaciens	.23 ± .03[a]	.17	.97
S. ruminantium	.25 ± .05[a]	.14	.97
S. bovis	.46 ± .13[c]	.26	.94
Arithmetric mean	.25	.21	
SEM	.06		

a,b,c, $p<.01$

The DNA/protein (Figure 5) of ruminal bacteria growing at μ from .1 to 1.2 ranged from a low of .65 to a high of 1.5, with most values falling between .85 and 1.15. Thus, specific growth rate did not systematically affect bacterial DNA/Protein values.

RNA/protein values of S. bovis are plotted against specific growth rate (μ) in Figure 6. The values represented are the composite mean of 3 separate observations for growth on each of the substrates listed. The regression for RNA/protein vs μ for this organism was $Y=.28\mu + .26$, $r=.94$. The linear relationships appears to strictly adhere up to μ of approximately 1.5 after which a plateau is observed. The slope of this regression line is .24 as compared to .65 for the composite of the other six predominant ruminal strains tested. This indicates that at any given growth rate, less RNA is required by S. bovis than is required by other ruminal bacteria. Protein synthesis in this strain would appear more efficient than with any of the other six ruminal strains tested.

Streptococcus bovis also appears to retain RNA as it enters stationary phase provided a high RNA/protein ratio was established in the exponential phase of growth. This is evidenced by the relatively large standard deviation associated with the S. bovis mean (low RNA/protein values associated with low growth rates remained low, whereas high RNA/protein values associated with high growth rates remained high). The bacteria in the other two groups tend to assume a constant macromolecular composition in stationary phase regardless of growth rate during exponential growth.

Experiment 2

The effect of nutrient limitation on the RNA/protein ratio of several rumen bacteria is presented in Table 5. No significant species x treatment interaction was observed, although S. bovis tended to respond

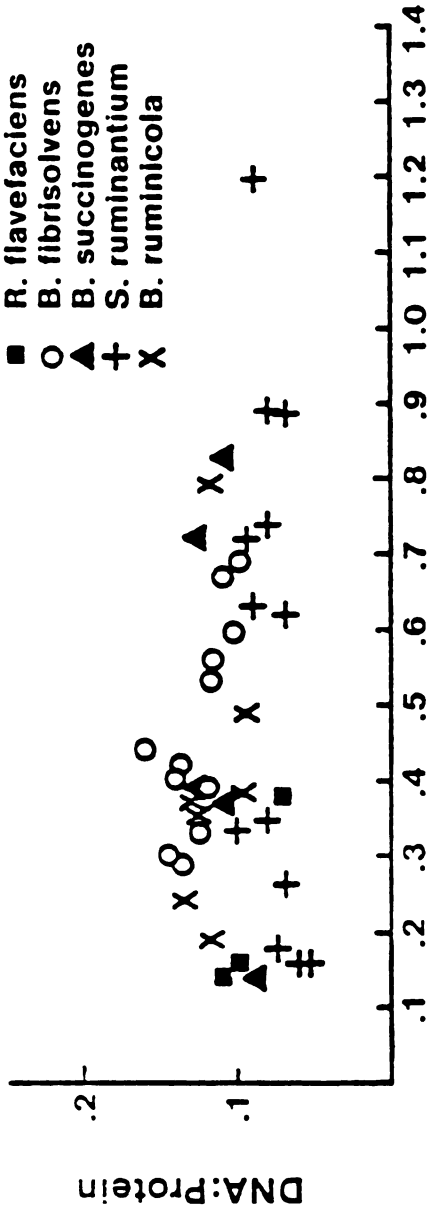

FIGURE 5. DNA:PROTEIN RATIOS AT SPECIFIC GROWTH RATES FROM .1 TO 1.4 DOUBLINGS PER HOUR FOR FIVE RUMINAL BACTERIA.

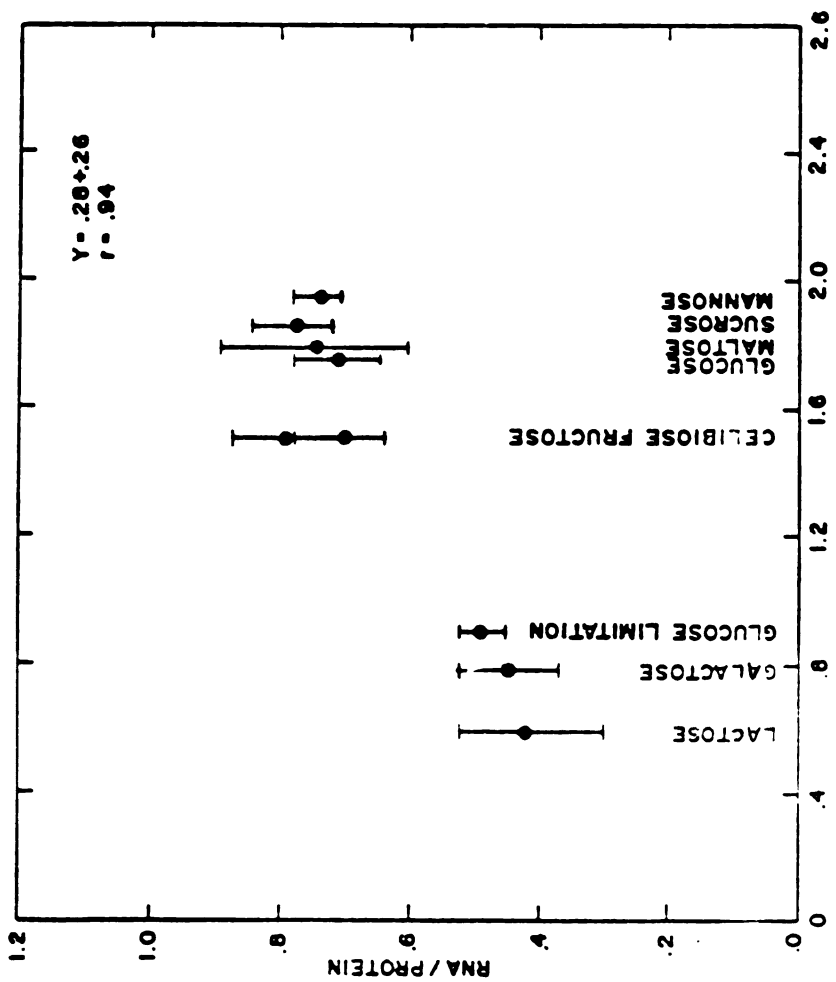

FIGURE 6. RNA:PROTEIN RATIO AT SPECIFIC GROWTH RATES FROM .4 TO 2.0 DOUBLINGS PER HOUR FOR STREPTOCOCCUS BOVIS.

Table 5. Effect of Nutrient Limitation on the RNA/Protein Ratio of Several Predominant Rumen Bacteria in the Stationary Phase of Growth.

	Species							
Treatment	S. bovis	B. ruminicola	S. ruminantium	B. fibrisolvens	B. succinogenes	R. flavefaciens	R. albus	SEM
Nutrient Sufficiency	.56	.29	.23	.25	.26	.19	.20	
Glucose Limitation	.21	.12	.12	.14	.08	.10	.07	
Nitrogen limitation	.65	.59	.33	.56	.56	.47	.39	
Mean	.47	.34	.23	.32	.30	.25	.22	.07

$P < .01$

Table 6. Nutrient Limitation as a Main Effect on the RNA:Protein Ratio of Ruminal Bacteria.

Treatment		RNA/Protein
Nutrient Sufficiency		.28[a]
Glucose Limitation		.12[b]
Nitrogen Limitation		.52[c]
	SEM	.08

a,b,c P<.01

Table 7. Species Variation as a Main Effect on the RNA:Protein Ratio of Ruminal Bacteria.

Species		RNA/Protein
S. bovis		.47
B. ruminicola		.34
S. ruminantium		.23
B. fibrisolvens		.32
B. succinogenes		.30
R. flavefaciens		.25
R. albus		.22
	SEM	.07

P<.01

differently to nutritional status than did the other six species. The RNA/protein value of stationary phase S. bovis was similar between nutritionally sufficient and nitrogen limited cultures whereas this ratio was much lower in nutritionally sufficient vs nitrogen limited cultures of the other microbial strains tested. The RNA/protein ratio of stationary phase cultures of seven rumen bacteria was affected by glucose ($P<.01$) and nitrogen limitation ($P<.01$) (Table 6). Nitrogen limited cultures had the highest ratio (0.52 across all species), followed by cultures grown with adequate energy and nitrogen (0.28) and glucose limited cultures (0.12). Significant species variation ($P<.01$) was also observed (Table 7). S. bovis had the highest overall mean stationary phase RNA/protein ratio. A group including B. ruminicola, B. succinogenes and B. fibrisolvens had intermediate value whereas S. ruminantium, R. flavefaciens and R. albus exhibited the lowest RNA/protein ratios across the three nutritional regimes.

Discussion

Physiological Status of Rumen Bacteria In Vivo

Schaechter et al. (1958) first reported that macromolecular composition of bacteria is determined by rate of growth. Since then, many other researchers have verified the validity of this concept (Neidhardt and Magasanik, 1960; Kjeldgaard and Kurland, 1963; Rosset et al., 1966; Dennis and Bremer, 1974; Quann et al., 1980). For a given microbial species, RNA/protein and RNA/DNA have been shown to be a function of growth rate. DNA/protein, however, is independent of specific growth rate (Leick, 1968). The macromolecular composition of ruminal bacteria reported in this study (as reflected in RNA/protien, RNA/DNA, and DNA/protein) is similar to other reported values for ruminal (Smith, 1969;

Mink et al., 1982; Russell, 1983) and nonruminal bacteria (Schaechter et al., 1958; Dennis and Bremer, 1974). As expected, RNA/protein and RNA/DNA increased with growth rate whereas growth rate had little effect on DNA/protein.

The DNA:RNA synthesis ratio has been suggested as a measure of community growth rate in complex bacterial ecosystems (Karl et al., 1981 a,b; Kirchman et al., 1982). John (1984) concluded that RNA/DNA in rumen bacteria followed the same general pattern as fermentation activity and microbial growth rate within the rumen. In a similar vein, RNA/protein is highly correlated with growth rate and physiological status of rumen bacteria in vitro. In this study, the relationship was not as strong over growth rates representative of in vivo conditions. However, it was still highly significant ($P<.05$) over a range of μ from .1-.5. Some general observations can be made by extrapolating from the plot of RNA/protein vs μ generated in vitro. First, RNA/protein approaching .2 clearly indicate that a bacterial population is growing very slowly if at all (Table 4). Second, RNA/protein less than .5 indicate that the bacterial population in the rumen is growing at a rate less than the maximum permitted by the genetic potential of the bacteria under optimal conditions. Fluctuations of RNA/protein due to experimental treatment may reflect variation in microbial growth rate, marked changes in population mix within the rumen or analytical error.

The results of the RNA/protein of rumen bacteria in vivo in a sheep study (Bates et al., 1985) are shown in Tables 8-11. A significant ($P<.001$) interaction of physical state (free vs bound) with diet was observed (Table 10). The RNA/protein of free floating bacteria isolated from corn fed sheep was higher ($P<.05$) than the ratio in free bacteria

Table 8. RNA:Protein Ratios of Free Ruminal Bacteria From Sheep Fed Three different Diets[1]

Hr. after feeding	Diet I[2]	Diet II[3]	Diet III[4]
0	.25 ± .01[a]	.25 ± .01[a]	.38 ± .02[a]
2	.29 ± .01[a]	.31 ± .02[a]	.36 ± .03[a]
4	.32 ± .01[a]	.34 ± .04[a]	.40 ± .03[a]
6	.31 ± .01[a]	.27 ± .04[a]	.52 ± .14[b]
8	.31 ± .01[a]	.27 ± .01[a]	.47 ± .09[a,b]
10	.29 ± .01[a]	.27 ± .01[a]	.40 ± .03[a]
12	.26 ± .01[a]	.27 ± .02[a]	.34 ± .03[a]
Mean	.29 ± .006[c]	.29 ± .011[c]	.41 ± .025[d]

[1]Bates et al., 1985
[2]Corn
[3]Corn-Corn silage
[4]Corn silage
[a,b]Values in columns (0-12h) not sharing common superscripts different at (P<.05).
[c,d]Values (in rows) not sharing common superscripts different at (P<.05). Differences at various sampling intervals across diets not tested.

Table 9. RNA:Protein Ratios of Adherent Ruminal Bacteria From Sheep Fed Three Different Diets[1].

Hr. after feeding	Diet Number I[2]	II[3]	III[4]
0	.24[a]	.13[d]	.21[a]
2	.22[a]	.15[a]	.18[a]
4	.22[a]	.18[a]	.24[a]
6	.22[a]	.13[a]	.22[a]
8	.22[a]	.15[a]	.22[a]
10	.21[a]	.16[a]	.23[a]
12	.22[a]	.12[a]	.22[a]
Mean	.23[c]	.15[d]	.21[c]
SEM	.007	.005	.005

[1]Bates et al., 1985
[2]Corn
[3]Corn-Corn silage
[4]Corn silage
[a,b]Values in columns (0-12h) not sharing common superscripts differnet at (P<.05).
[c,d]Values (in row) not sharing common superscripts different at (P<.05). Differences at various sampling intervals across diets.

Table 10. Interaction of Physical State and Diet on the RNA:Protein Ratios of Ruminal Bacteria From Sheep[1].

State	Diet I	Diet II	Diet III
Bound	.227	.147	.216
Free	.291	.288	.409

P<.001.
[1]Bates et al., 1985

Table 11. Overall Means Showing the Effect of Physical State, Diet and Time After Feeding on the RNA:Protein Ratio of Ruminal Bacteria From Sheep[1].

Item	RNA/Protein	N	Probability
State			
Free	.332	77	
Bound	.194	77	
SEM	.004		<.0001
Diet			
I	.259	42	
II	.217	56	
III	.313	56	
SEM	.004		<.001
Time after feeding[a]			
0	.243	22	
2	.252	22	
4	.286	22	
6	.279	22	
8	.280	22	
10	.263	22	
12	.242	22	
SEM	.004		<.14

[a]Shows a significant quadratic effect (P<.001).
[1]Bates et al., 1985

from the corn silage or corn-corn silage diet (.4 vs .29). Less marked variation was observed in the attached bacteria, but the RNA/protein of attached bacteria when corn-corn silage was fed was significantly lower ($P<.05$) than with either of the other diets (.15 vs .22).

Substrate limitation is generally accepted as the factor which most frequently limits microbial growth within the rumen (Hungate, 1966; Russell, 1984). Feeding a grain diet increases the concentration of soluble carbohydrate in rumen fluid (Takakashi and Nakamura, 1969). Increased RNA/protein of free floating bacteria when corn is fed may reflect greater substrate availability. If this is the case, two issues must be addressed:

1. The RNA/protein from corn fed sheep adherent bacteria does not differ from the RNA/protein of corn silage - attached bacteria
2. The RNA/protein from corn-corn silage fed sheep attached bacteria is depressed

Growth of adherent bacteria which colonize the surface of feed particles is supported by enzymatic attack of complex carbohydrates contained in plant material. Sugars that are released during the digestion process support a dense population of cells which form microcolonies (Costerton and Cheng, 1980). Growth in the center of a colony is limited by the sharp diffusion gradient which exists from outside to inside (Pirt, 1975). Soluble substrate concentration can increase (as in the case with high grain diets), but substrate availability within the microcolonies of attached bacteria may still be limited by restricted substrate diffusion into the center of the colony (Figure 7).

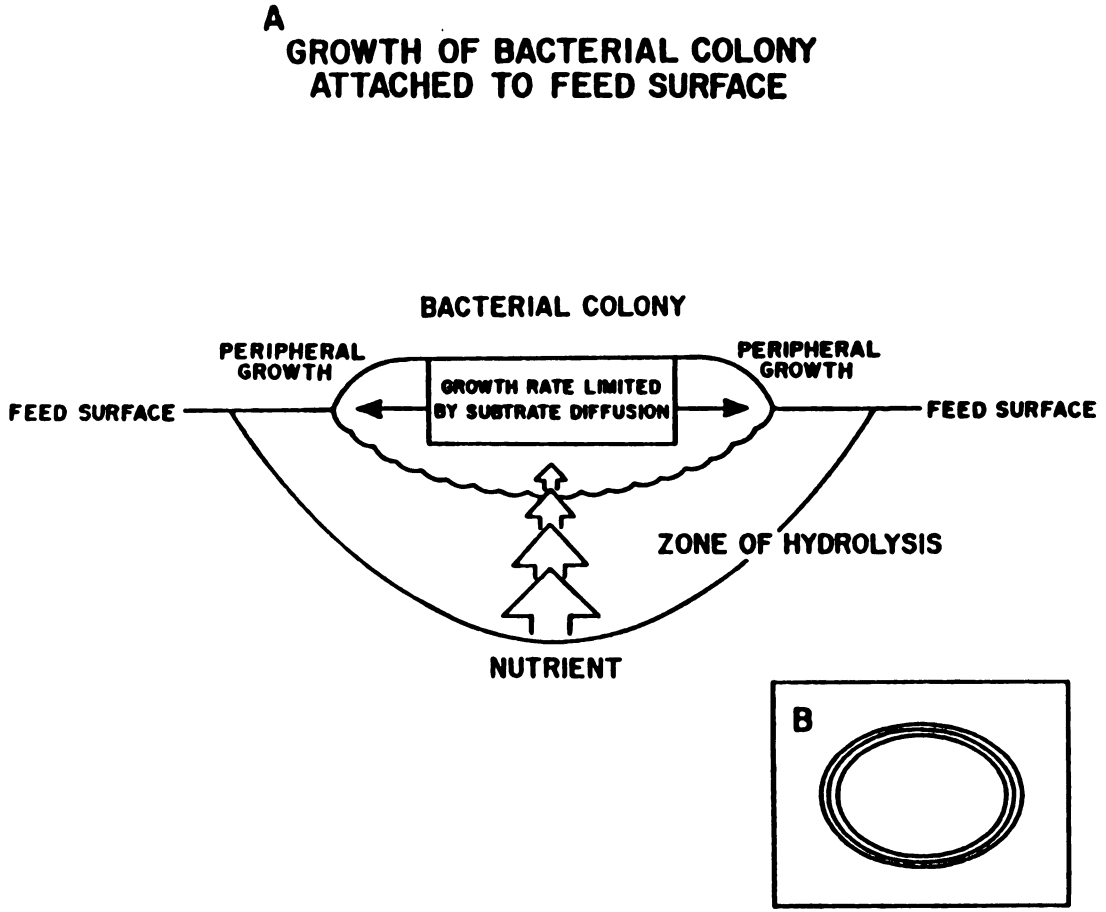

FIGURE 7A. GROWTH OF BACTERIAL COLONY ATTACHED TO A SOLID SURFACE CONTAINING NUTRIENTS.
7B. SHARP DIFFUSION GRADIENT EXISTS FROM THE EXTERIOR OF THE COLONY TOWARDS THE INTERIOR.

In addition, negative association effects have been reported when concentrates and roughages are fed in combination (Bergen, 1979). Depressed digestibility due to dietary interaction may explain the low growth rates indicated by low RNA/protein of adherent bacteria isolated from corn-corn silage fed sheep.

Variation in RNA-N/Total-N of rumen samples has been reported by a number of researchers. Diet, physical state and time after feeding have been implicated as factors which affect this ratio in rumen bacteria in vivo (Smith and McAllan, 1974; Czerkawski, 1976; Merry and McAllan, 1983). Czerkawski (1976) found that composition of microbial samples isolated from the rumen of sheep varied with diet and between samples taken before and 2 hr after feeding, although many of the differences disappeared when the results were expressed on a polysaccharide free basis. With sheep fed once per day, Smith and McAllan (1974) found that the RNA-N:Total-N ratios were lower for bacteria sampled just before feeding than 4-6 hrs later. Similar results were obtained when RNA/DNA were determined at various time intervals in sheep fed once a day, (John, 1984). Ruminal RNA pool size peaked at 3 hr post feeding while DNA reached a maximum at 6 hr. Overall, RNA/DNA was elevated over a 3-9 hr period after feeding. Cell composition with sheep fed hourly did not fluctuate and reflected the average daily values for sheep fed once daily (John, 1984). Marked differences have also been reported in the RNA-N/Total-N of bacteria associated with the solid and liquid fractions of rumen contents (Merry and McAllan, 1983). Merry and McAllan (1983) concluded that these differences may reflect different stages of growth or nutritional status, as well as bacterial species composition of the two fractions.

The RNA/protein of free floating from sheep (Gillett et al., 1982) and cattle (Barao et al., 1983) were also significantly affected by time after feeding. The free bacterial value obtained from grain fed sheep was significantly higher ($P<.05$) at 6 hr after feeding and then declined. In steers fed grain (Barao et al., 1983), the RNA/protein increased significantly ($P<.05$) for each 2 hr interval from 0-6 hr post feeding. These results are consistent with increased gas production (El Shazely and Hungate, 1965), volatile fatty acid production and P^{33} incorporation into bacterial cells (Bucholtz and Bergen, 1973) observed immediately after feeding.

Fluctuations in bacterial RNA/protein and RNA/DNA with time after feeding indicate that some bacteria in the rumen are in unbalanced growth with periods of shift up and shift down (Mandelstam and McQuillen, 1973). A continuous culture in steady state, on the other hand, is characterized by balanced growth in which every component of each bacterial cell within the culture is doubling at the same rate (Pirt, 1975). Clearly, fluctuations in the macromolecular composition of ruminal bacteria with diets fed once or twice daily (Gillett et al., 1982; John, 1984) represents a deviation from the continuous culture model for the rumen as proposed by Hungate (1966). Microbial growth kinetics in the rumen are more appropriately described by the repeated fed batch model (Pirt, 1975). In a repeated fed batch culture, microbial growth is characterized by growth transients similar to those found within the rumen.

The low RNA/protein of free ruminal bacteria isolated before feeding, and of adherent bacteria, indicate that a major proporation of the ruminal bacteria exhibit growth rates in vivo which are substantially lower than their maximal growth rates under optimal conditions. El Shazely and

Hungate (1965) predicted that rumen bacteria grow at a net average growth rate of .08 hr^{-1} in approximate agreement with average ruminal turnover time. When a bacterial culture ceases to grow because of nutrient limitation, chemical inhibition or physical stress, the RNA:protein ratio declines to a value predicted by the Y intercept of the regression of RNA/protein on μ (Table 4). This value was .23 for the composite regression (Figure 3) for six ruminal strains tested, which is slightly higher than the RNA/protein of adherent bacteria in sheep (.194). Several other lines of evidence provide support for the contention that bacteria in the rumen ecosystem spend considerable time at a low or static growth rate. These are high packing density of bacteria and protozoa in the rumen (Hungate, 1966), extensive attachment of bacteria to feed particles (Cheng and Costerton, 1980), relatively low adenylate energy charge observed in ruminal bacteria in vivo (Erfle et al., 1979), and extensive lysis, characteristic of bacteria in death phase, of ruminal bacteria observed in rumen contents (Nolan and Leng, 1972).

Metabolic Characteristics of Stationary Phase Cells

There are several important physiological implications of stationary phase. Static growth does not always mean that all hydrolytic activity by bacteria is curtailed, however net anabolic activities will be stopped. Continued metabolism of substrate occurs frequently during the stationary phase of batch cultures (Monod, 1949). Tempest and Niejssel (1978) reported that when glucose was pulse fed to a continuous culture growing at a low dilution rate, the rate of metabolism was increased above the value associated with classical operation of a chemostat. Growth efficiency of the pulsed cells was clearly diminished. Batch cultures of Ruminococcous flavefaciens grown with cellobiose as the primary substrate

attained maximum cell density with 40% of the substrate remaining (Pettipher and Latham, 1979); however, further increases in fermentation end products were noted until only trace amounts of sugar were detectable. Such cells represent the extreme in energetic uncoupling and inefficient utilization of substrate for growth.

Metabolic activity in the rumen may also be effected by bacterial growth stage. Pirt (1975) comments that "a feature of non growing organisms is their loss of enzyme activities . . . " and further speculates that these enzyme losses may be caused by turnover of intracellular protein which rises from <.5% hr^{-1} in growing bacteria to 5% hr^{-1} when growth ceases (Mandelstam, 1960). Enzymes active in the overall metabolic scheme in the rumen may be affected. Both aryl-B-glucosidase (Pettipher and Latham, 1979) and protease (Hazelwood et al., 1981) have been shown to decline in batch cultures of predominate ruminal strains as the cells enter stationary phase. Pettipher and Latham (1979) examined the influence of physiological status on hemicellulase, cellulase and pectinase in Ruminococcous flavefaciens. They concluded that there was no influence of stage of growth on any of these enzymes. Unfortunately, the bacteria were grown in glucose limited cultures (to avoid catabolite repression) and it is quite possible that this experimental design interfered with the ability of these researchers to detect an influence of physiological growth status. The pronounced intracellular proteolysis exhibited during stationary phase in energy sufficient cultures is absent if the cell is energy starved because intracellular proteolysis has an absolute energy requirement (St. John and Goldberg, 1978).

Experimental data on the production of several inducible catabolic enzymes were used to evaluate the dependence of rate of enzyme synthesis on the RNA content and specific growth rate of E. coli (Votruba et al., 1982). Specific rate of enzyme synthesis was proportional to cellular RNA at growth rates greater than .15. The rate of enzyme formation at a μ of zero was very low. The authors attributed this to an energy saving mechanism. A switch from translational control to transcriptional control of protein synthesis has also been shown to occur in mid exponential phase cultures of Klebsiella aerogenes (Bahramian and Hartley, 1980).

Energy charge values below .9 are frequently associated with non growing cells (Chapman and Atkinson, 1977). Ruminal energy charges of .681, .732, .773 and .827 were associated with dry matter digestibilities of 36.7, 48.2, 61.6 and 67.6% (Erfle et al., 1981), an actively growing rumen ecosystem is obviously required to optimize the fermentative capacity with the rumen.

Regulation of the Protein Synthesizing System in Prokaryotes

The growth rate of a bacterium is controlled by its nutritional, chemical and physical environment (Maaloe and Kjeldgaard, 1966; Nierlich, 1978). Efficient bacterial growth is often a reflection of how well a cell coordinates the size and activity of its protein synthesizing machinery with the catabolic and biosynthetic reactions that provide the adenosine triphosphate (ATP) and building blocks from which protein is synthesized (Inghram et al., 1983). Growth is considered to be a manifestation of a cell's ability to make protein because 1) protein comprises the bulk of cell dry weight, and 2) it is the primary constituent of enzymes that provide the catalytic basis of biochemical reactions. Bacterial cells are frequently under selective pressure to

out-compete neighboring cells for scarce nutrients such that efficiency of growth is a primary determinant which predisposes a cell towards survival. Thus, a basic tenet of bacterial physiology has arisen which states that control over the synthesis of the RNA components of the protein synthesizing system is central to the regulation of growth in the bacterial cell (Ingrahm et al., 1983).

This view is supported by the following observations:

1) A proportionality exists between RNA and growth rate.

2) The efficiency of a ribosome actively engaged in protein synthesis (expressed as average polypeptide chain growth rate) is independent of specific growth rate, except during slow growth or abnormal transients such as following B methyl-glucoside addition.

3) During normal transients (shift-up and shift down) the rate of rRNA accumulation may increase or decrease within a fraction of a minute whereas rate of protein synthesis only changes as the concentration of ribosomes in the cell changes.

4) The fraction of total protein that is ribosomal protein is positively correlated with steady state growth rate.

5) The synthesis of rProtein and rRNA is highly coupled

6) The synthesis of some of the protein factors involved in translation are balanced against the rate of ribosome synthesis. (Maaloe and Kjeldgaard, 1966; Nierlich, 1978; Maaloe, 1979 and Inghram, et al., 1983).

Both active and passive mechanisms have been proposed for the regulation of the genes coding for the protein synthesizing system. Active mechanisms are those in which some regulatory element directly influences the transcription of rRNA and rProtein genes. Guanosine

tetraphosphate has been implicated in this regard (Cashel, 1975). Travers (1976) has suggested that RNA polymerase is an allosteric protein subject to conformational changes by a variety of effectors. Maaloe (1979) has suggested that growth in an enriched medium results in repression of many of the pathways of intermediary metabolism. This may lead to a redistribution of RNA polymerase such that a greater proportion of the total transcription taking place is of rRNA and rProtein genes. He also suggests that at higher growth rates the function of weak promoters (that part of an operon to which RNA polymerase binds) will be saturated, again causing a redistribution of the binding of RNA polymerase to the stronger promoters associated with genes of the protein synthesizing system.

At about the time that Schaechter et al., (1958) were performing their classical studies on the relationship between macromolecular composition and growth rate, several laboratories observed that limiting amino acid availability resulted in discontinued synthesis of both RNA and protein in E. coli (Pardee and Prestige, 1956; Gros and Gros, 1958). In 1961, Stent and Brenner described certain mutant strains of E. coli which had lost the ability to control RNA synthesis in this manner. The discovery of guanosine - 5' - diphosphate-3'-diphosphate (ppGpp) and guanosine-5'-triphosphate- 3'-diphosphate (pppGpp) in wild type E. coli starved for essential amino acids (Cashel, 1969), the absence of these nucleotides in the mutant strains just described, and the observation that the concentration of these nucleotides varies with physiological state of the cell stimulated interest in these compounds as active regulators of protein synthesis.

When confronted with amino acid starvation most bacteria initiate what is referred to as the stringent response (Cashel, 1969). A depletion of

any species of aminoacyl tRNA triggers the stringent mechanism which is characterized by elevated ppGpp levels and a host of divergent cellular responses (Hazeltine and Block, 1973). These cellular responses are: Accumulation of stable RNA ceases; glucose respiration and glycolysis are repressed; membrane transport of glycosides and nucleobases is inhibited; phospholipid synthesis is depressed and the synthesis of cell wall components is curtailed (Cashel, 1975). PpGpp is truly a pleiotropic inhibitor of overall function (one gene product influences a number of diverse cellular activities).

Some processes are stimulated by ppGpp. These include glycogen synthesis, transcription of operons involved in amino acid biosynthesis and intracellular proteolysis (Cashel, 1975). While debate continues as to the exact role that this unusual nucleotide plays in regulating overall protein synthesis (Maaloe, 1979), much evidence has accumulated that implicate ppGpp in a number of enzymatic and transcriptional activities, including rRNA formation (Nierlich, 1978). PpGpp appears to be one of several means (i.e., cAMP, energy charge and absolute ATP concentration, catabolic reduction charge, anabolic reduction charge and a host of individual control mechanism involved with separate operons) by which a bacterial cell coordinates its cellular activities with the constraints imposed by a given external environment. As such, it should be anticipated that the exact nature of a cell's response to elevated ppGpp would vary in accordance with its interaction with the other regulatory mechanisms called into play.

Two mechanisms have been discovered by which ppGpp is synthesized. The first involves an enzyme called stringent factor and a ribosome bound ATP:GTP pyrophosphate transferase whose activity requires the presence of

mRNA and uncharged codon- specific tRNA. These requirements couple ppGpp synthesis by this mechanism to availability of amino acids and the rate at which they are removed for protein synthesis. Carbon source directly influences amino acid availability so ppGpp synthesis in relation to carbon source supply occurs via this route (Ingrahm et al., 1983).

The second system involves a relX gene product (Engel et al., 1979). This mechanism is much less defined, but seems capable of coupling ppGpp synthesis to energy downshifts (Engel et al., 1979).

Regardless of the route by which ppGpp is made, it is subject to degradation by a pyrophosphohydrolase which is the product of the spoT gene. Glucose exhaustion causes a rapid decrease in ppGpp degradation. An accumulation of ppGpp can be elicited by carbon source substitutions (Gallant et al., 1972) energy poisons (DeBoer et al., 1976) and membrane-perturbating agents such as Levallorphan (Boquet et al., 1973). Degradation of ppGpp in vivo is an energy dependent process and the maintenance of an intact transmembrane proton gradient has been implicated in its regulation. (Tetu et al., 1980).

In bacteria, the concentration of ppGpp varies inversely with growth rate (Cashel, 1975) consistent with the hypothesis that it serves as a metabolic inhibitor. Furthermore, growth and metabolic capacity of E coli in a recycling fermenter (100% cell recycle while whole fresh medium and spent broth are added and removed from the vessel at equal rates) have been shown to be dependent on relative ppGpp accumulation (Arbige and Chesbro, 1982). An analogous situation may exist in the rumen where dilution of rumen fluid and removal of fermentation products by absorption occur at a faster rate than outflow of microbes attached to feed particles.

RNA/Protein In Stationary Phase Cultures

In the normal bacterial culture undergoing unrestricted growth the rate of protein synthesis depends on the amount of rRNA such that

$d(P)/dt = K(r)$ where

P = protein/unit volume culture

r= rRNA/unit volume culture

and K = the rate constant expressing the rate of net protein synthesis/unit rRNA

The constant K reflects ribosomal efficiency and takes into account both synthetic and degrative processes.

A similar equation can be written for rRNA

$d(r)/dt = C(D)$ where

D = DNA/unit volume culture and

C = the rate constant describing rRNA synthesis/unit DNA.

During balanced growth $\{(r)/(P)\} = C/K$ (Koch, 1971). As a culture enters the declining phase of its growth cycle, the value C decreases proportionately more than K (Koch, 1971). This results in a decline in the ratio RNA/protein.

The application of equilibrium density gradient centrifugation to whole cells of E. coli has been used to gauge the effect of stage of growth on macromolecular composition (Patterson et al., 1980). This technique is based upon the assumption that RNA is intrinsically denser than other cellular constituents. The specific gravity of E. coli was found to vary markedly over the growth curve. Peak values were observed at mid-exponential phase and these regress back to starting values as the cell enters stationary phase. The authors conclude that rRNA production is halted prior to stationary phase and that rRNA is diluted as the cells

continue their final divisions. An isopyncnograph of a relaxed mutant (incapable of the stringent response) revealed that this strain is not capable of regulating its macromolecular composition in this manner. PpGpp has been shown to accumulate during stationary phase of both stringent and relaxed strains, albeit much more profusely in the stringent cultures (Kramer et al., 1981). An effect of medium composition on this has been demonstrated (Kramer et al., 1981). Interestingly, the most pronounced accumulation occurred with media containing either casamino acids or yeast extract as compared to minimal medium which was apparently deficient in complex nitrogenous materials.

RNA/Protein In Nutrient Limited Continuous Culture vs Batch Culture

Koch (1971) studied the regulation of growth in continuous cultures of E. coli. He found that cells growing at slow growth rates ($\leq .5$) had an excess of ribosomes beyond that necessary if all were maximally active. This was demonstrated by Koch and Deppe (1971) who showed that the rate of protein synthesis increased nearly ten fold within a few minutes of exposure to rich revitalization. Schaechter et al. (1958) studied this relationship in Salmonella typhimuruim and found that the slope and y intercept (predicted value at zero growth) of a regression of RNA (as a percent of mass) vs μ were considerably different when cells were grown in continuous culture as opposed to batch culture. The slope of the line is much less pronounced and the y intercept much higher when continuous culture data is compared to batch data. Thus, the regression of continuous culture data back to zero growth gives a RNA/unit dry mass (or RNA/protein) ratio which is much higher than that obtained for stationary batch cultures. Likewise, the slope and y intercept of RNA/protein vs μ of in vitro batch cultures of rumen bacteria follows this same pattern

when compared to RNA/protein vs μ as determined in continuous culture of various rumen bacteria (Mink et al., 1982; Russell, 1983). It seems entirely possible that an interaction of nutrient limitation with the normal regulatory properties which control the protein synthesizing system can account for the disparancy in these results. Certainly, Experiment 2 of these studies shows a marked effect of nutrient limitation on the size and composition of the protein synthesizing system, at least during transition to stationary phase.

RNA/Protein During Static Growth Caused By Nutrient Limitation

The results of Experiment 2 show that significant differences exist in the RNA/protein ratio observed in stationary phase cultures depending upon whether growth occurred in nutrient sufficient, glucose limited, or nitrogen limited medium.

The increase in RNA relative to protein seen during nitrogen limitation at first appears to be an anomoly. Cells entering stationary phase due to a nitrogen limitation would appear to be similar physiologically to those undergoing a shift down due to amino acid starvation. A response similar to the well defined stringent response would thus be expected, replete with rapid decline in RNA concentration. Indeed, a response of this nature has been characterized in stationary phase cultures grown in complex medium containing an adequate nitrogen supply (Kramer et al., 1981).

Why a similar stimulation of ppGpp production was not observed during growth transition due to a nitrogen limitation is not immediately clear (Kramer et al., 1981). It should be remembered, however, that the synthesis of ppGpp is related to the differential signal between rate of provision of aminoacyl tRNA's and the rate at which they are removed in

protein synthesis; that is, the ratio of uncharged to charged tRNA for a given species of tRNA. The rate of tRNA synthesis (a stable RNA species) is balanced against growth rate in a manner similar to rRNA (Inghram et al., 1983). During balanced growth, the rate of net tRNA synthesis is equal to μ. It follows, therefore, that the pool of tRNA in cells growing at high specific growth rates should be higher than the tRNA pool found in slower growing cells. The nutrient sufficient cultures in these studies all grew at a more rapid pace than did either of the nutrient limited counterparts.

One also must consider that both energy charge and absolute ATP concentrations decline during stationary phase (Chapman and Atkinson, 1977). Furthermore, the cells ability to maintain an intact transmembrane proton gradient diminishes (Jolliffe et at., 1981). This has a profound impact on a cells ability to transport amino acids and peptides. Aminoacylation of tRNA is an energy (ATP) dependent process (Lehninger, 1982). It would appear that the stationary phase culture is comprised of cells in which both intracellular amino acid pool (Langheinrich and Ring, 1976) and the ability to aminoacylate tRNA are depressed. The cells with the largest tRNA pool would possess the largest differential between charged and uncharged tRNA. This may explain the higher ppGpp concentrations found in stationary E. Coli grown in nutrient sufficient medium as compared to those grown in minimal medium deficient in amino acids and peptides (Kramer et al., 1981). Differences in RNA accumulation observed in stationary phase cultures of minimal bacteria are probably due, in part, to differences in ppGpp levels.

Decrease in cellular RNA as a survival mechanism during energy starvation appears to be a response widespread throughout the bacterial

kingdom and has been observed in Escherichia coli (Jacobsen and Gillespie, 1968; Kaplan and Apirion (1975), Peptococcous prevtii (Reece, et al., 1961), Pseudomonus aerogenosa (Gronlund and Campbell, 1965) and in the rumen microbes Megasphora elsdinii (Mink and Hespell, 1981a) and Selenomonas ruminantium (Mink et al., 1981b). The basic features of degradation during energy deprivation include 1) breakdown of ribosomes to monosomes followed by dissociation into 50 and 30 S sub-units; 2) attack by endoribonuclease and 3) further degradation of small pieces of RNA by the enzymes RNAase II and polynucleotidase (Kaplan and Aprion, 1975). Analysis of the starvation buffer of both glucose and NH_3 limited continuous cultures of Selenomonas ruminantium indicated that most of the RNA that is partially broken down is released as such into the starvation buffer (Mink and Hespell, 1981b; Mink et al., 1982). Cell viability studies show a direct correlation between the capacity of a mutant E. coli (possessing defective RNAase) to survive during starvation and its ability to degrade RNA, possibly because RNA degradation aids in the cells attempt to maintain energy charge (Knowles, 1977).

Both stringent and relaxed strains curtail stable RNA accumulation during energy source step downs by mechanisms that involve both RNA synthesis and degradation (Cashel, 1975) and another ppGpp mediated process has been implicated. The steady state level of ppGpp can be achieved by regulating the rate of ppGpp synthesis and/or its rate of decay. The spoT gene product in E. coli (ppGppase) is an enzyme which is ribosome associated (Fiil et al., 1977; Richter et al., 1979). It is inhibited by uncharged tRNA (Richter, 1980) and requires the existence of a transmembrane proton gradient to be fully active (degrading ppGpp molecules) (Deboer et al., 1975; Tetu et al., 1980). Glucose exhaustion

causes a rapid decline in ppGpp degradation while it has been argued that inhibition of ppGppase plays an important role in elevating ppGpp concentrations during conditions of energy limitation (Richter et al., 1979). Again, ppGpp regulates the amount of stable RNA synthesis and is probably involved, at least in part, in controlling the extent of RNA accumulation measured in experiment 2.

Both ppGpp and energy are also involved in regulating intracellular protein turnover, the extent of which can also effect ultimate the RNA: protein ratio. All other things considered equal, if the extent of protein turnover is raised appreciably, the resulting RNA:protein ratio will be higher. During nitrogen limitation, the RNA:Protein ratio should increase because proteolysis is elevated. Intracellular protein degradation in E. coli has the following properties: 1) it can increase or decrease during nutritional deprivation depending on the type of limitation involved (Pine, 1980); 2) amino acyl tRNA and ppGpp are involved in controlling rate of degradation (St. John and Goldberg, 1978); 3) abnormal proteins are degraded more rapidly than normal ones (Proutz et al., 1975; Goldberg, 1972a) and 4) inhibitors that block ATP formation diminish the rate of degradation (St. John and Goldberg, 1978). In cells starved for amino acids, the steady state level of ppGpp was varied using different amounts of tetracycline (an inhibitor of ppGpp formation). The rate of intracellular proteolysis was proportional to the concentration of ppGpp (Voellmy and Goldberg, 1980). Also, during exponential growth rel A+ spoT cells contained more ppGpp and showed a higher rate of protein catabolism then relA+ spoT+. This difference was eliminated following the addition of tetracycline (Voellmy and Goldberg, 1980). In energy restricted cells (relA+ spoT+) tetracycline caused a reduction in ppGpp

and a fall in proteolysis (Voellmy and Goldberg, 1980). PpGpp seems to signal the acceleration of proteolysis during both amino acid and energy deprivation. However, the dependence of intracellular proteolysis on ppGpp concentration is not the same under different sets of nutritional constraints (Voellmy and Goldberg, 1980).

The depletion of energy can have different effects. Partial restriction of energy supply through the addition of 2-4 Dinitrophenol in the presence of glucose stimulates proteolysis (Pine, 1980). There is, however, an absolute energy requirement which must be met for intracellular proteolysis to occur (Pine, 1972). Severe energy depletion by removal of energy source or poisoning of oxidative or fermentative processes may entirely eliminate intracellular protein breakdown (Pine, 1980). A number of ATP dependent proteases have been described (Etlinger and Goldberg, 1977; Roberts et al., 1977; Larimore et al., 1982) and there is speculation that these enzymes are the major effectors of intracellular protein degradation in bacteria (Pine, 1980; Larimore et al., 1982). The energy requirement of intracellular proteolysis appears to be directly related to a minimum ATP requirement (around 15% of normal ATP levels). The stationary phase cells grown in the glucose limited culture medium have probably depleted their ATP pool below this point. It must be remembered that those organisms are anaerobic and depend upon glucose primarily as an energy source.

In the final analysis, it would seem fitting that such wide values were observed for the RNA:protein ratio in ruminal bacteria grown under nutrient sufficient, energy limited and nitrogen limited conditions. Energy and amino acid availability would vary considerably under these three sets of conditions. The interplay between these two factors appears

to establish a ppGpp set point and modify a cell's final physiological response to ppGpp concentrations. Guanosine tetraphosphate has been isolated in the anaerobe Bacteroides thetaiotaomicron and is assumed to occupy a similar position in regulation of cellular physiology in anaerobes as has been proposed for aerobic strains (Glass et al., 1979).

RNA/Protein As An Ecological Determinant

The factors most frequently cited as influencing the ultimate population mix within a complex microbial ecosystem include:

1) Maximum growth rate
2) Affinity for available substrate
3) Maintenance expenditures and efficiency of cell yield (Y_S or Y_{ATP})
4) Versatility
5) Tolerance to pH and other inhibitory factors (Russell and Hespell, 1981)

A sixth determinant could be the relative maintenance, or lack thereof, of protein synthesizing capacity in cells entering the stationary phase of growth. This phenomenon is characterized by the RNA/protein ratio of a stationary phase culture.

Most of the carbon and energy consumed during bacterial growth goes towards the synthesis of proteins of one type or another (Inghram et al., 1983). The overall size of the protein synthesizing machinery, of which RNA as a part, if large and complex. It is comprised of ribosomes (RNA and protein), tRNA, mRNA, the enzymes that make and modify these molecules, aminoacyl-tRNA synthetases, and protein factors required for initiation, elongation and termination reactions. The composite of these factors constitutes over one half of the total mass of rapidly growing

cells (Maaloe, 1979). The synthesis and maintenance of ribosomes represents the single largest energy expenditure to the bacterial cell (Inghram et al., 1983).

Over the past fifteen years considerable attention has been given to the efficiency of protein synthesis in the prokaryote (Maaloe, 1979). Inside a given bacterial cell, a host of controls serves to regulate the polymerization of amino acids into protein. A debate has arisen with one group claiming that the efficiency of a ribosome is independent of specific growth rate, μ . They point to the correlation between RNA/protein and μ , among other things, as proof of the constant efficiency hypothesis (Maaloe, 1979; Inghram et al., 1983). Another proposal championed by Koch and his associates (Koch, 1971; Koch and Deppe, 1971) contends that slow growing cells carry an excess of RNA and that the polypeptide chain synthesis rate does not remain constant. These researchers point out that the rate of adjustment in protein synthesis following a shift up of a glucose-limited continuous culture of E. Coli exceeds the rate of synthesis of new ribosomes (Koch and Deppe, 1971).

The first group argues that the protein synthesizing system should never be bigger than that necessary to process available precursors (Inghram etal., 1983); the other argues that the "extra" RNA decreases lag phase upon shift up which transposes into a "head start" when compared against other "prudent but unwise" organisms that contain only enough ribosomes to be employed under the conditions in which the call is growing, especially starvation.

Two basic types of organisms appear to be found within the rumen. Most of the bacteria tested in these studies are adapted to grow under conditions of energy limitation. These cells economize on their

maintenance expeditures by cost cutting in the area of ribosome synthesis. Most bacterial cells behave in this way when they enter stationary phase (Patterson et al., 1980; Inghram et al., 1983).

S. bovis, on the other hand, occupies a completely different niche within the rumen. It is not particularly competitive in the normal rumen environment because it has such a low affinity for substrate molecules (Russell and Balwin, 1979). Yet it is maintained in low concentrations regardless of diet primarily because of its metabolic versatility. Also, it seems to possess the ability to retain a macromolecular composition indicative of the "readied" state referred to by Koch (1971). The relative proportion of RNA remains high in this organism for days after it enters stationary phase. Furthermore, the change in RNA/protein per unit change in specific growth rate is less for S. bovis than for any of the other species studied. This shows that, at a given growth rate, less RNA is required to synthesize a given unit of protein than with the other bacterial strains tested. Most bacteria are unable to sufficiently regulate nutrient uptake processes and will undergo substrate accelerated death when exposed to extreme shift-up conditions (Koch, 1971). This, however, is precisely the set of transient conditions in which S. bovis appears specifically adapted to thrive. This bacterium remains primed during periods of low or zero growth to resume a very rapid growth rate as soon as it is exposed to an environment rich in available soluble substrate. This accounts for the short lag time observed when stock cultures of this organism are reinnoculated into broth medium. It also explains the ability of this strain to dominate the rumen ecosystem when unusually high levels of energy substrates are available during rapid shifts to a high grain diet.

Nucleic Acid-N/Total-N as a Marker of Microbial Protein

The nucleic acid/protein values presented here are valuable in an assessment of the use of RNA as a marker for bacterial protein passage to the ruminant gut. Smith (1969) reported nucleic analysis for five strains of ruminal bacteria and concluded that RNA-N:total-N ratios were less variable between strains than were DNA-N:total-N ratios. Rate and stage of growth were not reported, however, which complicates interpretation of the reported data (Ingraham et al., 1983). Because RNA/protein is affected by several factors including species variation (Smith, 1969), bacterial fraction, diet and time-after-feeding under infrequent feeding conditions, researchers employing RNA-N/total-N as a marker should be cognizant of sources of variability in this technique. Parenthetically, the ratio RNA-N:total-N does not always accurately predict RNA/protein per se. For instance, as growth rate increases, the proportion of RNA in a bacterial cell also increases. Ribonucleic acid is approximately 14.8% nitrogen (Ling and Buttery, 1978) and this nitrogen will be included in any estimation of total nitrogen. True microbial protein passing to the small intestine will be overestimated by RNA-N/total-N when the RNA: protein ratio of ruminal bacteria is high.

DNA/protein values were quite constant irrespective of specific growth rate. These results contradict the earlier contention of Smith (1969) that RNA would be a more useful microbial marker then DNA. Thus, DNA would appear to be a much more suitable marker of bacterial flow to the lower gut than RNA. Leedle and Hespell (1983) have also shown that DNA, expressed as percent of bacterial dry matter, was quite constant in mixed ruminal bacterial cultures when grown under different conditions in short

term incubations. Wolstrup and Jensen (1978) used DNA as a marker and concluded that its use gave representative estimates of microbial in the rumen.

Macromolecular composition of rumen bacteria as a marker of microbial function and production in the rumen has much potential. Accurate assessment of those factors that influence ratios of cellular components is a prerequisite to interpretation of experimental results of research in which cellular composition has been used as an internal marker.

CHAPTER II

FACTORS AFFECTING PROTEOLYTIC ACTIVITY OF BACTEROIDES RUMINICOLA GA33

Review of Literature

Regulation of Bacterial Metabolism

Bacterial metabolism involves over a thousand separate biochemical reactions (Inghram et al., 1983). Some of these provide energy for the cell growth and others are involved in the manufacture and polymerization of cellular materials. The relative rates of these reactions must be coordinated if the cell is to optimize its energy expenditures. A number of general observations indicate the extent to which this occurs in most bacteria: 1) most monomers utilized during a cell's anabolic processes are produced in exactly the amount required, ie., the rate of amino acid biosynthesis matches the rate of amino acid polymerization into protein; 2) when a bacterium is grown in a complex medium which contains preformed cellular materials, such as amino acids, endogeneous synthesis of those compounds ceases; 3) enzymes in certain catabolic pathways are synthesized only if the substrate of the pathway is present and 4) if two substrates are available, often the preferred substrate which supports the fastest growth is metabolized first (Inghram et al., 1983). The metabolic controls that are involved can be divided into two broad classes: Control on enzyme activity and control of gene expression (Mandelstam and McQuillen, 1973).

Control of enzyme activity involves the binding of a metabolite, called an effector, to an enzyme thereby causing it to gain or lose catalytic activity. The binding of the effector molecule at the allosteric site of the regulated enzyme causes a conformational change which results in modulation of enzymatic activity. The effect of this modulation can either be on the Vmax or K_s of the enzymatic reaction. Frequently, the end product of a given reaction sequence feeds back to inhibit the first enzyme involved. This is called end-product inhibition.

Control of enzyme synthesis is also a means by which bacterial cells regulate their metabolic processes. In this type of regulation, the increased concentration of some compound in the medium is found to cause an increase (induction) or decrease (repression) in the synthesis rate of the regulated enzyme. An enzyme is referred to as constitutive if the gene product remains essentially constant regardless of external stimuli.

Often a number of functionally related enzymes are induced or repressed as a group. Those structural genes for which transcription frequency is regulated as a unit are referred to as an operon (Jacob and Monod, 1961). A number of different regulatory mechanisms have been described which include induction, repression and autogenous regulation (Goldberger, 1979).

In addition to regulatory mechanisms involving individual operons, there are mechanisms by which bacteria regulate the expression of several different operons at the same time. These allow an organism to coordinate a set of responses to a given external stimuli. One such mechanism is catabolite repression (Magasanik, 1961). This mechanism involves positive regulation of a group of functionally related operons by the catabolite repressor protein (CRP) and cyclic AMP (cAMP) (Goldberger, 1979). The

operons subject to catabolite repression are the catabolic operons which contain genes for the metabolic pathways by which various substrates are degraded. The binding of the CRP-cAMP complex to a specific site located adjacent to the promoter gene greatly increases the productive binding of RNA polymerase to the promoter gene thus resulting in transcription of the operon. Frequently, the enzymes encoded in catabolic genes controlled by catabolite repression are repressed when a bacterium grows in the presence of a preferred substrate. Cyclic AMP levels remain low as long as the bacterium utilizes the preferred substrate. The binding of the CRP-cAMP complex is required for active transcription of the regulated operon to occur (Perlman and Paston, 1968).

Two additional mechanisms have been proposed to explain preferential utilization of substrate. Catobolite inhibition involves interference by the preferred substrate with the transport and utilization of other substrates (McGinnis and Paigen, 1973). A second proposed mechanism, phosphotransferase system - mediated repression, involves inducer exclusion at the level of the cell membrane (Russell, 1984).

Another nucleotide, ppGpp, may play an even more central role in integrating the metabolic activities of bacterial cells. The regulatory mechanism involved is called stringency and is characterized by a ceasation of rRNA synthesis during periods of amino acid starvation. The stringent response involves many biological activities in addition to rRNA synthesis. Guanosine tetraphosphate causes an overall inhibition of cellular metabolism.

Regulation of Exporotease in Non Ruminal Bacteria

Bacteria are unable to transport proteins across their cell membrane yet many strains are capable of utilizing exogeneous protein sources (Law,

1980). These bacteria secrete protease outside the cell membrane either as free enzymes or as enzymes bound at sites within the cell envelope (Law, 1980). The products of exoprotease action are transportable amino acids and peptides.

A diverse set of metabolic controls have been implicated in the regulation of exoprotease production by nonruminal bacteria. Much attention has been devoted to the regulation of exoprotease in Bacillus species (Priest, 1977) and a control mechanism has been proposed to explain exoprotease synthesis during stationary phase (Coleman et al., 1975; Abbas and Coleman, 1977). Many gram positive bacteria synthesize and release extracellular protease during the post exponential phase of growth. Control at the transcriptional level is envisioned to involve weak promotion of exprotease mRNA and unfavorable competition for RNA polymease binding during the exponential phase of growth. Tran- scription of RNA associated with growth related processes ceases during the declining phase, resulting in an increase in the accessibility of RNA polymerase. Nutrient limited growth conditions are implicit to this model design (Coleman et al., 1975).

Additional control mechanisms operate in many species. There is evidence for catabolite repression of protease synthesis (Lichtfield and Prescott, 1970; Boethling, 1975; Juffs, 1976; Wiersma et al.,1978; Whooley et al., 1983). Repression by high levels of amino acids has also been reported (Daatselaar and Harder, 1974; Wiersma et al., 1978). Low concentrations of peptides or specific individual amino acids are sometimes required for maximal expression of activity (Wiersma et al., 1978; Ratcliffe et al., 1982). High ammonia concentrations have been shown to inhibit the exoprotease of Pseudomonas aeruginosa (Whooley et

al., 1983). In many gram negative species exprotease production is growth associated, at least under certain nutritional regimes, and a complex system of controls seems to be required for the final expression of exoprotease activity.

Regulation of Proteolytic Activity in the Rumen

It is well recognized that microorganisms in the rumen are responsible for degrading dietary protein to carbon skeletons and ammonia (Blackburn, 1965). Early attempts to enumerate and isolate proteolytic rumen bacteria employed differential media containing casein and ammonia (Blackburn and Hobsen, 1960; Fulghum and Moore, 1963). While initial results indicated the involvement of falcultative organisms (Blackburn and Hobsen, 1960), more recent studies employing strict anaerobic technique showed that the primary proteolytic bacteria in the rumen are predominant ruminal species (Blackburn and Hobsen, 1962; Fulghum and Moore, 1963). Approximately 10% of the total rumen isolates from sheep were proteolytic (Blackburn and Hobsen, 1962), whereas 38% of bovine rumen isolates were shown to possess proteolytic activity (Fulghum and Moore, 1963). Proteolytic isolates include Bacteroides ruminicola, Bacteroides amylophilus, Butyrivibrio species (including fibrisolvens), Selenomonas ruminantium and Lachnospira multiparus (Blackburn and Hobsen, 1962; Fulghum and Moore, 1963; Bladen et al., 1961). Streptococcus bovis has recently been added to this list (Russell et al., 1981; Hazelwood et al., 1983).

Ruminal proteases have been reported to be primarily cell bound (Nugent and Mangen, 1981). Cell wall bound, periplasmic and membrane bound proteases accessible to substrates from outside the cell have been characterized in several nonruminal and ruminal species (Blackburn and Hullah, 1974; Law, 1980). Degradative enzymes are located in the

periplasmic space in many gram negative bacteria (Heppel, 1971). Kopecny and Wallace (1982) reported that most ruminal protease activity is associated with coat and capsular material such that gentle disruption released most of the activity. Protease activity was also found in the cell envelope fraction. Allison (1970) observed that most gram positive bacteria produce extracellular enzymes that are actively secreted into the growth medium and concluded that the most important protease producing bacteria in the rumen must be gram negative.

Two gram negative organisms, Bacteroides amylophilus and Bacteroides ruminicola, have been the most intensively studies rumen bacteria. B. amylophilus produces both cell bound and free proteolytic activity which amounts to 80% and 20% of the total activity during exponential growth, respectively (Blackburn, 1968; Blackburn and Hullah, 1974). The protease of B. amylophilus was not affected by the cysteine protease inhibitor iodoacetate, yet was inhibited 87% in the presence of the serine protease inhibitor disopropyl fluorophosphate. During exponential growth the cell bound protease remains firmly bound to cellular components (including RNA). The proportion of cell free activity increases in stationary phase and a disappearance of cell bound actively coincides with this increase.

When a culture of Bacteroides ruminicola R8/4 was grown with Fraction 1 protein, specific activity of the cell associated protease was maximal during midexponential phase but declined to 23% of the maximal value by stationary phase (Hazelwood et al., 1981). The effect of EDTA on the proteolytic activity of Bacteroides ruminicola was inhibitory and the primary proteolytic activity of this organism is characteristic of a cysteine protease (Hazelwood and Edwards, 1981). The effects of protease inhibitors on the proteases of mixed ruminal bacteria also indicated that

most of the extracellular proteolytic activity found in rumen contents is severely inhibited by the cysteine protease inhibitor p-chloromercuribenzoate (Kopecny and Wallace, 1982). The overall pattern of inhibition was qualitatively similar to that observed with Bacteroides ruminicola. Studies on pure cultures of ruminal bacteria have indicated that Bacteroides ruminicola is one of the most important proteolytic rumen microorganisms (Bryant, 1977).

There have been few studies on regulation of exoprotease production by ruminal bacteria. Blackburn (1968) characterized the protease of Bacteroides amylophilus as constitutive because the activity was not induced or repressed by protein, peptides or amino acids in the growth medium. Protease production by this organism was shown to be proportional to ammonia and maltose concentration over the range in which these nutrients were growth limiting. A follow up study reported that casein at concentrations greater than 3% (w/v) caused substrate inhibition of the protease liberated from B. amylophilus by mechanical disintegration. Proteolysis of Fraction 1 protein by B. ruminicola R8/4 was also inhibited by high substrate concentrations (Hazelwood et al., 1981).

Protease production in continuous cultures of B. amylophilus varied with growth rate (Henderson et al., 1969). One peak of activity was found at low growth rate and another near the maximum growth rate of the culture. Clarke and Lilly (1969) observed a similar activity profile (vs. dilution rate) for amidase in Pseudomonas aeroginosa and attributed the early peak to an interplay between induction and catabolite repression.

Soluble carbohydrates have been shown to inhibit proteolysis during in vitro incubations of proteins with rumen contents (Blackburn, 1965). For instance, the addition of 0.5% (w/v) cellobiose reduced the degradation of

soy protein from 86.9%, when no sugars were included, to 4.4% (6 hour incubations). Soluble starch addition did not result in this same response (Blackburn and Hobsen, 1960). Russell et al., (1983) incubated mixed rumen bacteria with four different levels of mixed carbohydrate. They concluded that carbohydrate availability had little influence on proteolysis. But in these experiments, carbohydrate concentrations were always limiting. Catabolite regulation could well have been maximally stimulated in all four experimental conditions.

The Effect of Monensin on Nitrogen Metabolism in Ruminants

Research has indicated that the ionophore, monensin, may have a protein sparing effect in the ruminant. Ruminal ammonia is decreased by monensin (Chalupa, 1980) suggesting monensin either depresses overall cell numbers in the rumen or has some direct effect on both protease and deaminase activity (Van Nevel and Demeyer, 1977).

The escape from ruminal degradation (protein bypass) of preformed dietary protein in the presence of monensin has been investigated in vivo. Protein bypass was increased from 22 to 55% in five different experiments (Bergen and Bates, 1984). Associated with these studies were estimates of microbial protein (MP) flows to the small intestine and the efficiency of microbial growth or protein synthesis (E_{MCP}; g microbial crude protein/100 g total rumen organic matter digested). It is clear from these results that monensin depressed total microbial flow and E_{MCP}. Similar conclusions based on in vivo passage studies were drawn by Poos et al., (1979). Van Nevel and Demeyer (1977) reported that total and net microbial growth yields in in vitro rumen fermentations were depressed by monensin while substrate fermentation was unaffected, resulting in a lower growth efficiency. Whereas the depression in bacterial growth yield by

monensin in in vitro rumen fermentations can not be attributed to a change in the turnover rate of the fermentation, such a connection may possibly be drawn for the in vivo observations. Results from in vivo digesta passage studies and from many in vitro continuous culture fermentations with both ruminal and nonruminal microorganisms showed that growth yield efficiency (E_{MCP}) is reduced as the turnover rate of the fermentation system declines (Owens and Isaccson, 1977; Bergen et al., 1982). The lowered E_{MCP} is due to an increased maintenance requirement of the microorganism at lower growth rates (Stouthamer and Bettenhausen, 1973). Dinius et al. (1976) and Lemenager et al., (1978) reported that monensin caused a significant depression in the turnover of rumen contents (which would reduce E_{MCP}), while others have obtained less clearcut results (Van Nevel and Demeyer, 1979). Isichei (1980) observed an increased abomasal dry matter flow when steers were fed monensin, but also observed the decline in E_{MCP}.

The objectives of the studies reported in this chapter were to identify factors that affect the proteolytic activity of a key ruminal proteolytic bacterium, Bacteroides ruminicola; and to assess the interaction between monensin and the normal functioning of these regulatory processes.

Materials and Methods

Reagents

Azocasein, commercial protease (alkaline protease from Bacillus subtilis), dithiothreiotol, 2 deoxyglucose and α methyl-D- glucoside was purchased from Sigma Chemical Company. Monensin was a kind gift of Dr. E.

L. Potter, Lilly Research Laboratories, Greenfield, IN. Levallorphan was a gift of Dr. R. A. Scott, Hoffmann-LaRoche, Rahway, NJ.

Organism

A preliminary study indicated that Bacteriodes ruminicola GA33 had the highest exoprotease activity of any of the organisms stored in stock culture. This observation coupled with the suggestion of Bryant (1977) that Bacteroides ruminicola was one of the main proteolytic bacterial species in the rumen led to the decision to study this organism in more detail.

Media

The basal medium used in most of the following experiments is presented in Table 12. Modifications were made to this medium according to the objectives of the experiment. Five carbohydrate sources were tested. They were glucose, maltose, lactose, cellobiose and soluble starch. When monensin was included, it was added as .1% (v/v of a .25 mg% (w/v)) monensin solution in ethanol (final concentration 2.5 μg/ml). Energy limiting conditions were created by limiting glucose to .08% (w/v) of the culture medium while nitrogen limiting conditions were created by limiting Trypticase to .01% (w/v).

Continuous Culture of Microorganisms

Two chemostats were constructed in a fashion similar to Issacson (1973). Each system was composed of a source of O_2 free CO_2, medium reservoir, pump, fermenter, magnetic stirrer, effluent collection vessel, heating tape, temperature probe and temperature controller.

A heating pad was placed under this reservoir to help rid the reservoir medium of dissolved gas. Three pieces of 8mm glass tubing were inserted through the rubber stopper to serve either as a gas inlet, medium

Table 12. Basal Medium Used in the Protease Experiments

Medium Component	Concentration
Carbohydrate Source	.5% (w/v)
Yeast Extract	.05% (w/v)
Trypticase	.2% (w/v)
Mineral 1 Solution[b]	3.75% (v/v)
Mineral 2 Solution[c]	3.75% (v/v)
Hemin Solution[2]	.1% (v/v)
Volatile Fatty Acid Solution[e]	.1% (v/v)
Na_2CO_3 Solution[f]	5.0% (v/v)
Cysteine HCl Solution[g]	2.5% (v/v)
Clarified Rumen Fluid[h]	10.0% (v/v)

[a]Carbohydrate sources varied and included glucose, maltose, lactose, cellobiose and soluble starch.

[b]An aqueous solution containing 6.0 g H_2PO_4 per liter.

[c]An aqueous solution containing 12.0 g NaCl, 6.0 g KH_2PO_4, 6.0 g $(NH_4)_2\ SO_4$, 1.2 g $CaCl_2$ and 2.5 g $MgSO_4\ 7\ H_2O$ per liter.[4]

[d]A .1% (w/v) solution of hemin prepared according to Holdeman et al., (1977).

[e]Solution containing per liter: Acetic acid, 548.4 ml; propionic acid, 193.5 ml; N-butyric acid, 129.0 ml; N-valeric acid; 32.3 ml; isovaleric acid, 32.3 ml; isobutyric acid 32.3 ml; and DL-2 methylbutyric acid 32.3 ml.

[f]An 8% (w/v) aqueous solution of Na_2CO_3.

[g]An aqueous solution containing 2.5% (v/v) cysteine - HCl prepared according to Holdeman et al., (1977).

[h]Prepared according to Holdeman et al., (1977).

outflow port or gas outlet. The later was fitted with a sterile cotton filter. Carbon dioxide was continuously bubbled through the medium to saturate it with CO_2.

Two types of pumps were used. A Cole Palmer parastallic pump (Model #7553-10) fitted with a Masterflex^R head (Model #7014-20) was used in one system. A small piece of tygon tubing was placed into the head. Connections were made by cutting off two disposable glass syringes (GlasPak) and inserting the neck into the smaller tygon tube and the base into the butyl rubber. A FMI piston pump (RP G150) was used in the other system. Sterilization of the immediate fittings in contact with each pump was achieved by pumping a solution of Zepherin^R Chloride (Winthrop Laboratories) followed by 2% glutyraldehyde - .3% $NaHCO_3$ solution through the component in need of sterilization. This was followed by a wash with sterilized deionized water.

The fermenter was a Belco (#1970) continuous culture spinner flask (Slyter et al., 1964). Modifications included the addition of a serum bottle sampling port at the base of the fermenter.

Temperature control was achieved by regulating the current supplied to a GlasCol^R heating tape wrapped around the fermenter. A YSI (Model 74) temperature controller. An ace bandage was wrapped around the heating tape to insulate the system.

A magnetic stirrer (Fisher Flex a Mix^R) and a teflon coated magnetic stirring bar provided constant mixing within the fermenter.

Effluent was collected in a 9 liter pyrex Erlenmeyer flask fitted with an inflow port and a gas exit plugged with a sterile cotton fiber. Gas flow was monitored by observing the bubbles coming out a line leading from the gas outlet to a beaker of water. Glucose - limited basal medium

(Table 12) was made in the 9 liter Pyrex carboy. The sterilized components were assembled and gas flow was started within one hour of autoclaving. Sterilized Na_2CO_3 and cysteine HCl solutions were added at this time. A chalky precipitate usually formed but this went away with continued bubbling of the CO_2. Stoppers were wired and the medium allowed to equilibrate with CO_2 for 12 hours.

Preliminary Observations

Preliminary experimentation with the protease of Bacteroides ruminicola GA33 indicated the following:

1) Anaerobic conditions gave the highest proteolytic activity. Assays that were performed aerobically with dithiothreotol gave values around 80% of the maximum. Aerobic assays gave the lowest values of all.

2) The use of .02% dithiothreotol gave values nearest to those obtained anaerobically.

3) The enzyme showed a broad range of pH's over which activity could be measured with an optimum at around pH 6.7.

4) Sonication increased overall proteolytic activity around 50% when compared with whole cell suspension activity.

5) Toluene disruption gave high but inconsistent values.

6) EDTA decreased activity, but no additional activity was achieved by using a complex mineral buffer.

7) The distribution of cell-associated and supernatant activity was similar to that of whole rumen contents (Barao, 1983). A shift was observed towards increased supernatant values during stationary phase. This has also been previously reported (Hazelwood et al., 1981).

8) Hydrolyzation of certain batches of azocasein released a dye which

exhibited first order decay kinetics for 24 hours after the assay was stopped. The decay process was appreciably slowed after this point.

Assay

Protease was measured according to Hazelwood and Edwards (1981) using azocasein as the substrate. The reaction mixture contained one ml azocasein solution (3% (w/v)) in (.1M potassium phosphate buffer (pH 6.7)) and two ml of a homogenous whole cell suspension (in .1M potassium phosphate buffer (pH 6.7) with .02% (w/v) dithiothreitol). Trichloracetic acid (TCA) (to a final concentration of 5% (w/v)) was added to control tubes at time zero and to assay tubes after each 2 hr of incubation. Both the blanks and assay tubes were allowed to sit on ice for at least thirty minutes following the addition of the TCA. The assay tubes (12 ml polyropylene tubes, 101mm x 16mm; Michigan State University Biochemistry Stores # 29800) were centrifuged in a 24 hole centrifuge head (Sorvall RSM24) and one ml of supernatant was removed. This was mixed with one ml 1N NaOH and the absorbance recorded at 440 mM on a Gilford spectrophotometer.

A concentration of 1% (w/v) azocasein was required in the assay mixture to saturate the enzyme and optimize the activity with approximately 5 mg protein per assay mixture.

A protease standard curve was prepared using a commercial protease (Sigma # P5380) possessing 12 units of activity per milligram. A unit is defined by Sigma as that amount of protease that will hydrolyze casein to produce color equivalent to 1.0 mM of tyrosine per minute (pH 7.5 and 37 degrees; color formation by Folin - Ciocalteu reagent). A stock enzyme solution (.1M Tris, pH7.5) which contained 12 milliunits/ml was incubated

with 1% azocasein (w/v) for two hours at 37°C and A_{440} plotted against mU activity.

Specific activity was expressed on the basis of protein or dry matter. Protein in whole cell suspensions were measured following hydrolysis with 1N NaOH at 90°C for 10 minutes. The Hartree (1973) modification of Lowry (Folin-phenol) procedure was used to measure protein.

A standard curve of culture dry matter vs optical density (at 600 nm) was prepared for both glucose sufficient and glucose limited cultures. These curves were linear through culture optical density of .8 (A_{600}). Preparation of a culture prior to drying was done according to Issacson (1973). Thirty milliters of culture were mixed with 8.4 ml formalin solution (37% formaldehyde - .85% NaCL (w/v)) and centrigued at 45,000 x g for 20 minutes to collect the bacterial pellet. This pellet was washed with water, transferred to an aluminum drying pan and dried at 100°C overnight. A comparison of moisture absorption during gravimetric determination of cell dry weight showed that this procedure was more suitable than cell collection via filtration. The optical density of a culture of Bacteroides ruminicola GA33 was plotted against mg dry matter/ml. This curve was used to predict dry matter of the cells harvested during the exponential phase of their growth cycle. Dry matter of stationary phase cultures was determined directly by ovendrying at 100 degrees centigrade.

General Experimental Protocol

The following procedure was used to obtain and process samples. Thirty milliters of culture were harvested into 40 ml polypropylene centrifuge tubes containing .01 g dithiothreiotol. These samples were

centerfuged at 45,000 x g for 20 minutes and the resulting pellet washed with .1M potassium phosphate buffer (pH 6.7) which contained .02% dithiothreotol. The samples were then placed on ice. A homogeneous suspension of the cell pellet was achieved by suspending the pellet in the buffer and vortexing vigorously for one minute against a glass stirring rod. Two milliters of the resulting whole cell suspension were utilized in the assay.

Experiment 3

Bacteroides ruminicola GA33 was grown in 300 ml basal medium containing glucose, maltose, lactose, cellobiose or soluble starch. Inocula were obtained from cultures grown in medium of identical composition and represented 1% of the final volume of experimental cultures.

Growth of bacteria was monitored by measuring culture turbidity at 600nm. Both five and thirty ml samples were removed by pipette at various time intervals. All of these manipulations were carried out under strict anaerobic conditions using the method of Hungate (1966). The 30 ml samples were processed as described in the experimental protocol. All analysis were done in triplicate. Protease activity was recorded as activity present in the cells isolated from five mls of culture. The combination of growth and protease activity was plotted for each culture.

Experiment 4

A 5 x 2 x 2 x 2 factorial design was used to test the effect of sugar, monensin and stage of growth on the specific activity of general exo-proteolytic activity of B. ruminicola GA33 expressed per mg protein or per mg dry matter.

The protocol of this experiment was similar to that of experiment three. Bacteroides ruminicola GA33 was grown in batch cultures of basal medium containing the same sugar combinations used in experiment three with and without monensin. Duplicate cultures were grown for a given sugar-monensin combination and duplicate samples were obtained from each of these cultures. All analyses were performed in triplicate and read against a blank prepared by processing two ml of the phosphate buffer with one ml azocasein solution as the zero time control. A comparison of blanks prepared with whole cell suspensions or the .1M potassium phosphate buffer gave similar extinctions at 440 nm.

Cells were harvested at both mid-exponential growth (defined as growth to an optical density of .3-.5 at 600 nm) and stationary phase (48 hr cultures). Specific activity was expressed as milliunits per milligram dry matter (mU/mg dm) or milliunits per milligram protein (mU/mg protein). The results were analyzed by analysis of variance using the regression approach (Gill, 1978). Analyses were performed using the general linear models procedure of SAS (Freund and Littel, 1981).

Experiment 5

The protocol in experiment 5 was exactly like experiment 4 except that the three treatments were nutrient sufficiency (basal medium - .5% glucose), glucose limitation (basal medium - .08% glucose) and nitrogen limitation (.01% Trypticase).

Experiment 6

Experiment 6 tested for the involvement of catabolite repression and stringency in the regulation of exoprotease of Bacteroides ruminicola

GA33. The experimental approach was based on an experiment described by Dobrogoz (1983).

The growth of Bacteroides ruminicola on basal medium (glucose) has been previously described (Experiment 3). Supplemental cyclic AMP (5 mM), levallorphan 5 mM) and a combination of 1% (w/v) 2-dexoyglucose and 1% (w/v) α methylglucoside were added to three experimental cultures. These additions were made early in the exponential phase. A control culture was also tested.

Growth of the supplemented and unsupplemented cultures were then followed for six hours. Samples were collected at 30 minute intervals and analyzed for exoprotease as previously described. Bacterial growth and protease activity of each culture was plotted against time. The protease activity was expressed as that present in the cellular portion of each 30 ml sample.

Experiment 7

Continuous cultures of Bacteroides ruminicola GA33 were grown at a variety of dilution rates (.02 - .5) in basal medium [.08% glucose (w/v)]. Optical density of the culture had stabilized by 5 turnovers indicating steady state conditions. Protease expressed as mU/mg dry matter was plotted against dilution rate for the composite data from a total of 11 sampling periods.

Results

Experiment 3

The influence of substrate and stage growth on exoprotease production by Bacteroides ruminicola GA33 are seen in the growth-activity curves (Figures 8-12). The activity curve is seen to cross the growth curve of most of these cultures at an optical density (600nm) between .8 and 1.2.

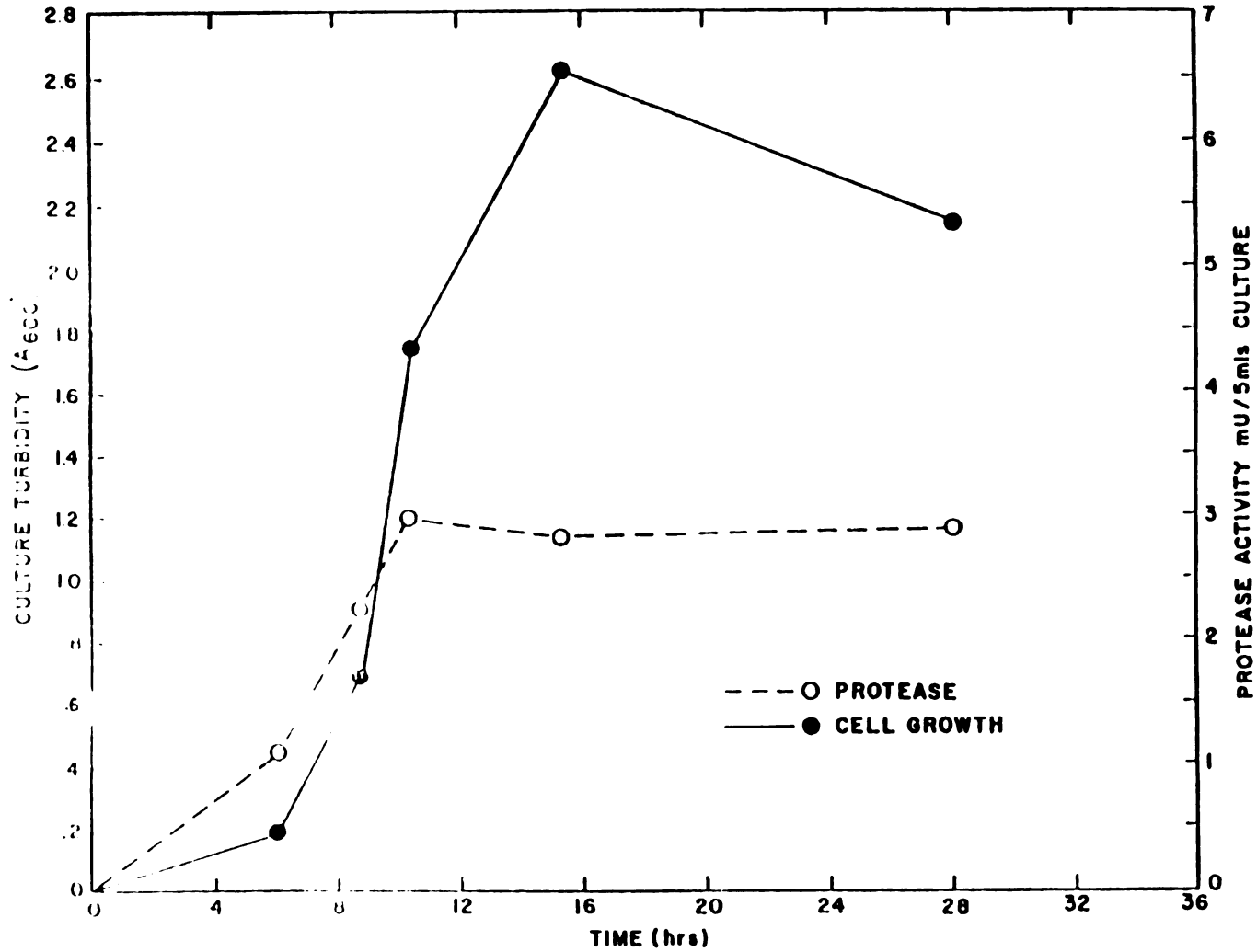

FIGURE 8. RELATIONSHIP BETWEEN GROWTH AND EXOPROTEASE PRODUCTION BY BACTEROIDES RUMINICOLA GA33 GROWN ON GLUCOSE.

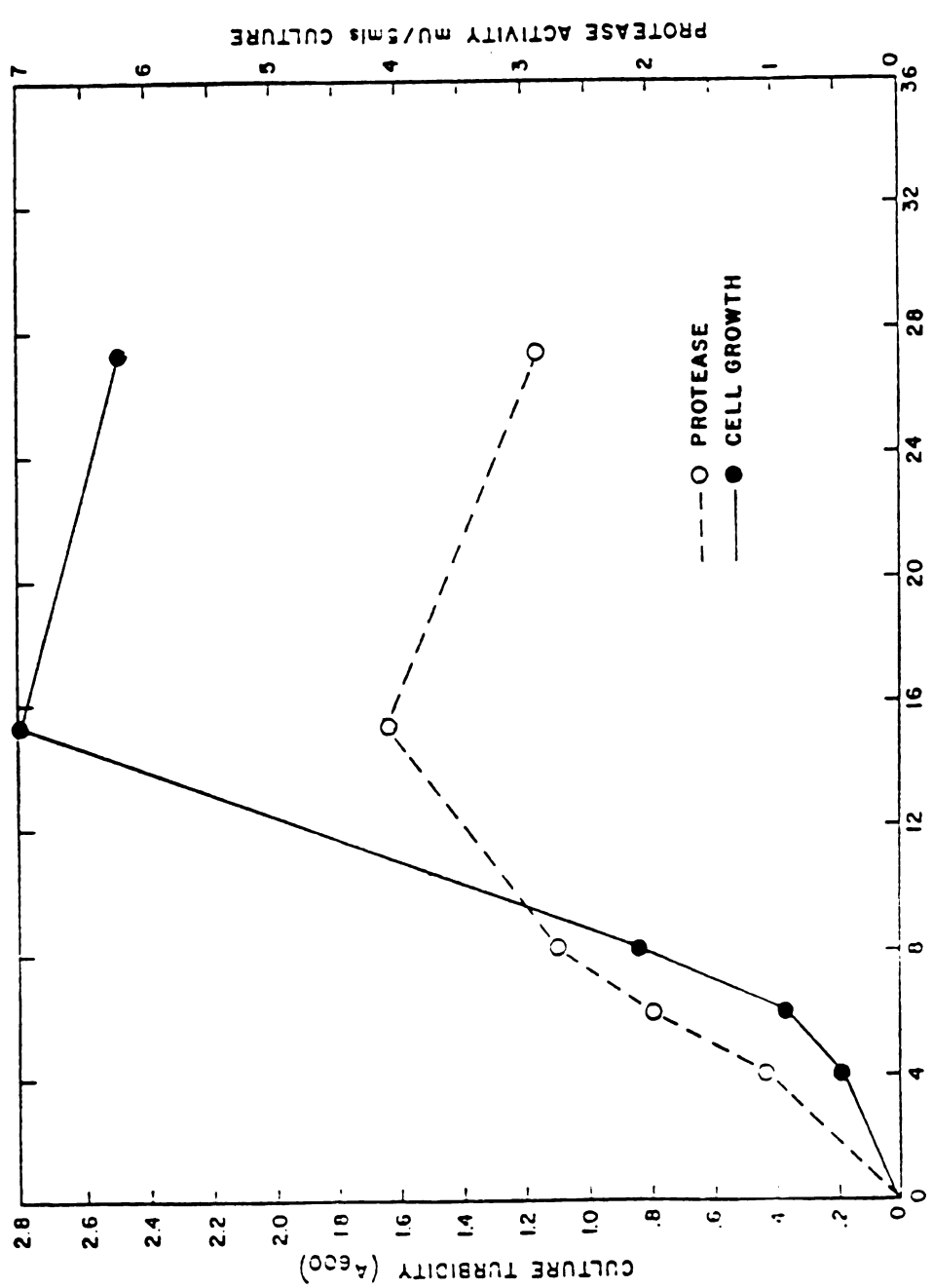

FIGURE 9. RELATIONSHIP BETWEEN GROWTH AND EXOPROTEASE PRODUCTION BY BACTEROIDES RUMINICOLA GA33 GROWN ON CELLOBIOSE.

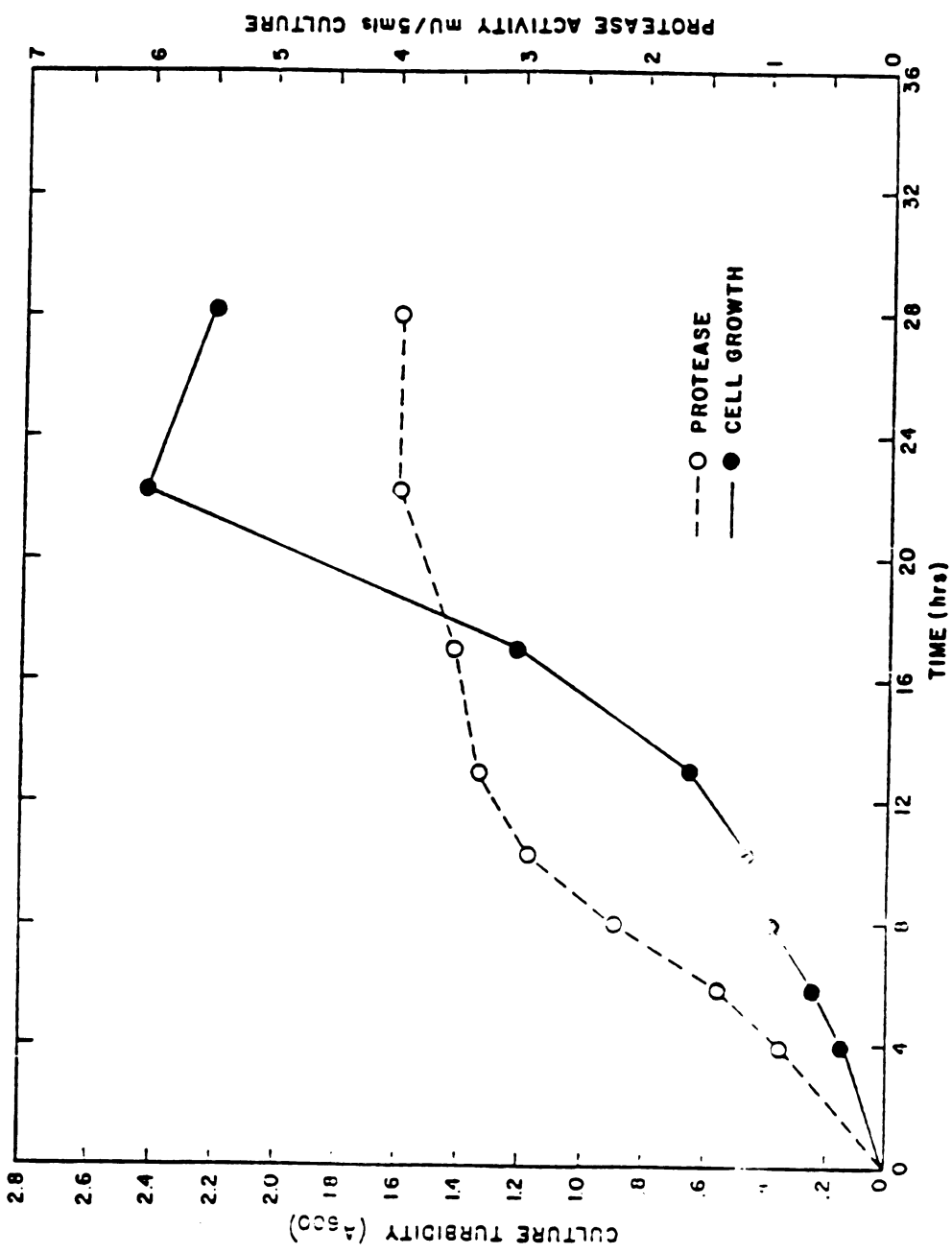

FIGURE 10. RELATIONSHIP BETWEEN GROWTH AND EXOPROTEASE PRODUCTION BY BACTEROIDES RUMINICOLA GA33 GROWN ON MALTOSE.

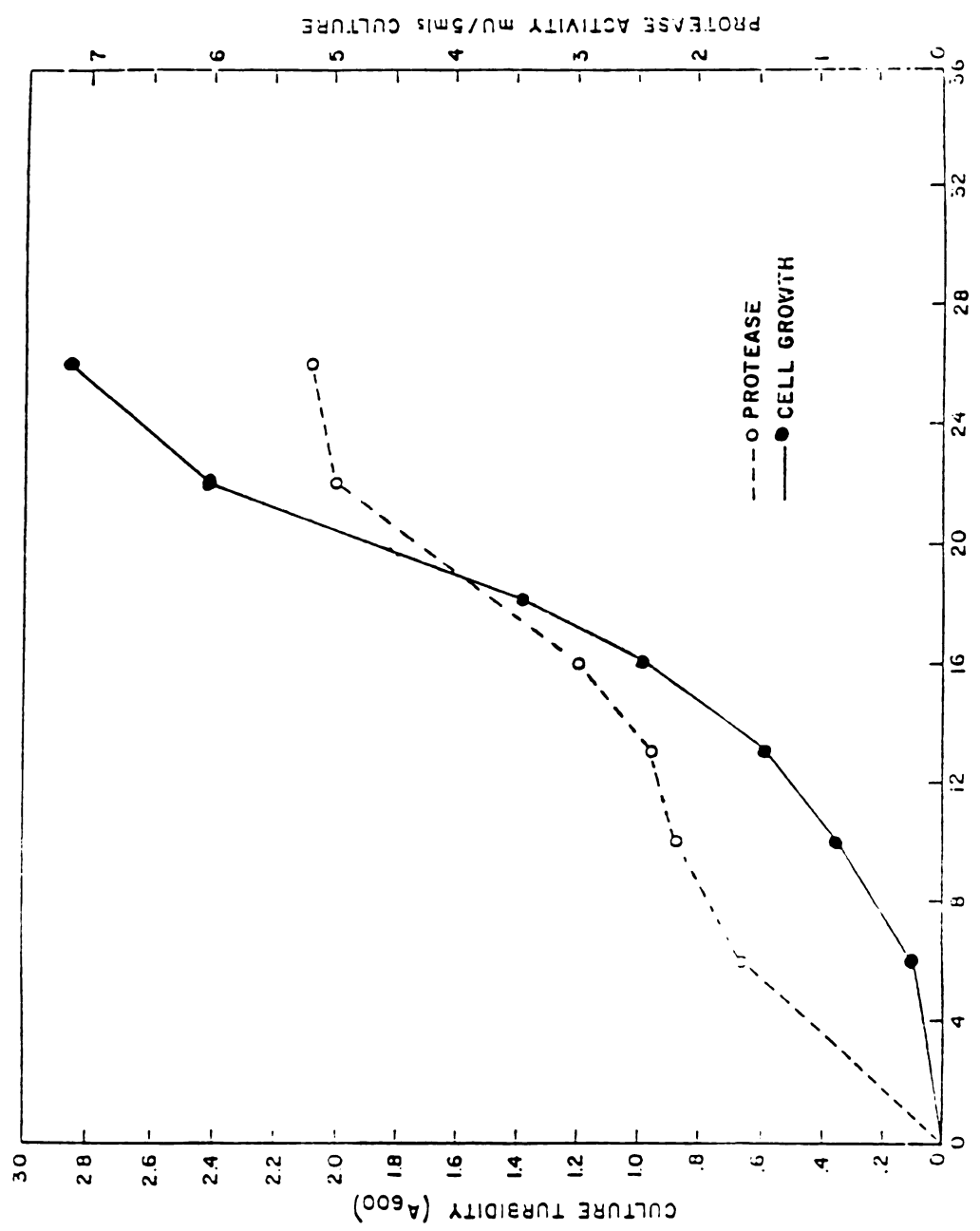

FIGURE 11. RELATIONSHIP BETWEEN GROWTH AND EXOPROTEASE PRODUCTION BY BACTEROIDES RUMINICOLA GA33 GROWN ON LACTOSE.

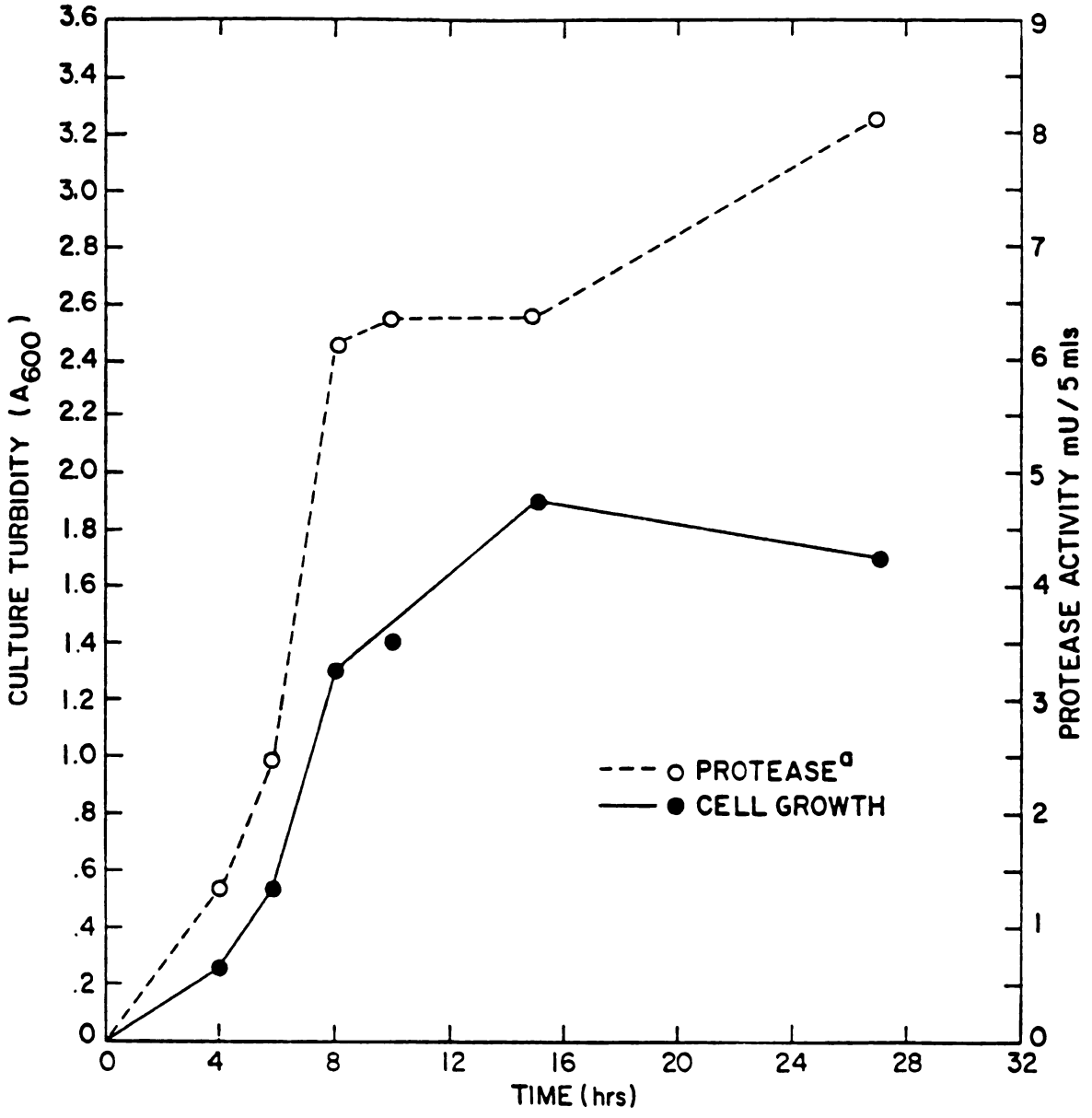

FIGURE 12. RELATIONSHIP BETWEEN GROWTH AND EXOPROTEASE PRODUCTION BY BACTEROIDES RUMINICOLA GA33 GROWN ON SOLUBLE STARCH.

This indicates a decline in the specific activity of exoprotease as the cultures enter the declining phase of their growth cycle. A decrease in activity was seen in four of the stationary phase cultures.

The sugars that supported fastest growth (glucose and cellobiose) resulted in the lowest absolute activity. Two sugars that supported intermediate growth rates (maltose and lactose) had activity curves intermediate in their final values. Soluble starch, with which the activity curve never crossed the growth curve, shows the highest protease activity. This activity increased somewhat during the stationary phase. Growth on soluble starch ceased at a comparatively low optical density of 1.8.

Experiment 4-6

The results of Experiment 4 are shown in Table 13. A significant interaction ($P<.001$) again was observed between growth substrate and stage of growth (Table 14). With all substrates, except soluble starch, protease activity substantially declined during stationary phase. When B. ruminicola GA33 was grown on soluble starch, protease activity remained relatively stable (decline from 4.78 to 3.99 mU/composite mg protein- mg dry matter) during shift from exponential to stationary phase.

Growth of B. ruminicola GA33 on soluble starch is initially very rapid, but ceases at a culture turbidity below that on other substrates (final optical density of 1.6 - 1.8). This may be an indication that B ruminicola GA33 undergoes substrate accelerated death when grown on soluble starch. Substrate accelerated death has been attributed to a cell's inability to adequately regulate transport processes during transition from nutrient deficient to repleted conditions. This defect

Table 13. The Effect of Substrate, Monensin and Stage of Growth on Exoprotease Activity of B. Ruminicola GA33

		Glucose		Maltose		Lactose		Cellobiose		Soluble Starch	
		+M[a]	-M	+M	-M	+M	-M	+M	-M	+M	-M
Exponential	mU/mg protein	8.6	7.9	8.6	11.2	15.8	11.3	5.0	8.6	10.9	5.8
	mU/mg dm	3.8	2.9	3.5	3.4	3.3	3.9	3.0	3.6	3.0	2.6
Stationary	mU/mg protein	5.7	4.8	3.0	6.3	-	4.1	5.7	4.5	7.2	11.9
	mU/mg dm	1.1	1.7	.4	2.1	-	2.0	1.1	1.1	1.6	5.4

[a] 2.5 μg monensin/ml

may ultimately result in a depletion of critical metabolic intermediates, thereby simulating an energy starved state (Koch, 1971).

An interaction also was observed between nutritional status of the growth culture and its stage of growth (Table 15). Glucose limited cultures did not experience the decline in stationary phase proteolytic activity observed with the other two nutritional treatments (Experiment 5). It appears that when culture growth ceases due to a limitation of energy, exoprotease activity stabilizes at a value comparable to that observed during exponential growth.

Further evidence for this conclusion is derived from the response of B. ruminicola GA33 to addition of inhibitors of glucose transport (Figure 13). Addition of α methylglucoside and 2 deoxyglucose to an exponentially growing culture caused an immediate ceasation of growth. Proteolytic activity stabilized and remained constant over a six hour period. Derepression of exoprotease activity due to increased cAMP (catabolite repression) appears to be ruled out because no net increase in activity was recorded. Indeed, little response to exogenous cAMP was observed (Figure 14). Rather, it can be concluded that, during energy starvation, something interferes with the mechanism that normally depresses exoprotease activity of stationary phase cultures (Table 16).

A clue may be found in the response of B. ruminicola GA33 to levallorphan (Figure 15). This compound, which stimulates ppGpp production in enteric bacteria, severely perturbed the exoprotease activity of this microorganisms. Although the activity vs growth curve (following levallorphan addition) showed a cyclical pattern (first stimulation and then repression of activity), final proteolytic activity appeared to be depressed. Nitrogen starvation, which should stimulate

Table 14. Interaction of Substrate and Stage of Growth on Exoprotease Activity[a] of B. Ruminicola GA33

Sugar	Stage of Growth	
	Exponential	Stationary
Glucose	5.26	1.79
Cellobiose	5.72	1.96
Maltose	6.92	1.97
Lactose	7.78	2.12
Soluble Starch	4.78	3.99

P<.001
[a]mU/mg protein - dry matter (least square means)

Table 15. Interaction of Nutritional Status and Stage of Growth on Exoprotease Activity[a] of B. Ruminicola GA33

Nutritional Status	Stage of Growth	
	Exponential	Stationary
Adequite	5.4	2.1
Glucose Limitation	6.6	5.3
Nitrogen Limitation	3.4	.7

P<.001
[a]mU/mg protein - dry matter (least square means)

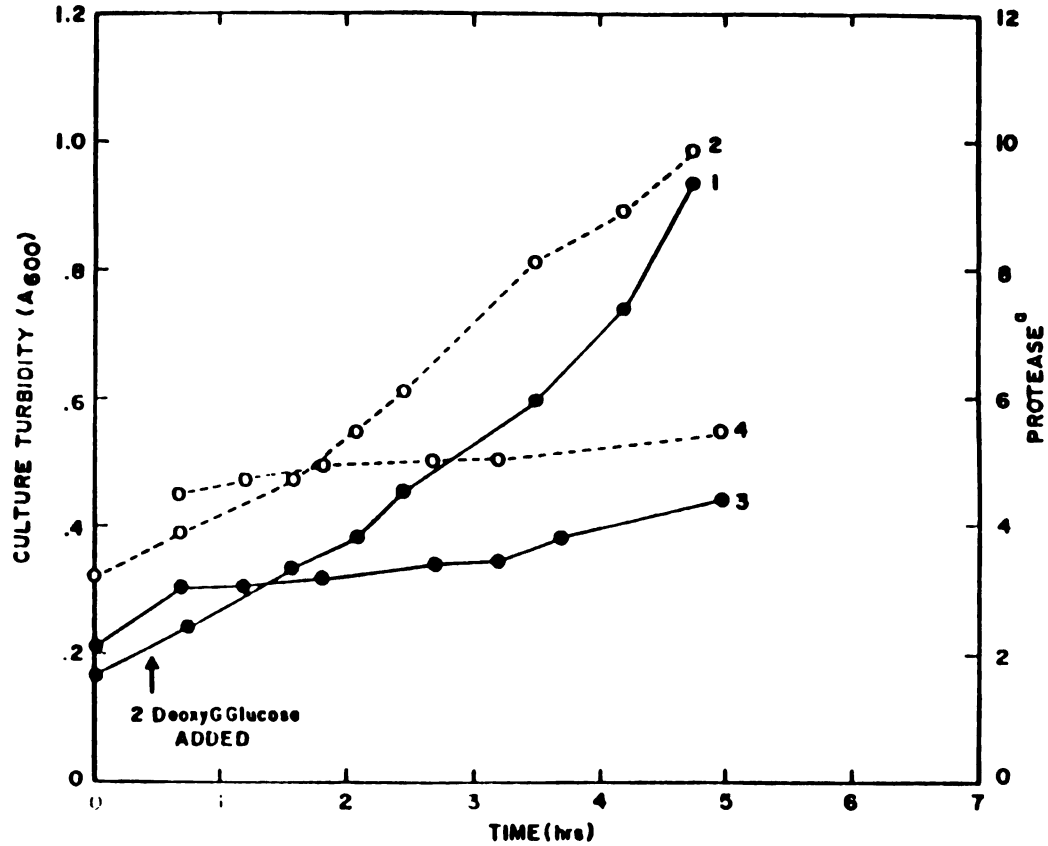

FIGURE 13. EFFECT OF ADDITION OF 2-DEOXYGLUCOSE ON EXOPROTEASE OF BACTEROIDES RUMINICOLA GA33. (LINE 1, CONTROL GROWTH; LINE 2, CONTROL PROTEASE; LINE 3, TREATMENT GROWTH; LINE 4, TREATMENT PROTEASE)

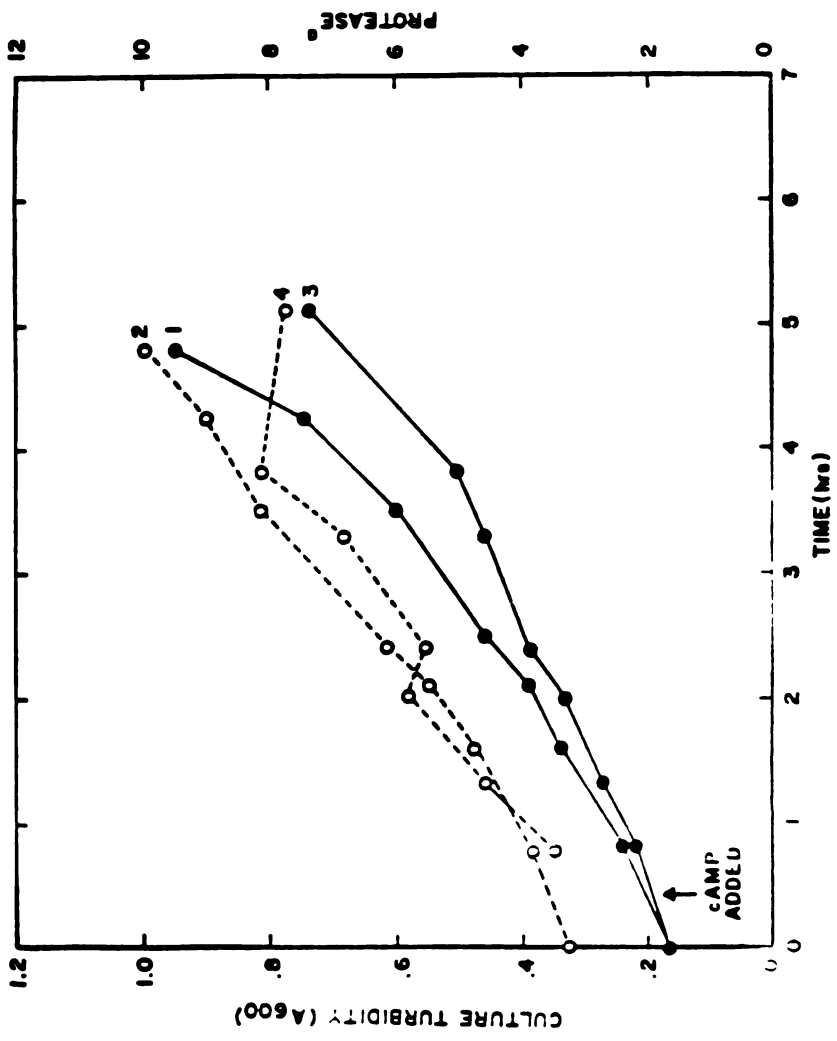

FIGURE 14. EFFECT OF ADDITION OF CYCLIC AMP ON EXOPROTEASE OF BACTEROIDES RUMINICOLA G33. (LINE 1, CONTROL GROWTH; LINE 2, CONTROL PROTEASE; LINE 3, TREATMENT GROWTH LINE 4, TREATMENT PROTEASE)

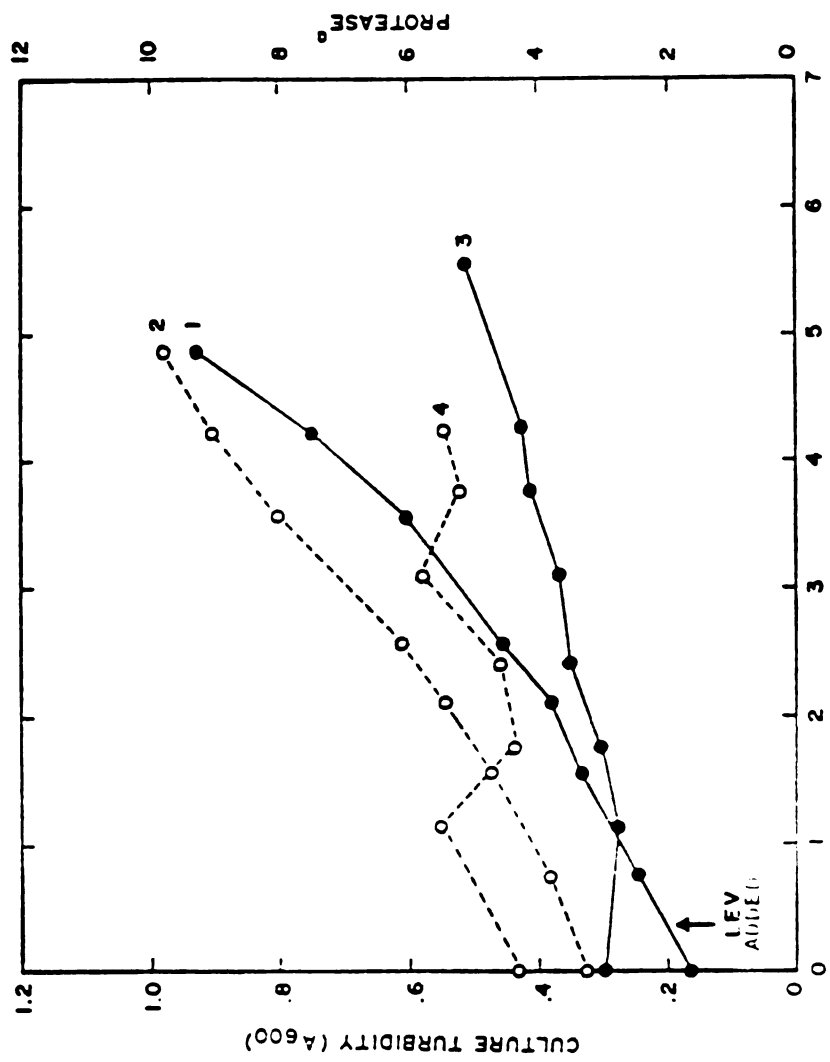

FIGURE 15. EFFECT OF ADDITION OF LEVALLORPHAN ON EXOPROTEASE OF BACTEROIDES RUMINICOLA GA33. (LINE 1, CONTROL GROWTH; LINE 2, CONTROL PROTEASE; LINE 3, TREATMENT GROWTH; LINE 4, TREATMENT PROTEASE)

ppGpp production as well, also resulted in a depression of protease activity in stationary phase cultures (Table 15).

A stimulation of ppGpp production should result in an increase in intracellular proteolysis (St. John and Goldberg, 1978). Guanosine polyphosphates accumulate during stationary phase of stringent E. coli (Kramer et al., 1981) and may be associated with an increase in intracellular proteolysis and decreased exoprotease normally observed in stationary phase cultures of B. ruminicola (Mandelstam and McQuillen, 1973). In nonruminal bacteria, intracellular proteolysis has been shown to be an energy dependent process (St. John and Goldberg, 1978). Thus, starvation for energy could conceivably result in an accumulation of ppGpp (Cashel, 1975) without a corresponding increase in intracellular proteolysis.

Cellular protein as a percent of total cell mass during nutrient sufficient, glucose limited and nitrogen limited growth of B. ruminicola GA33 is shown in Table 17. This value increased from 34% to 44% as glucose limited cultures entered stationary phase. Glucose limitation has been shown to decrease the RNA:protein ratio of B. ruminicola (Bates and Bergen, 1984). Nonprotein cell components must turn over faster than protein in this bacterium when it is in the glucose limited state, suggesting a depression of intracellular proteolysis.

Table 16. Effect of Stage of Growth on the Exoprotease Activity[a] of B. Ruminicola GA33.

Stage of Growth	Specific Activity		Mean
	mU/mg protein	mU/mg dry matter	
Exponential	9.18	3.42	6.22
Stationary	3.49	2.55	2.93
SEM			.42

P<.001
[a]Least square means

Table 17. Cellular Protein as a Percent of Total Cell Mass During Nutrient Sufficiency, Glucose Limitation and Nitrogen Limitation

Treatment	Stage of Growth	
	Exponential	Stationary
Nutrient Sufficiency	37%	34%
Nitrogen Limitation	29%	29%
Glucose Limitation	34%	44%

Table 18. Influence of Growth Substrate on Exoprotease Activity[a] of B. Ruminicola GA33.

Substrate	Specific activity[b]	μ[c]
Glucose	3.48	.49
Cellobiose	3.74	.43
Maltose	4.45	.13
Lactose	5.74	.17
Soluble Starch	4.45	.43
SEM	.78	

$P<.01$
[a]Least square means
[b]mU/mg protein - dry matter
[c]Specific growth rate

Growth substrate as a main effect is seen in Table 18. In general, as growth rate increased, exoprotease activity declined. A relationship between bacterial growth rate and proteolytic activity has been previously reported (Wiersma et al., 1978).

Monensin was not significant as a main effect although exoprotease activity tended to decline with monensin when expressed on a dry matter basis (Table 19). In addition, an interaction between substrate and monensin was observed (Table 20). Monensin decreased exoprotease activity of B. ruminicola with all substrates except glucose (with which activity increased). The decline for maltose monensin and soluble starch monensin was proportionately smaller when specific activity was expressed on a protein as opposed to a dry matter basis.

Experiment 7

Two peaks were observed in a plot of dilution rate (D) vs exoprotease activity (Figure 16). One peak occurred at D = .18, the other at a dilution rate approaching the maximum growth rate of the organism.

Table 19. Influence of Monensin on the Exoprotease Activity of B. Ruminicola GA33

Treatment	Specific Activity	
	mU/mg protein	mU/mg dry matter
Control	7.6	2.9
Monensin[a]	7.8	2.3

[a]2.5 g/ml

Table 20. Interaction of Monensin and Growth Substrate on Exoprotease Activity[a] of B. Ruminicola GA33

Substrate	Treatment	
	Control	Monensin
Glucose	4.3	4.8
Maltose	5.8	3.9
Lactose	5.3	
Cellobiose	4.5	3.7
Soluble Starch	6.4	5.7

P<.01
[a]mU/mg protein - dry matter

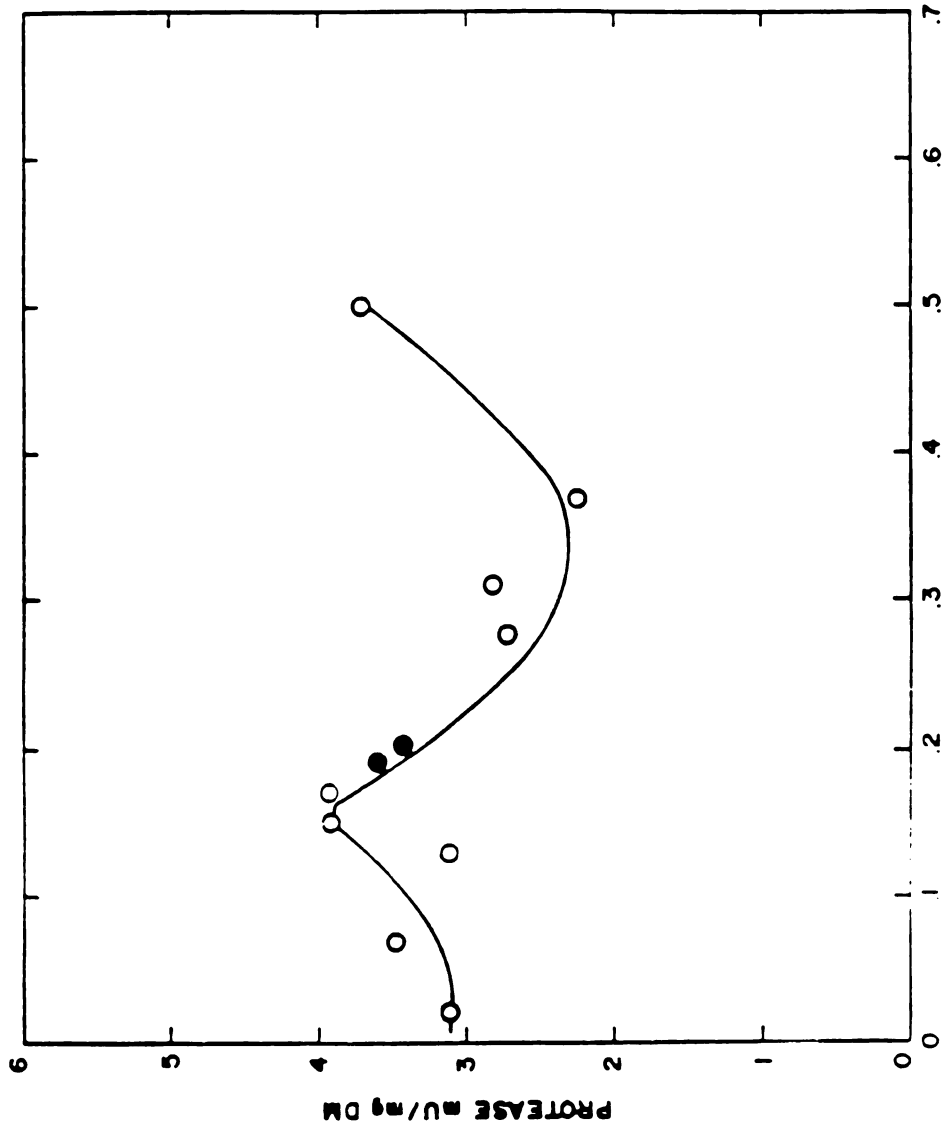

FIGURE 16. EXOPROTEASE OF BACTEROIDES RUMINICOLA GA33 EXPRESSED AS A FUNCTION OF DILUTION RATE OF GLUCOSE LIMITED CONTINUOUS CULTURE.

Discussion

Factors Affecting Exoprotease of B. Ruminicola GA33

A number of factors are involved in coordinating the rate of exoprotease synthesis in B. ruminicola GA33 with changes in environmental conditions. Transition from exponential to stationary growth elicits a decline in protease levels. Several authors have suggested that turnover of individual enzymes is effected by the rate of intracellular protein turnover (Pine, 1972; Pirt, 1975). No consistant change in protein composition of Bacteroides ruminicola was observed during shifts in growth stage. Nevertheless, considerable evidence has accumulated to support the hypothesis that enzyme concentrations in stationary cells decline due to high rates of intracellular proteolysis (Pine, 1980).

During starvation cellular protein continues to provide peptides for the endogenous synthesis of macromolecular components (Pine, 1980). Microbial cells usually do not contain one specialized protein that can serve as a protein store during starvation. Rather, a large number of cell proteins serve in this capacity (Pine, 1980). Decay of enzymatic activity has been demonstrated for alkaline phosphatase and amino acid synthetase in E. coli during amino acid starvation (Willets, 1967) and B. galactoside permease during stationary phase (Nath and Koch, 1970). One third of the proteins of E. coli appear to be susceptible to proteolytic attack during periods of starvation (Pine, 1980). During diauxie growth transition from one carbon source to another, the total rate of breakdown of all cell proteins goes up (Willets, 1967). This is probably due to transient increase in ppGpp production (Voellmy and Goldberg, 1983).

Enterobacteria shed a vesicle containing protein complexed with microcapsular lipopolysaccharide and phospholipid into the growth medium

(Williams and Niedhardt, 1969). An involvement of this phenomenon with protein turnover and extracellular protein release has been suggested (Pine, 1972). The release of cellulase and xylanase by Bacteroides succinogenes has been shown to occur by this mechanism (Forsberg et al., 1981). Bacteroides ruminicola is also a gram negative bacteria. A substantial increase in exoprotease activity found in culture supernatant has been reported for stationary cultures of this organism (Hazelwood et al., 1981).

The amount of cell protein that is mobilized by intracellular proteolysis is influenced by the type of starvation (Pine, 1980). This is reflected in divergent stationary phase RNA/ratios reported for ruminal bacteria (Bates et al., 1985). Proteolysis is particularly high during nitrogen starvation (Mandeltam, 1960) and has been shown to occur at 5% per hour in normal stationary phase cells (Mandelstam, 1960; Pine, 1980). Because proteolysis has an absolute energy requirement, the depletion of intracellular ATP below a certain critical value stops all further protein break down (Pine, 1980). An ATP dependent protease has been implicated in the degradation process (Larimore et al., 1982). Some of the differences observed with nutrient limited cultures may be attributable to differences in the extent of intracellular proteolysis in these cultures.

Levallorphan has been shown to increase ppGpp production in bacteria (Boquet et al., 1973). The fluctuation of protease observed following levallorphan addition is probably due to an accumulation of ppGpp. Guanosine tetraphosphate is highly correlated with intracellular proteolysis. The rapid increase in activity observed immediately following levallorphan addition may reflect 1) a direct influence of ppGpp on exoprotease transcription, 2) an increased transcription of

intracellular protease which somehow contributes to exoprotease or, 3) allosteric activation of existing enzyme. The protease responsible for the breakdown of normal cell proteins in E. coli and S. typhimurium do not correspond to any of the known proteases of these organisms. This would seem to rule out the second option (Miller et al., 1976).

Guanosine tetraphosphate has been shown to increase transcription of certain operons that impart adaptive characteristics (Nierlich, 1978). Peptides released during proteolysis are transported and utilized directly by Bacteroides ruminicola (Pittman and Bryant, 1964). An increase in exoprotease production would help a cell adapt to an amino acid deficient environment.

A direct effect on the existing metabolic machinery can not be ruled out. When wild type E. coli were given tetracycline (which inhibits ppGpp production) the rate of intracellular protein degradation returned to basal levels without any appreciable lag (Voellmy and Goldberg, 1980). It was suggested that ppGpp may serve as an allosteric effector or it may render certain cell proteins more susceptible to degradation.

The cyclical nature of the protein response to levallorphan may give a clue as to the nature of this response. An increase in exoprotease is immediate and is coupled to a slight decline in culture optical density. This is followed by a period of rapid decline in protease during which optical density increased somewhat. Then comes another steep rise in activity followed by a decline.

The initial increase was immediate and occured during a period where there was no net protein synthesis. Thus, some direct activation must be involved. The decline which follows may involve autocatalysis where the protease autolyzes itself, or it may involve some type of feedback

mechanism. The initial rate of proteolysis probably results in saturation of intracellular amino acid pools and a subsequent decline in ppGpp concentration. As the levels of ppGpp decline, the stimulus for proteolysis would disappear. Because of the artificial conditions imposed by levallorphan, ppGpp synthesis may be reinstituted at pool levels that normally would not elicit a response such that the cycle then can repeat itself. A lag is required before protease activity starts to rise. During this period a perceptible increase in cell mass occurs. This lag period would seem to be a period during which synthesis of new enzyme occurs. Thus, both direct and transcriptional mechanisms appear to be involved.

Regulation of exoprotease in Bacteroides ruminicola GA33 appears to be a highly complex process which involves a number of interacting mechanisms. The regulation displayed over a range of dilution rates in continuous culture utilized the composite of all the regulatory mechanisms at the cells disposal. The complexity of this process is apparent in the plot of protease activity vs dilution rate in the glucose limited continuous culture of Bacteroides ruminicola (Figure 16).

Four general patterns of enzyme production in continuous culture are shown in Figure 17 (Wiersma et al., 1978). Constitutive synthesis is shown in curve A. The rate of constitutive enzyme production is a function of the product of cell concentration and growth rate (Wiersma et al., 1978). Such a profile has been observed for the extracellular protease produced by Propionebacterium acnes.

A non linear relationship between enzyme produced and dilution rate is generally found for inducible enzymes (curve B), especially when the inducer is the limiting nutrient. An enzyme controlled by catabolite

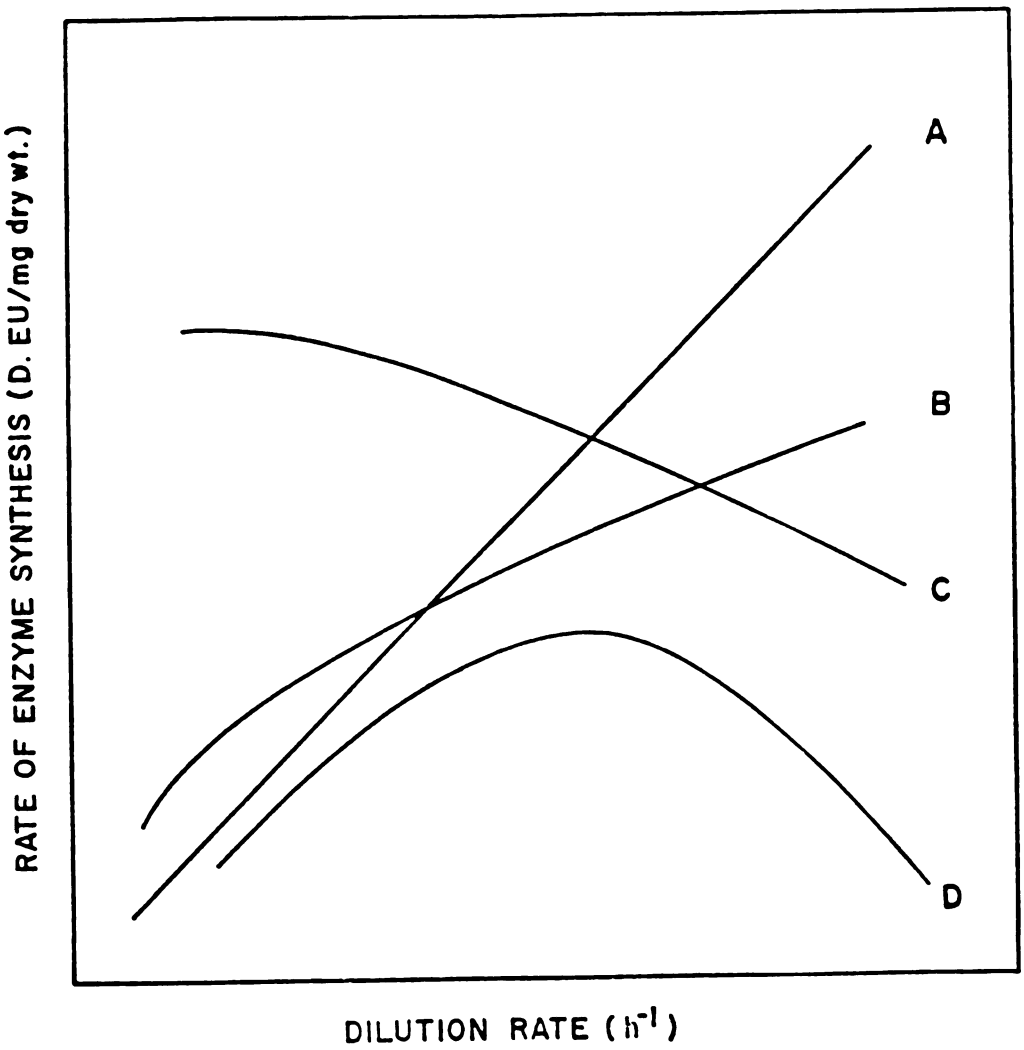

FIGURE 17. FOUR PATTERNS OF ENZYME PRODUCTION IN BACTERIA GROWN IN CONTINUOUS CULTURE (WIERSMA ET AL., 1973).

repression by the growth limiting nutrient is shown in curve C. The rate of enzyme production declines with increased dilution rate because the rate of substrate utilization increases at the higher dilution rate. Curve D has been attributed to a combination of regulatory processes including induction and repression (Clarke and Lilly, 1969).

The activity profile of the glucose limited continuous culture of Bacteroides ruminicola was very similar to that for maltose limited continuous cultures of Bacteroides amylophilus (Henderson et al., 1969). One peak in activity is seen at a dilution rate of .18 and another at .5. This later value is approaching the maximum growth rate of this organism. This profile indicates the involvement of some type of induction and repression. Induction by amino acids and peptides could not be demonstrated in batch cultures of this organism. The nature of the inducer remains undefined. Regulation by catabolite repression maybe responsible for the descending slope of the curve after D = .18.

Effect of Diet on Proteolytic Activity in the Rumen

The rate of hydrolysis of protein in the rumen is influenced by diet. Typically, proteolysis is elevated when high roughage diets are fed (Ganev et al., 1979). Nugent and Mangen (1981) recently reported that the rate of hydrolysis of alfalfa fraction 1 protein was nine times higher when sheep were fed alfalfa than when fed a hay-concentrate mix.

Substrate limitation is generally accepted as an important factor that limits microbial growth in the rumen (Hungate, 1966). Ruminal sugar concentrations should be elevated when grain is fed (Takakashki and Nakamura, 1969). Thus, the results of these in vivo studies are consistant with the observation that proteolytic activity of B. ruminicola is higher during glucose limited growth than when glucose is not limiting.

Why is There a Decrease in Ruminal Protease During Monensin Feeding?

A protein sparing effect has recently been attributed to monensin (Van Nevel and Demeyer, 1977; Poos et al., 1979). Decline in rumen ammonia is routinely observed when ionophores are used as feed additives (Van Nevel and Demeyer, 1977; Chalupa, 1980). Short (1978) reported inhibition of proteolysis in the presence of monensin using mixed rumen cultures in vitro. Barao (1983) observed a depression in both protease and deaminase activity in rumen bacteria in vivo when monensin was fed. An increase in the proportion of dietary protein escaping ruminal degradation has frequently been observed with diets containing monensin (Isichei, 1980; Poos et al., 1979). Thus, monensin may increase both the quantity and/or quality of protein reaching the lower gastrointestinal tract for digestion and absorption.

Several researchers (Chen and Wolin, 1979; Wallace et al., 1981) have established that a shift in the microbial ecology of the rumen results when ionophores are fed. Gram positive bacteria (The predominant acetate, hydrogen and formate producers in rumen) are inhibited by monensin (Chen and Wolin, 1979). Gram negative strains, many of which produce succinate, are not inhibited to the same degree (Chen and Wolin, 1979; Henderson et al., 1981). Dickerson (personal communication, 1983) observed a similar response to lasalocid using pure cultures of rumen bacteria in vitro. It has been suggested that ionophores cause a diversion of reducing equivalents from methane production to propionate production as a consequence of the selection process which allows gram negative organisms to proliferate and dominate the rumen fermentation (Chen and Wolin, 1979).

One explanation for the observed shift lies in the physical structure of gram negative bacteria. The outer membrane of gram negative species

serves as a penetration barrier which protects these cells from antibiotics (Kadner and Bassford, 1978). The growth energetics of gram negative strains may also be involved (Bergen and Bates, 1984). Given the inefficiency of anaerobic fermentative processes (Hungate, 1966), the availability of ATP is often considered the limiting factor controlling rate and extent of bacterial growth in the rumen (Hungate, 1966). Collapse of the proton gradient catalyzed by monensin would impinge on available ATP supplies to varying degrees (depending upon the mechanism by which protons are expelled from the cell). Those bacteria which couple this process to electron transport would protect their valuable ATP stores and have a selective advantage over strains which depend heavily on direct utilization of intracellular ATP. Anaerobic respiration in general and the fumarate reductase system in particular, is more prevalent in gram negative microorganisms. Thus, many of the rumen bacteria selected for by ionophores possess the capacity to generate a proton motive force by electron transport thereby minimizing the contribution required by the intracellular ATP supply.

Research conducted in the early 1960's indicated that the primary proteolytic organisms in the rumen are gram negative (Blackburn and Hobsen, 1962). Some gram positive strains have been implicated (Blackburn and Hobsen, 1960; Russell et al., 1981), but these should be inhibited by monensin (Chen and Wolin, 1979). It would seem that if a simple bacterial shift explains all monensin effects on the ruminal fermentation, ruminal proteolysis should actually increase during monensin feeding.

The observation that total proteolytic activity in the rumen did not change with different dietary protein levels lead Allison (1970) to speculate that many rumen bacteria possess the constitutive property for

at least a low level or protease production. This observation, coupled with research that indicated that protein, peptides or amino acids did not induce protease in Bacteroides amylophilus, has lead to the widely held view that protease production in the rumen is not subject to metabolic control. This view (in light of current data) may be too simplistic. Soluble carbohydrates have been shown to inhibit proteolysis in in vitro experiments using mixed rumen cultures (Blackburn, 1965). A 10-fold increase in the velocity of protease has been observed in rumen contents from sheep fed fresh cut alfalfa as opposed to those fed a mixture of hay and grain (Nugent and Mangen, 1981). Energy status of rumen bacteria appears to play a role in the regulation of proteolytic activity, while monensin may interfere with this regulatory process.

The inclusion of monensin with cultures grown on different energy substrates resulted in a diverse physiological response. Some general trends were seen. These included:

1) No direct effect of monensin on the exoprotease enzyme could be demonstrated.

2) Monensin tended to decrease exoprotease when expressed as mU/mg protein.

3) Monensin cultures had a low proportion of protein as percent cell mass which indicates extensive intracellular proteolysis.

4) A decline in both mU/mg protein and mU/mg dry matter were observed with monensin when maltose or soluble starch were the growth supporting substrate as compared to control cultures. These are the predominate energy substrates for rumen microorganisms on a high concentrate diet.

The physiological response of a bacterium exposed to monensin would seem to be much like the response seen with respect to nitrogen starvation

(Table 15). Nitrogen limiting conditions resulted in a depression of protease activity in Bacteroides ruminicola GA33 during stationary phase.

The mechanism behind this response probably has something to do with the protonophoric nature of monensin under most culture conditions (Bergen and Bates, 1984). The maintenance of an intact proton motive force is essential if a cell is to process ppGpp normally (Tetu et al., 1979). A decline in the proton motive force results in a decrease in the rate of ppGpp degradation and thus an increase in ppGpp accumulation. The decline in PMF may also influence the ability of the bacterial cell to transport amino acids or peptides (Booth and Hamilton, 1980). Monensin diminishes the yield response Bacteroides ruminicola normally displays to inclusion of peptides in the growth medium (Russell, 1983).

CONCLUSIONS

1. Macromolecular composition of rumen bacteria may be used as a marker of microbial function and production in the rumen. Ribonucleic acid:protein ratios of rumen bacteria studied in vitro were highly correlated (R^2 = .74) with specific growth rate, μ . As μ increased, RNA/protein and RNA/DNA values increased for all organisms. The overall regression (six organisms) for RNA/protein vs was $Y = .62\mu + .23$, r = 86. The DNA/protein of ruminal bacteria was not affected by variation in growth rate.

2. The RNA:protein ratio of stationary phase cultures of seven predominant rumen bacteria was affected by glucose and nitrogen limitation ($P<.01$). Nitrogen limited cultures had the highest ratio (0.52 across all species), followed by cultures grown with adequate nutrition (0.28) and glucose limited cultures (0.12).

3. The regression of RNA/protein on μ for Streptococcus bovis established the following linear relationship, $Y = .28\mu + .26$, $r = .94$. The slope of this regression line was lower (.28 vs .62) than the slope of the composite regression six other ruminal strains. This indicates that, at a given growth rate, S. bovis more efficiently synthesizes protein than other predominant rumen bacteria.

4. An interaction ($P<.001$) was observed between growth substrate and stage of growth as factors that affect exoprotease activity of B. ruminicola GA33. A similar interaction ($P<.01$) was observed between

nutritional status and stage of growth. It is concluded that energy starvation interferes with the mechanism that normally depresses ($P<.01$) exoprotease activity of stationary phase cultures of B. ruminicola GA33.

5. Several factors including rate of growth ($P<.01$), stage of growth ($P<.01$) and nutritional status ($P<.01$) have been identified that influence the exoprotease activity of B. ruminicola GA33. The plot of exoprotease activity vs dilution rate for a glucose limited continuous culture of B. ruminicola indicated that a complex interacting set of regulatory controls may be involved in regulating the proteolytic activity of this organism.

APPENDIX

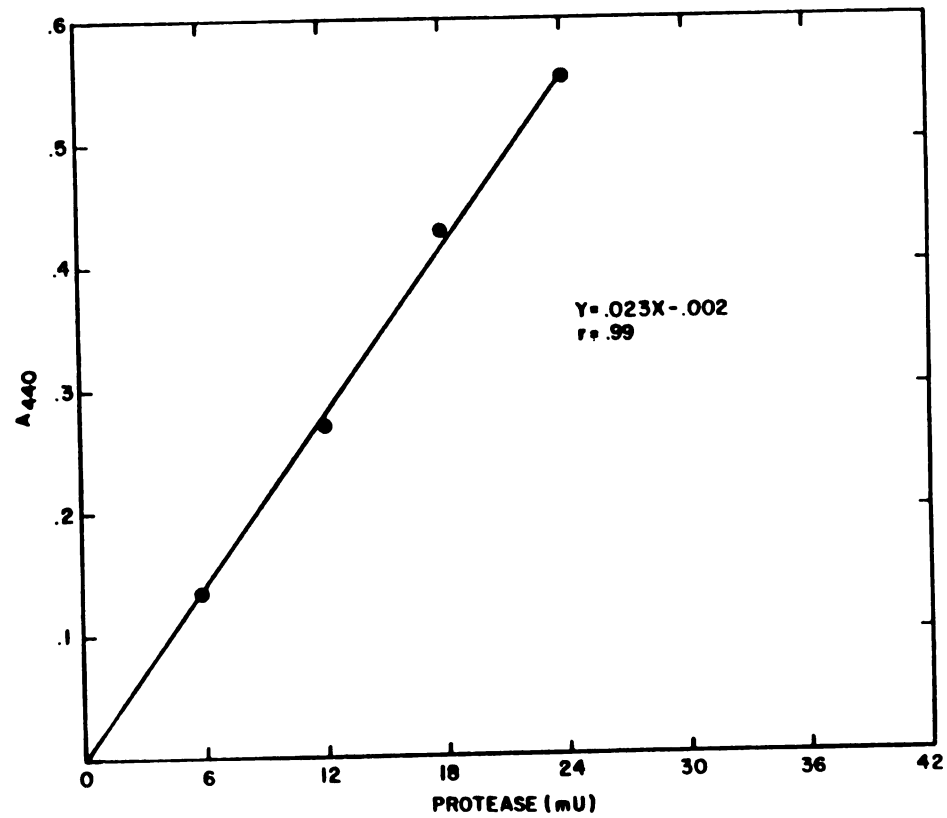

FIGURE A1. STANDARD CURVE OF PROTEASE (SIGMA # P5380) ACTIVITY POSSESSING 12 UNITS OF ACTIVITY PER MILLIGRAM.

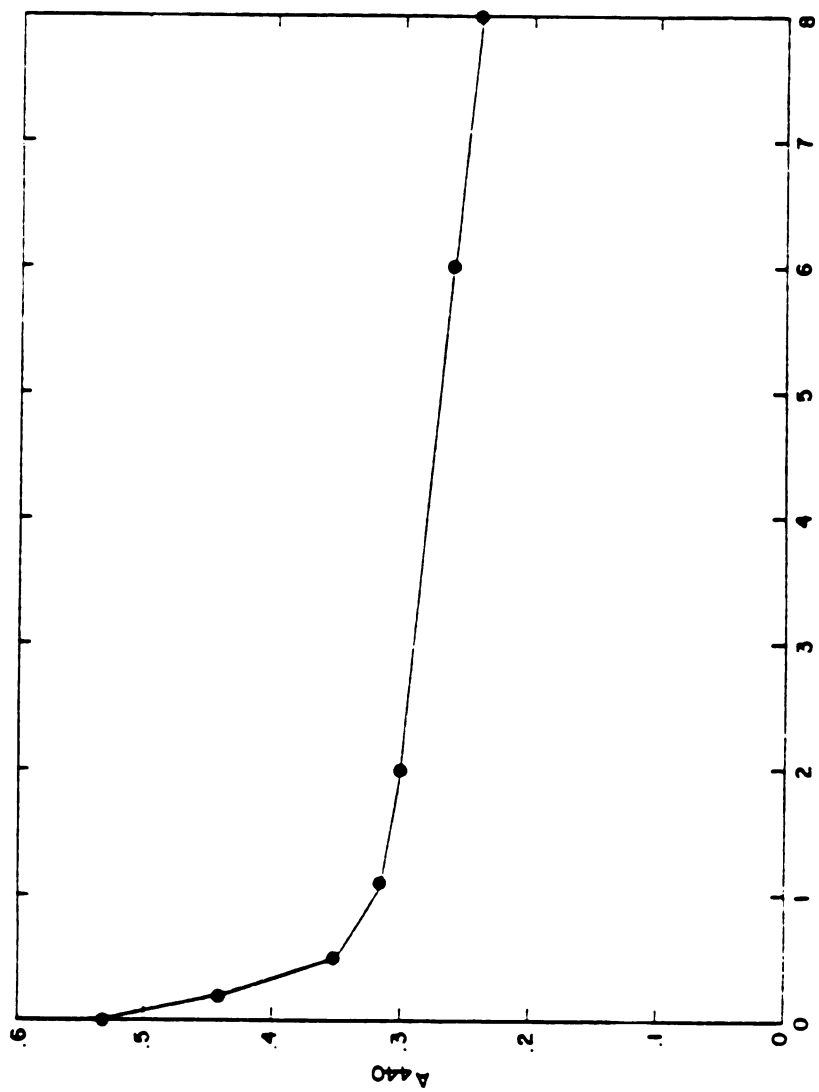

FIGURE A2. DECAY OF HYDROLYZED AZO DYE (STORAGE IN 5% TCA AT 0° C)

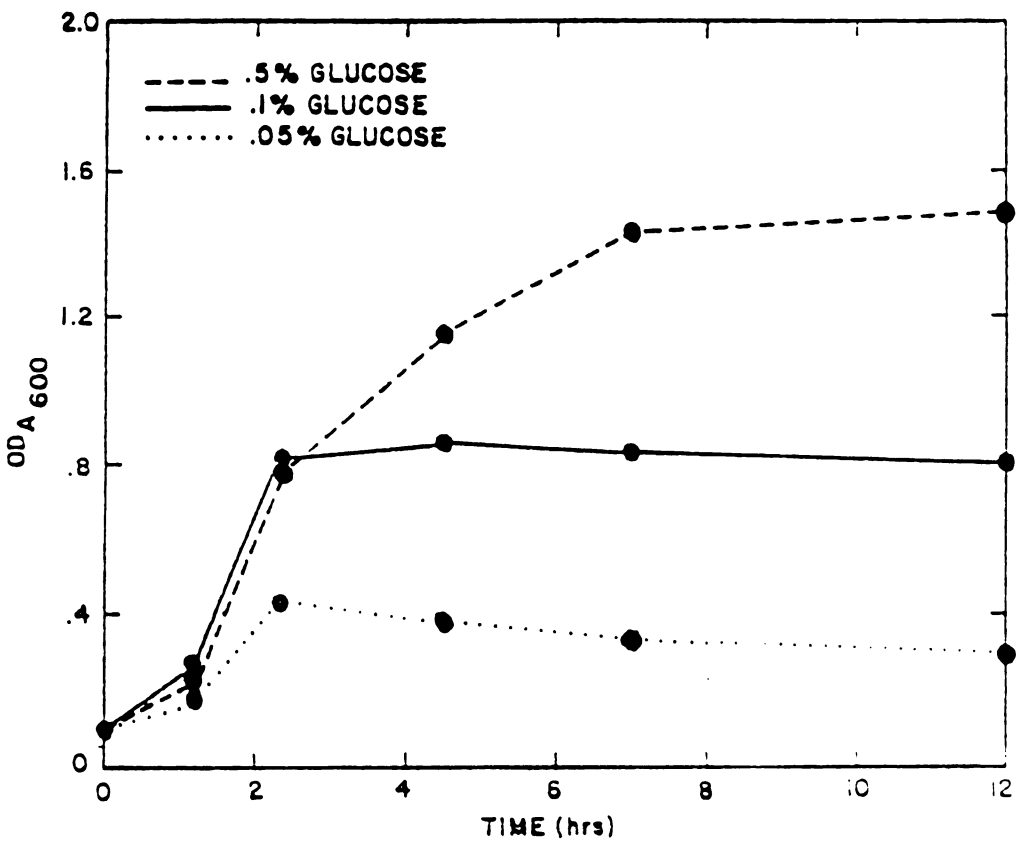

FIGURE A3. EXAMPLE SHOWING THE DETERMINATION OF A KS VALUE (GLUCOSE) FOR S. RUMINANTIUM.

Table A1. Comparison of Protease Activity in Several Ruminal Bacteria

Trial	Species	Protease (mU/mg protein)
1	Bacteroides ruminicola GA33[a]	3.1
	Bacteroides ruminicola B.4[a]	2.4
	Selenomonas ruminantium GA192[a]	1.4
	Selenomonas ruminantium ATCC19189[a]	2.2
	Butyriuibrio fibrisolvens H106[a]	2.3
	Streptococcus bovis 24[a]	3.0
2	Bacteroides ruminicola GA33[b]	4.9
	Bacteroides ruminicola B.4[b]	3.7
	Butyrivibrio Fibrisolvens D1[b]	2.3
	Bacteroides ruminicola B.4[a]	1.8
	Butyrivibrio Fibrisolvens D1[a]	1.0

[a]Tested using glucose sufficient stationary phase cells in a whole cell suspension containing dithiothreiotol.

[b]Tested using glucose limited stationary phase cells in a whole cell suspension containing diethiothreiotol.

Table A2. Effect of Cell Concentration on Azocasein Degradation[a] by Bacteroides Ruminicola GA33.

Culture Conditions	Volume of WCS used in assay		
	1 ml	.5 ml	.25 ml
Glucose Limitation	5.5	2.9	1.4
Glucose Sufficiency	2.3	1.0	.7

[a]mU protease activity

Table A3. Effect of Concentration of Azocasein Used in the Assay on Protease Activity of B. Ruminicola GA33.

Concentration of Stock Solution				
.5%	.1%	2%	3%	5%
1.0	2.1	3.3	4.5	4.8

[a]mU/mg protein

Table A4. Decay of Different Concentrations of Hydrolyzed Azo Dye[a]

Concentration	Time (hrs)			
	0	5	31	%Decline
High	.527	.440	.315	40%
Intermediate	.188	.158	.125	34%
Low	.108	.086	.063	42%

Table A5. Comparison of Dry Matter Yield (mg/ml) When Cells Were Harvested With or Without Formaldehyde-NaCl Pretreatment[a]

	Culture Medium	
	glucose sufficiency	glucose limitation
F-NaCl	1.984 ± .023	.500 ± .024
None	1.942 ± .054	.488 ± .021

[a]Thirty milliliter culture suspension grown until culture turbidity had stabilized were mixed with 8.4 ml formalin solution (37% formaldehyde - .85% NaCl, w/v) and centrifuged at 45.000 x g for 20 minutes to collect the bacterial pellet.

Table A6. Comparison of Absorption of Moisture During Gravitimetric Determination of Cell Dry Weight Using Filters, Metal Pans of Glass Tubes[a]

Filter[b]	Glass tube	Pan	Pan with paper lab
1.00±1.2mg(23)[c]	.000033±.000066mg(30)	.10±.08mg(20)	.40±.29mg(23)

[a]Increase in weight after equilibration with ambient surroundings.
[b]Membrane filter (47 mm, DAWP, .45 m) (Millipore Co. Inc., Bedford, MA).
[c]Number of samples measured indicated in parenthesis.

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