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FEASIBILITY OF USING TOTAL PURINES AS A MICROBIAL MARKER

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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* * * * *

The Ohio State University
1997

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ABSTRACT

A procedure for measuring purine content of mixed rumen bacteria was adapted for use in measuring purines in smaller size pure culture samples of rumen bacteria. Using adenine and guanine, alone or in mixture, as standards, recoveries were quite variable. The variation was traced to solubility of the silver salt of adenine in the solution used to wash the precipitate. With this corrected, recoveries of the purines or yeast RNA, either alone or in combination, were above 99%. Purine concentration and cell numbers were determined for ten pure cultures of ruminal bacteria: Butyrivibrio fibrosolvens, D16f. H10b, and H17c; Fibrobacter succinogenes B21a; Lachnospira multiparas D25e; Lactobacillus lactis ARD26e; Prevotella ruminicola H15a; Ruminococcus albus 7; Ruminococcus flavefaciens B34b; and Streptococcus bovis ARD5d. Three large batches of cells were grown with each organism and three separate analyses were carried out with each large batch to measure bacterial, purine and protein concentrations. The coefficient of variation for the MPN assay (bacterial concentrations) was 55.86%, for purine analysis 5.25% and for protein 6.52%. Considerable variation occurred when purine and protein concentrations were compared as amount per cell; however, more consistent values were obtained on a dry matter basis. Purine to protein ratios ranged from 0.023 to 0.1299 for the pure cultures. The mean value was found to be 0.0883 which was 2.8 times higher than the value determined for samples of mixed bacteria separated from rumen fluid (0.0313). The value determined for the mixed bacterial sample is similar to previously...
reported values, and has been used to estimate microbial protein at the duodenal level. Based on the ratio obtained with the ten pure cultures, it is probable that microbial protein has been overestimated in most of the previous reports. Limited studies have indicated a probable contamination of the mixed bacterial sample with feed particles containing protein, which results in lower purine to protein ratios.
DEDICATION

Dedicated to my Parents and Family
ACKNOWLEDGMENTS

I am very grateful to my friend and advisor, Dr. Burk Dehority, who gave me intellectual guidance and his encouragement, patience and support in my education. I appreciate also the guidance and support of Steve Loerch: particularly for his willingness to help me. Thanks are extended to Mike Lilburn for his friendship and serving on my committee.

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Appreciation is also addressed to Faculty, Staff Members and Graduate Students of the Animal Sciences Department. I express my sincere gratitude to Patricia Tirabasso, Fay Smith, Francis Fluharty, Beverly Fisher, Kenneth McClure, Bert Bishop, Pat Sachariat and Della Bardall for their help in this journey.

Finally, I am extremely grateful to my parents and family for their love, blessing, patience and goodwill through the whole period of my studies.
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FIELD OF STUDY

Major Field: Animal Science
Studies in Animal Nutrition and Rumen Microbiology
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INTRODUCTION

Research in ruminant nutrition has demonstrated that in addition to digesting structural carbohydrates in feeds, the rumen microorganisms are also an important source of protein to the host. The rumen microbiota is diverse by nature and the individuals occupy several different niches (Hungate, 1966). Moreover, the microorganisms differ not only in population size but also in their contribution to the host. However, in spite of all that is known about the rumen fermentation, there is still a need to estimate microbial biomass when investigating the overall digestive process and the nutritive value of the feedstuffs. For calculating feed requirements under practical feeding conditions (NRC, 1985; ARC, 1984) a reliable estimate of the contribution of microbial protein to the host animal is very important.

Estimation of microbial growth in the rumen and the flow of their protein to the small intestine is very dependent on a reliable microbial marker. Although some work has been done in this regard, using diaminopimelic acid (Purser and Buechler, 1966; Hutton et al., 1971), aminoethylphosphonic acid (Ankrah et al., 1989), nucleic acids (McAllan and Smith, 1969; Cecava et al., 1990b; Arambel et al., 1982), ATP (Wallace and West, 1982) and total purines (Zinn and Owens, 1986; Ushida et al., 1985; Aharoni and Tagari, 1991) as internal markers, and \(^{35}\)S (Stern and Hoover, 1979), \(^{15}\)N (Firkins et al., 1987), and \(^{32}\)P (Van Nevel and Demeyer, 1977) as external markers, none of these
cellular components or incorporated isotopes are ideally suitable as a microbial marker. The ideal microbial marker should: (1) not be present in the feed; (2) not be absorbed; (3) be biologically stable; (4) have a relatively simple assay procedure; (5) occur in a similar percentage between the various types of microbes, i.e., bacteria, protozoa and fungi; (6) be a constant percentage of the microbial cell in all stages of growth; and (7) all forms should flow at the similar rate, i.e., free and bound (Dehority, 1995).

At this time, based on several critical analyses (Broderick and Merchen, 1992; Stern et al. 1994), the total purine method (Zinn and Owens, 1986) and incorporation of $^{15}\text{N}$ have been suggested as the best available procedures to quantify microbial protein yields under practical conditions.

To estimate microbial protein at the duodenal level requires a bacterial sample from the rumen for a standard, which needs to be representative of the microbial population. At present, there is no information about the total purine contents of pure cultures of rumen bacteria and their relationship to the values measured with mixed bacterial samples isolated from the rumen. This information is needed before any reliable estimates can be made concerning the quantity of microbial protein passing to the duodenum.
LITERATURE REVIEW

Microbial markers

It has been estimated that up to 80% of the daily amino acid requirements of ruminants are supplied by microbial proteins flowing to the small intestine (Hutton et al., 1971; Ibrahim and Ingalls, 1971; Sniffen and Robinson, 1987; Stern et al., 1994). Hence, due to the significant contribution of the microbial protein to the animal, their quantitation is considered to be paramount to accurately predict the protein available for utilization by the animal. The digesta that reaches the small intestine contains proteins derived from three different sources, i.e., microbial proteins synthesized in the rumen, feed protein which has passed undegraded through the rumen, and endogenous protein (abomasal secretions and desquamated epithelial cells). If an appropriate marker is present in one of these fractions, then from the total content of protein in the sampled digesta, the proportion of that fraction can be determined by measuring the concentration of the marker.

The use of a microbial marker is based on the premise that a given component of the microbial cell is proportional to the cell numbers, or cell weight, but not to the size (Dehority, 1995). Microbes that are "growing" are increasing in number, accumulating in colonies of hundreds or thousands, or populations of billions. Hence the total mass of the bacterial population is usually roughly proportional to the number of cells in the
population; the rate of increase in the population's mass is often a measure of its reproductive rate. An ideal microbial marker should be: (1) found only in the microbes (does not occur in feed); (2) be biologically stable, i.e., not affected by the digestive tract or its microbes; (3) have a simple, specific and sensitive assay procedure; (4) be a constant percentage of the intact cell in all stages of growth, and finally, (5) all forms of the marker should flow at similar rate, i.e., whether free, bound in a cellular portion of a ruptured cell or within the intact cell. Several different markers have been used to assess the microbial contribution to total protein content in the intestine; however, due to some inherent errors these methods have been of questionable value. At present, there is not a satisfactory method available to determine this microbial fraction, and therefore, the validity of any microbial marker is difficult to substantiate (Broderick and Merchen, 1992; Dehority, 1995; Garret et al., 1980; Theurer, 1980). 

Dehority (1995) also pointed out, that any measurement of microbial protein passing from the rumen is further complicated by the fact that three types of microorganisms are involved, i.e., bacteria, protozoa and fungi. Rate and extent of growth differ among these microorganisms, and, is affected by total rumen volume, rate of passage out of the rumen, type of feed, level of intake and feeding frequency (Hoover et al., 1976; Merchen et al., 1986). Rumen volume and rate of passage can readily be determined; however, rate of microbial passage is confounded because some organisms attach to particulate matter and do not pass at the fluid rate (Hungate et al. 1971; Akin et al., 1974). Although there are reliable procedures to estimate the concentrations of bacteria, protozoa and fungi (Dehority and Grub. 1980; Dehority et al. 1989; Dehority.
and to measure particulate matter or dry matter turnover (Hungate, 1966), at present there is not an appropriate method for estimating the numbers of attached microorganisms (Bauchop and Clarke, 1976; Orpin and Letcher, 1978). For estimating total microbial protein reaching the duodenum separate markers would be needed for bacteria, protozoa and fungi (Dehority, 1995).

In most of the marker studies reported in the literature, when the investigators have been interested in measuring passage of microbial protein, the ratio of marker to nitrogen (N) is determined in a reference bacterial or protozoal fraction (physically isolated from rumen contents) and in the duodenal digesta. Microbial protein is calculated as the product of total N times the factor 6.25. This factor is based on the average value of 16% N for most proteins. However, about 30% of the microbial N is found as non-protein nitrogen in the indigestible cell wall. Also, true microbial protein contains about 15% N, which gives a correction factor of 6.67 instead of 6.25. Thus a significant overestimation generally occurs (Hespell and Bryant, 1979; Van Soest, 1994). Furthermore, both protozoa and fungi appear to contain less than half the concentration of nitrogen per unit of dry matter found in bacteria (Weller, 1957; Kemp et al., 1985; Gulati et al., 1989). Therefore, if microbial composition, i.e., percentage of bacteria, protozoa and fungi, were to vary markedly between the reference standard isolated from the rumen contents and the population in the duodenal contents, considerable error could be introduced into estimates of microbial protein (Dehority, 1995).

Markers used to estimate microbial protein concentration can be differentiated in two major types, i.e., an external labeled component which is uniformly incorporated
into all new microbial growth and internal, a compound inherently present in the microorganisms (Stem and Hoover, 1979; Broderick and Merchen, 1992; Stern et al., 1994; Dehority, 1995). Dehority (1995) compiled a list of some of the more commonly used internal and external markers, and included a summary of their major drawbacks from the ideal marker (Table 1 and 2).

Nucleic acids (NA) or purines, DAP, AEP, D-alanine and general amino acid profile of digesta are among the most widely used of the internal microbial markers. However some of these chemical components have been found to have unequal distribution between bacterial and protozoal cells as well as occurring in feedstuffs. In addition, AEP, DAP and D-alanine, are primarily associated with cell walls. Cell walls have been found to be more resistant to digestion than the cell content, therefore digesta may become enriched with this microbial cell component which results in overestimation of the microbial mass outflow (Owens and Goetsch, 1988). For the external markers there are additional disadvantages, i.e., using either $^{15}$N or $^{35}$S isotopes involves complicated and costly assay procedures (Stern and Hoover, 1979; Broderick and Merchen, 1992). Also, radioactive tracers ($^{35}$S or $^{32}$P) require adherence to all the precautions involved in animal trials with radioactive isotopes (Dehority, 1995).

Dehority (1995) pointed out that in most of the reviews dealing with markers for estimating microbial growth, fungi have been almost completely ignored. Orpin (1981) used chitin as a fungal marker, but assumed a constant percentage of this compound regardless of age or species. Later, although no verification of the procedure has been reported, Akin (1987) and Gay et al., (1988). used enzymatic procedures (chitinase and
<table>
<thead>
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<th>Microbial fraction(s) identified</th>
<th>Major deviations from the ideal marker</th>
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<td>DAPA</td>
<td>Bacteria</td>
<td>Percent composition varies between species</td>
<td>1</td>
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<tr>
<td></td>
<td></td>
<td>Occurs in both feedstuffs and protozoa</td>
<td>2, 3</td>
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<tr>
<td></td>
<td></td>
<td>Composition varies between free, bound and cell associated DAP with time after feeding</td>
<td>4</td>
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<tr>
<td></td>
<td></td>
<td>Rate of catabolism may vary with DAP location</td>
<td>5</td>
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<td>Bacteria</td>
<td>Similar deviations as for DAP except it was not found in feedstuffs</td>
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<td>Large variation in analysis</td>
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<td></td>
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<td>Occurs both in bacteria and feedstuffs</td>
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2 DAPA = 2,6-diaminopimelic acid; AEP = 2-aminoethylphosphonic acid; Nucleic acids = RNA and DNA.

Table 2. External markers which have been used to estimate microbial and protein concentration

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<td>Bacterial and protozoal pools are not enriched equally</td>
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</tr>
<tr>
<td>$^{35}$S</td>
<td>Bacteria and protozoa</td>
<td>Not all microbial amino acid sulfur arises from sulfate or sulfide</td>
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<tr>
<td>$^{32}$P</td>
<td>Bacteria and protozoa</td>
<td>Cell composition changes during microbial growth</td>
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1From: Dehority (1995)

2References: (1) Firkins et al., 1987; (2) Gawthorne and Nader, 1976; (3) Stern and Hoover, 1979; (4) Van Nevel and Demeyer, 1977.
chitin synthase) to estimate fungal biomass and protein content of fungal cultures, respectively.

Because of the variation within and between microorganisms the use of microbial markers has proven to be unsatisfactory. Nevertheless, Broderick and Merchen (1992) proposed use of the total purine method. This issue was critically evaluated by Stern et al. (1994), who suggested the use of purine or $^{15}$N as microbial markers to estimate the protein concentration in digesta. However, they did suggest that more research was needed to determine which one of these markers is the most reliable.

The nucleic acids in the ruminant stomach

Gaussères and Fauconeau (1965) developed a method to quantitate NA in foods, digestive contents and microorganisms using a ratio of adenine plus guanine, measured from deoxyribonucleic acid (DNA), to total nitrogen. However, their procedure was criticized because the methodology involves elaborate separations of the bases and other nucleic acid breakdown products (Smith, 1969). They compared the composition of rumen contents, rumen microbes and foodstuffs and concluded that most of the DNA in rumen content was of microbial origin. Similarly, it was observed that between 50 to 70% of the total and protein N of the duodenal contents were of microbial origin, as derived from lysine and DNA content of the feed (Temler-Kucharski and Gaussères, 1965).

Ellis and Pfander (1965) found that a considerable amount of the microbial nitrogen, ranging between 13.8 to 18.4% was from nucleic acid origin. Also highly
significant correlations were observed for total nucleic acids ($r=0.80$) and RNA ($r=0.72$) with total microbial nitrogen. These authors also showed that RNA and DNA increased in samples of rumen fluid incubated in vitro and together accounted for about $15\%$ of the microbial nitrogen formed.

Smith et al. (1968) working with calves fed different diets, disregarding the variation among calves and independent of the amount of N in the diet, reported a strong direct correlation between concentration of NA-N and total N in samples of rumen fluid. The proportion of NA-N to total N was found to range between 9.5 to 15.5%.

Plant material entering into the rumen contains significant amounts of NA and their concentration varies considerably with the type of plant (Cotta and Russell, 1997). However, McAllan and Smith (1968) reported that the amount of NA in samples of rumen fluid were always higher (at least five times higher) than could be explained by the levels of this compounds consumed in the diet. When pure RNA and DNA were added, either in the rumen or incubated in vitro with rumen fluid, they were very rapidly degraded (10% remained after 45 min of incubation). It was reasoned that dietary NA probably last in the rumen as long as they are protected within the cellular structure of the foodstuffs and those detected in the rumen fluid were mainly of microbial origin. This idea was supported by the fact that despite differences in RNA:DNA ratios in the diet, these variations were not evident in the samples of rumen fluid taken shortly after feeding (McAllan and Smith, 1968; Smith and McAllan, 1970a, Smith and McAllan, 1970b).
Leedle et al. (1982) monitored the diurnal variations in the cellular dry weight and composition of bacteria in ruminal contents and observed that the cellular constituents within the bacterial population show little diurnal variation. DNA levels varied between 1 to 2% of the dry weight, RNA levels were 10-fold higher than DNA levels, with the highest RNA values occurring in the period of expected maximum bacterial growth (2 h after feeding). In this study, protein and carbohydrate contents accounted for about 40 and 10%, respectively of the dry weight.

Smith and McAllan (1970a) presented evidence of a rapid degradation of pure NA added to the rumen; however, they stipulated that some dietary NA could contribute appreciably to the NA in rumen fluid particularly when the dietary source is protected by resistant cell structures or by processing. Some feedstuffs contain much lower levels of purines than ruminal microorganisms. but others, such as fish meal, have a total purine:N ratios that resemble those of the rumen microbes (Broderick and Merchen, 1992). Since significant amounts of dietary materials often escape degradation in the rumen, it is likely that some NA associated with these undegraded dietary fractions contribute to the duodenal digesta NA (Ling and Buttery, 1978).

McAllan and Smith (1973) working with cell-free rumen fluid observed that the initial degradation of the NA in the rumen occurs through the action of nucleases which, although of microbial origin, are extracellular. The absence of purine nucleotides, purine and pyrimidine bases and nucleosides, and the possible presence of small amounts of pyrimidine mononucleotides among the products formed by these extracellular enzymes, indicates that ribonucleases and deoxyribonucleases are endonucleases. In that same
study, the purine and pyrimidine bases were fairly resistant to degradation in vitro, while only trace amounts of the added NA, or their derivatives were observed in rumen fluid. Duodenal digesta samples were found to contain xanthine, hypoxanthine, uracil, pyrimidine, mono- and oligonucleotide material and thymine. The bases xanthine, hypoxanthine, uracil and thymine appeared to be relatively resistant to microbial degradation in the rumen. However, although guanine, adenine or cytosine were not detected in samples of rumen contents when pure NA were infused into the rumen, some dietary adenine and small amounts of cytosine have been reported to flow out the rumen (Schelling and Byers, 1984).

Cotta (1990) grew pure cultures of ruminal bacteria on purified media containing either RNA, DNA, ribose, deoxyribose, adenosine, guanosine, uridine or cytidine, and found that *Selenomonas ruminantium* HD4, GA192 and D were able to use RNA for growth but not DNA. *Prevotella ruminicola* D13d grew on nucleosides but not on the free bases or ribose. *S. ruminantium* strains were able to use either nucleosides or ribose for growth, but not the bases. *Selenomonas* strains, but not *Prevotella* were able to use nucleosides as N sources. The production of extracellular nucleases involved in the breakdown of DNA has been reported by Flint and Thomson (1990) for *P. ruminicola, F succinogenes, S. ruminantium,* and *L. multiparus.*

Protozoa can contribute significant amounts of protein to the total microbial protein produced in the rumen (Coleman, 1975). However, its contribution to the total microbial N in duodenal digesta has not been well defined (Williams and Coleman, 1988). The contribution of protozoal protein to the host is still controversial, primarily
because there is not an appropriate protozoal marker. Only indirect evidence is available since it is difficult to isolate and maintain pure cultures of rumen protozoa (Shelling et al., 1982). Ling and Buttery (1978), using RNA and DNA values, observed that there was a considerable variation between RNA-N:total N ratio for bacteria and particularly for protozoa. The differences were not related to the animal, diets or period of sampling. Marked differences were found in DNA-N:total N ratios (mg/g) for bacteria (30.9 ± 3.8) and protozoa (4.3 ± .09). Czerkawski (1976) working with very "clean" cultures of protozoa and bacteria from rumen fluid found the NA:protein ratio of the protozoa to be 96% that of the bacteria, which represented a very small difference between these two types of microorganism. RNA:total N values for mixed protozoa have been found to be relatively lower than those for mixed bacteria (Ling and Buttery, 1978).

Coleman (1968, 1980) found that *Entodinium caudatum* incorporate adenine, guanine and uracil into the cell. Several investigations (Coleman and Laurie, 1974; Coleman and Laurie, 1977; Coleman and Sandford, 1979) indicated that protozoa are active users of bacterial NA. Therefore, the ingestion of bacterial cells becomes an important source of these compounds. Limited information is available about the utilization of NA for other than entodiniomorphid protozoa, and nothing is known about the metabolism of NA by holotrich protozoa (Cotta and Russell, 1997).

Although consistently lower, the proportion of NA to total N that reach the duodenum has been observed to be paralleled with that found in rumen fluid. The differences have been attributed to additive endogenous N from the abomasal secretions, largely because there was not a significant degradation of NA in the abomasum (Smith et
al., 1968). Furthermore, McAllan and Smith (1973) found that the ultrafiltrable products formed from the degradation of NA in the rumen disappeared much faster than could be accounted by digesta flow alone. Koenig (cited by Schelling et al., 1982) indicated that except at 3 h postfeeding, when low amounts were detected, there was a negligible bypass of alfalfa NA bases with the digesta.

Although it is questionable (Smith, 1975; Stern and Hoover, 1979; Craig et al., 1987), it is generally accepted that either the chemical composition or microbial marker ratios are similar in the fluid and particle associated bacteria. Based on this assumption, most of the material collected as a representative sample of rumen microorganisms is generally obtained from the liquid fraction of rumen contents after several differential centrifugations. Therefore, most of the protozoa, large bacteria, clumps of bacteria and bacteria which remain firmly attached to feed particles may be not included in the standard (Weller et al., 1958). Since a significant proportion of the bacteria in the rumen are closely associated with the solid fraction (Cheng et al., 1977; Craig et al., 1987; Dehority and Grub, 1980; Cecava et al., 1990b), isolation of the microbial cells from ruminal or duodenal digesta is one of the most difficult aspects of estimating microbial proteins and this problem occurs for all of the microbial marker methods (Stern and Hoover, 1979).

In pure cultures, DNA tends to reflect the number of organisms present, independently of their stage of growth, whereas the amount of RNA is more associated with protein synthesis (Price, cited by Smith, 1969). DNA:total N ratio varies markedly between different organisms and particularly between bacteria and protozoa (Smith.
1969). Changes in the microbial composition and stage of growth were originally considered to be responsible for changes in DNA:total N ratios; however, Bates et al. (1985) working with pure cultures of ruminal bacteria *in vitro* and with mixed ruminal bacteria *in vivo*, and using NA:protein ratios, found that DNA:protein ratios were largely independent of growth rate *in vitro*. RNA:protein ratios were affected by bacterial growth rate *in vitro*, and affected by the diet and time after feeding *in vivo*. DNA:protein ratio was suggested as a better marker for microbial biomass in digesta passage studies than RNA:protein (Wolstrup and Jensen, 1978; Bates et al. 1985). Arambel et al (1982) observed that DNA:total N ratios (mg/g) in bacteria decreased from 27.2 to 20.09 for cattle fed low and high concentrate diets, respectively. In the same investigation, using pure cultures of rumen bacteria, it was found that DNA-N:total N (mg/g) ratio differed between gram-positive (8.8) and gram negative (18.9) bacteria. However, RNA-N:total N ratios were similar between these two group of bacteria.

Since RNA appears to be a more constant proportion of total microbial N, it was considered consequently to be a more reliable marker than DNA or total nucleic acid to estimate microbial protein. When the mean value for RNA: total N ratio in ruminal microorganisms was compared with mean values for this ratio in samples of digesta from calves, it was estimated that about 70 and 60 % of the non-ammonia nitrogen (NAN) was of microbial origin in rumen contents and duodenal digesta, respectively (Smith, 1969). McAllan and Smith (1984) determined the microbial-N flow at the abomasum or duodenum using DAPA or RNA as markers. Results with DAPA were more variable and significantly lower, by about 25%, than those based on RNA; however, they gave
similar relative patterns when different dietary treatments were compared. By using RNA:protein ratios, Bates et al. (1985) found differences in the composition of both free and particle associated bacteria. The average RNA:protein ratio for free bacteria was much higher (0.33) than for the attached bacteria (0.20). Merry and McAllan (1983) found that both RNA-N:total N and DAP-N:total N values were significantly lower in particle associated than in liquid associated bacteria.

From nucleic acids to purines

Early attempts to estimate nucleic acids from ruminant digesta, bacteria and protozoa preparations, plant leaves and other feedstuffs were unsuccessfully made by McDonald (1954). He used several of the available procedures for measuring nucleic acids and developed a procedure which allowed determination of the purine bases adenine and guanine, using acid hydrolysis followed by precipitation of the purines as the silver salts, separation of the bases by paper chromatography and final estimation by spectrophotometry. This procedure had a very low coefficient of variation (2-6%).

Although several different procedures were used to quantitate NA in animal tissues, rarely were they used to estimate NA in rumen digesta. Thus, Ellis and Pfander (1965) developed a procedure to estimate NA by using the phosphorus content of DNA and RNA assuming a ratio phosphorus to N of 8:15.3. On the other hand, using a simple method for precipitation and extraction, Topps and Elliot (1965) measured the concentration of NA in strained rumen fluid; however, the procedure presented some
difficulties largely associated with the presence of interfering materials (McAllan and Smith, 1969).

As mentioned earlier, Gaussères and Fauconneau (1965) developed a procedure to separate RNA and DNA in a sample. The sample was subjected to three extraction processes which involved ethanol, trichloroacetic acid, and a mix of methanol-chloroform. From these residues, RNA was determined by the fractional separation of its mononucleotides after hydrolysis with sodium hydroxide, and DNA by chromatography of its purine bases. This procedure was criticized for being time consuming and unsatisfactory for routine use (McAllan and Smith, 1969).

After an extensive investigation, McAllan and Smith (1969) developed a procedure to measure DNA and RNA in samples of digesta. Mainly, the method involved several steps for extraction and hydrolysis with solutions of acids and hydroxides, followed by specific colorimetric assays for the pentose components of nucleic acids (Figure 1) (i.e., diphenylalanine and orcinol reactions for DNA and RNA, respectively). Not only were these methods long and laborious, but they also lacked both accuracy and precision (Zinn and Owens, 1982; Ushida et al., 1985; Zinn and Owens, 1986).

Koenig et al. (1979) using high pressure liquid chromatography (HPLC) measured bacterial purine and pyrimidine concentrations in mixed rumen bacterial cultures in vitro as biomass indicator. Individual bases were correlated to microbial protein during the growth and death phases and the respective coefficients of correlations
Figure 1 Schematic outline of the standard procedure used for the extraction and estimation of nucleic acids in digesta samples (from McAllan and Smith, 1969)
were: uracil and xanthine, 0.9959, 0.9829; thymine 0.9837, 0.9218; hypoxanthine, 0.9878, 0.6562; guanine, 0.9964, 0.9744; cytosine, 0.9987, 0.9255; and adenine 0.9986, 0.9753. The authors concluded that these high correlation coefficients were a good indication of the potential use of certain purine and pyrimidine bases as microbial markers. Jackson (cited in Zinn and Owens, 1982 and Zinn and Owens, 1986), verified the oxidative procedure of Marshak and Vogel (1951), which allow the measurement of purine and pyrimidine bases without prior isolation of the NA and without the presence of the interfering compounds. Not only were the bases quantitatively recovered and remained stable under the hydrolytic conditions, but also the methodology was found to be relatively rapid to other procedures. The only drawback in Jackson’s experiment was the expense of using HPLC (Zinn and Owens, 1982; Zinn and Owens, 1986).

Zinn and Owens (1982) finally proposed an analytical procedure to quantify purine bases in feed and digesta. Their procedure was a combination of hydrolysis of the sample by perchloric acid to liberate the free bases (Marshak and Vogel, 1951) and separation of the free bases by precipitation from the interfering compounds with silver nitrate in acid solution (Kerr and Seraidarian, 1945) (Figure 2). The purines were then solubilized and quantitated by spectrophotometry. Ushida et al. (1985) later modified the purine procedure by including a filtration step after the sample hydrolysis, and then used the procedure to measure microbial nitrogen flow into the ruminant duodenum. The authors not only evaluated the accuracy of the method by comparing the results with those determined by HPLC, but also by comparing it with other marker methods (DAPA and $^{35}$S incorporation). The results of the spectrophotometric method were comparable
Figure 2. Schematic of operating principle in silver nitrate precipitation procedure (from Zinn and Owens, 1982)
with those measured by HPLC and recoveries range between 90-101%. When using
defaunated sheep, no differences were found in the flow of microbial nitrogen to the
duodenum from those values obtained by using the other markers. Otherwise,
Berchielli et al. (1995b) who measured the flow of microbial nitrogen to the duodenum,
found that DAPA gave the higher (73.2%) values as compared with $^{35}$S (55.6%) or
purines (51.1%). As well, Berchielli et al. (1995a) measured the efficiency of microbial
synthesis in steers using DAPA, purines and $^{35}$S as microbial markers. The values
estimated from purines and $^{35}$S were not significantly different, but these two methods
differed from DAPA. Likewise, Illg and Stern (1994) found that total purines as a
microbial marker was a better estimator of microbial protein, less complicated and was
less expensive than the DAPA procedure.

Later, Zinn and Owens (1986) published more details about their procedure and
pointed out that most of the materials that interfered with NA estimations were removed.
An average of purine recovery of 98.6% was obtained when yeast-RNA was hydrolyzed
alone or together with casein, corn starch or solka floc. The authors pointed out that
since purines are present in both RNA and DNA, the purine:N ratio should be more
constant than the RNA:N ratio.

Although of lesser magnitude, the purine analysis in duodenal samples suffers
from some of the drawbacks which affect the NA estimations. For instance, additional
purine from dietary and desquamated epithelial cells origin could increase the possibility
of overestimation of microbial purine. Some free purine bases had been observed in
abomasal samples (Ha and Kennelly, 1984); however, it appears that purines are rapidly
metabolized by rumen microorganisms in the rumen (Schelling and Byers, 1984). Calsamiglia et al. (1996) found that dietary purines were almost completely degraded by ruminal microbes \textit{in vitro}. Microbial degradation of feed purine N ranged from 81 to 110% of the purine N intake. From this study, it was concluded that dietary purine contamination was a minor factor affecting calculations of bacterial N flow to the duodenum.

Freezing of ruminal fluid before isolation of bacteria can result in losses of RNA and alter estimates of bacterial N flow to the duodenum (McAllan and Smith, 1983; Ha and Kennelly, 1984; Cecava et al., 1990\textit{b}). However, purine contents or N:purine ratio of mixed bacteria were not affected by freezing and thawing before separation of bacteria, or by the presence of some naturally occurring enzymes (Ha and Kennelly, 1984; Cecava et al., 1990\textit{b}). Some aspects related to the structure of the membrane of the gram-negative bacteria rather than the gram-positive were thought to explain the losses of NA during freezing storage of the samples (Hsu and Fahey, 1990). McAllan and Smith (1983) postulated that the physical effect of freezing and thawing can not only disrupt the bacterial cells, but also release some bacterial nucleases. The latter aspect was supported by the fact that no significant losses of RNA were observed in frozen abomasal contents where pH was approximately 2.5. The low pH would inactivate bacterial nucleases that have optima activity at pH values similar to those found in the rumen.

In several investigations (Merry and McAllan, 1983; Firkins et al., 1987; Cecava et al., 1990\textit{b}; Whitehouse et al., 1994), composition of the fluid-associated bacteria (FAB), particle-associated bacteria (PAB) or mixed populations have been found to be
different. Cecava et al. (1990b) using the purine:N ratio observed that FAB ratios gave lower arithmetic estimation of N flow to the duodenum as compared with the estimated flows using PAB or mixed bacterial fractions. Firkins et al. (1987) reported that although organic matter and N tended to be higher for PAB than FAB, purine:N ratios were similar between PAB and FAB. Moreover, lower values for this ratio were observed for protozoa (55.4% of that FAB) compared to bacteria. In the same study, the contribution of protozoa N to duodenal NAN was estimated to be appreciable (27%).

The most probable number procedure

A fundamental criterion in most ruminant studies is to obtain a reliable quantitation of the extensive microbial populations found in the rumen. In general, this appears to be a simple task; however, in practice this objective is not so readily accomplished. One of the main problems arises from the fact that an assortment of microorganisms are involved, both viable and non-viable within a diversity of niches in the rumen. The first prerequisite in enumeration of microbial populations in the rumen is how to obtain a representative sample. The concentrations determined, regardless of the procedure, are no better than the representative validity of the sample.

Although methods of direct counting are relatively easy to carry out, not only is it cumbersome to distinguish between viable and non-viable cells, but also it is difficult to count the clumped bacteria or bacteria attached to small feed particles (Hungate, 1966).

Living cells can be estimated by growing them in liquid, solid media or membranes filters. Underestimation of all the microbial populations present is possible
when the nutritional requirements of a given organism are incomplete. Even though, there are some problems affecting the enumeration of viable organisms, these types of counting methods are still the preferred choice (Herbert, 1990).

Dilution to extinction is the basis of a method called the most probable number (MPN) method in which the sample is diluted to the extent that no bacteria remain. Using statistical probabilities, it is assumed that there is a 95% chance that the bacterial population follows an ideal dilution curve, and concentration can be estimated by growth in specific dilutions (Tortora et al. 1989). The extent of dilution required and the number of positive tubes at different dilutions are then used to determine the MPN of organisms by reference to probability tables. The MPN method suffers from one major drawback, i.e., a very large sampling error (Herbert, 1990). Although precision is rather low when small numbers of tubes are inoculated from each dilution (Cochran, 1950), increasing the number of tubes per dilution can markedly improve the estimate (Parnov, 1972).

MPN procedures have been used for many years to enumerate coliforms and other microorganisms in water and food products (Chipley, 1987). Macy et al. (1982) used the MPN method (liquid medium) to count cellulolytic bacteria from gastrointestinal tract of rats and observed an improvement in precision as compared with the roll-tube method (agar medium). Dehority et al. (1989) was able to count both total and cellulolytic bacteria in ruminal content using a single medium MPN procedure. No differences were observed between the MPN and the classical roll-tube procedures. Although both procedures require the same amount of time for sample preparation, medium preparation and inoculation of the tubes, counting requires less time with the
MPN technique (Dehority et al., 1989; Shockey and Dehority, 1989; Obispo and Dehority, 1992). Further, the use of computers programs enables the investigators to calculate the MPN and confidence limits for any combination of dilution levels, sample volumes and number of replicates (Klee, 1993).

**General conclusions**

Based on the large contribution that bacterial protein can make to the nutrition of the host animal, it is desirable to have a method for measuring this criteria. However, the accuracy of estimating microbial protein passage through the gastrointestinal tract is dependent on an appropriate marker. At present, several microbial markers have been used to assess bacterial production in the rumen, but basic defects have ruled out their acceptability. Although all the criteria of an ideal microbial marker are not met, the procedure for measuring total purines proposed by Zinn and Owens (1982) and later modified by Ushida et al. (1985), appears promising. Early experiments carried out in our laboratory here indicated that this procedure is simple, sensitive, precise and relatively reasonable in cost. It is expected that changes in bacterial biomass should be associated with changes in microbial counts; however, information in this area is extremely limited. Also, no information is available about total purine content in pure cultures of rumen bacteria. This study was undertaken to: (1) determine total purine concentrations in pure cultures of rumen bacteria; (2) to establish the relationship between bacterial numbers and biomass; and (3) to compare purine and protein concentrations between pure cultures and mixed bacterial samples isolated from the
rumen which are normally used as the standard for estimating microbial protein in the duodenum.
MATERIALS AND METHODS

Organisms

Ten strains of rumen bacteria were used in the present study: three strains of *Butyrivibrio fibrosolvens* (D16f, H10b and H17c), *Fibrobacter succinogens* B21a, *Lachnospira multiparus* D25e, *Lactobacillus lactis* ARD26e, *Prevotella ruminicola* H15a, *Ruminococcus albus* 7, *Ruminococcus flavefaciens* B34b, and *Streptococcus bovis* ARD5d. Characteristics of these organisms are as follows:

*Butyrivibrio fibrosolvens* D16f

This organism was isolated by Dehority (1969) from a $10^6$ dilution of rumen contents obtained from a steer maintained on alfalfa hay, using a selective pectin medium. It is a gram negative motile curved rod varying in size from 0.5 to 0.6 µm by 1.0 to 2.0 µm.

*Butyrivibrio fibrosolvens* H10b

*B. fibrosolvens* H10b was isolated from a $10^8$ dilution of rumen ingesta from a steer maintained on mixed hay, using a selective xylan medium (Dehority, 1966). H10b is a motile curved rod, gram-negative, varying in size from 0.4 to 0.6 µm by 0.7 to 2.0 µm.
Butyrivibrio fibrosolvens H17c

*B. fibrosolvens* H17c was isolated from a 10^{-7} dilution of rumen ingesta from a steer maintained on mixed hay, using a selective xylan medium (Dehority, 1966). H17c is a slightly curved rod, gram-negative, varying in size from 0.4 to 0.6 μm by 0.7 to 2.0 μm. Motile, tending to occur in pairs joined end to end.

Fibrobacter succinogens B21a

*Fibrobacter succinogens* B21a was isolated from a 10^{8} dilution of a 24 hour *in vitro* fermentation mixture, inoculated with rumen contents from a steer fed mixed hay (Dehority, 1963). This strain is a gram-negative, nonmotile rod, appearing regularly as singles, varying in size from 0.5 to 1.5 μm in length and 0.5 μm in width and showing bipolar staining.

Lachnospira multiparus D25e

*L. multiparus* D25e was isolated from a 10^{8} dilution of rumen ingesta obtained from a cannulated steer maintained on alfalfa hay, using a selective pectin medium (Dehority, 1969). It is a weakly gram-positive, motile rod 0.4 to 0.5 μm in width and varying from 1.2 to 2.5 μm in length. Although single cells can be observed, it generally occurs as long chains of cells joined end to end. In roll tubes, the organism shows colonies with a wooly appearance.
*Lactobacillus lactis* ARD26e

*Lactobacillus lactis* ARD26e was isolated from a 10⁶ dilution of rumen contents obtained by stomach tube from an Alaskan reindeer (*Rangifer tarandus* L.), fed dried lichens (Dehority, 1975). ARD26e is a gram-positive to variable nonmotile straight rod, with dimensions varying from 0.6 to 0.75 μm wide and 1.0 to 8.0 μm long. The organism tends to form filaments.

*Prevotella ruminicola* H15a

*Prevotella ruminicola* H15a was isolated from a 10⁷ dilution of rumen ingesta from a steer maintained on mixed hay using a selective xylan medium (Dehority, 1966). The organism is gram-negative, coccoid to oval in shape and ranges in size from 0.5 to 0.6 μm. Usually occurs as singles or pairs. Occasionally short chains are present.

*Ruminococcus albus* 7

*Ruminococcus albus* 7 was isolated from a 10⁸ dilution of rumen contents from a cow fed an alfalfa hay-grain diet (Bryant et al., 1958). It is a nonmotile gram-positive coccus, occurring singly with diameter ranging from 0.7 to 1.2 μm.

*Ruminococcus flavefaciens* B34b

*Ruminococcus flavefaciens* B34b was isolated from a 10⁴ dilution of a 24 hour *in vitro* fermentation mixture, inoculated with rumen contents from a steer fed mixed hay (Dehority, 1963). *R. flavefaciens* B34b is gram-variable, coccoid in shape, nonmotile.
averaging 0.7 µm in diameter and occurring in very long chains. This strain produce a yellow pigment.

*Streptococcus bovis* ARD5d

*S. bovis* ARD5d was isolated from a 10⁶ dilution of rumen contents obtained by stomach tube from an Alaskan reindeer (*Rangifer tarandus* L.), fed dried lichens (Dehority, 1975). ARD5d is gram-positive, coccoid to oval in shape, nonmotile, facultatively anaerobic, with dimensions of 0.5 to 0.8 µm in length and 0.5 µm in width.

**Experimental protocol**

The stock cultures used for this study were grown in rumen fluid-glucose-cellobiose agar (RGCA) slants (Bryant and Burkey, 1953) and stored at -72 °C. Once revived, they were maintained by transferring onto fresh RGCA slants daily, or stored at 4 °C to slow their growth activities when not used for short periods of time. The anaerobic culture techniques used throughout the experiments were similar to those described by Hungate (1950), and modified by Dehority (1969). Essentially all procedures were carried out under a stream of O₂ free CO₂ and culture tubes were closed with rubber stoppers.

For purine analysis, pure cultures of rumen bacteria were grown in a 500-ml round-bottom flask containing 300 ml of the experimental medium. The medium (Appendix A) was designed to meet all the nutritional requirements of each strain and was devoid of purines. Inoculum for the large flask consisted of two 16 x 150 mm test
tubes containing 5.0 ml of the same media as in the flask. The tubes were inoculated with a loop of cells taken from a fresh slant and grown for 15-20 h. Before and after inoculation, 5 ml aliquots of the culture media were removed from the large flask and placed in sterilized 16 x 150 mm culture tubes to monitor the bacterial growth. Changes in optical density (OD) were measured at 600 nm in an Spectronic® 20 spectrophotometer. Cells were harvested when OD in the small tube reached 1.0 ± 0.1. For some strains, OD plateaued at a lower value and cells were harvested when no further increase in OD was observed in subsequent ½ hour readings.

Three separate three-tube MPN assays were carried out to quantify the viable number of bacteria in the flask (Dehority et al., 1989). For each MPN assay, one ml sample of the fermentation medium was serially diluted to $10^{-11}$ with anaerobic dilution solution (ADS) (Appendix B). Since some organisms were observed to grow in clumps, the ADS tubes were mixed (vortex) extensively between dilutions.

During the dilution and inoculation steps of the MPN assays, the remaining medium in the flask was placed into iced water to stop bacterial growth. After cooling for approximately 30 min, four 25-ml samples were taken for total purine and total protein analysis. Each sample was placed in a 50-ml Teflon² centrifuge tube. Samples were centrifuged at 12,000 x g for 30 min and the supernatant carefully removed by aspiration. The remaining pellet was washed twice with 0.9% (w/v) saline solution and

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¹ Spectronic® 20 spectrophotometer: Bausch & Lomb. 820 Linden Avenue. Rochester, NY. 14625

² Teflon tube (fluorinate ethylene propylene): Nalge Company, a subsidiary of Sybron Corp. Box 20365 Rochester, NY 14602-0365.
dried to constant weight in a convection oven at 80 °C. The four tubes assigned for protein analysis were pre-weighed, in order to determine dry weight of the bacteria sample.

For mixed cultures from the rumen, whole rumen contents were collected, either from animals at the slaughter house or through a ruminal cannula. Samples were strained through four layers of cheesecloth to obtain ca. 600 ml of rumen fluid, which was centrifuged at 200 x g for 10 min to separate protozoa and particulate material. The supernatant was strained again through four layers of cheesecloth, collected and kept in a 1000 ml round-bottom flask and gassed with a oxygen free CO₂ for at least 15 min. From this point, the sample was treated the same as the pure cultures, except that the bacterial pellet was washed three times with saline solution until the supernatant was completely clear and transparent. The MPN medium for mixed cultures was similar to that for the pure cultures except that four carbohydrates (glucose, cellobiose, maltose and xylose) were used (Appendix C).

Chemical analysis

Determination of total purines

Purines were determined following the procedure described by Zinn and Owens (1986), with modifications to account for the limited quantity of sample available. The general procedure is described below; however, several steps proved to be unreliable and subsequent modifications are described under results. One-half (0.5) ml of 70% HClO₄ was added to the sample in a 50-ml screw cap Teflon tube and incubated in a 95 °C water
bath for 1 h. Tubes were mixed every 15 min to break up the charred sample. The 
hydrolyzed samples were diluted with 3.5 ml of 0.0285M NH₄H₂PO₄ solution and placed 
in the water bath for 15 more min. The tubes were centrifuged at 25,000 x g for 15 min 
to sediment the charred material and the supernatant filtered through a Whatman GF/D 
fiberglass filter (Ushida et al. 1985). One half (0.5) ml from the hydrolyzed sample was 
transferred into a 15 ml PPCO³ centrifuge tube and 0.5 ml of 0.4M AgNO₃ solution plus 
9 ml of 0.2M NH₄H₂PO₄ buffer solution were added and the mixture allowed to stand 
overnight at 5 °C. The resulting precipitate was collected by centrifuging the tubes at 
25,000 x g for 30 min and carefully removing the supernatant by aspiration. The pellet 
was washed with distilled water which had been adjusted to pH 2 with H₂SO₄ and 
recentrifuged as before. The silver precipitate was dissolved by adding 10 ml of 0.5N 
HCl and incubating 30 min in a water bath at 95 °C. After centrifugation at 25,000 x g 
for 25 min, the supernatant was filtered through a Whatman No. 541 paper filter and read 
at 260 nm in a Genesis™5 spectrophotometer⁴. A 1:1 mixture of the pure bases adenine⁵ 
and guanine⁶ was used as standard (explained in the results). From a stock solution 
containing 1000 µg/ml of pure purine bases, 8 ml were placed into a Teflon centrifuge 
tube and after dried in the convection oven. From that point on the standard was treated 

³PPCO (polypropylene copolymer) centrifuge tube: Nalge Company, a subsidiary of 
Sybron Corp. Box 20365 Rochester, NY 14602-0365.

⁴Spectrophotometer Spectronic® “Genesis™5: Milton Roy Company. 820 Liden Avenue, 
Rochester, NY. 1425.

⁵Adenine (6-aminopurine): Sigma Chemical Co. P.O. Box 14508 St. Louis, MO 63178.

⁶Guanine (2-amino-6 hydroxypurine): Sigma Chemical Co. P.O. Box 14508 St. Louis, 
MO 63178.
the same as the sample. Using the final filtrated from this tube, the standards (µg/ml) 0.2.5, 7.5, 15, 25 and 35, were prepared by diluting the corresponding aliquots in 0.5N HCl. A separate tube containing 8 ml of 0.5N HCl was treated similar to the purine mixture and used as a zero for the standard. The 0.5N HCl solution was used as a blank.

**Protein analysis**

Bacterial protein was determined by the Folin reaction (Lowry et al., 1951). In this colorimetric assay, copper sulfate in strong alkali reacts with protein to form a blue colored complex. The complex is formed between Cu²⁺ and four nitrogen atoms from adjacent peptide chains. The blue color can be observed as it gradually develops (the biuret reaction). By using this reaction it is possible to detect proteins in the range of 200 to 2000 µg. However, a much stronger color reaction is observed when the Folin reagent (phosphomolybdate-phosphotungstate) is subsequently added. The Folin reagent is reduced by tyrosine and tryptophan residues. This procedure is called the Lowry assay, which takes the advantage of both color reactions and detects protein levels greater than 20 µg. Bovine serum albumin (BSA) is generally used as the standard protein because it has an average proportion of the amino acids tyrosine and tryptophan.

Dried bacterial samples in Teflon tubes were treated as described by Hanson and Phillips (1981), i.e., complete solubilization of the bacterial protein was accomplished by adding 1N NaOH to the tubes and incubating for 30 min in a 90 °C water bath. The solubilized proteins were diluted, assuming that between 50 to 60% of the dry matter was protein, into the concentration range of the standard curve. The standard was treated
similarly. From a stock solution containing 10 mg/ml of bovine serum albumin (BSA\textsuperscript{7}). 1 ml was transferred into the screw-cap Teflon tube and treated with 1 ml of 2N NaOH. After cooling, 18 ml of distilled water were added to the standard to obtain a concentration of BSA in solution of 500 µg/ml. Reagents and standards are described in Appendix D. One ml of the diluted sample was transferred into a clean 16 x 150 mm test tube. Five ml of reagent C were added, mixed well and allowed to stand for at least 10 min at room temperature. One half (0.5) ml of reagent D was added and the contents were immediately mixed. After standing 30 min at room temperature, the absorbance was measured at 750 nm in an Spectronic® "Genesis5" spectrophotometer. A similar procedure was used for the standards.

Statistical analyses

Data for purine and protein measurements were analyzed by ANOVA using the general linear model of SAS (1991) in a complete randomized design, or the paired t-test (Steel and Torrie, 1980). Means were compared with Duncan’s multiple range test when the F-value was statistically significant (Steel and Torrie, 1980). Significance was set at the P < 0.05 level.

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\textsuperscript{7} BSA: Sigma Chemical Co. P.O. Box 14508 St. Louis, MO 63178.
RESULTS AND DISCUSSION

Adaptation of the purine analysis

Using the procedure of Zinn and Owens (1986), preliminary attempts to measure total purine content in very small quantities of yeast RNA or bacteria were disappointing, in that considerable variation occurred between replicate samples. Consequently, several experiments were carried out to find out which steps in the procedure were responsible for this variation. Since our sample sizes were to be small, adjustments were made in the volumes of added reagents; however the ratios of one to another remained the same.

There was some question about the reliability of a standard for the analysis, and OD at 260 nm was used as the criteria to estimate the recovery of purines in the initial phases of these validation studies.

In a first experiment, the effect of acid hydrolysis on recovery of the purine bases Ad and Gu, alone or in a 1:1 mixture was investigated. From stock solutions containing 100 µg/ml in 0.5N HCL of the pure bases or the mixture, two aliquots of 0.2, 0.4, 0.6 and 0.8 ml were added to individual screw cap test tubes, which were then dried in a convection oven at 80 °C. To one of the dried samples, 1 ml of 70% perchloric acid and 7ml 0.0285M NH₄H₂PO₄ were added. The other sample was treated similarly, except that it was incubated 1h in a 95 °C water bath prior to the addition of the buffer solution. Optical density of the resulting solutions was read at 260 nm and recovery was based on
a standard of the purines bases in 0.5N HCL. The results shown in Table 3, indicated that the concentration of the purine bases was not affected by the addition of perchloric acid, either at room temperature or after heating at 95 °C for 1 hour. Mean recovery in perchloric acid was 97.6 %, with values ranging from 95.8 to 102.8%. Values ranged from 97.8 to 118.0 % in the perchloric acid plus heat treatment with a mean recovery of 102.7%.

Since perchloric acid is a strong mineral acid, it was thought that its concentration might have an effect on recovery of the pure bases after hydrolysis. However, no changes in OD were observed when 50 μg of the pure bases were incubated 1h at 95 °C in a water bath with 5 ml of either 6N or 11.7N (70%) solutions of perchloric acid. The resulting OD values (mean ± S.E) were: adenine . 0.774 ± .02 and 0.781±.01; guanine, 0.629±.005 and 0.626±.006 for the 6N and 11.7N solutions, respectively. Thus, reducing the acid concentration had no effect on OD of the two purines.

After hydrolysis, the next steps are precipitation with silver nitrate, washing of the precipitate with water (pH 2) and finally solubilization of the purine bases from the silver complex with 0.5N HCL solution. Experiments were designed to individually evaluate these steps on the recovery of the purine bases.

According to Marshak and Vogel (1951), yeast RNA contains Ad to Gu in a ratio of 1:1.3; therefore, a mixture with this proportion of purines was included in the recovery experiments. From stock solutions containing 100 μg/ml of the purine bases, 2.0 and 8.0 ml aliquots were placed into screw cap test tubes and dried in a convection oven at 80 °C.
Table 3. Effect of perchloric acid on the recoveries (%) of the purine bases adenine (Ad), guanine (Gu) or a Ad:Gu (1:1) mixture.

<table>
<thead>
<tr>
<th>Purine</th>
<th>Concentration (µg/ml)</th>
<th>Treatment</th>
<th>Recovery</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Perchloric acid</td>
<td>Perchloric acid + heat</td>
<td></td>
</tr>
<tr>
<td>Adenine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>97.6</td>
<td>105.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>98.4</td>
<td>100.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>97.5</td>
<td>99.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>95.4</td>
<td>97.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>97.2</td>
<td>100.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guanine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>100.0</td>
<td>118.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>97.0</td>
<td>104.0</td>
<td></td>
<td></td>
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<tr>
<td>7.5</td>
<td>96.0</td>
<td>104.0</td>
<td></td>
<td></td>
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<tr>
<td>10.0</td>
<td>97.7</td>
<td>103.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>97.7</td>
<td>107.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad:Gu (1:1 mixture)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>102.8</td>
<td>105.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>95.8</td>
<td>98.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>96.4</td>
<td>98.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>96.0</td>
<td>97.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>97.8</td>
<td>99.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall mean</td>
<td>97.6</td>
<td>102.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The dried purines plus 0.5 ml of 70% perchloric acid were incubated in a 95 °C water bath for 1 h. after which 3.5 ml of 0.0285M NH₄H₂PO₄ was added. One half (0.5) ml of the buffered hydrolysate was diluted with 9.5 ml of distilled water and OD read at 260 nm (Treatment 1 or Control). A second 0.5 ml of the hydrolysate was treated according to the procedure described by Zinn and Owens (1986) (Treatment 2), i.e. 0.5 ml of 0.4M AgNO₃ and 9.0 ml of 0.2M NH₄H₂PO₄ solutions were added and the mixture was allowed to stand overnight to precipitate the purine bases. After centrifugation at 25,000 x g for 25 min. the supernatant was discarded and the sedimented pellet washed with water (pH 2) and recentrifuged as above. Once again the supernatant was discarded and the pellet was dissolved in a hot water bath (95 °C) with 10 ml of a 0.5N HCl solution and OD read at 260 nm. Comparisons between treatments were made based on differences in OD, assuming that OD and concentration are proportional. Considerable loss of purines appeared to occur in the precipitation and washing steps after the hydrolysis of the purines (Table 4). These losses were much higher for Ad than for Gu. This difference could also be seen in the Ad:Gu mixtures. When Gu was increased from 1 to 1.3 in the mixture, a concomitant increase in recovery was observed. Obviously, losses occurred with both purines in the precipitation, washing and redissolving steps: however, losses were much greater with adenine.

To determine at what point the loss of purines occurred, i.e., in the precipitation or the washing steps, an experiment was set up to read OD of the corresponding supernatants, wash solutions and precipitated purines (redissolved in 0.5 N HCl) at 260 nm. From the stock solution containing 100 µg/ml of either Ad or Gu, 4-ml aliquots
<table>
<thead>
<tr>
<th>Purine</th>
<th>Purine concentration (µg/ml)</th>
<th>Treatment¹</th>
<th>Change in OD with precipitation steps</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenine</td>
<td>2.5</td>
<td></td>
<td>0.205</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>0.882</td>
</tr>
<tr>
<td>Guanine</td>
<td>2.5</td>
<td></td>
<td>0.140</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>0.588</td>
</tr>
<tr>
<td>Ad:Gu(1:1)</td>
<td>2.5</td>
<td></td>
<td>0.169</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>0.733</td>
</tr>
<tr>
<td>Ad:Gu(1:1.3)</td>
<td>2.5</td>
<td></td>
<td>0.175</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>0.695</td>
</tr>
</tbody>
</table>

¹Treatment: 1 = hydrolysis only; 2 = Complete procedure
were placed in twelve screw cap test tubes and dried in a convection oven at 80 °C. An extra tube, containing 4 ml of 0.5N HCL solution, was also dried and used as a blank. After drying, the samples and the blank were treated according to the procedure described in the last experiment for treatment 2, i.e., the complete procedure of Zinn and Owens (1986), except that the supernatants at each step during precipitation and washing were saved and OD read at 260 nm. The stock solution, diluted to a final concentration of 5.0 μg/ml was used as standard. Based upon OD readings (Figure 3), it was found that after precipitation only a negligible amount of both adenine and guanine remained in the supernatant solution. Guanine was almost completely recovered in the precipitate, whereas less than 50% of the adenine was present. However, over 50% was present in the wash of the silver salt of adenine. For both Ad and Gu, adding the purine and wash optical densities exceeded the standard, which can possibly be explained by the OD observed in the guanine wash solution. Since recovery in the precipitate was approximately 100%, this OD in the wash solution could be an artifact caused either by some impurities or as a consequence of reading OD in different solvents. Using the concentration of the standard, the final recoveries for adenine and guanine in the precipitate were: 48 and 99.25%, respectively. Clearly, washing the silver precipitate of adenine accounted for almost all the loss of the purine. In contrast, the silver salt of guanine was not affected by washing.

Kerr and Seraidarian (1945) observed that the acidity of the precipitating solution was an important factor in the recovery of purines from a mixture, i.e., they used this property to separate nucleosides from free purines. Based on this and without an
Figure 3. Effect of washing the pellet upon the recovery of the purine bases adenine and guanine
apparent evaluation. Zinn and Owens (1986) used acidified water (pH 2) in order to clean the pellet from impurities. Since washing was found to be the major factor in the loss of adenine, an experiment was carried out to investigate if this loss was pH related. For this experiment, 4-ml aliquots of stock solutions containing 100 μg/ml adenine or guanine were placed in sixteen screw cap tubes and dried in a convection oven at 80 °C. Four washing treatments were used: water at pH 2, pH 3 and pH 4 and without washing (control). Four extra tubes containing 4.0 ml of a 0.5N HCl were included as blanks, one for each treatment. The tubes were incubated 1 h in a 95 °C water bath with 0.5 ml of 70% perchloric acid and then 3.5 ml of 0.0285M NH₄H₂PO₄ were added and the tube reincubated for 15 more minutes in the water bath. After cooling, 0.5 ml of the hydrolysate was placed in a 15-ml polypropylene copolymer (PPCO) centrifuge tube. 0.5 ml of 0.4M AgNO₃ and 9.0 ml of 0.2M NH₄H₂PO₄ were added and the tubes were allowed to stand overnight in the refrigerator. After centrifugation at 25,000 x g, the supernatant was decanted into clean test tube and OD read at 260 nm. Next, two tubes each were washed with water at pH 2, 3 and 4 or were not washed. The tubes were recentrifuged as described above, the wash supernatant decanted and OD read at 260 nm. The purine precipitates were redissolved by heating 30 min in a 95 °C water bath with 0.5N HCl and OD was read at 260 nm. From the stock solutions of adenine and guanine, a standard containing 5.0 μg/ml was prepared and used as standard for relative comparisons. The OD readings for the supernatant and the washing solutions are summarized in the Table 5. Solubilization of the silver salt of adenine decreased as the pH of the washing solution increased, while pH appeared to be without effect on the
Table 5. Optical density readings of supernatant and washing solutions for the purine bases adenine and guanine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Solution</th>
<th>Purine</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Adenine</td>
<td>Guanine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>pH 2</td>
<td>1</td>
<td>0.020</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>pH 2</td>
<td>2</td>
<td>0.218</td>
<td>0.032</td>
<td></td>
</tr>
<tr>
<td>pH 3</td>
<td>1</td>
<td>0.020</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td>pH 3</td>
<td>2</td>
<td>0.153</td>
<td>0.026</td>
<td></td>
</tr>
<tr>
<td>pH 4</td>
<td>1</td>
<td>0.018</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>pH 4</td>
<td>2</td>
<td>0.072</td>
<td>0.032</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>0.022</td>
<td>0.018</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

1 Treatment: pH2, pH3 and pH 4 = distilled water acidified with sulfuric acid. Control = without washing.
2 Solution: 1= supernatant, 2= washing solution.
silver salt of guanine. Recoveries after redisolving the pellet in 0.5N HCl solution for Ad and Gu were estimated against the standard prepared from the stock solution (Figure 4). The recoveries (mean ± S.E) were: adenine; 53.5 ± 2.76, 69.8 ± 3.9, 87.6 ± 3.67 and 102.0 ± 0.1; and guanine; 91.5 ± 1.6, 96.7 ± 1.6, 87.7 ± 7.94 and 97.0 ± 4.4, for the treatments pH 2, pH 3, pH 4 and no-washing, respectively.

Since negligible amounts of purines were lost in the precipitation step, the use of the precipitating solution to wash the sedimented pellet from impurities appeared to be a feasible solution. Six (6) 4ml samples each from the Ad and Gu stock solutions (100 µg/ml) were placed into screw cap test tubes and assigned to the following treatment: T1= washing of the sedimented pellet with precipitating solution (Appendix E) and T2= no washing of the sedimented pellet. All other steps were the same as in the last experiment. Again, a 5.0 µg/ml standard for adenine and guanine were prepared from the stock solutions. The results (Figure 5) show that recovery of the purine bases adenine and guanine was almost 100% when using the precipitation solution to wash the pellet. The recoveries (mean ± SE) were: adenine, 100.8 ± 0.10 and 97.2 ± 0.74; guanine, 99.1 ± 2.20 and 97.7 ± 2.03, for unwashed and washed treatments, respectively.

In the procedure for total purines proposed by Zinn and Owens (1986) yeast RNA is recommended as a standard. In the literature, the values for purine are often referred to as purine yeast RNA equivalent. As mentioned earlier, from the values reported for Marshak and Vogel (1951) the ratio adenine to guanine for yeast extract RNA is calculated to be 1:1.3. Some information reported by Luria (1960) indicates that in
Figure 4. Effect of the washing solution pH on recovery of the precipitated purine bases adenine and guanine
Figure 5. Use of the precipitation mixture as washing solution on recovery of the purine bases adenine and guanine
different bacteria the molar ratio of Ad to Gu varies between 0.5 and 2.5 and for *E. coli* about 1.0. Ushida et al. (1985) determined this ratio to be 1:1.41 in isolated mixed bacteria from which was not different from the same ratio measured in yeast RNA (1:1.32). In order to have a reliable standard for this study, it was decided to use a mixture of the pure purine bases, adenine and guanine. However, to do this, it was necessary to determine which of the Ad to Gu ratios, 1:1 or 1:1.3 was more closely related to the composition of the yeast RNA currently used as a standard. Based on the values reported by Marshak and Vogel (1951), the total amount of Ad plus Gu was about 22% of the dried weight of the yeast RNA. Therefore, this value was used for calculating the concentrations of total purine in yeast RNA. Stock solutions were prepared of yeast RNA, adenine and guanine in 0.5N HCl, from which appropriate aliquots were placed into screw cap test tubes as working standards (0, 2.5, 5.0, 7.5, and 10 μg/ml). After hydrolysis and cooling as before, the tubes were centrifuged at 15,000 x g for 10 min to sediment most of the charred material (particularly in the yeast RNA tubes) and the supernatant filtrate through a Whatman GF/D fiberglass filter (Ushida et al., 1985). One half (0.5) ml of this filtrate was placed in a PPCO centrifuge tube and 0.5 ml of 0.4M silver nitrate and 9.0 ml of 0.2M NH₄H₂PO₄ solutions were added. The tubes were sealed with parafilm, gently shaken and allowed to stand overnight in the refrigerator at 5 °C. The tubes were then centrifuged at 25,000 x g for 25 min and the supernatant carefully aspirated without disturbing the pellet. The pellet was washed with

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*Yeast RNA stock solution (w/v): add 0.455 g of yeast RNA to 100 ml volumetric and make to volume with 0.5N HCl. This solution contains 1000 μg/ml of purine.*
the precipitation mixture (Appendix E) and centrifuged as before. The washing solution was then carefully removed by aspiration. Ten (10) ml of 0.5 N HCl were added and the tubes were incubated 30 min in a 95 °C water bath to redissolve the purines. After centrifugation at 25,000 x g for 25 min. to remove any sediment, the supernatant was filtered through a Whatman 541 filter paper and OD read at 260 nm. Also, OD at 280nm was measured to determine the presence of contaminants such as tryptophan or tyrosine (Sambrook et al., 1989). A ratio of OD at 260/280 above 1.8 signifies the absence of these components. The values for these ratios were (mean ± SE): 1.919 ± 0.018, 1.953 ± 0.016 and 1.969 ± 0.016 for the final extracts of the standards 1:1, 1:1.3 and yeast RNA, respectively.

The OD readings for each standard were regressed against its corresponding concentrations (µg/ml). The results were analyzed by the general linear model of SAS (1991) using the linear model:

\[ y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_1 x_2 + \epsilon \]

described by Ott (1984) to compare the slopes of two regression lines, where: \( x_1 = \) concentration of purine, \( x_2 = 1 \) if the standard is yeast RNA, but 0 is used for the mixture. The expected value for \( y \) is \( E(y) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_1 x_2 \). By substituting the values, the expected OD values for a given concentration of the standard mixture became: \( E(y) = \beta_0 + \beta_1 x_1 \), and for the yeast RNA: \( E(y) = \beta_0 + \beta_1 x_1 + \beta_2 + \beta_3 x_1 \). These two expected values represent linear regression lines. The parameters in the model can be interpreted in terms of the slopes and intercepts associated with these regression lines. Then, \( \beta_0 = y \)-intercept for standard mixture regression line, \( \beta_1 = \) slope of the standard mixture regression line, and \( \beta_2 = \) difference in the \( y \)-intercepts of the regression lines for yeast RNA and the standard.
mixture, and $\beta_3 =$ differences in slopes of the regression lines for yeast RNA and the standard mixture. The results of this analysis indicated that the slopes of the regression lines between yeast RNA and the two Ad:Gu ratios (1:1 and 1:1.3) were similar (Figure 6). Based on these results, i.e., ease of preparation and the cleaner hydrolyzed solution obtained with the purines as compared with the yeast RNA, the standard used for all further analyses in this investigation was a mixture of the purine bases adenine and guanine in a ratio 1:1.

The proposed standard, 1:1 Ad to Gu ratio, was used to determine the concentrations of purines in four recovery experiments. To 3 of 6 screw cap test tubes containing 750 μg of yeast RNA, 400 μg of a mixture of 1:1 Ad:Gu were added. After drying at 80 °C in a convection oven, the samples were analyzed as previously described to determine the total purine content in the tubes. The overall recovery (mean ± SE) for the 4 experiments was 100.6 ± 3.2%, with individual values ranging from 99.3 ± 0.06 to 104 ± 0.02% (Figure 7).

The commonly accepted procedure for estimating total purines in rumen bacteria (Zinn and Owens, 1986 and Ushida et al., 1985) was modified to measure very small amounts of purines in samples of pure cultures of ruminal bacteria. This modified procedure was adopted for all further analyses to determine purine contents. An schematic outline of the modified procedure is as follows:

1. Add a small bacterial sample (0.01-0.05g) into a 50-ml screw cap Teflon centrifuge tube and dry.

2. Add 0.5 ml of (70%) HClO₄, tightly cap tube (use Teflon seals) and incubate 1h in a 95 °C water bath and mix occasionally to break up the charred material.
Figure 6. Standard curves for yeast RNA and mixtures of pure adenine and guanine (ratios 1:1 and 1.1.3)
Figure 7. Recovery of purine mixture (1:1) added to yeast RNA
3. Remove tube from the water bath. add 3.5 ml of dilute buffer (0.0285M NH$_4$H$_2$PO$_4$), mix and reinsert the tube in the 95 °C water bath for 15 more min.

4. Remove tube from the water bath. centrifuge at 15,000 x g for 10 min and filter the supernatant through a Whatman GF/D fiberglass filter.

5. Transfer 0.5 ml filtrate into 15-ml polypropylene copolymer centrifuge tube. add 0.5 ml 0.4M AgNO$_3$ and 9.0 ml of buffer (0.2M NH$_4$H$_2$PO$_4$). Seal with parafilm and shake. Allow the tubes to stand overnight in the refrigerator (5 °C).

6. Centrifuge at 25,000 x g for 25 min. Carefully aspirate supernatant and discard. Be careful not to disturb the pellet while performing these steps.

7. Wash pellet with 10.0 ml of the precipitation solution (Appendix E) and repeat step 6.

8. Add 10.0 ml of 0.5N HCl. Cover tube with parafilm and mix thoroughly.

9. Place a marble over the end of the tube and incubate 30 min in a 95 °C water bath.

10. Remove tube from the water bath, seal with a very clean rubber stopper and mix at high speed and centrifuge at 25,000 x g for 25 min. (A pinkish precipitate may form).

11. Filter supernatant through a Whatman 541 paper filter and read OD at 260 nm.

- For standards: place 8.0 ml from a stock solution (1000 µg/ml total purine) of adenine and guanine at 1:1 ratio in 0.5N HCl, into a 50-ml screw cap centrifuge Teflon tube. Treat similarly to the sample. Prepare the working standards by diluting aliquots of the final filtrate (from step 11, contains 100 µg/ml) with 0.5N HCl to give a total purine concentration of 2.5, 7.5, 15.0, 25.0 and 35.0 µg/ml. For the blank, dry 8.0 ml of 0.5N HCl and then process similarly to the sample.
Analysis of pure cultures of rumen bacteria

Figure 8 shows the results of two experiments each with *R. albus* 7 and *B. fibrisolvens* D16f in which bacterial numbers were correlated with purine concentrations as determined in various amounts of the fermentation media. Although numbers and purine concentrations were highly correlated within each experiment, marked differences occurred between the experiments.

Since it is known that the methods for estimating bacterial numbers are quite variable (Herbert, 1990), it was decided that all analyses would be run in triplicate. In other words, three separate subsamples would be taken from the fermentation flasks for each of the analyses (numbers, purine and protein). From these data, coefficients of variation (CV) would then be calculated. Results of these experiments, using ten different pure cultures of rumen bacteria, are shown in Table 6. The cultures were grown as described in Material and Methods and harvested in the upper third of the log phase of growth. Typical growth curves for the different species and strains are shown in the Appendix F. Coefficients of variation for the MPN determination ranged from 28.95 to 76.53%, whereas CV for purine analysis ranged only from 2.63 to 10.76%. The range in CV for the protein analysis (Lowry procedure) was from 2.28 to 15.95%. Overall, the mean coefficients of variation were 5.25, 6.52 and 55.86% for purine, protein and MPN analysis, respectively. Thus precision was markedly better for both the purine and protein analysis than for the MP determination.

Table 7 presents the actual data obtained in the three experiments for each of the ten pure cultures of rumen bacteria. Cell concentrations ranged from $43.33 \times 10^8$ per ml
Figure 8. Total purine concentration vs numbers of Ruminococcus albus 7 and Butyrivibrio fibrisovens D16f
Table 6. Coefficients of variation for the MPN (bacterial concentration), purine and protein analyses

<table>
<thead>
<tr>
<th>Bacteria¹</th>
<th>MPN (concentration of bacteria)</th>
<th>Purine</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>45.09 ± 17.00</td>
<td>4.73 ± 1.42</td>
<td>2.28 ± 0.83</td>
</tr>
<tr>
<td>ARD26e</td>
<td>76.53 ± 21.92</td>
<td>5.86 ± 2.43</td>
<td>3.19 ± 0.75</td>
</tr>
<tr>
<td>ARD5d</td>
<td>59.37 ± 16.48</td>
<td>4.45 ± 1.50</td>
<td>7.36 ± 1.56</td>
</tr>
<tr>
<td>B21a</td>
<td>66.88 ± 19.77</td>
<td>10.76 ± 1.41</td>
<td>15.95 ± 2.13</td>
</tr>
<tr>
<td>B34b</td>
<td>72.09 ± 12.12</td>
<td>3.43 ± 0.57</td>
<td>3.48 ± 0.92</td>
</tr>
<tr>
<td>D16f</td>
<td>41.73 ± 14.71</td>
<td>3.70 ± 0.30</td>
<td>4.18 ± 1.22</td>
</tr>
<tr>
<td>D25e</td>
<td>42.85 ± 0.0</td>
<td>7.34 ± 3.67</td>
<td>10.03 ± 2.40</td>
</tr>
<tr>
<td>H10b</td>
<td>72.92 ± 20.93</td>
<td>6.80 ± 1.15</td>
<td>5.33 ± 2.35</td>
</tr>
<tr>
<td>H15a</td>
<td>28.95 ± 15.70</td>
<td>2.63 ± 0.98</td>
<td>9.52 ± 1.44</td>
</tr>
<tr>
<td>H17c</td>
<td>52.20 ± 5.91</td>
<td>2.83 ± 0.67</td>
<td>3.92 ± 0.50</td>
</tr>
<tr>
<td>Overall mean</td>
<td>55.86 ± 5.09</td>
<td>5.25 ± 0.79</td>
<td>6.52 ± 1.35</td>
</tr>
</tbody>
</table>

¹ Bacteria: 7 = R. albus; ARD26e = L. lactis; ARD5d = S. bovis; B21a = F. succinogenes; B34b = R. flavefaciens; D25e = L. multiparus; D16f, H10b and H17c = B. fibrisolvens; H15a = P. ruminicola
Table 7. Bacteria, purine and protein concentrations, and purine-protein ratios of ten strains of ruminal bacteria

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Concentration of bacteria</th>
<th>Protein</th>
<th>Purine</th>
<th>Purine:protein ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/cell (x 10^-8)</td>
<td>% dry matter</td>
<td>µg/cell (x 10^-8)</td>
<td>% dry matter</td>
</tr>
<tr>
<td>7</td>
<td>7.74</td>
<td>39.86(^b)</td>
<td>72.49(^a)</td>
<td>3.12(^b)</td>
</tr>
<tr>
<td>ARD26e</td>
<td>14.70</td>
<td>12.50(^{cd})</td>
<td>29.58(^{bc})</td>
<td>1.37(^c)</td>
</tr>
<tr>
<td>ARD5d</td>
<td>38.96</td>
<td>7.34(^{cd})</td>
<td>34.74(^{bc})</td>
<td>0.92(^{cd})</td>
</tr>
<tr>
<td>B21a</td>
<td>10.38</td>
<td>33.81(^b)</td>
<td>28.95(^{bc})</td>
<td>0.77(^{cd})</td>
</tr>
<tr>
<td>B34b</td>
<td>5.07</td>
<td>57.87(^a)</td>
<td>45.29(^b)</td>
<td>5.41(^a)</td>
</tr>
<tr>
<td>D16f</td>
<td>2.43</td>
<td>132.31</td>
<td>44.58(^{b})</td>
<td>9.83</td>
</tr>
<tr>
<td>D25e</td>
<td>0.33</td>
<td>617.57</td>
<td>19.01(^c)</td>
<td>54.89</td>
</tr>
<tr>
<td>H10b</td>
<td>17.25</td>
<td>8.23(^{cd})</td>
<td>18.07(^{c})</td>
<td>0.77(^{cd})</td>
</tr>
<tr>
<td>H15a</td>
<td>43.33</td>
<td>5.29(^d)</td>
<td>21.94(^{bc})</td>
<td>0.49(^d)</td>
</tr>
<tr>
<td>H17c</td>
<td>24.60</td>
<td>15.34(^c)</td>
<td>36.27(^{bc})</td>
<td>1.25(^{cd})</td>
</tr>
<tr>
<td>Mean(^d)</td>
<td>20.25</td>
<td>22.53</td>
<td>35.09(^{f})</td>
<td>1.76</td>
</tr>
</tbody>
</table>

\(^1\)Bacteria: 7 = R. albus; ARD26e = L. lactis; ARD5d = S. bovis; B21a = F. succinogenes; B34b = R. flavefaciens; D25e = L. multiparus, D16f, H10b and H17c = B. fibrisolvens; H15a = P. ruminicola.

\(^2\)Concentration of bacteria (number x 10^9/ml).

\(^3\)Dry matter basis.

\(^4\)Mean: minus D16f and D25e for concentration of bacteria and µg/cell of purine and protein (see text).

\(^{abcd}\)Means within columns with the same superscript are not different (P<0.05).

\(^5\)Standard error: protein (% DM) = 11.8; purine (% DM) = 0.86 and Purine:protein ratio = 0.02
for *Prevotella ruminicola* H15a down to $0.33 \times 10^8$ cells/ml for *Lachnospira multiparus* D25e. *L. multiparus* D25e and *B. fibrisolvens* D16f were observed to form cotton like clumps in liquid media which were very difficult to disperse by shaking the flask or mixing in the dilution tubes. Underestimation of the true concentration would be expected if the clumps of cells could not be broken up into a uniform suspension. This would be the probable explanation for the low concentrations with *L. multiparus* D25e ($0.33 \times 10^8$ cells/ml) and *B. fibrisolvens* D16f ($2.43 \times 10^8$ cells/ml). In turn, concentrations of purines or protein expressed on a per cell basis would be elevated, as can be seen in Table 7. Because of this bias, D25e and D16f were omitted from the calculations for overall mean and differences between means for concentration of bacteria and purine and protein concentration per cell.

Protein concentration per cell ($\mu$g/cell $\times 10^8$) was found to vary significantly between species, with the highest value occurring in *R. flavefaciens* B34b (57.87) and the lowest in *P. ruminicola* H15b (5.29). Although the two species of *B. fibrisolvens* (H10b and H17c) were numerically different, the difference was not significant (8.23 versus 15.34). *R. albus* and *F. succinogenes* were similar in their protein contents (39.86 and 33.81 $\mu$g/cell respectively) and *L. lactis* ARD26e (12.50 $\mu$g/cell) and *S. bovis* ARD5d (7.34$\mu$g/cell) were not different from *B. fibrisolvens* H10b (8.23 $\mu$g/cell). The overall mean for protein content per cell (excluding D16f and D25e) was calculated to be $22.53 \times 10^8$ $\mu$g/cell.

When protein contents were calculated on a percent of dry matter (DM) basis, the concentration of protein ranged from 72.49% in *R. albus* to 18.07% in *B.*
fibrisolvens H10b. *R. flavaeiaciens* B34b. and *B. fibrisolvens* D16f were similar in their protein contents, 45.29 and 44.58%, respectively and differed from the other organisms (P < 0.05). The concentration of protein in *L. multiparus* D25e (19.01%) was the same as in *B. fibrisolvens* H10b. An intermediate major group consisted of *S. bovis* ARD5d (34.74%), *B. fibrisolvens* H17c (36.27%), *L. lactis* ARD26e (29.58%), *F. succinogenes* B21a (28.95%) and *P. ruminicola* H15a (21.94%). The overall mean for protein concentration for these ten species of ruminal bacteria was found to be 35.09% of the DM.

Hungate (1963) working with continuous cultures of *Ruminococcus albus* found that the polysaccharide content of this organism can vary markedly. Protein content of bacteria varies according to species and concentration of carbohydrates inside the cell (Dufva et al., 1982; Arambel et al., 1982). Arambel et al. (1982) found significant differences in the nitrogen content between gram-negative and gram-positive rumen bacteria. Since nitrogen content of true microbial protein has been found to be about 15% (Van Soest, 1994; Dehority, 1995), the factor 6.67 is used to calculate protein from nitrogen content in bacteria. The mean value found in this study for protein (35.09%) is slightly lower than the mean value calculated from the data of Dufva et al. (1982). 43.3%; Firkins et al. (1987). 49.0% for particle-associated, and 42.8% for fluid-associated bacteria, harvested from the rumen, and those values reported by Hespell and Bryant (1979) for numerous gram-positive bacteria (60.0%) and gram-negative bacteria (52.4%), which compared well with their values for the mostly gram-negative mixed ruminal bacteria (54.0%). Arambel et al. (1982) reported an average value of 68.7% for
protein concentration in mixed ruminal bacteria, which is considerably higher than the other reports. Determination of protein from nitrogen content tends to overestimate the protein values in bacterial samples, mainly because of the non-protein which occurs in the cells (Herbert et al., 1971).

Purine content per cell varied considerably among species and ranged from 5.41 µg/cell x 10^{-8} in *R. flavefaciens* B34b to 0.49 µg/cell x 10^{-8} in *P. ruminicola* H15a. The intermediate values found for this estimation were in descending order as follows (µg/cell x 10^{-8}): *R. albus* 7, 3.12; *L. lactis* ARD26e, 1.37; *B. fibrisolvens* H17c, 1.25; *S. bovis* ARD5d, 0.92; *F. succinogenes* B21b, 0.77 and *B. fibrisolvens* H10b, 0.77. B34b and 7 differed from each other and all other organisms (P < 0.05). ARD26e and H15a also differed from each other (P < 0.05), and overlapped with the remaining organisms. The overall mean excluding D16f and D25e, was found to be 1.76 x 10^{-8} µg/cell.

Purine content as a percentage of the DM was also found to differ (P<0.05) among the species under study (Table 7). Purine contents for *S. bovis* ARD5d (4.29%) and *R. flavefaciens* B34b (4.17%) were not different from the concentration found in *R. albus* 7 (5.57%) which was the highest value observed for this criterion. Purine concentrations for *S. bovis* ARD5d and *R. flavefaciens* B34b were similar to those found in *L. lactis* ARD26e (3.21%), *B. fibrisolvens* D16f (3.20%), and *B. fibrisolvens* H17c (2.94%). Purine contents for *L. multiparus* D25e (2.36%), *B. fibrisolvens* H10b (1.50%) and *P. ruminicola* H15a (1.89%) were comparable to the concentration found for *F. succinogenes* B21a (0.69%) which was the lowest concentration of purine.
observed. The mean purine concentration for the ten strains of rumen bacteria was 2.98% of the dry matter.

Purine to protein ratios were calculated on a % of dry matter basis. The results are shown in the last column of Table 7. The ratio for *S. bovis* ARD5d (0.1239), *L. lactis* ARD26e (0.1093) and *R. flavefaciens* B34b (0.0913) did not differ (P < 0.05) from *L. multiparus* D25e (0.1299), which had the highest purine:protein ratio. Furthermore, the ratios for *S. bovis* ARD5d and *R. flavefaciens* B34b and *L. lactis* ARD26e were comparable to the purine:protein ratios for *B. fibrisolvens* H10b (0.0851) and *P. ruminicola* H15a (0.0847). Except for *S. bovis* ARD5d and *L. multiparus* D25e the purine:protein ratio of the above listed organisms did not differ (P < 0.05) from *B. fibrisolvens* H17c (0.0804), *B. fibrisolvens* D16f (0.0781) and *R. albus* 7 (0.0761). The lowest purine:protein ratio was found with *F. succinogenes* B21a (0.0237). The mean value for purine to protein ratio of these ten organisms was 0.0883.

Differences in composition between gram-negative and gram-positive bacteria suggested that this might influence the purine to protein ratio. When the purine:protein ratios were ranked in a descending order and matched with their gram stain reaction (Table 8), a tendency was observed for the gram-negative bacteria to have relatively lower purine:protein ratios than the gram-positive bacteria.

Genetic variation within bacterial populations can affect the specific growth rate of a given bacteria in a given substrate (Russell et al., 1979). In addition, changes in growth media, growth rate or growth phase at which cells are collected can alter the
### Table 8. Association between purine:protein ratios and the gram stain

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Purine:protein ratio</th>
<th>Gram stain</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. multiparus</em> D25e</td>
<td>0.12990&lt;sup&gt;a&lt;/sup&gt;</td>
<td>weak +</td>
</tr>
<tr>
<td><em>S. bovis</em> ARD5d</td>
<td>0.12397&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td><em>L. lactis</em> ARD26e</td>
<td>0.10930&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>+ to ±</td>
</tr>
<tr>
<td><em>R. flavefaciens</em> B34b</td>
<td>0.09130&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>±</td>
</tr>
<tr>
<td><em>B. fibrisolvens</em> H10b</td>
<td>0.08510&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td><em>P. ruminicola</em> H15a</td>
<td>0.08477&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td><em>B. fibrisolvens</em> H17c</td>
<td>0.08040&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td><em>B. fibrisolvens</em> D16f</td>
<td>0.07817&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td><em>R. albus</em> 7</td>
<td>0.07615&lt;sup&gt;c&lt;/sup&gt;</td>
<td>- to ±</td>
</tr>
<tr>
<td><em>F. succinogenes</em> B21a</td>
<td>0.02373&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a,b,c,d</sup> Means within column with the same superscript are not different (P < 0.05).
composition of the microbial cell (Hespell and Bryant, 1979). Differences in specific growth rate have also been associated with significant changes in the RNA:protein ratio. Bates et al. (1985) working with six strains of ruminal bacteria found that 74% of the changes in the RNA:protein ratio were due to changes in the specific growth rate between the organisms studied. In the present study, bacteria were harvested in the upper third of the log or near stationary phase. The specific growth rate was calculated from the OD readings at 600 nm in the log phase of the bacterial growth as follow:

\[ n = \frac{\log OD_t - \log OD_0}{\log 2} \]

where \( n \) = number of generation, \( OD_t \) = optical density at selected period time in the log phase and \( OD_0 \) = optical density at any time at the beginning of log phase. Then generation time is calculated by:

\[ G = \frac{T}{n} \]

where \( T \) = time period. Finally, doubling per hour is calculated by the inverse of the generation time \( (G') \). Figure 9 shows the relationship between purine:protein ratio and specific growth rate for nine of ten pure cultures of rumen bacteria used in the present study. F. succinogenes B21a was omitted from the graph based on the fact that its purine:protein ratio was drastically lower than all the other organisms. An explanation for this lower value for B21a is not immediately obvious. In general, the purine:protein ratio increased as doubling per hour increased \( (r = 0.80) \). This finding appear would support the previous observation by Bates and coworkers (1985) for the changes in RNA:protein ratio and specific growth rates. However, it should be noted that at least six of the organisms are grouped quite closely and the regression is markedly influenced by the remaining three. Although more studies would need to be conducted, some of the
$y = 0.0632 + 0.0428^2x$

$r^2 = 64$

Figure 9. Relationship between specific growth rate and purine to protein ratio
changes (64%) in purine:protein ratios among species could be explained by changes in the specific growth rate among species and strains of rumen bacteria.

**Analysis of mixed ruminal bacteria**

To determine microbial protein at the duodenal level, a constant ratio of microbial marker to microbial protein must be assumed both in the rumen and duodenum. Hespell and Bryant (1979) had hypothesized that the bacterial composition of a population of mixed rumen bacteria may not differ from that observed with other non-rumen bacteria. Since the bacteria used in this study are true ruminal bacteria, it was reasoned that the overall mean for purine:protein ratio from the pure cultures (0.0883) should correspond to the purine:protein ratio obtained from a mixed culture of ruminal bacteria. Therefore, samples of rumen bacteria prepared as described in Materials and Methods were analyzed to compare with the pure culture values. The results of this comparison are show in Table 9. Five different samples of mixed rumen bacteria were obtained from animals fed varying diets. Bacterial numbers, as well as purine and protein contents were determined and by using the general linear model of SAS (1991) in a complete randomized design, the data was analyzed by dividing the animals according to the type of diet fed, i.e., cow, sheep and steer 3 were placed in the forage diet group and the steers 1 and 2 in concentrate diet group.

Bacterial concentrations were observed to range from $2.13 \times 10^9$ per ml for steer 3 (forage diet) to $28.3 \times 10^9$ for steer 2 (concentrate diet). Bacterial concentrations were higher ($P < 0.05$) for animals fed concentrate ($22.5 \times 10^9$) as compared to those fed
Table 9. Bacterial numbers, purine and protein contents, and purine:protein ratios (Pur:Prot) of mixed bacterial samples isolated from rumen contents

<table>
<thead>
<tr>
<th>Animal</th>
<th>Type of diet</th>
<th>Bacterial numbers (x 10^6)</th>
<th>Purine</th>
<th>Protein</th>
<th>Pur:Prot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>µg/cell(x10^9) % DM</td>
<td>µg/cell (x10^9) % DM</td>
<td></td>
</tr>
<tr>
<td>Cow</td>
<td>Forage &amp; Concentrate</td>
<td>3.50</td>
<td>0.98</td>
<td>0.74</td>
<td>54.40</td>
</tr>
<tr>
<td>Sheep</td>
<td>Alfalfa pellet</td>
<td>2.54</td>
<td>2.49</td>
<td>1.55</td>
<td>70.00</td>
</tr>
<tr>
<td>Steer 3</td>
<td>Forage</td>
<td>2.13</td>
<td>1.16</td>
<td>1.20</td>
<td>32.70</td>
</tr>
<tr>
<td>Mean forage</td>
<td></td>
<td>2.72b</td>
<td>1.54</td>
<td>1.16</td>
<td>52.37</td>
</tr>
<tr>
<td>Steer 1</td>
<td>Concentrate</td>
<td>16.70</td>
<td>2.87</td>
<td>1.76</td>
<td>87.70</td>
</tr>
<tr>
<td>Steer 2</td>
<td>Concentrate</td>
<td>28.30</td>
<td>1.88</td>
<td>1.66</td>
<td>54.30</td>
</tr>
<tr>
<td>Mean concentrate</td>
<td></td>
<td>22.50a</td>
<td>2.37</td>
<td>1.71</td>
<td>71.00</td>
</tr>
<tr>
<td>Overall mean</td>
<td></td>
<td>10.63</td>
<td>1.88</td>
<td>1.38</td>
<td>59.82</td>
</tr>
</tbody>
</table>

1 Animal: the ruminal contents of sheep and steer 3 were obtained through ruminal cannula. For the cow and steers 1 and 2, at slaughtering.

ab Means within columns with the same superscript are not different (P < 0.05).
forage \((2.72 \times 10^9)\). The overall mean value for bacterial concentration was \(10.6 \times 10^9\) per ml.

Concentration of purine ranged from \(0.98 \times 10^8 \mu g/cell\) in the sample from the cow fed forage and concentrate, to \(2.87 \times 10^8 \mu g/cell\) in steer 1 fed the all concentrate diet. No statistical differences were found between animals fed forage \((1.54 \times 10^8 \mu g/cell)\) and concentrate \((2.37 \times 10^8 \mu g/cell)\) diets, and the overall mean of \(1.88 \times 10^8 \mu g/cell\) was very close to the mean value determined for the pure cultures of rumen bacteria \((1.76 \times 10^8 \mu g/cell)\).

No statistical differences were observed in purine content on a dry matter basis when the values were analyzed according to the type of diet fed. Values ranged from 0.74% in the sample from the cow fed forage and concentrate, to 1.76% in steer 1 fed and all concentrate diet. The overall mean was 1.38%.

Concentration of protein per cell ranged from \(32.7 \times 10^8 \mu g/cell\) in the sample from steer 3 fed all forage, to \(87.7 \mu g/cell \times 10^8\) in steer 1 fed all concentrate. The mean values between diets did not differ. Both the mean value for protein concentration per cell from forage \((52.37 \times 10^8 \mu g/cell)\) and concentrate \((71.0 \times 10^8 \mu g/cell)\) rumen samples were 2 to 3 times higher than the mean value determined for pure cultures in the present study \((22.53 \times 10^8 \mu g/cell)\). The overall mean was \(59.82 \times 10^8 \mu g/cell\). Protein contents in bacteria from the animals fed forage diets \((39.41\%)\) tended \((P < 0.08)\) to be lower than those fed concentrate diets \((50.98\%)\). In general, protein values were fairly constant across animals and the mean value was 44.04%.
When calculating the purine to protein ratio for mixed cultures from the rumen of animals fed forage (0.0297) or concentrate (0.0336) no statistical differences were observed. The highest and the lowest value were 0.0356 and 0.0181 for steer 3 and the cow, respectively. Both animals were fed forage type diets. The overall mean for the purine:protein ratio in bacterial samples isolated from rumen contents was 0.0313. This ratio is almost 3 times lower than average ratio determined previously for the ten pure cultures (0.0883). The mean value for purine:protein ratio found in this study for mixed rumen bacteria was surprisingly close to the mean of ratios calculated from data reported by Perez et al. (1996), 0.032 for mixed bacteria isolated from ruminal content of ewes fed varying quantities of barley (0, 40%, 73% and 100%) with alfalfa hay (Appendix G). A similar ratio, 0.0328, was calculated from the data reported by Zinn and Owens (1986) for mixed ruminal bacteria isolated from steers.

The majority of studies in the literature report concentrations of purine for mixed bacteria as yeast RNA equivalent. The concentration of purines (adenine + guanine) and nitrogen in yeast RNA has been estimated to be about 22% and 15%, respectively (Marshak and Vogel, 1951). Also, the percentage of nitrogen in adenine and guanine is 51.82 and 46.34%, respectively. If a 1:1 ratio of adenine to guanine is assumed, then an average of these nitrogen values (49.08%) can be used to calculate purine concentrations from data reported as yeast RNA equivalent. Using the above mentioned assumptions, purine:protein ratios were calculated from the data reported by Calsamiglia et al. (1996), working with mixed rumen bacteria in a fermentor (Appendix H). The overall mean of purine concentration for eight different diets was 1.41%. similar to the average purine concentration.
content estimated for mixed cultures in the present study. Calculations for protein content resulted in values somewhat higher than those found for mixed bacteria reported previously in Table 9. Values ranged from 51.9% to 59.8%, with a mean of 55.3%, as compared to 44.04% in this study. The calculated purine:protein ratios indicate that these values range from 0.024 to 0.027 and were very consistent across treatments (CV = 5.08%). The mean value for the purine:protein ratios was calculated to be 0.0256, which was markedly lower than the values determined in the present study for pure cultures (0.0883) and slightly less than for mixed cultures isolated from the rumen (0.0313). Similar values to those calculated from the data of Calsamiglia et al. (1996) were calculated from the data reported by Firkins et al (1987) for particle- (0.0256) and fluid-associated bacteria (0.0262) from rumen contents.

The overall purine to protein ratio determined with pure cultures is considerably higher than those determined in isolated mixed bacteria, both, in the present investigation and in the literature. If one assumes that only bacterial purine is measured in the analysis, i.e., there is no purine from feed particles or any other sources, then some source of nitrogen other than bacterial must be present in the sample. This would consequently lower the purine:protein ratio. Using the purine concentration per cell from pure culture data, the theoretical concentration of bacteria in the mixed standard was calculated from purine concentration. This gave estimates of bacterial numbers which ranged from $1.41 \times 10^9$ to $30.2 \times 10^9$ cells/ml which corresponds with the actual concentrations determined by MPN. No statistical differences were observed between
the actual mean (10.63 ± 5.18 x 10^9 cells/ml) and the calculated mean (13.01 ± 6.56 x 10^9 cells/ml).

Using the purine analysis as modified for bacteria, purine content was measured in four different forages. Table 10 shows the purine contents on a dry matter basis for these four forages, harvested at different stages of maturity. It can be observed that the mean value for these forage samples (0.345%) is markedly lower than the value determined for both the pure cultures (2.98%) and mixed cultures (1.38%).

Comparing the average protein content (µg/cell) determined with the pure cultures (2.253 x 10^-7) and the same values obtained from mixed cultures suggests that there is a significant amount of protein in the mixed cultures which is not of bacterial origin (Table 11). Overestimates ranged from 31.1 to 74.3% with a mean of 58.06%.

To investigate this further, two additional samples of rumen contents were obtained from the slaughter house from steers fed concentrate diets. These were processed as before (see Materials and Methods) except that after sampling for MPN, the bacteria were washed with 0.9% saline solution until the supernatant was clear. The bacteria were resuspended to a known volume with saline solution and samples were taken for purine and protein analysis as described previously. Three 3-ml subsamples were also placed in tared aluminum pans to determine dry matter. The remaining bacterial suspension was lyophilized. The lyophilized bacteria were analyzed for neutral detergent fiber (NDF) (Goering and Van Soest, 1970), starch (Fleming and Reichert, 1980) total nitrogen (Leco FP 2000 nitrogen analyzer) and ash (Table 12). Results of these later analyses were corrected for the added salt by subtracting out the difference between the
Table 10. Purine concentrations of four forages samples at different maturity stages

<table>
<thead>
<tr>
<th>Forage</th>
<th>Maturity stage</th>
<th>Purine, % DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa</td>
<td>Bud</td>
<td>0.360</td>
</tr>
<tr>
<td>Fescue</td>
<td>Pre bud</td>
<td>0.350</td>
</tr>
<tr>
<td>Orchardgrass</td>
<td>Hay</td>
<td>0.390</td>
</tr>
<tr>
<td>Ryegrass</td>
<td>Hay</td>
<td>0.280</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>0.345</td>
</tr>
</tbody>
</table>
Table 11. Comparison of protein content per cell in mixed and pure cultures of rumen bacteria

<table>
<thead>
<tr>
<th>Animal</th>
<th>Bacterial numbers per ml ((x\ 10^9))</th>
<th>Protein µg/ml</th>
<th>Protein content (µg/cell (x\ 10^7))</th>
<th>Mean protein content from pure cultures (µg/cell (x\ 10^7))</th>
<th>% overestimate of µg protein per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>3.50</td>
<td>1902.32</td>
<td>5.44</td>
<td>2.253</td>
<td>58.58</td>
</tr>
<tr>
<td>Sheep</td>
<td>2.54</td>
<td>1778.83</td>
<td>7.00</td>
<td>2.253</td>
<td>67.81</td>
</tr>
<tr>
<td>Steer 1</td>
<td>16.70</td>
<td>14638.80</td>
<td>8.77</td>
<td>2.253</td>
<td>74.31</td>
</tr>
<tr>
<td>Steer 2</td>
<td>28.33</td>
<td>15377.60</td>
<td>5.43</td>
<td>2.253</td>
<td>58.51</td>
</tr>
<tr>
<td>Steer 3</td>
<td>2.13</td>
<td>696.27</td>
<td>3.27</td>
<td>2.253</td>
<td>31.10</td>
</tr>
<tr>
<td>Mean</td>
<td><strong>10.64</strong></td>
<td><strong>6878.76</strong></td>
<td><strong>5.98</strong></td>
<td><strong>2.253</strong></td>
<td><strong>58.06</strong></td>
</tr>
</tbody>
</table>
### Table 12. Analysis samples of mixed bacteria isolated from rumen contents of two steers fed concentrate diets.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bacterial numbers per ml ($x 10^9$)</th>
<th>Protein µg/cell $x 10^7$</th>
<th>% DM Protein</th>
<th>Pur:Prot ratio</th>
<th>Protein ($N \times 6.67$)</th>
<th>N</th>
<th>NDF</th>
<th>Starch</th>
<th>Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steer 4</td>
<td>9.30</td>
<td>13.10</td>
<td>48.82</td>
<td>1.69</td>
<td>0.0346</td>
<td>50.89</td>
<td>7.63</td>
<td>7.19</td>
<td>2.28</td>
</tr>
<tr>
<td>Steer 5</td>
<td>8.40</td>
<td>16.40</td>
<td>51.50</td>
<td>1.19</td>
<td>0.0231</td>
<td>50.96</td>
<td>7.64</td>
<td>7.60</td>
<td>2.70</td>
</tr>
<tr>
<td>Mean</td>
<td>8.85</td>
<td>14.75</td>
<td>50.16</td>
<td>1.44</td>
<td>0.0289</td>
<td>50.93</td>
<td>7.64</td>
<td>7.40</td>
<td>2.49</td>
</tr>
</tbody>
</table>

1 Protein: (Lowry, 1951)
dry matter measured in the protein analysis and that obtained from the aluminum pans. Bacterial numbers (8.85 x 10^-9 cells/ml) were numerically similar to the overall mean (10.63 x 10^-9 cells/ml) found for the previous samples of mixed ruminal bacteria. However, the values were somewhat lower than might have been expected with a concentrate diet (Table 9). The purine:protein ratio was also slightly lower than the mean from the previous samples, i.e., 0.0289 versus 0.0313.

Protein concentration, as determined by the Lowry analysis, gave values similar to those reported previously for mixed cultures sampled from animals with a similar type of diet. The overall mean was 50.16% of the dry matter. Nitrogen content was determined to be 7.64% of the DM, which when converted to protein (N x 6.67) gave a value of 50.93%, surprisingly similar to the protein content values determined by the Lowry procedure.

Neutral detergent fiber (NDF) constituted 7.40% of the dry matter of these bacterial samples from rumen content. This value probably reflects the presence of some microscopic plant particles which sediment with the bacteria in the process of separation from rumen contents. Oluobokun et al. (1988) found that over a period of 24 hours, bacterial samples from the fluid fraction of rumen contents contained 2.4 to 3.2% of OM as acid detergent fiber (ADF). The presence of ADF was assumed to be an indicator of plant contamination.

The bacterial samples contained 2.49% of starch on DM basis. This value is similar to the carbohydrate value reported by Ingraham et al. (1983) of 2.5% of the DM. Polysaccharides are present in the bacterial cell as a reserve materials. For instance, it
has been found that the total amount of glycogen and other polysaccharides varies in *Aerobacter* and *Salmonella* from less than 10% to over 30% of the DM, depending on the nature and composition of the media and on the growth phase (Luria, 1960). Merry and McAllan (1983) suggested that less than 4% of the mannose and galactose measured in the bacterial suspensions were from feed contaminants.

The mineral content of these two samples of mixed cultures of rumen bacteria was 24.65% of the dry matter. Ash concentrations for mixed cultures of rumen bacteria were observed to range from 12.0 to 21.6% of the DM by Olubobokun et al. (1988). Higher ash values (27.9 to 31.7% of DM) were estimated from the reported data of Perez et al. (1996). Slightly higher values (10.7 to 38.5% of DM) were estimated from the data reported by Illg and Stern (1994). An estimation from the data of Cecava et al. (1990b) indicates that 17% of the bacterial sample DM was ash. Merry and McAllan (1983) found that ash concentration was 15.7% and 8.66 of DM for rumen bacteria isolated from fluid- and solid-associated bacteria, respectively. Luria (1960) has reported an ash of concentration of 12.75% of DM for *E. coli*. It has been hypothesized (Storm and Ørskov, 1983) that the applied gravity forces during the separation of bacteria from rumen contents are pulling down some mineral elements that may be elevating the ash value in the isolate bacterial samples.

In general, the combined analyses account for 86.9% of the dry matter of the bacterial samples (Table 12). Based on the compositional data for mixed ruminal bacteria reported by Hespell and Bryant (1979), it would appear that the remaining DM could be accounted for by polysaccharide content (glycocalyx) and lipids. As previously
mentioned, much of the variation in the composition of bacteria has been related to changes in the carbohydrate content (Hungate, 1963; Arambel et al., 1982; Dufva et al., 1982). Lipids can constitute a significant proportion of the bacterial membranes, for example lipid concentrations and lipid-associated compounds make up about 12.5% of the DM in *E. coli* B/r cells grown in glucose minimum medium (Ingraham et al., 1983). Moreover, compositional variation in the lipopolysaccharide content is different between gram-negative and gram-positive bacteria (Hebert, 1990). Since bacterial types change with the diet (Hungate, 1966), it is evident that variations in composition may occur.

The calculations used in the estimation of Table 11 were applied to the last samples of mixed rumen bacteria. Overestimations for protein contents per cell were found to be much higher than those reported previously (84.6% versus 58.06%). Although the values on a DM basis for protein concentrations (50.16% and 44.04%) were comparable to the values previously mentioned from the literature for similar mixed cultures, they were also slightly higher than the value determined in this study for pure cultures of rumen bacteria (35.09%).
GENERAL DISCUSSION

Adaptation of the purine analysis

The procedure for total purine estimation described by Zinn and Owens (1986) was modified to analyze small samples of pure culture of rumen bacteria. However, large variations were observed in recovery experiments, which made it necessary to carry out a series of stepwise experiments to determine at just which step of the procedure losses were occurring. Ushida et al. (1985) had found that abnormal values were obtained when wet samples of duodenal digesta or bacterial samples were hydrolyzed, due to a dilution effect of the perchloric acid by the water and the organic material in the sample. Another possibility would be that since perchloric acid is a strong mineral acid, it would have a deleterious effect on very small amounts of the purine bases. However, it was found in the present study that neither perchloric acid concentration nor the hydrolysis step were affecting the recoveries of pure purine bases.

By monitoring the OD in the precipitating and washing supernatants, it was observed that a large amount of adenine (over 50%) was lost during the washing step. In contrast, no losses in guanine were observed by washing the silver precipitate. Although the guanine wash gave a low OD reading, recovery in the silver precipitate of guanine was approximately 100%. When the OD for the silver precipitate of adenine was added to the OD of the wash the resulting value was greater than 100%, by the approximate
amount found in the guanine wash solution. Aharoni and Tagari (1991) also observed losses of purines that ranged from 10 to 56% when using the washing solution proposed in the original method of Zinn and Owens (1986). However, they concluded that the proportion of the losses were related to the concentration of purine in the sample ($r^2 = 0.88$).

Kerr and Seraidarian (1945) emphasized the importance of the pH of the precipitation solution for separating of purine bases from mixtures with nucleosides. Free purines are precipitated by silver nitrate in acid solution at pH values between 1.5 and 2. Based on this observation, Zinn and Owens (1986) proposed the use of acidified water (pH 2) to wash the silver precipitate. Since the losses of adenine and guanine were markedly different, the effect of different acidified washing solutions on their recoveries was investigated. When OD was read in the corresponding washing solution supernatants, once again the results showed that appreciable amounts of the silver precipitate of adenine were solubilized. In contrast, little if any of the silver salt of guanine was lost. Adenine losses were inversely related to the pH of the solution, i.e., larger losses were found at the lower pH. Recoveries increased about four fold as the pH of the washing solution increased from 2 to 4.

Kerr and Seraidarian (1945) treated the washing solution acidified with H$_2$SO$_4$ with a 0.02M silver nitrate solution to help in the recovery of free purines. Also, Aharoni and Tagari (1991) suggested the addition of a low concentration of silver nitrate to the washing to increase recoveries of purines. In the present investigation, since only negligible losses of purine occurred in the precipitation step, the precipitating solution
(pH 2.3) was used to wash the sedimented purines. The results of this study indicated almost complete recovery of both purines (adenine, 97.2% and guanine, 97.7%).

Although yeast extract RNA has been used satisfactorily as standard (Zinn and Owens, 1986), the pure bases have also been suggested for this purpose. The main concern was that the amount of charred impurities after acid hydrolysis of yeast RNA could interfere in the analysis. Although Ushida et al. (1985) included a filtration step (filtration using glass filters) to eliminate the solid black particles, some very small black particles were not retained by the filter.

Several investigations had indicated that the proportion of adenine to guanine is variable in different species of bacteria (Luria, 1960). However, Ushida et al. (1985) found that their proportion in isolated rumen bacteria did not differ significantly from that in yeast extract RNA, i.e., 1:1.3 adenine to guanine. Aharoni and Tagari (1991) used a 1:1 molar ratio of adenine and guanine dissolved in 0.5N HCl as a standard solution in their analyses. An experiment was carried out to determine which adenine to guanine proportion (1:1 or 1:1.3) was most closely related to the composition of the yeast extract RNA currently used as a standard. The OD readings were regressed against the known concentrations and the slopes compared to establish if there were any differences. Based on previous information the purines adenine and guanine comprise approximately 22% of the DM of yeast RNA (Marshak and Vogel, 1951), this value was used to calculate total purines in the yeast RNA. No differences were found between the regression lines for yeast RNA or adenine to guanine in ratios of 1:1 or 1:1.3. Both mixtures of adenine and guanine (1:1 and 1:1.3 ratio) yielded a clear hydrolysate as
compared with that obtained from yeast RNA. The ratio 1:1 was much easier to prepare and therefore chosen as the standard for all future analyses. It was interesting that the calculated amount of purines from yeast RNA agreed very closely with the values obtained using known amounts of purified purines.

Having decided upon a suitable standard, a final recovery experiment was conducted by adding a mixture of adenine and guanine (1:1 ratio) to yeast extract RNA. Recoveries over 99% were obtained in four experiments, confirming the reliability of the modified procedure.

**Analysis of pure cultures of rumen bacteria**

When different amounts of the fermentation media were analyzed, bacterial numbers were correlated with the purine concentrations within experiment; however, large variations were observed between experiments. When precision of the MPN assay and purine were compared, it became obvious that the variation in the MPN assay severely compromised the possibility of using purine concentration to estimate bacterial numbers. The large variability in the MPN assay agrees with the observation of Herbert (1990), that large variations can occur in estimating bacterial concentrations. Since MPN is an estimate based on probability, slight variation in sampling can result in major differences in bacterial concentrations, as evidenced by the large standard error of the MPN means (Bond, 1994).

Bacterial numbers were also observed to be significantly affected by clumping of the cells in liquid medium. *B. fibrisolvens* D16f and *L. multiparus* D25e formed
aggregates or clumps in the liquid media which resembled cotton. The lowest numbers of cells per ml were obtained for these species. Resulting in unreliable estimations of protein and purine concentrations per cell. Although there were significant differences between species, by removing these two organisms which grew in clumps, concentrations of purine and protein per cell were relatively homogeneous across bacterial species and strains.

When bacterial numbers were eliminated from the calculations, purine and protein concentrations (as % DM) were very well correlated (r= 0.81) (Figure 10). Samples were taken when bacteria in the fermentation flask were in the log phase of growth, i.e., all the biochemical constituents are being synthesized at the same relative rates. In balanced growth, a good parallelism among these two biomass indicators would be expected (Ingraham et al. 1983).

Purine concentrations, reported either as amount per cell or percentage of the DM, were found to vary considerably among these rumen bacteria. More work using additional strains and species, is needed to understand the nature of this variability. For example, among the organisms used in this study the gram-negative bacteria tended to have lower purine:protein ratios. In addition, composition of bacteria can be affected by several factors such as media composition, phase of the bacterial growth at the time of sampling, and genetic variation within organisms which in turn produces changes in the reproduction rate of the bacterial cell (Hespell and Bryant. 1979; Russell et al., 1979; Bates et al.. 1983).
Figure 10 Relationship between purine and protein concentrations as percentage of the dry matter in ten strains of pure culture of rumen bacteria
Comparison of purine:protein ratios between pure and the mixed cultures

When samples of mixed populations of rumen bacteria were analyzed, either in the present study or reported in the literature, purine:protein ratios were markedly lower than the mean ratio of 0.0883 found for the ten species of rumen bacteria. Although purine from feed particles or from other sources might be carried along with the bacterial fraction isolated from the rumen contents (Van Soest, 1994), the estimations of numbers from purine content per cell were fairly close to the actual values determined for the five samples of mixed rumen bacteria analyzed in the present study (Table 9). The possibility that purine from feed particles is affecting the purine:protein ratio in the bacterial sample isolated from the rumen seems rather remote. First, the purine content of forages is quite low, and second the probability of intact plant cells remaining for any length of time in the rumen is also low. Even though more work is needed, a comparison between the average protein contents from the pure cultures and similar values from the samples of mixed rumen bacteria appears to indicate that the value affected in the purine:protein ratio is probably protein concentration. This could be an indication that non-bacterial-protein sources are involved. Feed contamination has been considered a serious problem particularly when centrifugal techniques are used (Van Soest, 1994). The analyses of the two extra samples of mixed rumen bacteria were clearly significant. Protein content, either as determined by the Lowry procedure or calculated as from N % of DM (x 6.67) was quite similar and consistent with those values found previously for animals fed the concentrate diet. However, as observed in Table 11, when compared to the mean value of protein content per cell from the pure cultures, the results substantiate the idea of
microbial protein overestimation in the mixed rumen bacterial sample. Although not as much an overestimation as observed in protein per cell, higher values of protein, as % of DM, were observed in the mixed culture samples compared to pure cultures. The analyzed samples showed a considerable proportion of NDF that averaged 7.4% of the sample DM. The presence of NDF in the isolated bacterial sample indicates that some feed contaminants were collected during the isolation process, because pure bacteria are soluble in neutral or acid detergent solutions (Van Soest. 1994). The same conclusion was reached by Olubobokun et al. (1988) when they found that up to 3.2% of the organic matter (OM) in the rumen bacterial sample was ADF.

The ash concentration observed in these mixed bacterial samples (24.65% of DM), was consistent with values reported in the literature for mixed ruminal bacteria (Olubobokun et al., 1988; Cecava et al., 1990b; Ilg and Stern, 1994). In contrast, much higher values were observed by Perez et al (1996). Van Soest (1994) pointed out that higher ash values in the bacterial sample would indicate contamination in some degree with fine particles other than bacteria. A similar conclusion was reached by Storm and Ørskov (1983). In order to make valid comparisons, a complete compositional analysis of pure cultures is needed. However, the information available from *E. coli* indicates that the mineral content in pure bacteria is somewhat lower, ranging from 1% (Ingraham et al. 1983) to 12.75% of DM (Luria, 1960).

The analyses carried out in this study for two samples of mixed bacteria isolated from rumen fluid only comprises about 87% of the DM. However, the results clearly
seem to indicate that the collected sample is contaminated. Quite possibly the remaining DM is carbohydrate or lipids in nature, i.e., capsular material etc.

Since determination of protein at the duodenal level depends primarily on a reliable bacterial standard isolated from the rumen and on a constant ratio of microbiol marker to microbial protein, using a contaminated bacterial sample can result in aberrant values regarding the contribution of the bacterial protein to the host animal. In this study, when comparing the purine to protein ratios determined for the pure cultures (0.0883) and that obtained from the mixed cultures isolated from the rumen (0.0313), a 3-fold difference was observed. This means, that if the later ratio is used to estimate bacterial protein at the duodenal level, bacteria protein would be overestimated by about the same magnitude.

In the rumen, bacterial cells will presumably be in various stages of growth. Since all of the pure culture data was collected from bacteria in the late log phase of growth, there is a possibility that the differences between the pure and mixed cultures may reflect differences in stage of growth. However, the data presented by Craig et al. (1987) for samples of mixed rumen bacteria collected at different times after feeding would not support this hypothesis. Purine to protein ratios calculated from their data ranged from 0.0399 before feeding to 0.0267 at 1 hour after feeding. The overall mean for hourly samples obtained between 0 and 13 h after feeding was 0.0323, which compares quite closely to the present value of 0.0313 for the mixed bacteria.
CONCLUSIONS

1. The procedure of Zinn and Owens (1986) for measuring purine content in mixed rumen bacteria was scaled down for use in measuring the purine content of pure cultures of rumen bacteria.

2. Because of poor recovery of added purines, the various steps were investigated and it was found that the silver salt of adenine, but not guanine was soluble in the wash solution. Use of the precipitating solution eliminated this loss.

3. Using this modification, recoveries of a mixture of pure adenine and guanine (1:1) added to yeast RNA were above 99%.

4. Because of the variation in bacterial numbers between experiments, three separate analyses were run for measuring bacterial numbers, purine content and protein content. Coefficients of variation were: bacterial numbers (MPN assay) = 55.86%; purine analysis = 5.25%; protein analysis = 6.52%.

5. Concentrations of bacteria and their purine and protein contents were determined on ten pure cultures of rumen bacteria. Although considerable variation occurred when purine/cell or protein/cell values were compared between the different organisms, the coefficients of variation for the purine to protein ratio (as % of DM) were much lower.

6. When the purine to protein ratio was determined for samples of mixed bacteria isolated from rumen contents, a marked difference from the mean value determined for the ten pure cultures was observed, i.e., 0.0313 versus 0.0883. The value for mixed rumen bacteria (0.0313) was very similar to that reported by other investigators.
7. Use of the purine to protein ratio measured in mixed rumen bacteria to calculate microbial protein in duodenal contents would overestimate bacterial protein almost three fold, as compared to the value obtained with pure cultures.

8. Preliminary results indicated that the mixed bacterial sample was probably contaminated with feed material which contained protein, thus lowering the purine to protein ratio.
APPENDIX A. CELLOBIOSE MEDIUM

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount in 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral solution A(^1)</td>
<td>20.00 ml</td>
</tr>
<tr>
<td>Mineral solution B(^2)</td>
<td>20.00 ml</td>
</tr>
<tr>
<td>Resazurin(^3) (0.1% solution)</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>Hemin(^4) (0.1% solution)</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>VFA, mixture(^5)</td>
<td>0.45 ml</td>
</tr>
<tr>
<td>Sodium Carbonate(^6) (12% solution)</td>
<td>3.30 ml</td>
</tr>
<tr>
<td>Cysteine-HCl(^7) (3% solution)</td>
<td>3.30 ml</td>
</tr>
<tr>
<td>Vitamin mixture(^8)</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>51.75 ml</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0.10 g</td>
</tr>
<tr>
<td>Trypticase</td>
<td>0.16 g</td>
</tr>
</tbody>
</table>

\(^1\)Mineral solution A: 0.45% KH\(_2\)PO\(_4\) in distilled water (Scott and Dehority, 1965).

\(^2\)Mineral solution B: 0.45% NaCl; 0.45% (NH\(_4\))\(_2\)SO\(_4\); 0.033% CaCl\(_2\)•H\(_2\)O; 0.025% MgSO\(_4\); 0.01% MnSO\(_4\); 0.01% FeSO\(_4\)•7H\(_2\)O; 0.01% ZnSO\(_4\)•7H\(_2\)O; 0.001% CoCl\(_2\)•6H\(_2\)O in distilled water (Scott and Dehority, 1965).

\(^3\)Resazurin, 0.1% solution: Add 100 mg of resazurin to 100 ml volumetric flask and diluted to volume with distilled water.

\(^4\)Hemin solution: 100 mg of hemin is dissolved in 0.02% sodium hydroxide and diluted to final volume of 100 ml with distilled water.

\(^5\)VFA mix solution (v/v): 54.84% acetic acid; 18.75% propionic acid; 12.90% butyric acid; 3.23% isobutyric acid; 3.23% n-valeric acid; 3.23% α-CH\(_3\)-butyric acid (Caldwell and Bryant, 1966).
Sodium carbonate solution (12%): 36 g of anhydrous Na\textsubscript{2}CO\textsubscript{3} plus distilled water to final volume of 300 ml measured in a large graduate cylinder. Transfer to a 500 ml round-bottom flask and gassed with N\textsubscript{2} for at least 30 min. Tube 5 or 10 ml aliquots under N\textsubscript{2} and autoclaved at 15# for 20 min.

Cysteine-HCl solution (3%): add 300 ml of distilled water to 500 round-bottom flask. Heat to dispel gasses while gassing with N\textsubscript{2}. After 30 min, add 9.0 g of cysteine•HCl. Continue gassing for 15 min and tube 5 or 10 ml aliquots under N\textsubscript{2}. Autoclave at 15# for 20 min.

Vitamin mixture: 0.02% pyridoxine•HCl; 0.02% riboflavin; 0.02% thiamine•HCl; 0.02% nicotinamide; 0.02% Ca-d-pantothenic acid; 0.001% p-aminobenzoic acid; 0.0005% folic acid; 0.0005% d-biotin; 0.00005% cyanocobalamin (Scott and Dehority, 1965).
APPENDIX B. COMPOSITION AND PREPARATION OF ANAEROBIC DILUTION SOLUTION

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount in 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral solution I(^1)</td>
<td>15.00 ml</td>
</tr>
<tr>
<td>Mineral solution II(^2)</td>
<td>15.00 ml</td>
</tr>
<tr>
<td>Resazurin (0.1% solution)(^3)</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>65.73 ml</td>
</tr>
<tr>
<td>Sodium Carbonate (12% solution)(^3)</td>
<td>2.50 ml</td>
</tr>
<tr>
<td>Cysteine-HCl (3% solution)(^3)</td>
<td>1.67 ml</td>
</tr>
</tbody>
</table>

\(^1\) Mineral solution I (w/v): 0.3\% K\(_2\)HPO\(_4\) (Bryant and Burkey, 1953).

\(^2\) Mineral solution II (w/v): KH\(_2\)PO\(_4\), 0.3\%; (NH\(_4\))\(_2\)SO\(_4\), 0.6\%; NaCl, 0.6\%; MgSO\(_4\), 0.06\%; CaCl\(_2\), 0.06\% (Bryant and Burkey, 1953)

\(^3\) See Appendix. A.

Preparation:
Gas with CO\(_2\), heat almost to boiling over burner. When fairly well reduced, add 12\% sodium carbonate and 3\% cysteine-HCl solutions and keep gassing until reduced (colorless). Tube anaerobically under CO\(_2\) in 9.0 ml aliquots. Autoclave in tubes at 15\# for 20 min.
### APPENDIX C. COMPLETE MEDIUM

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount in 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral solution A</td>
<td>20.00 ml</td>
</tr>
<tr>
<td>Mineral solution B</td>
<td>20.00 ml</td>
</tr>
<tr>
<td>Resarzurin (0.1% solution)</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>Hemin (0.1 % solution)</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>VFA, mixture</td>
<td>0.45 ml</td>
</tr>
<tr>
<td>Sodium Carbonate (12% solution)</td>
<td>3.30 ml</td>
</tr>
<tr>
<td>Cysteine-HCl (3% solution)</td>
<td>3.30 ml</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>51.75 ml</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0.10 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.10 g</td>
</tr>
<tr>
<td>Maltose</td>
<td>0.10 g</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.10 g</td>
</tr>
<tr>
<td>Trypticase</td>
<td>0.16 g</td>
</tr>
</tbody>
</table>

1. See Appendix A for composition of solutions.
APPENDIX D. REAGENTS AND STANDARD FOR PROTEIN ANALYSIS
(LOWRY ASSAY)

Reagents:

A  Solution of 2% Na₂CO₃
B  0.5 g CuSO₄•5H₂O in 100 ml of 1% (w/v) aqueous solution of sodium tartrate
C  Just before use, mix 50 ml of A and 1 ml of B
D  1N solution of Folin & Ciocalteu’s phenol reagent

1 Folin & Ciocalteu’s phenol reagent (2N): Sigma Chemical Co. P.O. Box 14508, St. Louis, MO 63178

Standards:

<table>
<thead>
<tr>
<th>Total amount of BSA (µg)</th>
<th>Amount (ml) from a BSA dilution</th>
<th>Amount of distilled water (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>100</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>200</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>300</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>500</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

1 BSA final dilution obtained after incubation in hot with 2N sodium hydroxide.
APPENDIX E. WASHING SOLUTION FOR PURINE METHOD (400 ml)

A. Mix 2.5 ml HClO₄ and 17.5 ml of 0.0285M NH₄H₂PO₄

B. 20 ml Mix A
   20 ml 0.4M AgNO₃
   360 ml 0.2M NH₄H₂PO₄

On a percentage basis:

- 5% Mix A
- 5% 0.4M AgNO₃
- 90% 0.2M NH₄H₂PO₄
APPENDIX F. GROWTH CURVE OF:

*B. fibrisolvens* D16f

Optical density, 600nm

Hour after Inoculation
Growth curve *B. fibrisolvens* H10b

- **Optical density, 600nm**
- **Hour after inoculation**

- 0.00
- 0.03
- 0.05
- 0.14
- 0.229
- 0.37
- 0.4
- 0.45

- 0
- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
Growth curve *B. fibrisalvens* H17c
Growth curve *F. succinogenes* B21a
Growth curve *L. parovulgaris* D25e

Optical density, 600nm

Hour after inoculation
Growth curve *L. lactis* ARD26e
Growth curve *P. ruminicola* H15a

Optical density, 600nm

Hour after Inoculation
Growth curve *R. albus* 7
Growth curve *R. flavescens* B34b

- Optical density, 600nm
- Hour after inoculation
Growth curve of S. bovis ARD5d.
### APPENDIX G. Protein and purine concentrations and purine to protein ratios in bacterial samples isolated from ewes fed on 550 g of lucerne (Medicago sativa) hay as sole feed or supplemented with 220, 400, or 500 g of barley

<table>
<thead>
<tr>
<th>Diet</th>
<th>Protein*, g/g OM</th>
<th>Purine*, g/g OM</th>
<th>Pur:prot</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.685</td>
<td>0.0225</td>
<td>0.0328</td>
</tr>
<tr>
<td>220</td>
<td>0.664</td>
<td>0.0242</td>
<td>0.0364</td>
</tr>
<tr>
<td>400</td>
<td>0.645</td>
<td>0.0212</td>
<td>0.0329</td>
</tr>
<tr>
<td>550</td>
<td>0.590</td>
<td>0.0153</td>
<td>0.0259</td>
</tr>
<tr>
<td>Mean</td>
<td>0.646</td>
<td>0.0208</td>
<td>0.0320</td>
</tr>
</tbody>
</table>

2Protein: N x 6.67 (Van Soest, 1994).
3Purine: values for adenine and guanine were converted from µmol/g OM to g/g OM by using their correspondents molecular weights (adenine = 135.14 g and guanine = 151.13).
APPENDIX H. Calculations of purine and protein percentage, and purine to protein ratio from fermenter bacteria from the data reported by Calsamiglia et al. (1996)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Purine N, % DM</th>
<th>Purine(^2), % DM</th>
<th>Purine N:total N ratio</th>
<th>Total N(^3)</th>
<th>Protein(^4), % DM</th>
<th>Purine:protein ratio(^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.780</td>
<td>1.589</td>
<td>0.087</td>
<td>8.966</td>
<td>59.800</td>
<td>0.027</td>
</tr>
<tr>
<td>SBM</td>
<td>0.733</td>
<td>1.493</td>
<td>0.089</td>
<td>8.236</td>
<td>54.934</td>
<td>0.027</td>
</tr>
<tr>
<td>LSBM</td>
<td>0.641</td>
<td>1.306</td>
<td>0.080</td>
<td>8.013</td>
<td>53.443</td>
<td>0.024</td>
</tr>
<tr>
<td>CGM</td>
<td>0.607</td>
<td>1.237</td>
<td>0.078</td>
<td>7.782</td>
<td>51.906</td>
<td>0.024</td>
</tr>
<tr>
<td>BM</td>
<td>0.665</td>
<td>1.355</td>
<td>0.081</td>
<td>8.210</td>
<td>54.760</td>
<td>0.025</td>
</tr>
<tr>
<td>HFM</td>
<td>0.684</td>
<td>1.394</td>
<td>0.081</td>
<td>8.444</td>
<td>56.324</td>
<td>0.025</td>
</tr>
<tr>
<td>FM</td>
<td>0.744</td>
<td>1.516</td>
<td>0.087</td>
<td>8.552</td>
<td>57.040</td>
<td>0.027</td>
</tr>
<tr>
<td>MBN</td>
<td>0.683</td>
<td>1.392</td>
<td>0.084</td>
<td>8.131</td>
<td>54.233</td>
<td>0.026</td>
</tr>
<tr>
<td>Mean</td>
<td>0.692</td>
<td>1.410</td>
<td>0.083</td>
<td>8.292</td>
<td>55.305</td>
<td>0.025</td>
</tr>
</tbody>
</table>

\(^1\) Treatment: Control = urea and tryptone; SBM= soybean meal; LSBM= lignosulfonate-treated SBM; CGM= corn gluten meal; BM= blood meal; HFM= hydrolyzed feather meal; FM= fish meal; MBM= meat and bone meal.

\(^2\) Purine , % DM: (purine N % DM x 100) ÷ 49.08.

\(^3\) Total N: Purine N ÷ Purine N:total N ratio.

\(^4\) Protein, % DM: Total N x 6.67.

\(^5\) Purine:protein ratio: Purine % DM ÷ Total protein.
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