ABSTRACT

The predominant ruminal bacteria that were obtained from a 10⁸ dilution of ruminal fluid could be maintained as a mixed population for long periods as long as the bacteria were provided with a complex mixture of carbohydrates. Growth of predominant ruminal bacteria in carbohydrate-limited, ammonia-excess, continuous cultures (0.07/h) had a low requirement for maintenance energy, but the non-growth energy dissipation of ammonia-limited, carbohydrate-excess, predominant ruminal bacteria was approximately 10-fold higher (0.96 vs. 0.09 mg of hexose equivalent/mg of protein per h, respectively). Mathematical derivations indicated that this additional nongrowth energy dissipation could be accommodated by an energy spilling function that was independent of the growth rate. Peptides and amino acids had little impact on the yield of carbohydrate-limited, ammonia-excess, continuous cultures (0.07/h), but amino N greatly increased the growth rate and yield of excess-energy batch cultures. The change in growth rate and yield that was dependent on amino N indicated that the energy-excess batch cultures had the same capacity to spill energy as did the ammonia-limited, carbohydrate-excess, predominant ruminal bacteria (0.80 vs. 0.86 mg of hexose equivalent/mg of protein per h, respectively). When the energy-excess batch cultures were provided with amino N, the growth rate increased, the difference in anabolic and catabolic rates was smaller, and less energy was spilled.

(Key words: ruminal bacteria, peptides, amino acids, energy)

Abbreviation key: CNCPS = Cornell Net Carbohydrate and Protein System, ES = energy spilling, Kd = degradation rate, PRB = predominant ruminal bacteria.

INTRODUCTION

Ruminant feeding systems have generally been based on the assumption that energy should be given first consideration (19), but there is increasing evidence that amino acid supply can, in many cases, limit the production of high producing ruminants (24). Recent work (3) has stressed the importance of escape, or bypass, protein in meeting amino acid requirements, but microbial protein is generally the dominant source of amino acids passing into the small intestine (25). The 1989 NRC (21) recommendations for dairy cattle used a static equation to predict microbial protein flow from the rumen and assumed that ruminal microorganisms always had the same growth efficiency.

Most bacteria can utilize ammonia as a source of N, but many bacteria prefer preformed amino acids (2, 6, 10, 12). The impact of preformed amino acids on the growth efficiency of ruminal bacteria has not been clear-cut. In vitro batch cultures of mixed ruminal bacteria grew 60 to 400% more efficiently when amino acids or peptides, respectively, were provided (1, 17), but the responses of carbohydrate-limited cultures were much less (32) or even negligible (14). Hume (9) noted a 30% increase in microbial flow from the rumen when casein was added to a diet that was based on urea, but ruminally degraded protein has, in many cases, provided no benefit (13, 20, 27).

In the 1970s, Stouthamer (34) estimated the amount of ATP that would be needed to produce bacterial DM and concluded that amino acids should have little, if any, effect on the efficiency of bacterial growth. However, the impact of amino acids on maintenance energy requirements was not considered in those theoretical calculations. Most bacteria grow faster when amino acids are provided (18), and maintenance represents a small proportion of total energy utilization when growth rates are rapid (26).

Recent work with the ruminal bacterium Streptococcus bovis indicated that amino acids, by allowing the cells to more closely match their anabolic and catabolic rates, could decrease wasteful energy spilling (ES) reactions (29). Futive proton cycles of S. bovis can consume as much as 50% of the total ATP.
production (4, 28, 36), but the impact of ES on other ruminal bacteria has not been assessed. The following experiments were designed to ascertain the effect of protein hydrolysates on growth rate, growth efficiency, maintenance, and ES reactions of mixed ruminal bacteria.

MATERIALS AND METHODS

Organisms

Ruminal contents were obtained from a 650-kg, nonlactating, ruminally fistulated dairy cow. Timothy hay was fed at 0800 and 1600 h daily, and water was available for ad libitum intake. Ruminal contents were squeezed through four layers of cheesecloth into an Erlenmeyer flask and allowed to stand for 1 h (anaerobic, 39°C). After gas production had buoyed small feed particles to the top and after protozoa had settled to the bottom, bacteria were anaerobically transferred from the center of the flask to a Hungate tube (Bellco Glass, Inc., Vineland, NJ). The ruminal fluid was diluted 10^8-fold in basal media containing (per liter) 292 mg of K$_2$HPO$_4$, 292 mg of KH$_2$PO$_4$, 480 mg of (NH$_4$)$_2$SO$_4$, 268 mg of NH$_4$Cl, 480 mg of NaCl, 100 mg of MgSO$_4$ $\cdot$ 7H$_2$O, 64 mg of CaCl$_2$ $\cdot$ 2H$_2$O, 4 g of Na$_2$CO$_3$, 600 mg of cysteine-HCl, 250 mg of Na$_2$S, vitamins and minerals (5), 3.28 mg of 2-mercaptoethanesulfonic acid, 5 mmol of acetic acid, 2 mmol of DL-2-methylbutyric acid, 2 mmol of isovaleric acid, 2 mmol of isobutyric acid, 2 mmol of valeric acid, 15 g of Trypticase (BBL Microbiology Systems, Cockeysville, MD), 1680 mg of soluble starch, 700 mg of cellobiose, 420 mg of sucrose, 500 mg of xylose, 500 mg of arabinose, and 200 mg of pectin. *Selenomonas ruminantium* H18 (kindly provided by H. Strobel, University of Kentucky, Lexington), a rapid succinate fermenter, and the methanogens *Methanobrevibacter ruminantium* strain M1 and *M. smithii* strain PS were grown in basal medium without the carbohydrate mixture and with 2 g/L of Trypticase, 2 g/L of yeast extract, 1.3 g/L of formic acid, and 2 g/L of polyoxyethylenesorbitan monooleate under an H$_2$ and CO$_2$ headspace. Carry-over effects of Trypticase were eliminated prior to each growth experiment by transferring the mixed ruminal bacteria twice in media that did not contain Trypticase. Batch incubations for the determination of growth rates and yields were carried out in basal media; the total concentration of mixed carbohydrates was reduced to 2 g/L but was maintained in the same proportions as previously listed. Enzymatic hydrolysates of casein (Trypticase; BBL Laboratories), soybeans (US Biochemical Corp., Cleveland, OH), or gelatin (US Biochemical Corp.) (Table 1) were added in varying concentrations, as indicated in the figures. Continuous cultures were grown anaerobically (0.07/h at 39°C) in a fermentor (F1000; New Brunswick Scientific Co., Edison, NJ) under conditions limited in energy or N. The volume of the culture vessel was 360 ml, and at least a 98% turnover of medium was allowed to pass through the culture vessel before sampling.

Cell Growth

The mixed ruminal bacteria were maintained by transferring the culture every 48 h (20% inoculum) into the basal medium and incubating the bacteria at 39°C until growth ceased. *Selenomonas ruminantium* H18 was grown in basal medium; 1 g/L of Trypticase and 0.5 g/L of yeast extract replaced the vitamins and minerals, and 4 g/L of glucose replaced the carbohydrate mixture. *Methanobrevibacter ruminantium* strain M1 and *M. smithii* strain PS were grown in basal medium without the carbohydrate mixture and with 2 g/L of Trypticase, 2 g/L of yeast extract, 1.3 g/L of formic acid, and 2 g/L of polyoxyethylenesorbitan monooleate under an H$_2$ and CO$_2$ headspace. Carry-over effects of Trypticase were eliminated prior to each growth experiment by transferring the mixed ruminal bacteria twice in media that did not contain Trypticase. Batch incubations for the determination of growth rates and yields were carried out in basal media; the total concentration of mixed carbohydrates was reduced to 2 g/L but was maintained in the same proportions as previously listed. Enzymatic hydrolysates of casein (Trypticase; BBL Laboratories), soybeans (US Biochemical Corp., Cleveland, OH), or gelatin (US Biochemical Corp.) (Table 1) were added in varying concentrations, as indicated in the figures. Continuous cultures were grown anaerobically (0.07/h at 39°C) in a fermentor (F1000; New Brunswick Scientific Co., Edison, NJ) under conditions limited in energy or N. The volume of the culture vessel was 360 ml, and at least a 98% turnover of medium was allowed to pass through the culture vessel before sampling.

Analyses

Optical density was measured at 15-min intervals with a Gilford 260 spectrophotometer (600 nm, 18-mm tubes; Gilford Instrument Lab. Inc., Oberlin, OH). Carbohydrate disappearance from cell-free supernatants was measured by the anthrone method (8). Samples were centrifuged (7500 × g for 20 min at 5°C), and cell-free supernatants were stored at −15°C. Cell pellets were washed twice with 0.9% NaCl, concentrated, and stored at −15°C. Fermentation acids were determined by HPLC with a liquid chromatograph (Beckman 334, Beckman Instruments Inc., Fullerton, CA) equipped with a 156 refractive index detector (Altex Scientific Inc., Berkeley, CA) and an organic acid column (HPX-87H; Bio-Rad Laboratories, Richmond, CA). The sample size was 20 μl, the eluant was 0.0065 M H$_2$SO$_4$, the flow rate was 0.5 ml/min, and the column temperature was 50°C. Cell protein was measured by the method of Lowry et al. (16) after the cell pellets were boiled for 15 min in 0.2N NaOH. Cellular polysaccharide was determined by the anthrone method using glucose as the standard (8).

Statistical Analyses

All samples were performed in duplicate or in triplicate. Simple one-way ANOVA was used to test for statistical significance of mean estimates of cell
### TABLE 1. The AA composition of casein (Trypticase), soybean, and gelatin hydrolysates.

<table>
<thead>
<tr>
<th>AA</th>
<th>Casein</th>
<th>Soybean</th>
<th>Gelatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>5.0</td>
<td>4.3</td>
<td>9.4</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.0</td>
<td>7.5</td>
<td>9.2</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>5.8</td>
<td>11.9</td>
<td>4.4</td>
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<tr>
<td>Cystine</td>
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<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Glutamic acid</td>
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<td>21.2</td>
<td>10.4</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.0</td>
<td>4.4</td>
<td>23.2</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.7</td>
<td>2.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Isoleucine</td>
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<td>4.1</td>
<td>1.8</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.1</td>
<td>7.1</td>
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<tr>
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<td>4.8</td>
</tr>
<tr>
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<td>Phenylalanine</td>
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<tr>
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<tr>
<td>Valine</td>
<td>6.4</td>
<td>4.3</td>
<td>2.5</td>
</tr>
</tbody>
</table>

(\% of total AA)

1Data provided by the manufacturers (casein and gelatin: United States Biochemical Corp., Cleveland, OH; Trypticase: BBL Microbiology Systems, Cockeysville, MD).

The PRB grew rapidly without lag on the mixed carbohydrates, and the growth rate was constant during the 4-h growth period (Figure 1). When the carbohydrates were depleted, the cells stopped growing (data not shown). The specific growth rate increased ($P < 0.05$) when protein hydrolysate was added to the medium (Figure 1), but the response varied when the protein hydrolysates were varied (Figure 2a). Casein and soybean hydrolysates caused a very large increase in the specific growth rate ($P < 0.05$), but the response to gelatin hydrolysate was less ($P < 0.05$). The protein hydrolysates also caused an increase in carbohydrate yield (Figure 2b), and, once again, the response to gelatin was less than the response to casein or Trypticase ($P < 0.05$). Gelatin hydrolysate had a much less balanced amino acid composition than did casein or Trypticase, was deficient in branched-chain amino acids and histidine, and was highly enriched with glycine and proline (Table 1).

There was a positive relationship between specific growth rate and yield; 73% of the variation in yield that was dependent on amino N could be explained by an increase in specific growth rate (Figure 3). At a specific growth rate less than 0.4, the yield was dependent on ammonia N, and yield at specific growth rate of 0 must be 0. When the reciprocal of the carbohydrate yield was plotted against the reciprocal of the specific growth rate using the derivation of Pirt (26),

![Figure 1. Growth of predominant ruminal bacteria in basal medium (39°C); 2 mg of mixed carbohydrates/ml were the energy source, and either NH₃ (●) or NH₃ plus 1 mg of casein hydrolysate/ml (○) was the source of N.](image-url)
the apparent maintenance energy requirement (the slope) was 2.6 mg of carbohydrate/mg of cell protein per h, and the theoretical maximum growth yield (the reciprocal of the intercept) was 0.34 mg of protein/mg of carbohydrate (Figure 4). The carbohydrate yield was confounded because carbohydrates were used as a carbon source for growth, and some of the protein hydrolysate was fermented. The VFA yield more accurately reflected energy availability. The maximum growth yield of VFA yield was similar to the maximum growth yield of the carbohydrate yield (0.30 vs. 0.34 mg of protein/mg of hexose equivalent, respectively), but the value for maintenance energy was 3-fold lower (0.9 vs. 2.6 mg of carbohydrate/mg of cell protein per h, respectively).

When PRB were grown in continuous culture under conditions that limited energy (excess ammonia), the VFA yields were similar to those predicted by Isaacson et al. (11) (Figure 5). Carbohydrate-limited PRB that had an excess of ammonia did not show a response to protein hydrolysate \((P > 0.05)\). When the PRB were grown in continuous culture under ammonia-limiting conditions, the \(1/yield\) value was 4-fold greater than that when PRB were grown in continuous cultures under carbohydrate limitation. These values were near the line predicted by changes in yield and specific growth rate that were dependent on amino N.

**DISCUSSION**

With the advent of continuous culture techniques in the 1950s, it became apparent that bacteria util-
Figure 4. A double reciprocal plot of 1/yield (Y) versus 1/
specific growth rate (μ) for predominant ruminal bacteria showing
changes in growth rate and yield that were dependent on amino N
when casein (○), soybean (●), or gelatin (△, ▲) hydrolysates
were provided. The regression lines were \( y = 2.9 + 2.6x \) (\( r^2 = 0.73 \))
and \( y = 3.5 + 0.90x \) (\( r^2 = 0.58 \)) for carbohydrate yield (closed
symbols) and VFA yield (open symbols), respectively. The VFA
yield is expressed as hexose equivalents. The assumption is that 2
acetate, 2 lactate, 2 propionate, 2 succinate,
or 1 butyrate can be
derived per hexose. The dotted line represents the theoretical maxi-
mum growth yield and maintenance values of Isaacson et al. (11).

Figure 5. A double reciprocal plot of 1/VFA yield (Y) versus 1/
specific growth rate (μ) showing changes in growth rate and yield
that were dependent on amino N for predominant ruminal bacteria
when casein (○), soybean (●), or gelatin (△) hydrolysates were
provided (see Figure 4). The VFA yields of slow-growing (0.07/h),
carbohydrate-limited (●), or carbohydrate-excess (x) predominant
ruminal bacteria are also shown. Slow-growing, carbohydrate-excess
predominant ruminal bacteria were limited by ammonia. The dotted line shows the values of Isaacson et al. (11).

When Isaacson et al. (11) grew mixed ruminal
bacteria in liquid phase continuous cultures, they
observed an m value of 0.047 mg of hexose fermented/
mg of cells per h, and the \( Y_G \) was 0.50 mg of cells/mg
of glucose fermented. Carbohydrate-limited continu-
ous cultures of PRB had yields that were similar to
those reported by Isaacson et al. (11), and under
these conditions, protein hydrolysate had little effect
on yield (Figure 5).

Coefficients for maintenance energy are typically
measured under carbohydrate-limiting conditions, but
work with the nonruminal bacterium *Klebsiella aerogenes*
indicated that cultures with sufficient car-
bohydrates had inflated m (22, 23). The variation in
maintenance energy was a lively subject in the 1970s
(35, 37), and the terms “overflow metabolism”, “slip
reactions”, “uncoupling”, and “ES” were all used as
hypothetical explanations of energy dissipation. Re-
cent work (30) has indicated that ES can be caused
by futile cycles of potassium, ammonium, or protons
through the cell membrane. Because standard text-
books of biochemistry routinely indicate that “cells
are capable of regulating their metabolic reactions
and the biosynthesis of their enzymes to achieve max-
mum efficiency and economy” [page 12; (15)], the
importance of ES was only recently recognized.
Energy spilling appears to be a phenomenon that can

occur any time bacteria have an excess of energy, and spilling reflects an imbalance of catabolic and anabolic rates (30). Ammonia-limited, continuous cultures of PRB had a much lower yield than did carbohydrate-limited PRB, and this difference indicated that PRB had the capacity to spill energy (Figure 5).

Based on the observation that the apparent \( m \) of ammonia-limited cultures was higher (Figure 5), the basic yield equation could be modified:

\[
\frac{1}{Y} = \left( \frac{m}{\mu} \right) + \left( \frac{1}{Y_G} \right),
\]

to include an ES function:

\[
\frac{1}{Y} = \left( \frac{m}{\mu} \right) + \left( \frac{ES}{\mu} \right) + \left( \frac{1}{Y_G} \right),
\]

where ES is defined as the slope of the carbohydrate-excess Pirt plot minus the slope of cells limited in energy (\( m \)). The carbohydrate-excess batch cultures grew less efficiently when they were deprived of amino N, and the ES based on amino N deprivation was similar to the ES based on ammonia limitation (0.80 vs. 0.86 mg of carbohydrate/mg of protein per h, respectively) (Figure 5).

Energy-excess PRB always had lower yield than the PRB that were limited in energy, but this difference could be modified by the amount and type of N (Figure 6a). When specific growth rate was being regulated by the amount of ammonia, the impact of ES was very great, and additional ammonia caused a very large increase in yield. The response to amino N was not as great, and yield only increased when the degradation rate (\( K_d \)) of the carbohydrate was greater than the \( m \) allowed by ammonia. These observations are consistent with the idea that ES is due to an imbalance of anabolic and catabolic rates (30).

The Cornell Net Carbohydrate and Protein System (CNCPS) uses \( m \) and \( Y_G \) to predict the yield of ruminal bacteria (31). All ruminal bacteria are assigned the same \( Y_G \), but the \( m \) of bacteria fermenting nonstructural carbohydrates is 3-fold greater than the \( m \) of bacteria fermenting structural carbohydrates (0.3 and 0.1 mg of carbohydrate/mg of protein per h, respectively). In this regard, the yield of bacteria that ferment nonstructural carbohydrates is discounted by a higher \( m \), but these bacteria are stimulated by amino N availability. The CNCPS predicts \( m \) from the first-order \( K_d \) of carbohydrate:

\[
\mu = K_d,
\]

but the effect of N on \( \mu \) is ignored. If N is limiting, then

\[
\mu < K_d.
\]
The CNCPS adjusts the yield of the bacteria that ferment nonstructural carbohydrates with an empirical function of peptide and amino acid stimulation, but this function stimulates yield even at very low Kd (Figure 6b). Because amino N only increased the yield of PRB when carbohydrate was in excess and when the specific growth rate permitted by ammonia was less than Kd (Figure 6a), the CNCPS might be overpredicting the benefit of amino N when the rate of carbohydrate fermentation is low (Figure 6b). The modification of the CNCPS to include an ES term might provide a basis for estimating the microbial growth even if total N is restricted.

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REFERENCES
