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Growth inhibition of the rumen bacterium *Selenomonas ruminantium* by ammonium salts

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Summary. The objective of this study was to determine the maximum ammonium source concentration tolerated by *Selenomonas ruminantium* and its metabolic response to high ammonium source concentrations. The ammonia-nitrogen half-inhibition constant (K_i) in defined basal medium was 239 mM for NH_4Cl, 173 mM for NH_4OH, 153 mM for $(NH_4)_2SO_4$ and 110 mM for NH_4HCO_3. Reduction in continuous culture maximal growth rate was similar to the reduction in the batch culture logarithmic growth rate for the respective NH_4Cl concentrations. Cell yield when expressed as Y_{ATP} decreased for 150 and 200 mM NH_4Cl. The NH_3-N K_i estimates are in line with inhibiting concentrations observed for other bacteria and suggest that energy efficiency is reduced when the NH_3-N concentration is increased.

Introduction

The consequences of high rumen ammonia-nitrogen (NH_3-N) concentration in ruminants have been extensively documented (National Academy of Sciences 1976). Un-ionized NH_3 is absorbed from the reticulorumen as well as the omasum, small intestine and cecum. When the rate of NH_3-N absorption from the digestive tract exceeds the capacity of the liver to convert it to urea, NH_3-N accumulates in the blood and is potentially lethal to the animal. Rumen NH_3-N concentrations have been reported as high as 92 mM in sheep (Hungate 1966), yet a rumen fluid NH_3-N concentration of 57 mM is listed as toxic to the animal. Only a few studies have yielded results on bacterial responses to NH_3-N concentrations greater than an optimal minimum concentration. Kang-Meznarich and Broderick (1981) reported a trend toward reduced bacterial yields as NH_3-N increased from 10 mM to 21 mM. However, Hume et al. (1970) found no decrease in microbial protein yield at NH_3-N concentrations up to 22 mM nor did Leibholz and Kellaway (1980) at NH_3-N concentrations up to 23 mM. Likewise, Slyter et al. (1979) observed no microbial response to 16 mM NH_3-N but obtained an increase in total volatile fatty acid (VFA) concentration and animal nitrogen retention. Satter and Slyter (1974) did not detect significant changes in in vitro microbial protein and VFA production from the point of initial NH_3-N accumulation (1 mM) to the highest NH_3-N concentration employed (57 mM).

Nothing is known of individual rumen bacterial activities as NH_3-N increases to the upper levels tolerated by the animal. For our studies we have focused on the rumen microorganism *Selenomonas ruminantium* because it is a prominent species in the rumen and actively cross-feeds with some of the functionally important bacterial species including cellulolytics and methanogens. The purpose of the present study was to determine an NH_3-N half-inhibition constant (K_i) for *S. ruminantium* and evaluate the metabolic response within this estimated range of NH_3-N concentrations.

Materials and methods

Organism and media. S. ruminantium subsp. *lactilytica* strain HD_4 (HD_4) was obtained from M. P. Bryant (University of Illinois, Urbana, Ill., USA) and maintained as described previously (Ricke et al. 1988). The anaerobic technique described by Bryant (1972) was used for all manipulations during the studies. A basal medium was used for all studies (Schaefer et al. 1980; Ricke and Schaefer 1990), except that 0.5 gm·l^{-1} of yeast extract (YE; Difco Laboratories, Detroit, Mich., USA) replaced *p*-amino benzoic acid and biotin in all continuous culture incubations and some batch culture incubations.

Batch culture experiments and continuous culture apparatus. The salt concentrations tested ranged from 0 to 600 mM and included 16 separate concentrations of each salt. *S. ruminantium* HD_4 has

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the ability to use the medium reductant cysteine as its sole nitrogen source. Therefore, *S. ruminantium* inocula were pre-adapted to the absence of added ammonium salt via two serial transfers of 0.1 ml inoculum to 4.0 ml basal medium followed by overnight growth periods. Three incubations, each consisting of triplicate tubes, non-agitated in a 37° C waterbath, were completed for each salt treatment. Culture growth was monitored on a Spectronic 70 spectrophotometer (Bausch and Lomb, Rochester, N.Y., USA) as absorbance at 600 mm (A_{600}) with a light path length of 10 nm. Dry weight and A_{600} values of *S. ruminantium* HD_4 increased (data not shown) linearly ($r=0.88$) through an A_{600} range of 0.2 to 1.4. All A_{600} values recorded here fall within this range and therefore were taken to be true biomass responses. Batch culture maximum specific growth rate was calculated by taking the slope of the linear portion of the natural log-transformed optical density versus time curve.

The chemostat apparatus was constructed and operated as described by Mink and Hespell (1981) with the following modification. The growth chamber was a five-port pyrex glass vessel with a three-way pyrex teflon stopcock molded to it as an effluent port. A constant temperature of 38.5° C was maintained in the growth vessel. The sequence of NH_4Cl concentration treatments was 0, 150, 200 and 100 mM and nine dilution rates ranging from 0.1 to 0.65 h^{-1} were randomly selected within each NH_4Cl treatment. Dilution rates for each NH_4Cl concentration were statistically pooled into four ranges (0.10 ± 0.04; 0.18 ± 0.03; 0.29 ± 0.04 and 0.65 ± 0.03 h^{-1}). In the presence of supplementary NH_4Cl, glucose was the growth-limiting nutrient. The growth vessel was initially inoculated at 0 mM NH_4Cl from a batch culture pre-adapted to that medium. The apparatus was left intact and the culture was maintained in the growth vessel for the entire series of NH_4Cl concentration treatments. The maximal growth rate in the chemostat was calculated as a function of wash-out (Jannasch 1969). Chemostat samples for metabolite analysis were collected by diverting the effluent flow to a container immersed in ice and constantly flushed with CO_2.

Samples were obtained following filtration of effluent through a 0.45-μm pore size membrane filter. Filtered samples for all liquid chromatographic analyses were acidified with 50 μl of 36 N sulphuric acid per millilitre (Brotz and Schaefer 1987). Duplicate injections of each sample were made on a 300 × 7.8 mm HPX-87H Aminex ion exclusion column. Standard curves for glucose, succinate, lactate, formate, acetate, propionate, butyrate and valerate were linear ($R^2=99.9\%$) for concentrations from 0.5 to 10 mM. Steady-state growth was verified by frequent monitoring of effluent optical density and samples were never taken before three volume turnovers. Cell dry weights were obtained by harvesting cells after overnight growth then fixing them with 0.8% formal saline (Schaefer et al. 1980).

Results and discussion

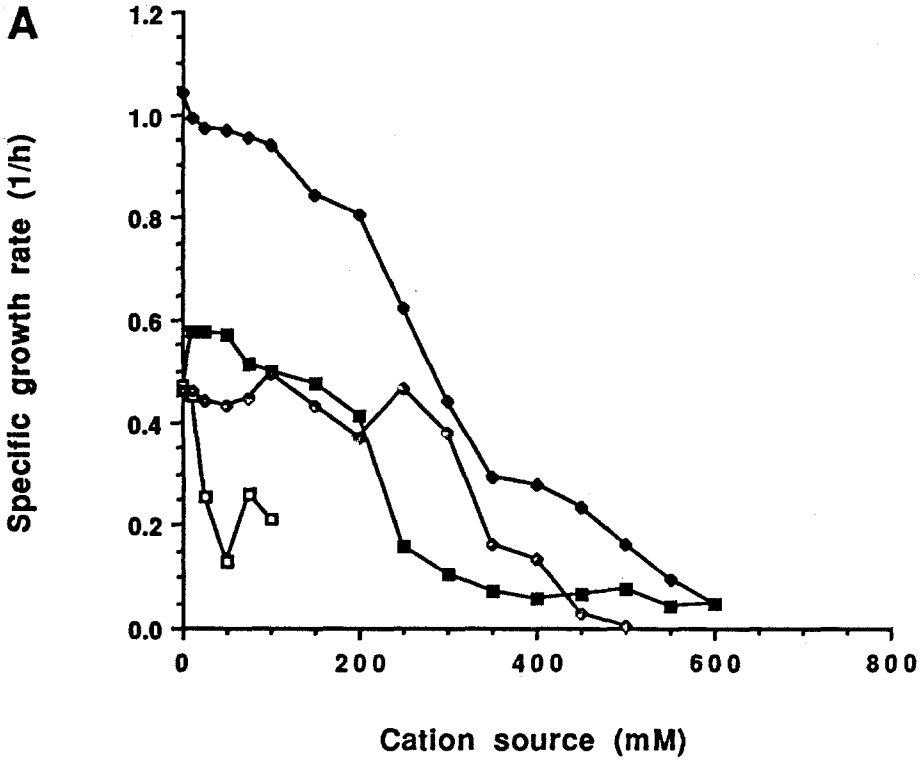
Determination of a half-inhibition constant

An NH_3-N half-inhibition constant (K_i) was determined from the relationship between ammonium source concentration and growth rate of HD_4. K_i is defined as the substrate concentration associated with the half-maximal specific growth rate in the descending leg of the overall substrate concentration versus growth rate relationship (Powell 1965). It was decided to test ammonium source concentrations above 100 mM to determine the concentrations at which K_i and complete inhibition of growth occurred. Primary emphasis was placed upon NH_4Cl, but the importance of anion species was addressed by substituting $(NH_4)_2SO_4$, NH_4OH or NH_4HCO_3 as the NH_3-N source. The initial medium pH (6.40 to 6.70) was not altered by adding high amounts of any of the ammonium-anion combinations except for NH_4OH additions, which increased the pH from 6.7 to 7.2 between 150 and 200 mM and to 8.2 at 300 mM, and NH_4HCO_3, which increased the pH to 6.85 and 7.00 for 50 and 75 mM, respectively.

The plots for medium, cation and anion effects on growth rate are shown in Fig. 1A and B. Maximal optical density (data not shown) began to decline at approximately 150 to 200 mM for all treatments. Bacterial growth in 100–200 mM NH_4Cl accounted for a utilization of 4–6 mM NH_3-N. Visual inspection of the figures reveals that the growth rate was halved around 300 mM for NH_4Cl in YE medium and NaCl, 200 mM for NH_4Cl, and 100 mM for CH_3NH_2Cl. Among the various ammonium anions, bicarbonate appeared to be most inhibitory (100 mM) with sulfate and hydroxide appearing to be somewhat less inhibitory (200 mM).

To statistically compare the growth responses of HD_4 to different basal media, cations and anions, a K_i value was calculated from each data set. The approach taken was to regress growth rate directly on salt concentration for each replicate incubation, omitting all concentrations that completely inhibited growth. Estimates of K_i obtained with this approach are presented in Table 1. They reveal that complexity of the culture medium and the anion associated with ammonium influence NH_3-N toxicity. Strain HD_4 was least sensitive ($P<0.05$) to NH_4Cl in YE medium and NaCl in defined (DF) medium, and K_i estimates for NaCl and NH_4Cl in DF medium were not different. The three ammonium-anion combinations (SO_4^{-2}, OH^-, and HCO_3^-) were more inhibitory than NH_4Cl, and HD_4 was most sensitive to CH_3NH_2Cl ($P<0.005$).

Basal medium containing YE doubled the growth rate of HD_4 and increased its tolerance to NH_4Cl. This observation highlights the importance of defining environmental conditions in which NH_3-N toxicity is being quantified. Control treatments for the chloride anion of NH_4Cl were NaCl and CH_3NH_2Cl. These controls were appropriate because rumen bacteria are regarded as slight halophiles (Caldwell and Hudson 1974) and preliminary evidence with CH_3NH_2Cl had shown it not to affect growth rate of *S. ruminantium* strain D at a ratio of 1 mM NH_4Cl to 10 mM CH_3NH_2Cl (data not shown). The higher and lower K_i estimates of these two respective control treatments indicate that the K_i estimate for NH_4Cl was not solely a consequence of its chloride anion. The high concentration of NaCl tolerated by HD_4 agrees with the results of Caldwell and Hudson (1974). The much lower K_i estimate for CH_3NH_2Cl is consistent with the results of Servin-Gonzalez et al. (1987), who found that 100 mM CH_3NH_2Cl caused complete inhibition of nitrogen-limited *Escherichia coli* growth. The lower K_i estimates for the other ammonium-anion combinations suggest and interaction between NH_4^+ and its counter ion. Addition of OH^- and HCO_3^- in combination with NH_4^+ increased the medium pH, which presumably caused formation of the more toxic NH_3 species. Koster and Koomen (1988)



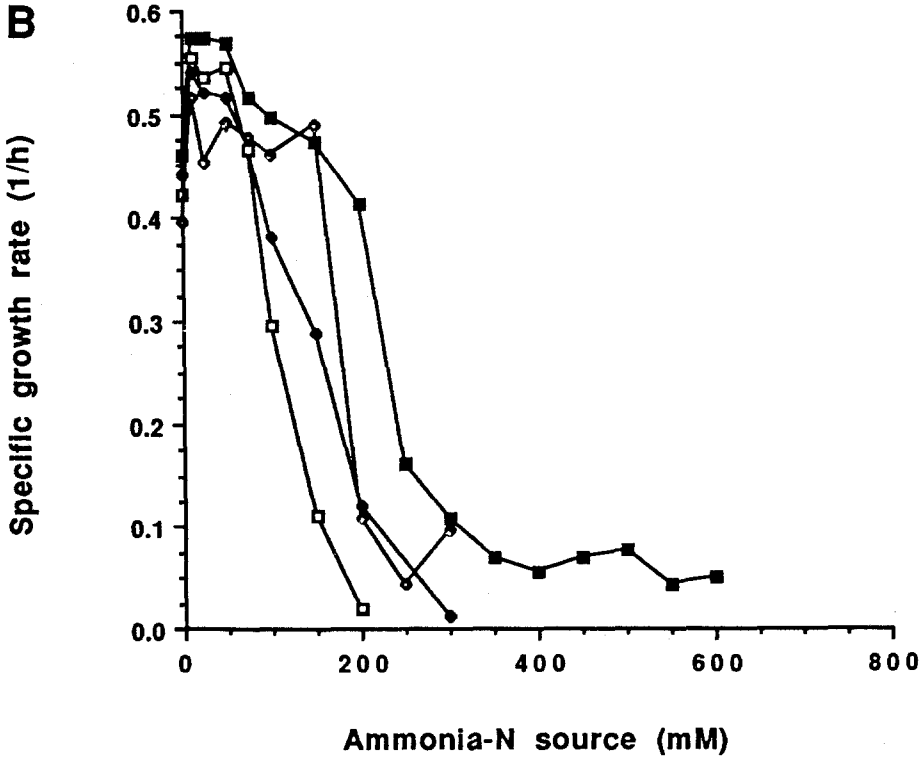


Fig. 1 A,B. Effects of medium, cation and anion on batch culture specific growth rates of *Selenomonas ruminantium* HD_4. Each point is the average of three incubations of triplicate tubes. The NH_4Cl data are the same for both plots. The culture medium did not contain yeast extract (YE) unless indicated otherwise. **A** Medium and cation effects on growth rate: ◆, YE + NH_4Cl; ■, NH_4Cl; □, CH_3NH_2Cl; ◇, NaCl. **B** Anion effects on growth rate: ■, NH_4Cl; ◆, $(NH_4)_2SO_4$; □, NH_4HCO_3; ◇, NH_4OH

observed that increasing the pH from 7.0 to 7.8 appeared to enhance NH_3-N inhibition of the maximal growth rate of hydrogenotrophic methanogens. An optimal upper pH for selenomonad maximum growth has not been reported. The lower K_i for $(NH_4)_2SO_4$ is probably not due to pH since the initial medium pH remained fairly constant (6.55 to 6.70) through the entire range of $(NH_4)_2SO_4$ additions. Sprott and Patel (1986) found equivalent inhibitions for chloride and sulfate in the presence of equimolar NH_3-N in growth studies with any of their NH_4Cl-sensitive methanogens. Therefore, either the selenomonad strain is more sensitive to sulfate than chloride anions or ammonium and sulfate represent an antagonistic combination of ions.

Table 1. Estimates of ammonia half-inhibition constant (K_i) for *Selenomonas ruminantium* HD_4 in batch culture

Treatment	Medium[a]	K_i(mM)[b]
NH_4Cl	YE	302[c]
NaCl	Basal	275[c,d]
NH_4Cl	Basal	239[d]
$(NH_4)_2SO_4$	Basal	153[e]
NH_4OH	Basal	173[e]
NH_4HCO_3	Basal	110[e,f]
CH_3NH_2Cl	Basal	86[f]

[a] See text for description
[b] Standard error of the mean = ±13
[c,d,e,f] Means with unlike superscripts differ ($P<0.005$)

Table 2. Effect of ammonium chloride on maximum growth rate in batch versus continuous cultures of *S. ruminantium* HD_4

NH_4Cl concentration (mM)	Batch culture (h^{-1})	Continuous culture (h^{-1})
0	1.04±0.01[a,b]	1.22±0.06[e,f]
100	0.94±0.02[c]	1.06±0.12[e,f]
150	0.84±0.02[d]	1.32±0.12[f]
200	0.81±0.02[d]	0.87±0.09[g]

[a] Standard error of the mean ($n \geq 2$)
[b,c,d] Means in the same column with unlike superscripts differ ($P<0.001$)
[e,f,g] Means in the same column with unlike superscripts differ ($P<0.05$)

Effect of NH_4Cl on maximum growth rate estimated in batch and continuous cultures

Estimates of maximum specific growth rate determined in batch and continuous culture systems are shown in Table 2. The control treatment was YE medium with no added NH_4Cl. Comparing the respective growth rate maxima, both batch culture and continuous culture estimates followed comparable decreases as NH_4Cl concentration was increased, except for 150 mM where continuous culture growth rate was increased above the control. These results are consistent with the earlier batch culture results (Fig. 1). For the same NH_4Cl concentration, maximum growth rates in continuous culture were faster ($P<0.006$) than those in batch culture.

Effect of NH_4Cl on cell metabolism in continuous culture

Since steady-state conditions could not be achieved at 400 mM and the K_i was approximately 200 mM, concentrations of 0, 100, 150, and 200 mM NH_4Cl were chosen. At dilution rates less than or equal to 0.29 h^{-1}, there was complete utilization of the available glucose (data not shown). Increases in dilution rate resulted in increased lactate, and decreased acetate, propionate and succinate concentrations ($P<0.05$). This relationship between dilution rate and fermentation product pattern has been reported previously for *S. ruminantium* (Scheifinger et al. 1975; Wallace 1978).

The effects of NH_4Cl on HD_4 metabolism are shown in Table 3. More glucose disappeared as NH_4Cl was increased to 100 and 150 mM, but then decreased when NH_4Cl was increased to 200 mM ($P<0.05$). Lactate and acetate concentrations were not influenced by NH_4Cl concentration nor were there noteworthy effects of NH_4Cl concentration on propionate and succinate. An $NH_4Cl \times$ dilution rate interaction ($P<0.0005$) was seen with propionate concentration, which decreased as the dilution rate was increased, but less so for all NH_4Cl levels above 0 mM NH_4Cl (data not shown). Cell dry weight concentration was reduced for NH_4Cl concentrations above 100 mM. The data for carbon recovery and redox balance indicate that oxidized carbon com-

Table 3. Effect of NH_4Cl on metabolite concentration, cell yield and energy partitioning in continuously cultured *S. ruminantium* HD_4

Metabolite	NH_4Cl (mM)			
	0	100	150	200
	(mM)			
Glucose disappearance	12.9 ±0.4[a,b]	14.2 ±0.5[b,c]	14.9 ±0.6[c]	11.5 ±0.5[d]
Lactate	11.4 ±0.9	10.1 ±1.1	14.9 ±1.4	11.0 ±1.0
Acetate	4.4 ±0.3	4.9 ±0.4	4.8 ±0.5	4.5 ±0.4
Propionate	2.9 ±0.2[b]	2.8 ±0.2[b]	2.6 ±0.3[b,c]	2.1 ±0.2[c]
Succinate	2.0 ±0.3[b]	4.1 ±0.4[c]	2.7 ±0.5[b]	4.3 ±0.4[c]
Cell dry weight (g/l)	0.41±0.02[b]	0.47±0.02[c]	0.36±0.03[b]	0.36±0.02[b]
Carbon recovery (%)	78.6 ±1.7[b]	77.2 ±2.1[b]	81.6 ±2.7[b]	99.2 ±2.0[c]
Redox balance[e]	0.4 ±0.1[b]	1.2 ±0.2[c,d]	1.0 ±0.2[b,c]	1.6 ±0.2[d]
$Y_{glucose}$[f]	32.1 ±1.1[b]	33.2 ±1.4[b]	24.6 ±1.7[c]	32.3 ±1.2[b]
Y_{ATP}[g]	14.6 ±0.4[b]	14.4 ±0.5[b]	11.0 ±0.6[c]	11.6 ±0.5[c]

[a] Standard error of the mean
[b,c,d] Means in the same row with different superscripts differ ($P<0.05$)
[e] Quotient for oxidized/reduced fermentation products
[f] Grams of dry cells/mole of glucose used
[g] Grams of dry cells/mole of ATP produced; moles ATP/mole acid (De Vries et al. 1970): 1, lactate; 2, acetate; 2, propionate; and 2, succinate; *S. ruminantium* forms propionate via the randomizing pathway (Paynter and Elsden 1970)

pound(s), e.g. CO_2, are missing at the 0 mM NH_4Cl concentration and reduced compound(s), e.g. H_2, are missing at 100 and 200 mM NH_4Cl. The ratio of oxidized to reduced products was determined by coding (Wolin 1960) the products as follows: succinate, +1; propionate, −1; and lactate and acetate, 0; multiplying each by its respective concentration and then dividing the sum for oxidized products by the absolute value of the sum for reduced products.

Energy partitioning can be assessed under steady-state conditions by determining the amount of energy diverted away from growth to maintenance functions. The maintenance energy coefficient (Pirt 1965) was calculated as the slope of the relationship between reciprocal dilution rate and reciprocal $Y_{glucose}$ or Y_{ATP}. However, this approach failed because the double reciprocal transformation yielded a poor linear relationship ($R^2 < 0.30$ for $Y_{glucose}$ and $R^2 < 0.90$ for Y_{ATP}) between dilution rate and either $Y_{glucose}$ or Y_{ATP}. Therefore, we elected not to report maintenance energy coefficients for these data sets and rely on the actual cell yields as an indicator of microbial energy status for the different NH_3-N concentrations. $Y_{glucose}$ was only decreased ($P < 0.001$) for 150 mM NH_4Cl and all were considerably less than the theoretical maximum growth yield of 105 g cells · mol^{-1} glucose for HD_4 reported by Russell and Baldwin (1979). The 150 and 200 mM NH_4Cl concentrations lowered the Y_{ATP} ($P < 0.0002$) below the values calculated for 0 and 100 mM NH_4Cl, and all were less than the theoretical maximum yield of 26 g cells · mol^{-1} ATP calculated for HD_4 (Russell and Baldwin 1979).

In conclusion, *S. ruminantium* is tolerant to at least twice the highest reported rumen NH_3-N concentration. This does not rule out different NH_3-N toxicities and/or differences in requirements for tolerance by some rumen organisms. Rumen NH_3-N concentration is not only known to be subject to rumen sampling location, time of sampling and rumen fluid volume (Wohlt et al. 1976) but may be quite different in rumen fluid versus microhabitats surrounding a feed particle (Allison 1980; Hoover 1986). Obviously, a wider survey of rumen organisms, especially comparisons between attached and unattached organisms, is required. It is also apparent that increasing NH_3-N influences energy efficiency in this strain of *S. ruminantium* such that a detectable decrease in cell yield is observed. It follows that such a shift in rumen microbial metabolism due to changing NH_3-N concentration, if prevalent in the microbial population at lower NH_3-N concentrations, would have a significant bearing on the host animal's interaction with its rumen products.

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