Studies on the Nitrogen Requirements of Some Ruminal Cellulolytic Bacteria

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Knowledge of the nitrogen compounds utilized for growth of ruminal cellulolytic bacteria is of considerable importance to an understanding of the nitrogen economy of ruminants and of factors affecting ruminal cellulose digestion.

Work with mixed cultures of bacteria from the rumen has shown that cellulose digestion occurs with ammonia or urea, which rapidly yields ammonia, as the sole source of nitrogen (Burroughs *et al.*, 1951). These workers and others showed that cellulose digestion was stimulated if protein was included and that ammonia or urea stimulated cellulose digestion above that obtained with protein as nitrogen source. Others have shown that certain amino acids can replace the protein in stimulation of cellulose digestion. These included valine, leucine, and isoleucine (MacLeod and Murray, 1956), valine, leucine, isoleucine, and proline (Dehority *et al.*, 1957) and proline, alanine, and methionine (Trenkle, Cheng, and Burroughs, 1957). Hall *et al.* (1954) obtained stimulation of cellulose digestion above that obtained with ammonia with materials believed to be peptides, whereas Bentley *et al.* (1954) obtained similar results with xanthine, uracil, guanine, and adenine. In a survey of nitrogen compounds replacing urea as the sole source of nitrogen for cellulose digestion by the mixed population, Belasco (1954) found that amides of certain monocarboxylic acids, guanidine, creatinine, creatine, uric acid, and allantoin were quite active.

In work with mixed cultures, it was never certain whether or not the nitrogen compounds stimulating cellulose digestion had a direct effect on cellulolytic organisms. The work of MacLeod and Murray (1956) and Dehority *et al.* (1957, 1958) indicated that volatile fatty acids such as *n*-valeric plus isovaleric acids could replace the amino acids when urea was the only nitrogen source.

Both groups of workers suggested that the amino acids were stimulatory because they were converted by other ruminal organisms to the active volatile fatty acids. Indeed, work with pure cultures indicates that many cellulolytic strains of the genus *Ruminococcus* and *Bacteroides succinogenes* require volatile fatty acids such as isovaleric or isovaleric plus *n*-valeric acids for growth even when supposedly adequate levels of amino acids are available (Allison, Bryant, and Doetsch, 1958; Bryant and Doetsch, 1955); and it is also known that other ruminal microorganisms produce these acids from amino acids (El-Shazly, 1952; Dehority *et al.* 1958; Bladen, Bryant, and Doetsch, 1961) and *n*-valerate can be produced from carbohydrate (Elsden *et al.*, 1956).

Although a considerable number of pure cultures of cellulolytic bacteria of undoubted significance in the rumen have been isolated in recent years, relatively little work has been done on their nitrogen requirements for growth. Fletcher (1956) and Ayers (1958) considered amino acids (casein hydrolyzate) to be essential for growth of the type strain of *Ruminococcus albus* and a strain of *Ruminococcus flavefaciens*, respectively, when grown in media also containing ammonia. The latter strain also required adenine and guanine. Allison, Bryant, and Doetsch (1959) found that very little UL-C^{14} chorella protein hydrolyzate was incorporated into cells of *R. flavefaciens* strain C94, grown with ammonia as the main source of nitrogen, and suggested that the organism synthesizes most of its amino acids in preference to utilization of exogenous preformed amino acids. Unpublished data of Allison indicate that this strain will grow with ammonia as the sole source of nitrogen. *B. succinogenes* did not require any amino acid for growth, grew quite well with only cysteine and ammonia present in the medium, and required ammonia for growth even in the presence of 19 amino acids, purines, and pyrimidines (Bryant, Robinson, and Chu, 1959). Gill and King (1958) found no absolute requirement for any single amino acid by a cellulolytic strain of the genus *Butyrivibrio* but no growth occurred when cysteine was the only amino acid available. Quite good growth was obtained with only histidine, isoleucine, methionine, lysine, cysteine, tyrosine, and valine available. It was of interest that large amounts of ammonia were assimilated and this was not appreciably affected by the amount of casein hydrolyzate in the medium.

The purpose of the present work was to explore the nitrogen requirements of strains of *R. albus* and *R. flavefaciens* and to obtain further information on the nitrogen compounds utilized for growth of *B. succinogenes*.

Materials and Methods

Except as indicated below, materials and methods were as described elsewhere (Bryant and Robinson, 1961). Unless otherwise indicated, the organisms used were *R. albus* strain 7, *R. flavefaciens* strain C94, and *B. succinogenes* strain S85.

The anaerobic solution used to prepare washed inoculum was modified to contain Na_2SO_3 in place of $(NH_4)_2SO_4$.

All experimental media were as shown in table 1 of Bryant and Robinson (1961) except that nitrogen-containing ingredients with the exception of B-vitamins were varied. In the first part of this work (tables 1 and 2 and figures 1 and 2), the nitrogen-containing ingredients were sterilized separately, combined in the appropriate mixtures, freed of O_2, and added to the tubed medium in either 1- or 2-ml amounts to bring the medium to final volume (5 ml) and concentration of ingredients. The B-vitamins were added to the medium before it was sterilized.

Individual solutions of L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-histidine·HCl·H_2O, L-isoleucine, L-lysine·HCl, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-

TABLE 1

Effect of ammonia on the growth response of Ruminococcus albus and Ruminococcus flavefaciens in media containing 17 amino acids, purines, and pyrimidines

Amount of Each Amino Acid	$(NH_4)_2SO_4$	Growth (OD × 100)	
		R. albus	*R. flavefaciens*
%			
0.01*	Added	91 (19†)	53 (46)
0.01	Added‡	90 (19)	48 (48)
0.01	Deleted‡	3 (41)	4 (43)
0.02	Added‡	75 (21)	50 (48)
0.02	Deleted	6 (41)	5 (46)

* Cysteine·HCl·H_2O, 0.05 and 0.10 per cent, for 0.01 and 0.02 per cent levels of amino acids, respectively. All media contained 1.0 mg per 100 ml each of adenine·SO_4, guanine·HCl, uracil, xanthine, and thymine.

† Hours of incubation to reach maximal OD.

‡ Na_2SO_4 was substituted for $(NH_4)_2SO_4$ in an equimolar amount.

TABLE 2

Effect of some nitrogen sources on growth of some strains of Ruminococcus albus and Ruminococcus flavefaciens

Strains	Growth (OD × 100) in Medium No.:*				
	1	2	3	4	5
Ruminococcus albus					
D89	97 (18†)	4 (26)	95 (18)	100 (20)	100 (19)
B199	69 (42)	9 (44)	69 (42)	92 (61)	100 (57)
$B_3$37	67 (40)	7 (25)	69 (39)	56 (76)	57 (33)
20	40 (58)	3 (94)	65 (49)	45 (60)	70 (47)
Ruminococcus flavefaciens					
$B_1$46	44 (45)	6 (42)	50 (45)	60 (56)½	55 (42)
FDI	34 (137)	4 (66)	44 (95)	20 (137)	24 (95)
C94	39 (71)	6 (42)	56 (60)	60 (50)	61 (42)

* Medium 1 contained 17 amino acids (0.01 per cent of each except for 0.05 per cent of cysteine·HCl·H_2O), purines, and pyrimidines (as in table 1) and $(NH_4)_2SO_4$ as nitrogen sources. Medium 2 was medium 1 but with Na_2SO_4 in place of $(NH_4)_2SO_4$. Medium 3 was medium 1 minus purines and pyrimidines. Medium 4 was medium 3 minus amino acids. Medium 5 was medium 3 with casein hydrolyzate in place of amino acids.

† Hours of incubation to reach maximal OD.

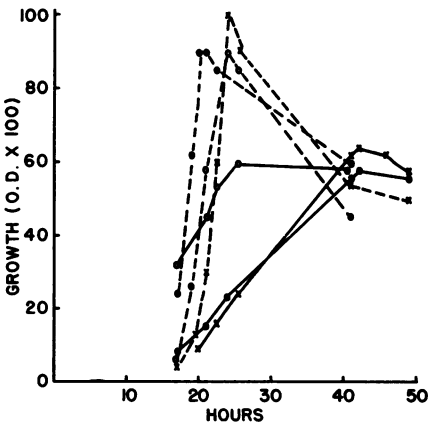

Figure 1. An experiment on the growth response of *Ruminococcus albus* (– – –) and *Ruminococcus flavefaciens* (——) in media with ammonia as nitrogen source (×), ammonia plus 0.01 per cent of each of 16 amino acids and 0.05 per cent of cysteine·HCl·H_2O (●), and ammonia plus casein hydrolyzate (○).

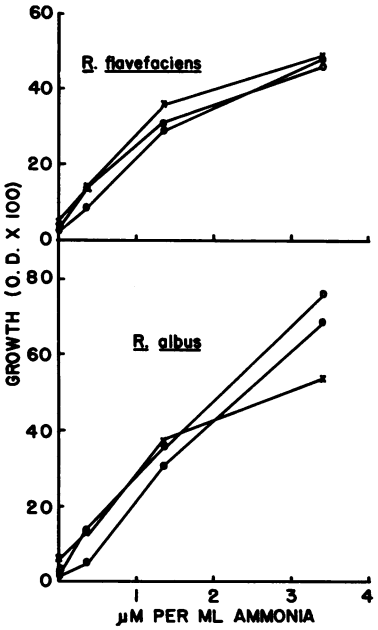

Figure 2. An experiment on the growth response of *Ruminococcus albus* and *Ruminococcus flavefaciens* to different concentrations of ammonia in media containing 0.02 per cent (w/w) of each of 16 amino acids and 0.1 per cent of cysteine·HCl·H_2O (×), half this amount of amino acids (○), and no amino acids (●).

tryptophan, L-valine, further purified "vitamin-free" acid hydrolyzed casein (Bryant and Robinson, 1961), glutathione, $(NH_4)_2SO_4$, and KNO_3 were prepared in screw-cap bottles, autoclaved at 15 lb for 5 min, and held in the refrigerator. Adenine·SO_4 and guanine·HCl were dissolved in 1 N HCl and uracil, xanthine, and thymine were dissolved in 1 N KOH and sterilized as above. Solutions of asparagine, glutamine, and urea were sterilized by filtration through sintered glass or a Millipore filter.

Various mixtures of the nitrogen compounds were freed from oxygen by heating in a boiling water bath and then equilibrating with CO_2 before being added to tubes of medium. The heat-labile materials, urea, asparagine, and glutamine were equilibrated well with CO_2 without heating to drive out O_2.

In later work, 0.01 per cent of each of the amino acids, other than cysteine (table 3 and figure 4), and Trypticase (table 4 and figure 3) were added to the media before they were sterilized.

The specific nitrogen-containing constituents of media are given in the Results.

In the determination of ammonia and "protein" nitrogen of cultures, aliquots were diluted with 20 per cent trichloroacetic acid (TCA) to a final concentration of 5 per cent TCA. After overnight refrigeration, the material was centrifuged at about 6000 × *g* for 10 min and ammonia nitrogen was determined on the supernatant fluid. The precipitate was washed twice with 5 per cent TCA and the pellet was suspended in 6 N HCl, sealed into the tube with a flame, and placed in an oven overnight at 105 to 110 C to render the nitrogen soluble. Total nitrogen was determined utilizing the digestion and nesslerizing procedure outlined by Umbreit, Burris, and Stauffer (1957). Ammonia nitrogen was determined by microdiffusion in Conway units from saturated K_2CO_3 to 0.1 N H_2SO_4 and nesslerizing as above. Cell nitrogen was determined on cells centrifuged and washed twice in a solution containing 0.85 per cent NaCl and 0.2 per cent formaldehyde. The method of Umbreit *et al.* (1957) was used. Aliquots of cultures were analyzed for ammonia nitrogen and cell or protein nitrogen when they had reached approximately 0, ¼, ½, ¾, and maximal growth as measured by optical density (OD).

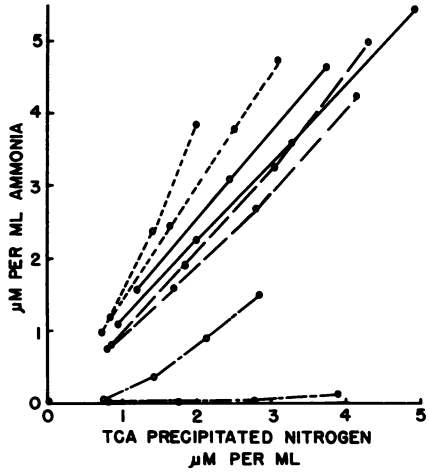

Figure 3. Relation of ammonia nitrogen uptake and 5 per cent TCA-precipitated nitrogen produced during growth in media containing high and low levels of enzymatic hydrolyzate of casein (Trypticase). *Ruminococcus albus* (——); *Ruminococcus flavefaciens* (-----); *Bacteroides succinogenes* (– – –); *Streptococcus bovis* (– – –). One per cent Trypticase medium (●); 0.1 per cent Trypticase medium (○).

Results

Results in figure 1 and table 2 confirm the unpublished results of Allison (1958) indicating that *R. flavefaciens* strain C94 utilizes ammonia as its sole source of nitrogen other than the small amount of nitrogen from B-vitamins and show that all other strains of ruminococci studied had this characteristic.

Growth of most strains was better, particularly as judged by the time required to reach maximal OD, when the medium contained casein hydrolyzate. However, growth was often quite good without either casein hydrolyzate or amino acids. The difference in time required to reach maximal growth appeared to be due mainly to lag rather than the growth rate in the logarithmic phase (figure 1).

Figure 1 also shows the rapid drop in OD after the maximum has been reached as indicated elsewhere (Bryant and Robinson, 1961).

Experiments shown in tables 1 and 2 show that ammonia was essential for growth of all strains of ruminococci studied even in the presence of 17 amino acids, purines, and pyrimidines. The small amount of growth obtained when no ammonia was added to the media can be attributed to the small amount of ammonia always present in media as a contaminant. Much of this was introduced as a contaminant of amino acid solutions. Previous experiments had resulted in a

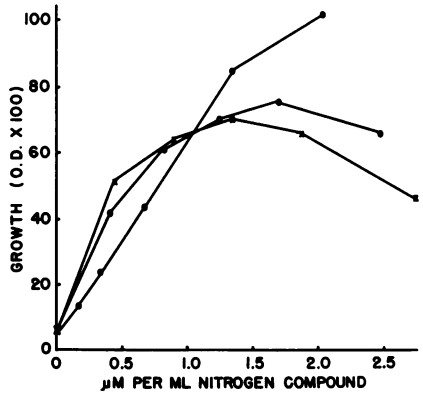

Figure 4. An experiment on the growth of *Bacteroides succinogenes* in media containing 0.01 per cent of each of 17 amino acids and different concentrations of NH_3 (●), glutamine (○), and asparagine (×).

similar demonstration of an ammonia requirement for *B. succinogenes* (Bryant *et al.*, 1959).

Experiments indicated that about 4 μM per ml NH_3 (added as $(NH_4)_2SO_4$) were required for optimal growth as measured by OD of both *R. albus* and *R. flavefaciens* when different concentrations were added to medium 3 (table 2) minus $(NH_4)_2SO_4$.

TABLE 3

Effect of various single nitrogen compounds on growth of Ruminococcus flavefaciens strain C94, Ruminococcus albus strain 7, and Bacteroides succinogenes in a basal medium containing 0.01 per cent of each of 17 amino acids and a growth limiting concentration (1 μM per ml) of ammonia

Nitrogen Compound	Growth (OD × 100)		
	R. flavefaciens	*R. albus*	*B. succinogenes*
*Experiment 4:**			
None	21 (30†)	20 (29)	73 (21)
$(NH_4)_2SO_4$, 7 μM	60 (41)	80 (27)	105 (25)
Tryptophan, 2 μM	26 (34)	23 (24)	73 (24)
Tryptophan, 4 μM	23 (41)	24 (25)	33 (37)
Experiment 5:			
None	24 (24)	20 (23)	70 (20)
$(NH_4)_2SO_4$, 7 μM	55 (27)	89 (24)	108 (21)
Uracil, 2 μM	21 (24)	18 (22)	50 (19)
Uracil, 4 μM	5 (19)	18 (22)	37 (20)
Xanthine, 2 μM	21 (25)	14 (22)	50 (19)
Thymine, 2 μM	21 (24)	23 (24)	48 (18)
Thymine, 4 μM	18 (41)	15 (22)	35 (20)
Adenine, 2 μM	2 (19)	20 (41)	15 (50)
Adenine, 4 μM	1 (19)	3 (24)	2 (24)
Asparagine, 2 μM	22 (24)	18 (22)	95 (24)
Asparagine, 4 μM	23 (24)	18 (22)	97 (25)
Experiment 6:			
None	22 (27)	21 (24)	66 (17)
Glutamine, 2 μM	28 (27)	28 (24)	110 (23)
Glutamine, 4 μM	31 (27)	30 (24)	110 (22)

* Other compounds tested that had no apparent effect on growth at either the 2 or 4 μM level were as follows: experiment 1, glutamic acid, aspartic acid, arginine, and alanine; experiment 2, glycine, histidine, lysine, methionine, and leucine; experiment 3, phenylalanine, threonine, valine, isoleucine, and proline; experiment 4, serine, cysteine, glutathione, and KNO_3; experiment 5, urea.

† The figures in parentheses refer to the hours of incubation required to obtain maximal OD.

TABLE 4

Some data on ammonia nitrogen fixed and cell nitrogen produced during growth of Ruminococcus flavefaciens, Ruminococcus albus, Bacteroides succinogenes, and Streptococcus bovis in a medium containing ammonia and 1 per cent Trypticase as nitrogen sources

R. flavefaciens		*R. albus*		*B. succinogenes*		*S. bovis*	
Cell-N	NH_3-N	Cell-N	NH_3-N	Cell-N	NH_3-N	Cell-N	NH_3-N
μM per ml							
0.45	1.16	1.02	1.28	0.27	0.70	0.88	0.02
1.21	2.38	2.48	2.51	1.06	1.66	2.77	−0.24
1.81	3.38	3.84	4.18	1.99	2.98	3.81	0.01
2.78	4.65	4.80	4.66	3.64	5.43	4.46	0.02

It was evident (figure 2) that similar and quite large concentrations of ammonia were essential to a certain level of growth regardless of the concentration of amino acids. The slightly increased growth in media containing amino acids was due to the contamination of the amino acids with ammonia. If the α-amino nitrogen of only one of those amino acids present in the lowest molar concentration (0.48 μM per ml) had been utilized in place of ammonia, it should have resulted in an increase of a minimum of 0.1 OD units in the medium with the low level amino acids as compared to the medium with none.

The results (figure 2) also show that the high level of amino acids (0.02 per cent of each) and ammonia inhibited growth of *R. albus*. This inhibition of growth by the higher level of amino acids was more evident in both species at all three levels of ammonia when time to reach maximal OD was considered. The values were 32 and 40 hr, 28 and 41 hr, and 38 and 67 hr for *R. albus* and *R. flavefaciens* at the 0, 0.01, and 0.02 per cent levels of amino acids, respectively, at the 3.4 μM per ml level of ammonia. Similar differences between the cultures with the high level of amino acids and those with the low level or no amino acids were evident at the 0.34 and 1.36 μM per ml concentration of ammonia.

Results of another series of experiments (table 3) showed that the growth of neither the ruminococci nor *B. succinogenes* was stimulated by various single additions of larger amounts of nitrogen compounds other than ammonia to medium containing a growth-limiting amount of ammonia and 0.01 per cent of each of 17 amino acids. Exceptions to this were the apparent stimulation of *B. succinogenes* by the amides, glutamine and asparagine. The results also showed that some of the nitrogen compounds were quite inhibitory to growth in the concentrations used. These concentrations were such that approximately maximal growth of the ruminocci would have been obtained if one mole of nitrogen had been available per mole of the higher level of compound added.

Data in figure 4 show that a good growth response of *B. succinogenes* was obtained to low levels of glutamine and asparagine when no ammonia was added to the medium but growth never was as abundant at higher levels of amide as with ammonia. Also, growth on amides was much slower than on ammonia. The points of maximal OD shown in figure 4 were reached in 16 to 18 hr for ammonia, 32 to 37 hr for glutamine, and 56 to 67 hr for asparagine. The difference between glutamine and ammonia was due both to a longer lag in growth and a slower logarithmic growth rate on glutamine, whereas the difference between amides was due mainly to a longer lag before growth on asparagine.

The results to this point (tables 1, 2, and 3 and figure 2) indicated that *R. albus*, *R. flavefaciens*, and *B. succinogenes* had a large growth requirement for ammonia and that many other nitrogen compounds could not replace it. It was suggested that the organisms could not utilize organic nitrogen for growth. To obtain further evidence on this point, attempts were made to directly determine ammonia uptake and cell nitrogen produced during growth.

Results in table 4 show that, with *R. albus*, cell nitrogen produced was approximately equivalent to ammonia nitrogen fixed in a medium containing a high level of amino acids and peptides. With *R. flavefaciens* and *B. succinogenes*, the amount of ammonia fixed considerably exceeded the apparent cell nitrogen produced. However it was noted that there was often a considerable drop in turbidity of the cells of *B. succinogenes*, suggesting lysis during the centrifugation and washing procedure. This was believed to explain much of the low values obtained for cell nitrogen as compared to ammonia uptake, at least with this organism.

Streptococcus bovis was included as a control which, it was assumed, would utilize amio acid and/or peptide nitrogen in preference to ammonia nitrogen during growth. It is known that this organism does not produce ammonia from amino acids (Niven, Smiley, and Sherman, 1942) but is capable of utilizing ammonia as its sole source of nitrogen for growth (Wolin, Manning, and Nelson, 1959; Prescott, Williams, and Ragland, 1959). The data (table 4) indicate that the assumption was correct.

Further experiments were done in which TCA-precipitable nitrogen was determined in place of cell nitrogen and both high amino acid (1.0 per cent Trypticase[1]) and low amino acid (0.1 per cent Trypticase) growth media were used (figure 3).

In the case of the cellulolytic organisms, ammonia nitrogen fixed exceeded the TCA-precipitable nitrogen produced and this occurred regardless of the amino acid level. On the other hand, the control organism, *S. bovis*, did not fix an appreciable amount of ammonia nitrogen except in the low amino acid medium.

The results (table 4 and figure 3) suggest that *R. flavefaciens* may have excreted considerable non-ammonia nitrogen because ammonia fixed was considerably greater than either cell nitrogen or TCA-precipitable nitrogen synthesized.

Discussion

The eight strains of ruminococci studied, five of *R. albus* and three of *R. flavefaciens*, are representative of 10 strains studied previously for vitamin requirements (Bryant and Robinson, 1961). Five other strains could not be grown in media of known composition.

The facts that all strains studied required ammonia for growth in the presence of purines, pyrimidines, and 17 amino acids and could utilize ammonia as the sole source of nitrogen, other than that in B-vitamins, suggest that this genus is much more homogeneous in nitrogen requirements than in other characteristics such as B-vitamin requirements (Bryant and Robinson, 1961), fermentation products, and other characteristics (Bryant, 1959). However, it is possible that some of the five strains that could not be grown in media of known composition have more exotic nitrogen requirements. Also, the work of Fletcher (1956) and Ayers (1958) suggested that a strain of *R. albus* in one of *R. flavefaciens*, respectively, required amino acids (protein hydrolyzates) for growth. The latter strain also required adenine and guanine for growth, whereas no other strains of ruminal cellulolytic bacteria so far studied are even stimulated by these compounds.

It is of considerable interest that many strains of the heterogeneous group of cellulolytic bacteria that appear to be of most significance in the rumen, that is, the ruminococci, *B. succinogenes*, and members of the genus *Butyrivibrio*, appear to have similar and relatively simple nitrogen requirements. All strains of *B. succinogenes* so far studied are similar to the ruminococci in that ammonia was utilized as the sole source of nitrogen and appeared to be essential for growth (Bryant *et al.*, 1959). No single amino acid was essential for growth of a strain of *Butyrivibrio* and a large amount of ammonia was assimilated regardless of the amino acid level in the growth medium (Gill and King, 1958). No attempt was made to determine whether this organism could be grown in the absence of ammonia.

The following facts show that *B. succinogenes*, *R. albus*, and *R. flavefaciens* cannot utilize nitrogen from many exogenous, preformed compounds for synthesis of quantitatively important and essential cellular constituents which are synthesized with utilization of ammonia nitrogen. (a) Ammonia was essential for growth of ruminococci (tables 1 and 2) and *B. succinogenes* (Bryant *et al.*, 1959) in media containing amino acids, purines, and pyrimidines; (b) similar and high levels of ammonia were essential for growth of ruminococci regardless of the level of an amino acid mixture added to the medium (figure 2); (c) large amounts of many single nitrogen sources did not significantly stimulate growth of ruminococci or *B. succinogenes* above that obtained by a growth-limiting concentration of ammonia in a medium containing amino acids (table 3); and (d) ammonia fixation was approximately equal to or was greater than cell nitrogen or TCA-precipitable nitrogen synthesized when determined at four different times during growth in media

[1] Baltimore Biological Laboratory, Inc., Baltimore, Maryland.

containing both high and low levels of amino acids and peptides (figure 3 and table 4). The finding of Bladen *et al.* (1961) that, of many strains of ruminococci and *B. succinogenes* tested, none produced ammonia from protein hydrolyzate tends to substantiate this conclusion, as do the results of Allison *et al.* (1959) indicating that *R. flavefaciens* strain C94 incorporated into cell protein only a very small amount of C^{14} from protein hydrolyzate (2.7 per cent of that incorporated by *Escherichia coli* for a similar amount of growth in the same medium except that glucose was substituted for cellobiose).

These facts also make it seem highly likely that all but possibly a very small amount of cell nitrogen of these organisms is synthesized from exogenous ammonia even when large amounts of exogenous, preformed organic nitrogen is present in the growth medium. It is possible but doubtful that the strains convert ammonia to other nitrogen compounds but utilize mainly other preformed organic nitrogen, if available, for cell synthesis. Direct experimental evidence on this possibility must involve experiments with compounds containing N^{15}.

Although few species of microorganisms have been studied for this characteristic, the authors have found reference to no species other than the present ruminal species and ruminal strains of *Lactobacillus bifidus* (Phillipson, Dobson, and Blackburn, 1959) that appear to utilize ammonia in preference to a mixture of exogenous amino acids for a major part of their cell nitrogen. The present study indicates that *S. bovis*, another organism common to the rumen, prefers mixtures of amino acids and/or peptide. Roberts *et al.* (1957) have shown that *E. coli*, *Torulopsis utilis*, and *Neurospora crassa* utilize the carbon of preformed amino acids in preference to synthesizing them from glucose carbon and, in competition experiments between single N^{14}-amino acids and N^{15}-ammonia, it was indicated that a large share of cell nitrogen in *E. coli* came from amino acids such as aspartic acid and alanine. Warner (1956) showed that another strain of *E. coli* preferentially utilized N^{14}-lysine rather than N^{15}-ammonia for formation of cell lysine even when 96 per cent of the nitrogen of the medium was in the form of ammonia. It would be of interest to determine whether or not some of the autotrophic bacteria prefer to synthesize amino acids from ammonia rather than utilizing preformed amino acids.

Cellulolytic bacteria utilizing ammonia rather than amino acids for cell nitrogen synthesis would appear to be well adapted to survival in the rumen. The rumen almost always contains relatively large amounts of ammonia and the level of free amino acids and peptides is almost always low and falls rapidly within 2 or 3 hr after the animal is fed (Annison and Lewis, 1959). Bacteria utilizing energy sources more readily available than cellulose, for example *S. bovis*, may assimilate most of the soluble organic nitrogen. The ability to use preformed amino acids for growth may have been lost by the cellulolytic organisms because it was of little value for their survival in the rumen.

Although data in table 3 and figure 4 indicate that *B. succinogenes* can utilize the nitrogen of glutamine and asparagine for growth, it is possible that these compounds as such are not metabolized. It is well known that the amide nitrogen of these compounds is easily converted to ammonia by nonenzymatic reactions and, perhaps, this ammonia accounts for the growth obtained. However, two points suggest that this is not the case. (a) Control experiments indicated that only 0.33 μM per ml of ammonia was produced in 66 hr in the uninoculated medium (figure 4) containing 1.64 μM of glutamine, whereas growth corresponding to over 1.2 μM of ammonia uptake in 37 hr occurred when the medium was inoculated. If nonspecific materials produced during growth allowed ammonia production from glutamine at a faster rate than in the uninoculated medium, it is surprising that the ruminococci did not also respond to glutamine (table 3). (b) The amide nitrogen of asparagine is much more stable than glutamine yet the organism grew at similar logarithmic growth rates on the two compounds suggesting that the nitrogen became available at similar rates probably via enzymatic reactions.

Growth curves of *B. succinogenes*, grown in basal medium (table 3) with glutamine, glutamine plus limited ammonia (1 μM), or ammonia only added, suggested that the organism utilized ammonia preferentially. In the medium containing both nitrogen sources the growth rate was similar to that with ammonia only until growth slightly in excess of that obtained with 1 μM of ammonia only was obtained. At this point the growth rate decreased to that of glutamine. From the position of the break in the growth curve, it appeared that only about 0.16 μM of glutamine nitrogen was used during the use of 1.0 μM of ammonia. Similar results were obtained with asparagine except that the break in the growth curve more closely approximated that of 1 μM of ammonia only.

When 1 μM per ml of ammonia was added to the medium (table 3), a higher concentration of glutamine or asparagine (4 μM per ml) did not limit growth nearly to the extent of that obtained when no ammonia was added (figure 4). A possible explanation of this was that decomposition products of the amides might be inhibitory rather than the amides as such and these products were not present in critical amounts in the ammonia-containing medium because of the preferential utilization of ammonia or because of the shorter time interval for growth.

A comparison of the growth response of *B. succino-*

genes to the amides and ammonia (figure 4) suggest that both amide and amino nitrogen of the amides are utilized. However, more definitive experiments on this point are needed.

ACKNOWLEDGMENT

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SUMMARY

All strains of ruminococci studied, three of *Ruminococcus flavefaciens* and five of *Ruminococcus albus*, were shown to grow with ammonia as the sole source of nitrogen other than that in B-vitamins. Ammonia was essential for their growth in the presence of 17 amino acids, 3 purines, and 2 pyrimidines. Similar amounts of ammonia were necessary for growth of *R. albus* strain 7 and *R. flavefaciens* strain C94 regardless of the amounts of amino acids in the medium. None of many single nitrogen compounds tested would increase growth of these strains above that obtained with a growth-limiting concentration of ammonia (1 μM per ml) in a medium also containing 17 amino acids. These compounds included 17 amino acids, 3 purines, 2 pyrimidines, glutathione, NO_3, urea, asparagine, and glutamine. *Bacteroides succinogenes* strain S85, previously shown to require ammonia in the presence of amino acids, grew with the latter two compounds added as nitrogen sources in place of ammonia but grew at a slower rate than with ammonia.

Experiments on strains 7, C94, and S85 indicated that similar amounts of ammonia were fixed during growth in media containing high and low levels of amino acids and peptides (Trypticase) and the amount of ammonia fixed was about equal to or greater than the amount of cell nitrogen or trichloroacetic acid-precipitable nitrogen synthesized. A control organism, *Streptococcus bovis*, did not fix an appreciable amount of ammonia except on the low amino acid medium.

The results make it seem likely that *B. succinogenes*, *R. albus*, and *R. flavefaciens*, important digesters of cellulose in the rumen, synthesize all but possibly a very small amount of their cellular nitrogen compounds from exogenous ammonia even when large amounts of preformed organic nitrogen are present in the growth medium.

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A Slit Sampler for Collecting T-3 Bacteriophage and Venezuelan Equine Encephalomyelitis Virus

I. Studies with T-3 Bacteriophage

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Continuous sampling of air to detect pathogens is essential in the conduct of hazardous operations and in monitoring infectious disease wards, and could have significance in instances of massive air pollution. Sampling devices employing liquid collecting media for the impingement of air-borne microorganisms, although quite satisfactory for laboratory use in the sampling of air for short periods of time, do not meet the requirements for satisfactory sampling over long periods. Problems associated with liquid impingers are (a) evaporation of the collecting fluid, (b) the relatively small volume of air passing through the sampler limits these devices to sampling of relatively highly concentrated aerosols, and (c) the high air velocity is detrimental to the survival of certain organisms after 10 to 15 min of operation (Shipe, Tyler, and Chapman, 1959; Tyler, Shipe, and Painter, 1959). These disadvantages have been overcome in the sampling of bacterial aerosols by the use of slit samplers in which the organisms are impacted directly upon a solid collecting medium which also serves as a nutrient during incubation of the sample. Many studies have shown these samplers to be highly efficient in the collection and quantitation of bacterial cells and spores in aerosols of low to moderate concentrations (Bourdillion, Lidwell, and Schuster, 1948; DuBuy, Hollaender, and Lackey, 1945; Decker and Wilson, 1954; Kuehne and Decker, 1957; and Decker *et al.*, 1958). Sampling times and air volumes are both increased markedly over the maximum obtainable with liquid impingers.

If a suitable solid medium of sufficiently low melting point to permit liquefaction and dilution at temperatures tolerated by the virus could be used, the slit sampler would possess the theoretical capability of sampling viral aerosols.

It is the purpose of this paper to report on (a) the ability of the slit sampler to recover aerosolized virus particles when using gelatin collecting media, (b) the comparison of the slit sampler with the all-glass impinger for collection of aerosolized virus particles, (c) the effect of culture plate aeration on sampling virus aerosols with the slit sampler, and (d) the use of the slit sampler for simultaneous collection of bacterial and viral aerosols by modifying a standard culture plate and using two different collecting media.

MATERIALS AND METHODS

The slit sampler (figure 1) developed by Decker and Wilson (1954) was used in these studies to sample T-3 bacteriophage aerosols for 1-hr periods. This sampler is operated by drawing air by vacuum through the slit opening in the top of the sampler at a rate of 1 cu ft per min. The virus particles in the air are impacted on the surface of a solid gelatin medium contained in a plastic culture plate (150 by 20 mm), which is rotated at a uniform rate beneath the slit by a timer mechanism located in the base of the sampler. A 1-hr timer, which allowed the plate to make 1 revolution per hr, was used in this sampler.

Aerosolization of T-3 bacteriophage. *Escherichia coli* bacteriophage T-3 suspensions were produced in Petri dishes on a solid F medium (Adams, 1959). The lysates were harvested from the surfaces of the agar plates by washing with a few milliliters of distilled water. The lysates were purified by centrifugation at moderate