Effect of ammonia concentration on rumen microbial protein production in vitro

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1. The effect of ammonia concentration on microbial protein production was determined in continuous-culture fermentors charged with ruminal contents obtained from steers fed on either a protein-free purified diet, a maize-based all-concentrate diet, or a forage–concentrate (23:77) diet. Urea was infused into the fermentors to maintain various concentrations of ammonia in the incubating mixtures.

2. Under nitrogen-limiting conditions, microbial protein yield measured as tungstic acid-precipitable N (TAPN) increased linearly with supplementary urea until ammonia started to accumulate in the incubating ingesta. Increasing the ammonia concentration beyond 50 mg NH_3-N/l had no effect on microbial protein production.

3. The molar proportions of volatile acids produced were not affected by the level of urea supplementation. Total acid production was decreased slightly under N-limiting conditions, but not to the same extent as microbial protein production.

4. Estimated yield of microbial dry matter/mol ATP produced averaged 15·6 when non-limiting N as urea was provided with the purified diet.

5. These results suggest that addition of non-protein N supplements to ruminant rations are warranted only if the prevailing concentration of ruminal ammonia is less than 50 mg NH_3-N/l ruminal fluid.

It has long been recognized that supplemental non-protein nitrogen is not well utilized in ruminant rations containing relatively large amounts of protein or non-protein N, or rations which are low in digestible energy. What is not known is the level of dietary protein below which addition of non-protein N to the diet improves animal performance, or above which it has little or no benefit. The dietary protein level at which point further addition of non-protein N to the ration ceases to be of benefit varies with the digestible energy content of the ration and the extent to which dietary protein is degraded to ammonia. The total amount of non-protein N that can be utilized will depend upon the amount of fermentable energy available for microbial growth and the amount of ammonia derived from dietary and salivary sources.

It can reasonably be assumed that dietary non-protein N will be of little benefit to the ruminant unless it is first converted into ammonia, and then utilized for microbial protein synthesis in the rumen. If this is so, it is then important to know what concentration of ruminal ammonia is necessary for maximal microbial growth. Maintenance of ruminal ammonia concentration in excess of the bacterial requirement would be unnecessarily costly.

The purpose of this study was to determine the concentration of ammonia necessary for maximal growth of rumen micro-organisms. This would allow a definitive understanding of when to expect benefit from non-protein N supplementation of ruminant diets varying in protein and digestible-energy content.

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Table 1. *Compositions of the three diets given to steers and added to continuous-culture fermentors*

| | Diet | | |
Ingredient (g/kg)	Purified (Expts 1, 2, 3)	Concentrate (Expts 4, 5)	Forage–concentrate (Expts 6, 7)
Cracked maize	—	929	453·9
Starch (soluble)	300	—	—
Cerelose	300	—	244
Molasses (dry)	—	51	68
Lucerne hay (ground)	—	—	113
Timothy hay (ground)	—	—	113
Wood pulp	320	—	—
Trace mineral salt	—	10	8
Dicalcium phosphate	—	10	—
Mineral mix*	60	—	—
Refined soya-bean oil	20	—	—
Sodium sulphate	—	—	0·1
(mg/kg)			
Retinol	52·8	6	10·56
Cholecalciferol	0·55	0·0625	0·11
DL-α-tocopheryl acetate	210	—	—

* See Oltjen, Slyter, Williams & Kern (1971).

EXPERIMENTAL

Six continuous-culture fermentors, similar to those described by Slyter, Nelson & Wolin (1964), were used to simulate rumen conditions. The fermentors were charged with 500 ml rumen fluid obtained through a rumen fistula from a steer given a diet similar to the mixture added to the fermentor. The rumen fluid was strained through two layers of cheesecloth before it was introduced into the vessels.

Three different substrate mixtures were used, as shown in Table 1. The purified diet was used in Expts 1, 2 and 3. A total of 17·0 g of substrate dry matter (DM) was put into the fermentor daily. In order to maintain as constant a fermentation as possible, the soluble components (starch and cerelose) of this ration were mixed with the buffer solution and infused continuously. The balance of the ration, containing wood pulp, minerals, soya-bean oil and vitamins, was added in two equal portions of 3·40 g each at 08.00 and 17.00 hours. In addition, 22 ml of a urea solution were infused continuously to supply a crude protein equivalent of 40–328 g/kg total ration.

The concentrate diet was used in Expts 4 and 5, and was added four times daily, with 3·23 g diet DM being given at 08.00, 12.30 and 17.00 hours, and 4·83 g at 23.00 hours. Since none of the ingredients of the concentrate diet could be infused, more frequent feeding was needed in these experiments to reach steady-state conditions approaching those of Expts 1, 2 and 3. In addition, 22 ml urea solution were infused continuously to attain a crude protein equivalent of 113–282 g/kg total ration.

A forage–concentrate diet was used in two experiments. A total of 14·6 g of diet DM was added daily to the fermentor. The cerelose and sodium sulphate were infused continuously, and 2·4 g of the remaining ingredients were added at 08.00, 12.30 and

Table 2. *Composition (g/l) of stock buffer solutions used in the continuous-culture fermentors*

Ingredient	High pH	Low pH
Na_2CO_3	6·2	—
$NaHCO_3$	—	4·9
K_2CO_3	8·03	—
$KHCO_3$	—	5·85
$Na_2HPO_4.12H_2O$	4·65	—
$NaH_2PO_4.H_2O$	—	1·8
K_2HPO_4	2·25	—
KH_2PO_4	—	2·04
NaCl	0·47	0·47
KCl	0·57	0·57
$MgCl_2.6H_2O$	0·1278	0·1278
$CaCl_2.2H_2O$	0·0001	0·0001
Distilled-water	to 1 l	to 1 l

17.00 hours, and 3·65 g at 23.00 hours. Varying amounts of urea were infused in 22 ml water to attain a crude protein equivalent of 74–333 g/kg total ration.

Different buffer solutions were used for each level of urea infused to offset the effect of increasing ammonia concentration and to maintain a similar pH. This was achieved by mixing the high- and low-pH stock buffer solutions, shown in Table 2, in proportions ranging from 0 to 100 % of low-pH buffer, with percentage of low-pH buffer increasing as crude protein content of the fermentor substrate increased. Sixty vol. of mixed stock buffer were diluted to 100 vol. with tap water and infused into the fermentor at the rate of 760 ml/d.

Each experiment lasted for 9 d. Effluents from the first 3 d were discarded, for this was considered to be a transition period. Analyses of effluent were made on composite samples obtained on days 4 and 5, days 6 and 7, and days 8 and 9. Similarly, gas production was not recorded during the first 3 d, but the gas was collected and measured every 2 d thereafter. It appeared that some transitional changes were still taking place on days 4 and 5, particularly in those fermentors receiving supplementary N at low levels, therefore only results obtained from days 6, 7, 8 and 9 are reported.

Microbiological methods

The numbers of entodiomorph protozoa were determined (Slyter & Putnam, 1967) in Expt 4 from culture contents collected at 10.00 hours on day 8 and the numbers of cellulolytic bacteria were determined in samples collected at 11.00 hours on day 8 in Expt 2. The cellulolytic bacteria assay was similar to that described by Slyter, Kern, Weaver, Oltjen & Wilson (1971). Dilutions of 10^{-7}, 10^{-8} and 10^{-9} were tested.

Analytical methods

Ammonia (Conway, 1950) and volatile fatty acid (Baumgardt, 1964) determinations were made on the supernatant fraction of centrifuged effluent. Total protein in the effluent was measured as tungstic acid-precipitable N (TAPN) (Shultz & Shultz, 1970). Gas collected from the fermentors was analysed with a Model 29 Fisher-

Table 3. *Proportion of volatile fatty acid (VFA) (mol/100 mol VFA), total production of VFA (mmol/d), methane production (mmol/d) and tungstic acid-precipitable nitrogen (TAPN) production (mg/d) during incubation of rumen contents of steers given a purified, concentrate or forage–concentrate diet in a continuous fermentor*

(Mean values and standard deviations)

	Diet					
	Purified (Expts 1, 2, 3)		Concentrate (Expts 4, 5)		Forage–concentrate (Expts 6, 7)	
End-product	Mean	SD	Mean	SD	Mean	SD
Proportion of total VFA						
Acetate	49·4	4·6	55·9	1·3	56·9	4·6
Propionate	29·2	3·8	27·5	2·6	22·3	4·8
Isobutyrate	0·05	0·1	0·4	0·2	0·3	0·3
Butyrate	16·0	4·3	9·8	0·2	14·5	6·2
Isovalerate	0·2	0·5	4·8	0·9	2·8	2·2
Valerate	3·9	2·0	1·4	0·5	2·4	0·8
Total VFA*	68·4	3·3	91·2	4·5	67·0	4·9
Total VFA (mol/kg organic matter added to fermentor)*	4·4	0·2	6·5	0·3	4·8	0·3
Methane*	16·0	1·8	18·9	2·0	17·9	0·5
TAPN*	252	12	238	40	251	55

* Only results obtained from fermentors with > 50 mg NH_3-N/l effluent were used in calculating these values.

Hamilton gas partitioner (Fisher Scientific, 7722 Fenton St, Silver Spring, Maryland) containing a di-2-ethylhexylsebacate (30 % DEHS on 60–80 mesh Chromosorb P, Fisher Scientific) and a molecular sieve (filled with 42–60 mesh molecular sieve 13X, Fisher Scientific) column attached to a thermal conductivity cell detector.

RESULTS

The molar proportions of volatile fatty acids produced as well as total acid production are shown in Table 3. Total acid production values in Table 3 were calculated only from those fermentors that maintained NH_3-N > 50 mg/l. In all experiments, the final pH of all cultures, which was measured at 10.30 hours on day 9, was between 6·0 and 7·0.

Methane production when NH_3-N > 50 mg/l was 16·0, 18·9 and 17·9 mmol/d with the purified, concentrate and forage–concentrate diets, respectively. The amount of TAPN in the fermentor effluents was approximately equal for all diets when NH_3-N was > 50 mg/l, but was more variable for the natural diets.

Fig. 1 shows the relationship between crude protein content of the mixture added to the fermentor (DM basis), the TAPN output of the fermentor and ammonia concentration of the incubation mixture. Each point is an average of observations obtained from one fermentor during the last 4 d of a 9 d incubation.
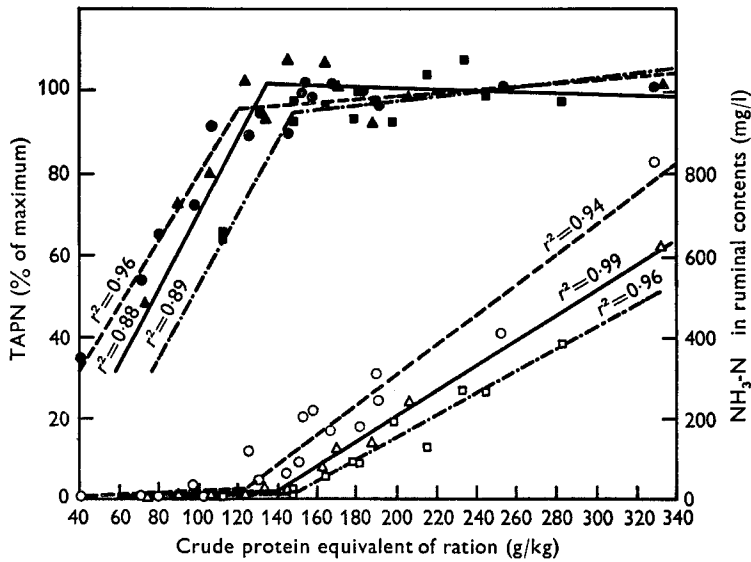
Fig. 1. Relationship between ammonia concentration (NH_3-N) of continuous-culture fermentor contents (open symbols) and output of tungstic acid-precipitable nitrogen (TAPN) (closed symbols) when either a purified ($\bigcirc$ and $\bullet$), all-concentrate ($\square$ and $\blacksquare$) or forage–concentrate (23:77) ($\triangle$ and $\blacktriangle$) mixture was added to the fermentor.
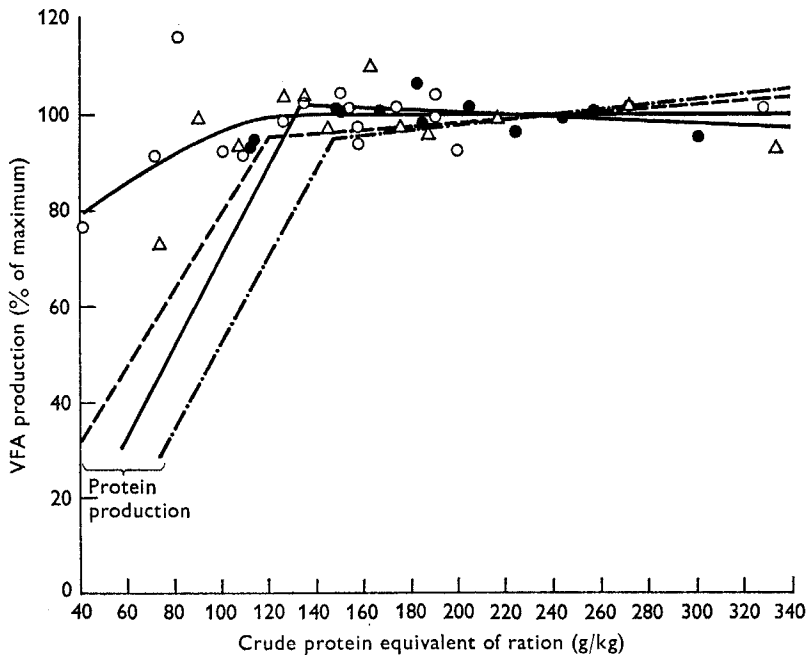

Fig. 2. Relationship between volatile fatty acid (VFA) production by continuous-culture fermentor contents and crude protein content of either a purified ($\bigcirc$), all-concentrate ($\bullet$) or forage–concentrate (23:77) ($\triangle$) mixture added to the fermentor. Protein production from Fig. 1 is included to facilitate comparison between protein and volatile acid production.

Protein output by the fermentors increased as the level of urea supplementation was increased, and then levelled off. Further increases in the amount of supplementary urea were without effect on protein output. The levelling-off of protein output coincided with the point when ammonia began to accumulate. NH_3-N in excess of approximately 50 mg/l of fluid contents had no effect on protein content of the fermentor effluent, even when NH_3-N concentration was increased to 800 mg/l.

The effect of low N supplementation on total volatile fatty acid production is shown in Fig. 2. Total volatile fatty acid production did not decline as much as did protein output by the cultures when N was limiting for the fermentation. Protein output, obtained from Fig. 1, is repeated in this figure to facilitate comparison. There was no consistent effect of ammonia concentration on the relative amounts of volatile fatty acids produced. Methane production paralleled total volatile acid production when NH_3-N < 50 mg/l.

<div align="center">DISCUSSION</div>

The molar proportions of volatile acids produced in vitro were close to those which have been observed in ruminal fluid in vivo with similar diets (Church, 1969; Oltjen, Slyter, Williams & Kern, 1971), showing the similarity between in vivo and in vitro fermentations.

Total acid production ranged from 4·4 to 6·5 mol/kg organic matter fed. If expressed on a DM basis, these values would be about 5 % less. They agree well with determinations of fatty acid production made in vivo. As summarized by Esdale, Broderick & Satter (1968), values for volatile fatty acid production ranging from 3·7 to 8·8 mol/kg ration DM have been obtained in vivo.

From stoichiometric considerations (Hungate, 1965), methane production (Table 3) was 101 %, 84 % and 91 % of theoretical production for the purified, concentrate and forage–concentrate rations when N was not limiting. In making these calculations, it was assumed that glucose was the sole substrate, and that isobutyrate and isovalerate were derived from valine and leucine (Dehority, Johnson, Bentley & Moxon, 1958). Since microbial cell mass is usually more highly reduced than the substrate, which in this example was glucose, methane production typically would have to be slightly less than predicted in order to maintain an oxidation–reduction balance (Hungate, 1965).

In addition to finding the expected ratios of end-products of fermentation, the following observations are taken as evidence that a relatively normal rumen microbial population was maintained in vitro. The numbers of cellulolytic bacteria for the purified diet (Expt 2) were 0·13, 2·4, 3·9, 1·1 and 7 × 10⁸/g fermentor content for the cultures given increasing amounts of urea. Except for the culture where N was most limiting, these counts are similar to those observed when the same diet was given to steers (Slyter, Oltjen, Kern & Weaver, 1968). Entodiomorph protozoa were counted in Expt 4 and were maintained in greater numbers (9500, 4540, 5320, 9440, 16380 and 4915/g fermentor content with increasing infusion of urea) over a longer time interval than observed previously (Slyter *et al.* 1964; Slyter & Putnam, 1967). For

the forage–concentrate diet, routine microscopic examination indicated an active protozoal population throughout the study.

When the purified diet was used, all the TAPN in the effluent would be of microbial origin because the diet was devoid of protein. With the natural diets, some protein would be expected to escape microbial degradation, and would appear in the effluent as TAPN. In view of this, the fermentor output of TAPN was lower than expected for the concentrate and forage–concentrate rations (Table 3). Approximately 67 % of the N in the unsupplemented natural diets was recovered in the combined TAPN and ammonia fractions in the fermentor effluent, suggesting that there may have been a significant amount of N in non-precipitable peptides, amino acids and other non-protein N products. In contrast, 86 % of the supplemental urea N was accounted for in the TAPN and NH_3-N fractions for the natural and purified rations.

To accurately distinguish microbial protein from protein of dietary origin is difficult, and it was concluded that less error would result if total TAPN were determined. Thus, by assuming that proteolytic activity would not vary significantly (Hungate, 1966) in the natural diets, a constant fraction of the dietary protein could be expected to escape degradation with any given diet. Any difference in TAPN in the effluent could then be considered to be of microbial origin. To facilitate comparisons between diets, TAPN in the effluent was expressed as a percentage of maximum output.

It appears that, once ammonia starts to accumulate, the growth of bacteria utilizing ammonia is not enhanced by increasing ammonia concentration. The failure to increase further the TAPN output by the fermentors, as well as the linear increase in ammonia concentration with increased urea supplementation, both support this conclusion. A concentration of 50 mg NH_3-N/l is enough to support maximum growth rates of rumen bacteria. The precise limiting concentration is perhaps closer to 20 mg NH_3-N/l, but use of the higher value gives a slight margin of excess.

It is worth noting that excessively high levels of ammonia, up to 800 mg NH_3-N/l, did not inhibit microbial growth. Levels far in excess of what would normally be found in the rumen were maintained in several of the fermentors without the slightest indication that microbial growth was being inhibited.

Under in vitro conditions and with the diets used in these experiments, ammonia started to accumulate when N equivalent to 110–140 g crude protein/kg diet (DM basis) was added. Ammonia accumulated earlier with the purified diet than with the natural diet because virtually all the N in the purified diet would go through the ammonia pool, whereas some of the protein in the natural diet escaped degradation and was not deaminated to ammonia. Additionally, the point of ammonia accumulation is determined by the amount of fermentable energy. Acid production/kg organic matter added to the fermentor was in the sequence: purified ration < forage–concentrate < concentrate (Table 3), and this coincided with the sequence of ammonia accumulation. Because there is neither absorption nor recycling of N in vitro, as would occur in vivo (Cocimano & Leng, 1967), it does not necessarily follow that ammonia would start to accumulate at the same dietary protein level in vivo.

The values in Fig. 1 were submitted to a least squares regression analysis employing intercept and slope shifters according to the following model:

$$\hat{Y} = \alpha + b_1 X + b_2 D + b_3 (D \times X),$$

where $\hat{Y}$ is a set of ordinate values, X is the crude protein value, and D is a dummy variable, assigned a value of either 0 or 1 (Draper & Smith, 1966). As shown in Fig. 1, r^2 values for each set of values ranged between 0·88 and 0·99, indicating a very good fit between the computed regression lines and the individual values. The intercepts for the TAPN and ammonia plots were at 120 and 119, 135 and 142, and 147 and 150 g crude protein/kg ration DM for the purified, forage–concentrate and concentrate rations. This good agreement underlines the close relationship between the achievement of maximum TAPN production and the beginning of accumulation of NH_3-N. The intercepts on the NH_3-N plot for each of the three rations occurred at 18·6, 20·1 and 17·3 mg NH_3-N/l rumen fluid.

Total acid production under N-limiting conditions was variable, but a slight decrease was noted (Fig. 2). It was certainly not as large as the decrease in TAPN output. Fermentation uncoupled to growth under N-limiting conditions has been demonstrated with *Bacteroides amylophilus* (Henderson, Hobson & Summers, 1969). This underlines the hazard of predicting microbial protein yield from volatile fatty acid production when N nutrition is inadequate for bacteria. Volatile fatty acid production could be expected to parallel total digestibility of a ration. From in vivo experiments it appears that DM digestibility is reduced only 0–30 % (Campling, Freer & Balch, 1962; Weston, 1967; Hume, Moir & Somers, 1970; Ørskov, Fraser & McDonald, 1971, 1972; Winter & Pigden, 1971) when rations containing 30–120 g crude protein/kg are given. This is a modest decrease compared with the 50 % decrease in TAPN when similar diets were fermented in vitro.

For purposes of comparison with previously reported studies, the yield in microbial DM(g)/mol of estimated ATP production (Y_{ATP}) was estimated when the purified diet was used. In obtaining these values, it was assumed that all the TAPN represented microbial crude protein, and that 65 % of the bacterial dry weight was crude protein (Hungate, 1966). It was also assumed that 2·3 mol ATP were produced per mol acid produced, a slightly higher value than that of approximately 2 suggested by Walker (1965), and more nearly equal the 2·4 value suggested by Baldwin (1970). With these assumptions, an average Y_{ATP} estimate of 15·6 ± 0·9 was obtained when N was not limiting the fermentation (Fig. 3). This is approximately 20 % higher than values obtained in other in vitro studies (Walker & Nader, 1968), but agrees well with what has been observed in vivo by Hogan & Weston (1970) when their results were adjusted for protozoal protein. Under N-limiting conditions, Y_{ATP} values decreased substantially.

In a continuous-culture system with the rumen micro-organism, *Bacteriodes amylophilus* (Henderson *et al.* 1969), cell growth was limited when the uninoculated medium contained less than 64 mg NH_3-N/l. The prevailing concentration of ammonia in the inoculated media was not given, but it must have been very low. Most bacteria are capable of scavenging ammonia from very dilute solutions.
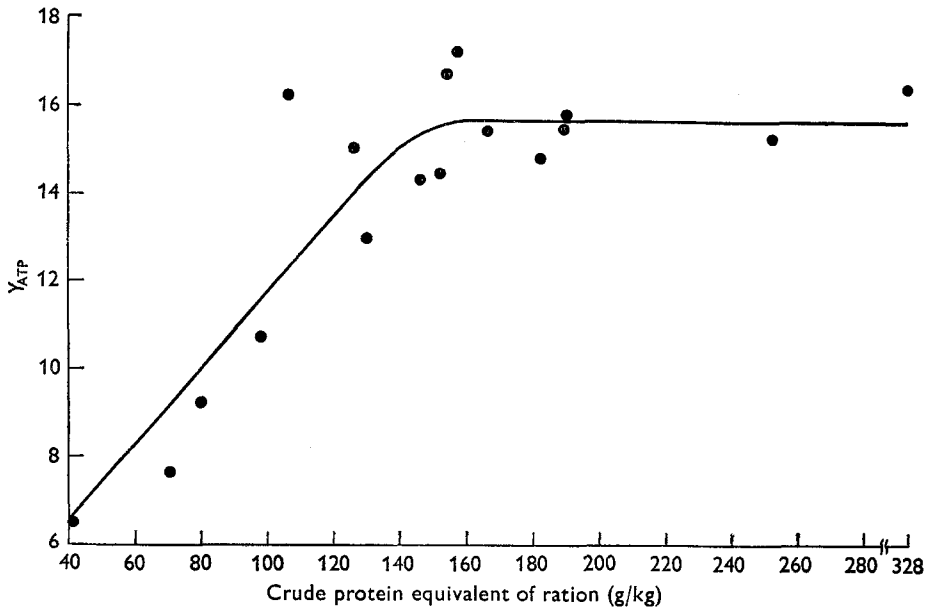
Fig. 3. Microbial growth, as g microbial dry matter/mol ATP produced (Y_{ATP} values), obtained when a purified diet mix containing urea at various levels was added to continuous-culture fermentors.

Hume *et al.* (1970), feeding mature wethers on a purified diet containing urea as the N source, suggested that the amount of TAPN passing through the omasum increased until dietary N reached a point somewhere between 9·5 and 18·2 g/kg ration DM, equivalent to a crude protein level of 59–114 g/kg. Ruminal ammonia concentrations at these two levels of dietary N were 88 and 133 mg NH_3-N/l rumen fluid, respectively. This suggested, therefore, that microbial protein synthesis would be stimulated by increasing ammonia concentration up to a point somewhere between these two levels.

Ørskov *et al.* (1971, 1972) were unable to show a statistically significant increase in the amount of protein passing through the abomasum of sheep given basal diets of barley (90 and 100 g crude protein/kg DM) supplemented with urea at different levels. The latter study (Ørskov *et al.* 1972) suggested that supplementing a basal barley diet with urea up to an equivalent of 120 g crude protein/kg DM was beneficial to young lambs, however, in that live-weight gain increased up to that point. Ruminal ammonia concentration was not reported, but the concentration of ammonia in abomasal fluid was. Comparison of results from the two experiments reported by Ørskov *et al.* (1972) suggests that maximum growth occurred when abomasal fluid contained between 40 and 80 mg NH_3-N/l fluid. It is reasonable to expect that the ruminal and abomasal ammonia concentrations were similar, since Hume *et al.* (1970) found little difference in the ammonia concentration between ruminal and omasal fluid.

Since the majority of rumen bacteria use ammonia as a N source (Bryant &

Robinson, 1962), it follows that availability of ammonia will be an important determinant of microbial protein production. It is essential to know what concentration of ammonia will support maximal microbial growth in order to make judgments regarding utilization of non-protein N. From this study it appears that once ammonia starts to accumulate in the rumen and exceeds 50 mg NH_3-N/l rumen fluid, nothing is gained by further supplementation with non-protein N.

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