The nutritive value of rumen micro-organisms in ruminants

4. The limiting amino acids of microbial protein in growing sheep determined by a new approach

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1. Four experiments were carried out to identify and quantify the limiting amino acids (AA) in rumen microbial protein (RMP).

2. A method was developed which involved first, an assessment of the efficiency of utilization of absorbed AA-nitrogen (*U*) of RMP, defined as the retention of AA-N from RMP absorbed from the small intestine, and second, addition of a mixture of AA similar to the absorbed AA profile in a quantity defined by the *U* of RMP and equal to $(1-U)/U$. Third, it involved removal of each AA in turn and measurement of the resultant N retention. Using this approach it was possible to calculate both the order and extent of AA limitations in RMP.

3. Apart from methionine which was found to be the most limiting AA, only lysine, arginine and histidine reduced N retention when omitted, and accordingly only these AA were limiting in RMP.

4. The method is discussed in detail and the amount of supplementary AA required to utilize RMP fully is calculated.

Although ruminants usually absorb a mixture of dietary, microbial and endogenous amino acids (AA) simultaneously, the microbial protein usually accounts for the largest proportion of the total AA-nitrogen entering the small intestine of ruminants. It is therefore meaningful to determine the limiting AA of the rumen micro-organisms (RMO) separately because the AA composition of microbial protein is relatively constant (Storm & Ørskov, 1983). Unfortunately, AA requirements are not easily measured in large animals such as ruminants, and progress towards the elucidation of the limiting AA in this important class of livestock has been slow and laborious. Although an indication of the limiting AA may be obtained by comparing the animal's AA composition with that of the absorbed AA profile, such estimates frequently relate to only a few specific conditions (Williams & Hewitt, 1979). Furthermore, even when AA profiles are available for RMO, the difficulty of measuring AA absorption and its varying extent make comparisons based on AA profiles alone (Buttery & Cole, 1977) uncertain.

Given the complex AA and N metabolism of the ruminant and its microbes, it is difficult to identify and quantify the limiting AA in any particular diet. The standard approach of identifying the first limiting AA and then determining its optimum concentration in a diet suffers from serious disadvantages which make the precise level of requirement of any particular AA difficult to define.

Experiments to identify those AA which are limiting, other than the major AA, are not easy to design. Harper (1959) suggested a method whereby individual AA were omitted in turn. This method has been useful in giving basic qualitative information on limiting AA, and on problems of imbalance. In the present paper we describe a method to determine quantitatively the order of limitation of essential AA in RMO or, more precisely, the limiting AA in the AA absorbed from the small intestine, in sheep nourished by infusions of volatile fatty acids and given RMO as the only source of protein. The non-AA-N in the isolated RMO is 0·20 of the total N and the true digestibility of AA-N in RMO in the small intestine has been found to be 0·85 (Storm *et al.* 1983*a*). The proportion of absorbed AA-N in RMO-N is therefore $0{\cdot}80 \times 0{\cdot}85 = 0{\cdot}68$. The efficiency of utilization of absorbed AA-N (*U*), i.e. the

retained N expressed as a proportion of the absorbed AA-N, is 0·80 (Storm & Ørskov, 1982). In a preliminary experiment (Expt 1) it was shown that N was limiting for the conditions used to test AA responses and under these conditions therefore it is reasonable to assume that the difference between U of the protein studied and 1·0 is due to a deficiency of one or more essential AA. It follows that if the protein were to be correctly supplemented with the limiting amino acid or acids, the utilization could be increased to a value close to 1·0. It also follows that the extent of the deficiency of the most limiting AA of the protein is defined by $(1-U)/U$. This would mean that the deficiency of the most limiting AA could be corrected by increasing its supply by $a \times (1-0{\cdot}8)/0{\cdot}8$ where a is the amount of AA absorbed from the basal input of RMO. Instead of adding more RMO we made a mixture of AA similar in composition to the absorbed AA from microbial protein and tested whether the N retention was similar to that found when 25% more of the microbial protein was added. Having ascertained that this was indeed the case, each AA was subsequently removed in turn from the mixed AA supplement and the respective N retentions determined. The most limiting AA should accordingly, if completely removed, give a N retention equal to that observed with the basal input of RMO. More important is the fact that the N retention observed when each AA is removed in turn should represent the extent to which that particular AA was limiting in the test protein. If N retention were not altered by removal of a particular AA, this would indicate that the AA in question was not limiting in microbial protein. Therefore it appeared possible to determine both the order and extent of all AA limitations by this method.

MATERIALS AND METHODS

Four experiments were conducted. In the first two, AA supplementation was tested and methodological studies were undertaken. In the third and fourth experiments the order of limitation determined in Expt 2 was confirmed by gradual rather than total omission of the limiting AA.

Animals

Expts 1 and 2. Four Suffolk × (Finnish Landrace × Dorset Horn) castrated male lambs about 2 months of age and with an average live weight of 21 kg were used.

Expts 3 and 4. Six lambs of similar breed to those used in Expt 1 were used. They had an average live weight of 26 kg.

Infusion procedure

Each lamb was fitted with an abomasal catheter and a rumen cannula according to the procedure described by Ørskov *et al.* (1979). The intragastric nutrition procedure described by Ørskov *et al.* (1979) was used except that isolated rumen micro-organisms (Storm & Ørskov, 1983) were used instead of casein for abomasal infusions. Volatile fatty acids in the molar proportions 0·65 acetic, 0·25 propionic and 0·10 butyric were infused to supply a total of 750 kJ/kg body-weight $(W)^{0{\cdot}75}$ per d.

N supplementation

Expt 1. A Latin-square design was planned with four different N inputs, all calculated as g N/kg $W^{0{\cdot}75}$. Treatment 1, infusion of 0·9 g RMO-N; treatment 2, as treatment 1 plus a value calculated as $0{\cdot}9 \times 0{\cdot}68 \times 0{\cdot}25$ of a mixture of AA similar in composition to the AA of absorbed RMO-N. This addition was included to supply essential AA (g/kg freeze-dried RMO): arginine 5·5, histidine 1·6, isoleucine 5·9, leucine 8·0, lysine 9·0, methionine 4·1, phenylalanine 10·8, threonine 5·6, valine 6·1, tryptophan 2·0, and non-

essential AA: alanine 9·9, glutamic acid 19·5, glycine 7·5, serine 6·4, cysteine 1·6, tyrosine 7·4. Treatment 3, as treatment 2 but with non-essential AA omitted; treatment 4, high level of RMO-N of 1·3 g N/kg $W^{0.75}$ per d.

Expt 2. Since in Expt 1 there was no significant difference in N retention between the supplement containing essential and non-essential AA and that containing only the essential AA, the four sheep were given 0·9 g N/kg $W^{0.75}$ plus 0·25 times that in the absorbed amount of each essential AA from RMO (as in treatment 3 described previously). Each essential AA was then omitted in turn. Since N retention was not altered by omission of tryptophan, phenylalanine and isoleucine, these acids did not limit *U* (see Table 3, p. 617). These AA were also omitted from the mixture in the later stages of Expt 2.

Expt 3. The six lambs were divided into two groups of three and two 3×3 Latin-square designs were planned to compare three treatments. The treatments comprised 1 and 4 as for Expt 1 and the third treatment was the RMO level of 0·9 g N/kg $W^{0.75}$ supplemented with the four AA found to be essential and limiting in Expt 2, namely arginine, histidine, lysine and methionine.

Expt 4. The six lambs were initially given the RMO level of 0·9 g N/kg $W^{0.75}$ supplemented with arginine, histidine, lysine and methionine. Instead of removing each AA completely they were given either in the full quantity (0·25 as before) or as one-third or two-thirds of this amount. Three lambs were used for gradual omission of methionine and lysine and three lambs for gradual omission of histidine and arginine.

Management. Each infusion period lasted 6 d. Urine and faeces were collected and the N retention was measured over the last 3 d. The urine was collected into 400 ml 5 M-sulphuric acid.

Since the length of periods and the small amount of faeces excreted did not allow an estimation of digestibility, the apparent N digestibility was calculated for each sheep during the whole experiment, assuming that the pure AA included were completely digestible. The N retention was calculated as the digestible N intake from RMO plus AA-N minus the urinary N.

RESULTS

Expt 1. The difference in the N retention of lambs receiving the low basal and the AA-supplemented treatment was highly significant ($P < 0·001$) and the high basal treatment gave a N retention which was significantly greater than any of the other treatments (Table 1). There was almost no difference betwen the lambs given only essential AA and those given the full complement of both essential and non-essential AA. The efficiency of utilization of the full complement of AA was $(160-57)/(1011-883) = 0·805$, similar to the utilization of absorbed AA from the RMO (Storm & Ørskov, 1982; Storm *et al.* 1983*b*). These responses indicated that experiments based on an RMO intake of 0·9 g N/kg $W^{0.75}$ would be conducted under N-limiting conditions. This was the case for the subsequent experiments.

Expt 2. As mentioned earlier, all essential AA were included in Expt 1. In the first part of Expt 2 it was found that omission of tryptophan, phenylalanine and isoleucine did not cause any reduction in N retention (Table 2).

The effects of omission of seven other essential AA were examined in the second part of the experiment (Table 2). Leucine and valine also gave no reduction in N retention. With threonine there was a small but non-significant reduction in N retention. When arginine and histidine were omitted the reduction in N retention compared with the mean of the basal values reached significance ($P < 0·05$), while for lysine and methionine the reduction in N retention was highly significant ($P < 0·001$) (Table 2).

Table 1. *Expt 1. The effect of different levels of rumen micro-organisms (RMO) in the infusate on nitrogen retention and the effect of adding mixtures comprising either all amino acids (AA) or essential AA only calculated to supply an addition of 25% of AA absorbed from the RMO given at the low level*

(Each value is the mean of four observations)

Treatment*	N infused (mg/kg $W^{0.75}$)	N retention (mg/kg $W^{0.75}$)
Low level of RMO (low basal)	883	57
Low level of RMO + essential and non-essential AA	1011	160
Low level of RMO + essential AA only	956	154
High level of RMO (high basal)	1320	280
SE of mean		16

W, body-weight. * For details, see p. 615.

Table 2. *Expt 2. The effect of removing different essential amino acids (AA) on the nitrogen retention in lambs receiving a basal infusate of rumen micro-organisms (RMO) and the mixture of essential AA (S1) described on pp. 614–615. The AA mixture designated as S2 contained all essential AA less tryptophan, phenylalanine and isoleucine*

(Each value is the mean of four observations)

Treatment*	N infused (mg/kg $W^{0.75}$)	N retention (mg/kg $W^{0.75}$)	SE
RMO + S1	965	159	15
RMO + S1 − tryptophan		162	19
RMO + S1 − phenylalanine		169	21
RMO + S1 − isoleucine		158	11
RMO + S1	963	149	16
RMO + S2	955	155	14
RMO + S2 − leucine		147	11
RMO + S2 − valine		151	21
RMO + S2 − arginine		119	10
RMO + S2 − histidine		112	9
RMO + S2	955	148	15
RMO + S2 − threonine		138	19
RMO + S2 − lysine		79	6
RMO + S2 − methionine		36	13
RMO + S2	955	149	14
RMO + S2 − leucine − valine		152	14

W, body-weight. * For details, see p. 615.

Expt 3. Only the four essential AA which had been shown to influence N retention in Expt 2 were included in the supplement. The difference in N retention between the basal and the supplemented infusates was highly significant ($P < 0.001$) and the high basal level gave N retention values significantly greater ($P < 0.001$) than those in the other two treatments (Table 3).

Expt 4. This experiment was in two parts (see Table 4). Methionine and lysine were partly omitted from the infusate given to one group of three lambs and histidine and arginine from

Table 3. *Expt 3. The effect on nitrogen retention of infusing two levels of rumen micro-organisms (RMO) compared with the addition of four essential amino acids (AA) determined to be limiting*

(The level of inclusion was based on an addition of 25% of those present in the absorbed AA from RMO at the lower input. The essential AA were methionine (met), lysine (lys), histidine (his) and arginine (arg). Each value is the mean of six observations)

Treatment*	N infused (mg/kg $W^{0.75}$)	N retention (mg/kg $W^{0.75}$)
Low level of RMO (low basal)	887	52
Low level of RMO+met, lys, his and arg	928	175
High level of RMO (high basal)	1324	291
SE		17

W, body-weight. * For details, see p. 615.

Table 4. *Expt 4. The effect of gradual removal of different essential amino acids (AA) on the nitrogen retention in lambs receiving a basal infusate of rumen micro-organisms (RMO)*

(The essential AA mixture (S3) contained methionine, lysine, arginine and histidine. The results of the trial where methionine and lysine were removed are means of three observations. Two lambs only were used when arginine and histidine were removed)

Treatment*	N infused (mg/kg $W^{0.75}$)	N retention (mg/kg $W^{0.75}$)	SE
RMO+S3	909	146	18
RMO+S3−one-third methionine		108	19
RMO+S3−two-thirds methionine		68	32
RMO+S3	909	139	13
RMO+S3−one-third lysine		140	13
RMO+S3−two-thirds lysine		117	21
RMO+S3	909	204	19
RMO+S3−one-third arginine		201	12
RMO+S3−two-thirds arginine		200	30
RMO+S3	909	209	18
RMO+S3−one-third histidine		206	12
RMO+S3−two-thirds histidine		199	33
RMO+S3		193	29

W, body-weight. * For details, see p. 615.

that given to another group of three. However, one lamb of the latter group died due to a mechanical failure of the buffer system and was not replaced. As methionine was gradually omitted there was a linear decrease in N retention for each decrement; with lysine there was no change in N retention when the first decrement was imposed but clearly some reduction with the second decrement, although the difference was not significant. In the second part, when histidine and arginine were omitted, results were less satisfactory since only two animals were tested. The N retention achieved with the basal level was higher than that observed in the other three lambs and there was no reduction when one-third or two-thirds of histidine or arginine were omitted.

Table 5. *Calculation of the amount of supplementary amino aid (AA) required to achieve the maximum nitrogen retention in relation to absorbed AA-N in rumen micro-organism (RMO) or per MJ metabolizable energy (ME) in feed assuming a yield of 1·25 g N/MJ ME (Agricultural Research Council, 1980)*

	Cysteine + methionine	Lysine	Arginine	Histidine
AA present in RMO* (g/kg)	20·4	47·2	28·7	9·9
Supplement (g/kg RMO)	4·1	9·0	5·5	1·6
Absorbed profile* of AA from RMO (g/kg AA)	35	84	52	14
Supplemented AA profile (1·25 × absorbed profile)	4·37	10·50	6·50	1·75
Proportion of supplement utilized	1·00	0·73	0·26	0·32
Supplement required				
g/kg absorbed AA	8·7	15·3	3·3	1·1
g/kg RMO-N	42·3	67·8	15·0	5·4
mg/MJ ME	53	85	19	7

* Storm *et al.* (1983*b*).

DISCUSSION

Methodology

The method developed here involves (1) the assessment of U (i.e. the efficiency of utilization of absorbed AA-N) of a given protein, (2) the assessment of the absorbed AA profile of that protein (e.g. in the small intestine), (3) the formulation of a mixed AA supplement derived as the proportion $(1-U)/U$ of the individual AA which is truly absorbed from the protein, and (4) the assessment of responses obtained (N retention, N gain etc.), when the test animals are given the basal protein, together with the mixed AA supplement, but from which individual AA has been sequentially omitted.

From a comparison of these individual responses the minimal supplement of each individual AA necessary to ensure the full utilization of the basal supplemented protein is derived.

The advantages of the method can be summarized briefly: (1) the size of the final mixed AA supplement is the smallest supplement necessary to ensure full utilization of basal protein plus AA supplement, (2) the order in which the individual AA are investigated is immaterial, (3) co-limiting AA pose no experimental problems, (4) each AA has to be investigated just once (with one trial each) in order to arrive at both the relative order and the extent of limitation of that AA, (5) all the limiting AA of the protein are classified in this way, (6) the size of response is linearly related to the extent of the AA limitation and therefore measurable if the limitation is at all significant, (7) the sensitivity of response remains high and uniform throughout the experiment, (8) the number of individual trials necessary is equal to the number of essential AA+3 (test protein given at high level and at low basal level, with and without complement of non-essential AA), (9) the plan of the whole experiment may be designed efficiently beforehand, (10) the proposed method can be used with any type of diet and animal, including non-ruminants, provided that the U of absorbed AA can be obtained.

As mentioned earlier, a simple technique of AA omission was first used by Harper (1959) but only as a method of studying AA imbalance in rats. A somewhat similar approach was used by Egan & Rogers (1978) in a study of AA imbalance in lambs. Pelaez & Walker (1979) and Phillips & Walker (1978, 1979) also used the omission method to identify the order of

some of the secondary limiting AA, but not to estimate the extent of limitation of any of these.

A method which appears to be somewhat more similar in concept to the present one, was that of Bender (1960), who found that the biological value of whole-egg protein was still close to 1·0 even when it was diluted by 15% of non-essential AA. He then made up mixtures of AA similar in composition to this, and omitted in turn 50% of each essential AA to determine the relative AA requirements of rats for growth. In contrast, our method may be used to determine the minimal supply of all individual essential AA needed to optimize the utilization of any supplemented feed protein, and thus closely approximates the limiting AA of such a protein.

It must be emphasized that our method, like any other AA supplementation method, relies heavily on using the appropriate level of supplementation. In our method we specify that it must be linearly related to the efficiency of utilization of absorbed AA-N (U) of the test protein, through the expression $(1-U)/U$, throughout the area of investigation. Furthermore, by remaining within this area, problems of AA imbalance, which appear to occur only when AA are given well in excess of that which could be expected due to the natural limitation imposed by sub-optimal AA comparison, are avoided.

Calculation of order and extent of limitations

From the individual values used to derive Tables 1 and 2, it is possible to calculate the proportional reponse to total omission of the different AA. This was expressed as the proportion of the response to supplementation with the complete AA mixture (over the basal) which was lost when that specific AA was omitted from the supplement. Thus when methionine was omitted, the response lost was 1·04 (SE 0·11) (i.e. all the response). Similarly, the reduction in N retention for the omission of lysine was 0·73 (SE 0·06), for arginine 0·26 (SE 0·01) and for histidine 0·32 (SE 0·03). In Table 5 the value of 1·00 was used for methionine.

From the values of Storm & Ørskov (1983) we have the AA content per kg RMO (Table 5). The RMO contained 102 g N/kg dry matter. It is then possible to calculate the supplement per kg RMO-N or per AA absorbed. It is also possible to calculate the amount of supplement per MJ metabolizable energy (ME) in diets for ruminants assuming that 1·25 g microbial N is formed per MJ ME consumed (Agricultural Research Council, 1980). Therefore, theoretically it should be possible to improve considerably the utilization of microbial protein, provided of course, that the AA are given in such a way that the rumen is by-passed, or that they are protected from rumen degradation. It is also possible to assess the sources of supplementary protein which can complement microbial protein most efficiently. It is then possible to find supplements of proteins or AA which, due to their complementary effect on RMO, could have a biological value in excess of 1·0.

The finding here that methionine was the most limiting AA in RMO is in agreement with observations of AA supplementation of urea-based purified diets and measurements of N retention (Nimrick *et al.* 1970; Ørskov & Fraser, 1970; Ørskov, 1982) and observations using plasma AA concentrations (Richardson & Hatfield, 1978; Mathers & Miller, 1979). The finding here that lysine was the second limiting AA is less well supported in the literature. Also, histidine and arginine have not normally been implicated, although Bergen *et al.* (1968) found that by using the plasma AA concentration as the indicator, histidine could sometimes be limiting. Tao *et al.* (1974) found responses to arginine supplementation and also observed that there was an optimum value for parenterally infused lysine–arginine.

The experimental approach used here gives quantitative information on limiting AA but it must be stressed that this can only be assessed when the utilization of the absorbed AA is known. The technique described has equal application to ruminants and non-ruminants.

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