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Use of stable isotopes to measure *de novo* synthesis and turnover of amino acid-C and -N in mixed micro-organisms from the sheep rumen *in vitro*

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Protein synthesis and turnover in ruminal micro-organisms were assessed by stable-isotope methods in order to follow independently the fate of amino acid (AA)-C and -N in different AA. Rumen fluid taken from sheep receiving a grass hay–concentrate diet were strained and incubated *in vitro* with starch–cellobiose–xylose in the presence of NH_3 and 5 g algal protein hydrolysate (APH)/l, in incubations where the labels were $^{15}NH_3$, [^{15}N]APH or [^{13}C]APH. Total ^{15}N incorporation was calculated from separate incubations with $^{15}NH_3$ and [^{15}N]APH, and net N synthesis from the increase in AA in protein-bound material. The large difference between total and net AA synthesis indicated that substantial turnover of microbial protein occurred, averaging 3·5 %/h. Soluble AA-N was incorporated on average more extensively than soluble AA-C (70 *v.* 50 % respectively, $P=0{\cdot}001$); however, incorporation of individual AA varied. Ninety percent of phenylalanine-C was derived from the C-skeleton of soluble AA, whereas the incorporation of phenylalanine-N was 72 %. In contrast, only 15 % aspartate-C + asparagine-C was incorporated, while 45 % aspartate-N + asparagine-N was incorporated. Deconvolution analysis of mass spectra indicated substantial exchange of carboxyl groups in several AA before incorporation and a condensation of unidentified C_2 and C_4 intermediates during isoleucine metabolism. The present results demonstrate that differential labelling with stable isotopes is a way in which fluxes of AA synthesis and degradation, their biosynthetic routes, and separate fates of AA-C and -N can be determined in a mixed microbial population.

Amino acids: Carbon skeletons: Rumen: Sheep

Non-ruminant animals cannot synthesise some of their amino acids (AA), which must therefore be supplemented in their diet (McDonald *et al.* 1995). In contrast, ruminant animals do not have such a dietary requirement (Virtanen, 1966), because the microbes that inhabit the rumen can form all of their AA *de novo*, and these then become available for use in the small intestine after the microbes are digested by the host enzymes. Some AA are metabolised in the rumen more rapidly than others (Chalupa, 1976; Broderick & Balthrop, 1979) and some AA are incorporated into microbial protein to a greater extent than others (Armstead & Ling, 1993; Atasoglu *et al.* 1999). It has been demonstrated that providing the ruminal microbes with AA stimulates the growth rate and growth yield of ruminal bacteria (Maeng *et al.* 1976; Cotta & Russell, 1982; Argyle & Baldwin, 1989; Cruz Soto *et al.* 1994; Atasoglu *et al.* 1999; Carro & Miller, 1999), but the precise combination of AA that cause this stimulation has not been yet elucidated.

Until now, labelling experiments that were aimed at determining how much microbial AA is formed from NH_3 and how much is derived from pre-formed AA have generally used either $^{15}NH_3$ or ^{14}C-labelled AA as a marker (Allison, 1969; Nolan *et al.* 1976; Nolan & Stachiw, 1979; Armstead & Ling, 1993; Atasoglu *et al.* 1999) or ^{14}C-labelled acetate, propionate, CO_2 or bicarbonate (Allison, 1969; Sauer *et al.* 1975). The present study was undertaken to determine the fluxes of both C and N into microbial AA in the same experiment, and to calculate turnover rates for microbial total and individual AA. The results provide new insight about AA synthesis and turnover in ruminal micro-organisms.

Materials and methods

Animals and diets

Four ruminally fistulated adult sheep received a mixed diet comprising grass hay, barley, molasses, fish meal, and minerals and vitamins, at 500·0, 299·5, 100·0, 91·0 and 9·5 g/kg DM respectively, fed in equal meals of 500 g at 08.00 and 16.00 h. Ruminal digesta samples were taken before feeding in the morning from each sheep in order to minimise peptides and AA in the inoculum. The rumen contents were kept at 39°C and strained through a linen cloth before use.

Abbreviations: AA, amino acid; ape, atom percent excess; APH, algal protein hydrolysate; I, isotopomer correction factor.

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Incubations with rumen fluid in vitro

Strained rumen fluid was added under CO_2 to a buffer–mineral solution (1:2, v/v; Menke & Steingass, 1988) at 39°C. Samples of diluted rumen fluid (30 ml) were then added to plastic tubes (130 × 40 mm) fitted with Bunsen valves. The energy source was 200 mg soluble starch–cellobiose–xylose (1:1:1, by wt).

Incorporation of ^{15}N- or ^{13}C-labelled algal peptides and $^{15}NH_3$

The experiment comprised a set, one per sheep, of three parallel incubations. The three parallel incubations were chemically and microbiologically identical, but contained different stable isotope labels. Diluted rumen fluid + the carbohydrate mixture (starch–cellobiose–xylose) was incubated under CO_2 at 39°C with: (A) unlabelled algal protein hydolysate (APH) + $^{15}NH_3$; (B) [^{13}C]APH and unlabelled NH_3; (C) [^{15}N]APH and unlabelled NH_3. The APH preparations, consisting mainly of small peptides and AA, were obtained from Celtone (Martek Biosciences Corporation, Columbia, MD, USA). $^{15}NH_4Cl$ (98 % ^{15}N) was from Sigma (Poole, Dorset, UK). The total concentration of APH was 5 g/l in all incubations, with [^{13}C]- and [^{15}N]APH diluted fivefold with unlabelled APH before use. Samples (5 ml) were removed into 5 ml 100 g TCA/l at 0 and 8 h of the incubation, then stored at 4°C.

Isotopic and analytical measurements

Pellets from TCA-treated samples were obtained by centrifuging at 28 000 ***g*** for 15 min and washing once with 50 g TCA/l. Supernatant fractions from cultures were kept at 4°C. The pellets were resuspended in 10 ml 0·5 M-NaOH and heated at 100°C for 30 min to solubilise the cell walls. The NaOH-treated samples were then centrifuged at 28 000 ***g*** for 15 min and the liquid fraction was retained for further analytical measurements.

NaOH-treated samples (2 ml) were neutralised with 2 ml 0·5 M-HCl to which 4 ml TCA (100 ml/l) was then added. The samples were centrifuged at 28 000 ***g*** for 15 min and the supernatant fractions were discarded. The pellets were resuspended in 6 M-HCl. Acid hydrolysis was carried out as described by Atasoglu *et al.* (1998). ^{15}N and ^{13}C enrichments in individual AA were determined by GC–MS as described by Calder & Smith (1988). The isotopomer measured in most analyses was $M_r + 1$ for AA labelled with ^{15}N and M_r + number of C atoms (n) for AA labelled with ^{13}C. Deconvolution analysis, whereby the relative concentration of all isotopomers is determined (rather than only $M_r + n$) was carried out in three 8 h samples of protein-bound AA from different sheep. The mathematical procedures were the same as those described by Campbell (1974). AA concentrations of supernatant fractions and microbial cells were determined by ion-exchange chromatography after hydrolysing them in 6 M-HCl (Atasoglu *et al.* 1999). ^{15}N enrichment in NH_3 was determined by incorporating $^{15}NH_3$ into norvaline using glutamate dehydrogenase and 2-oxopentanoate as described by Nieto *et al.* (1996). Total N and ^{15}N were determined as described previously (Atasoglu *et al.* 1999).

Calculations

The incubation sets were: (A) APH + $^{15}NH_3$; (B) [^{13}C]APH + NH_3; (C) [^{15}N]APH + NH_3. The experimental measurements used in calculations were as follows:

P_t AA concentration in protein-bound material at time t (mmol/l);
S_t AA concentration in soluble material at time t (mmol/l);
A_t NH_3 concentration at time t (mmol/l);
m_t ^{15}N enrichment in protein-bound AA at time t, incubation set A (atom % excess (ape)/100);
n_t ^{15}N enrichment in protein-bound AA at time t, incubation set B (ape/100);
c_t ^{13}C enrichment in protein-bound AA at time t, incubation set C (ape/100);
p_t ^{15}N enrichment in NH_3 at time t, incubation set A (ape/100);
q_t ^{15}N enrichment in NH_3 at time t, incubation set C (ape/100);
x_t ^{15}N enrichment in soluble AA at time t, incubation set A (ape/100);
y_t ^{15}N enrichment in soluble AA at time t, incubation set C (ape/100);
z_t ^{13}C enrichment in soluble AA at time t, incubation set B (ape/100);
n number of C atoms in AA;
I_a molar proportion of isotopomer $M_r + a$ in protein-bound AA at 8 h;
Σ sum of ^{13}C in isotopomers from $M_r + 1$ to $M_r + n$.

The value of c_t measured above was only from the isotopomer fully labelled with ^{13}C. In order to compensate for different enrichments in other isotopomers of the AA molecule, an isotopomer correction factor was calculated, as follows:

$$^{13}\text{C atoms in } M_r + n \text{ isotopomer} = n \times I_n,$$

$$\text{total } ^{13}\text{C atoms incorporated} = \Sigma n \times I_n \text{ (i.e. } n \times I_n + (n-1)\ I_{n-1} + \ldots + I_1),$$

$$\therefore \text{ isotopomer correction factor } I = \Sigma n \times I_n / n \times I_n. \quad (1)$$

Net microbial AA synthesis (W) was calculated using protein-bound AA concentrations at 0 and 8 h:

$$\text{net AA synthesis} = P_8 - P_0. \quad (2)$$

Microbial AA-N from NH_3 was calculated from ^{15}N incorporated from $^{15}NH_3$ into protein-bound material in incubation A:

$$^{15}\text{N incorporated from } NH_3 \text{ (mmol/l)} = (P_8 m_8 - P_0 m_0),$$

$$\text{average } ^{15}\text{N enrichment in } NH_3 = (p_8 + p_0)/2,$$

$$\therefore \text{ microbial AA-N from } NH_3 \text{ (mmol/l)} = 2(P_8 m_8 - P_0 m_0)/(p_8 + p_0). \quad (3)$$

Microbial AA-N from soluble AA was calculated from ^{15}N incorporated directly from soluble N into protein-bound material, including ^{15}N passing through the NH_3 pool, in incubation C:

total soluble AA-^{15}N incorporated into protein-bound material (mmol/l) $= (P_8n_8 - P_0n_0)$.

However, ^{15}N may have been incorporated via NH_3, *viz.* ^{15}N-algal AA-N $\rightarrow$ $^{15}NH_3$ $\rightarrow$ ^{15}N-labelled microbial AA-N:

$$\text{average } ^{15}\text{N enrichment in } NH_3 = (q_8 + q_0)/2.$$

Therefore, from the earlier estimation of microbial AA-N from NH_3:

$$\begin{aligned} &^{15}\text{N entering microbial-AA from } NH_3 \text{ (mmol/l)} \\ &= ((q_8 + q_0)/2)(2(P_8m_8 - P_0m_0)/(p_8 + p_0)) \\ &= (q_8 + q_0)(P_8m_8 - P_0m_0)/(p_8 + p_0), \end{aligned}$$

$$\begin{aligned} \therefore\ &^{15}\text{N entering microbial AA-N directly from soluble} \\ &\text{AA-N (mmol/l)} = (P_8n_8 - P_0n_0) - (q_8 + q_0) \\ &\qquad \times (P_8m_8 - P_0m_0)/(p_8 + p_0), \end{aligned}$$

$$\text{average } ^{15}\text{N enrichment in soluble AA} = (y_8 + y_0)/2,$$

$$\begin{aligned} \therefore\ &\text{N entering microbial AA-N directly from soluble} \\ &\text{AA-N (mmol/l)} = 2((P_8n_8 - P_0n_0) - (q_8 + q_0) \\ &\qquad \times (P_8m_8 - P_0m_0)(p_8 + p_0))/(y_8 + y_0). \end{aligned} \tag{4}$$

Total microbial AA-N synthesis (T) was calculated as:

$$\begin{aligned} &\text{microbial AA-N from } NH_3 \\ &\quad + \text{microbial AA-N from APH.} \end{aligned} \tag{5}$$

Turnover rate (%/h) was calculated as from the difference between total microbial AA-N synthesis and net microbial AA-N synthesis, based on the average AA concentration during the incubation period:

$$\begin{aligned} &\text{microbial biomass turnover (mmol/l)} \\ &= \text{total microbial AA-N synthesis} \\ &\quad - \text{net microbial AA-N synthesis} \\ &= T - W, \end{aligned}$$

$$\text{average AA concentration (mmol/l)} = (P_8 + P_0)/2,$$

$$\begin{aligned} \therefore\ &\text{turnover rate of AA-N (\%/h)} \\ &= 200\ (T - W)/8\ (P_8 + P_0) \\ &= 25\ (T - W)/(P_8 + P_0). \end{aligned} \tag{6}$$

Microbial AA-C incorporated from soluble AA was calculated from incubation B, using ^{13}C incorporated into protein-bound AA, the ^{13}C enrichment of soluble AA, and the isotopomer correction factor for protein-bound AA:

$$\begin{aligned} &\text{algal AA-}^{13}\text{C incorporated into protein-bound} \\ &\text{material as the } M_r + n \text{ isotopomer (mmol/l)} \\ &= (P_8c_8 - P_0c_0)/100, \end{aligned}$$

$$\text{average enrichment in soluble AA} = (z_8 + z_0)/2,$$

$$\begin{aligned} \therefore\ &\text{microbial AA-C incorporated from soluble} \\ &\text{AA as the } M_r + n \text{ isotopomer (mmol/l)} \\ &= 2(P_8c_8 - P_0c_0)/(z_8 + z_0), \end{aligned}$$

$$\begin{aligned} \therefore\ &\text{total soluble AA-C incorporated into microbial} \\ &\text{AA (mmol/l)} = 2I(P_8c_8 - P_0c_0)/(z_8 + z_0). \end{aligned} \tag{7}$$

Proportion of microbial AA-C derived from APH AA-C was calculated as:

$$\begin{aligned} &\text{microbial AA-C from APH/} \\ &\text{total microbial AA synthesis.} \end{aligned} \tag{8}$$

Proportion of microbial AA-N derived from APH AA-N was calculated as:

$$\begin{aligned} &\text{microbial AA-N from APH/} \\ &\text{total microbial AA synthesis.} \end{aligned} \tag{9}$$

AA catabolism was calculated in two ways. The first was from soluble AA concentrations and net microbial AA synthesis:

$$\text{AA disappearance (mmol/l)} = S_8 - S_0,$$

$$\begin{aligned} \therefore\ &\text{net AA catabolism (mmol/l)} \\ &= \text{AA disappearance} \\ &\quad - \text{net microbial AA synthesis} \\ &= S_8 - S_0 - P_8 - P_0. \end{aligned} \tag{10}$$

The second calculation was made from the changes in enrichment of ^{15}N in NH_3 in incubations A and C: in incubation 1, the enrichment in NH_3 declined due to NH_3 produced from unlabelled soluble AA. Thus,

$$\text{initial } ^{15}\text{N in } NH_3 = p_0A_0,$$

$$\text{final } ^{15}\text{N in } NH_3 = p_8A_8,$$

$$\begin{aligned} \therefore\ &^{14}\text{N released into } NH_3 \text{ pool (mmol/l)} \\ &= (1 - p_8)A_8 - (1 - p_0)A_0. \end{aligned}$$

In incubation 3, the enrichment in NH_3 increased due to NH_3 produced from ^{15}N-labelled soluble AA. Thus,

$$\text{initial } ^{15}\text{N in } NH_3 = q_0A_0,$$

$$\text{final } ^{15}\text{N in } NH_3 = q_8A_8,$$

$$\begin{aligned} \therefore\ &^{15}\text{N released into } NH_3 \text{ pool (mmol/l)} \\ &= q_8A_8 - q_0A_0. \end{aligned}$$

The average enrichment in soluble AA-N $= (y_8 + y_0)/2$, where y_8 and y_0 refer to the mean enrichments in soluble

AA in incubation C,

$$\therefore\ NH_3 \text{ formed from AA catabolism (mmol/l)} = 2(q_8A_8 - q_0A_0)/(y_8 + y_0). \quad (11)$$

AA-C in soluble AA derived from protein-bound AA-C was calculated from the dilution of ^{13}C in soluble AA in incubation B:

$$^{12}C \text{ increase in soluble AA-C} = (z_0 - z_8)S_8,$$

$$\text{average } ^{12}C \text{ enrichment in protein-bound AA} = (2 - c_8)/2,$$

$$\therefore \text{ AA-C released into soluble AA-C from protein-bound AA-C (mmol/l)} = 2((z_0 - z_8)\ S_8)/(2 - c_8). \quad (12)$$

AA-N in soluble AA derived from protein-bound AA-N was calculated from the dilution of ^{14}N in soluble AA in incubation C:

$$^{14}N \text{ increase in soluble AA-N} = (y_0 - y_8)S_8,$$

$$\text{average } ^{14}N \text{ enrichment in protein-bound AA} = (2 - n_8)/2,$$

$$\therefore \text{ AA-N released into soluble AA-N from protein-bound AA-N (mmol/l)} = 2((y_0 - y_8)\ S_8)/(2 - n_8). \quad (13)$$

Statistical analyses

Combined SEM values for the twelve AA analysed were generated using Genstat (Genstat 5 Committee, 1987; Oxford, UK). All analyses were carried out in triplicate, and the average value calculated. The mean values across the four sheep were then calculated, and compared by a paired *t* test (*n* 4).

Results

The incorporation, biosynthesis and catabolism of AA by mixed ruminal micro-organisms were measured in an 8 h incubation *in vitro* in which soluble starch–cellobiose–xylose formed the energy source for growth, and APH and NH_3 were N sources available to the micro-organisms. The origin and fate of AA-N and -C were determined using parallel incubation mixtures containing stable-isotope labels in $^{15}NH_3$, [^{15}N]APH and [^{13}C]APH. The measurements were carried out on the twelve AA whose isotopomers were most readily measured by the GC–MS technique. Some AA were destroyed by acid hydrolysis, while others were present at concentrations too low for accurate analysis.

The mean increase in protein-bound AA concentration during the fermentation was from 0·60 to 1·01 mmol/l, an increase of 67 % (Table 1). The increases for individual amino acids ranged from 96 (alanine) to 50 (serine) %. Analysis of total protein-bound N (Table 2) indicated that the proportional increase was 83 %.

Table 1. Primary measurements of concentrations and isotopic enrichments of protein-bound amino acid (AA) and soluble AA in mixed ruminal micro-organisms incubated *in vitro* with starch–cellobiose–xylose for 8 h*

	Concentration (mmol/l)				Enrichment (atom % excess)							
	Protein-bound AA		Soluble AA		Incubation A†		Incubation B†			Incubation C†		
					^{15}N-Protein-bound AA	^{13}C-Protein-bound AA	^{13}C-Soluble AA	^{15}N-Protein-bound AA	^{15}N-Soluble AA			
Amino acid	0 h (P_0)	8 h (P_8)	0 h (S_0)	8 h (S_8)	8 h (m_8)	8 h (c_8)	0 h (z_0)	8 h (z_8)	8 h (n_8)	0 h (y_0)	8 h (y_8)	
Ala	0·72	1·40	1·95	1·55	9·73	2·86	18·90	12·29	9·02	17·89	14·05	
Gly	0·88	1·51	1·97	1·21	7·67	4·33	19·89	15·78	8·39	17·85	15·49	
Val	0·60	1·07	1·15	0·67	7·73	5·20	20·46	17·48	9·26	17·70	15·65	
Leu	0·77	1·22	1·46	0·66	6·63	5·23	19·19	16·65	8·49	17·89	15·97	
Ile	0·44	0·72	0·61	0·28	7·73	2·60	15·03	12·13	6·93	17·81	15·11	
Pro	0·33	0·57	1·02	0·29	4·82	3·98	19·05	12·04	8·11	18·48	17·04	
Ser	0·18	0·27	0·67	0·27	7·85	2·10	17·75	9·54	7·10	16·90	14·68	
Thr	0·17	0·32	0·87	0·26	4·43	4·44	18·13	14·36	9·25	16·79	15·20	
Phe	0·42	0·66	0·60	0·25	7·50	3·86	18·59	15·57	7·59	16·06	14·98	
Asp	1·08	1·64	1·29	0·58	12·33	0·77	17·90	9·93	4·71	16·58	13·78	
Glu	0·97	1·60	1·83	0·55	13·45	1·72	17·18	9·92	5·50	17·28	13·72	
Lys	0·68	1·13	1·70	1·10	2·26	4·18	16·89	16·70	3·57	6·31	6·82	
Mean‡	0·60	1·01	1·26	0·64	7·68	3·44	18·25	13·53	7·32	16·46	14·37	
SEM§	0·09	0·14	0·15	0·13	0·91	0·41	0·42	0·84	0·54	0·95	0·74	

* For details of procedures and calculations, see pp. 253–256.
† Incubation A: $^{15}NH_3$; incubation B: ^{13}C-labelled algal protein hydrolysate; incubation C: ^{15}N-labelled algal protein hydrolysate.
‡ Mean values of estimations made with samples of rumen fluid from four sheep.
§ Combined SEM of values calculated for all amino acids.

Table 2. Primary measurements of concentrations and isotopic enrichments of ammonia in a culture of mixed ruminal micro-organisms incubated *in vitro* with starch–cellobiose–xylose for 8 h*
(Mean values and standard deviations)

Incubation time (h)	Microbial N (mmol/l)		NH_3 concentration (A_t) (mmol/l)		^{15}N enrichment in NH_3 (atom % excess)			
					Incubation A (p_t)†		Incubation C (q_t)†	
	Mean‡	SD	Mean‡	SD	Mean‡	SD	Mean‡	SD
0	15·0	0·8	17·9	1·2	52·29	3·04	3·17	0·61
8	27·4	0·8	17·4	1·9	31·91	2·89	6·74	0·35

* For details of procedures, see p. 254.
† Incubation A, $^{15}NH_3$; incubation C, ^{15}N-labelled algal protein hydrolysate.
‡ Mean estimations made with samples of rumen contents from four sheep.

The concentrations of all soluble AA decreased during the incubation, though none was exhausted (Table 1). The greatest decreases were for glutamic acid, glycine and lysine (1·28, 0·76 and 0·60 mmol/l respectively), while the smallest decreases occurred with isoleucine and phenylalanine (0·33 and 0·35 mmol/l). The ^{15}N enrichment in protein-bound AA in incubation A ($^{15}NH_3$) was highest with aspartic acid and glutamic acid, and lowest with threonine and proline; the apparent low enrichment in lysine occurs because only the $M_r + 2$ isotopomer was measured in this analysis; thus, the incorporation of single N atoms was not recorded. The complementary incubation C, in which the ^{15}N was present in the form of APH, showed the opposite pattern, namely that aspartate and glutamic acid were enriched least; the lysine value was anomalous for the same reason as before.

The ^{13}C enrichment in protein-bound AA during incubation B again reflected the greater *de novo* synthesis of asparate and glutamic acid (Table 1). Leucine and valine had the highest enrichment. However, these values are misleading, because they reflect only AA fully labelled in all C atoms in the AA C-skeleton ($M_r + n$). The pattern of enrichment in different isotopomers was analysed by a procedure known by mass spectroscopists as 'deconvolution', using three randomly selected 8 h samples of protein-bound material. In all cases except phenylalanine, the fully labelled AA was the most abundant isotopomer, but the proportion of the other isotopomers varied between 15 (threonine) and 66 (isoleucine) % of the total. The isotopomer correction factor I was used to calculate total C incorporation into protein-bound C (Table 4).

The enrichment of ^{13}C in soluble AA decreased in incubation B (Table 1). The decrease was greatest for serine, asparate and glutamic acid and least for lysine. For ^{15}N enrichment in soluble AA, in incubation C, the greatest decreases occurred in alanine, aspartate and glutamic acid.

The ^{15}N enrichment in NH_3 in incubation A declined by 39 % during the incubation, while ^{15}N enrichment in NH_3 in incubation C increased (Table 2). The observation that the ^{15}N enrichment in NH_3 in incubation C was 3·17 ape at zero time indicated that the ^{15}N label was not present entirely in AA in the APH.

The results presented in Tables 1, 2 and 3 were used to calculate the fate of NH_3 and AA in several ways (Table 4). Total AA-N synthesis was calculated as the sum of AA synthesis from NH_3 and AA incorporation from soluble AA: on average, total AA synthesis was 0·63 mmol/l, while net AA synthesis was 0·43 mmol/l, indicating a turnover rate of 3·53 %/h. The calculated turnover rates of isoleucine ($P=0·030$) and lysine ($P=0·016$) were significantly less than the average, and alanine ($P=0·050$) and valine ($P=0·008$) were greater than the average. The average

Table 3. Deconvolution analysis of distribution of ^{13}C-labelling in each carbon atom of individual amino acids at 8 h*
(Mean values)

Amino acid	Enrichment of ^{13}C in different isotopomers of amino acid of molecular mass M_r (mol %)†									Isotopomer correction factor I
	$M_r + 1$	$M_r + 2$	$M_r + 3$	$M_r + 4$	$M_r + 5$	$M_r + 6$	$M_r + 7$	$M_r + 8$	$M_r + 9$	
Ala	0·13	0·51	2·23							1·17
Gly	0·82	3·30								1·12
Val	0·21	0·15	0·21	1·31	3·56					1·36
Leu	0·24	0·38	0·07	0·41	2·09	3·66				1·61
Ile	0·75	1·37	0·06	0·27	1·09	1·81				1·94
Pro	0·28	0·13	0·14	0·57	3·00					1·22
Ser	0·24	0·22	1·26							1·18
Thr	0·21	0·04	0·29	3·10						1·09
Phe	0·05	0·09	0·02	0·01	0·07	0·17	0·77	3·03	2·33	2·49
Asp	0·27	0·14	0·18	0·60						1·45
Glu	0·35	0·28	0·10	0·50	1·49					1·43
Lys	0·06	0	0·44	0·58	1·69	3·11				1·65
SEM										0·17

* For details of procedures, see p. 254.
† Three randomly selected samples from 8 h incubations, from each of three different sheep, were subjected to mass spectral deconvolution analysis (results are the mean values of these determinations).

Table 4. Microbial amino acid (AA) synthesis, turnover and catabolism in mixed ruminal micro-organisms incubated *in vitro* with a mixture of starch, cellobiose and xylose for 8 h*
(Mean values for triplicate cultures from each of four sheep)

Amino acid	Net microbial AA synthesis (mmol/l); 1†	Microbial AA-N from NH_3 (mmol/l); 3†	Microbial AA-N from soluble AA (mmol/l); 4†	Total AA-N synthesis (mmol/l); 5†	Turnover rate (%/h); 6†	Microbial AA-C from soluble AA (mmol/l); 7†	Proportion of AA-C derived from soluble AA; 8†	Proportion of AA-N derived from soluble AA; 9†	Net AA catabolism (mmol/l); 10†	AA-C released into soluble AA from protein-bound AA (mmol/l); 12†	AA-N released into soluble AA from protein-bound AA (mmol/l); 13†
Ala	0·68	0·32	0·72	1·04	4·24	0·30	0·29	0·69	0·10	0·104	0·062
Gly	0·63	0·28	0·70	0·98	3·59	0·41	0·42	0·72	0·34	0·051	0·030
Val	0·47	0·20	0·55	0·75	4·16	0·40	0·53	0·74	0·09	0·020	0·014
Leu	0·45	0·19	0·57	0·76	3·98	0·57	0·75	0·75	0·23	0·017	0·013
Ile	0·28	0·13	0·27	0·41	2·79	0·27	0·66	0·68	0·06	0·008	0·008
Pro	0·24	0·07	0·25	0·31	2·10	0·18	0·57	0·79	0·56	0·021	0·004
Ser	0·09	0·05	0·11	0·16	3·93	0·05	0·31	0·69	0·35	0·022	0·006
Thr	0·15	0·03	0·18	0·21	3·33	0·10	0·45	0·84	0·51	0·010	0·004
Phe	0·24	0·12	0·30	0·41	3·97	0·37	0·90	0·72	−0·02	0·008	0·003
Asp	0·56	0·48	0·40	0·88	2·96	0·13	0·15	0·45	0·58	0·046	0·017
Glu	0·63	0·51	0·45	0·96	3·26	0·29	0·30	0·47	0·99	0·040	0·020
Lys	0·45	0·06	0·58	0·64	2·64	0·46	0·72	0·91	0·14	0·002	−0·006
Mean	0·41	0·20	0·42	0·63	3·53	0·29	0·50	0·70	0·33	0·029	0·015
SEM‡	0·06	0·05	0·06	0·09	0·20	0·05	0·08	0·04	0·09	0·008	0·005

*For details of procedures, see p. 254.
† Equation number (for details, see pp. 254–256).
‡ Combined SEM of values calculated for all amino acids.

proportion of protein-bound AA derived from soluble AA-C was 50 % (after correction using I), while the proportion of AA-N derived from soluble AA-N was greater (70 %, $P=0{\cdot}001$). The proportions varied greatly with different AA. Ninety per cent of phenylalanine-C was derived from the C-skeleton of soluble AA, whereas the incorporation of phenylalanine-N was 72 %. In contrast, only 15 % asparate-C + asparagine-C was incorporated, while 45 % of asparate-N + asparagine-N was incorporated.

The difference between initial and final soluble-AA concentrations (Table 1) resulted partly from catabolism and partly from incorporation. The catabolic component (Table 4) revealed that catabolism was greatest for glutamic acid, aspartate and proline, and least for phenylalanine and isoleucine, where the calculation indicated net synthesis. NH_3 formed from AA catabolism (equation 11) was 3·93 mmol/l.

The isotopic enrichment in soluble amino acids decreased during the incubation, such that the average ^{13}C enrichment decreased from 18·3 to 13·5 ape and the ^{15}N enrichment fell from 16·5 to 14·4 ape (Table 1). A deconvolution analysis was not carried out on soluble amino acids. The decreases in enrichment in individual AA indicated that a substantial quantity of alanine was released into soluble AA, while no lysine and little isoleucine were released (Table 4).

Discussion

The results reported here demonstrate that it is possible, using multiple stable isotope labelling, to carry out simple incubations which can generate a wealth of information about AA fluxes, both synthetic and degradative, in the mixed microbial population of the rumen. The results provide information about net and total microbial growth, enabling turnover rates, both of individual and total AA, by ruminal micro-organisms, to be determined. The results also indicate how the metabolism of AA-N and AA-C-skeletons should be considered separately and may lead to different conclusions, and how ^{13}C distribution in the C-skeletons provides information about the metabolic origin of the microbial AA. Perhaps most importantly, the values for C incorporation show which AA C-skeletons are incorporated, and which are formed at least partly by *de novo* synthesis. The hope is that, by understanding the way in which AA are synthesised and incorporated, we will be able to identify key AA that limit ruminal fermentation.

Net AA synthesis was calculated from the difference between protein-bound AA at the beginning and end of the incubation. Total microbial growth was calculated from the combined incorporation of ^{15}N from $^{15}NH_3$ in one incubation mixture and ^{15}N from soluble ^{15}N-labelled AA in an incubation mixture that was identical except for the isotope and/or the molecules that were labelled. The turnover of microbial AA was then calculated simply by dividing the difference between total and net AA synthesis by the time of incubation, and dividing by the average biomass during the incubation period. As samples were only taken at 0 and 8 h, the progress of the fermentation at intermediate times was not established. However, previous

work showed that the microbial growth was more similar to a linear rather than exponential progression and that carbohydrate was not exhausted at 8 h (Atasoglu *et al.* 1999). Therefore, the calculated turnover rates are unlikely to be greatly in error. The average value for the twelve AA examined was 3·53 %/h. Similar calculation of C-skeleton turnover was not possible, because the total synthesis of AA-C, unlike the total synthesis of AA-N, was not determined. Nor was the re-entry of ^{13}C-labelled degradation products from ^{13}C-labelled AA degradation measured, so equation 7 does not include that flux. However, the magnitude of that flux would be expected to be small for most AA. Breakdown rates of different individual bacterial species in the rumen varied from 5–29 %/h, and these rates decreased greatly when protozoa were removed (Wallace & McPherson, 1987). The lower turnover rate measured here may be because rumen bacteria growing in the mixed population may be less susceptible to breakdown than bacteria that have been grown in pure culture *in vitro* for a prolonged period.

The ^{15}N tracer experiments done in sheep by Nolan and his colleagues (Nolan, 1975; Nolan & Stachiw, 1979) demonstrated that microbial protein breakdown and resynthesis was highly significant *in vivo*. Nolan & Stachiw (1979) noted that 'the total production of micro-organisms was at least twice the net outflow' in the rumen of Merino sheep fed a low-quality roughage, following estimates of 30 % total N incorporation being recycled in the rumen of sheep, also using [^{15}N]urea and $^{15}NH_4SO_4$ (Nolan *et al.* 1976). Van Nevel & Demeyer (1977) used ^{32}P incorporation to estimate total growth of ruminal micro-organisms *in vitro*, and calculated that 50 % total N incorporated into microbial protein was recycled. Subsequent experiments demonstrated that the difference was due in large part to the bacteriolytic activity of ciliate protozoa (Demeyer & Van Nevel, 1979). In the present experiment, turnover as a proportion of total synthesis was, from Table 4, (0·63 – 0·41)/0·63 = 35 %.

The present experiment also provides quantitative data on the turnover of individual AA. The turnover of individual AA varied, with alanine turnover greater than others, and proline and lysine lower. Alanine appears to have a highly significant role in AA synthesis in mixed ruminal bacteria. Shimbayashi *et al.* (1975) and Blake *et al.* (1983) indicated that alanine was the first AA to be formed from NH_3, and free alanine concentrations were higher than those of other AA at high NH_3 concentrations in the sheep rumen (Wallace, 1979). Here, also at high prevailing NH_3 concentrations, alanine-C and -N from microbial protein was lost to soluble AA much more extensively than other AA. This observation may only reflect the ease of interconversion of alanine to pyruvate and acetyl-CoA, and the central role of pyruvate in glycolytic as well as AA metabolism (Sauer *et al.* 1975). Deconvolution analysis was not carried out on the soluble AA, so we cannot be sure about the origin of this alanine. Nevertheless, microbial alanine was predominantly present as the fully labelled isotopomer (Table 3); if the released alanine was the same, this indicates a substantial leakage or export of alanine from microbial cells. Alanine concentrations declined proportionally less than any other among the free soluble AA, consistent with a small flux of alanine from microbial AA to soluble extracellular AA.

Glutamic acid and aspartic acid were the most abundant AA in microbial protein, as is found usually (Purser & Buechler, 1966; Sauer *et al.* 1975; Storm & Ørskov, 1983; Wallace, 1994). It should be noted that, as acid hydrolysis removes the amide-N of glutamine and asparagine, the concentrations of glutamic acid and aspartic acid quoted here actually reflect the sums of (glutamic acid + glutamine) and (aspartic acid + asparagine) respectively. The concentrations of the other AA were also fairly typical of previous studies, except serine, which in the present study was 17 % of the concentration of glutamic acid, whereas in other studies the value was closer to 40 %. Changes in microbial AA can occur according to diet and microbial population (Wallace, 1994). Serine concentration is particularly variable, with some bacteria having half the serine concentration of mixed microbial species (Purser & Buechler, 1966; Sauer *et al.* 1975; Storm & Ørskov, 1983; Wallace, 1994). Nevertheless, such a low proportion of serine appears to be atypical.

The labelling of different substrates in different incubations enabled the loss of soluble AA to be quantified in terms of incorporation and catabolism. Some AA, although their concentration declined during the incubation (Table 1) were incorporated but little was degraded. These AA included isoleucine, phenylalanine, lysine and to a lesser extent leucine. Others, particularly AA of the glutamic acid family, were catabolised much more extensively, such that glutamic acid catabolism was almost as great as its incorporation. The minimal catabolism of these AA is not obvious from simple AA concentrations at the beginning and end of the incubation (Table 1), nor was it evident from the analyses carried out by Chalupa (1976) or Broderick & Balthrop (1979); these analyses measured only AA loss and did not distinguish between catabolism and incorporation. The low catabolism of these acids is consistent with their C-skeleton being required, either as the AA or as precursor short- and branched-chain fatty acids (Allison, 1965, 1969; Robinson & Allison, 1969; Russell & Sniffen, 1984; Gorosito *et al.* 1985; Mir *et al.* 1986). In the experiments of Armstead & Ling (1993), only leucine, tyrosine and phenlyalanine were labelled in microbial protein when a ^{14}C-labelled AA mixture was added; the low AA concentration added (about 10 mg/l compared with 5 g/l used here) may explain the different levels of incorporation.

Analysis of more than one isotopomer of each AA is a daunting task for more than a few samples, so routinely only the fully ^{13}C-labelled ($M_r + n$) isotopomers were monitored by GC–MS. A small number of samples was analysed for the complete array of isotopomers in the 8 h samples, and the results of this deconvolution analysis were applied to the experimental data. The results indicated that extensive metabolism of C-skeletons occurred before incorporation of AA, such that less than half of the AA were incorporated without modification of the C-skeleton. With alanine, glycine, proline, serine, threonine, asparate and glutamic acid, the fully labelled C-skeleton ($M_r + n$) was far more abundant than other isotopomers; this reflects the ease with which, following deamination or

transamination, the AA-C-skeleton enters the main metabolic pathways. With valine, leucine, isoleucine and pheylalanine, $M_r + (n - 1)$ isotopomer was abundant, a consequence of the transamination and decarboxylation to the $(n - 1)$ fatty acid by some species and the subsequent carboxylation and amination of the fatty acids by other species (Allison, 1965, 1969; Robinson & Allison, 1969; Amin & Onodera, 1997; Hungate & Stack, 1982). Lysine also had a high enrichment in $M_r + (n - 1)$, consistent with the exchange of C with diaminopimelic acid (Masson & Ling, 1986; Onodera & Kandatsu, 1973; Onodera *et al.* 1991). In isoleucine, the $M_r + 2$ isotopomer was present at 76 % of the $M_r + n$ isotopomer, and was more abundant than the $M_r + (n - 1)$ isotopomer, indicating a pattern of metabolism similar to the two-C (not known) and four-C (thought to be 2-oxobutyrate) condensation suggested by Sauer *et al.* (1975). The two-C group may even be derived from another AA, such as glycine.

It has been known for a long time that the supplementation of AA results in increased microbial growth rate and microbial growth yield of most species of ruminal bacteria in the rumen (Maeng *et al.* 1976; Russell, 1983; Argyle & Baldwin, 1989; Carro & Miller, 1999). The stimulation is achieved when a complete mixture of AA is supplied to the microbes, but no single one AA or groups of AA can give the full benefit of adding a complete mixture (Maeng *et al.* 1976; Argyle & Baldwin, 1989). The previous studies involved the supplementation of an *in vitro* fermentation by individual AA or groups of AA. However, it is possible that a small number of AA might limit microbial growth, but because there are twenty AA, it would clearly be difficult to cover all possible combinations of AA as supplements to a defined medium. The present study provides an alternative approach to the problem. One might predict that those microbial AA that are incorporated most from pre-formed AA may be the ones that limit fermentation most in the rumen. Almost all phenylalanine-C in microbial protein was derived from soluble AA. The phenylalanine-C-skeleton has been recognised for a long time as being difficult for ruminal bacteria to synthesise (Allison, 1965; Amin & Onodera, 1997); indeed, phenylacetic acid or phenylpropionic acid are essential for growth of *Ruminococcus albus* (Hungate & Stack, 1982; Stack *et al.* 1983). Similarly, the total formation of leucine- and isoleucine-C from soluble AA confirms the limiting nature of C-skeleton synthesis in these AA (Allison, 1969; Oltjen *et al.* 1971; Russell & Sniffen, 1984), particularly for plant cell wall digestion (Gorosito *et al.* 1985; Mir *et al.* 1986). In contrast, only 18 % of aspartate-C was formed from soluble AA, reflecting the central position of oxaloacetate in microbial metabolism and its ease of transamination. In general, the proportion of AA-N incorporated from soluble AA was higher and less variable than AA-C, and the latter may therefore be a better indicator of *de novo* synthesis. The ease with which an AA is transaminated may not reflect the difficulty of synthesising its C skeleton. The high incorporation of both AA-C and -N into lysine suggests that its synthesis may also limit microbial growth, possibly partly due to difficulty in C-skeleton synthesis and partly due to low transamination.

Implications

The main limitations of the analysis reported here are that only twelve AA were analysed and that only a single set of incubation conditions was investigated. Thus, some of the AA essential to the animal and possibly some of the key AA for microbial growth were not analysed, and growth conditions under which AA catabolism might be greatly altered were not investigated. Nevertheless, it has been demonstrated that the methodology provides a powerful means of analysing AA metabolism in mixed ruminal micro-organisms, which enabled the measurement of AA turnover, the separate incorporation of AA-N and -C, and transformations within C-skeletons. The implication might also be drawn that two or three of the AA may be particularly limiting microbial growth in the rumen, although past experience suggests that the full beneficial effects of AA supplementation to ruminal micro-organisms can only be realised with a full complement of AA found in microbial protein (Maeng *et al.* 1976; Argyle & Baldwin, 1989; Atasoglu *et al.* 1999).

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