A comparison of methods for the estimation of microbial nitrogen in duodenal digesta of sheep

BY R. C. SIDDONS, D. E. BEEVER AND J. V. NOLAN*

The Grassland Research Institute, Hurley, Maidenhead, Berkshire SL6 5LR

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1. Six sheep, each fitted with a rumen cannula and re-entrant cannulas in the proximal duodenum and distal ileum, were given two diets (600 g dry matter (DM)/d) consisting of either grass silage (32·1 g nitrogen/kg DM) or dried grass (18·3 g N/kg DM). A net loss of N occurred between mouth and duodenum with the silage diet, indicating extensive ruminal degradation of dietary N, compared with a net gain on the dried-grass diet. Consequently, despite higher N intakes when silage was given, N flow at the duodenum was similar for both diets.

2. The proportion of microbial N in duodenal digesta N was estimated using diaminopimelic acid (DAPA), [^{35}S]methionine (^{35}S), ^{15}N-enriched non-ammonia-N (^{15}NAN) and amino acid profiles (AAP) as microbial markers. Isotopic labelling of rumen micro-organisms was achieved by intraruminal infusions of $Na_2{}^{35}SO_4$ and $(^{15}NH_4)_2SO_4$.

3. A comparison of all methods was made based on the marker concentrations in microbial fractions isolated by differential centrifuagation of strained rumen contents. With both diets, DAPA gave the highest estimates and AAP the lowest. Estimates based on ^{35}S and ^{15}NAN were intermediate and did not differ significantly ($P > 0{\cdot}05$).

4. For the ^{15}NAN, ^{35}S and AAP methods, the effect of site of sampling of the microbial fraction, i.e. from rumen contents or duodenal digesta, was examined and in all instances mean estimates based on duodenally-derived microbes were higher. However, the differences were significant for only ^{15}NAN with both diets ($P < 0{\cdot}001$), for ^{35}S with the dried grass ($P < 0{\cdot}05$), and for AAP with the silage ($P < 0{\cdot}05$). Estimates based on duodenally-derived microbes were higher ($P < 0{\cdot}05$) using ^{15}NAN than those obtained using ^{35}S with both diets.

5. Depending on the method used for estimating microbial N, estimates of the efficieny of microbial N synthesis in the rumen (g microbial N flow at duodenum/kg organic matter apparently digested in the rumen) ranged between 16 and 38 for the silage diet and 10 and 46 for the dried grass diet. Similarly, estimates of feed N degradability in the rumen ranged between 0·62 and 0·97 for the silage and 0·00 and 0·93 for the dried grass.

Digesta entering the small intestine of ruminants contains protein originating from three different sources, i.e. microbial protein synthesized in the rumen, feed protein which has passed undegraded through the rumen, and endogenous protein in the form of abomasal secretions and desquamated epithelial cells. If a suitable marker exists which is present in only one of the three fractions, the proportion of that fraction can be estimated by marker dilution, by measuring the marker concentration in an isolated sample of the fraction and in unfractionated duodenal digesta. The proportion can then be used in conjunction with measurements of total protein flow to the duodenum to obtain the duodenal flow of that particular fraction.

A number of different markers have been used in this respect to identify microbial protein in duodenal digesta. Two commonly used, naturally-occurring markers are diaminopimelic acid (DAPA, Weller *et al.* 1958) and ribonucleic acid (RNA, McAllan & Smith, 1971). DAPA is a constituent of the cell wall of many species of bacteria, but is absent from protozoa and plant material whilst RNA is present in bacteria, protozoa and feed and its use as a microbial marker is dependent on no feed RNA reaching the duodenum. As an alternative to naturally-occurring markers, isotopic labelling of rumen microbes by intraruminal infusion of ^{35}S (Beever, Harrison *et al.* 1974) or ^{15}N (Mathison & Milligan, 1971) has been used to identify microbial material in duodenal digesta. A somewhat different approach was proposed by Evans *et al.* (1975) in which the amino acid profiles (AAP) of

* Present address: The University of New England, Armidale, NSW 2351, Australia.

feed, microbial and endogenous protein are used as markers. This method has the advantage that it apportions duodenal digesta protein into its three constituent fractions whereas, using specific microbial markers, estimates of feed protein reaching the duodenum can only be derived as the difference between total protein flow at the duodenum and microbial protein flow with or without an arbitary allowance for endogenous protein. Consequently, errors in estimating microbial protein will result in errors in estimates of feed protein degradability in the rumen.

Studies in which different methods have been compared (McMeniman, 1975; Walker & Nader, 1975; Kennedy & Milligan, 1978; Ling & Buttery, 1978; Smith *et al.* 1978; Tamminga, 1978; Amos *et al.* 1979) have shown estimates of microbial N to vary with the marker used. Furthermore, the differences between methods were not consistent between studies, suggesting that dietary conditions may have a differential effect on the markers. Therefore, the present study was undertaken to compare the five most commonly used methods, i.e. DAPA, RNA, ^{35}S, ^{15}NAN and AAP, when used simultaneously to estimate microbial protein in duodenal digesta from sheep given two all-forage diets at low levels of intake. However, since the study was completed it has been found (A. B. McAllan, personal communication) that freezing and thawing of rumen microbes, as occurred in this study, can result in losses of RNA and therefore estimates based on RNA have not been included. For three of the methods (^{35}S, ^{15}NAN, AAP) the effect of site of sampling of the microbial fraction, i.e. from rumen contents of duodenal digesta, was also investigated. A preliminary account of the findings has been published (Siddons *et al.* 1979).

EXPERIMENTAL

Animals

Six Suffolk × Halfbred wethers weighing 45–55 kg and each fitted with a rumen cannula and re-entrant cannulas in the proximal duodenum and distal ileum were used. They were housed individually in metabolism crates in a temperature-controlled animal house with continuous lighting.

Diets

Grass silage was prepared from primary growth perennial ryegrass (*Lolium perenne* cv. S23), which was harvested with a double-chop precision forage harvester and ensiled without additive in a 2-t capacity butyl rubber silo. The silo was opened after 220 d and the contents transferred to polyethylene bags (600 g dry matter (DM)/bag), blast frozen at −33° for 48 h and then stored at −15° until fed. Dried grass was prepared from another primary growth perennial ryegrass (*Lolium perenne* cv. S23) sward, which was harvested with a double-chop precision forage harvester, dried in a high temperature rotary drum drier and cobbed (Thomson *et al.* 1972). Each diet was given at a daily level of 600 g DM/sheep in twenty-four equal hourly portions.

Experimental procedure

The experiment consisted of two periods, each of 7 weeks duration, with an intervening rest period of 5 weeks during which the sheep were kept in resting pens. All sheep received the grass silage in period 1 and the dried grass in period 2. No drinking-water was supplied but, instead, tap-water was infused continuously into the rumen at the rate of 20 ml/h in period 1 and 40 ml/h period 2. During the first 2 weeks of each period, 'donor' digesta was collected from the experimental sheep and stored at −15°, to be used subsequently to replace digesta removed during the collection period. At the beginning of the third week, ruthenium phenanthroline (Ru-ph, Tan *et al.* 1971; Beever *et al.* 1978) was included in the tap-water infusion (7·2 mg Ru-ph/d). After 4 d of Ru-ph infusion, $Na_2{}^{35}SO_4$ (200 μCi/d,

48 mg Na_2SO_4/d) was also included in the tap-water infusion and, in addition, $(^{15}NH_4)_2SO_4$ (960 atoms ^{15}N/1000 atoms total N; 53 mg ^{15}N/sheep per d) was infused (20 ml/h) into the rumen via a separate infusion line. The Ru-ph^{35}S and ^{15}N infusions were continued for 48 h. After 36, 42 and 48 h of infusion, samples of rumen contents, free from large particulate matter, were collected manually by suction through an indwelling sampler with a gauze filter and a microbial fraction prepared from each sample as described below. With both diets, the [^{35}S]methionine specific activity and ^{15}NAN enrichment of the microbial fractions was found to be relatively constant, indicating that steady-state conditions for ^{35}S and ^{15}N had been achieved. The three samples per animal were bulked for other analyses. A proportion of the total duodenal digesta flow was also collected during the last 12 h of infusion, using automatic sampling machines (Canaway & Thomson, 1977) and a representative sample taken for the preparation of a microbial fraction and the remainder freeze-dried. Microbial fractions were prepared from rumen contents and duodenal digesta by low-speed centrifugation at 1500 ***g*** for 10 min, followed by high-speed centrifugation of the supernatant fraction at 20000 ***g*** for 30 min at 4°. The sedimented microbes were washed twice with distilled water, stored at −15° and thawed before analysis.

Analytical methods

The DM content of the silage was determined by toluene distillation. Total N content of all samples was determined by Kjeldahl digestion, followed by an automated alkaline phenate-hypochlorite colorimetric procedure which was also used to measure the ammonia content of feed and digesta samples after extraction with 0·1 M-sulphuric acid. The buffer soluble N content of the feeds was determined by incubating 0·5 g DM in 20 ml buffer (McDougall, 1948) at 39° for 1 h. Insoluble material was removed by filtration and the N content of the filtrate analysed. The amino acid composition of feed, digesta amd microbial samples was measured, after hydrolysis with 6 M-hydrochloric acid, by automated ion-exchange chromatography (Moore & Stein, 1951) and methionine was measured as methionine sulphone after performic acid oxidation (Moore, 1963). The specific radioactivity of [^{35}S]methionine in duodenal digesta and microbial fractions was determined at The Grassland Research Institute, Hurley, using the procedure described by Beever, Harrison *et al.* (1974); the ^{15}N enrichment of non-ammonia-N (^{15}NAN) was determined at The University of New England, Armidale, using the procedure described by Nolan & Leng (1972) and Kempton *et al.* (1979), and the RNA and DAPA contents were determined at The National Institute for Research in Dairying, Reading, by the procedures described by McAllan & Smith (1969) and Smith *et al.* (1978). The Ru concentration of digesta and infusion solutions was measured by X-ray fluorescence spectrometry (Evans *et al.* 1977).

Calculation of results

Flow (g/d) of nutrients at the duodenum was calculated as Ru infused (mg/d) multiplied by the nutrient:Ru value (g/mg) in duodenal digesta. For the ^{35}S, ^{15}NAN and DAPA methods, the proportion of microbial N in duodenal digesta N was calculated by dividing the marker:N value in duodenal digesta by the marker:N value in the isolated microbial fraction, and the proportion was multiplied by total N flow at the duodenum to give microbial N flow. For the AAP method, microbial N flow at the duodenum was calculated by multiplying microbial amino acid flow at the duodenum (total amino acid flow × proportion of microbial amino acids as predicted by the AAP method) by the N:amino acid value of the microbial fraction.

Statistical analysis

For each diet, a paired t test was used to compare results obtained using either rumen or duodenal microbial fractions. Analysis of variance and Newman Keul's test were used for comparisons between methods within a diet, and for comparisons between diets.

RESULTS

The DM content of the silage and dried-grass diets was 217 and 895 g/kg fresh weight respectively. Total N content of the silage (32·1 g/kg DM) was higher than that of the dried grass (18·3 g/kg DM) as was the proportion of total N soluble in buffer (silage 0·74; dried grass 0·20). Ammonia accounted for 0·11 of silage total N and only 0·01 of the dried grass total N, whilst amino acids accounted for a higher proportion of the non-ammonia-N (NAN) of the dried grass (0·80) than of the silage (0·68).

Amounts of organic matter (OM), total N, NAN and amino acids ingested and flowing at the duodenum when sheep were given the two diets, are shown in Table 1. Organic matter intakes were similar for both diets and the amount flowing at the duodenum did not differ significantly ($P > 0·05$) between diets. Intakes of total N, NAN and amino acids were higher when silage was given, whereas amounts of these constituents flowing at the duodenum tended to be higher with the dried grass, but only the difference in amino acid flow was statistically significant ($P < 0·05$). Net losses of total N, NAN and amino acids occurred between mouth and duodenum when silage was given, compared with net gains when dried grass was given.

The concentrations of different microbial markers in microbial fractions isolated from either rumen contents or duodenal digesta and in duodenal digesta are presented in Table 2. [^{35}S]methionine specific activity of either the rumen microbial fraction or the duodenal microbial fraction did not differ significantly ($P > 0·05$) between diets. The ^{15}NAN enrichment of both fractions, on the other hand, was significantly ($P < 0·001$) higher when dried grass was given. For both diets, [^{35}S]methionine specific activity and ^{15}NAN enrichment were significantly ($P < 0·01$) lower in the duodenal microbial fraction than in the rumen microbial fraction; the mean concentration of the two isotope tracers in duodenal microbes being 0·76 and 0·75 respectively of their concentrations in rumen microbes when silage was given and 0·85 and 0·84 respectively when dried grass was given. The DAPA concentration in the rumen microbial fraction tended to be higher when dried grass was given than when silage was given but the difference was not significant ($P > 0·05$).

The amino acid compositions of the feeds, microbial fractions and duodenal digesta are presented in Tables 3 and 4. Apart from a lower arginine and higher aspartate content in the silage, the amino acid composition of the two feeds was similar. There were also no marked differences between diets in the amino acid composition of the rumen microbial fraction, the duodenal microbial fraction or the duodenal digesta. For each diet, differences between the amino acid composition of the rumen and duodenal microbial fractions were small, but for a number of amino acids they were statistically significant. The amino acid composition of bovine pepsinogen (Chow & Kassell, 1968), which was used to represent endogenous protein, is also included in Table 3. It differs from the other fractions, particularly in respect of its high serine content and low alanine, histidine and lysine contents.

The fractionation of the amino acids in duodenal digesta into feed, microbial and endogenous origins by the AAP method is shown in Table 5. When the amino acid composition of the rumen microbial fraction was used, the microbial component was estimated to comprise 0·39 of total amino acids in duodenal digesta when silage was given and 0·19 of the total when dried grass was given. Feed and endogenous amino acids comprised 0·36 and 0·25 of the total respectively with the silage diet and 0·67 and 0·14 of the total respectively with the dried grass. Replacing the amino acid composition of the rumen microbial fraction with that of the duodenal microbial fraction had no significant

Table 1. *Intake and flow at the duodenum of organic matter, nitrogen and total amino acids for sheep given silage or dried grass*

(Mean values for six sheep)

	Grass silage	Dried grass	SEM
Intake (g/d)			
Organic matter	563	566	
Total N	19·5	11·0	
Ammonia-N	2·2	0·1	
Non-ammonia-N	17·3	10·9	
Total amino acids	96·6	64·8	
Flow at duodenum (g/d)			
Organic matter	240	271	11·2
Total N	15·5	16·5	0·72
Ammonia-N	1·5	1·1	0·18
Non-ammonia-N	14·0	15·5	0·63
Total amino acids	64·8	80·4	4·08*

* $P < 0·05$.

Table 2. *Concentration of different markers in microbial fractions isolated from either rumen contents or duodenal digesta, and in duodenal digesta from sheep given grass silage or dried grass*

(Mean values with their standard errors for six sheep)

	Grass silage		Dried grass	
	Mean	SE	Mean	SE
[^{35}S]methionine specific activity (Bq/g) in				
Rumen microbial fraction	465	24·5	473	13·2
Duodenal microbial fraction	355	18·5	402	17·3
Duodenal digesta	267	16·5	248	11·5
^{15}N enrichment of non-ammonia-N (mmol/mol NAN) in				
Rumen microbial fraction	1·99	0·05	3·16	0·14
Duodenal microbial fraction	1·50	0·09	2·64	0·14
Duodenal digesta	1·00	0·07	1·57	0·08
Diaminopimelic acid-N (mg/g total N) in				
Rumen microbial fraction	5·26	0·207	5·87	0·473
Duodenal digesta	3·97	0·201	4·56	0·317

($P > 0·05$) effect on the relative proportions of the three components for the dried-grass diet, but significantly ($P < 0·05$) increased the microbial contribution (0·49) and decreased the feed contribution (0·27) for the silage diet

The proportion of microbial amino acids in duodenal digesta obtained using the AAP method was used in conjunction with the N:amino acid value for the microbial fraction to obtain estimates of the proportion of microbial N in duodenal digesta N (see *Calculation of results*). These are presented in Table 6, together with estimates obtained using the other three markers. Based on the composition of the rumen microbial fraction the proportion of microbial N in duodenal digesta N ranged between 0·32 and 0·76 when silage was given

Table 3. *Amino acid composition (g amino acid/kg total amino acid) of feed, microbial fractions and duodenal digesta from sheep given grass silage*

(Mean values with their standard errors for six sheep)

			Duodenal digesta		Microbial fraction†				
					Rumen		Duodenal		Statistical significance of difference
	Endogenous‡	Feed	Mean	SE	Mean	SE	Mean	SE	
Aspartate	12·3	12·9	10·5	0·15	11·9	0·12	11·2	0·07	**
Threonine	7·3	4·9	5·8	0·05	5·8	0·06	5·2	0·05	**
Serine	12·1	4·9	5·3	0·10	5·5	0·08	5·5	0·14	NS
Glutamate	10·9	12·0	12·8	0·17	14·1	0·14	14·0	0·07	NS
Proline	4·1	5·9	4·3	0·05	3·6	0·08	4·3	0·13	**
Glycine	6·0	5·3	6·0	0·04	6·0	0·03	6·1	0·03	*
Alanine	3·3	9·2	7·1	0·04	7·6	0·05	7·5	0·06	NS
Valine	6·7	7·3	6·8	0·08	5·4	0·09	5·3	0·09	NS
Isoleucine	9·5	6·9	7·8	0·13	5·5	0·06	5·7	0·02	NS
Leucine	7·6	9·1	8·4	0·20	7·4	0·04	7·7	0·08	*
Tyrosine	7·4	3·0	5·2	0·03	5·3	0·03	4·9	0·03	***
Phenylalanine	5·7	5·3	6·1	0·07	4·9	0·06	4·7	0·07	NS
Histidine	0·7	1·6	2·1	0·02	1·8	0·04	2·5	0·06	***
Lysine	2·7	7·6	4·8	0·13	8·7	0·18	8·0	0·14	NS
Arginine	2·4	2·2	4·7	0·01	4·5	0·07	4·9	0·16	*
Methionine	1·3	2·0	2·0	0·01	2·3	0·04	2·6	0·14	*
Total N (g/kg total AA)		202	242	5·5	196	3·0	187	7·1	NS

NS, not significant ($P > 0·05$). * $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.
Paired *t* test used to test significance of difference between rumen microbes and duodenal microbes.
† Microbial fraction isolated by differential centrifugation of either rumen contents or duodenal digesta.
‡ Composition of bovine pepsinogen as given by Chow & Kassell (1968).

Table 4. *Amino acid composition (g amino acid/kg total amino acid) of feed, microbial fractions and of duodenal digesta from sheep given dried grass*

(Mean values with their standard errors for six sheep)

	Feed	Duodenal digesta		Microbial fraction†				Statistical significance of difference
				Rumen		Duodenal		
		Mean	SE	Mean	SE	Mean	SE	
Aspartate	9·7	10·1	0·15	11·8	0·07	11·7	0·31	NS
Threonine	4·9	5·8	0·12	6·0	0·04	5·3	0·08	**
Serine	5·0	5·5	0·12	5·0	0·06	5·0	0·07	NS
Glutamate	14·7	13·1	0·18	13·7	0·20	13·8	0·16	NS
Proline	5·7	4·6	0·09	3·8	0·21	3·9	0·21	NS
Glycine	6·1	5·6	0·05	6·2	0·05	5·6	0·09	**
Alanine	7·6	7·4	0·10	7·5	0·06	7·5	0·15	NS
Valine	6·0	6·9	0·05	5·8	0·11	5·3	0·10	*
Isoleucine	6·4	6·5	0·11	6·5	0·13	6·1	0·09	*
Leucine	9·0	9·4	0·22	7·2	0·02	8·4	0·11	***
Tyrosine	3·7	5·0	0·12	5·1	0·09	5·2	0·08	NS
Phenylalanine	5·8	5·8	0·07	5·1	0·03	4·9	0·08	*
Histidine	2·0	1·9	0·12	1·6	0·08	1·9	0·05	**
Lysine	6·6	6·2	0·18	7·9	0·10	7·8	0·57	NS
Arginine	5·4	4·7	0·13	4·6	0·07	5·3	0·53	NS
Methionine	1·6	1·7	0·08	2·2	0·18	2·7	0·12	*
Total N (g/kg total AA)	169	212	8·4	201	4·1	235	5·9	***

NS, not significant ($P > 0·05$). * $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.
Paired t test used to test significance of difference between rumen microbes and duodenal microbes.
† Microbial fraction isolated by differential centrifugation of either rumen contents or duodenal digesta.

and between 0·15 and 0·80 when dried grass was given. The ranking of methods was similar for both diets with AAP giving the lowest estimates, DAPA the highest and ^{35}S and ^{15}NAN intermediate. AAP gave significantly ($P < 0·05$) lower estimates than all other methods with the dried-grass diet but did not differ significantly ($P > 0·05$) from ^{35}S with the silage diet. With both diets, DAPA gave significantly ($P < 0·05$) higher estimates than the other three methods and ^{35}S tended to give lower estimates than ^{15}NAN but the differences were not significant ($P > 0·05$).

In addition to differences between methods, there were also differences within a method, depending on whether the microbial fraction was isolated from rumen contents or duodenal digesta. For all three methods (^{35}S, ^{15}NAN, AAP), for which such a comparison was made, estimates obtained using the duodenal microbial fraction were higher and the differences were significant for ^{15}NAN ($P < 0·001$) with both diets, for ^{35}S ($P < 0·05$) with the dried grass and for AAP ($P < 0·05$) with the silage. Furthermore, whereas estimates of microbial N based on ^{35}S or ^{15}NAN did not differ significantly ($P > 0·05$) when using the rumen microbial fraction, estimates based on ^{15}NAN were significantly ($P < 0·05$) higher than those based on ^{35}S when using the duodenal microbial fraction.

The effect of using different methods for estimating microbial N on estimates of the efficiency of microbial N synthesis in the rumen, i.e. g microbial N at duodenum/kg OM apparently digested in the rumen, and on estimates of feed N degradability in the rumen is shown in Table 7. Efficiency of microbial N synthesis varied between 16 and 38 with the silage diet and between 10 and 46 with the dried grass. Only for the AAP method based on either the rumen or duodenal microbial fraction and for ^{15}NAN based on the rumen

Table 5. *Proportion of amino acids of feed, microbial and endogenous origins in duodenal digesta of sheep given grass silage or dried grass as predicted by the amino acid profile method*

(Mean values with their standard errors for six sheep)

	Grass silage					Dried grass				
	Rumen microbes†		Duodenal microbes†		Statistical significance of difference	Rumen microbes†		Duodenal microbes†		Statistical significance of difference
	Mean	SE	Mean	SE		Mean	SE	Mean	SE	
Feed	0·36	0·027	0·27	0·032	*	0·67	0·027	0·68	0·030	NS
Microbial	0·39	0·026	0·49	0·033	*	0·19	0·036	0·18	0·041	NS
Endogenous	0·25	0·005	0·24	0·009	NS	0·14	0·015	0·15	0·015	NS

NS, not significant ($P > 0·05$). * $P < 0·05$.

Paired *t* test used to test significance of difference between values obtained using the amino acid composition of rumen or duodenal microbes.

† Using the amino acid composition of the microbial fraction isolated by differential centrifugation of either rumen contents or duodenal digesta.

Table 6. *Estimates, using different marker methods, of the proportion of microbial nitrogen in duodenal digesta nitrogen of sheep giving either grass silage or dried grass*

(Mean values for six sheep)

	Grass silage			Dried grass		
	Rumen microbes‡	Duodenal microbes‡	Statistical significance of difference†	Rumen microbes‡	Duodenal microbes‡	Statistical significance of difference†
Amino acid profile	0·32[a]	0·38[a]	*	0·18[a]	0·20[a]	NS
[^{35}S]methionine	0·41[ab]	0·46[a]	NS	0·35[b]	0·44[b]	*
^{15}N-enriched non-ammonia-N	0·45[b]	0·60[b]	***	0·47[b]	0·56[c]	***
Diaminopimelic acid	0·76[c]	—	—	0·80[c]	—	—
SEM	0·037	0·031	—	0·044	0·034	—
df	15	10	—	15	10	—

NS, not significant ($P > 0·05$). * $P < 0·05$, *** $P < 0·001$.

a, b, c. For each column, values with different superscript letters were significantly different ($P < 0·05$).

† For each method within each diet, a paired t test was used to test significance of difference between values based on rumen microbes and those based on duodenal microbes.

‡ Estimates based on marker concentrations in microbial fractions isolated by differential centrifugation of either rumen contents or duodenal digesta.

Table 7. *Estimates, using different methods for measuring microbial nitrogen, of the efficiency of microbial N synthesis (g microbial N at duodenum/kg organic matter apparently digested in the rumen) and the proportion of feed N degraded in the rumen when sheep were given either grass silage or dried grass*

(Mean values for six sheep)

		Efficiency of microbial N synthesis			Feed N degradability†		
		Silage	Grass	SEM	Silage	Grass	SEM
Amino acid profile	R	16·2	9·8	1·71*	0·62	0·00	0·051***
	D	19·0	10·8	2·27*	0·66	0·02	0·054***
[^{35}S]methionine	R	19·9	19·9	1·87 NS	0·68	0·26	0·036***
	D	22·3	24·7	3·32 NS	0·72	0·38	0·056**
^{15}N-enriched non-ammonia-N	R	21·7	26·2	1·24*	0·70	0·43	0·046**
	D	29·4	31·6	2·18 NS	0·83	0·57	0·031**
Diaminopimelic acid	R	37·9	44·9	4·83 NS	0·97	0·93	0·078 NS

R, estimates based on the composition of rumen microbes.

D, estimates based on the composition of duodenal microbes.

NS, not significant ($P > 0·05$). * $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.

† $1-\left(\frac{\text{NAN flow at duodenum (g/d)} - \text{microbial N flow (g/d)} - 1·5 \text{ g/d endogenous NAN}}{\text{Feed N intake (g/d)}}\right)$.

microbial fraction was there a significant ($P < 0·05$) difference between the diets. However, whereas the AAP method gave lower values for the dried grass, ^{15}NAN gave lower values for the silage.

The proportion of feed N degraded in the rumen was calculated assuming 1·5 g/d endogenous NAN at the duodenum (Harrop, 1974). It ranged between 0·62 and 0·97 for

the silage and between 0·00 and 0·93 for the dried grass. There was no significant ($P > 0·05$) difference between diets when DAPA was used to estimate microbial N, whilst with all other methods feed N degradability was significantly ($P < 0·01$) lower for dried grass.

For both efficiency of microbial N synthesis in the rumen and feed N degradability, differences between methods within a diet were the same as for the values relating to the proportion of microbial N in duodenal digesta (Table 6).

DISCUSSION

The two main determinants of protein flow to the ruminant small intestine are feed protein degradability and efficiency of microbial protein synthesis in the rumen. Values for both are normally derived from estimates of microbial N flow at the duodenum and both have been found to vary under different dietary conditions (Agricultural Research Council, 1980). The purpose of the present study was to examine to what extent such variation may be attributed to the use of different methods for estimating microbial N. RNA is one of the more commonly used microbial markers and was originally included in the present study. However, it was subsequently found (A. B. McAllan, personal communication) that freezing and thawing, to which the rumen microbial fractions were subjected, can result in losses of RNA. Therefore, estimates of microbial N based on RNA, which tended to be slightly higher than those based on DAPA, have been omitted. The microbial RNA-N : N values obtained were, in fact, similar to other reported values (McAllan & Smith, 1971; Smith *et al.* 1975; Ling & Buttery, 1978) and the RNA method has, in general, tended to give higher estimates of microbial N than other methods (Ling & Buttery, 1978; Smith *et al.* 1978; Tamminga, 1978). Whilst this may be due to the presence in duodenal digesta of undegraded feed RNA or of RNA arising from the desquamation of epithelial cells in the abomasum, it is not known to what extent the loss of RNA on freezing of the microbial fraction may be a contributory factor.

The two diets used in the present study were chosen on the basis of previous studies which have shown that the N fraction of untreated silages is readily degraded by rumen micro-organisms (Beever *et al.* 1977), whereas the N of dried grass appears to be less susceptible to microbial degradation in the rumen (Beever, Cammell *et al.* 1974). When DAPA was used to estimate microbial N, N degradability values were high ($> 0·90$) for both diets, suggesting that at least for the dried-grass diet the method may have over-estimated microbial N. The AAP method, on the other hand, gave very low degradability values for the dried grass, suggesting that it may under-estimate microbial N. Degradability values obtained using ^{35}S or ^{15}NAN were intermediate and, as would be expected from the N solubility of the two diets, were higher for the silage than for the dried grass.

Depending on the method used for estimating microbial N, values for the efficiency of microbial N synthesis in the rumen varied considerably for both diets. In a comprehensive review of published values (Agricultural Research Council, 1980) efficiency values were found to range between 14 and 61 and it was concluded that, although on theoretical grounds there are good reasons for efficiency to vary, it was not possible to show any clear difference due to class of ruminant or type of diet. From the findings in the present study, it would appear that much of the variation may be attributed to the use of different methods for measuring microbial N. It is of interest to note that many of the higher values quoted by the Agricultural Research Council (1980) were obtained using DAPA as the microbial marker, which also gave the highest values in the present study.

In order to obtain reliable estimates of microbial N using the marker-dilution technique, either the microbial marker must be uniformly distributed throughout all microbial species or the isolated microbial fraction must be representative of those microbial species leaving

the rumen. A microbial fraction isolated by differential centrifugation is unlikely to be representative because of the loss of protozoa and particle-bound bacteria during low-speed centrifugation. The extent to which such unrepresentative sampling may affect estimates of microbial N will depend on the variability of marker concentration between different species. DAPA concentration in different bacterial species can vary quite considerably and it is also absent from protozoa (Synge, 1953; Work & Dewey, 1953; Rhuland, 1960; Czerkawski, 1976). Isotopic markers such as ^{35}S or ^{15}N are also unlikely to be uniformly distributed throughout all microbial species because labelling of different micro-organisms will vary according to the extent to which they incorporate preformed amino acids of dietary origin. Microbial incorporation of both ^{35}S and ^{15}N, when infused into the rumen as $Na_2{}^{35}SO_4$ or $(^{15}NH_4)_2SO_4$ respectively, has been shown to vary with the amount and type of protein in the diet (Pilgrim *et al.* 1970; McMeniman *et al.* 1976).

Because DAPA is absent from protozoa and therefore does not take into account the protozoal contribution to microbial flow, it is generally considered to under-estimate microbial N. However, with both diets, estimates of microbial N based on DAPA were higher than those based on either ^{35}S, ^{15}NAN or AAP. In contrast, Ling & Buttery (1978) and Walker & Nader (1975) obtained lower estimates with DAPA than with ^{35}S, whilst McMeniman (1975) found that the two methods gave very similar results. It has been suggested (Nikolic & Javanovic, 1973) that DAPA may over-estimate microbial N because of the preferential degradation of bacterial cell contents in the rumen. If this did occur, DAPA-containing cell wall fragments present in the rumen would also be present in the isolated microbial fraction giving high DAPA-N:N values. The DAPA-N:N values of the microbial fractions in the present study were in fact slightly lower than values (range 6·10–7·13 mg/g) reported by other workers (Weller *et al.* 1958; Ling & Buttery, 1978; Tamminga, 1978). For the DAPA method to give estimates of microbial N lower than those obtained with ^{35}S, the DAPA-N:N values for the isolated microbial fractions would have had to be greater than 9·61 and 13·10 for the silage and dried-grass diets respectively.

When using DAPA to estimate microbial N it is usual to isolate the microbial fraction from rumen contents, whereas with isotopic markers the microbial fraction is usually isolated from duodenal digesta. The latter procedure has the advantage that attainment of steady-state plateau conditions with respect to the specific activity of the label in the microbes is not necessary, whilst a further advantage would appear to be sampling of those microbes which reach the duodenum, although it is not known to what extent they may have been modified by lysis or proteolytic attack during passage through the abomasum. Morphological differences between rumen and duodenal microbes have been observed and the $^{35}S:NAN$ value also tended to be lower in duodenal microbes, although the difference was not significant (Mathers & Miller, 1980). In the present study, both [^{35}S]methionine specific activity and ^{15}NAN enrichment were significantly lower in duodenally-derived microbes than in ruminally-derived microbes and consequently estimates of microbial N based on marker concentration in duodenal microbes were higher. Irrespective of site of sampling of the microbial fraction, estimates of microbial N based on ^{35}S tended to be lower than those based on ^{15}NAN, whereas Kennedy & Milligan (1978) found the opposite, which led them to suggest that the secretion of organic ^{35}S compounds into the omasum and abomasum may cause the ^{35}S method to over-estimate microbial N, although it would seem more likely that ^{15}NAN secretion into the abomasum, possibly in the form of urea, might be greater than the secretion of ^{35}S-labelled organic compounds.

When first describing the AAP method, Evans *et al.* (1975) showed that it gave estimates similar to DAPA but lower than RNA, whereas in the present study, values obtained using AAP were much lower than those obtained with all other methods. The method is based on a number of assumptions which are probably not valid. For example, the amino acid

profile of the endogenous fraction is based on bovine pepsinogen (Chow & Kassell, 1968), whereas it is likely to contain a number of other proteins such as albumen, globulins and mucoproteins (Harrop, 1974). Feed protein reaching the duodenum is assumed to have the same amino acid composition as dietary protein, whereas it has been shown that the amino acid composition of the readily-degradable fraction of ruminant feeds is different from that of the less-degradable fraction (MacGregor *et al.* 1978; Schingoethe & Ahrar, 1979). In addition, the ion-exchange chromatography technique used for estimating amino acid is subject to quite large errors and if, as in the present study, the amino acid profiles of the feed, microbes and duodenal digesta are not appreciably different, the method would be quite sensitive to errors in amino acid analysis.

Of the methods examined, ^{35}S and ^{15}NAN gave the most reasonable and therefore probably the most reliable estimates of microbial N. However, estimates obtained using the two methods did differ significantly and also the site of sampling of the microbial fraction had a significant effect. It is not possible to say which procedure yields the most accurate estimates, because no absolute standards exist. Of the two methods, ^{15}NAN involves fewer analyses and was found to have lower coefficient of variation. However, since this study was completed, Mathers & Miller (1980) have published a simplified ^{35}S procedure, reducing the number of analyses and therefore probably increasing the precision of the method. ^{35}S does have the advantage that it is considerably less expensive, although ^{15}N, on the other hand, being non-radioactive, is not subject to the restriction placed on the use of radioactive materials and therefore can be used in all situations.

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