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The simultaneous use of ribonucleic acid, ^{35}S, 2,6-diaminopimelic acid and 2-aminoethylphosphonic acid as markers of microbial nitrogen entering the duodenum of sheep

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1. Three sheep, each fitted with a ruminal cannula and duodenal re-entrant cannulas were given three isonitrogenous, isoenergetic diets in a Latin-Square design. Each diet contained (/ kg) approximately 400 g N as white fish meal, soya-bean meal or urea and approximately 600 g dry matter (DM) was barley grain. The diets were fed continuously and supplied about 28 g N/d.

2. Total duodenal digesta was collected manually for 72 h and the proportions of microbial N in that digesta were simultaneously estimated for all sheep using RNA, radioactive sulphur (^{35}S), diaminopimelic acid (DAPA) and aminoethylphosphonic acid (AEPA) as markers.

3. Three of the estimation methods showed that the variable source of dietary N had the greatest (RNA $P < 0.05$, ^{35}S $P < 0.005$, DAPA $P < 0.1$) effect on the proportions of microbial N in duodenal digesta, though differences between sheep accounted for some variation.

4. These methods also ranked the diets in the order: urea > soya-bean meal > fish meal with respect to the proportions of digesta N that were microbial in origin; the respective mean values for these diets with the different markers were: RNA 0·98, 0·70, 0·56; ^{35}S 0·92, 0·64, 0·54; DAPA 0·80, 0·47, 0·42.

5. AEPA was found to be present in substantial quantities not only in isolated rumen protozoa, but also in dietary and bacterial material; an observation that invalidated its further use as a protozoal marker.

6. Calculations using values obtained from the ^{35}S procedure showed that the proportions of dietary N degraded within the rumen were 0·38, 0·43 and 0·89 for the white fish meal, soya-bean meal and barley respectively.

7. The marker methods are compared and their advantages and disadvantages (real and apparent) are discussed. It is concluded that where microbial N estimates of a more general and comparative nature are required, the use of RNA as a marker is probably adequate. Where information for more exacting purposes is required, the use of ^{35}S appears to be more appropriate.

Effective nitrogen utilization by the ruminant involves supplying the small intestine with sufficient protein, composed of the correct proportions of amino acids, to satisfy the productive requirements of the animal. Since many nitrogenous compounds can be both degraded and synthesized in the rumen, an adequate description of the N reaching the duodenum requires measurement of the contributions made by undegraded dietary, bacterial, protozoal and endogenous fractions.

Endogenous fractions have rarely been quantified. Research in this area has mainly been concerned with estimating the dietary and microbial fractions of digesta. The methods used have generally relied upon the measurement of the concentration of some microbial component (or marker) in the digesta. By relating this to the marker concentration in the actual micro-organisms, an estimate of the proportion of microbial material in the digesta is obtained.

The markers used have included nucleic acids, measured as total RNA+DNA (Coelho da Silva, Seeley, Thomson, Beever & Armstrong, 1972), RNA (McAllan & Smith, 1972), or DNA (Temler–Kucharski & Gaussères, 1965). The microbial incorporation of isotopic

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Table 1. *The composition (g/kg fresh wt), laboratory analysis (dry matter (DM) basis) and DM intake of the fish meal (F), soya-bean meal (S) and urea (U) diets*

Diet ...	F	S	U
Ingredient			
Barley grain (N, 18·1 g/kg)	610	550	685
Barley straw	150	150	150
Molassed peat*	100	100	100
White fish meal (N, 96·4 g/kg)	90		
Soya-bean meal (N, 66·1 g/kg)		150	
Urea			15
Vitamins and minerals†	50	50	50
DM (g/kg)	879	885	883
N (g/kg)	25·1	25·8	24·1
Gross energy (MJ/kg)	18·2	18·2	18·1
DM intake (g/d)	1120	1090	1160

* Nutramol 20; Rumenco Ltd, Burton-on-Trent.

† Sodium chloride–steamed-bone flour–limestone–trace mineral and vitamin supplement (3 : 3 : 3 : 1, by wt); trace mineral and vitamin supplement contained (mg/kg): 44 manganese, 41 iron, 38 zinc, 2 copper, 1·3 cobalt, 1·5 retinol equivalent, 0·03 cholecalciferol equivalent.

labels such as ^{15}N (Pilgrim, Gray, Weller & Belling, 1970; Mathison & Milligan, 1971) and ^{35}S (Beever, Harrison & Thomson, 1972; Hume, 1974) have also been used. These methods give measures of material that is considered to be primarily microbial in origin. Bacterial contributions have been measured by 2,6-diaminopimelic acid (DAPA) (Hutton, Bailey & Annison, 1971) and the protozoal contributions by 2-aminoethylphosphonic acid (AEPA) (Hagemeister, 1975). These five components are commonly regarded as the most useful markers presently available. Unfortunately each has inherent disadvantages. In particular, the use of ^{15}N is associated with such high cost and complex analytical techniques that its application has been strictly limited. The remaining four markers (nucleic acid, ^{35}S, DAPA and AEPA) have not been reported as simultaneously compared methods for estimating microbial N passing to the duodenum. This is considered to be an important exercise since no calibration procedure can exist and hence a single method cannot be regarded as providing definitive results. Therefore the most satisfactory way of assessing the methods is to compare them under common conditions. This is a report of such a comparison between techniques using RNA, ^{35}S, DAPA and AEPA.

EXPERIMENTAL

Animals and their management

Three crossbred wether sheep (Q, K and W) of the same age (about 3 years) and the same weight (60–65 kg) were used. Each was fitted with a permanent cannula in the rumen and re-entrant cannulas in the proximal duodenum. The sheep were housed in metabolism cages under approximately constant environmental conditions and continuous lighting.

Diets

Three isoenergetic, isonitrogenous diets were compounded and pelleted through a 12·5 mm die. Approximately 400 g total N/kg diet was supplied as white fish meal, soya-bean meal or urea. The composition, laboratory analysis and daily allowance of each diet is given in Table 1.

Experimental design and sample preparations

Each sheep was given, at 1 h intervals, each diet according to a 3 × 3 Latin-Square design. There were no feed refusals. Water was constantly available. Throughout the entire experiment a solution of sodium sulphate (0·5 g $Na_2SO_4.H_2O$/l) was infused into the rumen of each sheep via a multi-branched infusion tube at a rate of approximately 40 ml/h.

Each period of the experiment consisted of approximately 30 d. After at least 5 d on full ration intake (see Table 1), approximately 1500 ml rumen digesta was withdrawn from each animal, pooled, mixed and one-third of the mixed digesta was returned to each sheep in order to minimize any residual treatment effects and to present initially a common microbial population to each animal. On day 22, samples of mixed rumen bacteria and protozoa were isolated from each sheep by a procedure based on that of Ibrahim, Ingalls & Bragg (1970). All samples were judged to be pure by microscopic examination. From day 25, total duodenal digesta was collected manually for the next 72 h as described by Oldham & Ling (1977). At the commencement of each 72 h duodenal digesta collection, $Na_2\ ^{35}SO_4$ (The Radiochemical Centre, Amersham, Bucks.) was added to the rumen infusate so that it contained approximately 1 μCi ^{35}S/ml. Additional samples of duodenal digesta collected at 48, 56, 64 and 72 h were prepared as whole digesta (*W*) and microbial (*M*) fractions by methods similar to those described by Harrison, Beever & Thomson (1972). The day after completion of duodenal digesta collections, microbial isolates were again prepared from rumen digesta of each sheep.

Methods of analysis

The dry matter (DM) of dietary samples was determined by drying for 18 h at 105°; all other samples were freeze-dried. The bacterial and protozoal isolates obtained before and after duodenal digesta collections, were combined and the duodenal digesta was bulked on a 12 h basis. Total N was determined by the Kjeldahl method (Association of Official Agricultural Chemists, 1965) and the gross energy of the diets was measured by adiabatic bomb calorimetry (Association of Official Agricultural Chemists, 1965).

RNA analysis. The RNA content of diets, duodenal digesta and microbial isolates was extracted by the sodium chloride procedure of Guinn (1966) and assayed by the orcinol reaction (Kerr & Seraidarian, 1945), as previously outlined by Ling & Buttery (1976). Standards of RNA (baker's yeast RNA, type XI; Sigma Chemicals, Kingston-upon-Thames, Surrey) in a range up to 300 μg produced a linear calibration curve and additions of this RNA to duodenal digesta resulted in recoveries of 95·5 ± 2·5% (mean ± SE for four observations).

^{35}S analysis. Samples (50–100 mg DM) of *W* and *M* fractions were hydrolysed in 200 ml 6 M-hydrochloric acid by refluxing in an oil-bath for 22 h. After cooling, an internal standard of 2·5 μmol DL-norleucine was added. The solution was then filtered and evaporated to dryness under reduced pressure at 37° in a rotary evaporator. The residue was washed three times with 10 ml portions of 0·1 M-HCl. Finally, the dried residue was dissolved in 5 ml distilled water, passed through a 0·45 μm filter (Millipore SA, Molsheim, France) and stored at 0°.

Methionine contents were estimated using an automatic amino acid analyser (Evans Electro-Selenium, Halstead, Essex; Model 294) with a 335 mm × 9 mm i.d. column containing Aminex A5 resin (Biorad, Richmond, Calif., USA). The method was that for physiological fluids described by Atkin & Ferdinand (1970) and the methionine concentrations were calculated with reference to the norleucine internal standard. Samples of hen egg-white lysozyme (Grade 1; Sigma Chemicals) were hydrolysed alone and with 100 or

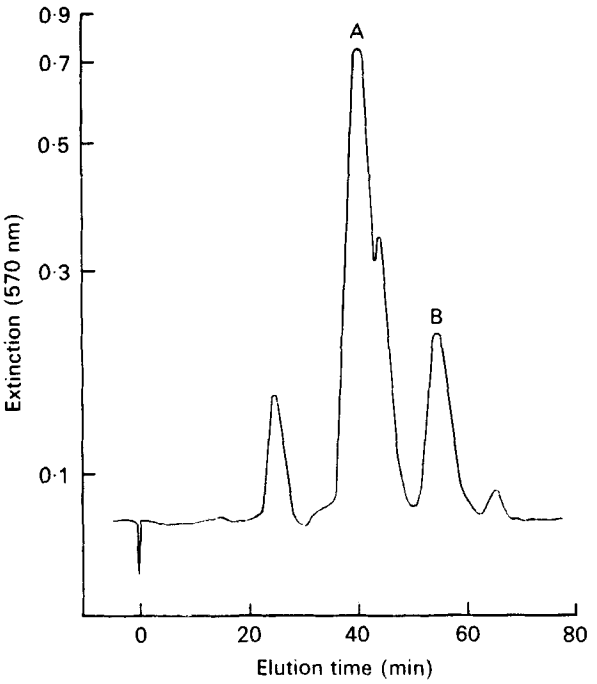

Fig. 1. The fractionation pattern of a hydrolysate of isolated rumen protozoa eluted from a cation-exchange chromatography column at 54° with a sodium buffer, pH 2·13 (for details of procedures, see p. 169). A was caused by coloured condensation products and B was due to 2-aminoethylphosphonic acid (AEPA). The sample size of the hydrolysate applied to the column was 0·1 ml and this contained 0·043 μmol AEPA.

200 mg maize starch and analysed according to this procedure. By comparison with the amino acid values for the standard protein (Jollès, 1967), satisfactory methionine recoveries of 101·7 ± 0·5% (mean ± SE for six observations) were obtained. In addition, the elution of oxidation products of methionine was not observed.

[^{35}S]methionine activity was determined by collecting the appropriate fractions using a cation-exchange resin column similar to that described for methionine analysis. Samples of DL-[1-^{14}C]methionine (The Radiochemical Centre) were eluted after a period of between 50 and 65 min. When hydrolysed digesta samples were applied to the column, no other ^{35}S-labelled compounds were eluted after a period of between 40 and 80 min, therefore fractions were collected for this period and assayed for [^{35}S]methionine activity. The fractions (each approximately 1·5 ml collected in a 2·4 min period) were taken up in 10 ml scintillant (2000 ml Triton X-100 (Sigma Chemicals); 1000 ml toluene; 12 g diphenyloxazole; 0·3 g 1,4-bis, 2-(phenyloxazolyl)-benzene) and radioactivity measured using a liquid scintillation counter (Tracerlab Corumatic 200; ICN, Walton-on-Thames). Quenching was corrected by the external-standard channel-ratio facility. Radioactivity was corrected for isotopic decay to a common date and the specific activity in all *W* and *M* samples was calculated.

DAPA analysis. The method used was based on that of Hutton *et al.* (1971). The hydrolysis procedure was essentially as described previously, except 400 mg samples of diets, digesta, isolated bacteria or protozoa were used. Eluted standards of DL-DAPA (Sigma Chemicals) produced a linear calibration curve and hydrolysis recoveries of DAPA (up to 0·4 μmol) added to duodenal digesta were satisfactory at 96·9 ± 1·4% (mean ± SE for six observations).

AEPA analysis. The samples were those hydrolysed and prepared for DAPA analysis and analysed using the automatic amino acid system described for methionine, except that a 395 mm column was used at 54°. The eluting buffer was prepared from the following: sodium hydroxide 330 g, citric acid 840 g, polyoxyethylene lauryl ether 30 g, 6 M-HCl 428 ml, thiodiglycol 200 ml, methanol 1200 ml. These were made up to 40 l with deionized water and finally adjusted to pH 2·13 with HCl. The buffer elution rate was 0·59 ml/min and detection was by the ninhydrin reaction recorded at 570 nm. Regeneration of the resin was by 0·2 M-NaOH.

There are several ninhydrin-positive substances that can be eluted before aspartic acid in a system such as that described. Some, such as phosphoserine and phosphorylethanolamine would be destroyed by the acid-hydrolysis procedure, though others could still interfere. However, during the development of this system, the pH and temperature were often altered, but AEPA was always eluted as a single symmetrical 'peak', whether recorded at 570 nm or 440 nm. Therefore under the conditions described, AEPA can be separated with an elution time of approximately 52 min as shown in Fig. 1.

Standards of AEPA (Sigma Chemicals) up to 0·15 μmol were eluted from the column and produced 'peak' areas that resulted in a linear standard curve. This was used for estimating the AEPA concentration of all hydrolysed samples. Recoveries of AEPA (up to 0·15 μmol) added to duodenal digesta and carried through the hydrolysis procedure were satisfactory at 98·0 ± 3·1 % (mean ± SE for six observations).

Protozoal counting. On the 2 d that microbial isolates were prepared, ciliate protozoa were counted. Strained rumen fluid samples (5 ml) were collected at 07.00, 11.00, 15.00 and 19.00 hours on the earlier sampling days and at 10.00 hours on the later sampling days. These were added to 10 ml portions of glycerol–water–formalin (500 : 450 : 50, by vol.). Protozoal suspensions were pipetted onto a counting chamber (Neubauer improved; Hawksley, London) and four randomly-selected fields of view (magnification × 10) were counted. Since five samples were taken from each sheep given each diet, the protozoal numbers (Table 6) were the means of twenty counted fields.

Statistical analysis. The results were, where appropriate, analysed as a 3 × 3 Latin Square. Missing values were calculated for Tables 3 and 6 where insufficient sample was available for analysis.

RESULTS

Duodenal digesta DM *and total N.* The mean daily passage of both these components is shown in Table 2. Values for both components were always significantly less ($P < 0·025$) for sheep Q.

RNA marker procedure. The RNA-N and DNA-N values were calculated on the assumption that RNA and DNA contain 14·8 and 14·0 % N respectively. Table 3 shows that considerable variation existed for RNA-N: total N for bacteria and particularly for protozoa, but these differences were not related to the effects of sheep, diets or periods of sampling. DNA-N: total N values for bacteria and protozoa were calculated from estimates of the extracted nucleotides using the diphenylamine reaction (Burton, 1956). However, relatively large variations about the mean (± SE) value for DNA-N : total N (mg/g) (bacteria 30·9 ± 3·8; protozoa 4·3 ± 0·9; 9 and 8 observations respectively) were evident. Therefore bacterial RNA-N was considered the most appropriate marker (a finding in common with the conclusion of Smith & McAllan (1970)).

The duodenal passage of RNA-N varied from 0·49 to 2·63 g/d, though the results given in Table 3 are the mean values for 3 d. The individual mean values for bacterial RNA-N : total N were used to calculate the proportions of microbial N in duodenal digesta for each sheep. These proportions were found to be markedly affected ($P < 0·05$) by the variable

Table 2. *The mean daily passage of dry matter and total nitrogen (g/d) at the duodenum of sheep given fish meal (F), soya-bean meal (S) and urea (U) diets**

Sheep	Diet	Dry matter	Total N
Q	F	387	14·28
	S	487	19·27
	U	462	15·40
K	F	616	20·22
	S	556	21·64
	U	592	20·23
W	F	562	20·58
	S	526	26·77
	U	633	24·50
Mean	F	522	18·36
	S	523	22·56
	U	562	20·04
SE (2 df)		10·9	0·42
CV		3·51	3·54

CV, coefficient of variation.
* For details of composition, see Table 1.

Table 3. *Values for RNA-nitrogen: total N (mg RNA-N/g N) of isolated, mixed rumen bacteria and protozoa, the mean daily duodenal passage of RNA-N (g/d) and microbial N: total N of digesta entering the duodenum as determined by the RNA marker procedure* for sheep given fish meal (F), soya-bean meal (S) and urea (U) diets†*

(Mean values with their standard errors for three daily observations)

		RNA-N: total N			Microbial N: total N	
Sheep	Diet	Bacteria	Protozoa	Duodenal RNA-N	Mean	SE
Q	F	91·8	40·8	0·73	0·55	0·029
	S	63·8	49·2	1·00	0·81	0·029
	U	99·3	41·6	1·35	0·88	0·089
K	F	85·8	64·0	1·07	0·62	0·049
	S	97·3	45·6	1·32	0·63	0·060
	U	86·3	38·8	1·88	1·08	0·038
W	F	108·9	60·2	1·15	0·52	0·033
	S	95·7	48·6	1·68	0·66	0·037
	U	100·4	ND	2·40	0·97	0·037
Mean	F	95·5	55·0	0·98	0·56	
	S	85·6	47·8	1·33	0·70	
	U	95·3	48·4	1·88	0·98	
SE (2 df)		5·24	1·73‡	0·08	0·05	
CV		9·85	4·86	9·29	11·39	

ND, not determined because of insufficient sample; CV, coefficient of variation.
* For details, see p. 167.
† For details of composition, see Table 1. ‡ 1 df.

dietary N source. In one animal, the proportion of microbial N in the digesta was estimated to be greater than unity.

^{35}S *marker procedure.* The relevant mean values are shown in Table 4. The specific activities of the *W* and *M* fractions for each sheep within each 24 h sampling period were (with the possible exception of sheep K given the urea diet) quite uniform. The specific activity ratio, *W*: *M* gives only the proportion of duodenal methionine that is of microbial

Table 4. *The specific activities of methionine in the whole (W) and microbial (M) fractions of duodenal digesta (disintegrations/min per μmol methionine) and W : M; the duodenal passage of total methionine (g/d) and methionine : total N of the M fractions, the duodenal passage of total N (g/d) and microbial N: total N of digesta entering the duodenum as determined by the ^{35}S marker procedure* for sheep given fish meal (F), soya-bean meal (S) and urea (U) diets*†

(All values refer to the final 24 h of each 3 d duodenal digesta collection period; mean values with their standard errors for four 6 h observations for duodenal digesta and for four observations for methionine)

		W		*M*						Microbial N: total N	
Sheep	Diet	Mean	SE	Mean	SE	*W* : *M*	Total methionine	Microbial methionine: total N	Total N	Mean	SE
Q	F	604	29	1234	96	0·489	1·58	0·095	15·57	0·52	0·018
	S	407	16	812	26	0·501	2·80	0·097	26·49	0·55	0·024
	U	878	24	1312	33	0·669	3·33	0·172	14·11	0·92	0·031
K	F	334	13	713	27	0·469	2·31	0·104	20·50	0·51	0·030
	S	540	24	898	16	0·599	2·52	0·109	19·37	0·71	0·024
	U	2163	229	2289	243	0·945	2·90	0·125	23·29	0·94	0·016
W	F	386	6	788	49	0·489	2·33	0·100	19·41	0·59	0·018
	S	1226	30	1444	34	0·848	3·28	0·156	26·88	0·66	0·020
	U	252	21	1143	26	0·867	3·33	0·119	26·56	0·91	0·032
Mean	F	441		912		0·482	2·07	0·100	18·49	0·54	
	S	691		1051		0·649	2·87	0·121	24·25	0·64	
	U	1098		1581		0·827	3·19	0·139	21·32	0·92	
SE (2 df)		26·2		10·5		0·009	0·25	0·021	1·49	0·11	
CV		6·10		1·54		2·31	15·98	30·13	12·11	2·65	

CV, coefficient of variation.

* For details, see p. 167.

† For details of composition, see Table 1

Table 5. *Values for diaminopimelic acid (DAPA)-nitrogen: total N (mg DAPA-N/g N) of isolated, mixed rumen bacteria, the mean daily duodenal passage of DAPA-N (mg/d) and bacterial N: total N of digesta entering the duodenum as determined by the DAPA marker procedure* for sheep given fish meal (F), soya-bean meal (S) and urea (U) diets†*

(Mean values with their standard errors for three daily observations)

Sheep	Diet	Bacterial DAPA-N: total N	Duodenal DAPA-N	Bacterial N: total N	
				Mean	SE
Q	F	10·40	37·1	0·25	0·007
	S	7·91	39·2	0·26	0·072
	U	4·95	76·8	1·01	0·009
K	F	5·63	57·3	0·50	0·019
	S	5·14	76·4	0·68	0·053
	U	6·18	97·8	0·79	0·042
W	F	5·66	58·7	0·50	0·028
	S	6·84	84·3	0·46	0·020
	U	8·07	119·5	0·60	0·009
Mean	F	7·23	51·0	0·42	
	S	6·63	66·6	0·47	
	U	6·40	90·8	0·80	
SE (2 df)		1·04	6·27	0·08	
CV		26·73	15·09	25·57	

CV, coefficient of variation.
* For details, see p. 168.
† For details of composition, see Table 1.

origin. To calculate the quantity of N of microbial origin, the ratio, methionine : total N of *M* and the total daily passage of methionine at the duodenum (calculated from the appropriate DM passage and the methionine contents of *W*) were taken into consideration as follows:

$$\text{total microbial N} = \frac{\text{specific activity of } W}{\text{specific activity of } M} \times \frac{\text{duodenal methionine passage}}{\text{methionine : total N for } M}.$$

Since ^{35}S measurements were made for only 24 h (that is between 48 and 72 h of the duodenal digesta collections), all calculations were based on values related to that period (Table 4).

The estimated microbial N fractions were subject to some variation due to sheep differences ($P < 0{\cdot}1$), but again it was the diets that exhibited the greatest influence upon this measurement ($P < 0{\cdot}005$). A 'period' effect ($P < 0{\cdot}1$) was also apparent.

DAPA marker procedure. The relevant values are recorded in Table 5. Marked variations were found for DAPA-N : total N of isolated rumen bacteria, but again these could not be related to the effect of animals, diets or periods. No DAPA was detected in the diets. Though traces of DAPA were detected in protozoal samples, these were far too low to quantify (less than 0·05 mg DAPA/g protozoal DM).

The daily duodenal passage of DAPA varied from 0·16 to 0·87 g, but the 3 d mean values are given in Table 5. The individual values for DAPA-N : total N of isolated bacteria were used to calculate the duodenal digesta N fractions of bacterial origin. These latter values were only slightly ($P < 0{\cdot}1$) influenced by the differences between the diets. The proportions of bacterial N in the digesta of sheep Q given the fish-meal and soya-bean diets were considered to be particularly small; whereas when the urea diet was fed to this animal, this fraction was exceedingly large.

AEPA marker procedure. Table 6 shows that in common with the other procedures described here, the individual values for marker : total N (in this instance AEPA-N : total N

Table 6. *Values for aminoethylphosphonic acid (AEPA)-nitrogen: total N (mg AEPA-N/g N) of the diets, isolated, mixed rumen protozoa and bacteria, the mean daily duodenal passage of AEPA-N (mg/d) and the rumen ciliate populations ($\times 10^{-5}$/ml) for sheep given fish meal (F), soya-bean meal (S) and urea (U) diets**

AEPA-N: total N

Diets	
F	0·490
S	0·467
U	0·528

Sheep	Diet	AEPA-N: total N		Duodenal AEPA-N	Ciliates
		Protozoa	Bacteria		
Q	F	2·29	0·50	20·2	22·8
	S	2·17	1·09	33·2	41·2
	U	2·51	0·23	18·5	4·4
K	F	1·59	0·23	23·1	14·7
	S	2·85	0·61	28·9	32·1
	U	1·80	0·35	24·5	29·6
W	F	2·11	0·26	20·5	11·3
	S	1·77	0·21	21·6	6·2
	U	ND	ND	18·7	2·7
Mean	F	2·00	0·33	21·3	16·3
	S	2·26	0·64	27·9	26·5
	U	1·72	0·22	20·6	12·2
SE (2 df)		0·06†	0·03†	2·56	9·5
CV		4·31	106·32	19·09	89·72

ND, not determined because of insufficient sample; CV, coefficient of variation.
* For details of composition, see Table 1.
† 1 df.

of protozoa) varied substantially. In addition, Table 6 displays two surprising observations, i.e. that considerable concentrations of AEPA were found in both dietary and bacterial material. Appropriate calculations suggested that the proportions of N reaching the duodenum of protozoal origin were between 0·46 and 0·79. These were considered to be unrealistically high and, in view of the previous observations, it seemed most probable that the AEPA measured at the duodenum did not all originate from the passage of protozoal material.

DISCUSSION

Of the four marker procedures for estimating the proportion of microbial N used in this study, three (RNA, ^{35}S and DAPA) produced values related to the variable source of N in the diets. These values, with respect to outflow of microbial N from the rumen, were in the order urea > soya-bean > fish meal. But while these marker methods produced the same order for the diets, the actual values each produced were not equivalent.

The use of RNA as a marker depends on the assumption that all of this component isolated at the duodenum is of microbial origin. Pure RNA has been shown to be rapidly and almost totally degraded within the rumen (Smith & McAllan, 1970). However, diets contain RNA; in this present study the values obtained were (mg RNA-N/g DM): fish meal 0·91, soya-bean meal 1·01, urea 0·96. Since significant amounts of dietary materials often escape degradation in the rumen, it is not unlikely that the RNA associated with these undegraded dietary fractions also contributes to the duodenal digesta RNA. The fact that

values of greater than 1·0/d were obtained for the proportion of microbial N with the urea diet would suggest this to be a real problem. Another deficiency of the RNA procedure is its ill-defined consideration of a protozoal fraction in digesta. The ratio RNA-N : total N used in the present calculations was derived from bacteria only. Although it is probable that bacteria always account for the greater proportion of the microbial N reaching the duodenum, an adequate marker procedure should also be sensitive to protozoal N contributions. Where the latter are substantial, the use of RNA-N : total N for bacteria only would underestimate the duodenal microbial N since Table 3 shows that the values for RNA-N : total N of protozoa were lower than those of bacteria. Unfortunately, it is not realistic to adjust the ratio to compensate for protozoal contributions, since the latter constitute an unknown part of the microbial N fraction to be measured.

The 'RNA' analytical method used in this study is quite different from the one most widely adopted in ruminant studies, i.e. that of McAllan & Smith (1969). Nevertheless, the method we used is considered to be simple (a high-speed centrifuge, spectrophotometer, water-bath and test-tubes are the only equipment needed), rapid (twenty samples analysed/d) and quantitative (as shown by recovery values). However, the method, as used in this study, estimated RNA in the presence of extracted DNA. This has been shown to cause an over-estimation of RNA when determined by the orcinol reaction (see Munro & Fleck, 1966). When investigated here, additions of pure DNA (calf thymus, type V; Sigma Chemicals) to pure RNA produced a linear response with a 16% over-estimation of RNA when RNA : DNA values of 3 : 1 (by wt) were used; that is the approximate value for RNA : DNA for all materials analysed in this study.

Two further considerations would, however, support the contention that the RNA extraction and assay procedure used here was satisfactory. First, estimates of total nucleic acids in all isolated microbial samples were made from their extinctions at 260 and 280 nm, using a nomogram (Warburg & Christian, 1942). Not only were the ultraviolet spectra of the extracted nucleotides fairly free from interfering substances, but they produced results for total nucleic acids that were on average 13% lower than those values obtained by the diphenylamine plus orcinol reactions (a value similar to that of 16% already discussed). Second, the mean value for RNA-N : total N for bacteria from this present study (0·092) is 21% (or again similar to the value of 16%) higher than that (0·076) reported by Smith & McAllan (1974) as determined in sheep by the generally accepted technique of McAllan & Smith (1969).

This over-estimation in the RNA assay could be simply overcome by subjecting the extracted nucleotides to alkaline hydrolysis and fractionation as in the Schmidt & Thannhauser (1945) procedure or by the use of a simple ion-exchange resin column to purify the ribonucleotide fraction before the orcinol reaction. Even so, it should be understood that there are doubts as to the ability of any of the available analytical techniques to produce absolute nucleic acid values (see Munro & Fleck, 1966). But this does not necessarily invalidate their use for marker analysis. All that is required of an extraction and assay method is that it should produce results that are consistently proportional to the actual amounts of RNA present in both microbial and digesta samples.

The ^{35}S procedure used in this study is but one variant of the general method described by Harrison *et al.* (1972); Hume (1974) and Beever, Harrison, Thomson, Cammell & Osbourn (1974). The rationale and limitations have been discussed by these authors and in most instances, the potential shortcomings have been satisfactorily answered. Central to the method is the isolation by centrifugation of a duodenal *M* fraction that should have the same composition as the organisms that leave the rumen. However, the mean value for methionine : total N of the *M* fractions (0·120) was different from that of the isolated rumen bacteria and protozoa (0·111 and 0·103 respectively; Ling, 1976). At least two simple reasons

could account for this disparity; the centrifugation technique may not be satisfactorily isolating fractions representative of the flora and fauna that pass out of the rumen, and these fractions most probably undergo some form of modification while passing from the rumen to the duodenum. More detailed evidence is required concerning this aspect of the technique.

DAPA has been extensively used as a marker of bacterial N and its major limitations have been cited by Hutton *et al.* (1971) and Mason & White (1971). Its effectiveness depends upon the precision with which the measured duodenal DAPA reflects the rumen outflow of bacterial N. In common with the work of Synge (1953), this study did not detect DAPA in the dietary material, though traces were detected in protozoa. These latter were probably due to residues from engulfed bacteria and bacterial contamination of the isolated protozoa. While these facts may suggest that DAPA is a satisfactory marker, little is known of the metabolism of bacterially-bound DAPA released in the rumen from, for example, lysed bacteria, or of that engulfed by protozoa. Nikolić & Jovanović (1973) have reported higher DAPA-N : N values in some rumen digesta samples than in bacterial isolates; these might have been caused by unrepresentative sampling or intraruminal degradation that was preferential for cell contents. Furthermore, the decarboxylation of DAPA by protozoa has been demonstrated in vitro (Onodera & Kandatsu, 1974; Onodera, Shinjo & Kandatsu, 1974). In addition the fate of any DAPA absorbed from the small intestine is uncertain. These processes and their effects upon DAPA passage require further investigation.

A common objection to DAPA as a marker is that its concentration varies from bacterial species to species. Some do not contain DAPA at all (Work & Dewey, 1953), whereas DAPA-concentrating bacterial mutants have been identified (Kase, Hagino & Nakayama, 1970). Accumulation of such species would lead to variation in the ratio, marker-N : total N of the isolates. To overcome this and other potential causes of variations (which are probably applicable to the use of all marker substances), marker-N : total N values should be measured concurrently in the duodenal digesta and the appropriate microbial isolate for each animal. This was done in the present study where considerable variation between isolates was found (Table 5). Such variation may simply be a reflexion of the wide range of rumen environments that existed as evidenced by the diversity in numbers of ciliate protozoa (Table 6) and the molar proportions of VFA (Ling, 1976). Even though the over-all mean DAPA-N : N value from the present study (6·75) was similar to reported values of 6·04 (Hogan & Weston, 1970), 7·02 (Bird, 1972) and 6·38 (Ulyatt, MacRae, Clarke & Pearce, 1975), which have been determined from animals given a wide range of diets, the identification of these variations as biological or otherwise is necessary before any universal marker constants can be applied to all situations with confidence. This criterion is relevant to the use of all microbial N marker systems and as such warrants further attention.

Several procedures for determining AEPA in ruminant digesta have been reported. Abou Akkada, Messmer, Fina & Bartley (1968) separated AEPA by a combination of column and paper chromatography and estimated it by the ninhydrin reaction. Two similar methods have been described by Czerkawski (1974) and El-Shazly, Nour & Abou Akkada (1975). Both these methods depend on the separation of AEPA from inorganic phosphates by column chromatography, the hydrolysis of the C–P bond of AEPA and the colorimetric determination of the resultant phosphate. These methods utilize unsophisticated equipment but must be considered tedious and labour intensive.

There are three reports of automated column chromatography procedures similar to that described here. Ibrahim, Ingalls & Bragg (1970) reported an erroneous elution time for AEPA. Although this was subsequently corrected (Anon, 1973), it does mean that the AEPA estimates of protozoal N in the ruminant by Ibrahim & Ingalls (1972) are invalid. The procedure described by Mackie (1973) and the analytical method proposed recently by

Hagemeister (1975) are similar in principle to that described here, which is considered to be both simple and accurate and capable of analysing eight samples/d.

Although these several AEPA analytical procedures exist, there have been no detailed reports of their use in estimating the passage of protozoal N into the duodenum of the sheep.

However, this study has shown AEPA to be an unsatisfactory marker of protozoal fractions, primarily because it is not unique to the protozoa of the rumen. Kandatsu & Horiguchi (1962) were unable to detect AEPA in plant tissue. But the only published analysis of a complete diet is that of Abou Akkada *et al.* (1968). They concluded that AEPA was absent from the one diet they used. This is not in accord with the findings for the diets used in the present study. The source of this dietary AEPA is not readily explicable. AEPA has been detected in the lipid and protein fractions of a great variety of invertebrates (see Kittredge & Roberts, 1969). It has also been found in various tissues of ruminants (Kandatsu & Horiguchi, 1965; Shimizu, Kakimoto, Nakajima, Kanazawa & Sano, 1965; Tamari & Kametaka, 1973) and humans (Alhadeff & Daves, 1971). In view of this widespread distribution of AEPA, its detection in the dietary material of the present study was not unexpected; nor was its presence in bacteria of the rumen, since the greater part of their N is supplied by dietary and protozoal sources.

Rumen bacteria contained varying amounts of AEPA, ranging from a trace (estimated at 0·03 mg AEPA/g DM) to 0·68 mg AEPA/g DM. The detection of significant amounts of bacterial AEPA is contrary to the findings of Abou Akkada *et al.* (1968); El-Shazly *et al.* (1975) and Hagemeister (1975), but in agreement with those of Czerkawski (1974). The latter reported values for AEPA-N : total N for bacteria of 0·01–0·55, or somewhat less than the range of 0·21–1·09 found in the present study. The source of this AEPA was not identified, but it could arise from the diet, lysed protozoa or maybe even endogenously. The protozoal source may have been of importance here since there was a close correlation between the bacterial concentrations of AEPA and the size of the accompanying ciliate population (r 0·91, $P < 0·001$).

Protozoal AEPA concentrations displayed a considerable range. Part of this variation may have been due to the fact that some protozoal species possess different values for AEPA-N : total N (Abou Akkada *et al.* 1968). However, the mean value of $2·13 \pm 0·15$ (mean $\pm$ SE for eight observations) obtained in the present study was similar to the values of 1·80 and 1·91 reported by Czerkawski (1974) and Hagemeister (1975) respectively, but very different from the value of 19·58 determined by Abou Akkada *et al.* (1968). Nevertheless, the apparent ubiquity of AEPA, as found in the present study, jeopardizes its validity as a protozoal N marker.

The fact that the remaining three procedures have limitations suggests that they would not produce absolute values, against which all other values could be truly compared. It should be noted that the results from the RNA and DAPA marker methods are the mean values for a 3 d period of measurements, whereas the ^{35}S values were obtained in a 24 h period only. Even so, consideration of the coefficients of variation (CV) associated with the procedures (Tables 3–5) would suggest that the ^{35}S marker method is the most reproducible method (CV 2·65), then, in decreasing order, the RNA (11·39) and DAPA (25·57) marker methods. A similar conclusion might be drawn from an examination of the correlation coefficients obtained from these procedures; i.e. ^{35}S *v.* RNA (r 0·916), ^{35}S *v.* DAPA (r 0·824) and RNA *v.* DAPA (r 0·530). Thus, if for the sake of expediency (though not without some theoretical and experimental justification, as already discussed) the ^{35}S procedure is assumed to give the most reliable results, further comparison between the three methods can be more readily made.

Fig. 2 shows the relationship between the microbial N results as determined by these methods. The slopes of both lines, RNA and DAPA *v.* ^{35}S, were not significantly different

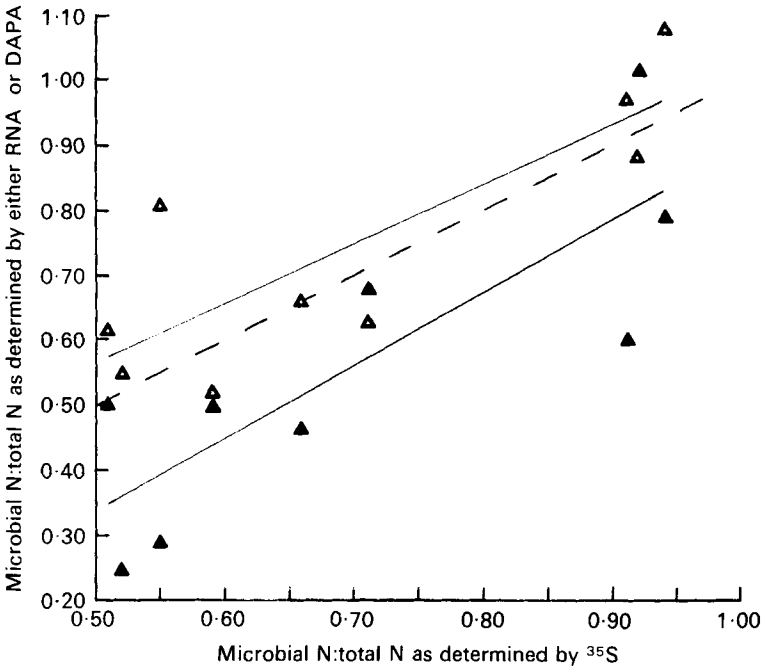

Fig. 2. The relationship between the values for microbial nitrogen: total N of digesta entering the duodenum of sheep as determined by the RNA(y) and the ^{35}S(x) marker methods (△) ($y = 0{\cdot}916x + 0{\cdot}105$; residual SD 0·117), and as determined by the DAPA(y) and the ^{35}S(x) marker methods (▲) ($y = 1{\cdot}124x - 0{\cdot}227$; residual SD 0·148) (for details of procedures, see pp. 167–168) in sheep given diets with different N sources (for details of diets, see Table 1). (– – –), $x = y$.

from 1 or from each other. However, fitting the parallel lines to these results did show that the intercepts were different ($P < 0{\cdot}001$). Therefore, in terms of estimating proportions of microbial N, Fig. 2 demonstrates that the RNA procedure produced similar, but generally higher values than that of the ^{35}S procedure. By contrast, the use of DAPA produced significantly lower values, presumably in part due to the omission of a protozoal N component.

Comparisons of the values obtained in this study with those reported in the literature are usually confounded by differences in marker estimation techniques, animals and particularly dietary composition. The report of Nikolić & Jovanović (1973) is a good example of this problem. However, there is a rapidly increasing amount of relevant information. It has become conventional to report results of various dietary proteins in terms of the proportions that are degraded in the rumen when that protein is a major constituent of a diet. Table 7 shows these mean values (0·38, 0·43 and 0·89 for white fish meal, soya-bean meal and barley respectively) obtained from the ^{35}S procedure of the present study. The calculations of Table 7 assume that the non-microbial N at the duodenum when the urea diet was given, is the contribution of undegraded dietary barley and endogenous N components. Since this amount (1·71 g N/d) could be accounted for by endogenous N alone (Phillipson, 1964), the proportion of barley N degraded in the rumen could vary from 0·89 to 1·0. These figures agree well with the values of 0·92 (Sutton, Smith, McAllan, Storry & Corse, 1975) and 0·89 (Mathers & Miller, 1977) determined by RNA and ^{35}S procedures respectively.

A wider range of reported values exists for other N sources. Using ^{35}S marker techniques, Hume (1974) reported values of 0·29 and 0·39 for fish meal and soya-bean meal in semi-

Table 7. *Calculation of the proportions of white fish meal, soya-bean meal and barley that are degraded in the rumen of sheep given fish meal (F), soya-bean meal (S) and urea (U) diets**

(Mean values from ^{35}S marker procedure)

Diet ...	F	S	U
N intake g/d	28·02	28·16	27·97
N at duodenum g/d	18·49	24·25	21·32
Microbial N: total N at duodenum	0·54	0·64	0·92
Microbial N at duodenum g/d	9·98	15·52	19·61
Non-microbial N at duodenum g/d	8·51	8·73	1·71
Fish meal N or soya-bean meal N escaping degradation g/d	6·80	7·02	—
Intake of fish meal N or soya-bean meal N g/d	11·05	12·21	—
Proportion of fish meal N or soya-bean meal N degraded	0·38	0·43	—
Intake of barley N g/d			16·29
Proportion of barley N degraded			0·89

* For details of composition, see Table 1.

purified diets, whereas Miller (1973) has suggested values of 0·30 and 0·55. The problem of compiling a comprehensive list of dietary proteins is amply illustrated by the fact that Miller (1973) proposed values for fish meal of either 0·50 or 0·30 depending whether it was the white or Peruvian variety. Additional difficulties could be expected with all dietary proteins, since their degradation characteristics would depend upon numerous factors such as the level of food intake (and hence the period of retention within the rumen), protein source (as illustrated by the fish meal varieties), age (particularly relevant to forages), mode of preparation (such as heating and grinding) and animal differences (as found in the present study). Of additional importance are the proportions and degradation characteristics of other major nitrogenous components in the diet. Clearly, a more rigorous classification of dietary N sources will be necessary if such information is to be useful for prediction purposes.

The choice of which marker method to use to quantify the amount of microbial N flowing from the rumen is another problem. Since the majority of microbial N is bacterial in origin, it does seem likely that DAPA will continue to be widely used. However, the protozoal contributions to duodenal digesta N should not be dismissed as unimportant. Nevertheless, independent assessment of this fraction is limited by the lack of an adequate protozoal component marker. Where microbial values are required, the choice of method therefore becomes either RNA or ^{35}S. This study has shown that both produce similar results when applied to concentrate diets.

Theoretically, the ^{35}S procedure is more satisfactory, though procedurally the RNA method, particularly as described in this study, is considerably less complex and therefore more attractive for rapid, though perhaps less reliable estimations. Further comparative investigations using a wider range of dietary types may indicate additional limitations of these procedures, but at the present time, the choice of either will probably be determined by the purpose of the experiment and the analytical facilities available.

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