Determination of rumen microbial-nitrogen production in sheep: a comparison of urinary purine excretion with methods using ^{15}N and purine bases as markers of microbial-nitrogen entering the duodenum

BY J. F. PEREZ, J. BALCELLS, J. A. GUADA AND C. CASTRILLO

Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, M. Servet 177, Zaragoza 50013, Spain

(*Received 10 November 1994 – Revised 20 June 1995 – Accepted 16 August 1995*)

The present study compares estimates of rumen microbial-N production derived from duodenal flow measurements (^{15}N and purine bases) with those from measurements of the urinary excretion of purine derivatives. Four Rasa Aragonesa ewes fitted with simple cannulas in the rumen and proximal duodenum were used. Four diets consisting of 550 g lucerne (*Medicago sativa*) hay/d as sole feed or supplemented with 220, 400 and 550 g rolled barley grain/d were given in a 4 × 4 random factorial arrangement. Duodenal digesta flows were determined by the dual-phase marker technique during continuous intraruminal infusions of Co-EDTA and Yb-acetate. Microbial contribution to the non-NH_3 N (NAN) flow was estimated from ^{15}N enrichment and purines:N ratio in duodenal digesta and bacterial fractions isolated from the rumen content. Whole tract organic matter (OM) digestibility and duodenal flow of OM and NAN increased ($P < 0.001$) with the level of barley supplementation. Digestible OM intake ranged from 19·0 to 42·7 g/kg metabolic weight ($W^{0.75}$) and the duodenal flow of purine bases and the urinary excretion of allantoin increased linearly ($P < 0.001$) from minimum values of 7·47 (SD 1·524) and 4·65 (SD 0·705) mmol/d respectively on the basal diet to 18·20 (SD 1·751) and 11·62 (SD 0·214) mmol/d on the 400 g barley diet; a further increase in barley supplementation decreased both variables (13·50 (SD 2·334) and 8·77 (SD 0·617) mmol/d respectively). Urinary excretion of uric acid and hypoxanthine showed a slight but significant increase ($P < 0.05$) over all levels of barley. Molar recoveries of duodenal purine bases as purine derivatives or allantoin in the urine were 0·78 (SD 0·156) and 0·65 (SD 0·130) respectively. The increase on barley supplementation significantly augmented microbial-N, but large differences between microbial markers employed were observed. Mean values of microbial-N estimated from the duodenal purine bases or urinary allantoin excretion were on average 18 and 29% lower than those measured by ^{15}N.

Microbial synthesis: Purine excretion: Sheep

Yield of microbial protein from the rumen is the largest element of uncertainty associated with the estimation of protein supply in the new Feed Evaluation Systems for ruminants (Webster, 1992). Protein reaching the duodenum is predicted from fermentable metabolizable energy (FME) intake, but recognizing that the efficiency of microbial synthesis varies with the level of feeding. Additional sources of variation may be incorporated into this approach as long as they are identified and their effects properly quantified. Current measurements of rumen microbial production, based on duodenal flow estimates, are of limited value in this respect due to the need for fistulated animals and assumptions involved in the flow of digesta and in the microbial markers used.

Recently, non-invasive methods based on the urinary excretion of purine derivatives (PD) have been developed to estimate microbial protein supplied to the duodenum (Chen

et al. 1990*a*; Balcells *et al.* 1991). These methods assume that duodenal nucleic acids are mostly of microbial origin (McAllan & Smith, 1973) and after intestinal digestion and absorption, purine base catabolites are proportionally recovered in the urine, mostly as allantoin but also as hypoxanthine, xanthine and uric acid. However, the usefulness of these methods needs to be validated by verification of predicted responses and comparison of estimates with alternative techniques. The urinary excretion of allantoin has been found to respond to rumen degradable N deficiency (Balcells *et al.* 1993*b*), monensin supplementation (Dewhurst & Webster, 1992) and type of carbohydrate fermentation (Balcells *et al.* 1993*a*). Also, good relationships between urinary allantoin and duodenal microbial N flow have been reported, although the equimolar recovery of duodenal purines as urinary PD differed greatly between estimates obtained with different microbial markers (Lindberg *et al.* 1989; Puchala & Kulasek, 1992). In the present experiment, values of microbial N flowing into the duodenum obtained from indirect measurements (PD excretion) were compared against values obtained in cannulated animals using purine bases as internal, or ^{15}N as external, markers.

MATERIALS AND METHODS

Animals and diets

Four Rasa Aragonesa ewes, averaging 37·4 (SD 3·15) kg live weight, each fitted with both a simple rumen cannula (45 mm internal diameter) and a T-shape cannula in the proximal duodenum, were used. Four weeks after surgery, animals were individually penned under continuous lighting and randomly allocated to four dietary treatments in a 4×4 complete crossover design. Treatments consisting of 550 g lucerne (*Medicago sativa*) hay/d were given as a sole feed or supplemented with increasing amounts of rolled barley grain (220, 400 and 550 g/d). In addition, each ewe received 10 g of a commercial vitamin–mineral complex with the following declared content (g/kg): P 130, S 30, Ca 180, Mg 80, Cl 8·2, Na 55, Mn 3, Fe 2·5, Zn 3·9, Cu 0·5, Co 0·014, I 0·076, Se 0·006, retinol 130 mg, cholecalciferol 10 mg (Pradial ovino, Nanta, Madrid, Spain). Daily rations were distributed automatically in twelve equal meals with free access to water. The compositions of the lucerne hay and rolled barley were (g/kg DM): organic matter (OM), 887·5 and 976·4; crude protein (CP), 189·1 and 127·4; neutral-detergent fibre (NDF), 455 and 205·8; acid-detergent fibre (ADF), 341·8 and 47·7; acid-detergent lignin (ADL), 260·5 and 33·4 respectively.

Experimental procedures

Each experimental period lasted for 20 d allowing 10 d from dietary changeover for adaptation and 10 d for experimental measurements. The following schedule was employed: continuous infusions of flow markers from days 11 to 19, collections from days 11 to 16 (faeces) and days 14 to 16 (urine), isotope infusion ($(^{15}NH_4)_2SO_4$) from days 14 to 18, collection of duodenal digesta from days 17 to 18. Finally, sampling of rumen content for isolation of rumen bacteria and characterization of rumen fermentation was performed on days 19 and 20 respectively.

Yb acetate (254 mg Yb-Ac/l; 20 ml/h) and Co-EDTA (416·7 mg Co-EDTA/l; 20 ml/h) were continuously infused into the rumen through two independent lines by means of a peristaltic pump (Gilson Minipuls 2, Villiers le Bel, France). Faeces (collected daily) were dried at 60° for 48 h and 20% daily samples pooled on an individual basis, ground through a 1 mm screen and stored at room temperature. Urine was collected daily by means of urethral catheter (Foley 18Ch 6·0 mm, balloon 10 ml) in buckets containing 100 ml 1 M-H_2SO_4 to keep pH < 2. Urine weight and specific gravity were recorded daily and four individual subsamples (2%) stored immediately at $-20°$. On day 14 labelled $(NH_4)_2SO_4$

(10 + atom % ^{15}N, Isotec Inc, Ohio, USA) calculated to supply 25 mg ^{15}N/d was infused for 120 h into the rumen along with the Co infusate. Duodenal digesta samples (150–200 ml) were collected at 6 h intervals for 48 h and pooled on an individual animal basis. Composite samples of whole duodenal digesta and particulate matter (Faichney, 1975) obtained after centrifugation (1000 *g* for 5 min) were freeze dried for subsequent analyses. On day 19, marker infusion was stopped and between 500 and 600 ml rumen content was withdrawn from each ewe and a rumen bacterial fraction was isolated from the rumen liquid as follows: collected whole rumen contents were squeezed through eight layers of cheesecloth and digesta solids were combined with an equal volume of saline solution (9 g NaCl/l) at 37°, mixed gently, and squeezed again to remove residual fluid-associated bacteria; bacteria were isolated from the filtrate by centrifugation at 500 *g* for 5 min, followed by two consecutive centrifugations of the supernantant fraction at 20000 *g* for 20 min at 4°. The microbial pellet was freeze-dried for subsequent analysis. Finally, rumen contents were sampled at 3, 9, 15, 21 and 33 h after the end of the flow marker infusion, pH recorded and three samples taken and filtered to remove coarse particles. One was frozen and stored until Co analysis, and the other two were acidified with HCl (10 ml rumen fluid/10 ml 0·2 M-HCl) or H_3PO_4 (4 ml rumen fluid/1 ml 0·51 M-H_3PO_4, 50 mM-3 methyl valerate) and stored at −20° until their analysis for NH_3-N and volatile fatty acids (VFA) concentrations. Liquid outflow rate was obtained from the dilution curve of Co concentration in the rumen fluid.

Analytical procedures

DM was determined by drying (105°) samples to constant weight, and OM by ashing at 550° for 8 h. NDF, ADF and ADL contents were determined by the procedures of Goering & Van Soest (1975), using amylase (*EC* 3.2.1.1) for hydrolysing concentrate samples. The total N content of feed samples and non-NH_3-N (NAN) of duodenal digesta samples were determined by the Kjeldahl method, using Se as catalyst. NH_3-N concentration in rumen fluid samples was determined by the same method after direct distillation with sodium tetraborate. Marker (Co, Yb) concentrations were analysed by atomic emission spectrophotometry (AES-ICP, Perkin Elmer P-40, Uberlingen, Germany) after hydrolysis of freeze-dried digesta samples (1 g sample/15 ml of acid dilution, 2·33 M-$HClO_4$–10·5 M-HNO_3, 1:4, v/v) at 150° for 1 h followed by 2 h at 300° (A. Vega and D. P. Poppi, personal communication), and directly in the supernatant fraction of centrifuged (2500 *g* for 20 min) rumen liquid samples.

VFA concentrations in deproteinized rumen fluid were determined by GLC, following the method proposed by Jouany (1982). PD in acidified urine were determined by HPLC (Balcells *et al.* 1992). Adenine and guanine in rumen bacteria (20 mg) and duodenal digesta samples (40 mg) were determined by the same HPLC technique, after their acid hydrolysis with 2 ml 2 M-perchloric acid (PCA) at 100° for 1 h, adding 0·75 μmol of allopurinol and neutralizing immediately with 4·5 M-KOH (Martin Orue *et al.* 1995). The isotope enrichment of ^{15}N was determined using mass spectrophotometry (VG PRISM II, IRMS hooked in series to a DUMAS-style N analyser EA 1108, Carlos Erba, Milan, Italy).

The proportion of microbial-N in duodenal NAN was calculated from the ^{15}N:N enrichment ratio and the ratio of purine bases (PB) content between duodenal digesta and bacterial fraction. The amounts of PB absorbed were calculated from the relationship between the urinary allantoin excretion and the duodenal PB flow established by Balcells *et al.* (1991). Microbial-N was estimated from the determined bacterial composition (see Table 5; N:PB ratio) and assuming all PB in duodenal digesta were of microbial origin.

Statistical analysis

Values were examined by ANOVA as a randomized design with sheep considered as a random factor. Treatment sums of squares were partitioned in two orthogonal contrasts to test linear and quadratic responses to barley supplementation, and Duncan's new multiple range test was used to compare treatment means. Differences between microbial markers were tested as paired comparisons and responses in urinary allantoin and PD excretion to the duodenal flow of purine bases were calculated by regression analysis (Steel & Torrie, 1960).

RESULTS

Intake and digestibility

All diets were consumed without refusals and therefore average feed intake of total diet is shown in Table 1, together with whole tract digestibility and duodenal flow of OM. Digestible OM intake (DOMI) increased from approximately 0·8 to 1·8 times the maintenance requirement (Agricultural Research Council, 1984) providing a wide range of rumen-fermentable OM as a substrate for microbial synthesis in the rumen. Both whole-tract OM digestibility and duodenal flow of OM increased ($P < 0·001$) with the level of barley supplementation, but whereas OM digestibility showed progressively smaller increases, the duodenal flow of OM reached a maximum at 400 g barley supplementation, showing a significant quadratic response ($P < 0·05$ and $P < 0·01$ respectively). The proportion of digestible OM that was apparently digested in the rumen (DOMR) was on average 0·61 and differences between treatments were significant ($P < 0·05$), since the highest level of barley supplementation rendered a value substantially higher (0·72) than other treatments.

Rumen fermentation variables

Mean values of pH, VFA and NH_3-N concentration in the rumen fluid, together with the liquid dilution rate are presented in Table 2. Rumen pH showed a small but significant decrease from 6·81 to 6·20 ($P < 0·001$) as the level of barley supplementation increased. NH_3-N concentration in rumen liquid was not significantly changed by the experimental treatment, averaging 177 mg/l. Total VFA concentration in rumen fluid increased linearly ($P < 0·001$) with the dietary level of barley. Acetic acid molar proportion decreased ($P < 0·001$) from 0·768 on the non-supplemented diet to 0·658 with the highest level of barley supplementation, whereas there were increases ($P < 0·01$) in the concentrations of propionic (from 0·165 to 0·194) and butyric acids (from 0·072 to 0·142). Liquid dilution rate showed a quadratic response ($P < 0·05$) to the level of barley supplementation, reaching a maximum at intermediate levels of supplementation (220 and 400 g/d).

Rumen microbial synthesis

Barley supplementation of the basal diet up to 400 g/d promoted a significant increase in both duodenal flow of PB (from 7·5 to 18·2 mmol/d) and urinary excretion of allantoin (from 4·6 to 11·6 mmol/d, Table 3). The increase was followed by significant declines to 13·5 and 8·8 mmol/d at the highest level of supplementation (550 g/d). The duodenal flow of NAN increased linearly with experimental treatment although no further increases were achieved above 400 g barley, the quadratic component of the response also being significant ($P < 0·05$). Variations in the urinary excretion of total PD reflected those for allantoin, although there were also slight but significant increases ($P < 0·05$) in uric acid (from 0·46 to 0·66 mmol/d) and hypoxanthine (from 0·76 to 1·06 mmol/d) excretion.

In Table 4 are presented average flows of microbial-N, estimated either from ^{15}N and PB as microbial markers or from the urinary allantoin excretion. The duodenal flow of

Table 1. *Mean amounts of dry matter (DM) and organic matter (OM) consumed and flowing at the duodenum for ewes given 550 g lucerne* (Medicago sativa) *hay/d as sole feed or supplemented with 220, 400 or 550 g barley; whole-tract digestibility of OM and the proportion of digestible OM apparently digested in the rumen are also shown*†

Diet...	0	220	400	550	SE	Significance of effects: lin	Significance of effects: quad
Intake (g/d)							
DM	498	700	859	994	—	—	—
OM	440	630	780	929	—	—	—
Digestible OM	277	431	555	680	3·8	***	NS
Duodenal flow (g/d)							
OM	264	403	495	440	17·3	***	**
OM digestibility (%)	63·10	68·39	71·20	73·16	0·713	***	*
Proportion of digestible OM digested in rumen	63·20	57·90	51·62	71·94	4·806	NS	*

Lin, linear; quad, quadratic.
* $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.
† For details of procedures, see pp. 700–702.

Table 2. *Mean values of pH, ammonia-nitrogen and volatile fatty acid (VFA) concentration in the rumen fluid, and dilution rate in ewes fed on 550 g of lucerne* (Medicago sativa) *hay/d as sole feed or supplemented with increasing amounts of barley (220, 400, 550 g)*†

Diet...	0	220	400	550	SE	Significance of effects: lin	Significance of effects: quad
pH	6·85	6·55	6·33	6·20	0·087	***	NS
Ammonia-N (mg/l)	170·7	165·4	177·7	201·8	21·56	NS	NS
Total VFA (mmol/l)	74·5	84·1	92·3	100·9	5·68	***	NS
Main VFA proportions (%)							
Acetic acid	76·78	71·91	69·67	65·78	0·812	***	NS
Propionic acid	16·47	15·96	16·87	19·41	0·438	**	***
Butyric acid	7·25	12·13	13·44	14·81	0·892	***	**
Liquid dilution rate (% per h)	7·2	8·2	8·5	7·0	0·54	NS	*

Lin, linear; quad, quadratic.
* $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.
† For details of procedures, see pp. 700–702.

microbial-N was significantly ($P < 0·001$) affected by the experimental treatments, however there were large differences between methods of estimation. Mean values of microbial-N estimated from PB and urinary allantoin were on average 18 and 29% lower than those measured by ^{15}N. Differences were significant ($P < 0·01$) in all treaments and ranged in the case of PB from 14 to 22% and in the case of allantoin from 24 to 35%. Differences between PB- and allantoin-based estimations were also significant ($P < 0·05$). When estimated from ^{15}N, microbial-N flow increased from 6·21 to 14·0 g/d ($P < 0·001$) in response to the first two levels of supplementation, but no further increases were achieved by increasing barley supplementation above 400 g/d. However, the quadratic component

Table 3. *Urinary excretion of purine derivatives (PD) and duodenal flows of purine bases (PB) from ewes fed on 550 g of lucerne* (Medicago sativa) *hay/d as sole feed or supplemented with 220, 400, 550 g/d of ground barley grain*†

Diet...	0	220	400	550	SE	Significance of effects: lin	Significance of effects: quad
Urinary excretion (mmol/d)							
Allantoin	4·65	7·06	11·62	8·77	0·315	***	***
Hypoxanthine	0·76	0·91	0·96	1·06	0·069	*	NS
Xanthine	0·14	0·12	0·14	0·09	0·023	NS	NS
Uric acid	0·46	0·59	0·59	0·66	0·080	*	NS
Total PD	5·88	8·68	13·47	10·59	0·261	***	***
Duodenal flow PB (mmol/d)							
Co-EDTA/Yb-Ac‡	7·47	12·13	18·20	13·50	0·675	***	***
Allantoin (a)§	6·59	9·63	15·29	11·83	0·312	***	***
Total PD (b)§	7·15	11·11	17·62	13·85	0·375	***	***

Lin, linear; quad, quadratic.
* $P < 0·05$, *** $P < 0·001$.
† For details of procedures, see pp. 700–702.
‡ Measured using the double marker technique of Faichney (1975).
§ Estimated using the urinary excretion of allantoin (a) (Balcells *et al.* 1991), and of total PD (b) (Chen *et al.* 1990).

Table 4. *Duodenal flow of non-ammonia nitrogen (NAN) and microbial-nitrogen estimated from ^{15}N, purine bases or allantoin excretion, and microbial yield expressed as g N/kg organic matter apparently digested in the rumen (ADOMR) in ewes fed on 550 g lucerne* (Medicago sativa) *hay/d as sole feed or supplemented with increasing amounts of barley (220, 400, 550 g)*†

Diet...	0	220	400	550	SE	Significance of effects: lin	Significance of effects: quad
Duodenal flow							
NAN (g)	12·6	17·59	21·65	20·92	0·710	***	*
Microbial-N (g)‡							
^{15}N	6·21	8·98	14·04	14·36	0·810	***	NS
Purine bases	4·82	7·19	12·01	11·72	0·520	***	NS
Allantoin	4·28	5·82	10·08	10·85	0·573	***	NS
Microbial yield (g N/kg ADOMR)							
^{15}N	37·17	37·32	52·20	29·40	5·600	NS	NS
Purine bases	29·16	29·49	45·11	24·30	3·800	NS	*
Allantoin	27·80	23·40	38·08	22·60	1·920	NS	NS

Lin, linear; quad, quadratic.
* $P < 0·05$, *** $P < 0·001$.
† For details of procedures, see pp. 700–702.
‡ Estimated from direct measurements (^{15}N, purine bases) and from the urinary excretion of allantoin (Balcells *et al.* 1991), using as reference isolated microbial composition.

of the response did not reach statistical significance. A similar response was recorded when microbial-N was estimated from PB or urinary allantoin excretion, although absolute values were proportionally lower. The average efficiency of microbial-N synthesis in the rumen (g microbial-N/kg OM apparently digested in the rumen) amounted to 39·0, 32·0

Table 5. *Organic matter (OM), nitrogen, purine bases, purine:nitrogen ratio and ^{15}N enrichment in bacterial samples isolated from ewes fed on 550 g of lucerne* (Medicago sativa) *hay/d as sole feed or supplemented with 220, 400 or 550 g of barley*†

						Significance of effects	
Diet...	0	220	400	550	SE	lin	quad
OM (mg/g)	683·2	644·3	696·3	721·1	38·56	NS	NS
N (mg/g OM)	102·7	99·5	96·7	88·4	3·07	*	NS
Purine bases (μmol/g OM)							
Adenine	88·0	86·7	75·5	53·5	6·43	***	NS
Guanine	70·8	82·9	72·8	53·7	6·13	NS	*
Adenine + guanine	158·8	169·7	148·4	107·2	12·03	*	*
Purine bases:N (μmol/mg N)	1·55	1·70	1·53	1·21	0·108	*	*
^{15}N:N (‰)	1·506	1·278	1·006	0·702	0·0953	***	NS

Lin, linear; quad, quadratic.
* $P < 0·05$, *** $P < 0·001$.
† For details of procedures, see pp. 700–702.

and 28·0 g/kg when estimated from ^{15}N, PB and urinary allantoin respectively, and differed significantly between markers and methods of estimation ($P < 0·05$).

Mean values of bacterial composition (OM, N and PB content, and ^{15}N enrichment) are presented in Table 5. Significant differences among treatments were recorded in all the studied components, except OM. Total N content and ^{15}N enrichment were higher in the basal diet and decreased linearly with the level of supplementation ($P < 0·05$ and $P < 0·001$ respectively). Adenine content showed a similar response, but the decrease in guanine and total purines (adenine + guanine) was only evident ($P < 0·05$) with the highest level of supplementation. Hence the purine:N ratio was significantly lower ($P < 0·05$) on this diet (1·21 μmol/mg) compared with the rest of the treatments (1·62 μmol/mg).

DISCUSSION

Microbial nitrogen estimation from direct measurements (^{15}N v. purines)

Barley supplementation led to a significant increase of NAN flow into the duodenum, except with the highest level of supplementation where it levelled off. The response was mainly explained by the increased contribution of microbial-N to duodenal NAN flow, that ranged from 50 to 69% while the non-microbial NAN flow remained more constant, about a mean value of 7·3 g/d, when using ^{15}N as microbial marker. The lack of response on the highest level of supplementation may be attributed to the reduced dilution rate of rumen fluid observed on this treatment associated with a wasteful turnover of nutrients and the lysis of microbial cells (Harrison & McAllan, 1980), and to the likely higher maintenance requirements of the amylolytic flora enhanced by high grain rations (Russell, 1984). The lower microbial yield was reflected in a lower efficiency of microbial synthesis on this treatment compared with other supplemented diets, although differences did not reach statistical significance.

Whatever the experimental diet studied, estimations of microbial-N flow to the duodenum and efficiency of microbial-N synthesis were modified significantly by the microbial marker employed, suggesting that much of the variation found in the literature (Agricultural Research Council, 1984) is associated with the use of different microbial markers (Siddons *et al.* 1982; Whitelaw *et al.* 1984). In general it is accepted that values of

microbial-N, estimated using nucleic acids or PB as microbial markers, tend to overestimate microbial-N flow due to the presence of undegraded dietary RNA in duodenal digesta (Smith *et al.* 1978). Ling & Buttery (1978) cited that overestimation in RNA estimations could be due to the interference by DNA when the former is determined by the orcinol reaction. On the other hand the presence of ribonucleases during freezing and thawing of samples can directly affect polynucleotide analysis (Siddons *et al.* 1982). Many of the errors in extraction and fractionation of nucleic acids in biological material can be avoided by direct determination of PB (Ha & Kennelly, 1984; Zinn & Owens, 1986).

Microbial-N flows estimated from PB (see Table 4) were significantly lower than those estimated by the ^{15}N procedure. Similar differences have been previously reported by Firkins *et al.* (1987) and have been attributed to an inadequate isotope distribution and/or to difficulties in obtaining a representative sample of rumen microbial pools.

After 72 h of intraruminal continuous infusion of ^{15}N, it can be assumed that ^{15}N enrichment of the isolated bacterial fraction and duodenal digesta have nearly reached a plateau (Broderick & Merchen, 1992), and therefore a minimal variability in the measurement due to sampling procedure might be expected. However, an irregular distribution of the isotope among the different microbial-N pools cannot be neglected, because isotope distribution would vary according to the ability of the different microbial populations to incorporate preformed amino acids or peptides of dietary origin.

Isolation of the microbial fraction from the rumen liquid phase by differential centrifugation is unlikely to be representative of the whole biomass reaching the duodenum, due to the loss of protozoa and particle-bound bacteria during the low-speed centrifugation. Although a decline in the purity of the microbial preparation cannot be discounted, differences between values estimated from PB and ^{15}N might be explained by differences in microbial composition. Firkins *et al.* (1987) reported a much lower purine:N ratio in protozoa than in bacteria isolated from the rumen liquid phase. It is likely that bacteria account for the greatest proportion of microbial-N in duodenal digesta. However, both nucleic acids and PB would underestimate microbial synthesis depending on the contribution of protozoal-N to the duodenal flow.

In relation to particle-bound bacteria, whose contribution to duodenal flow may be as high as 0·75 of the total amount (Craig *et al.* 1987*b*), several authors (Merry & McAllan, 1983; Olubobokun *et al.* 1988; Legay-Carmier & Bauchart, 1989; Cecava *et al.* 1990) have reported lower purine:N ratios in particle-bound bacteria than liquid-associated bacteria, but the contribution of both fractions to duodenal flow is still not clear. Obtaining a rumen microbial sample representative of the total biomass flowing to the intestine is complicated by: (1) the differential distribution of microbial species between phases (Craig *et al.* 1987*a*; Olubobokun *et al.* 1988) and (2) the difficulty of obtaining a consistent and reliable sample of particle-bound bacteria; current methods for detaching microbes from digesta content show a low efficiency (less than 52% of adherent bacteria may be removed, Craig *et al.* 1987*a*; Olubobokun & Craig, 1990) and many of them produce a differential lysis during the detaching process (Legay-Carmier & Bauchart, 1989). Hence, studies of microbial synthesis in the rumen are always linked to the lack of representativity of the microbial sample.

Microbial protein synthesis estimated from urinary purine derivative excretion

Urinary excretion of allantoin and total PD increased with the level of barley in line with the higher DOMI. This increase was mainly explained by the significant reponse of allantoin excretion, although the intermediate metabolites, hypoxanthine and uric acid, showed a lower but significant increase. Present observations agree well with early works (Fujihara *et al.* 1987; Chen *et al.* 1990*a*) although in previous results from our laboratory

(Balcells *et al.* 1991, 1993*a*) allantoin was the sole PD responsive to exogenous purine input. It is difficult to explain that a significant fraction of exogenous PB can by-pass the liver, since this organ in sheep is the main compartment containing xanthine oxidase (*EC* 1.2.3.2) and uricase (*EC* 1.7.3.3), and PB coming from the gut must be deaminated and oxidized to allantoin before they reach peripheral circulation (Zöllner, 1982).

The lowest allantoin excretion recorded on the basal diet (317 μmol/kg metabolic weight ($W^{0.75}$) was higher than the reported endogenous losses in sheep (Giesecke *et al.* 1984; Chen *et al.* 1990*b*; Balcells *et al.* 1991), and exceeded the threshold level required to maintain a constant urinary recovery of the duodenal purine supply (Balcells *et al.* 1991). Thus, it seems that the response of urinary allantoin to the increased DOMI could be attributed to an exogenous input of PB. On the other hand, the low content of PB in rolled barley and their high rumen degradability, as observed in a previous study (Zelaya, 1994), precludes any significant contribution of dietary PB to the duodenal flow. Therefore it is likely that the increase in duodenal PB input promoted by barley supplementation may be explained in terms of microbial supply.

As expected, there was a close relationship between duodenal flow of PB (adenine + guanine) and urinary excretion of PD (r 0·85) or allantoin (r 0·83) supporting the potential use of such compounds as estimators of the duodenal flow of PB. When urinary excretion of PD (mmol/d) was related to duodenal flow of PB (mmol/d), the following equation was obtained:

$$PD = 0{\cdot}57\ (\text{SE}\ 0{\cdot}102)\ PB + 2{\cdot}3\ (\text{SE}\ 1{\cdot}34) \quad (r\ 0{\cdot}85, \text{RSD}\ 1{\cdot}62, n\ 16).$$

The regression coefficient obtained was apparently lower than values obtained by Chen *et al.* (1990*a*) (0·76, assuming a PB digestibility of 0·91), but differences were not significant ($P > 0{\cdot}05$). In fact, when estimated values of duodenal flow from the response models (Chen *et al.* 1990*a*; Balcells *et al.* 1991) were compared against values obtained from direct measurements (Table 3), urinary excretion of total PD gave consistent estimations of duodenal flow of PB, showing no differences between the two groups of values ($P > 0{\cdot}05$). However, duodenal flow of PB estimated from the urinary excretion of allantoin, considering this compound as a single derivative responsive to duodenal input, was underestimated, depending on the contribution of non-allantoin PD to the urinary excretion, suggesting that using allantoin as a single compound can result in an oversimplification of the method.

It has been suggested that PD excretion might constitute a simple and non-invasive method to predict microbial yield from the rumen. Our results support this proposal and show that it provides reliable values of the duodenal flow of PB, although attention should be paid to the relationship between PB and N concentration in the ruminal microorganisms.

The authors are grateful to Dr J. I. Bonafonte and J. Sopena for the insertion of the cannulas, and R. Weightman for the correction of this manuscript; thanks are due also to Javier Lanzas for laboratory assistance.

This work was supported by the CICYT funded Project Gan-1050/91 CO2.

REFERENCES

Agricultural Research Council (1984). *The Nutrient Requirements of Ruminant Livestock*. Slough: Commonwealth Agricultural Bureaux.

Balcells, J., Fondevila, M., Guada, J. A., Castrillo, C. & Surra, J. C. E. (1993*a*). Urinary excretions of purine derivatives and nitrogen in sheep given straw supplemented with different sources of carbohydrates. *Animal Production* **57**, 287–292.

Balcells, J., Guada, J. A., Castrillo, C. & Gasa, J. (1991). Urinary excretion of allantoin and allantoin precursors by sheep after different rates of purine infusion into the duodenum. *Journal of Agricultural Science, Cambridge* **116**, 309–317.

Balcells, J., Guada, J. A., Castrillo, C. & Gasa, J. (1993*b*). Rumen digestion and urinary excretion of purine derivatives in response to urea supplementation of sodium-treated straw fed to sheep. *British Journal of Nutrition* **69**, 721–732.

Balcells, J., Guada, J. A., Peiró, J. M. & Parker, D. S. (1992). Simultaneous determination of allantoin and oxypurines in biological fluids by high-performance liquid chromatography. *Journal of Chromatography* **575**, 153–157.

Broderick, G. A. & Merchen, N. R. (1992). Markers for quantifying microbial protein synthesis in the rumen. *Journal of Dairy Science* **75**, 2618–2632.

Cecava, M. J., Merchen, N. R., Gay, L. C. & Berger, L. L. (1990). Composition of ruminal bacteria harvested from steers as influenced by dietary energy level, feeding frequency, and isolation techniques. *Journal of Dairy Science* **73**, 2480–2488.

Craig, W. M., Broderick, G. A. & Ricker, D. B. (1987*a*). Quantitation of microorganisms associated with the particulate phase of ruminal digesta. *Journal of Nutrition* **117**, 56–62.

Craig, W. M., Brown, D. R., Broderick, A. & Ricker, D. B. (1987*b*) Post-prandial composition changes of fluid and particle-associated ruminal microorganisms. *Journal of Animal Science* **65**, 1042–1048.

Chen, X. B., Hovell, F. D. DeB., Ørskov, E. R. & Brown, D. S. (1990*a*). Excretion of purine derivatives by ruminants: effect of exogenous nucleic acid supply on purine derivative excretion by sheep. *British Journal of Nutrition* **63**, 131–142.

Chen, X. B., Ørskov, E. R. & Hovell, F. D. DeB. (1990*b*). Excretion of purine derivatives by ruminants: endogenous excretion, differences between cattle and sheep. *British Journal of Nutrition* **63**, 121–129.

Dewhurst, R. J. & Webster, A. J. F. (1992). Effects of diet, level of intake, sodium bicarbonate and monensin in urinary allantoin excretion in sheep. *British Journal of Nutrition* **67**, 345–353.

Faichney, G. J. (1975). The use of markers to partition digestion within the gastrointestinal tract of ruminants. In *Digestion and Metabolism in the Ruminant* pp. 277–291 [I. W. MacDonald and A. C. I. Warner, editors]. Armidale: University of New England Publishing Unit.

Firkins, J. L., Berger, L. L., Merchens, N. R., Fahey, G. C. & Mulvaney, R. L. (1987). Ruminal nitrogen metabolism in steers as affected by feed intake and dietary urea concentration. *Journal of Dairy Science* **70**, 2302–2311.

Fujihara, T., Ørskov, E. R., Reeds, P. J. & Kyle, D. J. (1987). The effect of protein infusion on urinary excretion of purine derivatives in ruminants nourished by intragastric nutrition. *Journal of Agricultural Science, Cambridge* **109**, 7–12.

Giesecke, D., Stangassinger, M. & Tiemeyer, W. (1984). Nucleic acid digestion and urinary purines metabolites in sheep nourished by intragastric infusion. *Canadian Journal of Animal Science* **64**, 144–145.

Goering, H. K. & Van Soest, P. J. (1975). *Forage Fiber Analysis. Agricultural Research Service. Agricultural Handbook* no. 379. Washington, DC: US Department of Agriculture.

Ha, J. K. & Kennelly, J. J. (1984). Influence of freeze-storage on nucleic acid concentrations in bacteria and duodenal digesta. *Canadian Journal of Animal Science* **64**, 791–793.

Harrison, D. G. & McAllan, A. B. (1980). Factors affecting microbial growth yields in the reticulo-rumen. In *Digestive Physiology and Metabolism in Ruminants*, pp. 205–226 [Y. Ruckebush and P. Thivend, editors]. Lancaster: MTP Press.

Jouany, J. P. (1982). Volatile fatty acid and alcohol determination in digestive contents, silage juices, bacterial cultures and anaerobic fermentor contents. *Sciences des Aliments* **2**, 131–144.

Legay-Carmier, F. & Bauchart, D. (1989). Distribution of bacteria in the rumen contents of dairy cows given a diet supplemented with soya-bean oil. *British Journal of Nutrition* **61**, 725–740.

Lindberg, J. E., Bristav, H. & Manyenga, A. R. (1989). Excretion of purines in the urine of sheep in relation to duodenal flow of microbial protein. *Swedish Journal of Agricultural Research* **19**, 45–52.

Ling, J. R. & Buttery, P. J. (1978). 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. *British Journal of Nutrition* **39**, 165–179.

McAllan, A. B. & Smith, R. H. (1973). Degradation of nucleic acids in the rumen. *British Journal of Nutrition* **29**, 331–340.

Martin Orue, S. M., Balcells, J., Guada, J. A. & Castrillo, C. (1995). Endogenous purine and pyrimidine derivative excretion in pregnant sows. *British Journal of Nutrition* **73**, 375–385.

Merry, R. J. & McAllan, A. B. (1983). A comparison of the chemical composition of mixed bacteria harvested from the liquid and solid fractions of rumen digesta. *British Journal of Nutrition* **50**, 701–709.

Olubobokun, J. A. & Craig, W. M. (1990). Quantity and characteristics of microorganisms associated with ruminal fluid or particles. *Journal of Animal Science* **68**, 3360–3370.

Olubobokun, J. A., Craig, W. M. & Nipper, W. A. (1988). Characteristics of protozoal and bacterial fractions from microorganisms associated with ruminal fluid or particles. *Journal of Animal Science* **66**, 2701–2710.

Puchala, R. & Kulasek, G. W. (1992). Estimation of microbial protein flow from the rumen of sheep using microbial nucleic acid and urinary excretion of purine derivatives. *Canadian Journal of Animal Science* **72**, 821–830.

Russell, J. B. (1984). Factors influencing competition and composition of the rumen bacterial flora. In *Herbivore Nutrition in the Subtropics and Tropics*, pp. 313–345 [M. C. Gilchrist and R. I. Mackie, editors]. Craighall, South Africa: The Science Press Ltd.

Siddons, R. C., Beever, D. E. & Nolan, J. V. (1982). A comparison of methods for the estimation of microbial nitrogen in duodenal digesta of sheep. *British Journal of Nutrition* **48**, 377–389.

Smith, R. H., McAllan, A. B., Hewitt, D. & Lewis, P. E. (1978). Estimation of amounts of microbial and dietary nitrogen compounds entering the duodenum of cattle. *Journal of Agricultural Science, Cambridge* **90**, 557–568.

Steel, R. G. D. & Torrie, J. H. (1960). *Principles and Procedures of Statistics*. London: McGraw-Hill Book Company, Inc.

Webster, A. J. F. (1992). The metabolizable protein system for ruminants. In *Recent Advances in Animal Nutrition*, pp. 93–110 [P. C. Garnsworthy, W. Haresign and D. J. A. Cole, editors]. Oxford: Butterworth-Heinemann.

Whitelaw, F. G., Eadie, J. M. Bruce, L. A. & Shand, W. J. (1984). Microbial protein synthesis in cattle given roughage–concentrate and all-concentrate diets: the use of 2,6-diaminopimelic acid, 2-aminoethylphosphonic acid and ^{35}S as markers. *British Journal of Nutrition* **52**, 249–260.

Zelaya, J. C. (1994). Degradación ruminal de las bases púricas de origen alimentario (Ruminal degradation of dietary purine bases). MSc Thesis, Instituto Agronómico Mediterraneo de Zaragoza.

Zinn, R. A. & Owens, F. N. (1986). A rapid procedure for purine measurement and its use for estimating net ruminal protein synthesis. *Canadian Journal of Animal Science* **66**, 157–166.

Zöllner, N. (1982). Purine and pyrimidine metabolism. *Proceedings of the Nutrition Society* **41**, 329–342.

Printed in Great Britain