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Estimation of microbial protein synthesis efficiency in sheep using purine nitrogen index and nitrogen capture efficiency

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ABSTRACT

An experiment was conducted to evaluate the efficiency of utilization of rumen degradable nitrogen (RDN) for optimum microbial protein synthesis in sheep by the use of possible potential parameters such as purine nitrogen index (PNI) and nitrogen capture efficiency (NCE), which was estimated from purine derivatives excretion in spot urine samples. Sheep (36) were divided into 4 groups of 9 each and the animals in all groups were fed finger millet straw (FMS) as a basal diet and groundnut cake (GNC) as a nitrogen source. The animals in group 1(G1) were fed with *ad lib.* FMS. Animals in groups 2, 3 and 4 (G2, G3 and G4) were offered GNC@ 14, 18, and 23 g RDN/kg digestible organic matter (DOM) along with FMS. The experimental feeding was continued for 1 month followed by a metabolism trial of 7days. A total of 4 spot urine samples from each sheep were collected at 6 h intervals each day during last week of 1 month feeding period. The total purine derivatives (mmol/litre) excretion and purine derivatives; creatinine (PDC) index recorded were 17.52 and 38.07; 20.53 and 50.09; 24.30 and 51.10 and 18.65 and 39.17, respectively in G1, 2, 3 and 4, respectively. The microbial purine absorption, the calculated microbial nitrogen (MN) supply (g/d) was comparable among the RDN supplemented groups (G2, G3 and G4). PNI and NCE values recorded were 0.19 and 1.40; 0.19 and 1.05and 0.13 and 0.62, respectively in G2, G3 and G4. The results indicated that 14 g RDN from GNC may be adequate for optimum microbial protein synthesis in sheep fed on FMS based diet. Further, PNI and NCE estimated in spot urine of sheep fed *ad lib.* appeared to be useful in evaluation of ruminant diets.

Key words: Fingermillet, Groundnut-cake, Microbial protein synthesis, Nitrogen, Sheep

The methods generally used for determining microbial protein production depend on the use of natural microbial markers such as RNA (ribonucleic acid) and DAPA (di-amino-pimelic acid) or isotopes ^{35}S, ^{15}N or ^{32}P (Martin-orue *et al.* 2000). These techniques are complex, tedious and difficult to practise extensively under field conditions and require ruminally and post ruminally fistulated animals (Broderick and Merchen 1992). The method based on measurement of urinary purine derivatives (PD) overcomes the problems of earlier methods (Chen *et al.* 1995b). Analysis of spot urine samples for PD and creatinine and linking the information to feed intake, makes possible to study the nutritional adequacy in ruminant livestock under field conditions (Nashlai *et al.* 2000). The present study was undertaken to explore the possibility of using potential parameters such as Purine nitrogen index (PNI) and Nitrogen conversion efficiency (NCE) which was estimated from spot urine samples to evaluate the efficiency of utilization of Rumen degradable nitrogen (RDN) for optimum microbial protein synthesis in sheep fed on finger millet straw based diet.

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MATERIALS AND METHODS

Animals and feeding: Nellore Jodipi rams (36) were allocated into 4 groups of 9 each and fed finger millet straw (FMS) as a basal roughage source. Groundnut cake (GNC) was used as a rumen degradable nitrogen source. The RDN content of GNC was calculated by estimating the dry matter (DM) and crude protein (CP) disappearing from samples in nylon bags (Pore size 40 µm) of size 100 mm × 170 mm incubated in the rumen of crossbred fistulated steers for 3, 6, 9, 12 and 24 h (Mehrez and Orskov 1977). The residual CP values were transformed to natural logarithms and then subjected to linear regression (Snedecor and Cochran 1989). The degradation constant was calculated at 3 to 12 h period. The effective degradability was calculated for an assumed outflow rate of 5%/h (Miller 1980). The RDN provided by straw was not taken into account for calculating the total RDN of the diet. The animals in group 1 (control/ unsupplemented-G1) were fed with *ad lib* finger millet straw (FMS). Animals in groups 2, 3 and 4 (G2, G3 and G4) were

offered GNC once daily in the morning (8:00 AM) to provide 15, 22 and 30 g RDN/kg digestible organic matter (DOM) of FMS, respectively. Fresh clean water was made available at all times. All the experimental animals were dewormed and kept in individual pens with feeding and watering arrangements.

Experimental period, sample collection and preparation: The experimental feeding period was continued for one month followed by a 7-day metabolism trial. Representative samples of feeds offered, left over and faeces were collected for 7 days and preserved for further analysis.

Spot collection of urine and preservation: Four spot urine samples per animal per day was collected at 6 h intervals, i.e. each day (24 h) was divided into 4 blocks at 6 h intervals, starting immediately before feeding (designated as 0 h) during last week of 1 month feeding period. The urine excreted by animals in each block was collected, mixed, measured and a fixed sample (10–15 ml based on volume collected during the period), was immediately transferred into plastic vials each containing sufficient quantity of 10% H_2SO_4 to maintain pH below 3. Like that the spot urine samples were collected for 7 days and these samples were bulked to give finally one composite sample from each animal. These urine samples were diluted with distilled water in such a way that the concentration in the final samples would fall with in the range of standards (5 to 50 mg/litre) used in the assays for estimation of purine derivatives. These samples were diluted to avoid precipitation of uric acid during storage. These diluted samples were centrifuged and filtered through surgical gauze and stored at –20°C before actual analysis takes place.

Chemical analysis

Feeds and faecal samples: The proximate composition of the feeds and faeces were carried out as per AOAC (1995). Further, the feed samples were analyzed for neutral detergent fibre (NDF) and acid detergent fibre (ADF) (Goering and Van Soest 1970).

Purine derivatives analysis in urine: The spot urine samples collected were thawed and used for assays for estimation of purine derivatives such as allantoin, uric acid, hypoxanthine and xanthine. Purine derivatives and creatinine content in urine samples were analyzed as per Resines *et al.* (1992) using HPLC system (Waters) with minor modifications. The thawed urine samples were, sonicated using Sonics vibra cell model sonicator, centrifuged and filtered through a millex- HV 0.45 µm pore size filter and a 5 µl of the filtrate was injected into HPLC column.

HPLC analysis was performed with Water HPLC system equipped with automatic gradient controller, two 510 model pumps, a column heater, and a UV detector using a reversed-phase column. Quantification was based on the integration of peak areas using waters millennium 32 software.

Chromatographic conditions: The mobile phase was 10µm potassium phosphate buffer (pH 4.0). Before use, the mobile phase was filtered through a 0.20µm pore size PTFE membrane and further degassed by sonication. The flow rate was 0.5ml/min, the column was maintained at 26°C and the absorbance detector was set at 218nm. The purine derivatives and creatinine peaks were identified by their retention times and co-elution with authentic standards and quantified by comparison of the peak areas of the samples with those of authentic standards.

Measurement of purine derivatives parameters

PDC index: The PDC index, PD excretion thereby the microbial protein synthesis was estimated as per the equations of Chen *et al.* (1992a, 1995a) and IAEA (1999) .

Microbial purine absorption: The amount of microbial purines absorbed (X m mol/day) corresponding to the PD excreted (Y m mol/day) was calculated based on relationship derived by Chen *et al.* (1990).

Intestinal flow of microbial nitrogen: The intestinal flow of microbial N (g N/d) from the microbial purines absorbed (X mmol/d) was calculated on the premises of Chen and Gomes (1992). The efficiency of microbial N supply (EMNS) was expressed as grams of microbial N per kg of digestible OM apparently digested in the rumen (DOMR), which is assumed to be 65% of digestible OM intake (DOMI) (ARC 1984). The purine nitrogen index (PNI) and nitrogen Conversion Efficiency (NCE) were calculated only in RDN supplemented groups (G2, G3 and G4) according to method reported by Chen *et al.* (1999).

Statistical analysis: The statistical analysis of data was carried out as per Snedecor and Cochran (1989). The data obtained were subjected to one-way analysis of variance. Pair-wise significance was done by Duncan multiple range test using statistical software SPSS.

RESULTS AND DISCUSSION

The FMS contained 90.82, 5.1, 0.50, 73.01 and 52.79% organic matter (OM), crude protein, ether extract, NDF and ADF, respectively, whereas the values in corresponding order for GNC were 93.62, 44.05, 5.27, 21.88 and 18.90 respectively. The RDN content in GNC was 3.96%, whereas RDN content of FMS was not taken in to consideration for calculation. Body weight of Nellore rams used in the study ranged from 17.01 to 17.39 kg. Though the diets were formulated to supply 15, 22 and 30 g RDN/kg DOM of straw in animals of G2, G3 and G4, the corresponding actual intakes were 18, 25, and 34 g RDN/kg DOM of straw alone and 14, 18, and 23 g RDN/kg DOM of total ration (total DOMI from straw and GNC), respectively.

As the supplementation level increased from G1 to G4, the total DMI and OMI increased from G1 to G3, hence experimental animals in supplemented groups ate slightly higher DM and OM when compared to unsupplemented/ control group of animals (G1). The total DMI in

Table 1. Effect of different RDN levels on intake and digestibility of finger millet straw based diet in sheep

	G 1	G 2	G 3	G 4	SEM	Significance of effect
Body weight						
kg	17.01±0.62	17.27±0.74	17.39±0.70	17.26±0.75	0.34	0.98
kg $W^{0.75}$	8.37±0.23	8.46±0.27	8.51±0.26	8.45±0.28	0.12	0.98
DM intake (g/day)						
Groundnut cake	0.00±0.00^{a}	80.06±0.00^{b}	108.91±0.00^{c}	136.84±0.00^{d}	8.64	0.000
FMS	411.79±9.68^{c}	348.47±6.19^{b}	335.10±1.61^{b}	307.03±3.81^{a}	7.11	0.000
Total	411.79±9.68^{a}	428.52±6.19^{ab}	444.02±1.61^{b}	443.87±3.81^{b}	3.68	0.002
OM intake (g/day)						
Groundnut cake	0.00±0.00^{a}	74.93±0.00^{b}	101.94±0.00^{c}	128.08±0.00^{d}	8.09	0.000
FMS	373.99±8.79^{c}	316.48±5.62^{b}	304.34±1.46^{b}	278.84±3.46^{a}	6.46	0.000
Total	373.99±8.79^{a}	391.41±5.62^{b}	406.28±1.46^{b}	406.93±3.46^{b}	3.49	0.000
Total intake (g/day $W^{0.75}$)						
DM	49.31±0.89	50.97±1.37	52.61±1.72	52.89±1.53	0.72	0.27
OM	44.79±0.81	46.56±1.25	48.14±1.57	48.49±1.40	0.66	0.18
Digestibility (%)						
DM	54.45±1.41^{a}	58.23±0.93^{b}	58.60±0.64^{b}	56.80±1.26^{ab}	0.59	0.05
OM	56.47±1.66^{a}	59.98±0.99^{ab}	60.41±0.59^{b}	59.20±1.13^{ab}	0.61	0.09

a, b, c, d values with different superscripts within the row differ significantly.

supplemented groups were comparable but the G1recorded significantly (P< 0.002) lower intakes. The similar trend was recorded in total OMI also. The DMI and OMI from straw was significantly (P<0.001) lower in G4 compared to G2 and G3 among the supplemented groups, since the animals in these groups have already met the DM requirements more than the recommended levels (ICAR 1985).

Dry matter intake and organic matter intake/kg $W^{0.75}$ was not significantly different among different experimental groups. The sheep had mean values of DMI ranging from 49.31 ± 0.90 to 52.89 ± 1.53/kg $BW^{0.75}$ which were comparable to the value of 50 g/kg $BW^{0.75}$ suggested by NRC (1985) for non-productive sheep and maintenance requirement of adult sheep weighing 20 kg (ICAR 1985).

The DMD and OMD % were comparable among the supplemented groups but significantly (P< 0.05) higher than the unsupplemented (G1) group indicating that supplementation would have got beneficial effect in increasing the digestibility. The higher DMD and OMD % observed in supplemented groups when compared to unsupplemented groups (Table 1) might be owing to RDN consumption thereby availability of required rumen degradable nitrogen for optimum microbial growth in supplemented groups. Improved *in-vitro* digestibility was observed when locally available protein sources were supplemented @15 g RDN/kg DOM on finger millet straw based diets by Chandrasekharaiah *et al.* (2002, 2003).

Purine derivatives, creatinine, PDC index, microbial nitrogen supply

The mean concentration of allantoin, uric acid, hypoxanthine + xanthine, total PD and creatinine (m mol/lit) excretion is presented in Table 2. The mean allantoin concentration recorded was higher in RDN supplemented groups than in G1, although the differences were non significant. The mean uric acid and hypoxanthine plus

Table 2. Urinary purine derivatives (PD), creatinine excretion and PDC index as affected by different RDN levels in sheep fed finger millet straw based diet

	G 1	G 2	G 3	G 4	SEM	Significance of effect
Allantoin (mmol/lit)	13.79±3.27^{a}	17.56±1.62^{ab}	21.42±2.42^{b}	15.66±1.98ab	1.24	0.16
Uric acid (mmol/lit)	2.73±0.36	2.29±0.39	2.26±0.14	2.37±0.42	0.17	0.77
Hypoxanthine+xanthine	1.01±0.42	0.70±0.23	0.61±0.08	0.63±0.22	0.13	0.69
Total PD (mmol/lit)	17.52±3.40^{a}	20.55±2.12^{ab}	24.30±2.42^{b}	18.65±2.51^{b}	1.34	0.31
Creatinine (mmol/lit)	3.92±0.40	3.54±0.35	4.25±0.36	4.00±0.58	0.21	0.72
Creatinine (mmol/kg $W^{0.75}$)	0.05±0.005^{a}	0.09±0.01^{b}	0.11±0.01^{b}	0.11±0.01^{b}	0.007	0.006
PDC index	38.07±7.76	50.09±3.87	51.10±7.72	39.17±1.83	2.99	0.26

a, b, values with different superscripts within the row differ significantly.

Table 3. Microbial purine absorption, microbial nitrogen supply (MNS), efficiency of microbial N supply (EMNS), purine nitrogen index (PNI) and nitrogen conversion efficiency (NCE) in sheep fed different RDN levels on finger millet straw based diet

	G 1	G 2	G 3	G 4	SEM	Significance of effect
Microbial absorption (g/day)	1.89±0.55[a]	6.09±0.51[b]	6.20±1.0[b]	4.64±0.25[b]	0.42	0.000
MNS (g/day)	1.38±0.40[a]	4.43±0.37[b]	4.51±0.72[b]	3.37±0.18[b]	0.31	0.000
MNS (g/kg DOMI)	6.73±2.13[a]	18.83±1.46[b]	18.33±2.85[b]	14.05±0.78[b]	1.25	0.000
MNS (g/kg DOMR)	10.36±3.28[a]	28.97±2.25[b]	28.20±4.39[b]	21.61±1.20[b]	1.92	0.000
PNI	-	0.19±0.0.02	0.19±0.03	0.13±0.02	0.01	0.11
NCE	-	1.40±0.12[c]	1.05±0.17[b]	0.62±0.03[a]	0.09	0.001
MN: UN	-	2.79±0.0.22	3.18±1.38	1.56±0.24	0.47	0.36

a, b, values with different superscripts within the row differ significantly.

xanthine concentrations were not significantly different among experimental groups. The mean PD concentrations were also not significantly different among experimental groups. The PD excretion in animals followed the similar trend as that of the allantoin excretion since allantoin was quantitatively major component of purine derivatives excreted in urine.

The concentration of creatinine (m mol/litre urine) was not significantly different among the experimental groups. However, the creatinine concentration when expressed in terms of creatinine excretion/kg $BW^{0.75}$, it was significantly ($P<0.006$) different among the experimental groups. The observed measurements of creatinine/kg $BW^{0.75}$ of 0.05 ±0.01 to 0.12±0.02 mmol/kg $BW^{0.75}$ for sheep was lower than the range of values 0.40±0.06 to 0.502±0.10 m mol/kg $BW^{0.75}$ observed in European sheep (Hovel *et al.* 1987, Dewhurst 1989, Chen *et al.* 1991, Lindberg and Jacobsson 1990 and Chen *et al.* 1995a).

The lower creatinine concentration observed in the present study when compared to the reported values may be due to lower body weight of the animal, as the creatinine excretion is a function of protein mass of the animal or metabolic body weight of the animal. It is presumed that excretion rate of creatinine is relatively constant in healthy animals and remains independent of level of feed intake (Chen *et al.* 1992b, 1995a). The creatinine excretion varies widely with the breed or species of animals but usually independent of the level of intake or duodenal infusion of purine bases (IAEA 2002).

The proportions of the individual purine derivatives in total PD excretion were not constant. This observation agrees with the results of the previous studies by Chen *et al.* (1990, 1992b). The fluctuations noted for urinary concentrations of allantoin, uric acid and xanthine plus hypoxanthine and consequently total PD and creatinine in spot urine samples could have been due to the influence of urine volume associated with that of drinking water intake (Chen *et al.* 1992a).

The mean PDC index values were increased as the supplemental level increased from G1 to G3, although the differences were nonsignificant. The lower or higher PDC index values observed in respective groups was mainly due to corresponding lower or higher creatinine excretion coupled with the metabolic body weight of the animals in the concerned groups of sheep. It appears that when sheep were fed under *ad lib.* conditions, PD in spot urine may provide a practical indicator of microbial protein supply status (Chen *et al.* 1995a). The PDC index in spot urine of sheep fed *ad lib.* was subject to little diurnal variations and was highly correlated with the daily output of PD. The ratio in spot urine samples could be used under farm conditions as a practical indicator of microbial protein supply in sheep. The mean of multiple spot samples may reflect more accurately the standing level of purine excretions for the animal on a particular feeding regimen than collecting few samples over several days (Shingfield and Offen 1998).

The estimated microbial purine absorption, microbial nitrogen supply (MN) per day, MNS (g/kg DOMI) and EMNS was significantly ($P<0.001$) lower in G1 than G2, G3 and G4, which were comparable (Table 3), indicating that optimum RDN from the GNC would have been available for microbial fermentation, thereby providing matching nitrogen energy requirement to rumen microbes which would have provided desired rumen environment for increased microbial protein synthesis in G 2. The MN supply when expressed as MNS/kg DOMI or MNS/kg DOMR (EMNS), it remained high in G2, and around 180% (3 times) more MN was synthesized in G 2, when compared to unsupplemented group. EMNS was ranged from 10.36 to 28.97 g N/kg DOMR as against the value, 32 g N/kg DOMR adopted by the ARC (1984). The values observed in the present study are in the range of reported values, i.e. 14 to 49 g/kg DOMR (AFRC 1992) and 18.1 to 46.7 g N/kg DOMR (Chen *et al.* 1995b) except for G1indicating that FMS needs to be supplemented with minimum RDN for optimum microbial protein synthesis. The purine bases absorption in gut showed that the RDN content supplied from GNC had a strong impact on microbial protein (MP) yield and, consequently on the MP supply to the host animal. The MN yield (g/d) obtained in this study is corroborated well with

the reported values (2.7 to 9.12 MN g/d) of Mupangwa *et al.* (2000) and Chikagwa-Malunga *et al.* (2009) in sheep.

PNI and NCE

Very limited literature is available with regards to PNI and NCE of ruminant diets. PNI values observed were comparable among the RDN supplemented groups. However, NCE values were significantly (P<0.001) different among RDN supplemented groups. Higher NCE values were observed in G2 followed by G3 and G4, respectively. It is suggested that PNI can potentially be used as an indictor of the efficiency with which degradable dietary nitrogen is converted to microbial protein in the rumen. (Chen *et al.* 1999). The excretion of PD in urine provides an estimation of the intestinal flow of microbial protein, and therefore, PNI effectively corresponds to the amount of microbial protein produced in the rumen relative to the nitrogen loss in the urine. If a diet or a dietary regimen has high nitrogen capture efficiency (NCE) proportionally more rumen degradable nitrogen is converted to microbial protein and less nitrogen is excreted in urine resulting in a high PNI. Conversely, if a diet has poor conversion efficiency, proportionally less dietary nitrogen is converted to microbial protein and more is excreted in urine, resulting in a low PNI. However, in the present study, higher NCE values in G2 and comparable PNI values among G2, G3 and G4, indicated that 14 g level of RDN/kg DOM may be adequate for optimum microbial protein synthesis in sheep fed on finger millet straw based diets.

The results indicated that the PDC index, PNI, NCE measured in spot urine of sheep fed *ad lib.* could be used under farm conditions as a practical indicator of microbial protein supply in sheep provided uniformity maintenance in spot urine collection is maintained, multiple collections (more number of days) are made and creatinine should have been estimated previously from these animals (al least from same breed) by total urine collection method. However, more studies are yet required to define the sensitivity and usefulness of this spot test method. Further it is indicated that 14 g RDN/kg DOM or 21 g RDN/kg DOMR may be adequate for optimum microbial protein synthesis in the rumen on finger millet straw based diets in sheep.

REFERENCES

AFRC. 1992. Agricultural Food and Research Council. *Energy and Protein Requirements of Ruminants*. Wallingford. CAB international, United Kingdom.

AOAC. 1995. *Official Methods of Analysis*. 16 edn, Vol.1. Association of Official Analytical Chemist. Washington, DC, USA.

ARC. 1984. *The Nutrient Requirements of Ruminant Livestock*. Agricultural Research Council. Supplement 1. Wallingford, CAB International, United Kingdom.

Broderick G A and Merchen N R. 1992. Markers for quantifying microbial protein synthesis in the rumen. *Journal Dairy Science* **75**: 2618.

Chandrasekharaiah M., Sampath K T, Prakash C and Praveen U S. 2002. Effect of supplementation of different concentrate ingredients on *in vitro* NDF digestibility of finger millet straw. *Animal Nutrition and Feed Technology* **2**(2): 169–76.

Chandrasekharaiah M, Sampath K T, Praveen U S and Prakash C. 2003. Improving the digestibility of finger millet straw by strategic supplementation through locally available concentrate ingredients and green fodders/top feeds. *Indian Journal of Animal Sciences* **73** (10): 1184 –86.

Chen X B and Gomes M J. 1992. Estimation of microbial protein supply to sheep and cattle based on urinary excretion of purine derivatives: An overview of the technical details. *Rowett Research Institute*. University of Aberdeen, UK. Occasional publication.

Chen X B, Chen Y K, Franklin M F, Orskov E R and Shand W J.1992b. The effect of feed intake and body weight on purine derivative excretion and microbial protein supply in sheep. *Journal of Animal Science* **70**: 1534–42.

Chen X B, DeB F D, Hovell, Orskov E R and Brown D S. 1990. Excretion of purine derivatives by ruminants: effect of exogenous nucleic acid supply on purine derivatives excretion by sheep. *British Journal of Nutrition* **63**: 131.

Chen X B, Grubic G, Orskov E R and Osuji P. 1992a. Effect of feeding frequency on diurmal variation in plasma and urinary purine derivatives in steers. *Animal Production* **55**: 185–91.

Chen X B, Kyle D J, Orskov E R and Hovell F D. 1991. Renal clearance of plasma allantoin in sheep. *Experimental Physiology* **76**: 59–65.

Chen X B, Mejia A T, Kyle D J and Orskov E R. 1995a. Evaluation of the use of the purine derivative: creatinine ratio in spot urine and plasma samples in ruminants: Studies in sheep. *Journal of Agricultural Science* (*Cambridge*) **125**: 137–43.

Chen X B, Subba D B, Orskov E R and Jayasuriya M C N. 1999. Purine nitrogen index, potentially a new parameter for rapid feed evaluation in ruminants. *IAEA-IECDOC* **109**: 97–110.

Chen X B, Susmel P, Stefanon B and Orskov. 1995b. On the use of purine derivatives in spot urine, plasma and milk samples as indicators of microbial protein supply in sheep and cattle. *Proceedings of the 7th International Symposium on Protein Metabolism and Nutrition*. Vale de santarem-portugal.

Chikagwa- Malunga S K, Adesogan A T, Szabo N J, Littell R C, Phatak S C, Kim S C, Arriola K G, Huisden C M, Dean D B and Krueger N A. 2009. Nutritional characterization of Mucuna pruiens. 3. Effect of replacing soybean meal with Mucuna on intake, digestibility, N balance and microbial protein synthesis in sheep. *Animal Feed Science and Technology* **148**: 107–23.

Dewhurst R J. 1989. 'Studies on energy and nitrogen metabolism in the rumen investigation of less invasive techniques for these studies.' Ph.D thesis. University of Bristol.

Goering H K and Van Soest P J. 1970. *Forage Fibre Analysis. Handbook No.379*. ARS USDA, Washington. DC.

Hovell F D DCB, Orskov E R, Kyle D J and MAcLead N A. 1987. Under nutrition in sheep nitrogen repletion N depleted sheep. *British Journal of Nutrition* **57**: 77–88.

IAEA. 1999. Nuclear based technologies for estimating microbial protein supply in ruminant livestock. IAEA-TECDOC-1093, Vienna.

IAEA. 2002. Report of final Research Co-ordination Committee Meeting (IAEA). Vietnam.

ICAR. 1985. *Nutrient Requirements of Livestock and Poultry*. Indian Council of Agricultural Research, New Delhi

Lindberg J E and Jacobsson K G. 1990. Nitrogen and purine metabolism at varying energy and protein supplies in sheep sustained on intragastric infusion. *British Journal of nutrition* **64**: 359–70.

Martin-Orue, Balulls S M J, Guada J A and Fondevila M. 2000. Microbial nitrogen production in growing heifers. Direct measurements of duodenal flow of purine bases versus urinary excretion of purine derivatives as estimation procedures. *Animal Feed Science Technology* **88**: 171–88.

Mehrez A Z and Orskov E R. 1977. A study of the artificial bag technique for determining the digestibility of feeds in the rumen. *Journal of Agricultural Science, Cambridge* **88**: 645–50.

Miller E L. 1980. *Vicia faba* 3/4 Feeding Value, Processing and Viruses. *Protein Value of Feedstuffs for Ruminants*. (Ed.) Bond D A. Martinus. Nijhoff, The Hague.

Mupangwa J F, Ngongoni N T, Topps J H, Acamovic T, Hamudikuwanda H, Nalovu L R. 2000. Dry matter intake, apparent digestibility and excretion of purine derivatives in sheep fed tropical legume key. *Small Ruminant Research* **36**: 261–68.

Nashlai I V, Osuji P O, Umunna N N. 2000. Effect of form and quality of feed on the concentration of purine derivatives in urinary spot samples, daily microbial N supply and predictability of intake. *Animal Feed Science and Technology* **85**: 223–38.

NRC. 1985. *Nutrient Requirements of Sheep*. Revised 6th edn. National Academy press, Washington, DC.

Resines J E, Arin M J and Diez MT. 1992. Determination of creatinine and purine derivatives in ruminants urine by reversal phase high performance liquid chromatography. *Journal of Chromatography* **607**: 199–202.

Shingfield K J and Offen N W. 1998. Evaluation of spot urine sampling technique to asses urinary purine derivatives excretion in lactating dairy cows. *Animal Science* **66**: 557–68.

Snedecor G W and Cochran W G. 1989. *Statistical Methods*. 8th edn. Oxford and IBH publishing Co., Kolkata.