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Nuclear based technologies for estimating microbial protein supply in ruminant livestock

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FOREWORD

The Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture through its Co-ordinated Research Projects (CRPs), has been assisting national agricultural research systems in Member States to develop and apply nuclear and related techniques for improving livestock productivity. The programmes have focused on animal nutrition, animal reproduction and more recently on animal nutrition/reproduction interactions with emphasis on smallholder farming systems.

The measurement of microbial protein supply to ruminant livestock has been an important area of research in ruminant nutrition. An estimate of microbial protein contribution to the intestinal protein flow is important for estimating the protein requirement of ruminant animals. Understanding the process of microbial protein synthesis has been difficult however, and due to the lack of simple and accurate methods for measuring microbial protein production in vivo, the methods used are based on complex microbial markers which require surgically prepared animals.

Under a Technical Contract awarded to the Rowett Research Institute, United Kingdom, a simple calorimetric technique was developed to analyse purine derivatives in urine. Using this test, knowledge of endogenous excretion and other factors affecting the production and excretion of purine derivatives, makes it possible to predict microbial protein supply to the host animal. However, the method was developed mainly for European breeds of cattle and sheep. Cattle and sheep differ markedly in their endogenous excretions and there is evidence to suggest that zebu (*Bos indicus)* cattle and buffaloes differ in their rate of purine excretion and may require a prediction model different to that of European breeds.

As a result of a consultants meeting held in May 1995 to advise the Joint FAO/IAEA Division on the feasibility of using nuclear and related techniques for the development and validation of techniques for measuring microbial protein supply in ruminant animals, an FAO/IAEA Co-ordinated Research Project on Development, Standardization and Validation of Nuclear Based Technologies for Measuring Microbial Protein Supply in Ruminant Livestock for Improving Productivity was initiated in 1996, with a view to validating and adapting this technology for use in developing countries.

To assist scientists participating in the CRP, a laboratory manual containing experimental protocols and methodologies for standardization and validation of the urine purine derivative technique and the development of models to suit local conditions, was published as IAEA-TECDOC-945.

The present publication contains the final reports from participants in Phase 1 of the project presented at the second research co-ordination meeting held in Vienna from 24 to 28 August 1998.

Mr. M.C.N. Jayasuriya, Consultant to the Joint FAO/IAEA Division, was responsible for this publication.

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SUMMARY OF THE CO-ORDINATED RESEARCH PROJECT ON DEVELOPMENT, STANDARDIZATION AND VALIDATION OF NUCLEAR BASED TECHNOLOGIES FOR ESTIMATING MICROBIAL PROTEIN SUPPLY IN RUMINANT LIVESTOCK FOR IMPROVING PRODUCTIVITY

M.C.N. Jayasuriya

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1. BACKGROUND

A major constraint to animal production in developing countries is poor nutrition due to inadequate or fluctuating nutrient supply. This results in low rates of reproduction and production as well as increased susceptibility to disease and mortality.

The smallholder farmers in developing countries have limited resources available for feeding their livestock. Unlike those in developed countries, they are unable to select their basal diet according to requirement for production. Therefore, the strategy for improving production has been to maximize the efficiency of utilization of the available feed resources in the rumen by providing optimum conditions for microbial growth, and then by supplementation, to provide dietary nutrients to complement and balance the products of digestion to meet requirement.

Microbial cells formed as a result of rumen degradation of carbohydrates under anaerobic conditions are a major source of protein for ruminants. They provide the majority of the amino acids that the host animal requires for tissue maintenance, growth and production. In roughage-fed ruminants, micro-organisms are virtually the only source of protein. Therefore, a knowledge of the microbial contribution to the nutrition of the host animal is essential to developing feed supplementation strategies for improving ruminant production. While this factor has been recognized for many years, it has been extremely difficult to determine the microbial protein contribution to ruminant nutrition.

The methods generally used for determining microbial protein production depend on the use of natural microbial markers such as RNA (ribonucleic acid) and DAPA (diamino-pimelic acid) or of isotopes ^{35}S, ^{15}N or ^{32}P. However, these methods involve surgical intervention such as post-rumen cannulation and complex procedures that require accurate and quantitative information on both digesta and microbial marker flow.

The idea of using microbial purine compounds as a specific marker for the rumen microbial biomass was first suggested in 1954. Since micro-organisms have high concentrations of purine-containing compounds such as RNA and DNA relative to concentrations in plant and mammalian cells, and since purines in dietary and endogenous materials are rapidly degraded by microbial enzymes in the rumen, it is highly likely that negligible concentrations of purines are found in the digesta leaving the rumen. Therefore, any purines present in digesta in the small intestine can be expected to be only of microbial origin and can be considered to be specific markers for the microbial fraction.

A calorimetric technique using enzymatic procedures was developed for measuring purine derivatives (PD) in urine under a Technical Contract. With knowledge of the amount of PD excreted in the urine, the microbial protein supply to the host animal can be estimated. The principle of the method is that nucleic acids leaving the rumen are essentially of microbial origin. The nucleic acids are extensively digested in the small intestine and the resulting purines are absorbed. As only a small amount of the absorbed purines are utilized by the animal, the remainder are metabolised forming hypoxanthine, xanthine, uric acid and allantoin, and these metabolites are excreted, mainly in the urine. Therefore, with an understanding of how urinary excretion responds to purine absorption (i.e. the response curve of PD excretion to purine input into the intestines), the microbial purine outflow from the rumen and hence microbial nitrogen supply to the animal can be estimated from the measured excretion of PD in the urine. Current evidence suggests that milk and other body fluids are not appropriate for developing prediction equations.

2. OBJECTIVES AND SCOPE OF THE PROJECT

The PD excretion method is simple, non-invasive and does not require surgical preparation of the animal. It is being used in many laboratories, e.g. for comparisons between different dietary regimes in European breeds of cattle and sheep, and shows good agreement with other methods used for measuring microbial yield, including the methods based on the determination of digesta flow markers and microbial markers such as ^{15}N and ^{35}S. However, the relationships between microbial yield of purines from the rumen and urinary excretion of PD may differ between different breeds and species of ruminants. Therefore, there is a need to determine whether the purine excretion method can provide precise predictions of microbial protein supply in non-European breeds of cattle and other ruminants. This and other aspects need to be studied in a variety of laboratories to provide data for a general model which will generate prediction equations for each breed and species of animal. Some refinements of the technique and development of a robust and inexpensive method for analysing urine will also be necessary before the method can be widely applied at the farm level.

It is important to recognise that there are some potential sources of error. There is a relatively small loss of PD via non-renal routes that may not be a constant fraction of the total loss from the plasma pool. If the renal and non-renal losses from the body purine pools differ between different types of ruminants the accuracy of prediction of microbial flow from the rumen from urinary purines may be affected. For example, the slope of the line relating urinary PD to digestible organic matter intake (DOMI), which reflects this loss-partition ratio, has been shown to differ between sheep, cattle and buffaloes. This ratio may vary with type of diet and other factors (e.g. glomerular filtration rate (GFR), kidney function, digesta flow rate through the gut etc.). The purine excretion method depends on the assumption that purines reaching the intestine are derived exclusively from rumen micro-organisms. There are reports that dietary purines can escape rumen breakdown in certain dietary components, e.g. fish meal. If materials known to escape degradation in the rumen form a major part of the diet, then results obtained with the method should be interpreted with caution. Prediction equations also depend on a value for digestion and absorption of purines in the small intestine. A review of the available literature suggests that a value of 0.85 is appropriate and this value has been adopted for present use. As already noted, it is possible that this value might vary with the diet or for other reasons, and is subject to modification if new information becomes available.

In order to standardise and validate the urinary PD excretion technique and determine its potential value as a tool for estimating microbial protein supply to ruminant livestock in developing countries, an FAO/IAEA Co-ordinated Research Project (CRP) was initiated in 1996 with the following objectives:

(a) To refine and standardise the purine excretion technique for measuring microbial protein supply in ruminant livestock
(b) To validate the above technique for indigenous zebu *(Bos indicus)* cattle and their crosses with exotic breeds and buffaloes in developing countries, using nuclear and related techniques
(c) To support the use of purine excretion technique as a robust and inexpensive method for estimating rumen microbial protein supply for developing feeding strategies for improving productivity of ruminant livestock in developing countries.

The programme was to be carried out in two phases. During Phase I which was to last for 3 years, the technologies developed based on research by the Rowett Research Institute, UK, and other European laboratories, were to be refined, standardised and validated for indigenous zebu *(Bos indicus)* cattle and their crosses with exotic breeds *(Bos taurus* × *Bos indicus)* and buffaloes. Assuming success in Phase I, then during Phase II, the validated technique would be

applied at field level to estimate the supply of rumen microbial protein and the information generated used to assist Member States in developing feeding strategies.

2.1. Objectives of Phase I

Phase I of the project had two major objectives.

(1) To standardize, validate and extend the methodology for use in buffaloes, *Bos indicus,* and their crosses with European breeds

Appropriate methodology and models for the estimation of microbial protein supply based on urinary purine excretion have been established for European breeds of cattle and sheep. However, limited information available suggests that the current prediction model/equations may not be wholly suitable for other types of ruminants (e.g. buffaloes, *Bos indicus* cattle, *Bos indicus* × *Bos taurus*) and therefore these may need to be adjusted to improve the precision of prediction of microbial protein yield in these breeds.

The information required for defining the prediction equation included:

(i) The contribution of basal purine excretion (endogenously produced purines) to the total excretion of PD

(ii) The tissue profiles for enzymes involved in purine metabolism (blood, intestine and liver)

(iii) The relationships between rate of purine absorption from the gut and rate of excretion of PD in the urine

(iv) The renal to non-renal partition ratio and its variability in different breeds.

(2) To establish a database of values for purine N:total N in mixed rumen micro-organisms.

The calculation of microbial protein supply based on purine absorption requires the measurement of the ratio of purine N:total microbial N in mixed rumen micro-organisms. The limited information currently available suggests that this is relatively constant, but further evaluation of this ratio is desirable. Therefore, there is a need to establish a database of values for purine N:total N in rumen micro-organisms from ruminants on a range of diets and under different conditions in a number of different laboratories.

2.2. Objectives of Phase II

During Phase II of the Programme the fully validated urinary PD technique will be applied at farm level to estimate the microbial protein production in ruminant livestock.

Once standardized and validated the technique can be used as a diagnostic tool by National Agricultural Research Systems (NARS) to indicate whether an animal or group of animals have been underfed or fed on an imbalanced diet, thus enabling corrective action.

The technique can be used also by extension services to assist farmers in developing feeding strategies which ensures the maximum protein supply to animals. This will be particularly relevant for milking cows in peri-urban farming systems where protein supply may be most critical and where inexpensive microbial protein would be much cheaper than un-degraded protein.

3. IMPLEMENTATION OF PHASE I

On the basis of applications received from Research Institutes and Universities in both developed and developing countries, 4 Research Contracts and 4 Research Agreements were awarded in 1996. The Research Contracts were awarded to institutes in Malaysia, Indonesia, Venezuela and Turkey and Research Agreements to Australia, Italy and Spain. The Rowett Research Institute in the United Kingdom, received a Technical Contract to continue with the development and refinement of protocols and methodologies for measurement of urinary PD and

to assist other developing country laboratories with the analysis and interpretation of experimental data.

The First Research Co-ordination Meeting (RCM) of the CRP was held at Gadjah Mada University in Yogyakarta, Indonesia, from 5 to 9 August 1996. During this meeting it was concluded that PD excretion technique had great potential to provide a simple and scientifically valid method for predicting the flow of microbial nitrogen from the rumen, provided that there is a clear understanding of differences amongst ruminant species of the variation in purine N:total N in rumen microbes, the excretion of exogenous and endogenous purines and renal and non-renal routes of excretion of PD. Accordingly, experimental protocols were developed under 4 areas of activity, namely a) the response in PD excretion to microbial protein supply due to different levels of feed intake, b) the determination of endogenous PD excretion, c) the estimation of purine N:total N in rumen microbes and d) enzyme profile studies. These studies used both radio active and stable isotopes (^{14}C and ^{15}N) as well as non-isotopic techniques such as chemical assays.

As a part of the contribution of the Technical Contract the Rowett Research Institute compiled all available information on procedures and methodologies used in the standardization and validation of the urinary PD technique. The detailed descriptions of methods involving both radioactive and stable isotopes as well as non-isotopic techniques were published in 1997 as an IAEA-TECDOC (IAEA-TECDOC-945). This ensured that all participants of the CRP used standardized methodologies and procedures which provided a high degree of uniformity in experimental procedures and allowed for the central evaluation of research.

The Second RCM was held in Vienna, Austria, from 24 to 28 August 1998. At this meeting each participant submitted a technical report which summarized the studies conducted and results achieved since the first RCM in 1996. These are presented as country reports in this technical document. The group also formulated conclusions and recommendations on the basis of Phase I studies, which are also presented.

4. CONCLUSIONS AND RECOMMENDATIONS - PHASE I

4.1. Conclusions

4.1.1. The response in PD excretion to microbial protein supply due to different levels of feed intake.

Studies carried out with *Bos indicus*, *Bos taurus, Bibos banteng* (Bali cattle) and swamp buffaloes showed that there was a linear increase in PD excretion with increasing levels of feed intake in all species at all locations, verifying the value of PD excretion as a predictor of microbial purine outflow from the rumen. The slopes of these relationships reconfirmed that there were different responses between species probably due to different partitioning of PD removal from the blood by renal and non-renal routes. Studies using ^{14}C or ^{15}N labelled uric acid in Indonesia, Turkey, Malaysia and Venezuela showed that recoveries of labelled PD in urine were 80-85% and hence non-renal losses were 15-20%.

4.1.2. Determination of endogenous PD excretion

The levels of PD excretion were determined in fasting animals in order to determine the endogenous level of PD excretion and PD:creatinine ratio that existed when no microbial purines were flowing out of the rumen. Rates of endogenous excretion have now been established with confidence for buffaloes, *Bos taurus, Bos indicus* and *Bos banteng* cattle and sheep under this CRP.

4.1.3. Estimation of purine N:total N ratio in rumen microbes

Methods for estimating rumen microbial protein or N outflow using PD excretion rate rely on the knowledge of the ratio of purine N:total N in microbial material leaving the rumen. The ratios obtained during Phase I (e.g. in Zebu cattle 12.2% ± 1.25) were relatively constant,

unaffected by diet and within the range reported in the literature. It is concluded that the value previously recommended (11.6%) is sufficiently consistent to be applied in most situations except when concentrate:roughage ratios in the diet are >0.5-0.6, and when protozoa represent a major fraction of the microbial material in duodenal digesta.

Although existing information indicates that particle-associated (PA) bacteria contribute significantly to the duodenal flow, the low recovery of the bacterial isolates obtained in the studies made in Phase I (only 19% ± 0.69 and 20.1% ± 5.39, of the original adherent micro-organisms were released) shows that there is no need to change the original protocol.

4.1.4. Enzyme profile studies

The aim of obtaining enzyme profiles of individual animal species was successfully completed. Xanthine oxidase (XO) was determined in the gut, liver and blood of buffaloes and cattle. The results show a difference in the xanthine oxidase activity in different tissues and can justify the use of different values for endogenous excretion of PD and urinary recovery in the prediction equations for different species. The patterns of XO enzyme activity in different tissues in these species confirm results obtained previously. An interesting finding was that Bali cattle had extremely low xanthine oxidase activity in the intestinal tissues. However, this finding needs to be reconfirmed because of the uniqueness of the results.

4.1.5. Renal and non-renal excretion of purine derivatives

Excretion of PD by renal and non-renal routes was successfully evaluated using both ^{14}C and ^{15}N tracers. The results were used to refine the models used for predicting microbial flow into the small intestine from urinary PD excretion rate. Estimates of glomerular filtration rate (GFR) based on U:P (urine:plasma) ratios of creatinine showed that this parameter is also related to level of feed intake, providing reasons for the different ratios of partitioning of PD between renal and non-renal routes of loss from the blood.

Preliminary models were developed for all species based on the recovery of labelled PD (nuclear technique) and the measures of endogenous excretion.

For buffaloes:

$$Y = 0.74\ X + 0.337\ W^{0.75}$$

For Kedah-Kelantan (KK) cattle

$$Y = 0.68\ X + 0.275\ W^{0.75}$$

For Zebu crosses

$$Y = 0.84\ X + 0.236\ W^{0.75}$$

For Ongole cattle

$$Y = 0.85X + 0.132\ W^{0.75}$$

For Bali cattle

$$Y = 0.86\ X + 0.145\ W^{0.75}$$

where Y = PD excretion (mmol/day), X = absorbed purine bases (mmol/day) and W = live weight (kg).

By applying the model equations, and allowing for the purine-N:total N ratio in rumen micro-organisms, the total microbial outflow from the rumen was predicted. When microbial flow was predicted at different levels of feed intake, the corresponding microbial flow estimates per unit of DOMI were within the expected range found in the literature. The new knowledge concerning endogenous excretion helps to improve the confidence in the prediction models. Further work now being conducted in established laboratories (e.g. Malaysia and Indonesia) will undoubtedly improve the level of confidence in prediction of the absolute levels of microbial

flow. The method can, however, now be used with confidence in the field for comparing the microbial flows between diets or across different environmental or other treatments.

The knowledge of endogenous excretion can also be used as a diagnostic aid. For example, animals with a endogenous excretion similar to, or only slightly above the endogenous value established for their species will have a dysfunctional rumen or are simply not eating adequately which requires adjustment to the feeding management.

4.2. Recommendations

4.2.1. Use of PD technique under field conditions

The urinary PD technique for estimating rumen microbial protein production is now ready for use under field conditions and should be evaluated in various developing countries and under different livestock production systems. By using 'spot' urine samples, analysing for PD and creatinine and linking the information to feed intake and feed analysis, it will be possible to study the nutritional adequacy in ruminant livestock and to group animals into 'categories' according to the efficiency of their rumen function.

The models developed should be put into use for those species for which the models were successfully validated in Phase I.

4.2.2. Evaluation of colorimetric techniques for 'on-farm' use

The colorimetric techniques developed for estimating uric acid, allantoin and creatinine concentrations in urine should be evaluated for field or 'on-farm' use as simple indicators of the 'nutritional status' of animals. The final objective should be the availability of a method which can readily be used by farmer advisors or extension workers to identify major problems of nutrition that result in a grossly inefficient rumen digestion of feed and a low level of microbial protein supply to the host animal.

4.2.3. Verification of the techniques under local conditions

Controlled feeding experiments should be undertaken by new Research Contract holders in their own regions to verify the techniques in their own setting. They should also determine the existing base level of nutritional adequacy so that the impact of the new technology in their countries can be measured. It will be important to determine whether the use of the new technique results in improvements in the efficiency of locally available feed resource use.

4.2.4. Assessment of impact on animal productivity

An assessment of the impact of the urinary PD technique in the field will be expected at the end of the CRP. A procedure for this assessment will be developed at the next RCM scheduled for early 2000.

COUNTRY REPORTS

PREDICTION OF RUMEN MICROBIAL OUTFLOW BASED ON URINARY EXCRETION OF PURINE DERIVATIVES

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Abstract

PREDICTION OF RUMEN MICROBIAL OUTFLOW BASED ON URINARY EXCRETION OF PURINE DERIVATIVES.

The method for predicting microbial protein outflow from the rumen based on the excretion of purine derivatives (PD) in the urine is being increasingly used by nutritionists. In contrast to methods that depend on estimates of digesta flow, the PD method does not require animals to be fitted surgically with cannulae into the gut, and studies can be performed with minimal disturbance to the experimental animals. Methods of analysis of PD have been improved and standardized. Certain assumptions, however, are required that could lead to errors when this method is used to predict microbial protein outflow from the rumen. The need for further investigation of these assumptions by means of isotopic tracers and other techniques is examined.

1. INTRODUCTION

Estimation of the flow of digesta from the rumen (or of any component of the digesta) has always been a difficult problem for ruminant nutritionists. The majority of estimates of digesta outflow from the rumen have been made using surgically modified animals. Several such methods have been used. Commonly, digesta leaving the rumen are either quantitatively collected, or representative samples obtained and their contribution to the total outflow is estimated by reference to a non-absorbable gut marker that passed through the gut at a known rate. These methods involve surgical intervention and the animals are frequently disturbed while digesta samples are collected.

Researchers usually want to estimate the rate of microbial or 'escape' protein outflow rather than total digesta outflow. Determination of microbial outflow (or outflow of a microbial component such as microbial crude protein) require obtaining accurate quantitative information on both digesta and microbial marker flow. Digesta consist of a heterogeneous mixture of dietary, microbial and endogenous materials. Markers that are specific for microorganisms are required and errors associated with these markers further reduce the accuracy of the final estimates of the rate of microbial outflow. External markers such as ^{15}N and ^{35}S, and internal markers such as L-alanine, diaminopimelic acid have been used. Purine compounds have been evaluated and are the subject of the discussion below.

The idea of using microbial purine compounds as a specific marker for the rumen microbial biomass was suggested by McDonald in 1954 [1] and by others [2-5]. Purines are heterocyclic ring structures (nitrogenous bases) with varying functional groups. The purine bases, adenine and guanine are found in both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The purine nucleotides (adenylate and guanylate) are the building blocks of the nucleic acids, DNA and RNA, and consist of, (a) a purine base which is heterocyclic nitrogenous compound, (b) a sugar (ribose in RNA, deoxyribose in DNA) and (c) a

phosphoryl group ester-linked to the sugar. Adenosine and guanosine are the corresponding nucleosides formed when the phosphate groups are removed.

Micro-organisms have high concentrations of purine-containing compounds (RNA and DNA) relative to concentrations in plant and mammalian cells. Moreover, purines in dietary and endogenous materials are, in general, rapidly degraded by microbial enzymes in the rumen [6]. They are therefore likely to be present in only negligible concentrations in digesta leaving the rumen. The microbial purines, on the other hand, remain intact in living microbial cells and pass via the abomasum to the small intestine. The purines present in digesta entering the small intestine can therefore be expected to be almost totally of microbial origin. Microbial purines are, therefore, specific markers of the microbial fraction. If the concentrations of purines in the whole digesta and in a pure sample of the microbial biomass are expressed as a ratio, the result is an estimate of the fraction of the digesta that is of microbial origin. If digesta flow rate is determined using cannulated animals and non-absorbable gut markers as mentioned above, the flow rate of crude protein or any other microbial component in the digesta can also be determined from its ratio relative to the purine concentration.

The use of purines as microbial markers has special appeal by eliminating the need for estimation of digesta flow rate. When microbial materials containing purines enter the abomasum and small intestine, they are degraded enzymatically to nucleotides and purine bases [7]. These are then absorbed into the body of the animal. Although these purine compounds may be incorporated into tissues, the amount absorbed greatly exceeds tissue requirements and the majority is excreted via the kidney. The rates of purine derivatives (PD) in the urine therefore tend to closely reflect, and can therefore be used to predict, the flows of microbial purines into the intestines [8]. If the concentration of purines in a pure sample of the mixed microbial biomass is known, as well as the predicted flow rate of purines from the rumen, then the flow of microbial biomass can be simply determined.

2. PRINCIPLE OF THE PURINE-BASED ESTIMATION OF RUMEN MICROBIAL OUTFLOW

The methodology of this technique has been greatly advanced and an example of the use of the technique for sheep [9] is as follows.

Absorption of purines (X, mmol/d) is assumed to be related to urinary PD excretion (Y, mmol/d) according to the equation:

$$Y=0.84X + (0.150W^{0.75}).\exp(-0.25X)$$

The equation allows for a non-linearity in the relationship that is thought to be due to a urinary component of PD of endogenous origin that is related to live weight (W) and decreases as an animal's plane of nutrition increases from sub-maintenance to maintenance level (Figure 1).

Experimentally determined values for the daily PD excretion (Y) and live weight (W) are entered and the equation is solved to give a prediction of purines absorbed (X) (Alternatively, the values could be determined from the figure). After allowing for net purine digestibility in the small intestine (assumed to be 0.83), a value for purine entry into the intestines is calculated (X/0.83). The purines entering the small intestine (assumed to contain 70 gN/mol) are considered to be derived entirely from rumen microbes with a purine-N: total N ratio of 0.116:1. Thus, a prediction of microbial N outflow from the rumen (gN/d) is given by 70X/(0.116 x 0.83 x 1000), which simplifies to 0.727X.

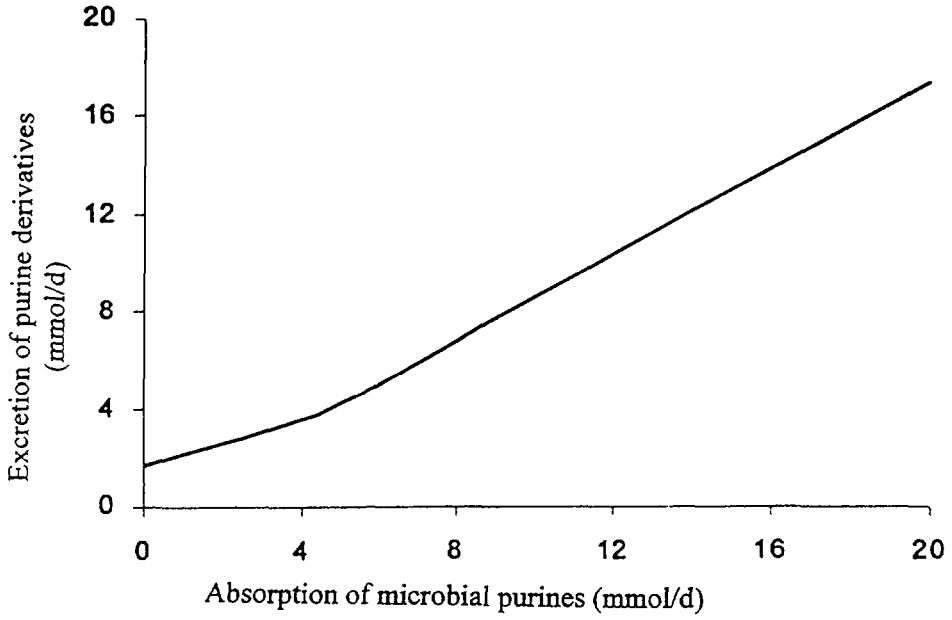

FIG.1. Relationship between excretion of purine derivatives in urine and absorption of dietary purines from the gut of a 35 kg sheep [10].

The method assumes that the relationship between rate of absorbed purines and rate of excretion of PD, intestinal digestibility of purines, and the ratio of purine:N in mixed rumen microorganisms are constants. These assumptions may not be entirely valid under all circumstances and are discussed further below. Alternative models for predicting the rate of absorption of microbial purines and of microbial outflow rate from the rumen from urinary PD excretion rate have been proposed [11-14]. An alternative to predicting values for net microbial synthesis is simply to compare the urinary PD excretion rates between treatments.

3. DEGRADATION OF DIETARY PURINES AND FORMATION OF MICROBIAL PURINES

Nucleic acids are synthesized by bacteria and protozoa and RNA and DNA represent the majority of purine compounds in rumen microorganisms. Of the total N present in rumen bacteria, nucleic acids and proteins comprise 13-19% and 75-85%, respectively. On average, purine-N:total N was 9.6% in bacteria and 4.8% for protozoa [15]. In mixed rumen bacteria from steers, RNA-N:total N ratios were 7.7% (± 0.2 sd) [16]. When predicting rumen microbial outflow from estimates of the rate of flow of purine compounds from the rumen, it would be more convenient if a constant factor was universally applicable. Unfortunately, this is unlikely to be the case. The ratio of RNA:total N increases as bacterial growth rate in the rumen increases [17], with RNA-N:total N varying more than DNA-N:total N [18]. Bates and co-workers [19] found that RNA:protein ratios in mixed rumen bacteria increased with increasing specific growth rate: the ratios were also affected by diet and time after feeding and there was an interaction between diet and free bacteria *vs* particulate bacteria. The total N

content and the ratio of RNA-N:total N were lower in fluid-phase bacteria than in particle-associated bacteria [20] even though the latter might be expected to grow more slowly than the former. An extensive study of ruminal microorganisms was made with continuous fermenters using a basal feed supplemented with various protein-rich supplements [21]. The ratio of purine N:total N in bacteria averaged 8.3% and was not affected by the type of supplement: however, liquid and solid dilution rates were not varied in this experiment. From the above, it can be seen that some reports indicate that purine-N:total N ratios in mixed bacteria may be lower than the 11.6% used in the previous example. However, it should also be recognised that estimates of the ratio have at times been based on non-specific colorimetric methods for estimating purine concentration and such estimates need to be interpreted with caution.

4. ESCAPE OF DIETARY PURINES TO THE SMALL INTESTINE

In general, it appears that free nucleic acids and derivatives from the diet are rapidly degraded in the rumen. Nucleic acids in hay were also rapidly degraded when incubated in rumen contents [22]. In a study of digestion of feeds in continuous fermenters, dietary purine content (%DM) in a variety of meals ranged from 0.03 in blood meal to 0.08 in fishmeal. Escape of feed purine N averaged 1.7% (± 2.9 sd) of total purine N flow. These and other studies suggest that, because of the relatively low concentrations of purines in most feeds, the escape of feed purine N is generally unlikely to affect predictions of microbial N flow from the rumen based on purine content of digesta. There may, however, be exceptions to this generalisation. One study demonstrated that up to 15% of the RNA entering the small intestine of young steers given a hay/concentrate diet (50/50) was of non-microbial origin [23]. Estimates of the percentage escape of dietary purines in sheep given a mixed diet of vetch-oat hay and concentrate (2:1) by the *in situ* method were 5-17% for lucerne hay, barley, gluten feed, sunflower meal and maize, 11-23% for meat meal and soybean meal, and 20-40% for fishmeal and brewery distillers grains, respectively [24].

5. DIGESTION AND ABSORPTION OF MICROBIAL PURINE COMPOUNDS

There is little change in the flow of nucleic acids in digesta between the rumen and duodenum [25]. Microbial nucleic acids entering the small intestine (of which about 60-70 % is RNA) are extensively degraded to mononucleotides [7], and enzymes capable of removing the phosphate groups from the mononucleotides to form the nucleosides are also present. It is clear that a variety of nucleases and related enzymes occur in the small intestine of ruminants and these degrade nucleic acids to nucleosides. McAllan [18] hypothesised that the rate of removal of the sugar to release the free base was the rate-limiting step for the complete degradation of purine compounds in the intestines.

Pancreatic secretions of ruminants are particularly high in ribonuclease (RNAase) which ensures that microbial nucleic acids are extensively degraded in the small intestine [26]. These enzymes catalyse the hydrolysis of phosphodiester bonds and release poly- or mono-nucleotides [27]. Two phosphodiesterases have been isolated from intestinal mucosa which are essentially exonucleases, the first attacking polyribonucleotides on one end of the nucleic acids and liberating the nucleoside-5-phosphates in a stepwise manner, and the second liberating nucleoside 3-phosphates from the other end. Intestinal 5'-nucleotidase and a non-specific alkaline phosphatase have also been found in ruminants. These nucleosides or their breakdown products, adenine, guanine and ribose are absorbed. Condon *et al.* [28] found that

adenine and guanine were completely absorbed from the small intestine whereas nucleosides were less completely absorbed. Mammalian tissues also have purine nucleoside phosphorylases that would allow nucleosides, if they are absorbed as such, to be further degraded within the body.

Net digestibility coefficients for nucleic acids in the small intestine of ruminants were 80-90% for RNA and 75-85% for DNA [18]. McAllan [27] reported that true digestibility for nucleic acids infused into the duodenum of steers was 97% and Chen and co-workers [10] reported a digestibility of 91% for an infused source of microbial purines. Adenine was also completely absorbed from the small intestine of lambs [28, 29]. A value of 0.83 for net digestibility is used in the example of calculations given previously. However, true digestibility is arguably more appropriate because purines flowing from the ileum are likely to be derived from endogenous sources and should therefore be considered as part of the non-renal excretion of purines.

6. TISSUE PURINE TURNOVER

6.1. Intracellular purine turnover

Studies of recovery in the urine of purine metabolites following the infusion of nucleic acid products into the rumen indicate that the majority of the absorbed purines are eventually excreted. However, Ellis and Bleichner [25] concluded that about 70 % of the nucleosides or bases absorbed from the gut are not immediately excreted in the urine. When ^{14}C-labelled RNA, adenine or guanine were infused into the abomasum, 10% of the radioactivity appeared in tissue nucleic acids [28].

Smith and co-workers [17] injected *E. coli* or mixed cultures of rumen bacteria, labelled their adenine and guanine by growing them on 8-^{14}C-adenine, and found that the label became incorporated into tissue nucleic acids. They found that only 3-5% of the radioactivity was excreted in faeces and only 15% in the urine in the first 2 days. They concluded that at least 5% of the purine compounds from the bacteria were incorporated into the liver, kidney and spleen and argued that muscle would contain a further 20%. Other workers [29] have also provided evidence for the incorporation of absorbed purines into tissues. They injected rumen bacteria labelled with ^{14}C-adenine into the rumen of lambs and found about 48% of the radioactivity was present in tissues 48 h later. Incorporation of ^{14}C-adenine by such reactions was also reported by Kahn and Nolan [30]. These studies indicate that some of the absorbed purines are usually incorporated into tissues rather than being immediately oxidised and it is clear that absorbed purines are not simply degraded in the body and then excreted via the kidneys. Rather they take part in synthetic and catabolic reactions that constitute the intracellular turnover of nucleic acid components in tissues. With this in mind, it is not surprising that, when purine compounds were infused into the small intestine of intragastrically maintained sheep, the responses in blood and urine purine concentrations were delayed for 2-3 hours [31]. These response lags may be due to biochemical regulation of tissue purine turnover reactions. Lags may also be due to physical sequestering of PD in tissues because intravenously administered ^{14}C-allantoin has been retained in tissue pools for extended periods [32].

Within cells, DNA is strongly conserved and has a slow turnover rate. In contrast, some types of RNA (e.g. messenger RNA) are more rapidly turned over. During catabolism, DNA and RNA are hydrolysed by nucleases and diesterases to oligonucleotides and then to mononucleotides and nucleosides. Nucleosides can be salvaged or further degraded by cleavage of the ribose moiety to yield the free bases. Catabolic pathways predominate in the

metabolism of purines in ruminants. However, adenine and guanine can be incorporated into purine nucleotides, catalyzed by phosphoribosyl transferases. The latter has been found in the liver of cattle [33]. Nucleoside phosphorylases that catalyse the formation of nucleosides from purine bases and ribose-1-phosphate also occur [33].

Purine nucleotides can be synthesized in three ways: by *de novo* synthesis, by reconstruction from purine bases by addition of ribose phosphate (*salvage*) or by phosphorylation of purine nucleosides to the corresponding nucleotides [34]. The *de novo* and *salvage* pathways are considered to be more important quantitatively in mammals and both involve phosphoribosylpyrophosphate (PRPP) as an essential precursor. The latter is formed from ribose-5-phosphate and ATP by the action of ribose-5-phosphate pyrophosphokinase. The first complete purine nucleotide, inosine monophosphate (IMP) is formed in a pathway starting from PRPP during which formate, glutamine, glycine and aspartate are involved. IMP does not accumulate in the cell but is converted to AMP and GMP, and then to ADP and ATP and GDP and GTP, respectively. PRPP is also involved in the salvage pathway by which mononucleotides are formed.

Purine base + PRPP → ribonucleoside-5'-phosphate + PP_i

The PP_i released is rapidly hydrolysed to inorganic phosphate in a coupled reaction catalyzed by inorganic pyrophosphate so that the formation of ribonucleosides by the salvage pathway is irreversible.

Nucleotides are catabolised within cells by several types of intracellular nucleotidases that are under strict regulatory control. Another type of nucleotidase is attached to the outer surface of the outer membrane of many types of cells and dephosphorylates purine ribonucleoside monophosphates to the corresponding nucleosides that are then transported into the cell on specific transporters.

The general pathways for degradation of purine compounds are well known and uric acid and allantoin are the principal end-products in most ruminant species. The purine nucleosides, inosine and guanosine are readily cleaved by purine nucleoside phosphorylase which is found in many tissues. Inosine is phosphorolyzed to yield hypoxanthine and guanosine to guanine. Hypoxanthine and guanine are then converted to xanthine by xanthine oxidase and amino hydrolysase, respectively. Xanthine is then oxidised to uric acid, again by the action of xanthine oxidase. Finally, uric acid is oxidised by the liver enzyme, urate oxidase or uricase, which is a copper protein, to allantoin. Even though it is considered to be a non-salvageable end-product, allantoin is not quantitatively recovered in the urine after being injected intravenously in sheep [31].

6.2. Metabolism of absorbed purines

The intestinal mucosa is the first possible site for the degradation of absorbed purines. The mucosal cells of buffaloes and cattle are rich in xanthine oxidase (EC 1.2.3.2) which increases the potential for oxidation of absorbed purines before they enter the bloodstream, and this reduces the potential for salvage. Sheep mucosa in contrast has only trace amounts of this enzyme [35] so that a higher blood concentrations of xanthine and hypoxanthine can be expected in sheep than in cattle or buffaloes. Purines entering the blood are again subject to oxidation by xanthine oxidase and uricase. Xanthine oxidase is present in buffalo and cattle plasma but not in sheep plasma [36], whereas uricase is not present in cattle blood but is present in sheep blood, although with relatively low activity [37]. In sheep, the liver has quite high activities of both enzymes, and this is the principal site of purine catabolism in this species.

7. RENAL AND NON-RENAL REMOVAL OF PURINE COMPOUNDS FROM THE BLOOD

If purine absorption, and eventually microbial purine outflow from the rumen, are to be predicted from PD excretion then, ideally, the absorbed purines should be excreted quantitatively via the kidney. However, the results of various studies indicate that purines administered into the gut or into the bloodstream, though often variable, are not quantitatively recovered in the urine. There are several reasons why urinary recovery may be incomplete.

- Some PD may be salvaged, resulting in increases in storage of purines in tissues.
- Some PD may be secreted via saliva into the gut.
- Some PD may enter the gut by non-salivary routes.
- Some purines may be excreted in milk.

Chen and co-workers [38] analyzed saliva of sheep by a colorimetric method and, assuming a daily secretion of saliva of 10 L/d, estimated that the secretion of PD in saliva was about 10% of the daily excretion in urine. Surra and others [39] on the other hand, analyzed sheep saliva by a reverse-phase HPLC method that was probably less prone to non-specific reactions and found allantoin and total PD in much lower concentrations. These workers later concluded that salivary excretion of PD accounted for only 0.3% of urinary excretion [40]. When Kahn and Nolan [30] injected ^{14}C-allantoin intravenously into sheep only negligible amounts of radioactivity were found in saliva, also indicating that allantoin excretion via this route was low. The non-recovery of absorbed purines is not as yet fully explained. However, transfer of purines into the gut does occur by several routes. Xanthine, hypoxanthine and uric acid are transported through intestinal tissues of hamsters [41] and uric acid is transferred into the gut of humans [42]. Allantoin has been found in the bile of the dog [43] and non-renal loss of purines was closely related to plasma purine pool size [44], indicating that the non-renal excretion is a concentration-dependent process. Various workers have investigated the excretion of PD in milk [45]. Excretion is generally less than 5% of the urinary excretion and does not appear to be reliably related to urinary excretion [46].

The fractional loss of PD via the kidneys suggested in the example for sheep given above (0.81) may not be applicable under all conditions. Work with tropical cattle and buffaloes [36, 47] suggests that lower values may be applicable to these species. The aspects discussed above are shown in Figure 2.

8. RELATIONSHIPS BETWEEN PURINE ABSORPTION AND EXCRETION IN URINE

If relationships between purine absorption and urinary purine excretion are to be used for prediction purposes, any variability occurring under different conditions also needs to be predictable. These relationships are curvi-linear [35, 48], probably reflecting the degree of biochemical feedback on *de novo* synthesis which is a source of endogenous purines and the balance between the utilisation of absorbed purines by tissues, and the degree of salvage of PD released within tissue cells. These factors are likely to be most affected when absorption of purines is relatively low. The distribution in gut, liver and other tissues of enzymes such as xanthine oxidase that are responsible for degrading potentially salvagable purines will also be a major factor, diverting purines produced from tissue nucleotide turnover into the oxidation pathway.

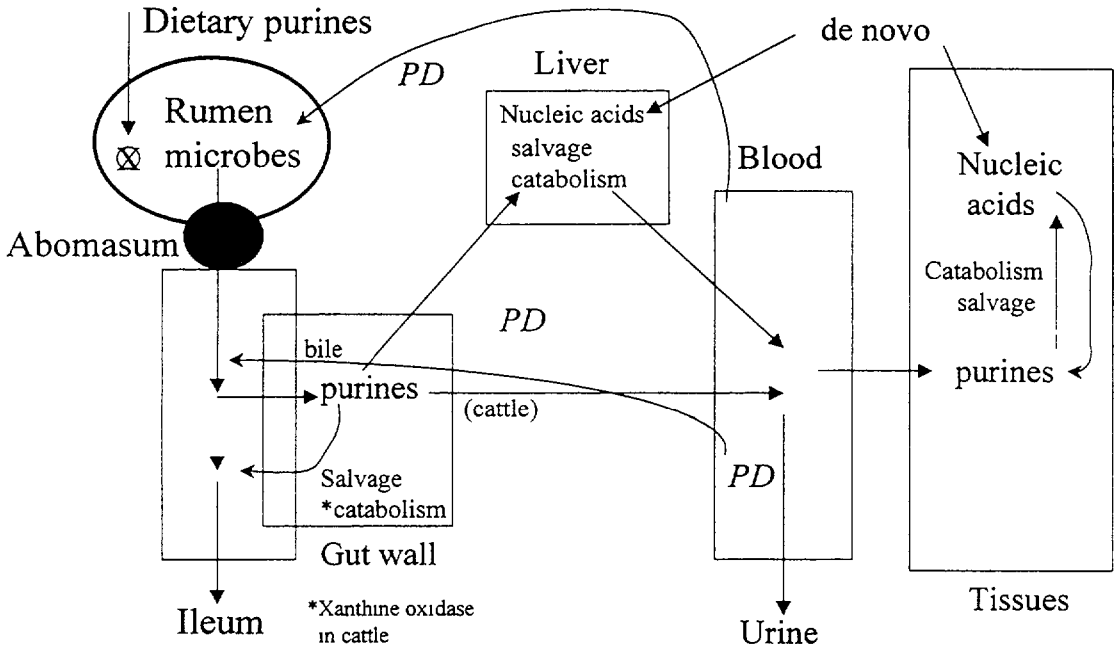

FIG.2. A representation of the fluxes of purine compounds from the rumen into the intestines and turnover of absorbed purines and excretion of oxidation end-products in ruminants.

Estimates of the extent of endogenous release of purines have been made using the technique of maintaining animals by intragastric infusion of nutrients, or by replacing normal digesta entering the intestines with digesta devoid of purines. The endogenous PD excretion is higher in cattle, per unit of metabolic weight, than in buffaloes and sheep, and is not inhibited as purine absorption increases. Sheep, goats, pigs and humans are similar. A consequence of these factors is that endogenous secretion of purines is always present in cattle, and the relationship between purine absorption and urinary PD excretion is more nearly linear [11, 34]. A further consequence is that different prediction equations will be required for different species of livestock [15]. Pertinent information has been collected by various groups in recent years, and some of the results are published in this IAEA TECDOC.

9. COMPARISON OF METHODS OF ESTIMATING MICROBIAL OUTFLOW FROM THE RUMEN

A comparison was made of estimates of rumen microbial N outflow from the rumen of duodenally cannulated sheep given a daily ration of 550 g lucerne hay, or the same ration plus either 220, 400 or 550 g rolled barley [49]. Estimates were made from measurements of digesta flow into the duodenum coupled with ^{15}N or purines as markers specific for the microbial fraction, or by using the method based on urinary PD excretion. Appreciable differences were found between the methods used, with predictions based on the urinary PD being 18-29% lower than values based on digesta flow. Currently, there is no method available that can be guaranteed to give a true measure of microbial flow from the rumen. However, the differences between the results in this comparison probably indicate that some of the assumptions used in the PD excretion method were incorrect in the conditions of these experiments. An alternative method of standardization of the PD technique is to compare its predictions with estimates of microbial outflow from the rumen based on feeding standards models that summarize results of many different studies using a variety of methods. An elegant demonstration of the potential of this kind of validation was presented by Susmel [14].

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QUANTIFICATION AND CHEMICAL COMPOSITION OF MIXED BACTERIA HARVESTED FROM SOLID FRACTIONS OF RUMEN DIGESTA: EFFECTS OF DETACHMENT PROCEDURE

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Abstract

QUANTIFICATION AND CHEMICAL COMPOSITION OF MIXED BACTERIA HARVESTED FROM SOLID FRACTIONS OF RUMEN DIGESTA: EFFECTS OF DETACHMENT PROCEDURE.

Four ewes were given two diets made up with two ratios of rolled barley grain and ammonia treated straw (80/20, diet C and 20/80, diet R). The animals were fed twice a day. Two microbial markers, purine bases (PB) and ^{15}N, were used as internal and external markers, respectively. Ruminal bacteria from the liquid (LAB) and solid (SAB) fractions of digesta were harvested from samples obtained at 1 and 6 h after feeding. Bacteria were separated from the particulate material by cooling plus homogenisation (B), by applying tertiary butanol (TB) or methylcellulose (M), or by changes of temperature (CHT). The most effective procedures to remove bacteria from the solid phase were M and CHT treatments. CHT, however, showed the highest level of losses and the lowest total recovery of the bacterial pellet. There were no differences between B and TB treatments in either detaching efficiency or total recovery ratio of adherent bacteria. Ratio of recovery of detached material as a bacterial pellet was 32.0, 32.2, 33.3 and 27.8% for B, TB, M and CHT treatments, respectively. Diet did not interact with detaching efficiency of the experimental treatments although concentration of total N (g/100g OM) and PB (μmol/g OM) were higher in pellets obtained with diet C (9.11 and 125) than with diet R (8.20 and 107), respectively ($P < 0.05$). Postpandrial differences were not significant. Bacterial samples extracted from the liquid phase contained significantly ($P < 0.001$) more total N (9.21 vs 8.51), PB (160.5 vs 104.3) and PB/N (1.73 vs 1.23 μmol/mg) than those samples extracted from the solid phase. There were no differences in the chemical composition of the microbial sample after detachment by B, TB and M treatments but the bacterial extract obtained after CHT treatment showed significant changes in PB, N content and PB/N ratio ($P < 0.01$).

1. INTRODUCTION

The N-feed evaluation systems for ruminants are largely dependent on estimates of rumen microbial protein synthesis and its contribution to the chyme. Rumen microbial production is usually estimated by using either naturally occurring microbial markers (diaminopimelic acid or nucleic acids) or isotopes (^{15}N or ^{35}S) incorporated during microbial growth. The harvested bacteria must be representative of the ruminal population and the marker:non-ammonia-N (NAN) ratio must be constant if these methods are to provide valid data. Current methods frequently measure liquid-associated bacteria (LAB) and assume that this easily-measured fraction is representative of all the microbes leaving the rumen.

Available information shows that solid-associated bacteria (SAB) are predominant in the rumen contents [1, 2] and probably in duodenal digesta [3]. However, if bacterial species [4] and their chemical composition [5] are largely different between SAB and LAB, the marker ratios of SAB must be taken into account.

Different procedures have been described recently to detach pure SAB-extract from the solid material in digesta. Physicochemical and mechanical treatments have been described [4-6], but the recovery of the microbial population after detachment has not been reported, which raises doubts about whether the purified microbial material is representative of the ruminal population.

The aim of this study was to investigate the efficiency of removal of SAB from rumen particles using several detachment procedures and to determine the total microbial recovery and its chemical composition. A further objective was to analyse changes in these parameters in relation to diet and time after feeding.

2. MATERIALS AND METHODS

2.1. Animals and diets

Four 3-year old non-lactating Manchega ewes (average body weight 45 ± 0.3 kg) fitted with a permanent rumen cannula (10 cm, i.d.) fed on two different rations (80:20, diet R and 20:80, diet C) of 3% ammonia-treated barley straw and barley grain supplemented with 25 g/kg of casein were used in this experiment. The chemical composition of feeds is given in Table I. Feeds were given daily in two equal meals at 9 and 17 h, restricted to 8 MJ of metabolizable energy intake per day (1 kg fresh matter/day for diet R, and 0.75 kg fresh matter/day for diet C) to ensure a rapid intake and to avoid feed refusals. Each diet was offered in one experimental period to the four animals. Each experimental period consisted of 4 weeks of adaptation followed by one week of measurements.

TABLE I. CHEMICAL COMPOSITION OF FEEDS

	Straw	Barley
Dry matter (g/kg)	900	895
Organic matter (g/kg)	945	977
Crude protein (g/kg)	101	121
Neutral detergent fibre (g/kg)	752	225
Acid detergent fibre (g/kg)	405	47
Metabolizable energy (MJ/kg)	7.9	13.2
Purine bases (μmol/g DM)	2.98	7.91
^{15}N (% of total N)	0.3695	0.3718

DM basis

^{15}N-Ammonium sulphate (10 + at. % ^{15}N, Isotec, OH, USA) was used as a microbial marker (40 mg ^{15}N/animal/d) diluted in distilled water and infused directly through the rumen cannula by means of a peristaltic pump at a flow rate of 0.4 ml/min for 9 days.

2.2. Sample collection and isolation of microbial population

Samples were obtained on days 5, 7 and 9 of the isotope infusion. Rumen contents, mostly liquid (600 ml), was sampled 1 and 6 h after the morning feeding from the ventral sac by means of a manual vacuum pump. An additional 200 g of solid digesta were taken from the

upper mat and added to the sample. Composite samples from the four animals obtained at each time and collection day were pooled, and fluid and particulate material were obtained as follows (Figure 1). The whole sample was squeezed through four layers of surgical gauze and a 50 μm nylon filter to trap particulate matter. Solid residue obtained was re-suspended in two times its weight of saline solution (0.85%, 39°C). After shaking, the suspension was filtered again to obtain the solid phase (Residue 1). The washing solution was added to the strained rumen fluid, constituting the liquid phase, (Filtrate 2) and liquid-associated bacteria (LAB) were isolated from 600 ml of this rumen fluid by differential centrifugation (500 × *g* for 5 min followed by two consecutive centrifugations of the supernatant fractions at 20 000 × *g* for 20 min at 4°C).

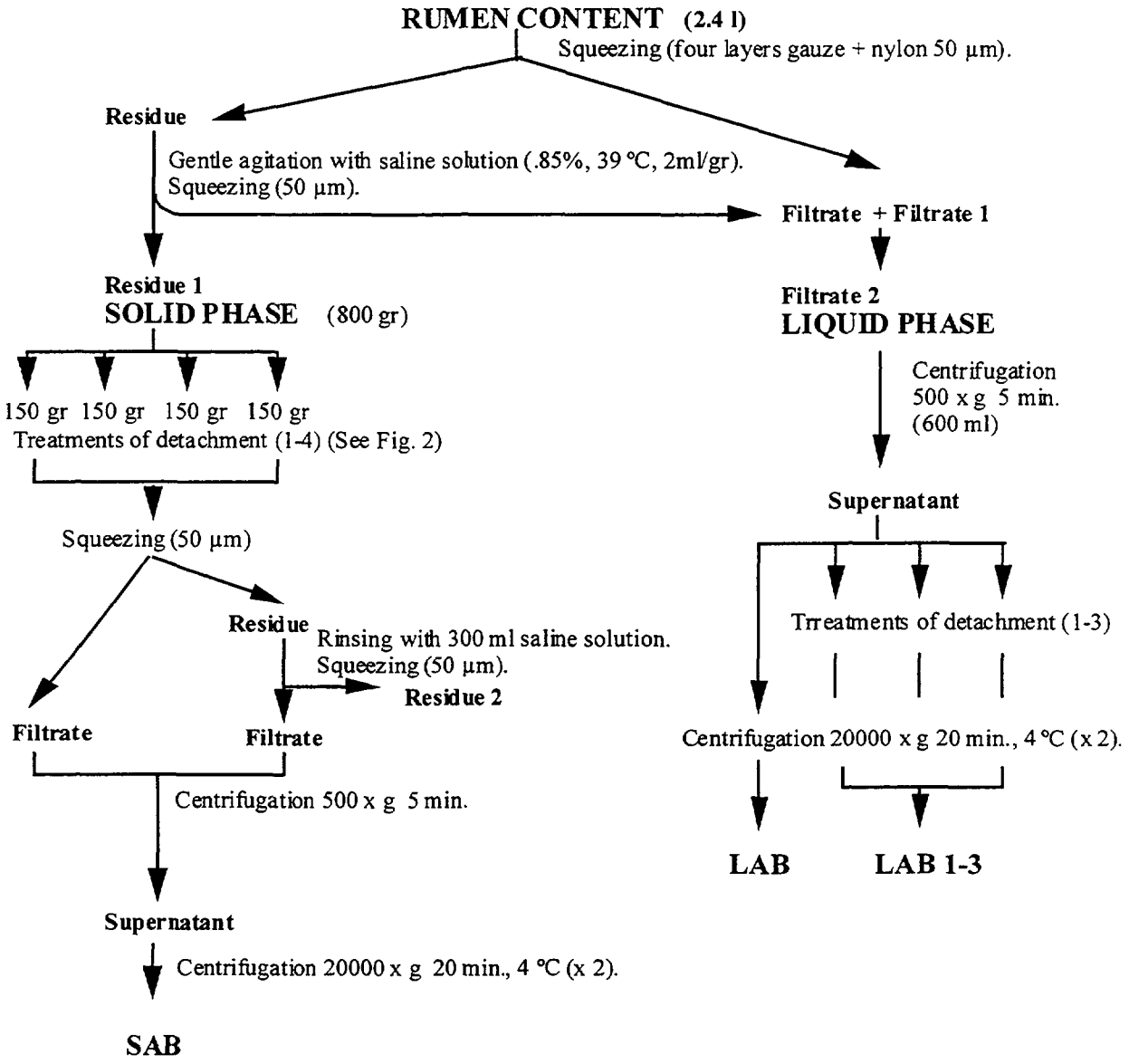

FIG.1. Procedure to isolate solid associated bacteria (SAB) and liquid associated bacteria (LAB) from rumen contents.

To obtain the SAB, solid material was fractionated into four sub-samples (150 g) and the following detachment procedures were used (Figure 2).

(a) Basal treatment (B): solid sample was diluted in 0.85% of saline solution (450 ml, 4°C) kept at 4°C for 24 h and homogenised six times for 30 s with a Waring Blender, based on the method of Dehority and Grubb [6]. Cooling and homogenisation was considered as a basal treatment and included in all the other treatments.

(b) Tertiary butanol (TB): basal treatment but including 1% of tertiary butanol in the saline solution, based on the method of Whitehouse *et al.* [7].

(c) Methylcellulose (M): solid material was diluted in saline solution plus 0.1% of methylcellulose at 39°C, shaking by hand for 5 min before B was applied (based on Minato and Suto [8]

(d) Changing temperatures (CHT): after treatment B, the homogenised suspension was heated in a 60°C water bath for 10 min, shaken vigorously and then cooled in an ice bath for 10 min. This procedure was repeated twice Temperature inside the bottle never rose above 37°C.

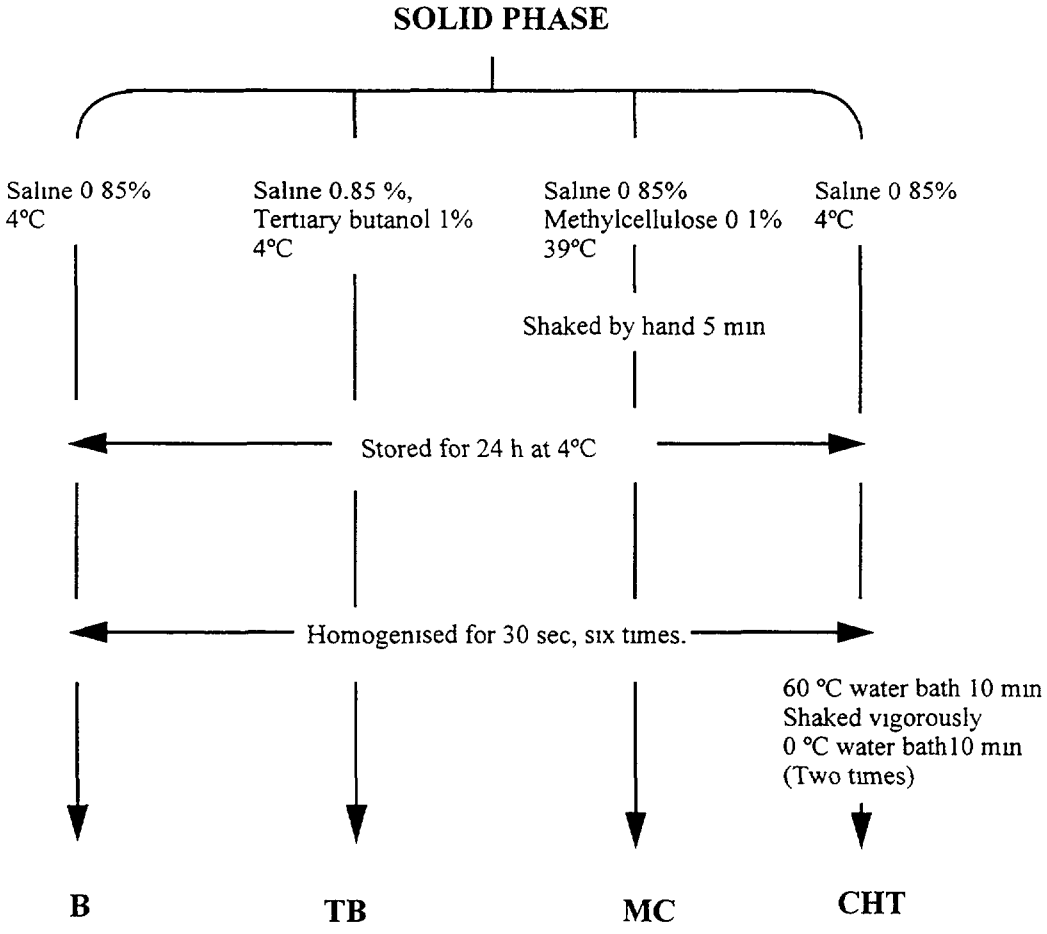

FIG 2 Detaching treatments used in removing solid associated bacteria from the solid phase

After each treatment, the samples were strained through four layers of surgical gauze plus 50 µm nylon filter. Particulate material was resuspended in saline solution (300 ml), mixed, shaken and strained again, and the washing liquid added to the previous filtrate. The volume of total filtrate and the weight of the particulate residues (Residue 2 in Figure 1) were

recorded. The adherent population (SAB) was obtained from the filtrates by differential centrifugation as described for LAB. Microbial pellets and particulate residues (Residues 1 and 2) were freeze-dried and their dry matter determined.

To determine whether the physicochemical or mechanical treatments had any effect on bacterial composition, different LAB extracts were obtained after applying each of these extracting treatments to 600 ml of centrifuged (500 × *g* for 5 min) liquid phase without diluting with saline solution. The methylcellulose treatment was not checked because it was impossible to dissolve in rumen liquid without modifying the treatment.

The last day of the collection period rumen fermentation was characterised. The pH from the rumen fluid was recorded immediately before the morning feed and 4 h later. Simultaneously, two subsamples were taken, acidified with HCl (25 ml rumen fluid/25 ml 0.2 M HCl) or H_3P0_4 (4 ml rumen fluid/1 ml 0.5 M H_3P0_4, 50 mM-3 methylvalerate) and stored at -20°C until analysis for NH_3-N and volatile fatty acids (VFA), respectively.

2.3. Analytical procedures and calculations

Dry matter (DM), organic matter (OM), Kjeldahl-N from Residue 1 and 2 (Figure 1) and the different microbial extracts were measured using AOAC [9] procedures. Adenine and guanine (purine bases, PB) were determined by HPLC according to Balcells *et al.* [10] with the modification of Martin Orue *et al.* [11]. Prior to ^{15}N analysis, ammonia-N from residues samples was eliminated drying the samples at pH>10 [2]. The isotope enrichment of ^{15}N was determined by mass spectrophotometry (VG PRISM 11, IRMS hooked in series to a DUMAS-style N analyser EA 1108). The VFA concentrations in deproteinised rumen fluid were determined by GLC, following the method proposed by Jouany [12] and ammonia concentration was determined by Kjeldhal procedure after direct distillation with sodium tetraborate.

The proportion of detached microorganisms was calculated as the loss of purine bases (μmoles) or atoms in excess of ^{15}N in the particulate residue after detachment.

The percentage detachment = {1-([DM Residue 2 × % marker in Residue 2]/[DM Residue 1 × % marker in Residue 1])} × 100.

The percentage recovery from detached micro-organism = ([DM in microbial pellet × % marker in microbial pellet])/([DM Residue 1 × % marker in Residue 1]-[DM Residue 2 × % marker in Residue 2]) × 100.

The total recovery = ([% Recovery from detached] × [% of Detachment])/100.

The effectiveness of the different procedures for removing bacteria was determined assuming that all the purine bases in the solid digesta were of bacterial origin. When ^{15}N was used as microbial marker, it was also assumed that the isotope enrichment in the original material did not change throughout rumen fermentation or changes were negligible in relation to microbial enrichment [13].

2.4. Statistical analyses

The experimental design was a complete block design in a split-plot arrangement of treatments with three repeated measurements (days). Since animals were adult, non-lactating, non-pregnant and feed-restricted and maintained in a controlled environment, the period effect was assumed to be negligible. Diet and times post-feeding were the whole plot treatment and were tested against their interaction as the error term. The detachment treatment was the sub-plot treatment and was tested against the residual. The sums of squares were further partitioned by orthogonal contrasts to analyse differences in detachment method. The contrast were distributed as follows: C1, basal (B) vs the others; C2, chemical (TB, M) vs physical

treatments (CHT); C3, between chemicals TB vs M. To check the effect of detachment treatment on cellular integrity, LAB was included in the analysis and contrasted against all the different SAB obtained. All analysis were performed using SAS [14] statistical package.

3. RESULTS

3.1. Rumen fermentation variables

The rumen pH values were similar between diets before feed administration but the decrease post-feeding was more pronounced with diet C (6.7 vs 5.3 for diet R and diet C, respectively, P <0.05). Ammonia concentration was higher in diet C than in diet R (21.1 vs 15.4 mg N/100 ml) and increased for both diets due to time post-feeding (11.95 vs 24.58 mg N/100 ml at 0 and 4 h, respectively). Diet did not affect total VFA concentration in the rumen liquor but the concentration was higher (P <0.01) after 4 h than before feeding. This effect was more pronounced with C (interaction of diet x sampling time P <0.01). Concentration of acetate was lower in diet C (P <0.01) and those of propionate and butyrate apparently higher than in diet R.

3.2. Efficiency of detachment treatments for SAB removal and recovery of detached microbes

Since rumen environment changes induced in this study by diet or time after feeding did not modify either removal efficiency or marker recovery, only average values for each detachment treatment and microbial marker are presented in Table II.

TABLE II. PERCENTAGES OF DETACHMENT, RECOVERY OF DETACHED AND TOTAL RECOVERY OF MICROBIAL PURINES AND ^{15}N FROM RUMEN PARTICLES AFTER APPLYING DIFFERENT DETACHING TREATMENTS (DETACHING TREATMENTS AND CALCULATION ARE DESCRIBED IN THE TEXT).

Detachment	Detachment		Recovery of detached		Total recovery	
treatments	Purines	^{15}N	Purines	^{15}N	Purines	^{15}N
B	60.7	60.3	31.8	32.1	18.8	19.4
TB	59.5	59.3	31.8	32.5	18.7	19.2
M	66.2	66.3	33.0	33.5	21.3	22.0
CHT	71.1	68.3	25.1	30.5	17.3	20.9
RSD	7.20	10.62	10.63	2.12	5.39	0.69
C1	*	NS	NS	NS	NS	NS
C2	**	NS	NS	NS	NS	NS
C3	*	NS	NS	NS	NS	**

B, basal treatment; TB, tertiary butanol; M, methyl cellulose; CHT, changes of temperature
C1, C2, C3, orthogonal contrasts: C1:B vs TB, M and CHT; C2:TB and M vs CHT; C3:TB vs M
RSD, Residual Standard Deviation.
NS, not significant; *, P <0.05; **, P <0.01

Purine bases were removed from the solid digesta at 60.7, 59.5, 66.2 and 71.1% (RSD 7.20) whereas the isotope was at 60.3, 59.3, 66.3 and 68.3% (RSD 10.62) using B, TB, M and CHT, respectively. No significant differences between estimations derived from both markers were detected.

Orthogonal contrast analysis did not show significant differences in SAB removal between treatments when ^{15}N data were considered. However, in terms of purine bases, the treatments TB, M and CHT further improved the SAB-removal ($P < 0.05$) obtained by cooling plus homogenisation (B). Changes of temperature (CHT) showed a higher level of SAB-removal than TB plus M ($P < 0.01$) and M higher than TB ($P < 0.05$). No marked differences were detected in the recovery of detached markers.

Total recovery ranged from 17 to 22% and was modified significantly by the detachment treatment in ^{15}N terms ($P < 0.05$). CHT promoted the highest level of marker dislodgement (71.1 and 68.3% for PB and ^{15}N, respectively) but also the lowest level of recovery of detached material (25.1 and 30.5 for PB and ^{15}N, respectively). Hence total recovery obtained with this treatment was similar (^{15}N and PB) to B or TB. The effect of M on total recovery, using ^{15}N as microbial marker, was reflected in the significance of differences among B vs TB, M and CHT ($P < 0.05$) and M vs TB ($P < 0.01$). Methylcellulose treatment improved total recovery of SAB, although its effect did not reach statistical significance in PB terms.

3.3. Chemical composition of microbial extracts

Table III presents the amounts of the different chemical constituents (OM, g/100g DM; N, g/100g OM; PB, μmol/g OM; PB/N, μmol/mg; ^{15}N, % of total N) found in LAB and SAB extracts. No differences were detected between SAB and LAB (76.0 vs 74.0), although microbial population isolated in animals fed concentrated diets apparently had a higher level of OM than animals eating roughage (79.2 vs 72.0).

Total N concentrations (8.51 vs 9.21), PB (104 vs 161), PB/N (1.23 vs 1.73) were lower in SAB than LAB extracts ($P < 0.001$). Total N and PB content were lower in animals fed R than C diets (8.20 vs 9.11 and 107 vs 125; $P < 0.05$), although values for PB/N were not affected by rumen environment. Nitrogen content in the bacterial extract tended to increase with time after feeding but no change was detected in PB content or PB/N ratio. Isotope enrichment of the microbial extract was significantly higher in LAB than in SAB (0.6299 vs 0.5834; $P < 0.05$) although differences between diets did not reach statistical significance. (0.6324 vs 0.5530).

No marked differences were observed in the chemical composition among bacteria extracted by different detachment treatments. Although treatment of solid digesta with changes of temperature (CHT) resulted in a bacterial pellet that showed a higher content of N (CHT vs. M and TB; $P < 0.01$), the effect was more pronounced in bacteria extracted in diet C (interaction of treatment × diet, $P < 0.05$) and PB content and PB/N-ratio were significantly lower in this treatment (CHT vs. M and TB; $P < 0.01$).

In order to determine whether the physico-chemical or mechanical treatments applied had any effect on bacterial composition, comparisons were made between bacteria before and after treatment. LAB was used for this purpose and bacteria from the centrifuged liquid phase were harvested before and after treatment. Comparisons are presented in Table IV. Only the treatment CHT seemed to have an adverse effect on PB and PB/N ratio, with lower values than in untreated bacteria (150 and 1.56 vs 172 and 1.79, respectively).

TABLE III. CHEMICAL COMPOSITION OF THE DIFFERENT BACTERIAL EXTRACTS OBTAINED FROM 4 EWES, 1 AND 6 HOURS AFTER FEEDING TWO DIETS (LOW (R) AND HIGH (C) % OF CONCENTRATE), FROM THE RUMEN FLUID (LAB) OR FROM THE SOLID DIGESTA (SAB) AFTER APPLYING DIFFERENT DETACHING TREATMENTS (DIETS AND DETACHING PROCEDURES ARE DESCRIBED IN THE TEXT).

		Detaching treatments	Diet R 1 h	Diet R 6 h	Diet C 1 h	Diet C 6 h	E 1	E 2	D	T	C1	C2	C3	C4
OM	LAB		69.2	65.7	80.0	80.9	3.87	5.00	NS	NS	NS	NS	NS	NS
(g100 g DM)	SAB	B	75.3	67.7	80.8	75.7								
		TB	75.0	69.9	80.2	79.2								
		M	74.2	73.2	81.7	77.0								
		CHT	76.2	74.0	77.8	78.5								
N	LAB		8.59	9.17	9.17	9.90	0.158	0.237	*	NS	***	NS	**	NS
(g/100 g OM)	SAB	B	7.89	8.14	8.69	9.01								
		TB	7.82	8.18	8.82	8.97								
		M	7.88	8.19	8.93	8.89								
		CHT	7.94	8.17	9.25	9.41								
PB	LAB		133	145	166	198	10.6	23.2	*	NS	***	NS	**	NS
(μmol/g OM)	SAB	B	107	111	108	120								
		TB	105	111	109	118								
		M	94	103	115	114								
		CHT	78	79	94	103								
PB/N	LAB		1.55	1.59	1.80	1.99	0.126	0.240	NS	NS	***	NS	**	NS
(mmol/g)	SAB	B	1.36	1.36	1.23	1.33								
		TB	1.34	1.36	1.23	1.31								
		M	1.20	1.26	1.29	1.28								
		CHT	0.98	0.96	1.02	1.10								
^{15}N	LAB		0.653	0.645	0.584	0.638	0.0349	0.009	NS	NS	***	NS	NS	NS
(% of tot. N)	SAB	B	0.644	0.625	0.537	0.544								
		TB	0.646	0.628	0.538	0.547								
		M	0.638	0.620	0.533	0.542								
		CHT	0.617	0.608	0.532	0.535								

B, basal treatment; TB, tertiary butanol; M, methyl cellulose; CHT, changes of temperature; E1, Residual Standard Deviation of Diet x Time as error term; E2, Residual Standard Deviation of Residual error term; D, T, statistical significance of Diet and Time after feeding effect; C1, C2, C3, orthogonal contrasts: C1:LAB vs SAB; C2:B vs TB, M and CHT; C3:TB and M vs CHT; C4:TB vs M; NS, not significant; *, $P < 0.05$; **, $P < 0.01$

TABLE IV. CHEMICAL COMPOSITION OF THE BACTERIAL FRACTION OBTAINED FROM THE RUMEN LIQUID FROM 4 EWES FED TWO DIFFERENT DIETS (LOW (R) AND HIGH (C) % OF CONCENTRATE), WITH AND WITHOUT APPLYING DIFFERENT DETACHING TREATMENTS (DETACHING TREATMENTS ARE DESCRIBED IN THE TEXT).

	Treatment	Diet R	Diet C	RSD	Diet	Treatment
N	Without treatment	9.17	9.90	0.540	**	NS
(g/100g OM)	B	9.24	9.94			
	TB	9.16	9.99			
	CHT	9.12	9.93			
BP	Without treatment	145	198	23.68	***	NS
(µmol/g OM)	B	147	201			
	TB	141	190			
	CHT	122	178			
BP/N W	Without treatment	1.59	1.99	0.168	***	NS
(µmol/mg)	B	1.58	2.01			
	TB	1.54	1.89			
	CHT	1.34	1.78			

B, basal treatment; TB, tertiary butanol; M, methyl cellulose; CHT, changes of temperature
RSD, Residual Standard Deviation.
Diet, Treatment: Statistical significance of effect diet and effect treatment
NS, not significant; *, P <0.05; **, P <0.01

4. DISCUSSION

Microorganisms have many associations with solid material [15, 16] so, an ideal protocol for dissociating the entire adherent fraction probably requires a combination of several methods to detach the microbes without substantially modifying microbial integrity. In the present work, the following treatments (or combinations of them) were studied: cooling, vigorous agitation (homogenisation), abrupt changes of temperature and addition of either methylcellulose or tertiary butanol. In order to evaluate the detachment efficiency of the different physical and chemical methods, we considered one of them as a reference. Cooling and homogenisation was used as the reference method that was applied to all the samples in order to check the efficiency and the additive effects of the additional treatments.

Homogenisation and cooling have been shown to be a useful procedure to increase removal of SAB from ruminal digesta by disrupting adherence or releasing the trapped microorganisms [5, 17]. Dehority and Grubb [6] demonstrated that colony count increased significantly as ruminal content was cooled from 0 to 8 h. They concluded that chilling may alter cell-to-cell and cell-to-food particle attachments. Estimates of the removal of SAB, made using purine bases or ^{15}N, averaged 60%, a value similar to the 65% obtained by Merry and McAllan [5]. However, it was higher than the less-than-50% obtained by Legay-Carmier and Bauchart [18] and Martin *et al.* [19]. Notably in all these cases DAP was used as a bacterial marker, and since DAP is associated with the bacterial cell-wall, it is possible that some DAP remained on feed particles if some cell lysis occurred during the detachment procedure.

Alcohols have been used as detaching agents because of their ability to remove the bacterial polysaccharide capsule [20]. Fletcher [21] reported decreases in bacterial attachment as concentration of butanol increased from 0.2 to 2%, results that were recently confirmed by Whitehouse *et al.* [7], who found that 1% tertiary butanol added to pH 2, Tween 80 and methanol treatments increased removal of SAB from 61 to 66% using purine bases as a microbial marker. However, the addition of 1.0% tertiary butanol to the basal treatment (cooling plus homogenisation), in the present experiment, did not have any significant effect on either PB or isotope removal. The use of alcohols as detaching agents is dependent on their reaction with the lipid structure of the cellular membrane, breaking the non-covalent bonds in the hydrophobic regions of lipids [22]. It may cause cell lysis in both attached and free-floating bacteria and hence losses of cellular contents. If this effect is significant, then the recovery of purine bases in the deposit after centrifugation would be much lower than the values obtained with the basal treatment. Total recovery of purine bases was low (18.7%), but not different than values obtained by simply cooling and homogenising (18.8% for PB). On the other hand, if the losses of cellular content are consistent, then the chemical composition of the bacterial pellet would be altered because of the disproportionality between the cell wall and cytoplasmic content. This effect was not detected in the SAB extracts obtained with TB compared with B, or when both treatments were applied over rumen liquid (LAB).

Methylcellulose inhibits adhesion and causes detachment of adherent bacteria [23, 24]. However it is not known whether this is due to binding inhibition, competitive binding [25] or surfactant qualities [16]. Minato and Suto [8, 26] reported that bacteria attached to cellulose powder at 38°C were eluted with an aqueous (0.15%) solution of methylcellulose. Whitehouse *et al.* [7] reported an increase in bacterial removal from 62 to 83%, using purine bases as the microbial marker, when methylcellulose (0.1%) was added to Tween 80 and methanol. Even though the detachment level obtained in the present experiment was lower than that reported by Whitehouse *et al.* [7], methylcellulose increased SAB-removal over cooling plus homogenisation from 60 to 66%, as determined by both purine bases and ^{15}N, supporting the ability of the methods to detach bacteria described in previous reports. Methylcellulose allows a high SAB-removal without apparently altering cell integrity (compared to cooling plus homogenisation) as shown by the similar relationship of PB/N in the microbial pellet (Table III) to that obtained after the basal treatment. Therefore, the total recovery of SAB in the bacterial pellet after treatment with methylcellulose reached the highest value (21.3 and 22.0% in terms of PB and ^{15}N).

Minato and Suto [26] suggested that adhesion can be decreased significantly by either lowering the temperature to 4°C or by raising it above 38°C [16]. The mechanisms of this response have not yet been established and no experimental data about this procedure are available. Change of temperature was included as an experimental treatment and it has been shown to increase removal of SAB to the same extent as methylcellulose. However, whereas treatment M did not apparently have any additional effect on cell integrity compared with treatment B, changes of temperature showed the lowest level of recovery of detached purine bases. The fact that differences were more pronounced with PB as an intracytoplasmatic marker, suggests a possible adverse effect on cell integrity. In addition, microbial pellet from CHT treatment showed a significantly low PB/N ratio probably reflecting the different compositions of the cell wall and cytoplasmic contents. These results indicate that the use of CHT treatment with the aim of obtaining a representative pellet of bacteria associated to the solid phase of the rumen content can not be recommended.

In this study, diet or time after feeding did not alter the dislodgement effect of the experimental treatments. However, diet modified the chemical composition of the bacterial extract. Total N content (g/100g OM) of bacteria associated with both phases of rumen contents from sheep given diet C (9.11) was higher than with diet R (8.20). The effect of diet

on N content is open to discussion. McAllan and Smith [27] reported that in mixed rumen bacteria, the N content declined while carbohydrate content increased as the concentrate content of the diet was increased. In other studies, however, diet did not affect the N content of bacteria isolated from the liquid [28] or the solid phase [29]. Martin *et al.* [19] reported changes only in N content of SAB. Probably it is difficult to correlate directly the N content and the concentrate level [30] because these effects would respond to several factors such as species differences (G + vs G -) [28] microbial growth rate [31] and effects of diet [27].

In relation to the effect of time after feeding on total N content, other authors have reported that microbes (mainly from the liquid phase) can store polysaccharides rapidly after feeding decreasing their protein content just after feeding [32]. We observed this trend in N concentration of the bacterial extracts, which was lower at 1 h (8.50 g/100g OM) than at 6 h (8.80 g/100g OM) after feeding.

Nucleic acid/N ratio has been reported as an index of RNA activity and bacterial growth and RNA/N has been reported to increase during growth of pure cultures of ruminal [33] and non-ruminal bacteria [34]. Susmel *et al.* [35] also demonstrated such variation in RNA content in LAB with dry cows given a concentrate diet. However, this effect was not confirmed by Arambel *et al.* [28] who found a decrease in RNA concentration in the microbial extract with increasing levels of concentrate given to cattle. Our findings show an increase in purine bases with diet C, particularly in LAB, which was higher than the increase in N content, resulting in an increase in the ratio PB/N. On the other hand our results suggest a trend (though not-significant) to increase PB content (μmol/g OM) in SAB across time, from 1 h (101) to 6 h (107) after feeding.

Among bacterial samples extracted from the different phases, our findings confirm the marked differences found in N and PB content and ^{15}N enrichment of SAB compared with LAB that have been extensively cited in literature. The higher content of LAB may reflect different stages of growth and nutrition as well as changes in microbial composition of the different ruminal populations [19, 28, 36-38].

Comparing bacterial samples extracted from solid material, no differences were detected between those extracts obtained with the basal treatment and those obtained after alcohol or methylcellulose treatment, and only changes of temperature gave an extract with a lower content of PB and higher content of N and ^{15}N enrichment as reported previously. Again, in LAB untreated or after detachment treatment, only the CHT-pellet showed changes in chemical composition confirming the adverse effect of such treatment on cell integrity.

Methods of estimating the efficiency of rumen microbial yield rely on marker techniques and specially in N:marker ratio of rumen microbes. LAB has been the common reference, but N:marker in SAB may differ with those values obtained in LAB and hence SAB-contribution needs to be taken into account. However, the low recovery of particle-associated PB or ^{15}N raises doubts about the representativity of such extract with regard to the total population of adherent microorganisms.

Effectively, total recovery as SAB-pure extract varied from 17.3 to 21.3% and from 19.2 to 20.9%, in PB and ^{15}N terms respectively, indicating that a significant proportion was lost during the process. Results also showed that this ratio was independent of the type of diet and the time after feeding. Craig *et al.* [2, 32], using ^{15}N as a microbial marker reported an increasing recovery of the isotope in the microbial pellet from 32% at 1 h to 46% at 10 h after feeding. Olubobokun *et al.* [29] using DAP as a bacterial marker recovered 32.9 and 34.0% of the original microbes when rumen was sampled 2 h after feeding, although the ratio increased to 50.3 and 54.0% 23 h later for a concentrate and alfalfa hay diets, respectively. This low recovery of microbial marker in the pellet result from protozoal and bacterial aggregate losses during the first centrifugation (500 × *g* for 5 min) or an incomplete precipitation of the

detached bacteria, although processes of bacterial lysis due to physical treatment, aeration or other manipulations can not be excluded.

The variability reported in the recovery of adherent micro-organism may be partially attributable to differences in the original solid material and/or the extraction procedure, but in any case the main fraction of the detached microbial material was lost throughout the experimental procedure.

In summary, results of this study and other workers indicate that existing procedures render only a small fraction of the total adherent population. It is easy to assume that the effect of the whole isolation process had a different effect on bacterial species and therefore SAB-pure extract would not fully represent the adherent population.

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ESTIMATION OF RUMEN MICROBIAL PROTEIN PRODUCTION FROM URINARY PURINE DERIVATIVES IN ZEBU CATTLE AND WATER BUFFALO

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Abstract

ESTIMATION OF RUMEN MICROBIAL PROTEIN PRODUCTION FROM URINARY PURINE DERIVATIVES IN ZEBU CATTLE AND WATER BUFFALO.

Two experiments were conducted in order to develop equations for predicting rumen microbial protein production for indigenous Kedah-Kelantan (KK) cattle and swamp buffaloes in Malaysia, using urinary purine derivatives (PD) excretion rates. Endogenous PD excretion rates determined by a fasting procedure for KK cattle and swamp buffalo were 275 and 370 μmol/kg $W^{0.75}$/day, respectively. Urinary PD excretion rate per kg digestible organic matter intake (DOMI) for KK cattle was higher than that for swamp buffalo, reconfirming the earlier findings. Glomerular filtration rate, allantoin and uric acid tubular load and PD re-absorption rate for swamp buffalo were generally higher than those for KK cattle. However, due to the large variations among animals within species, these parameters were not significantly different between species. Nevertheless, the higher PD re-absorption in swamp buffalo provides support for the earlier postulation that the lower urinary PD excretion rate of swamp buffalo was due to their higher recycling of plasma PD as compared to KK cattle. Labelled 8-^{14}C uric acid was used to estimate the ratio of renal to non-renal PD excretion. The recovery rates of the radioactive tracer via the renal route for both species were much lower than values reported previously for unlabelled PD for European cattle.

1. INTRODUCTION

Urinary purine derivatives (PD) excretion rate is widely used to predict rumen microbial protein production in ruminant livestock. Prediction equations have been developed based on European cattle [1] and sheep [2]. There is evidence to suggest that the urinary PD excretion rates of Zebu cattle and water buffaloes (*Bubalus bubalis*) differ from those of the European cattle [3, 4]. Therefore, it is pertinent to develop species specific models for the prediction of microbial protein yield in Zebu cattle and water buffalo.

Two experiments were conducted to develop prediction models for the Malaysian Kedah-Kelantan (KK) cattle (*Bos indicus*) and swamp buffaloes (*Bubalus bubalis*). The objective of the first experiment was to estimate the rate of endogenous PD excretion in the two species. The objectives of the second experiment were to: (i) measure the response of PD excretion to digestible organic matter intake (DOMI) and (ii) measure the proportion of plasma PD excreted in the urine. The present paper reports some results from both experiments.

2. MATERIALS AND METHODS

2.1. **Experiment I** Fasting trial

The experiments were conducted according to procedures described in the laboratory manual [5]. In the first experiment, 6 male KK cattle of 12-14 months age and 6 swamp buffaloes of similar sex and age were used. The average body weight of cattle and buffaloes were 108 ± 9.0 and 141 ± 17.7 kg, respectively. The experimental diet consisted of 40% oil palm frond and 60% concentrate pellets, with an energy value of 8.1 MJ ME and 123 g of CP/kg DM. The animals were fed 1% DM of body weight daily for 2 weeks. The amount of feed offered was then reduced stepwise within 2 days (0.5 and 0.25% body weight, respectively) before fasting commenced. The animals were fasted for a total of six days. Urine from each individual animal was collected daily, over a total period of 10 days, including the 6 days of fasting. One blood sample per animal was taken each morning at about 0900 hours during the fasting period. Urine and blood samples were processed and stored according to the procedures described in the laboratory manual [5], for further analysis.

2.2. **Experiment II** Feeding trial

The second experiment was conducted two months later using 4 animals per species drawn from the 6 animals used in the above fasting trial. Prior to the actual trial, the animals were fed individually at *ad libitum* for 1 week to determine the "lowest level of intake" for each species to ensure that all animals allocated later for the highest intake level (L4) were able to consume all the feed offered to them. A double 4 × 4 Latin Square (one for each species) was used for the experiment. The experiment consisted of four 21-day feeding periods and four feeding levels (see Table I - calculation based on 40, 60, 80 and 95% of the "lowest level of intake" of each species determined earlier). During the last 10 days of each period (test period), total urine and faeces excreted were collected daily. On the third day of each test period, 2 cattle and 2 buffaloes (animals that were allocated to treatments L1 and L3) were given a single intravenous administration of 8-^{14}C uric acid (Amersham Life Science – code CFQ9786). Blood samples were collected as in the fasting trial, once before injection of the tracer to determine the background activity, followed by hourly sampling for the next 5 hours and thereafter, at a longer interval, until 96 h post injection.

TABLE I. TREATMENT LEVELS FOR FEEDING TRIAL

Treatment	Buffalo		Cattle	
	Oil palm frond	Concentrate	Oil palm frond	Concentrate
	(kg DM/day)			
Level 1	0.85	1.44	0.84	1.26
Level 2	1.27	1.91	1.12	1.68
Level 3	1.49	2.09	1.40	2.10
Level 4	1.91	2.86	1.68	2.52

Urine and blood samples were processed and stored in a manner similar to that in the fasting trial. Faecal samples were also processed and stored for further analysis according to procedure described earlier [5].

3. MEASUREMENTS

Faeces and feed were analyzed for DM and OM to enable the calculation of DM and OM digestibilities. The urine was analyzed for total N, creatinine and purine derivatives (allantoin, uric acid, xanthine and hypoxanthine) by HPLC following the procedure of Balcells *et al* [6]. Blood plasma samples were also analyzed for creatinine and PD in a manner similar to the urine samples. Urinary PD excretion rates were regressed against their respective digestible organic matter intake (DOMI) using linear regression expressed as a deviation from the endogenous PD excretion determined in Experiment I.

4. RESULTS AND DISCUSSION

4.1. Experiment I Fasting trial

4.1.1. Endogenous PD excretion

The daily PD excretion rates of KK cattle and swamp buffalo recorded during pre-fasting and fasting periods are shown in Figure 1. The average daily endogenous PD excretion rates (Table II) for the last five days of fasting for KK cattle was significantly lower ($P < 0.05$) than that for swamp buffalo (275 and 370 μmol/kg $W^{0.75}$/day, respectively) but both values were slightly higher than those reported earlier [3, 4]. As in previous studies, allantoin remained to be the principal urinary PD; 82.9 and 87.3% of total PD, respectively for KK cattle and swamp buffalo. The uric acid content in the urine samples of swamp buffalo was significantly higher than that of KK cattle (15.5 vs 9.6% of total PD).

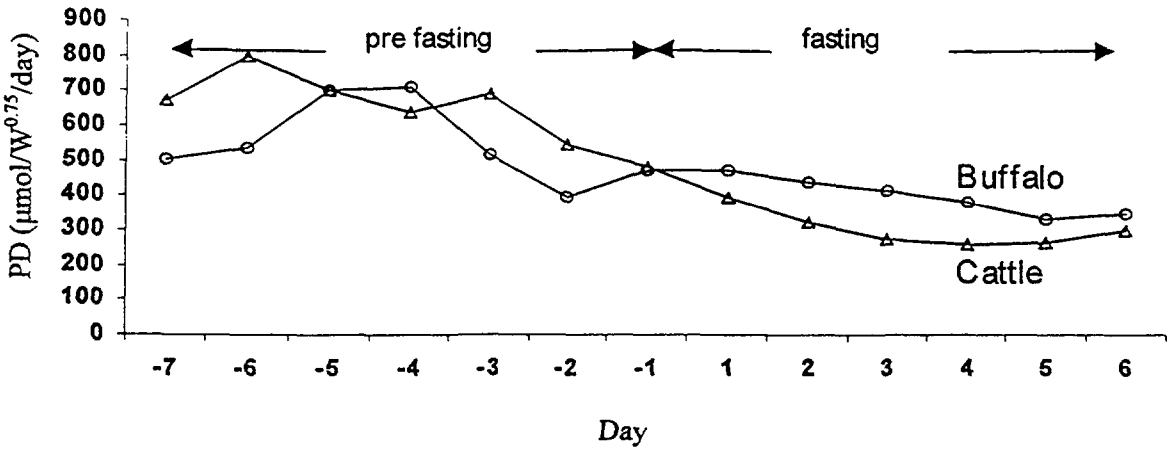

FIG.1. Excretion of urinary PD in buffalo and cattle during pre-fasting and fasting periods.

4.1.2. Glomerular filtration rate (GFR) and PD re-absorption rate

The GFR, tubular load and creatinine excretion and PD re-absorption rates during pre-fasting and fasting are shown in Table III. GFR of swamp buffalo and KK cattle were 334 and 266 L/day, respectively, during pre-fasting. The values decreased to about half during fasting (178 and 95 L/day, respectively). During both periods, the re-absorption of total PD was higher for swamp buffalo than for KK cattle, but the differences were not statistically significant.

TABLE II. DAILY URINARY EXCRETION OF PD IN SWAMP BUFFALO AND KK CATTLE DURING PRE-FASTING AND FASTING

Parameter	Buffalo	Cattle	SED	Significance
	(μmol/kg $W^{0.75}$/day)			
Pre-fasting				
Allantoin	507.3	608.8	58.51	NS
Uric acid	75.1[a]	54.6[b]	8.04	*
Hypoxanthine	6.1 [a]	12.2 [b]	2.71	*
Xanthine	21.0	26.3	2.45	NS
Total PD	609.5	701.9	65.25	NS
Fasting				
Allantoin	307.7 [a]	240.7 [b]	29.79	*
Uric acid	56.4 [a]	26.0 [b]	5.14	**
Hypoxanthine	1.6	2.5	1.87	NS
Xanthine	4.4	2.5	1.87	NS
Total PD	370.0 [a]	274.8 [b]	30.08	**

SED, Standard Error of Difference
NS, Not significant
*, Means with different superscripts within rows are significantly different (P <0.05)
**, Means with different superscripts within rows are significantly different (P <0.01)

TABLE III. DAILY GLOMERULAR FILTRATION RATE, CREATININE EXCRETION IN URINE AND PLASMA, TUBULAR LOAD AND EXCRETION AND RE-ABSORPTION OF PD DURING PRE-FASTING AND FASTING

Parameter	Buffalo	Cattle	SED	Significance
Pre-fasting				
GFR (L/d)	333.5	265.8	70.2	NS
Urine creatinine (mmol)	27.18	18.32	4.616	NS
Plasma creatinine (mmol/L)	0.084	0.081	0.012	NS
Allantoin tubular load (mmol)	79.8	56.4	21.0	NS
Uric acid tubular load (mmol)	7.8	5.5	2.3	NS
Allantoin re-absorption (mmol)	58.4	46.0	18.1	NS
Uric acid re-absorption (mmol)	5.1	3.7	2.3	NS
Total PD re-absorption (mmol)	66.6	51.3	20.7	NS
Fasting				
GFR (L/d)	178 [a]	95 [b]	23.7	*
Urine creatinine (mmol)	21.34	15.18	3.607	NS
Plasma creatinine (mmol/L)	0.141	0.177	0.017	NS
Allantoin tubular load (mmol)	44	30	7.5	NS
Uric acid tubular load (mmol)	17 [a]	11 [b]	2.6	*
Allantoin re-absorption (mmol)	29	21	6.7	NS
Uric acid re-absorption (mmol)	15	11	2.6	NS
Total PD re-absorption (mmol)	44	32	8.8	NS

SED, Standard Error of Difference
NS, Not significant
*, Means with different superscripts within rows are significantly different (P <0.05)

4.2. **Experiment II** Feeding trial

4.2.1. PD excretion rate

The relationships between urinary PD excretion rates and DOMI for the two species are shown in Figure 2. Allantoin was found to be the principal PD in the urinary samples of both species (Table IV). The results of the present study reconfirmed the earlier reports that urinary PD excretion rate per kg DOMI for cattle is higher than that for buffalo [3, 4]. However, the present values were higher than those previously reported for the same two species, by the previous workers.

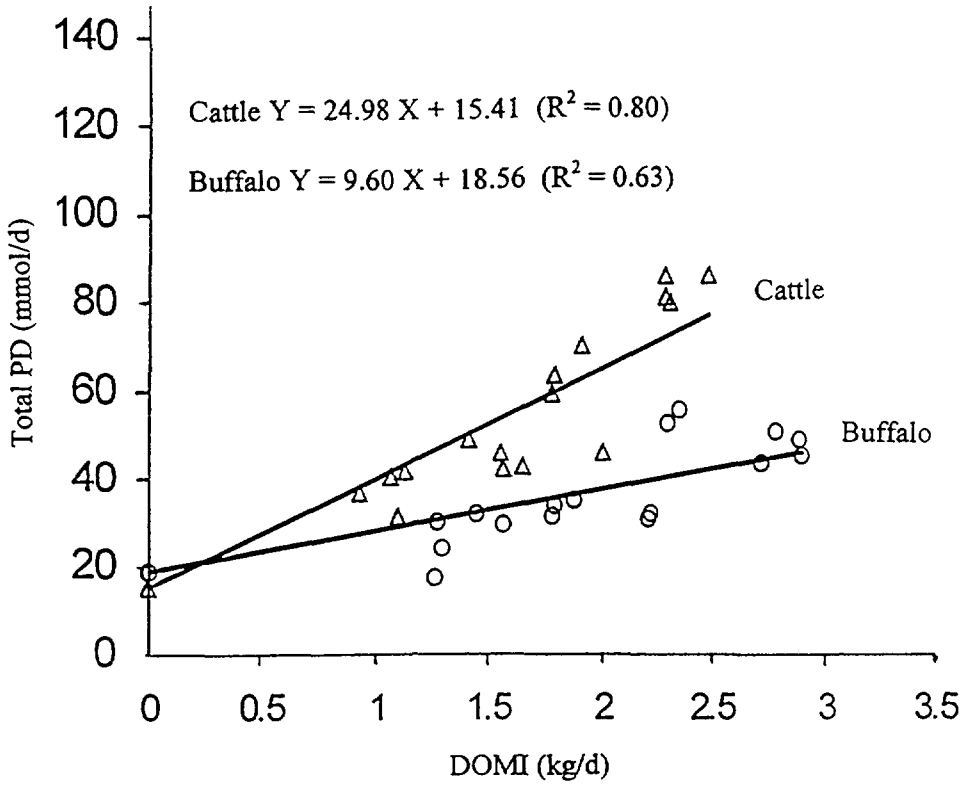

FIG.2. Urinary PD of KK cattle and buffalo as a function of DOMI.

4.2.2. Glomerular filtration rate (GFR)

Generally, GFR increased with increasing feeding level in both species. The GFR recorded in this experiment were 3 to 8 times higher than that recorded in the fasting trial. Irrespective of species, total PD re-absorption increased with increasing level of feeding. GFR values of swamp buffalo were generally higher than KK cattle. However, because of the large variation among animals within species, the differences between the two species were not statistically significant (Table V). Higher GFR, allantoin and uric acid tubular load and PD re-absorption recorded for the buffalo lend support to the earlier postulation that the lower urinary PD excretion rate of buffalo was due to its higher recycling of plasma PD as compared to cattle [4].

TABLE IV. MEAN BODY WEIGHT AND DAILY PD AND CREATININE EXCRETION IN KK CATTLE AND SWAMP BUFFALO UNDER DIFFERENT FEEDING LEVELS

Feeding level	L1	L2	L3	L4	SED	Significance
Cattle						
Body weight (kg)	147	147	155	158	7.40	NS
Allantoin (μmol/kg$W^{0.75}$)	670^{c}	877^{bc}	1252^{b}	2113^{a}	206.4	**
Uric acid (μmol/kg$W^{0.75}$)	116^{b}	142^{ab}	118^{ab}	239^{a}	37.1	*
Hypoxanthine (μmol/kg$W^{0.75}$)	10.2	4.9	16.9	24.0	7.19	NS
Xanthine (μmol/kg$W^{0.75}$)	56.3	26.5	38.7	55.4	19.7	NS
Total PD (μmol/kg$W^{0.75}$)	852.5^{c}	1050^{bc}	1480^{b}	2431^{a}	200.0	**
Creatinine (mmol/kg$W^{0.75}$)	1.08^{b}	1.60^{ab}	1.27^{b}	2.18^{a}	0.29	*
Buffalo						
Body weight (kg)	184	188	193	197	7.12	NS
Allantoin (μmol/kg$W^{0.75}$)	416^{a}	417^{a}	774^{b}	1550^{bc}	256.4	**
Uric acid (μmol/kg$W^{0.75}$)	105	119	106	173	30.88	NS
Hypoxanthine (μmol/kg$W^{0.75}$)	2.9	10.5	6.9	12.3	8.67	NS
Xanthine (μmol/kg$W^{0.75}$)	16.6	31.6	44.2	26.8	16.3	NS
Total PD (μmol/kg$W^{0.75}$)	540.1^{a}	609.0^{a}	931.0^{ab}	1762.1^{abc}	266.8	**
Creatinine (mmol/kg$W^{0.75}$)	1.42	1.45	1.83	1.98	0.65	NS

SED, Standard Error of Difference
NS, Not significant
*, Means with different superscripts within rows are significantly different ($P < 0.05$)
**, Means with different superscripts within rows are significantly different ($P < 0.01$)

4.2.3. Proportion of plasma PD excreted in urine

Measurements of the proportion of plasma PD excreted in urine could provide an explanation of the discrepancy in the urinary PD excretion rates between cattle and buffalo reported earlier [3, 4]. In this experiment, ^{14}C-uric-acid was injected intravenously and used to estimate the recovery of PD by the renal route. The urinary recovery of unlabelled PD has been found to be about 85% for European cattle [7] but there is no similar estimation for buffaloes. Recoveries of PD with the labelled uric acid were lower; 41 and 35% for KK cattle and swamp buffaloes, respectively. When analyzing the urine, the total radioactivity was separated into that associated with PD and other labelled components using the "C_1" and "C_2" separations [5]. Total recovery of radioactivity was about 72 and 69% for swamp buffaloes and KK cattle, respectively. The compounds containing radioactivity were not identified but are most likely to be degradation products of allantoin such as urea (any urinary urea that was degraded could have given rise to labelled bicarbonate but this would not have been retained after the urine was mixed with H_2SO_4 that was used as a preservative). Assuming that the non-PD radioactivity in urine was present as urea, this urea is likely to have been produced by the degradation of allantoin and it should, therefore, be considered to be part of the urinary PD excretion.

TABLE V. EFFECT OF FEEDING LEVEL ON DAILY GLOMERULAR FILTRATION RATE, TUBULAR LOAD AND RE-ABSORPTION OF PD IN BUFFALO AND KK CATTLE

Parameter	L1	L2	L3	L4	SED	Significance
Cattle						
GFR (L/d)	389	380	556	709	115	NS
Allantoin tubular load (mmol)	45[a]	45[a]	67[a]	114[b]	156	**
Uric acid tubular load (mmol)	32	23	35	47	12	NS
Allantoin re-absorption (mmol)	11	10	16	24	5	NS
Uric acid re-absorption (mmol)	22	15	28	38	10	NS
Total PD re-absorption (mmol)	33[a]	26 [a]	44 [a]	64[b]	8	**
Buffalo						
GFR (L/d)	411[a]	421[a]	722[b]	850 [b]	81	**
Allantoin tubular load (mmol)	29	53	56	94	23	NS
Uric acid tubular load (mmol)	28	44	41	83	24	NS
Allantoin re-absorption (mmol)	12	41	31	63	21	NS
Uric acid re-absorption (mmol)	22	47	37	92	27	NS
Total PD re-absorption (mmol)	35	89	68	155	41	NS

SED, Standard Error of Difference
NS, Not significant
**, Means with different superscripts within rows are significantly different (P <0.01)

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ESTIMATING RUMEN MICROBIAL PROTEIN SUPPLY FOR INDIGENOUS RUMINANTS USING NUCLEAR AND PURINE EXCRETION TECHNIQUES IN INDONESIA

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Abstract

ESTIMATING RUMEN MICROBIAL PROTEIN SUPPLY FOR INDIGENOUS RUMINANTS USING NUCLEAR AND PURINE EXCRETION TECHNIQUES IN INDONESIA.

The microbial protein supply to ruminants can be estimated based on the amount of purine derivatives (PD) excreted in the urine. Four experiments were conducted to evaluate the PD excretion method for Bali and Ongole cattle. In the first experiment, six male, two year old Bali cattle (*Bos Sondaicus*) and six Ongole cattle (*Bos Indicus*) of similar sex and age, were used to quantify the endogenous contribution to total PD excretion in the urine. In the second experiment, four cattle from each breed were used to examine the response of PD excretion to feed intake. ^{14}C-uric acid was injected in one single dose to define the partitioning ratio of renal:non-renal losses of plasma PD. The third experiment was conducted to examine the ratio of purine N:total N in mixed rumen microbial population. The fourth experiment measured the enzyme activities of blood, liver and intestinal tissues concerned with PD metabolism.

The results of the first experiment showed that endogenous PD excretion was 145 ± 42.0 and 132 ± 20.0 μmol/kg $W^{0.75}$/d, for Bali and Ongole cattle, respectively. The second experiment indicated that the proportion of plasma PD excreted in the urine of Bali and Ongole cattle was 0.78 and 0.77 respectively. Hence, the prediction of purine absorbed based on PD excretion can be stated as $Y = 0.78\ X + 0.145\ W^{0.75}$ and $Y = 0.77\ X + 0.132\ W^{0.75}$ for Bali and Ongole cattle, respectively. The third experiment showed that there were no differences in the ratio of purine N:total N in mixed rumen microbes of Bali and Ongole cattle (17% vs 18%). The last experiment, showed that intestinal xanthine oxidase activity of Bali cattle was lower than that of Ongole cattle (0.001 vs 0.015 μmol uric acid produced/min/g tissue) but xanthine oxidase activity in the blood and liver of Bali cattle was higher than that of Ongole cattle (3.48 vs 1.34 μmol/min/L plasma and 0.191 vs 0.131 μmol/min/g liver tissue). Thus, there was no difference in PD excretion between these two breeds. Liver uricase in Bali and Ongole cattle was 1.46 and 1.17 nmol/min/g tissue, where as no activity was detected in the intestinal tissue and blood.

1. INTRODUCTION

The major constraint to improving animal production in tropical countries is under-nutrition due to inadequate or fluctuating nutrient supply. Therefore, the strategy for improving production has been to maximize the efficiency of utilization of the available feed resources.

The utilization of roughages as a feed resource for ruminant livestock depends mainly on the efficiency of its microbial fermentation in the fore stomach. Moreover, in such diets rumen microbes constitute the main source of digestible protein to the host animal.

The efficiency of rumen fermentation and rumen microbial protein output can be easily estimated from the amount of purine derivatives (PD) excreted in the urine. This method has been developed with European breeds of cattle [1] and sheep [2], although experimental evidence suggest significant differences in purine metabolism amongst ruminant species [3].

The objective of the present study was to investigate whether Bali cattle (*Bos sondaicus)* and Ongole cattle (*Bos indicus)* have PD excretion patterns different to European breeds, and if so to establish response models between duodenal flow and renal excretion of PD for these two breeds. In order to facilitate this, four experiments were conducted to determine (i) the endogenous contribution of PD to urinary excretion, (ii) the activity of xanthine oxidase (XO) of liver, blood and intestinal tissue as a key enzyme in purine metabolism and (iii) the specific relationship between uptake and renal excretion of purine compounds.

2. MATERIALS AND METHODS

2.1. Experiment I Estimation of endogenous PD excretion (Fasting trial)

2.1.1. Animals and diets

Six, two year old male Bali cattle and six male Ongole cattle were used in this experiment. The feed consisted of King grass (a *Pennisetum* hybrid) harvested after 42 days of planting.

2.1.2. Experimental procedure and sample collection

All animals were kept in individual pens and fed *ad libitum* for one week and weighed before moving to the metabolism cages. *Ad libitum* feeding was continued for one week and urine samples were collected daily. On the last day, two blood samples were taken at 0800 and 1500 h. After one week of urine collection, the feed was reduced gradually within two days to 60 and 30%, followed by fasting for 6 days. During the fasting period, urine collection was continued and the blood samples were taken every two days. Urine and blood samples were processed and stored for subsequent analysis according to procedures described in the IAEA TECDOC [4].

2.2. Experiment II The response of purine excretion to feed intake and measurement of the proportion of plasma purine derivatives excreted in the urine

2.2.1. Animals and diets

Four animals from each breed used in the fasting trial (Experiment I) were used in this experiment. All animals were fed twice daily at 0800 and 1500 h with King grass as in Experiment I.

2.2.2. Experimental procedure and sample collection

During the preliminary period, all animals were fed at *ad libitum* level of intake for over a week to determine the lowest intake amongst the animals of the same breed. This level of intake was defined as the "voluntary intake" for that breed. During the experimental period four animals of each breed were fed at four fixed levels namely 95, 80, 60 and 40% of "voluntary intake". The treatments were allocated according to a 4x4 Latin Square design. All animals were kept in individual metabolism cages. During each feeding period, feed samples were obtained daily and made into composite samples.

Each feeding period lasted for 3 weeks. Urine and faeces were collected during the last 10 days (collection period) of each feeding period. On the third day of each collection period each animal was given a single dose of 8-^{14}C uric acid (280 μCi/animal in 45 ml solution)

intravenously via a jugular catheter. The tracer administration was performed in the morning just before feeding. Blood sampling was carried out using the jugular catheter at 1, 2, 3, 4, 6, 7, 8, 14, 20, and 26 h after tracer administration. Blank blood samples were taken just before tracer administration to determine the background activity.

Urine, faeces and blood samples collected were processed and stored for further analysis according to procedure described earlier [4].

2.3. Experiment III The measurement of the ratio of purine nitrogen to microbial nitrogen in mixed rumen microorganisms

2.3.1. Animals and feeding

Three year old, male Bali and Ongole cattle which were used in Experiments I and II, were used as donors of rumen fluid. The body weight of animals were recorded. Animals were kept in individual pens and were fed King grass *ad libitum*. The grass was offered twice a day at 0800 and 1500 h. Composite samples of feed were kept for nutrient analyses.

2.3.2. Sample collection

Rumen fluid samples were collected from each animal at 3-6 h after feeding in the morning. The samples were transferred into a warm vacuum flask and taken to the laboratory for processing.

Processing of rumen fluid samples for the preparation of microbial matter for purine analysis was carried out according to the procedure described in the IAEA-TECDOC [4].

2.4. Experiment IV The measurement of xanthine oxidase and uricase activity in plasma, liver and intestinal tissue

2.4.1. Sample collection and processing

The intestinal mucosa and liver samples were taken from 2 year old, male Bali and Ongole cattle. The materials were obtained from a slaughter house in Yogyakarta for Ongole cattle and Denpasar for Bali cattle, respectively.

2.4.2. Preparation of tissue extracts

Blood samples were taken from the same animals used in the previous experiments (Experiment I and Experiment II), into four, 10 ml heparinised tubes and were centrifuged for 10 min at 2 500 *g*, at 4°C. The plasma obtained was transferred into the vials and used for assaying enzyme activity within 2 h.

About 100 g of liver tissue were taken from the slaughtered animals and the material was transferred to the laboratory in a polythene bag stored in ice. The samples were washed in cold 0.15 M KCl solution, blotted dry and frozen immediately until analyses.

The first 30 cm segment of the small intestine was obtained and the lumen washed with cold 0.15 M KCl solution and then with 0.05 M HEPES buffer (pH 7.5) containing 0.25 mM EDTA and 0.25 mM. PMSF. The segment of the intestine was cut length wise, opened flat and the mucosal cells were isolated by scrapping them with a spatula. The mucosal cell samples were weighed and 1 g was homogenized in 9 ml of the HEPES-EDTA-PMSF buffer. The extract was centrifuged at 40 000 *g* for 30 min at 4°C. The supernatant was dialysed for 24 h against the HEPES-EDTA-PMSF buffer. The contents of the dialysis tubing were centrifuged at 40 000 *g* for 30 min at 4°C and the supernatant was stored at 4°C.

Further preparation of tissue extracts for measuring xanthine oxidase (XO) and uricase enzyme activity was as described in the IAEA-TECDOC [4].

2.5. Measurements

Feed and faeces samples from all four experiments were analyzed for DM and nitrogen according to standard procedure. Blood and urine samples were analyzed for allantoin, uric acid and ^{14}C activity according to procedure described in the IAEA TECDOC [4]. Creatinine analysis was carried out according to procedure described by Hawk *et al.* [5]. Total urinary nitrogen was determined by the Kjeldahl method.

The XO activity in plasma and extracts of intestinal mucosa and liver samples was measured as the rate of uric acid production when xanthine was incubated with plasma or tissue extracts. The activity of uricase was measured as the rate of uric acid disappearance when uric acid was incubated with plasma or tissue extracts. Uric acid production or uric acid disappearance was measured by spectrophotometer, where OD was read at 292 nm [4].

3. RESULTS AND DISCUSSION

3.1. Experiment I

3.1.1. Estimation of endogenous PD excretion

The daily excretion of urinary PD, creatinine and total nitrogen in Bali and Ongole cattle are presented in Table I. The mean excretion of total PD was 460 (± 231) and 541 (± 75.6) μmol/kg $W^{0.75}$/d respectively, for Bali and Ongole cattle fed *ad libitum* and the difference was not significant. The relative proportion of urinary allantoin and uric acid in the PD of Bali and Ongole cattle during *ad libitum* feeding were 0.86 and 0.14 and 0.85 and 0.15, respectively (Figure 1).

TABLE I. URINARY PD, CREATININE AND NITROGEN EXCRETION IN BALI AND ONGOLE CATTLE DURING FASTING AND WHEN FED *AD LIBITUM*

	Breed of Cattle		SE	Significance
	Bali	Ongole		
Ad libitum (μmol/kg $W^{0.75}$/d)				
Allantoin	395.1 ± 205.1	461.7 ± 62.9	64.11	NS
Uric acid	65.1 ± 26.2	78.9 ± 12.7	8.40	NS
Purine derivatives	460.3 ± 231.3	540.6 ± 75.6	69.02	NS
Creatinine	861.8 ± 17.0	720.0 ± 49.0	51.66	NS
Nitrogen *	715.0 ± 72.0	735.0 ± 51.0	25.43	NS
Fasting (μmol/kg $W^{0.75}$/d)				
Allantoin	111.9 ± 35.0	101.6 ± 12.5	11.36	NS
Uric acid	33.5 ± 8.4	30.7 ± 9.2	3.84	NS
Purine derivatives	145.4 ± 42.0	132.3 ± 20.0	13.85	NS
Creatinine	828.5 ± 179.6	639.1 ± 99.7	60.82	**
Nitrogen*	348.5 ± 79.4	360.1 ± 102.5	45.67	NS

* mg/kg $W^{0.75}$/d

NS, Not significant; **, P <0.01

When feed allowance was reduced from *ad libitum* (100%) to 60, 30 and 0% (fasting), PD excretion decreased rapidly as seen in Figure 2.

The endogenous PD excretion of Bali cattle, based on the last 3 days of fasting was 145 μmol/kg $W^{0.75}$/d, while for Ongole cattle it was 132 μmol/kg $W^{0.75}$/d. Xanthine and hypoxanthine were not detectable in urine samples, thus PD included only allantoin and uric acid. The proportional contribution of both compounds to total PD during fasting were respectively, 0.77 and 0.23 for Bali cattle and 0.82 and 0.18 for Ongole cattle (Figure 1). Values in both breeds were similar to those reported by Chen *et al.* [3] for European breeds.

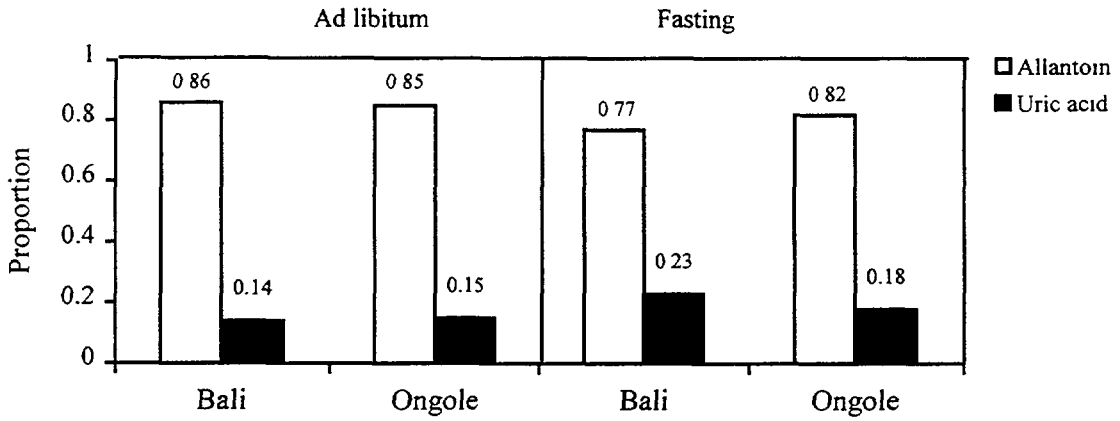

FIG.1. The proportion of allantoin and uric acid in PD in Bali and Ongole cattle during fasting and ad libitum feeding.

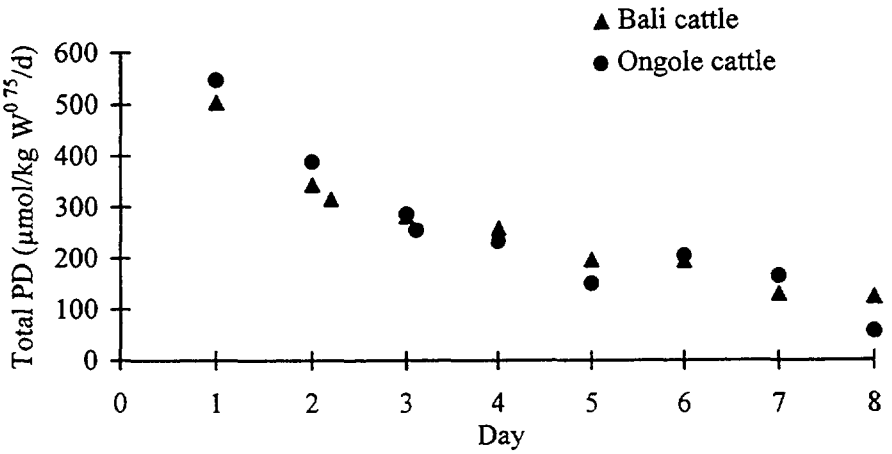

FIG.2. PD excretion in the urine of Bali and Ongole cattle during fasting period.

There was no difference in urinary nitrogen excretion of Bali and Ongole cattle fed, either at *ad libitum* intake or during fasting (Table I). Endogenous nitrogen excretion was 715 and 735 mg/kg $W^{0.75}$/d for Bali and Ongole cattle, respectively at *ad libitum* feeding while they were 348 and 360 mg/kg $W^{0.75}$/d, respectively during fasting. The proportion of allantoin to total nitrogen excretion decreased from 8.7 to 5.1% in Bali and from 9.9 to 4.5% in Ongole cattle. The proportion of uric acid to total nitrogen excretion was not influenced by the treatments. It represented 1.5 and 1.6% in Bali and 1.8 and 1.4% in Ongole cattle for *ad libitum* feeding and fasting, respectively. The results are in the range of 2.2-22.8% and 0.60-1.81%, for proportion of allantoin:total N and uric acid:total N excretion respectively, as reported by Bristow *et al* [6].

Urinary creatinine excretion in Bali cattle was higher ($P < 0.01$) than that of Ongole cattle during the fasting period (829 vs 639 μmol/kg $W^{0.75}$/d), while the opposite trend was true during the feeding period.

3.1.2. Glomerular filtration rate (GFR)

The plasma PD, GFR, tubular load and reabsorption of PD are shown in Table II. Plasma allantoin concentration was affected by the dietary treatment. It was significantly lower ($P < 0.01$) both in Bali and Ongole cattle during fasting compared to *ad libitum* feeding (99 and 72 μmol/L vs 146 and 174 μmol/L). There was no difference in plasma PD concentration between Bali and Ongole cattle when fed *ad libitum* but plasma allantoin and PD concentration in Bali cattle were significantly higher ($P < 0.01$) than those of Ongole cattle during the fasting period. The uric acid concentration was not influenced by the dietary treatment.

The GFR in Bali cattle was higher ($P < 0.01$) than that of Ongole cattle (1265 vs 948 L/d) when they were being fed *ad libitum.* It decreased almost 50% in both cases as a result of fasting (650 vs 538 L/d) but with no significant difference between the breeds. No differences were observed in tubular load of allantoin, uric acid and PD between Bali and Ongole cattle during *ad libitum* feeding but during fasting Bali cattle showed a significantly ($P < 0.05$) higher rate of PD tubular load compared to Ongole cattle (81.9 vs. 50.3 μmol/d).

The PD reabsorption during both fasting and *ad libitum* feeding periods tended to be higher in Bali cattle (though not significantly) than in Ongole cattle (86.5 vs 81.6% and 86.5 vs 80.3%, for Bali and Ongole cattle, respectively).

3.2. Experiment II

3.2.1. Response of PD excretion to feed intake

Urinary PD excretion in Bali and Ongole cattle fed at different levels of intake are shown in Table III. The daily excretion of allantoin and uric acid in both breeds showed a positive response to the level of intake. For Ongole cattle PD excretion was correlated to digestible organic matter intake (DOMI) according to the following equation (Figure 3).

$$Y = 11.30\,X + 8.89 \quad (R^2 = 0.74;\ n = 16;\ P < 0.01)$$

For Bali cattle PD excretion was correlated to DOMI according to the following equation but the correlation coefficient was not significant.

$$Y = 10.04\,X + 8.36 \quad (R^2 = 0.14;\ n = 16;\ NS)$$

TABLE II. GFR, TABULAR LOAD AND REABSORPTION OF PD IN BALI AND ONGOLE CATTLE DURING FASTING AND *AD LIBITUM* FEEDING

	Breed of Cattle		SE	Significance
	Bali	Ongole		
Ad libitum				
Plasma (μmol/L)				
Allantoin	146.3 ± 23.3	173.5 ± 23.8	10.5	NS
Uric acid	23.1 ± 4.5	24.8 ± 6.7	2.6	NS
Purine derivatives	169.4 ± 26.7	198.2 ± 24.4	11.6	NS
Creatinine	43.8 ± 10.4	50.1 ± 3.7	3.2	NS
Urine creatinine (mmol/d)	54.7 ± 11.4	47.3 ± 3.6	3.5	NS
GFR (L/d)	1265 ± 149	948 ± 111	53.8	**
GFR ($L/W^{0.75}/d$)	19.9	14.5	0.9	***
Tabular load (mmol/d)				
Allantoin	182.9 ± 23.4	164.9 ± 31.9	12.5	NS
Uric acid	28.7 ± 2.8	23.4 ± 6.9	2.4	NS
Purine derivatives	211.6 ± 24.7	188.4 ± 33.6	13.2	NS
Reabsorption (mmol/d)				
Allantoin	158.1 ± 20.5	134.7 ± 34.2	12.6	NS
Uric acid	24.6 ± 3.0	18.3 ± 7.2	2.5	NS
Purine derivatives	182.7 ± 21.6	152.9 ± 36.0	13.3	NS
Reabsorption (%)				
Allantoin	86.6 ± 5.5	80.7 ± 5.3	2.2	NS
Uric acid	85.7 ± 5.2	76.3 ± 6.2	2.5	NS
Purine derivatives	86.5 ± 5.3	80.3 ± 5.1	2.3	NS
Fasting				
Plasma (μmol/L)				
Allantoin	98.7 ± 13.0	71.7 ± 11.7	5.5	**
Uric acid	26.9 ± 6.9	23.2 ± 2.6	2.4	NS
Purine derivatives	125.6 ± 14.9	94.9 ± 12.3	6.1	**
Creatinine	74.6 ± 14.1	71.3 ± 10.5	5.1	NS
Urine creatinine (mmol/d)	46.9 ± 9.8	37.9 ± 6.6	3.5	NS
GFR (L/d)	650.9 ± 2.0	538.3 ± 95.0	64.5	NS
GFR ($L/W^{0.75}/d$)	11.5 ± 3.7	9.1 ± 1.4	1.1	NS
Tabular load (mmol/d)				
Allantoin	65.5 ± 22.3	37.8 ± 3.8	7.2	NS
Uric acid	16.5 ± 3.2	12.5 ± 2.7	1.3	NS
Purine derivatives	81.9 ± 23.4	50.3 ± 5.7	9.3	*
Reabsorption (mmol/d)				
Allantoin	57.4 ± 20.3	30.6 ± 4.7	6.6	NS
Uric acid	13.9 ± 3.6	10.6 ± 2.7	1.4	NS
Purine derivatives	71.3 ± 21.1	41.2 ± 6.2	6.9	NS
Reabsorption (%)				
Allantoin	83.0 ± 3.8	79.8 ± 5.5	2.1	NS
Uric acid	53.5 ± 1.9	83.7 ± 3.6	1.3	NS
Purine derivatives	86.5 ± 3.4	81.6 ± 3.9	1.6	NS

NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

TABLE III. URINARY PD AND CREATININE EXCRETION IN BALI AND ONGOLE CATTLE FED AT DIFFERENT LEVELS OF INTAKE

	Level of feed intake (%)				SE	Significance.
	95	80	60	45		
Bali cattle (mmol/d)						
Allantoin	23.0 ± 13.2	17.7 ± 8.8	15.6 ± 4.7	12.3 ± 3.3	1.9	*
Uric acid	4.7 ± 2.1	3.9 ± 1.4	3.0 ± 0.9	2.3 ± 0.4	0.4	*
Purine derivatives	27.7 ± 15.2	21.7 ± 10.3	18.6 ± 5.7	14.7 ± 3.7	2.3	*
Ongole cattle (mmol/d)						
Allantoin	25.7 ± 2.8	23.1 ± 3.2	16.5 ± 3.7	14.7 ± 2.1	0.9	**
Uric acid	4.5 ± 0.6	4.0 ± 1.1	2.9 ± 0.3	2.2 ± 0.5	0.3	*
Purine derivatives	30.3 ± 3.2	27.2 ± 2.9	19.4 ± 3.9	16.9 ± 2.3	0.9	**

*, P <0.05; **, P <0.01

The extrapolated endogenous PD excretion for Ongole cattle was close to endogenous PD excretion obtained from the fasting trial (8.89 vs 9.10 mmol/d). However, for Bali cattle using all four animals gave a poor and non-significant correlation between PD excretion and DOMI, presumably because of the high variability amongst the animals. It appeared that two animals had low PD excretion rates while the other two had high rates of excretion. Separating them into two groups gave better correlations (Figure 3). The extrapolated endogenous PD excretion for animals with high excretion rates was similar to the endogenous PD excretion obtained from the fasting trial (10.7 vs 10.6 mmol/d) but was far from close for the animals with lower excretion rates (0.10 vs 10.62 mmol/d).

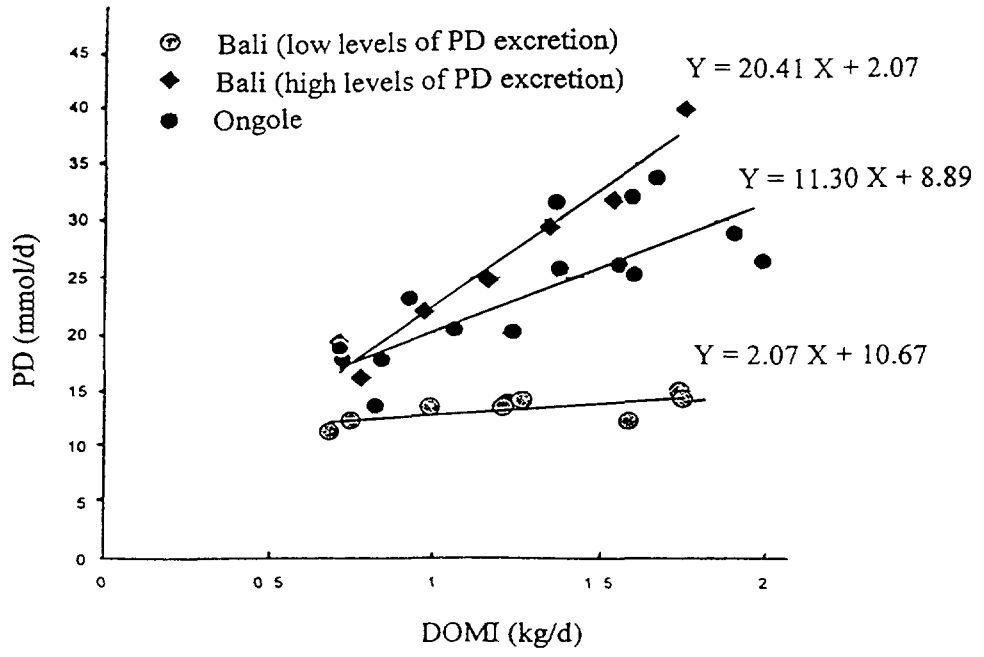

FIG.3. Relationship between PD excretion and digestible organic matter intake (DOMI) in Bali and Ongole cattle.

There were no differences in allantoin, uric acid and PD excretion between the breeds. The plasma PD of Bali cattle tended to be lower than that of Ongole cattle, but the GFR was not different between the two breeds. This can be explained by the data of PD reabsorption (Table II) which was higher in Bali than in Ongole cattle (77.6% vs 75.39%), since there were no effects on urine PD excretion between the breeds.

3.2.2. Glomerular filtration rate

Plasma PD, creatinine, GFR, tubular load and reabsorption of PD are given in Tables IV and V. Plasma allantoin, uric acid and PD of both breeds were not significantly affected by the level feed intake.

The GFR of Bali cattle was not affected by the level of feed intake but in Ongole cattle level of feed intake had a significant effect on the GFR ($P < 0.01$). However, when the values were expressed on the basis of metabolic body weight, the GFR of Bali cattle decreased significantly ($P < 0.01$) with decreasing level of feed intake, while that of Ongole cattle was not affected.

TABLE IV. GFR, TABULAR LOAD AND REABSORPTION OF PD IN BALI AND ONGOLE CATTLE

	Breed of Cattle		SE	Significance
	Bali	Ongole		
Plasma (μmol/L)				
Allantoin	91.5	109.6	8.8	**
Uric acid	27.3	29.3	3.7	NS
Purine derivatives	117.7	136.1	9.9	NS
Creatinine	92.0	89.4	4.9	NS
Urine creatinine (mmol/d)	68.9	60.6	7.1	NS
GFR (L/d)	758.5	701.1	58.4	NS
GFR (L/ $W^{0.75}$/d)	10.9	9.5	0.9	*
Tabular load (mmol/d)				
Allantoin	70.8	70.6	10.1	NS
Uric acid	20.5	20.3	3.0	NS
Purine derivatives	91.4	95.2	12.0	NS
Reabsorption (mol/d)				
Allantoin	53.4	54.8	8.0	NS
Uric acid	16.9	16.9	2.9	NS
Purine derivatives	70.4	71.7	9.5	NS
Reabsorption (%)				
Allantoin	75.4	72.7	1.9	NS
Uric acid	82.9	81.5	2.4	NS
Purine derivatives	77.7	75.3	2.5	NS

NS, not significant; *, $P < 0.05$; **, $P < 0.01$

3.2.3. Digestibility of the feed

Dry matter and organic matter digestibilities of King grass given to Bali and Ongole cattle are given in Table VI. The digestibilities were not affected by level of feed intake and were not different between the breeds, presumably because feed intake was below energy maintenance.

TABLE V. GFR, TABULAR LOAD AND PD REABSORPTION IN BALI AND ONGOLE CATTLE FED AT DIFFERENT LEVELS OF INTAKE

	Level of feed intake				SE	Significance
	95%	80%	60%	40%		
Bali cattle						
Plasma (μmol/L)						
Allantoin	110.2	85.1	88.8	81.8	7.0	NS
Uric acid	28.3	28.8	27.1	25.3	1.5	NS
Purine derivatives	138.5	117.5	108.9	110.4	6.9	NS
Creatinine	87.9	88.7	88.7	102.7	3.7	NS
Urine creatinine (mmol/d)	76.8	70.2	64.8	63.7	2.6	*
GFR (L/d)	891.0	792.5	717.5	633.0	58.3	NS
GFR ($L/W^{0.75}/d$)	12.7	11.2	10.4	9.1	0.9	**
Tabular load (mmol/d)						
Allantoin	101.2	70.9	58.5	51.6	9.4	*
Uric acid	24.4	22.6	19.3	15.8	1.8	NS
Purine derivatives	125.6	94.7	77.8	67.4	10.8	*
Reabsorption (mmol/d)						
Allantoin	78.2	53.1	42.9	39.3	7.7	*
Uric acid	19.7	18.7	16.2	13.4	1.5	NS
Purine derivatives	97.9	71.8	59.2	52.7	8.4	*
Reabsorption (%)						
Allantoin	78.3	75.9	72.5	74.9	1.8	NS
Uric acid	80.9	81.7	84.4	84.6	0.6	*
Purine derivatives	79.1	77.8	75.6	78.1	1.1	NS
Ongole cattle						
Plasma (mmol/L)						
Allantoin	123.0	104.5	109.6	101.2	10.2	NS
Uric acid	23.2	29.2	31.3	33.3	4.6	NS
Purine derivatives	135.2	133.6	140.9	134.5	12.2	NS
Creatinine	81.8	85.0	92.2	98.2	3.3	*
Urine creatinine (mmol/d)	61.0	70.9	54.7	56.0	2.6	*
GFR (L/d)	759.0	835.2	602.8	607.5	36.9	**
GFR ($L/W^{0.75}/d$)	10.3	11.4	8.3	8.2	1.0	NS
Tabular load (mmol/d)						
Allantoin	91.8	86.3	65.2	56.0	4.9	**
Uric acid	17.4	24.7	19.4	19.9	2.9	NS
Purine derivatives	109.3	110.9	84.6	75.9	5.7	**
Reabsorption (mmol/d)						
Allantoin	66.1	63.2	48.7	41.3	2.8	*
Uric acid	12.9	20.6	16.5	17.7	4.7	NS
Purine derivatives	79.0	83.8	65.2	58.9	5.6	NS
Reabsorption (%)						
Allantoin	71.5	72.6	73.5	73.2	2.1	NS
Uric acid	73.4	80.7	83.3	88.5	3.1	NS
Purine derivatives	71.9	75.2	76.3	77.7	1.5	NS

NS, not significant; *, $P < 0.05$; **, $P < 0.01$

TABLE VI. NUTRIENT DIGESTIBILITY OF KING GRASS FED TO BALI AND ONGOLE CATTLE AT DIFFERENT LEVELS OF INTAKE

	Level of feed intake (%)				SE	Significance
	95	80	60	40		
Bali cattle						
DM digestibility (%)	58.7	58.4	60.3	59.7	1.9	NS
OM digestibility (%)	62.0	62.1	64.2	64.3	2.0	NS
DOMI (kg/d)	1.7	1.4	1.1	0.7	0.02	**
Ongole cattle						
DM digestibility (%)	60.0	58.0	57.8	60.7	2.6	NS
OM digestibility (%)	63.2	61.9	61.8	65.1	2.2	NS
DOMI (kg/d)	1.8	1.5	1.1	0.8	0.3	**

NS, not significant; **, P <0.01

3.2.4. Nitrogen balance and ^{14}C uric acid excretion

Nitrogen excretion of Bali and Ongole cattle are shown in Table VII. Faecal and urine nitrogen were significantly affected by the level of feed intake (P <0.01), but the nitrogen excretion was not affected by the breed type. The nitrogen balance decreased when the intake of digested organic matter was reduced. The regression between nitrogen excretion (Y) and DOMI (X) were Y = - 19.27 + 2.88 X and Y = - 21.36 + 6.72 X respectively, for Bali and Ongole cattle. The extrapolated endogenous nitrogen excretion of Bali and Ongole cattle were -19 and -21 g/d respectively. Those values were higher than the nitrogen excretion during the fasting period (-46 and -45 g/d, respectively for Bali and Ongole cattle). This was probably due to higher nitrogen catabolism during fasting compared to the level of protein used for production of glucose precursors.

TABLE VII. THE EFFECT OF LEVEL OF FEED INTAKE ON NITROGEN BALANCE OF BALI AND ONGOLE CATTLE

	Level of feed intake (%)				SE	Significance
	95	80	60	40		
Bali cattle (g/d)						
Nitrogen intake	47.3	39.8	29.8	19.9	0.8	***
Fecal nitrogen	18.4	16.0	9.9	6.7	0.8	***
Urine nitrogen	34.8	30.1	28.8	23.1	1.8	**
Nitrogen balance	-5.9	-6.1	-8.9	-9.9	1.8	NS
Ongole cattle (g/d)						
Nitrogen intake	45.5	41.7	31.4	20.8	0.7	***
Fecal nitrogen	17.7	16.2	11.2	9.1	1.3	***
Urine nitrogen	33.9	30.9	29.5	25.0	1.2	**
Nitrogen balance	-2.2	-5.5	-9.3	-13.3	2.5	NS

NS, not significant; **, P <0.01; ***, P <0.001

The concentration of ^{14}C uric acid in the plasma of both breeds, decreased exponentially with time. After 24 h, tracer disappearance from the plasma of Bali and Ongole cattle fed at the level of 95 and 60% of voluntary intake were not different. On the other hand, tracer recovery in the urine of cattle fed at the level of 95% voluntary intake was higher than cattle fed at the level of 60% (85.6 vs 74.4% and 84.7 vs 55.8% for Bali and Ongole cattle, respectively) (Table VIII).

Based on these results it can be presumed that when animals were fed at a lower level of intake, PD excretion via renal route decreased while excretion via non-renal routes increased. The ratio PD excretion via renal:non-renal routes in Bali and Ongole cattle fed at 90% level intake were 85.5:14.4 and 84.7:15.3, respectively, while when the animals were fed at 60% level of intake, the ratio was found to be 74.4:15.6 and 55.8:44.2, respectively.

TABLE VIII. RECOVERY OF ^{14}C URIC ACID IN THE URINE OF CATTLE RECEIVING TWO LEVELS OF FEED INTAKE

Recovery ^{14}C (%)	Bali		Ongole	
	Level of intake (%)			
	95	60	95	60
Total ^{14}C	85.6 ± 10.4	74.4 ± 12.4	84.7 ± 8.9	55.8 ± 29.9
^{14}C Purine Derivatives	77.8	66.0	76.6	50.2
^{14}C as other compounds	7.8	8.2	8.1	5.7

The presumption that PD in the plasma can be excreted via renal and non-renal routes is supported by results reported by Chen *et al.* [7]. They stated that concentration of allantoin and uric acid in sheep plasma was 52 and 6 μmol/L while in saliva it was 120 and 16 μmol/L. They also stated that the presence of allantoin and uric acid in sheep saliva demonstrated that purine derivatives in the plasma can be recycled via salivary secretion to the rumen. Chen *et al.* [8] also stated that the proportion of absorbed exogenous purine excreted as derivatives in the urine was 0.84.

3.3. Experiment III

Nitrogen and nucleic acid content in rumen microbes are listed in Table IX. The table shows that the amount of microbial matter was 1.021 and 0.77 g/L for Bali and Ongole cattle, respectively. The nitrogen content of Ongole rumen microbes was slightly higher than that of Bali cattle (6.3 vs 5.9% DM).

The RNA content of the microbes from the two breeds was very similar (19.2 for Bali cattle and 20.3 for Ongole cattle), but appear to be higher than values reported by other workers [9]. This could be due to errors associated with RNA determination. Not withstanding this discrepancy, based on the nitrogen content in microbes and nitrogen in RNA, it could be calculated that the ratio of purine N:microbial N in mixed rumen microbes was 0.42 ± 0.06 for Bali cattle and 0.43 ± 0.04 for Ongole cattle, respectively. The ratio obtained can be used to calculate microbial nitrogen production based on the amount of PD excretion.

The ratio of N-RNA:N-total microbes of Bali cattle were not significantly different from that of the Ongole cattle.

Based on the assumption that purine base content was equal to pyrimidine base, it can be stated that the ratio of purine N:total N was about 0.21 and 0.22 for Bali and Ongole cattle, respectively. The values were higher than the purineN:total N in the microbes (0.116) as reported by Chen *et al.* [8]. This could be partly due to the above mentioned errors associated with the purine N determination in the microbes.

3.3.1. Xanthine oxidase activity

Figures 4, 5 and 6 show the uric acid production from plasma, liver and intestinal tissue extracts in Bali and Ongole cattle as an index of XO activity. The rate of uric acid produced by the enzyme was 1.74 μmol/min and 0.67 μmol/min for Bali and Ongole cattle, respectively. These values gave an activity of 3.48 and 1.34 μmol/min/L for plasma in Bali and Ongole cattle. The xanthine oxidase activity in plasma of Ongole cattle was nearly the same as that reported by Chen *et al.* [3], (1.13 μmol/min/L) while xanthine oxidase activity in the plasma of Bali cattle was higher.

TABLE IX. NITROGEN AND RNA CONTENT OF RUMEN MICROBES IN BALI AND ONGOLE CATTLE

Breed	Microbe (g/L)	N-microbe	RNA (% DM)	N-RNA	Ratio of N-RNA:N-Microbe
Bali	1.02 (± 0.12)	5.90 (± 0.08)	19.20 (± 0.34)	2.49 (± 0.34)	0.42 (± 0.06)
Ongole	0.77 (± 0.20)	6.30 (± 0.47)	20.82 (± 2.80)	2.71 (± 0.41)	0.43 (± 0.04)

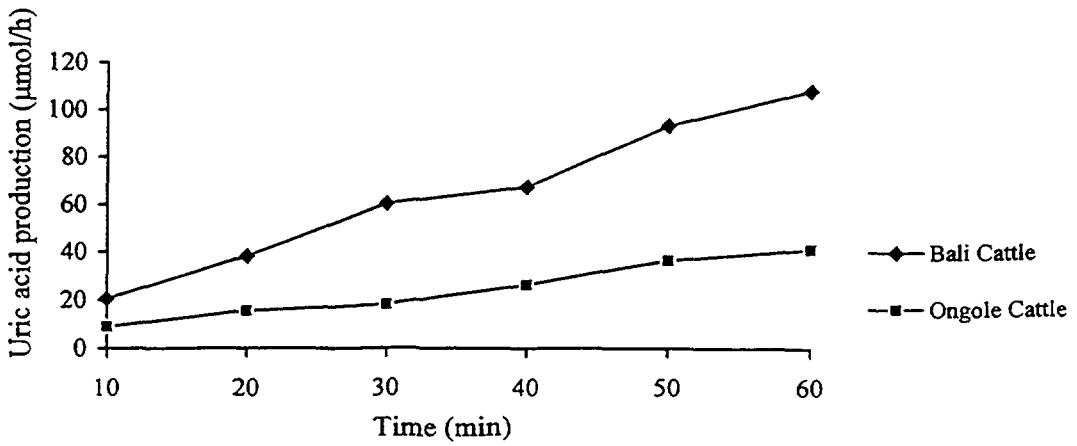

FIG.4. Xanthine oxidase activity in the plasma of Bali and Ongole cattle.

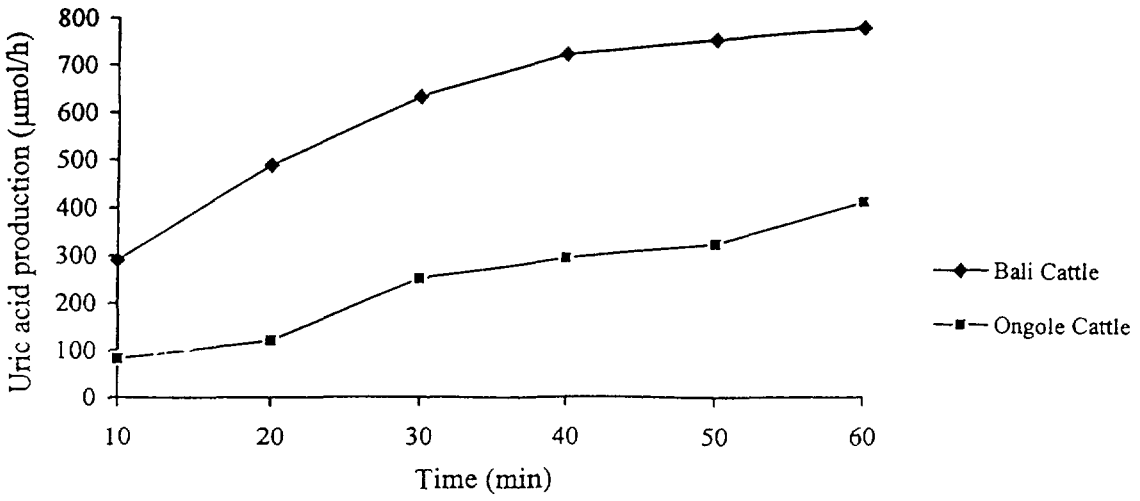

FIG.5. Xanthine oxidase activity in the liver of Bali and Ongole cattle.

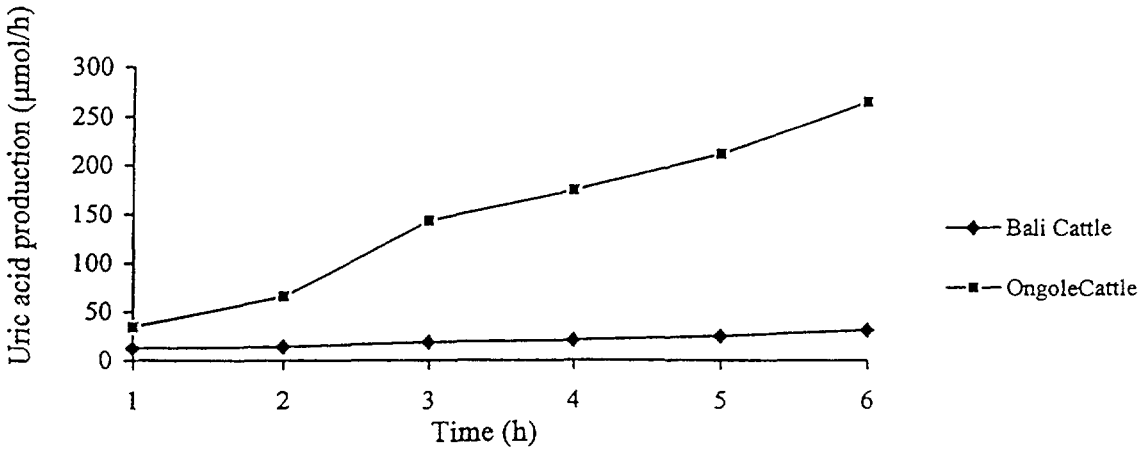

FIG.6. Xantine oxidase activity in the intestinal mucosa of Bali and Ongole cattle.

The liver XO activity in Bali and Ongole cattle was 0.191 and 0.131 µmol/min/g tissue, respectively, while the activity of the intestinal mucosa was 0.001 and 0.015 µmol/min/g tissue. The XO activity in intestinal mucosa of Ongole cattle was in the range described by Chen *et al* [3] in European cattle. It appears that XO activity converts most of the absorbed purines into uric acid and therefore became unavailable for tissue nucleic acid synthesis. In Bali cattle, however, the activity of xanthine oxidase was low and therefore absorbed purines could have entered the liver unchanged and became available for salvage. The low XO activity in gut mucosa of Bali cattle would have been compensated by much higher liver and blood enzyme activity resulting in similar PD excretion in the two species.

3.3.2. Uricase

There was a slight activity of uricase in plasma of both breeds (Figure 7). These results were in agreement with the results reported by Chen *et al.* [3]. They stated that there was no metabolism of uric acid in cow or pig plasma, indicating the absence of uricase. They showed that in sheep plasma, there was slight uricase activity.

It seems that there was no uricase activity in the intestinal mucosa of both Bali and Ongole cattle. The rate of uric acid degradation by liver uricase of Bali and Ongole cattle was 0.035 and 0.028 μmol/h per reaction mixture. This is equivalent to an activity of 1.46 and 1.17 nmol/min per g tissue.

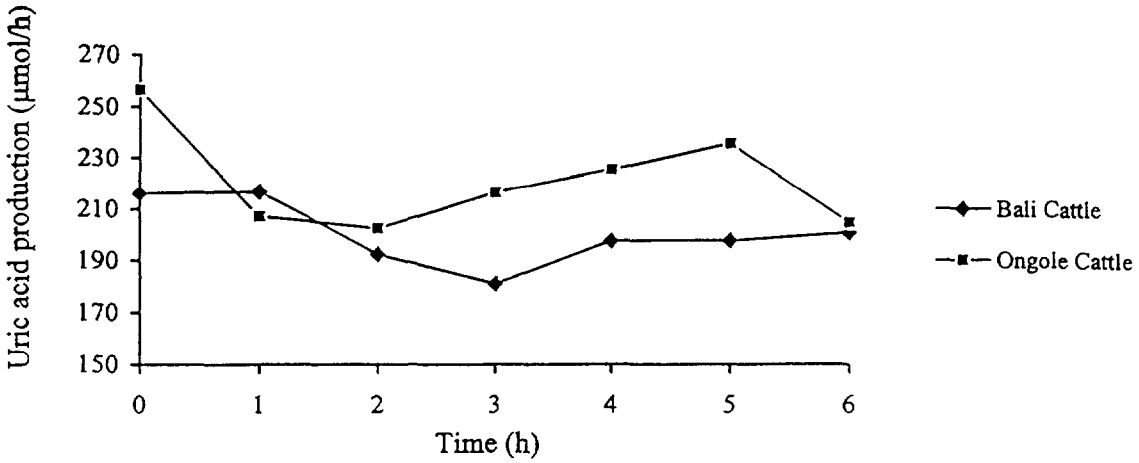

FIG.7. Uricase activity in the plasma of Bali and Ongole cattle.

4. CONCLUSIONS

These experiments showed that the endogenous PD excretion in both Bali and Ongole cattle were lower than endogenous PD excretion reported by Chen *et al.* [1], therefore the equation between PD excretion and PD absorbed which has been postulated by Chen *et al.* [8] should be adjusted. The endogenous PD excretion for Bali cattle was 145 μmol/kg $W^{0.75}$/d while for Ongole cattle it was 132 μmol/kg $W^{0.75}$/d.

These experiments demonstrated that not all of the PD in the blood is excreted in the urine and therefore the PD excretion via non-renal routes should be taken into account when PD excretion is used to predict microbial protein syntheses in the rumen. The proportion of plasma PD excreted in the urine of Bali and Ongole cattle fed 90% level of intake was 0.78 and 0.77 respectively. At 60% level of feeding the proportion of pasma PD excreted was 0.66 and 0.50 for Bali and Ongole cattle respectively.

The prediction of purine absorbed based on PD excretion can be stated as: $Y = 0.78\ X + 0.145\ W^{0.75}$ and $Y = 0.77X + 0.132\ W^{0.75}$ for Bali and Ongole cattle respectively. The coefficient of 0.21 and 0.215 found for the ratio of purine N:total N in mixed rumen microbes of Bali and Ongole cattle, could be used for calculating microbial N production.

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URINARY EXCRETION OF PURINE DERIVATIVES AS AN INDEX OF MICROBIAL PROTEIN SUPPLY IN CROSS-BRED (*Bos indicus* × *Bos taurus*) CATTLE IN TROPICAL ENVIRONMENT

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Abstract

URINARY EXCRETION OF PURINE DERIVATIVES AS AN INDEX OF MICROBIAL PROTEIN SUPPLY IN CROSS-BRED (*Bos indicus* × *Bos taurus*) CATTLE IN TROPICAL ENVIRONMENT.

Four experiments were carried out to establish a response model between urinary excretion of purine derivatives (PD) and microbial production in *Bos indicus* × *Bos taurus* cross-bred cattle: LZ, MZ and HZ (3/8, 1/2 and 5/8 *Bos indicus*, respectively). The fasting PD excretion was considered as endogenous excretion and amounted to 268 (± 85.1), 294 (± 128.1) and 269 (± 68.4) μmol/kg $W^{0.75}$ for LZ, MZ and HZ, respectively. Urinary recovery of absorbed purine bases (PB) was calculated as the urinary recovery of a single dose of intrajugular infused uric acid (1,3-^{15}N). In HZ crossbred cattle 83% (± 20.3)of infused uric acid was recovered in the urinary PD. The relationship between duodenal purine absorption (X, mmol/d) and urinary PD excretion (Y, mmol/d) was defined in HZ crossbred cattle as $Y = 0.83\ X + 0.269W^{0.75}$ (± 85.1), assuming that the endogenous contribution was constant and independent of the exogenous PB supply. The activity of xanthine oxidase (EC 1.2.3.2.) was determined in HZ and MZ and was found to be higher in the liver (0.62 and 0.66 units/g, respectively) than in intestinal mucosa (0.09 and 0.03 units/g, respectively), whereas xanthine oxidase activity was practically absent in plasma of both cross breeds. The ratio PB:total N was determined in microbial extracts taken from rumen fluid of cows fed Bermuda grass *(Cynodon dactylon)* as the sole diet or supplemented (ratio of 80:20, grass:supplement) with gluten feed, soybean hulls or *Gliricidia* species and were found to range from 1.52-1.62 μmol PB/mg N.

1. INTRODUCTION

Urinary excretion of purine derivatives (PD: i.e. allantoin, uric acid, hypoxanthine and xanthine) has been used as an index to estimate rumen microbial synthesis in sheep, goats and cattle [1-5]. This is a non-invasive method that only requires urine collection and so is suitable for use under farm conditions to monitor the status of microbial protein supply to the animal. Published methods refer only to the above cited species and it would be desirable to extend them to other species or breeds of economic significance in tropical areas. However, probable species differences in the metabolism of purine bases may prevent the direct application of the existing model [6].

The aim of this study was to obtain a response model to predict microbial protein supply in *Bos indicus* × *Bos taurus* crossbred cattle on the basis of: (i) the endogenous contribution of PD to urinary excretion, (ii) the relationship between duodenal absorption and urinary excretion of purine derivatives and (iii) tissue profile of xanthine oxidase (EC 1.2.3.2.) as a key enzyme of purine base metabolism [7]. In the present trails, changes in the concentration of purine bases in microbial N as induced by diet, were also studied.

2. MATERIAL AND METHODS

2.1. **Experiment I** Urinary excretion of PD during fasting

2.1.1. Animals and diets

Eighteen male, crossbred (*Bos taurus* × *Bos indicus*) cattle of approximately 2 years of age were used in this experiment. The crossbred cattle were either 3/8 *Bos indicus* (LZ) (271 ± 8.1 kg LW); 1/2 *Bos indicus* (MZ) (246 ± 7.7 kg LW) or 5/8 *Bos indicus* (HZ) (366 ± 19.8 kg LW). Six animals from each category were housed individually in metabolism cages and were fed hay (*Cynodon dactylon*) *ad libitum,* for 15 days (adaptation period). Thereafter, feed intake was reduced every 2 days and feed was offered at 60, 30, and 0%, of the previously registered *ad libitum* intake (restriction period), and then fasted for 7 days (fasting period).

2.1.2. Sample collection

On day 7 of the adaptation period urine was collected into buckets containing 200 ml 1M-H_2SO_4 in order to maintain the pH of urine below 3. The daily collection of urine was weighed, density measured and 1% of total daily excretion was diluted to 1 L with distilled water. On day 14, blood samples were taken from the tail vein every 8 h. Thereafter, blood samples were taken every 24 h until the end of the fasting period. Blood samples (15 ml) were centrifuged at 1 500 rpm for 15 min and plasma was frozen for subsequent analyses.

2.2. **Experiment II** Urinary excretion of PD and isotope recovery at different levels of feed intake

2.2.1. Animals and diets

Four crossbred cattle (HZ) with a mean live weight of 328 ± 11.7 kg were used. Animals were individually housed and randomly allocated to four dietary treatments in a 4x4 Latin Square design. Bermuda grass hay (*Cynodon dactylon*) was given at four levels: 95 (D95), 80 (D80), 60 (D60) or 40% (D40) of *ad libitum* (120 g DM/kg $W^{0.75}$) intake.

2.2.2. Experimental procedure and sample collection

Each experimental period lasted for 21 days, allowing 11 days for dietary change over and 10 days for experimental measurements. The following schedule was employed: Urine and faeces were collected from day 12 to 21 and isotope infusion was carried out from day 18 to 21. Faeces was collected daily, homogenised and sampled (10% of total excretion) and stored at 4°C until analysis. The urine was collected daily in buckets containing an acidic solution (as in Experiment I) and weighed. Specific gravity was recorded and urine was sampled and stored. On day 18, 200 mg of labelled uric acid (1,3-^{15}N 98%+; Isomed, Madrid, Spain) were diluted with 100 ml of 50% (v/v) glycerol/saline solution and made alkaline to pH 8 with 2M NaOH solution to obtain complete solubilization of the uric acid. The uric acid solution was autoclaved and slowly infused through the jugular vein to two animals on each of the treatments D40 and D95. After isotope infusion, urine was sampled every 6 h during 4 days, weighed, specific gravity recorded and 2 sub-samples (2%) stored at -20°C. Blood samples were also taken every 12 h as described in Experiment I.

2.3. **Experiment III** Xanthine oxidase activity in plasma, liver and intestinal tissue

Samples of plasma, liver and intestinal tissues were taken from six animals (3, HZ and 3, MZ cross-breds) at a local slaughter house for measuring xanthine oxidase activity.

Blood samples were collected into heparinized tubes and centrifuged at 3 000 × *g* for 15 min. Plasma samples were analysed within the same day. The procedure for collection, processing and extraction of liver and intestinal samples were as described in IAEA-TECDOC-945 [8]. Activity of xanthine oxidase was measured as the rate of uric acid production when xanthine was incubated with tissue extracts [8].

2.4. **Experiment IV** Microbial composition of rumen samples

Four, five year old zebu crossbred cows (MZ) (348 ± 48.5 kg), each fitted with a rumen cannula (12 cm Ø) were used in this study. Animals were randomly allocated to four dietary treatments in a 4 x 4 Latin Square design. Treatments consisted of four experimental diets: Bermuda grass hay, given as the sole diet (C, 100%) or at 80% and supplemented with either 20% gluten feed (GF), soybean hulls (SH) or dry *Gliricidia* foliage (*Gliricidia sepium*, G). Animals were housed in individual pens and were fed *ad libitum* (120 g/kg $W^{0.75}$/d) twice a day at 8.00 and 16.00 h, for 2 weeks. On the last day rumen contents were sampled.

The whole rumen contents (500 ml) were squeezed through surgical gauze to remove solid material. Liquid associated bacteria were isolated from the filtrate by centrifuging at 500 × *g* for 5 min, followed by two consecutive centrifugations at 20 000 × *g* for 20 min at 4°C. The microbial extracts were freeze dried for subsequent analysis.

2.5. **Analytical procedure**

Dry matter was determined by drying the samples to constant weight at 105°C and organic matter by ashing at 550°C for 8h. NDF and ADF contents were determined by the procedures of Goering and Van Soest [9]. The total nitrogen content was determined by the Kjeldahl method. Allantoin in acidified urine was determined by colorimetry [10]. Adenine and guanine (PB) in samples of rumen bacteria (15 mg) were determined either by HPLC, after acid hydrolysis with 2 ml 2N-perchloric acid at 100°C for 1h and the addition of 0.20 µmol of allopurinol and immediate neutralisation with 4.5 M KOH [11] or by silver ion precipitation following Zinn and Owens [12]. Creatinine concentration was measured using picric acid [13]. Uric acid was measured by the bound uricase method [8]. The isotope enrichment of ^{15}N was determined using gas Chromatography/Mass Spectrometry following Chen *et al.* [14].

3. RESULTS AND DISCUSSION

3.1. **Urinary excretion of PD during fasting**

The mean daily excretion of urinary PD (allantoin, uric acid and total PD) are presented in Table I. The effect of fasting on PD excretion is shown in Figure 1. Restriction of feed lead to a rapid decrease in urinary excretion of allantoin and total PD, reaching a stable basal value after about 5 d. In the present paper this value was considered as the endogenous contribution to urinary excretion, assuming that duodenal flow of purine bases would represent only a minor fraction.

PD excretion during fasting from day 12 to 14 averaged 266 ± 75, 274 ± 28.7 and 268 ± 42.4 µmol/kg $W^{0.75}$ for HZ, MZ and LZ breed types, respectively. There were no differences

between the breed types. Fasting PD excretion showed a range between 160.5 and 394.2 μmol/kg $W^{0.75}$ and the CV of daily measurements were 17.3, 15.8 and 17.2% for LZ, MZ and HZ, respectively. Creatinine excretion was independent of the animal type and was 384 ± 56.1 μmol/kg $W^{0.75}$.

TABLE I. DAILY URINARY EXCRETION OF PD (μmol/kg $W^{0.75}$) IN THREE DIFFERENT TYPES OF ZEBU × EUROPEAN CROSS-BRED CATTLE (LZ, MZ, AND HZ), PRIOR TO FASTING (PF), UNDER RESTRICTED FEEDING (R) AND DURING FASTING (F)

	Breed type		
	LZ ± sd	MZ ± sd	HZ ± sd
Allantoin			
PF	450.9 ± 170.4	408.9 ± 125.1	452.1 ± 140.7
R	285.3 ± 95.2	339.2 ± 115.2	346.5 ± 166.0
F	236.5 ± 67.7	259.8 ± 127.7	244.5 ± 88.5
Uric acid			
PF	30.2 ± 4.3	31.4 ± 3.0	24.8 ± 1.5
R	30.4 ± 2.5	31.2 ± 1.9	25.1 ± 2.0
F	32.3 ± 2.6	33.9 ± 3.0	24.8 ± 1.5
Total PD			
PF	481.1 ± 171.9	440.4 ± 125.9	476.9 ± 141.2
R	315.7 ± 95.0	370.4 ± 115.7	371.6 ± 167.5
F	268.8 ± 68.4	293.7 ± 128.1	269.4 ± 85.1

Basal excretion of PD during fasting was higher than values reported for sheep (136 to 202 μmol/kg $W^{0.75}$) [1, 15-17] and goats (195 μmol/kg $W^{0.75}$) [3], and lower than values reported for cattle (455 to 609 μmol/kg $W^{0.75}$) [18-20]. Osuji *et al.* [21] reported much lower values of 172 μmol/kg $W^{0.75}$ and 108 μmol/kg $W^{0.75}$ for zebu and zebu cross-bred cattle, respectively. In swamp buffalo, Liang *et al* [22] found a similar PD excretion level during fasting, for both allantoin (228 μmol/kg $W^{0.75}$) and uric acid (152.6 μmol/kg $W^{0.75}$) with a total PD excretion during fasting of 380 μmol/kg $W^{0.75}$. It is not possible to determine whether the differences are due to genetical or to methodological effects but lower PD excretion could be related to a differential capability of N re-utilization depending on the species [6] or breed type.

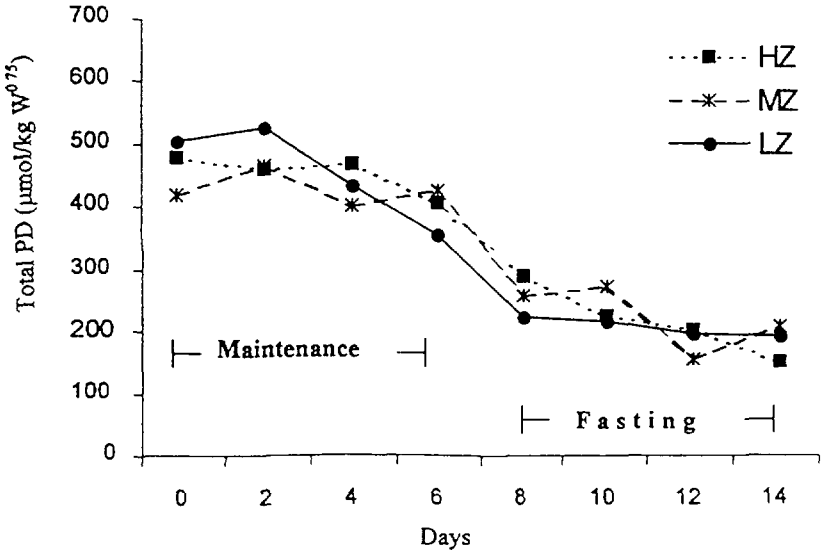

FIG.1. Daily urinary excretion of PD in cross-bred Bos indicus x Bos taurus cattle (LZ, MZ and HZ) during feed restriction and fasting.

3.2. Urinary excretion of PD at different levels of feed intake

The daily urinary excretion of PD at different levels of intake are presented in Table II. As in Experiment I, allantoin and uric acid accounted for all urinary PD. The PD excretion responded significantly to digestible organic matter intake (DOMI). This increase is mainly explained by the significant response in alloantoin excretion, although intermediate metabolites such as uric acid showed a lower but significant increase. When urinary excretion of PD (mmol/d) was plotted against DOMI, the following equation was obtained.

$$Y = 7.69 (\pm 4.2) + 5.69 (\pm 1.68) X$$

(where n = 16, RSD = 5.22 and r = 0.67)

Although in all cases, values were characterised for high residual variations (CV= 18%), the slope of the regression indicates that the rate of PD excretion per unit of DOMI (5.67 ± 1.68 mmol/kg DOMI) was much lower than values reported for *Bos taurus* (18.5mmol/kg DOMI) [20] or buffalo (8.3 mmol/kg DOMI) [22]. Individual variation adjustment procedure together with the use of different experimental conditions may explain partially the variation registered, yet results seem to suggest that *Bos indicus* shows a lower response to intake of digestible organic matter in terms of urinary allantoin excretion.

TABLE II. INTAKE (kg/d) OF DRY MATTER (DM), ORGANIC MATTER (OM) AND DIGESTIBLE ORGANIC MATTER (DOM) AND URINARY EXCRETION OF CREATININE AND PD (μmol/kg $W^{0.75}$) IN ZEBU CATTLE AT DIFFERENT LEVELS OF FEED INTAKE

	Level feed intake (%)					
	40	60	80	95	SEM	Significance
Intake						
DM	3.50	5.60	7.38	8.85	2.08	**
OM	2.84	4.74	6.26	7.51	1.82	**
DOM	1.35	2.16	2.84	3.41	0.80	**
PD excretion						
Allantoin	15.8	17.0	20.2	20.6	3.01	**
Uric acid	1.95	2.02	2.10	2.12	0.18	NS
Total PD	17.9	19.1	22.3	22.7	2.78	*
Creatinine	533.5	468.3	487.0	431.5	68.2	NS

Statistical significance of the effect of diet (D)
NS, Not significant; * , P <0.05, ** , P <0.01

3.3. Isotope recovery

The relationship of the response model between PD excretion and purine absorption has been defined by infusing known amounts of nucleic acid through the abomasum or duodenum and monitoring the excretion of PD in urine. In cattle, this relationship has been described as a linear model Y= a + bX (where Y = urinary PD and X = duodenal PB [2, 23], "a" the intercept representing the endogenous contribution and "b" the proportion of plasma PD excreted in

urine and representing the incremental recovery in urine of absorbed PB. Such a parameter could be estimated by direct recovery of labelled-PD as uric acid when it is administered directly through the jugular vein [8]. In Table III, the amount of labelled uric acid infused and PD (allantoin plus uric acid) recovered together with isotope recovery in urine are presented.

TABLE III. RECOVERY AS URINARY PD (ALLANTOIN PLUS URIC ACID) OF LABELLED URIC ACID (1,3-^{15}N) INFUSED THROUGH THE JUGULAR VEIN IN TWO CROSSBRED ZEBU CATTLE (HZ) FED DIFFERENT LEVELS OF BERMUDA GRASS (*Cynodon dactylon*)

Animal	^{15}N Uric acid infused (μmol/animal)	^{15}N Urinary recovery (μmol/animal)	Recovery (%)
1	1.07	0.961	88
2	1.05	0.822	78
Mean ± SEM	1.06 ± 0.1	0.891 ± 0.2	83 ± 20.6

The concentration of ^{15}N-uric acid in plasma decreased exponentially due to its excretion in urine as well as by oxidation to allantoin. Therefore, 36 h after the injection of the labelled material no enrichment was detected in the urine samples. With the reservation that the number of animals was small, the results showed that 84% of plasma ^{15}N-uric acid was excreted in urine. The authors are unaware of data in zebu cattle, but those corresponding to *Bos taurus* seem to be in a similar range. Thus, Verbic *et al* [2] found a recovery of 77% and Beckers and Thewis [23] reported a recovery of 74%. Lower values have also been reported by McAllan *et al* [24] in *Bos taurus* cattle with 65 and 40%, where intraduodinally infused adenine and guanine were recovered as urinary metabolites. Although the recovery ratio agrees well with the proportion of renal and non-renal partitioning proposed by Chen *et al* [16] (84 and 16% respectively), such recovery levels do not explain the low response in PD excretion to DOMI obtained in this species. It is necessary to emphasise that when comparing both methodological approaches the isotope recovery trial does not take into account digestion, absorption and metabolism of duodenal nucleic acid.

3.4. Xanthine Oxidase activity in plasma, and liver and intestinal tissues

Figure 2 (A, B and C) shows the increase in uric acid when xanthine was incubated with different tissue extracts as a measure of their xanthine oxidase (XO) activity. Estimated values of XO activity (units/min/g tissue) in plasma and intestine are presented in Table IV. The XO activity was much higher in the liver than in the intestine (0.62 and 0.66 vs 0.09 and 0.03 for HZ and MZ, respectively) whereas plasma showed only traces of XO activity. The differences between tissues confirmed previous results in sheep [5], cattle [16], buffalo [6] and rabbits [25]. However zebu cattle, *Bos taurus* cattle and sheep showed much higher values in the liver than in the gut, while in buffalo and rabbit there was very little activity in the liver. When data were analysed individually for each tissue, HZ showed a consistently higher XO activity in the gut than in MZ ($P < 0.05$) while data from liver and plasma were more variable.

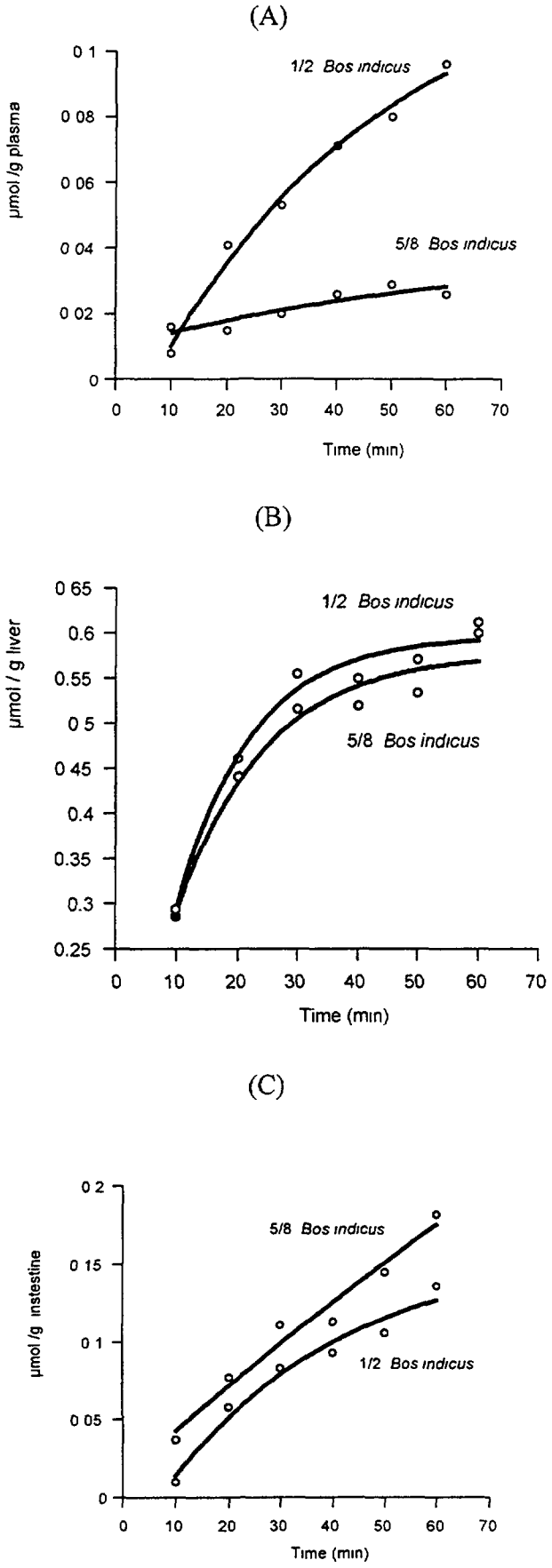

FIG.2. Production of uric acid when xanthine was incubated with plasma (A) liver (B) or extract of intestinal mucosa (C) of MZ (1/2) and HZ (5/8) Bos indicus × Bos taurus crossbred cattle.

TABLE IV. ACTIVITY OF XANTHINE OXIDASE (EC 1.2.3.2) IN PLASMA, LIVER AND INTESTINAL MUCOSA OF MZ AND HZ *BOS INDICUS* × *BOS TAURUS* CROSSBRED CATTLE.

Tissue	Breed type	Animals 1	2	3	Mean	SD
Plasma	HZ	0.92	0.77	0.83	0.83	0.075
(unit/L)	MZ	3.16	8.33	7.71	6.37	2.82
Liver	HZ	0.29	0.85	0.72	0.62	0.29
(unit/g wet tissue)	MZ	0.45	0.76	0.76	0.66	0.17
Intestine	HZ	0.09	0.09	0.08	0.09	0.006
(unit/g wet tissue)	MZ	0.02	0.02	0.04	0.03	0.092

3.5. Composition of microbial extracts

Table V shows the composition (N, mg/g DM; PB, μmol/g DM and PB/N ratio) of microbial samples extracted from the liquid fraction of rumen fluid. PB was analysed by both colorimetry [12] and by HPLC methods [11]. In general, there was a tendency for colorimetric methods to overestimate PB values, but differences between colorimetric and HPLC methods were not statistically significant. Such overestimation could be explained by the non-specificity of the calorimetric methods.

TABLE V. COMPOSITION OF MICROORGANISMS ISOLATED FROM THE LIQUID FRACTION OF RUMEN CONTENTS IN ZEBU CROSSBRED CATTLE FED *AD LIBITUM* BERMUDA GRASS GIVEN AS THE SOLE DIET (C, 100%), OR AT 80% AND SUPPLEMENTED EITHER WITH 20% OF GLUTEN FEED, SOYA BEAN HULLS OR DRY GLIRICIDIA FOLIAGE (*Gliricidia sepium*).

Diet	N (g/kg)	Purine Bases μmol/mg N (mgPB-N/100 mg N) HPLC [11]	Zinn & Owens [12]
Hay (*Cynodon* spp)	8.25 ± 1.57	1.56 (11.0)	1.77 (12.4)
Gluten feed	7.69 ± 1.38	1.61 (11.3)	1.93 (13.5)
Soybean hulls	7.81 ± 0.79	1.52 (10.6)	1.80 (12.6)
Gliricidia foliage	7.29 ± 1.33	1.55 (10.8)	1.46 (10.2)
RSD 1	-	3.46	0.18
RSD 2	-	2.33	0.21

Type of diet affected PB content (μmol/g DM) and microbial extracts taken from animals fed hay or gluten feed, showed lower values than those taken from animals fed soybean hulls or Glyricidia ($P < 0.05$). When values were expressed as μmol/mg N there was no difference among extracts.

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MEASURING MICROBIAL PROTEIN SUPPLY FROM PURINE EXCRETION IN YERLI KARA CATTLE IN TURKEY

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Abstract

MEASURING MICROBIAL PROTEIN SUPPLY FROM PURINE EXCRETION IN YERLI KARA CATTLE IN TURKEY.

The urinary excretion of purine derivatives (PD) was measured in four Yerli Kara (*Bos indicus*) bulls in two experiments, a fasting experiment lasting for 7 days and the other, where animals were given a diet containing 30% wheat straw and 70% compounded feed at four levels of intake (40, 60, 80 and 95% of voluntary feed intake). In the second experiment, which was carried out according to a 4x4 Latin Square design, four animals receiving 60 and 95% levels of intake were also given a single injection of 8-^{14}C-uric acid via a jugular catheter. In addition to the above two experiments, the activity of xanthine oxidase and uricase in plasma, liver and intestinal mucosa obtained from Yerli Kara cattle was also determined.

In the first experiment, fasting PD excretion averaged 0.691 (± 0.053) mmol/kg $W^{0.75}$/d. Glomerular filtration rate (GFR), tubular load and net re-absorption of allantoin between pre-fasting and fasting were statistically significant ($P < 0.05$). In the second experiment the recovery of injected 8-^{14}C-uric acid as total PD was 72.5 and 89.9% for 60 and 95% feeding levels, respectively. The average recovery was 81%. Plasma kinetics measured by 8-^{14}C-uric acid indicated that the total compartment pool size was 214.0 (± 43.8) and 250.3 L (± 29.5) for 60 and 95% feeding levels, respectively. GFR, tubular load and net re-absorption of uric acid and allantoin were not affected by feed intake. The allantoin:PD molar ratios changed between 0.78 to 0.93 for the four levels feed intake. There were significant correlations between PD excretion (mmol/d), and DDMI (kg/d) and DOMI (kg/d) ($r = 0.99$, $P < 0.01$). The rate of PD excretion as a linear function of feed intake was 16.4 mmol/kg $W^{0.75}$ DDMI, 19.8 mmol/kg DDMI and 22.7 mmol/kg DOMI. Xanthine oxidase and uricase activities were; 1.34 (± 0.72) and 0.44 (± 0.05), and 0.13 (± 0.03) and 0.08 (± 0.03) unit/g fresh tissue in liver and intestinal mucosa, respectively. In plasma xanthine oxidase activity was 5.0 (± 1.2) unit/L while uricase activity was absent.

1. INTRODUCTION

The purine excretion technique has been developed and used to estimate microbial protein supply in European breeds of cattle and sheep [1-3]. Species differences in purine metabolism of ruminants are attributed to the level of endogenous purine excretion, the utilization ability of exogenous purines and enzyme profiles which are involved in purine degradation [4, 5]. The urinary excretion of purine derivatives (PD) (i.e. allantoin, uric acid, hypoxanthine and xanthine) by different ruminants has been shown to be positively but not linearly, related to digestible organic matter intake (DOMI) [6]. Only allantoin and uric acid

are present in cattle. This is because in cattle, the activity of xanthine oxidase in the blood and tissues convert xanthine and hypoxanthine into uric acid prior to excretion in the urine [7].

The current prediction models may not be suitable for all types of ruminants, and may need to be refined and standardised to extend the methodology to breeds of cattle other than European.

The objective of this study was the development and standardization of the prediction equation for the estimation of microbial protein supply for indigenous (Yerli Kara) cattle in Turkey. For this reason PD excretion, the response of PD to feed intake and the proportion of plasma PD excreted in the urine were studied, along with the tissue profiles of enzymes involved in purine metabolism.

2. MATERIALS AND METHODS

2.1. **Experiment I** Estimation of basal PD excretion (Fasting trial)

2.1.1. Animals and feeding

Four bulls (indigenous Yerli Kara cattle) between the ages of 2-5 years and of average live weight 346 ± 79 kg were used in this experiment. They were given, 4.0 kg/d of a diet containing 3.0 kg compounded feed (12 MJ ME/kg DM) and 1.0 kg/d wheat straw (6 MJ ME/kg DM), for three weeks. The compounded feed containing 67% barley, 20% sunflower cake, 10% wheat bran, 1% salt, 1.5% marmer dust and 0.5% mineral and vitamin mixture, was offered in two equal meals at 0830 and 1630 h. Straw was offered at mid day. At the beginning of the fourth week daily feed intake was reduced stepwise within 3 days (60, 30, and 0%, on day 1, 2 and 3, respectively), and thereafter the animals were fasted for 7 days. During the experimental periods the animals were kept in metabolism cages and fitted with urine collection aprons.

2.1.2. Urine and plasma sampling and analysis

After 2 weeks of adaptation total urine was collected daily into containers containing 10% sulphuric acid (final pH of urine was kept below 3), weighed, diluted and sub-sampled. Urine samples were stored at -20°C until analysed. During week 3 of adaptation and fasting periods, a 10 ml blood sample was taken daily into heparinized vacutainers from each animal, at a fixed time (1400 h). Plasma was stored at -20°C until analysis.

Urine samples were analysed for total N [8], and urine and plasma samples were analysed for allantoin, uric acid and creatinine [9]. Urine, allantoin and creatinine excreted were expressed as μmol/kg $W^{0.75}$/d. Basal nitrogen excretion was calculated in mg/kg $W^{0.75}$/d. Glomerular filtration rate (GFR) and renal clearance of allantoin and uric acid were estimated using urine and plasma allantoin, uric acid and creatinine concentrations [10].

2.2. **Experiment II** Response of PD excretion to feed intake and measurement of the proportion of plasma PD excreted in the urine

2.2.1. Animals, feeding and treatments

The same bulls used in Experiment I were housed in metabolism cages and fitted with urine collection aprons. Animals were fed a mixed diet containing 30% wheat straw and 70% compounded feed, as in Experiment I. The diet contained 90% DM, its N and OM contents were 124 and 950 g/kg DM, respectively. The diet was offered twice daily at 0830 and 1630 h, in two equal meals. Fresh water was available freely. During the preliminary period, the diet was given at *ad libitum* level of intake. Voluntary intake was measured for each animal

over a period of 2 weeks. After the preliminary period, animals were fed at 4 fixed levels as 95, 80, 60 and 40% of the voluntary intake. Voluntary intakes were 8 kg/d for three bulls and 13 kg/d for the other. The treatments were allocated according to a 4 × 4 Latin Square design. Each feeding period lasted for 3 weeks.

2.2.2. Urine and plasma sampling and analysis

During the last 10 days of each feeding period urine was collected daily, and during the first 3 days of the collection period faeces were also collected in addition to urine. On the 3rd day of each sample collection period, the animals were given an intravenous administration of a tracer (8-^{14}C-uric acid, 52 mCi/mmol, Amersham Inst. Code CF Q9786) by single injection via a jugular catheter. Due to the high cost of the tracer, its kinetics was only measured in 2 treatments (95 and 60% of the voluntary intake). 1 ml aliquots of tracer solution (300 μCi) was prepared and stored at -20°C. Aliquots were diluted to 50 ml with saline before injection. Each animal received 45-47 ml of tracer (270-282 μCi). Urine was collected after 6h, and thereafter daily until day 7 of the dose administration, and sub-sampled. Blood samples were collected at 0.5, 1, 2, 3, 4, 6, 8, 12, 16, 24 and 28 h after tracer administration. Plasma and diluted urine sub-samples were stored at -20°C until analysis. The activity of tracer solution was estimated from the established linear response curve of counted activity vs amount of tracer solution added.

Urine and plasma samples were analysed for allantoin, uric acid and creatinine [9]. Total N in urine and faecal samples [8] and DM and OM in faecal samples were also determined. Urine allantoin, uric acid and creatinine (mmol/d) and plasma allantoin, uric acid and creatinine (mmol/L) were measured and GFR and renal clearance of PD were calculated using plasma and urine values.

Urine and plasma sample preparation and analysis for ^{14}C-activity were made according to the procedures given in the IAEA TECDOC [10]. Recovery of administered 8-^{14}C-uric acid in urine was calculated as the proportion of the administered dose excreted in the urine. Fractional rates of clearance from the blood and pool size of compartments in the blood were estimated using plasma 8-^{14}C counts, following the method proposed by Chen and Franklin [11].

2.3. Experiment III Measurement of xanthine oxidase and uricase activities in plasma, liver and intestinal tissues

Samples of plasma, liver and intestinal tissues were taken either at the local slaughter house (liver and intestine) or at the Lalahan Livestock Research Institute (plasma), Ankara. All samples were collected randomly with respect to nutritional status, age, or sex.

2.3.1. Samples and preparation of tissue extracts

Jugular blood samples collected from six Yerli Kara bulls into heparinized tubes, were centrifuged at 1 500 *g* for 15 min at 4°C and plasma was used for the assay within 2h.

Liver samples were taken from four bulls and washed in cold 0.15 M KCI solution, 30 min after slaughtering, blotted dry and frozen immediately. 3 g of liver sample was homogenized in 27 ml 0.5 mM EDTA in 0.05 M KH_2 PO_4 (pH 7.5) in a glass homogenizing tube with a Teflon pestle. The other steps of extraction were as described by Chen *et al.* [5].

Intestinal and liver samples were taken from the same animals. The first 30 cm segment of the small intestine was taken and the lumen washed with cold 0,15 M KCI solution and then with 0,05 M N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffer (pH 7.5) containing 0.25 mM EDTA and 0.25 mM phenylmethylsulphonyl fluoride (PMSF). The segment of intestine was cut length wise and mucosal cells were taken with a spatula. 4 g

of mucosal cell sample was homogenized in 36 mL of the HEPES-EDTA-PMSF buffer and centrifuged, and the supernatant was dialysed at 4°C for 24 h against the same buffer. The contents of the bag were centrifuged at 40 000 *g* for 30 min at 4°C and the supernatant was used for determining the enzyme activity.

2.3.2. Xanthine oxidase and uricase activities

The activity of xanthine oxidase (XO) was measured as the rate of uric acid produced when xanthine was incubated with plasma, liver or intestinal extracts. The activity of uricase was measured as the rate of uric acid disappearance when uric acid was incubated with plasma, liver or intestinal extracts. The activity of the enzymes was measured by spectrophotometric methods [10]. One unit of XO activity was defined as 1 μmol uric acid produced per min at 37°C. One unit of uricase was defined as 1 μmol uric acid decreased per min at 37°C.

2.4. Statistical analysis

The relationship between PD excretion and feed intake; plasma PD and creatinine concentrations and urinary PD and creatinine excretion were examined by linear regression analysis. Single-factor ANOVA was performed to compare the differences between PD, creatinine and total nitrogen excretion measurements between the feeding and fasting periods in Experiment I, and between the four levels of feed intake in Experiment II. The same analysis was carried out to compare differences between recoveries of injected 8-^{14}C uric acid, plasma kinetic parameters (Experiment II), GFR and tubular load of PD in Experiments I and II. The statistical analysis was aided by Minitab.

3. RESULTS

3.1. Experiment I Estimation of basal PD excretion (Fasting trial)

Xanthine and hypoxanthine were not detected in Yerli Kara bull urine. Therefore, the sum of allantoin and uric acid is regarded as the total PD excretion. Excretion of allantoin, uric acid, total PD, creatinine and total nitrogen were different during pre-fasting and fasting periods (Table I). The average excretion of total PD, and the relative proportions of allantoin and uric acid in total PD were, 1011.8 (± 237) and 691 (± 53) μmol/kgW $^{0.75}$/d, 0.93 and 0.94 and 0.07 and 0.06, for pre-fasting and fasting periods, respectively. The ratios of urinary PD-N:total-N during pre-fasting and fasting were significantly different ($P < 0.001$); the average values were 0.88 (± 0.10) and 0.58 (± 0.10), respectively.

TABLE I. COMPARISON OF DAILY URINARY EXCRETION OF PD, CREATININE AND TOTAL NITROGEN IN FOUR BULLS DURING PRE-FASTING AND FASTING

	Pre-fasting		Fasting	
	Mean	SD	Mean	SD
Allantoin (μmol/kg W$^{0.75}$/d)	942.0^{a}	239.5	651.9^{a}	44.4
Uric acid (μmol/kg W$^{0.75}$/d)	73.3^{b}	18.2	39.5^{b}	13.9
PD (μmol/kg W$^{0.75}$/d)	1011.8^{c}	237.5	691.4^{c}	53.5
Creatinine (μmol/kg W$^{0.75}$/d)	322.0^{d}	92.0	427.0^{d}	30.9
Nitrogen (mg/kg W$^{0.75}$/d)	354.0	96.0	407.4	61.2
PD-N:total-N (μmol/kg W$^{0.75}$/d)	0.88^{e}	0.10	0.58^{e}	0.10

$^{a, b, c, d, e}$ Mean values with the same superscripts in the same row are significantly different (a, c, e, $P < 0.001$; b, $P < 0.01$; d, $P < 0.05$)

TABLE II. GFR AND RENAL CLEARANCE OF PD IN BULLS DURING PRE-FASTING AND FASTING

	Pre-fasting (n=12)		Fasting (n=12)	
	Mean	SD	Mean	SD
GFR (L/d)[a]	614.0[d]	100.0	454.9[d]	92.0
Tubular load (mmol/d)[b]				
Allantoin	494.6[e]	16.0	233.4[e]	52.9
Uric acid	28.1	10.0	21.7	3.0
Net re-absorption (mmol/d)[c]				
Allantoin	421.0[f]	16.0	176.5[f]	47.1
Uric acid	20.8	10.0	18.4	3.4

[a] GFR (L/d) = Creatinine excretion in urine (mmol/d) ÷ plasma creatinine (mmol/L)
[b] Tubular load of purine derivatives (mmol/d) = GFR (L/d) x plasma PD (mmol/L)
[c] Net re-absorption of purine derivatives (mmol/d) = Tubular load (mmol/d) - excretion in urine (mmol/d)
[d, e, f] Mean values with the same superscripts in the same row are significantly different (d, $P < 0.05$; e, f, $P < 0.01$)

3.2. Experiment 2 Response of PD excretion to feed intake and measurement of the proportion of plasma PD excreted in the urine

Table III shows the recovery of injected dose of 8-^{14}C-uric acid in urine of four bulls fed at two feeding levels. The mean recovery was 72.5 (± 14.9) and 89.8% (± 3.9), for 60 and 95% levels of feed intake. Differences between the two periods were not significant. The plasma kinetic parameters such as fractional rates and pool size of compartments 1 and 2 and total pool size are shown in Table IV. The mean values of fractional rates (K_{21}, K_{12}), compartment pool size (V_1, V_2) and the sum of the V_1 and V_2 were not significantly different between the two feeding levels 60 and 95% of voluntary intake. The total pool size of compartments 1 and 2 at 60 and 95% feeding level were 214.0 (± 43.8) and 250.3 (± 29.5) L, respectively.

The GFR and renal clearances of allantoin and uric acid were not affected by level of feed intake and therefore the data are not presented here.

TABLE III. RECOVERY OF INJECTED DOSE OF 8-^{14}C-URIC ACID IN URINE OF FOUR YERLI KARA BULLS FED AT TWO DIFFERENT FEEDING LEVELS

Level of feeding			
60%		95%	
BW (kg)	Recovery (%)	BW (kg)	Recovery (%)
274	56	480	86
395	64	402	88
410	84	420	95
418	83	442	92
Mean	72.5		89.8
SD	± 14.9		± 3.9

The effect of level of feeding on daily excretion of allantoin, uric acid, creatinine, total PD and total urinary nitrogen are shown in Table V. The differences between the four

TABLE IV. PLASMA KINETICS OF 8-^{14}C-URIC ACID INJETED INTO THE BLOOD OF FOUR BULLS FED WITH A DIET CONTAINING 30% WHEAT STRAW AND 70% COMPOUNDED FEED AT TWO DIFFERENT FEEDING LEVELS

Body weight	Feeding level (FL)	Daily feed intake	Fractional rate		Compartment pool size (L)		
(kg)	(%)	(kg)	K21[a]	K12[b]	V1[c]	V2[d]	V[e]
410	60	4.80	1.607	0.916	80.4	141.0	221.4
420	95	7.60	0.596	0.520	123.5	141.7	264.3
274	60	4.80	2.048	0.979	48.6	101.6	150.2
280	95	7.60	2.373	1.218	80.0	156.0	236.0
418	60	7.80	2.645	1.116	75.4	171.1	246.6
442	95	12.35	1.388	0.703	89.5	176.7	266.2
402	60	4.80	1.582	1.218	103.4	134.4	237.8
402	95	7.60	1.387	0.657	85.0	179.6	264.7
Mean	60		1.97	1.06	76.9	137.0	214.0
SD			0.50	0.14	22.5	28.5	43.8
Mean	95		1.44	0.78	94.5	163.5	250.3
SD			0.73	0.31	19.7	17.9	29.5

SD, Standard deviation

[a, b] Fractional rate from compartment 1 to 2 or 2 to 1

[c, d] Pool size of compartments 1 and 2

[e] Pool size of compartment 1+2

treatments were statistically significant (P <0.05). The daily total nitrogen excretion and the ratio of PD-N:total-N increased with feed intake.

There were significant correlations between PD excretion (mmol/d) and DDMI (kg/d) and DOMI (DOMI, kg/d). These relationships could be expressed as:

$$PD = 10.6 (\pm 8.7) + 19.8 (\pm 2.03) \text{ ---- for DDMI } (r = 0.99, P < 0.01) \quad (1)$$

$$PD = 10.8 (\pm 8.0) + 22.7 (\pm 2.15) \text{ ---- for DOMI } (r = 0.99, P < 0.01) \quad (2)$$

The mean values for the intercepts of equations 1 and 2 indicated an excretion of 10.6 and 10.8 mmol PD/d at zero feed intake which were equivalent to 0.156 and 0.158 mmol/d kg $W^{0.75}$/d.

TABLE V. EXCRETION OF URINARY PD, ALLANTOIN, CREATINE, URIC ACID AND TOTAL NITROGEN IN YERLI KARA BULLS FED AT FOUR DIFFERENT LEVELS OF INTAKE

Purine derivatives	Level of feed intake (DOMI, kg/d)			
	2.06 (± 0.68)	3.26 (± 0.66)	4.14 (± 1.09)	4.87 (± 1.76)
Total PD (mmol/d)	60.3^{a} (± 11.8)	85.6^{b}(± 20.2)	98.4^{c} (± 17.7)	120.5^{d} ± 25.6)
Allantoin (mmol/d)	53.9^{a} (± 15.1)	79.2^{b} (± 19.7)	91.6^{c} (± 15.9)	95.1^{d} (± 4.8)
Creatinine (mmol/d)	15.4^{a} (± 4.8)	24.2^{b} (± 7.6)	21.2^{c} (± 7.3)	31.1^{d} (± 9.9)
Uric acid (mmol/d)	4.6^{a} (± 1.3)	6.4^{b} (± 1.8)	8.1^{c} (± 2.5)	12.0^{d} (± 4.6)
Total N (g/d)	35.8^{a} (± 10.7)	51.2^{b} (± 25.9)	36.2^{c} (± 9.2)	43.7 (± 24.7)

Standard deviations within parenthesis
Mean values within a row with different superscripts are significantly different (P <0.05)

3.3. **Experiment III** Measurement of xanthine oxidase and uricase activities in plasma, liver and intestinal tissue

Table VI shows the activities of xanthine oxidase (XO) and uricase in plasma, liver and intestinal tissue of Yerli Kara cattle. The uricase activity was not detectable in the plasma and reached a low value in the intestinal mucosa.

TABLE VI. ACTIVITIES OF XANTHINE OXIDASE (E.C.1.2.3.2) AND URICASE (EC.1.7.3.3) IN PLASMA, LIVER AND INTESTINAL TISSUE OF YERLI KARA CATTLE

	Xanthine oxidase		Uricase	
	Mean	SD	Mean	SD
Plasma (unit/L)	5.0 (n = 6)	1.2	---	---
Liver (unit/g wet tissue)	1.34 (n = 4)	0.72	0.44 (n = 4)	0.05
Intestine (unit/g wet mucosal cell)	0.13 (n = 4)	0.03	0.08 (n = 4)	0.03

4. DISCUSSION

4.1. Estimation of basal PD excretion

The profile of PD excretion in the urine of Yerli Kara cattle was similar to other breeds of cattle [2, 4, 5]. The proportion of allantoin:total PD (0.94 ± 0.015) during fasting was higher than the range (0.86-0.91) previously observed in cattle [2, 12]; similar values were also obtained from pre-fasting period (0.93). Tissue enzyme profile (Table VI) supported this observation with the high XO activities in plasma and liver.

Daily PD excretion during the fasting period was different from the pre-fasting period (Table 1). PD excretion during fasting (691 μmol/kg $W^{0.75}$/d), could be taken as an estimate of the basal PD excretion, which was higher than the values of 428, 514 and 456 μmol/kg $W^{0.75}$/d reported elsewhere [2, 4, 13], but close to the value of 705 μmol/kg$W^{0.75}$/d reported by Funaba *et al.* [14]. Lowering the intragastric infusion of energy into sheep to 25% of maintenance requirement resulted in an increase in PD excretion by 28% in the work reported by Giesecke *et al.* [15]; similarly our work showed that endogenous PD excretion increased by 46% when the animals were changed from a pre-fasting diet to a fasting diet.

The proportions of total PD and nitrogen excretion to daily urinary creatinine excretion of bulls were relatively constant during pre-fasting and fasting period. The mean PD-N:total-N values of pre-fasting and fasting imply that this ratio may be used for identification of the feeding level. The average GFR (L/d) values were significantly different between fasting and pre-fasting periods ($P < 0.05$) (614 ± 100 for pre-fasting vs 454.9 ± 92 for fasting). The results obtained imply that plasma PD may provide a better indication of the influx of PD into blood or of the output of PD in urine during pre-fasting than fasting of Yerli Kara cattle.

TLA and NAA (mmol/d) were different between pre-fasting and fasting periods. Net re-absorption of allantoin during the pre-fasting period was higher than during fasting; only 76% of TLA was re-absorbed during fasting compared to 85% during pre-fasting. However, contrary to this, net re-absorption of uric acid (mmol/d) was 85% of TLU during fasting as compared to 74% during pre-fasting. This indicates that uric acid is converted into allantoin in the intestine or the liver by the enzyme uricase. Uricase enzyme profile of Yerli Kara cattle (Experiment III) supports this findings. There was no uricase activity in plasma, however liver and intestine uricase activities were 0.44 ± 0.05 and 0.08 ± 0.03 unit/g wet tissue, respectively.

4.2. Response of purine excretion to feed intake and measurements of the proportion of plasma purine derivatives excreted in the urine.

The average recoveries of injected 8-^{14}C-uric acid in urine of four bulls fed at 60 and 95% of voluntary intake were 72.5 (± 14.9) and 89.8% (± 3.9), respectively. The recoveries at two different levels of feed intakes were not significant but positively related to intake. The average recovery for all four bulls was 81.2 % (mean of 16 measurements).

The percentage recovery of tracer in this study was in agreement with those reported for cattle by other workers [2, 7]. Plasma kinetic parameters of the tracer were not different at different levels of feed intake. Total compartment pool size was 214.0 L (± 43.8) for 60% feeding level and 250.3 L (± 29.5) for 95% feeding level. GFR, TLU and NAA were not significantly affected by intake restriction, although there was a tendency for these parameters to increase with the decrease in feed intake. This tendency is consistent with data reported by Daniel *et al.* [17], who showed that GFR was affected by intake.

There was a significant increase in PD excretion to increasing level of feed intake. The relationship was linear (r = 0.99, P <0.01) and the intercept which represents the endogenous contribution of PD to urinary excretion was 10.6 (± 8.7) and 10.8 (± 8.0) mmol/d for DDMI and DOMI, respectively. When expressed on the basis of metabolic body weight the endogenous contribution was 219 μmol/kg $W^{0.75}$/d. This was lower than the measured fasting PD excretion of 691 μmol/kg $W^{0.75}$/d in the fasting trial. The slopes of the regression lines (19.8 mmol/kg DDMI or 22.7 mmol/kg DOMI) were close to previously reported values in *Bos taurus* cattle [17, 18]. The molar proportions of allantoin:PD varied from 0.78 and 0.93 between the lowest and highest level of intake. The mean molar ratios 0.90:0.10 was similar to previously reported values (0.90:0.10) [5, 19]. Since the ratios of daily excreted PD and total nitrogen to creatinine were not different between feed intakes, they could not be the basis for the variation of efficiency in Yerli Kara. The ratios of daily excreted PD-N to total-N increased with feed intake, this proportion might be used as a feed intake index.

4.3. Xanthine oxidase and uricase activities in plasma, liver and intestinal tissues

The results indicate that Yerli Kara cattle have higher XO activities in plasma, liver and intestinal tissues compared to values reported for European cattle [4]. Xanthine oxidase activities in plasma and intestine were lower but higher in liver than values reported for buffaloes [5]. The patterns of tissue distribution of XO activities in native cattle (Table VII) were in agreement with the results of Chen *et al.* [5] and Al-Khalidi and Chaglassion [20]. The higher XO activities in the plasma, liver and intestinal tissue suggests that extensive conversion of exogenous purines into uric acid takes place in the course of absorption, thus making absorbed exogenous purines unavailable for direct incorporation into tissue nucleotides. This conclusion has also previously pointed out by Balcells *et al.* [21].

Uricase activity was not detected in plasma of Yerli Kara cattle, in agreement with previous results of Chen *et al.* [5, 21]. The absence of uricase activity in plasma could explain the relatively high concentrations of uric acid in plasma, but apparently in contrast with a high level of allantoin. Liver uricase activity was found to be higher than in the intestinal mucosa (0.44 and 0.08 unit/g wet liver and intestine, respectively). According to this result uricase activity appears to be confined to mucosal tissue in the digestive tract, and to the liver at metabolic level.

In conclusion, it appears that the PD excretion is related to feed intake and PD excretion could be used as an index to estimate microbial protein supply as well as to measure DDMI or DOMI in Yerli Kara cattle. The endogenous excretion was estimated as 0.691 mmol/kg $W^{0.75}$/d and the average recovery of injected 8-14C-uric acid in urine was 0.81. Hence the following model is suggested for Yerli Kara cattle of Turkey.

PD excretion (Y mmol/d) = 0.81 X (PD absorption, mmol/d) + (0.691 $W^{0.75}$).

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MEASUREMENT OF THE PROPORTION OF PLASMA PURINE DERIVATIVES EXCRETED IN THE URINE OF SHEEP

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Abstract

MEASUREMENT OF THE PROPORTION OF PLASMA PURINE DERIVATIVES EXCRETED IN THE URINE OF SHEEP.

Four sheep were used to measure the proportion of plasma allantoin excreted in the urine at three levels of intake. The sheep were fed a mixed ration at 800, 1200 and 1600 g air dry matter per animal/d during three periods, using an incomplete Latin Squre design. Each period consisted of 10-days adaptation and 7-days measurement. After the adoptation, each animal was injected via a jugular catheter, a single dose of 30 μCi of 4,5-^{14}C-allantoin as a tracer. Urine collection was made 5 days before tracer injection and until 7 days after tracer injection. Plasma samples were taken at different intervals after the tracer injection. The proportion of plasma allantoin which is excreted in the urine was measured as the recovery of dosed ^{14}C-allantoin in the urine. Glomerular filtration rate (GFR) was estimated from creatinine clearance. There was no significant difference in the recovery of plasma allantoin between levels of intake but there was a considerable variation ($P < 0.05$) between individual animals. The recovery in the four sheep were 88.0, 79.9, 72.4 and 73.0%. This animal variation may be partly explained by the difference in GFR in these animals (6.96, 5.5, 5.0 and 4.32 L/kg $W^{0.75}$/day in the four sheep, respectively). GFR tended to increased with feed intake. However, variation in GFR in the same animal did not seem to affect the proportion of plasma allantoin excretion.

1. INTRODUCTION

Purine derivative excretion (PD) in the urine is used as an index to estimate the amount of microbial protein supplied to ruminants [1-3]. It has been consistently shown that PD produced in the body is not completely disposed of via renal excretion. The proportion at which plasma PD is excreted in the urine may explain differences between species animals in the excretion rate of PD [4-6]. The objectives of this work were: i) to estimate the proportion at which plasma PD is excreted in the urine of sheep using ^{14}C-allantoin as a tracer, ii) to examine whether this proportion is affected by level of feed intake, and iii) to examine whether the variation in the proportion is related with the glomerular filtration rate (GFR) of sheep.

2. MATERIALS AND METHODS

2.1. Animals and management

Four female rumen-cannulated Suffolk crossbred sheep (approx. 1 year old, body weight 50 to 58 kg) were used. The sheep were kept in metabolism crates with unrestricted access to clean drinking water and daily feeding at 0800 and 1600 h. The feed contained 50% hay, 30% barley, 10% molasses, 9% fishmeal and 1% mineral and vitamin premix. Continuous ventilation and lighting were provided. The animals were maintained in this condition through out the experiment.

2.2. Experimental design

The experimental design in this study was an Incomplete Latin Square. Three levels of feed intake (800, 1200, and 1600 g/d of 92% dry matter) were allocated as treatments to 4 sheep over 3 periods. There was a 10-day adaptation period for each feeding level (period).

2.3. Tracer solution and injection

4, 5-^{14}C-allantoin (purity 0.96 and specific activity of 6 μCi/mmol) was used as the tracer in this experiment. In each period, 30 μCi of the tracer was injected into the jugular vein of each animal. Therefore, the injected dose contained 4 mg of cold allantoin (allantoin carrier) and 4 mg of tracer dissolved in 200 ml medical saline. Tracer solution was sterilised before injection. The animals had a jugular catheter inserted 24 h before dosing the single injection of the tracer. 40 ml of tracer solution was injected into each animal within 2-3 min. After injection, the catheter was flushed with heparinised saline.

2.4. Sample collection

2.4.1. Blood

10 ml of blood was withdrawn to a heparinised vacutaner from each sheep via jugular catheter at the following times: 1 h before injection, 1-8 h hourly and thereafter at 22, 24, 28, 30, 46, 52, 96, 120 and 144 h after injection. The blood samples were centrifuged at 1 500 *g* for 20 min and plasma samples stored at -20°C.

2.4.2. Urine

Complete collection of urine was made 5 days before injection and 7 days after the tracer injection. The first 6-h urine after the injection was also collected. Urine was collected in 10% H_2SO_4 so that the pH was below 3. The urine samples were diluted and stored at -20°C.

2.5. Sample analysis

2.5.1. Measuring ^{14}C- allantoin activity in urine

Measurement of ^{14}C- allantoin activity was made according to the procedure described in the IAEA-TECDOC [7]. The proportion of plasma allantoin excreted in the urine was estimated as the recovery in the urine of the injected ^{14}C-allantoin, i.e. the ^{14}C radioactivity present in allantoin in urine expressed as a proportion of the total activity injected.

2.5.2. Purine derivatives in urine

Urine samples prior to tracer injection were analysed for xanthine, hypoxanthine and uric acid by Autoanalyzer [8] and allantoin by HPLC [9], respectively.

2.5.3. Creatinine in plasma and urine

Urine and plasma were analysed for creatinine [10, 11] and GFR was estimated from creatinine clearance and calculated as: GFR (L/d) = Daily creatinine excretion (mmol/d) ÷ plasma creatinine concentration (mmol/l).

3. RESULTS AND DISCUSSION

3.1. PD excretion to feed intake

Digestibilities of organic matter (OM) or dry matter (DM) of the feed were not significantly affected by the level of intake, as shown in Table I. Urinary excretion of PD was correlated with the digestible OM intake ($P < 0.05$) although the mean values of the three intake levels did not reach significance due to large variation between animals.

TABLE I. FEED INTAKE, FEED DIGESTIBILITY AND DAILY URINARY EXCRETION OF PD

Intake level (g/d)	DM Digestibility (%)	OM Digestibility (%)	Digestible DM Intake (g/d)	Digestible OM Intake (g/d)	PD excretion (mmol/d)	PD excretion (mmol/kg $W^{0.75}$/d)
800	79.2	80.4	575	537	12.47	0.588
1200	75.3	76.5	823	769	17.98	0.884
1600	69.5	71.2	939	885	20.95	1.134
SED	3.7	3.5	67	62	3.88	0.215
F test	NS	NS	**	**	NS	NS

NS, Not significant ; **, = $P < 0.01$

3.2. Measurement of the proportion of plasma PD excreted in the urine

^{14}C radioactivity in the urine sharply increased following the injection of the tracer but fell to the background level within 3 days, indicating that all of the dosed ^{14}C radioactivity was cleared from the body within that short time period. The urinary recovery of the injected ^{14}C-allantoin did not reach significance between the three levels of intake (Table II). However, there were significant differences ($P < 0.05$) between animals (Table III). It appears that the proportion at which plasma allantoin is excreted in the urine is not so much a feed related variable, but is more an animal variable.

The recovery of the injected ^{14}C-allantoin in the urine was not complete, indicating that only a fraction of the plasma allantoin is excreted in the urine. The values observed in the this work (72–88% for the four sheep) were within the same range of values for the recovery plasma PD or allantoin excreted in the urine reported by Chen *et al.* [3, 12] observed using different experimental approaches. Secretion into the rumen via saliva may account for part of the unrecovered ^{14}C-allantoin injected, since ^{14}C activity was detected in the saliva of the sheep in this work (detailed procedure not described here). ^{14}C activity was not detected in the rumen fluids probably due to breakdown of allantoin by rumen microbes.

TABLE II. THE PROPORTION OF THE DOSED ^{14}C-ALLANTOIN EXCRETED IN URINE AND GFR AT 3 LEVELS OF FEED INTAKE

Intake level (g/d)	Recovery	Creatinine excretion (mmol/kg $W^{0.75}$/day)	GFR (L/kg $W^{0.75}$/day)
800	78.6	436	4.88
1200	77.1	488	5.45
1600	79.3	495	6.05
SED	5.9	71.2	0.92
F test	NS	NS	NS

NS, Not significant ; *, = $P < 0.05$; **, = $P < 0.01$

TABLE III. THE PROPORTION OF THE DOSED ^{14}C-ALLANTOIN EXCRETED IN URINE AND GFR IN THE 4 SHEEP

Sheep	Body weight (kg)	Recovery (%)	Creatinine excretion (mmol/kg $W^{0.75}$/day)	GFR (L/kg $W^{0.75}$/day)
A	54.5	88.0	580	6.96
B	51.2	79.9	448	5.57
C	54.1	72.4	437	5.00
D	55.4	73.0	427	4.32
SED		3.5	72.1	0.709
F test		**	NS	*

NS, Not significant ; *, = P <0.05; **, = P <0.01

3.3. Relationship between GFR and PD excretion

Table II and III show the daily excretion of creatinine excretion and GFR at 3 levels of intake and in the 4 animals. GFR tended to increase with feed intake although difference between the mean values at the 3 levels of intake did not reach significance. However, GFR for the different animals was significant (P <0.05). Figure 1 shows that variation in GFR may account for part of the differences between animals in the recovery of dosed ^{14}C-allantoin. Animals with a higher GFR tended to have a higher recovery of ^{14}C-allantoin in the urine (see Figure 1 and Table III). It is interesting to note that within each animal, however, GFR (which increased with feed intake) does not affect the recovery factor.

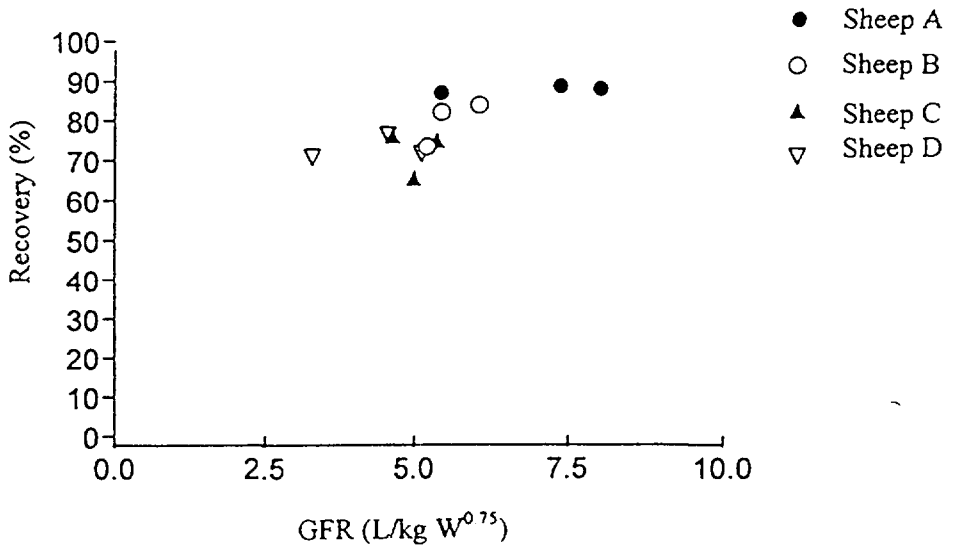

FIG.1. The recovery of ^{14}C-allantoin plotted against the GFR in the 4 sheep.

In summary, the proportion at which plasma allantoin is excreted in the urine of sheep, determined by the tracer method, was not affected by the levels of feed intake, but was more affected by animal variation. The animal variation may be partly explained by the difference in GFR in these animals. However, variation in GFR in the same animal did not seem to affect the proportion.

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DEVELOPMENT OF A NEURAL NETWORK MODEL TO PREDICT THE EXCRETION OF PURINE DERIVATIVES IN THE URINE OF COWS

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Abstract

DEVELOPMENT OF A NEURAL NETWORK MODEL TO PREDICT THE EXCRETION OF PURINE DERIVATIVES IN THE URINE OF COWS.

A Neural Network Model to predict the urinary excretion of purine derivative nitrogen (UPDN) in cows is presented. The input variables of the model are dry matter intake (DMINT), NDF intake (NDFINT), total soluble nitrogen (SP), total soluble non-protein dry matter (SNPDM), total degradable nitrogen (DCP), total degradable non-protein dry matter (DNPDM), hourly available CP in the rumen (HACP), hourly available non-protein dry matter (HANPDM), three different gross indexes of synchronization, namely SYNCA (SP/SNPDM), SYNCB (DCP/DNPDM) and SYNCK (HACP/HANPDM) and two variables describing some metabolic aspects of purine derivative excretion such as live weight of the cow (LW) and milk yield (MILKY). The Model developed uses the Multi Layer Perceptron (MLP) utility, with 13 nodes in the input layer, 8 nodes in the hidden layer and 1 node in the output layer. The Model performances have been tested over 24 observations not previously used to train the model. When compared to a linear regression approach, the Neural Network model showed better performance but under predicted the daily excretion of UPDN for values around 20 g/day. When evaluated in terms of behaviour and depicted scenario the model responded to changes of live weight (LW) and milk yield (MILKY) and to modifications of the pattern of nutrients supplied to rumen microbes.

1. INTRODUCTION

Several models aimed at describing the extent of microbial protein synthesis in the rumen and its response to dietary and animal parameters have been developed. These approaches can be dynamic and mechanistic [1, 2], empirical [3, 4] or static and mechanistic [5].

The urinary excretion of purine derivatives (PD) represents a useful tool to predict the yield of microbial protein in the rumen [6] and has been validated as an indicator in dry and lactating cows [7]. Different approaches have been defined to link PD excretion and microbial synthesis in the rumen in cattle. Some authors [8] have proposed that purine derivatives excretion can be partitioned into an endogenous contribution, which is a function of the metabolic live weight of the animal, and an exogenous contribution which is directly related to microbial purines absorbed in the intestine. In this case the body mass is the sole factor that is recognised to affect endogenous PD contribution. Other authors [9] have proposed that endogenous PD excretion can vary according to the physiological status of the cattle, distinguishing an alternative endogenous PD excretion for dry and lactating cows.

In all these models the endogenous contribution is subtracted from total urine PD excretion in order to estimate the exogenous fraction related to microbial synthesis; however, as the regulation of purine degradation in tissues and organs presents a high degree of complexity, several factors could affect the endogenous excretion of these metabolites, thus the latter contribution could be different from that expected according to the models described.

An alternative approach to study the complex metabolic events concerning PD excretion could be the use of a learning model, such as the Neural Networks.

Neural Networks can be used for real-world applications such as engineering, sensor processing and data analysis [10], and Berg et al. [11] recently used Neural Networks to develop a model for the prediction of pork carcass composition from electromagnetic scans.

A Neural Network typically consists of nodes (*processing elements*) related each other by connections. Each node has a local memory and a *transfer function* which usually has a subfunction called the *learning law*; this latter responds to input signals arriving to the node and adapts the input-out behaviour of the transfer function [10].

The urinary excretion of PD is expressed on weight or molar basis when describing the effect of different factors such as nutrition, species and physiological state of the animal. The same excretion can be referred to as urinary purine derivative nitrogen (UPDN) to obtain a simple synthetic measure to be converted into rumen microbial nitrogen yield. Studies at our Department have shown that UPDN is related to rumen microbial nitrogen yield when estimated by linear regression [9].

An attempt to overcome the liner approach is presented here through the development of a neural network model to predict UPDN excretion in cattle. Such a prediction will be based on aspects affecting UPDN excretion such as metabolic parameters and the pattern of nutrients available in the rumen, which respectively influence the endogenous and exogenous proportion of total UPDN.

2. MATERIAL AND METHODS

2.1. Neural Network: an overview

The present model has been designed adopting a Multi-Layer Perceptron Neural Network, a procedure included in the NEURAL CONNECTION 2.0 software application [12, 13]. Multi-Layer Perceptron (MLP) is a supervised neural network that implements the mapping between the input and output data presented during the *model training*. The *processing elements* (nodes) of the Neural Network are grouped in three different layers: input, hidden and output layers. Nodes within the same layer are not connected to each other "horizontally", but have "vertical" connections to those of the preceding and following layers (Figure 1).

The model assigns a *weight* to each connection between nodes. Weights are modified according to the *learning law* that operates during the Neural Network training process. The inputs to any of the nodes are the result of the input data and the associated weights, the output from a node is the result of input handling by the transfer function. Neural networks have to be trained, and MLP has a training process that follows a supervised training where the network is supplied with an input vector $\mathbf{x}$ and produces an output vector $\mathbf{y}$: each input $\mathbf{x}_i$ is entered in the network with the correct output $\mathbf{y}_i$ as a series of correct input/output pairs [10].

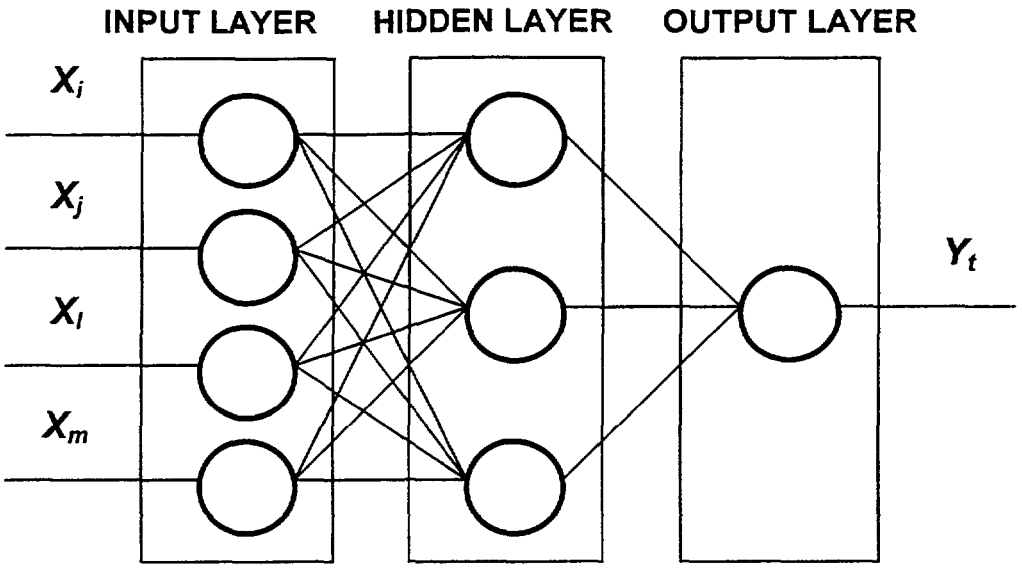

FIG.1. A Schematic diagram of a Neural Network.

In NEURAL CONNECTION 2.0 [12, 13] the training process of the MLP proceeds as follows. First the model randomly sets small initial values for weights and biases, then a training pattern is applied to the input layer and forwarded to the hidden and the output layers. The difference between the *actual* and the *correct* output values is calculated and, in order to reduce the distance between the two outputs, changes in the connection values are automatically established by the software and back propagated from the output layer to the input layer. In the training process the input pattern $\mathbf{X_p}$ that is related to the *correct* output pattern $\mathbf{T_p}$ is presented to the MLP. The initial values of the weights are set randomly to calculate the output from each node in a layer. The output from a node k in the second layer is:

$$\mathbf{z_k = f \sum w_{jk}\, x_j}$$

where **f** is the transfer function of the node, $\mathbf{w_{jk}}$ is the weight between nodes j and k, and $\mathbf{x_j}$ is the input value.

The weights between nodes are adjusted according to the back propagation process as follows:

$$\mathbf{w_{jk}\,(s+1) = w_{jk}\,(s) + h d_{pk}\, z_{pk}}$$

where $\mathbf{w_{jk}\,(s)}$ is the weight between nodes j and k at step s, **h** is the activation function, $\mathbf{d_{pk}}$ is the error of the pattern p at node k and $\mathbf{z_{pk}}$ is the output value at the node k.

The training process can be evaluated in terms of accuracy by comparison of the *correct* output (i.e. the output values used for training) and the *actual* output (that is an estimation of the correct output) of the model. The model is targeted to achieve the lowest error between actual outputs and correct outputs, that represents the best solution of the model.

Two data sets are required to evaluate the model. The data set used to let the network learn the solution of the problem is called the *training data set*. An additional independent input/output data set should be used to test the accuracy of the model. This second data set is called the *validation data set*. The validation process requires the correct interpretation of results. By processing continuously to the lowest training error, the network can also learn errors associated with the training data set. In other words, the model can lose the ability to generalize, in a situation that is defined by the term *over training*. In this case, the network has

learned too precisely the "noise" that accompanies training data and the validation error increases while the training error tends to flatten. The validation data set is then required to monitor the training cycle.

2.2. Input variables, training and validation data set

Data were collected from seven different N digestibility trials with dry and lactating cows, performed at the University of Udine (82 observations) and at the Federal Research Institute for Agriculture in the Alpine Regions in Irdning (48 observations), giving a total of 130 observations. The available measurements were DM, CP and NDF content, intake and digestibility. Moreover, ruminal degradability of CP and non-protein dry matter (NPDM) for the diets offered was also estimated by in situ nylon bag technique [14].

To develop the model, different input variables were considered. Some were used because of their importance in describing ruminal degradability and the availability of nutrients for microbial synthesis in the rumen, (dry matter intake, NDF intake, total soluble nitrogen (SP), sum of the "a" fractions for CP - total soluble non-protein dry matter (SNPDM), sum of the "a" fractions of NPDM - total degradable nitrogen (DCP), sum of the "b" fractions for CP - total degradable non-protein dry matter (DNPDM), sum of the "b" fractions for NPDM - hourly available CP in the rumen (HACP), sum of the b*c terms for CP - hourly available NPDM (HANPDM), sum of the b*c terms for NPDM. Some of these variables were also combined to calculate three different gross indexes of synchronization between carbohydrates and protein availability in the rumen, namely SYNCA (SP/SNPDM), SYNCB (DCP/DNPDM) and SYNCK (HACP/HANPDM). To link the excretion of UPDN to the intermediate metabolism of the animal, further variables were taken into consideration, such as live weight of the cows (LW) and milk yield (MILKY).

The main dietary and animal parameters, the PD excretion along with microbial nitrogen synthesis estimated according to the Italian PDI system [14] of the complete data set are summarised in Table I.

TABLE I. MAIN DIETARY AND ANIMAL PARAMETERS, PD EXCRETION AND MICROBIAL NITROGEN ESTIMATION

	Min	Max	Mean	SD	CV (%)
DM intake (kg/d)	6.92	23.95	12.91	3.69	28.6
NDF intake (kg/d)	3.66	9.66	6.01	1.36	22.6
Soluble crude protein (SOLP) (g/d)	107	1398	541	278	51.4
Soluble non-protein DM (SNPDM) (g/d)	610	4922	2324	1028	44.2
Potentially degradable crude protein (DCP) (g/d)	319	2873	1095	526	48.0
Potentially degradable non-protein DM (DNPDM) (g/d)	2854	11721	6302	1831	29.1
SYNCA	0.113	0.532	0.248	0.107	42.9
SYNCB	0.079	0.280	0.170	0.048	28.1
SYNCK	0.021	0.456	0.166	0.080	48.4
Live weight (LW) (kg)	494	800	622	60.57	9.7
Milk yield (MILKY) (kg/d)	0	35.8	12.3	9.69	8.8
Microbial N [(1)] (g/d)	27	514	194	99.1	51.0
Urinary PD nitrogen (UPDN) (g/d)	3.1	24.7	10.5	4.57	43.5

[(1)] estimated according to the Italian PDI system [14]

3. RESULTS AND DISCUSSION

3.1. Development of the neural model

The complete set of 130 observations was randomized into three groups: *training*, *validation* and *testing* data sets. The training data set was used to let the model learn the case study, the validation data set was used to monitor the model error during the learning process and avoid *over training*, the testing data set was finally used to evaluate the performance of the trained model. This latter partition of the complete data set in three different groups was aimed at conducting an *internal validation* of the neural network model; thus the use of the *test data set* was not meant to validate the model. In fact alternative observations from different experiments would have been needed for a classical validation of the model.

Several combinations of the number of nodes in the hidden layer were tested to choose the best Neural Network Model for the data set provided. The number of nodes adopted for the hidden layer depends on the final performance of the model. Thus, increasing the number of nodes can help the model to learn further underlying features of the data set, resulting in a better performance of the neural network. On the other hand increasing further the number of nodes could cause a decrease of the performance of the model as the network could start to learn the *background noise* associated with the data used for the model training.

For the transfer function of the nodes in the hidden layer we adopted a sigmoid function which is a smooth non-linear function with a continuos positive first derivative value. The parameter we adopted to select the different possible models was the standard error (SE) of the output performed by the model on the *testing data set.*

Figure 2 shows how the standard error for testing, training and validation data sets changed according to the number of nodes provided for the hidden layer. As a consequence of the approach we chose the Neural Network with 8 nodes in the hidden layer, as this had the lowest SE for the testing data set. Therefore, the model we adopted was a 13 - 8 -1, a conventional indication for a Multi Layer Perceptron neural network consisting of an input layer with 13 nodes, an hidden layer with 8 nodes and an output layer with a single node.

The ability of the Neural Network model to predict daily UPDN excretion is presented in Figure 3, showing observed (OBS, g/d) and predicted (PRED, g/d) values over the test data set. The function describing the observed vs predicted (OBS = 0.470 + 0.928 × PRED; SE ± 2.329) had average determination coefficient of $R^2 = 0.641$, but the regression coefficient was not significantly different from 1.0 indicating a correct prediction. Figure 4 shows the residuals of the model and the OBS values are also plotted, revealing a tendency of the model to under predict daily UPDN excretion for values around 20 g/day.

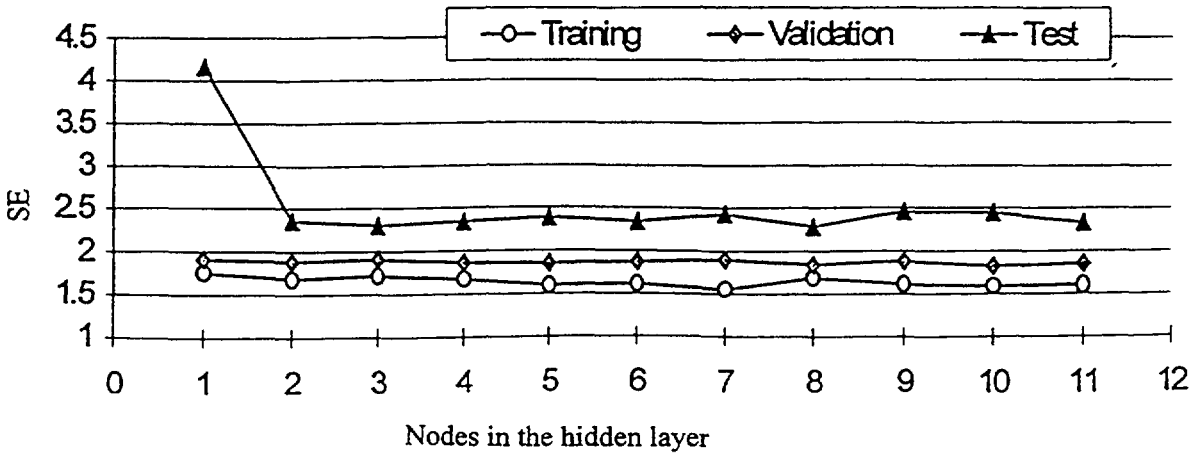

FIG.2. Standard error trend of the Neural Network Model.

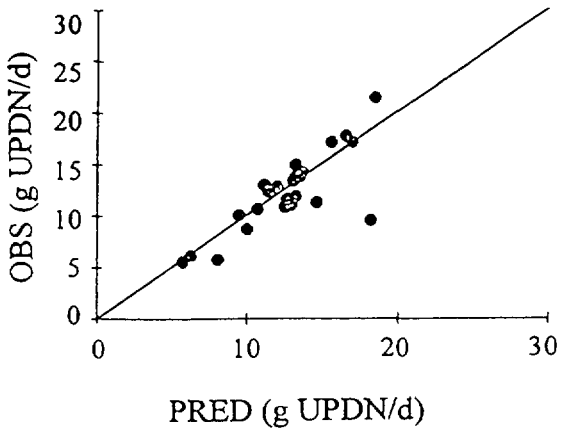

FIG.3. Neural Net Work model prediction.

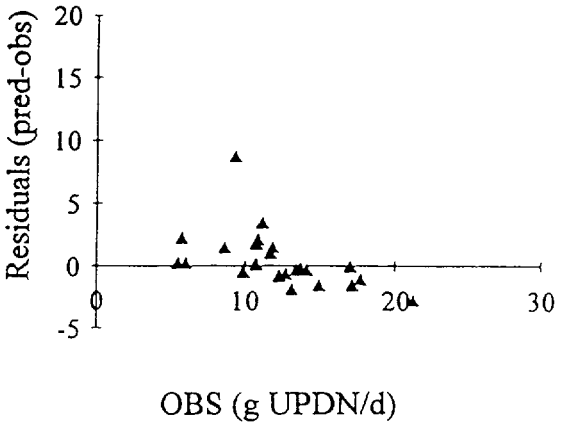

FIG.4 Residuals with Neural Network model

The choice of adopting a Neural Network model to predict the daily UPDN excretion in cows was also evaluated by comparing the alternative results obtained by two approaches.

First we used the same observations used for training the Neural Network to obtain a linear multiple regression based on 13-input variables and we tested the prediction ability of the latter on the same testing data set used to measure the Neural Network 13-8-1 model performances. Results of this approach are shown in Figures 5 and 6. In the former, observed values (OBS) of daily UPDN excretion and values predicted (PRED) by the multiple regression equation are plotted (OBS = 0.551 + 0.737 × PRED; R^2 = 0.657; SE ± 2.276). In this case the multiple linear regression over predicts the daily PDN excretion, as the regression coefficient b_1 is significantly different from 1.0 ($P < 0.05$). The over prediction can also be appreciated in the plot of residuals vs observed values presented in Figure 6.

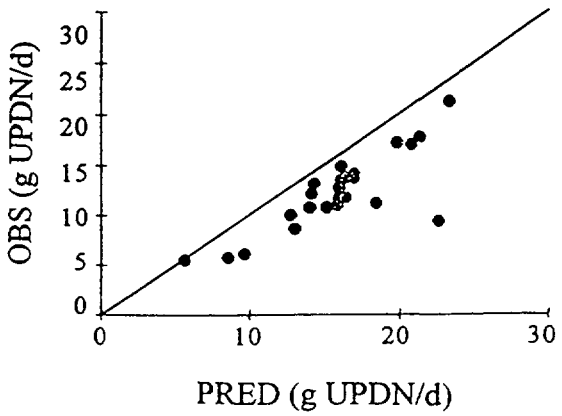

FIG.5. Linear regression prediction.

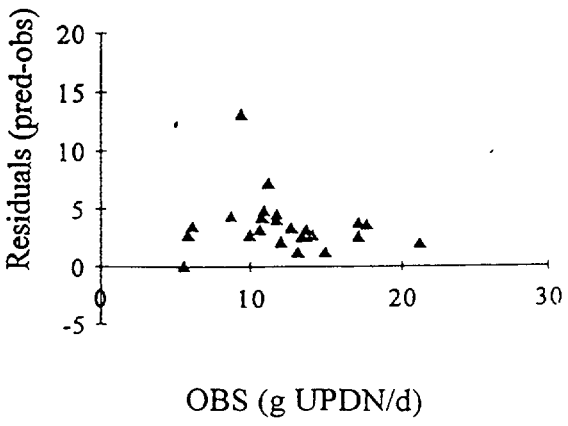

FIG.6. Residuals with linear regression prediction.

We then applied the following empirical equation developed previously at the University of Udine [9] to evaluate the Neural Network.

$$UPDN\ (mg/W^{0.75}) = 12.25 + 29.1 \times L + 44.15 \times TMN$$

This linear equation is aimed at predicting the excretion of purine derivatives nitrogen in the urine of cows from the physiological status of the cows (L, dry or lactating) and total microbial nitrogen synthesis in the rumen (TMN) estimated by the Italian PDI system [14]. The empirical equation was applied to the same testing data set used to evaluate the Neural Model.

The plot of the UPDN excretion predicted by the empirical equation vs the observed values (OBS = 4.084 + 0.561 × PRED; R^2 = 0.388; SE ± 3.040) is shown in Figure 7. The residuals of this prediction are plotted in Figure 8, showing that the equation over predicts UPDN and that the plot of residuals is similar to that of the multiple linear regression seen in Figure 6.

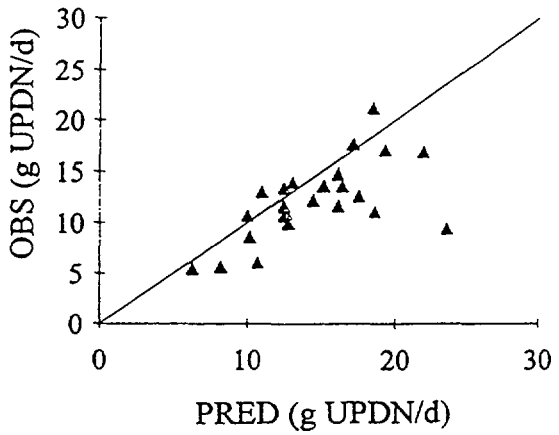

FIG.7. Empirical equation prediction.

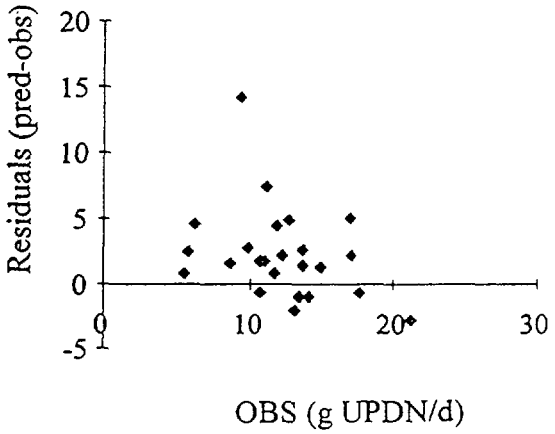

FIG.8. Residuals with empirical equation.

3.2. Model behaviour and scenario

3.2.1. Metabolic aspects

In order to describe the behaviour of the present Neural Network model and the scenario it is able to represent, we plotted two different graphs showing different aspects of the purine derivatives excretion. In Figure 9 we have plotted the model output when changes in the animal's live weight (LW) and daily milk yield (MILKY) are imposed and the other input variables are held constant. It can be noticed that the model predicts a higher UPDN excretion as a consequence of an increased live weight and milk yield.

This scenario is typical of the model developed but relies also on physiological basis. The urinary excretion of PD is dependent on body mass [8] and yielding cows undergo high body tissue turn over particularly during early lactation. Such an increase in tissue turnover may reasonably drive a high endogenous UPDN excretion. We tried to isolate endogenous UPDN excretion related to milk production from that related to the live weight of the cows. For this reason we set LW to zero and changed MILKY to generate a linear relationship between UPDN and MILKY. The equation UPDN = 11.926 + 0.052 × MILKY (SE ± 0.025;

R^2 = 0.998), showed that UPDN increased by 52 mg/kg of milk produced. Conversely, to investigate the effect of LW on UPDN excretion, we set MILKY to zero, varying LW to obtain a linear function. The equation UPDN = 10.974 + 0.002 × LW (SE ± 0.023; R^2 = 0.995), showed that UPDN increased by 2 mg/kg LW. Clearly the latter estimations of 52 and 2 mg of UPDN have to be considered as relative outputs of the model for MILKY and LW variations individually, but despite this limitation it is worth noting that the model was sensitive to these metabolic factors.

When expressed in terms of millimoles, the endogenous PD excretion represented by the present model is equal to 0.268 mmol/kg/$W^{0.75}$ and 0.928 mmol/kg milk produced. These figures can be compared to the endogenous PD excretion estimations presented previously in the literature of 0.385 mmol/kg$W^{0.75}$ [8], and 0.219 mmol/kg$W^{0.75}$ and 0.738 mmol/kg$W^{0.75}$ for dry and lactating cows respectively [9]. In comparison with available literature the neural network model appeared to give reasonable and robust estimation of endogenous contribution of PD excretion.

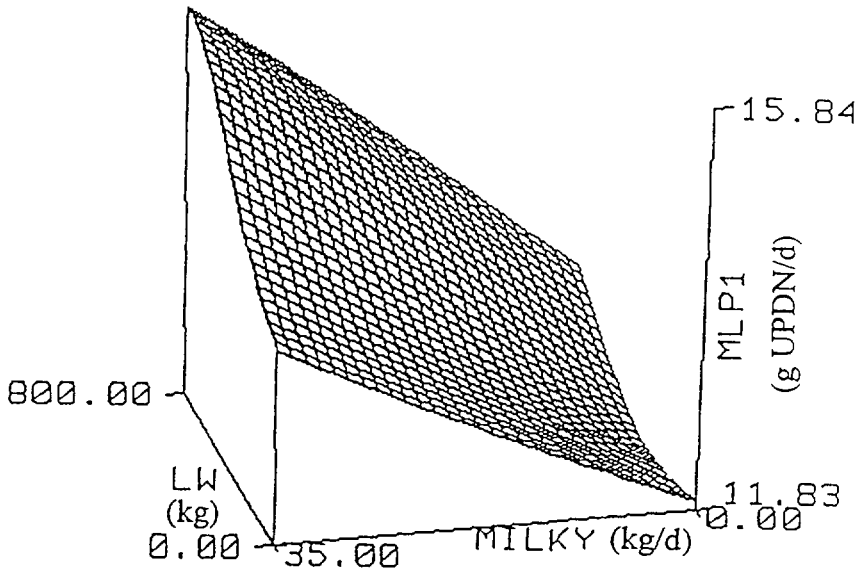

FIG.9. Neural Network model behaviour: Model output (MLP1; g UPDN/d) with respect to variations in live weight (LW; kg) and milk yield (MILKY; kg/d).

3.2.2. Supply of nutrients for rumen microbial population

The model reaction to variation of some ruminal parameters is presented in Figures 10 and 11. Among these, more relevance was given to indexes of synchronization of nutrient availability in the rumen, i.e. nitrogen and carbohydrates, considering their recongnized importance for microbial growth [15, 16].

The model output was investigated by varying dry matter intake (DMINT) and rumen synchronization of the soluble fractions, SYNCA (Figure 10) and when variations in dry matter intake and rumen synchronization of hourly available protein and non-protein dry matter (SYNCK) were imposed (Figure 11). The model was consistently sensitive to variations in dry matter intake. Figure 10 shows that the output UPDN is larger when an increased intake is accompanied by a synchronous availability of the soluble fractions in the rumen, at least at low levels of DMINT. Figure 11, however, shows that the output increases when hourly availability of the non-protein dry matter tends to exceed that of proteins. This latter behaviour was likely due to the fact that hourly available protein in the rumen of the training data set did not limit microbial synthesis.

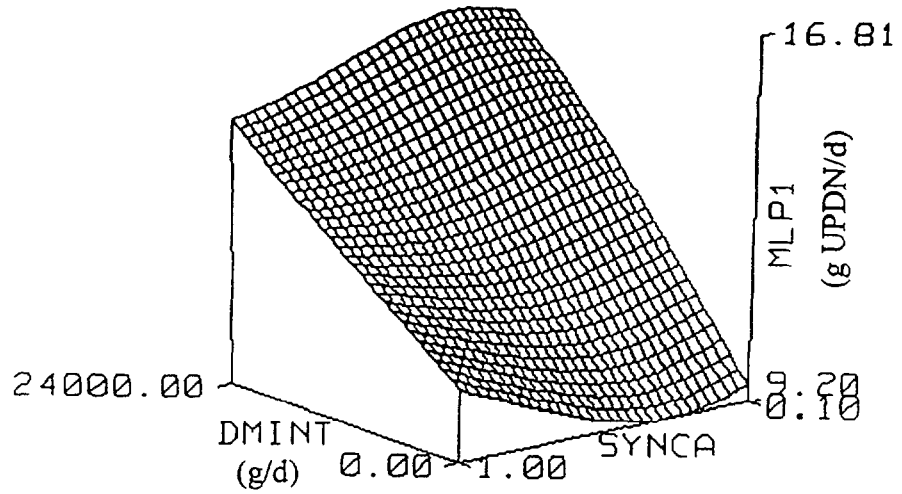

FIG.10. Neural Network model behaviour: Model output (MLP1; g UPDN/d) with respect to variations in dry matter intake (DMINT; g/d) and synchronisation of the soluble fractions (SYNCA; g/g).

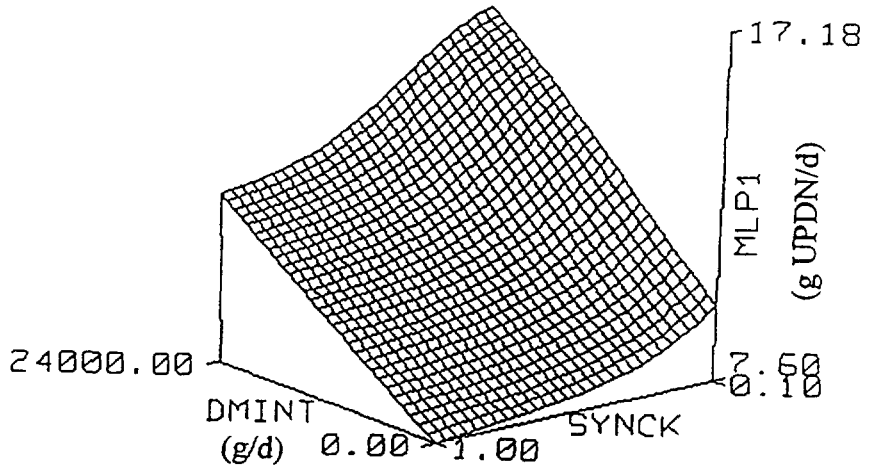

FIG.11. Neural Network model behaviour: Model output (MLP1; g UPDN/d) with respect to variations in dry matter intake (DMINT; g/d) and synchronisation of hourly available protein and non-protein dry matter (SYNCK; g/h / g/h).

4. CONCLUSIONS

The model developed represents an attempt to apply a Neural Network approach to the excretion of purine derivatives in the urine of cows. Although the model showed better performance when compared to classical linear regression approaches, further data from different experiments would be needed to validate the model. The scenario depicted by the model is promising because the model is able to react to variations in the metabolic status of the cow and patterns of nutrients supplied to rumen microbial population. According to the model presented here other factors apart from live weight should be considered to define the fraction of urinary PD that is of endogenous origin. Milk yield and stage of lactation deserve further investigation to clarify their role. Therefore, this model can be useful in the study of physiological principles affecting UPDN in cows.

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PURINE NITROGEN INDEX, POTENTIALLY A NEW PARAMETER FOR RAPID FEED EVALUATION IN RUMINANTS

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Abstract

PURINE NITROGEN INDEX, POTENTIALLY A NEW PARAMETER FOR RAPID FEED EVALUATION IN RUMINANTS.

The concept of a new parameter 'Purine Nitrogen Index (PNI)' for feed evaluation in ruminants is discussed. PNI refers to the ratio of purine derivative (PD) nitrogen to total nitrogen in urine. It is suggested that PNI can potentially be used as an indicator of the efficiency with which degradable dietary nitrogen is converted to microbial protein in the rumen. The excretion of PD in the 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 regime has a high conversion efficiency, proportionally more rumen degradable nitrogen is converted to microbial protein and less nitrogen is excreted in the urine, resulting in a high PNI. Conversely, if a diet has a poor conversion efficiency, proportionally less dietary nitrogen is converted to microbial protein and more is excreted in the urine, resulting in a low PNI. Preliminary data from six experiments involving 34 sheep confirmed a positive correlation between PNI and the nitrogen conversion efficiency, and suggested that a dietary regime with a PNI lower than 0.08 for sheep appeared to be a less efficient in the production of microbial protein and have a greater loss of nitrogen in the urine. PNI can theoretically be determined in spot urine samples, and has the potential to serve as a 'dipstick' method for the rapid evaluation of ruminant feeds. However, more research with a mathematical modelling approach is required to evaluate and develop the concept further.

1. INTRODUCTION

During microbial fermentation, part of the dietary nitrogen is converted to NH_3, a proportion of which is captured by rumen micro-organisms for the synthesis of microbial protein. The remaining NH_3 is absorbed from the rumen and is finally excreted, as a source of nitrogen, in the urine. Microbial protein is an important source of protein for ruminants. The efficiency with which rumen degradable nitrogen (RDN) in the diet is converted to microbial protein determines the overall utilisation efficiency of ruminant diets on one hand and the loss of nitrogen in the urine on the other. A technique which provides a rapid indication of this efficiency is desirable and will benefit future feeding systems with an orientation for improved biological efficiency and reduced waste secretion to the environment.

If a diet or a dietary regime has a high nitrogen conversion efficiency (NCE), more microbial protein is produced and less nitrogen is excreted in the urine. Therefore, the ratio of intestinal flow of microbial protein nitrogen to urinary nitrogen excretion (MN:UN ratio) will

be higher and *vice versa*. With the assumption that other factors (e.g. protein degradability, intake of undegradable dietary protein) remain unchanged, it is expected that the MN:UN ratio is positively correlated with the NCE. This ratio, however, is difficult to measure.

Urinary PD refer to the sum of allantoin, uric acid, xanthine and hypoxanthine excreted in the urine. The excretion of PD provides an indirect measurement of the intestinal flow of microbial protein [1]. Results of recent work [2-4] showed that the estimates of microbial protein nitrogen based on PD excretion were in close agreement with the direct measurements using microbial markers. Therefore, by replacing the term 'microbial protein nitrogen' (MN) in the expression 'MN:UN' with PD nitrogen (PDN), the ratio of PDN:UN should also be correlated with NCE. This ratio is hereafter referred to as "Purine Nitrogen Index" (PNI). Therefore, PNI refers to the proportion of total urinary nitrogen that is present in the form of PD.

PNI can be easily determined. Moreover, since it is a ratio of two chemical components in the urine, it can theoretically be determined from spot urine samples provided that there is little diurnal variation, a feature required for applications under farm conditions. If the relationship between PNI and NCE is established, determination of PNI from urine samples may provide a rapid indication of the efficiency with which rumen RDN in the diet is converted to microbial protein.

The objectives of this work were to: examine the relationship between the PNI and NCE based on data generated from a range of experiments and assess the diurnal variability of PNI measurements based on spot urine samples to evaluate the feasibility for application under farm conditions when complete urine collection may not be feasible.

2. MATERIALS AND METHODS

Three experiments (Experiments I, II and III) were specifically conducted for obtaining information on PNI. Data were also collated from three experiments (Experiments IV, V and VI) previously conducted in our laboratory. Full details are provided for Experiments I, II and III but only a brief description of the treatments are provided for Experiments IV, V and VI since they have already been published elsewhere [1].

2.1. Animal experiments

2.1.1. Experiment I

A total of 12 female Finn/Dorset × Dorset sheep, approximately one year old and of average body weight of 51 ± 6 kg, were used. The sheep were randomly allocated into 4 groups of 3 each. The four groups of animals were fed with the following four diets: i) basal diet of grass cubes, primarily of rye grass, ii) basal diet supplemented with 8 g urea/d, iii) basal diet supplemented with 16 g urea/d and vi) basal diet supplemented with 173 g rolled barley DM/d. The intake of the grass cubes was identical in all 4 groups at 1000 g/d air dry weight (963 g DM/d). With the urea-supplemented diets, the required amount of urea was dissolved in minimum quantity (24 ml water) of water and sprayed on the grass cubes and well mixed, prior to feeding. The feed was offered in two equal meals twice daily, at 0800 and 1600 h, respectively. The grass cubes contained 909 g DM/kg air dry weight and 28.5 g N and 897 g OM, per kg DM. The estimated RDN contents of the four treatments were: 42.4, 51.0, 9.5 and 37.5 g RDN/kg digestible organic matter apparently fermented in the rumen (DOMR).

The animals were allowed an adaptation period of 10 (two groups) or 17 (other two groups) days before a 7-day measurement period. During the latter period, the animals were housed in metabolism cages for collection of urine and faeces. Total urine was collected at 24

h intervals between Day 1 and 5, and at 2 h intervals between days 6 and 7. The daily urine production was collected into plastic containers containing approximately 200 ml of 10% H_2SO_4, diluted to 5.5 litres with water and sub-sampled. The 2-hourly collection of urine was made with the aid of a fraction collector. Urine excreted was immediately delivered by a continuously running pump into bottles containing 20 ml 10% H_2SO_4 situated on the collector. The 2-h urine can be regarded as a spot urine sample. Sub-samples of both daily and spot urine were stored at -20 °C. Total faecal samples were collected for seven days. A 10% portion of the daily faecal output was kept and bulked at the end of period for each animal. The faecal samples were freeze-dried and stored until analysis.

2.1.2. Experiment II

The same 12 sheep used in Experiment I were used. At end of Experiment I, the sheep were randomly re-grouped into 4 groups of 3 each. They were allocated to one of the following 4 diets: i) basal diet of grass cubes (as in Experiment I), ii) basal diet supplemented with 82 g DM/d of pre-washed fishmeal, iii) basal diet supplemented with 164 g DM/d of pre-washed fishmeal and vi) basal diet supplemented with 346 g DM/d of rolled barley as used in Experiment I. The estimated RDN contents of the four treatments were: 42.4, 45.3, 48.0 and 34.7 g RDN/kg DOMR. The length of adaptation and measurement periods and procedures for urine collection and faecal sampling and processing were as in Experiment I.

2.1.3. Experiment III

Four female sheep (Suffolk cross) of average body weight 46 ± 1.4 kg were used. The animals were fed with a mixed diet containing 50% hay, 30% rolled barley, 10% molasses, 9% fishmeal and 1% minerals and vitamins (hereafter referred to as 'GP' diet). The diet contained 920 g DM/kg air dry weight and 20.2 g N and 925.9 g OM, per kg DM. The estimated RDN content was 23.3 g RDN/kg DOMR. The diet was offered at 3 levels, 800, 1200 and 1600 g/d (air dry weight) to 4 sheep, allocated according to a 3 × 4 design. Each period representing an intake level consisted of 10 days adaptation and 12 days collection. The feeding and housing conditions were similar to Experiment I and II. Total urine and faeces were collected at 24 h intervals. Procedures for urine collection (total only) and faecal sampling and processing were as in Experiment I.

2.1.4. Experiment IV

Nineteen crossbred Suffolk wether sheep with body weights ranging from 22 to 73 kg were all offered 820 g DM/d of the GP diet (same composition as in Experiment III but from a different batch of ingredients). The rumen digesta outflow rates in those animals varied due to different levels of feed intake relative to body weight. Experimental details have been previously presented [1].

2.1.5. Experiment V

Four Blackface × Suffolk wether sheep of average body weight 41 ± 1.5 kg were fed with ammonia-treated barley straw alone or supplemented with either sugar beet pulp or barley each at 20 or 40%. Experimental details have been previously presented [5].

2.1.6. Experiment VI

Five wether sheep of average body weight 58 ± 14.2 kg were given hay supplemented with urea plus either molasses or three levels of a rice polishings. Experimental details have been previously presented [6].

2.2. Measurements and calculations

In all six experiments, RDN intake, digestible organic matter intake (DOMI), and daily excretions of total urinary nitrogen and PD were measured. The microbial nitrogen production (i.e. intestinal flow of microbial protein nitrogen) was estimated based on daily output of PD.

The NCE was calculated as "microbial nitrogen production (g/d) expressed as a proportion of RDN intake (g/d)". The value may be greater than 1 when the RDN intake was low relative to that of DOMI, due to a net flow of urea-N from plasma to the rumen for conversion to microbial protein.

The efficiency of microbial protein supply (EMPS) was expressed as "microbial nitrogen production (g) per kg DOMR". DOMR was taken as 0.65 of the measured digestible organic matter intake. While NCE reflects how efficient RDN is used, EMPS reflects how efficient organic matter is used for synthesis of microbial protein.

2.3. Chemical analysis

Details of chemical analysis for Experiments I-III are described as follows, but those for Experiments IV-VI were as in the cited original publications [1, 5, 6]. Dry matter and ash contents of the diets and faecal samples were determined according to AOAC [7]. Nitrogen in the urine was determined using the method described by Davidson *et al.* [8]. Rumen degradability of OM and nitrogen was determined by incubating the feed samples in the rumen of three separate sheep according to the Nylon bag technique [9], and the effective degradability was calculated from the measured potential degradability assuming a rumen digesta outflow rate of 5%/h. Urinary PD were measured as the sum of allantoin, uric acid, xanthine and hypoxanthine. Allantoin was determined using a HPLC [10], and the other components using an Auto Analyzer [11]. Creatinine in urine was determined using the method of Larsen [12].

2.4. Statistical analysis

Analysis of variance was carried out to examine the effects of urea, barley and fishmeal supplementation (Experiments I and II) and the effect of intake levels on PNI and NCE. The possible relationship between the PNI and NCE was examined by regression analysis. The statistical work was aided with the computer program GENSTAT 5.

3. RESULTS

3.1. PNI, NCE and EMPS

3.1.1 Experiment I

Results are shown in Table I. There were significant differences ($P < 0.05$) in PD excretion and thus the estimated microbial nitrogen supply. NCE (ranging from 0.209 to 0.437) decreased significantly with urea supplementation. PNI ranged from 0.027 to 0.048. Urea supplementation at the higher level had a significantly lower PNI than the other treatments. Barley supplementation did not show a significant effect on either NCE or PNI. The EMPS decreased with high level of urea supplementation.

TABLE I. TOTAL PD, PNI, MN, EMPS AND NCE IN 12 SHEEP FED BARLEY AND UREA SUPPLEMENTS WITH GRASS CUBES AS BASAL DIET (MEAN OF 3 SHEEP IN A 5-DAY PERIOD)

Diet	DOMI (kg/d)	Total PD (mmol/d)	N-excretion (g/d)	Microbial protein nitrogen		PNI (fraction)	NCE (fraction)
				MN (g N/d)	EMPS (g N/kg DOMR)		
B1	0.620	8.73	10.18	7.6	19.5	0.048	0.367
C1	0.510	9.69	13.86	8.4	25.0	0.039	0.437
U1	0.470	8.15	12.99	7.1	22.5	0.040	0.292
U2	0.560	6.67	13.62	5.8	14.7	0.027	0.209
SED	0.046	1.68	1.23	1.58	5.69	0.010	0.078
F-test	P <0.001	NS	P <0.001	NS	P <0.05	P <0.05	P <0.001

3.1.2. Experiment II

Results are shown in Table II. NCE ranged from 0.291 to 0.373 and PNI from 0.042 to 0.051. Fishmeal or barley supplementation did not have significant effects on either NCE, PNI or EMPS.

TABLE II. TOTAL PD, PNI, MN, EMPS AND NCE IN 12 SHEEP FED BARLEY AND FISHMEAL SUPPLEMENTS WITH GRASS CUBES AS BASAL DIET (MEAN OF 3 SHEEP IN A 5-DAY PERIOD)

Diet	DOMI (kg/d)	Total PD (mmol/d)	N excretion (g/d)	Microbial protein nitrogen		PNI (fraction)	NCE (fraction)
				MN (g N/d)	EMPS (g N/kg DOMR)		
B2	0.760	9.69	11.59	8.2	18.0	0.047	0.373
C2	0.510	7.87	9.43	6.4	19.3	0.046	0.344
FM 1	0.480	7.47	8.48	6.1	19.4	0.051	0.317
FM 2	0.540	8.05	10.85	6.7	18.8	0.042	0.291
SED	0.031	1.67	1.02	1.40	4.08	0.007	0.062
F-test	P<0.001	NS	0.001	P<0.05	NS	NS	NS

3.1.3. Experiment III

Results are shown in Table III. PD excretion and thus the estimated microbial N supply increased significantly with level of intake. However, EMPS tended to be higher with the highest level of feed intake, but the difference was not significant. NCE ranged from 0.616 to 0.738 and PNI from 0.07 to 0.083.

TABLE III. TOTAL PD, PNI, MN AND NCE IN 4 SHEEP FED GP DIET AT 3 LEVELS OF INTAKE (MEAN OF 4 SHEEP AT 7 DAYS COLLECTION PERIOD)

Intake level	Treatment	DOMI	Total PD	N-excretion	Microbial protein nitrogen		PNI	NCE
		(kg/d)	(mmol/d)	(g/d)	MN (g N/d)	EMPS (g N/kg DOMR)	(fraction)	(fraction)
1	GP1	0.575	9.06	7.58	7.6	20.6	0.070	0.738
2	GP2	0.780	11.17	8.65	9.6	18.7	0.083	0.616
3	GP3	0.897	15.58	10.61	13.4	23.0	0.082	0.651
	SED	0.035	0.808	1.36	0.704	1.3	0.0108	0.031
	F-test	P<0.01	P <0.01	P <0.001	P <0.01	NS	NS	P <0.05

3.1.4. Experimentts VI-VI

The ranges of PNI, NCE and EMPS are listed in Table IV. All three variables had a larger spread within individual experiments than in Experiments I-III.

TABLE IV. THE RANGE OF NCE, PNI AND EMPS FOR DATA COLLATED FROM EXPTERIMENTS IV-VI

	Number of observations	NCE (fraction)	PNI (fraction)	EMPS (g N/kg DOMR)
Experiment IV	19	0.194 - 0.964	0.024 - 0.160	7.0 - 35.8
Experiment V	5	0.309 - 0.515	0.034 - 0.064	19.1 - 22.6
Experiment VI	4	0.854 - 1.034	0.095 - 0.118	16.2 - 18.6

The pooled data from all six experiments showed that PNI was positively correlated with NCE (Figure 1). Linear effect was significant (P <0.001), but quadratic effect was not.

$$PNI = -0.002\ (0.006\ se) + 0.130\ (0.010\ se)\ NCE\ (n = 39,\ R^2 = 0.811)$$

There was a trend for PNI to increase with the EMPS (Figure 2), but the data points were more scattered ($R^2 = 0.435$) than in Figure 1. Most of the data points had a EMPS values of 20-25 g microbial N/kg DOMR.

$$PNI = -0.0059\ (0.0137\ se) + 0.0037\ (0.0007\ se)\ EMPS\ (n = 39,\ R^2 = 0.435)$$

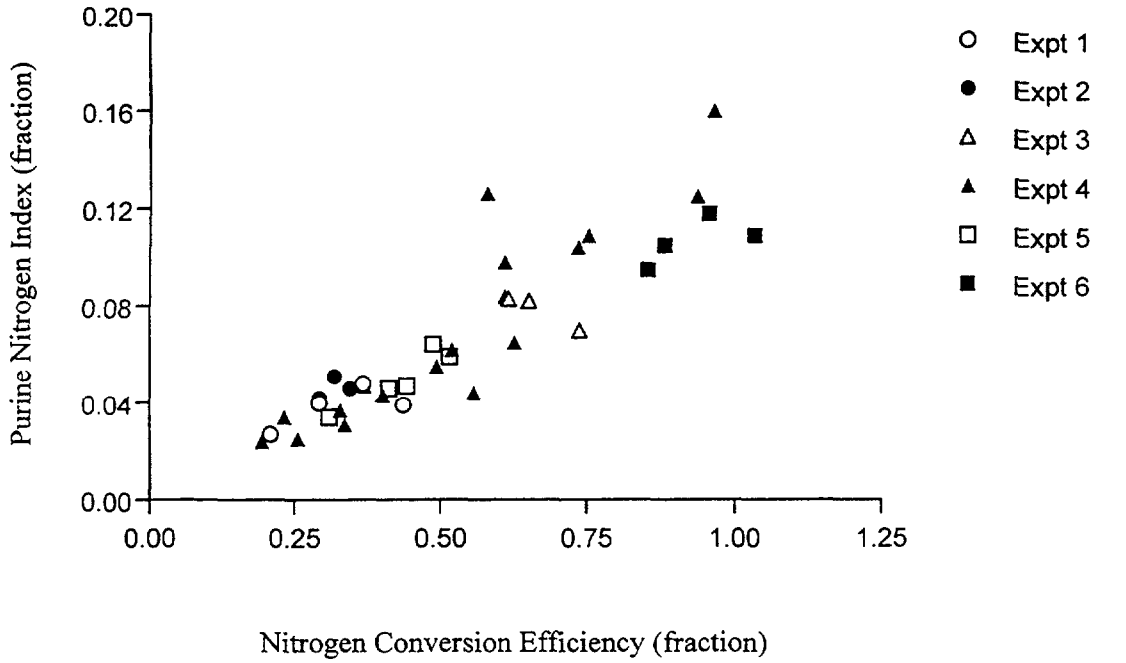

FIG.1. Pooled data from all six experiments showing the relationship of PNI to NCE.

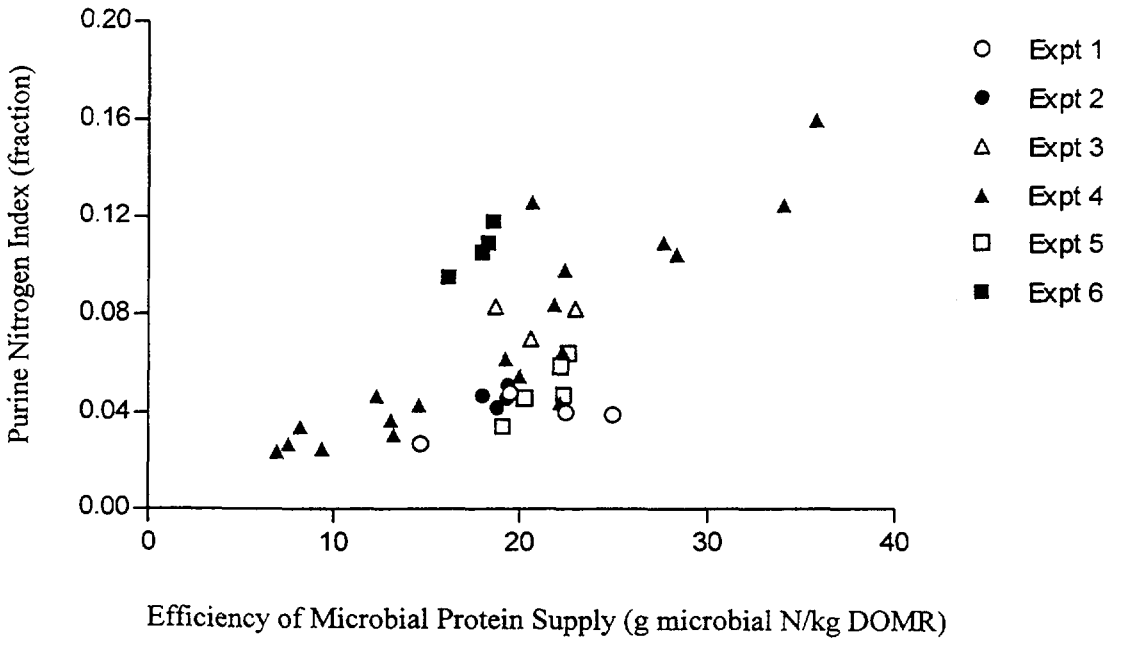

FIG.2. Pooled data from all six experiments showing the relationship of PNI to EMPS.

3.2. Diurnal variation in PNI

From the data (12 animals, each measured during two periods of 2-h urine collection for 24 hours) in Experiment I and II, there was no clear pattern of diurnal variation in PNI, although with urea and fishmeal supplemented diets, PNI decreased after feeding. However, the variability (in terms of coefficient of variation (CV) among the 12, 2-h measurements) was relatively large; CV averaged 19.7% (± 11.5) (n = 24). The ratio of PD:creatinine (mmol/mmol) was also measured. Its variability was much smaller (CV = 10.4% ± 4.0) than PNI (ratio of PD:N), indicating that N output in the urine was more variable than PD output.

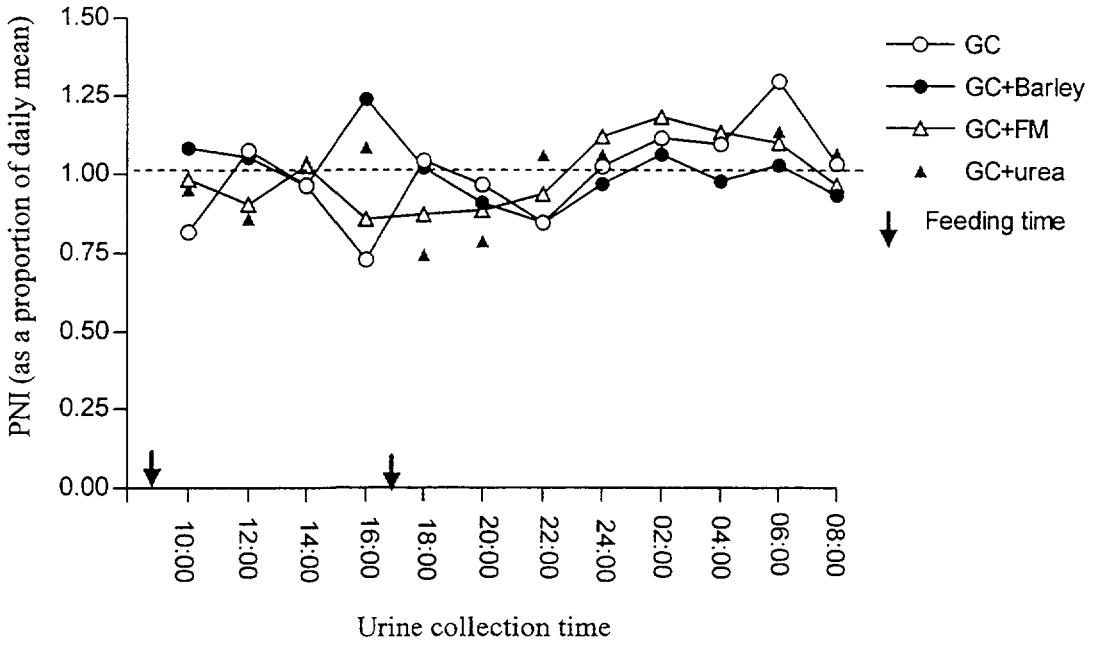

FIG.3. Diurnal variation in PNI as affected by dietary supplements.

4. DISCUSSION

4.1. Assumptions made in this work

In the six experiments reported here, microbial protein N production was not measured directly but estimated based on PD excretion. It can thus be criticised that the relationship between NCE and PNI has some element of auto-correlation since PD excretion as a variable is present in both terms. However, microbial protein N flow is an independent variable that can be measured by other methods, such as those based on ^{35}S or RNA as microbial markers. The concept of using PNI to indicate microbial N production relative to RDN intake should therefore still be valid.

The NCE values in this work should not be taken as absolute but as relative, again since microbial N production was not measured directly. Results of several studies have shown that there was a close agreement, and a linear relationship, between microbial protein nitrogen estimated by PD excretion and direct measurements based on isotopic and microbial markers [3, 4]. Therefore, the NCE thus calculated should be rather close to, or linearly correlated

with, the true values. Further experiments should be conducted in the future in which direct measurements of microbial N production are made to calculate NCE.

4.2. PNI and NCE relationship and other factors affecting PNI

As expected, over the NCE range of 0.19-1.03, PNI, which ranged from 0.024-0.160, was positively correlated with the NCE based on the pooled data of six experiments. Within Experiments 1, 2, 3 and 6 individually, this relationship could not be revealed since the ranges of either NCE or PNI were too small relative to the error of the regression.

The data from the six experiments showed that PNI was linearly correlated with the NCE without considering other factors. However, it is known that, apart from NCE, endogenous N output, nitrogen intake, protein degradability, digestibility of protein, and inefficiency of absorbed amino acids can all affect the value of PNI. The interrelationship between these factors are shown in Figure 4. In order to understand the intrinsic relationship between PNI and NCE, a mathematical modelling approach is required. Here we make some attempt to derive an equation that relates PNI and NCE.

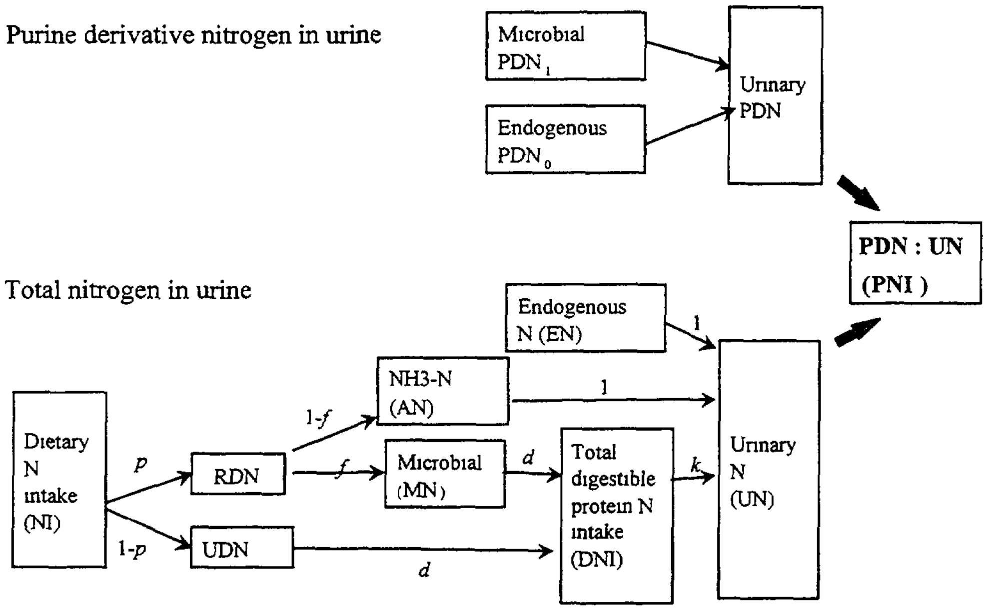

FIG.4. An illustration of factors affecting PNI.

Total nitrogen excretion in the urine (UN) is determined by endogenous nitrogen excretion (EN), intake and digestibility (d) of total protein, i.e. microbial protein (MN) plus un-degraded dietary protein (UDN), the proportion of the absorbed amino acids that is not retained but excreted in the urine (k) and absorbed ammonia nitrogen (AN). Here it is assumed that all of the AN is excreted in the urine and that MN and UDN have the same digestibility.

UN = EN + d k (MN+UDN) + AN ....(1)

Equation (1) can be re-arranged as:

$$1 = \frac{EN}{UN} + dk\frac{MN}{UN} + dk\frac{UDN}{UN} + \frac{AN}{UN}$$

The terms UDN/UN and AN/UN can be replaced by MN/UN, taking into account protein degradability (p), and NCE (f):

$$1 = \frac{EN}{UN} + dk\frac{MN}{UN} + dk\frac{(1-p)MN}{p \times f \times UN} + \frac{(1-f)MN}{f \times UN}$$

The above equation can finally be re-arranged as:

$$\frac{MN}{UN} = \left(1 - \frac{EN}{UN}\right) \times \left[\frac{pf}{(dk + p - dkp) + (dk - 1)pf}\right] \quad \ldots\ldots(2)$$

The urinary excretion of PD nitrogen is a function of the endogenous purine excretion and absorption of microbial biomass. Derived from the equations of Chen *et al.* [1, 13], PDN (g/d) in sheep can be expressed as a function of microbial N supply (MN, g/d):

$$PDN = 0.0647\ MN + 0.0084\ W^{0.75} e^{-0.34\ MN} \quad \ldots\ldots(3)$$

$$\text{Thus } PNI = \frac{PDN}{UN} = 0.0647\frac{MN}{UN} + \frac{0.0084W^{0.75}e^{-0.34MN}}{UN}$$

We are unable to derive a simple equation whereby PNI can be calculated. The following equation gives an approximation:

$$PNI \approx \left(1 - \frac{EN}{UN}\right) \times \left[\frac{0.0647pf}{(dk + p - dkp) + (dk - 1)pf}\right] + 0.024 \quad \ldots\ldots(4)$$

At an extreme situation where the animal does not have any exogenous input of nitrogen or microbial nitrogen,

PDN_0=0.0084 g/kg $W^{0.75}$ per day and

UN_0=EN = 0.350 g/kg $W^{0.75}$ per day [14].

$$\text{Thus } PNI = \frac{0.0084W^{0.75}}{0.350W^{0.75}} = 0.024$$

In Equation (4), the term $\left(1 - \frac{EN}{UN}\right)$ (referred to as A) represents the proportion of total urine nitrogen (UN) that is not endogenous. Its value increases with nitrogen intake. The value can not be determined easily but may be estimated based on the creatinine:N ratio at fasting (R_0) and in the same urine where PNI is calculated as (R_1): A = 1-R_1/R_0. In Equation 4, d, k and p are parameters that can be measured or already published in the literature. Figure 5 shows the simulated values of PNI at different A values and the observed values, both plotted against the observed NCE (f in the equations). The parameters used in the simulation were: d = 0.85 (from Storm *et al.* [15]), p = 0.80, k = 0.25 (calculated as 1-0.75, the later is reported value for the efficiency of utilisation of apparently digested amino acid nitrogen in the small intestine, recommended by ARC [14]).

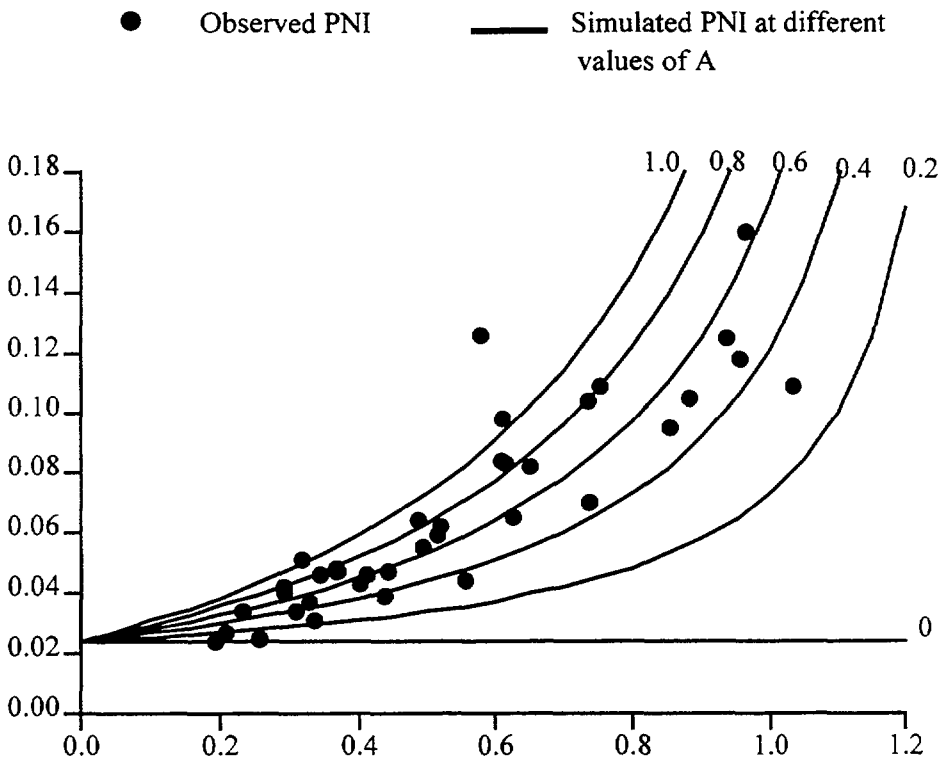

FIG.5. PNI values calculated based on Equation 4.

It should be stated that the mathematical modelling presented here is only tentative and has yet to be improved. However, the modelling exercise indicates that in order for the PNI to provide an indication of the NCE, other parameters such as, dietary protein degradability and dietary nitrogen intake, should also be considered or measured.

4.3. Changes in PNI in response to dietary treatments

The responses of PNI to dietary treatments in Experiments I and II were as expected. Urea supplementation at 16 g/d lead to an increased urinary nitrogen output and thus a lower PNI.

4.4. Use of spot urine samples

The feasibility of using PNI measurements in spot urine samples are subject to two criteria. First, there should be relatively little diurnal variability in the PNI. Second, the PNI measurements made based on spot samples must be correlated with those based on daily urine collection. Results from Experiments I and II can be used to evaluate the first criterion. The CV for the spot measurements was 20%, based on which the least significant difference (LSD) between two treatments can be estimated. If four spot measurements are made (n = 4) from each treatment, the LSD should not be lower 33% in order to reach statistical significance at $P < 0.05$ level. If three or two measurements are made, the LSD needs to be 42

and 64%, respectively ($LSD = t\sqrt{\frac{2}{n}} \cdot CV$, where t = the tabulated t distribution at (2n-1) degree of freedom; n = number of spot measurements from which the mean is derived). Although there is no clear pattern of diurnal variation, the data did indicate that PNI could be affected by time of feeding if the diet contains high content of a highly degradable N source. Taking this into consideration, multiple samples would be required in order to derive a measurement of PNI as representative as possible, and it is recommended to spread the sampling for different times after feeding. There is no appropriate data in this work to evaluate the second criterion. This is because in Experiments I and II when spot urine sampling was made, the ranges of the PNI values based on daily urine collection, and of those based on spot urine collection, were both too small to evaluate the correlation.

4.5. Potential application, limitations and future work

PNI is unique in that it provides an indication of efficiency at which RDN is converted to microbial protein, as well as the potential cost of N waste to the environment that a feeding regime may incur. PNI can be measured readily particularly if urine spot samples can be used.

PNI could be used as one of the criteria to help formulate ruminant diets that are biologically more efficient and produce less N waste. A practical application would be to set an empirical threshold criteria for a specific group of ruminants, and diets with PNI values lower than this threshold are graded as unsatisfactory with respect to N utilisation. For example, based on the preliminary data from this work, a diet with a PNI lower than 0.08 for sheep would seem unsatisfactory. In this system, PNI is effectively used as a semi-quantitative parameter and its application would not be detrimentally affected by the 20% variability from spot measurement. Therefore, where complete urine collection is not plausible, measurements of PNI may be made based on an incomplete but major fraction of the urine, or multiple spot urine samples. PNI may also be measured in digestion studies, the data of PNI would complement the data of efficiency of microbial N supply per kg organic matter fermented, and provide an indication of the efficiency of utilisation of nitrogen.

Measurement of PNI alone is however not sufficient to assess the scale of NCE as indicated in Equation (4). PNI measurements should therefore be used together with other parameters such as protein degradability and dietary nitrogen intake.

The limitations of the PNI are: i) the index does not offer any explanation as to the cause of poor efficiency, ii) the variations as noted in this work indicate that PNI is not sensitive for detecting small differences. The latter limitation could be overcome by using it in a 'grouping' system.

5. CONCLUSIONS

In this work, we conceptually propose a new parameter, PNI, which could potentially provide a simple and rapid means for assessing the efficiency of conversion of dietary nitrogen to microbial protein. Used in conjunction with other existing parameters, it would be particularly useful in research into improving ruminant feeding efficiency at the rumen level and reducing nitrogen waste to the environment. Some preliminary data are presented in this work, but further experiments with direct measurements of microbial nitrogen production need to be made to validate the concept and to provide information for application. The mathematical model which relates PNI with NCE and other parameters also needs further development.

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MEASUREMENT OF PURINE DERIVATIVES IN THE URINE OF SOME RUMINANT SPECIES

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Abstract

MEASUREMENT OF PURINE DERIVATIVES IN THE URINE OF SOME RUMINANT SPECIES.

The application of published high performance liquid chromatography (HPLC) methods for the determination of PD in urine of cattle, sheep, buffaloes (*Bubalus bubalis*) and arabian camels (*Camelus dromedarius*) was investigated.

Urine was taken from two water buffaloes, two camels, three cows and four sheep, all fed at maintenance level. Total nitrogen content in urine was determined using a micro-Kjeldahl procedure. Allantoin, uric acid and creatinine levels were determined colorimetrically while xanthine and hypoxanthine concentrations were determined by HPLC.

Relative proportion of allantoin ranged from 74 ± 7 to 91 ± 1% in camels and cattle, respectively. Uric acid proportion was very low in camel urine (1.7 ± 1) but ranged from 3.7 ± 3 to 9.2 ± 1% in sheep and cows, respectively. Xanthine + hypoxanthine ranged from 11 ± 3 to 25 ± 7% in buffalo and camels, respectively. Total PD:Creatinine ratio (mol/mol $W^{0.75}$) was 118 ± 15, 46 ± 17, 37 ± 9 and 33 ± 5 for cattle, camels, buffaloes and sheep respectively.

The adoption of a single method for the simultaneous detection of all derivatives proved difficult due to elution of polar coextractives at the same retention times as the peaks of allantoin, uric acid and creatinine.

1. INTRODUCTION

The urinary excretion of purine derivatives (PD) has been validated as an index of microbial protein yield in the ruminant species [1-3]. Chen *et al.* [4] reviewed different analytical procedures for the determination of PD concentrations in urine. Colorimetric methods have low technology requirements but are usually based on the Rimini-Schryver reaction that is not specific for allantoin [5, 6]. HPLC methods can be applied to detect allantoin, uric acid, xanthine and hypoxanthine [7, 8], or allantoin by a pre-column derivatization procedure that converts it to glyoxilic acid [9]. This latter technique is simple and fast but it only measures the converted allantoin. Previous work from our own lab [10] was entirely based on colorimetric and chemical techniques. Compared to HPLC techniques, these methods are less sensitive to small variations in PD concentrations and vulnerable to matrix effects. However, a good correlation between both methods have been obtained [11].

A trial was set up with the objective of evaluating two published HPLC methods. They were modified for the simultaneous determination of all PD in urine of four ruminant species: cattle, sheep, buffalo (*Bubalus bubalis*) and one-humped camel (*Camelus dromedarius*), and to provide some preliminary information on their PD profiles.

2. MATERIAL AND METHODS

2.1. Animals and management

2.1.1. Buffaloes

Three dry buffaloes (live weight (LW) = 671 kg ± 65) which had been bred in Italy were fed two iso-nitrogenous diets (CP = 15.2% of DM) formulated as follows, on DM basis. Diet 1: 55% lucerne hay, 25% barley grain, 14% maize, 5% wheat straw and 1% soybean meal and Diet 2: 35% maize silage, 21% barley grain, 18% lucerne hay, 10% wheat bran, 9% soybean meal and 7% wheat straw. Experimental diets were given at maintenance level in two consecutive periods. Each experimental period lasted for 3 weeks with the first two weeks for adaptation and last week for sample collection. Urine was collected for 4 days from each animal during each period and from the total of 24 samples collected, 6 were selected (i.e. third day-samples of each animal, for the two periods) for analysis.

2.1.2. Camels

Two one-humped camels (LW = 290 ± 77 kg) were kept in individual stalls and fed at maintenance level (3.5 kg/d, DMI) a diet based on oat hay (95.2% DM; CP = 9% of DM). Spot urine samples were collected during 3 consecutive days at consistent sampling times.

2.2.3. Cattle

Six urine samples were collected from three dry Simmental cows (LW = 621 ± 67 kg) kept in individual stalls and fed at maintenance level (6.9 kg/d, DMI) a diet based on fescue hay supplemented with a compounded feed (CP = 13.9% of DM).

2.2.4. Sheep

Four Bergamasca sheep (LW = 65 ± 7 kg) were kept in metabolic cages and fed at maintenace level a basal diet based on fescue hay (1.5 kg/d, DMI) supplemented with a compound feed (0.3 kg/d, DMI). Urine was collected for 15 h a day (1700-0800 h) and 6 samples were selected from two consecutive days of collection from the 4 sheep.

2.2. Urine collection

All samples were collected into acidified containers (4N H_2SO_4 for buffaloes, 2M HCl for sheep, cattle and camels) in order to keep the pH of urine below 3 and immediately diluted (water:urine ratio of 1:4) to avoid the precipitation of uric acid during storage.

2.3. Analytical procedure

Urine samples were analysed for total N content (micro-Kjeldahl procedure), allantoin [6], uric acid [12] and creatinine [13]. Xanthine plus hypoxanthine were determined following the method proposed by Resines *et al.* [7] with the following modifications:

Standards were diluted with ammonium phosphate (solvent A) as described by Czauderna and Kowalczyk [8]. Wavelength was set at 254 nm. The pump was programmed as below:

Time	0	10	11	19	20
%A	100	100	50	50	100
%B	0	0	50	50	0

The HPLC conditions used were: A 300 × 4.9 mm reverse-phase NOVAPAK C_{18} column, (Waters, Milford, MA) along with a 20 × 3.9 mm NOVAPAK C_{18} guard column (Waters, Milford, MA,) were fitted to a Perkin-Elmer (series 200), connected to a ISS 100 auto sampler and lc95 UV-VIS detector (Perkin-Elmer, Norwalk, Connecticut, USA).

3. RESULTS AND DISCUSSION

3.1. HPLC methods

Two available methods to detect PD directly with a single injection and without derivatization were considered. A summary of the characteristics of both methods is given in Table I.

TABLE I. CHARACTERISTICS OF THE HPLC METHODS UNDER STUDY

Features	Resines *et al.* [7]	Czauderna and Kowalczyk [8]
Conditions	Isocratic	Gradient
Columns	Novapack C18 reversed-phase (300 mm x 3.9 mm I.D., 4 μm particles size)	Novapack C18 (150 mm x 3.9 mm I.D., 4 μm particles size) Chrompack (100 mm x 3 mm I.D., 4 μm particles size) precolumn pellicular packing material, reversed phase C18 (10 mm x 6 mm I.D.)
Mobile phase	10 mM potassium phosphate buffer (pH = 4)	binary gradient program: eluent A (ammonium phosphate 2.5 mM; pH = 3.5 with 10% phosphoric acid) eluent B (eluent A plus methanol 95/5 v/v)
Flow rate	0.5 mL/min	0.4 mL/min
Wavelength	218 nm	205 nm
Temperature	25°C (stabilized)	17-25°C (unstabilized)
Standards dissolved in:	water	solvent A

Standards were easily dissolved in the solvent 'A' described by Czauderna and Kowalczyk [8], therefore mobile phases of this method was chosen. A single column (300 mm long) [7] connected to a pre-column was chosen instead of the two 150 mm Novapack columns plus the 100 mm Chrompack column and a 10 mm pre-column connected in series, as suggested by Czauderna and Kowalczyk [8] because of the cost and technical complexity of this option.

A standard solution for each PD was prepared and injected to test the response of the method, and clear peaks were detected at 205 nm, with allantoin appearing at 2.93, creatinine at 3.73, uric acid at 5.48, hypoxanthine at 6.15 and xanthine at 7.44 minutes. Choosing the best wavelength to detect different substances within the same run is critical. The best absorption for the metabolites ranges between 200 and 300 nm. Resines *et al.* [7] suggested 218 nm while Czauderna and Kowalczyk [8] used 205 nm. Our preliminary tests with a

spectrophotometer indicated that metabolites could be divided into two groups: one that showed best linear response to changes of concentration at 200 nm (allantoin, uric acid and creatinine) and the other showing best results at 260 nm (xanthine and hypoxanthine).

A chromatogram from the injection of a mixture of the standards with wavelength set at 205 nm [7] is shown in Figure 1.

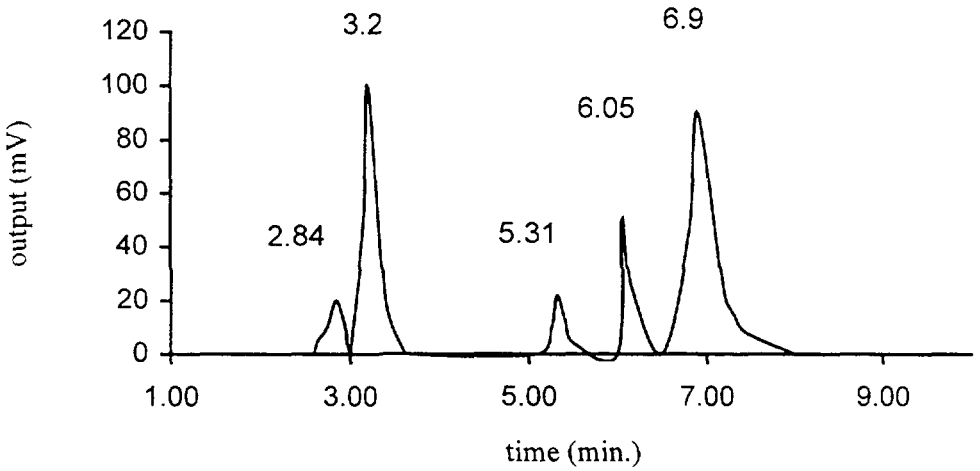

FIG.1. Chromatogram of the standard mixture, with peaks for allantoin (2.84 min), creatinine (3.2 min), uric acid (5.31 min), hypoxanthine (6.05 min), and xanthine (6.9 min).

However, when injecting urine samples the peaks for allantoin, creatinine and uric acid were not clearly detected because they overlapped with unidentified compounds. One of the reason for this is the high polarity of allantoin and uric acid that requires very low wavelenghts and long columns to separate allantoin from other chemical compounds present in the urine. These compounds have chemical properties which are comparable to those of allantoin, therefore the separation of the latter PD was complicated by the "background noise" generated in the column by the chemically-competitive compounds [4]. It may be possible to achieve better separation using solid phases more suitable for polar compounds and multi-wavelength detectors.

These suggestions can be adopted to elucidate the metabolism of PD studying every derivative to the most detailed level for research purposes, however the approach is necessarily expensive. A further level of application of PD is their practical use for the evaluation of the efficiency of feed utilization by ruminants in developing countries. This scenario conflicts with the above mentioned high-tech approaches, requiring simple and cheap measures, and often applying these measures to species that render a daily total urine collection virtually impossible. Therefore, possible suggestions would include the conversion of all derivatives to the more oxidized form, i.e. allantoin, expressing total PD as units of allantoin and using colorimetric or HPLC approaches as the two measures seem to be strictly related [11]. Moreover, the spot sampling technique, crucial for a widespread adoption of PD technique at the farm level particularly with grazing animals, is still capable of detecting only large differences in rumen microbial nitrogen yield [14].

Due to the analytical retrictions described above it was decided to analyse allantoin, uric acid and creatinine by standard methods, as reported in the material and methods, limiting the use of the modified HPLC method to xanthine and hypoxanthine. The absorbance of 254 nm was chosen because it was virtually equal to the one giving the best absorbance rates for

hypoxantine and the same adopted in our department for the analysis of allantoin by HPLC with pre-column derivatization [9].

3.2. Purine derivative partitioning

The concentration of PD in the urine of cattle, sheep, buffalo and camel are shown in Table II. PD concentrations were consistent with our previous findings for cattle and sheep. Camel urine showed the lowest uric acid concentration although an extensive precipitation of uric acid during sample storage could be not discarded, even when deposits of urate salts were not detectable under microscopic examination.

TABLE II. CONCENTRATION OF PD IN URINE SAMPLES OF DIFFERENT SPECIES

	Buffaloes mean ± sd	Camels mean ± sd	Cattle mean ± sd	Sheep mean ± sd
Allantoin (mg/L)	391.9 ± 147.2	952.2 ± 333.1	1801.1 ± 364.9	2364.7 ± 1074.4
Uric acid (mg/L)	43.7 ± 20.6	17.7 ± 11.4	180.3 ± 32.1	84.2 ± 47.3
Xanthine (mg/L)	35.5 ± 7.9	157.2 ± 69.2	ND	93.3 ± 36.2
Hypoxanthine (mg/L)	15.9 ± 18.1	167.9 ± 147.8	ND	512.4 ± 234.8
Total PD (mg/L)	486.9 ± 174.2	1294.9 ± 456.9	1981.5 ± 385.3	3054.7 ± 1307.2
Total N (g/L)	8.8 ± 1.78	6.4 ± 3.9	9.9 ± 2.0	12.7 ± 4.2

ND= not detected

Table III presents the relative proportions of the different derivatives. Allantoin concentration in cattle urine (91%) was similar to our previous findings [10]. Sheep and camel urine contained 74-76% allantoin whereas buffalo urine showed a intermediate value (80%). Neither xanthine nor hypoxanthine were detected in cattle urine while a significant proportion (20%), was found in sheep urine. Differences in urinary PD profiles has been previously defined using both colorimetric [1] and HPLC methods [15] among species, reflecting differences in xanthine oxidase distribution as a key enzyme in shifting PD towards allantoin and uric acid which cannot be recycled back to intermediate metabolites [16].

TABLE III. PROPORTION OF ALLANTOIN, THE SUM OF XANTHINE + HYPOXANTHINE, AND THE PURINE NITROGEN INDEX (PNI) IN THE URINE OF DIFFERENT SPECIES.

	Buffaloes	Camels	Cattle	Sheep	root MSE	Pr > F
Allantoin *	80.2 ^{b}	73.7 ^{c}	90.8 ^{a}	76.1 ^{bc}	4.88	0.0001
Xanthine + Hypoxanthine *	10.9 ^{b}	24.6 ^{a}	-	20.2 ^{a}	5.07	0.001
PNI **	0.019 ^{b}	0.088 ^{a}	0.071 ^{a}	0.086 ^{a}	0.02	0.0001

* = as percentage of total PD concentration;

** = PD nitrogen as a fraction of total urinary nitrogen.

When comparing the urine of buffaloes to those of cattle the former had a realively high xanthine plus hypoxanthine contribution to total PD, higher than the values previously reported by Liang *et al.* [17] and Chen *et al.* [18]. Chen *et al.* [18] also demonstrated that xanthine oxidase profile in buffalo was similar to that in cows indicating the capability to convert almost all salvageable PD to uric acid. Differences may be explained by breed selection of Italian-bred buffaloes as well as the methodological approaches used in the different studies.

Camels showed a high proportion of xanthine plus hypoxanthine in their urine whereas uric acid proportion was very low. Bakker *et al.* [19] postulated that this metabolic profile would provide high antioxidant activity to scavenge radicals generated by the stressful exposure of these animals to the extreme chill of desert nights.

Table III also presents the ratio between purine-N and total-N (Purine Nitrogen Index, PNI) in the urine. This parameter has been proposed by Chen and Jayasuriya [20] as an index of the utilization of nitrogen in the rumen (PD nitrogen) compared to the amount wasted in the urine.

Camels, cattle and sheep had comparable values of this index, while buffaloes showed lower values. Indeed, PNI may reflect differences in feed-N conversion efficiency and/or endogenous PD excretion, which in turn is the result of enzymatic activity in blood and tissues reflecting differences in metabolic activity among species. In this sense Liang *et al.* [17] compared swamp buffaloes to indigenous Malaysian cattle and suggested a lower conversion of digestible DMI into urinary allantoin. Similar results were obtained by Chen *et al.* [18], who also indicated that differences may be explained by the efficiency of microbial synthesis or digestibility of microbial purines.

In absence of total urine collection, PD:creatinine ratio may provide an index to detect differences in PD excretion assuming that creatinine excretion depends only on the body mass and is constantly excreted through the day. Each derivative and the total PD concentration was expressed as a ratio to creatinine concentration which in turn had to be divided by metabolic live weight (MLW) to allow comparisons between species. This double correction is required for two reasons:

- to render PD excretion independent of the time of sampling, because creatinine and PD have similar daily fluctuations [19]. The ratio can than be adopted to evaluate spot urine sampling
- to correct for the animal body mass that is linearly and positively related to the urinary excretion of creatinine [20].

Table IV presents PD:creatinine ratio for the four species given a similar feeding regime. No difference was observed between sheep, buffalo and camel. However, cattle showed significantly higher ($P < 0.001$) PD:creatinine values than the other species. Allantoin:creatinine ratio was similar to that of PD:creatinine ratio whereas uric acid and salvageable PD (hypoxanthine plus xanthine):creatinine ratio was lower in buffaloes than in sheep and camels.

This ranking is the result of dietary and endogenous aspects which were not fully considered in this preliminary study, but literature consistently reported higher values of endogenous PD excretion in cattle than sheep [15] and low values of endogenous excretion and conversion of excreted PD to microbial protein in the rumen of buffaloes [18].

Very few data are available to describe the metabolism of PD and their relationship to ruminal microbial protein synthesis in one-humped camel, because of the difficulty of collecting urine and measuring microbial protein synthesis in these animals. However, these preliminary results seem to indicate that this species has a purine metabolism that is more comparable to sheep and buffaloes rather than to cattle.

TABLE IV. RATIO OF PD:CREATININE RATIO FOR THE FOUR SPECIES ($W^{0.75}$)

	Buffaloes	Camels	Cattle	Sheep	MSE	Significance
Allantoin:creatinine	29.2 ^{b}	32.4 ^{b}	107.5 ^{a}	24.8 ^{b}	10.01	0.0001
Uric acid:creatinine	3.0 ^{b}	0.7 ^{c}	10.1 ^{a}	1.1 ^{c}	0.76	0.0001
Xanthine + Hypoxanthine:creatinine	4.4 ^{b}	12.7 ^{a}	-	7.5 ^{ab}	4.89	0.030
PD:creatinine	36.6 ^{b}	45.8 ^{b}	117.6 ^{a}	33.3 ^{b}	12.42	0.0001

ratios are expressed as mmol/mmol

4. CONCLUSIONS

The preliminary investigations presented here has shown that the adoption of a single method for the simultaneous detection of all purine derivatives is difficult due to elution of polar coextractives at the same retention times as the peaks of PD and creatinine. The determination of allantoin and uric acid is particularly difficult because of the chemical properties of these compounds that cause a fast elution from the column. Therefore, further studies are required to investigate methods based on a single direct injection of the sample to detect all purine derivatives.

Even though the trial was not directly aimed at comparing species, some conclusions can be drawn to address further research. Water buffaloes need further investigation because of their potential economic interest and the lack of information regarding purine metabolism. In addition, more detailed studies are required to clarify PD metabolism of one-humped camels.

The contribution of the different PD to total daily excretion, the estimation of endogenous contribution to total PD excretion, and the conversion factors for estimation of microbial protein production under different dietary regimes and physiological conditions require to be elucidated in these two latter species. The spot sampling technique, crucial for the widespread adoption of PD approach on-farm in developing countries, needs further research to fully define its effectiveness for predicting differences in rumen microbial protein supply.

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