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Estimation of rumen microbial protein production from purine derivatives in urine

A laboratory manual for the FAO/IAEA Co-ordinated Research Programme on Development, Standardization and Validation of Nuclear Based Technologies for Measuring Microbial Protein Supply in Ruminant Livestock for Improving Productivity

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FOREWORD

The Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture has a long history of supporting isotope aided research programmes for improving animal productivity in developing countries. These programmes have focused on animal nutrition, animal reproduction and more recently on animal nutrition/reproduction interactions with emphasis on small holder farming systems. This approach has lead to the identification and alleviation of a number of problems associated with the feeding and reproductive management of ruminant livestock in developing countries.

The measurement of microbial protein supply to ruminant livestock has been an important area of research in ruminant protein nutrition. An estimate of microbial protein contribution to the intestinal protein flow has been of significant importance when estimating 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 and require cannulated animals.

Under a Technical Contract awarded to Rowett Research Institute, United Kingdom, through the FAO/IAEA Co-ordinated Research Programme on Development of Feed Supplementation Strategies for Improving the Productivity of Dairy Cattle under Smallholder Farms in Africa, a simple colorimetric technique has been developed to analyse purine derivatives in urine. Using this information, the knowledge of endogenous excretion and other factors affecting the production and excretion of purine derivatives, it is possible to predict microbial protein supply to the host animal. This method has been 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 *Bos indicus* and buffaloes differ in their rate of purine excretion and require a different prediction model.

In May 1995, the Joint FAO/IAEA Division held a consultants meeting to advise on the feasibility of using nuclear and related techniques for the development and validation of techniques for measuring microbial protein supply in ruminant animals. After reviewing the current state of knowledge, the meeting discussed the usefulness and application of the purine excretion method in relation to other currently available methods. The meeting was of the opinion that, with some 'fine tuning' and validation to suit developing country situations, it could be used to estimate microbial protein supply in ruminant livestock and thereby develop feeding strategies for improving productivity. This method is particularly well suited for diagnostic use on-farm and for research in laboratories with limited equipment and technical expertise. On the basis of the above expert consultation an FAO/IAEA Co-ordinated Research Programme 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 to suit developing country situations.

This laboratory manual contains the methodologies used in the standardization and validation of the urine purine derivative technique for estimating microbial protein supply to the rumen. It includes descriptions of methods that involve both radioactive and stable isotopes as well as non-isotopic techniques such as chemical assays, since it has been recognized that while isotopic tracer methods provide a powerful tool for nutrition research they cannot and should not be used in isolation.

The IAEA and FAO would like to thank all Research Agreement and Research Contract holders for their contribution towards the preparation of this manual. Special thanks are due to X.B. Chen of the Rowett Research Institute, United Kingdom, for compiling the detailed experimental protocols presented in this manual. This manual is being published as an IAEA-TECDOC so that experimental protocols useful for measuring urine purine derivatives and the methodology for development of models to suit local conditions would be available to other scientists undertaking work on microbial protein supply in ruminant livestock. The IAEA officer responsible for this publication was M.C.N. Jayasuriya of the Joint FAO/IAEA Division.

EDITORIAL NOTE

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FAO/IAEA CO-ORDINATED RESEARCH PROGRAMME ON DEVELOPMENT, STANDARDIZATION AND VALIDATION OF NUCLEAR BASED TECHNOLOGIES FOR MEASURING MICROBIAL PROTEIN SUPPLY IN RUMINANT LIVESTOCK FOR IMPROVING PRODUCTIVITY

1. OBJECTIVES AND SCIENTIFIC SCOPE OF THE PROJECT

1.1. Introduction

Under-nutrition due to inadequate or fluctuating nutrient supply is a major constraint to animal production in developing countries. Poor nutrition results in low rates of reproduction and production as well as increased susceptibility of livestock to disease.

The smallholder farmers in developing countries have limited resources available for feeding their ruminant livestock. Unlike those in developed countries, they are unable to select their basal diet according to requirement for production. The strategy for improving production has therefore 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 requirement.

Microbial cells formed as a result of rumen digestion 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 paramount 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 radioisotopes ^{35}S, ^{15}N or ^{32}P. However, the need to use post-ruminally cannulated animals and complex procedures to determine digesta flow are major limitations.

A colorimetric technique using enzymatic procedures has been developed for measuring purine derivatives in urine. With knowledge of the amount of purine derivatives 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 purine derivative 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 purine derivatives in the urine. Current evidence suggests that milk and other body fluids are not appropriate for developing prediction equations.

The purine 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, 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 purine derivatives 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 it 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 purine derivatives 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 purine excretion to digestible dry matter (DM) intake, 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). The purine excretion method depends on an 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.

The FAO/IAEA Co-ordinated Research Programme has the following objectives:

(a) To refine and standardize the purine excretion technique for measuring microbial protein supply in ruminant livestock.

(b) To extend the above technique for indigenous Zebu cattle (*Bos indicus*), their crosses with European breeds (*Bos taurus*), and buffaloes in developing countries, using nuclear and other related techniques.

(c) To make the purine excretion technique available as a robust and inexpensive method for estimating rumen microbial protein supply which can be used for developing feeding strategies to improve productivity of ruminant livestock in developing countries.

The Programme will be carried out in two phases. In Phase 1, which will last for 2 years, the technology developed based on research at the Rowett Research Institute, UK and other European laboratories, will be refined and validated for indigenous Zebu cattle (*Bos indicus*), their crosses with European breeds and buffaloes. In Phase 2, which will last for 3 years, the validated technique will be applied at field level to estimate the supply of rumen microbial protein and the information will be used to develop feeding strategies in developing countries.

1.2. Objectives of Phase 1

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

Appropriate methodology and model 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) and therefore the prediction

equations currently available 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 includes:

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

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

(iii) The relationships between rate of purine absorption from the gut and rate of excretion of purine derivatives 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 nitrogen (N):total nitrogen in mixed rumen microbes

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. In order to ensure that differences in the ratio, if any, are due to the animals and conditions, and not to analytical errors, all analyses should be done using an identical protocol (developed by the Rowett Research Institute, UK).

1.3. Objectives of Phase 2

During Phase 2 of the Programme the fully validated urine purine technique will be applied at farm level to estimate the microbial protein production in ruminant livestock and the information will be used in developing feeding strategies for improving productivity.

Once standardized and validated the technique can be used as a diagnostic tool by National Agricultural Research Services (NARS) to indicate whether an animal or group of animals have been underfed or fed on an imbalanced diet, thus enabling them to take 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 the animals. This will be particularly relevant for milking cows where the protein supply may be most critical and where inexpensive microbial protein would be much cheaper than undegraded protein.

2. BACKGROUND INFORMATION RELATING TO THE OBJECTIVES OF THIS RESEARCH PROGRAMME

2.1. How does the urinary excretion of purine derivatives relate to the microbial protein supply in ruminants?

Most ruminant feeds contain negligible amounts of nucleic acid. In the rumen, dietary nucleic acids are extensively broken down by micro-organisms. Therefore the nucleic acid arriving at the lower gut for digestion and absorption by the animal is essentially of microbial origin, i.e. nucleic acids from the microbial cells. In cattle, absorbed purines are almost completely converted into uric acid during passage across the intestinal mucosa before reaching the liver. Uric acid can then be converted into allantoin. Allantoin and uric acid are referred to as 'purine derivatives' (PD). In cattle, the daily excretion of PD is linearly correlated with the amount of microbial purines absorbed. If we assume that the ratio of protein to purine in mixed microbial population is constant, then PD excretion provides an index for the calculation of the intestinal flow of microbial protein.

2.2. How to estimate microbial protein supply from purine derivative excretion ?

To estimate the microbial protein supply from purine derivative excretion the following information is required.

- The response between PD excretion in urine and the absorption of microbial purines.
- The ratio of total-N : purine-N in mixed rumen microbes.

Here we use the information available for European cattle to illustrate how we use PD excretion to estimate microbial protein flow. PD excretion (Y mmol/d):

$$Y = 0.85X + (0.385\ W^{0.75}) \qquad (1)$$

where X = purine absorption (mmol/d), and $W^{0.75}$ = metabolic body weight (kg) of the animal.

Once Y is determined, X can be calculated. For example, a steer of 321 kg live weight excreted 153 mmoles of PD per day. The amount of microbial purines absorbed can be calculated using equation (1) as:

$$153 = 0.85X + (0.385 \times 321^{0.75})$$
$$X = (153 - 0.385 \times 321^{0.75}) \div 0.85$$
$$= 145.7 \text{ mmol/d.}$$

Then microbial N (nitrogen) yield is calculated using:

$$Microbial\ N(gN/d) = \frac{X(mmol/d) \times 70}{0.116 \times 0.83 \times 1000} = 0.727X \qquad (2)$$

The following factors are used in Equation (2):

(i) Digestibility of microbial purines is assumed to be 0.83. This is taken as the mean digestibility value for microbial nucleic acids based on observations reported in the literature.
(ii) The N content of purines is 70 mg N/mmol.

(iii) The ratio of purine N· total N in mixed rumen microbes is taken as 11.6:100.
Therefore, the intestinal flow of microbial N for the steer can be estimated as 106 g N/d.

Please note, the parameters in Equation (1) may not apply to animals in the tropics. The objectives of the present project are to establish Equation (1) for indigenous cattle and buffaloes, and the value of purine N total N in Equation (2).

2.3. Can the established equation derived from European cattle be used for other species of ruminants?

No Available information indicates huge differences between species in their responses of PD excretion to purine absorption For example, buffaloes excrete less PD per unit feed intake than *Bos taurus* cattle. Therefore a different equation may need to be used.

2.4. How to establish equations for different species?

One method would be to infuse various levels of purines into the abomasum of the animal and monitor the PD excretion in urine The equations for European sheep and cattle were established using this method. This process however is time-consuming.

Previous studies have enabled us to develop models to describe PD excretion (Y mmol/d) in relation to purine uptake (X mmol/d) It is now clear that in zebu cattle and buffaloes, the relationship is likely to be: $Y = e + b X$, where 'e' is the endogenous excretion in urine, and 'b' corresponds to proportion of PD entering the plasma that is excreted in the urine The endogenous excretion 'e' could be estimated by fasting excretion; and the 'b' can be determined by tracer techniques.

2.5. A model describing the relationship between purine absorption and purine derivative excretion in urine

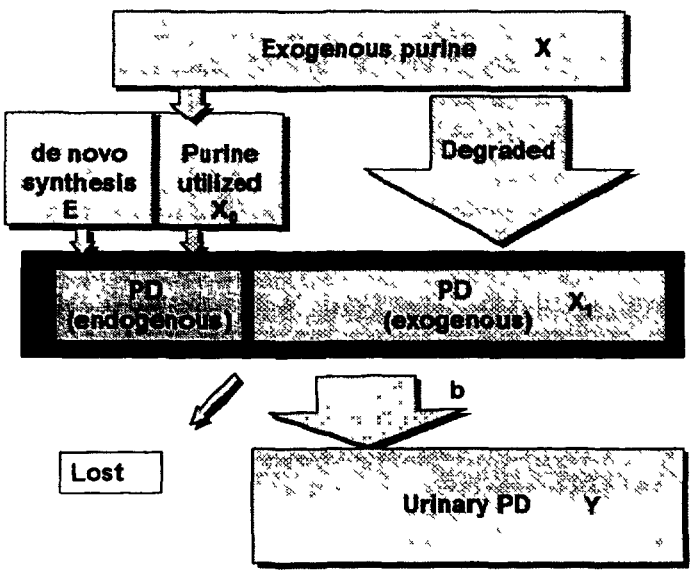


FIG 1. A model describing the relationship between purine absorption and purine derivative excretion in urine [1].

The above model which is general to all species including sheep, describes the relationship between PD excretion (Y) in the urine and the exogenous purine uptake (X) taking into account the contribution from the endogenous purines. Let's assume that X mmol/d of exogenous purines are absorbed from the gut and enter the liver. X_0 mmol/d of the exogenous purines could be utilized (more precisely, incorporated into tissue nucleic acids) to replace part or all the endogenous purine loss. The rest of the exogenous purine X_1 will be converted into PD which then enters the blood. The animal has an obligatory loss of purines leading to the production of PD. We call this fraction 'endogenous PD'. In order to replace this loss, the animal will resort to the *de novo* synthesis to produce purines from amino acids (the amount referred to as E in the diagram). However, if there is exogenous purine available for utilization, E will be reduced accordingly. The endogenous purine loss is equal to the sum of E and X_0. Therefore, the total amount of PD entering the blood can be calculated as E+X.

Not all of the PD in the blood is excreted in the urine. It is known that some can be disposed of by secretion into the gut either via saliva or direct secretion into the gut lumen. The proportion of total PD in the blood that is excreted in the urine is defined as 'b' in the diagram.
Therefore the PD excretion in urine:

$$\begin{aligned} Y &= b\,(E+X) \\ &= bE + bX \end{aligned} \tag{3}$$

When exogenous purine input is zero (i.e. X=0), bE is the measured endogenous excretion.

In cattle, there is little utilization of exogenous purines (i.e. X_0 is small), therefore bE in Equation (3) can be taken as a constant. An equation derived from cattle experiment of Verbic *et al.* [2] is:

$$Y = (0.385\, W^{0.75}) + 0.85X \tag{4}$$

In sheep, bE decreases as X increases. In the following equation, E is empirically defined as a exponential function of X:

$$Y = (0.150\, W^{0.75}\, e^{-0.25X}) + 0.84X \tag{5}$$

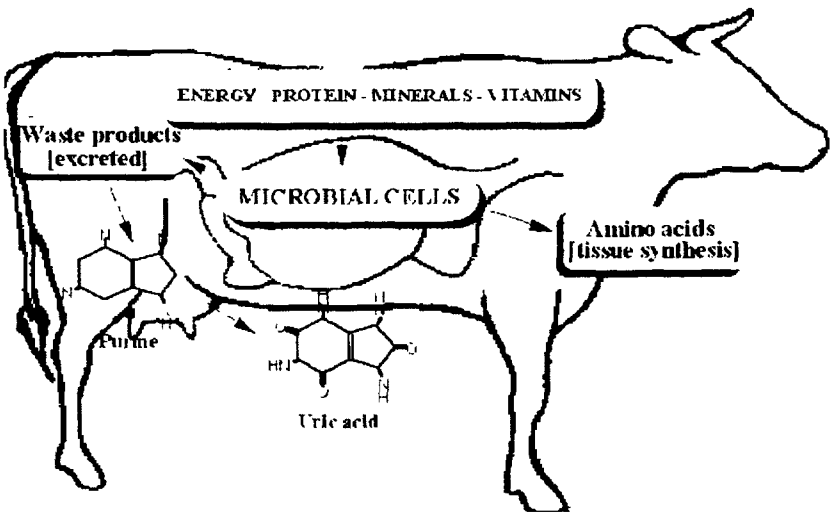
It is interesting to note that the value of 'b' is similar for cattle (0.85) and sheep (0.84). Available information indicates that the 'b' value is lower with buffaloes and zebu cattle. Whether the equation is linear (as in cattle) or non-linear (as in sheep) can be qualitatively determined by the status of xanthine oxidase activity in the intestinal mucosa. A high activity of xanthine oxidase in the intestinal mucosa makes exogenous purine unavailable for utilization. Since the net endogenous contribution is constant, the purine input-output response equation is linear.

REFERENCES

[1] CHEN, X.B., HOVELL, F.D.D., ØRSKOV, E.R., BROWN, D.S., Excretion of purine derivatives by ruminants: effect of exogenous nucleic acid supply on purine derivative excretion by sheep, Brit. J. Nutr. **63** (1990) 131-142.

[2] VERBIC, J., CHEN, X.B., MACLEOD, N.A., ØRSKOV, E.R., Excretion of purine derivatives by ruminants: effect of microbial nucleic acid infusion on purine derivative excretion by steers. J. Agri. Sci. Camb. **114** (1990) 243-248.

EXPERIMENTAL DESIGNS, SAMPLING AND LABORATORY METHODS



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1 MEASUREMENT OF PURINE AND NITROGEN CONTENTS OF MIXED RUMEN MICROBIAL POPULATIONS

1.1. Objectives

- To examine the variability of the ratio of nucleic acid (purines) N content in mixed rumen microbial populations from sheep and cattle
- To establish a database for the purine N ratio of mixed rumen microbial samples from animals under various dietary conditions

1.2. Outline of working protocol

1. All participating laboratories should collect and process the rumen digesta samples using the given protocol
2. Yeast RNA (e g Sigma R6625) will be used as the Standard for measuring purine content in microbial samples It will be analysed for purine content at more than one laboratory using high performance liquid chromatography (HPLC) before the Standard is distributed to participants
3. The individual participants will analyse the microbial samples according to the standard procedure provided (see below) The result, therefore, will be expressed as yeast RNA equivalent Since the purine content of the yeast RNA Standard is known, the purine content of the microbial samples can be calculated
4. N content of samples of mixed rumen microbes will be determined by the method available at each laboratory The yeast RNA Standard will also be analysed as a 'control' to check possible errors due to methods of analysis
5. The data will then be combined and analysed collectively

1.3. Sampling and sample preparation

Sampling procedure

1. Make sure that all information about the animals (sheep or cattle) and feeding is recorded (please refer to the sample recording sheet) Samples will be collected from 3-5 animals for each diet Use diets that will be used in future experiment or locally relevant
2. One sample will be taken from each animal 3-6 h after feeding (Sample size >500 ml) More samples can be taken on different days
3. Transfer the samples in thermos vacuum flasks to keep them warm Prepare the rumen fluid samples for analysis as soon as possible

Note: When sampling rumen fluid, do not use a sampling device which has a small inlet or a gauze attached to the inlet, because representative sampling is difficult to achieve

Sample preparation

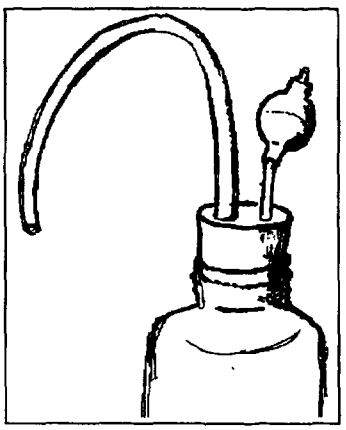
1. Strain the rumen fluid through two layers of surgical gauze to remove large feed particles
2. Transfer the remaining fluid to 250 ml plastic centrifuge bottles
3. Centrifuge at 500 *g* for 5-10 min Transfer the supernatant to small centrifuge tubes
4. Centrifuge at 20 000 *g* for 20 min (longer if centrifuge can not achieve 20 000 *g*)
5. Remove the supernatant (remove as much as possible)
6. Wash the sediment with 200 ml saline solution
7. Repeat steps 4 and 5
8. Make sure the washed sediment does not contain feed particles (check by microscopic examination)
9. Freeze the samples (sediment) for freeze-drying
10. Freeze dry and store the sample as powder At least 10 g of each sample is prepared

Reagents

- saline: 0.85% NaCl in water.

Equipment

- Rumen fluid sampling equipment (see picture).
- Thermos vacuum flasks.
- Centrifuge.
- Freeze-drier.
- Surgical gauze.



A "home made" sampling pump

Analysis (see later sections)

Analyse samples for:

- Total N by Kjeldahl method
- RNA by Zinn & Owen method [1]
- Purines (adenine and guanine) by HPLC [2, 3]

Note about the procedure used for the isolation of rumen micro-organisms

The sample preparation procedure used in this section is only intended to obtain a sample of microbial cells. The microbial cells so obtained may be derived proportionately more from liquid phase than from the solid phase. A more complex procedure is required if liquid and solid phase microbes are to be isolated completely.

1.3.1. Record sheet for rumen fluid samples

Name of participant: _____

Sample Identification: _____

Animal (type/species)	Breed	_____
	Sex	_____
	Age	_____
	Live weight	_____
Diet and Feeding	Diet composition	_____
	Intake (Approx DMI)	_____
	Feeding time and frequency	_____
	Other treatments applied	_____
Sampling	Date of sampling	_____
	Time (h after feeding)	_____
	Sample size	_____
	Sampling method	_____
Analysis	RNA content (mg yeast RNA equivalent/g DM)	_____
	Purine content (μmol/g DM)	_____
	N content (g/g DM)	_____

NOTES e g origin of sample etc

DMI - dry matter intake

1.4. Hydrolysis of samples for the determination of adenine and guanine contents using HPLC

Reagents

- HCl 1.25 N.
- $HClO_4$ 0.5M (PCA) (e.g. Aldrich 32043).
- KOH 3 N.
- Allopurinol, Oxypurinol or Caffeine 1 g/100 ml, used as internal standard.

Acid hydrolysis of samples [2]

1. Weigh samples (100 mg rumen microbes) into screw-cap culture tubes, in duplicate.
2. Add 1.25 ml internal standard solution and 5 ml 1.25 N HCl.
3. Tightly cap tubes and place in a boiling water bath (or oil bath) for 1 h.
4. Cool in tap water and transfer the samples to centrifuge tubes.
5. Add 3.75 ml 0.5M PCA and keep at room temperature for 30 min.
6. Centrifuge at 900 *g* for 20 min.
7. Cool in ice for 15 min.
8. Adjust to pH 7 using 3 N KOH and repeat step 7.
9. Centrifuge at 30 000 *g* (18 000 rpm) for 15 min.
10. Transfer the supernatant to 25 ml volumetric flasks.
11. Adjust the volume to 25 ml with distilled water.
12. Store the samples at -20°C for HPLC analysis.

1.5. Measurement of purine in rumen microbial samples [1]

Reagents

- $HClO_4$ - 70% v/v (PCA).
- $NH_4H_2PO_4$ - 0.2 M (23 g/L).
- $NH_4H_2PO_4$ - 28.5 mM (143 ml above solution and dilute to 1 litre).
- HCl - 0.5N (41.85 ml reagent grade concentrated HCl /L).
- pH 2 distilled water (adjusted pH to 2 with H_2SO_4).
- $AgNO_3$ - 0.4 M (6.9 g/100 ml) (e.g. Sigma S 0139).

When making up the $NH_4H_2PO_4$ buffers, no adjustment of pH is necessary.

Procedure

1. Weigh samples into 25 ml screw-cap culture tubes, in duplicate (for rumen microbial samples use 250 mg samples).
2. Add 5 ml 70% PCA, tightly cap the tubes, mix the contents well and incubate in a water bath at 90-95°C (or oil bath) for 1 h.
3. Cool in tap water and add 15 ml of 28.5 mM $NH_4H_2PO_4$ buffer. Mix and return tubes to the 90-95°C water bath for 15 min.
4. Filter through Whatman No.4 or No.1 filter paper.
5. Transfer 0.25 ml filtrate into centrifuge tubes, add 0.25 ml $AgNO_3$, 4.5 ml of 0.2M $NH_4H_2PO_4$ buffer and allow to stand in the dark overnight at 5°C (or >1 h).
6. Centrifuge at 12 000 *g* for 10 min, and remove the supernatant (4.2 ml). Make sure that pellet is not disturbed.
7. Wash the pellet with 4.2 ml pH 2 distilled water and repeat step 6.
8. Add 5 ml 0.5 N HCl, mix by vortex mixer.

9. Cover tubes with marbles and incubate in 90-95°C water bath for 30 min.
10. Centrifuge at 12 000 *g* for 10 min.
11. Read absorbance of supernatant at 260 nm. (For Standards, dilute 1:20 with pH 2 water before reading. Samples normally do not need dilution, however, if the OD reading is too high i.e. > the upper limit of the standard curve, it is necessary to dilute the supernatant from Step 10 accordingly so that the reading is within the standard range).

Standards

Use yeast RNA (e.g. Sigma R 6625) as the Standard. Four levels, 100, 200, 300 and 400 mg, of the Standard are carried through the procedure and dilute 1:20 with pH 2 water before step 11. The following may be used for the dilution: take 0.5 ml supernatant from Step 10 into a glass test tube, add 10 ml pH 2 water and mix by vortex mixer.

1.5.1. An example of a standard curve

Weight of standard (mg)		Dilution	Optical density (OD)		
1	2		1	2	Mean
0.0	0.0	20	0.000	0.000	0.000
107.8	105.6	20	0.142	0.139	0.141
197.3	208.4	20	0.275	0.282	0.279
299.5	300.2	20	0.424	0.400	0.412
403.6	411.0	20	0.556	0.567	0.564

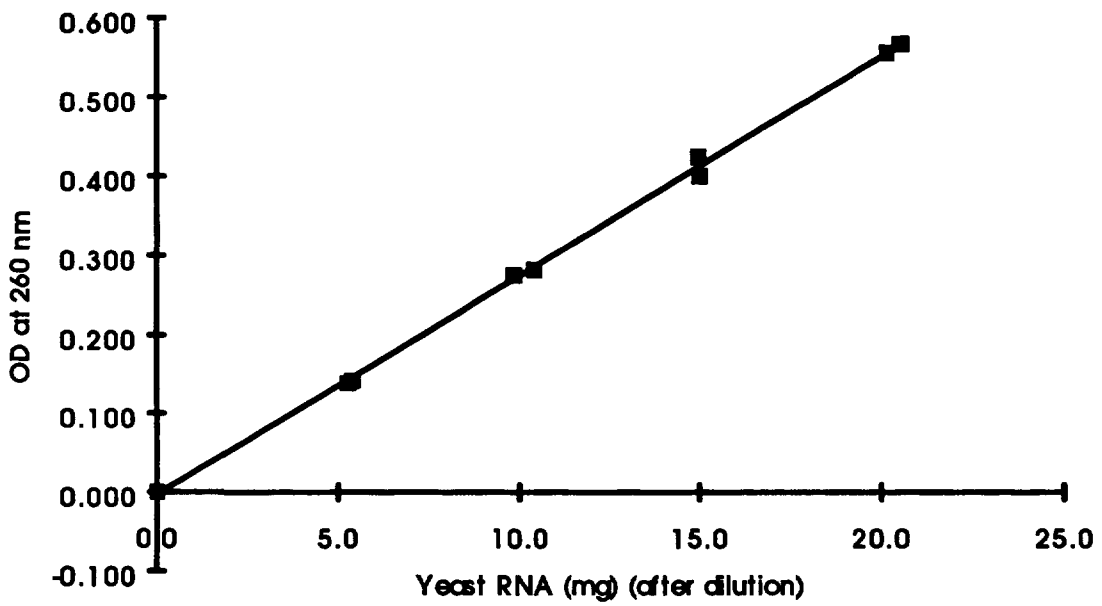


FIG. 1. Standard curve for yeast RNA (mg of yeast RNA vs OD at 260 nm).

Intercept = 0.00278
Slope = 0.2767
Correlation coefficient (r) = 0.999

The standard curve is linear over the concentration range.

1 5 2 An example of a record sheet with data

Date

Sample	Sample weight (mg)		Dilution	Optical Density		mg yeast RNA/g sample			
	1	2		1	2	1	2	mean	% diff
1	567 5	517 6	1	0 249	0 239	15 8	17 6	16 7	10 8
2	569 8	520 2	1	0 214	0 206	13 8	15 1	14 4	9 1
3	504 8	514 3	1	0 035	0 032	2 7	2 7	2 7	1 9
4	533 2	519 7	1	0 038	0 038	2 8	2 8	2 8	2 6
5	520 4	518 9	1	0 026	0 028	2 0	2 0	2 0	0 3
6	503 7	523 8	1	0 030	0 037	2 4	2 3	2 3	3 9
7	511 0	543 8	1	0 021	0 045	1 7	1 6	1 6	6 2
8	533 3	549 4	1	0 034	0 037	2 5	2 4	2 5	3 0

Programme for calculation

A computer program for the calculation can be provided on request

1.6. Nitrogen content of mixed rumen micro-organisms

On the assumption that the method for determination of total N has already been established in each of the participating laboratories, it is not necessary that all laboratories use the same method The samples will be analysed using the existing methods for the total N However, Standards and quality control samples will be provided centrally so that errors associated with variation in methodology are minimized

Ash content of microbial isolate samples will need to be determined if value for N content per unit organic matter is to be reported

The yeast RNA Standard could be used as an inter-laboratory control Some cross-checking of a subset of samples between laboratories is desirable

REFERENCES

[1] ZINN, R A , OWENS, F N , A rapid procedure for purine measurement and its use for estimating net ruminal protein synthesis Can J Anim Sci **66** (1986) 157-166

[2] CHEN, X B , HOVELL, F D D , ØRSKOV, E R , BROWN, D S , Excretion of purine derivatives by ruminants effect of exogenous nucleic acid supply on purine derivative excretion by sheep Brit J Nutr **63** (1990) 131-142

[3] BALCELLS, J , GUADA, J A , PEIRO, J M , PARKER D S , Simultaneous determination of allantoin and oxypurines in biological fluids by High-performance liquid chromatography J Chromatography **575** (1992)153-157

2. MEASUREMENT OF XANTHINE OXIDASE AND URICASE ACTIVITY IN PLASMA, LIVER AND INTESTINAL TISSUE

2.1. Objective

The objective is to provide some qualitative information about the tissue distribution of xanthine oxidase and uricase in the animals studied. The activities of xanthine oxidase in the intestine mucosa, the liver and blood affect the magnitude and pattern of PD excretion in the urine.

2.2. Outline of working procedure

1. Blood samples will be collected from at least 3 experimental animals of each species and assayed for enzyme activity within 2 h of collection.
2. Liver and intestinal tissue samples can be collected from a slaughter house from at least 3 different animals of the same species as the experimental animals. The tissue samples are kept in a polythene bag stored in ice, before assaying for enzyme activity, which should be carried out as quickly as possible.

2.3. Sampling and preparation of tissue extracts

Reagents

1. 0.05 M KH_2PO_4 (pH 7.5).
2. 0.15 M KCl.
3. 0.5 mM ethylenediaminetetracetic acid (EDTA) in 0.05 M KH_2PO_4
4. 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).

Equipment

1. High speed centrifuge.
2. Glass tissue homogeniser with a Teflon pestle (e.g. Jencons 15-ml size, Cat. No 361092 or Fison TKW-300-030T or TWK-400-070K).
3. Dialysis (membrane) tubing (e.g. Visking, inflated diameter 19.0 mm, or Fison Cat. TWT-400 070M).

Procedure

The procedure is a modification of the method by Furth-Walker & Amy [1].

Blood samples

1. Collect 20 ml of jugular blood into 2, 10-ml heparinised tubes.
2. Centrifuge at 2,500 *g* (4000 rpm) for 10 min at 4°C.
3. Use plasma for the assay within 2 h.

Liver samples

1. Collect 50-100 g of liver tissue (from animals in slaughter house). Transfer in ice to the laboratory as quickly as possible.
2. Wash in cold 0.15 M KCl, blot dry and freeze immediately if analysis is to be carried out on a different day.

3. Homogenise 1 g of liver in 9 ml of 0.5 mM EDTA in 0.05 M KH_2PO_4 (pH 7.5) in a glass homogenising tube with a Teflon pestle.
4. Centrifuge the extract at 40 000 *g* for 30 min at 4°C.
5. Dialyse the supernatant against the same EDTA-KH_2PO_4 buffer at 4°C for 24 h.
6. Centrifuge the contents of the dialysis bag at 40 000 *g* for 30 min at 4°C.
7. Use the supernatant for the assay. Store the supernatant at 4°C if the assay is to be carried out next day.

Intestinal tissue samples

1. Collect intestinal samples from a slaughter house. Take the first 30 cm segment of the small intestine.
2. Wash the lumen with cold 0.15 M KCl and then with 0.05 M HEPES buffer (pH 7.5) containing 0.25 mM EDTA and 0.25 mM PMSF.
3. Cut the segment of intestine length wise, open flat and scrape the mucosal surface carefully with a spatula in order to isolate the mucosal cells.
4. Homogenise 1 g of mucosal cells in 9 ml of the HEPES-EDTA-PMSF buffer, centrifuge the extract at 40 000 *g* for 30 min at 4°C.
5. Dialyse the supernatant at 4°C for 24 h against the HEPES-EDTA-PMSF buffer.
6. Centrifuge the contents of the dialysis tubing at 40 000 *g* for 30 min at 4°C.
7. Use the supernatant for the assay. Store the supernatant at 4°C if the assay is to be carried out next day.

2.4. Measurement of xanthine oxidase activity

The activity of xanthine oxidase (XO) is measured as the rate of uric acid production when xanthine is incubated with plasma or tissue extracts.

Reagents

1. 100 % (w/v) trichloroacetic acid (TCA).
2. 0.05 M KH_2PO_4 (pH 7.5) buffer (adjust pH with KOH or H_3PO_4).
3. Substrate solution: 1.5 mM xanthine, 4.3 mM L-histidine and 1.0 mM potassium oxonate in 0.02 M NaOH. L-histidine is added to remove the possible inhibition of XO by excess xanthine [2] while potassium oxonate is added to inhibit uricase [3].
4. Uric acid Standard solutions: 30 - 360 μM uric acid.

Equipment

- Water bath.
- UV spectrophotometer.
- High speed centrifuge.

Procedure

1. Into test tubes, add 0.5 ml of blank or sample (plasma, liver or mucosa extract), 3 ml 0.05 M KH_2PO_4 (pH 7.5) buffer and 0.5 ml substrate solution. Use 0.5 ml of 0.05 M KH_2PO_4 (pH 7.5) as a blank for plasma sample, 0.5 ml 0.5 mM EDTA in 0.05 M KH_2PO_4 (pH 7.5) as a blank for liver samples and 0.5 ml of 0.05 M HEPES buffer (pH 7.5) containing 0.25 mM EDTA and 0.25 mM PMSF as a blank for intestinal samples. Do each sample and blank in duplicate (14 tubes are needed for each sample so that 2 tubes can be removed at 6 different times during incubation).
2. To two tubes, add 0.5 ml 100% (w/v) TCA and follow Steps 4 and 5.

3. Incubate the reaction mixture of other tubes at 37°C in water bath for up to 60 min. From the time of commencement of incubation, remove two tubes at 10 min intervals and terminate the reaction by addition of 0.5 ml 100% (w/v) TCA.
4. Centrifuge the mixture at 40 000 *g* for 30 min at 4°C.
5. Read OD of the supernatant at 292 nm.

Calculation

1. Establish a Standard curve using uric acid standard solution in place of the sample. The OD vs concentration relationship for uric acid may not be linear. Use a quadratic model ($Y = c_1 + c_2X + c_3X^2$) if not linear.
2. Calculate the amount of uric acid produced based on the uric acid Standard curve. Describe the amount of uric acid produced (U μmol) as a mono-exponential function of incubation time (t, min):

   $$U = a + b\,(1-e^{-kt})$$

   where 'U' is the cumulative production of uric acid (μmol), 'a' is the initial amount (μmol) of uric acid present in the reaction system, 'b' the potential production of uric acid (μmol), and 'k' the fractional rate of uric acid production.
3. Calculate the rate of uric acid production as b·k (μmol/min). One unit of XO activity is defined as 1 μmol uric acid produced per min at 37°C with excess substrate.

2.5. Measurement of uricase activity

The activity of uricase is measured as the rate of uric acid disappearance when uric acid is incubated with plasma or tissue extracts.

Reagents

1. 0.67 M pH 9.3 glycine buffer.
2. Uric acid solution: 357 μmol/l (60 mg/L) as substrate solution.
3. 100% (w/v) trichloroacetic acid (TCA).

Equipment

- Water bath.
- UV Spectrophotometer.
- High speed centrifuge.
- Eppendorf tubes.

Procedure

1. Mix 1 ml glycine buffer, 1 ml blank, plasma, liver or intestinal extracts and 0.5 ml uric acid substrate solution.
   As a blank, use 0.05 M KH_2PO_4 (pH 7.5) for plasma samples, 0.5 mM ethylenediaminetetracetic acid (EDTA) in 0.05 M KH_2PO_4 (pH 7.5) for liver samples, 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) for intestinal samples. Do each sample and blank in duplicate (14 tubes are needed for each sample so that 2 tubes can be removed at 6 different times during incubation).
2. To two tubes, add 0.5 ml 100% (w/v) TCA and follow Steps 4 and 5.

3. Incubate the reaction mixture of other tubes at 37°C in water bath for up to 6 h. From the time of commencement of incubation, remove two tubes at 1 h intervals and terminate the reaction by addition of 0.5 ml 100% (w/v) TCA.
4. Centrifuge the mixture at 40 000 *g* for 30 min at 4°C.
5. Read OD of the supernatant at 292 nm. Use uric acid ranging from 30-357 μmol/L (5-60 mg/L) as Standards.

Note: This procedure has not been well tested and may need modification.

Calculation

It is the same as the calculation for xanthine oxidase activity described earlier. The uric acid content in the mixture decreases due to the presence of uricase.

REFERENCES

[1] FURTH-WALKER, D., AMY, N.K., Regulation of xanthine oxidase activity and immunologically detectable protein in rats in response to dietary protein and iron. J. Nutr. **117** (1987) 1697-1703.

[2] MURAOKA, S., Studies on xanthine oxidase 2. Biochimica et Biophysica Acta **73** (1963) 27-38.

[3] HASHIMOTO, S., A new spectrophotometric assay method of xanthine oxidase in crude tissue homogenate. Analytical Biochemistry, **62** (1974) 426-435.

[4] CHEN, X.B., ØRSKOV, E.R., HOVELL, F.D.DeB., Excretion of purine derivatives by ruminants: endogenous excretion, differences between cattle and sheep. Brit. J. Nutr. **63** (1990) 121-129.

[5] CHEN, X.B., SAMARAWEERA, L., KYLE, D.J., ØRSKOV, E.R., ABEYGUNAWARDENE, H., Urinary excretion of purine derivatives and tissue xanthine oxidase activity in buffaloes, with special reference to differences between buffaloes and *Bos taurus* cattle. Brit. J. Nutr. **75** (1996) 397-407.

3. ESTIMATION OF BASAL PURINE EXCRETION (FASTING TRIALS)

3.1. Objectives

- To quantify the contribution of basal purine excretion (originating from the degradation of tissue nucleic acids) to the total excretion of PD.
- To establish basal purine excretion, creatinine excretion and glomerular filtration rate (GFR).

3.2. Experimental procedure

Animals

Select animals from indigenous Zebu (*Bos indicus*) cattle, *Bos taurus* × *Bos indicus* crosses and buffaloes. The types of animals used in each location may vary. However, it is important to have at least two species in the experiment at each site for comparative purposes.

Use either all male or all female animals. However, it is easier to use male animals for urine collection. Avoid using lactating animals. Body weight of animals should be recorded before and after the fasting period.

Type of feed and feeding

There is no requirement for different laboratories to use the same feed. However, a relatively good quality ration should be used. The same diet will be used for the feeding trial (see Section 4.3). The animals should be fed twice a day.

The feed should be analysed for DM content, N content (g/kg DM) and organic matter content (g/kg DM) using standard procedure.

Facilities required

- Metabolism stalls which allow complete collection of urine and the separation of urine from faeces.
- Centrifuge.
- Freezer.

Materials

Heparinised vacutainers, needles and needle holder.

Protocol

1. At least 6 animals are required from each species for the fasting experiment.
2. The animals will be fed at a fixed level of intake equivalent to energy maintenance, for at least 2 weeks before starting the sampling period. At the beginning of sampling period animals should be moved to metabolism stalls to facilitate total collection of urine.
3. Daily urine collections will be made for 1 week. Towards the end of the week, 3 blood samples (each about 20 ml) will be taken in one day, using vacutainers.
4. The feed allowance to the animals will then be reduced in steps within 2 days (e.g. 60%, 30%, 0% on days 1, 2 and 3). Collection of urine will be continued.
5. The animals will be fasted for 6 days or if possible for a longer period. Daily urine collection will be continued over the whole period. One blood sample will be taken each day at a fixed time (e.g. afternoon) during this period.

Note: Previous experience with fasting experiments have indicated that 6 days may not be sufficient for the urinary excretion of purine derivatives to reach a steady state and therefore a longer duration may be required. During the fasting period, close veterinary monitoring of animal health is essential, probably by daily measurement of β-OH-butyrate. Fasting should be terminated if an animal develops ketosis or other health problems.

3.3. Urine collection and preparation

1. Urine is collected into a container with approximately 500 ml of 10% H_2SO_4. The final pH of urine should be below 3. It is essential to acidify the urine in order to prevent bacterial destruction of purines in the urine. Check the pH on the first day of collection and make adjustments in the amount of acid used, if necessary. Slight excess of acid will not matter.
2. Record the weight of urine. The daily urine output may vary with individual animals and dietary regime, between 5-20 litres for cattle. Add tap water to a constant final weight (e.g. 20 kg*), so that the final volume of diluted urine is the same for all animals every day.

   * *If it is difficult to handle large quantities of liquid, take an accurate 1% representative sample of urine and dilute it to a fixed volume (e.g. 1 litre).*

3. Mix the diluted urine thoroughly, filter through glass wool or surgical gauze, take a sub-sample of about 60 ml and store at -20°C in 3 or 4, 20-ml bottles or vials. Label the bottles or vials with date and animal number. Keep 20 ml of diluted urine for purine analysis. If other analyses are required, take a larger sample at step 3.

3.4. Faeces collection and preparation

No collection of faeces is required for this trial.

3.5. Plasma sampling and preparation

1. Take blood samples (20 ml) from the jugular vein using heparinised vacutainers.
   Gently invert the tube several times after sampling.
2. Centrifuge the sample at about 1500 *g* for 20-30 min. Transfer the plasma into storage tubes and store at -20°C.

For determination of allantoin in plasma by HPLC method [1], a fraction of the sample can be treated as follows. PCA is used to precipitate plasma protein and KOH to remove the excess of PCA. Steps 2 and 3 below can also be used for colorimetric method of allantoin determination.

1. Transfer 1 ml of plasma in Eppendorf tubes, add 100 µl of 1 µM allopurinol.
2. Add 100 ml of 4M PCA, centrifuge at 10 000 *g* for 5 min and neutralise using 4 M KOH.
3. After 10 min remove the potassium perchlorate by centrifugation (2000 *g* for 10 min) and keep the deproteinized plasma at -20°C.

3.6. Measurements

1. The urine should be analysed for:
   (a) total N.
   (b) purine derivatives: i.e. allantoin and uric acid.
   (c) creatinine.
2. The plasma samples should be analysed for:
   (a) purine derivatives, i.e. allantoin and uric acid.
   (b) creatinine.

3.7. Results

1. Purine derivative excretion during fasting period. It is expected that the excretion would decrease with duration of fasting. Express the results as 'µmol/kg $W^{0.75}$ per day'.
2. Estimates of basal N excretion. Express the results as 'mg N/kg $W^{0.75}$ per day'.
3. Creatinine excretion. Express the results as 'µmol/kg $W^{0.75}$ per day'.
4. Glomerular filtration rate (GFR). It can be calculated as:

   GFR (L/d) = creatinine excretion in urine (mmol/d) ÷ plasma creatinine concentration (mmol/L)

5. Renal clearance of purine derivatives

   Tubular load of allantoin (mmol/d) = GFR (L/d) × plasma allantoin concentration (mmol/L)

   Net re-absorption of allantoin (mmol/d) = tubular load (mmol/d) – excretion in urine(mmol/d)

   The same parameters can be calculated for uric acid.

3.8. Limitations of the technique

The fasting excretion of PD can only provide an indication of the endogenous excretion. This is because fasting can affect the animal's metabolism and the PD excretion may be lower than when the animal receives a maintenance nutrient supply.

REFERENCE

[1] BALCELLS, J., GUADA, J.A., PEIRO, J.M., PARKER, D.S., Simultaneous determination of allantoin and oxypurines in biological fluids by High-performance liquid chromatography. J. Chromatography 575 (1992)153-157.

4. RESPONSE OF PURINE EXCRETION TO FEED INTAKE AND MEASUREMENT OF THE PROPORTION OF PLASMA PURINE DERIVATIVES EXCRETED IN THE URINE

4.1. Objectives

- To examine the response of PD excretion to feed intake.
- To define the renal:non-renal partitioning ratio of plasma purine derivative (PD) and its variability in different breeds. Based on the partitioning ratio and the fasting excretion, an equation which relates purine absorption to PD excretion in urine can be established.

4.2. Outline of working protocol

Animal experiments should be conducted at various sites. Two species should be used, but the trials using each species can be done at different times. The experiment should be performed according to a set protocol, including the administration of tracer.

4.3. Experimental procedure

Animals

Four animals each, from two different species (same as those used in the fasting trial) should be used in this work. The animals used in the fasting trial are preferred.

Type of feed and feeding

There is no requirement for the different laboratories to use the same feed. However, a relatively good quality diet should be used, so that high level of feed intake could be achieved. Animals will be fed twice daily. During the preliminary period, all animals will be fed at *ad libitum* intake. The lowest intake among all animals of the same species is set as the 'voluntary intake'.

The feed should be analysed for DM content, N content (g/kg DM) and organic matter content (g/kg DM) using standard procedure.

Protocol

The 4 animals will be fed at 4 fixed levels. The highest level of intake will be 95% of 'voluntary intake', determined in the preliminary period. The other 3 levels will be 80%, 60% and 40% of the 'voluntary intake'. The treatments will be allocated according to a 4 × 4 Latin square design (see Section 4.3.1). Each feeding period will last for 3 weeks. During the last 10 days of each feeding period, urine and faeces will be collected daily. On the 3rd day of each sample collection period, the animals will be given an intravenous administration of a tracer either by continuous infusion over 10 h or by single injection* (see Section 6 on Tracer study). Urine samples will be kept frozen before analysis or sent to a designated laboratory for analysis.

* *Due to the high cost of tracer, the tracer kinetics will only be done in 2 treatments: Treatments 1 and 3.*

4.3.1. Proposed experimental design

Treatments (1, 2, 3 and 4) will be allocated to 4 animals in 4 periods according to 4 × 4 Latin square design. Treatments 1, 2, 3 and 4 are 95%, 80%, 60 and 40% of 'voluntary intake', respectively.

	Animal A	Animal B	Animal C	Animal D
Period 1	1	2	3	4
Period 2	2	4	1	3
Period 3	3	1	4	2
Period 4	4	3	2	1

4.4. Urine collection and preparation

1. Urine is collected into a container with approximately 500 ml of 10% H_2SO_4. The final pH of urine should be below 3. It is essential to acidify the urine in order to prevent bacterial destruction of purines in the urine. Check the pH on the first day of collection and make adjustments in the amount of acid used, if necessary. Slight excess of acid will not matter.
2. Record the weight of urine. The daily urine output may vary with individual animals and dietary regime, between 5-20 L for cattle. Add tap water to a constant final weight (e.g. 20 kg*), so that the final volume of diluted urine is the same for all animals every day.

   * *If it is difficult to handle large quantities of liquid, take an accurate 1% representative sample of urine and dilute it to a fixed volume (e.g. 1 L).*

3. Mix the diluted urine thoroughly, filter through glass wool or surgical gauze, take a sub-sample of about 150 ml and store at -20°C in 3 or 4, 20-ml bottles labelled with date and animal number. Keep one sample of about 100 ml for tracer measurement. 20 ml of the diluted urine is sufficient for purine analysis. However, if other analyses are required, take a larger sample at step 3.

4.5. Faeces collection and preparation

1. Collect total faeces daily. Record weight. Mix well and take a 10% sub-sample and keep in a polyethylene bag stored at 4°C.
2. At the end of each sampling period, bulk the daily faecal samples for each animal. Mix well and take a sub-sample of about 10%.
3. Measure DM, OM and N contents of the sub-samples. DM content determination can be done immediately after the sampling.

4.6. Plasma sampling and preparation

1. At least 3 blood samples should be taken from each animal during each treatment period. For treatments involving tracer administration, if possible, take a series of blood samples, one before tracer dosing and a few afterwards (at 0.5, 1, 2, 3, 4, 8, 24 and 36 h). This will provide additional information on the kinetics of the tracer in the blood. Blood should be taken from the jugular vein by venepuncture or by using heparinised vacutainers (20 ml sample). Gently invert the tube a couple of times after sampling.
2. Centrifuge the sample at 1500 *g* for 20-30 min. Transfer the plasma into labelled storage tubes.
3. Store the plasma (about 12 ml) in 2 tubes for each sample at -20°C. One sample could be used for creatinine and PD measurement and the other for measuring tracer kinetics (see Section 6.4). Please also refer to Section 3.5 for sample preparation.

4.7. Measurements

1. The urine should be analysed for:
   (a) total N.
   (b) purine derivatives: i.e. allantoin and uric acid.

(c) creatinine.
(d) tracer (see section on Tracer study).

2. The plasma samples should be analysed for:
(a) purine derivatives, i.e. allantoin and uric acid.
(b) creatinine.
(c) tracer (see section on Tracer study).

3. Faecal samples should be analysed for:
(a) dry matter.
(b) organic matter.
(c) total N.

4.8. Results

(1) The response of purine derivative excretion to level of feed intake.
(2) More measurements for establishing creatinine excretion, GFR and renal clearance of purine derivatives.
(3) The kinetics of purine derivatives in the blood (see Section 6.6 for calculation).
(4) The proportion of plasma purine derivatives excreted in the urine.

The calculations are as in Section 3.7.

5 DETERMINATION OF PURINE DERIVATIVES IN URINE

For sheep urine, purine derivative determination includes analysis of xanthine, hypoxanthine, uric acid and allantoin. However, for cattle urine, only the analyses of uric acid and allantoin are required since xanthine and hypoxanthine are present in trace quantities.

5.1. Published methods for determination of purine derivatives

Methods for the chemical analysis of purine derivatives using various instruments such as spectrophotometer, autoanalyzer and HPLC, are given in Table I. In the next two sections only methods based on spectrophotometer are described. A comprehensive review of methods used for the determination of allantoin is given by Chen *et al.* [1].

TABLE I. PUBLISHED METHODS FOR THE DETERMINATION OF PURINE DERIVATIVES (THE NUMBERS REFERS TO PAPERS LISTED IN THE REFERENCE SECTION)

	Allantoin	Uric acid	Xanthine + hypothanthine
Spectrophotometer	[2] [3] [4]	[3] [5] [6] [7]	[5] [8]
Auto-Analyser	[5] [8] [9]	[8]	[8]
HPLC	[10] [11] [12]	[11] [13]	[11]

5.2. Dilution of urine samples for analysis

The urine samples which have been previously diluted before storage need further dilution. The next dilution should be such that the concentrations of purine derivatives in the final sample would fall within the range of the standards used in the assays (5-50 mg/L for both uric acid and allantoin) The dilution factor needed therefore depends on the feed intake, and thus the microbial protein supply of the animal An estimate of the dilution factor required can be calculated based on the following:

1. feed intake (fresh or DM)
2. daily urine output (volume)
3. body weight (not so necessary for sheep urine).

The calculations are illustrated as follows (the same for sheep and cattle unless specified) A spreadsheet template using Lotus 123 or Excel can be used for this calculation

(1) Calculation of Microbial N (MN) yield

MN = 32g/kg DOMR [14]

where DOMR= Feed intake × DM content × OM content × OM digestibility × 0.65

(2) Calculation of the equivalent amounts of purine absorbed (P_a) by the animal

P_a (mmol/d) = MN (gN/d) − 0 727

(3) Calculation of total purine derivative excretion (PD_e mmol/d)

For sheep: $PD_e = 0.84\ P_a + 2$ (assume the endogenous contribution = 2 mmol/d)

For cattle: $PD_e = 0.85\ P_a + 0.385 \times W^{0.75}$

where $W^{0.75}$ = metabolic body weight in kg

On average, allantoin and uric acid account for 80% and 15% of the total PD. Therefore, their daily outputs can be calculated.

(4) Calculation of the dilution factors

The concentration of allantoin and uric acid in the original urine can be calculated as:

= daily output ÷ volume of urine

The required dilution factor can then be calculated as:

= original concentration ÷ target concentration.

The target concentration is between 10-40 mg/L for both allantoin and uric acid.

As an example to illustrate the above calculation, for a steer of 300 kg body weight, consuming 8 kg fresh feed and excreting 10 L/d of urine, the urine requires to be diluted between 10-30 times for uric acid assay and between 40-160 times for allantoin assay.

Based on the values obtained for the individual samples, a common dilution factor could be used for all urine samples.

Animals in tropics tend to have a lower excretion of purine derivatives than European animals at a given feed intake. In this case, a dilution factor of half the calculated value may be used for dilution. For example, if the above calculation indicates that a dilution of 40 time is required, then a 20 time dilution may be adopted for urine from tropical animals.

If precipitates are visible in the sample of urine, place the sample in an ultrasonic bath for 20 min to break up the particles before dilution. It is strongly advised not to filter the urine using filter paper since the particles may be uric acid precipitates.

5.3. Determination of allantoin by colorimetry

Principle

This is based on the calorimetric method described by Young and Conway [1]. In this procedure, allantoin is first hydrolysed under a weak alkaline condition at 100°C, to allantoic acid which is hydrolysed to urea and glyoxylic acid in a weak acid solution. The glyoxylic acid reacts with phenylhydrazine hydrochloride to produce a phenylhydrazone derivative of the acid. The product forms an unstable chromophore with potassium ferricyanide. The colour is read at 522 nm.

Reagents

1. 0.5 M NaOH.
2. 0.01 M NaOH.
3. 0.5 M HCl.

4 0 023 M Phenylhydrazine hydrochloride (freshly prepared before use)
5 0 05 M Potassium ferricyanide (freshly prepared before use)
6 Concentrated hydrochloric acid (11 4 N) cooled at -20°C for at least 20 min before use
7 Alcohol bath, 40% (v/v) alcohol, kept at -20°C (40% NaCl solution instead of alcohol solution may also be used)
8 Allantoin (e g from Sigma or BDH)

Equipment

1 Spectrophotometer
2 Boiling water bath If you have a temperature-controlled water bath, you may use polyethylene glycol (PEG MW 400) solution instead of water in the bath and set the temperature at 100°C This way the temperature can be better controlled since PEG has a boiling point greater then 100°C
3 Ultrasonic bath (optional)

Standards

Prepare a 100 mg/L stock solution of allantoin Dilute it to give working concentrations of 10, 20, 30, 40, 50 and 60 mg/L It is preferable to prepare the Standard solution in a larger volume and store each working Standard as small aliquots at -20°C

Procedure

This procedure requires critical timing of the reactions The reading of standard and sample ODs must be done within the shortest possible time-span, since OD decreases with time Therefore, no more than 10 samples in duplicate should be processed in each run A set of Standards and a blank (using distilled water) in duplicate, are also processed

1 Pipette 1 ml of sample, Standard or distilled water (blank) into 15 ml tubes
2 Add 5 ml of distilled water and 1 ml of 0 5 M NaOH Mix well using a vortex mixer
3 Place the tubes in the boiling water bath for 7 min Remove from the boiling water and cool the tubes in cold water
4 Add to each tube 1 ml of HCl (0 5 M) The pH after adding the HCl must be in the range 2-3 *
5 ** Add 1 ml of the phenylhydrazine solution Mix and transfer the tubes again to the boiling water for exactly 7 min
6 Remove tubes from the boiling water and place immediately in the icy alcohol bath for several min***
7 Pipette 3 ml of concentrated HCl (operate in a fume cupboard) and 1 ml of Potassium ferricyanide Perform this for all samples within the shortest possible time span
8 Mix thoroughly and transfer to 4 5 ml cuvettes at room temperature
9 Read the absorbance at 522 nm after exactly 20 min+ Once started, do it as quickly as possible (because the colour will fade gradually) It is important that OD for samples and standards be read within the shortest possible time span

* *If this is the first time you do this assay, you need to check the pH Add more HCl if necessary, the same amount can then be used in later runs*

** *Steps 5-9 should not be interrupted*

*** *The idea is to slow down the reaction by reducing the temperature The use of an alcohol/water mixture is to achieve a temperature below zero for the liquid*

\+ *In places where ambient temperature is high, the waiting time could be shortened However, it is important that the same duration is used for all samples and all runs*

Standard curve and calculation

The standard curve is linear Therefore, we can fit a linear regression between the known allantoin concentrations (standards) (X) and the corresponding OD (Y) The slope of the line is usually 0 16-0 18 Calculate the concentration of the unknowns based on this equation

Note: Formaldehyde causes over-estimation of allantoin by this method Therefore if the animals are fed with feeds treated with formaldehyde, the method may not be suitable Acetate also gives slight over-estimation

5.4. Determination of xanthine and hypoxanthine by enzymatic method

Principle

In this method, xanthine and hypoxanthine are enzymatically converted to uric acid and thus determined as uric acid which is monitored by its absorbance at 293 nm The OD at 293 nm increases after the enzyme treatment The net increase in OD is then used for the calculation of the amount of uric acid formed based on the uric acid standard curve

Reagents

1 KH_2PO_4 buffer, 0 2M pH 7 35, adjust pH with either H_3PO_4 or KOH
2 L-histidine 4 3 mM
3 Xanthine oxidase, add 25 µl of the solution (e g catalogue No X-1875 - 50 unit in 2 6 ml) to 3 ml of the buffer
4 Uric acid

Equipment

1 Spectrophotometer
2 Water bath
3 Ultrasonic bath (optional)

Standards

Prepare a 100 mg/L stock solution of uric acid (add about 100 µl of 0 6 N NaOH to 1 litre of the solution to help dissolve the uric acid) Dilute it to give working concentrations of 20, 40, 60, 80 and 100 mg/L Store each working standard as small aliquots at -20°C

Procedure

1 Pipette 1 ml of urine, standard or blank (distilled water) into test tubes All samples and standards are done in duplicates Distilled water is used as the blank Prepare two sets
2 Add 2 5 ml phosphate buffer
3 Add 0 35 ml L-histidine solution Mix well
4 To one set, add 150 µl of buffer To the other set add 150 µl of the XO solution Mix well and incubate at 37°C for 60 min*
5 Read OD at 293 nm

**The conversion of xanthine and hypoxanthine to uric acid is complete when the OD of the samples remains constant*

Standard curve and calculations

1. Use OD of the standards without XO added for the construction of uric acid standard curve. Transform both X and Y into natural logarithmic function. Fit the Ln (Y) into a linear function of Ln (X).

2. Calculate the ΔOD for the samples, i.e. the difference between two sets with and without XO addition: ΔOD = OD with XO - OD without XO.

3. Calculate the corresponding concentration of uric acid from ΔOD based on the above standard curve. This increment in uric acid concentration corresponds, on a molar basis, to the sum of xanthine and hypoxanthine present in the samples.

4. Estimate the approximate contribution of OD reading from the xanthine (OD_X) in the set withoutXO based on a pre-determined xanthine standard curve*. For practical purposes, use the concentration of xanthine plus hypoxanthine calculated in Step 3 to calculate OD_X.

5. Re-adjust the ΔOD (i.e. $\Delta OD_2 = \Delta OD + OD_X$), and repeat from step 3 once.

Note: The activity of xanthine oxidase can be inhibited by excess amount of substrate in the sample. The addition of L-histidine reduces this inhibition.

* *Xanthine can also absorb UV at 293 nm although the absorbance is 10 times lower than uric acid at the same concentration. Hypoxanthine does not absorb UV at this wavelength. To correct for the absorbance due to xanthine in the sample set without addition of XO, we need to generate a standard UV absorbance curve for xanthine. This can be done the following way:*

Use xanthine solutions of concentrations ranging from 10 to 50 mg/L, and go through the above procedure (without XO added). Fit the OD of xanthine (OD_X) into a linear function of xanthine concentration. A xanthine standard may not be needed for every run.

5.5. Determination of uric acid by uricase method

Principle

The procedure was described by Fujihara *et al.* [2]. This method is similar to that proposed by Praetorius and Poulsen [12]. Uric acid absorbs UV at 293 nm, although other compounds may also absorb at this wavelength. When samples are treated with uricase, uric acid is converted to allantoin and other compounds that do not absorb UV at 293 nm. Therefore, the reduction in OD reading after treatment with uricase is correlated with the concentration of uric acid in the sample. After treatment, the OD of the standards should be zero if the conversion is complete.

Reagents

1. KH_2PO_4 buffer, 0.67 M, pH 9.4. Adjust the pH with KOH.
2. Uricase from porcine liver (e.g. SIGMA Cat. No U-9375, 19 unit/g solid). Prepare an enzyme solution of 0.12 unit/ml buffer.
3. Uric acid.

Equipment

Spectrophotometer
Water bath
Ultrasonic bath (optional)

Standards

See preparation of uric acid standards in the previous section In this case, prepare standard working concentrations of 5, 10, 20, 30 and 40 mg/L

Procedure

1 Pipette 1 ml of urine or standard or blank (distilled water) into 10 ml tubes Mix with 2 5 ml phosphate buffer Prepare two sets of tubes
2 To one set, add 150 µl buffer and to the other add 150 µl of uricase solution Mix well
3 Incubate in the water bath at 37°C for 90 min
4 Remove from water bath, mix and transfer the solutions to cuvettes and read the OD at 293 nm If the enzymatic conversion is complete, the OD of the standards with uricase added should be zero If not, incubate in water bath for an additional 30 min and read again

Standard curve and calculation

1 Standard curve is curvilinear When both X and Y are transformed to Ln functions, Ln (Y) is linearly correlated to Ln (X) Use the OD reading of the set without addition of uricase for the construction of standard curve Please refer to the previous section for construction of standard curve

2 Calculate the net reduction in OD (ΔOD) for the samples due to uricase treatment ΔOD = OD without enzyme - OD with enzyme

3 Calculate the uric acid concentration from ΔOD based on the established standard equation (as in 1)

5.6. Daily excretion of purine derivatives

Calculate the excretion of allantoin, uric acid, and xanthine plus hypoxanthine, using SI units (mmol/d) The proportions of individual components of the total PD are normally

- *sheep urine* allantoin 60-80%, uric acid 30-10%, xanthine plus hypoxanthine 10-5% As the total excretion increases, the proportion of allantoin increases

- *cattle urine* allantoin 80-85%, uric acid 20-15% Within the same animal, the proportions are relatively constant, but there seems to be variation between animals

REFERENCES

[1] CHEN, X B , MATUSZEWSKI, W , KOWALCZYK, J , Determination of allantoin in biological cosmetic and pharmaceutical samples J AOAC International **79** (1996) 628-635
[2] YOUNG, E G , CONWAY, C F , On the estimation of allantoin by the Rimini-Schryver reaction J Biol Chem **142** (1942) 839

[3] FUJIHARA, T., ØRSKOV, E.R., REEDS, P.J., The effect of protein infusion on urinary excretion of purine derivatives in ruminants nourished by intragastric nutrition. J. Agric. Sci. Camb. **109** (1987) 7-12.
[4] BORCHERS, R., Allantoin determination. Analytical Biochemistry **79** (1977) 612-613.
[5] PENTZ, E.I., Adaptation of the Rimini-Schrywer reaction for the measurement of allantoin in urine in the AutoAnalyzer. Analytical Biochemistry **27** (1969) 333-342.

[6] FOSSATI, P., PRENCIPE, L., BERTI, G., Use of 3,5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenoazone chromogenic system in direct enzymic assay of uric acid in serum and urine. Clinical Chemistry **26** (1980) 227-231.
[7] PRAETARIUS, E., POULSEN, H., Enzymatic determination of uric acid with detailed directions. Scan. J. Clin. Inv. **3** (1953) 273.
[8] CHEN, X.B., MATHIESON, J., D,F., DEB. HOVELL, REEDS, P.J., Measurement of purine derivatives in urine of ruminants using automated methods. J. Sci. Food Agric. **53** (1990) 23-33.
[9] LINDBERG, J.E., JANSSON C., A rapid automated analysis of allantoin in ruminant urine. Swedish J. Agri. Res. **19** (1989) 163-167.
[10] CHEN, X.B., KYLE, D.J., ØRSKOV, E.R., Measurement of allantoin in urine and plasma by high-performance liquid chromatography with pre-column derivatisation. J. Chromatography, **617** (1993) 241-247.
[11] BALCELLS, J., GUADA, J.A., PEIRO, J.M., Simultaneous determination of allantoin and oxypurines in biological fluids by high-performance liquid chromatography. J. Chromatography **575** (1992) 153-157.
[12] DIEZ, M., T., ARIN, M.J., RESINES, J.A., Simultaneous determination of allantoin and creatinine in urine by a rapid reversed-phase liquid chromatography method. J. Liquid Chromatography **15** (1992) 1337-1350.
[13] LUX, O., NAIDOO, D., SALONIKAS, C. Improved HPLC method for the simultaneous measurements of allantoin and uric acid in plasma. Ann. Clinical Biochem. **29** (1992) 674-675.
[14] AGRICULTURAL RESEARCH COUNCIL, The Nutrient Requirements of Ruminant Livestock, Suppl. No.1, Commonwealth Agricultural Bureaux, Slough (1984).

6 TRACER STUDIES

This will be part of the experiment (feeding trial) described in the previous section The reason for describing it as a separate section is that there are several options to choose from based on analytical facilities for the tracer measurement There will be choice of tracer used and method of administration

6.1. Options

1 Either stable isotope ^{15}N or radioactive ^{14}C tracer can be used The advantage of using ^{15}N tracer is its ease of handling (including transportation) However it requires a larger dose and is therefore more expensive ^{14}C tracer has the advantage that it is easy to measure Because a larger sample size can be used for counting, less tracer is needed for injection ^{14}C-6- uric acid should not be used as the ^{14}C will be lost when uric acid is converted to allantoin

2 ^{15}N labelled uric acid or allantoin can be used Labelled allantoin is exceedingly more expensive (>£5000/g for ^{15}N allantoin) than labelled uric acid (about £900/g ^{15}N uric acid)

3 Single-injection or continuous infusion may be used Single-injection is easier to manage, however, continuous infusion may need to be employed in practice if ^{15}N-uric acid is used since a relatively large volume will need to be administrated The highest uric acid concentration in the tracer solution injected in about 100 mg/ L Therefore, if 100 mg tracer is to be injected, it needs to be delivered in 1000 ml solution If continuous infusion is used, the solution will be delivered by a peristaltic pump via a jugular catheter over a period of 10 h or longer Continuous infusion approach will give additional information of the rate of PD influx to the plasma if measurement is taken at steady state (by 4-6 h)

6.2. Tracers

^{15}N tracer

The ^{15}N tracer will most likely be 1,3-^{15}N uric acid (99% ^{15}N at 1 and 3 positions), which is commercially available After administration to the blood, it is expected that the enrichment will be present in the urine and blood as 1,3-^{15}N uric acid, 1,3-^{15}N-allantoin and ^{15}N-urea (Figure 1)

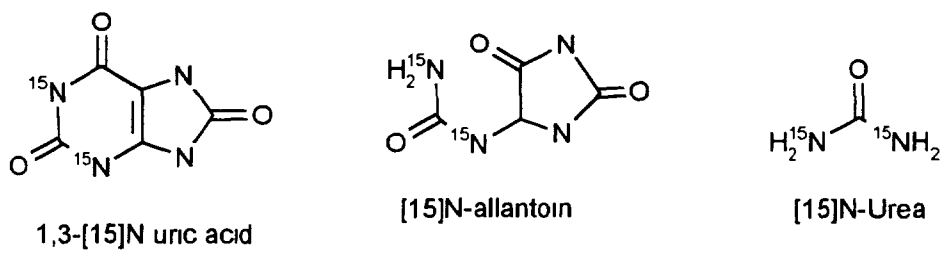


FIG 1 Enrichment positions of uric acid, allantoin and urea with ^{15}N

^{14}C tracer

The ^{14}C tracer used will be 8-^{14}C uric acid, solid, having a specific activity of 30 mCi/mmol and 95% purity Its metabolic pathway is shown in figure 2 After administration to the blood, it is expected that radioactivity will be present as 8-^{14}C uric acid and 8-^{14}C allantoin in urine or blood If secreted into the gut, 8-^{14}C uric acid and 8-^{14}C allantoin will be converted to ^{14}C urea and most of the ^{14}C finally lost in the form of $^{14}CO_2$

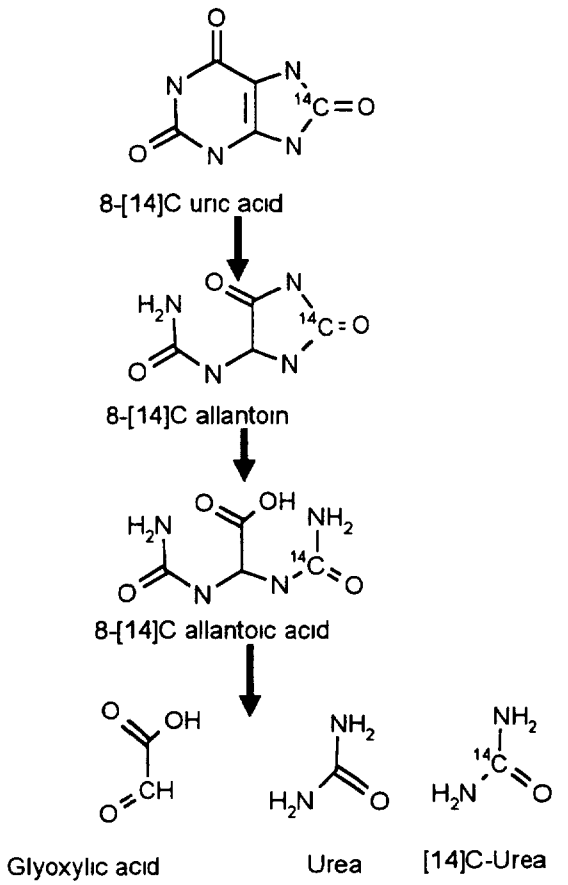


FIG 2 The metabolic pathway of uric acid and allantoin

6.3. Tracer solution preparation and administration

6 3 1 Using ^{15}N - uric acid as tracer

The quantity of tracer required for each animal varies with the daily purine derivative excretion The amount will be such that the final ^{15}N enrichment in the urinary uric acid and allantoin will be about no less than 3% (preferably 5%) in excess of natural abundance Basically, the dose should be 3% of the total purine derivative excretion per day

Materials required

Peristaltic pump or preferably Harvard syringe pump
PVC tubing and tubing connectors

Preparation of tracer solution

The procedure is similar to that used for the ^{14}C-uric acid tracer solution, except that the solution will contain higher concentration of uric acid (80-100 mg/L) Add drops of NaOH to aid dissolution of uric acid (<100 µl of 0 6 N NaOH for 1 litre solution) The volume of tracer solution to be prepared varies with the intended dose of tracer

Tracer administration - continuous infusion

Insert a jugular catheter 1-2 days before tracer administration Start the tracer administration in the morning

The tracer solution will be administered by infusion over a period of several hours

1 Insert a jugular catheter (see description later) 1-2 days before infusion of tracer
2 Set up the peristaltic pump (or Harvard syringe pump) with silicon or PVC transmission line Set the required flow rate so that the required amount of tracer solution is delivered in 10 h (or even longer)
3 On the day of administration, transfer the tracer solution to room temperature
4 Record the weight (W_1) of the bottle containing tracer solution
5 Start infusion At the end of infusion record the weight (W_2) again The volume infused is W_2-W_1
6 Flush the catheter with saline containing 100 unit/ml heparin
7 Keep the remaining tracer solution at -20°C, so that the tracer can be analysed together with the urine (and plasma) samples

6 3 2 Using ^{14}C - uric acid as tracer

Each injection will be 280 µCi per animal, to be administered in 45 ml of solution Due to the poor solubility of uric acid in water and the need to reduce the volume of injection, no cold uric acid is included in the tracer solution (concentration of uric acid in the solution will be 36 7 mg/L, radioactivity 6 222 µCi/ml) The calculation is shown as below

Sp Activity of tracer (mCi/mmol), corrected for 95% purity	28 5
Total amount of tracer (mg)	29 47
Dose per injection (µCi)	280
Expected dose volume per injection (ml)	45
Amount of tracer per injection (mg)	1 651
Final uric acid concentration (mg/L)	36 68
Radioactivity (µCi/ml)	6 222

Since the preparation and administration of tracer needs to be done with great accuracy, the following special steps should be taken

Preparation of tracer solution

If two animals are to be injected, prepare 100 ml of solution (3 67 mg tracer), If four animals are to be injected, prepare 200 ml of solution (7 34 mg tracer)

Prepare the tracer solution one day before administration

1 Prepare 1 litre of 0 85% NaCl solution (saline) in distilled water

2 Carefully weigh 3 67 mg of the tracer into a 100-ml volumetric flask, dissolve completely with saline If necessary, add a drop of 1 N NaOH to help dissolution Top up to the volume with saline Mix thoroughly
3 Prepare one 100-ml or two 50-ml autoclaving bottles with Suba-seal rubber stoppers Record the tare weights of the bottle(s) Transfer the tracer solution using a syringe attached with a 0 2 μm pore size syringe filter Weigh the bottle(s)
4 Autoclave the tracer solution Weigh the bottle(s)
5 Store the tracer solutions at 4°C before injection

Note: It may be possible to achieve sterilization in an alternative way autoclave the bottle with stopper (in step 3 above) Then fill the solution through the sterile syringe filter

The radioactivity of the tracer solution prepared can be measured as follows
1 Add 10, 20, 40, 60, 80 and 100 μl of the tracer solution each into a 7 ml scintillation vial, add 4 ml scintillation fluid for counting of ^{14}C activity
2 Establish a linear response curve of measured activity vs amount of tracer solution added The slope of regression line gives the ^{14}C activity per μl of tracer solution This value will be used for the calculation of actual amount of ^{14}C activity injected to the animals

Tracer administration - single dose injection

It is strongly recommended that the tracer solution is injected via a jugular catheter, although direct injection into the vein is possible The jugular catheter can be put in 1-2 days before (see later section for materials needed for the catheter preparation) Perform the tracer administration in the morning The catheter can be used for subsequent blood sampling

The tracer solution will be administered as a single injection

1 Transfer the tracer solution to room temperature
2 Use a 60-ml, or smaller size, sterile syringe Load the tracer solution Record the weight (W_1)
3 Inject the tracer solution via the jugular catheter Record the weight (W_2) The volume injected is W_2-W_1
4 Repeat 2 and 3 until sufficient amount (approx 45 ml) of tracer has been injected
5 Flush the catheter with saline containing 100 unit/ml heparin
6 Keep the remaining tracer solution at -20°C, so that the tracer can be analysed together with the urine samples

6.4. Sampling and analysis

For the measurement of the proportion of purine derivatives entering the plasma that is excreted in the urine, only urine samples are required Basically, we need to calculate what proportion of the administered labelled uric acid (either ^{15}N or ^{14}C -uric acid) is excreted in the urine as the sum of labelled uric acid and labelled allantoin The calculation is illustrated in Section 6 5

Daily urine will be collected as described in Section 4 4

Urine samples from the day of administration are used to determine the output of labelled uric acid and labelled allantoin The sum of daily outputs (from day one until the tracer is absent) is calculated Express the sum as proportion of the total tracer administered

Based on previous experiments, it is expected that the total recovery of the dosed tracer as labelled uric acid and allantoin would be 40-90% Over 60% of the dosed tracer will be excreted in the

urine on day 1 of dosing. By day 7, all the dosed tracer will be completely cleared from the body. Since uric acid and allantoin are non-utilisable waste products, it is unlikely that the uric acid tracer can be retained in the body.

Sample analysis and preparation:

Using 8-^{14}C uric acid as the tracer, the possible forms of ^{14}C are uric acid, allantoin, urea and CO_2. In order to measure the ^{14}C activity from uric acid and allantoin only and not from urea and CO_2, a separation procedure is used. The procedure will involve the following steps:

1. Mix the urine sample well. If there are precipitates, place the sample in an ultrasonic bath for 20 min. Take a representative subsample, dilute the urine to a concentration of about 100 mg allantoin or 50 mg uric acid per litre.
2. Take 0.5 ml of the diluted urine into a 7 ml scintillation vial, add 4 ml scintillation fluid for counting of total ^{14}C activity (C_1). Mix well by vortexing and ensure that there is no separation between the urine and the scintillation fluid.
3. Mix 0.9 ml of the diluted urine, and 30 µl 2N NaOH in an Eppendorf tube. After mixing the urine and NaOH solution, the pH should be between 12-13. Add 0.3 ml a solution containing 10 g Hg acetate and 100 g Na acetate per litre. Leave the mixture to stand 4-6 h in ice or at 4°C overnight. Centrifuge at 20 000 g for 20 min and take 0.5 ml supernatant into a scintillation vial for counting of radioactivity (C_2) as in step 2. C_2 refers to radioactivity present in compounds other than allantoin and uric acid. Urea can not be precipitated by Hg acetate.
4. Calculate the ^{14}C radioactivity present in allantoin and uric acid based on C_1 and C_2.

Note that the amount of 2N NaOH used in step 3 above may need to be adjusted according to the acidity of the urine samples. The final pH of urine after adding NaOH should be 11-13, at which pH, 99% of allantoin and 100% uric acid are precipitated.

Plasma samples taken before and after tracer dosing can be measured for the total ^{14}C activity using steps 2 and 4 above. The value obtained will also include contribution from ^{14}C urea and CO_2 if these are present.

It is well known that urine colour and presence of excess amount of organic matters in the samples can affect the ^{14}C counting efficiency, commonly referred to as 'Quenching' effect. Preliminary tests by adding ^{14}C uric acid to plasma and diluted urine showed the absence of quenching with these samples. However, it is recommended that the quenching effect should be checked. Urine samples can be diluted by different factors and the counts compared.

^{15}N uric acid and allantoin will be determined by gas chromatography coupled with mass spectrometry (GC-MS). The procedure will involve the following steps:

1. Urine samples are diluted and desalted by using an anion exchange column (AG1-X8, chloride form).
2. The eluent from the column is then derivatised to form the tertiary-butyldimethylsilyl (TBDMS) derivatives of allantoin and uric acid for analysis by GC-MS.
3. The ^{15}N enrichments of uric acid and allantoin are quantified using selective ion recording (SIR) by monitoring *m/z* 398, 399 and 400 ions for allantoin and *m/z* 567 and 569 ions for uric acid.
4. Accurate determination of the concentrations of allantoin and uric acid in the samples is made using isotopic dilution technique (by addition of known quantities of ^{15}N- uric acid as an internal standard).

Note: the procedures given above have yet to be further tested as part of the research activities of the project.

Measurement

^{14}C by scintillation counter
^{15}N uric acid and allantoin, by GC-MS

6.5. A model for the measurement of the renal disposal of purine derivatives in ruminants using tracers

Model description

Uric acid pool

U Uric acid entering the plasma (mmol/d)
f_a The conversion of uric acid into allantoin (the conversion occurs in the circulation) (mmol/d)
f_b Excretion of uric acid in the urine (mmol/d)
f_c Loss of uric acid by non-renal route (mmol/d)
[U] Pool size of (unlabelled) uric acid in the circulation (mmol)

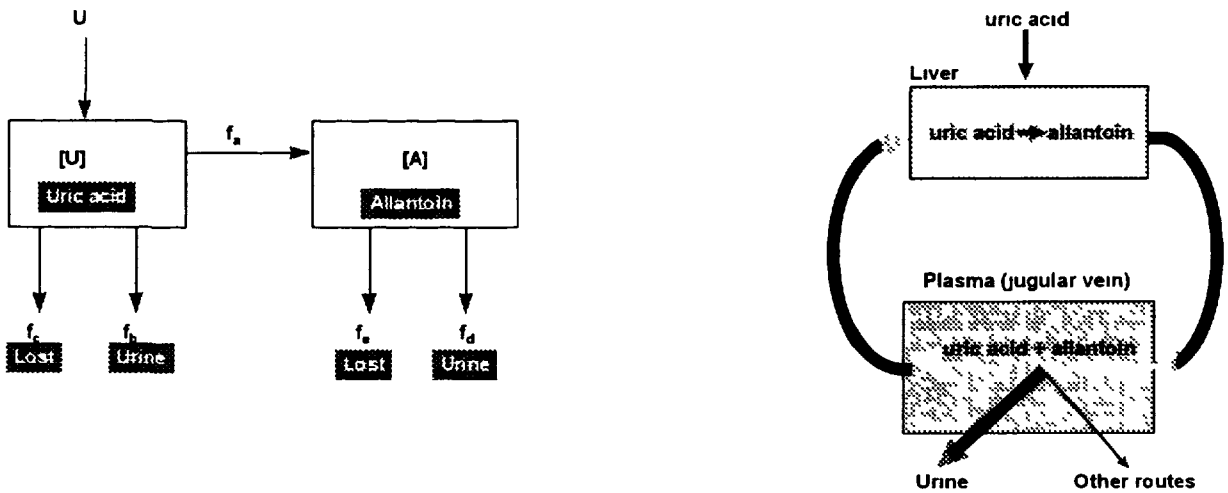


FIG 3 A model for the measurement of renal disposal of purine derivatives in ruminants using tracers

Allantoin pool:

f_a influx of allantoin (from conversion of uric acid) (mmol/d)
f_d Excretion of allantoin in the urine (mmol/d)
f_e Loss of allantoin by non-renal route (mmol/d)
[A] Pool size of (unlabelled) allantoin in the circulation (mmol)

Objectives

To calculate the following ratios

1 Proportion of the uric acid in the plasma that is excreted in the urine, i e , $\frac{fb}{fb + fc}$

2 Proportion of the allantoin in the plasma that is excreted in the urine, i e $\frac{fd}{fa}$

Assumptions

1 The proportion of plasma allantoin excreted in the urine is the same as the proportion of plasma uric acid excreted in the urine

Here it is assumed that once uric acid or allantoin are filtered into the glomeruli, these are quantitatively excreted in the urine We know this is true for allantoin, but it is not known whether this is the case for uric acid in sheep and cattle

$$\frac{fb}{fb + fc} = \frac{fd}{fd + fe} = y \qquad \text{(Assumption 1)}$$

Equations

$$U = fa + fb + fc \qquad (6)$$

$$fa = fe + fd \qquad (7)$$

$$U = fb + fc + fe + fd \qquad (8)$$

$$U = \frac{fb}{y} + \frac{fd}{y} = \frac{fb + fd}{y} \qquad (9)$$

$$y = \frac{fb + fd}{U} \qquad (10)$$

Following injection of a known dose of 8-^{14}C-uric acid, 'y' can be determined as the recovery in the urine (after total urine collection for up to 5 days) of 8-^{14}C -uric acid plus 8-^{14}C -allantoin It can be calculate as

(^{14}C-uric acid excreted in urine + ^{14}C-allantoin excreted in urine) –^{14}C-uric acid dosed

The fraction of tracer lost by non-renal route is the sum of 'f_c' and 'f_e' The ^{14}C in this fraction of uric acid or allantoin is likely to be converted into urea and CO_2 or other carbon skeleton There is a chance for these compounds to be recaptured by rumen microbes and re-enter the system However, in practice, the proportion of ^{14}C to become ^{14}C-purines is insignificant Therefore, we can neglect the fact that 'f_c' and 'f_e' can re-enter the plasma as 'U' A similar argument can be made when ^{15}N is used as tracer

Limitations

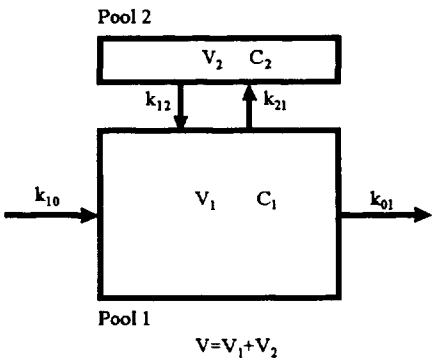
The calculation relies heavily on Assumption 1

6.6. Kinetics of plasma purine derivatives

Following a single injection of ^{14}C-uric acid or allantoin into the blood, the decline of ^{14}C activity can give an estimate of the fractional rate of clearance of purine derivatives from the blood. Plasma samples are taken at intervals as in Section 4.6. The radioactivity (C) in the plasma samples is most likely to follow a double exponential function of time (t):

$$C = Ae^{-u_1 t} + Be^{-u_2 t} \tag{11}$$

Equation (1) suggests that we are dealing with a two-compartment system. Here we assume that the system consists of a closed compartment and an open compartment:



In each compartment, the pool size is V, radioactivity per ml is C. k's are the specific flow rate constants. It is not clear what Compartment 2 exactly refers to, but Compartment 1 refers to plasma where measurements are made. k_{01} is the fractional rate at which the dosed ^{14}C is cleared from the plasma. If we assumed that the ^{14}C is present all in the form of uric acid or allantoin, then k_{01} is the fractional clearance rate for the compound in question.

Two differential equations can be written for the two compartments:

$$\frac{dC_1}{dt} = k_{12}\frac{V_2}{V_1}C_2 - (k_{21} + k_{01})C_1 \tag{12}$$

$$\frac{dC_2}{dt} = k_{21}\frac{V_1}{V_2}C_1 - k_{12}C_2 \tag{13}$$

The above equations can be solved to give the following solutions:

Initial radioactivity in Compartment 1 after dosing: $C_0 = A + B$

$$k_{12} = u_1 + \frac{A(u_2 - u_1)}{C_0}$$

$$k_{01} = \frac{u_2 u_1}{k_{12}}$$

$$k_{21} = (u_1 + u_2) - (k_{12} + k_{01})$$

$$V_1 = \frac{Q}{C_0}$$ where Q is the radioactivity dosed.

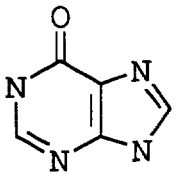
$$V_2 = \frac{V_1 k_{21}}{k_{12}}$$

$$V = V_1 + V_2 = \frac{Q}{C_0}\left(1 + \frac{k_{21}}{k_{12}}\right)$$

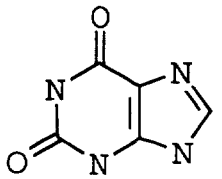
7 MISCELLANEOUS INFORMATION

7.1. Structure and molecular weights of purine derivatives

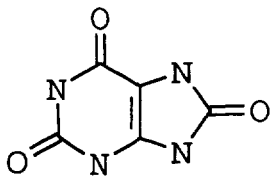
Hypoxanthine
MW 136



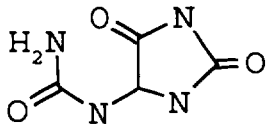
Xanthine
MW 152



Uric acid
MW 168



Allantoin
MW 158



7.2. Unit conversion of centrifugal force

To calculate Relative centrifugal force (RCF g) from rotation per minute (rpm) use the following formulae

$$RCF(g) = \left(\frac{rpm \times 2\pi}{60}\right)^2 \times \frac{r}{980}$$

$RCF\ (g) = 1118 \times 10^{-8} \times r \times rpm^2$ (r in cm)

$RCF\ (g) = 2840 \times 10^{-8} \times r \times rpm^2$ (r in inches)

7.3. Preparation for jugular catheter

Materials

1. Tubing for sampling PVC (e g SV55, Medical grade, animal tested tubing, id 0 80 and od 1 20 mm) Cut about 90 cm long, mark at 30 cm from one end
2. Needle for introducing tubing into the vein (14 G-2") Use 19G-1 5" needle for end of catheter and connection between tubing
3. Heparin saline 100 unit/ml for day use and 1000 unit/ml for overnight use
4. Bandage (a) for attaching catheter to neck (prepared in the shape of a wing), (b) for protecting catheter (e g use Tubigrips)
5. Plastic fittings (with caps) to seal the catheter
6. Marinol blue (1 50 in water) as disinfecting solution
7. 75% alcohol for general cleaning
8. Cotton swabs

Procedure

1. Insert the needle to the jugular vein. Carefully feed 30 cm of PVC tubing (e.g. Medical grade, animal tested tubing, id. 0.80 and od. 1.20 mm) into the jugular (i.e. to the point of marking). Inject some heparin solution to the tubing.
2. Remove the needle. Seal the tubing with a plastic end fitting.
3. Fix it with the wing plaster to the neck using glue.
4. Cover the catheter and plaster with Tubigrip.

7.4. List of some useful materials

Description	Quantity
Dialysis membrane * MW cut off 12-14K.	1 role (30 m)
Tissue grinder (homogeniser) *	1
Glass tubes with screw caps, 26 × 100 cm	20 tubes
Disposable syringe filter, sterile, 0.2 μm pore size	1 pack of 50
N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES)	10 g
Phenylmethylsulphonylfluoride (PMSF)	5 g
Xanthine	10 g
Xanthine oxidase **	50 unit
Uricase	25 unit
Uric acid	25 g
Allantoin	25 g
L-histidine	25 g
Potassium ferricyanide	100 g
Phenylhydrazine hydrochloride	2 × 25 g
Oxonic acid (potassium salt)	5 g
RNA (from yeast)	100 g

* Can be any model.
** No need to order if working with cattle urine.

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