

*International Feed Resources Unit  
Rowett Research Institute, Bucksburn Aberdeen AB2 9SB, UK  
Occasional Publication 1992*

**ESTIMATION OF MICROBIAL PROTEIN SUPPLY TO SHEEP AND CATTLE  
BASED ON URINARY EXCRETION OF PURINE DERIVATIVES  
- AN OVERVIEW OF THE TECHNICAL DETAILS**

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Edited and reprinted September 1995

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## INTRODUCTION

Measurement of microbial protein supply to ruminants has been an important area of study in ruminant protein nutrition. An estimate of microbial protein contribution in the intestinal protein flow is incorporated into the new protein evaluation systems already being used in different countries. The supply of microbial protein to the animal per unit of feed ingested, usually expressed as g microbial N/kg digestible organic matter fermented in the rumen (DOMR), varied by almost 4 folds (14-60 gN/kg DOMR, ARC 1984). This variation is due to the influence of various factors relating to the diet or rumen environment. The effects of many of these factors have not yet been conclusively demonstrated or quantitatively defined.

Limiting the progress in the understanding of microbial protein synthesis is the lack of simple and accurate methods of measuring microbial protein production *in vivo*. The methods usually used are based on determination of microbial markers, such as RNA, DAPA and <sup>35</sup>S. These methods however, involve complicated procedures of measuring digesta flow and require the use of post-ruminally cannulated animals. It is therefore difficult in practice to conduct extensive *in vivo* studies on microbial protein synthesis.

The method based on measurement of purine derivatives (PD) in urine overcomes the disadvantages of the above methods. It is simple (it only requires a total collection of urine) and non-invasive (it does not require any surgical preparation of the animal) and has the potential to be further simplified for use under farm conditions.

This article provides an introduction to, or an overview of the method of estimating microbial protein supply to sheep and cattle based on daily excretion purine derivatives. The emphasis is to provide the technical details which can be followed by students, and at the same time, attach as much background information as possible.

## PRINCIPLE OF THE METHOD

The principle of this method is simple. Nucleic acids leaving the rumen are essentially of microbial origin. This is because ruminant feeds usually have a low purine content, most of which undergo extensive degradation in the rumen as the result of microbial fermentation. Absorbed nucleic acid purines are degraded and excreted in the urine as their derivatives, hypoxanthine, xanthine, uric acid and allantoin (Fig. 1). The excretion of the PD is directly related to the purine absorption (See Fig. 2). With the knowledge of the purine-N: total-N ratio in microbial biomass, microbial N absorption can be calculated from the amount of purine absorbed which is estimated from urinary PD excretion.

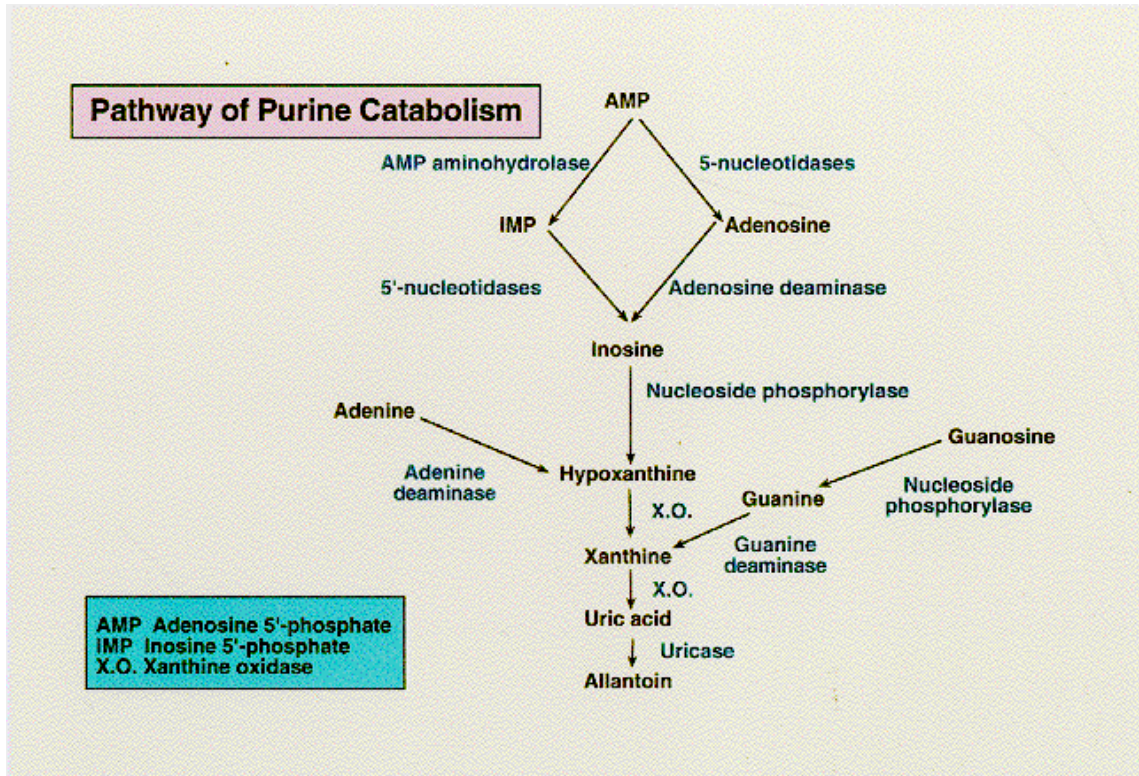


Fig 1. Degradation of purine nucleotides and formation of purine derivatives

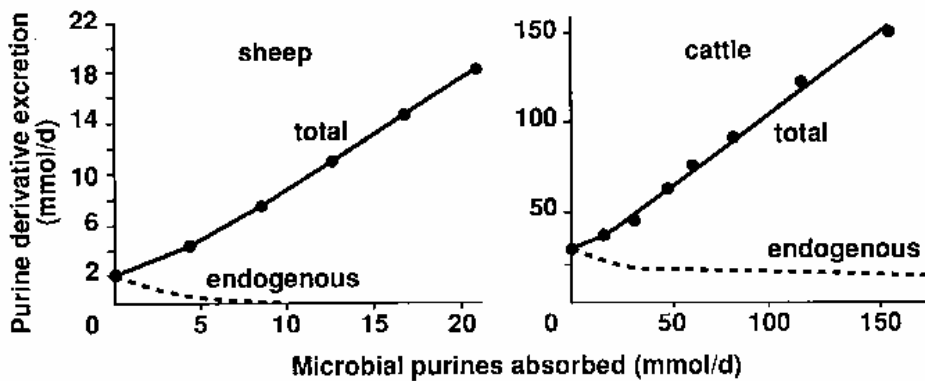


Fig.2 Urinary excretion of purine derivatives in sheep and cattle in relation to the amount of microbial purines absorbed (from Chen et al. 1990 and Verbic et al. 1990). The dotted line represents the net contribution of purine derivatives from degradation of tissue nucleic acids.

### BRIEF BACKGROUND OF PURINE METABOLISM IN RUMINANTS

An overview of this section is given schematically in Fig.3. Microbial nucleic acids leaving the rumen undergo extensive digestion in the small intestine. No apparent digestion occurs in the abomasum. In the small intestine, purine nucleotides are hydrolyzed into purine nucleoside and free bases. Both forms can be absorbed from the intestine. There has been very few studies made to measure the

digestibility of microbial purines. However, it can be regarded as being the same as the digestibility of nucleic acids since purine nucleoside and free bases can be readily absorbed. The digestibility of microbial nucleic acids is about 85%.

### Estimation of microbial protein supply to ruminant by purine derivative excretion in urine

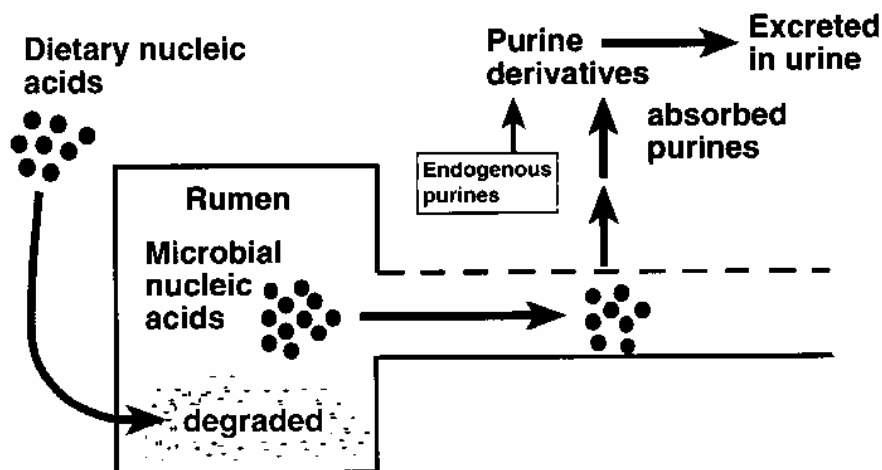


Fig.3 Schematic presentation of the principle of the method.

Purine nucleosides and free bases absorbed from the intestinal lumen are subjected to degradation as well as utilization in the intestinal mucosa. This is where sheep and cattle differ from each other (see Fig. 4). In cattle, there is a very high activity of xanthine oxidase in the intestinal mucosa, which can convert practically all the absorbed purines into uric acid. The result is that the absorbed purines enter the liver as uric acid and therefore are not available for utilization by the animal for incorporation into tissue nucleic acids. In sheep, the activity of xanthine oxidase is negligible and therefore, absorbed purines can enter the liver unchanged and are available for incorporation into the tissue nucleic acids (this process is usually called *salvage*). Both the salvage and degradation enzymes are very active and there could be competition for the substrates between salvage and degradation pathways. The result is that, those absorbed purines that have not been incorporated into the tissue nucleic acids are completely converted into their metabolic end products, hypoxanthine, xanthine, uric acid and allantoin.

The PD entering the circulation blood can also be from the degradation of tissue nucleic acids. We call this fraction "endogenous PD". Measurement of the magnitude of endogenous excretion has been made with the aid of the technique of intragastric infusion, or the technique of replacement of digesta entering the small intestine. The endogenous excretion of PD is three times higher in cattle than in sheep per kg of metabolic weight ( $150$  and  $530 \mu\text{mol/kg W}^{0.75}$  per day for sheep and cattle respectively). Cross species comparison is also interesting. Sheep, goats, pigs and humans are very similar in the magnitude of endogenous excretion expressed on basis of metabolic weight. The difference in tissue distribution of xanthine oxidase may be the reason for these species differences. Cattle have high xanthine oxidase activities in most tissues including the blood, whereas sheep have low xanthine oxidase activities in most tissues and none in the blood. The high xanthine oxidase activity will divert more of the purines released from the tissue nucleic acid degradation away from the salvage pathway into the degradation pathway.

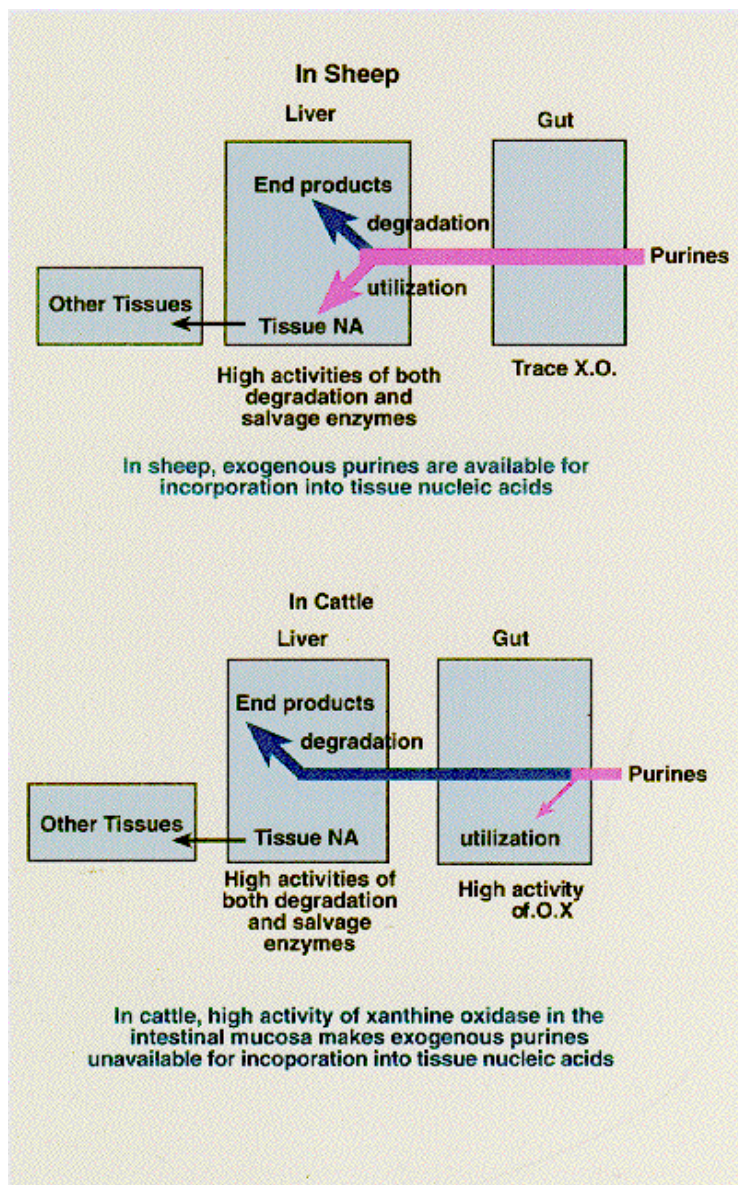


Fig.4 Difference between sheep and cattle in the utilization of exogenous purines

When using the daily excretion of PD to calculate the amount of exogenous purine absorbed by the animal, we need to correct for the contribution from the PD of endogenous origin. In sheep, when we take into account the amount of exogenous purines utilized by the animal, the net endogenous contribution (i.e. the endogenous loss minus exogenous purines utilized) is reduced. Therefore, as the amount of exogenous microbial available to the animal increases, the net endogenous contribution decreases to practically zero (Fig. 2). The response curve of PD excretion vs purine absorbed is not linear as shown in the work of Chen *et al.* 1990 and Balcells *et al.* 1992. In cattle, since the exogenous purines are not available for utilization by the animal, the purine loss must be replaced by *de novo* synthesis from amino acids. As a result, there is always a net endogenous contribution to the total excretion in urine (Fig. 2). The response curve of PD excretion vs purine absorbed is linear. It is now clear that we need to use different equations for sheep and cattle to calculate the purine absorption from the daily excretion of PD. Two equations proposed by this laboratory based on data available so far will be discussed in a later section. The studies of Balcells *et al.* (1991, 1992) also derived a similar equation for sheep and confirmed the general model for cattle. However, more work is still needed to assess whether all parameters adopted in the equations will apply to all situations.

PD (from both the endogenous and exogenous sources) entering the blood are cleared rapidly. The clearance rate constant is about 30%/h. Urinary excretion is the primary route for the disposal of these purine end products. In sheep and cattle, uric acid and allantoin, once filtered into the glomerular, are quantitatively excreted in the urine. There is very little secretion and reabsorption of these compounds in the nephric tubes. Thus the urinary excretion of PD is a function of the plasma concentration and glomerular filtration rate (GFR). It should be noted that, allantoin, uric acid, xanthine and hypoxanthine are all present in sheep urine, but only allantoin and uric acid in cattle urine. This may be because in cattle the high activity of xanthine oxidase in the blood and tissues (also kidney) converts xanthine and hypoxanthine into uric acid prior to excretion in the urine.

Some of the PD in the blood can also be disposed of by none-renal routes, e.g. by secretion into the gut via saliva or across the gut wall, by secretion with the milk. Once allantoin and uric acid are secreted into the gut, they can not re-enter the blood for secretion into the urine. This is due to degradation by microbes in the rumen and/or by those associated with the gut wall. Another possibility is that they are not absorbed from the gut at all. The amount of PD that are lost by none-renal routes is probably also a function of the plasma concentration of PD and thus is proportional to the total amount entering the blood. We incorporate this partitioning factor between renal and the none-renal disposal of plasma PD in the equations to relate purine excretion with purine absorption. The value of 0.85:0.15 is adopted based on data available so far. However, whether this value will apply to all feeding situations remains to be investigated. Results of recent work using buffaloes (Chen *et al.* 1996a) and Zebu cattle indicates in tropical animals a low proportion of plasma PD is excreted in the urine.

### LIMITATIONS OF THE METHOD

- 1) In the calculation, it is assumed that there is little dietary nucleic acid reaching the small intestine. This could be true with most diets, but may not be so when animals are fed with rations containing large amount of fishmeal.
- 2) The calculation of microbial N from purine content assumes that the ratio of purine: total N in mixed microbial population is constant.
- 3) Equations are species specific. It has been clearly shown that sheep, cattle and possible other species differ in their purine metabolism. A review of the species differences is given by Stangassinger *et al.* (1994). The implication is that different models should be used for these species to relate PD excretion with intestinal flow of microbial protein. So far more information is available for sheep, cattle and goats, and little in other species.
- 4) At this stage, the values of microbial N flow calculated from PD excretion should not be taken as absolute values although results of limited number of experiments showed values obtained by the PD method were in good agreement with other methods. Nevertheless, this method is best used to compare differences in intestinal microbial N flow between dietary treatments.

### SAMPLE COLLECTION

- (1) The idea is to collect all the urine produced by the animal. Good separation of urine from faeces is essential.

- (2) To obtain a more realistic measurement of the daily PD excretion, urine collection should be made for more than 5 days. This helps to reduce the error due to the 'end-of-collection' variation in urine output from the animal.
- (3) The collection can be made as a bulk for the whole period. However, where analysis facility allows, it is better to make the urine collection daily. Daily collections will provide us with additional information on the variability of the daily measurement. From our experience, this day-to-day variation is usually about 10%.
- (4) Urine is collected into a container with approximately 100 ml of 10% H<sub>2</sub>SO<sub>4</sub>. The final pH of urine needs to be below 3. It is essential to acidify the urine to prevent bacterial destruction of PD in urine. It may be necessary to check the pH on the first day of collection and make necessary adjustment of the amount of acid used accordingly. Slight excess of acid will not matter.
- (5) PD concentration in straight urine is very high and precipitation (particularly of uric acid) can occur during storage. This will make it difficult for representative sampling for analysis. Dilution by 3-4 times will prevent the occurrence of precipitation. We normally dilute the urine just after collection (i.e. before storage) as follows:
  - a) Record the original weight of the urine. The daily urine output may vary with individual animals and diets, 0.5-2 l and 20-30 l for sheep and cattle, respectively. Add tap water to a constant final weight (4 kg for sheep, 50 kg or even higher for cattle †). So the final volume of diluted urine is the same for all animals and every day. The advantages are: (1) it is easy to bulk the daily samples if needed (just by taking an equal volume of all daily samples); (2) it is easy for the analysis since all samples can be further diluted by a same factor.

† If it is difficult to handle large quantity of liquid, collect a representative sample of urine and dilute it by a known factor (4-5 times).
  - b) Mix the diluted urine thoroughly, filter some urine through glass wool or surgical gauze, take a subsample of about 20 ml and store it in labeled bottle (e.g. date and animal number) at -20°C.
  - c) Twenty ml of the diluted urine is plenty for purine analysis, but take enough for other analysis. For example, for the determination of N in the diluted urine, it is necessary to increase the sample size accordingly.

## **DETERMINATION OF PURINE DERIVATIVES**

### **Dilution of urine samples**

The urine samples which have been previously diluted before storage (as detailed before) still need further dilution. The next dilution should be to such an extent that the concentrations in the final samples will be 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 microbial protein supply of the animal. We can get a guideline by doing some rough calculations. The idea of these calculations is: (1) estimate the daily microbial protein supply from data of feed intake, based on the relationship between microbial N production in the rumen and the quantity of digestible organic matter fermented in the rumen (DOMR): 32 g microbial N/kg; (2) estimate the equivalent amount of

microbial purines absorbed by the animals and thus the daily excreted of PD to be expected; (3) estimate the approximate concentrations of PD in urine and compare these with the target concentrations.

Information needed (the values do not need to be precise):

- feed intake (fresh or DM).
- daily urine volume, i.e. volume of the diluted urine, or the corrected volume if the urine has been diluted by a certain factor.
- body weight (not so necessary for sheep).

The calculations are illustrated as follows (the same for sheep and cattle unless specified). If you perform this calculation routinely, you can build a spreadsheet template using Lotus 123 or Excel for doing so. You may contact the authors for a worksheet already made.

[Example] A sheep consuming 1.6 kg fresh feed, urine collected was 1.2 l/d of straight urine, but diluted to 4 l (urine volume used for the calculation is therefore 4 l, not 1.2 l).

1) Calculation of DOMR

$$\text{DOMR} = \text{Feed intake} \times \text{DM content} \times \text{OM content} \times \text{OM digestibility} \times 0.65$$

In this example we assume: DM content of the diet 90%; organic matter (OM) content (DM basis) 88%; and an estimated digestibility of the OM 60%.

$$\text{DOMR} = 1.6 \times 0.9 \times 0.88 \times 0.60 \times 0.65 = 0.494 \text{ (kg/d)} \quad \dots\dots\dots (1)$$

2) Calculation of Microbial N (MN) yield:

$$\text{MN} = 32\text{g/kg DOMR (ARC 1984)}$$

In this case:

$$\text{MN} = 32 \times 0.494 = 15.808 \text{ (g/d)} \quad \dots\dots\dots (2)$$

3) Calculation of the equivalent amounts of purine absorbed ( $P_a$ ) by the animal:

$$P_a \text{ (mmol/d)} = \text{MN (gN/d)} \div 0.727.$$

In this case:

$$P_a = 15.808 \div 0.727 = 21.744 \text{ (mmol/d)} \quad \dots\dots\dots (3)$$

4) Calculation of total PD excretion ( $PD_e$  mmol/d)

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

cattle  $PD_e = 0.85 P_a + 0.385 \times W^{0.75}$  (where  $W^{0.75}$  = metabolic weight in kg)

In this case:

$$PD_e = 0.84 \times 21.744 + 2 = 20.265 \text{ (mmol/d)} \quad \dots\dots\dots (4)$$

Allantoin excretion ( $A_e$ )

$$A_e \text{ (mmol/d)} = PD_e \times 0.85 \quad \text{(i.e. 85\% of the } PD_e \text{ is allantoin)}$$

In this case:

$$\begin{aligned} A_e &= (4) \times 0.85 = 20.265 \times 0.85 = 17.225 \text{ (mmol/d)} \\ &= 17.225 \times 158 = 2721.590 \text{ (mg/d)} \quad \dots\dots\dots (5) \end{aligned}$$

Uric Acid excretion ( $UA_e$ )

$$UA_e \text{ (mmol/d)} = PD_e \times 0.15 \quad \text{(i.e. 15\% of the } PD_e \text{ is uric acid)}$$

In this case:

$$\begin{aligned}
 UA_e &= (4) \times 0.15 = 20.265 \times 0.15 = 3.040 \text{ (mmol/d)} \\
 &= 3.040 \times 168 = 510.678 \text{ (mg/d)} \dots\dots\dots (6)
 \end{aligned}$$

5) Calculation of the dilution factors

Allantoin

$A_e \text{ (mg/L of urine)} = A_e \text{ (mg/d)} \div \text{urine produced daily}$

In this case:

$$A_e = (5) \div 4 = 2721.590 \div 4 = 680.4 \text{ (mg/L)}$$

To obtain a concentration between 10 and 40 mg/L it is necessary diluted the urine by a factor in the follow range:

To 10 mg/L:  $680.4 \div 10 = 68.4$  times

To 40 mg/L:  $680.4 \div 40 = 17.0$  times

i.e., approximately between 17 and 68

Uric acid

Follow the same procedure to allantoin using the (6) value. The range of the dilution factor will be between 3 and 12 times.

Based on the values obtained for the individual samples, a common dilution factor could be used for all urine samples. In our laboratory, we dilute the urine samples in two steps, first dilution for uric acid analysis and a further dilution for allantoin analysis. In the present example, we would dilute 5 times for uric acid and further 11 times ( $5 \times 11 = 55$ ) for allantoin assay.

Animals in tropics excrete less PD than European animals at a given feed intake. In this case, a value half the calculated value may be used for dilution. For example, if the above calculation indicates that a dilution of 40 time is needed, then a 20 time dilution may be adopted for urine from tropical animals.

**List of published methods for determination of purine derivatives**

Methods for the chemical analysis of purine derivatives using various instruments, spectrophotometer, AutoAnalyzer and High-Performance Liquid Chromatography (HPLC), are given in Table 1. In the next two sections only method based on spectrophotometer are described. A comprehensive review on the methods for the determination of allantoin has been given by Chen et al. (1996a).

Table 1: Published methods for the determination of purine derivatives. (The numbers refers to papers listed in the reference section 2.)

	Allantoin	Uric acid	Xanthine+hypoxanthine
Spectrophotometer	1,2,8	2,3,9	3,4
AutoAnalyzer	3,4,10	4	4
HPLC	5-7	6,11	6

References cited in Table 1

1. Young, E. G. and Conway, C. F.(1942). On the estimation of allantoin by the Rimini-Schryver reaction. *Journal of Biological Chemistry* 142, 839.
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### Determination of allantoin by a colorimetric method

#### Chemistry:

The manual method adopted to measure allantoin is based in the calorimetric method described by Young and Conway (1942). In this procedure, allantoin is hydrolyzed firstly under a weak alkaline condition at 100°C, to allantoic acid which is further degraded to urea and glyoxylic acid in weak acid solution. The glyoxylic acid then reacts with phenylhydrazine hydrochloride to produce a phenylhydrazone of the acid. The product can then form an unstable chromophore with potassium ferricyanide. The colour is read at 522 nm.

#### Apparatus

- (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. The temperature can be better controlled since PEG has a boiling point greater than 100°C.
- (3) Ultrasonic bath (optional).

#### Chemicals

- (1) Sodium hydroxide (NaOH) 0.5 M.
- (2) NaOH 0.01 M.
- (3) Hydrochloric acid (HCl) 0.5 M.
- (4) Phenylhydrazine hydrochloride 0.023 M freshly prepared before use.
- (5) Potassium ferricyanide 0.05 M freshly prepared before use.
- (6) Concentrated Hydrochloric acid (11.4 N) cooled at -20°C at least 20 min before use.
- (7) Alcohol bath, 40% (v/v) alcohol, kept at -20°C. You may use 40% NaCl solution instead of alcohol solution.
- (8) Allantoin (From Sigma or BDH).

### *Standards*

Prepare a stock allantoin solution 100 mg/L. Dilute it to give working concentrations of 10, 20, 30, 40, 50 and 60 mg/L.

(1) Weigh 50 mg of allantoin and transfer it to a 500 ml volumetric flask. Dissolve in about 100 ml 0.01 M NaOH, and top up to volume with distilled water. The addition of NaOH is only to help to dissolve allantoin.

(2) To prepare 50 ml of the working standards 10, 20, 30, 40, 50 and 60 mg/L respectively, pipette 5, 10, 15, 20, 25 and 30 ml of stock solution into 50 ml volumetric flasks and make up to volume with distilled water. If you do this analysis routinely, it is better to prepare the standard solution in larger volumes.

(3) Store each working standards as small aliquots in the freezer. Only the necessary quantities are thawed and any left over discarded. This ensures that fresh standards are used for each analysis run.

### *Preparation*

Before the analysis:

- (1) Put the alcohol bath into the freezer over night.
- (2) Put the concentrated hydrochloric acid into the freezer just before the beginning of the analysis.
- (3) Switch on the water bath.
- (4) Check the samples. If any precipitate is visible, place the samples in an ultrasonic bath for 20 min to break up the particles.
- (5) Prepare the fresh solutions of Phenylhydrazine hydrochloride and Potassium ferricyanide.

### *Phenylhydrazine hydrochloride and Potassium ferricyanide solutions:*

Prepare solutions of phenylhydrazine hydrochloride and Potassium ferricyanide on the day of analysis (keep these solutions in fridge before use). Fifty ml is enough for 10 samples in duplicate: Weigh 0.1663g of phenylhydrazine hydrochloride, dissolve in a small beaker and transfer to a 50 ml volumetric flask. Top up to volume with distilled water. Weigh 0.835g Potassium ferricyanide and transfer to a 50 ml volumetric flask, dissolve and make up to volume with distilled water.

### *Procedure*

This procedure requires critical timing of the reactions. The reading of standards and samples OD must be done within a shortest possible time-span, since OD decrease with time (see Fig. 5). 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 processed.

- (1) Pipette 1 ml of sample, standard or distilled water (blank) into 15 ml tubes.
- (2) Add 5 ml of distilled water.
- (3) Add 1 ml of 0.5 M NaOH.
- (4) Mix the contents of the tubes by vortexing.
- (5) Put the tubes in the boiling water bath for 7 min.
- (6) Remove from the boiling water and cool the tubes in cold water.
- (7) Add to each tube 1 ml of HCl (0.5 M). The pH after adding the HCl must be in the range 2-3<sup>†</sup>.

- (8)<sup>‡</sup> Add 1 ml of the phenylhydrazine solution. Mix and transfer the tubes again to the boiling water for exactly 7 min.
- (9) Remove from the boiling water and dump it immediately into the icy alcohol bath for several min<sup>§</sup>.
- (10) Pipette 3 ml of concentrated HCl (operate in a fume cupboard) and 1 ml of Potassium ferricyanide. Perform this for all samples within a shortest possible span. Start the timer.
- (11) Mix thoroughly and transfer some to 4.5 ml cuvettes at room temperature.
- (12) Read the absorbance at 522 nm after exactly 20 min<sup>¶</sup> on the timer. Once started, do it as quickly as possible (because the colour will disappear.) It is important that OD for samples and standards be read at a shortest possible time span.

<sup>†</sup> If this is the first time you have done this method, you need to check the pH. Add more HCl if necessary, the required amount can be followed in later runs.

<sup>‡</sup> Steps 8-12 should not be interrupted.

<sup>§</sup> The idea is to slow down the reaction by reducing the temperature. The use of alcohol and water mixture is to achieve a temperature below zero for the liquid.

<sup>¶</sup> In places where ambient temperature is high, this waiting time could be shortened. However, it is important that the same duration is used consistently for all samples and all runs.

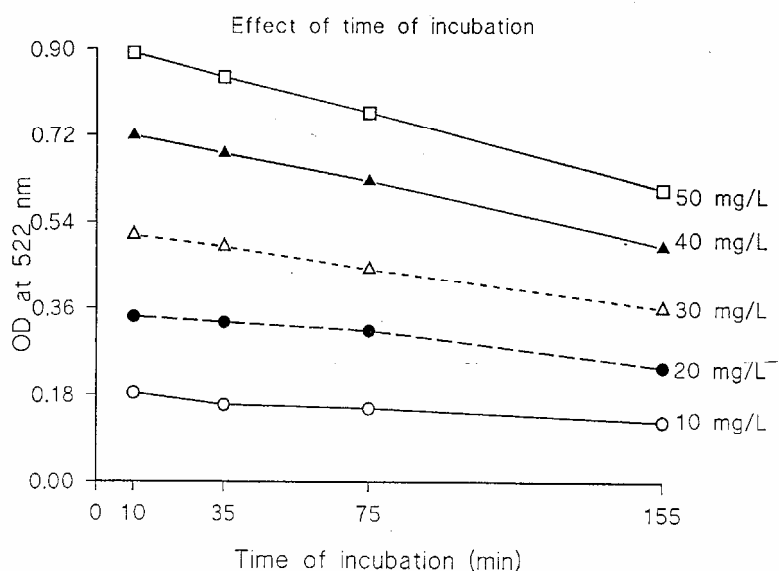
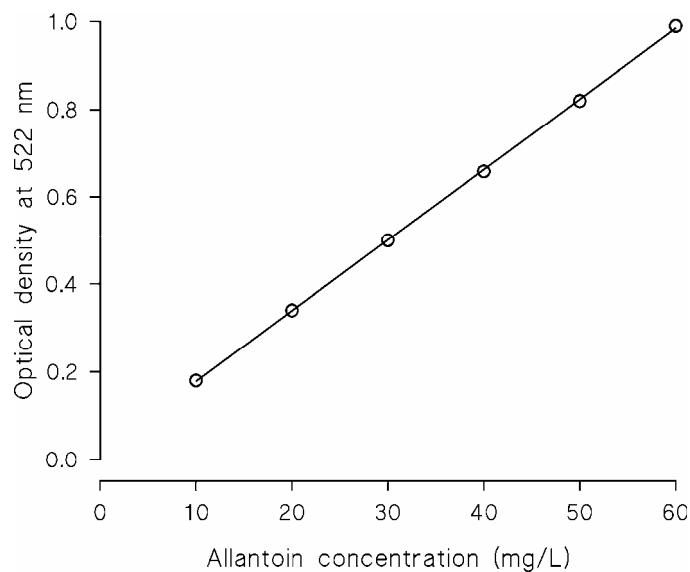


Fig.5 Influence of time of incubation on the optical density readings in allantoin assay.

#### Calculation

##### Standard curve:

The standard curve is linear (See Fig. 6). 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 unknown based on this equation.



*Fig. 6 Allantoin standard curve*

#### *Notes*

Formaldehyde strongly affects this method. Therefore if the animals are fed with feeds treated with formaldehyde, the method may not be suitable.

Borchers (1977) proposed another colorimetric method for determination of allantoin based on a different chemistry. Experience from our laboratory indicates that this method is not specific and usually gives higher values than those obtained using Young and Conway (1942) method. The method by Young and Conway (1942) is nevertheless the most commonly used method for allantoin determination.

### **Determination of xanthine plus 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. When the urine samples are treated with xanthine oxidase, xanthine and hypoxanthine are converted to uric acid. There should be an increase in OD at 293 nm after the enzyme treatment. The net increase is then used for the calculation of the amount of uric acid formed based on the uric acid standard curve.

#### *Apparatus*

- Spectrophotometer.
- Water bath.
- Ultrasonic bath (optional).

#### *Chemicals:*

- (1)  $\text{KH}_2\text{PO}_4$  buffer, 0.2M pH 7.35, adjust the pH with either  $\text{H}_3\text{PO}_4$  or KOH.
- (2) L-histidine 4.3 mM (66.8 mg/100 ml)
- (3) Xanthine Oxidase, add 25  $\mu\text{l}$  of the solution from Sigma (Catalog No. X-1875. 50 unit in 2.6 ml) to 3 ml of the buffer.
- (4) Uric acid.

#### *Standards preparation:*

Prepare a stock uric acid solution of 100 mg/L. Dilute it to give working concentrations of 20, 40, 60, 80 and 100 mg/L.

- (1) Weigh 50 mg of uric acid and transfer it to a 500 ml volumetric flask. Dissolve in about 100 ml 0.01 M NaOH, and make to volume with distilled water. The addition of NaOH helps to dissolve uric acid.
- (2) To prepare 50 ml of the working standards 10, 20, 40, 60 and 80 mg/L, measure respectively, 5, 10, 20, 30 and 40 ml of stock solution into 50 ml volumetric flasks and make to volume with distilled water.
- (3) Store each working standards as small aliquots in freezer. Only the required amounts are thawed and any left over discarded. This ensures that a fresh set of standards are used for each analysis run.

#### *Preparation*

Before the analysis do not forget to:

- (1) Set the water bath to 37°C.
- (2) Check the samples. If some precipitate is visible, place in an ultrasonic bath for 20 min to break up the particles.

#### *Procedure:*

- (1) Pipette 1 ml of urine or standard or distilled water into a cuvette. 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.
- (4) In one set, add 150  $\mu$ l of buffer, in the other set add 150  $\mu$ l of the XO solution. Mix well and incubate at 37°C for 60 min<sup>†</sup>.
- (5) Read OD at 293 nm.

<sup>†</sup> 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 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) (see Fig 8).
- (2) Calculate the  $\Delta$ OD for the samples, i.e. the difference between two sets with and without XO addition:  $\Delta$ OD = OD with XO - OD without XO.
- (3) Calculate the corresponding concentration of uric acid from  $\Delta$ OD based on the above standard curve.
- (4) Estimate the contribution of OD reading from the xanthine ( $OD_x$ ) in the set without XO based on a pre-determined xanthine standard curve §.
- (5) Re-adjust the  $\Delta$ OD (i.e.  $\Delta$ OD<sub>2</sub> =  $\Delta$ 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 can reduce this inhibition.

§ As shown in Fig 7, 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. We can do it in this 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. We may not need to do xanthine standard for every run.

At UV 293 nm, the absorbance (OD) for uric acid is about 10 times higher than xanthine. Hypoxanthine does not absorb UV at this wavelength. Over the range of concentrations used (0-50 mg/L for uric acid, and 0-30 mg/L for xanthine), relationship between UV absorbance and concentration is curvilinear for uric acid, but linear for xanthine. See Fig 7.

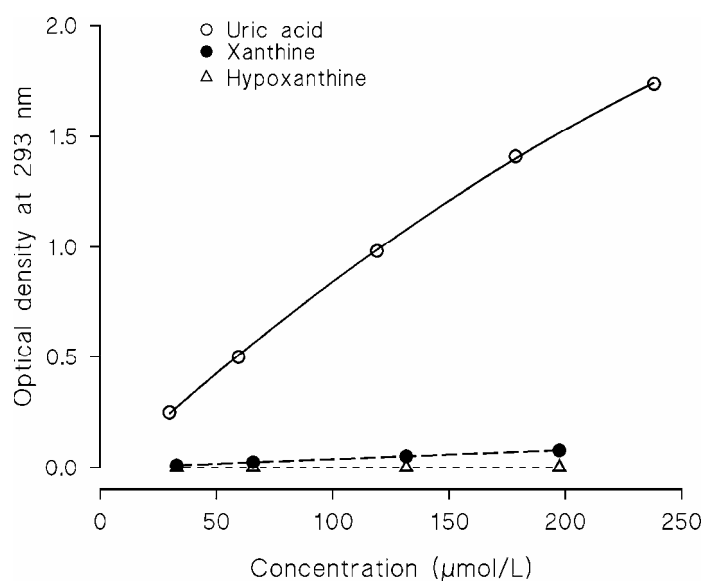


Fig. 7 Absorbance of the uric acid, xanthine and hypoxanthine at 293 nm

#### Standard curve for uric acid:

The uric acid standard curve is not linear, but when both X (concentration) and Y (OD) are transformed into  $\ln(X)$  and  $\ln(Y)$ ,  $\ln(Y)$  becomes a linear function of  $\ln(x)$ . See Fig. 8. This relationship is then used for the calculation of the concentrations of the samples from their OD readings.

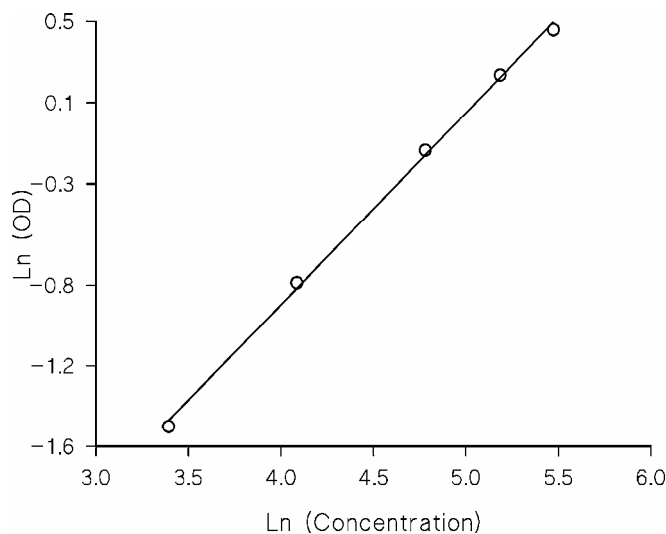


Fig. 8 Standard curve for uric acid assay. Both axis are expressed as a function of Ln.

### Determination of uric acid by uricase method

#### Principle

The manual method adopted to measure the uric acid was described by Fujihara *et al.* (1987). This method is very similar to that proposed by Praetorius and Poulsen, (1953). 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 degraded in allantoin and other compounds that do not absorb UV at 293 nm. Therefore, the reduction in OD reading after treatment with uricase is related with the concentration of uric acid in the sample. After treatment, the OD of the standards should be zero if the conversion is complete.

#### Apparatus

- Spectrophotometer.
- Water bath.
- Ultrasonic bath (optional).

#### Chemicals

- (1)  $\text{KH}_2\text{PO}_4$  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).
- (3) Uric acid.

#### Standards preparation

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.

#### Enzyme preparation

Dilute the enzyme in the buffer to obtain a concentration of 0.12 U/ml. To preserve the enzyme activity, keep the enzyme solution in fridge.

#### Preparation

Before the analysis do not forget to:

- (1) Put the water bath at 37° C.
- (2) Check the samples. If some precipitate is visible, place in an ultrasonic bath for 20 min to break up the particles.

### *Procedure*

Two sets of standards and a blank (using distilled water) and samples in duplicate, are prepared.

- (1) Pipette 2.5 ml of urine or standard or water into a 10 ml tubes.
- (2) Add 1 ml phosphate buffer.
- (3) Mix well the content of the tubes by vortexing.
- (4) In one set, add 150  $\mu$ l buffer and in the other add 150  $\mu$ l of uricase solution.
- (5) Mix again by vortexing, and incubate in the water bath at 37°C for 90 min.
- (6) Remove from water bath, mix and transfer again the solutions to the 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 as in Fig.7. When both X and Y are transformed to Ln functions, Ln (Y) is linearly correlated to Ln (X), as shown in Fig.8. 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 ( $\Delta$ OD) for the samples due to uricase treatment.  $\Delta$ OD = OD without enzyme - OD with enzyme

(3) Calculate the uric acid concentration from  $\Delta$ OD based on the established standard equation (as in (1)).

## **CALCULATIONS**

### **Daily excretion of purine derivative:**

(1) Calculate the excretion of allantoin, uric acid, and xanthine plus hypoxanthine, using the unit of mmol/d.

(2) The total PD excretion (i.e. sum of all compounds) is used for the estimation of microbial protein supply. This should therefore be presented in the result section.

In sheep, the sum of all 4 compounds, and in cattle the sum of allantoin and uric acid, since xanthine and hypoxanthine are present in trace quantities. The proportion of the individual components expressed as the percentage of the sum are approximately in this order:

(1) sheep: allantoin 60-80%, uric acid 30-10%, xanthine plus hypoxanthine 10-5 %. As the total excretion increases, the proportion of allantoin increases. This is also the reason for not using allantoin excretion alone to calculate the microbial protein supply.

(2) cattle: allantoin 80-85%; uric acid 20-15%. Within the same animal, the proportions are very constant, but there seems to be variation between animals.

(3) With dairy cows, allantoin and uric acid are also secreted with the milk. The daily amount secreted is equivalent to about 5% of that excreted in the urine. The concentrations of these compounds in milk seem very constant between and within animals and not are affected by

microbial protein supply. One may ask 'Can we use the milk purine derivatives to estimate microbial protein supply?'. The idea is good but available data indicates that this does not work.

For the calculation of microbial protein flow, the total output of PD is use. So if you have the data for output of PD in milk, just add the PD output in milk to the urine output. Then use it for the next calculation. If you do not have the milk PD data, you can make a small correction for it. The PD concentration in milk may be subjectively taken as about 1 mmol/L. Therefore the output is 1 × milk yield (L/d). This number only give an estimate of the PD output in milk. It is not accurate but it is very small anyway.

### Calculation of microbial N supply

#### *Purine absorption and PD excretion*

Different equations are used for sheep and cattle to describe the quantitative relationship between absorption of microbial purines (X mmol/d), and excretion of PD in urine (Y mmol/d):

$$\begin{aligned} \text{sheep } Y &= 0.84X + (0.150 W^{0.75} e^{-0.25X}) \dots\dots\dots (1) \\ \text{cattle } Y &= 0.85X + (0.385 W^{0.75}) \dots\dots\dots (2) \end{aligned}$$

where  $W^{0.75}$  represents the metabolic body weight (kg) of the animal. These two equations are also plotted as in Fig.2.

The slopes of 0.84 and 0.85 in Equations (1) and (2) respectively, represent the recovery of absorbed purines as PD in urine. The component within parenthesis represents the net endogenous contribution of PD to total excretion after correction for the utilization of microbial purines by the animal. In cattle, the endogenous contribution is taken as a constant at 0.385 mmol /kg  $W^{0.75}$  per day. In sheep, this reduces to zero as exogenous purines are utilized and *de novo* synthesis of purines is phased out.

#### *Calculation of daily purine absorption*

Based on Equations (1) and (2) for sheep and cattle, respectively, the amount of exogenous purines absorbed can then be estimated from the daily excretion of PD.

*Sheep:* the calculation of X from Y based on Equation (1) can be performed by means of the Newton-Raphson iteration process, as shown below:

$$X_{(n+1)} = X_n - \frac{f(X_n)}{f'(X_n)} \dots\dots\dots (3)$$

where  $f(X) = 0.84X + 0.150 W^{0.75} e^{-0.25X} - Y$   
 and the derivative of f(X):  $f'(X) = 0.84 - 0.038 W^{0.75} e^{-0.25X}$

#### [Example]

A sheep of 60 kg (W) excreted 5.0 mmol/d PD (Y). We give an initial value  $X_1 = Y + 0.84 = 5.952$  to put in Eqn (3) to calculate  $X_2$ . Now we obtain  $X_2 = 4.838$ . Next we put  $X_2$  back to Eqn (3) to calculate  $X_3$  ( $X_3=4.790$ ), and so on.  $X_4$  and  $X_5$  can be calculated as 4.790 and 4.790 respectively. We will notice that, as the iteration process goes on,  $X_n$  approaches a constant value, which is the answer. In this example, the amount of microbial purines absorbed is therefore 4.79 mmol/d.

*Cattle:* the calculation is straight forward.

$$X = (Y - 0.385 \times W^{0.75}) \div 0.85 \dots \dots \dots (4)$$

[Example]

A steer of 321 kg live weight, the daily excretion of purine derivatives was 153 mmol/d. The amount of microbial purines absorbed can be calculated as:  $(153 - 0.385 \times 321^{0.75}) \div 0.85 = 145.7$  mmol/d.

*Calculation of intestinal flow of microbial N*

The following factors are used for the calculation of intestinal flow of microbial N (g N/d) from the microbial purines absorbed (X mmol/d) (Equation 5):

- 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 (Table 2).
- 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.

$$\text{Microbial.N (gN / d)} = \frac{X (\text{mmol / d}) \times 70}{0.116 \times 0.83 \times 1000} = 0.727 X \dots \dots \dots (5)$$

Note It should be born in mind by the user of this method that the calculation of protein supply from purine absorption involves the assumption that the purine:protein ratio in mixed rumen microbes is unchanged by dietary treatments. Whether this assumption is valid remains the subject of debate until enough data are generated to assess this argument. Therefore, the values should not be taken as absolute measurements.

Table 2 Disappearance of microbial nucleic acid (NA) N from the small intestines of ruminants.

References	Animals	Disappearance %		
		NA-N	RNA-N	DNA-N
Condon et al. (1970)	Sheep	-	82	
Smith and McAllan (1971)	Calves	80	85	75
Coelho da Silva et al. (1972a)	Sheep	85-93		
Coelho da Silva et al. (1972b)	Sheep	72-82		
Roth and Kirchgessner (1979)	Calves	97-98		
Storm et al. (1983)	Sheep	86	87	81
Overall mean		83		

## Presentation of results

The results of the calculated microbial protein supply are expressed as 'g microbial N per day' and/or as 'g microbial N per kg digestible organic matter intake (DOMI)'. The latter is effectively an expression of the efficiency of microbial protein supply. The efficiency can also be expressed as 'g microbial N per kg digestible organic matter apparently fermented in the rumen (DOMR)' to facilitate comparison with the reports in the literature, here DOMR can be assumed to be 0.65 of the DOMI, based on the report of ARC (1984). An example is given in Table 3.

Table 3 Effect of defaunation on purine derivative (PD) excretion, rumen microbial N supply and N balance of two steers (means of 5 days).

Treatment	Body weight (kg)	DOMI (kg/d)	PD excretion† (mmol/d)	Microbial N	
				g N/d‡	g N/kg DOMR¶
<u>Defaunated</u>					
Steer A	321	5.44	153 ± 6.6	106	30.0
Steer B	404	6.74	156 ± 12.4	104	23.6
<u>Refaunated</u>					
Steer A	343	5.63	134 ± 2.1	88	24.6
Steer B	423	6.63	136 ± 7.0	85	19.8

† PD as sum of uric acid and allantoin. Mean ± S.E. of five measurements

‡ Calculated as based on Equation (2) and (4).

¶ DOMI (digestible organic matter intake). DOMR (organic matter apparently fermented in the ruminants) was taken as 0.65 DOMI (ARC, 1984).

## USE OF SPOT SAMPLES

This article has so far dealt with the method based total urine collection. A lot of work has also been conducted to study the possibility of urine spot urine, plasma or milk samples for the estimation of microbial protein supply in order to extend the use of the technique to animals out door. A review has been made by Chen *et al.* (1995 ab).

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