

Comparison of Methods for the Measurement of Digesta Flow and Microbial Protein Supply in Sheep

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DECLARATION

This thesis has been composed by myself and has not been accepted in any previous application for a degree. The work has been done by myself and all sources of information have been acknowledge by means of references.

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DEDICATION

A mi familia, novia y amigos que con su apoyo constante estuvieron presente en los momentos más difíciles durante la realización de esta tesis.

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ABSTRACT

The excretion of purine derivatives (PD) in urine has been used as an index for the estimation of microbial nitrogen (MN) supply in ruminants. This method is simple and does not require surgical intervention of the animal. There has been however little work carried out to compare the results of this method with other methods of MN supply estimation based on intestinal flow of microbial markers. The objective of this work was to compare the PD method with a method using RNA as a microbial marker.

A method for determining digesta flow was needed for the RNA method. It was suggested that natural occurring alkanes could be a useful marker for digesta kinetics. In a preliminary experiment, digesta flow from 3 sheep was determined using alkanes as feed marker, and using the commonly-used 'double-marker' technique ($^{51}\text{Cr-EDTA}$ and $^{103}\text{Ru-complex}$ as liquid and solid markers respectively). There was a good agreement in the results of the two methods ($Y = -1.9 \text{ (SE 34.0)} + 0.99 \text{ (SE 0.06)}X$ $r^2 = 0.97$) where $Y =$ digesta flow (g/d) estimated with the double-marker technique and $X =$ digesta flow (g/d) estimated with alkanes. The results therefore indicated that n-alkane can be reliably used as a marker for the estimation of digesta flow.

Five sheep (body weight 38-47 kg) fitted with duodenal or jejunal and also with ileal cannulae were fed pelleted grass-nuts at three levels, 560, 930, and 1300 g DM/d. The treatments (feeding levels) were allocated in two 3 x 3 Latin Square design (one of which was incomplete). The animals were fed once hourly by a continuous feeder. Urine and faecal collections were made. Urine samples were analysed for purine derivatives (allantoin, uric acid, xanthine and hypoxanthine). Digesta samples were taken from the duodenal or jejunal cannula and from the ileal cannula. N-alkane naturally present in the feed was used as marker of digesta flow.

PD excretion ranged 6.99 - 18.38 mmol/d for the 560 - 1300 g DM intake per day. The estimated microbial N supply was 5.55 - 15.90 g/d; whereas, for the RNA method, the estimation of MN supply was 6.21 - 18.65 g/d within the same range of DM intake.

It was noted that animals fitted with jejunal cannulae were unsuitable for the determination of microbial flow using the RNA method since substantial digestion and absorption of RNA occurred between the duodenum and jejunum. The available data showed MN flow estimated

based on RNA method using duodenal digesta was in good agreement (r^2 of 0.96) with that based on the PD method. $Y = 1.70 \text{ (SE 1.13)} + 1.07 \text{ (SE 0.11)X}$, where $Y =$ (g MN/d) based on RNA method and $X =$ (g MN/d) based PD method.

Estimates of intestinal flow of MN by either methods was positively correlated with the level of feed intake. The efficiency of the MN supply (i.e. g MN per kg digestible organic matter fermented in the rumen) also increased with the level of intake.

The results of this work indicated that the PD method provides reliable estimates of MN supply in sheep.

1. INTRODUCTION

MICROBIAL PROTEIN AS A PROTEIN SOURCE FOR RUMINANTS

Ruminants obtain their protein in two sources, feed protein that escapes degradation in the rumen, and secondly microbial protein synthesised in the reticulo-rumen. Much of the dietary protein entering the rumen is degraded by the micro-organisms. The extent of protein degradation varies with types of protein, treatments and the residence time in the rumen. Proteolytic digestion releases oligopeptides, which are then broken down to dipeptides and amino acids. Amino acids are further hydrolysed to organic acids, ammonia and carbon dioxide. The micro-organisms utilise the ammonia, amino acids and small peptides to produce microbial protein. The ammonia (and possibly some of the amino acids) that are not utilised by the microbes are absorbed through the rumen wall, and carried to the liver in the blood stream. In the liver these compounds (mainly the ammonia) are converted into urea. Urea can pass to the kidney to be excreted, or it can return to the rumen via saliva and possibly, some absorption may occur from the blood through the rumen wall may occur. In the rumen, urea is hydrolysed to ammonia and re-utilised by the microbes to produce microbial protein. In this way there is a form of nitrogen salvage.

Microbial fermentation in the rumen enables the ruminant to utilise poor quality forage or non-protein nitrogen (NPN), that could not be utilised by the host animal, and to transform them to a good digestible protein of a reasonable constant composition of amino acids. A great part, and sometimes all, of the protein arriving to the small intestine is of microbial origin. It can contribute from 0.42 to 0.93 of the total protein available to the host animal (Djouvinov and Todorov, 1994) the rest is composed of undegraded dietary proteins.

Storm and Ørskov (1983) found that the total microbial amino acid nitrogen comprised 0.81 of the total microbial N, this agrees with the mean value proposed by INRA (1978) of 0.80. The mean value of the true digestibility of the microbial amino acids proposed by INRA (1989) was 0.80, and Storm and Ørskov (1983) found a true N digestibility of the micro-organisms and in the small intestine of 0.815. According to the AFRC (1993) the microbial crude protein (MCP) (total N x 6.25) was estimated to consist of 0.75 of true protein (amino acids) and the rest, consisting of nucleic acids. The digestibility of this true protein was taken to be 0.85 by ARC (1980;1984), AFRC (1993) (slightly higher than 0.80 and 0.815 proposed by INRA (1989) and

Storm and Ørskov(1983) respectively). Therefore, the amount of digestible microbial true protein (DMTP) becomes:

$$\text{DMTP (g/d)} = 0.75 \times 0.85 \times \text{MCP (g/d)} = 0.6375\text{MCP}$$

where: true protein = 0.75 of microbial nitrogen (MN)

digestibility = 0.85 of microbial nitrogen (MN)

To calculate the efficiency of utilisation of the DMTP the AFRC (1993) proposed different values for the amino acid utilisation efficiency for various production purposes:

Maintenance	$k_{nm} = 1.00$
Growth	$k_{ng} = 0.59$
Pregnancy	$k_{nc} = 0.85$
Lactation	$k_{nl} = 0.68$
Wool	$k_{nw} = 0.26$

Thus to know the efficiency of utilisation of DMTP for any type of production, the value of 0.6375MCP needs to be multiply by one of the values of k mentioned above.

FACTORS AFFECTING MICROBIAL SUPPLY

The supply of microbial protein, as an important source of protein for the ruminants, can be affected by several factors:

Energy supply

Energy supply is normally the first limiting factor to microbial protein synthesis. Microbial activity is directed towards the generation of ATP for the maintenance and growth of the microbial population. ATP is derived from fermentation of carbohydrate, yielding VFA, and the process in turn produces microbial cells by incorporating amino acids and non- protein nitrogen (NPN). The fermentation of the carbohydrates is dependent of its digestibility and its degradation rate. A feed source with high digestibility and a fast degradation rate would give the animal a good supply of energy; therefore, a good microbial supply. Rumen microbial protein production is generally determined by the total intake of fermentable carbohydrates. (Gomes *et al*, 1994) demonstrated, in an experiment, that the supplementation of starch concentrate, on a straw diet, up to 19% starch, increased both voluntary food intake and the supply of microbial protein per kg of DOMI. It is known that sucrose also increases the microbial growth in the

rumen and it has been demonstrated (Chamberlain *et al*, 1993) that it can be almost three times more effective than starch which is the traditional way of animal supplementation.

Nitrogen supply

The ammonia concentration in the rumen is of vital importance for the microbial degradation and synthesis of protein. If there is a deficit in protein in the diet, the concentration of ammonia, in the rumen, will be low and the growth of rumen organisms will be retarded as a consequence the breakdown of carbohydrates will be slow (McDonald, 1995). But if there is not a sufficient amount of energy, that the micro-organisms require to synthesise their protein, and excessive amount of ammonia would be excreted in the urine resulting a loss in N. Hence, many rumen micro-organism can live just with ammonia source as long as they have some carbon skeleton of branch chain fatty acids as energy source (Wallace, 1991).

Passage rate

The rate of passage is one of the major factors affecting the efficiency of microbial production (Van Soest ,1994). By increasing the intake the rate of passage is increased. In addition, increasing the rate of passage reduces the engulfing of protozoa over bacteria because the protozoa have less opportunity of contact with microbes if they stay for a shorter period of time in the rumen giving, in this way, more microbial cells to the host animal. Since roughage intake increases the rate of passage (Van Soest, 1994), addition of forage to high-concentrate diets increases the microbial supply (Chen *et al*, 1992).

Defaunation

Removal of protozoa from the rumen (defaunation) would increase microbial cell production because of the reason mentioned previously. Table 1 shows some effect of defaunation over microbial N yield.

Rumen pH

An abundant rapidly fermentable energy supply could be detrimental to microbial protein production. Rapidly fermentable carbohydrate produces propionate and ultimately lactate. If there is an excess of energy supply the microbes would increase lactate production, hence the pH would decrease. At a low pH there is a reduction in the microbial yield.

Table 1.1. Effect of defaunation on rumen microbial N yield.

Reference	Microbial Yield (g N/kg FOM)	
	Faunated	Defaunated
Rowe <i>et al.</i> , 1985	49.6	57.3
Meyer <i>et al.</i> , 1986	27.4	42.7
Ushida <i>et al.</i> , 1986 ^a	26.9 ^b	60.6
	37.3 ^c	59.2
Kayouli <i>et al.</i> , 1986 ^d	34.9	49.6
	18.2	40.7

* nitrogen incorporated in microbial cells

^a Data obtained using DAPA were used.

^b Lucerne diet.

^c Alkaline treated straw diet.

^d Data from individual refaunated and defaunated animals were used.

(Demeyer and Tamminga, 1987 cited by Demeyer, 1991)

Minerals

The requirement of the micro-organisms for minerals could be another factor affecting its production. Microbial growth in the rumen is dependent on the availability of major minerals as well as trace minerals in the rumen to allow for normal cell function and metabolism (Hvelplund, 1991). Some examples are sulphur, for the synthesis of sulphur containing amino acids and some micro-elements like cobalt for the synthesis of vitamin B₁₂. Addition of sodium and potassium increased dilution rate in dairy cattle and hence the microbial efficiency (Ruilaba, 1984 cited by Hvelplund, 1991). Durand and Komisarczuk, (1988) stressed that mineral supply should be equal

to the amount of energy available for fermentation and therefore the mineral requirement should be related to fermentable organic matter in the rumen.

ESTIMATION OF MICROBIAL PROTEIN CONTRIBUTION IN VARIOUS PROTEIN EVALUATION SYSTEMS

The amount of microbial protein available to the animal is usually calculated based on the fermentable energy intake. To estimate the amount of energy made available for microbial growth due to the anaerobic fermentation in the rumen, energy supply should be, ideally, expressed as moles of ATP. In practice it can be assumed that the amount of organic matter fermented in the rumen is a measure of energy supply. Hence, the organic matter apparently digested in the rumen (DOMR) was adopted (*ARC, 1980*). The ARC (1980) adopted a mean value, of digested organic matter, in the feeds of about 19.0 MJ/kg. This is not true for all feed, but it is for the most common ruminant feeds e.g. low in fat and of average protein content.

In Table 1.2 there are values of microbial N yields expressed in terms of DOMR have been taken from ARC (1980). There is a wide range apparently between the values (15 -45g MN/kg DOMR) but (*ARC, 1980*) has adopted a mean value of 30g of microbial N/Kg of DOMR for all diets, whether given to sheep or cattle.

Table 1.2. Mean microbial nitrogen yield/kg organic matter apparently digested in the rumen (ARC, 1980).

	Cattle	Sheep
Pasture		
Grass	27	21
Grass/ clover	26	-
Clover	-	32
Dried grass		
Chopped	-	30
Ground and pelleted	-	25
Dried legumes		
Chopped	-	36
Others		
Hay (legume)	-	44
Silage (untreated)	-	45
Silage (treated with HCHO)	-	15
Straw(alkali-treated, ground)	-	44
Mixed rations containing		
>500 g roughage/Kg	33	32
<500 g roughage/Kg	33	27
All-concentrated diets	-	33
Semi-purified diets	-	34
Mean of above values	30	32

The ARC (1984) expanded this information for cattle and sheep citing data for 262 different diets. They found a mean value for microbial N yield of 32g/ kg DOMR. The summary of this data is given by Table 1.3.

Table 1.3. Proportion of digestible organic matter apparently digested in the rumen and microbial N flow to duodenum per kg of organic matter apparently digested in the rumen ARC (1984).

Animal	Diet	Microbial N (kg/ DOMR)
Sheep	Hay	30.0 ± 11.71
	Hay with concentrates	30.8 ± 7.13
	Concentrates	26.1 ± 7.46
	Fresh grass or legumes forage	37.8 ± 10.62
	Dried grass or legume forage	49.2 ± 8.24
	Grass silage	19.7 ± 6.80
	Grass silage with concentrates	25.4 ± 4.96
	All	34.3 ± 13.96
Cattle	Hay with concentrates	28.6 ± 7.28
	Concentrates	13.8 ± 4.96
	Grass silage	26.8 ± 3.04
	Grass silage with concentrates	36.0 ± 4.66
	Maize silage with concentrates	45.4 ± 4.21
	All	29.0 ± 9.49
Cattle and sheep	All	32.1 ± 12.62

The *Fermentable Metabolisable Energy* (FME) system was proposed by the AFRC (1993) as a new unit of measurement of energy. Since the microbial protein production depends a great deal on the outflow rate of digesta from the rumen, the AFRC (1993) tables microbial crude protein production at three levels of feeding (L) (as proposed by the ARC (1984) for the measurement of the outflow rates) for the recommended levels of microbial crude protein synthesis (MCP):

All animals at maintenance level of feeding	(L = 1)	9g MCP/MJ of FME
Growing sheep and cattle	(L = 2)	10g MCP/ MJ of FME
Late pregnancy or lactating ewes and lactating dairy cows where L is intake expressed as a multiple of maintenance.	(L = 3)	11g MCP/ MJ of FME

So, the AFRC (1993) proposed the following microbial yield (Table 1.4) according to the level of feeding and with the FME as the unit of energy.

Table 1.4. Microbial protein yield (g/ MJ FME) as a function of level of feeding (AFRC 1993).

Level of feeding (L)	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5
MCP yield (y)	8.8	9.5	10.0	10.5	10.9	11.2	11.5	11.8

These values were derived from the equation:

$$y \text{ (g/MJ FME)} = 7.0 + 6.0(1 - e^{-0.35L})$$

As has been shown, there are many different factors affecting the microbial yield, the French PDI system (see INRA, 1989) takes into account only for the most important factors (energy and N supplies, assuming the other requirements are met). The PDI system uses the fermentable organic matter (FOM) as a new parameter in order to make a better estimate of the amount of rumen energy available while still being available for a large set of feeds.

To cover the enormous variety feeding situations, the PDI system included more than 400 different diets originating from many different laboratories (Table 1.5). From their data they estimated an average for the microbial N flow of 23.2 g MN/kg FOM intake or 145 g CP/kg FOM intake.

Table 1.5. Prediction of protein flow to duodenum according to the N (g) intake per kg of fermentable organic matter (FOM). (INRA, 1989).

Laboratories	Diets ¹	N intake FOM g/kg	Duodenal flow (g NAN/kg FOM) Range
Sheep			
Hurley	FG,GS,H	26 to 64	35 to 69
Palmerston	FG,GS	49 to 66	42 to 54
Adelaide	H,FG	30 to 77	20 to 50
Alberta	H,GP	24 to 51	37 to 58
Hannah	MD (H,GS)	22 to 58	23 to 50
Aberdeen	SSD	22 to 52	27 to 44
Theix	MD (H)	24 to 77	30 to 58
Blacktown	MD (H)	22 to 63	33 to 61
Dairy cows			
Wisconsin	MD, H, GS	43 to 66	32 to 73
Rennes	MD (MS)	28 to 67	33 to 60
Lelystadt	MD (30% H)	29 to 60	38 to 60
Copenhagen	MD, SSD	27 to 64	40 to 64
Rostock	MD (MS)	34 to 54	31 to 51
Braunschweig	MD (MS, H)	31 to 45	34 to 47
Kiel	MD (H), FG	25 to 49	31 to 48
Rennes	FG	30 to 82	29 to 51

¹ FG: fresh grass, GP: grass pellets, GS: grass silage, MS: maize silage, H: hay, DG: dehydrated grass, MD: mixed diet (main forages), SSD: semi synthetic diet.

METHODS FOR DETERMINATION OF MICROBIAL PROTEIN FLOW

There are several methods used to determine the microbial supply to the host animal. Most of these methods are based on determination of a microbial marker. They could be internal markers, which is present in the microbial cell as part of the cell structure e.g. ribonucleic acid (RNA) in all microbial cells, diaminopimelic acid (DAPA) present in bacteria, or they could be external markers which are incorporated into the microbial cell structure during its growth e.g. ³⁵S, ¹⁵N, ³²P. In these methods, the proportion of microbial nitrogen in the total nitrogen of duodenal digesta is calculated by comparing the ratio of marker to nitrogen in isolated rumen microbes with the same ratio in the duodenal digesta. Thus, all of these methods require the use of surgically-prepared animals to obtain the digesta samples turning them inappropriate in some circumstances. The principle, advantages and disadvantages of these methods are discussed

briefly. The purine derivative in the urine is described in more details as an alternative of these methods.

External Markers

Inorganic ¹⁵Nitrogen (¹⁵N)

The method is based on the incorporation of nitrogen from ¹⁵N labelled ammonia (Stern and Hoover, 1979). The ¹⁵N incorporation into microbes could be from either (¹⁵NH₄)₂SO₄ (Pilgrim *et al.*, 1970) or ¹⁵NH₄Cl (Mathieson and Milligan, 1971). The method has several advantages, as cited by Broderick and Merchen (1992).

- 1) ¹⁵N is not environmental hazardous because it is a stable isotope.
- 2) Generally, the feedstuffs do not contain ¹⁵N, so it could be dosed intraruminally with ¹⁵N ammonium salts and, in short-term studies, it will label only the microbial N.
- 3) Bacterial N is labelled directly by direct incorporation of ¹⁵NH₃ and protozoa are labelled indirectly by bacterial predation.
- 4) ¹⁵N is relatively inexpensive.

The inconvenience of this method is the tedious task of isolating the N fraction under study prior to analysis using the isotope ratio mass spectrometry (IRMS) (Broderick and Merchen, 1992). Also the cost of the IRMS is very high, so this is for another disadvantage.

³⁵Sulphur (³⁵S)

³⁵S has been the radioisotope most used to distinguish between microbial and dietary protein (Stern and Hoover, 1979). The use of ³⁵S was first proposed by Henderickx (1961). Similar to ¹⁵N, the ³⁵S can be incorporated into microbial biomass by ruminal infusion. ³⁵S is incorporated into bacterial protein via de novo synthesis of cystine and methionine. The ³⁵S also will be incorporated into other sulphur compounds, such as coenzyme A (Broderick and Merchen, 1992). Eventually, ³⁵S will indirectly label protozoal proteins in the same way as with ¹⁵N. A representative sample of rumen microbes must be then isolated to provide the standard ³⁵S : bacterial crude protein (BCP) ratio which to estimate the BCP flow from the rumen.

A possible limitation of the ³⁵S method is the error introduced due to the direct incorporation of dietary sulphur amino acids into the microbial fraction. There is also an assumption of a similar ratio of sulphur amino acids to total protein for microbial and dietary material (Nikolic, 1977

cited by Stern and Hoover, 1979). Although the radioactivity emissions are low, and ^{35}S has a relatively short half-life (87 days), the radioactivity accumulates in tissues and milk, thus constraining their use for food (Broderick and Merchen, 1992).

Though the technique has some limitations it also has some definite advantages. Quantitative recovery of the sulphur amino acids is not essential, as the technique is based on a ratio of tracer : BCP. Also, the technique is capable of determining total microbial protein synthesis rather than just bacterial or protozoal protein synthesis as with DAPA and AEP respectively.

$^{32}\text{Phosphorus}$ (^{32}P)

Bucholtz and Bergen (1973) observed that phosphorus uptake and incorporation into microbial phospholipids was highly correlated ($r=.98$) to ruminal protein synthesis thus they used ^{32}P as a tracer for ruminal microbes. However, ^{32}P has similar restriction as ^{35}S , except that its radioactivity represents a higher environmental danger (Broderick and Merchen, 1992).

Internal Markers

2-6-Diaminopimelic Acid (DAPA)

DAPA was used by Weller *et al.* (1958) to estimate the rate of synthesis of bacterial protein. DAPA is an amino acid found in the bacterial cell walls. The advantage of this technique is that DAPA is absent in plant cell walls. The DAPA method consists in estimating the ratio of DAPA : N in the mixed rumen bacteria (sampled from the rumen) and the amount of DAPA in the digesta (sampled from the abomasum or proximal duodenum). From these values the amount of bacterial nitrogen in the digesta can be calculated (Hogan and Weston 1970 cited by Stern 1979). The problem with the method is that DAPA is only found in the cell wall of bacteria and not in the whole cell. This means that the concentration in the total ruminal bacteria protein would vary with growth condition that alter mean cell size (Broderick and Merchen 1992). Larger cells would have a lower ratio of cell wall (proportional to surface area) to protoplasm (proportional to volume) and decrease DAPA : N ratios. Since the accuracy of the method is dependent on a constant DAPA : N ratio among various microbial species, or the maintenance of a constant ratio of microbial species in the rumen (Stern and Hoover, 1979), but it has been shown by Work and Dewey (1953) that the ratio of DAPA : N varies between species of bacteria. This means that for every animal on every diet a reliable estimation of the DAPA : N ratio of the rumen bacteria has to be made. A further problem with DAPA is that substantial amounts of DAPA

can be found in some feed sources (Rahnema and Theurer 1986, Theurer 1982). The authors found that the DAPA : N ratios in common feedstuffs were 18 to 40% of those found in isolated ruminal bacteria. DAPA has also been found in protozoa (Czerkawski 1974, Rahnema and Theurer 1986) partly because there was some bacteria contamination during the isolation of protozoa, but mainly because of the presence of digested bacteria engulfed by protozoa during bacteria predation (Coleman 1975). Broderick and Merchen (1992) said that dead bacteria could remain attached to the food particles, coming out of the rumen, despite catabolism of their protoplasm. Thus the cell walls of the bacteria would arrive at the small intestine, but no bacterial protein, giving in this way an overestimation of the protein supply. To be able to estimate the microbial protein supply with the DAPA method, a measurement of digesta flow is needed.

2-Aminoethylphosphonic Acid (AEP)

AEP is an amino acid that was isolated by Horiguchi and Kandatsu (1959) and that appeared to be found only in protozoa and animal tissue. In protozoa, AEP occurs both in lipids and in protein; in animals it is found mainly in lipids and is almost certainly due to the digestion and absorption of protozoal matter (Kittredge and Roberts 1969; Rhuland 1960, cited by Czerkawski 1974). The AEP is characterised by its P-C bonds that are very resistant to acid hydrolysis. Thus, the AEP can serve as a protozoal marker but, as the DAPA, it has some disadvantages. Horigane and Horiguchi (1990) found that there is a widespread distribution of AEP among ruminal bacteria and the presence of AEP in feedstuffs. They stated that AEP and other aminophosphonic acids are incorporated by ruminal bacteria. Plants can also contain AEP and other aminophosphonic acids taken from soil micro-organisms and some fish meal may contain AEP originated from phytoplankton. Whitelaw *et al.* (1984) also found large amounts of AEP in bacterial samples, in defaunated sheep, and in the animal's feed.

(Stern and Hoover, 1979) have suggested that DAPA and AEP can be utilised together to estimate total microbial protein synthesis in the rumen to get a more approximate result than with DAPA alone.

Ribonucleic Acid (RNA)

The RNA technique relies on the assumption that nearly all dietary RNA is degraded in the rumen (McAllan and Smith, 1973). McAllan and Smith (1973) used the ratio of RNA to total

nitrogen in rumen fluid and rumen microbes to estimate the extent of conversion of dietary nitrogen to bacterial and protozoal nitrogen. However, measuring the digesta flow, total microbial nitrogen leaving the rumen can be determined with the use of RNA (Zinn and Owens, 1986). Since, isolation of nucleic acid from digesta is difficult, lacking in both precision and accuracy, Zinn and Owens (1986) adopted a method of separating the purines and pyrimidines (base components of nucleic acids) by direct hydrolysis with perchloric acid (Marshak and Vogel, 1951). Silver has been shown quantitatively to precipitate free purines (Kerr and Seraidarian 1945). Zinn and Owens (1986) procedure combines this purification procedure with the hydrolysis procedure, removing any interfering components and permits simple and precise quantitation of the nucleic acids.

Since, it is assumed that all RNA found in the rumen is of microbial origin, microbes are also isolated from the rumen to determine the nucleic acid N : total bacterial N ratio. The same parameter is also used in the digesta, so that combining it with the digesta flow, total amount of microbial N can be estimated.

There are some problems that apply to this technique. If dietary nucleic acids are not entirely degraded in the rumen it may overestimate the microbial protein flow. This can be enlarged if dietary protein and nucleic acid turn insoluble by exposure to heat or chemical treatment (Buttery and Cole, 1977). Apart from the dietary nucleic acid, old epithelium cells may also contribute to the non-microbial nucleic acid in the rumen. Another problem with the RNA method is the variability of the RNA : total N ratio of mixed bacteria due to diet and environment (Smith and McAllan, 1974). Limited passage of protozoa from the rumen could influence the determination of microbial synthesis (Zinn and Owens, 1986) underestimating it.

Purine Derivatives:

Topps and Elliott (1965) found in sheep that urinary allantoin excretion was correlated with the ruminal concentration of nucleic acids; so they suggested that allantoin in urine could serve as a useful index of the production of nucleic acids in the rumen. Validation of this model was provided by Chen (1989) who used animals nourished by intragastric infusions of VFA (rumen) and casein (abomasum) with known amounts of purines (as nucleic acids) infused into the abomasum. Chen (1989) concluded that the urinary excretion of the purine derivatives could be used to estimate the microbial protein available to the ruminant.

The principle of the method is simple. Ruminant feeds are usually low in nucleic acid content and, as it was mentioned above, nucleic acids are almost entirely degraded by the rumen microbes. Therefore, nucleic acids entering the small intestine are effectively entirely of microbial origin. Once in the small intestine, the microbial nucleic acids are degraded and absorbed as purine nucleosides and free bases. These two products can be reutilize for the synthesis of tissue nucleic acid and/or metabolised into their derivatives (allantoin, uric acid, xanthine and hypoxanthine) depending on the activities of enzymes involved in purine salvage and purine degradation pathways. These purine derivatives are mainly excreted in urine, and the excretion is directly related to purine absorption. Therefore, if the ratio of purine : microbial N, and the digestibility of purines are known, then microbial N absorbed from the small intestine can be calculated from the amount of purines absorbed, which is estimated from purines derivatives excreted in the urine. However, tissue nucleic acids turnover generates purine derivatives (endogenous purines) and thus contributes to the total excretion in urine. Measurement of the proportion of endogenous excretion has been made with the aid of the technique of intragastric infusion (Chen, 1989), or the technique of replacement of digesta entering the small intestine (Balcells *et al.* 1991).

The advantage of this method is that it is simple and non-invasive. It only requires total urine collection and does not require any surgical preparation of the animal. Determination of digesta flow is also not needed with this method, but is required by all the other methods outlined above. The purine derivative (PD) method avoids the implication of having to measure digesta flow, and thus overcomes many of the disadvantages of the other methods.

The PD method has some limitations. A low content of dietary nucleic acids could be true for most of the ruminant diets, but it is not so in the case of fishmeal. Another limitation is that the calculation of microbial N from purine content assumes that the ratio of purine : total N in mixed microbial population is constant. In addition, the equations to relate purine derivative excretion to microbial protein supply as used in this method is species specific, and different models have to be used for different species.

A lot of work has been carried out with sheep. In sheep, it has been demonstrated (Chen, 1989; Chen *et al.* 1990a; Balcells, 1991) that the endogenous contribution of purine derivatives to total excretion of purines derivatives is not constant as it is in cattle (Verbic *et al.* 1990). Chen *et al.* (1990a) indicated that when no endogenous purines were provided, purines lost by tissue

turnover are completely replaced by *de novo* synthesis. However, when the animal was given an increasing exogenous supply, biosynthesis was gradually replaced by utilisation (salvage) of exogenous purines, and became completely inhibited with abundant supply of exogenous purines. Integrating all of these concepts, Chen *et al.* (1990a) quantified a relationship between the excretion of purine derivatives (Y)(mmol/day) and absorption of exogenous purines (X)(mmol/day). Thus, the following equation was obtained:

$$Y = 0.84X + 0.150 W^{0.75} e^{-0.25X}$$

where Y is the urinary excretion of total purine derivatives of both endogenous and exogenous origin (mmol/d); X is absorbed exogenous purine (mmol/d); 0.84 (SE 0.007) represents the proportion of absorbed plasma purine derivatives excreted in the urine; 0.150 is the endogenous derivative excretion (mmol/kg $W^{0.75}$ per day) measured when the animal had no supply of exogenous purine; 0.25 (SE 0.044), is a constant defining the rate of replacement of *de novo* synthesis of purines by exogenous purines.

Balcells *et al.* (1991) using the technique of full replacement of duodenal digesta flow arrived to a similar equation:

$$Y = 0.87X + 0.210 W^{0.75} e^{-0.14X} \quad (\text{residual SD} = 1.15)$$

where Y is the excretion of total purine derivatives (mmol/d); X represents the absorbed purines (mmol/d); 0.14 (SE 0.056) is a constant defining the rate of replacement of *de novo* synthesis of purines by exogenous purines.

MEASUREMENT OF DIGESTA FLOW

Determination of digesta flow is required in several of the above methods for estimation of microbial protein supply. There are several methods for digesta flow measurement which they are briefly discussed here.

Digesta flow can be considered in terms of velocity, flow rate or rate of passage (Warner, 1981). Velocity (distance per time) is applicable only to tubular segments of the gastro intestinal (GI) tract, where it provides a measure of gut motility; whereas, flow rate refers to the volume of mass of digesta passing a point in the GI tract per unit of time and its measurement in association with

particular analyses allows estimates to be made of the extent of digestion, absorption and/or secretion occurring in defined segments of the tract (Faichney, 1993).

Measurement of digesta flow requires some degree of surgical preparation of the animal; although, flow through the total tract can be estimated from the recovery of markers in the faeces and the use of mathematical markers (e.g. Grovum 1973). However digesta flow from specific compartments (e.g. from the rumen to the abomasum or the duodenum) and to measure the flow of digesta components, then cannulation of the GI tract is required, so that samples can be taken for analysis. Most of the studies of digesta flow involve either simple cannulae or re-entrant cannulae

Re-entrant cannulae divert digesta flow outside the body and allow direct measurement by total collection (Mac Rae, 1975). Collection procedures which involve the diversion, sampling and return of digesta tend to affect digesta flow. Partial collection needing the use of an indigestible marker whose recovery can be used to calculate the flow rate can be used, but the re-entrant cannula can still affect digesta flow (Faichney, 1993).

Simple cannulae are normally 'T' shaped in which diversion of the digesta is not complete as with the re-entrant cannula. Most of the digesta flows normally through the base of the cannula while some it is diverted to the outside enabling the sampling of the digesta. The part of the digesta diverted to the outside is not always a representative sample of it. Thus, when animals are prepared with simple 'T' cannulae in the small intestine, indigestible markers are also required to measure digesta flow at the point of cannulation. There is a wide variety of markers that can be used which have to be selected as far as possible according to the criteria of an 'ideal marker'. Namely:

1. It must be indigestible: meaning that it should not change its own composition and it should not be absorbed.
2. It has to be able to mix well with the digesta
3. It has to behave like digesta
4. It should not to change the digesta characteristics: it should not increase or decrease the digestibility of the feed; it should not alter the flow dynamics and it should not interact with the digesta so as to affect the measurement of either the marker or the materials in the digesta.
5. Has to be easily measured

6. Has to be easily dosed
7. It has to be reasonably cheap and easily obtained
8. Its use must not have environmental consequences.

There are two general types of markers: internal markers and external markers. Internal markers are components of the feedstuff which are indigestible, whereas external markers are inert compounds added to the feedstuff. Markers can be also classify according to the phase (liquid or solid) of the digesta they label. To decide which kind of markers to employ it has to be compare to the characteristics of an ideal marker cited above. Here are some examples of some commonly used markers.

Liquid Phase Markers

Polyethylene Glycol (PEG)

PEG is a liquid and an external marker. It was first introduced by Sperber, Hyden and Ekman (1953, cited by Downes and McDonald, 1964). They found that the polymer with mean molecular weight of 4000 was not degraded in the gut or absorbed.

A full mathematical treatment of the principles involved in the use of soluble markers to measure the rate of flow and volume of rumen fluid was given by Hyden (1961, cited by Downes and McDonald, 1964) and has indicated some limitation of the method. He found that PEG gave a satisfactory estimate of rumen fluid volume (where PEG space was approximately 95% of the water in the rumen). In experiments of long duration, the flow rate could be calculated from the rate of fall in concentration of the marker; and in short-term experiments, the flow could be estimated from the amount of reference substance that had disappeared from the rumen during the experiment and the mean concentration of the marker in the rumen fluid.

However, a serious limitation in the use of PEG is the lack of a specific, sensitive and accurate method of analysis. Downes and McDonald (1964) reported that the analysis for PEG is non-specific, and it is known that substances in the gut interfere and that appreciable errors occur even under good analytical conditions. They added that it is not practicable to estimate PEG in low concentrations, hence one is obliged to use a large dose or to run an experiment for a short period of time. The fact that PEG is precipitated by certain feeds high in tannins (Kay, 1969) and can bind to particulate matter (Sutherland *et al.*, 1962 cited by Teeter and Owens, 1983) can be another important disadvantage.

¹⁵Cr-Ethylenediamine tetraacetic acid (¹⁵Cr-EDTA)

¹⁵Cr-EDTA is also a soluble and external marker. Unlike PEG, its radioactivity is selected as a mean for sensitive and completely specific analysis. Downes and McDonald (1964) concluded that the very important advantage of ¹⁵Cr-EDTA is that the analytical procedure is quite specific and highly accurate over wide range of concentrations. As ⁵¹Cr emits γ -rays (0.323 MeV) it can be counted easily in a scintillation counter with very simple preparation of the specimens for analysis.

One disadvantages they found of ⁵¹Cr-EDTA is that it is absorbed slightly and subsequently excreted in the urine but appropriate correction could be made for this. Another disadvantage could be attributed to the fact that it is radioactive although ¹⁵Cr is considered to be one of the less hazardous isotopes according to the International Atomic Energy Agency (1959, cited by Downes and McDonald, 1964) considering that it has a half-life of 27.8 days.

Solid Phase Markers

Lignin

An internal marker (a plant constituent) would be the most convenient, and presumable accurate, marker, especially for grazing animals. Lignin is often regarded as being indigestible but it was shown by Csonka *et al.* (1929) that lignin digested resulting from enzyme action in the stomach. There have been many published works on lignin, and its digestibility varies within a wide range. Hale *et al.* (1940) also found that digestibility of lignin varied from 5.1 to 23.7% and that none of the digestion took place in the rumen. Furthermore, Galyean *et al.* (1979) found that total tract digestion of lignin was approximately 52% when steers were fed 84% corn diets. Porter and Singleton (1971) found that as much as 10.2% of the dietary lignin disappeared from the alimentary tract and concluded that the digestion of lignin was mainly in the stomach.

The fact that lignin is not completely indigestible makes it questionable to use it as a marker. It is not only the digestibility the problem but also the analytical method used to estimate its concentration. Fahey and Jung (1983) found that using the gravimetric method (acid detergent lignin and permanganate lignin) two-thirds of the total lignin disappearance occurred in the rumen, whereas approximately one-third of total lignin disappearance occurred in the lower digestive tract. When lignin was determined by spectrophotometer (acetyl bromide soluble lignin

method), just the reverse occurred (38% of the potentially digestible lignin disappeared in the rumen, 62% in the lower digestive tract). Fahey *et al.* (1979) explained that the discrepancy could be due to the variation in the extent of solubilization of different plant lignin in acetyl bromide. Fahey and Jung (1983) concluded that choice of analytical method and extent of recovery may drastically alter interpretation of digesta flow measurements calculated using lignin as a reference.

Ytterbium

Ytterbium (Yb) is a rare earth element and rare earth have been used to label particulate matter directly because they possess adsorptive properties (Kyker, 1962). Marker for particulate matter should mimic the flow characteristics of the particulate matter . Adsorption of markers by particulate matter permits direct measurement of particle behaviour, assuming that the marker does not influence digestion and passage.

Teeter *et al.* (1984) showed that Yb has a good affinity of binding to the feed. They demonstrated that adsorbing increased as the concentration of Yb increased but it reached to a plateau as the binding sites were saturated. If administration of Yb continued after saturation of these binding sites, migration of Yb to other feed or ruminal components could happened.

The adherence of the marker to the feed is important as well as its strength to bind to it. Teeter *et al.* (1984) utilising water dialysis to wash the feed samples showed that feed-Yb complexes dissociated with 0.1 to 0.6% of bound Yb being released each hour. They expected similar dissociation in the rumen to occur, though the rate of exchange of water with the dialysis was more severe (0.47/h), thus migration of a fraction of the marker would be expected. To study this migration *in vivo* Teeter *et al.* (1984) used nylon bags containing a feed with high binding affinity and capacity for Yb (prairie hay) and fed animals with Yb-labelled corn. They found that 0.18% of the Yb in the corn had migrated to the hay 96h after dosing.

These workers also found that the affinity of soluble organic compounds (glycine, lysine, glucose, sucrose, lactate and acetate) for Yb also affect the binding of Yb to the feed particles. If organic compounds have low affinity for Yb, Yb can bind with the added particulate matter. Affinity of solute for Yb and a low rate of Yb release from the soluble compound will decrease the quantity of Yb available to bind with added feeds.

Addition of Yb to feed components decreased *in vitro* and *in situ* disappearance of dry matter components that could have been caused by antibacterial activity of Yb (Teeter *et al.*, 1984). The effect of Yb over the feedstuff digestibility does not agree with criterion iv.

Chromic Oxide (Cr₂O₃)

Cr₂O₃ is an external solid phase marker. It is recovered quantitatively in the faeces enabling its use as a marker for digestion studies. However, the physical properties of Cr₂O₃ have little correspondence to those of the solid digesta fraction.) The relative density of Cr₂O₃ is considerably greater than that of digesta and Faichney (1972) considers likely that Cr₂O₃ would then to move largely adjacent to the gut mucosa. Thus the concentration of Cr₂O₃ in a sample taken from a simple cannula would depend on the site of the cannula, the viscosity of digesta and the rate at which digesta flow from the cannula. This difference in physical property of the Cr₂O₃ makes it overestimate the digesta flow (Drennan, Holmes and Garret, 1970, cited by Faichney, 1972). Faichney (1972) concluded that Cr₂O₃ is not satisfactory as a single marker for studies of digesta flow when digesta samples are taken from a simple cannula. He added that Cr₂O₃ cannot be used in association with a soluble marker to allow corrections to be made for sampling errors because it behaves independently of the particulate matter in the digesta.

Cr-mordanted fibre

Another way of utilising Cr as a marker is by mordanting it with fibre. Before adding Cr to the fibre, the fibre has to be washed thoroughly to eliminate the soluble part of the fibre that could associate with the Cr. Uden *et al.* (1980) found a recovery of Cr ranging from 96-99% and the recovery from these mordants increased with increasing levels of mordanted Cr added to the fibre. In an *in vivo* trial they found a faecal recovery of Cr of 99.5%. These made them conclude that Cr-mordanted fibre fulfilled most criteria as a particulate marker. However, the treatment renders the fibres undegradable in the rumen and overestimates the mean retention time since the fibres are broken down to smaller particles only by physical action (Elamin-Eliman, 1983).

Plant Wax Alkanes

In 1934 Chibnall *et al.* (cited in Dove and Mayes, 1991) demonstrated the presence of *n*-alkanes in the cuticular wax of plants. Dove and Mayes (1991) cited several features of these alkanes.

1. The carbon-chain lengths of the main alkanes detected (by gas-liquid chromatography) are usually in the range C₂₅ (pentacosane) to C₃₅ (pentatriacontane). Shorter chain-length alkanes can be detected but are usually present in much smaller quantities.
2. In most plants, the alkanes are present with odd-numbered carbon chains in much greater amounts than the even-numbered alkanes.
3. While C₂₉ (nonacosane), C₃₁ (hentriacontane) and C₃₃ (trtriacontane) alkanes are the dominant alkanes in all species, there are marked species differences and between species in the levels and patterns of alkanes.

Grace and Body (1981, cited by Dove and Mayes, 1991) showed that the cuticular long-chain (C₁₉-C₃₂) fatty acids in herbage were quantitatively recovered in faeces, and they suggested that these compounds could be used as indigestible internal markers. Mayes and colleagues (Mayes and Lamb, 1984; Mayes *et al.*, 1986a, 1986b, 1986c; Mayes *et al.*, 1988 cited by Dove and Mayes, 1991), following this work, suggested that the plant cuticular alkanes could be used as internal markers to estimate digestibility.

Furthermore, the natural alkanes, with their strong association with the digesta solids and quantitative duodenal recovery, appears to be also an appropriate and useful markers for studying the kinetics of particulate digesta flow (Dove and Mayes, 1991). As mentioned previously, for estimating digesta flow rates at different sites along the digestive tract, the marker must be completely recoverable and show a marked affinity for either the solid or liquid phase. The close association of the natural alkanes with digesta solids suggests they could be used as solid-phase markers. Their quantitative recovery at the duodenum (Mayes *et al.*, 1988) (Table 1.6) shows their potential as a digesta flow marker.

Table 1.6. Recoveries of *n*-alkanes at the duodenum and terminal ileum in sheep (Mayes *et al.*, 1988).

Alkane	Recovery (%)			
	Duodenum		Terminal Ileum	
	Mean	S.E	Mean	S.E
C ₂₅	112.8	4.09	51.9	2.71
C ₂₇	103.7	3.87	62.6	2.50
C ₂₉	99.7	3.54	74.5	2.24
C ₃₁	96.5	3.40	81.5	2.14
C ₃₃	98.8	3.48	87.5	2.09
C ₃₅	101.3	3.52	97.7	2.19

With these results Mayes *et al.* (1988) demonstrated that the disappearance of alkanes was mainly due to the absorption in the small intestine and that the rumen microflora appear to be incapable of synthesising long-chain alkanes. It means that the recovery of the alkanes in the duodenum is almost complete as it can be seen.

Ruthenium (Ru)

The use of Ru was proposed by Tan *et al.* (1971) as an alternative marker to lignin for studies of digestion in sheep. Ru was dosed as complex of (Ru-phenanthroline) into the rumen of sheep and they reported that there was not any significant absorption of Ru in the digestive tract (93-102% administered into the rumen was recovered from the faeces and 0.4% or less was recovered from the urine). The adsorption of Ru onto food particles was very fast, the quantity adsorbed in 1 hr was 90% or more of that adsorbed over 24 hr. Although Ru was found inappropriate for marking the feed they concluded that, with intraruminal infusion, Ru is a satisfactory marker for digestion studies in sheep and the fact that it is simple to prepare made it even more attractive.

Using Ru as a radioactive isotope (^{103}Ru) makes the measurement more rapid and accurate. The isotope is relatively cheap; its half-life of 40 days is long enough to enable several studies to be carried out with a single batch of the ^{103}Ru complex, but short enough to permit safe disposal of samples after storage. The level of dosing need not to be high, thus does not affect the rumen microbial population (Tan *et al.* 1971).

Furthermore, considering the good adsorption of the marker by the particulate fraction of the digesta, together with the radiation characteristics of ^{103}Ru , they suggested that the marker may be used in combination with ^{51}Cr -EDTA in studies of digestion in the stomach of sheep.

Dual Phase Markers

Hogan and Weston (1967, cited by Faichney 1972,1980) pointed out that it is difficult to obtain samples of digesta containing not only particulate matter but also dissolved substances in the same proportions as are present in the digesta leaving the abomasum or flowing through the duodenum. Any single marker (solid or liquid) may not be present in a sample of digesta in the same concentration as in the digesta as a whole and is therefore unlikely to provide a valid estimate of the flow of digesta. They suggested that the problem can be overcome by using two markers, one of which remains in solution while the other is intimately associated with the

particulate matter, so that corrections can be made for sampling errors. The problem of these ‘ two-marker ’ method is that it requires that the markers used be ideal, i.e. they must associate exclusively with the solid or liquid phase (Faichney, 1980). Thus, Faichney (1975) suggested the use of a ‘ double-marker ’ (dual-phase marker) method in which it does not require that the markers associate exclusively with one of the phases. The method consists in knowing the ratio liquid to solid marker infused and correcting to the same ratio when sampled. The method requires a steady-state conditions, though, established by feeding continuously or frequently at fixed intervals. Theoretically, this method does not apply when only one or two meals are given, although Faichney (1980) demonstrated that the overestimation of the flow using the ‘ double-marker ’ method with animals given one meal per day was so small that it suits for practical purposes. The markers that are normally used for this method are the $^{51}\text{Cr-EDTA}$ and ^{103}Ru (as complex) as a liquid and solid phase marker respectively.

2. OBJECTIVES OF THE PRESENT WORK

The main objective of the present work was to compare the method for the estimation of microbial N flow based on purine derivative excretion in the urine with the different method based on digesta flow and microbial marker measurements in sheep. RNA was chosen as the microbial marker in the latter method, because the purine derivative method can be considered as an extension of the RNA method. The animals were given different levels of feed intake in order to create a range of microbial N production. The work is described as Experimental 2.

Taking in consideration that the RNA method requires digesta flow measurements from the rumen to the duodenum, a digesta flow marker had to be employed. It was an advantage to use alkanes naturally present in feed as the digesta flow marker since no dosing of external markers was required. A second objective of this work therefore was to validate the use of natural alkanes present in the feed as digesta flow marker. This method was compared with a commonly-used double-marker method ($^{51}\text{Cr-EDTA}$ and $^{103}\text{Ru-complex}$ as liquid and solid markers respectively). This work was carried out before the main experiment and is described as Experiment 1.

3. MATERIALS AND METHODS

EXPERIMENT 1: VALIDATION OF ALKANES AS A DIGESTA FLOW MARKER

Animals and management

Three sheep were fitted with duodenal and ileal cannulae. They were kept in metabolic cages and fed hourly with continuous feeders. They had access to water at all times. The sheep were fed with Lucerne at two different levels of intake e.g. 800 and 1200 g/day. Digesta flow measurements were made by Double Marker Technique and by alkanes as feed marker.

Measurement of digesta flow

Double Marker Technique for measurement of digesta flow

The procedure described by Faichney (1975) was used. A mixture of the isotopes ^{51}Cr (as EDTA) and ^{103}Ru (as the Ruthenium phenanthroline complex) was dosed at the rate of approximately 3×10^6 cpm of each isotope per day. The tracers were sprayed on the feed which was offered at hourly intervals. Prior to the hourly dosing, the animals were given a 'boost' dose equivalent to one day's dose given in one-hour feed portion, in order to build up the tracer concentration quickly.

Sampling of digesta started on the fourth day of dosing. Sampling was carried out via plastic T-piece cannulae at the ileum and duodenum on alternate days for a total of 4 days. About 250 ml of sample was collected over the course of the day, and frozen until ready for counting of radioactivity.

Two ml of digesta were pipetted, in triplicate, into tared counting tubes and weighed. The sampling was carried out with the contents continuously stirred. About 30-40 ml of digesta was also centrifuged to obtain the supernatant (45 min. at 13,920 relative centrifuge force) and 2 ml (in triplicate) weighed into counting tubes.

Counting was carried out in a χ -counter (Canberra-Packard Cobra) for 10 min per sample. Background samples and samples of dosing solution were also counted along with tubes containing ^{51}Cr and ^{103}Ru separately to check for spill-up of ^{51}Cr (400-700 keV) into the ^{103}Ru region (240-400 keV) and spill-down from the ^{103}Ru into the ^{51}Cr region. All counts and weights

were then entered into a spreadsheet to calculate the total digesta flow. The method of calculation was as described by Faichney (1975).

n-Alkanes for measurement of digesta flow

Dosing of alkanes was not needed since internal alkanes from the feed were used. The digesta samples obtained as described above were used. The samples were freeze-dried prior to the analysis.

The n-alkane contents of digesta and feed samples were determined as follows. About 0.5g of dried, ground samples were accurately weighed, in duplicate, into 120mm x 20mm borosilicate glass culture tubes. A solution of C₃₄ (0.8 mg/g) was added by weight (about 0.22 g) to each tube as an internal standard, followed by 7 ml ethanolic KOH (1M). The tubes were capped and heated for 16h at 90°C in a dry-block heater allowing the samples to saponify. After partial cooling (to 50-60°C) 7 ml heptane was added and the tube was capped and shaken gently. Water (2 ml) was added and the tube was restoppered and shaken vigorously. After separation into two liquid layers the top (non-aqueous) layer was transferred to a scintillation vial with a polyethylene Pasteur pipette. Another 7 ml heptane was added to the tube and the extraction repeated, adding the top later to the same scintillation vial. The solution in the vial was evaporated to dryness on a dry-block heater fitted with a sample concentrator blowing air into the vial. The extracts was re-dissolved in 1.5 ml heptane, with warming, and applied to a small column containing silica gel (Keisegel 60, 70-230 mesh) with a bed volume of 5 ml. The hydrocarbons were eluted into a second scintillation vial by the addition of 12 ml n-heptane to the column. After evaporation the dryness the hydrocarbons were re-dissolved in 0.4 ml heptane and, after warming, transferred to an autosampler vial for analysis by gas chromatography. The feed samples were treated in a similar manner to the digesta with the exception that larger sample size (1.5 g), and greater quantities of liquid reagents (10 ml ethanolic KOH, 3 ml water and 2 x 10 ml heptane) were used.

EXPERIMENT 2: MEASUREMENT OF INTESTINAL FLOW OF MICROBIAL N USING METHODS BASED ON RNA AND PURINE DERIVATIVES

Animals

Five Blackface castrated male sheep (body weight 38-47 kg) were used. They were all of approximately one year of age. Two had been fitted with jejunal and ileal cannulae, and three with duodenal and ileal cannulae. Cannulae were plastic T-piece (Sheilgreen Services, 2 Private Road, Gorebridge, Midlothian EH234 HG) and of about 12 mm internal diameter. The duodenal cannulae were located approximately 6- 10 cm distally from the pylorus. The ileal cannulae was located in the terminal ileum.

Housing and Management

Prior to the experiment, the animals were kept in individual pens. They were fed twice daily at 08:00 and 16:00 h with the same amount of feed as that to be given in period 1 of the experiment. Three days before the experiment, they were transferred to metabolism cages. There the animals were then fed hourly with continuous feeders. The daily feed allowance was thus divided into 24 equal meals. Fresh water was freely available at all times. The animals were kept in the cages until the end of the experiment. The metabolism cages were equipped with urine and faeces separators.

Diet

All of the animals were fed with pelleted grass-nuts (primarily of Rye grass). The approximate analysis of the grass-nut diet is shown in Table 3.1:

Table 3.1. Approximate analysis of the grass pellets used in the experiment.

Dry matter (DM)	929 g/kg
Organic matter	903 g/kg DM
Nitrogen	2.67 g/kg DM

Treatments and Design

Three levels of feeding were imposed: 600, 1000 and 1400g/d (air dry). The diets were allocated to the 5 animals according to two 3 x 3 Latin Square designs (the second of which was incomplete) (see Table 3.2):

Table 3.2. Allocation of treatments to sheep.

<u>Sheep</u>			<u>Sheep</u>	
2571	3126	2573	3124	3125

Period I	600	1000	1400	600	1000
Period II	1000	1400	600	1400	600
Period III	1400	600	1000	1000	1400

Each period was of 19 days. The first 10 days of each period were to allow the animals to adjust to the level of intake. During days 11-19, samples were collected as detailed below. In the first week of the third period Sheep 2571 was withdrawn from the experiment due to some problems with the ileal cannula. Sheep 3124 & 3125 were not able to complete period II & III due to shortage of time within this project.

Sample collection

Urine

Total urine was collected during days 11-15. The urine was collected into plastic pans containing about 300ml acetic acid to ensure a final pH of between 3 and 4 to prevent bacterial destruction of the purine derivatives in the urine. Each days collection of urine was and diluted with water and made up to about 4 kg (if the volume of the undiluted urine was less than 4 kg) and weighed. This dilution was made to prevent precipitation of uric acid in the urine during storage. The diluted urine was mixed thoroughly and filtered through glass wool. Subsamples (about 40 ml) were then taken and stored at -20°C for subsequent analyses of purine derivatives.

Faeces

Faeces were collected at the same time as the urine. The daily faecal collection was put in plastic bags and weighed. The bags, with faeces, were labelled and stored at 5°C. At the end of each period faeces collected from each sheep over the 5-day period were bulked, mixed thoroughly and subsampled for DM, OM and N analysis.

Digesta

Digesta samples were taken after the last excreta sample of each period (i.e. on days 16-19). During the first 2 days (16 and 17) ileal samples were obtained. The next 2 days (18 and 19), samples were taken from the duodenum (or jejunal). About 250 ml of samples were obtained during the day by attaching plastic bags (18 x 13cm) at the mouth of the cannulae. It took nearly 8 h to collect enough ileal digesta whereas it only took 1-2 h to collect the duodenal digesta.

Sampling was thus done only once a day. The two-day samples were bulked and freeze-dried for subsequent analysis of alkanes and RNA.

Rumen microbes

Samples of mixed rumen microbes were required. Since the animals were not fitted with rumen cannula for sampling of rumen digesta, the samples were obtained at end of experiment when the sheep were slaughtered. About 2 L of rumen digesta were transferred into thermal flasks immediately after the sheep were slaughtered. The samples were then strained with 4 layers of surgical gauze to remove the large feed particles. The strained liquid was put into a beaker which was kept in an incubator at 39 °C for 1 h to allow the light feed particles to rise to the surface. The floating feed particles were then removed using a vacuum pump. The remaining liquid was centrifuged and its supernatant removed. The pellet was washed with a buffer solution and centrifuged again. The remaining pellet were examined under a microscope to check the presence of feed particles. The process of centrifugation was done about 4 to 5 times until the feed particles were essentially removed. The sample was then freeze-dried. They were then stored as powder for subsequent N and RNA analysis.

Determination of MN flow based on RNA method

RNA contents of microbial isolates and digesta samples were determined according to the method of Zinn and Owens (1985). Samples of isolated rumen microbes or digesta were hydrolysed with perchloric acid to liberate the purines and pyrimidines. Silver nitrate was then added to the samples and stored in the refrigerator overnight to allow precipitation of the purines. Then the samples were centrifuged and the supernatant was removed. Centrifugation was done several times to make sure that the precipitated pellets contained only purines. The purines were then taken back into solution with 0.5 N hydrochloric acid. The absorbance of the solution was then read at 260 nm with a spectrophotometer (Unicam 8625, UV/VIS Spectrometer, Unicam Limited, Cambridge-UK). Yeast RNA containing known quantities of purines was used as a standard. The absorbance of the microbial samples were then compared with the standards. To determine the RNA content of the samples (microbes and digesta) the optical density (OD) of each sample was read in the spectrophotometer and related to the yeast RNA to obtain the quantity of RNA in the sample.

The ratio of RNA : N in the rumen isolated microbes and in the digesta were measured. the intestinal flow of microbial N (g/d) was calculated according to the following equation:

$$MN = \frac{N \times R_2}{R_1} \times F$$

where N is the nitrogen content in the digesta (g N/g DM); R₂ represents the N : RNA ratio in the digesta; R₁ is the N: RNA ratio in the isolated rumen microbes; and F (g DM/d) represents the digesta flow.

To measure the digesta flow natural alkanes in the feeds were utilised as markers. The alkane used was the C₃₃. Although it was not the most abundant alkane in the feed, it was present in considerable amounts (see Table 3.3) and was less subject to absorption due to its relatively long chain-length.

Table 3.3. Alkanes concentration in feed (mg/g DM)

	Alkanes (mg/g DM)					
	C ₂₅	C ₂₇	C ₂₉	C ₃₁	C ₃₃	C ₃₅
Feed	26.65	47.97	174.42	238.48	86.81	8.82

Determination of MN flow based on Purine Derivatives method

The daily output of PD (including allantoin, uric acid, xanthine and hypoxanthine) in the urine was determined. Chemical analysis of PD is described later.

The amount of exogenous purines absorbed by the animals was then estimated from the daily excretion of PD, based on the following equation which relates the absorption of microbial purines (X mmol/d) with the excretion of PD in urine (Y mmol/d):

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

where W^{0.75} represents the metabolic body weight (kg) of the animal.

The calculation of X from Y, based on the equation, was done by means of the Newton-Raphson iteration process in an Excel spreadsheet program. Having calculated X, the microbial N flow was estimated. The following factors were used for the calculation of intestinal flow of microbial N (g/d) from the microbial purines absorbed (X mmol/d):

- 1) Digestibility of microbial purines between the duodenum and ileum is assumed to be 0.83.
- 2) The N content of purines is 70 mg N/mmol.

3) The ratio of purine N : total N in mixed rumen microbes is taken as 11.6 : 100

$$\text{Microbial N} = \frac{X \times 70}{0.116 \times 0.83 \times 1000} = 0.727X$$

Allantoin was measured using the high-performance liquid chromatography (HPLC) and pre-column derivatization. The description of this method is given by Chen *et al.* (1993). In the derivatization procedure, allantoin is converted to glyoxylic acid which forms a hydrazone with 2,4-dinitrophenylhydrazine. The derivative was measured by HPLC using a reversed-phase C₁₈ column. Detection wavelength was 360 nm. Determination of uric acid, xanthine and hypoxanthine was determined using the phosphotungstic acid method as described by Chen *et al.* (1990b). In this method; xanthine and hypoxanthine were converted with xanthine oxidase to uric acid and thus determined as uric acid.

Chemical analysis

Dry matter was determined by drying the samples in an oven at 100°C for 48 h. The organic matter was determined by ashing the samples in a furnace at about 550°C overnight.

Nitrogen was determined by the Macro Nitrogen Analyser (Foss Electric UK Ltd., Millfield Lane, Ind. Estate, Wheldrake, York). The analysis involves oxidative combustion of the sample at >1000°C which converts the nitrogen in the sample to N₂ gas this being measured by thermal conductivity.

4. RESULTS

EXPERIMENT 1

Digesta flow by the Double-marker method

The results of digesta flow measurements calculated with the double-marker technique are shown in Table 4.1.

Table 4.1. Measurement of digesta flow using ^{103}Ru -complex and ^{51}Cr -EDTA.

Sample Type	Intake (g DM/d)	Digesta Flow (g DM/d)
Duodenal	800	490
Duodenal	800	450
Duodenal	1200	720
Duodenal	1200	740
Duodenal	1200	670
Ileal	800	290
Ileal	800	330
Ileal	1200	500
Ileal	1200	500
Ileal	1200	470

Digesta flow by the Alkane method

The concentration of alkanes found in the feed and in the digesta are given in Table 4.2. The natural alkanes utilised for determination of digesta flow were C₂₉ and C₃₁, since they were found in greater quantities in the feed than the rest of the natural alkanes.

Table 4.2. Concentration (mg/kg DM) in the major alkanes in digesta and feed samples.

Sample type	Intake (g DM/d)	Alkane (mg/kg DM)		
		C ₂₉	C ₃₁	C ₃₃
Duodenal	800	177	228	76.8
Duodenal	800	185	238	79.7
Duodenal	1200	174	228	78.5
Duodenal	1200	183	236	78.8
Duodenal	1200	182	235	78.6
Ileal	800	183	274	103.3
Ileal	800	214	294	103.0
Ileal	1200	185	267	97.7
Ileal	1200	194	277	100.9
Ileal	1200	205	299	109.7
Feed		107	139	47.3

Digesta flow rate (g DM/d) was calculated according to the following equation:

$$\text{Digesta Flow} = \frac{I \times A_F}{A_D}$$

where: I is the intake (g DM/d); A_F is the concentration of alkanes in the feed (mg /kg DM); and A_D is the concentration of alkanes in the digesta (mg /kg DM).

Since there is some absorption of the alkanes from the duodenum to the ileal and its rate of absorption is determined by the alkanes chain length, a correction factor (CF) has to be utilise in the digesta flow calculation depending on the number of carbons of the chain. The values of CF used for C_{29} , C_{31} and C_{33} were 0.745, 0.815 and 0.875 respectively (Mayes, 1989) respectively. Thus, to calculate the digesta flow for the ileal section is then:

$$\text{Digesta Flow} = \left(\frac{I \times A_F}{A_D} \right) \times CF$$

The digesta flow measurements estimated using C_{29} , C_{31} and C_{33} respectively are given in Table 4.3. The estimates from the 3 different alkanes were consistent.

Table 4.3. Comparison of double-marker technique with the natural alkanes in the measurement of digesta flow.

Sample Type	Intake (g DM/d)	Digesta Flow (g DM/d)			Average
		C_{29}	C_{31}	C_{33}	
Duodenal	800	487	487	493	489
Duodenal	800	465	467	475	469
Duodenal	1200	742	732	723	732
Duodenal	1200	706	707	720	711
Duodenal	1200	709	711	722	714
Ileal	800	350	331	321	334
Ileal	800	299	308	321	310
Ileal	1200	518	509	509	512
Ileal	1200	496	491	492	493
Ileal	1200	468	455	453	459

Comparison of the methods

The digesta flow data based on the double-marker method (Y g DM/d) was linearly correlated with those based on the alkane method (X g DM/d). The following equations show the correlation equations for estimates based on C₂₉, C₃₁ and C₃₃ respectively.

$$\text{Based on C}_{29}: Y = -2.95 (\text{se } 37.27) + 0.99 (\text{se } 0.07) X \quad r^2 = 0.96 \quad n = 10$$

$$\text{Based on C}_{31}: Y = -0.24 (\text{se } 31.48) + 0.99 (\text{se } 0.06) X \quad r^2 = 0.97 \quad n = 10$$

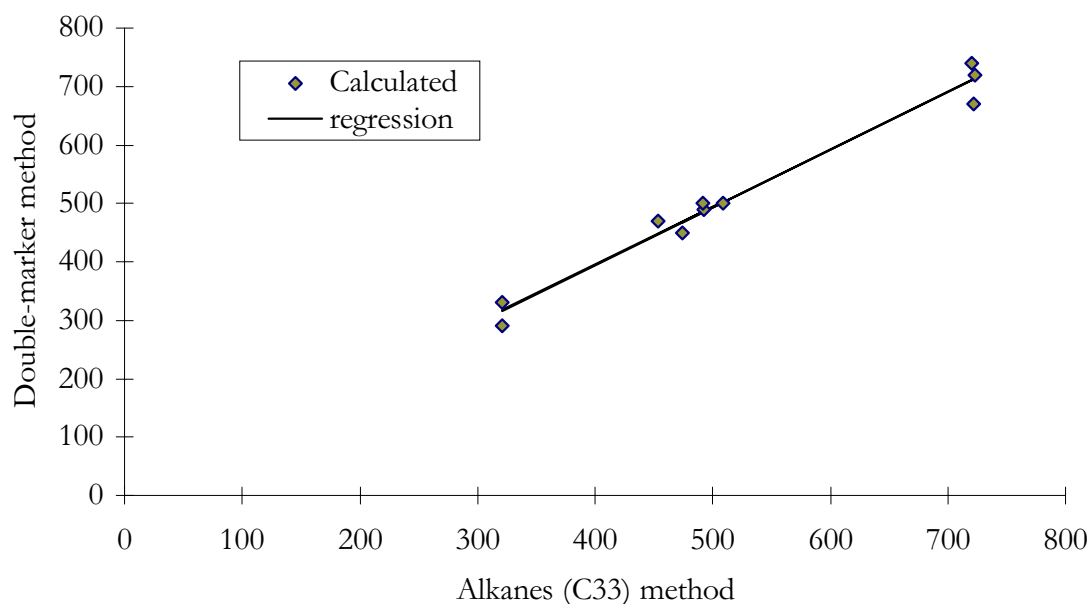
$$\text{Based on C}_{33}: Y = -0.53 (\text{se } 28.57) + 0.99 (\text{se } 0.05) X \quad r^2 = 0.98 \quad n = 10$$

Based on average of C₂₉, C₃₁ and C₃₃

$$Y = -2.32 (\text{se } 31.46) + 0.99 (\text{se } 0.06) X \quad r^2 = 0.97 \quad n = 10$$

The values of intercept in any of the above equations was not significantly different from zero and the values of slope were all close to 1. Therefore, the digesta flow estimated by any of the C₂₉, C₃₁ and C₃₃ alkanes were in good agreement with that by the double-marker method. By comparing the correlation coefficient, C₃₃ gave the best estimates.

Figure 4.1 Comparison of alkanes (C33) and double-marker technique for estimation of digesta flow



Having validated the alkane method, it was decided to use this method in Experiment 2 in which digesta flow needed to be measured for the estimation of digesta flow using RNA method as a microbial marker.

EXPERIMENT 2

Microbial N supply estimated using the purine derivatives in urine

The results of PD excretion and the estimates of microbial N are tabulated in Table 4.4..

Table 4.4. MN flow (g/d) estimated by the PD (mmol/d) excreted in the urine in relation to the DM intake (g/d).

Sheep	DMI (g/d)	BW (kg)	PD (mmol/d)	Estimated purine absorption (mmol/d)	MN (g/d)
2571	560	42	6.99±0.52	7.91	5.75
2573	560	47	7.00±0.81	7.89	5.73
3124	560	40	7.03±1.28	7.98	5.80
3126	560	40	6.77±0.23	7.64	5.55
2571	930	42	11.61±0.78	13.73	9.98
2573	930	47	13.17±1.03	15.61	11.35
3125	930	38	12.49±0.76	14.80	10.76
3126	930	40	13.03±0.56	15.45	11.23
2573	1300	47	17.10±0.97	20.34	14.79
3126	1300	40	18.38±2.12	21.87	15.90

It can be seen that the PD excretion increased with DMI. There was little variation between animals.

Determination of microbial N supply using the RNA method

The results of the RNA and N contents found in the mixed rumen microbes and in the digesta samples are presented in Table 4.5 and 4.6. There was no clear influences of intake level on the RNA:N ratio of mixed rumen microbes.

It was noted that the RNA concentrations in samples (Table 4.6) taken from the jejunum were considerably lower than those from the duodenum, presumably due to rapid digestion of RNA. The animals with jejunal cannulae were thus not useful to estimate the supply of microbial N to the animal based on the RNA method.

Table 4.5. Concentration of RNA (mg/g DM) and N (mg/g DM) in isolated rumen microbes.

Animal	DMI (g/d)	RNA (mg/g DM)	N (mg/g DM)	RNA:N ratio
3125	560	36.45	60.34	0.60
3126	560	48.78	79.31	0.62

2571	930	54.25	68.09	0.80
2573	930	48.72	67.89	0.72
3124	1300	44.34	63.32	0.70
mean				0.69 ± 0.08

Table 4.6. Concentration of RNA (mg/g DM) and N (mg/g DM) in digesta to estimate MN (g/kg DM).

Sample type	Animal	DMI (g/d)	RNA (mg/g DM)	N (mg/g DM)	RNA:N ratio	MN content (g/kg DM)
Duodenal	3124	560	17.38	43.53	0.40	25.31
Duodenal	3126	560	11.52	40.94	0.28	16.77
Duodenal	3125	930	15.01	37.40	0.40	21.85
Duodenal	3126	930	13.61	30.08	0.45	19.82
Duodenal	3126	1300	11.71	24.88	0.47	17.05
Jejunal	2571	560	6.33	42.11	0.15	9.22
Jejunal	2573	560	4.61	37.24	0.12	6.71
Jejunal	2571	930	5.35	37.01	0.14	7.79
Jejunal	2573	930	4.34	41.13	0.11	6.32
Jejunal	2573	1300	3.77	34.98	0.11	5.49
Ileal	3124	560	1.68	24.44	0.07	2.45
Ileal	3126	560	2.43	23.22	0.10	3.54
Ileal	3125	930	2.56	22.49	0.11	3.73
Ileal	3126	930	3.01	24.70	0.12	4.38
Ileal	2573	1300	2.88	22.01	0.13	4.20
Ileal	3126	1300	2.13	21.90	0.10	3.10

The production of MN was calculated knowing the ratio of RNA : N of the rumen microbes and digesta. To know the actual supply of MN arriving to the duodenum, this value had to be multiply by the digesta flow. The digesta flow was estimated based on C₃₃ alkane in duodenal samples. Results are shown in Table 4.7. Both the digesta flow and the MN flow increased with DMI.

Table 4.7. MN flow (g/d) estimated with the RNA method, and digesta DM flow estimated with natural alkanes in feed.

Animal	DMI (g/d)	Digesta DM flow (g/d)	MN flow (g/d)
3124	560	356.57	9.02
3126	560	388.34	6.51

3125	930	609.53	13.32
3126	930	685.36	13.59
3126	1300	1093.70	18.65

Comparison of the two methods

The data of the animals with jejunal cannulae were not utilised in the comparison of the methods of measuring microbial N supply because of the reason mentioned previously, namely the absorption of RNA between the duodenum and the jejunum. Although there were a limited number of animals to do the comparison of the methods, there was enough data to compare them at the three different levels of intake. The supply of microbial N (g/d) estimated with both methods is plotted in Figure 4.2.

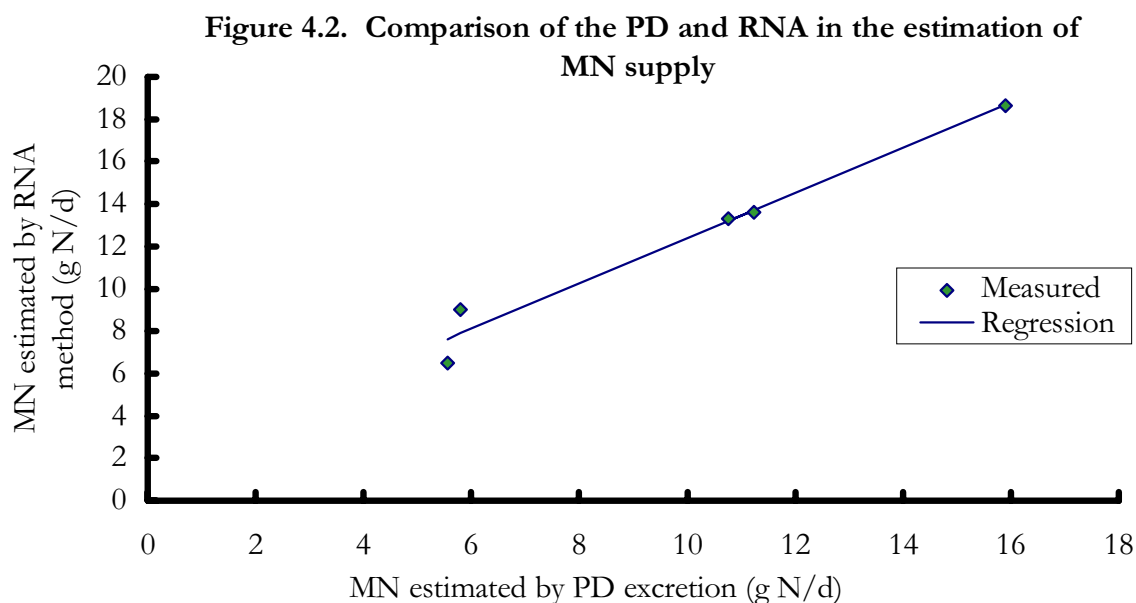


Figure 4.2 shows that results from the two methods were highly correlated. The relationship between PD method (X g N/d) and RNA method (Y g N/d) was derived from five observations and is described by the following equation:

$$Y = 1.70 \text{ (SE 1.31)} + 1.07 \text{ (SE 0.11)} X \quad (r^2 = 0.97).$$

The intercept of the equation indicates that the estimates of RNA method were consistently 1.7 g/d higher than those by the PD method.

Digestibility of RNA and N

The apparent digestibility of RNA from the duodenum to the ileum was calculated according to the equation:

$$\text{Digestibility} = \frac{\text{RNA flow at duodenum} - \text{RNA flow at ileum}}{\text{RNA flow at duodenum}}$$

The RNA flow at duodenum was calculated as: RNA concentration of duodenal digesta × digesta flow rate entering the duodenum. Similarly, the RNA flow at ileum was calculated as: RNA concentration of ileum digesta × digesta flow rate entering the ileum.

The apparently digestible N was also measured (in the same section of the tract) using the same calculation method as that for RNA digestibility. The results of digestibility of RNA and N are presented in Table 4.8.

The RNA digestibility averaged 0.88 ± 0.03 and was slightly higher than the purine digestibility of 0.83 used in the equation for the estimation of MN flow based on purine derivative excretion. Purine digestibility was not measured in this experiment. The apparent N digestibility averaged 0.52 ± 0.09 and was lower than RNA digestibility. The N digestibility decreased with a higher level of intake. However, the RNA digestibility remained relatively constant.

Table 4.8. Apparent N and RNA digestibility estimated between the duodenum and ileum.

Animal	Intake	Digestibility	
		RNA	N
3124	600	0.93	0.61
3126	600	0.85	0.60
3125	1000	0.87	0.55
3126	1000	0.85	0.43
3126	1400	0.88	0.42
Average		0.88 ± 0.03	0.52 ± 0.09

Effect of DM intake on microbial N supply

As expected, the intestinal flow of microbial N, estimated by either of the two methods, increased with the level of feed intake (see Figure 4.3 and Figure 4.4). Microbial N flow ranged

from about 5 - 18 g N/d. The regression for MN supply in relation to the DMI according to both methods were:

$$\text{PD method: } Y = -2.05 (\text{SE } 0.37) + 13.89 (\text{SE } 0.41)X \quad r^2 = 0.99$$

$$\text{RNA method: } Y = -0.45 (\text{SE } 1.50) + 14.65 (\text{SE } 1.66)X \quad r^2 = 0.95$$

where Y= MN supply (g/d) and X= DM intake (kg/d)

Figure 4.3. Microbial N supply according to DMI estimated by PD method

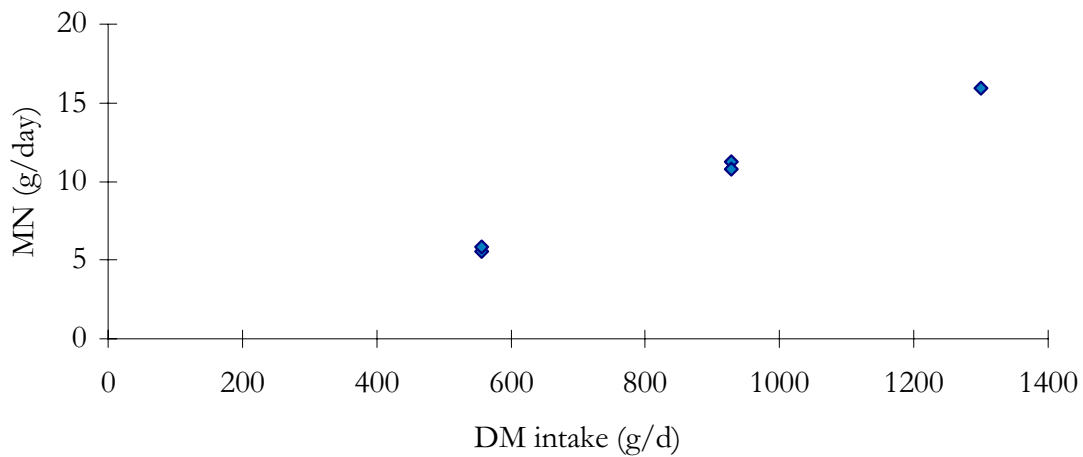


Figure 4.4. Microbial N supply in relation to DMI estimated by RNA method

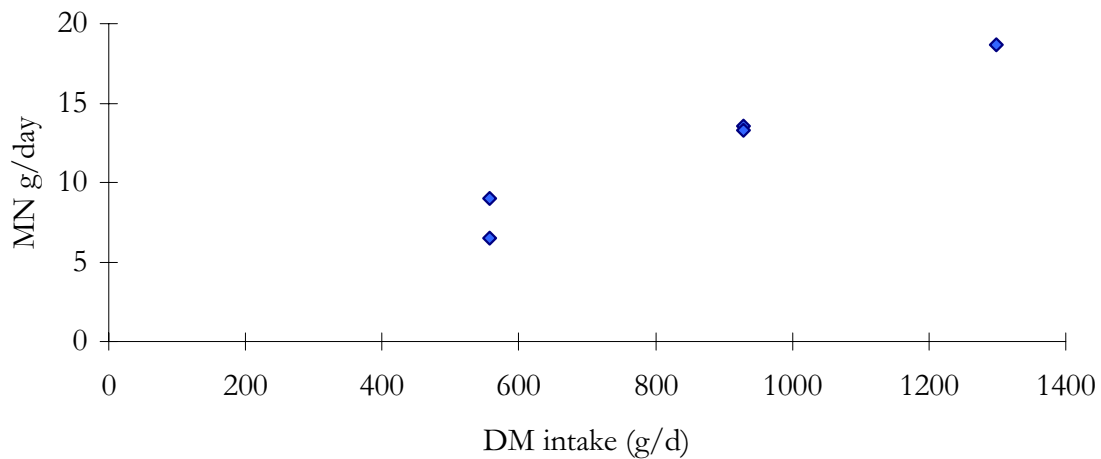


Table 4.9 shows the data of microbial N supply expressed as per kg of digestible organic matter fermented in the rumen (DOMR) This expression is commonly used in the literature as the ‘efficiency of microbial N supply’.). The DOMR was calculated as digestible organic matter

intake (DOMI) x 0.65 according to ARC (1984). The average values for the efficiency of microbial N supply was 33.8±10.3 g MN/kg DOMR estimated based on the purine derivative method, and 42.5±7.2 g MN/kg DOMR estimated based on the RNA method. As shown in Figures 4.5 and 4.6, the efficiency of microbial N supply also increased with feed intake. The regression equations:

$$\text{PD method} \quad Y = 15.38 (\text{SE } 1.48) + 0.022 (\text{SE } 0.002)X \quad r^2 = 0.98$$

$$\text{RNA method} \quad Y = 27.01 (\text{SE } 6.06) + 0.018 (\text{SE } 0.007)X \quad r^2 = 0.70$$

where Y= Efficiency of MN (MN g/kg DOMR) and X= DM intake (kg/d)

Table 4.9. Efficiency of MN supply estimated by the RNA and PD method.

Intake (g/d)	Animal	DOMI (kg/d)	Efficiency (MN g/kg DOMR)	
			based on PD method	based on RNA method
600	3126	0.202	27.45	32.21
600	3124	0.216	26.89	41.84
1000	3126	0.322	34.91	42.23
1000	3125	0.291	36.99	45.78
1400	3126	0.370	42.98	50.40
			33.8 ± 10.31	42.5 ± 7.21

Figure 4.5. Efficiency of MN supply estimated by the PD method

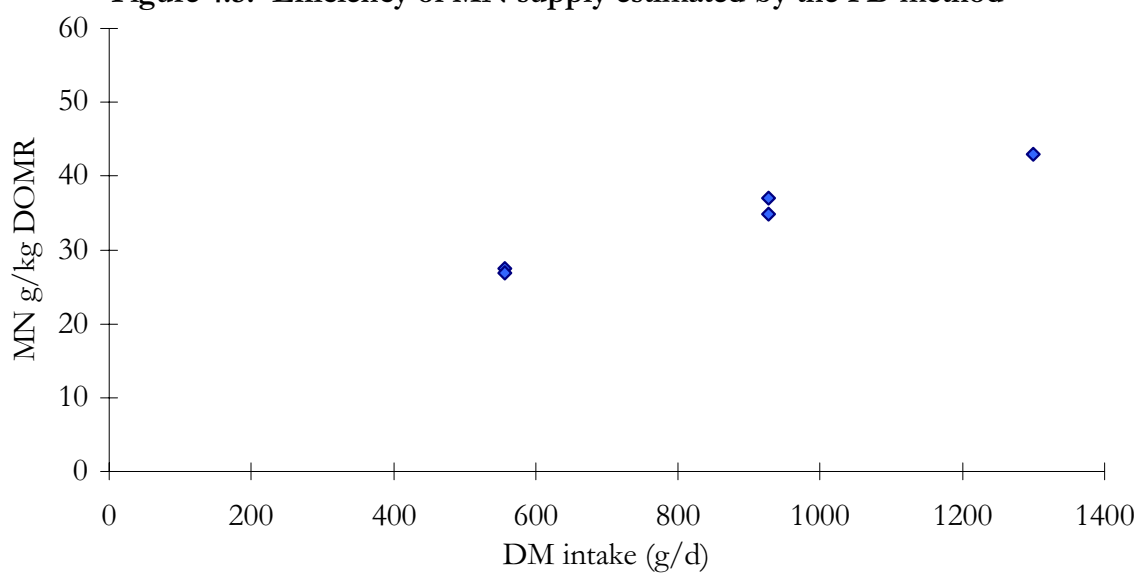
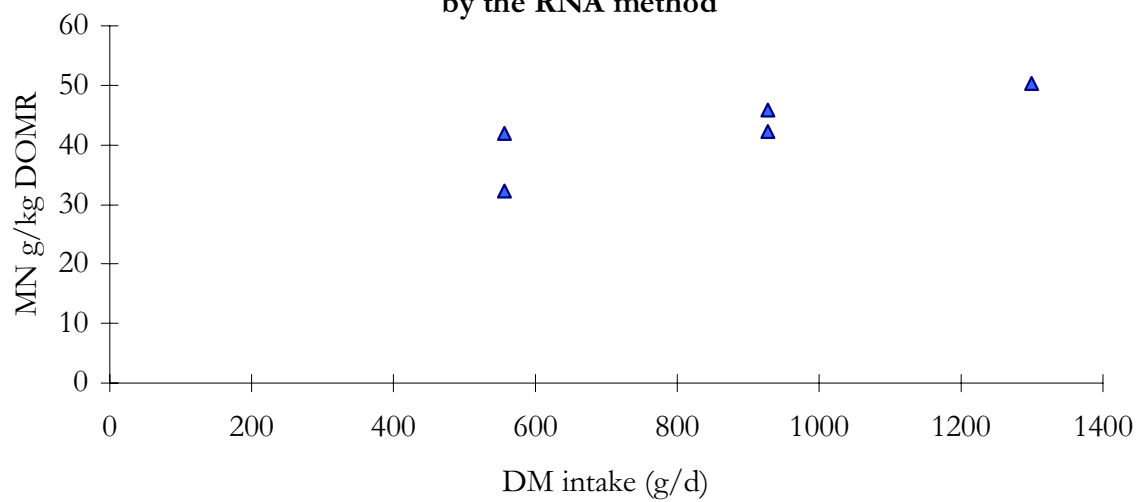


Figure 4.6. Efficiency of MN supply in relation to DMI estimated by the RNA method



5. DISCUSSION

N-ALKANE FOR DIGESTA FLOW MEASUREMENT

Estimates of digesta DM flow based on alkane C₂₉, C₃₁ or C₃₃ were in good agreement with those based on the commonly-used double-marker method. The present work demonstrates the validity of using alkane C₂₉, C₃₁ and particularly C₃₃ as reliable markers to estimate digesta flow. The advantage of the use of natural alkanes is that there is no need for dosing of external markers. With externally dose markers, poor mixing of the markers with the feed particles could be a problem. However, with the alkane method, such a problem is overcome because alkanes are constituents of the feed. The alkane method is advantageous not only in that it cost less and simple but also in that it is not environmental hazardous comparing with the double-marker technique.

The feeds (Lucerne in Experiment 1, and Rye grass in Experiment 1) used in the present work contained adequate amounts of natural long-chain alkanes. With feeds containing low amounts of long-chain natural alkanes, it is possible to dose synthetic alkanes. However this would make it more laborious and the problem of dosed alkanes mixing well with the food, mentioned by Dove and Mayes (1991), will reduce its advantages over other markers.

EFFECT OF INTAKE ON EFFICIENCY OF MICROBIAL N SUPPLY

Both the RNA and PD methods were sensitive in detecting the changes in MN supply when there was a change in the levels of intake. The efficiency of MN supply (g MN/kg DOMR) was not constant but also increased positively with the level of feed intake. The results agree with the observation by Chen *et al.* (1992) which indicated that the efficiency of MN supply increased with levels of intake. The effect of feed intake on the efficiency of microbial supply was considered in the AFRC (1993) protein evaluation system (Table 1.4). It was suggested that the higher efficiency of microbial N supply associated with higher level of intake was due to a higher digesta passage rate.

COMPARISON OF RNA AND PD METHOD

The results comparing the PD and RNA methods demonstrated that there is a good correlation of the method with a slope of close to 1. The intercept of 1.70 showed that the RNA method estimated consistently about 2 g more MN than the PD method. Within the range of MN estimated (about 5-18 g MN/d), this difference remained rather constant independently of the level of intake. It is not clear what factor that contributed to the constant differences between the RNA method and PD method.

From the limited number of observations available in this work, it is evident that the PD method gave estimates of microbial N flow that were highly correlated with and close to estimates by an separate method. It is difficult to judge which of the methods gave the most accurate results. The values for the efficiency of microbial N supply estimated by the PD method (33.8 gN/kg DOMR) was closer to the average value reported in the literature (32 gN/kg DOMR) than the RNA method (42.5 gN/kg DOMR). There are many steps involved in the RNA method, and each step could contribute to the final precision of the results. The simplicity and the non-invasive nature of the PD method make this method more advantageous than the RNA method.

It is recognised that the present work provided only a limited number of observation. It is desirable to extend the work with more animals to enable a closer examination of the agreement between the PD method and other methods. However, it is certain at this stage that the PD method can be used reliably to compare differences in microbial N supply between different dietary regimes.

DIGESTION OF RNA AND N

There was a large extent of digestion of RNA between the duodenum and ileum (mean digestibility of 88%). The results were similar to the findings of Ben-Ghedalia (1981), who noted an RNA digestibility of 83.9% at a distance of 7 meters from the pylorus. In contrast to the RNA digestibility, the apparent digestibility of N was unexpectedly low (52%). This may be due to the contribution of the endogenous N at the ileum samples.

It was found considerably less amount of RNA concentration in the jejunum than in the duodenum. The results demonstrated clearly that RNA was absorbed mainly in the section between the duodenum and jejunum. This agrees with the results by Ben-Ghedalia (1981) were he demonstrated that more than 50% of the degraded RNA disappeared in the first meter of the small intestine. Animals with jejunal cannulae were used in this experiment because a rapid

digestion and absorption of RNA before this point was not expected. Therefore, the flow of RNA at the jejunum does not reflect the total flow of RNA from the rumen.

Due to limitation of time, the purine digestibility was not measured in Experiment. However, it is expected to be similar to the digestibility of RNA. The purine digestibility of 83% (obtained from the literature) was used for the calculation of microbial N supply based on PD method. If a value of 88% were to be used instead, the microbial N supply estimates would be about 5% lower than the values listed in Table 4.4.

CONCLUSION

The present work indicated that n-alkanes C₂₉, C₃₁, and particularly C₃₃ can be used as reliable markers for the estimation of digesta flow in sheep. The results of the digesta flow measurements were practically identical to those measured by the double-marker technique. Lucerne and Rye grass contained sufficient quantities of these alkanes enabling estimation of digesta flow without dosing of external alkanes.

There was a good correlation between the estimates of microbial N supply based on PD excretion in the urine and those based on intestinal flow of RNA. The RNA method gave a constant 1.7 gN/d microbial N higher than the PD method. However, the slope of regression was close to 1. Estimates of the efficiency of microbial N supply (g microbial N/kg DOMR) based on the PD method was closer to the values reported in the literature.

There was a rapid digestion and absorption of RNA between duodenum and jejunum. Apparent digestibility of RNA between the duodenum and ileum averaged 88%, whereas that of N was only 52%.

The results of this work indicate that purine derivative excretion in urine can be used as a reliable indicator of microbial N supply in sheep. The fact that it is simple and requires no surgical preparation of the animal makes it a useful tool to study factors affecting microbial protein supply in ruminants.

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